Clearance of *Aspergillus fumigatus* is impaired in the airway in allergic inflammation

Running Title: Clearance of *Aspergillus fumigatus* is impaired in asthma

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ABSTRACT

Background: *Aspergillus fumigatus* (*Af*) sometimes colonizes and persists within the respiratory tree in some asthmatics. To date, the precise reasons why the clearance of *Af* is impaired in patients with asthma remain unknown.

Objective: To characterize the effects of allergic airway inflammation on clearance of *Af*.

Methods: Control and *Dermatophagoides farinae* (*Df*) allergen-sensitized Balb/c mice were intranasally infected with *Af*. After 2 and 9 days infection, pathology, fungal burden and cytokine profile in lung tissue were compared. In a different set of experiments, the phagocytotic activity of alveolar macrophages (AM) and their pathogen recognition receptors (PRRs) expression were also determined.

Results: *Af* conidia and neutrophilic airway inflammation disappeared by day 9 after infection in control mice. In *Df*-sensitized mice, *Af* conidia and both neutrophilic and eosinophilic airway inflammation persisted at day 9 after infection. When compared to control mice, *Df* allergen-sensitized mice showed significant increases in IL-5 and decreases in IL12 and IFN-γ in lung tissues at day 2 after infection. Most importantly, compared with *Af* infected non *Df* sensitized mice, IL-17 in lung tissues was significantly reduced in *Df* allergen sensitized *Af* infecetd mice at day 2 after infection,

while it significantly increased at day 9. AM isolated from *Df* allergen-sensitized mice exhibited significant decreases in the phagocytotic activity and the expression of both TLR4 and Dectin-1 compared to those in control mice.

Conclusions: In the airway of allergic individuals, Th2-dominant immunity potentially affects the expression of PRRs and attenuates cellular defense against *Af*. Prolonged IL-17 production could also play an important role.

Introduction

Aspergillus fumigatus (*Af*) is a ubiquitous saprophytic mold[1] that forms airborne spores (conidia) that are ubiquitously found in the environment. Humans are thought to inhale hundreds of conidia daily. Inhaled conidia bind soluble receptors, for example, pentraxin-3 and lung surfactant protein D, that enhance inflammatory responses and are then phagocytosed by pulmonary macrophages.[2, 3] Some swollen conidia expressing more ß-glucan on their surfaces are recognized by dectin-1 and TLR2, resulting in the induction of host Th17 response. Consequently, recruited neutrophils and alveolar macrophages kill the conidia.[4-7] In this way, in immune-competent hosts, inhaled *Af* are killed and cleared by cells of the pulmonary immune system immediately. However, in a subset of asthma and cystic fibrosis patients, *Af* colonizes and persists within the respiratory tree and allergic bronchopulmonary aspergillosis (ABPA) occurs. To date, the precise reasons why the clearance of *Af* is impaired in patients with asthma remain unknown. Although many animal studies have indicated that innate immunity plays a critical role in anti *Af* response, several lines of evidence support T cell participation in host defense. In addition, it was recently reported that IL-4, a key cytokine in Th2

differentiation, inhibits both Th1 and Th17 differentiation.[8, 9] Thus, we hypothesized that the Th2-skewed immunity in a murine model of asthma may contribute to impairment of Th1 and Th17 response against *Af*. In the present study, we addressed these issues by comparing fungal burden between *Af* infected control mice (Af mice) and *Af*-infected mite-sensitized murine model of asthma (Af-Df mice), and then, in a different set of experiments, comparing several cytokines, including IL-12, IL-4, IL-23, IFN-γ, IL-5 and IL-17 production between Af mice and Af-Df mice. In addition, the phagocytotic activity against *Af* and expression of pathogen recognition receptors (PRRs) on alveolar macrophages were compared in alveolar macrophages isolated from untreated naïve mice and mite allergen-sensitized mice.

Methods

Preparation of Af conidia

Af MF-13 isolated from the sputum of a patient with pulmonary aspergilloma was prepared for intranasal infection as described previously.[10] *Af* MF-13 was subcultured on Sabourand dextrose agar (Becton Dickinson, Cockeysville, MD) at 30ºC for 7 days. Conidia were then harvested with sterile saline containing 0.02% Tween 80 (Wako Pure Chemical Industries, Tokyo, Japan). The suspension was filtered through a 40-μm cell strainer (Falcon, Tokyo, Japan) to separate conidia from contaminating mycelia and was verified microscopically (100% resting conidia). The suspension was then counted in a hemocytometer and diluted with sterile saline.

Experimental protocol

Female BALB/c mice (age, 5 and 10 wk) were purchased from Charles River (Yokohama, Japan) and housed in a specific pathogen-free facility. As illustrated in **Figure 1**, mice were immunized twice intraperitoneally on days 1 and 14 with 0.5 mg of *Dermatophagoides farinae* (*Df*: crude extract of mite: LG-5339; Cosmo Bio, Tokyo,

Japan) precipitated in aluminum hydroxide. Mice were then challenged intranasally (i.n.) with either 50 μg/50 μL *Df* allergen (Df-Af group) or PBS (Af group) on days 14, 16 and 18. Both groups of mice were i.n. infected with 1×10^5 *Af* conidia on days 19, 21 and 23. Either 2 days (day 25) or 9 days (day 32) after infection, two groups of mice were sacrificed to obtain bronchoalveolar lavage fluid (BALF) and lung tissues. Procedures were reviewed and approved by Nagasaki University School of Medicine Committee on Animal Research. All experiments were repeated at least three times.

Bronchoalveolar lavage and lung pathology

BAL was conducted with 1 mL of PBS in the immediate postmortem period. Obtained BAL samples were centrifuged. Differential cell counts were performed using cytocentrifuged BAL samples stained with May-Grünwald-Gìemsa. Formaldehyde fixative was gently infused through the lavage catheter set in the trachea. Resected lungs were fixed for an additional 24 h and embedded in paraffin. Sections (4 μm) were stained with hematoxylin and eosin (HE). After BAL, paraffin-embedded lung tissues were prepared for hematoxylin and eosin (HE) and gomeri methenamine-silver (GMS)

staining. For fungal-burden examination, numbers of CFU per lung tissue were calculated as described elsewhere.[10]

Analysis of cytokines concentrations in homogenized lung

Lung homogenates were prepared by homogenizing a freshly excised lung. Concentrations of IL-12, IL-4, IL-23, IFN-γ, IL-5 and IL-17 in homogenized lung samples were measured by enzyme-linked immunosorbent assay, in accordance with the manufacturer's directions (Endogen, Wobum, MA).

Phagocytic function of alveolar macrophages

In a different set of experiments, alveolar macrophages (AM) were prepared from naïve mice without any treatment and a murine model of asthma, which were prepared as mentioned above. Lung tissues were chopped with sterile scissors and digested in a 37°C water bath for 2 hours in digestion buffer containing 1.5 mg/mL collagenase A (type 1A; Boehringer Mannheim, Mannheim, Germany), and were filtered with a metal mesh. After washing with RPMI-1640 medium (Gibco-BRL Life Technology, Inc.,

Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (hereafter referred to as cRPMI) three times, cells were resuspended in cRPMI. Mononuclear cells were isolated using a density gradient method with Ficoll (Amersham Pharmacia Biotech). FBS was put into a dish, which was then incubated at 37°C for 15 min. After FBS was discarded, suspended cells were placed in this dish and incubated at 37°C overnight. Thereafter, cells in the dish were collected using PBS containing EDTA. Aliquots (1 mL) of cell suspension (10^6 cells/mL) were mixed with 1 mL of *Af* suspension (10^6 cells/mL) opsonized with 100 μL of normal serum, and were incubated for 60 min at 33°C. Ten minutes before completion of incubation, methylene blue (0.01%) was added. Two hundred conidia were then examined and the number of phagocytosed conidia was counted in three representative regions. Results are expressed as an index representing the percentage of phagocytosed *Af* conidia.

Analysis of expression of TLR4 and Dectin-1 on AMs

In order to determine the effects of PRR expression on AMs on phagocytotic activity, the expression of TLR4 and Dectin-1 on AMs was determined by Real Time RT-PCR. In a different set of experiments, alveolar macrophages (AM) were prepared from Af mice and Df-Af mice on days 25 and 32, as mentioned above. Total RNA was also isolated from each group of AM with TRIzol (Life Technologies, Gaithersburg, MD) using the method recommended by the supplier. A High-Capacity cDNA Archive Kit (Applied Biosystems, Tokyo, Japan) was used to synthesize cDNA from 2 μg of total RNA and 200 ng of cDNA was amplified by primers complementary to the published sequences of murine TLR4, Dectin-1 and control GAPDH. Quantitative real-time PCR was performed on an ABI 7500 (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems). Probes (IDT) labeled with 5' FAM and 3' TAMRA modifications were used at a final concentration of 0.9 mM, and primers were used at 0.2 mM (GIBCO BRL). The PCR program was as follows: 50°C for 2 min and 95°C for 10 min, then 95°C for 15 s and 60°C for 1 min for 40 cycles. Specific signals were normalized against the signals from constitutively expressed GAPDH. Data are presented as relative mRNA units and represents the average of at least three values ±

112 SEM.

113

114 ***Statistical analysis***

115 Results are expressed as means ± standard error of mean (SEM). Differences between

116 groups were examined for statistical significance using repeated-measures ANOVA

117 with a Bonferroni multiple comparison test. p values of <0.05 were considered to be

118 significant.

119

120

Results

Pulmonary inflammation

Representative pulmonary pathologies of the two groups of mice sacrificed on day 25 and 32 following *Af* infection are shown in **Figure 2**. Neutrophilic inflammation was only observed on day 25, and disappeared by day 32 in Af mice. The airways of Df-Af mice exhibited both neutrophilic and eosinophilic inflammation on day 25, which persisted on day 32. Pathological changes were confirmed in a quantitative manner by BAL (**Table 1**). In Df-Af mice, total cell counts were significantly elevated when compared to Af mice on day 32. Irrespective of sacrifice day, airway eosinophils were significantly elevated in Df-Af mice when compared to Af mice. Airway neutrophils were significantly reduced in Df-Af mice when compared to Af mice on day 25, while they were significantly higher in the former compared to the latter on day 32.

Aspergillus fumigatus pathology, fungal burden and phagocytosis

Representative pulmonary pathologies (GMS) of the two groups of mice sacrificed on

days 25 and 32 following *Af* infection are shown in **Figure 3**. *Aspergillus fumigatus* conidia were only found on day 25 and disappeared by day 32 in Af mice. However, *Aspergillus fumigatus* conidia persisted in the airway of Df-Af mice on day 32, and some of these conidia had germinated (**Figure 4**). Quantitative evaluation of fungal burden in lung tissue demonstrated that a significantly higher number of *Af* were present on both days 25 and 32 in Df-Af mice, as compared to Af mice (**Figure 5**). To determine the mechanisms of increased fungal number in Df-Af mice, phagocytosis of *Af* conidia by AM isolated from naïve mice without any treatment and AM isolated from the murine model of asthma was compared. In comparison with AM isolated from naïve mice, those from the murine model of asthma showed a significant decrease in phagocytosis (**Figure 6**). In comparison with AM isolated from Af mice, on day 25, TLR4 and dectin-1expression on AM isolated from Df-Af mice on day 25 showed a significant decrease. In comparison with AM isolated from Af mice on day 32, dectin-1 expression on AM isolated from Df-Af mice at day 32 showed a significant increase (**Figure 7**).

Cytokine profile in lung homogenate

Analysis of cytokine concentrations in lung homogenates, as shown in **Figure 8,** revealed that, in comparison with Af mice sacrificed on day 25, Df-Af mice sacrificed on day 25 showed significant increases in Th2-like cytokines (IL-4 and IL-5) and significant decreases in both Th1-like (IFN-γ and IL-12) and IL-17 production. In Af mice, Th1-like and Th17-like (IL-23 and IL-17) cytokines significantly decreased on day 32, as compared to those on day 25. Th2-like cytokines in Df-Af mice significantly decreased on day 32 when compared to day 25, but were still significantly higher than in Af mice. In marked contrast, IL-17 levels in Df-Af mice increased significantly on day 32 when compared to those on day 25, and Th17-like cytokines in Df-Af mice became significantly higher than those in Af mice.

Discussion

In this study, we showed that after *Af* infection, production of cytokines involved in protective immunity against *Af* and phagocytotic activity of AM was lower in a murine model of allergic asthma. Experimental studies indicate a critical role for macrophages in conidial defense.[11, 12] It has also been reported that TLR4 on macrophages is required for an optimal immune response to *Af* in vivo.[13] In contrast, neutrophils play a predominant role in killing hyphae.[14, 15] In addition, other innate immune cell subsets contribute to antifungal defense. For example, pulmonary dendritic cells transport conidia to draining mediastinal lymph nodes in order to activate fungus-specific T-cells and when *Af* arrives in the airways, *Af*-specific T cells are rapidly primed and fully differentiated into IFN-γ-producing Th1 $CD4^+$ T cells in immune-competent mice. Thereafter, inhaled conidia are rapidly cleared from the airway.[16] In our study, after infection with *Af* conidia, IL-12 and IFN-γ production in the airway and expression of TLR4 on AM were reduced in a murine model of asthma when compared with control mice. In addition, the phagocytosis of *Af* conidia by AM isolated from the murine model of asthma was impaired when compared with AM isolated from untreated naïve mice.

185 This may be due to the preexisting Th2-skewed immunity in asthmatic airways, which
186 inhibit Th1 cytokine production against *Af* infection and relatively lower IFN-γ
187 condition, leading to reduced phagocytosis of *Af* conidia via poor expression of TLR4
188 on AM.

189 In addition to pre-existing Th2-skewed immunity prior to *Af* infection, IL-17 levels
190 became higher following *Af* infection in Df-Af mice. Recently, it was reported that
191 excess Th17 immunity attenuates antifungal immune defense.[17, 18] It has also been
192 reported that Th17 response was initiated thorough the recognition of β-glucan, which
193 increases on the surface of fungi during their growth from conidia to hyphae.[19] In
194 addition, we previously reported that high levels of ligand for dectin-1 receptors induce
195 upregulation of these receptors on antigen-presenting cells and enhanced signaling.[20] It
196 is likely that preexisting Th2 immunity attenuated Th1 immunity, which permitted
197 colonization of conidia in the asthmatic airway in the present study. Subsequently, the
198 growth of conidia to hyphae could further stimulate dectin-1 in the host, thus resulting
199 in higher levels of IL-17/IL-23 production. Persistent colonization of *Af* may keep
200 significantly higher levels of IL-17/IL-23 in the asthmatic airway when compared to

those in controls by continuous stimulation of dectin-1 signaling. It has also been reported that Th17 immunity not only attenuates Th1 immunity, but also up-regulates Th2 immunity.[17, 21] It has also been reported that both protease secreted from *Af* and IL-17 induce enhanced MUC5AC gene expression in airway epithelial cells.[22,23] Collectively, the present study suggests that preexisting Th2-skewed immunity in asthma permits *Af* to colonize in the airway by inhibiting innate anti-fungal defense. Once colonized in the airway, *Af* stimulats excess expression of Dectin-1 and Th17 immunity, which further enhances *Af* colonization by upregulating Th2 immunity and overproduction of mucus in a vicious circle.

On the other hand, other investigators reported that in the murine model of asthma, the ingestion potential of conducting airway neutrophils is enhanced when compared to control mice.[24] Interestingly, in our study, we also found that although colonization of *Af* in the airway of the murine model of asthma was seen, penetration of *Af* into the airway epithelial barrier and dissemination of *Af* into the airway was not seen. The reason for this may be that the enhanced phagocytotic activity of neutrophils in the murine model of asthma controlled the development of colonization of *Af* to dissemination of *Af* in the

217 airway.

218 A distinct characteristic feature of Df-Af mice includes neutrophilic airway
219 inflammation in the present study. In this regard, several studies have indicated that
220 IL-17 is important for neutrophilic inflammation in patients with acute airway
221 inflammation.[25-27] Airway neutrophils were also associated with IL-17 in the lung
222 tissues in the present study. A key characteristic of fungal-associated asthma is the
223 increased severity of asthma. Neutrophilic airway inflammation caused by *Af* may at
224 least partially explain the increased severity of fungus-associated asthma. Indeed,
225 current anti-inflammatory therapies for asthma, including inhaled corticosteroids, are
226 effective in managing eosinophilic airway inflammation, but have little or no impact on
227 neutrophilic airway inflammation.[28, 29] Accordingly, additive treatment, which has an
228 impact on neutrophilic inflammation, is required for fungus-associated asthma. Thus,
229 the development of therapeutic modality targeting IL-17 for the treatment of fungus
230 associated asthma is a critical issue in the future.

231 However, our study has several limitations. First, we only describe the results for mice,
232 and it is uncertain whether these results can be applied to humans. In addition, although

we hypothesized that Th2-dominant immune response may contribute to the impairment of Th1 response against *Af* challenge, we did not directly show whether specific Th2 response inhibition in the murine model of asthma improves the Th1 response against *Af* challenge.

In conclusion, these results support the mechanism of *Af* colonization in the asthmatic airway. Mite allergen sensitization concomitant with *Af* infection enhanced the Th2-dominant immune response in the airway, wherein Th1 response against *Af* conidia infection was attenuated and Th17 response against *Af* was promoted, both of which impair anti-fungal defense and permit further colonization of *Af* in the asthmatic airway. Th17-associated neutrophilic airway inflammation may be involved in the pathogenesis of steroid-resistant severe asthma with fungal sensitization.[30]

Figure legends

Figure 1. Experimental protocol. BALB/c mice were each immunized intraperitoneally on days 1 and 14 with 0.5 mg of *Df* precipitated in aluminum hydroxide. Af group: Mice were sham-sensitized intranasally (i.n.) with PBS on days 14, 16 and 18 and infected i.n. with *Af* on days 19, 21 and 23. Df-Af group: After immunization with *Df*, mice were challenged i.n. with *Df* allergen on days 14, 16 and 18. Subsequently, mice were infected i.n. with *Af* on days 19, 21 and 23. On days 25 and 32, all mice were sacrificed. (n=6 for each)

Figure 2. Pulmonary pathology (HE). Lung tissues were obtained from each group. Representative HE stained photomicrographs of lung tissues from each group (n=6 for each) are shown. A: Af at day 25; B: Af on day 32; C: Df-Af on day 25; and D: Df-Af on day 32.

Figure 3. Pulmonary pathology (GMS). Lung tissues were obtained from each group. Representative GMS stained photomicrographs of lung tissues from each group (n=6 for each) are shown. A: Af on day 25; B: Af on day 32; C: Df-Af on day 25; and D: Df-Af on day 32.

Figure 4. Form of *Aspergillus fumigatus* conidia found in lung tissue from Df-Af mice on day 32. Representative GMS-stained high resolution photomicrographs of *Aspergillus fumigatus* conidia in lung tissue from Df-Af mice on day 32 showed conidia germination.

Figure 5. *Aspergillus fumigatus* fungal burden. Fungal burden in lung tissue from both groups was quantitatively evaluated. Results are expressed as means (n=6 for both groups) ± SEM. ***P* < 0.01 vs. Af.

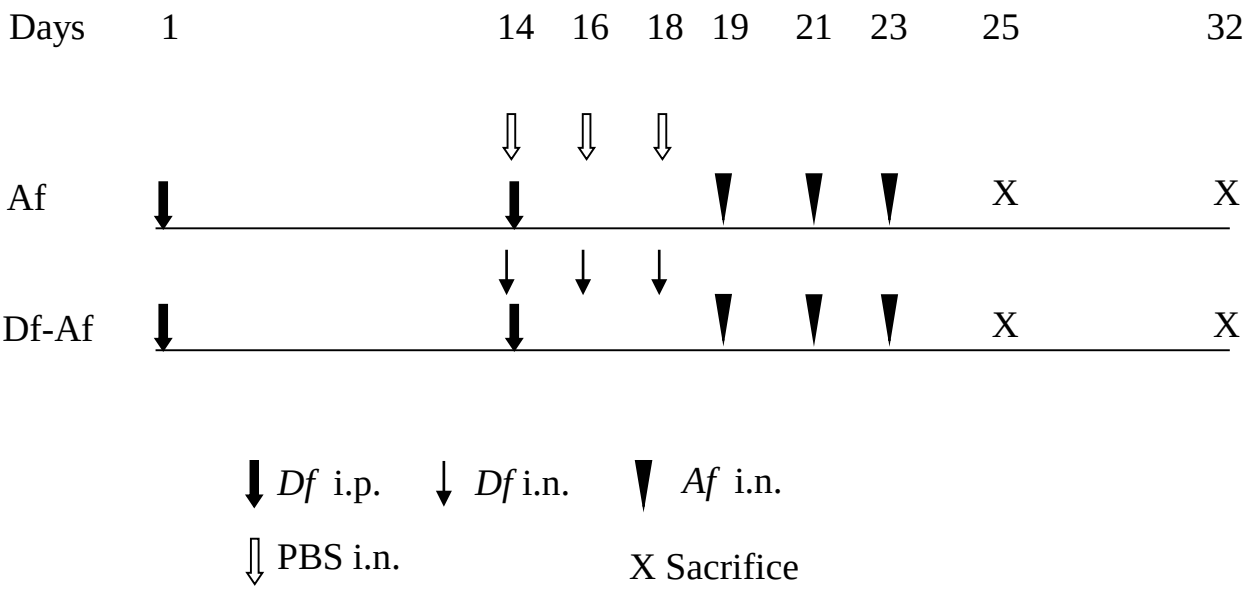
Figure 6. AM phagocytotic activity against *Af* conidia. AM isolated from naïve and a murine model of asthma groups of mice were cultured with *Af* conidia. Number of phagocytosed conidia in each mouse was counted. Results are expressed as an index representing the percentage of phagocytosed *Af* conidia. Bars represent mean values (n=6) ± SEM. *p < 0.01 vs. naïve mice.

Figure 7. Quantitative analysis of TLR4 and Dectin-1 mRNA expression in AM. Expression of TLR4 and Dectin-1 mRNA of AM isolated from Af-mice and Df-Af mice

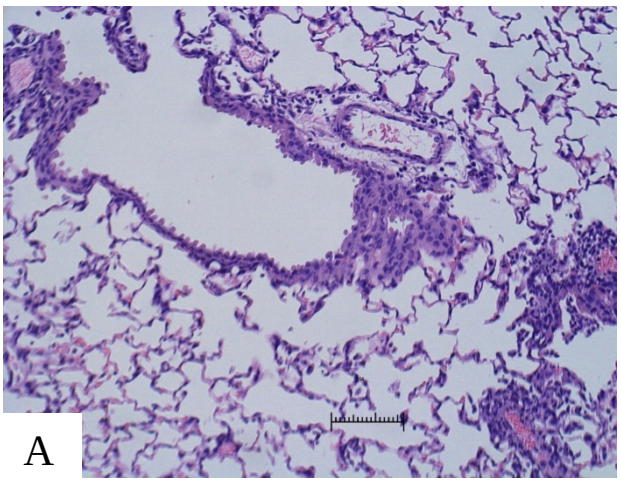
on day 25 and 32 was determined by quantitative real-time RT-PCR and is depicted as the number of transcripts per 10^3 copies of the housekeeping gene GAPDH. Data from experiments with cells from each of the groups are summarized and presented as mean (n=8 for each group) values ± SEM. *$p < 0.01$ vs. Af-mice.

Figure 8. Cytokine profile in lung homogenates. Cytokine concentrations in lung homogenates in each mouse were determined by ELISA. Bars represent mean values (n=6) ± SEM. *$p < 0.01$ vs. Af, †$p < 0.01$ vs. Day.

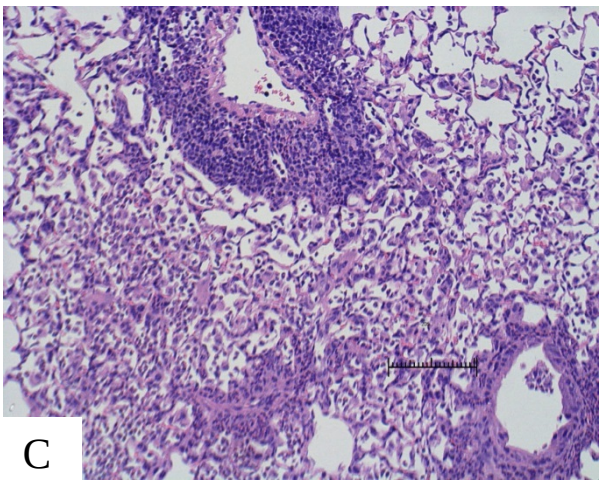
Figure 1


Days
1
14
16
18
19
21
23
25
32
Af
X
X
Df-Af
X
X
Df i.p.
Df i.n.
Af i.n.
PBS i.n.
X Sacrifice

Figur 2



A



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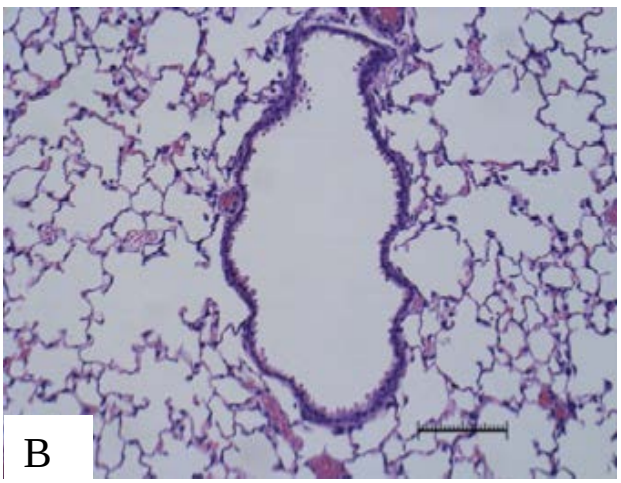
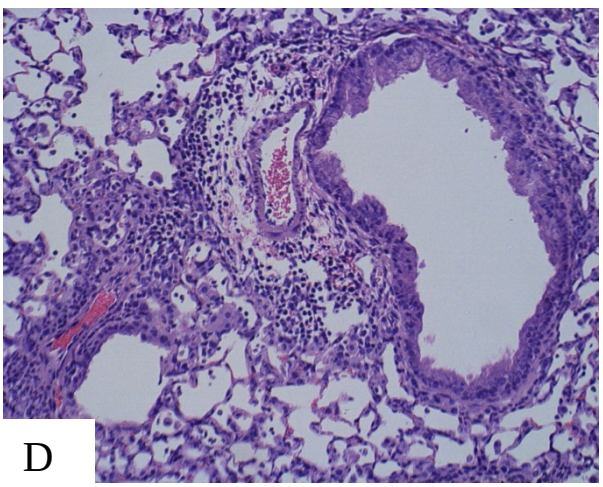
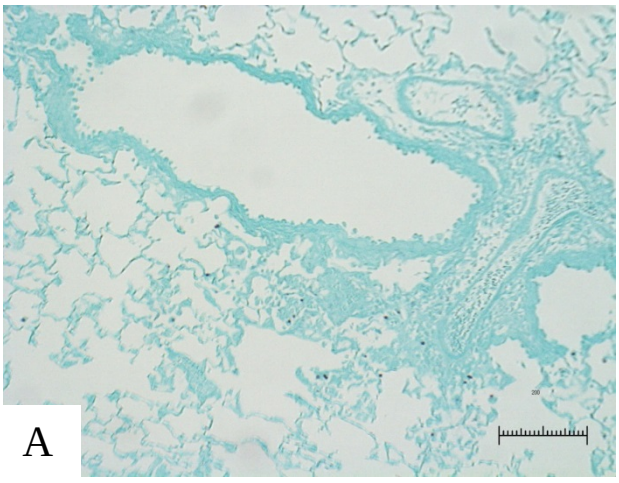


B

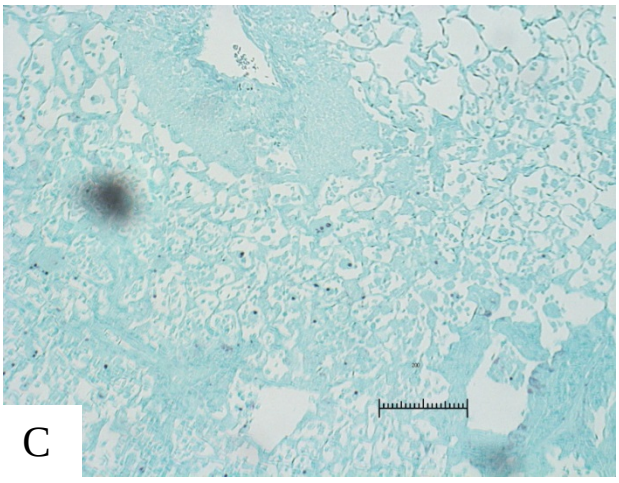


D

Figure 3



A



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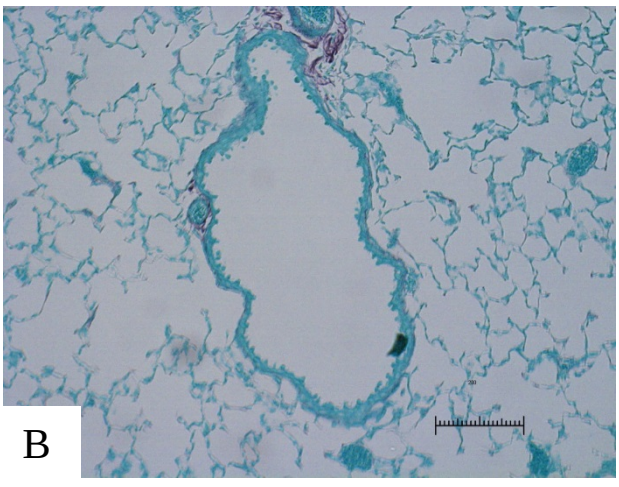

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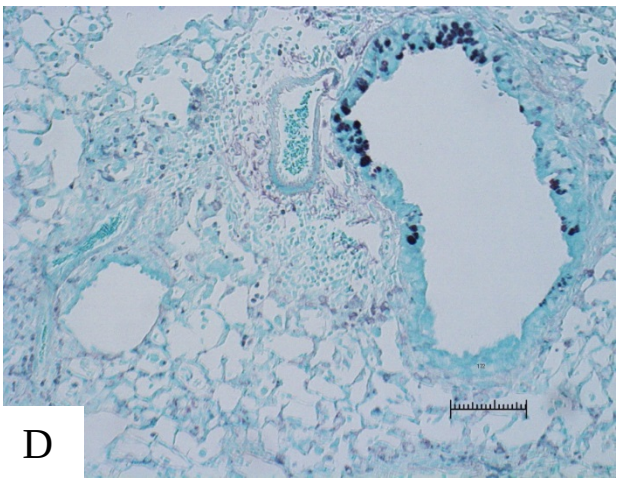
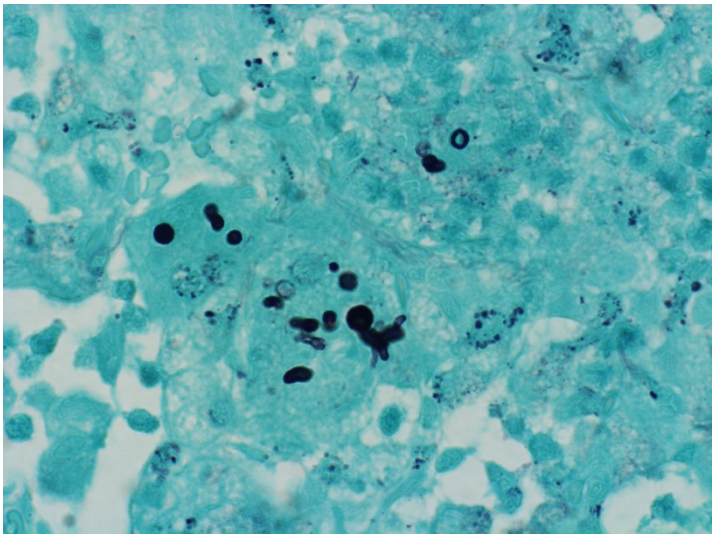


D

Figure 4



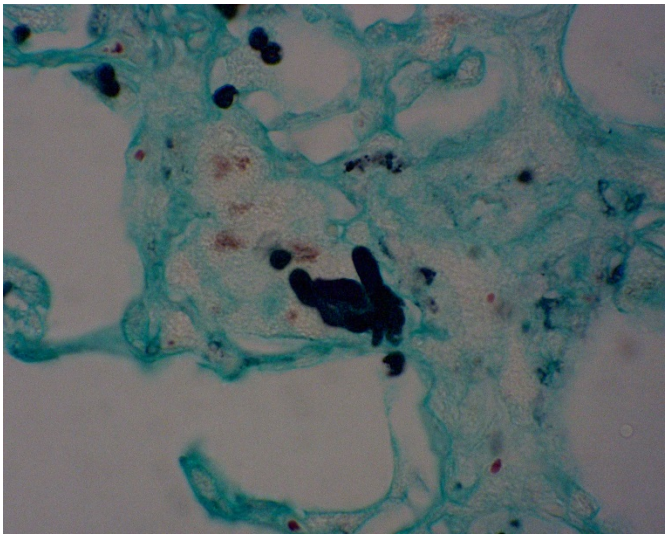


Figure 5

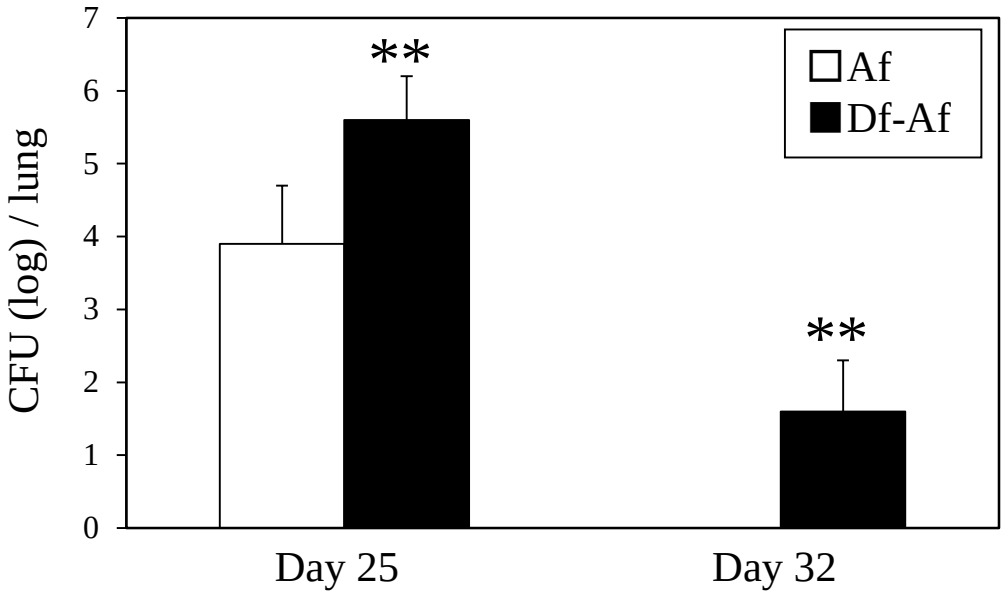


Figure 6

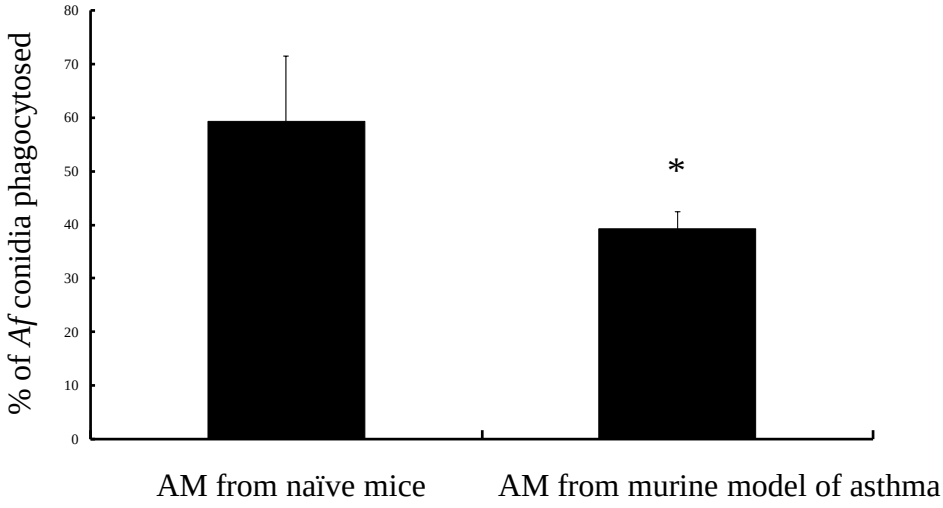
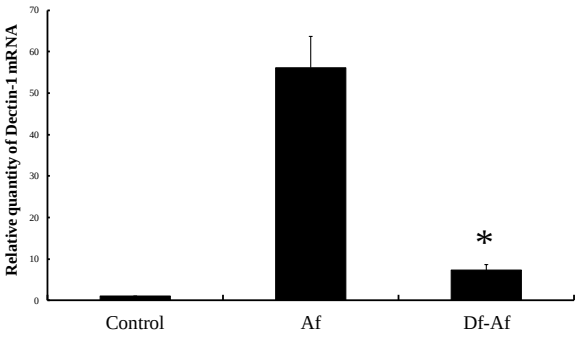
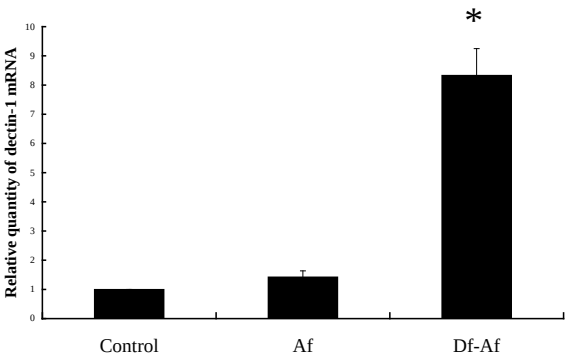

% of *Af* conidia phagocytosed
80
70
60
50
40
30
20
10
0
*
AM from naïve mice
AM from murine model of asthma

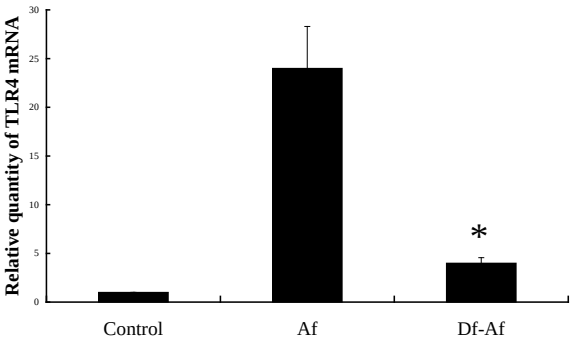
Figure 7



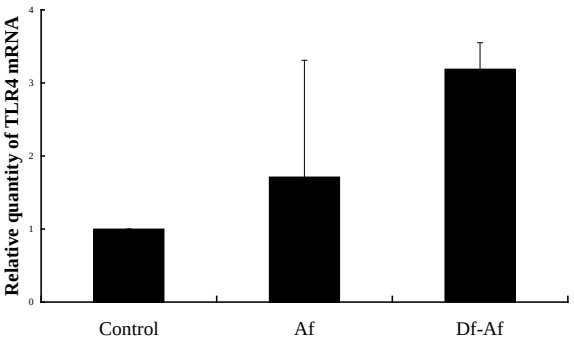
At day 25



At day 32



At day 25



At day 32

Figure 8

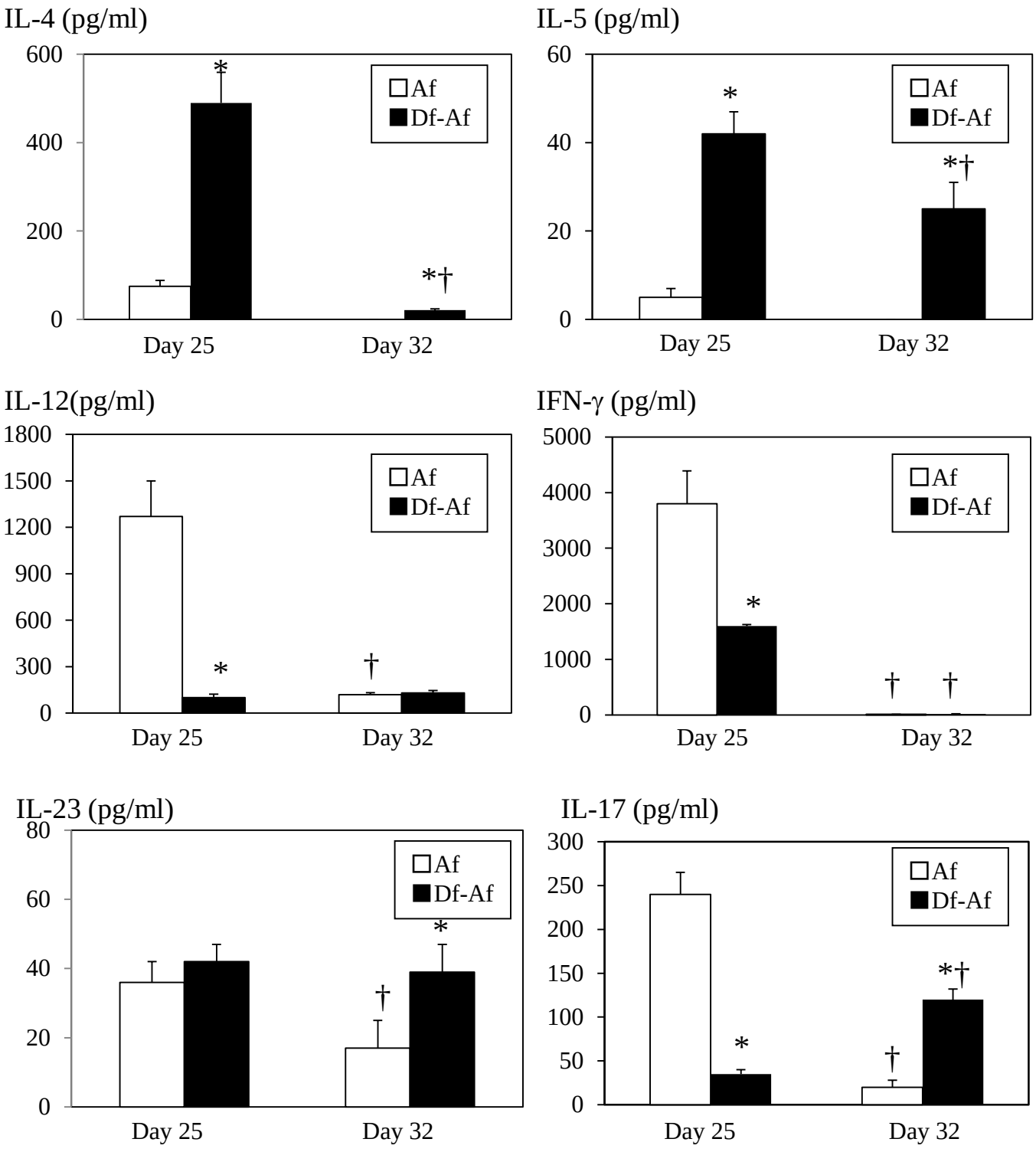

IL-4 (pg/ml)
600
400
200
0
Af
Df-Af
*
*†
Day 25
Day 32
IL-5 (pg/ml)
60
40
20
0
Af
Df-Af
*
*†
Day 25
Day 32
IL-12(pg/ml)
1800
1500
1200
900
600
300
0
Af
Df-Af
*
†
Day 25
Day 32
IFN-γ (pg/ml)
5000
4000
3000
2000
1000
0
Af
Df-Af
*
† †
Day 25
Day 32
IL-23 (pg/ml)
80
60
40
20
0
Af
Df-Af
†
*
Day 25
Day 32
IL-17 (pg/ml)
300
250
200
150
100
50
0
Af
Df-Af
*
†
*†
Day 25
Day 32

Table 1. Differential cell counts in BALF.

	Macrophages ($\times 10^5$ cells)	Neutrophils ($\times 10^5$ cells)	Lymphocytes ($\times 10^5$ cells)	Eosinophils ($\times 10^5$ cells)
Af at day 25	13.8±3.6	12.6±7.8	1.7±0.2	1.1±0.7
Af at day 32	6.8±2.5*	0.4±0.8*	1.0±0.5*	0.5±0.2*
Df-Af at day 25	12.6±4.1	9.8±3.2	4.4±5.1*	6.2±1.7*
Df-Af at day 32	11.3±3.9*†	6.8±2.9*†	3.4±1.9*†	4.1±2.0*†

Results are expressed as means (n=6 for each group) ± SEM.

*$P < 0.01$ vs. Af at day 25, †$p<0.01$ vs. Af at day 32.