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Aspergillus and asthma – any link?

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The innate immune and acquired immune responses are not separate, parallel systems but form interdependent components of a single integrated immune response. This is nicely highlighted by an expanding database demonstrating that the innate immune response provides the acquired immune response with information about the origin of the antigen and the type of response required via pattern recognition receptors (PRRs). *Aspergillus* is among a growing list of allergens that can aggravate asthmatic responses. Significant pulmonary pathology is associated with *Aspergillus*-induced allergic and asthmatic lung disease characterized by increased Th2 cytokine generation, IgE and IgG, eosinophilia, airway hyper-responsiveness and airway remodeling. Experimental data from a model of chronic fungal asthma demonstrate that thymus associated and regulated chemokine (TARC/CCL17) and macrophage derived chemokine (MDC/CCL22), working via CCR4, directly impair the innate anti-fungal immune response, thereby promoting the maintenance of acquired Th2-mediated asthmatic disease. Both chemokines appear to accomplish this by regulating the expression of PRRs such as toll like receptors (TLRs) and triggering receptor expressed on myeloid cells (TREM-1) by immune cells. Thus, the link between *Aspergillus* and asthma appears to reside in the magnitude and appropriateness of the host innate immune response, and ongoing research is revealing promising targets for therapy.

Keywords *Aspergillus*, asthma, chemokine, chemokine receptor, toll like receptors

Introduction

The increased incidence and prevalence of asthma in the developed world has raised major concerns and concerted research efforts. Without a clear understanding of the etiopathogenesis of asthma, a number of hypotheses have emerged to explain this alarming change in public health. The hygiene hypothesis is among the most heavily cited and draws upon the interesting connection that exists between the innate and acquired immune system. Essentially, as this hypothesis outlines, the increase in asthma reflects the increase in hygiene within the developed world. While this development has all but eliminated many infectious illnesses such as cholera, the lack of pathogen exposure in early childhood appears to predispose to allergy and asthma later in life. No official list of 'protective' pathogens has been formulated, but according to the hygiene hypothesis any organism that evokes a strong type-1 cytokine response may prevent the development of asthma later in life [1]. Clearly, the lack of appropriate environmental pathogen exposure cannot entirely explain the rise in allergy and asthma, nor does this hypothesis explain the rise of Th1-type autoimmune diseases and the anti-allergic effect of strong Th2-cytokine mediated parasitic infections. Considerable research is presently directed toward understanding genetically driven susceptibility to allergy and asthma [2].

Role of *Aspergillus fumigatus*

Although other fungal genera including *Bipolaris*, *Curvularia*, *Cladosporium*, *Penicillium* and *Alternaria*

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are also suspected culprits in the growing epidemic of allergy and asthma [3–5], the airborne fungal pathogens that have received the greatest research attention are found in the genera *Aspergillus.* Based on worldwide sampling of indoor and outdoor ambient air, *Aspergillus* is among the most prevalent of airborne fungal spores. *Aspergillus* poses a significant problem in hospital and home environments where it readily infects patients with various forms of immunodeficiency. In a recent study, Englehart *et al*. [6] detected mean indoor and outdoor *A. fumigatus* counts of 8.1 cfu/m^3 and 9.4 cfu/m^3, respectively. Alarmingly, their study also showed that portable air filtration units only reduced indoor airborne *Aspergillus* spore counts by approximately 30%, indicating that even filtered air poses a major threat to immunocompromised patients. *Aspergillus* also affects individuals with intact immune systems. For example, the intrapulmonary growth and persistence of *A. fumigatus* elicits a chronic hypersensitivity reaction in the lung that is commonly referred to as allergic bronchopulmonary aspergillosis (ABPA). Several features of ABPA mirror those of asthma, including severe airway hyperactivity, remodeling (i.e., mucus hypersecretion and fibrosis) and inflammation characterized by increased type-2 cytokine levels, eosinophilia, and $CD4^2+$ T cell activation. ABPA is typically present in only 1%–2% of chronic asthmatics, a finding that may be related to the fact that colonization of the lung by *Aspergillus* is rarely observed in immunocompetent individuals. However, the lack of *Aspergillus* colonization in the asthmatic lung does not rule out a major exacerbating role for this fungus in the vast majority of other asthmatics that do not present with clinically defined ABPA. Although clinical data are clearly lacking to support this statement, animal studies are providing important clues as to the nature of the interaction between *Aspergillus* and the asthmatic lung.

Experimental studies in mice

Drawing upon experimental studies using various strains and genetic variations of mice, it is apparent that the allergic lung provides a permissive environment for the accumulation of fungal material within immune cells resident to the lung. In our experience, the introduction of as many as 5.0×10^6 *A. fumigatus* spores into genetically intact mice does not lead to invasive disease [7]. However, in mice that have been previously sensitized to *Aspergillus* or other allergens (e.g. cockroach antigens), entire spores or partially degraded fungal material persists within lung mononuclear cells. This phenomenon is not observed in non-allergic mice exposed to the same number of *Aspergillus* spores [7]. The data we have collected so far strongly suggest that the persistence of fungal material in the lungs of allergic mice promotes the chronicity of allergic airway hyperreactivity and inflammation, since the elimination of this fungal material from these mice reverses these major features [8].

Chemokines are major immune factors that affect almost every type of immune cell and are responsible for modulating the clearance of *A. fumigatus* from the lungs of immunodeficient [9] and *A. fumigatus*-sensitized mice [8]. Chemokines are a superfamily of about 50 proteins that are divided into four major families that reflect the number of cysteine residues present in the N terminus of the protein and the relative juxtaposition of these residues. Accordingly, the first subfamily is comprised of CC chemokines (referred to as CC chemokine ligands [CCLs] [10]), which contain two adjacent cysteine residues in the N terminus. One cysteine group characterizes the C chemokine group, the CXC group is characterized by two cysteines separated by an amino acid, and fractalkine or C-X3-C chemokine is membrane associated and appears to function as an adhesion-like molecule. Chemokines bind unique G-protein coupled receptors and the chemokine receptor nomenclature follows that of the particular chemokine subfamily that it binds to. For example, CC chemokine receptor-4 (CCR4) only binds CC chemokines, and is shared by CCL17 and CCL22 [11]. Nevertheless, closer examination of these two CCR4 ligands reveals distinct patterns of expression and stability, and hence CCL17 and CCL22 have markedly different roles during inflammatory events such as asthma and allergy [12,13]. One important distinction between these CCR4 ligands is highlighted by the ability of CCL17 to prevent the activation of macrophages [14] whereas CCL22 is a potent activator of macrophages in many tissue sites [15].

Experimental strategies that have been successfully employed to eliminate fungal material from the lungs of *Aspergillus* sensitized mice include eradicating IL-13 [16] or CCL5-responsive cells [17], and genetic deletion of CCR1 [18], CXCR2 [19], or CCR4 [20]. Consistent to all of these therapeutic modalities is the finding that the resolution of allergic airway inflammation and hyper-reactivity is directly linked to enhancement of the pulmonary innate immune response elicited by macrophage and/or neutrophils. Further evidence supporting the importance of the innate immune response directed against *Aspergillus* in the allergic lung is highlighted by the observation that A. *fumigatus*-sensitized mice deficient in CCR2 (the receptor for

CCL2) are highly susceptible to *Aspergillus* growth and these mice exhibit markedly enhanced allergic airway disease [21]. Table 1 summarizes the allergic and anti-fungal events regulated by chemokines and chemokine receptors during experimental fungal asthma.

Role of CCR4 in chronic fungal asthma due to *Aspergillus*

The role of CCR4 in experimental allergic airway disease remains controversial, given findings showing no role for this receptor in acute allergic responses to ovalbumin (OVA) [22], while targeting of either of its two ligands markedly attenuates many features of OVA-induced allergic airway disease [23,24]. Although mice genetically deficient in CCR4 (CCR4 −/−) develop OVA-induced allergic airway disease similarly to their wild type counterparts [25], our findings in these mice were particularly revealing in regard to the critical importance of CCR4 in the link between innate and acquired immunity during chronic fungal asthma [20]. At all times after the conidia challenge in wild type mice, CCR4 was prominently expressed on the airway epithelium and in mononuclear cells present in the alveolar space and around inflamed airways.

Fig. 1 highlights the magnitude of CCR4 expression in whole lung sections stained at day 14 after the conidia challenge into *A. fumigatus*-sensitized mice. *Aspergillus*-sensitized CCR4 −/− mice exhibited enhanced serum amounts of IgE and IgG in response to the conidia challenge compared with the wild type (CCR4+/+) groups at days 3 and 7 after the conidia challenge, but the IgE response was not maintained in CCR4 −/− mice when examined at day 30 after conidia. The CCR4 −/− mice had a greatly accelerated elimination of fungal material, which stains black in Gomori methenamine silver (GMS)-stained slides. Fungal material was present in CCR4+/+ mice at all three examinations (days 3, 7, and 30), while fungal material was only present in the knockouts at day 3 after conidia. Airway hyper-responsiveness was measured using a direct ventilation Buxco plethysmograph system and it was apparent that CCR4 −/− mice exhibited a biphasic airway response. At day 3, these mice showed markedly enhanced airway responses to a systemic methacholine challenge, whereas at the later time points (i.e. days 7 and 30 after conidia) the airway response was significantly attenuated in the knockout group compared with the control group at the same time after conidia. A biphasic change in Th2 cytokines was also observed in CCR4 −/− mice; immunoreactive whole lung levels of IL-4, IL-5 and IL-13 were significantly elevated at day 3 whereas at day 30 the levels of all three cytokines were significantly lower compared with the appropriate whole lung sample from the wild type groups. Whole lung IFN-γ levels were significantly elevated in the CCR4 −/− group relative to the CCR4+/+ group at day 3, but at day 30 no differences were noted between the wild type and knockout groups of mice. At all time points after conidia challenge, the numbers of eosinophils around the airways of CCR4 −/− mice were many-fold lower than the eosinophil quantity detected around the airways of the CCR4+/+ group. Thus, the introduction of live *Aspergillus* conidia into *Aspergillus*-sensitized mice lacking CCR4 initiated events that led to accelerated fungal clearance, enhanced IgE and IgG1, and exacerbated airway hyper-responsiveness at day 3. Nevertheless, this response was short-lived and most of the features of allergic airway disease including the airway reactivity, peribronchial eosinophilia and the Th2 profile were absent in *A. fumigatus*-sensitized CCR4 −/− mice at later times after the conidia challenge.

Table 1 Role of CC and CXC chemokine receptors on airway inflammation, airway remodeling and airway hyper-responsiveness during chronic fungal asthma in mice. The alterations in airway appearance, structure, and physiology summarized below were analysed at day 30 after *Aspergillus fumigatus* conidia challenge into *A. fumigatus*-sensitized mice

Chemokine receptor Gene knockout	Major ligands that bind chemokine receptor	Effect on airway inflammation	Effect on airway remodeling	Effect on airway hyper-responsiveness
CCR1	CCL3	⇓	↓	—
CCR2	CCL2	⇑	↑	∧
CCR4	CCL17, CCL22	⇓	↔	∨
CCR5	CCL5	⇓	↓	∨
CCR8	CCL1	⇓	↔	∨
CXCR2	CXCL2, CXCL3	⇓	↓	∨

Symbol schema:
Airway inflammation: ⇑ = increased, ⇓ = decreased.
Airway remodeling: ↑ = increased, ↔ = unchanged, ↓ = reduced.
Airway hyper-responsiveness: ∧ = increased, — = unchanged, ∨ = reduced.

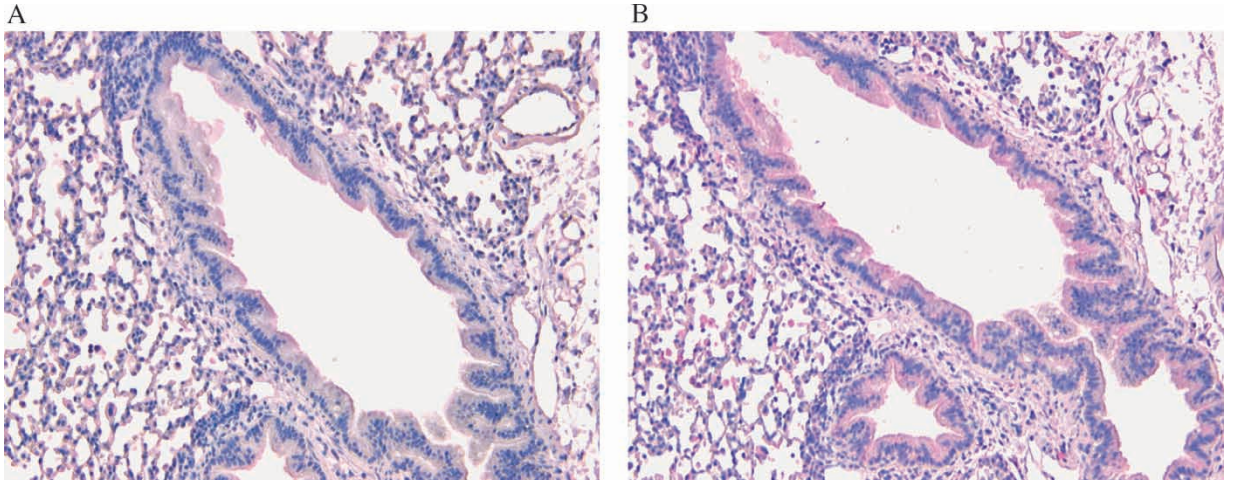

Fig. 1 Immunohistochemical identification of CCR4 ligand in whole lung sections from *Aspergillus fumigatus*-sensitized mice at day 14 after conidia challenge. (A) Control IgG staining; (B) CCR4 staining. The presence of CCR4 (red staining in right panel) was particularly prominent in the airway epithelium, and in mononuclear cells present around the airway and in the alveolar space. Control staining (using a control IgG) is shown in the left panel. Original magnification was ×200.

How does CCR4 activation modulate the innate and acquired immune responses to *Aspergillus*?

At present we are exploring the manner in which chemokine-chemokine receptor interactions regulate the magnitude and duration of innate and acquired immune responses directed against *Aspergillus*. As mentioned above, CCL17 has important immunomodulatory effects on macrophages [14] whereas CCL22 is a potent activator of macrophages [15]; it is not, therefore, immediately clear why CCR4 −/− mice rapidly clear *Aspergillus* from lung and other tissue compartments. Accordingly, we are exploring whether CCR4 activation (within either the macrophage or the neutrophil) affects the expression and/or function of given pattern recognition receptors (PRRs) that bind conserved molecular structures shared by microorganisms such as fungi. We have preliminary data showing that CCL17, but not CCL22, inhibits the expression of toll like receptor-2 (TLR2) and TLR4 by macrophages. This finding is significant in light of the importance of both TLRs in the innate immune response against *Aspergillus* [26,27]. More recently, we have begun to

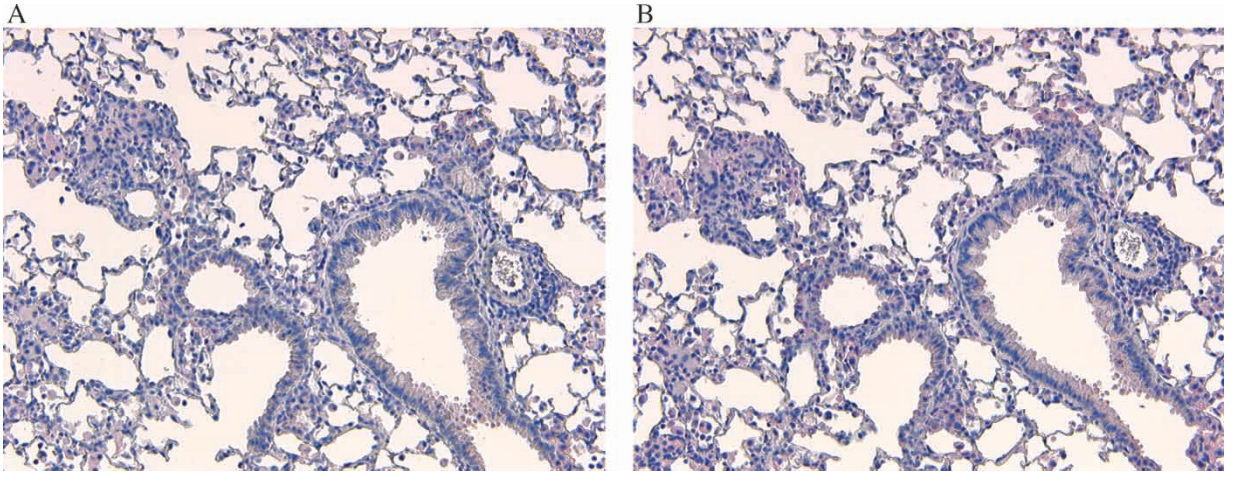

Fig. 2 Immunohistochemical identification of TREM-1 ligand in whole lung sections from *Aspergillus fumigatus*-sensitized mice at day 7 after conidia challenge. (A) Control IgG staining. (B) TREM-1 staining. The presence of TREM-1 (red staining in right panel) was prominent in mononuclear cells present around the airway and in the alveolar space. Control staining (using a control IgG) is shown in the left panel. Original magnification was ×200.

examine the hypothesis that the enhanced innate immune response observed in CCR4 −/− mice is related in part to the expression and/or activation of triggering receptor expressed on myeloid cells (TREM-1) [28]. TREM-1 belongs to a immunoglobulin superfamily that is restricted in its expression to neutrophils and monocytes [29]. The precise nature of the pathogens that trigger activation of TREM-1 and its intercellular adapter protein, DAP-12 [30], are not known but the presence of *Aspergillus* appears to strongly upregulate the expression of TREM-1 [31]. Prior to our studies, nothing was known about the role of TREM-1 in regulating the innate and acquired immune responses to *A. fumigatus*.

An immunohistochemical survey of whole lung sections from *A. fumigatus*-sensitized mice at various times after *A. fumigatus* conidia challenge revealed that TREM-1 was restricted in its expression to mononuclear and polymorphonuclear cells in the lung (Fig. 2). To explore the role of TREM-1 and DAP-12, we employed an adenoviral approach used recently by Nochi *et al*. [32]. Two adenovirus gene vectors were examined: the first contained a DAP-12 transgene (abbreviated AdDAP-12) and the second contained the extracellular domain of mouse TREM-1 and the Fc portion of human IgG1 (AdTREM-1Ig). With these adenovirus vectors this group showed that adenoviral-mediated DAP12 gene transfer sustained and enhanced zymosan-A-induced granuloma formation. In contrast, the soluble form of extracellular domain of TREM-1 remarkably inhibited zymosan A-induced granuloma formation [32]. When these vectors were administered concomitantly with the conidia challenge in *A. fumigatus*-sensitized mice, and airway disease was assessed at day 7 after conidia, those mice that received AdDAP-12 showed significantly less airway inflammation, airway hyper-responsiveness and fungal material compared with the group that received the control adenovirus. Conversely, TREM-1 blockade significantly enhanced allergic airway disease and the presence of fungal material in the lungs of AdTREM-1Ig-treated mice compared with the control adenovirus group at day 7 after virus and conidia challenge. The link between CCR4 and TREM-1 has yet to be confirmed, but we observed that lung lavage levels of CCL17 and CCL22 were significantly increased in the AdTREM-1Ig-treated group. CCL17, but not CCL22, lavage levels were reduced by the co-administration of AdDAP-12 with conidia 7 days previously. Thus, these findings show that TREM-1 and DAP-12 have significant roles in the elimination of *A. fumigatus* from the lungs of allergic mice, and this in turn directly impacts the magnitude of the allergic airway disease present in these mice. Further investigation is required to determine the manner in which CCL17 and/or CCL22 influence the expression and/or function of TREM-1 in the innate anti-fungal response.

Future directions

Given our experimental data showing that modulation of the pulmonary innate immune response via the targeting of pro-allergic factors described above, such strategies may be appropriate in the context of allergic asthma. The added benefit of modifying the innate immune response within the lung environment may also extend to the elimination of other persistent asthma-inducing agents including pollens, house dust mite, cockroach antigens, and viruses. The advent of interesting data pertaining to the asthma-modulating roles of PRRs, such as TLR2 [33] and, from our ongoing studies, TREM-1, suggest that other immunotherapeutic strategies may soon be available that enhance the ineffective or inappropriate immune responses elicited during allergy and asthma.

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