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Original Article

Obesity enhances Th2 inflammatory response via natural killer T cells in a murine model of allergic asthma

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Abstract: Background: Obesity increases the incidence of asthma, but mechanism between asthma and obesity isn't utterly understood. NKT cells are intermediary activist between the innate and adaptive immune. It may play an equally important role in both obesity and asthma. We studied an obese mouse model of allergic asthma to test whether NKT cells act as a linkage in the development of obesity with asthma. Methods: Balb/c mice were divided into control group (A), asthma model group (B), the obesity group (C) and obesity with asthma group (D), asthma model made by OVA. Obesity was induced. AHR were measured; HE staining of lung was made; NKT cells were detected and IL-4 and IFN-γ concentration were determined. Results: Lung histology showed airway inflammatory in obesity with asthma are significant than in asthma. IL-4 levels were increased compared with the control group. IFN-γ levels were decreased compared with the control group. More CD69+NKT cells of asthma group and obese asthma group correlated to the enhancement of airway inflammation and AHR. IFN-γ+NKT cells vary in different states not paralleling with CD69+NKT cells. Conclusion: The activity level of NKT cells in obesity with asthma mice enhances Th2 Inflammatory response by regulating IL-4 and IFN-γ secretion. The activation of NKT enhanced asthma TH2 inflammatory responce. NKT cells play an important role in the development of asthma in obesity.

Keywords: Bronchial asthma, airway inflammation, NKT cell, obesity

Introduction

The incidence of both obesity and asthma has increased in the past decades. Obesity has been identified to be the significant risk factor of asthma [1]. And asthma symptoms appear to be more severe in obese people, moreover obesity may also alter the efficacy of standard medications for asthma [2]. Asthma in obese individuals responds poorly to typical asthma medications thus leads to greater asthma-related healthcare utilization and reduce of life quality [3, 4]. Epidemiological data shows that obese or overweight individuals in China have increased progressively since the 1980s as a result of the fast pace of industrialize. Despite of the numerous epidemiological evidence linking asthma and obesity, the mechanistic link between these diseases is far from clear [5]. So additional researches still need finding out the biological basis for the connection between obesity and asthma.

As known adipose tissue isn't merely to store energy passively; it is an important endocrine organ with major effects on immune function [6]. Data from human and animal studies suggest that obesity is a pro-inflammatory state [7]. Many proinflammatory and anti-inflammatory factors, including the adipokinesleptin, adiponectin, resistin, and visfatin, as well as cytokines and chemokines, such as TNF-α, IL-6, monocyte chemoattractant protein 1, were produced or secreted by adipose tissue [8]. White adipose tissue (WAT) involves in producing adipocytokines including C-reactive protein (CRP), IL-6, IL-9, IL-18 [9]. Macrophages and mast cells are increased in obese WAT compared to lean

tissue [10, 11]. Saturated fatty acids can stimulate toll-like receptors (TLRs) [12] and lead to cardiometabolic deregulation [13].

Actually, asthma in obese individuals is not a single illness. Researchers identified there are at least two different phenotype of asthma in obesity. Early-onset asthma in obesity is accompanied with more atopic disease, increased IgE and airway hyper responsiveness (AHR). These patient appear to have allergic asthma that is complicated by obesity. In contrast, late onset asthma in obese individuals frequently has less atopy, lower levels of TH2 inflammation and AHR. These cases have asthma that has happened in the status of obese physical [14]. However, the mechanistic basis for anyone of above two phenotypes has not yet been well established. First of all, obesity induces multiple kind salters of mechanical and metabolic circumstances that directly act on airways. Also, systemic inflammation associated to obesity may link obesity and asthma. This systemic inflammation originate from the adipose tissue itself, leading to airway inflammation [15].

Natural killer T (NKT) cells are innate-like T lymphocytes which are capable of rapidly producing a mixture of TH1 and TH2 cytokines. NKT cells function as to link the innate and adaptive immune systems [16]. They have the ability to either promote or suppress immune responses. The NKT TCR is activated by glycolipid antigens through CD1d, while an alternative activation pathway is dependent on cytokine signaling from activated dendritic cells [17]. Cytokine rapidly produced from activating NKT cells may be of mixing, which TH1 or TH2 dominance depending on the microenvironment [18]. A study demonstrated that mice lacking NKT cells have reduced adipose tissue inflammation and improved glucose tolerance compared to wild-type mice when exposed to a high fat diet [19]. Lynch et al. have revealed that iNKT cells are abundant in human adipose tissue but are dropped in obesity [20]. NKT cells may play an important immune regulatory role in adipose tissue. Lynch L reported iNKT cells protect against the progress of metabolic syndrome and inflammation coming after a high fat diet challenge [21].

On the other hand, several studies in mice asthmatic models reported that NKT cells is necessary for AHR in asthma. In allergic asthma, AHR failed to occur in NKT cell-deficient mice (Ja182/2 mice and CD1d2/2 mice), even though eosinophilic airway inflammation to a certain extent exist. AHR was restored in the NKT cell-deficient mice by the adoptive transfer of NKT cells from wild-type but not from IL-4/IL-13-deficient mice [22]. Lisbonne M. et al. have demonstrated that iVα14 NKT cells intervene Th2 eosinophil airway inflammation and AHR in a model of asthma. NKT cells may mediate or magnify the Th2 response and AHR in allergic asthma [23]. They can aggravate allergic asthma by enhancing AHR and eosinophilia during the effector phase, but confer protection at disease onset, once they have been activated by their specific ligand a-galactosylceramide (a-GalCer) [24-26]. So it is inferred that iNKT cells exert deleterious or beneficial effects, depending on the stage of the immune response in which they are activated.

On the basis of the above, it has been seen that the same cells play an equally important role in both obesity and asthma. As we all know obesity and asthma are both chronic inflammatory diseases. Do NKT cells link obesity and asthma? Thus, we hypothesized that NKT cells may play an essential role in the development of obesity with asthma. Therefore we studied a obese mouse model of allergic asthma in which mice were fed with High fat diet to induce obesity, and were subsequently induced with ovalbumin making an asthmatic model. Specific airway resistance double-chamber plethysmography were measured; Pathological HE staining of lung airway inflammation and airway remodeling were made; liver NKT cells were detected; cytokines IL-4 and IFN-γ concentration were determined. Our study investigated the role of NKT in the mechanism of obesity with asthma.

Materials and methods

Animals

All animal care and experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Guangxi Medical University for Animal Experimentation, and followed the Guide for the Care and Use of Laboratory Animals. Animals were treated humanely and with regard to alleviation of suffering. Specific pathogen-free female BALB/C mice, 3-4 weeks of age, were obtained from

Laboratory Animal Center of Guangxi Medical University (Nanning, Guangxi, China). Mice were allowed to acclimatize to their new environment for one week, and were housed in an air-conditioned room at 23±2°C, 55±10% humidity, on a 12-h light/dark cycle and fed for 12 weeks with either a standard chow diet (70% carbohydrate; 20% protein, 10% fat) or a high-fat diet that induces obesity (29% carbohydrate, 16% protein, 55% fat).

Reagents

Ovalbumin (OVA), Grade V, was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Aluminum hydroxide was from Pierce. The following monoclonal antibodies from eBioscience were used: anti-mouse CD3 APC, anti-mouse CD1d PE, anti-mouse CD69 PE-CY5 and anti-mouse IL-4 TITC, anti-mouse IFN-γAF-488. Fixation Medium was obtained from invitrogen (Invitrogen, Camarillo, CA). Monensin, Ionomycin and Phorbol ester was purchased from Sigma-Aldrich. Monoclonal antibody-based mouse IL-4 and IFN-γ ELISA kit were from R&D (R&D system, Minneapolis, MN, USA).

Experimental protocols

Mice were randomly categorized into four experimental groups of six mice each: the normal control group (group A), the asthma model group (group B), the obesity group (group C) and obesity with asthma group (group D). The mice of group C and D were fed with a high-fat diet that induces obesity (D12451, Research Diets, Inc., New Brunswick, NJ; 35% carbohydrate, 20% protein, 45% fat). Group A and B were fed with a standard chow diet (70% carbohydrate; 20% protein, 10% fat). The mice of the group B and D were sensitized and challenged with Ovalbumin to make a murine asthma model. During 12 weeks, the body weight and length of all mice were measured.

Allergen sensitization and challenge

Mice were sensitized to 25 μg of ovalbumin (OVA) emulsified in 1 mg of aluminum hydroxide in a total volume of 200 μL on day 0, 7 and 14 by intraperitoneal administration. After the initial sensitization, the mice were challenged an aerosol of either PBS containing 6% OVA (weight/volume) or PBS daily in a closed chamber. Mice were studied 24 hours after the last aerosol challenge. Lung tissue, liver tissue and serum samples were harvested. Lung tissue were fixed with 10% formalin for HE stain. Liver tissue were stored at -80°C until further use for Flow Cytometric analysis.

Airway responsiveness

AHR was assessed using a double-chamber plethysmography device (TBL4500, BUXCO, USA) on the basis of the increase in the specific airway resistance (sRaw). In brief, the mice were exposed to nebulized PBS for 3 min to establish baseline sRaw values, followed by exposure to increasing concentrations of nebulized methacholine (6.25-25 mg/mL; Sigma; USA) using an Aerosonic ultrasonic nebulizer. Following each nebulization cycle, recordings were obtained for 3 min. The sRaw values measured during each 3-min sequence were averaged and expressed for each methacholine concentration. The increase in sRaw was calculated as follows: sRaw with each methacholine concentration-sRaw with PBS)/sRaw with PBS × 100%.

Histologic examination of lung tissue

Lungs were fixed in situ with 10% formalin. The lungs were then embedded with paraffin, cut into 4 μm sections, and stained with hematoxylin and eosin. Sections were examined blindly under light microscopy to determine the lung inflammation. Severity was assigned based on the number of inflammatory cell infiltrate layers around the airways and blood vessels. Determine the extent of mucus production, goblet cell hyperplasia in the airway epithelium according to the description by Henderson WJ et al [27]. Lung specimens were evaluated by two pathologists in a blind fashion.

Cytokine analysis in serum by ELISA

The cytokine concentrations of IL-4 and IFN-γ in serum were determined using commercially available ELISA kits. R&D Systems (MN, USA) DuoSet ELISA kits were used according to the manufacturer's instructions. The absorbance was measured at 450 nm by a microplate ELISA reader (Bio-Rad Labarotaries, CA, USA).

Preparation of single cell suspensions from liver

Single cell suspensions from liver tissues were made by mechanical disruption combined with

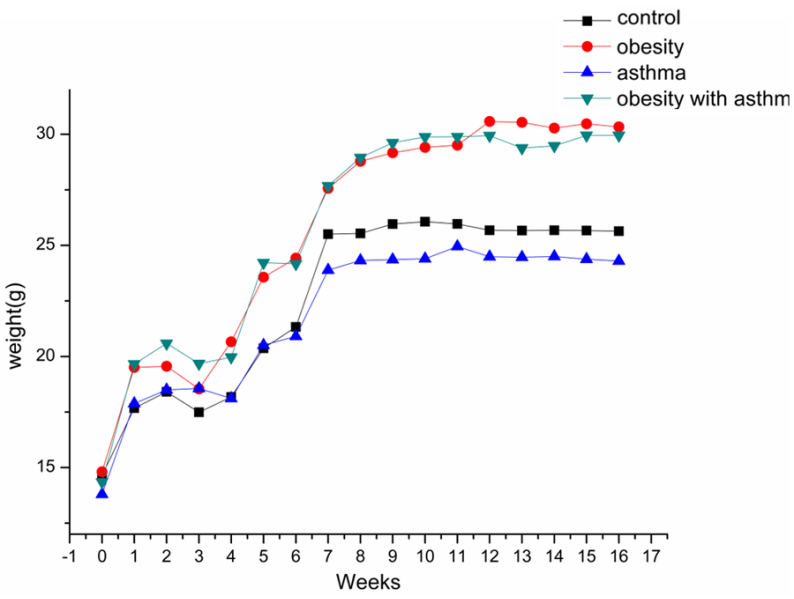

Figure 1. Effect of high fat diet on body weight. The mice on a high fat diet gained body weight more rapidly than the mice on a normal diet. The total body mass of high fat diet mice was substantially greater than that of normal diet mice. During the experimental period, the high-fat-fed mice's body weight increased significantly at 12 week.

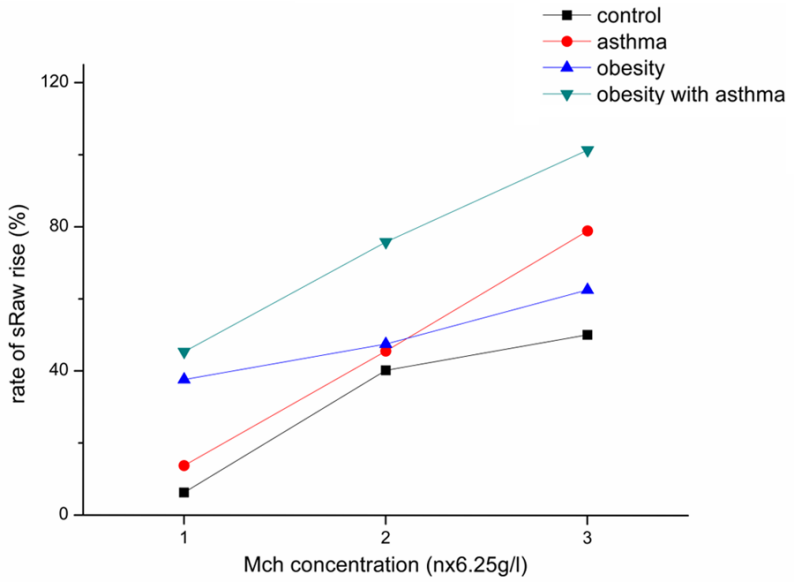

Figure 2. The growth of sRaw. The growth of sRaw shows a gradually increasing trend after sensitized and challenged with OVA in mice after inhalation of different concentrations of methacholine compared with the control group. The obese group mice show a significant growth of sRaw at low concentrations of methacholine (6.25 g/L), ($P < 0.01$), whereas sRaw has no significant increase at moderate concentrations (12.5 g/L) and high concentration (25 g/L), ($P > 0.05$). In obese with asthma mice, the growth of sRaw show a significant increase at each concentration of methacholine as compared to asthma group ($P < 0.01$).

enzymatic digestion. Firstly, liver was flushed with cold PBS until it became pale. The right lobe of liver was minced into small pieces using sterile scalpels. Then, the tissue was incubated in PBS containing 1 mg/mL collagenase IV and 10% fetal calf serum for 45 minutes at 37°C in a sterile polypropylene tube. After incubation, liver tissue was vigorously pipetted up and down to further dissolve remaining tissue clumps and then filtered using a 70 μm cell-strainer to obtain a single-cell suspension. Fluid filtered were centrifuged (500 × g for 10 min at 4°C). The cell pellet was washed and resuspended in 1 mL of PBS. Let the cell suspension remain for 3 min at room temperature to lyse RBCs, and then dilute with 9 mL of RPMI1640 medium. Centrifuge at 800 g for 5 min at 4°C and remove supernatant. Wash cells twice with 10 mL of RPMI1640 medium by centrifugation at 800 g for 5 min at 4°C, resuspend in FACS staining buffer and count the live cell number. Adjust to a cell concentration to 1 × 10^6 cells/mL by adding FACS staining buffer. The total number of cells was counted manually using a hemocytometer chamber (Fisher).

NKTcell surface markers and intracellular cytokine analysis by flow cytometry

Liver mononuclear cells were stained with fluorescein isothiocyanate-conjugated antibodies. Staining with isotype control antibodies was performed in all experiments. Prepare A, B, C, D tube for each specimen of 100 μL. For each sample to be analyzed, 1 × 106 cells were added appropriate

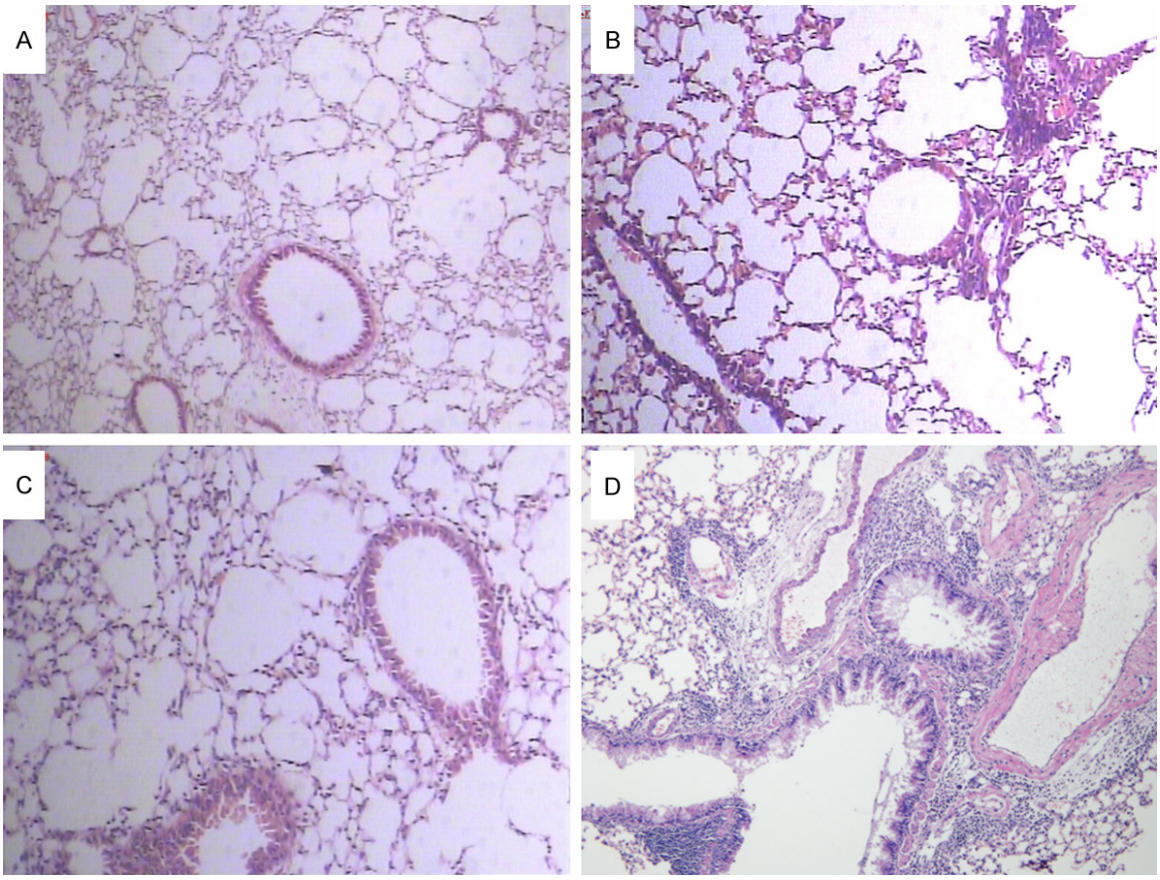

Figure 3. Histopathologic analysis of lungs. Lung samples were fixed with 10% formalin for 24 hours, and embedded in paraffin. Specimens were cut into 4 μm sections. The microsections were stained with hematoxylin-eosin (HE) to measure lung inflammation. Original magnification: × 200. Asthma and obese asthma mice showed eosinophilic inflammation compared to control mice. Obese mice without OVA challenge did not show eosinophilic inflammation. Histological examination showed inflammatory cell infiltration around the bronchioles, reduction of mucosal folds and airway remodeling. These changes are more significant in obese asthma group than in asthma group. It is even observed the performance of mucosa and muscle rupture and alveolar fusion in obese asthma BALB/c mice. A: The normal group; B: The asthma model group; C: The obesity group; D: The obesity with asthma group.

anti-mouse CD3 APC according to the manufacturer's instructions or the appropriate isotype controls. Tube B were added anti-mouse CD1d PE, anti-mouse CD69 PE-CY5. Cells were incubated for 15 minutes in the dark at room temperature. And then cells were added 100 μL Fixation Medium (Invitrogen, Camarillo, CA) and incubated for 15 minutes at room temperature. After once wash in PBS+0.1% NaN3+5% FBS, cells were centrifuged (350 × g for 5 min at 4°C) and resuspended. Then cells were added 100 μl Permeabilization Medium (Invitrogen, Camarillo, CA) and recommended volume of anti-mouse IL-4 FITC and anti-mouse IFN-γAF-488 according to the manufacturer's instructions or the corresponding isotype controls. Cells were incubated for 20 minutes in the dark at room temperature. After washing with PBS containing 0.1% NaN3 and 5% FBS, the cells were resuspended. Samples were analyzed using a FACSC alibur Becton Dickinson flow cytometer (Mountain View, CA). Data were processed with CELLQUESTPRO software (BD Biosciences, Mountain View, CA).

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 statistic software. Multiple groups were compared by one-way analysis of variance (ANOVA) followed by post-testing with LSD's multiple comparison of means. Histological scores were compared by the Kruskal-Wallis test. Statistical significance was set at $P < 0.05$.

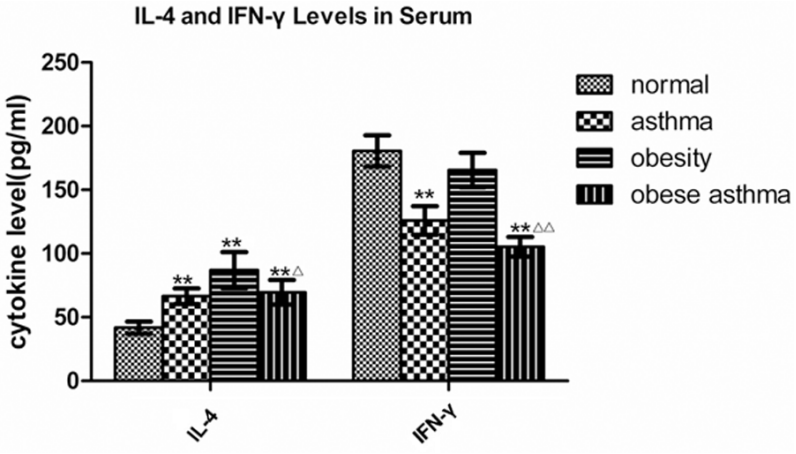

Figure 4. Cytokines (IL-4 and IFN-γ) levels in serum. The levels of IL-4 in serum of the obesity groups (86.95±14.2) were higher than those in the normal control group ($P < 0.01$). In asthma group and obese asthma group, IL-4 levels (66.48±6.13; 69.51±9.71) were largely increased compared with the normal control group ($P < 0.01$). Moreover, IL-4 concentration in obese mice was the highest. The IFN-γ levels in obesity group (165.62±13.54) were decreased compared with the normal control group ($P < 0.05$). In asthma group and obese asthma group, IFN-γ levels (125.97±11.29; 105.28±7.65) were significantly reduced compared with the normal control group ($P < 0.01$). And IFN-γ concentration in obesity with asthma group mice was the lowest.

Table 1. NKT Counting & Intracellular cytokines profile of NKT cells by Flow cytometry

Group	Number	CD1d+NKTcells	IL-4+NKTcells	IFN-γ+NKTcells	CD69+NKT cells
Normal	6	9.21±2.38	13.83±3.31	11.05±1.93	44.7±8.2
Asthma	6	20.08±3.32**	17.88±2.18**	7.2±0.68**	53.9±3.5*
Obesity	6	4.69±1.32**	31.12±2.36**	8.07±0.67**	25.6±2.8**
O & A	6	28.98±5.30**,#	24.41±4.72**,##	4.91±1.00**,#	77.3±8.2**,#

Date expressed as mean ± SD. **$P < 0.01$ and *$P < 0.05$ as compared to the normal control group (group A); ##$P < 0.01$ and #$P < 0.05$ as compared to the asthma model group (group B).

Results

Effect of high fat diet on body weight

The mice on a high fat diet gained body weight more rapidly than the mice on a normal diet. The total body mass of high fat diet mice was substantially greater than that of normal diet mice. During the experimental period, the high-fat-fed mice's body weight increased significantly than the lean mice at 12 weeks (**Figure 1**).

The growth of sRaw

The growth of sRaw show a gradually increasing trend after sensitized and challenged with OVA in mice after inhalation of different concentrations of methacholine compared with the control group. The obese group mice show a significant growth of sRaw at low concentrations of methacholine (6.25 g/L), ($P < 0.01$), whereas sRaw has no significant increase at moderate concentrations (12.5 g/L) and high concentration (25 g/L), ($P > 0.05$). Noticeably, in obese with asthma mice, the growth of sRaw shows a significant increase at each concentration of methacholine as compared to asthma group ($P < 0.01$, **Figure 2**).

Histopathologic analysis of lungs

Asthma and obese asthma mice showed eosinophilic inflammation compared to control mice in H&E stain. Obese mice without OVA challenge did not show eosinophilic inflammation. Histological examination showed inflammatory cell infiltration around the bronchioles, reduction of mucosal folds and airway remodeling. These changes are more significant in obese asthma group than in asthma group. It is even observed the performance of mucosa and muscle rupture and alveolar fusion in obese asthma BALB/c mice (**Figure 3**).

Cytokines (IL-4 and IFN-γ) levels in serum

The levels of IL-4 in serum of the obesity groups (86.95±14.2) were higher than those in the normal control group ($P < 0.01$). In asthma group and obese asthma group, IL-4 levels (66.48±6.13; 69.51±9.71) were largely increased compared with the normal control group ($P < 0.01$). Moreover, IL-4 concentration in obese mice was the highest. The IFN-γ levels in obesity group (165.62±13.54) were

decreased compared with the normal control group ($P < 0.05$). In asthma group and obese asthma group, IFN-γ levels (125.97±11.29; 105.28±7.65) were significantly reduced compared with the normal control group ($P < 0.01$). And IFN-γ concentration in obesity with asthma group mice was the lowest (**Figure 4**).

*NKT counting & intracellular cytokines profile of nkt cells by flow cytometry (**Table 1**)*

The percentage of CD1d+NKT cells, IL-4+NKT cells in the asthma model group were significantly higher compared with those in the normal control group ($P < 0.01$). In contrast, the percentage of IFN-γ+NKT cells in the asthma model group was lower obviously compared with those in the normal control group ($P < 0.01$). In the obese mice group, the percentage of CD1d+NKT cells, IFN-γ+NKT cells were significantly decreased compared with those in the normal control group ($P < 0.01$). And the percentage of IL-4+NKT cells were higher obviously compared with those in the normal control group ($P < 0.01$). Furthermore, in obese asthma mice group, the percentage of CD1d+NKT cells and IL-4+NKT were increased the most obviously ($P < 0.01$). The percentage of IFN-γ+NKT was decreased the most obviously ($P < 0.01$). IFN-γ+NKT cells vary in different states not paralleling with CD69+NKT cells.

Discussion

Bronchial asthma is a classical Th2-dominateddisease, with a chronic airway inflammatory related to the infiltration of activated eosinophils, neutrophils, airway epithelial cells, Th cells and mast cells. CD4 Th2 cells are activated by certain antigen, releasing Th2 associated- cytokines and then resulting in the synthesis of IgE, followed by mast cell degranulation and airway mucosa infiltration, all of which induce airway remodeling and AHR. Yet clinical and experimental researchers suggest asthma is of a complex and heterogeneous trait. In addition, non-Th2 factors such as IFN-γ, IL-17 and neutrophils are increased in the lungs of asthma, particularly patients with severe asthma or with corticosteroid resistant asthma [28]. Non-allergic asthma is believed to be associated with air pollutants, stress and obesity, independently of Th2 cells. Asthma phenotypes associated with obesity include early-onset allergic asthma and late-onset non-allergic asthma. There probably exists complex pathogenesis of the linkage. Obesity induces lots of mechanical, metabolic, and immunological changes, which influence the airways [29]. Preferentially, obesity probably alters adaptive immunity related to asthmatic airway inflammation. We propose that NKT cells are involved in both asthma and obesity by being the target and source of inflammatory cytokines.

Innate immunity, particularly NKT cells, NK cells and γδT cells, may be involved in causing airway inflammation in allergic animal. We have studied immunomodulatory effects of γδT cells in marine asthma models [30-32]. NKT cells are a unusual subgroups of T cells that recognize various lipid antigens in the context of CD1d molecules [33]. NKT cells can produce large amounts of various cytokines and chemokines and also demonstrate cytocidal activity when stimulated. NKT cells perform as an intermediary activist connect the innate and adaptive immune systems. On the other hand, upon activation with a-GC, NKT cells rapidly release cytokines (such as GM-CSF, TNF-a, IFN-γ, IL-4, IL-10 and IL-13 etc. [34]. And they can promote maturation of dendritic cells into APC that are capable of activating conventional αβ-T cells [35]. Nie et al. demonstrate that in the OVA-induced asthma model, α-GalCer administration and adoptive transfer of iNKT cells significantly augmented the Th2 inflammatory responses and increased serum levels of OVA-specific IgE and IgG1. These results suggest that NKT cells may serve as an adjuvant to enhance Th2 inflammatory response in an OVA-induced murine model of asthma [36]. AHR failed to occur in NKT cell deficient mice (Jα18-/-mice and CD1d-/-mice) and was restored in the NKT cell deficient mice by the adoptive transfer of NKT cells from wild type but not from IL-4/IL-13 deficient mice. The CD4+IL-4- and IL-13-producing NKT cell subset is indispensible for the development of AHR in some mouse models of allergic airways disease [37].

In the present study, we observed that in obesity with OVA-induced asthma mice, histological examination showed airway inflammatory are more significant than in asthma group. We investigated the cytokines (IL-4 and IFN-γ) levels in serum to evaluate the status of the Th1/Th2 balance. The production of cytokines in NKT is significantly faster than conventional T cells [38]. Our results illustrate that the serum

IL-4 levels were markedly increased compared with the normal control group in asthma group and obese asthma group. The serum IFN-γ levels were decreased compared with the normal control group. IFN-γ concentration in obese asthma mice was the lowest. In summary, this study indicated that NKT cells strengthened the Th2 bias in the obesity with asthma. CD69 is the activation markers on iNKT cells. We detected there were more CD69+NKT cells in the liver of asthma model group and obese asthma group, and this has been correlated to the enhancement of airway inflammation and AHR of asthma mice and obese asthma mice. These results indicate activated NKT cells are one of the factors aggravating airway inflammation and AHR. In only obesity individuals, CD69+NKT cells were reduced and IFN-γ concentration decreased. IFN-γ+NKT cells vary in different states not paralleling with CD69+NKT cells, indicating activated NKT cells are predominate in inducing airway inflammation and airway hyper responsiveness, which enhance asthma performance. Flow cytometry results also shown that in obese asthma mice, the percentage of CD1d+NKT cells and IL-4+NKT was increased the most obviously and the percentage of IFN-γ+NKT were decreased the most obviously. Since NKT cells coincides with the state of chronic, low-grade inflammation in of obesity and integrate proinflammatory cytokines, we speculated that NKT cells activation enhance airway inflammation in obesity with asthma through induction of Th2 deviation, thus contributing to the development of asthma in obesity.

In conclusion, NKT cells maturation and activation levels in obesity with asthma individuals enhances Th2 Inflammatory response by regulating IL-4and IFN-γ secretion. On the other hand, airway inflammation and airway hyper responsiveness of obese asthmatics exhibited a more severe symptoms and pathophysiological changes than unitary asthma. The activation of NKT cells exacerbated asthma TH2 inflammatory response. Therefore, NKT cells play an important role in the development of asthma in obesity. The NKT cells may be the novel therapeutic targets in the treatment of obese asthmatics.

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Disclosure of conflict of interest

None.

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