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Th17 cells in the setting of *Aspergillus* infection and pathology

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Innate and adaptive immune responses act to generate the most effective form of immunity for protection against *Aspergillus fumigatus*. The decision of how to respond is still primarily determined by interactions between fungi and cells of the innate immune system, but the actions of T cells will feed back into this dynamic equilibrium to regulate the balance between pro-inflammatory and anti-inflammatory signals. The enzyme indoleamine 2,3-dioxygenase, and tryptophan metabolites, acting as a bridge between dendritic cells and regulatory T cells, pivotally contribute to such a homeostatic condition by taming inflammatory responses. IL-23 and the newly described Th17 pathway, by means of negative regulation of tryptophan catabolism, play an inflammatory role previously attributed to uncontrolled Th1 response. Our data support a model in which IL-23/IL-17A/Th17-driven inflammation promotes infection and impairs antifungal immune resistance. Thus, modulation of the inflammatory response represents a potential strategy to stimulate protective immune responses to *Aspergillus*.

Keywords inflammation, *Aspergillus*, indoleamine 2,3-dioxygenase, Th17 cells, regulatory T cells

Introduction

Invasive infections caused by *Aspergillus* moulds have increased in frequency during the last two decades and are associated with high rates of morbidity and mortality, especially among immunocompromised patients [1,2]. Although immunocompetent and non-atopic subjects are relatively resistant to *Aspergillus* infections and diseases, *A. fumigatus* is associated with a spectrum of diseases in humans that include saprophytic colonization of pre-existing cavities (aspergilloma), allergic asthma, and allergic bronchopulmonary aspergillosis. These considerations imply that only certain host circumstances render the fungus pathogenic or innocuous for the host [1–3].

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The most important risk factor for invasive aspergillosis has historically been neutropenia. However, it also has been reported that recipients of hematopoietic stem cell transplants show a reduced neutropenia-related infection and an increased late-onset infection, concomitant with the occurrence of graft-versus-host disease [4]. These findings, together with the occurrence of aspergillosis in non-neutropenic patients [2,5], attest to the importance of specific defects in both the innate and adaptive immune mechanisms to the pathogenesis of the infectious process [6–8].

Intriguingly, severe fungal infections also occur in patients with immune reconstitution syndrome [9]. This is a condition seen in some cases of immunosuppression, in which the immune system begins to recover and results in quiescent or latent infections manifesting as opportunistic mycoses. The overwhelming inflammatory response paradoxically makes the symptoms of infection worse. Evidence suggests a close association between susceptibility to *A. fumigatus* infections by individuals who have weakened immune systems or a

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history of immune disorders, with heightened immune reactivity [10,11].

Although fungi are not mere passive participants in the infectious process, compelling evidence indicates that the mammalian immune system represents the most important determinant of the host-fungus interaction. Host-defense mechanisms against *Aspergillus* are numerous and range from relatively primitive and constitutively expressed non-specific defenses to sophisticated adaptive mechanisms that are induced specifically during infection [1,12]. These two arms of the immune system are linked through a variety of cross-regulatory pathways, the integration of which provides for the host the complex armamentarium of effector mechanisms for defense.

The multifunctional first line of host defence

Among the multiple effector mechanisms of the innate immune system, resident alveolar macrophages have long been recognized as the first line of defense, preventing hyphal germination and the consequent activation of inflammatory responses against the fungus [1]. In contrast, polymorphonuclear cells (PMNs) are the predominant immune cells in the acute stage of the infection. In this regard, PMNs may act as double-edged swords, as they are essential for pathogen eradication, but an excessive release of oxidants and proteases may be responsible for injury to the lung [13]. This implies that tight regulatory mechanisms are required to balance protection and immunopathology for efficient control of the fungus.

In this regard, *Aspergillus* and other fungi can exploit or subvert the host's inflammatory response [14], which may affect pathogenicity [15]. The host structures that recognize fungi, pattern recognition receptors (PRRs), play important roles in manipulating the immune response against the fungus, improving or decreasing the intensity of the inflammatory reactions often intended to sterilize the host to avoid infection. Among signaling PRRs, the Toll-like receptor (TLRs) family is undoubtedly involved in cell activation upon contact with pathogens [16]; TLR2, TLR4, and TLR9 have been implicated in host defense against *A. fumigatus* [13,17–21]. It is interesting that the finding of signaling through TLR2 and TLR4 in PMNs is associated with the induction of distinct activation programs in PMNs, eventually culminating in the occurrence of different patterns of fungal clearance and inflammatory pathology [13,17].Despite TLRs being proved to be crucial for detecting infection and activating the innate and adaptive immune systems, sustained TLR stimulation can result in chronic inflammation and is associated also with the development of certain autoimmune diseases [22,23]. Recently, soluble PRRs have gained interest because of the ability to modulate the intensity, as well as the efficacy of the innate response against the fungus. Upon TLR engagement, dendritic cells (DCs), macrophages, and other cell types secrete pentraxin 3 (PTX3) [24]. PTX3 localizes in neutrophil extracellular traps [25], recognizes microbial moieties, and opsonizes selected fungi and bacteria. PTX3-null mice are defective in the recognition of conidia by alveolar macrophages and DCs, as well as have inappropriate induction of an adaptive Th1 response [26]. Thus, PTX3 is a an example of an endogenous protein endowed with protection against *Aspergillus* attacks.

The privilege to be tolerant

Efficient responses to fungi require different mechanisms of immunity [12]. DCs are uniquely able to decode the fungus-associated information and translate it into qualitatively different T helper (Th) immune responses. Murine and human DCs phagocytose conidia and hyphae of *A. fumigatus* through distinct recognition receptors. The engagement of distinct receptors subsequently translates into disparate downstream signaling events, ultimately affecting cytokine production and co-stimulation.

DCs orchestrate the overall antifungal immune resistance in the lungs [18,27,28]. A dense network of DCs has been described in the respiratory tracts [29]. The evidence pulmonary DCs, through the production of IL-10, mediate unresponsiveness to respiratory antigens [30] suggests that the ability of DCs to modulate the appropriate T-cell responses to the invading pathogen may be affected by local immunoregulatory events. In the case of *Aspergillus*, by using distinct PRRs, including TLRs, murine pulmonary DCs are able to discriminate between conidia and hyphae in terms of induction of adaptive Th responses [18,27]. Adoptive transfer of different types of DCs activates protective and non-protective Th cells as well as regulatory T cells (Tregs), ultimately affecting the outcome of the infection in mice with aspergillosis. The infusion of fungus-pulsed or RNA-transfected DCs accelerates recovery of functional antifungal Th1 responses in mice with hematopoietic transplantation [31–33].

The inflammatory and anti-inflammatory state of DCs in response to *Aspergillus* is strictly controlled by the metabolic pathway of tryptophan catabolism and mediated by the enzyme indoleamine 2,3-dioxygenase (IDO) [34,35]. IDO is an intracellular heme-containing enzyme that catalyzes the initial, and rate-limiting, step

in tryptophan degradation into kynurenines along the so-called kynurenine pathway [36]. IDO is expressed in a variety of human tissues, including macrophages and DCs, and is induced in inflammatory states by IFN-γ. Recently, it has been shown that 'non-canonical' activation of the transcription nuclear factor-κB characterizes the peculiar events occurring in DCs upon the engagement of selected TLRs that culminates with the induction of IDO expression [37,38]. Localized depletion of tryptophan and the production of proapoptotic kynurenines are among the mechanisms potentially responsible for the multiple activities observed after IDO induction, and are directly associated with the induction of the immunological tolerance at the host-pathogen interface [35].

In experimental aspergillosis, IDO activity was induced in PMNs and DCs at sites of infection through IFN-γ- and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4)-dependent mechanisms. IDO blockade greatly exacerbated infections and the inflammatory pathology, and eliminated resistance to re-infection, which resulted in deregulated innate and adaptive immune responses caused by the impaired activation and function of suppressor $CD4^+CD25^+$ Tregs [34]. In aspergillosis, different types of Tregs actively participate in the induction of a state of protective tolerance [35]. Naturally occurring Treg cells (nTregs) originate in the thymus and survive in the periphery as natural regulators, whereas inducible (or adaptive) Treg cells (iTregs) develop from conventional CD4+ T cells that are activated under conditions of impaired costimulatory signaling or are induced by deactivating cytokines and drugs [39,40]. The contribution of different subsets of Tregs to the so-called protective tolerance is demonstrated by the fine control exerted over infection and allergy to the fungus. Early in infection, nTregs inhibited the antifungal effector and proinflammatory activities of PMNs. The degree of inflammation and amount of IFN-γ in the early phase of the infection set the stage for subsequent adaptive by conditioning the IDO-dependent tolerogenic program of plasmacytoid DCs (pDCs) and the subsequent activation and expansion of tolerogenic iTregs, which prevented allergy to the fungus through the production of IL-10 and TGF-β. Interestingly, it has been shown recently that the synthetic glucocorticoid dexamethasone exerts IDO-dependent protection in allergy to the fungus via the glucocorticoid-induced tumor necrosis factor receptor (GITR)-GITR ligand coreceptor system [37,38].

Intriguingly, experimental evidence has suggested that the fungus can interfere with the host tolerogenic program dictated by IDO activity in a morphotype-dependent manner. IDO activity was promoted by resting conidia of *Aspergillus* more so than swollen conidia or hyphae, which were actually potent inhibitors of IDO expression and function [34]. These findings imply that the fungus manipulates Treg function by promoting or subverting IDO-dependent tolerance, which may contribute to immune evasion or promotion of host inflammatory responses (Fig. 1).

How Th17 gets in shape

Prolonged inflammation is a hallmark of a wide range of chronic diseases and autoimmunity. For *Aspergillus*, in the last two decades, the immunopathogenesis of fungal infections and associated inflammatory diseases have been explained primarily in terms of Th1/Th2 balance, which is considered to be affected by a combination of different types of Tregs [12,35,41]. Until recently, CD4+ Th1 cells were considered responsible for the development of inflammatory responses to *Aspergillus* that were mediated by IL-12p70, the key cytokine driving Th1-cell differentiation.

The discovery of the IL-12 cytokine-family member IL-23 has led to a re-evaluation of this conceptual framework [42,43]. IL-23, although not directly involved in Th17 differentiation, plays an important role in maintaining Th17 effector function [44,45]. Several experimental studies and clinical investigations confirmed that IL-23-driven Th17 cells, rather than the Th1-cell subset, mediate the inflammatory responses of autoimmune or infectious origin [46–48]. IL-17A-producing T cells have been recognized as constituting a separate subset of T cells, termed Th17, in which different retinoic acid receptor-related orphan receptors, including (ROR)γt, mediate lineage specification [49]. Th17 cells are responsible for various organ-related autoimmune diseases [46,47]. In addition, both IL-23 and the Th17 pathway correlate with disease severity and immunopathology in diverse infections [50–52]. These studies suggest that IL-12 and IL-23 have distinct roles in promoting antimicrobial immune responses and diseases *in vivo*.

IL-23 and IL-17 have a multi-faceted largely negative role in aspergillosis

As mentioned above, IL-12 and IL-23 are members of a family of proinflammatory heterodimeric cytokines. They share a common p40 subunit linked to IL-12p35 (for IL-12p70) or IL-23p19 (for IL-23p70). IL-23 functions through a receptor complex composed of the IL-12Rβ1 subunit and a unique component, the IL-23R chain [53,54]. In mice with pulmonary aspergillosis, we have shown that the absence of IL-23 increased

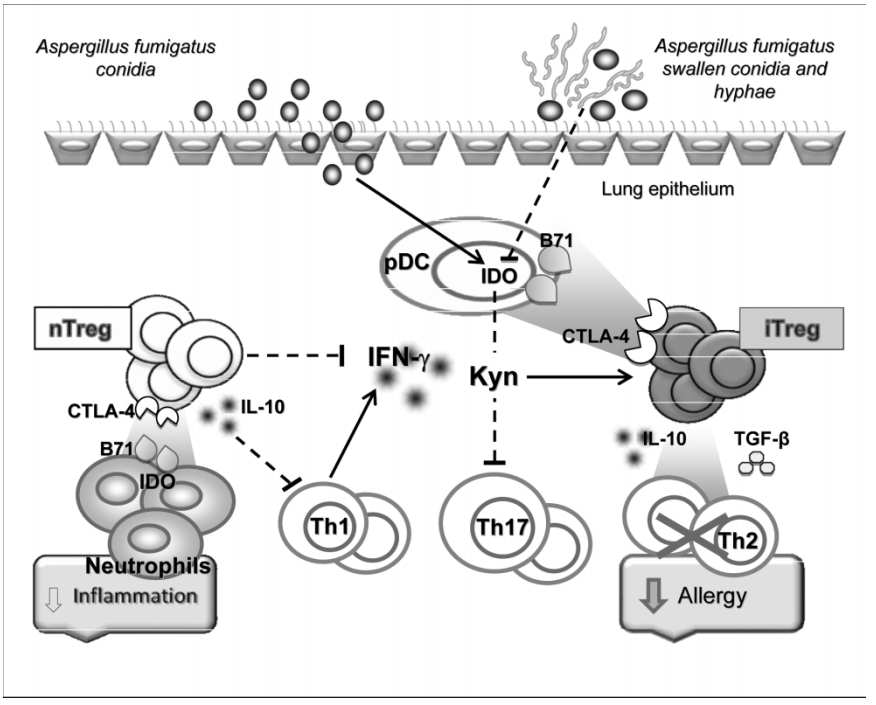

Fig. 1 Cross-talk between Treg subsets in *Aspergillus* infection and allergy. During the early phase of *Aspergillus fumigatus* infection, lung naturally-occurring (n) Tregs limit PMN's effector functions, and dampen inflammation through IL-10-dependent and CTLA-4/B71-reverse signaling mechanisms. The amount of IFN-γ produced affects the IDO-dependent tolerogenic program of plasmacytoid dendritic cells (pDCs) and the subsequent activation and expansion of tolerogenic induced (i) Tregs that prevent allergy to the fungus through TGF-β/IL-10-dependent mechanisms. Germinating conidia, through IDO inhibition, may promote the host inflammatory response and thus favour Th17 expansion.

IL-12 production, and the absence of IL-12 increased IL-23 production, and the latter condition increased susceptibility to infection [55]. In addition, blockade of IL-23 and IL-17 greatly increased antifungal resistance, as judged by a decreased fungal growth in the relevant target organs. Either treatment greatly ameliorated signs of inflammation, both clinically and at the tissue level [55].

Although signaling through IL-17R is required for PMN recruitment and host defense in bacterial pneumonia [56], the involvement of IL-17A in inflammatory lung disorders has also been reported [57]. Both IL-23 and IL-17 impaired the antifungal effector activities of PMNs even in the presence of IFN-γ, a finding that is suggestive the Th17 effector pathway prevails over the Th1 pathway. In addition, both cytokines induced the release of matrix metalloproteinase 9 and myeloperoxidase, which likely accounted for the significant inflammatory pathology associated to Th17 cell activation. Moreover, by subverting the tolerogenic program of PMNs, both cytokines counteracted IFN-γ-dependent activation of IDO [55]. These findings indicate that IL-23 and IL-17A, despite the important role in regulating PMN homeostasis and recruitment [57,58], may promote pathogen growth and infection as a result of the induced inflammatory pathology. These results may serve to accommodate the paradoxical association of chronic inflammatory responses with intractable forms of fungal infections, where fungal persistence occurs in the face of an ongoing inflammation.

The production of IL-12 was higher in IL-23-deficient mice and similarly, IL-23 higher in IL-12-deficient mice. Thus, both cytokines are cross-regulated in infection [55] and reciprocally regulated at the level of DCs [59]. However, because inflammatory DCs, more so than tolerogenic DCs, appear to produce IL-23 in response to the fungus, this implies the Th1/Th17 balance also depends on the reciprocal regulation by DCs subsets at different body sites. By perpetuating the activation of Th17 cells, IL-23 may contribute to the uncontrolled fungal growth and the resulting concomitant activation of nonprotective Th2 cells [55].

It is known that the differentiation of Th17 and Tregs are strictly linked [45]. In mice, TGF-β induces the differentiation of both Th17 and Tregs. However, IL-6 inhibits Tregs differentiation and resulting in a population constitutes of Th17 cells. Thus, IL-6 is known to divert murine generation of Tregs toward Th17 induction in the presence of TGF-β [45]. In line with previous findings on the protective role of IL-6 in aspergillosis [60], we found that neutralization of IL-6 did not promote resistance to *Aspergillus* infection (Table 1). Interestingly, we previously described an increased production of IL-17A in condition of IL-6 deficiency that, notably, was associated with the failure to activate antifungal effector functions, despite inflammatory cell

Table 1 Effect of cytokine administration on Aspergillosis.

Neutralization of:	Effect on infection	Ref.
IL-23	Improvement	[56]
IL-17A	Improvement	[56]
IL-6	Exacerbation	Unpublished
TGF-β	Improvement	Unpublished

recruitment into the lung [60]. In contrast, TGF-β neutralization during infection decreased resistance to infection and exacerbated inflammatory reactions and immunopathology (Table 1). Moreover, we have found recently that IL-1 signalling is essentially required for the activation of pathogenic Th17 cells by IL-6 and TGF-β [61]. Thus, opposite roles are apparently played by the IL-23/IL-17/TGF-β combination versus IL-6. Preliminary as they are, these data are consistent with a cytokine dependent pathway of immune protection against the fungus in which IL-23/IL-17A/TGF-β worsen the infection, while IL-6 plays a protective role.

TLRs provide the scenario in which Th17 takes place

As already mentioned, DCs produce IL-12 and IL-23 in response to the fungus *via* TLRs [55]. Because TLRs use distinct adaptor molecules that lead to different gene-induction programs, the production of IL-12 family members can be differentially regulated downstream of TLRs. We found that TLR2 and TLR4 are required for production of IL-23 by conventional DCs through the myeloid differentiation primary response gene 88 (MyD88), but not through the TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway. Notably, IL-23 appeared to be promoted even in the absence of TRIF [55]. The finding that IL-23 is produced in response to the fungus in conditions of high-threat inflammation has important implications as it points to IL-23 as an important molecular link between the inflammatory processes, fungal virulence, and pathogenicity.

Recently, we found that Toll IL-1R 8/Single Ig IL-1 related receptor (TIR8/SIGIRR) acted as a negative regulator of the Th17 pathway in aspergillosis and was essentially required to fine-tune the inflammatory and adaptive immune response to the fungus. Thus, TIR8/SIGIRR recruited at signaling receptor complexes is required intracellularly for fine-tuning the balance between protective immunity and immunopathology in aspergillosis [61]. The role of TLRs and cytokines in the activation of the Th17 pathway against the fungus are shown in Table 2.

Table 2 Pathogenic Th17 cell activation in Aspergillosis.

Genotype	Clinical features	Ref.
IL-12 p35 deficiency	Defective clearance Inflammation	[56]
TRIF deficiency	Defective clearance Inflammation	Unpublished
TIR8/SIGGIR deficiency	Defective clearance Inflammation	[62]
NADPH oxidase deficiency	Defective clearance Inflammation	[9]

IL-17 affects fungal morphology

An anticipated finding was that IL-17 shared a direct effect on fungi. The blockade of IDO in *Candida albicans* cells [62] induces fungal germination as opposed to IFN-γ [63]. Similar to what was observed for *Candida*, the inhibition of IDO induces strong germination of *Aspergillus* conidia as well as after exposure to IL-17A (unpublished observations). This finding suggests an action on fungal IDO, an enzyme that is highly responsive to signals from the mammalian host immune system [64]. No visible effects on fungal morphology were observed with IL-23 (Fig. 2). This was correlated with the presence of hyphae *in vivo*

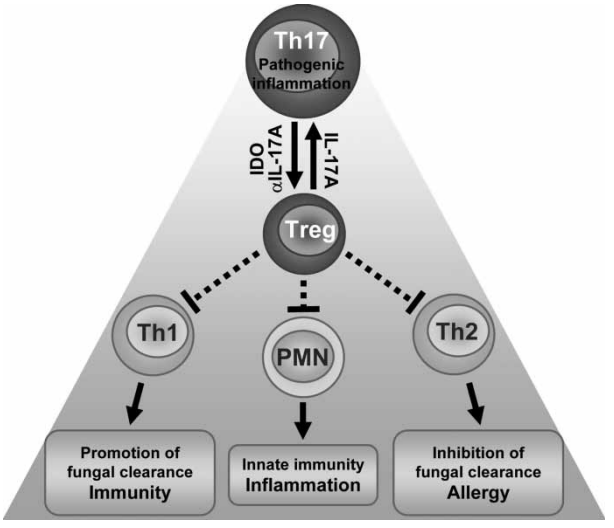

Fig. 2 IL-17A and IDO have opposite roles in Th17/Treg cell lineage decision. A delicate balance is established between Tregs, capable of fine-tuning protective innate and adaptive Th immunity to the fungus and the Th17 pathway, which can be responsible for the failure to resolve the infection in the face of an ongoing inflammation. IDO and kynurenins, in their capacity to induce Tregs and inhibit Th17, pivotally contribute to cell lineage decision in aspergillosis and highlight the emerging role of metabolic pathways in regulation of immunity and tolerance. In contrast, by promoting Th17, IL-17A may contribute to pathogenicity, despite inflammatory cell recruitment. Solid and dotted arrows, positive or negative signals, respectively.

in condition of Th17 activation, particularly in the lung of p35-deficient mice [55]. Therefore, the function of the Th17 pathway may go beyond its ability to promote inflammation and subvert antimicrobial immunity by also having a direct action on fungal morphology and virulence. This may translate in concomitant IL-4+ Th2 cell activation, known to be strictly dependent on high levels hyphal growth [65], and further prevent Th1 functioning. Thus, the Th17 pathway may contribute to pathogenesis of fungal infections, occurring in a fungus-autonomous fashion at sites of infection.

Tryptophan metabolites dampen immunopathology

Two major theories have been described to explain causally the role of IDO in tolerance induction. One theory posits that tryptophan breakdown suppresses T cell proliferation by decreasing the availability of the essential tryptophan [64]. The other theory assumes that downstream metabolites of tryptophan catabolism, known as kynurenines, act to suppress immune reactivity of effector T lymphocytes [36].

Recently, it has been shown that tryptophan metabolites were capable of inducing the Foxp3 (forkhead box P3) transcription factor, which directs the Treg lineage and suppresses the gene encoding RORγt *in vitro* and *in vivo* [8,66]. Thus, kynurenines seem to promote DC-supported generation of Foxp3+ cells [38,67]. This suggests the existence of an IFN-γ/IDO-dependent pathway leading to Th1/Treg cell activation that is crucial in promoting protective tolerance in infection.

Strikingly, defective IDO functioning has been found in a mouse model of chronic granulomatous disease (CGD). The most common autosomal recessive cause of CGD, affecting 25% of patients, is a defect in the p47phox subunit of NADPH oxidase [68]. Human CGD leukocytes display a hyperinflammatory phenotype, with increased production of specific cytokines in response to stimulation with TLR agonists. Due to the absence of superoxide, an impaired IDO activity might compromise *de novo* synthesis of cytoprotective NAD, microbial tryptophan starvation, and kynurenines production by phagocytes, all of which would be responsible for reduced antimicrobial defense and exaggerated inflammatory responses. CGD mice exhibit high levels of IL-17A production, largely secreted by a particular subset of γδ T cells, such as Vγ1+ cells, and IL-23, whereas IFN-γ, IL-10, and TGF-β are defective. Although IL-17A may be important for initiating an immediate and early neutrophil response to mucosal infections, blockade of IL-17A or IL-23, as well as replacement therapy with natural kynurenines, reversed the inflammatory phenotype and decreased the production of IL-17A (Table 2). Thus, surprisingly, downstream IDO metabolites may reduce immunopathology by disrupting the deleterious regulatory loop that underlines host inability to eradicate fungal infection [8] (Fig. 3).

Conclusions

The finding that IL-23 and IL-17 promote inflammation, while subverting protective antifungal immunity, may serve to accommodate the seemingly paradoxical association of chronic inflammatory responses with fungal persistence in the face of ongoing inflammation. In this scenario, unrestricted fungal growth will result from the activation of not only pathogenic Th17 cells, but also Th2 cells, whose activation is strictly dependent on fungal burden. However, because both IL-17A and IL-17F may contribute to the expression of airway inflammation and pulmonary hyperreactivity, free soluble IL-17A is increased in asthma, and allergic cellular and humoral responses are suppressed in IL-17-deficient mice [69]. These findings indicate that the Th17 pathway also may be directly involved in fungal-associated allergic diseases. This suggests that conditions of high-threat inflammation may represent a local

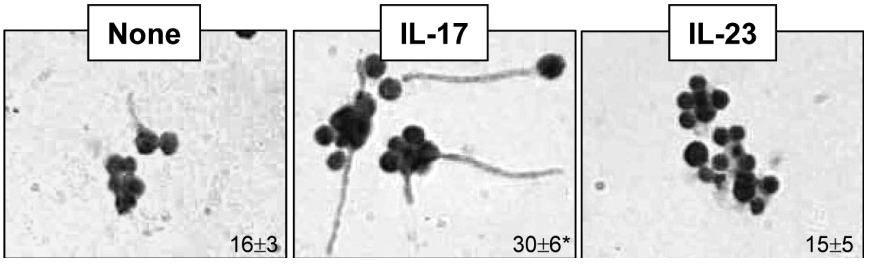

Fig. 3 IL-17 promotes fungal germination. *Aspergillus* conidia were exposed at 37°C in 5% CO_2 in sterile water to 100 ng/ml of recombinant (r) IL-17A or rIL-23 for 8 h before visualization of fungal morphology by crystal violet staining and light microscopy. Numbers refer to the percentages (mean ± SE) of germinating cells over a total of 400 cells counted. Magnification ×100. *$P < 0.05$, IL-17-treated vs untreated cells. Shown are the pooled results from 4 experiments.

host circumstance that predisposes to Th17 activation during *Aspergillus* infections and diseases. These new findings, by providing a molecular connection between the failure to resolve inflammation and lack of antifungal immune resistance, point to a novel strategy for the prevention of inflammatory immunity and allergy to *Aspergillus*. In this regard, IL-23/IL-17 blockade and tryptophan metabolites may prove to be potent regulators, capable of dampening an overzealous or heightened inflammatory host response to the benefit of eradication of the pathogen and host survival.

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