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ANIMAL MODELS OF ALLERGIC BRONCHOPULMONARY ASPERGILLOSIS

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1. ABSTRACT

Experimental animal models of Allergic Bronchopulmonary Aspergillosis (ABPA) serve several purposes. Both common and distinct pathological features occurring in natural and experimental diseases are of great interest as they serve to identify the key elements in the pathogenesis. Experimentally induced diseases can be modeled to understand the various parameters such as antigen and route of exposure, genetic background and the role of response modifiers in the disease process. Furthermore, animals with targeted gene-deletion or with insertion of transgenes have been studied to define the roles of specific cells, receptors and mediators in the pathogenesis. The resulting conclusions have been used to formulate hypothesis, which have to be tested for their application to human disease.

2. INTRODUCTION

Aspergillus fumigatus (Af) a widely distributed spore bearing fungus, causes multiple diseases in humans (1, 2). These diseases include invasive pulmonary aspergillosis, aspergilloma, and different forms of hypersensitivity diseases. There is increased exposure to growth of Aspergillus species and other molds in buildings due to airtight constructions. Where ever water leakage occurs that leads to excessive dampness and resultant fungal growth. In these buildings, the incidence and severity of chronic airway disease including asthma and allergic conditions appears to be increased (3-9). *Aspergillus* pneumonia and systemic aspergillosis occur primarily in patients who are immuno-suppressed (T-cell or phagocyte impairment), or who suffer from cystic fibrosis (10-13). The immunodeficiency detected in these patients

may be congenital, acquired or iatrogenic. Chronic granulomatons diseases, neutrophil dysfunction and severe immunodeficiency (e.g. due to infection with the AIDS virus) contribute to the development of the predominantly fatal infection with Af . The absence of a T helper 1 (Th1) cytokine response is thought to be the critical contributor to the susceptibility to infection with Af while antibody responses do not appear to be critical for protection (14).

Hypersensitivity lung diseases include allergic asthma, hypersensitivity pneumonitis, allergic fungal sinusitis and allergic bronchopulmonary aspergillosis (ABPA); all result from the exposure to allergens of Af. *Aspergillus* spores on inhalation trigger an IgE mediated allergic inflammatory response in the bronchial airways leading to bronchial obstruction and asthma (15). The immune response to Af antigens in these asthmatic patients is characterized by a Th2 response (16-20). Hypersensitivity pneumonitis is characterized by dyspnea due to pulmonary restriction and "influenza like" syndrome due to fever and fatigue (15). Serum IgE titers are usually very low in hypersensitivity pneumonitis and eosinophilia is often insignificant. During the acute phase of hypersensitivity pneumonitis infiltration of neutrophils have been detected, while during the chronic phase the inflammatory cells are represented predominantly by T-cells and macrophages. This disease is the result of a predominant Th1 type of response in contrast to other allergic diseases caused by *A. fumigatus* (15, 21).

Hypersensitivity lung disease due to Af develops from sensitization with fungal allergens that are present in the environment. Development of allergy to Af antigens depends on the mode and frequency of exposure. Sensitization to Af antigens usually occurs in combination with other aeroallergens. In atopic individuals exposure to fungal spores and hyphal fragments leads to the production of specific IgE (19-21).

Allergic bronchopulmonary aspergillosis (ABPA) is a disease primarily occurring in patients with asthma or with cystic fibrosis (22-24). This disease is characterized by a variety of clinical and immunological responses to antigens of Af, which colonizes the bronchial tree of patients. Some immunological manifestations are peripheral blood eosinophilia, immediate cutaneous reactivity to Af antigen, elevated total serum IgE, precipitating antibody to Af, elevated specific serum IgE and IgG antibodies to Af, and increased serum Interleukin (IL)-2 receptor concentrations (1, 25, 26). The hyphae of Af that grow saprophytically in the bronchial lumen result in persistent bronchial inflammation and lead to bronchiectasis in patients with asthma. The bronchiectasis is frequently proximal in the central two-thirds of lung field on high-resolution examinations.

Animal models have been developed to study the precise function of cells and mediators in the pathogenesis of allergy or infection with Aspergillus species.

3. MODELS USING *A. FUMIGATUS* EXTRACT & *A. FUMIGATUS* CONIDIA

Currently over 20 recombinant allergens from *A. fumigatus* have been cloned and expressed. In spite of the recombinant antigens available in pure form, soluble, crude Af antigen extract is still most commonly used for the induction of the experimental disease by intranasal instillation in animals. Spores, or plastic beads coated with crude antigen extract challenged intranasally are good inducers of respiratory pathology as well (27-29). In order to combine the infectious and allergic features of ABPA, recent models have employed priming with Af antigen extract followed by challenge with live Af conidia (30-32). The crude extract is a mixture of culture supernatant and mycelial extract. The exclusive use of infectious material such as spores has been reported as well (33, 34). Most protocols use exposures to Af antigens over a period of 2-5 weeks, the longest reported experiments span over 10-12 weeks (35).

Most of the major antigens associated with ABPA appear to be constituents of the crude extract (36-39). Analysis of the crude extracts by Western blotting using sera from patients with ABPA reveal bands which correspond in size to known antigenic components, including Asp fI, a ribotoxin, proteases, and carboxidases (32,33). In addition, crude extracts can contain toxic molecules including hemolysin, fumitremorgin, fumigallin, helvolic acid, and gliotoxin (40, 41). Extracts containing Asp f 1 are highly toxic for mice, as are some of the other toxins. Thus, the extract is highly antigenic and contains biologically active substances.

Interestingly, potent sensitization to the extract occurs in the absence of exogenous adjuvants independently of the route used for priming (36, 42). The various toxins and enzymes such as proteases present in the extract may serve as adjuvants, perhaps by inducing epithelial damage and allowing normally excluded antigens to bypass the mucosal barrier. As proteases have been implicated to induce Th2 responses, they may be involved in skewing the response to *A. fumigatus* to a more allergic phenotype (43-46).

3.1. Immune and inflammatory response

In *Aspergillus* induced allergy in humans, a strong T helper (Th) 2 response with pronounced antibody production and eosinophilia is detected (17, 19, 47-52). Mice sensitized with Af antigen recapitulate the antibody response (predominantly of IgE, and IgG1 isotypes), the eosinophilia and the response of Th2 cells (35, 36). Furthermore, primed mice that are challenged with Af antigens intranasally develop prominent airway hyperreactivity (42), goblet cell hyperplasia (53) and peribronchial, subepithelial fibrosis (31, 54). Therefore, the mouse models of injury due to challenge with Af antigen in sensitized mice allow to address the question which cells and mediators are most critical for each of the different signs of injury.

3.1.1. Lung inflammation

Exposure of mice with Af antigens (or conidia) induces a strong eosinophilic inflammation in the lungs that persists over several weeks (27-29, 34, 35, 37, 42, 55, 56). Microscopic changes differ in severity depending on the route of sensitization, the frequency of sensitization, the form of the antigen, and the mouse strain. Pathologic lesions tend to be multifocal but can become more diffuse in severely affected animals. Airway lesions are characterized by emigration of eosinophils and

Table 1. Type of inflammation, immune response and airway response during the time course of sensitization with *A. fumigatus* antigens

Mouse Strain	Time Points (Time Periods) of Analysis				
	1	2	3	4	5 (may be seen at 4)
C57BL/6	neutrophils	neutrophils, (fewer relative to #1)	eosinopils, Th2 cells	as # 3 & serum IgE	as # 4 & airway hyperreactivity
129 SvEv	neutrophils	neutrophils & eosinophils	eosinophils, Th2 cells	as # 3 & serum IgE	as # 4 & airway hyperreactivity

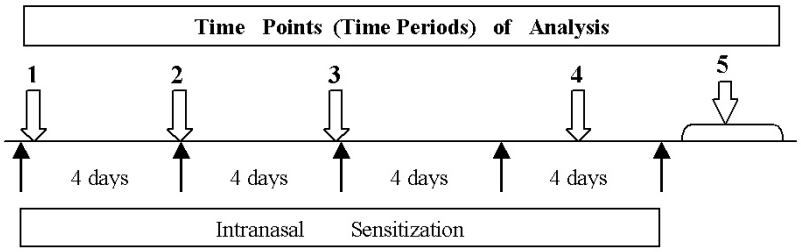

Figure 1. Outline of the intranasal sensitization process.

mononuclear cells into the lumen, goblet cell hyperplasia, and in severe cases epithelial metaplasia and mucus accumulation. Inflammatory infiltrates are found in the interstitial tissues of the airways, blood vessels, and alveoli. They consist primarily of lymphocytes and eosinophils, accompanied by smaller numbers of macrophages, plasma cells, and neutrophils. In cases of severe lung inflammation, alveolar septa are thickened by inflammatory cells and multinucleated giant cells accumulate. Accumulation of collagen is frequently noted at the sub-epithelial area. Fibrotic lesions appear to increase in chronic models of exposure to Af antigen or Af conidia. Lesions disappear within a few weeks after exposure to Af antigen, lesions persist for a longer time in mice challenged with Af conidia most probably due to the persistence of antigen as the clearance from the lungs of the Af organisms proceeds at a slow rate (27).

Analysis of bronchoalveolar lavage (BAL) samples reveals that naive mice have a strong neutrophil and macrophage response after the first intranasal exposure to Af antigens (55). Depending on the mouse strain, a predominant eosinopilic BAL exsudate is already seen after the second intranasal exposure to Af antigens (Table 1, Figure 1). 129 SvEv mice switch faster to eosinophilic inflammation as compared to C57BL/6 mice (G. Grunig, unpublished). The degree of eosinophilia tends to be very high, eosinophils comprise more than 50 % of the BAL cells. Three to 4 days after the second exposure to Af antigens, significantly increased numbers of T cells capable of expressing cytokines are present in the BAL fluid as well as in the lung tissues, both in C57BL/6 mice and in 129SvEv mice (G. Grunig, unpublished). Activated macrophages and some neutrophils are also constituents of the BAL in primed mice challenged with Af antigen. If intranasal exposures to Af antigens are continued for a prolonged period of time, numbers of mononuclear cells in the BAL fluid increase and numbers of eosinophils decrease although they remain well above saline treated controls. In the lung tissues, the accumulation of mononuclear cells is more prominent relative to eosinophils.

Recent studies have addressed the molecular pathway involved in the migration of inflammatory cells into the airways (Table 2) (57). Egression of inflammatory cells into the airway lumen is critically dependent on the expression of the metalloproteinase MMP2 (57). In mice lacking MMP2, inflammatory cells accumulate in the lung tissues. These mice are prone to Af antigen induced asphyxiation (57). These data indicate a protective effect of the egression of inflammatory cells into the airway lumen.

Analysis of the role of single cytokines (e.g. IL-5) (36, 53), chemokines (e.g. RANTES/ CCL5) (58), or enzymes (MMP2 (57)) has allowed to determine how these molecules affect inflammation. Further research is needed to determine how the co-ordinate expression of cytokines, chemokines, proteinases and adhesion molecules orchestrates the inflammatory response.

3.1.2. Airway hyperreactivity

Sensitization with Af antigens consistently induces very strong airway hyperreactivity (29, 36, 42, 53, 56). The relative potency of Af antigens in inducing airway hyperreactivity appears to be increased compared to other antigens, such as ovalbumin. Af antigens have been used successfully to induce airway hyperreactivity in all of the inbred strains of mice tested thus far (C57BL/6, 129 SvEv, BALB/c and crosses of these strains). In mice that are sensitized via the intranasal route, airway hyperreactivity is clearly apparent following the fourth or fifth intranasal challenge (Table 1) (G. Grunig, unpublished). While we understand the cytokine requirements for airway hyperreactivity, the molecular pathway that leads to airway hyperreactivity is unknown (59-61).

3.1.3. Airway remodeling

Challenge of primed mice induces very pronounced goblet cell hyperplasia and mucus overproduction (27, 53,

Table 2. Molecules that are critical determinants of inflammation and cell recruitment in response to *A. fumigatus* antigens or *A. fumigatus* organisms

Molecule	Mode of Activity	Role in Af induced lung disease
MMP2 (57)	Metalloproteinase	Removal of inflammatory cells by facilitating egression into the airways
ICAM-1 (37)	Adhesion Molecule (ligand for LFA1 & MAC1(CD11a/b-CD18)	Correlated with the numbers of T cells that accumulate in the lungs
CCR5 and RANTES (58)	Chemokine receptor and ligand	Accumulation of T cells in the lungs, inflammation
C10 / CCL6 (100)	Chemokine (ligand of CCR1)	Critical for development of inflammation
CCR1 (54)	Chemokine receptor (for among others RANTES, MIP1α)	Critical for T cell and macrophage response, and for clearance of Af organisms
CCR2 (31) and MCP-1 (101)	Chemokine receptor and ligand	Macrophage recruitment and response, neutrophil recruitment, critical for clearance of Af organisms in primed mice.
CXCR2 (30, 110)	Chemokine receptor (for among others GRO/KC, LIX)	Neutrophil recruitment, critical for clearance of Af organisms in naïve mice. Clearance of Af organisms normal in primed mice but critical for the regulation of the T cell response and airway hyperreactivity.

61, 62). In mice that are sensitized via the intranasal route, goblet cell hyperplasia is clearly apparent a few days after the second exposure to Af antigen. In mice that were primed intraperitoneally, large numbers of goblet cells are apparent 24-48 hours following intranasal exposure to Af antigens. T cells by making IL-13 are critical for the induction of goblet cell hyperplasia in mice challenged with Af antigens (61) or Af conidia (59, 60). However, the molecular pathway by which mucus overproduction is induced in mice challenged with Af antigen is unknown. Critical components may include the EGF receptor pathway (63) or calcium-activated chloride channels (64).

Subepithelial fibrosis is another hallmark of airway remodeling. This fibrosis is particularly prominent in mice chronically exposed to Af antigens, or Af conidia (27). Relatively little is known about the molecular events that contribute to subepithelial fibrosis in mice exposed to Af antigens. IL-13 appears to play an important role (60), however, Af antigen induced fibrosis also occurs independently of IL-13 (32).

3.2. T cell response

To our surprise, T cells and the mediators released by T cells are the most critical components of the response that induce the asthma phenotype (antibody response, eosinophilia, airway hyperreactivity, goblet cell hyperplasia, and sub-epithelial fibrosis) in mice challenged with Af antigens. The role of T cells was demonstrated using mice with targeted disruption of the recombinase activating genes (RAG deficient) (53). As recombinase is critically involved in the gene rearrangement of immunoglobulins and T cell receptors, and these molecules in turn are critically important for B and T cell development, RAG deficient mice are devoid of mature B and T cells. After exposure to Af antigens, RAG-deficient mice failed to develop significant lung lesions or airway hyperreactivity. When RAG-deficient mice were reconstituted with highly purified CD4+ T cells from naïve mice, sensitization induced eosinophilic inflammation of the lungs and airway hyperreactivity (53). Thus CD4+ T cells are essential for the development of peribronchial, perivascular, and alveolar lesions and also for airway hyperreactivity in experimental ABPA. The lack of severe lesions in RAG-deficient mice exposed to *Aspergillus* was surprising, as the components of the Af antigen extract are thought to have the capability to induce tissue injury and to activate cells of the innate immune system. An inflammatory component was observed in RAG-deficient mice exposed to Af antigens because quite a substantial number of eosinophils were found in broncho-alveolar lavage (BAL) samples (about 20-30 fold more than in unchallenged mice, and approximately 3-5 fold less than in sensitized wild type mice) (53).

As predicted from the predominant IgE and IgG1 responses induced by sensitization with Af antigens, the dominant T cell response in wild type mice to Af antigens is of the Th2 type (14). The T cell response has been analyzed using different methods. Upon restimulated *in vitro* with immobilized anti-CD3 antibodies, cell suspensions from sensitized but not from control mice produce significant amounts of Th2 cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13 (28, 42). The type 1 cytokine, Interferon gamma (IFN-gamma), on the other hand, was not made at increased levels when controls and sensitized mice were compared. Similar results have been obtained from the analysis of lung T cells for the presence of intracellular cytokines, or from the examination of bronchoalveolar lavage fluids for secreted cytokines (42). Compared with controls, sensitized animals have a larger number of Th2 lymphocytes in the lung tissue, and antigen challenge induces the release of Th2 cytokines into the airway lumen. Immunohistochemical analysis has shown that exposure to Af antigens induces peribronchial and perivascular infiltration of T cells that make IL-4, or IL-5 (38).

Little is known about the molecular determinants of T cell migration and homing in the lungs of mice exposed to Af antigens. ICAM-1, an adhesion molecule of

Table 3. Determinants of the immune response to *A. fumigatus* antigens or *A. fumigatus* organisms –cytokines and complement

Molecule	Mode of Modulation	Role in Af induced lung disease
IL-13 (60, 61)	Inhibitors	• Inhibits all signs of asthma (airway eosinophilia, airway hyperreactivity, goblet cell hyperplasia, peribronchial fibrosis) as long as lesions are associated with Th2 responses. Does not induce Th1 responses.
IL-4 (29, 36, 40)	Inhibitors, gene deficient mice	• When given prior to priming with Af antigen, development of Th2 response inhibited and development of Th1 response enhanced. Inhibition of IgE, and airway eosinophilia.
IL-5 (36, 53, 98)	Inhibitors, gene deficient mice	• Absolutely critical for the infiltration of the lungs with eosinophils and for airway eosinophilia.
IL-10 (42, 90)	Gene deficient mice	• Role dependent on the genetic background and on the route of sensitization. • Inhibits inflammation and cytokine production (both Th1 and Th2 cytokines). • Inhibits response that promotes clearance of Af organisms.
IFNγ (61, 42, 90 105, 106)	Model of mixed T cell responses, gene deficient mice	• Changes the phenotype of inflammation and airway hyperreactivity (more variable, less eosinophils, less goblet cells), changes the importance of critical mediators (IL-13 inhibition has a much attenuated effect), critically important for the clearance of Af organisms.
Complement C3 (65)	Gene deficient mice	• Critical for the full development of the Th2 response, antibody response, and airway hyperreactivity

he immunoglobulin superfamily, may be a candidate as the number of T cells in the lung tissues of mice exposed to Af antigens were positively correlated with ICAM-1 expression (Table 2) (37). The chemokines that bind to the chemokine receptors CXCR2 and CCR5 may likewise be involved, as mice deficient in these chemokine receptors have decreased numbers of T cells in the airways following challenge with Af conidia (Table 2) (30,58).

The molecular events through which Af antigens prime for strong Th2 responses have become more clear recently. Utilizing the model of transferring T cells from naïve mice into RAG deficient mice, we discovered that the response in these mice was of a mixed kind (Th1 and Th2 cells accumulated in the lungs at approximately even ratios, although differences were seen among individual mice) (61). We first tested whether the numbers of transferred T cells, or the activation state of the transferred T cells (quiescent – P-selectin negative; or activated – P selectin positive) was critical for the development of the T cell response. We found that numbers or types of T cells that were transferred only marginally affected the outcome of the T cell response (G. Grunig, unpublished). Instead, molecules of the innate immune system appear to be critical for the outcome of the T cell response to Af antigens. The complement cascade is one of these critical components (Table 3) (65). Mice deficient in the complement component C3 had reductions in Th2 cytokine secretion, in IgE and IgG1 antibody levels and did not develop airway hyperreactivity. These mice did not develop a Th1 response to Af antigens because no IFN-gamma could be detected and IgG2a antibody titers were not increased (65). Other components of the innate immune system, for example Toll Like Receptors (TLR), specifically TLR2 (66), or TLR4 (67) appear to be also critical for the regulation of the cytokine response to Af organisms.

In conclusion, to our surprise, T cells are effectors of the asthma phenotype in response to Af antigens (Figure 2). As elaborated in the next section, in the lungs of Af antigen challenged mice, T cells make cytokines that directly interact with epithelial cells, fibroblasts, smooth muscle cells, and endothelial cells (68), as well as with cells of the immune system. Furthermore, the type of the T cell response that develops to Af antigens appears to be dependent on the environment of antigen presentation as determined by the innate immune system.

3.3. Cytokine response

Since many years, cytokines like Interleukin (IL)-4, 5, 10, 13, or Interferon gamma (IFN-gamma) have been recognized as critical determinants of T cell responses (69-71). T helper 2 (Th2) cells are important for protection against helminth infection and when produces in a dis-regulated manner induce asthma and allergies. Th2 cells make among others IL-4, 5, 10 and 13. Th1 cells make IFN-gamma and are important for protection against intracellular pathogens and when produced in a dis-regulated manner induce chronic inflammation (e.g. inflammatory bowel disease). T regulatory cells (Tr1 or Th3) suppress the effects of Th1 and Th2 cells. T regulatory cells make IL-10 and TGF beta. However, all of these cytokines can also be made in large, biologically relevant quantities by cells other than T cells such as NK cells, eosinophils, or basophils. Therefore, frequently, IL-4, 5, or 13 are called 'type 2 cytokines' while IFN-gamma is called a 'type 1 cytokine'.

3.3.1. IL-13

IL-13 is the major mediator of the Af antigen induced airway hyperreactivity, lung inflammation, airway eosinophilia, goblet cell hyperplasia, and airway fibrosis (Table 3) (59-61). This was demonstrated using different types of IL-13 inhibitors: a neutralizing antibody to IL-13 (60), or a construct of two high affinity receptor chains (IL-13 receptor alpha 2, IL-13R alpha2 molecules) coupled to the Fc moiety of an antibody molecule (IL-13R alpha2-Fc) (61). The anti-IL-13 antibody neutralizes the activity of IL-

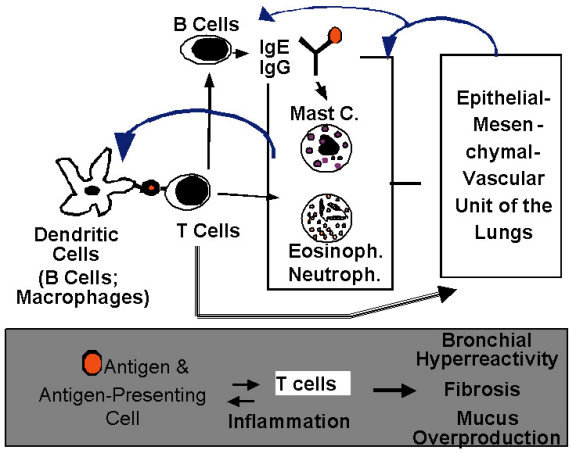

Figure 2. Injury induced by *Aspergillus fumigatus* antigens: Schematic representation of the cellular components of the pathogenesis. T cells by making cytokines, e.g. IL-13, IL-4, IL-5 are critical effector cells of the lung injury. T cells interact with lung resident cells (e.g. epithelial cells), and with the other cells of the immune system. The T cell response is initiated by antigen presented by antigen presenting cells (e.g. dendritic cells). The response of B cells, eosinophils, mast cells, and neutrophils is an important determinant of the environment of antigen presentation. Lung resident cells are critical determinants of the inflammatory response by producing chemokines and other mediators.

13 (60). The soluble IL-13R alpha2-Fc molecule has very high affinity for IL-13 and also has a low release rate of the bound IL-13, thereby being very effective at neutralizing IL-13 *in vivo* (72-75). These inhibitors were highly effective when given prophylactically (to primed mice during the time of intranasal challenge with Af antigen) (61), or when given therapeutically after lung challenge had been initiated (60). The role of IL-13 binding cells was examined using IL-13 coupled to Pseudomonas exotoxin (IL-13PE). Il-13PE binds to cells that have the IL-13 receptor and delivers a cytotoxic molecule that kills the cells. The destruction of cells that bind IL-13 by using IL-13PE also inhibited the asthma phenotype in response to Af antigens (59). In contrast, neutralization of IL-4 (using an antibody) during the intranasal challenge period in primed mice, only had mild effects, e.g. reduction in the numbers of BAL eosinophils, or reduction of IgE antibody titers (36,60, G. Grunig, unpublished). Mice given anti-IL-4 antibody developed a concomitant Th1 response with elevated levels of IFN-gamma and IL-12 (60). Therefore, the reduction in eosinophil numbers by anti-IL-4 antibody treatment may be due to the down-regulation of IL-5 production due to the relative reduction of Th2 cells in these mice, or due to a shift in the chemokine balance in these lungs that follows shifts in the balance of Th2 to Th1 cytokines. IL-13 neutralization, while potently inhibiting the asthma phenotype, failed to show enhanced induction of cytokines associated with Th1 responses (IFN-gamma or IL-12) (60).

The clear hierarchy of the functions of IL-4 and IL-13 was surprising. Following exposure to Af antigens in mice both IL-4 and IL-13 show significant increases in the lungs (60, G. Grunig, unpublished). Furthermore, both cytokines have a large overlap in their biological functions (76). IL-4 reacts with a broader variety of cells, including T and B cells, while in the mouse, IL-13 does not react with T or B cells due to the absence of the critical IL-13 specific receptor chain (76). Therefore, the expectation was that neutralization of IL-13 alone would only have minor effects on the Af antigen induced asthma phenotype. The clear hierarchy – critical role of IL-13 as effector molecule of Af antigen induced changes in the lungs – could be due to a tight temporal and spatial regulation of the secretion of IL-13 and IL-4, or due to a tight regulation of the expression of the IL-4 and IL-13 receptors (77). IL-13 and IL-4 share the IL-4 receptor alpha (IL-4Ralpha) chain, and partially, the IL-13 receptor alpha 1 (IL-13R alpha1) chain; IL-4 but not IL-13 utilizes the IL-2 receptor gamma (common gamma) chain, while IL-13 but not IL-4 binds IL-13R alpha2 (77, 78). The expression of IL-13R alpha1 was shown to be elevated in the airways of primed mice after exposure to Af conidia while the expression of the IL-13R alpha2 chain was constitutive in the lungs from naive mice and downregulated in primed mice exposed to Af conidia (59, 60).

The signaling pathway used by IL-13 to induce airway eosinophilia and goblet cell hyperplasia involves the IL-4Ralpha chain with subsequent activation (phosphorylation) of the STAT6 signaling molecule (32). In contrast, the chronic airway hyperreactivity and peribronchial fibrosis that develop in response to Af conidia were not dependent on STAT6. However, in STAT6 deficient mice challenged with Af conidia, depletion of IL-13 binding cells with IL-13PE decreased airway hyperreactivity, and further reduced inflammation but not airway fibrosis (32).

The dominant role of IL-13 was seen in the mouse models that were characterized by development of Th2 responses in the lungs as shown before using other models of Th2-induced lung injury (74, 75, 79). In contrast, blockade of IL-13 was relatively much less effective in a model of Af antigen-induced lung injury characterized by mixed T cell responses (Table 3) (61). T and B cell-deficient RAG-/- mice that were reconstituted with T cells from naïve wild type mice developed mixed T cell responses (simultaneous presence of Th1 and Th2 cells) in the lungs to challenge with Af antigen. Blockade of IL-13 in these mice resulted in a trend of lower airway hyperreactivity, modestly reduced goblet cell hyperplasia, but did not reduce airway inflammation. The reduced effect of the IL-13 inhibitor is most probably due to a change in the type of inflammation. Most likely, a distinct set of cytokines, chemokines, and cells is induced in lungs injured by mixed T cell responses. This inflammation due to mixed T cell responses was characterized by increased neutrophils and IL-6 when compared to inflammation induced by a polarized Th2 response. NK cells are also likely to be major constituents of inflammation induced by mixed T cell responses (61).

3.3.2. IL-4

IL-4 is critical for the IgE response in mice sensitized with Af antigen (Table 3). The IgE response was

arrested or retarded by the administration of anti-IL-4 antibody (29, 36). However, no significant difference was detected in the levels of IgG1. A marked increase in the IgG2a antibody levels in these animals were also observed, indicating that anti-IL-4 antibody induced a more pronounced Th1 response, as IgG2a levels are IFN-gamma dependent. Thus, anti-IL-4 antibody treatment resulted in a suppressed Th2 response and an enhanced Th1 response. Similarly, a Th1 type of response was also detected in IL-4 deficient mice that were challenged with Af antigen. In these mice, no IgE was demonstrable after Af antigen challenge, but considerable enhancement in the levels of IgG2a were detected (29, 36). The lung injury observed in IL-4 deficient mice may be due to IL-13, or to eosinophil mediators and IFN-gamma, but not due to IL-4 and IgE (36). In this model we have observed eosinophil infiltration in the lung tissue, while surprisingly no mRNA for IL-5 was detected. IL-4 deficient mice challenged with Af antigen expressed mRNA for RANTES, suggesting a role for this chemokine in the recruitment of eosinophils (58). It is possible that eotaxin (80) and GM-CSF (38, 81) may also be involved in the recruitment of eosinophils and in the maintenance of these cells in the lungs.

3.3.3. IL-5

IL-5 is critical for the eosinophilia seen in mice sensitized with Af antigen (Table 3) (36,53). Neutralizing anti-IL-5-monoclonal antibody injected intraperitoneally partially abrogated the eosinophilia in all three compartments, namely peripheral blood, lung, and bone marrow, suggesting a major role for IL-5 in eliciting eosinophilia (36). Multiple anti-IL-5 antibodies were effective in maintaining baseline levels of blood eosinophils. Injection of multiple anti-IL-4 antibodies also down-regulated eosinophils in bone marrow, lungs, and peripheral blood though to a lesser extent than in mice injected with anti-IL-5 antibody (36).

3.3.4. IL-10

Endogenous IL-10 inhibited inflammation in mice sensitized with Af antigens (Table 3) (42) Depending on the mouse strain examined or on the route of priming, endogenous IL-10 inhibited the Th2 response to Af antigens, or both the Th2 and Th1 response to Af antigens (42). This finding is in keeping with the function of IL-10 as a potent immunosuppressive molecule that is constitutively produced by bronchial epithelial cells (82). IL-10 is a powerful inhibitor of Th1 responses in mice (83). IL-10 inhibits cytokine production of Th1 and Th2 lymphocytes in humans (84). IL-10 is thought to exert its suppressive function by inhibiting antigen presenting cells (84); such as dendritic cells (85) or macrophages (86). IL-10 is thought to be a major cytokine required for the development and function of regulatory T cells (87-89). While the major role of endogenous IL-10 in the response to Af antigen was to inhibit lung inflammation (42), the specific role of IL-10 varied depending on the mouse strain examined and on the route of sensitization. In a mouse (cross of C57BL/6 and 129 SvEv) that developed a mixed T-cell response (Th2 and Th1), endogenous IL-10 prevented mortality, and restricted the secretion of IL-5 and IFN-gamma into the airway lumen. In C57BL/6 mice primed intraperitoneally and challenged intranasally with Af antigens, endogenous IL-10 limited IL-5 production and lung eosinophilia. However, in the same mouse strain (C57BL/6), when the mice were exposed to Af antigens (primed and challenged) only by the intranasal route, endogenous IL-10 had no detectable effect on the response to Af antigens most probably due to compensatory mechanisms (42). In exaggerated T cell responses due to the lack of IL-10, IFN-gamma clearly appears to be harmful, as only the mouse strain that developed a strong mixed T cell response (IFN-gamma together with Th2 cytokines) to Af antigen showed increased mortality. Due to its potent immunosuppressive effects, IL-10 inhibits the clearance of microbial infections, including the clearance of Af organisms (90). Because of its complex role, the therapeutic use of IL-10 will most probably rely on the utilization of IL-10 expressing T regulatory cells, or on dendritic cells engineered to express IL-10.

3.4. Antibody response

The role of antibodies in the pathogenesis of the Af antigen induced asthma phenotype is still not clear. This question was addressed using mice with targeted deletions of the epsilon and mu-chains (53, 56). 129SvEv epsilon-chain-deficient mice are not capable of making IgE. C57BL/6 mu-chain-deficient mice do not only lack antibodies of all isotypes but also mature B cells. Sensitization with Af antigens via the intranasal route induced histological lesions in the lung, which were similar in quality and extent in both types of gene targeted mice relative to wild type mice. These lesions consisted of perivascular and peribronchial infiltrates, as well as some interstitial inflammation, and goblet cell hyperplasia (53, 56). Furthermore, airway hyperreactivity in response to challenge with Af antigens developed to a similar extent in primed B cell deficient mice as compared to primed wild type mice (53, 56). The numbers and type (Th2) of cytokine producing T cells in the lungs of sensitized B cell deficient mice was indistinguishable from wild type mice (53, G. Grunig, unpublished). In another model of asthma, using priming and challenge with Ovalbumin (OVA) B cell deficient mice developed eosinophilic airway and lung inflammation, as well as mucus overproduction (91) but airway hyperreactivity was diminished (92). In a monkey model of ABPA, allergic human serum or control serum was infused into primed animals. Inhalation of Af antigens led to the development of severe lung lesions typical of ABPA only in the monkey, which had received allergic human serum (93). However, this monkey failed to demonstrate airway hyperreactivity. Together, these data suggest that the antibody response to Af antigen is not critical for most or all of the Af antigen-induced changes in the lungs. This is surprising because of the strength and consistency of the antibody response in the animal models. Therefore, it is likely, that we do not yet understand the critical function of the antibody response in animals sensitized to Af antigen. It is possible that the major function of the antibody response is not in the effector arm of the immune response by orchestrating inflammation, but instead in the initiation and shaping of the T cell response by regulating and directing antigen presentation (Figure 2) (33, 94-96).

3.5. Eosinophil response

As eosinophil emigration into the airways and infiltration into the interstitium of the lungs is a prominent feature of human and experimental ABPA, many studies have addressed the role of eosinophils in the pathogenesis of the asthma phenotype induced by Af antigens (Figure 2). Thus far, the answer to this question is not clear.

Wild type mice exposed intranasally to Af antigens develop not only lung eosinophilia, but also blood and bone marrow eosinophilia (35, 36, 97-99). If these animals are treated with neutralizing antibodies to IL-5, the bone marrow, blood, and lung eosinophilia is abolished. GM-CSF is another cytokine that can activate and maintain eosinophils in the tissues. It has been shown that GM-CSF producing T cells accumulate in the lungs of animals exposed to Af antigens (38). However, GM-CSF must be ordered in hierarchy behind IL-5, because endogenous GM-CSF production cannot support eosinophilia in animals treated with neutralizing anti-IL-5 antibodies (36, 53, 98) or in IL-5 gene-deficient mice (53).

Although eosinophils have been shown *in vitro* to secrete mediators that are proinflammatory or cytotoxic, the role of these cells in the response to Af antigens is still unknown. Eosinophils were not critical for the asthma phenotype that developed in response to Af antigens. IL-5 deficient animals, or animals treated with anti-IL-5 antibodies developed the full spectrum of peribronchial, and perivascular inflammation (albeit without eosinophils) as well as goblet cell hyperplasia, and airway hyperreactivity (53). The inflammatory infiltrates consisting of mononuclear cells instead of eosinophils were just as substantial as in wild type mice. The changes in the airway epithelium (goblet cell hyperplasia) were still present to the full extent. Furthermore, eosinophils were not required to mediate airway hyperreactivity as IL-5 deficient animals or mice treated with neutralizing anti-IL-5 antibodies developed airway hyperreactivity similar to wild type mice when exposed to Af antigens (53). However, all these experiments were performed in short-term models of lung injury due to exposure to Af antigens. It is possible that the role of eosinophils is to promote lung injury that occurs in the chronic disease that stretches over the span of many years.

3.6. Chemokine response

Recently a number of chemokines and chemokine receptors were evaluated for their role in the pathogenesis of allergic aspergillosis (27, 30, 31, 54, 58, 80, 100, 101). The lungs of mice challenged with Af antigen or Af conidia express a number of chemokines, including C10 / CCL6, MDC / CCL22, MIP-1alpha / CCL3, MCP-1 / CCL2, RANTES/ CCL5, or eotaxin / CCL11. This large spectrum of expressed chemokines is remarkable, however, Th2 cytokines such as IL-13 induce the expression of a characteristic spectrum of chemokines in the lungs (102,103, G. Grunig, unpublished). Many of these chemokines are made by non-hematopoietic cells such as epithelial cells, fibroblasts, smooth muscle cells, or endothelial cells (Figure 1).

The role of chemokines and adhesion molecules in mediating lung eosinophilia has not been investigated thoroughly in experimental ABPA. It has been reported that mRNA for RANTES is upregulated following sensitization of mice with *A. fumigatus* antigen (20). It will be of great interest to determine the role of eotaxin as it has been shown to be a major chemoattractant for eosinophils in the lungs (46). MIP-1a, RANTES and MCP-4 / CCL13 are other chemokines which may be instrumental in eosinophil recruitment to the lungs (12,47-49). The ability of eotaxin to induce eosinophil accumulation is still dependent on IL-5 as the infusion of eotaxin at a dose, which will elicit eosinophilia in wild type mice, does not cause eosinophilia in IL-5 deficient animals (50).

3.6.1. C10 / CCL6

C10 signals through the chemokine receptor CCR1 and is chemotactic for T cells, B cells, and monocytes (100). C10 production is increased in macrophages after stimulation with IL-4, or IL-13 but not after stimulation with Th1 cytokines (104). Neutralization of C10 using a specific antibody abolished the induction of airway hyperreactivity, ablated the development of peribronchial and perivascular inflammation and reduced airway eosinophilia (Table 2). (100). These studies showed that C10 plays a major role in the orchestration of inflammation and airway hyperreactivity induced by Af antigen.

3.6.2. CCR1

CCR1 is the chemokine receptor for, among others, MIP-1alpha (MIP-1alpha) / CCL3, and RANTES/ CCL5. CCR1-deficient mice had increased numbers of lymphocytes and macrophages in the BAL fluid four weeks after challenge with Af conidia (Table 2) (54). IFN-gamma levels in the lungs of CCR1 deficient mice were significantly higher than in the wild type mice, and the Th2 response was attenuated. The Th2 inducible chemokines C10, eotaxin and MDC were significantly lower in CCR1 deficient as compared to wild type mice. Nevertheless, primed CCR1 deficient mice challenged with Af conidia developed airway hyperreactivity and serum IgE levels to a similar degree when compared to wild type mice. In contrast, CCR1 deficient mice appeared to clear the Af organisms more rapidly than wild type mice. This is in accord with data that show that Th1 responses and IFN-gamma are essential for the clearance of Af organisms (90, 105-108). CCR1 deficient mice did not develop goblet cell hyperplasia or subepithelial fibrosis (54). Together, these results clearly show that CCR1 is critical for the development of the T cell response and for airway remodeling in mice exposed to Af (54).

3.6.3. CCR2 & MCP-1 / CCL2.

CCR2 is among others the receptor for MCP-1. Studies using a neutralizing antibody to MCP-1 or mice deficient in CCR2 clearly showed phase dependent roles for MCP-1 and CCR2. Clearance of Af organisms was delayed in CCR2 deficient mice as well as in mice in which MCP-1 was neutralized right before the intranasal inoculation of conidia (Table 2) (31,101). Concomitant

with the decreased clearance, the mice had increased numbers of eosinophils in the bronchoalveolar lavage, increased airway inflammation, airway hyperresponsiveness, and sub-epithelial fibrosis. Immunoneutralization of MCP-1 during days 14 to 30 after conidia challenge in mice that had been sensitized with Af antigen did not affect clearance of the fungus. However, airway hyperresponsiveness, goblet cell hyperplasia, lymphocyte recruitment to the lungs, and lung IL-4 levels were attenuated. Overexpression of MCP-1 followed by challenge with Af conidia increased fungal clearance and decreased airway hyperreactivity and airway inflammation.

CCR2 deficient mice that were sensitized with Af antigens had increased serum IgE levels as compared to wild type mice (31). Upon challenge with Af conidia, CCR2 deficient mice had a progessive increase in the burden of Af organisms in the lungs. The CCR2 deficient mice had an initial reduction in the recruitment of macrophages into the airways and a persistent major defect in neutrophil recruitment together with increased recruitment of lymphocytes, and eosinophils (31). In the lungs, levels of Th2 cytokines (IL-5, IL-13) were increased as well as levels of eotaxin, RANTES and MDC, while IFN-gamma levels were decreased. The CCR2 deficient mice maintained much increased serum IgE levels, increased airway hyperreactivity, and subepithelial fibrosis as compared to wild type mice (31). Thus, CCR2 plays a critical role in the type of T cell response that develops to Af antigen and Af conidia (increased Th2 response, and decreased Th1 response). The exaggerated Th2 response together with the defect in neutrophil recruitment is most probably the cause for the decreased clearance of the organism (90, 105-108) and the persistent, increased asthma phenotype.

3.6.4. CXCR2

CXCR2 is the chemokine receptor for, among others, GRO/KC, and LIX/CXCL5. GRO/KC and LIX are neutrophil attractant chemokines (109). Inhibition of CXCR2 by neutralizing antibodies resulted in a drastic reduction in neutrophil recruitment into the lungs and concomitantly in a decreased clearance of organisms in a model of invasive pulmonary aspergillosis (Table 2) (110). CXCR2 deficient mice that were given Af conidia intratracheally succumbed to lethal invasive aspergillosis while wild type mice cleared the fungus. Surprisingly, CXCR2 deficient mice that were primed with Af antigen and then challenged with Af conidia were protected from infectious aspergillosis. In fact, primed CXCR2 deficient mice appeared to have a slightly increased clearance of the fungus when compared to wild type mice. The antibody response (IgE and IgG1) was increased in CXCR2 deficient mice throughout the experiment. CXCR2 had transiently (at early time points) increased airway hyperreactivity that was dependent on the neutrophil response. Throughout the experiment, CXCR2 deficient mice had lower levels of IL-4, IL-5, RANTES, and eotaxin in the lungs. Levels of IFN-gamma were slightly increased and levels of IP-10 / CXCL10, MIG / CXCL9, and neutrophil myeloperoxidase were significantly increased in CXCR2 deficient mice during the early phase of the anti-Af conidia response. IP-10 and MIG are typical chemokines induced in the course of a Th1 response (103), both chemokines utilize CXCR3 (109). The neutrophil response was dependent on the presence of IP-10 and MIG as shown using neutralizing antibodies (30). Four weeks after challenge with Af conidia, CXCR2 deficient mice had less goblet cell hyperplasia and less peribronchial and perivascular inflammation relative to wild type mice. However, granulomatous interstitial lesions were seen in CXCR2 deficient but not in wild type mice. These data demonstrate a complex modulation of the immune response to Af antigens and Af conidia that is mediated by CXCR2.

3.6.5. CCR5 and RANTES / CCL5

While RANTES is one of the ligands of CCR5, CCR5 is also only one of the receptors for RANTES. RANTES and CCR5 are critical for the recruitment of T cells. CCR5 deficient mice had an attenuated asthma phenotype in response to challenge with Af conidia (Table 2) (58). In CCR5 deficient mice, airway hyperreactivity developed only transiently and could be further abolished by neutralizing RANTES. CCR5 deficient mice showed transient goblet cell hyperplasia, and diminished subepithelial fibrosis. Inflammation in response to Af antigen was further decreased by neutralizing RANTES in CCR5 deficient mice. In CCR5 deficient mice, eotaxin and C10 expression were reduced in the lungs, as well as peribronchial inflammation. A similar, or slightly reduced degree of perivascular inflammation was seen in CCR5 deficient mice as compared to wild type mice. These data identify CCR5 and RANTES as molecules that are critically involved in orchestrating lung injury to Af antigen and Af conidia.

4. MODELS USING RECOMINANT ANTIGENS

The majority of the animal model studies of ABPA have been carried out in mice using crude Af antigen extracts or intact Af organisms, particularly the spores, or conidia. In recent years a number of relevant allergens from Af have been identified and the corresponding cDNAs have been cloned, sequenced and expressed (19, 51, 111-120). Several of these allergens bound specifically to serum IgE from patients with ABPA and their disease specificity has been established. However, only few of these major allergens have been investigated in murine models to understand their specific role in the pathogenesis of ABPA.

Four major allergens of Af namely Asp f 1, 3, 4, and 6 were investigated in a murine model (121). Mice exposed to Asp f 1, 3, and 4 showed inflammatory changes in the lungs and airway hyperreactivity. The immune responses demonstrated included elevated serum IgE, marked production of allergen specific IgE, peripheral blood, lung and BAL eosinophilia, and cytokines typical of a Th2 type. Asp f 6 failed to induce any airway hyperreactivity and invariably induced less inflammatory response in the lungs and an overall truncated antibody response. In another study, Asp f 2 was shown to induce diminished responses similar to what had been seen with Asp f 6. However, when Asp f2 was combined with Asp f

13, an alkaline serine proteinase, marked inflammatory, immune and airway responses were induced (Kurup VP, unpublished). Thus, it is clear that such studies will yield more useful information on the individual allergens and cumulative effects of the combination of different allergens. These studies will lead to a better definition of the pathomechanisms of the disease. This knowledge may eventually contribute to initiatives for controlling the disease.

5. IMMUNOTHERAPY

Specific immunotherapy and vaccination are probable means for controlling Th2-mediated diseases. However, the challenge with the immunotherapy to Af is that the disease has two components, an infectious and an allergic component. For example, immunotherapy aimed at establishing broad immunologic tolerance to Af antigens may result in increased susceptibility to infection.

5.1. Recombinant allergens & peptides

Selected recombinant allergens of Af were used to establish immunologic tolerance (122, 123). These candidate allergens for immunotherapy need further evaluation. As an alternative for intact or slightly modified recombinant allergens/ peptides from specific Af allergens have been evaluated (124). Two peptides, Peptide #5 and Peptide #10 derived from Aspf 1 were identified that induce distinct cytokine responses. Peptide #5 NGYDGNGKLIKGRTPI elicited a Th2 type cytokine response while Peptide #10 KVFCGIVAHQRGN elicited a Th1 cytokine response (124). Mice immunized with two other epitopes of Asp f 1 by the intravenous route protected mice from elaborating a harmful immune response upon subsequent challenge with Af antigen (122). While these results support the potential role for synthetic peptides as immunotherapeutic agents in allergic aspergillosis, this avenue requires further study.

5.2. Immunostimulatory oligonucleotides

In recent years, a number of studies have emphasized the usefulness of immunostimulatory CpG oligonucleotides (ISS-ODN, CpG-ODN) in vaccination against IgE mediated allergy (125-127). Immunization with DNA based vaccines such as plasmid DNA of major Af antigens or Af protein antigen co-administered with CpG oligonucleotides resulted in a predominantly Th1 biased immune response instead of the usual Th2 immune response. However, the mechanism by which CpG injected animals are protected from the development of Th2-mediated tissue injury is still not understood. Neutralization of major type 1 cytokines (e.g. IFN-gamma, or IL-12) did not abrogate the protective effect of CpG injections. However, increased amounts of CpG oligonucleotides were required to elicit a protective response in mice deficient in IFN-gamma and IL-12 (126). CpG injections also protected against Af antigen induced lung lesions (127). BALB/c mice were immunized intraperitoneally with alum precipitated Af culture filtrate antigens at three-day intervals for three times. This was followed by two intranasal exposures at five-day intervals and animals were sacrificed 24 hours after the last antigen challenge. For immune intervention, a second group of mice was immunized first with CpG oligonucleotides (50 microg/animal) followed by IP immunization with Af antigen. Two more doses of CpG oligonucleotides were administered in between the intranasal challenges. A third group of animals received only one dose of CpG oligonucleotides (35 microg/animal) after the last IP antigen immunization. On sacrifice, sera were studied for specific antibodies and peripheral blood for eosinophils. Lung inflammation, goblet cell hyperplasia, and antigen induced cytokine expression in the lungs were also analyzed. There was a three-fold increase in serum IgG2a in CpG oligonucleotides treated group compared to mice treated with antigen alone. Blood eosinophil counts in mice treated with CpG oligonucleotides were significantly less compared to mice in the antigen-only group. Peribronchial and perivascular eosinophilic infiltrates were reduced as well as goblet cell hyperplasia in mice treated with CpG oligonucleotides when compared to control mice. These data suggest that CpG oligonucleotides may represent a possible candidate for immunotherapy in allergic aspergillosis (127).

6. FUTURE PERSPECTIVES

Results of the murine model studies clearly demonstrate the central role of $CD4^+$ Th2 cells in the allergic response to Af. However, no animal model captures all of the aspects of Af induced hypersensitivity diseases such as ABPA. Furthermore, there is no animal model comparable to ABPA in cystic fibrosis patients. Although the major features of ABPA in cystic fibrosis and adult ABPA show similarities, they show considerable differences as well. Hence, any translation of the animal model results to the human disease requires additional studies in humans.

In experimental ABPA, along with the Th2 response, a Th1 immunopathological response can also occur. The type of T cell response that develops depends on the specific component of the antigen that is immunodominant, on genetic factors determined by the mouse strain, on the environment as determined by the innate immune response in which sensitization to allergen occurs, and on other, not yet well understood factors. The antibody response, eosinophil differentiation and chemotaxis, T and B-cell infiltration and activation are part of the overall response detected in the models and are comparable to human ABPA. The cytokines, chemokines, their receptors and various other factors produced by cells of the immune system are also needed for the full expression of the disease. Thus, a clear understanding of the mechanism of lung injury in the animal models may aid in devising strategies (e.g. altering or stopping of regulatory signals) that effectively control disease. Because effective therapeutic strategies for ABPA must accomplish downregulation of the allergic response and increasing the response required to clear Aspergillus organisms, a multi-faceted attempt may prove most beneficial.

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Abbreviations: ABPA- Allergic Bronchopulmonary Aspergillosis; Af- Aspergillus fumigatus; BAL- bronchopulmonary lavage; CCR- CCR-chemokine receptor; CXCR- CXC-chemokine receptor; IFN; Interferon; IL- Interleukin; R- receptor; Ig- immunoglobulin; Th- T helper

Key words: Animal Models, Allergic Bronchopulmonary Aspergillosis, ABPA, T cells, Cytokines, IL-13, IFN-gamma, Eosinophils, B cells, Chemokines, Chemokine Receptors, Review

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