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## Th2 Allergic Immune Response to Inhaled Fungal Antigens is Modulated By TLR-4-Independent Bacterial Products

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### Abstract

**SUMMARY**—Allergic airway disease is characterized by eosinophilic inflammation, mucus hypersecretion and increased airway resistance. Fungal antigens are ubiquitous within the environment and are well know triggers of allergic disease. Bacterial products are also frequently encountered within the environment and may alter the immune response to certain antigens. The consequence of simultaneous exposure to bacterial and fungal products on the lung adaptive immune response has not been explored. Here we show that oropharyngeal aspiration of fungal lysates (*Candida albicans*, *Aspergillus fumigatus*) promotes airway eosinophilia, secretion of Th2 cytokines and mucus cell metaplasia. In contrast, oropharyngeal exposure to bacterial lysates (*Pseudomonas aeruginosa*) promotes airway inflammation characterized by neutrophils, Th1 cytokine secretion and no mucus production. More importantly, administration of bacterial lysates together with fungal lysates deviates the adaptive immune response to a Th1 type associated with neutrophilia and diminished mucus production. The immunomodulatory effect that bacterial lysates have on the response to fungi is TLR4-independent but MyD88 dependent. Thus, different types of microbial products within the airway can alter the host's adaptive immune response, and potentially impact the development of allergic airway disease to environmental fungal antigens.

### Keywords

T helper cells; Lung inflammation; cytokines; immune responses; eosinophils

### INTRODUCTION

Allergic airway disease is characterized by eosinophilic inflammation, mucus hypersecretion and increased airway resistance [1–3]. This response results from antigen-specific Th2 cell activation characterized by IL-4, IL-5 and IL-13 production [4]. IL-4 and IL-5 stimulate eosinophil maturation and activation while IL-13 is an important mediator of mucus production [4,5]. Among inhaled allergens that can trigger this type of immune response are

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a number of fungal species [6]. These agents can stimulate allergic inflammation without causing active infection in the immunocompetent host [6–8]. It is unclear why airway exposure to fungal antigens leads to a Th2 immune response but may result from specific antigenic determinants of the fungi that preferentially stimulate Th2 responses. However, CD4 T cells from mice with transgenic T-cell receptors specific for a particular *Aspergillus* antigen differentiate *ex vivo* into IFN $\gamma$  producing Th1 cells rather than Th2 cells [9]. Alternatively, it could be the lung environment with its costimulatory cytokine milieu that predisposes to a Th2 immune response to fungal antigens. Interestingly, not all individuals develop allergic airway inflammation in response to inhaled fungal allergens despite ubiquitous exposure [10]. The 'hygiene hypothesis' proposes that lung exposure to certain bacterial components (e.g. CpG DNA) could modulate the Th2 immune response [11–13]. Thus, CD4 T cell immune response to specific protein antigens could be modulated by exposure to bacterial microbial products.

We have previously demonstrated that direct airway exposure to *Aspergillus fumigatus* lysates promotes a Th2 biased immune response in the lungs of mice [5]. This is similar to the immune response to *A. fumigatus* seen in humans with allergic bronchopulmonary aspergillosis (ABPA) [14] and is characterized by prominent lung eosinophilia and mucus production. It is also associated with antigen-specific CD4 T cell IL-4 production characteristic of a Th2 bias [5]. Using this animal model, we have now examined whether the Th2 immune response is unique to fungal antigens or whether it results from direct lung exposure to bacterial lysates as well. Here we demonstrate that, like *A. fumigatus*, *Candida albicans* antigens also promote Th2 inflammation characterized by airway eosinophilia and mucus production, whereas *P. aeruginosa* antigen exposure promotes a Th1 immune response characterized by neutrophilia without mucus production. Further, the presence of *P. aeruginosa* products are capable of modulating the adaptive immune response to fungal antigens independent of TLR4 signaling but dependent on MyD88 signaling.

## RESULTS

### ***A. fumigatus* and *C. albicans* induce eosinophilic airway inflammation with mucus hypersecretion**

We have previously demonstrated that direct inoculation of the lung with killed *A. fumigatus* hyphal antigens without previous immunization and in the absence of adjuvant induces a Th2 immune response characterized by airway eosinophilia and mucus production [5]. To determine whether this response is specific to *A. fumigatus* or common to other fungal antigens, we examined the pulmonary immune response to *Candida albicans*. In certain organs, live *C. albicans* infections have previously been shown to induce a Th2 immune response but the immune response to killed lysates in the lung has not been determined. Using a chronic exposure model in which mice are exposed to antigens three times over 14 days, we found that similar to *A. fumigatus*, killed *C. albicans* antigens also generated significant airway inflammation as measured by BALF cell number (Figure 1A). *C. albicans*-induced inflammation was also characterized by airway eosinophils with minimal numbers of neutrophils (Figure 1B) and marked airway epithelial mucus production compared to nonexposed mice (Figure 1C). Further, we found similar eosinophilic inflammation with mucus production to *Aspergillus niger* (data not shown). We conclude that exposure of the lung to fungal lysates results in a clear Th2 immune response characterized by accumulation of eosinophils and mucus production.

### ***P. aeruginosa* induces neutrophilic airway inflammation without mucus hypersecretion**

To determine whether the lung environment favors a Th2 bias to any microbial antigens or whether this type of immune response was specific to fungi, we examined the response to

bacterial antigens. *P. aeruginosa* is a Gram negative bacterium that is frequently associated with a variety of chronic lung diseases [15–21]. We therefore exposed mice to cellular lysates of *P. aeruginosa* and found that lung exposure also induced airway inflammation with high numbers of cells in BALF (Figure 2A). However, unlike the airway eosinophilia elicited by fungal antigens, the cellular composition in the BALF was largely neutrophils with very few eosinophils (Figure 2B). Additionally, despite the lung tissue inflammation observed after *P. aeruginosa* exposure, no airway epithelial mucus staining was observed (Figure 2C). Thus, direct lung exposure to *P. aeruginosa* induces an immune response in the lung characterized by neutrophilia instead of eosinophilia and with no mucus production. These results demonstrate that the lung environment alone is not sufficient to dictate the type of immune response generated to microbial pathogens.

### ***C. albicans* induces Th2 cytokine production while *P. aeruginosa* induces Th1 and Th17 cytokine production**

To define the *in vivo* CD4 T cell response to *C. albicans* and *P. aeruginosa*, we examined IFN $\gamma$ , IL-4, IL-5 and IL-13 production upon CD4 T cell restimulation with either *C. albicans* or *P. aeruginosa*. CD4 T cells from *C. albicans*-exposed mice were restimulated with *C. albicans* lysates and CD4 T cells from *P. aeruginosa*-exposed mice were restimulated with *P. aeruginosa* lysates. CD4 T cells from *C. albicans*-exposed mice produced higher levels of IL-4, IL-5 and IL-13 when compared to CD4 T cells from *P. aeruginosa*-exposed mice (Figure 3A, 3B and 3C) correlating with the eosinophilic lung inflammation and mucus production observed in the mice exposed to *C. albicans*. Interestingly, CD4 T cells from mice exposed to *P. aeruginosa* produced significantly more IFN $\gamma$  compared to CD4 T cells from mice exposed to *C. albicans* (Figure 3D), in agreement with the neutrophilic inflammation in the *P. aeruginosa*-exposed mice. Thus, lung exposure to fungi, but not to bacterial products, promoted CD4 T cell Th2 differentiation.

An additional subset of helper T cells, Th-17, has been recently described and are characterized by their ability to produce the proinflammatory cytokine IL-17. IL-17, produced primarily by T lymphocytes early in the course of bacterial infection, promotes neutrophil recruitment and survival [22,23], is released in response to antigen presenting cell-derived IL-23 and represents important cross-talk between the innate and adaptive immune systems [23]. We measured CD4 T cell IL-17 production in response to lung exposure to *C. albicans* and *P. aeruginosa* microbial products. *Ex vivo* restimulated CD4 T cells from mice exposed to *P. aeruginosa* produced high levels of IL-17 while CD4 T cells from *C. albicans*-exposed mice produced low levels (Figure 3E). These results correlate with the selective accumulation of neutrophils in *P. aeruginosa*-exposed mice but not in *C. albicans*-exposed mice. Thus, fungal products do not induce CD4 T cell responses characterized by IL-17 production.

### **The immune response to *C. albicans* is shifted from Th2 to Th1 by the presence of *P. aeruginosa***

In chronic bacterial infections of the lung, such as those associated with CF, fungi, such as *A. fumigatus* and *C. albicans* are often also detected [18,24–27]. While bacterial colonization is clearly detrimental and often treated aggressively [16,17,26], the effects of fungal airway infections on lung function, bacterial virulence or host response are almost completely unknown. To determine how the presence of bacteria can affect the host immune response, we examined the immune response in the lung to the combined exposure of directly administered *C. albicans* and *P. aeruginosa* microbial products. As expected, *C. albicans*, *P. aeruginosa* and the co-exposure to *C. albicans* and *P. aeruginosa* all resulted in airway inflammation as measured by BALF cell number when compared to nonexposed mice (Figure 4A). However, while *C. albicans* exposure resulted in predominantly

eosinophils accumulating in the airway, exposure to *C. albicans* and *P. aeruginosa* resulted in the immune response changing from eosinophilic to neutrophilic (Figure 4B). Both the percent and total number of eosinophils were greatly reduced upon co-exposure. Mice exposed to *C. albicans*, *P. aeruginosa* or both developed peribronchial airway inflammation on H&E staining when compared to nonexposed mice (Figure 4C). However, while PAS staining of the lung tissue demonstrated epithelial mucus production in *C. albicans* exposed mice (Figure 4C), exposure to both *C. albicans* and *P. aeruginosa* together resulted in an inflammatory response characterized by an absence of mucus production (Figure 4C). Lung mucus staining was further quantified by determining the expression of the *muc5ac* gene (a common mucin gene in the lung) [28] in the lung tissue using real time RT-PCR. *C. albicans* exposure induced a 25-fold increase in levels of *muc5ac* gene expression when compared to nonexposed control mice while exposure to *P. aeruginosa* or both *C. albicans* and *P. aeruginosa* resulted in less than a 4-fold increase in *muc5ac* gene expression (Figure 4D). These results demonstrate that the presence of bacteria does not significantly alter the magnitude of inflammation but rather dictates the type of inflammation elicited.

Recent studies using models of combine ovalbumin (OVA) and LPS sensitization and challenge have suggested that a Th1 dominant immune response to OVA, characterized by neutrophilia and  $\text{INF}\gamma$  production, results in a greater increase in airway hyperresponsiveness (AHR, PenH) in response to methacholine [29]. To determine if immunodeviating the immune response to *C. albicans* from eosinophilic to neutrophilic airway inflammation with *P. aeruginosa* had an impact on AHR, we measured airway resistance ( $R_N$ ) following methacholine challenge in mice exposed to PBS, *C. albicans*, *P. aeruginosa* or a combination of *C. albicans* and *P. aeruginosa* antigens. Airway exposure to *C. albicans* alone showed increased airway resistance ( $R_N$ ) when compared to PBS exposed controls (Figure 4E). Interestingly however, exposure to *P. aeruginosa*, either alone or in combination with *C. albicans* resulted in a significantly increased degree of AHR when compared to exposures to PBS or *C. albicans* alone. These results suggest that shifting of the immune response to *C. albicans* by *P. aeruginosa* leads to a more severe asthma phenotype, which may be the result of greater airway injury and more direct access of nebulized methacholine to underlying smooth muscle.

To determine whether the airway neutrophilia observed after co-exposure to *C. albicans* and *P. aeruginosa* is associated with differential airway cytokines, we measured IL-4 and  $\text{INF}\gamma$  in BALF after exposure to *C. albicans* and *P. aeruginosa*. The co-exposure to *C. albicans* and *P. aeruginosa* led to high levels of  $\text{INF}\gamma$  and low levels of IL-4 (Figure 5A), suggesting that *P. aeruginosa* may alter the differentiation of antigen specific CD4 T cells from Th2 to Th1. To test this hypothesis we then isolated splenic CD4 T cells from mice co-exposed to *C. albicans* and *P. aeruginosa* and restimulated them *in vitro* with *C. albicans* and found that antigen co-exposure resulted in high levels of  $\text{INF}\gamma$  while IL-4 was undetectable (Figure 5B). Similar results were found when CD4 T cells were isolated directly from the lung of mice co-exposed to *C. albicans* and *P. aeruginosa* (Figure 5C). Additionally, when splenic CD4 T cells from mice co-exposed to *C. albicans* and *P. aeruginosa* *in vivo* and restimulated with both microbial products *in vitro*, there was still preferential production of  $\text{INF}\gamma$  over IL-4 (Figure 5D). Taken together, these results support the conclusion that the Th2 immune response induced by fungal microbial products is redirected to a Th1 immune response in the presence of *P. aeruginosa* products.

### The adaptive immune response to *C. albicans* is Th1 and not Th2 in the presence of LPS

As in other Gram negative bacteria, the *P. aeruginosa* outer membrane contains abundant lipopolysaccharide (LPS) [30–32]. LPS has been shown to modulate CD4 Th1/Th2 responses in a dose dependent manner [33]. To determine if Th1-type responses elicited by exposure to *P. aeruginosa* lysates were similar to those elicited by purified LPS, we examined the

immune response against airway exposure to *C. albicans* antigens in combination with bacterial LPS. We selected our dose of LPS to be similar to the dose present in the cell lysates of *P. aeruginosa* and similar to levels used in other models of Gram negative infection [34–36]. As expected, *C. albicans*, LPS and coexposure to LPS and *C. albicans* together all induced airway inflammation (Figure 5A). Analysis of differential cell counts showed that exposure to LPS alone caused lung inflammation characterized largely by the presence of macrophages at the time of lung harvest with few neutrophils and no eosinophils (Figure 5B). However, while *C. albicans* stimulated an eosinophilic inflammatory cell infiltration, the addition of LPS to *C. albicans* resulted in an immune response in which neutrophilic inflammation was predominant (Figure 5B). Peribronchial lung tissue inflammation was observed on H&E staining after *C. albicans*, LPS and coexposure to LPS and *C. albicans*; however, epithelial mucus staining was only observed after *C. albicans* alone, was not present after LPS exposure and was abolished by the addition of LPS to *C. albicans* (Figure 5C). Mucus production was further quantified by measuring *muc5ac* gene expression, which demonstrated high levels of expression selectively in the *C. albicans* exposed mice, but not in LPS-exposed and LPS and *C. albicans* exposed mice, confirming the lung histology findings (Figure 5D). These results suggest that *C. albicans* induces a Th2 immune response *in vivo* with eosinophilic inflammation and mucus production while bacterial LPS is sufficient to alter this immune response to Th1 as characterized by neutrophil accumulation and a lack of mucus production. To confirm this, CD4 T cells were restimulated *ex vivo* after *in vivo* exposure to *C. albicans*, LPS or LPS and *C. albicans* coexposure. As expected, CD4 T cells from the *C. albicans* exposed mice produced high levels of IL-4 and low levels of IFN $\gamma$  indicating a Th2 bias (Figure 5E). Conversely, CD4 T cells from mice exposed to both LPS and *C. albicans* produced low levels of IL-4 and high levels of IFN $\gamma$  supporting the generation of a Th1 phenotype (Figure 5F). LPS alone did not result in significant IL-4 or IFN $\gamma$  production, indicating that the production of these cytokines is antigen-specific (Figure 5E, 5F). We conclude that the absence of LPS in the fungi favors a Th2-biased immune response.

### ***P. aeruginosa* alters the adaptive immune response to *C. albicans* independent of TLR4 but dependent on MyD88**

To determine if the Th1-biasing effect of *P. aeruginosa* on *C. albicans* immune response was simply due to the presence of LPS we used TLR4 deficient mice. TLR4 is a receptor for pathogen-associated molecular patterns (PAMPs) expressed by cells of the innate immune system that initiates intracellular signaling in response to LPS [37]. Since fungi do not contain LPS, *C. albicans* exposure in TLR4 deficient mice resulted in a Th2 biased inflammatory response similar to that seen in WT mice (Figure 6A, 6B). TLR4 deficiency did not affect airway inflammation induced by *C. albicans* as determined by total cell number and percent of eosinophils in BALF, indicating that the immune response to *C. albicans* is TLR4-independent. However, as expected, the total cell number and percent neutrophils in BALF of TLR4 deficient mice exposed to *P. aeruginosa* were reduced compared with the numbers in WT mice (Figure 7A, 7B), indicating that the type of immune response generated to *P. aeruginosa* is partially due to the presence of LPS. Interestingly, the absence of TLR4 did not prevent *P. aeruginosa* exposure from switching the immune response to *C. albicans* from eosinophilia to neutrophilia (Figure 7A, 7B). Furthermore, TLR4 deficiency did not prevent *P. aeruginosa* from inhibiting mucus production triggered by *C. albicans* (Figure 7C, 7D). These results indicate that the effect that *P. aeruginosa* has on *C. albicans* immune response is not due exclusively to the presence of LPS.

To determine if other members of the TLR family were important in immunodeviating the adaptive immune response to *C. albicans* by *P. aeruginosa*, we exposed wild type (WT) and MyD88 deficient (MyD88 $^{-/-}$ ) mice to a combination of *C. albicans* and *P. aeruginosa*



lysates. As expected, airway inflammation was severely compromised in MyD88<sup>-/-</sup> mice compared with WT mice (Figure 8A). Interestingly however, while WT mice have a predominance of airway neutrophils, MyD88<sup>-/-</sup> mice have an eosinophilic predominant airway inflammatory response suggesting that the immunodeviation induced by *P. aeruginosa* is dependent on MyD88 signals (Figure 8B). Additionally, CD4 T cells from MyD88<sup>-/-</sup> mice restimulated *in vitro* with *C. albicans* antigens appear to produce more IL-4 and less IFN $\gamma$  compared to CD4 T cells from WT mice (Figure 8C). Taken together, these results suggest that the immunodeviation of the adaptive response to *C. albicans* from Th2 to Th1 by *P. aeruginosa* is independent of LPS and TLR 4 but requires other MyD88-dependent signals.

## DISCUSSION

COPD, bronchiectasis and CF are often complicated by chronic airway infections [15,26,38–42]. *P. aeruginosa* is a commonly encountered bacteria and is typically treated aggressively with antibiotics, particularly at time of acute exacerbation [15–18]. Frequently, *P. aeruginosa* colonization of the airway is accompanied by colonization with different species of fungi such as *C. albicans* and *A. fumigatus* [24,25,27,39,43–46]. *In vitro* work has demonstrated that *P. aeruginosa* has a number of interactions with *C. albicans* [47,48]. Additional studies have suggested that the presence of *C. albicans* within the airway is a risk factor for contracting *P. aeruginosa* lung infection. Unlike bacterial infection, little effort is made clinically to treat fungal airway colonization and the fungi are often considered innocuous bystanders [16,17,26,27]. However, the presence of multiple microbes could affect the adaptive immune response to specific microbes. Little is known about the *in vivo* interaction between bacterial and fungal products and how it may impact on the immune response to these products. We have previously shown that direct airway exposure to *A. fumigatus* is sufficient to generate a Th2-biased immune response characterized by the accumulation of eosinophils and mucus production [5]. In this study, we show a similar type of response after exposure to antigens from *C. albicans*. Thus, direct exposure of lung to fungal antigens is capable of inducing an antigen-specific Th2 immune response. In contrast, we also show here that direct exposure of lung to bacterial antigens induces a Th1 type of immune response characterized by airway neutrophilia without mucus secretion.

Th2 immune responses are associated with infections by extracellular pathogens, while Th1 responses are associated primarily with intracellular pathogens [8]. However, in our studies we use killed pathogen lysates instead of infectious organisms, indicating that the type of immune response in the lung is not determined only by the specific cellular route of infection. Instead, the results shown here suggest that the presence of components specifically expressed in fungi, but not in bacteria are likely to be the cause of the Th2-biased response to fungal antigens. Alternatively, the absence in fungi of a specific component that promotes a Th1 response could result in a default Th2 immune response. Since the administration of *P. aeruginosa* together with *C. albicans* induces a Th1 response, the later mechanism is more likely to be the case.

LPS is a component of *P. aeruginosa* and other Gram-negative bacteria that rapidly triggers the innate immune response, but can also indirectly modulate Th1/Th2 bias probably indirectly through innate cytokine production [33]. Fungi lack LPS and our data show that the absence of TLR4 has no effect on the immune response induced by *C. albicans* airway exposure, further demonstrating that no contaminant LPS contributes to the Th2 immune response to fungal antigen. Thus, the lack of LPS in fungi could determine the lack of Th1 and default to Th2 response. Although somewhat controversial, LPS has been shown to affect infection with live *C. albicans* by increasing fungal-related mortality [49–51]. We show here that through direct exposure to the lung, LPS can also modify the immune

response to *C. albicans* lysates by deviating the immune response from Th2 to Th1. However, the Th1 bias observed when both *P. aeruginosa* and *C. albicans* antigens were administered together was not impaired in TLR4 deficient mice, indicating that components of *P. aeruginosa* other than LPS promote the Th1 bias when fungi are present. Our results in MyD88<sup>-/-</sup> indicate that TLRs other than TLR4 are involved. The airway epithelium expresses a variety of TLRs and may itself be an important component in the innate immune response to pathogens [52–54]. In addition to TLR4 the TLRs, TLR2, TLR5 and TLR9 play a central role in the stimulation of proinflammatory cytokine production from airway epithelium in response to *P. aeruginosa* [55–57]. Thus, signals from TLR2, TLR5 and TLR9 may be responsible for the Th1 bias seen after exposure to these products. *P. aeruginosa* also possesses a variety of virulence factors important in organism pathogenesis, including proteases, lipases and type III secretion factors that exploit the host environment and allow the organism to escape from tissues [58–61]. *P. aeruginosa* also has virulence factors that help it evade the immune response, notably polysaccharides such as alginate [62,63]. These virulence factors are most relevant in live infections with *P. aeruginosa* and are unlikely to play a significant role in our studies as the *P. aeruginosa* lysates were heat-killed there by inactivating the enzymatic activity required to impart virulence. Our results demonstrate that the Th1 response to *P. aeruginosa* was less robust in TLR4 deficient mice while the response to *P. aeruginosa* and *C. albicans* in these mice was not affected. This result suggests that the adaptive immune response to the combined microbial products is directed against *C. albicans* antigens rather than *P. aeruginosa* antigens, further demonstrating the ability of *P. aeruginosa* to immunodeviate the response to *C. albicans* independent of LPS and TLR4.

To date, the components of fungi that can promote the Th2 immune response remains unclear. Some studies have proposed that the presence of fungal-associated allergenic proteinase is responsible for the Th2 type of immune response to these microorganisms [64,65]. *A. fumigatus* antigens devoid of proteases are not sufficient to cause a Th2 immune response, but rather only with the addition of proteases does direct airway inoculation result in allergic disease [64]. Similarly, non-allergenic proteins such as ovalbumin can be made allergenic by the addition of fungal protease as an adjuvant [64]. However, our studies were performed with fixed or heat-killed extracts without active proteases, suggesting that enzymatic activity was not required to generate an immune response. Thus, although we cannot discard the involvement of proteases during fungal infection, they are not absolutely required for the induction of a Th2 immune response. The C-type lectin receptor (CLR) class of pattern recognition receptors (PRR), including dectin 1, are also important in regulating mammalian immune responses to a variety of fungi, including *C. albicans* [8]. Dectin 1 recognizes  $\beta$ -glucan, the complex carbohydrate common in fungal cell walls [66,67] and plays an important role in host defense against germinating fungal yeast [8,68,69]. Its role in the generation of an *in vivo* Th2 immune response to fungal lung exposure remains uncertain.

Additional factors that qualitatively influence the type of immune response are the route of fungal exposure, live versus killed organism and the phase of fungal growth [9,10,69,70]. *In vivo* fungal administration of conidia via the gut, via the lung or systemically has been shown to induce a Th1 immune response, which is necessary in the clearance of live organism [8]. If the conidia are killed, then the adaptive immune response is characterized by IL-4-producing CD4 T cell clones [70] suggesting that the host can distinguish between conidia that have the potential to grow and cause further damage and those that cannot [70]. Mannoproteins on the fungal cell wall have been shown to stimulate robust Th1 responses and thereby prevent disseminated infection [8,71]. Our work and that of others have clearly documented a Th2 immune response with fungal administration directly to lung and may be a consequence of the hyphal components of our fungal lysates [64,65].

Polymicrobial infections are common in chronic lung disease and a better understanding of the host's immune response to pathogens is essential in developing new therapies and guiding current treatment. Here we show the novel finding that in the lung *P. aeruginosa* is able to immunomodulate the host response to *C. albicans* from Th2 to Th1 in an LPS/TLR4 independent but MyD88 dependent mechanism.

## MATERIAL AND METHODS

### Reagents

*A. fumigatus* lysates, generously provided by Dr. Kieren Marr, Department of Medicine, Portland, Oregon, were generated by culture of Af293 isolate (5 days, 37°C) in RPMI 1640 (+10% FCS). The hyphal mat was harvested, sequentially washed with PBS, and disrupted by vortexing with glass beads. The slurry was subjected to paraformaldehyde (1%) for microbial inactivation. Protein concentration was measured using the Bradford assay (BioRad Laboratories, Hercules, CA). The product was concentrated using an endotoxin-free dialysis membrane with 10 kDa pore size (Pierce Biotechnology, Rockford, IL) to achieve a final protein concentration of 800–1000 µg/mL. *P. aeruginosa* strain PA14 [72] and *C. albicans* strain SC5314 [73] were grown in 100 ml of DMEM medium (#10-13-CV, Mediatech, Manassas, VA) in 250 ml flasks at 37°C for 16 h at 200 rpm. Flasks were inoculated from 5 ml overnight cultures. Cells were harvested by centrifugation, washed one time, then resuspended in 100 mM phosphate buffer (pH 7). Bacterial cells were lysed by ballistic disintegration via 6 1-min pulses with 0.2 mm glass beads using a Beadbeater (Biospec Products, Bartlesville, OK) with 1 min incubations on ice in between each pulse. *C. albicans* cells were similarly lysed except that 0.5 mm beads were used. Both bacterial and fungal lysates were heated at 65°C for 10 minutes to kill any remaining cells. The absence of any viable cells was confirmed by plating 100 µl of the lysate onto solid medium and inoculation of 500 µl of the lysate into liquid medium followed by incubation at 37°C for 24 and 48h. Viability experiments were performed using Luria broth medium for *P. aeruginosa*, and Yeast Extract/Peptone/Dextrose for *C. albicans*. The protein concentration of lysates was determined by the Bradford Assay (BioRad, Hercules, CA) and the lysate was diluted in phosphate buffer to a final concentration of 1 mg/ml. Lysates were stored frozen at –80°C prior to use.

### Mice

6–8-week-old male C57BL/6J mice were purchased from The Jackson Laboratories (Bar Harbor, ME). 6–8-week-old male TLR4<sup>–/–</sup> and MyD88<sup>–/–</sup> mice both on the C57BL/6J background were bred at the University of Vermont. All mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility at the University of Vermont. The Institutional Animal Care and Use Committee granted approval for all studies. Mice were anesthetized with i.p. pentobarbital sodium (70–90 mg/kg) prior to nonsurvival surgery performed on day 19 after initial microbial product exposure.

### Microbial product airway exposure protocol

Mice were briefly anesthetized (isoflurane by inhalation) and oropharyngeally exposed under direct vocal cord visualization to 5 µg of protein of either *A. fumigatus*, *C. albicans*, *P. aeruginosa* or *C. albicans* and *P. aeruginosa* cellular lysates or *P. aeruginosa*-derived LPS (Sigma, St. Louis, MO) or PBS in a volume of 40 µl on days 0, 7 and 14. This is a model of acute exposure to microbial products. Although a three administration protocol was used for the studies in this manuscript, a similar type of immune response of lesser magnitude was obtained after a single exposure to microbial products.



## BALF

Tracheas were cannulated with 2 cm of 22 gauge polyethylene tubing attached to a 23 gauge needle, 1 ml of cold PBS was instilled into the lungs and the BALF fluid was collected. Cells were pelleted and counted using an Advia cell counter (Bayer, Terrytown, NY) and supernatant was stored at  $-80^{\circ}\text{C}$  for cytokine analysis. Cells ( $50 \times 10^3$ ) were cytospun onto glass slides and stained with Hema-3 (Biochemical Sciences, Swedesboro, NJ). Two hundred cells per slide were counted and scored as macrophages, eosinophils, neutrophils or lymphocytes based on characteristic morphology and staining.

## Quantitative PCR analysis of gene expression

The right lung from each experimental mouse was removed and snap frozen in liquid nitrogen. It was then ground to a fine powder using a chilled mortar and pestle and total RNA was extracted using trizol and further purified using an RNeasy kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized from 1  $\mu\text{g}$  RNA using random primers and Superscript II reverse transcriptase (Invitrogen, San Diego, CA). Quantitative PCR was performed on cDNA with Assays on Demand predesigned *muc5ac* and *hypoxanthine-guanine phosphoribosyltransferase (HPRT)* primer/probe sets (Applied Biosystems, Foster, CA), using TaqMan Universal PCR Master Mix and the Applied Biosystems PRISM 7700 Sequence Detection System. The level of *muc5ac* expression was normalized to *HPRT* housekeeping gene levels and relative *muc5ac* mRNA levels were determined according to the comparative cycle threshold method (Applied Biosystems, ABI Prism 7700 Sequence Detection System, User Bulletin #2). Briefly, the threshold cycle ( $C_T$ ) was determined for *muc5ac* and *HPRT* in each sample. The  $\Delta C_T$  was calculated for each sample by subtracting the  $C_T$  of *HPRT* from the  $C_T$  of *muc5ac*. The baseline  $\Delta C_T$  was assigned to non-AHA exposed WT mice. The  $\Delta\Delta C_T$  values were calculated by subtracting the  $\Delta C_T$  of the baseline from the  $\Delta C_T$  of the experimental samples. The  $\Delta\Delta C_T$  values were transformed into relative values using the equation,  $\text{expression} = 2^{-\Delta\Delta C_T}$

## Lung histology

Lungs were processed for histological analysis as described previously [74] and stained with H&E and PAS according to routine procedures.

## Cell preparation and activation

Single-cell suspensions were generated from spleen by passing the disassociated tissue through a 70- $\mu\text{m}$  mesh, and lymphocytes were enriched by separation with Lymphocyte Separation Medium (MP Biomedicals, Solon, OH). Single cell suspensions were generated from lung by infusion of Type 1 collagenase (1 ml, 1 mg/ml, Gibco, Carlsbad, CA) into the cannulated trachea followed by mincing of lung tissue and incubation in 5 ml of additional type 1 collagenase for 15 minutes at 37 C. Tissue was further dissociated by repeated aspiration with a 14 gauge hollow needle and 60 ml syringe followed by filtering with a 70- $\mu\text{m}$  mesh. Cells were then spun at 600 rotations per minute for 6 minutes, washed with Hank's balanced salt solution (HBSS, Mediatech Inc., Manassas, VA) and resuspended in 5 ml of HBSS. CD4 T cells were isolated from the cellular suspensions by positive selection using CD4 magnetic microbeads (Miltenyi Biotec, Auburn, CA), according to the manufacturer's protocol and purity was confirmed (typically >93%) by surface CD4 and T-cell receptor staining and FACS analysis. Isolated CD4 T cells ( $2 \times 10^6$  cells/ml) were incubated in the presence of syngeneic antigen presenting cells (APC,  $4 \times 10^6$  cells/ml) and activated either by *A. fumigatus* cellular lysates (5  $\mu\text{g}/\text{ml}$ ), *C. albicans* cellular lysates (5  $\mu\text{g}/\text{ml}$ ), or *P. aeruginosa* cellular lysates (5  $\mu\text{g}/\text{ml}$ ) for 72 hours.

APCs were obtained from untreated mice by splenic T cell depletion with negative selection using Abs to CD4 (GK1.5) [75], CD8 and Thy-1 [76], and treatment with rabbit complement. The remaining cells were then treated with mitomycin C, as previously described [77], prior to incubation with the CD4 T cells.

### Determination of cytokine production in BALF and by CD4<sup>+</sup> T cells

ELISAs were performed on the BALF and the cell culture supernatant for IL-4, IL-5, IL-13, IL-17 and IFN $\gamma$  using Duosets (R&D Systems, Minneapolis, MN), according to manufacturer's recommendations.

### Determination of Pulmonary function

Mice were anesthetized, tracheotomized and mechanically ventilated for the measurement of respiratory impedance (Zrs) using the forced oscillation technique as previously described [78]. Mice were ventilated at a rate of 2.5 Hz, with a tidal volume of 0.2 ml and 3 cmH<sub>2</sub>O positive end-expiratory pressure (*flexiVent*; SCIREQ, Montreal, PQ, Canada). Impedance data were collected during regular ventilation to establish a baseline for each mouse, and inhaled methacholine (Sigma-Aldrich) in saline was then delivered via aerosol in successively increasing doses (0, 3.125, 12.5, and 50 mg/ml). Multiple linear regression was used to fit Zrs spectra derived from measured pressure and cylinder displacement to the constant phase model of the lung [79].

$$Z(f) = R_n + i\omega I + (G + iH_{ti}) / \omega^a$$

where  $Z$  is input impedance of the respiratory system,  $R_n$  is Newtonian resistance composed primarily of flow resistance of conducting pulmonary airways,  $I$  is the inertance of the gas in central airways,  $G$  represents dissipation of viscous energy in respiratory tissues,  $H_{ti}$  represents elastic energy storage in the tissues,  $f$  is frequency,  $i = \sqrt{-1}$ ,  $\omega$  is  $2\pi f$ , and  $a$  couples  $G$  and  $H_{ti}$ . Using this model, the dose response of  $R_n$  to methacholine was determined as an index of AHR.

### Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Statistical significance was determined by the Student's  $t$  test unless otherwise indicated. For all analyses  $p \leq 0.05$  was considered statistically significant.

### Acknowledgments

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### Abbreviations

<b>AHR</b>	Airway hyperresponsiveness
<b>R<sub>n</sub></b>	Airway resistance
<b>Zrs</b>	Respiratory impedance

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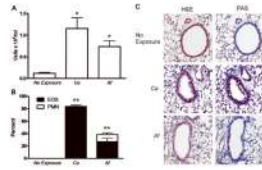
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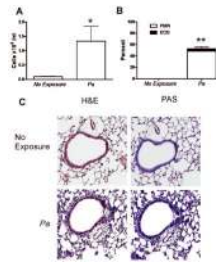


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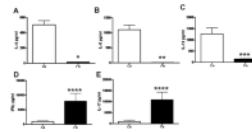
**FIG. 1.**

*A. fumigatus* (Af) and *C. albicans* (Ca) induce eosinophilic airway inflammation with mucus hypersecretion. C57Bl/6J mice were untreated (no exposure) or exposed to 5  $\mu$ g of either *A. fumigatus* or *C. albicans* on day 0, 7 and 14 with lung harvest on day 19. (A) BALF cell numbers. (B) Percentage cell type in BALF as determined by manual differential cell count of neutrophils (PMN) and eosinophils (EOS). Values represent the mean  $\pm$  SEM of 4 mice per group and are representative of experiments performed twice. Statistical significance was determined by one-way ANOVA; \* $p$ <0.01 compared with non-exposed mice; \*\* $p$ <0.0001 eosinophil percentages compared with those in non-exposed mice. (C) Lungs were inflation fixed, sectioned and stained with H&E and PAS. Representative images are 100x magnification.



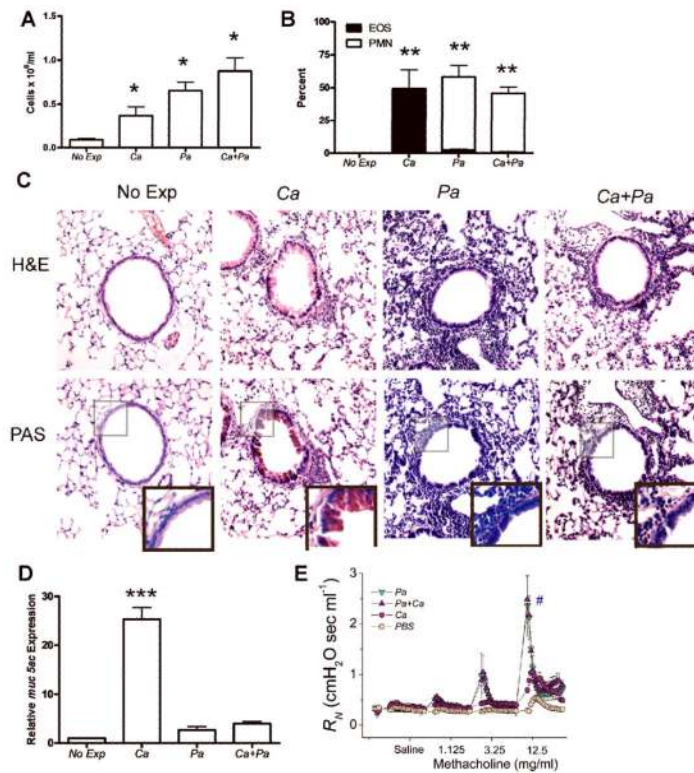
**FIG. 2.**

*P. aeruginosa* (Pa) induces neutrophilic airway inflammation without mucus hypersecretion. (A) BALF cell numbers after exposure to *P. aeruginosa* (5  $\mu$ g) according to the protocol described in Figure 1. Statistical significance determined by the students t-test, four mice per group, \* $p < 0.05$  compared with non-exposed mice. (B) Percentage cell type in BALF by cell type as determined by manual differential cell count of neutrophils (PMN) and eosinophils (EOS). Values represent the mean  $\pm$  SEM of 4 mice per group and are representative of experiments performed twice. Statistical significance was determined by one-way ANOVA; \*\* $p < 0.001$  compared with neutrophil percentages in non-exposed mice. (C) Lungs were inflation fixed, sectioned and stained with H&E and PAS. Representative images are 100x magnification.

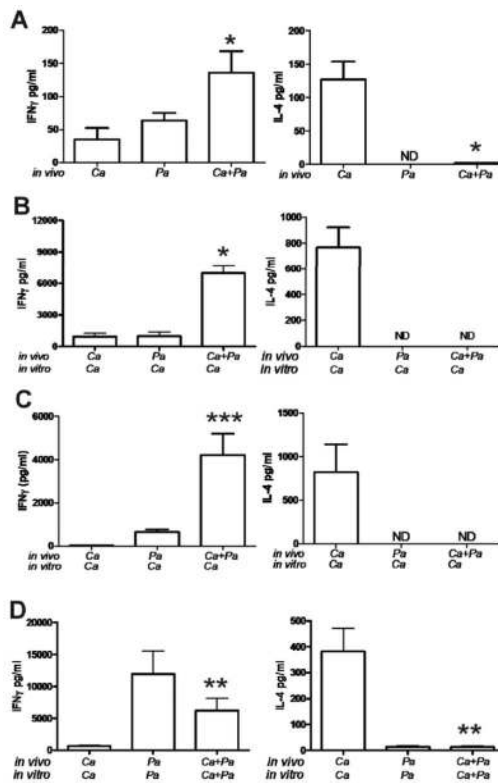
**FIG. 3.**

*C. albicans* (Ca) induces Th2 cytokine production while *P. aeruginosa* (Pa) induces Th1 and Th17 cytokine profile. (A) IL-4, (B) IL-5, (C) IL-13, (D) IFN $\gamma$  and (E) IL-17 production by *ex vivo* restimulated splenic CD4 T cells after *in vivo* exposure to *C. albicans* or *P. aeruginosa*, as determined by ELISA. *In vitro* data presented are the means  $\pm$  SEM of cytokine production by CD4 T cells isolated from a single mouse from a total of four individual mice per group. SEM represents the variation in cytokine production for the four individual mice. Statistical significance determined by the students *t*-test \*,  $p < 0.0001$ , \*\*,  $p < 0.001$ , \*\*\*,  $p < 0.01$ , \*\*\*\*,  $p < 0.05$  compared with values in *C. albicans* exposed mice. Results are representative of two individual experiments.

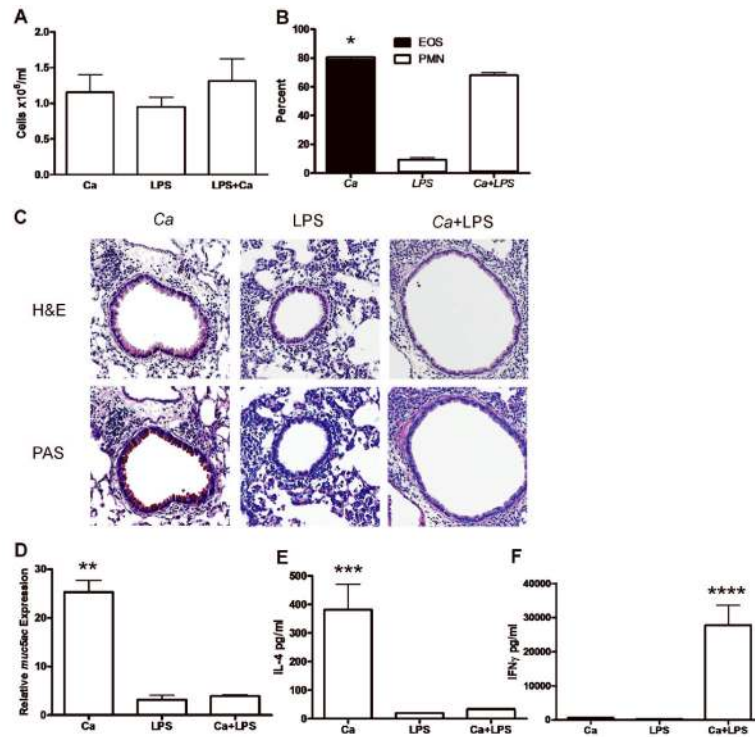


**FIG. 4.**

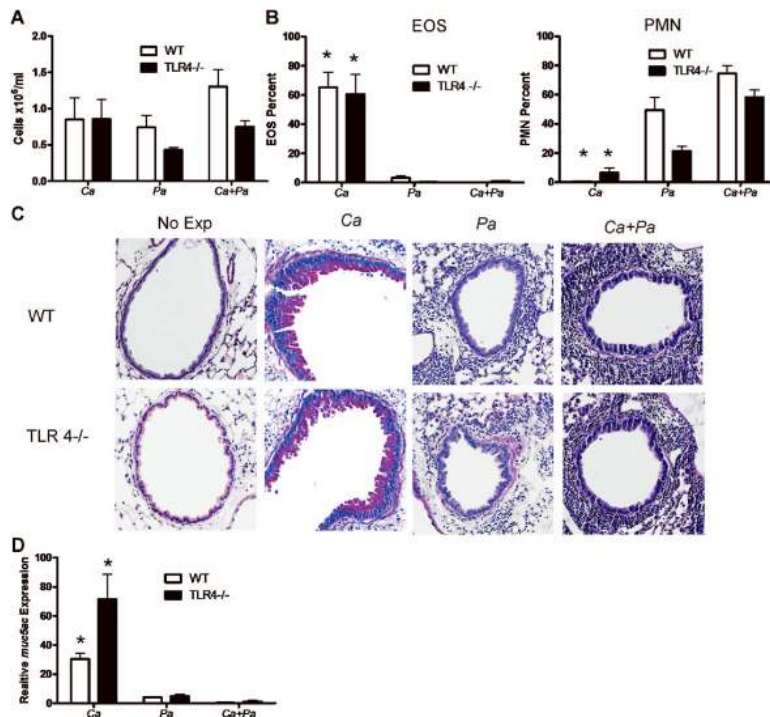
The adaptive immune response to *C. albicans* (Ca) is shifted from a Th2 toward Th1 by the presence of *P. aeruginosa* (Pa). **(A)** BALF cell numbers after exposure to either *C. albicans* or *P. aeruginosa* (5  $\mu$ g) or both using the protocol described in Figure 1. **(B)** BALF cell percentage by cell type as determined by manual differential cell count of neutrophils (PMN) and eosinophils (EOS). Values represent the mean  $\pm$  SEM of 4 mice per group and are representative of experiments performed twice. Statistical significance determined by one-way ANOVA; \*,  $p < 0.05$  compared with nonexposed mice, \*\*,  $p < 0.0001$  compared with EOS or PMN in non-exposed mice. **(C)** C57BL/6J mice were exposed to either *C. albicans*, *P. aeruginosa* or both as in Figure 1. Lungs were inflation fixed, sectioned and stained with H&E and PAS. Representative images are 100x magnification and PAS inlay are 200x magnification. **(D)** Real time RT-PCR using total lung tissue to detect *muc5ac* after oropharyngeal exposure to either *C. albicans* or *P. aeruginosa* or both. Expression of *muc5ac* is presented relative to expression in non-exposed C57BL/6J mice. Statistical significance determined by the students *t*-test, 4 mice per group and are representative of experiments performed twice; \*\*\*,  $p < 0.0001$  compared with either non-exposed, *P. aeruginosa* exposed or *P. aeruginosa* and *C. albicans* exposed mice. **(E)** Airway resistance in response to methacholine after oropharyngeal exposure to either phosphate buffered saline (PBS), *C. albicans* or *P. aeruginosa* or both. Data presented are the means + SEM of four mice per group. # $p < 0.05$  between *P. aeruginosa* or combined *P. aeruginosa* and *C. albicans* compared to PBS or *C. albicans* alone (two-way ANOVA).

**FIG. 5.**

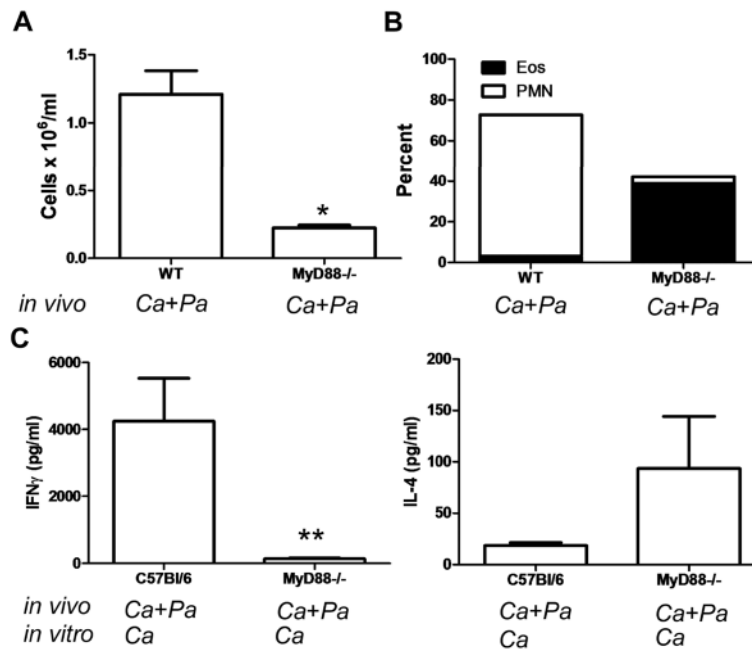
BAL and CD4 T cell cytokine production after exposure to *C. albicans* (Ca), *P. aeruginosa* (Pa) or both. (A) Production in BALF from mice exposed to *C. albicans* (Ca), *P. aeruginosa* (Pa) or both. Data presented are the means + SEM of four mice per group. \*\*,  $p < 0.05$  compared with values in *C. albicans* exposed mice. Production from (B) splenic CD4 T cells or (C) pulmonary T cells restimulated *in vitro* with *C. albicans* antigens after *in vivo* exposure to *C. albicans*, *P. aeruginosa* or both. *In vitro* data presented are the means + SEM of cytokine production by CD4 T cells isolated from a single mouse with a total of four individual mice per group. SEM represents the variation in cytokine production for the four individual mice. Statistical significance determined by the students *t*-test \*,  $p < 0.0001$ , \*\*\*,  $p < 0.01$  compared with values from *C. albicans* or *P. aeruginosa* exposed mice. (D) Production from splenic CD4 T cells restimulated *in vitro* with antigens from *C. albicans*, *P. aeruginosa* or both after *in vivo* exposure to *C. albicans* or *P. aeruginosa* or both. *In vitro* data presented are the means + SEM of cytokine production by CD4 T cells isolated from a single mouse with a total of four individual mice per group. SEM represents the variation in cytokine production for the four individual mice. Statistical significance determined by the students *t*-test; \*\*,  $p < 0.05$  compared with values in *C. albicans* exposed mice. For all panels in this figure, results are representative of two individual experiments.

**FIG. 6.**

The adaptive immune response to *C. albicans* (Ca) is Th1 and not Th2 in the presence of LPS. **(A)** BALF cell numbers after exposure to either *C. albicans*, LPS (5  $\mu$ g) or both following the protocol as described in Figure 1. There is no significant difference between groups. **(B)** Percentage cell type in BALF by cell type as determined by manual differential cell count of neutrophils (PMN) and eosinophils (EOS). Values represent the mean  $\pm$  SEM of 4 mice per group and are representative of experiments performed twice. \*,  $p < 0.0001$  EOS in *C. albicans* compared with EOS in LPS or *C. albicans* and LPS exposed mice. **(C)** Lungs were inflation fixed, sectioned and stained with H&E and PAS. Representative images are 100x magnification. **(D)** Real time RT-PCR using total lung tissue to detect *muc5ac* after oropharyngeal exposure to either *C. albicans*, LPS or both. Expression of *muc5ac* is presented relative to expression in non-exposed C57BL/6J mice. \*\*,  $p < 0.001$  *muc5ac* in *C. albicans* compared with LPS or Ca and LPS exposed mice. **(E)** Splenic CD4 T cell production of IL-4 by *ex vivo* restimulated CD4 T cells after *in vivo* exposure to *C. albicans*, LPS or both, as determined by ELISA. *In vitro* data presented are the means  $\pm$  SEM of one representative experiment of two with four mice per group. \*\*\*,  $p < 0.05$  IL-4 in *C. albicans* compared with values in LPS or *C. albicans* and LPS exposed mice. **(F)** Splenic CD4 T cell production of IFN $\gamma$  by *ex vivo* restimulated CD4 T cells after *in vivo* exposure to *C. albicans*, LPS or both, as determined by ELISA. *In vitro* data presented are the means  $\pm$  SEM of one representative experiment of two presenting combined data from four mice per group. \*\*\*\*,  $p < 0.05$  IFN $\gamma$  in *C. albicans* compared with values in *C. albicans* or LPS exposed mice.

**FIG. 7.**

*P. aeruginosa* (Pa) alters the adaptive immune response to *C. albicans* (Ca) independently of TLR4. (A) BALF cell numbers after exposure of C57BL/6J (WT) or TLR4<sup>-/-</sup> mice to either *C. albicans* or *P. aeruginosa* or both using the protocol described in Figure 1. (B) BALF cell percentage by cell type as determined by manual differential cell count of neutrophils (PMN) and eosinophils (EOS). Values represent the mean  $\pm$  SEM of 4 mice per group and are representative of experiments performed twice. Statistical significance was determined by two-way ANOVA; \*,  $p < 0.0001$  compared with values in *P. aeruginosa* or *C. albicans* and *P. aeruginosa* exposed WT or TLR4<sup>-/-</sup> mice. (C) Lungs were inflation fixed, sectioned and stained with PAS. Representative images are 100x magnification. (D) Real time RT-PCR using total lung tissue to detect *muc5ac* after oropharyngeal exposure to either *C. albicans*, *P. aeruginosa* or both. Expression of *muc5ac* is presented relative to expression in non-exposed C57BL/6J mice. Statistical significance was determined by two-way ANOVA to be  $p < 0.0001$ . \*,  $p < 0.0001$  compared with corresponding groups in *P. aeruginosa* or *C. albicans* and *P. aeruginosa* exposed WT or TLR4<sup>-/-</sup> mice.

**FIG 8.**

*P. aeruginosa* (Pa)-induced immunodeviation of the adaptive immune response to *C. albicans* (Ca) is MyD88 dependent. (A) BALF cell number after exposure of C57BL/J6 (WT) or MyD88<sup>-/-</sup> mice to *C. albicans* and *P. aeruginosa* using the protocol described in Figure 1. Statistical significance determined by the students *t*-test; \*,  $p < 0.001$ . (B) BALF cell percentage by cell type as determined by manual differential cell count of neutrophils (PMN) and eosinophils (EOS). Values represent the mean  $\pm$  SEM of 4 mice per group. Statistical significance was determined by one-way ANOVA to be  $p < 0.0001$ . (C) IL-4 and IFN $\gamma$  production as determined by ELISA using splenic CD4 T cells from C56Bl/6 or MyD88<sup>-/-</sup> mice restimulated *in vitro* with *C. albicans* antigens after *in vivo* exposure to both *C. albicans* and *P. aeruginosa*. *In vitro* data presented are the means + SEM of cytokine production by CD4 T cells isolated from a single mouse from a total of five individual mice per group. SEM represents the variation in cytokine production for the five individual mice. Statistical significance determined by the students *t*-test; \*\*,  $p < 0.01$  compared with values in C57Bl/6 mice exposed to *C. albicans* and *P. aeruginosa*.