Coevolution of $T_H^1$, $T_H^2$, and $T_H^{17}$ Responses during Repeated Pulmonary Exposure to *Aspergillus fumigatus* Conidia

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Aspergillus fumigatus, a ubiquitous airborne fungus, can cause invasive infection in immunocompromised individuals but also triggers allergic bronchopulmonary aspergillosis in a subset of otherwise healthy individuals repeatedly exposed to the organism. This study addresses a critical gap in our understanding of the immunoregulation in response to repeated exposure to A. fumigatus conidia. C57BL/6 mice were challenged intranasally with A. fumigatus conidia weekly, and leukocyte composition, activation, and cytokine production were examined after two, four, and eight challenges. Approximately 99% of A. fumigatus conidia were cleared within 24 h after inoculation, and repeated exposure to A. fumigatus conidia did not result in hyphal growth or accumulation of conidia with time. After 2 challenges, there was an early influx of neutrophils and regulatory T (T_{reg}) cells into the lungs but minimal inflammation. Repeated exposure promoted sustained expansion of the draining lymph nodes, while the influx of eosinophils and other myeloid cells into the lungs peaked after four exposures and then decreased despite continued A. fumigatus challenges. Goblet cell metaplasia and low-level fibrosis were evident during the response. Repeated exposure to A. fumigatus conidia induced T cell activation in the lungs and the codevelopment by four exposures of T_{H1}, T_{H2}, and T_{H17} responses in the lungs, which were maintained through eight exposures. Changes in CD4 T cell polarization or T_{reg} numbers did not account for the reduction in myeloid cell numbers later in the response, suggesting a non-T-cell regulatory pathway involved in dampening inflammation during repeated exposure to A. fumigatus conidia.

Allergic bronchopulmonary aspergillosis (ABPA) is characterized by early allergic and late-phase lung injury in response to repeated exposures to Aspergillus antigens, which are the consequence of persistent fungal colonization of the lungs (47, 60). The disease occurs primarily in patients who have skewed pulmonary immune responses, such as those found in atopic asthma or cystic fibrosis. The pulmonary immune response in these patients includes a strong T helper 2 (T_{H2}) response to the colonizing fungus. The underlying mechanism(s) by which Aspergillus induces T_{H2} responses in some patients, but not others, is presently unknown. If undiagnosed, ABPA can result in progressive lung damage, pulmonary fibrosis, and death (47, 60).

Aspergillus fumigatus conidia are frequently inhaled into airways at a rate of several thousand a day (41), and pulmonary exposure to large numbers of conidia is uncommon (24). Upon reaching the warm, moist environment of the lungs, the conidia lose their hyphophic properties and begin to germinate (34). Following conidial swelling and germ tube extension, the fungus develops invasive hyphae (54), and the cycle repeats itself. Although A. fumigatus can pose a serious threat to immunocompromised individuals, even relatively large doses of conidia pose little danger to immunocompetent hosts.

The immune response to inhaled A. fumigatus in healthy individuals is characterized by a complex interaction between innate and adaptive immune responses, both of which are activated upon exposure to the fungus (18, 31, 56, 58). Macrophages and neutrophils efficiently phagocytize inhaled conidia in the lungs, with neutrophils being absolutely essential, and conidial clearance can occur with minimal inflammation (7, 33, 44, 63, 66, 67, 70). Invasive infection of humans is associated with decreased gamma interferon (IFN-γ) production and poor T cell proliferation (27), and inhibition of IFN-γ or tumor necrosis factor alpha (TNF-α) enhances fungal invasiveness (2, 14, 43). A single inhalation of aerosolized A. fumigatus spores by mice can induce the expression of TNF-α, IFN-γ, interleukin 12 (IL-12), and IL-18 (10). In an adoptive transfer model, A. fumigatus-specific CD4+ T cells are rapidly primed in lung-associated lymph nodes by CCR2+ Ly6Cdim monocytes/dendritic cells, and these CD4+ T cells differentiate fully into IFN-γ-producing T_{H1} cells upon arrival in the airways (30, 62).

Most studies have focused on the host response following one exposure or a very limited number of exposures to A. fumigatus conidia. While repeated exposure to A. fumigatus conidia is common, little is known about the evolution and regulation of the host response to repeated exposure to A. fumigatus conidia. There is a significant body of literature on the pulmonary allergic response to a conidial challenge of mice previously sensitized by intraperitoneal injection of Aspergillus antigen extracts in an adjuvant (6, 40), which has provided much information about the mechanisms underlying T_{H2}-mediated pathological changes in the lungs. In addition, we have previously reported that two intranasal exposures to A. fumigatus conidia without a sensitizing event do not result in pulmo-

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nary allergic inflammation (51, 52). Our current study addresses a critical gap in our understanding of the regulation and evolution of adaptive immune responses to repeated exposures to *A. fumigatus* conidia. We set out to test the hypothesis that while two airway exposures to *A. fumigatus* conidia stimulate an innate response (neutrophils and macrophages) and begin priming for a Th1 response to the fungus, repeated exposures also stimulate the development of both Th1 and Th17 cells, which coincides with the development of a robust inflammatory response in the lungs.

**MATERIALS AND METHODS**

**Mice.** Wild-type (C57BL/6j) mice obtained from the Jackson Laboratories (Bar Harbor, ME) were housed under pathogen-free conditions in enclosed filter-top cages. Clean food and water were given *ad libitum*. The mice were handled and maintained using microisolator techniques, with daily veterinarian monitoring. All studies involving mice were approved by the University Committee on Use and Care of Animals at the University of Michigan.

*A. fumigatus* strain ATCC 13073 was grown on Sabouraud dextrose agar (SDA; Difco) for 14 days. Conidia were harvested by washing plates with sterile phosphate-buffered saline (PBS; pH 7.4) with 0.1% Tween 80 (PBS-Tween), followed by filtration of the suspension through two layers of sterile gauze to remove hyphae. Conidia were washed in PBS-Tween, counted with a hemocytometer, diluted to 10⁸ spores/ml in sterile PBS-Tween, and stored at 4°C for as long as 4 months. The conidial preps consisted of >99.9% resting conidia and did not contain appreciable numbers of swollen conidia. The viability of the conidial stocks, as assessed by dilution plating, remained reproducible and high.

**Intranasal challenge.** To achieve sedation, mice were injected intraperitoneally with 0.4 mg/ml xylazine (Lloyd Laboratories, Shenandoah, IA) and 10 mg/ml ketamine (Fort Dodge Animal Health, Fort Dodge, IA) in sterile saline (Hospira, Inc., Lake Forest, IL) based on weight. Following sedation, 20 µl of an *A. fumigatus* suspension was administered intranasally as a total of 2 × 10⁶ conidia per mouse per challenge.

**Lung histology.** Lungs were fixed by inflation with 10% neutral buffered formalin (Sigma). After paraffin embedding, 5-µm-thick sections were cut and stained with either hematoxylin and eosin (H&E) for histological analysis, periodic acid-Schiff stain (PAS) for the detection of mucus and goblet cell metaplasia, Masson’s trichrome stain for the detection of collagen deposition, or Grocott’s methamine silver (GMS) stain for the detection of conidia and hyphae (McClinchey Histology Lab, Stockbridge, MI).

**Lung digestion for whole-lung leukocyte enrichment.** Lungs from each mouse were excised, washed in PBS, minced, and digested enzymatically for 30 min in 15 ml/lung of digestion buffer (RPMI medium, 5% fetal calf serum, 1 mg/ml collagenase [Biohringer Mannheim Biochemical, Chicago, IL], and 30 µg/ml DNase [Sigma Chemical Co., St. Louis, MO]) as previously described (50). After enzymatic digestion, lungs were rinsed with 1 ml/lung of PBS, minced, and digested enzymatically for 30 min (2 × 10⁶ conidia/ml) with collagenase and hyaluronidase (McClinchey Histology Lab, Stockbridge, MI). At least four separate experiments (Fig. 2 to 8) or two separate experiments (Fig. 9) with 3 to 4 mice per group per experiment were performed. The quantitative data in each graph are from the cumulative analysis across the multiple experiments (i.e., the graphs do not show results for a single representative data set). All values are reported as means ± standard errors of the means. Differences between groups were evaluated by analysis of variance (ANOVA) with a posthoc test; a P value of <0.05 was considered statistically significant.

**RESULTS**

Cellular infiltrate and airway remodeling following repeated pulmonary exposure to *Aspergillus conidia*. To determine the host response to repeated exposure to *A. fumigatus* conidia, mice were challenged intranasally once a week for 2, 4, or 8 weeks with 2 × 10⁶ live *A. fumigatus* conidia and were analyzed 24 h after the final conidial challenge. This intranasal exposure dose is 5- to 100-fold lower than that typically used by labs in...
studies of invasive aspergillosis (43, 46, 63, 76). We prepared histological sections of the lungs and analyzed pulmonary inflammation (H&E), goblet cell metaplasia (PAS), and fibrosis (trichrome). Following two challenges, granulocytic infiltrates were beginning to become evident around the airways, with minimal changes in goblet cells or collagen deposition (Fig. 1A). After four challenges, the size of the inflammatory infiltrate had increased markedly around the airways and in the parenchyma, including large numbers of eosinophils and neutrophils and the formation of multinucleated giant cells (Fig. 1B). In addition, goblet cell metaplasia in the epithelium was now evident, along with low-level fibrotic changes (Fig. 1A). Following eight challenges, inflammatory infiltrates, goblet cell metaplasia, and fibrotic changes were still histologically evident, but all were either at the same level or a lower level than those observed at four challenges, though still greater than those observed at two challenges (Fig. 1A). Despite repeated challenges and diminished pulmonary inflammation, the survival rate over the course of 8 weeks was 100% (data not shown). Thus, repeated exposure of C57BL/6 mice to A. fumigatus conidia induced a marked pulmonary inflammatory response between 2 and 4 weeks of conidial challenge; however, the magnitude of the pulmonary inflammatory response did not continue to amplify despite continued A. fumigatus challenges.

Fungal clearance and expansion of cellular populations in the lungs and lymph nodes following two, four, and eight conidial challenges. We next addressed the question of whether the changes in the pulmonary inflammatory response with increasing exposures were accompanied by changes in conidial clearance. The numbers of viable conidia in the lungs were quantified at each time point, as described in Materials and Methods. Twenty-four hours after inoculation, the initial number of conidia ($2 \times 10^6$) administered to the lungs had been reduced to less than $2 \times 10^4$ viable conidia; levels of conidial clearance at 24 h postinoculation were identical for the mice in the two-, four-, and eight-challenge groups, regardless of differences in the inflammatory response between these groups.

**FIG. 1.** (A) Cellular infiltrate around the airways, goblet cell metaplasia, and collagen deposition following repeated intranasal exposure to Aspergillus fumigatus conidia. Lungs from nonchallenged mice (Untreated) and from mice challenged two, four, or eight times were fixed in formalin and embedded in paraffin blocks. Histological slices were then stained with either H&E, PAS, or Masson’s trichrome stain. Magnifications, $\times 400$ for H&E and PAS; $\times 200$ for trichrome. (B) Multinucleated giant cells were observed in the lungs of mice challenged four times with conidia. Magnifications are given below the images.
time points (Fig. 1 and 2A). By silver staining, we could occasionally identify very small numbers of conidia but never observed hyphal masses. Most commonly, we could not identify any significant amount of fungal material at sites of inflammation or other uninvolved regions of the lungs by silver staining (data not shown). We did occasionally observe rare germinating conidia in some mice (data not shown), which is worth noting because germinating conidia are metabolically active and reorganize the contents of their cell walls to expose immunostimulatory glucans (32, 70). Thus, approximately 99% of *A. fumigatus* conidia were cleared within 24 h of inoculation, and repeated exposure to *A. fumigatus* conidia did not result in hyphal growth or accumulation of conidia with time.

To provide a quantitative analysis of the kinetics of the pulmonary inflammatory response, leukocytes were isolated from enzymatically dispersed lungs of challenged mice 24 h after the final challenge at each time point. Two challenges stimulated the influx of a small number of leukocytes into the lungs and expansion of the draining mediastinal lymph nodes (Fig. 2B). Repeated challenges augmented pulmonary inflammation and lymph node lymphocyte numbers through 4 weeks. By 8 weeks, pulmonary inflammation had begun to wane with the additional challenges, although the numbers of lymphocytes in the draining lymph nodes remained elevated (Fig. 2B). Thus, repeated exposure promoted sustained expansion of the draining lymph nodes, while the pulmonary inflammatory response peaked and waned.

We next used flow cytometry to identify specific myeloid cell populations in the lungs and to further delineate the dynamics of the inflammatory response through eight weekly challenges with *A. fumigatus* conidia. Consistent with our histological analysis, we observed a significant difference in the number of granulocytes between untreated mice and those challenged twice (Fig. 3). However, mice challenged four times with conidia had 6-fold more eosinophils in the lungs than mice challenged twice (Fig. 3). By eight challenges, there were significantly fewer neutrophils and a trend toward fewer eosinophils in the lungs. The numbers of basophils in the lungs increased significantly between two and four challenges and then decreased slightly by eight challenges (Fig. 4). The monocyte/macrophage/DC population was numerically the largest cell population in the lungs. This heterogeneous population followed kinetics similar to those of the other myeloid cells, with a significant peak at four challenges and a significant decline by eight challenges. These data provide quantitative analyses that are consistent with the histological observations described above and confirm that, after peaking at four challenges, the influx of eosinophils and other myeloid cells did not continue to grow despite continued *A. fumigatus* challenges.

**Activation of CD4 T cells in the lungs and lymph node in response to repeated pulmonary challenges with conidia.** We
next examined the dynamics of the CD4 T cell response to repeated challenges with *A. fumigatus* conidia. First, we quantified CD4, CD8, and B lymphocyte levels in the lungs by flow cytometry as described in Materials and Methods. The number of CD4 T cells did not increase significantly following two challenges, but four challenges induced a 5-fold increase in the total number of CD4 T cells in the lungs, which remained elevated through eight challenges (Fig. 5). There was a similar influx of B cells into the lungs; however, CD8 T cell numbers remained relatively low throughout. In the draining lymph nodes, the CD4 T and B cell populations expanded as early as two challenges and continued to expand through four challenges but then leveled off and remained elevated through eight challenges (Fig. 5), concurrently with the increase in serum IgE levels at four and eight challenges over those for unchallenged controls (see Fig. 9A).

We hypothesized that differences in CD4 T cell activation might account for the development and waning of the pulmonary inflammatory response. Just two exposures to conidia resulted in an increase in the percentage of pulmonary CD4 T cells that were activated (CD44\textsuperscript{high} CD69\textsuperscript{+}), even though the number of CD4 T cells in the lungs did not increase significantly (Fig. 6). The proportion of activated CD4 T cells did not change significantly between two, four, and eight challenges (remaining high, approximately 70%). In the lymph node, the fraction of activated CD4 T cells was lower than that in the lungs and remained constant throughout the course of exposure, although the number of activated CD4 T cells increased coincidently with the increase in the total number of CD4 T cells in the lymph nodes (Fig. 6). The activation marker analysis supports the concept that an adaptive immune response begins to develop between two to four challenges and is sustained, despite the presence of less pulmonary inflammation, through eight exposures.

**Polarization of the CD4 T cell response during repeated challenges with conidia.** We next investigated whether changes in the polarization of the CD4 T\textsubscript{H1} cell response accounted for the development of the pulmonary inflammatory response by four challenges and its subsequent waning by eight challenges. Using intracellular flow cytometry, we observed that after two challenges, the only notable change was an increase in the number of IL-10\textsuperscript{+} CD4 T cells (Fig. 7A). By four challenges, there were marked increases in the numbers of IL-4\textsuperscript{+}, IL-17\textsuperscript{+}, and IFN-\gamma\textsuperscript{+} CD4 T cells. After eight challenges, the numbers of IL-4\textsuperscript{+}, IL-17\textsuperscript{+}, IFN-\gamma\textsuperscript{+}, and IL-10\textsuperscript{+} T cells all remained elevated in the lungs. Repeated *A. fumigatus* exposure also resulted in the recruitment of IFN-\gamma\textsuperscript{+} IL-17\textsuperscript{+} CD4 T cells, although this was a minor population (Fig. 7B). These data demonstrate that repeated exposure to *A. fumigatus* conidia induces the codevelopment of T\textsubscript{H11}, T\textsubscript{H12}, and T\textsubscript{H17} responses in the lungs by four exposures, that these responses are maintained through eight exposures, and that changes in CD4 T cell polarization do not account for the reduction in myeloid cell numbers later in the response.

We next investigated whether an increase in the ratio of regulatory to effector CD4 T cells could account for the waning
of the inflammatory response. In the draining lymph nodes, the percentage of regulatory T (T\textsubscript{reg}) cells within the pool of CD4 T cells was constant over the course of exposure to \textit{A. fumigatus} conidia. In the lungs, the percentage of T\textsubscript{reg} (CD25\textsuperscript{+} Foxp3\textsuperscript{+}) cells increased nearly 50% after two exposures, consistent with the observed increase in the number of IL-10\textsuperscript{+} CD4 T cells, suggesting a higher regulatory/nonregulatory ratio early in the response (Fig. 8). As the number of challenges increased, the total number of T\textsubscript{reg} cells also increased. However, the relative proportion of T\textsubscript{reg} cells dropped (i.e., an increase in the non-T\textsubscript{reg}/T\textsubscript{reg} cell ratio) concurrently with the development of the inflammatory response. Despite the presence of less inflammation, the ratio of non-T\textsubscript{reg} cells to T\textsubscript{reg} cells remained high at eight challenges, ruling out an expansion of the T\textsubscript{reg} population as a major factor in modulating the inflammatory response.

**Immune recall response to repeated pulmonary Aspergillus challenges.** In our final set of studies, we addressed whether the inflammatory response would progress or resolve after four challenges if there were no additional conidial challenges. Since the numbers of inflammatory cells in the bronchoalveolar lavage fluid were determined in these studies, they also provided quantitative data to address whether inflammation was similar in the airway and parenchymal pulmonary compartments. Both mice challenged four times and mice challenged eight times had significant leukocytic infiltrates in the airways (Fig. 9B). However, the infiltrate in the mice challenged four times was dominated by eosinophils, while that in the mice challenged eight times was dominated by macrophages (Fig. 9C). These results are similar to those seen for the whole-lung digests except that the diminished eosinophilia was more pronounced in the airways. If the mice were challenged four times and then left to rest for 4 weeks, the inflammatory response resolved to near baseline, except for the number of lymphocytes, which remained slightly elevated. We also elicited a recall response during the resolution phase by challenging a group of mice previously exposed four times after 2 weeks of resolution (Fig. 9B and C). The recall response was similar to the peak allergic response noted after 4 weeks in terms of
airway eosinophilia (Fig. 9C), suggesting that continued exposure throughout 8 weeks, in contrast to intermittent exposure, resulted in the attenuation of the allergic response in spite of the presence of primed cells capable of promoting a vigorous hypersensitivity response.

DISCUSSION

We have demonstrated that repeated exposure of an immunocompetent host to *A. fumigatus* conidia does not result in invasive aspergillosis or fatal disease but does result in the development of chronic pulmonary inflammation. Repeated exposure to *A. fumigatus* conidia induces the coevolution of TH1, TH2, and TH17 responses in the lungs by four exposures, and these responses are maintained through eight exposures. We observed striking increases in the numbers of IFN-γ- and IL-17-producing CD4 T cells between two and four challenges. IFN-γ IL-17 double-positive T cells are associated with inflammation in several models of autoimmunity and graft rejection (11, 15, 45, 48), and their presence suggests a role for IL-17 in maintaining the inflammatory response to *Aspergillus*. However, by eight exposures, myeloid cell recruitment is diminished, including that of airway eosinophils, and changes in CD4 T cell polarization or Treg numbers do not account for the waning inflammatory response with continued exposure. The total time of exposure is not a factor, because if, during the same 8-week period, exposures are stopped for 2 weeks, then inflammation resolves and a robust recall response occurs.

Repeated exposure to viable *A. fumigatus* conidia induced both CD4+ T_{H1} and T_{H2} cells. Several previous reports have demonstrated that a single exposure to *A. fumigatus* conidia leads primarily to the priming of CD4+ T_{H1} cells (9, 31) and that exposure to *A. fumigatus* hyphae or antigen extracts leads primarily to the priming of CD4+ T_{H2} cells (9, 36). Systemic priming and airway exposure to *A. fumigatus* antigens establish a T_{H2}-mediated allergic airway disease (AAD) that can sub-
in our study were administered (9, 31). The differential effect of dose and frequency of exposure to *A. fumigatus* conidia has clinical relevance, because *Th1*-associated hypersensitivity pneumonitis results from very high dose exposure, generally in an occupational setting (1, 20, 57, 65).

It is interesting that in spite of the presence of *Th2* and *Th1* cells, the CD4⁺ *T* cell-dependent hypersensitivity disease that developed after repeated exposure to conidia had many features of AAD, a *Th2* disease. *Th1* cells can abrogate *Th2* cell-mediated AAD (35); however, *Th1* responses to viral infections are known to exacerbate asthma symptoms (4, 42).

Furthermore, it has been demonstrated in mice that a *Th1*-promoting rhinovirus infection can augment the *Th2* response in AAD and that a preexisting *Th2* response augmented the *Th1* response to rhinovirus infection (3). The data generated in the present study support the emerging understanding that allergic diseases are not simply the result of an imbalance in counterregulatory *Th1* and *Th2* responses (73). Rather, current evidence suggests that both *Th2* and *Th1* responses are inflammatory and that both are subject to control by myriad regulatory mechanisms, such as those mediated by dedicated regulatory T cells.

Viable conidia could be detected in lung homogenates 24 h after each challenge, providing a sustained source of antigen or inflammatory stimuli during the evolving CD4⁺ T cell response despite a lack of hyphal formation. Neutrophils, a major cell type during the early phases of the response, are well known to play a central role in the host defense against *Aspergillus* (5, 29, 43, 44, 64) and were seen at all stages of the response, particularly at early stages prior to the engagement of the adaptive response. Levels of lung eosinophils, serum IgE, and IL-4⁺ CD4⁺ T cells, all hallmarks of a *Th2* response, were elevated at four and eight exposures. The chronic inflammatory response included the recruitment of antigen-presenting cells, such as basophils, dendritic cells, macrophages, CD4⁺ T cells, and B cells, all of which are capable of driving *Th2*, *Th1*, *Th17*, or regulatory T cell responses.

Expansion of the regulatory T cell population is important for limiting disease, because Treg cells ultimately aid in the clearance of fungi by limiting *Th1* inflammation (17) or dampening *Th2* hypersensitivity reactions (8, 16). Exposure to *A. fumigatus* conidia or fungal glucan has been reported to induce regulatory responses via Toll-like receptor 2 (TLR2) and defenacin-1 (19, 46). However, in aspergillosis studies using high doses of conidia (10⁶ spores per mouse), a strong *Th1* response occurs 1 week after the initial exposure (32), rather than the Treg response that we have reported here during repeated exposure to *A. fumigatus* conidia, and there is no subsequent *Th2* reaction.

There are several possible explanations, then, for the transition from tolerance to inflammation that we observed during repeated exposure to *A. fumigatus* conidia. One possibility is that the accumulation of innate cells eventually overwhelms the regulatory response. Chitin—which is generated following conidial germination—can drive an accumulation of innate cells, stimulating inflammation that ultimately leads to an allergic response (61). We describe here that viable conidia are still present 24 h after challenge and that germinating fungi can be detected in the lungs even when low concentrations of conidia are used. Thus, it is possible that chitin production

![FIG. 9. Characterization of the inflammatory response to prolonged continuous or intermittent *A. fumigatus* inhalational exposure.](http://iai.asm.org/Downloaded from http://iai.asm.org.org by University of Michigan Library)
results in an innate response that outpaces the tolerance response and eventually results in the engagement of Th2 adaptive immunity. Another possibility is that suppression of the Th1 response by Treg cells facilitates the development of the Th1 response. The Th1 response can be responsible for tissue damage (69), so it is possible that the immune system dampens the Th1 response to a dose of conidia that can easily be cleared by the innate immune system. Such Th1 control could, in turn, allow the expansion of a Th2 response, since Th1 and Th2 responses are often reciprocally regulated. The latter hypothesis is supported by our studies demonstrating increased numbers of IL-17-producing CD4 T cells during the response, because the Th17 response can be negatively regulated by IFN-γ (21).

Like the development of the Th2 response, the Th17 adaptive immune response during repeated exposure to A. fumigatus conidia may result from a combination of factors. The initial Th17 adaptive immune response may be triggered simply by the presence of conidia: like regulatory T cells, Th17 cells are promoted by fungal cell wall components via interaction with dectin-1 (23, 71). In addition, Treg cells can facilitate the differentiation of Th17 cells (37, 74), and regulatory T cells themselves can be converted to Th17 cells (59), a process that is facilitated by DC (55). Thus, the initial Th17 response seen following two challenges may be driven by the conversion of regulatory T cells to Th17 cells. This process would be aided by the dampened Th1 response, since the development of Th17 cells is inhibited by Th1 and Th2 cytokines. On the other hand, CD4 T cells already committed to the Th17 lineage are resistant to Th1- or Th2-mediated suppression (26), which could explain why we observed that IL-17-producing CD4 T cells continue to expand even during the Th2-driven inflammatory response that follows four challenges. The Th17 T cells in our study are also likely playing an active role in shaping the reaction to conidia, since Th17 regulates Th1 differentiation (49, 75) and dampens the production of indoleamine-2,3-dioxygenase (IDO). While IDO can inhibit Th1 activity, it also suppresses the cytotoxic potential of neutrophils. This would be consistent with the persistent number of viable conidia seen in the lungs even after eight challenges, because Th17-induced IDO reduction and subsequent neutrophil inhibition have been shown to inhibit fungal clearance (17).

We observed that Th17 cell levels increased during repeated exposures to A. fumigatus conidia, while Th1 cell levels remained steady or decreased. Previous studies have indicated that Th12 airway inflammation is enhanced by Th17 cells (72). However, in the context of repeated A. fumigatus exposures, it may be that the Th17 response, rather than suppressing the Th12 response, becomes the dominant adaptive response through attrition. Repeated exposure to an antigen leads to restimulation-induced death of CD4 T cells, but it has been reported in autoimmune disease models that Th17 cells are resistant to this form of apoptosis (30). To our knowledge, there have been no reports demonstrating that the Th17 response directly regulates Th12 responses, so the presence of Th17 cells does not explain the dampening of the inflammatory response between four and eight challenges. However, multiple reports have shown that IL-17 both exacerbates and attenuates inflammatory Th12 responses and that the function of IL-17 is timing dependent (28, 68).

Thus, the emergent Th17 response may arise as an imperfect immune compromise when an individual is dealing with repeated exposure to low levels of conidia. The Th17 response hinders Th1-mediated clearance of fungi and can promote severe neutrophil-mediated tissue inflammatory pathology associated with infection (17), but on its own the Th17 response has antifungal properties (22, 25, 38, 39). A persistent Th1 response would result in damage to the tissue of the lung, but its suppression facilitates an emergent Th17 response that does little to aid in the clearance of nonhyphal A. fumigatus. Therefore, it is likely that repeated pulmonary exposure to A. fumigatus conidia eventually leads to immune homeostasis and the induction of non-T cell regulatory pathways that result in the least possible tissue damage while still controlling conidial germination (12, 13).

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