Aim: To determine the roles of breast regression protein-39 (BRP-39) in regulating dendritic cell maturation and in pathology of acute asthma.

Methods: Mouse bone marrow-derived dendritic cells (BMDCs) were prepared, and infected with adenovirus over-expressing BRP-39. Ovalbumin (OVA)-induced murine model of acute asthma was made in female BALB/c mice by sensitizing and challenging with chicken OVA and Imject Alum. The transfected BMDCs were adoptively transferred into OVA-treated mice via intravenous injection. Airway hyperresponsiveness (AHR), inflammation and pulmonary histopathology were characterized.

Results: The expression of BRP-39 mRNA and protein was significantly increased in lung tissues of OVA-treated mice. The BMDCs infected with adenovirus BRP-39 exhibited greater maturation and higher activity in vitro. Adoptive transfer of the cells into OVA-treated mice significantly augmented OVA-induced AHR and eosinophilic inflammation. Meanwhile, BRP-39 further enhanced the production of OVA-induced Th2 cytokines IL-4, IL-5, and IL-13, but significantly attenuated OVA-induced IFN-γ production in bronchoalveolar lavage fluid.

Conclusion: In OVA-induced murine model of acute asthma, BRP-39 is over-expressed in lung tissue and augments Th2 inflammatory response and AHR. BRP-39 promotes dendritic cell maturation in vitro. Therefore, BRP-39 may be a potential therapeutic target of asthma.

Keywords: asthma; ovalbumin; bone marrow-derived dendritic cells (BMDCs); breast regression protein-39 (BRP-39); YKL-40; Th2 inflammation; airway hyperresponsiveness; bronchoalveolar lavage fluid

Introduction

Asthma is a chronic inflammatory disease that has been recognized throughout the world as a common respiratory disorder for more than two decades. It is characterized by local and systemic allergic inflammation and reversible airway obstruction, such as airway hyperresponsiveness (AHR), infiltration of eosinophils into the bronchial wall and lumen, and mucus hypersecretion in the airways[1]. The onset and progression of allergic asthma is accompanied by a complex series of overlapping and concurrent inflammatory responses in the lung, orchestrated by CD4⁺ Th2 lymphocytes[2]. These inflammatory responses are caused in part by both inflammatory cells and cytokines.

BRP-39 (human homolog YKL-40), as a chitinase-like protein (CLP), has been associated with a number of diseases characterized by inflammatory and tissue remodeling responses[3]. In cardiovascular disease, YKL-40 protein expression is observed in macrophages and smooth muscle cells in atherosclerotic plaques, with the highest expression in macrophages in the early lesion of atherosclerosis[4]. YKL-40 level is also elevated both in patients with type 1 and type 2 diabetes. Kwon et al found that serum YKL-40 level is correlated with symptom severity in patients with allergic rhinitis[5]. Human YKL-40 and mouse BRP-39 are both encoded by the chitinase 3-like-1 (CHI3L1) gene and are synthesized as 40 and 39 kDa proteins located on chromosomes 1 and 2 in human and mouse, respectively[6]. They lack chitinase activity and have been regarded as a prototype of CLP in mammals. Both forms of the protein are members of glycosyl hydrolase family 18, and the crystal structure of YKL-40 has been described[6–7]. Recently, many studies have focused on the regulatory function of BRP-39/YKL-40 in asthma and other allergic diseases. Lee et al found that YKL-40 plays a novel regulatory role in the
initiation and effector phases of Th2 inflammation and remodeling in mice\cite{8}. Otuka \textit{et al} reported that in patients with asthma only, sputum YKL-40 level was positively correlated with disease severity\cite{9}. Tang \textit{et al} have observed that serum YKL-40 was increased in Chinese patients with asthma, and its level correlated with exacerbation attacks, indicating that high serum YKL-40 may be a biological characteristic of the exacerbation of asthma\cite{10}. In Th2 inflammation, BRP-39/YKL-40 are involved in multiple stages of allergic responses, regulating sensitization and Th2 cytokine effector functions by stimulating dendritic cell (DC) accumulation and activation\cite{6}. Lee \textit{et al} also found that the expression of CD86 and CD40 in myeloid DCs (mDCs) was significantly decreased in lungs from sensitized and challenged BRP-39/− mice\cite{8}, suggesting that BRP-39 might play a critical role in the accumulation and activation of pulmonary dendritic cells. However, the detailed mechanisms underlying these functions of BRP-39/YKL-40 remain incompletely understood.

The purpose of this study is to determine whether BRP-39 expression in DCs affects their function and thereby regulates Th2 inflammation in allergic mice. A recombinant adoviral vector carrying the gene \textit{CHI3L1} (AdCHI3L1) was constructed and infected into BMDCs. We utilized a mouse model of acute asthma to recreate many features of the human disease in an effort to investigate the effect of BRP-39 on DCs and airway inflammation. The subsequent AHR, airway inflammation, and histological changes were observed.

Materials and methods

\textbf{Mice}

Female BALB/c mice, weighing 18-25 g, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained in a pathogen-free environment. All mice used were between 6 and 8 weeks of age and were age-matched within each experiment.

\textbf{Preparation of bone marrow-derived dendritic cells (BMDCs)}

DCs were prepared from bone marrow progenitors according to a published method with minor modifications\cite{11}. Bone marrow mononuclear cells were prepared from mouse (6–8 weeks old) tibial and femoral bone marrow suspensions on d 0 by depletion of red blood cells and then were cultured at a density of 2×10^6 cells/mL in 6-well plates in RPMI-1640 medium supplemented with 10% FBS, 20 ng/mL of recombinant mouse granulocyte-monocyte colony-stimulating factor (GM-CSF) and 1 ng/mL of recombinant mouse IL-4 (R&D Systems, Minneapolis, MN, USA). Non-adherent cells were gently pipetted out on d 2, and the medium was replaced with fresh culture medium. The remaining loosely adherent clusters were further cultured for subsequent experiments.

\textbf{Construction of recombinant adoviral vectors and adovirus packaging}

An over-expression vector was constructed as below. The mRNA sequence of the mouse \textit{CHI3L1} gene (NCBI: NM_007695.3) was inserted into an adoviral shuttle vector (pDONR221). Then, recombinant adovirus (AdCHI3L1) was generated by homologous recombination with an adoviral backbone plasmid (pAd/CNV/V5-DEST) and amplified in the 293T cell line. As a control, mock adovirus (AdMock) was made from the pAd/CNV/V5-DEST vector not carrying the transgene. After propagation in 293T cells, the recombinant viruses were purified from infected cells 24–36 h after infection by three freeze-thaw cycles followed by successive banding by cesium chloride density-gradient centrifugation. The purified viruses were dialyzed and stored at -80 °C until the experiment. Viral titers were measured by a standard endpoint dilution assay using 293T cells.

\textbf{Adovirus infection of DCs in vitro}

On d 5, BMDCs were collected and infected with AdMock or AdCHI3L1, then centrifuged at 60×g for 1 h, and incubated for one more day. The purity, infection efficiency, and surface marker expression of the BMDCs were analyzed by flow cytometry for MHC class II, CD80, and CD11c. The optimal multiplicity of infection (MOI) of adovirus infection was chosen by the evaluations of viability and the enhanced green fluorescent protein (EGFP) expression of infected DCs \textit{in vitro}. Infected and non-infected DCs were collected for the following functional assay.

\textbf{Sensitization, treatment, and challenge of mice}

BALB/c mice were divided into four groups: (i) Saline group: normal saline (NS) sensitized and challenged, (ii) OVA group: OVA sensitized and challenged, (iii) OVA-AdMock group: OVA sensitized and challenged with AdMock-DC treatment; and (iv) OVA-AdCHI3L1 group: OVA sensitized and challenged with AdCHI3L1-DC treatment. All DCs were loaded with OVA and matured with LPS (100 ng/mL) 24 h before collection. All mice were sensitized and challenged with chicken OVA (Sigma-Aldrich, USA) and Imject Alum (Thermo, USA)\cite{22}. Briefly, on d 0 and d 14, all animals were injected with 0.2 mL of a mixture containing 80 µg OVA, 0.1 mL NS and 0.1 mL Imject Alum. On d 23, Ad-DCs were adoptively transferred by intravenous injection. On d 24, d 25, and d 26, animals were challenged with 0.6% OVA aerosol for 40 min. AHR was measured 1 d after the last challenge (on d 27). Immediately after measuring AHR, cells in BALF were centrifuged for Wright-Giemsa staining, and BALF supernatant was collected to measure cytokine production by ELISA.

\textbf{Determination of airway responsiveness}

At 24-36 h after the last aerosol exposure, airway responsiveness to methylcholine (MeCh) was assessed by the Resistance and Compliance system (Buxco Electronics, USA)\cite{13}. Mice were first exposed to aerosolized saline and subsequently to increasing concentrations of aerosolized MeCh dissolved in isotonic saline. Following each nebulization, lung resistance (RI) was assessed for 3 min.
BALF and pulmonary histological analysis
Mice were intraperitoneally anesthetized with 100 mg/kg sodium pentobarbitone 24 h after the last OVA or saline challenge. The trachea was cannulated, and BALF was collected by injecting 0.3 mL phosphate-buffered saline three times into the left lung. Total leukocyte numbers were counted under microscopy. Differential cell counts were performed in a blinded fashion on cytopsin slides stained with Wright-Giemsa by counting at least 300 cells under immersion oil at ×1000 magnification. The absolute number of eosinophils was then calculated. The right lungs, without obtaining BALF, were fixed with 10% phosphate-buffered formalin, embedded, and sectioned. Tissue sections were stained with H&E (hematoxylin-eosin) to identify inflammation. With H&E staining, the nuclei of inflammatory cells were dyed dark blue, while the cytoplasm was dyed purple-red. The inflammatory cells accumulated around the bronchus, and blood vessels could be seen clearly. We quantified the inflammation of lung tissue by counting the inflammatory cells. The sections were examined in a blinded fashion under light microscope (×400).

Flow cytometry
BMDCs were stained with CD11c-PerCPcy5.5, and the purity of DCs was measured. The optimum MOI of adenovirus infection was determined according to the percentage of GFP-positive cells. BMDCs infected with AdMock or AdCHI3L1 were stained with CD11c-PerCPcy5.5, CD80-PE, and MHCII-APC at the same time for 30 min in the dark at 4 °C, then washed and centrifuged three times with phosphate-buffered saline (PBS). After the last centrifugation and discarding the supernatant, the cells were reconstituted and analyzed by flow cytometry (BD FACS Calibur, USA). The cells of interest were the CD11c-PerCPcy5.5-positive and GFP-positive ones, which were gated out for analysis. MFI was calculated by FlowJo 7.6. Pictures were drawn in GraphPad Prism 5.

Reverse transcription-polymerase chain reaction
The total RNA was extracted from the lung tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Then, 2 µg total RNA was reverse-transcribed into cDNA using MMLV reverse transcriptase (Promega, Madison, WI, USA) and oligo(dT) as a primer. PCR consisted of 95 °C for 3 min followed by 10 cycles of 95 °C for 10 s, 70 °C for 20 s, and 72 °C for 20 s, and then 30 cycles at 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s, with a final step of 72 °C for 10 min. As a control, cDNA samples were also amplified with actin-specific primers. The specific primer sequences were as follows: CHI3L1: 5′-CAGACGCCA-TCCACCTTT-3′ and 5′-TTTCCACCTCAGACAC-3′; actin: 5′-CGTTGACATCCGTAAAGACC-3′ and 5′-AA-CAGTCCGCTAAGAC-3′. After separation in agarose gels, the specific bands were quantified with a Biosens gel imaging system (Shanghai Bio-Tech, Shanghai, China). Relative mRNA levels were calculated by the 2^(-ΔΔCT) method.

Western blotting
Frozen lung tissue was lysed in protein extraction buffer containing protease inhibitors. Lysates were boiled after adding SDS loading buffer, and equal amounts of proteins were separated in 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. After blocking, the membrane was reacted with a mouse monoclonal antibody (MAB2649, R&D Systems, USA) and an actin monoclonal antibody and further incubated with the anti-mouse IgG horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Santa Cruz, USA) and washed. Detection was carried out with chemiluminescence (Beyotime, Shanghai, China). Protein expression was determined by photodensitometry with the Biosens gel imaging system (Shanghai Bio-Tech, Shanghai, China). Equal protein loading was confirmed by actin levels. For protein level analysis, we compared the change in BRP-39 expression between groups, normalized to actin expression.

ELISA of BALF and cytokine culture supernatant
The concentrations of IL-4, IL-5, IL-13, and IFN-γ in BALF and T lymphocyte culture supernatant were determined by using ELISA kits (eBioscience, San Diego, USA) according to the manufacturer’s recommendations. The BRP-39 expression in infected and non-infected DC culture supernatant was determined by ELISA (MC3L10, R&D Systems) according to the manufacturer’s instructions.

Statistical analysis
For all of the groups, data are presented as the mean±SEM. The results were analyzed by one-way ANOVA to identify significant differences between groups. The level of statistical significance was set at P<0.05, and all statistical calculations were performed using GraphPad Prism 5.

Results
Increased expression of BRP-39 in OVA-treated mice and OVA-treated BMDCs
BRP-39 is prominently induced in lung tissues during the course of OVA-induced Th2 inflammation[8]. To confirm this observation in our animal model, we detected the expression of BRP-39 in lungs from control mice and mice sensitized and challenged with OVA. The levels of both BRP-39 mRNA (CHI3L1) (Figure 1A) and protein (Figure 1B) were significantly increased in lung tissues of allergic mice compared with control mice. We also found elevated BRP-39 in plasma of allergic mice (Figure 1C). DCs are a dedicated lineage of white blood cells that initiate and control immunity. Maturation of DCs is accompanied by antigen uptake, processing and presentation[14]. We also demonstrated that OVA treatment enhanced the BRP-39 expression in BMDCs (Figure 1D) after confirming its basal expression. Our results indicate that BRP-39 expression was induced by OVA and played a potential role in OVA-induced Th2 inflammation in our model.

Over-expression of BRP-39 in BMDCs
We first assessed the expression of BRP-39 in BMDCs (Figure 1D) and confirmed that both the mRNA and protein was low under normal conditions in BMDCs. Pulmonary dendritic...
cells are considered to be a bridge between innate and adaptive immunity, playing a critical role in OVA-induced allergic inflammation[6, 8]. Because BRP-39 is thought to play a critical role in the accumulation and activation of pulmonary DCs, we were interested in exploring the role of BRP-39 in the regulation of DC function.

To over-express BRP-39 in BMDCs, we constructed a recombinant adenoviral vector (Figure 2A) that carried the mouse gene CHI3L1 (AdCHI3L1). We infected BMDCs with this AdCHI3L1 or its control (AdMock). BRP-39 protein was significantly increased in both cells and culture supernatant on the 3rd d after infection (Figure 2B, C, P<0.05). The purity of DCs and the optimum MOI were determined by flow cytometry. The purification of DCs we obtained was above 80%, and the optimal MOI was 100 (Figure 2D). DCs grew in clusters, as observed by light microscopy. By fluorescence microscopy, DCs infected with adenovirus fluoresced green due to EGFP, which means they expressed BRP-39 (Figure 2E).

BRP-39 promotes maturation of DCs in vitro
To determine whether over-expression of BRP-39 affects dendritic cell function, we used CD11c as a DC-specific surface marker and examined the changes in expression of CD80 and class II MHC on DCs by flow cytometry (Figure 3). The mean fluorescence intensities (MFIs) of CD80 and MHCII both increased in DCs infected with AdCHI3L1 relative to AdMock according to three independent experiments. These results demonstrate that BRP-39 plays a role in promoting DC maturation.

Adoptive transfer of BRP-39-over-expressing DCs augments OVA-induced allergic inflammation
To further investigate whether over-expression of BRP-39 in DCs could influence airway inflammation, 1×10^6 BMDCs infected with AdCHI3L1 or AdMock were adoptively transferred into mice by intravenous injection 1 d before OVA challenge. Mice were divided into a Saline group, an OVA group, an OVA-AdMock group and an OVA-AdCHI3L1 group. Interestingly, mice injected with AdCHI3L1 DCs had significantly more total cells and eosinophils in the BALF compared with AdMock-treated DCs (Figure 4A), whereas no significant difference in neutrophils or lymphocytes was observed among the three groups. The total numbers of leukocytes and eosinophils in the OVA group were significantly greater than those in the Saline group. Analysis of the pulmonary pathology, as shown by H&E staining, also revealed that the mice injected with AdCHI3L1 DCs had significantly more total cells and eosinophils in the BALF compared with AdMock-treated DCs (Figure 4B). These results demonstrate that adoptive transfer of BRP-39-over-expressing DCs augments OVA-induced allergic inflammation.

BRP-39-over-expressing DCs exacerbate AHR in allergic mice
To determine whether BRP-39-over-expressing DCs could affect AHR in allergic mice, we performed a lung function test to examine the AHR in response to MeCh. The OVA group had a significantly higher RI value at doses of 25 and 50 mg/mL compared with saline group (Figure 5, P<0.05), which means the comparison between OVA-AdMock and OVA-AdCHI3L1 based on the model of asthmatic mice. Compared with the OVA-AdMock group, mice in the OVA-AdCHI3L1 group developed higher RI in response to MeCh at the doses of 25 and 50 mg/mL (Figure 5, P<0.05). Our data suggest that
BRP-39-over-expressing DCs exacerbate AHR in allergic mice.

Adoptive transfer of BRP-39-over-expressing DCs influences the levels of Th1/Th2 cytokines in BALF of allergic mice

To evaluate the effect of BRP-39-over-expressing DCs on the pulmonary immune responses in allergic mice, the concentrations of IL-4, IL-5, IL-13, and IFN-γ in BALF were measured. IL-4, IL-5, and IL-13 were similar between the OVA and OVA-AdMock groups. IL-4, IL-5, and IL-13 in the OVA-AdCHI3L1 group were significantly elevated in comparison to the OVA-AdMock group (Figure 6). In contrast, IFN-γ was decreased in the OVA-AdCHI3L1 group. These data suggest that BRP-39 on DCs promotes the Th2 but attenuates the Th1 immune response in a murine model of asthma.

Discussion

BRP-39 (YKL-40) is widely expressed in mammals. BRP-39 is secreted by cultured articular chondrocytes, synovial cells, macrophages, epithelial cells, vascular smooth muscle cells, and some cancer cells. BRP-39 was identified and termed by Johansen et al. in a search for new bone proteins in 1989. Subsequent studies have revealed much about its structure and functions. Chupp et al. reported that serum YKL-40 was significantly elevated in patients with asthma. Goldman et al. also reported that compared with non-asthmatics, asthmatic children exhibited increased chitinase activity and increased YKL-40 levels in BALF. Lee et al have demonstrated that BRP-39-/- mice have markedly diminished antigen-induced Th2 responses but that...
epithelial BRP-39 can rescue the Th2 response. Subsequent publications have discussed the potential relationship between YKL-40 and Th2 inflammation[8]. Our results demonstrate that BRP-39 was not only induced in allergic inflammation but also played a critical role in promoting the Th2 inflammatory response in allergic mice, likely by enhancing the antigen-presenting ability of dendritic cells.

We initiated our study by over-expressing BRP-39 in DCs in a mouse model of asthma to examine how it influences the functions of DCs and subsequent inflammatory responses. DCs are the most powerful antigen-presenting cells (APCs) in the immune system. Several DC surface molecular markers (CD80, CD86, and MHCII) indicate their antigen-presenting ability. Antigens such as OVA can be processed and presented by DCs, which will initiate the proliferation of T lymphocytes. Lung DCs can integrate a variety of stimuli from allergens, microbial colonization, environmental pollution, and innate immune cells into a single signal for activating T lymphocytes of the adaptive immune system[28]. DCs transduced with an adenovirus vector expressing a secondary lymphoid chemokine (CCL21) have antitumor capabilities in a murine model of spontaneous bronchoalveolar cell carcinoma[29]. In allergen-induced inflammation, IL-10 and IL-12 gene-modulated DCs are effective in suppressing asthmatic airway inflammation through both immune deviation and immune suppression[30]. We also utilized transgenic DCs for BRP-39 over-expression in an asthma model. Our results demonstrate that modification of BRP-39 expression on DCs effectively modulated the allergic inflammation in a murine model of asthma.

Modified DCs enter the blood by migration. These cells stop and function where allergic antigen is deposited to stimulate naive T cells. Lee et al found that expression of CD86 and CD40 was significantly decreased in mDCs in lungs from OVA sensitized and challenged BRP-39−/− mice[8]. In contrast, com-
parable CD80 levels in mDCs were observed in the presence and absence of BRP-39. Our results demonstrate that BRP-39 expression critically regulates DC maturation in vitro (Figure 3) and in vivo (Figure 4–6). These data suggest that BRP-39 might play a role in the accumulation and activation of pulmonary DCs.

The present study also demonstrates that BRP-39 over-expressed in DCs plays a pivotal role in directing OVA-induced airway inflammation by promoting the Th2 but attenuating the Th1 response. Adoptive transfer of BRP-39-overexpressing DCs resulted in a significantly increased number of eosinophils. The Th2 cytokines IL-4, IL-5, and IL-13 were also elevated in the BALF of allergic mice, accompanied by decreased Th1 cytokine IFN-γ. Our results show BRP-39 to be a key protein in the pathogenesis of asthma, which may depend on the regulation of the Th1/Th2 balance. These findings imply that BRP-39 expression on DCs might induce Th0 cells to differentiate to Th2, which would consequently further augment the OVA-induced allergic inflammation. Therefore, BRP-39 may be a potential therapeutic target for asthma.

AHR is one of the hallmark features of asthma, which is defined as the abnormal increase in airflow limitation in response to a provoking stimulus. Our results show that adoptive transfer of BRP-39-over-expressing DCs can also increase the AHR in allergic mice. Park et al reported that asthmatic airway mechanical stress may contribute to enhanced YKL-40 levels. They also found that mechanical stress potently induces CHI3L1 expression, leading to increased secretion of YKL-40 protein in an EGFR- and MEK1/2-dependent pathway[18]. Whether YKL-40/BRP-39 directly affect or influence AHR by other factors, such as IL-13 or Muc5Ac, remains unclear and requires further investigation.

In conclusion, our data demonstrate for the first time that BRP-39 over-expression in DCs regulates DC maturation and function and subsequently promotes the Th2 immune response while attenuating the Th1 immune response in allergic asthma. AHR was augmented after BRP-39-over-expressing DCs were adoptively transferred to asthmatic mice, and elevated BRP-39 in lung tissue and plasma in asthmatic mice was observed. Our results indicate that BRP-39 plays a potential role in OVA-induced airway inflammation.

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Author contribution

Kai WANG designed research; Qian XU and Shou-jie CHAI performed research; Ying-ying QIAN contributed new reagents or analytic tools; Ying-ying QIAN and Min ZHANG analyzed data; Qian XU and Kai WANG wrote the paper.

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