Jagged2- $\gamma\delta$ T17 is Regulated by *Mycobacterium Vaccae* in Asthmatic Mice

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Research

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Abstract

Background: *Mycobacterium vaccae* nebulization imparted protective effect against asthma in a mouse model. The Jagged2- $\gamma\delta$ T17 signal transduction pathway plays an important role in bronchial asthma. However, the effect of *M. vaccae* nebulization on the Jagged2- $\gamma\delta$ T17 signal transduction pathway in mouse models of asthma remains unclear. **Methods:** In total, 30 female C57 mice were randomized to normal control (group a), asthma control (group b), *M. vaccae* nebulization prevention, and *M. vaccae* nebulization treatment (group d) groups. Asthma mice models were created using ovalbumin (OVA). The Notch signaling pathway was blocked by DAPT inhibitors. Airway hyperreactivity (AHR) was measured by noninvasive lung function tests. Histopathological analyses using blue-periodic acid Schiff along with hematoxylin and eosin were performed. Immunohistochemistry, immunofluorescence, and a Western blotting assay allowed for the detection of lung protein expressions, while spleen expressions of IL-17+ $\gamma\delta$ T+ cytokines were assessed with FLOW cytometry. One-way analysis of variance for within-group comparisons, the least significant difference t-test or Student-Newman-Keuls test for intergroup comparisons, and the nonparametric rank sum test for analysis of airway inflammation scores were used in the study. **Results:** Asthmatic mice models demonstrated downregulated Notch signaling pathway activation and decreased $\gamma\delta$ T cells and IL-17 cytokine secretion. There was also increased Jagged2 protein expression which correlated positively with $\gamma\delta$ T+IL-17+ secretion. In asthmatic mice, the expressions of Jagged2 and $\gamma\delta$ T17, along with airway inflammation and airway reactivity, were all decreased after *M. vaccae* exposure ($p < 0.05$). **Conclusion:** The Notch signaling pathway contributed towards asthma initiation and progression by facilitating $\gamma\delta$ T cells and IL-17 cytokines production. Inhaled *M. vaccae* led to a significant decrease in Jagged2 and $\gamma\delta$ T17 expressions in asthmatic mice, indicating its utility in asthma prevention.

1.0 Introduction

Bronchial asthma is a serious global health concern afflicting 3 billion people worldwide. The disease imposes a severe burden on health care systems. Currently, there are no effective measures to prevent and treatment the condition of asthma. Studies on the efficacy of immunotherapy during of asthma is at their nascent stage.

Asthma is a chronic inflammatory airway disease that manifests as several different clinical phenotypes, all of which involve a myriad of cells, including mast cells, T lymphocytes, eosinophils, and neutrophils^[1]. Current research has been focusing on the immunopathology behind asthma. Of interest is the $\gamma\delta$ T cell, which appears to be a crucial immune mediator in asthma^[2]. The $\gamma\delta$ T cell response triggers airway hyperreactivity (AHR) as well as airway eosinophilia and Th2 cell recruitment^[3]. $\gamma\delta$ T secretes IL-17, which is a key participant in airway inflammation, AHR, and airway remodeling in asthma^[4–8]. Existing research confirms that airway remodeling is modulated by IL-17 through activation of epithelial cells, airway smooth muscle cells, and fibroblasts^[9]. IL-17 promotes airway DC migration and activation, leading to AHR, increased eosinophils, mucus hypersecretion, and decreased IgE levels^[10]. Moreover, IL-17-induced

autophagy induces bronchial fibroblasts-mediated fibrosis^[11]. $\gamma\delta$ T17 cells are $\gamma\delta$ T cells that produce IL-17^[12]. Compared with other T lymphocytes, $\gamma\delta$ T secretes more IL-17 cytokines^[13]. Investigations have demonstrated that pro-inflammatory cytokines and the proportion of percentage of IL-17⁺ $\gamma\delta$ T cells are dampened upon RTA-408 exposure, leading to attenuated inflammation in the airways of asthma mice models stimulated by ozone^[14]. Prior research has established the involvement of $\gamma\delta$ T17 in asthma^[15]. Nevertheless, literature linking asthma and $\gamma\delta$ T17 cells are scarce. Our previous study confirmed the involvement of $\gamma\delta$ T17 in mice models of OVA-induced asthma^[15]. *M. vaccae* atomization prevents allergic bronchial asthma in mice models through reduction of AHR and airway inflammation^[16]. The $\gamma\delta$ T cells are critical regulators of allergic inflammation, AHR, and airway function in asthmatic mice models^[17]. The development of $\gamma\delta$ T cells is closely related to notch-hes1 and IL-17 production^[18]. However, little is known regarding how $\gamma\delta$ T cells work together with the Notch signaling pathway in asthma pathogenesis. Notch signaling strongly regulates lymphocyte activation and differentiation. The interaction of Notch receptor and ligand may promote cytokine production in addition to Th cell polarization and proliferation^[19–21]. Notch signaling pathway critically contributes towards asthma initiation. The allergic reaction appears to be mediated by Notch signaling that inhibits Th2 polarization and augments differentiation of Th1 cells, resulting in a proinflammatory Th1/Th2 balance in mice^[22]. Asthma pathogenesis is thought to rely heavily on Notch signaling-induced Th2 cell differentiation^[23, 24].

Other significant factors in asthma include the upregulation of the Jagged2 factor and subsequent Th2 cell differentiation, a phenomenon that is brought about by CD4T-augmented Th1 cell differentiation^[25, 26].

The current investigation utilizes a Notch signal pathway inhibitor to better study the role of Jagged2 in asthma pathogenesis. Experiments were performed on ova-induced asthma mice models, while the DAPT drug was used as a Notch signaling pathway inhibitor to detect changes in markers of inflammation in the $\gamma\delta$ T17 cells. To study whether Jagged2 is involved in the anti-inflammatory effects of aerosolized *M. vaccae* in $\gamma\delta$ T17 cells, different inflammatory responses were compared among the asthma, prevention, and treatment groups.

2.0 Experimental Materials And Animals

2.1 Experimental materials

Reagent OVA; Phorbol myristate ester (PMA) (Sigma); Ionomycin (sigma); aluminum hydroxide gel (Pearce, USA); *M. vaccae* f.u.22.5 μ g injection (Anhui Zhifei Longkema Biopharmaceutical Co., Ltd.); DATP (Selleck), immunohistochemistry, immunofluorescence, and Western blotting antibody Jagged2 (CST); Flow cytometry antibody percp-cy5-5cd3, APC $\gamma\delta$ T $\square$ PE-IL-17 $\square$ eBioscience, USA $\square$ Flow cytometry antibody monomycin, fixed / membrane breaking solution (eBioscience, USA). Microplate reader (Bio Rad, United States), ultrasonic atomizer wh-2000 (Guangdong Yuehua Medical Equipment, China),

pathological image analysis system (Leica, Germany), 5810R high-speed cryogenic centrifuge (Eppendorf, Germany), FACS canto II flowmeter (BD), and a self-made atomization inhaler were used.

2.2 Ethics statement

The study was performed in accordance with the guide for the care and use of laboratory animals of the National Institutes of Health, and approval was gained from the Animal Ethics Committee of Guangxi Medical University (Protocol number: 201711033). We using Chloral hydrate anesthesia and all efforts were made to minimize suffering

2.3 Animals

Healthy male C57 mice (about 6 weeks old weighing approximately 20g each) were supplied by the Animal Experimental Center of Guangxi Medical University and reared under specific pathogen-free conditions. Animals were allowed free access to food and water and were kept at appropriate room temperatures and humidity. A total of 5 mice groups (n=6 per group) were formed: treatment group (OVA + *M. vaccae*), prevention group (*M. vaccae* + OVA), blocking group (DAPT + OVA), asthma group (OVA), and the control group. 25mg OVA was emulsified in aluminum hydroxide gel on days 0, 7, and 14. The OVA emulsification was then diluted on days 21-28 and soaked in phosphate buffered saline (PBS) for 30 minutes prior to exposing the concoction to the mice. Mice in the blocking group were treated for 30 min with aerosolized DAPT (0.3 mg/kg) prior to OVA exposure. In the prevention and treatment groups, mice were treated with vaccinia aerosolized with 10mL of normal saline at days 21-28 and 28-35, respectively. Normal saline in place of all the above treatments was used for the control group. After the experiments, mice were sacrificed for specimen collection using intraperitoneal injection of 10% chloral hydrate (0.1 ml). All experiments were repeated twice.

2.4 Airway hyperreactivity in mice

Mice were stimulated by methacholine and placed in a testing cubicle equipped with a ventilation switch that was able to detect the special airway resistance (sRaw) value. Different concentrations of methacholine were used (6.25, 12.5, 25 and 50 mg/ml), with sRAW value compared against the value gained upon PBS stimulation.

2.5 Histopathological analysis

Lung samples were treated for 24 h with 4% paraformaldehyde, washed, dehydrated with ethanol gradient, and treated with paraffin. 4mm-thick slices were treated with hematoxylin & eosin as well as periodic acid-Schiff. An optical microscope (Olympus, Japan) was used to visualize the sections.

2.6 Immunohistochemical and immunofluorescence studies

4mm sections were dewaxed in xylene and rehydrated in graded alcohol. 3% hydrogen peroxide was used to block endogenous reactions. Tissue sections were then boiled for 10 min in 10 mM citrate buffer (pH 6.0) for antigen recovery, before being cultured in 5% goat serum albumin followed by anti-human Jagged2 antibody (1:2000; D4y1r, CST). Non-immune serum instead of a primary antibody was used in the negative control group. Samples underwent another 60 min incubation with secondary antibody bound to horseradish peroxidase at room temperature. The tissues were then stained with 3,3'-diaminobenzidine and hematoxylin blue. The control tissue sections and specimens were treated in unison. Protein detection was done via immunofluorescence and observed under an optical microscope (Olympus).

For Jagged2 staining, 2mm sections of air-dried, frozen lung tissue were treated for 20 min with cold methanol before being air-dried again. Lung tissues were then cultured for 10 min with PBS supplemented with 20% fetal bovine serum (FCS) before being permeabilized with 30 ml of 1.5% H₂O₂. PBS was then used to rinse the sections twice before they were incubated for another 10 min with avidin D solution. Biotin solution was used to block endogenous peroxidase, rinsed, and cultured overnight at 4°C with rabbit anti-mouse Jagged2 antibody (1:1500; D4y1r, CST). The following morning, sections were rinsed twice with PBS and exposed for 2 h to goat anti-mouse IgG secondary antibody in the dark. All sections were then observed under a fluorescence microscope (Olympus).

2.7 Western blotting

Lung tissue was homogenized on ice with 10 ml RIPA buffer containing PMSF. Samples were mixed with 5s protein sample buffer at a 4:1 ratio, boiled and denatured, and separated by a 10% SDS-PAGE gel. The protein strips were charged onto the membrane and sealed with 10% BSA at 4°C for 1 hour. Proteins were detected with anti-GAPDH (1:5000) and anti-Jagged2 (1:1500) antibodies overnight at 4°C, washed once with TBST, then re-incubated with secondary antibody (1:800) for another hour. The positive bands were analyzed by Licor Odyssey software.

2.8 Flow cytometry

Mouse lung tissue was digested with collagenase IV-containing RPMI 1640 media at 37°C (2 mL, 2.5g/L) (Gibco, USA). The partially digested lung and spleen tissue particles were ground with a 200mm mesh filter and centrifuged at 250g at 4°C for 5min. The supernatant was discarded and the granules were cultured in red cell lysis buffer for 4 min in the dark before being centrifuged for another 5 min at 250 g and 4°C. The supernatant was discarded, and the granules were rinsed with PBS. Cells were isolated in an incubator of 5% CO₂ and 37°C for 72 hours. Retained granules containing lung mononuclear cells

were re-suspended in RPMI 1640 media supplemented with 0.2% monensin, 1 mg/l ionomycin, 25 mg/l PMA, and 10% fetal bovine serum at a concentration of 106 cells/ml. The cell suspension was cultured for another 4 h at 5%CO₂ and 37°C before being centrifuged for another 5 min at 250 g and 4°C. The pellets were incubated in the dark for 30 min at 4°C with the APC anti-anti-γδT17 antibody and the percp-CY5-5 anti-CD3 antibody. PBS was then used to rinse the cells, before they were resuspended in Cytofix/Cytoperm solution (Becton Dickinson, USA). The cell suspension was then left in the dark for 20 min at 4 °C. Samples were then rinsed, incubated for 30 min with PE anti-IL-17 antibody, rinsed again with PBS twice before finally being suspended in 200mL PBS. the FlowJo 7.6 software (Becton-Dickinson) was used to perform flow cytometry analysis.

2.9 Statistical methods

SPSS22.0 software (IBM, USA) was used for statistical analysis, and Prism 5.0 software (GraphPad, USA) was used to generate graphs. Data were reported as mean ±SE. Analysis of variance was utilized to evaluate the differences between groups, followed by the post-hoc Fischer minimum significant difference (LSD) test for pairwise comparisons between groups. Sample correlations were evaluated using Pearson correlation. Statistical significance was determined when $P < 0.05$.

3.0 Results

3.1 Airway hyperreactivity in mice

Figure 1 shows that stimulation of 12.5mg/ml, 25mg /ml, and 50mg/ml of methacholine in the asthma group resulted in higher airway resistance in contrast to the control group ($P < 0.05$). There were also significant variability of the sRAW differences between the prevention and treatment groups, as well as in the control and blocking groups ($P < 0.01$).

3.2 Lung histopathological analyses

Lung histopathological analyses of the control group revealed that no abnormalities in bronchial and alveolar morphologies, no tracheal wall thickening, and no epithelial cell proliferation. There were also less inflammatory cells surrounding lung blood vessels and bronchi.

PAS staining of the airway epithelium revealed few goblet cells without mucus exudation. Those in the asthma group demonstrated bronchial lumen stenosis and wall thickening, obvious inflammatory cell infiltration in bronchus and perivascular lung tissue, as well as higher rates of goblet cell proliferation and mucus exudation in contrast to the control groups. Mice in the treatment, prevention, and blocking groups demonstrated much lower rates of inflammatory infiltrates in the bronchial and perivascular cells, as well as fewer amounts of goblet cells and mucus exudation in comparison to the asthma group (Table 1, Figure 2).

3.3 Immunohistochemistry, immunofluorescence and western blotting studies

Mice with asthma had more significantly elevated Jagged2 expressions in contrast to the control group ($P < 0.05$). Conversely, the treatment, prevention, and blocking groups revealed statistically significant lower Jagged2 expressions in contrast to the asthma group (Fig. 2, $P < 0.05$).

3.4 Correlation analysis of Jagged2 and $\gamma\delta$ T17 cells in mice spleen tissue

Mice of the asthma model group had increased proportions of $\gamma\delta$ T17 cells ($26.7 \pm 3.7\%$) in contrast to the control group ($8.1 \pm 0.5\%$). The number of $\gamma\delta$ T17 cells was $9.0 \pm 1.0\%$ in the DAPT + OVA, $10.8 \pm 1.4\%$ in the *M. vaccae* + OVA, and $11.0 \pm 0.9\%$ in the OVA + *M. vaccae* groups. Compared with the asthma group, the IL-17⁺ $\gamma\delta$ T ratio of T⁺ cells was low (Fig. 3, $P < 0.05$). The IL-17⁺ $\gamma\delta$ T percentage of T⁺ cells correlated positively with Jagged2 protein expression in lung tissue ($r = 0.63$, $P < 0.001$).

4.0 Discussion

Asthma involves several yet-to-be determined signal pathways. Previous studies have supported the Notch signaling pathway as a cornerstone of cell development, differentiation, proliferation, and survival^[27, 28]. Specifically, δ ligands are involved in Th1 cell development, while Jagged2 ligands are closely in Th2 cell development. Many studies in asthma show that the Notch signaling pathway stimulates Th2 cell differentiation^[20, 21, 29, 30]. Notch guides Th1 and Th2 cell differentiation while enabling T cell signal transduction^[22, 31]. Tu et al. highlights that asthma is modulated primarily through Th2 cell immunity^[28]. The central function of the Notch signaling pathway makes it an ideal therapeutic target in modulating Th2 cell immunity in asthmatic patients^[32]. Data shows that jagged2 is involved in immune regulation in allergic airway inflammation^[12, 32]. Studies have also shown that allergic asthma can be alleviated by inhibiting IL-4R α -Stat6 and the Jagged1/Jagged2-Notch1/Notch2 signaling pathways in mice^[11]. On the other hand, IL-17 exerts significant influence in activating and recruiting neutrophils, enhancing inflammatory cell infiltration, participating in airway remodeling, and promoting airway hypertrophy^[33–36]. $\gamma\delta$ T cells support the Th1/Th2 imbalance, implicating $\gamma\delta$ T cells in asthma development^[3, 37, 38]. $\gamma\delta$ T17 cells contribute towards the inflammatory response by producing pro-inflammatory cytokines and augmenting inflammatory messenger secretion.

Previous investigations have underscored the importance of $\gamma\delta$ T17 in asthma pathogenesis^[39]. Dysregulated Th1/Th2 cell proportions among the $\gamma\delta$ T cell population is implicated in asthma development in animal models. Aerosol inhalation of *M. Vaccae* corrects the Th1/Th2 imbalance in $\gamma\delta$ T cells in asthma, thus alleviating lung inflammation^[5]. It was also demonstrated that *M. vaccae*

inhalation is hypothesized to be able to prevent mice allergic bronchial asthma by suppressing inflammation and AHR^[16]. $\gamma\delta$ T17 cells participate in the inflammatory response by inducing inflammatory cytokine and messenger production and release. The current investigation establishes an asthma mouse model and evaluated the degree of airway reactivity, lung pathological manifestations, lung protein expression, and CD3 + $\gamma\delta$ T proportion of T + IL-17 + cells. Compared to the control group, airway reactivity, airway inflammation, IL-17, as well as $\gamma\delta$ T expression of T+ and jagged2 protein in the asthma group lung specimens increased significantly. We noted a positive correlation between Jagged2 protein and CD3 + $\gamma\delta$ T+IL-17+ cell populations. The Notch signaling pathway may regulate airway reactivity and airway inflammation in asthmatic mice through its receptor, Jagged2. Jagged2 and $\gamma\delta$ T17 cell secretion appear to be closely related in asthma pathogenesis. *M. Vaccae* appears to regulate the Notch / jagged2 signal pathway in alleviating asthma, representing a potential therapeutic candidate in this common respiratory condition.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Approval was gained from the Animal Ethics Committee of Guangxi Medical University.

CONSENT FOR PUBLICATION

manuscript is approved by all authors for publication.

AVAILABILITY OF DATA AND MATERIAL

Data set for the proteomic inventory and quantitative analysis, FLOW cytometry, and inflammatory response of mice during asthma below "attach files", include Figures and table.

COMPETING INTERESTS

The authors declare no conflict of interest.

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AUTHORS' CONTRIBUTIONS

Contributors Me, Yi-En Yao: acquisition of the data, analysis and interpretation of the data and drafting of the manuscript. Jian-Lin Huang: acquisition of the data, analysis and interpretation of the data and critical revision of the manuscript. Qi-Xiang Sun, Jing-Hong Zhang, Si-Yue Xu: technical support and critical revision of the manuscript for important intellectual content. Chao-Qian Li: study concept and design, drafting of the manuscript, obtained funding and study supervision

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Data is depicted in terms of mean $\pm$ S.E. of the percentage in relation to the control group (n=6). *P<0.05, **P<0.01, ***P<0.001 vs. control group; #P<0.05, RP<0.05, @P<0.05, ###P<0.01, \$\$P<0.01, &&P<0.01, ###P<0.001 vs. asthma group (ANOVA).

Figure 2

Lung histopathology was detected using Periodic acid-Schiff (PAS) and hematoxylin and eosin (HE) staining. The effects of inactivated *M. Vaccae* on ovalbumin (OVA)-triggered airway inflammation. Histological inspection of the normal control (a), asthma (b), γ -secretase inhibitor DAPT + OVA group (c), *M. vaccae* + OVA (d), OVA + *M. vaccae* (e). HE staining: original magnification 400x, scale bar=50 mm;

PAS staining: original magnification 400, scale bar=100 mm. **P<0.01, ***P<0.001 vs. control group; #P<0.05, &P<0.05, &P<0.05 vs asthma group (ANOVA).

Figure 3

The percentage of $\gamma\delta$ T in T17 cells of CD3+T cells was determined. Data is presented in terms of mean $\pm$ S.E. relative percentage of the control group (n = 6). T17 cells correlated positively with $\gamma\delta$ and Jagged2 cells (r = 0.63, P < 0.05). **P<0.01 vs control group; \$P<0.05, &P<0.05, ##P<0.01 vs. asthma group (ANOVA).

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