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Aberrant Tissue Localization of Fungus-Specific CD4⁺ T Cells in IL-10-Deficient Mice

Amariliz Rivera, Nichole Collins, Matthias T. Stephan, Lauren Lipuma, Ingrid Leiner, and Eric G. Pamer

Aspergillus fumigatus, a common environmental fungus, can cause lethal invasive infections in immunocompromised hosts. In immunocompetent individuals, however, inhaled A. fumigatus spores prime CD4⁺ T cells and activate immune responses that prevent invasive infection. Calibration of inflammatory responses to levels that prevent fungal invasion without inducing collateral tissue damage is essential for host survival, but the underlying regulatory mechanisms remain undefined. Although IL-10 is a validated regulatory cytokine that suppresses immune responses, and IL-10 deficiency or blockade generally enhances immune responses, we find that A. fumigatus-specific T cell frequencies are markedly reduced in airways of IL-10-deficient mice. T cell priming, proliferation, and survival were unaffected by IL-10 deficiency and did not account for decreased frequencies of A. fumigatus-specific T cells in the airways of IL-10-deficient mice. Instead, IL-10 deficiency results in redistribution of A. fumigatus-specific T cells from infected lungs to the gut, a process that is reversed by antibiotic-mediated depletion of intestinal microbes. Our studies demonstrate that disregulated immune responses in the gut can result in dramatic redistribution of pathogen-specific T cells within the host. The Journal of Immunology, 2009, 183: 631–641.

The respiratory system is exposed with great regularity to airborne particles, including pollens and microbial spores. Although most of these particles are innocuous and incapable of causing disease in the mammalian host, some, such as the spores of Aspergillus fumigatus, can cause lethal invasive infections when the immune system is compromised (1). Invasive aspergillosis (IA)² is a serious complication of immune suppression, with high mortality rates, even when optimal antifungal chemotherapy is provided (2, 3). IFN-γ-producing CD4⁺ T cells provide defense against IA and adoptive transfer of A. fumigatus-specific T cells can provide protection against invasive infection following bone marrow transplantation (4, 5). Aberrant Th2 immune responses to A. fumigatus, in contrast, can lead to the development of aspergillus-induced asthma, allergic bronchopulmonary aspergillosis and allergic sinusitis (6–8).

Humans and mice are, at baseline, highly resistant to A. fumigatus infection. Indeed, most mammals inhale hundreds if not thousands of A. fumigatus spores every day without any signs or symptoms of disease. Nevertheless, inhaled spores arrive in the airways wrapped in layers of β-glucan and other components that stimulate innate immune receptors (9–11) and cytokine responses (12), and enhance T cell priming against fungal protein Ags (13–16). In experimental models, depending on the route of exposure, the T cell response to A. fumigatus challenge of mice can be quite varied. For example, i.p. immunization with nonviable A. fumigatus extracts, followed by intratracheal challenge with viable spores, induces pulmonary pathology that is similar to human asthma and that depends on production of Th2 cytokines (17). In contrast, direct intratracheal challenge of naïve mice with live A. fumigatus spores induces rapid and robust Th1 CD4⁺ T cell responses (14). T cell priming occurs in draining mediastinal lymph nodes and is followed by explosive expansion of A. fumigatus-specific T cells (18). Upon trafficking to the lung, A. fumigatus-specific T cells differentiate into IFN-γ producing Th1 cells (18). Because even temporary compromise of the respiratory system is life threatening, modulating pulmonary inflammatory responses to a level that inactivates invading pathogens without impairing respiration is essential. Given the daily exposure to highly immunogenic A. fumigatus spores, it seems likely that the pulmonary immune system has evolved mechanisms to calibrate T cell responses to a level that does not compromise respiration. These mechanisms, however, remain undefined.

IL-10 is a cytokine that regulates inflammatory responses to microbial infection (19). IL-10 deficiency enhances in vivo clearance of Listeria monocytogenes from infected tissues and renders mice more resistant to this bacterial infection (20). Infection of IL-10-deficient mice with Toxoplasma gondii, in contrast, is lethal, despite enhanced clearance of this pathogen (21). In this setting, an overly robust immune response kills the host. CD4⁺ T cells responding to systemic T. gondii infection appear to be the major source of IL-10 in the spleens of infected mice (22, 23). IL-10 is also implicated as a regulatory cytokine during immune responses to inhaled Ags (24). Production of IL-10 by pulmonary dendritic cells induces CD4⁺ T cell tolerance following challenge with an inhaled protein (25), and IL-10-producing T...
cells induced by inhaled heat-killed *Listeria monocytogenes* and protein Ag have been shown, in vitro, to inhibit proliferation of CD4+ T cells (26). An inhibitory role for IL-10 production by T regulatory cells during pulmonary inflammation was demonstrated in mice with specific deletion of IL-10 in FoxP3-positive T regulatory cells (27). In these mice, loss of IL-10 expression resulted in increased allergic manifestations in response to challenge with a protein Ag, but the effects of IL-10 deficiency on Ag-specific T cell responses were not measured. It remains unclear, therefore, whether IL-10 directly or indirectly affects Ag-specific CD4+ T cell responses in the lungs following challenge with fungal spores.

We have investigated the effect of IL-10 on CD4+ T cell responses to infection with *A. fumigatus*. Adoptive transfer of naive, *A. fumigatus*-specific CD4+ T cells into wild-type (WT) or IL-10-deficient mice followed by intracheal challenge with live spores revealed a marked decrease in frequency of fungus-specific effector T cells in the airways of IL-10-deficient mice. This decrease could not be attributed to increased innate immune responses, enhanced fungal clearance, impaired trafficking to the lung, or increased apoptosis of fungus-specific T cells. Instead, in IL-10-deficient mice, fungus-specific T cells were diverted from the airways to the gut. Broad-spectrum antibiotic administration, by depleting the gut microbial flora, restored accumulation of *A. fumigatus*-specific CD4+ T cells in the airways of IL-10-deficient mice. Our studies suggest that pooling of effector CD4+ T cells in the gut of IL-10-deficient mice is capable of depleting effector T cells from sites of active infection.

**Materials and Methods**

**Mice and infection**

IL-10R2−/−, IL-10−/−, and C57BL/6J control mice were originally purchased from The Jackson Laboratory and subsequently bred and maintained under specific pathogen-free conditions at Memorial Sloan Kettering Research Animal Resource Center. Sex and age matched controls were used in all studies following institutional guidelines for animal care. *A. fumigatus*-specific CD4+ TCR-transgenic (tg) mice (Af3.16) have been previously described (18). Af3.16 mice were bred to RAG−/−. Thy1.1 to generate Af3.16.RAG−/− mice and to IL-10R2−/− mice to generate Af3.16.IL-10R2−/−/RAG−/− TCR-tg cells unable to respond to IL-10. *A. fumigatus* strain 293 is a clinical isolate and was originally provided by Michael Anderson. The fungus was cultured on Sabouraud Dextrose agar for 7–10 days prior to use in all experiments. Mice were infected intratracheally with 107 conidia/mouse as previously described (18). All mice survived the infection in excess of 2 mo.

**T cell proliferation, intracellular cytokine staining, and flow cytometry**

T cell proliferation assays were performed as previously described (18). In brief, CD4+ T cells were purified from the mediastinal lymph node (MLN) of infected mice and cultured with irradiated, T-depleted splenocytes, hyphal fragments, and the fungal growth inhibitor voriconazole (28). In some experiments, CD4+ T cells were stimulated with 1 μg/ml anti-CD3 and 0.5 μg/ml anti-CD28 Abs. Eighty-four hours after culture initiation, T cell proliferative responses were measured by thymidine incorporation and liquid scintillation. Staining for intracellular IFN-γ was performed on BALB/c cells that were stimulated with APCs and hyphal Ags as previously described (14, 18). Samples were stained with fluorescent Abs obtained from BD Bioscience or eBioscience and analyzed by flow cytometry on a BD LSR II. Further flow cytometric analysis was performed with FlowJo software (Tree Star).

**Th1 differentiation and retroviral transduction**

To generate Th1 cells, Af3.16.RAG−/−/RAG−/− TCR-tg CD4+ T cells were purified by magnetic cell sorting (Miltenyi Biotec) according to manufacturer’s instructions and cultured with T-depleted, irradiated splenocytes and hyphal fragments in the presence of mouse rIL-12 (10 ng/ml) and anti-IL-4 (5 μg/ml). Total spores were added on day 0 and 3 days later (day 3). Initial stimulation, cell cultures were split and supplemented with 50 U/ml mouse rIL-2 (R&D Systems). Murine leukemia virus-derived retroviral vectors for the expression of a click beetle luciferase-gfp or mouse Bcl-2 have been previously described (29, 30). Retroviral transduction was performed on Th1-differentiated Af3.16.RAG−/−/RAG−/− TCR-tg cells by spinoculation on retronectin-coated plates as previously described (31). Transduced T cells were expanded for an additional 3 days with 10% FCS RPMI 1640 medium supplemented with 50 U/ml rmIL-2.

**Adoptive T cell transfers**

Naive, CD4+ Af3.16 TCR-tg cells were isolated from lymph node and spleen of Af3.16 TCR-tg mice. A total of 2 × 107 Af3.16 TCR-tg cells were injected i.v. into naive recipients 1 day before infection. For trafficking studies, Th1 cultures were maintained on IL-2 for 6 days before i.v. delivery of 5 × 107 Th1 T cells into infected recipients. For imaging studies, Af3.16 TCR-tg cells were cultured for a total of 7 days and a total of 1–3 × 106 transduced Af3.16 TCR-tg CD4+ were transferred i.v. into previously infected recipients. The adoptive transfer of transduced T cells was timed to coincide with the initial entry of endogenously primed CD4+ T cells to the lung.

**In vivo bioluminescence imaging**

Animal imaging studies were performed on a Xenogen IVIS 200 system at the Memorial Sloan-Kettering Cancer Center Small Animal Imaging core facility. To visualize luciferase-expressing Af3.16 TCR-tg cells in vivo, recipient mice were injected with the CBR-luciferase substrate D-luciferin (* Xenogen*). Living Image software version 2.6 (Caliper LifeSciences) was used to acquire and analyze bioluminescent signals.

**Antibiotic treatment and statistical analysis**

Mice were treated with metrodinazole (1 g/l), neomycin sulfate (500 mg/l), and vancomycin (1 g/l) in drinking water for 3 wk before T cell transfers and infection. Statistical analysis was performed with Prism (GraphPad Software) software. A p value of <0.05 was considered significant. Error bars denote SEM.

**Results**

**Diminished *A. fumigatus*-specific CD4+ T cell responses in the absence of IL-10**

To determine whether IL-10 plays a regulatory role during pulmonary antifungal CD4+ T cell responses, WT and IL-10-deficient mice were infected with live *A. fumigatus* spores and CD4+ T cell responses were analyzed in MLN and airways 7 days following challenge, as previously described (14). *A. fumigatus*-specific CD4+ T cell priming, as measured by T cell proliferation upon in vitro restimulation with *A. fumigatus* hyphal Ags, was slightly diminished when MLN CD4+ T cells were isolated from IL-10−/− as opposed to WT mice (Fig. 1A). To examine whether diminished in vitro proliferation was unique to Ag stimulation, CD4+ T cells from naive IL-10−/− or control mice were stimulated in vitro with anti-CD3/CD28 Abs. The extent of thymidine incorporation by IL-10−/−/CD4+ T cells after a nonspecific, TCR stimulation was also slightly diminished as compared with control T cells (supplementary Fig. 1A) suggesting that diminished proliferation is not unique to our Ag. To determine why T cell proliferation of IL-10−/− CD4+ lymphocytes is diminished, we measured IL-2 levels in culture supernatants. The amount of IL-2 produced by IL-10−/− CD4+ T cells, as compared with control CD4+ T cells, was diminished 24 h after stimulation (supplementary Fig. 1B), providing a plausible mechanism for the reduced proliferative responses of *A. fumigatus*-specific CD4+ T cells in the absence of IL-10 production. In contrast to the small effect on proliferation, IL-10-deficiency markedly decreased the frequency of *A. fumigatus*-specific CD4+ T cells in the airways (Fig. 1B). Thus, contrary to expectation, IL-10-deficiency did not result in enhanced CD4+ T cell responses to respiratory fungal challenge.

The online version of this article contains supplemental material.
To characterize the contribution of IL-10 to T cell activation, proliferation, differentiation, and trafficking, we adoptively transferred naive Af3.16 TCR-tg T cells into WT mice or mice with a deletion of IL-10R2, directly or indirectly, we adoptively transferred naive Af3.16 TCR-tg T cell responses in IL-10−/− mice. CD4+ T cell responses in IL-10−/− and control mice were assessed 6 days after an intratracheal infection with 10⁷ live, A. fumigatus spores. A, CD4+ T cells were purified from the MLN of IL-10−/− control mice and cultured with APCs in the absence (■) or presence (○) of A. fumigatus hyphal Ags. Values shown are mean ± SD of six individual wells per group and are representative of three individual experiments. B, BALF cells were cultured with APCs with or without A. fumigatus Ags before intracellular cytokine staining and FACS analysis. Data shown are for CD4+ gated populations and are representative of results obtained in three individual experiments. Numbers represent the percentage of IFN-γ producing cells.

Decreased accumulation of A. fumigatus-specific CD4+ T cells in the airways of IL-10-deficient mice

To characterize the contribution of IL-10 to A. fumigatus-specific T cell activation, proliferation, differentiation, and trafficking, we adoptively transferred 2 × 10⁶ naive Af3.16 TCR-tg (A. fumigatus-specific) CD4+ T cells into IL-10−/− and WT control mice and then challenged recipient mice intratracheally with live fungal spores. Before infection, the total number of Af3.16 TCR-tg cells present in the MLN was undetectable by flow cytometry in both IL-10−/− and control mice. Af3.16 TCR-tg T cells were recruited to the MLN and expanded in response to infection in both IL-10−/− and IL-10−/− recipients (Fig. 2A). Consistent with our observation for endogenous T cell responses, Af3.16 TCR-tg T cell responses in the MLN were modestly diminished in the absence of IL-10 (Fig. 2B), with a 1.5-fold reduction in the total number of Af3.16 TCR-tg in IL-10−/− as compared with WT recipient mice (Fig. 2B). Diminished frequencies of A. fumigatus-specific CD4+ T cells in the MLN may be due to reduced production of IL-2 by activated T cells in IL-10−/− mice (supplementary Fig. 1B).

 Recruitment of Af3.16 TCR-tg T cells to the airways was markedly reduced in IL-10−/− recipient mice (Fig. 2C). On average, we detected a 9-fold reduction in the total number of Af3.16 TCR-tg T cells recovered from the airways of IL-10−/− mice as compared with control animals (Fig. 2D). Significantly decreased frequencies of A. fumigatus-specific CD4+ T cells were also detected in the lung parenchyma of IL-10−/− mice (Fig. 2E), indicating that diminished frequencies of fungus-specific CD4+ T cells in the airways reflect an overall deficit in the recruitment and/or retention of A. fumigatus-specific CD4+ T cells to the lung in the absence of IL-10 production. Differentiation of Af3.16 TCR-tg T cells into IFN-γ-producing CD4+ T cells, however, was similar in WT and IL-10-deficient mice (Fig. 2F), indicating that Th1 differentiation is IL-10-independent. These results demonstrate that IL-10 deficiency modestly diminishes expansion of A. fumigatus-specific T cells in draining lymph nodes and more dramatically decreases accumulation of specific T cells in the airways.

CD4+ T cells responding to fungal infection are not directly affected by IL-10

Many different cells, including T cells, respond to IL-10. Some studies indicate that IL-10 can enhance survival of T cells responding to Ag stimulation (32–35) while others demonstrate direct IL-10-mediated inhibition of CD8+ T cell expansion (36). To determine whether IL-10 enhances T cell accumulation in airways directly or indirectly, we adoptively transferred naive Af3.16 TCR-tg T cells into WT mice or mice with a deletion of IL-10R2, an essential component of the IL-10 receptor complex. Upon infection, Af3.16 TCR-tg CD4+ T cells expanded in the MLN of both IL-10R2−/− and WT controls (Fig. 3, A and B). Consistent with our studies using IL-10−/− recipient mice, Af3.16 TCR-tg T cell accumulation in the airways of IL-10R2−/− was markedly reduced in comparison to WT recipients (Fig. 3, C and D), suggesting that IL-10 does not act directly on responding CD4+ T cells. To definitively evaluate direct effects of IL-10 on A. fumigatus-specific T cells, we crossed Af3.16 TCR-tg mice with IL-10R2−/− mice to generate IL-10 unresponsive, A. fumigatus-specific CD4+ T cells (Af3.16-IL-10R2−/−). Naïve, Thy1 disparate, WT Af3.16 were mixed at a 1:1 ratio with Af3.16-IL-10R2−/− TCR-tg cells and cotransferred into congenic recipients. Upon pulmonary challenge with live A. fumigatus spores, Af3.16-IL-10R2−/− TCR-tg CD4+ T cells expanded and accumulated in the MLN to the same extent as their WT counterparts (Fig. 4, A and B). Moreover, Af3.16-IL-10R2−/− TCR-tg CD4+ T cells were normally recruited to the airways of recipient mice (Fig. 4, A and C). Th1 differentiation and IFN-γ production were similarly unaffected by impaired IL-10 signaling in fungus-specific CD4+ T cells (Fig. 4D). These results indicate that the effects of IL-10 do not result from direct stimulation of A. fumigatus-specific T cells but instead are due to IL-10-mediated effects on cells of the recipient mouse.

Fungal clearance, CD4+ T cell trafficking, and apoptosis are unaffected by IL-10 deficiency

IL-10 is a pluripotent cytokine, with many parallel effects that could potentially enhance antifungal CD4+ T cell responses. We reasoned that decreased accumulation of fungus-specific CD4+ T cells in the airways of IL-10-deficient mice might result from: 1) enhanced innate responses and accelerated fungal clearance from the lung, 2) aberrant T cell priming in the lymph node, 3) impaired recruitment of CD4+ T cells to the airways, or 4) increased CD4+ T cell trafficking, and apoptosis are unaffected by IL-10 deficiency.
T cell apoptosis in the airways. We performed a series of experiments to test each of these hypotheses. With respect to innate immune responses, the absence of IL-10 did not alter fungal burden or innate cell recruitment to the lung (results not shown). Thus, diminished accumulation of *A. fumigatus*-specific CD4$^+$ T cells in the airways of IL-10$^{-/-}$ mice could not be explained by more effective innate immune responses or limited Ag availability.

The extent of Af3.16 TCR-tg CD4$^+$ T cell proliferation as assessed by CFSE dilution was similar in WT and IL-10$^{-/-}$ mice, suggesting that T cell priming was unaltered in the absence of IL-10 (data not shown). With respect to in vivo T cell trafficking, i.v. transfer of primed Af 3.16 TCR-tg cells into infected WT and IL-10$^{-/-}$ recipient mice demonstrated similar entry of fungus-specific CD4$^+$ T cells to airways 16 h after T cell transfer (supplementary Fig. 2). These findings demonstrate that T cell trafficking from the bloodstream into the airways is not diminished by the absence of IL-10, suggesting that limited accumulation of fungus-specific CD4$^+$ T cells does not result from impaired entry to the infected lung of IL-10$^{-/-}$ mice.

To determine whether greater numbers of *A. fumigatus*-specific CD4$^+$ T cells undergo apoptosis in the absence of IL-10, we stained adoptively transferred Af3.16 TCR-tg T cells for the expression of Annexin V. We detected
slightly increased frequencies of apoptotic Af3.16 TCR-tg cells in IL-10−/− recipients, but these differences were not statistically significant (supplementary Fig. 3, A and B). To more directly address whether increased T cell death was leading to diminished fungus-specific CD4+ T cell accumulation, Af3.16 CD4+ TCR-tg cells were transduced to constitutively express Bcl-2 and cotransferred with control vector-transduced T cells into infected recipients. The ratio of Bcl-2:control, airway-infiltrating Af3.16 CD4+ T cells recovered from the MLN. FACS plots are gated on CD4+ T cells and are for individual mice representative of each group. B, Total number of Af3.16 TCR-tg CD4+ T cells in the MLN of IL-10R2−/− or control mice. Each symbol represents one mouse. C, Frequency of Af3.16 TCR-tg CD4+ T cells among CD4+ T cells recovered from the airways of IL-10R2−/− or control mice. FACS plots are gated on CD4+ cells and are for individual mice representative of each group. D, Total number of Af3.16 TCR-tg CD4+ T cells recovered from the airways (BALF) of IL-10R2−/− or control mice. Each symbol represents one mouse.

A. fumigatus-specific CD4+ T cells are redistributed to the gut of IL-10-deficient hosts

Because enhanced fungal clearance, diminished T cell priming, or enhanced T cell apoptosis could not adequately explain the diminished frequency of CD4+ T cells in IL-10−/− mice, we reasoned that T cells might localize to nonpulmonary sites in IL-10−/− mice. To address this possibility, we transduced Af3.16 TCR-tg cells with a retrovirus encoding the click beetle luciferase gene to enable in vivo imaging to assess the localization and survival of adoptively transferred T cells (29). Luciferase-expressing Af3.16 TCR-tg cells (Af3.16-luc) were i.v. transferred into infected IL-10−/− or WT recipients. Five minutes after T cell transfer, bioluminescence imaging revealed that nearly all Af3.16-luc T cells localized to the thoracic region of both IL-10−/− and control recipients, consistent with trapping in the pulmonary vasculature (Fig. 5, A and B). One hour after transfer, Af3.16-luc T cells were found to circulate throughout the body, with similar distribution patterns in IL-10−/− and WT recipients (Fig. 5, A and B). Three days after T cell transfer, however, the distribution of Af3.16-luc T cells was distinct in IL-10−/− and WT recipients (Fig. 5, C and D). Although bioluminescence was principally detected in the thoracic region of WT recipients, bioluminescence decreased in the thoracic and abdominal regions revealed lower thoracic signal with increased abdominal bioluminescence in IL-10−/− recipients (Fig. 5E). Quantification of bioluminescent signals detected over the entire animal demonstrated that total bioluminescence was not significantly different between IL-10−/− and control recipients (Fig. 5F). Quantification of bioluminescence in thoracic and abdominal regions revealed lower thoracic signal with increased abdominal bioluminescence in IL-10−/− hosts as compared with control recipients (Fig. 5E).
Af3.16 TCR-tg cells were present at reduced frequencies in IL-10−/− recipients as compared with control hosts (Fig. 5F). These observations suggest that the frequency of A. fumigatus-specific CD4+ T cells diminishes in the airways of infected IL-10−/− mice because of T cell redistribution to the gut.

Redistribution of A. fumigatus-specific CD4+ T cells in IL-10−/− mice is corrected by broad-spectrum antibiotic administration

IL-10-deficient animals have served as a useful model of inflammatory bowel disease (IBD) (19, 40, 41). Colitis in IL-10−/− mice is dependent on the gut flora (40), and diminishing the intestinal microbial flora with broad-spectrum antibiotics ameliorates intestinal inflammation (42–44). Although the animals used in our study did not have obvious signs of active colitis (such as rectal prolapse), we have observed the development of colitis in older IL-10−/− mice in our colony suggesting that the microbial flora in our animals is capable of inducing colitis in the absence of IL-10. Because signs of subclinical colitis have been documented to appear as early as 4 wk of age and A. fumigatus-specific CD4+ T cells in IL-10−/− mice are redistributed from the lungs to the intestines, we reasoned that intestinal inflammation might contribute to altered localization of fungus-specific CD4+ T cells. Therefore, IL-10−/− and control animals were treated with an antibiotic mixture of metronidazole, neomycin, and vancomycin (MNV), previously shown to greatly diminish the intestinal microbial flora (45, 46). Control and IL-10−/− mice were treated with MNV for 3 wk before adoptive transfer of Af3.16 TCR-tg CD4+ T cells and a pulmonary challenge with live A. fumigatus spores. As before, in mice not treated with antibiotics, Af3.16 TCR-tg cell frequencies were markedly decreased in the airways of IL-10−/− as compared with WT recipient mice (Fig. 6, A and B). In contrast, treatment with antibiotics reversed the effect of IL-10-deficiency and resulted in similar accumulation of A. fumigatus-specific CD4+ T cells in the airways of IL-10−/− and WT mice (Fig. 6, A and B). Moreover, the adoptive transfer of in vitro activated Af3.16 TCR-tg cells into A. fumigatus-infected, MNV-treated recipient mice resulted in similar recruitment into the airways of IL-10−/− and control mice (Fig. 6, C and D). These studies suggest that microbe-driven intestinal inflammation, as occurs in IL-10-deficient mice, leads to the redistribution of fungus-specific cells from the infected lung to the inflamed gut.

Discussion

The dangers of overly robust T cell responses to microbial infection are well known. In some infections, such as those caused by Helicobacter pylori or Hepatitis B virus, clinical disease is completely attributable to the host’s inflammatory response to the pathogen. The mammalian immune system has mechanisms in place to protect the host from overly exuberant immune responses. The cytokines TGF-β, IL-27, and IL-10 play particularly important roles as attenuators of innate and adaptive immune responses.
TGF-β deficiency is associated with severe autoimmune inflammatory responses (47, 48) and IL-27 deficiency is associated with overly robust T cell responses to *Toxoplasma gondii* and *Mycobacterium tuberculosis* infection (49, 50). Along similar lines, IL-10 deficiency enhances immune responses to *Listeria monocytogenes* and *Toxoplasma gondii* infection, in the latter case resulting in inflammatory responses that kill the host (20, 21). Given the important role for IL-10 in restraining innate and adaptive immune responses to microbial infections, we were initially surprised by our finding that IL-10 deficiency resulted in attenuated pulmonary *A. fumigatus* specific CD4⁺ T cell responses.

A potential role for IL-10 in regulating *A. fumigatus*-specific immune responses has been suggested by previous studies of acute and allergic responses to challenge with fungal Ags (51–55). In a mouse model of allergic bronchopulmonary aspergillosis, IL-10-deficient animals had increased mortality and increased airway inflammation following challenge with killed preparations of *A. fumigatus* (54). In contrast, in a mouse model of IA, IL-10-deficient mice were more resistant to challenge with *A. fumigatus* spores (52, 53). Polymorphisms in the promoter region of the IL-10 gene correlate with higher incidences of IA in patients with cystic fibrosis or following bone marrow transplantation (51, 56). In patients with established IA, higher levels of IL-10 secretion have been associated with poor prognosis and persistent invasive fungal disease (55, 57). Although IL-10 production has been correlated with IA, whether increased IL-10 production is a cause or consequence of fungal growth remains unclear. Nevertheless, these studies, in aggregate, suggest that IL-10 protects
against allergic disease, on the one hand, but enhances susceptibility to invasive fungal infections, in contrast.

Although our results seem to contrast with previously published findings demonstrating the suppressive function of IL-10 in *A. fumigatus*-mediated IA (52, 53), there are several differences that we believe account for our disparate findings. First, previous studies were performed in immunosuppressed animals or after i.v. delivery of spores, while we investigated pulmonary infection with live spores in the absence of immune suppression. It is possible that IL-10 impairs inflammatory responses to tissue-invasive fungal infections.

**FIGURE 6.** Treatment of IL-10−/− mice with broad-spectrum antibiotics restores fungus-specific CD4+ T cell accumulation in the airways. WT and IL-10-deficient recipients were treated with a mixture of broad-spectrum antibiotics metronidazole, neomycin, and vancomycin (MNV) for 3 wk before the adoptive transfer of naive Af3.16 TCR-tg cells (A and B) or in vitro primed Af3.16 Th1 TCR-tg cells (C and D). A, MNV treated and untreated control recipients were infected with live *A. fumigatus* spores and analyzed 6 days after infection. FACS plots are for representative mice from each group and are gated on CD4+ T cells recovered from the airways of infected hosts. B, Total number of Af3.16 CD4+ TCR-tg cells recovered from the airways of IL-10−/− (■) or control mice (□) in untreated (upper graph) or MNV-treated (bottom graph) recipients. Data in each bar are for a total of 8–10 mice from two separate experiments. C, Af3.16 Th1 TCR-tg cells were adoptively transferred into infected recipients. FACS plots are from representative mice in each group and are gated on CD4+ T cells recovered from the airways. D, Total number of Af3.16 Th1 TCR-tg cells recovered from the airways of IL-10−/− (■) or control mice (□) that were untreated (upper graph) or treated with MNV (bottom graph). Data in each bar are for a total of eight to ten mice from two separate experiments.
in immunocompromised hosts, but not during the initial response to inhaled spores. Second, our study focuses on *A. fumigatus*-specific CD4+ T cell responses while previous studies characterized more general aspects of the immune response. Adoptive transfer of naive *A. fumigatus*-specific T cells, as performed in our studies, provides detailed views of CD4+ T cell priming, differentiation, and trafficking, thus enabling direct quantitation and characterization of the effect of cytokine deficiency on T cell responses.

Our finding that fungus-specific CD4+ T cells accumulate or pool in the intestines of IL-10−/− mice was unexpected, since infection with *A. fumigatus* is largely confined to the lungs of infected mice. Adoptive transfer of activated effector CD4+ T cells into *A. fumigatus*-infected mice demonstrated that early trafficking to the lung and entry into the airways was IL-10-independent and thus did not account for the 90% reduction in the frequency of AF3.16 T cells in airways of IL-10-deficient mice. Although initial trafficking to the lungs may be similar in WT and IL-10-deficient mice, it is possible that recirculation of T cells differs. Little is known, however, about recirculation of effector T cells from tissues such as infected lungs after their initial arrival. It is possible, for example, that effector T cells re-enter the circulation from the lung and then either return to the lung or infiltrate other tissues. If this is the case, the number of T cells in any given tissue will reflect the rate of initial arrival, the rate of departure and, importantly, the rate of T cell return. If recirculating effector T cells encounter a sink, their likelihood of returning to the site of infection will decrease and T cell frequencies, in this case in the lung, will drop. We postulate that in IL-10-deficient mice, the gut serves as a sink where *A. fumigatus*-specific T cells pool, eventually depriving the lung of T cells.

Previous studies demonstrated that activated and effector memory CD8+ T cells preferentially migrate to nonlymphoid tissues including the lung, liver, and intestines (58–60), independent of the site of infection or the infectious agent (59, 60). Pulmonary infection with Sendai virus, for example, resulted in similar frequencies of virus-specific CD8+ T cells in the intestines and lung (60). Trafficking of activated CD4+ T cells into nonlymphoid tissues also occurs in the absence of inflammation (61). We believe it is likely, therefore, that activated, *A. fumigatus*-specific CD4+ T cells traffic to the intestines of WT and IL-10−/− mice but are selectively retained in the gut of IL-10−/− mice. It is possible that intestinal inflammation resulting from IL-10 deficiency or impaired IL-10 signaling leads to the aberrant retention of activated fungus-specific CD4+ T cells. We postulate that the retention of fungus-specific CD4+ T cells in IL-10−/− mice is mediated by the enhanced secretion of inflammatory cytokines and chemokines that occurs in response to the gut flora in the absence of IL-10. Enhanced production of several chemokines, including the T cell-attracting chemokine CXCL9, was detected before the appearance of histologic changes in two different mouse models of IBD including IL-10−/− mice (62). Enhanced expression of the adhesion molecules ICAM-1, VCAM-1, and MadCAM-1 has been observed in the colonic mucosa of IL-10−/− mice and may also facilitate the entry of T cells to the gut (63). Thus, it is possible that, during colitis development, there is a nonspecific recruitment of T cells due to alterations in adhesion molecule expression and chemokine production that attract T cells. Additional studies will be necessary to determine the relative contribution of adhesion molecules and chemokines to the recruitment of *A. fumigatus*-specific CD4+ T cells to the intestines of IL-10−/− mice.

The development of colitis in IL-10−/− and IL-10R2−/− is well documented, with detectable inflammation as early as 4 wk after birth (19, 40, 41, 64). The role of intestinal inflammation in retention of CD4+ T cells is further supported by reversal of gut pooling of *A. fumigatus*-specific CD4+ T cells and redirection to the airways when IL-10−/− mice were treated with broad-spectrum antibiotics. The beneficial impact of antibiotic treatment in mouse models of colitis (including IL-10−/− and IL-10R2−/− mice) has been documented (42–44). Moreover, it has been shown that diminishing the gut flora leads to the prevention and amelioration of ongoing inflammation (42–44). Deficiency in IL-10 production, however, may also lead to alterations in the gut flora and the outgrowth of bacterial populations that induce colitis. Consistent with this possibility, Madsen et al. (42) documented alterations in bacterial populations in the intestines of IL-10−/− mice before any evidence of colitis development. Thus, retention of *A. fumigatus*-specific CD4+ T cells in the gut of IL-10−/− mice might result from alterations in the gut flora that are reversed by antibiotic treatment. Our experimental evidence does not distinguish between these possibilities. Our results do not support the notion that *A. fumigatus* conidia are selectively retained in the gut of IL-10−/− mice, because multiple attempts to culture fungal spores from gut-draining lymph nodes were unsuccessful. Moreover, treatment of IL-10−/− mice with antibiotics that lack any antifungal activity restored the trafficking of *A. fumigatus*-specific CD4+ T cell to the airways. Thus, our experimental results are consistent with a required function for IL-10 in maintaining the intestinal mucosal integrity by either influencing commensal microbial populations or preventing the development of inflammation.

Is the observed retention of CD4+ T cells in the inflamed gut unique to *A. fumigatus*-specific CD4+ T cells or more broadly relevant? IBD in humans follows a chronic course of disease progression with periods of remission and relapse. The factors responsible for the relapses are incompletely understood but include alterations in treatment regimens, activation of eosinophils by environmental stimuli, and enteric or systemic infections (65, 66). Up to 40% of IBD exacerbations in pediatric patients were associated with pulmonary infections (67) and there is an increased incidence of asthma among patients with ulcerative colitis and Crohn’s disease (68, 69). Our finding that activated, pathogen-specific CD4+ T cells are retained in the gut of IL-10−/− mice may provide a partial explanation for the correlation of airway inflammation with IBD exacerbations. In this scenario, activation of pulmonary T cells by an allergen or pathogen would lead to their migration and retention at peripheral sites of inflammation. In the case of IBD patients, activated T cells would be retained in the inflamed gut where they might contribute to the pathogenesis of disease. Previous studies assessing upper respiratory infections in IBD patients have focused on viruses or bacteria. Given the abundance of *A. fumigatus* spores in the environment, future studies should focus on a potential role for *Aspergillus*-specific immune responses as an exacerbating factor for IBD.

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**Disclosures**

The authors have no financial conflict of interest.

**References**


Figure legends for supplementary figures:

Supplementary figure 1: Diminished proliferation and IL-2 production by IL-10−/− CD4+ T cells. (A) T cell proliferation was assessed by [3H]-thymidine incorporation on naïve CD4+ T cells purified from IL-10−/− or control mice that were stimulated with anti-CD3/CD28 antibodies. Values shown are mean ± SD of 12 individual wells per group and are representative of two separate experiments. (B) Culture supernatants from anti-CD3/CD28 stimulated CD4+ T cells were analyzed for the presence of IL-2 by ELISA 24 hrs after culture initiation. Values shown are mean ± SD of 6 individual wells and are representative of two separate experiments.

Supplementary figure 2: Normal early recruitment of activated A. fumigatus-specific CD4+ T cells to the airways of IL-10−/− mice. (A) Upper panel: experimental approach. Naive Af3.16 were primed in vitro prior to their intravenous delivery into infected IL-10−/− or control mice. Recipients were analyzed 16 hours after T cell transfer. Bottom panel: Frequency of Af3.16 TCR-tg CD4+ T cells recovered in the airways (BALF) and lung parenchyma of infected recipients. FACS plots are gated on CD4+ T cells and are for individual mice representative of 3 mice per group in two separate experiments. (B) Upper panel: experimental approach. Af3.16 TCR-tg CD4+ T cells were primed in vivo prior to their intravenous delivery into infected IL-10−/− and control recipients. CD4+ T cells (including congenically marked, Af3.16 TCR-tg cells) were recovered from the lymph node (MLN) of infected normal control mice by negative MACS sorting. Bottom panel: Frequency of Af3.16 TCR-tg CD4+ T cells recovered in the airways (BALF) and lung parenchyma of infected recipients. FACS plots are gated on CD4+ T cells and are for individual mice representative of 3 mice per group in two separate experiments.
Supplementary figure 3: Loss of Af3.16 TCR-tg from the airways of IL-10-deficient mice is not due to increased apoptosis. (A and B) Naïve, Af3.16 TCR-tg CD4⁺ T cells were transferred into IL-10⁻/⁻ or control mice one day prior to a pulmonary infection with live *A.fumigatus* spores. The frequency of dead (A) or apoptotic (B) Af3.16 TCR-tg CD4⁺ T cells was examined in the MLN, 6 days after infection. (C) Af3.16 CD4⁺ TCR-tg cells were primed in vitro and retrovirally transduced to enforce the expression of murine Bcl-2 or a control luciferase gene. Modified Af3.16 TCR-tg cells were mixed in vitro and adoptively transferred into infected IL-10⁻/⁻ or control hosts. Left panel: experimental approach. Right panel: Depicted plots are for cells recovered from the BALF of representative recipient mice and are gated on CD4⁺ or Af3.16 TCR-tg cells. (D) Frequency of Af3.16 CD4⁺ TCR-tg cells recovered from the BAL or lung parenchyma of recipient IL-10⁻/⁻ (black bars) or control mice (white bars). Bars are for a total of 5 mice per group. (E) Ratio of Bcl2/luciferase among transduced Af3.16 TCR-tg cells recovered from the airways or lung of IL-10⁻/⁻ (black bars) or control mice (white bars). Bars are for a total of 5 mice per group.
**Supplementary figure 1**

**A.**

CD4+ T cell proliferation

- No stimulation
- anti-CD3/CD28

- IL-10+/+
- IL-10-/+

p = 0.0009

**B.**

IL-2 pg/ml

- No stimulation
- anti-CD3/CD28

- IL-10+/+
- IL-10-/+

p = 0.007

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Supplementary figure 2: 

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A. naive Af3.16 TCR-tg 1.1/1.2

Th1 T cell activation in vitro

IL-10+/+

D6 infected recipients

IL-10−/−

B. naive Af3.16 TCR-tg 1.1/1.2

A. fumigatus

D6 infected recipients

MLN-CD4+

IL-10+/+

16 hrs FACS analysis

16 hrs FACS analysis

Suplementary figure 2: Rivera, et al
Supplementary figure 3

A. Frequency of dead Af3.16 T cells

B. Frequency of apoptotic Af3.16 T cells

C. Flow analysis

D. Frequency of AF3.16 TCR-tg cells

E. Ratio Bcl2/control