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Original Article

Diversity of Th1/Th2 immunity in mice with acute lung injury induced by the H1N1 influenza virus and lipopolysaccharides

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Abstract

Introduction: The polarization of T helper (Th) cells plays an important role in the inflammatory response, pathogen removal, and tissue damage processes of infectious acute lung injury (ALI). However, Th cell polarization in viral- or bacterial-mediated ALI is not well defined. Herein, an influenza virus (A/FM/1/47, H1N1) and lipopolysaccharide (LPS) were chosen to induce ALI in mice, and the resultant diversity of Th-cell polarization was explored.

Methodology: BALB/c mice were challenged intranasally with the influenza virus or LPS. Edema of the lung, infiltration of inflammatory cells (macrophages, neutrophils, and lymphocytes), oxidative stress, and signature cytokines of Th1 and Th2 cells were detected at 2 days post virus or LPS challenge.

Results: The mice exhibited increased capillary permeability accompanied by lung edema and protein-rich alveolar exudation after virus or LPS challenge. Additionally, excessive infiltration of inflammatory cells, robust oxidative stress, and cytokine production were observed in both mouse groups. However, there was conspicuous disparity in the inflammatory cell infiltration and cytokines between the virus- and LPS-challenged mice, where the infiltration in virus-challenged mice was mainly of macrophages and accompanied by robust Th1 cytokine elevation, whereas the infiltration in LPS-challenged mice was primarily of neutrophils and accompanied by robust Th2 cytokine elevation.

Conclusions: The Th cell polarization was skewed depending on whether ALI was induced by the influenza virus or LPS. The polarization in the virus-challenged mice was primarily toward a Th1 response, whereas that in the LPS-challenged mice was mainly toward Th2.

Key words: Th1/Th2; inflammation; acute lung injury; infection; polarization.

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Introduction

Bacteria or respiratory viruses are the main pathogens of respiratory tract infections, which easily develop into acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) [1-3]. Researchers have shown that the first 2 days of infection was the acute phase of infection [4], it is the critical time in which acute inflammation response, the formation of edema, and hypoxemia, ensues in influenza virus- or lipopolysaccharide (LPS)-challenged mice [4,5]. Mice, at this time, might rapidly develop either ALI/ARDS or infection resolution within 6 days [6]. Clinical research has also shown that the startup of the host's innate immune response occurs 2 days after the onset of illness [7]. This demonstrated that the immune response, especially in the acute stage, is critical for pathogen removal and is likely to affect the development of the infection or lead to tissue damage [8-10]. The immune response does all this by recruiting immune cells (including innate immune cells and T and B lymphocytes) to the site of infection, where they either clear the pathogens or cause an excessive inflammatory response [10,11].

T cells, which act as "warriors" in the body by regulating cellular immunity, are the main effector cells against infection. During an infection, the function of cytotoxic T cells is to clear the cellular infection, whereas that of T helper (Th) cells is to promote the inflammatory response via lymphokines, which are crucial in excessive inflammation and tissue damage. In general, Th0 cells are likely to polarize into Th1 or Th2 cells, which are the main response in cellular immunity and humoral immunity, respectively [12,13]. However, the polarization of Th cells is a complex and subtle procedure, being closely dependent on cellular homeostasis and influenced by a variety of factors, including the pathogen itself, animal species, gender, pregnancy, and cytokines [14]. The skewed polarization of Th cells might impact the recruitment of inflammatory cells and the immune response process

[15,16]. It is well known that the excessive inflammatory response induced by the infiltration of macrophages and neutrophils is the trigger for the increased microvascular permeability, protein-rich edema exudation, and lung edema in ALI [17-19]. However, there are different patterns of infiltration and dominant associations of inflammatory cells in different types of infectious ALI, and such pattern of infiltration might be the central link to Th cell initiation and polarization [15,16].

However, Th cell polarization in viral- or bacterial-mediated ALI is not yet fully understood. In this study, to better understand the differences of Th immunity in the early phase of ALI induced by viruses or bacteria, an influenza virus (A/FM/1/47, H1N1) and LPS were chosen to induce ALI in mice, and the resultant diversity of Th cell polarization in the challenged mice was explored.

Methodology

Ethics statement

The animal research was approved by the Laboratory Animal Services Center at Guangzhou University of Chinese Medicine (Guangzhou, China) (Approval ID SCXK (Guangdong) 2013-0034). The guidelines of the Animal Welfare and Ethics of the Institutional Animal Care and Use Committee were followed.

Virus and reagents

A mouse-adapted influenza virus (A/FM/1/47, H1N1) was provided by the Biosafety Level II laboratory of the Guangzhou University of Chinese Medicine. The virus was propagated at 36 °C in specific pathogen-free embryonated eggs for 48 h. The median lethal dose (LD_{50}) of the virus was titrated in mice, where that in the BALB/c mouse was $10^{-2.57}$/0.05 mL. LPS (from *Escherichia coli* O111:B4) was obtained from Sigma (St. Louis, MO, USA). The BD Cytometric Bead Array Mouse Inflammation Kit was obtained from BD Biosciences (San Diego, CA, USA). The Pierce BCA Protein Assay Kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Wright-Giemsa stain, malondialdehyde (MDA), superoxide dismutase (SOD), and catalase (CAT) kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Animals and experimental design

BALB/c mice (N = 90, either sex, approximately 4–6 weeks old) were acclimated in individual ventilated cages in a Biosafety Level II facility for 1 week before the experiments. The room was maintained at a temperature of 22 ± 2 C, with 50 ± 10% relative humidity and a 12-hours light/dark cycle. Food and water were provided *ad libitum*.

The mice were randomly divided into three groups: control group, LPS group, and H1N1 group. Following anesthetization using ether, the mice were intranasally administered 20 μg of LPS or $2LD_{50}$ of virus in 50 μL of phosphate-buffered saline (PBS) for the respective experimental groups, or 50 μL of PBS for the control group. For the survival test (n = 12), the body weight, and morbidity were recorded for 15 days. For the remaining experiments, the mice were sacrificed 2 days post LPS or virus administration, and their blood and tissues were collected for further analyses (n = 9).

Lung edema assessment

At 2 days after challenge, the mice were anesthetized with 1% pentobarbital sodium, the lungs were excised, blotted dry, weighed to obtain the wet weight, and then placed in an oven at 80 °C for 48 hours to obtain the dry weight. The ratios of lung weight to body weight and wet lung weight to dry lung weight were calculated to assess for tissue edema.

Cytokine measurement

Blood was collected and centrifuged at 3000 rpm for 10 minutes, following which the serum levels of interleukin-2 (IL-2), IL-4, IL-5, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) were measured by flow cytometry using the cytometric bead array technique. In brief, 50 μL of the supernatant or standard dilutions was added to 50 μL of mixed mouse inflammation capture beads, and then 50 μL of phycoerythrin-conjugated mouse inflammation detection reagent was added. Subsequently, the samples were incubated in the dark at room temperature for 2 hours. Then, 1 mL of wash buffer was added and the solution was centrifuged at 1300 rpm for 5 minutes, following which the supernatant was discarded and 300 μL of wash buffer was added to resuspend the pellet. All data were collected on a BD FACSCanto II Flow Cytometry System (BD Bioscience, USA), and the cytokine levels were analyzed using BD CBA software.

Inflammatory cell infiltration analysis

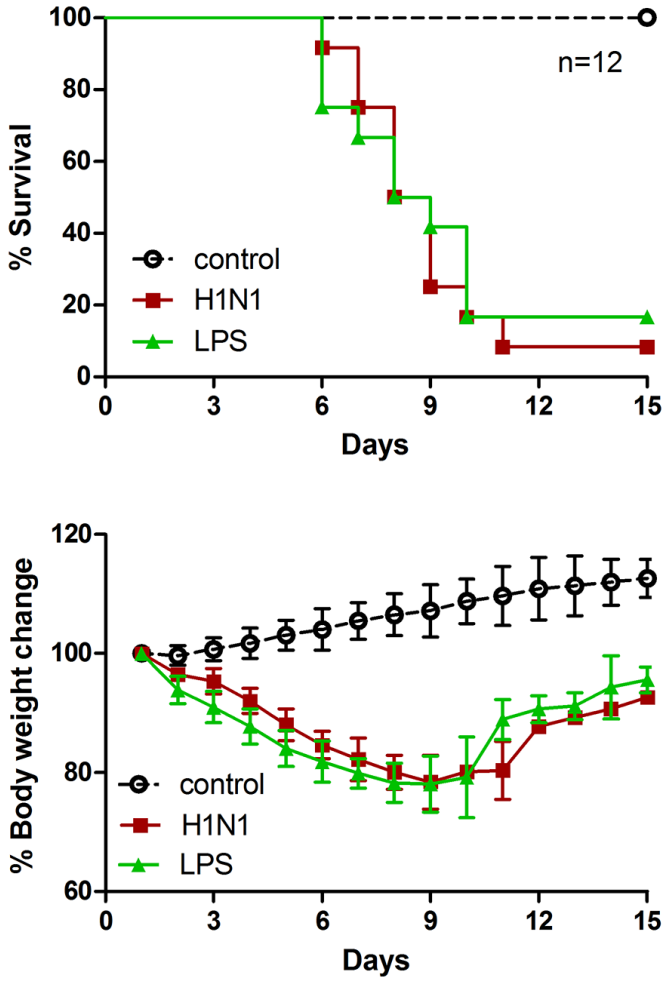
Bronchial alveolar lavage fluid (BALF) was collected from the mice at 2 days post challenge for enumeration of the inflammatory cells. In brief, each lung was lavaged three times with 500 μL of PBS (total volume, 1.5 mL). The BALF sample was then centrifuged at 300 × *g* for 10 minutes at 4 ° C and the

supernatant was stored at –80 ℃ for later measurement of the protein, SOD, MDA, and CAT concentrations. The cell pellet was washed with red blood cell lysis solution for 1 minute, and then centrifuged at 300 × *g* for 10 minutes and resuspended in 200 μL of PBS. The total cell count was determined using a hemocytometer. The differential cell count was determined by Wright-Giemsa staining, where macrophages, neutrophils, and lymphocytes were quantified by counting 200 cells per slide at 400× magnification.

Oxidative stress measurement

The BALF levels of MDA, SOD, and CAT were measured according to the instructions of the Jiancheng Bioengineering Institute. For SOD measurement, the supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 minutes, and then nitroblue tetrazolium was added. Thereafter, blue formazan formation was monitored at 550 nm. For MDA measurement, a solution of thiobarbituric acid and trichloroacetic acid was mixed with the supernatant and incubated in boiling water for 90 minutes. After cooling, the sample was centrifuged and the absorbance of the supernatant was read at 532 nm, with tetraethoxypropane used as the standard for comparison. For CAT measurement, hydrogen peroxide (H_2O_2) was added to a mixture of potassium phosphate buffer (pH 7.0) and supernatant. The rate of H_2O_2 decomposition was then measured at 240 nm.

Figure 1. The mortality of mice in LPS or virus challenged mice. Mortality (A) and weight loss (B) in 20 μg LPS or $2LD_{50}$ of virus (A/FM/1/47, H1N1) challenged mice in 15 days. Survival curves were derived by the Kaplan-Meier method and compared by log-rank test, *p < 0.05, **p < 0.01, (n=12).



Histopathology

The mouse lungs were separated and fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. The paraffin blocks were then sectioned (thickness, 4 μm) and the tissue slices were stained with hematoxylin and eosin. Histopathologic changes (i.e., capillary congestion, fibrin exudation, cell infiltration, and bronchial epithelial cell shedding) were measured under a light microscope.

Statistical analysis

All results are expressed as the mean ± standard deviation. Differences between groups were evaluated by one-way analysis of variance. Survival curves were derived by the Kaplan-Meier method and compared using the log-rank test. The level of significance was set at $p \leq 0.05$.

Results

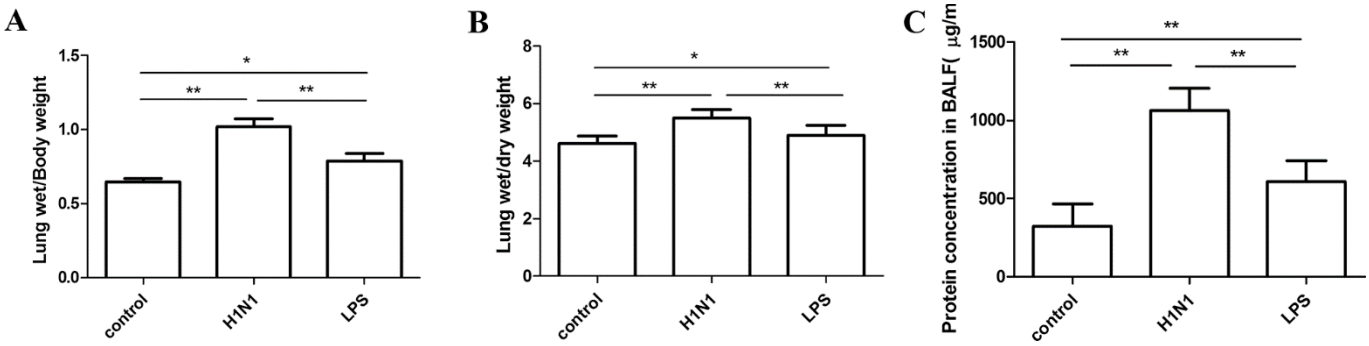
Mortality and symptoms

The mortality of the BALB/c mice challenged with 20 μg of LPS or $2LD_{50}$ virus was observed for 15 days. The mice in the H1N1 group exhibited weight loss, dull skin, and reduced activity from day 2, and began to die at day 6; the mice in the LPS group had similar symptom manifestations but with more dramatic weight changes (Figure 1B). The results showed that days 6 to 10 were the main period of death, and both the 20 μg LPS and $2LD_{50}$ virus resulted in an approximately 90% mortality rate within 10 days (Figure 1A).

Lung edema

The lung weight/body weight ratio, lung wet weight/dry weight ratio, and protein concentration in the BALF were measured to evaluate lung edema. The fluid effusion in the alveoli was increased in both the

Figure 2. Edema in virus or LPS challenged mice. Lung/body weight (A), lung wet/dry weight (B) and the proteins concentration in BALF (C). Mice were intranasally inoculated with 20μg of LPS or $2LD_{50}$ of virus (A/FM/1/47, H1N1), the lung was excised to evaluate the edema at 2 days after incubation. Data were analyzed by ANOVA, and values are expressed as the means ± S.D, *p < 0.05, **p < 0.01, (n = 9).



LPS- and virus-challenged mice, which showed high lung weight/body weight (Figure 2A) and lung wet/dry weight ratios (Figure 2B). Intense effusion in the alveoli was also observed and the BALF was rich in protein (Figure 2C).

Inflammatory cell infiltration

There was significant transmigration of inflammatory cells from the vessels to the alveoli after virus or LPS challenge, but the quantity was obviously different in both mouse groups. In brief, although the total number of cells was significantly elevated in both the H1N1 and LPS groups (Figure 3A), the percentages of macrophages and lymphocytes were higher in the virus-infected mice (Figure 3B). In contrast, neutrophils dominated in the LPS-challenged mice (Figure 3D), whereas the number of macrophages in this group was not obviously changed (Figure 3C).

Serum cytokine levels

The levels of serum cytokines were measured, with results demonstrating that the levels of IL-2, IFN-γ, and TNF-α were increased in the virus-infected mice (Figure 4A–4C); however, the IL-4 and IL-5 levels were not obviously changed, and were in fact lower than those in the control group (Figure 4D and 4E). In contrast, the levels of IL-4 and IL-5 in the LPS group were obviously increased (Figure 4D and 4E), whereas the levels of IFN-γ and TNF-α were elevated slightly (Figure 4B and 4C), and the level of IL-2 was decreased relative to the control levels (Figure 4A). In addition, the ratio of Th1 to Th2 cytokines (i.e., IFN-γ/IL-4) was dramatically elevated in the virus-infected mice (Figure 4F).

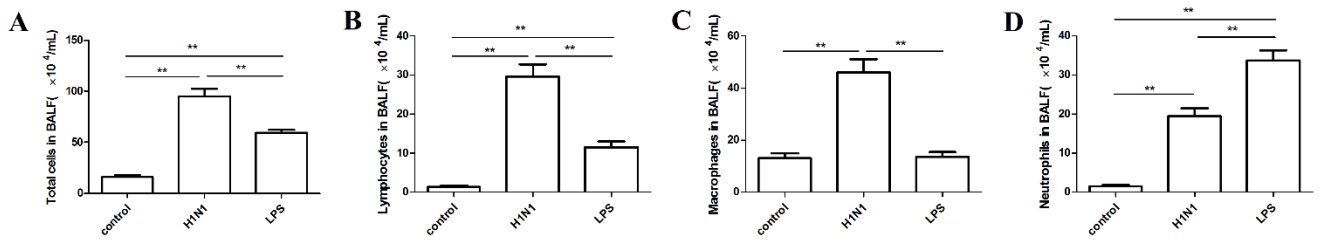
Oxidative stress

Markers of oxidative stress were observed in the BALF of virus- or LPS-challenged mice. The results showed that oxidation and antioxidation were disturbed in both mouse groups, with a decrease in the SOD and CAT levels and an increase in the MDA level (Figure 5A–5C). However, in terms of oxidative stress, there was no significant difference between the LPS- and virus-challenged mice.

Histopathology

The microscopy results showed that the lungs from the virus-challenged mice had apparent viral

Figure 3. Inflammatory cells infiltration in virus or LPS challenged mice. The total cell (A), lymphocyte (B), macrophage (C) and neutrophils (D) in BALF. Mice were intranasally inoculated with 20μg of LPS or $2LD_{50}$ of virus (A/FM/1/47, H1N1), the BALF was collected by Bronchial alveolar lavage of 1.5mL PBS at 2 days after incubation, the cell were counted by hemocytometer; the differential cell count was determined by Wright-Giemsa staining and quantified by counting 200 cells per slide at 400 × magnification. Data were analyzed by ANOVA, and values are expressed as the means ± S.D, *p < 0.05, **p < 0.01, (n = 9).



pneumonia and interstitial edema that was characterized by a shedding of bronchial epithelial goblet cells, alveolar telangiectasia, and presence of large numbers of red blood cells, as well as lymphocyte and neutrophil infiltration in the alveoli. In contrast, the lungs from the LPS-challenged mice were characterized by interstitial inflammatory pneumonia, with infiltration of the lymphocytes and neutrophils being primarily in the alveolar interstitium, and accompanied by slight edema and red blood cell infiltration (Figure 6).

Discussion

A number of research studies have shown that the first 2 days make up the acute phase or early stage of infection in virus- or LPS-challenged mice, and that the immune response or inflammation in this early stage is critical for edema formation and hypoxemia in these challenged mice [4,5]. Moreover, the ALI in these mice might either rapidly develop into ARDS within 6 days or improve depending on the immune response in the acute phase post infection [6]. Meanwhile, clinical research has also demonstrated that the host's innate immune response starts up at 2 days after the onset of illness [7]. In our present study, similar results were found, in that the death of mice occurred heavily from days 6 to 10 in the virus- or LPS-challenged mice (Figure 1A). Furthermore, a decline in body weight (Figure 1B), accompanied by ruffling of the fur, a hunched posture, and reduced activity, occurred from 2 days after challenge. The results showed that the pathological damage (Figure 6) and inflammatory cell infiltration (Figure 3) were strikingly different in the acute stage of ALI depending on whether the injury was induced by the influenza virus or LPS. Likewise, the Th cell polarization was obviously different, in that Th1 polarization was predominant in the influenza virus-challenged mice, whereas Th2 polarization was the priority in the LPS-challenged mice (Figure 4). In addition, both groups of mice showed a change in the balance of the oxidation/reduction status (Figure 5) and experienced lung edema (Figure 2).

It is well known that the Th cell acts as an important effector cell in the immune response and is critical in ALI [20-22]. The polarization of Th cells has a critical role in anti-infection [23], and both Th1 and Th2 cells are now known to mediate inflammation and pathogen killing as well as tissue damage [22]. The Th1 and Th2 cells are the two main subsets activated from the Th0 cell, and respond mainly in cellular immunity and humoral immunity, respectively [12,13]. It has been

Figure 4. Th1/Th2 cytokines in virus or LPS challenged mice serum. The level of IL-2 (A), IFN-γ (B), TNF-α (C), IL-4(D), IL-5 (E) and ratio of IFN-γ/IL-4 (F) in serum. Mice were intranasally inoculated with 20 μg of LPS or $2LD_{50}$ of virus (A/FM/1/47, H1N1), serum was obtained at 2 days after incubation, and the level of cytokines was measured by flow cytometry. Data were analyzed by ANOVA, and values are expressed as the means ± S.D, *$p < 0.05$, **$p < 0.01$, ($n = 9$).

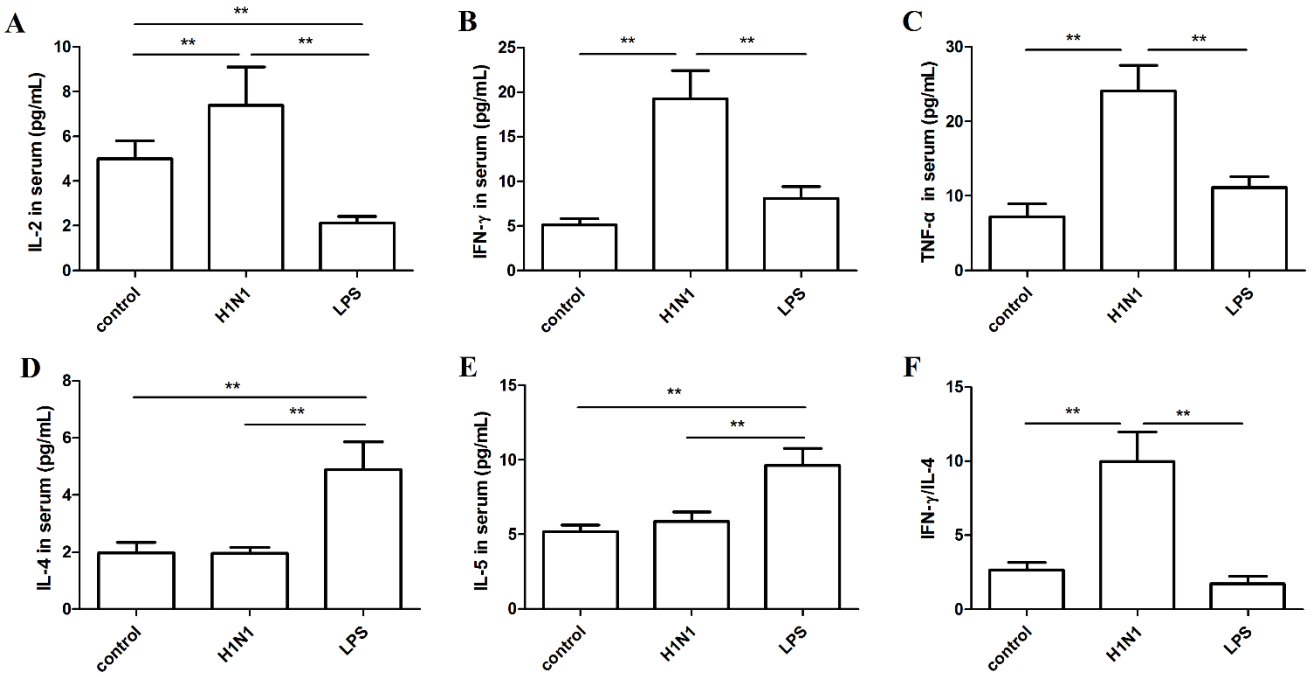
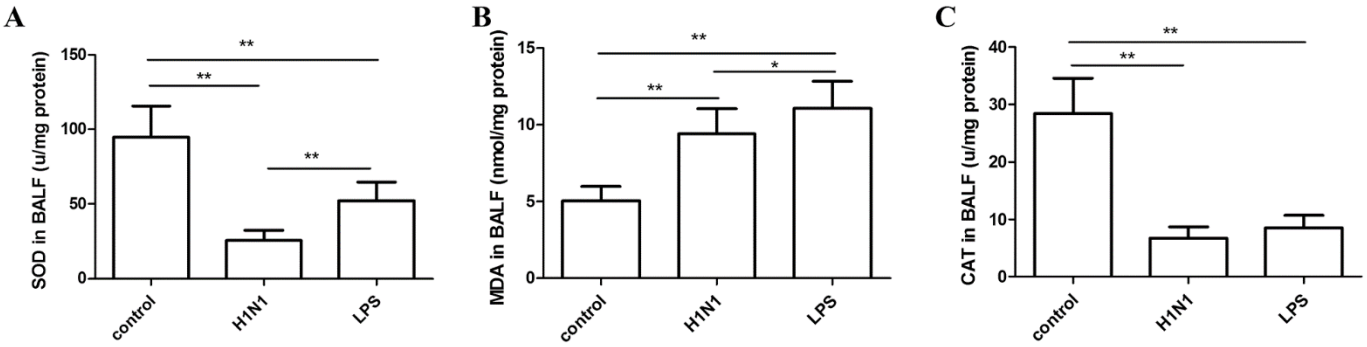
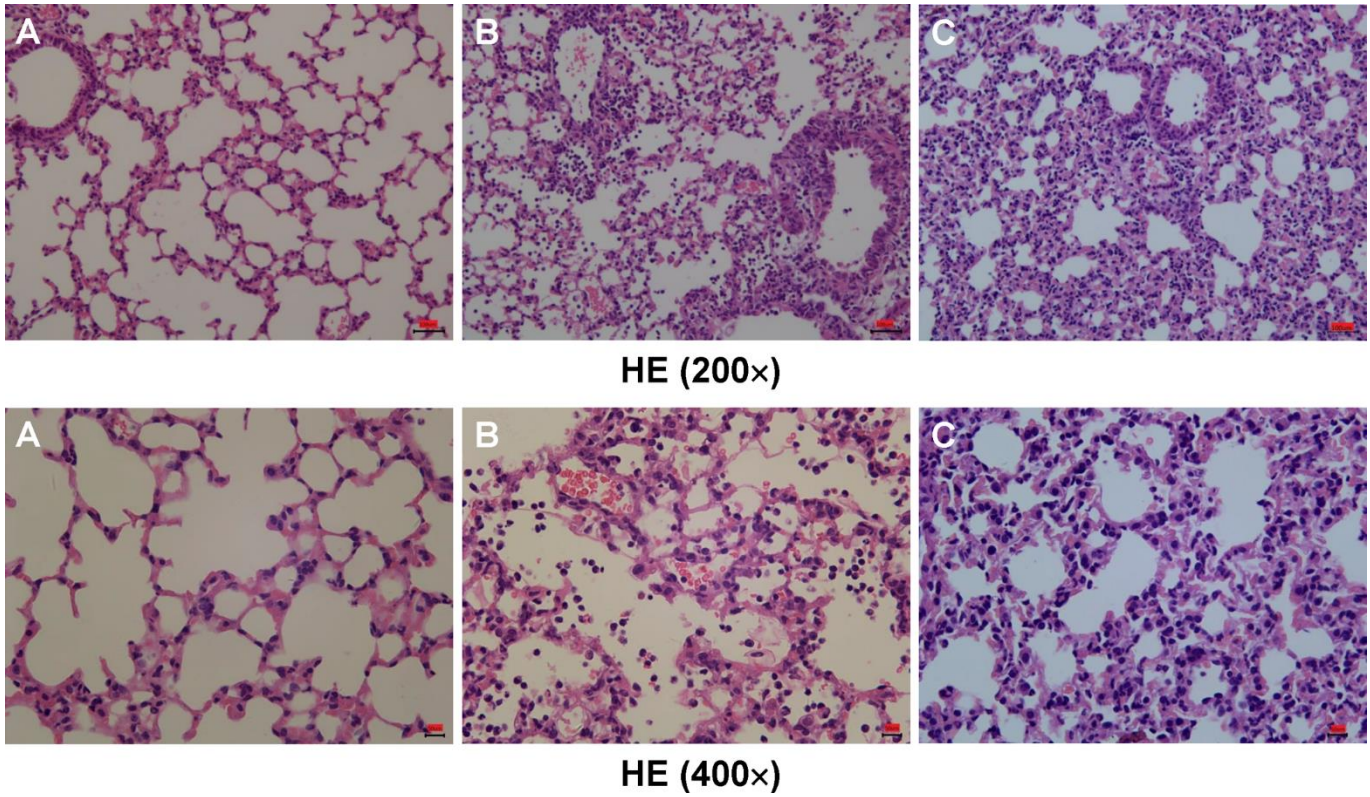


Figure 5. Oxidative stress in virus or LPS challenged mice. SOD (A) MDA (B) and CAT (C) in BALF. Mice were intranasally inoculated with 20μg of LPS or $2LD_{50}$ of virus (A/FM/1/47, H1N1), the level of SOD, MDA and CAT were measured by a spectrophotometer. Data were analyzed by ANOVA, and values are expressed as the means ± S.D, *p < 0.05, **p < 0.01, (n = 9).



demonstrated that the skew in polarization is usually mutually restrictive [24-26], being closely dependent on the nature of the infectious agent and cytokines [14,25,27]. Studies have shown that the cross-talk between antigen-presenting cells and pathogens is decisive in the polarization of Th cells. The cytokines secreted by epithelial cells or antigen-presenting cells after virus or LPS challenge are significantly different, impacting the polarization of Th cells [28,29]. In addition, Th cell polarization is also influenced by cytokines, where IFN-γ or IL-12 lead to the expression of T-bet, resulting in Th1 cell differentiation and the classical production of IFN-γ, TNF-β, and IL-10; whereas, in the presence of IL-4, Th2 cell development is mediated by GATA3, which in turn results in the production of IL-4, IL-5, IL-13, IL-10, and IL-21 [30-32]. In addition, the fully mature and produced IFN-γ or IL-4 (both signature cytokines that determine the Th cell polarization profile) induces a positive feedback

Figure 6. Pathological changes in virus or LPS challenged mice. HE, (200× and 400×) A: control, B: H1N1, C: LPS. Mice were intranasally inoculated with 20μg of LPS or $2LD_{50}$ of virus (A/FM/1/47, H1N1), lung was excised at 2 days after incubation and stain with HE. The pathological features of virus challenged mice were characterized by viral pneumonia and interstitial edema, while that's in LPS challenged mice was features of interstitial inflammatory pneumonia.



mechanism to enhance the Th1 or Th2 response, respectively [33,34].

Research has shown that Th1 cells mediate cellular immune responses against intracellular viruses and bacteria through the activation of macrophages and cytotoxic T cells. In contrast, Th2 cells mediate humoral immune responses by promoting B-cell proliferation, differentiation, and maturation and the production of antibodies, which are important for the host's defense against parasitic infections and contribute to the pathogenesis of allergy [28,35]. However, the overactivation or skewing of either pattern can directly or indirectly cause severe tissue injury [22]. The skewed polarization of Th cells in turn contributes to the transmigration of macrophages or neutrophils to the infection site in early acute infection, as well as cells with anti-inflammatory functions [15,16]. However, excessive immune cell infiltration contributes to alveolar cell and pulmonary capillary injury via the overproduction of cytokines and reactive oxygen species (ROS) [36-38], although the transmigration of macrophages, neutrophils, and special immune cells to the sites of infection is crucial in pathogen elimination [39,40]. It has been demonstrated that excessive inflammatory cells (e.g., lymphocytes, macrophages, and neutrophils) and the overproduction of cytokines or ROS are induced in early bacterial or viral infections, and this state might persist to the recovery phase [41,42]. Furthermore, the pathological process of ALI is closely related to the excessive inflammation mediated by inflammatory cell infiltration, such as alveolar cell and pulmonary capillary injury [37,41,43].

Conclusion

The finding demonstrating that the Th1 and Th2 responses were skewed in ALI depending on whether the lung injury was caused by the influenza virus or LPS. Likewise, the results showed that the polarization of the Th cell and the infiltration of inflammatory cells (macrophages and neutrophils) were strikingly different between the influenza virus- and LPS-challenged mice. In the influenza virus-infected mice, the main cytokine was IFN-γ, accompanied by the infiltration of macrophages. In contrast, the LPS-challenged mice had a much lower IFN-γ level, with neutrophil recruitment. Our results suggest that Th cell polarization might explain (at least in part) the diversity in the infiltration of inflammatory cells in influenza virus- and LPS-challenged mice. These results suggest that the polarization of Th1 and Th2 cell was the main power of the diversity of infiltration of macrophages and neutropils. Although, it seems that the other subset of helper T cells was activated by cytokines in simultaneously, and has a non-negligible role in the development of inflammation in infectious disease, however, mainly function in fungal, parasites infection or immunity tolerance. Additionally, we hypothesis that regulating the skewed polarization of helper T cells in early stage might contributes to reducing inflammation and lung pathological damage by impacting on subsequent inflammatory cell recruitment, remodeling cytokines might be a valid measure of the illness that characterized by excessive infiltration and inflammation. Although the dates are limited, our results indicate that the polarization of Th1 and Th2 may be important to discriminate the diversity immunity response in early stage of acute lung injury induced by virus or bacteria. Further studies should be planned to ascertain the decisive effect of Th cell polarization on inflammatory cells recruitment and inflammation.

Acknowledgements

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