Innate and adaptive immune response to chronic pulmonary infection of hyphae of *Aspergillus fumigatus* in a new murine model

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**Abstract**

**Purpose.** The pathogenesis of chronic pulmonary aspergillosis (CPA) has seldom been studied due partly to a lack of animal models. Since hypha is the main morphology colonizing the airway in CPA, it's critical to study the immune reaction to chronic pulmonary infection of hyphae of *Aspergillus fumigatus*, which also has seldom been studied in vivo before.

**Methodology.** We established a novel murine model of chronic pulmonary infection of hyphae by challenging immunocompetent mice with tightly-structured hyphae balls intratracheally, and described the ensuing immunoreaction to hyphae and conidia, and the pathogenesis of CPA.

**Results.** Our experiment proved that the hyphae balls could induce a chronic pulmonary infection for 28 days with a considerable recrudescence at day 28 post-infection. Lungs infected with hyphae balls were remarkable for the many neutrophils and macrophages that flooded into airway lumens, with peribronchiolar infiltration of leukocytes. There was a transient increase of Th2 cells and Th17 cells at day 7 post-infection in the lung tissue. In contrast, lungs infected with conidia showed no peribronchiolar infiltration of leukocytes, but an influx of a great number of macrophages, and a much less number of neutrophils in the lumen. Besides, conidia activated the co-response of Th1, Th2 and Th17 cells with an increase of Treg cells in the lung tissue (quite different from most previous studies).

**Conclusion.** We established a new murine model of chronic infection of hyphae to mimic the formation of CPA, and provide a new marker for different immune responses to hyphae and conidia.

**INTRODUCTION**

*Aspergillus fumigatus* is a ubiquitous mold that can release many asexual spores (conidia) into the air. Conidia are small enough (2–3 µm in diameter) to reach the pulmonary alveoli after inhalation [1, 2]. Immunocompetent persons can eliminate inhaled conidia by the mucociliary clearance and airway macrophages phagocytosis [1]. However, the function of clearance is compromised for patients with pre-existing structural pulmonary disease such as tuberculosis (TB), in which case the inhaled conidia begin to swell and germinate into hyphae, resulting in formation of CPA [3]. It has been reported that the 5 year estimated prevalence rate of CPA following TB reached 18 % and the 5 year mortality rate reached 50 % even after antifungal treatment [4].

In histopathology, hyphae are confined to the lung pre-existing cavity with no angioinvasion in CPA [5]. The mechanism of CPA remains unclear. Since the course of CPA endured the process of conidia germinating into hyphae that become the main morphology colonized in the airway [6], it is critical to study immune response to pulmonary hyphae infection to better understand the pathogenesis of CPA. However, the natural immune response of prolonged colonization of hyphae in the airways has also seldom been studied [7, 8]. In this study, our research team established the murine model by injecting tightly-structured hyphae balls into the lungs of mice intratracheally. These hyphae balls could remain for 28 days in the lung, with no invasion of lung parenchyma and blood vessel, indicating a...
chronic pulmonary infection of hyphae. We studied the roles of neutrophils, macrophages and T lymphocytes during this process.

METHODS

Fungal strain tagged with GFP and inoculum preparation

*A. fumigatus* wild-type strain *Af293* (purchased from FGSC, Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA) was transfected with the DNA sequence of green fluorescence protein (GFP). The plasmid pFN03 was exploited as the DNA vector. The primers were listed as: F: 5′-CCCTTAATTCAAGCTATCGATATGGGAGCTGGTGCCGCTGG-3′, and R: 5′-TCGAGGTGTCAGCGTTATCGATGAGGGTGAAGAGCATGTTT-3′. Fluorescence of conidia and hyphae was verified under the microscope. The GFP-tagged strain was cultured on solid YAG (yeast+agar+glucose) medium for 48 h; conidia were collected by Phosphate Buffer solution (PBS) (Hyclone), supplemented with 0.01 % tween 80 (Fisher Scientific) and filtered through eight layers of sterile carbasus. The concentration of conidia suspension was diluted into 5*10^8 ml⁻¹ after being counted by the haemocytometer. Two milliliters of conidia suspension were added into a 10 ml YAG fluid medium, and the new suspension was spun at a speed of 250 r.p.m. at 37 °C for 16 h. The suspension was filtered through metal mesh to select the hyphae balls with a diameter less than 250 µm. After standing for 1 min, the supernatant was removed and the suspension spun at 1000 r.p.m. The pellet was washed by 50 ml PBS (Hyclone) three times and resuspended in 1 ml PBS (Hyclone). After serial dilutions onto solid YAG medium and counting the colony-forming-units (c.f.u.), the concentration of the hyphae balls suspension was adjusted to 5*10^4 ml⁻¹.

Mice and infection model

The 8–12 week-old C57BL/6J female mice were purchased from Yangzhou University (Yangzhou, Jiangsu, China). Animals were treated in strict accordance with the Beijing Administration Rule of Laboratory Animals in China. All experimental protocols were reviewed and approved by the Animal Investigation Ethics Committee of Jinling Hospital (Permission ID: SYXK <military>2012-0047). To establish the infection model, the mice were anaesthetized by injecting 1 % pentobarbital sodium into cava abdominis (10 ml kg⁻¹), and then were hung vertically and intratracheally challenged with 50 µl 5*10^7 ml⁻¹ conidia suspension (C group), 5*10^4 ml⁻¹ hyphae balls suspension (H group), or PBS (Hyclone) (CN group).

Fungal burden and body weight change

The lungs were extracted at day 1, 7, 14, 21 and 28 post-infection (p.i.), weighed and ground by Tenbroeck tissue homogenizers (Wheaton) with a ratio of 1 g lung tissue : 5 ml physiological saline. The homogenates and their serial dilutions were sedimented to solid YAG medium and incubated under 37 °C. The c.f.u. were counted after 24 h incubation. The fungal burden was presented as log10 c.f.u. g⁻¹ of lung [9]. The body weights of mice were taken down every four days.

Distribution of *A. fumigatus* in the lung

The lungs were removed at day 1, 7, 14, 21 and 28 p.i. and washed by sterile PBS (Hyclone). The ex vivo live imaging was performed using the Kodak In-vivo FX PRO Imaging System to observe the distribution of *A. fumigatus* in the lungs very quickly (the wavelengths of excitation and absorption lights were 470 nm and 530 nm respectively).

Histopathology

After being observed with the live imaging system, lungs were fixed in 10 % (v/v) formalin overnight, embedded in paraffin, cut into 5 µm sections, and stained with Periodic acid-Schiff (PAS) stain.

Bronchoalveolar lavage

One ml sterile cold PBS (Hyclone) was used to lavage the lungs 5 times, then the suspension was spun at 2000 r.p.m. for 10 min. The pellet was resuspended in 800 µl ACK buffer (Milenyi) to lyse erythrocytes for 15 min, then the suspension was spun at 2000 r.p.m. for 10 min and the supernatant removed. The pellet was washed twice by PBS (Hyclone) and resuspended in 800 µl PBS (Hyclone). Cells were counted using a haemocytometer.

Lung digest

Lungs were removed after bronchoalveolar lavage, washed twice with sterile PBS (Hyclone) and cut into 1–2 mm³ pieces. These were put in the digestion buffer (RPMI 1640 supplemented with 5 % PBS and 150 U ml⁻¹ collagenase, Sigma) to digest for 60 min at 37 °C. The fluid was filtered by 70 µm cell strainer and spun at 2000 r.p.m. for 10 min. The supernatant was removed and the pellet was resuspended in 1 ml ACK buffer (Milenyi) to lyse erythrocytes for 15 min. Cells were washed twice with PBS (Hyclone) and resuspended in 800 µl PBS, then counted with the haemocytometer and divided into three polyethylene pipes.

Leukocyte analysis and T cell subset analysis

A volume of 250 µl of cell suspension from lungs and cells from bronchoalveolar lavage were used to analyse the amount of leukocytes. The Fc receptor was blocked by unlabelled anti-CD16/CD32 (BD Pharmingen) for 15 min, followed by cell staining with antibodies against cell surface components for 20 min at room temperature. The antibodies were purchased from eBioscience and listed as below: CD45-FITC, CD11b-PE, Gr-1-APC and F4/80-PerCP-Cy5.5. Cell subsets were defined as neutrophil: CD45⁺CD11b⁺Gr-1⁻, and macrophage: CD45⁺CD11b⁺F4/80⁺. Data acquisition was performed on the BD FACSCalibur.

Cells from lung digest were used to analyse the T cell subset. A volume of 250 µl of cells suspension was stimulated by cell stimulation cocktail (eBioscience) at the temperature of 4 °C for 16 h according to the manufacturer’s instructions before...
intracellular staining. The Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used in another 250 µl cells suspension to test the Treg cell according to the manufacturer’s instructions. Antibodies purchased from eBioscience were listed as below: CD4-FITC, IFN-γ-APC, IL-17a-PE, IL-4-PerCP-eFlour 710, CD25-PerCP-Cyanine5.5, CD3-APC and Foxp3-PE. T lymphocyte was defined as CD3+ cells and T cell subsets were defined as Th1: CD4+ IFN-γ+, Th2: CD4+ IL-4+, Th17: CD4+ IL-17a+, and Treg cell: CD3+CD4+ CD25+FOXP3+.

Statistical analysis
The differences between groups and time points in each group were evaluated by One-way analysis of variance (ANOVA) followed by Bonferroni post-tests. Repeated measures data of ANOVA were used to evaluate differences of between the variation tendencies of body weights of mice in three groups.

RESULTS

The fungal strain is tagged with GFP
The fluorescence intensity of conidia and hyphae was strong under the fluorescence microscope (Fig. 1a, b).

Hyphae balls are prepared with diameter less than 250 µm
The hyphae balls of A. fumigatus were observed under the microscope and showed compact spherical structure (Fig. 1c, d).

Hyphae balls induce chronic pulmonary infection and a recrudescence at day 28 p.i.
The distribution of A. fumigatus in the lung was shown in Fig. 2. There was no fluorescence of the lung in the CN group. The fluorescence of the lungs in C group could be seen at day 1 and day 7 p.i., and not after. Fluorescence intensity in H group was strong at day 1 and day 7, kept being weaker at day 14 and day 21, while it became stronger and diffuse at day 28 p.i. (Fig. 2). Fig. 3 showed the histopathology of PAS–stained lung tissues in each group at different time points. Fig. 3(a) illustrated the normal structure of bronchia in the control group. Histopathology of lung tissues in C group showed the conidia phagocytized by the macrophages at day 1 p.i. and abundant magenta tracheal secretions in the airway lumen at day 7 p.i. (Fig. 3b, c). In the H group, the PAS-stained sections showed magenta hyphae at each time point (Fig. 3d–h).

The results of fungal burden were shown in Fig. 4(a). The fungal burden of the mice infected with conidia decreased over time. Consistent with the distribution of hyphae in the lung, the fungal burden of H group began to decrease at day 14 and remained the same until day 21, with a considerable increase at day 28 p.i.

Fig. 4(b) showed the changes of body weights of mice. There was no significant difference between the variation tendencies of body weights of mice in three groups (P=0.621). In addition, mice in each group did not display any other clinical signs such as dark urine, wheezing, fur ruffling, or increased mortality.

Different recruitment of leukocytes induced by conidia and hyphae
The histopathology of lungs in the C group showed that the conidia were phagocytized by macrophages at day 1 p.i. with abundant secretions in the airway lumen at day 7 p.i. (Fig. 3b, c). There was no obvious inflammation afterwards in the C group. Histopathology in the H group showed that plenty of neutrophils and macrophages were recruited to the infection site at day 1 p.i. (Fig. 3d). The hyphae ball grew into a large hyphae block in which many neutrophils and macrophages infiltrated at day 7 p.i. (Fig. 3e). At day 14 and day 21 p.i., the quantity of hyphae decreased compared to day 7 p.i. (Fig. 3f, g), and the airway wall was destroyed at day 14 p.i. (Fig. 3f). The infection sites formed circumscribed foci that were infiltrated by neutrophils, macrophages and lymphocytes at day 21 and 28 p.i. (Fig. 3g, h). There was lung consolidation around the infection sites at day 28 p.i. (Fig. 3h).

Flow cytometry was used to evaluate the inflammation in and around the infected airway lumen, and the lung digest and bronchoalveolar lavage samples were detected (Figs 5, 6, 7 and S1–S4, available in the online Supplementary Material). In the C group, an influx of macrophages and fewer neutrophils were noted in the lumen. The number of leukocytes reached their peak at day 7 p.i. (Figs 5 and S1). There was no increase of neutrophils and macrophages compared to the CN group all over the experiment, while a moderate increase of lymphocytes at day 28 p.i. infiltrated around the lumen (Figs 6, S2 and S4).

In contrast, there was an infiltration of neutrophils, macrophages and lymphocytes around the lumen in the H group. The number of neutrophils reached the highest value at day 7 p.i. and decreased afterwards, and macrophages and lymphocytes began to increase at day 1 p.i., decreasing at day 7 p.i. (Figs 6, S2 and S4). Many neutrophils and macrophages flooded into the lumen and the amount of neutrophils and macrophages peaked day 7 p.i. (Figs 5 and S1).

Priming of T lymphocytes is different when induced by conidia and hyphae
T cell subsets of the digested lung were studied to evaluate the adaptive immune response to A. fumigatus (Figs 7, S3 and S4). In the C group, the amount of Th1, Th2 and Th17 cells began to increase at day 1, reached the greatest at day 7, decreased at day 14 p.i., and remained higher afterwards compared with the control group (Figs 7 and S3). However, in the H group, there was no statistically significant change of Th1 cells over the process of infection. There was a transient increase of Th2 cells at day 7 p.i. and a rise of Th17 cells in the meanwhile (Figs 7 and S3). The Treg cell kept being high from day 1 to day 21 p.i. in C group (Figs 7 and S4). In contrast, the number of Treg cell reached
the greatest at day 7 and decreased afterwards in the H group (Figs 7 and S4).

**DISCUSSION**

*A. fumigatus* can lead to varying clinical features depending on patients’ immunity conditions, such as invasive aspergillosis (IA), allergic bronchopulmonary aspergillosis (ABPA) and chronic pulmonary aspergillosis (CPA) [1]. CPA usually happens to those of normal immunity or minor immunity defect with pre-existing lung cavity, which makes it difficult to establish the animal model of CPA to study this disease of high mortality [3]. Histopathology of CPA shows that there is no invasion of lung parenchyma and blood vessel and the hyphae are confined to the pre-existing lung cavity [3]. In addition, the histopathology of infection sites may progress to consolidation with no effective treatment [5]. In our experiments, we employed immunocompetent mice to establish this model. The histopathology showed that the hyphae were confined to the infection airway lumen that could be considered as the pre-existing lung cavity and there was a pulmonary consolidation at day 28 p.i., which were consistent to the histopathology of CPA in humans. The results of pulmonary fungal distribution and pulmonary fungal burden in the H group indicated that the hyphae were cleared gradually from day 1 to day 21 p.i., followed by a recrudescence at day 28 p.i., which might mimic a recrudescence that is of high rate in CPA patients [3].

In contrast to animal models of IA, our murine model did not show any other clinical features of invasive infections such as weight loss, dark urine, wheezing, ruffling of fur, or increased mortality [10, 11]. To establish these animal models of IA, immunosuppression was induced to mimic the status of IA patients and the histopathology of IA showed invading of lung parenchyma by *A. fumigatus* [10, 11]. However, in our model, we exploited mice with normal immunity that was consistent to the condition of CPA patients and the histopathology was confined to the circumscribed foci, which indicated no invasive infection all along the course.

There’s another novel murine model of prolonged pulmonary colonization of hyphae, in which the immunocompetent mice were intratracheally injected with conidia embedded in agar beads [8]. Conidia in the agar beads could germinate and produce hyphae that emerge from within agar beads, inducing prolonged airway colonization of hyphae for 28 days [8]. In the experiment of evaluating the inflammation in lung tissue, the time points of Th17 and Treg cells reaching the peak value were later than ours, which might because the wall of agar limited the full interaction between hyphae and host cells. In addition, sterile beads induced significant changes of the number of Treg cell and the level of IL-4 compared to the uninfected mice, indicating that the immune response to agar could not be excluded. Fungal burden in this model decreased over time, suggesting that it was a process of clearance of hyphae. In

Fig. 1. (a, b) the strain was tagged with GFP and the fluorescence intensity of both conidia and hyphae was strong. Scale bars=10 µm; (c), (d) the hyphae balls under the microscope. (c) photograph was 100× magnified, scale bar=200 µm; (d) photograph was 400× magnified, scale bar=50 µm.
contrast, in our murine model, there was a recrudescence at day 28 p.i., which made our model more like a model of CPA. Although our murine model couldn’t completely explain the pathogenesis of CPA, because the pathogenic process of this model skipped the stage of conidia infection, it could illustrate the immune response to hyphae infection, the main course of CPA.

What’s more, our model provided an insight into the immune response to pulmonary infection of the nonsporulating A. fumigatus strains. Our laboratory has reported the first nonsporulating strain of A. fumigatus named Aij, which was isolated from an immunocompetent patient with a TB history [9]. The patient was infected with the hypha of this strain for 3 months. Previous studies showed the hyphae of Af293 and Aij led to the same histopathological manifestation, which was different from the histopathological manifestation induced by the conidia of Af293 [9], indicating the different immunoreaction induced by hyphae and conidia.

In our experiment, we demonstrated that hyphae and conidia could induce different immunoreactions, which is consistent with early studies [7, 9]. It has been reported that neutrophils and macrophages are the main effector cells that participate in confronting the pulmonary infection of conidia and hyphae [12, 13]. Resident alveolar macrophage (AM) is recognized as the first line against the invasion of A. fumigatus [14]. AMs can phagocytize and kill the inhaled conidia depending on the oxidative and non-oxidative mechanisms [15, 16]. Besides, chemokine and cytokines secreted by AMs including macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC) will recruit neutrophils [17]. Early studies showed that neutrophil was the main phagocyte against hyphae [18], but not phagocytize conidia [7], while a few studies showed that the neutrophils were also recruited to the lung when challenged with conidia both in immunocompetent and immunosuppressed mice [19–21]. Furthermore, some researches revealed that neutrophils were vital in preventing the

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**Fig. 2.** The hot and fluorescence images of lungs at different time points. The white arrows pointed to the A. fumigatus tagged with GFP. The figure showed the representative results from at least two independent experiments (total n=4–6 per time point in each group).
germination of conidia in vivo [19] and improving the survival rate of IA [22] and the neutrophilic phagocytosis event was essential in the killing of conidia [23]. In our study, there was an abundant number of macrophages, while much fewer neutrophils were recruited to the airway lumen in C group according to the results of flow cytometry. Further studies have to be conducted to investigate whether the minor increase of neutrophils was essential for the killing of conidia or was merely the collateral result of recruitment by chemokine and cytokines secreted by AMs.

For pulmonary hyphae colonization, AMs are recruited to surround the hyphae [7]. However, the hyphae are too large for AMs to phagocytize and neutrophils are employed to eliminate hyphae [20, 24–26], which coincides with results of histopathology and flow cytometry analysis in the H group that abundant neutrophils and AMs infiltrated in the hyphae balls and around the airway lumens. It was probably the decreasing infection sites, pulmonary consolidation around the infection sites, and bronchial obstruction, that the number of neutrophils, macrophages and lymphocytes in and around the infection sites evaluated by flow cytometry did not synchronize with the results of pulmonary fungal distribution, fungal burden and histopathology.

In spite of AM and neutrophil, natural killer cells (NK), dendritic cells (DC), eosinophil and mast cells also play different parts against different morphologies of A. fumigatus infection [27–33]. NK cells were proven to respond to hyphae, while not to conidia in vitro [27]. Eosinophils were shown to be another contributor to the clearance of A. fumigatus from the lung [29, 30]. Mature hyphae, but not conidia or germ tubes could induce the IgE independent degranulation of mast cells [31, 32].

DCs have been recognized as the bridge between innate and adaptive immune response, and a dense distribution of DCs has been described in the respiratory tract [34, 35]. It has been proven that DCs phagocytized both conidia and hyphae [36–39], migrated to the draining lymph nodes and spleen, underwent functional maturation, and induced

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**Fig. 3.** The histopathology of the PAS-stained lung tissue. (a) uninfected mice; (b), (c) mice infected with conidia, at day 1 and 7 p.i.; (d), (e), (f), (g), (h) mice infected with hyphae ball, at day 1, 7, 14, 21 and 28 days p.i. The black arrows pointed to the conidia phagocytized by macrophages and the red arrows pointed to hyphae. Photographs were 400× magnified, and the right below ones were 1000× magnified.

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**Fig. 4.** (a) the pulmonary fungal burden. Bars represented the mean±standard errors of the means from at least two independent experiments (total n=4–6 per time point in each group). *, P<0.05 compared to the previous time point. **, P<0.001 compared to the previous time point; (b) the changes of body weights of mice every four days. Bars represented the mean±SE of the means from at least two independent experiments (total n=6–8 per time point in each group).
selective Th priming of CD4+T lymphocytes that conidia induced increasing Th1 cells and hyphae activated Th2 cells [36]. There were few studies of the adaptive immune response to hyphae in vivo. In a murine model of allergic lung disease, the exposure to hyphae led to the priming of Th2 cells [40]. In our murine model of chronic pulmonary infection with hyphae, there was also a transient increase of Th2 cells at day 7 p.i., but no change of Th1 cells.

It is evident that exposure to conidia led to the priming of Th1 cells [41]. However, it has been reported that repeated exposure to conidia activated the co-response of Th1, Th2 and Th17 cells with an early increase of Treg cells in an ABPA murine model [42]. In addition, in a murine model of invasive aspergillosis, there were increases of both IFN-γ and IL-4 at day 2 and 7 after injection intravenously with a lethal dose of conidia [43], which was similar to our result that there was an increase of both of Th1 and Th2 cells in the first 7 days p.i. In contrast, there was a higher level of IFN-γ and lower level of IL-4 with sublethal dose than the group with lethal dose [43], which indicated that the priming of CD4+T subsets might be associated with the quantity of conidia and the times of challenge. Also, treatment with soluble IL-4 receptor cured more than 70% of the mice from primary infection and resulted in acquired resistance to a subsequent lethal infection, which proved that Th2 responses were deleterious in confronting the pulmonary infection of A. fumigatus [43].

Th17 cells were proven to be a contributor to resistance to cutaneous and systemic infection of Candida albicans [44, 45] and pulmonary infection of A. fumigatus [46, 47]. Treg cell was reported to enhance the production of IL-17 and activate the priming of Th17 cells [48, 49]. Our results show that Th17 cells and Treg cells were in collaboration against the pulmonary infection of conidia and hyphae of A. fumigatus.

**Conclusion**

This study established a chronic pulmonary colonization of hyphae of A. fumigatus to mimic the process of CPA formation. Our experiment proved that the hyphae could induce pulmonary infection and exist in the lung up to 28 days. Additionally, conidia and hyphae induced different innate immune responses that macrophage and neutrophil were

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**Fig. 5.** Detection of neutrophils and macrophages in the lumen at different time points. Bars represented the means±SE of the means from at least two independent experiments (total n=4–6 per time point in each group). *, P<0.05 compared to the uninfected group. **, P<0.001 compared to the uninfected group.

**Fig. 6.** Detection of neutrophils, macrophages and lymphocytes in the lung tissues at different time points. Bars represented the means±SE of the means from at least two independent experiments (total n=4–6 per time point in each group). *, P<0.05 compared to the uninfected group. **, P<0.001 compared to the uninfected group.
the main effector cells for hyphae and that macrophages were the main effector cells for conidia. Exposure to conidia activated co-response of Th1, Th2 and Th17 cells and hyphae activated response of Th2 and Th17 but no Th1.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

All the animal care and experimental protocols were reviewed and approved by the Animal Investigation Ethics Committee of Jinling Hospital (Permission ID: SYXK<military>2012–0047). Animals were housed in a SPF animal facility and treated in strict accordance with Beijing Administration Rule of Laboratory Animal, China.

**References**


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**Fig. 7.** Detection of T cell subsets in the lung tissues. Bars represented the means±SE of the means from at least two independent experiments (total n=4–6 per time point in each group). *, P<0.05 compared to the uninfected group. **, P<0.001 compared to the uninfected group.

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