

Respiratory syncytial virus infection increases regulated on activation normal T cell expressed and secreted and monocyte chemotactic protein 1 levels in serum of patients with asthma and in human monocyte cultures

Jenifer Gotera, MSc ^{*}; Maria Giuffrida, MSc [†]; Alibeth Mavarez ^{*}; Hector Pons, PhD [‡]; John Bermudez, MSc ^{*}; Mery Maldonado, MSc [§]; Luz Marina Espina, MSc, MSc ^{*}; Jesus Mosquera, MD ^{*}; and Nereida Valero, PhD ^{*}

^{*} Instituto de Investigaciones Clínicas Dr. Américo Negrette, Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela

[†] Cátedra de Inmunología, Escuela de Medicina, Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela

[‡] Centro de Cirugía y Medicina Experimental, Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela

[§] Cátedra de Bioquímica, Facultad de Odontología, Universidad del Zulia, Maracaibo, Venezuela

ARTICLE INFO

Article history:

Received for publication December 8, 2011.

Received in revised form February 28, 2012.

Accepted for publication March 5, 2012.

ABSTRACT

Background: Respiratory syncytial virus (RSV) infection is associated to episodic exacerbations of asthma involving alveolar macrophages and chemokine production.

Objective: The aim of this study was to determine the circulating levels of monocyte chemotactic protein 1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), and substance P (SP) in patients with and without asthma with acute respiratory RSV infection and the chemokine profile in RSV-infected monocyte cultures from normal individuals and individuals with asthma.

Methods: In this regard, 31 adult patients with acute respiratory infection (15 patients with asthma) were studied. MCP-1, RANTES and SP were measured in serum and in supernatants from monocyte cultures by enzyme-linked immunosorbent assay (ELISA).

Results: Increased levels of MCP-1 and RANTES were observed in serum from patients with asthma related to RSV infection. RSV-infected monocyte cultures from healthy individuals showed increased content of those chemokines, and monocyte cultures from patients with asthma showed increased expression of MCP-1.

Conclusion: These data show that RSV infection induces increased circulating level of chemokines in patients with asthma, and this finding could be mediated in part by the interaction virus-monocyte.

© 2012 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. All rights reserved.

Introduction

Respiratory syncytial virus (RSV) has been associated with asthma exacerbation.¹ Current hypotheses for the pathogenesis of virus-induced asthma exacerbations involve induction of inflammation and chemokine production.^{1,2} In this regard, monocyte chemotactic protein 1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), and substance P (SP), a neuromodulator, have been involved in inflammation of airways during RSV infection.^{2–4} Increased production of monocyte chemotactic proteins and RANTES have been found in nasal aspirates from children with infection by RSV^{2,5}; however, few studies have been published on the systemic chemokine re-

sponse in adult patients with and without asthma during RSV infection. Alveolar macrophages are present in large numbers in the lower airway,⁶ and circulating monocytes are the source of alveolar macrophages, determining its capacities to produce inflammatory and chemoattractant substances and the systemic expression of those chemokines during RSV infection. In addition, C-reactive protein (CRP), a marker of inflammatory activity, has been associated with increased blood levels of MCP-1 and RANTES, suggesting a chemokine-inducer activity in some pathologic conditions.^{7,8} Thus, the aim of this investigation was to determine the serum concentrations of MCP-1, RANTES, SP, and CRP in patients with and without asthma infected by RSV and the production of those substances by RSV-infected human monocyte cultures. RSV induced increased expression of RANTES and MCP-1 in patients with asthma and in human monocytes cultures from normal donors. Increased production of MCP-1 was observed in infected monocyte cultures from patients with asthma.

Reprints: Nereida Valero, PhD, Apartado Postal 23, Maracaibo 4001-A Zulia, Venezuela; E-mail: nere98@hotmail.com.

Disclosures: Authors have nothing to disclose.

Funding Sources: This manuscript was supported by Instituto de Investigaciones Clínicas “Dr. Américo Negrette,” Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela.

Methods

Subjects of study

Thirty-one male and female patients from an outpatient hospital at a university teaching hospital presenting with a clinical diagnosis of acute respiratory infection (15 patients with asthma and 16 without asthmatic antecedents; 36 to 73 years of age) were studied. The inclusion criteria were those individuals who had at least one respiratory symptom, such as cough, wheeze, running nose, sneeze, or snore, and who were suspected by a professional physician to be infected with RSV. We excluded individuals who had cardiac disease, immunodeficiency, and chronic inflammatory disease. Patients were not treated with antibiotics, anti-allergic medication, or steroid, and they were at least 15 days free of any kind of treatment when blood samples were obtained. Patients with asthma were selected according to the criteria of Global Initiative for Asthma (GINA) Program.^{9,10} Asthmatic crisis was not present (at least 1 month previously) when blood samples were taken. In addition to suggestive clinical diagnosis, RSV virus infection was confirmed by the presence of virus in specimens from nasopharynx and bronchoalveolar lavage. Viral replication was demonstrated in HEP-2 cell cultures (protocol -520-I; National Institute for Health, Bethesda, Maryland). Age- and sex-matched healthy individuals ($n = 10$) were used as controls. Blood samples were obtained from patients and controls and stored at -70°C until use. The Ethics Committee of Instituto de Investigaciones Clínicas Dr. Américo Negrette and FONACIT (Caracas, Venezuela) approved this study protocol, and written informed consent was obtained from all patients before blood collection.

Study design

Samples from bronchoalveolar lavage or nasopharyngeal lavage were obtained from studied patients for viral identification and isolation. Bronchoalveolar lavage was performed by inserting a bronchofiberscope tip in the segmental bronchus, and then 20 to 50 mL 0.9% NaCl solution was administered to a total volume of 150 to 200 mL. Each volume of fluid administered was immediately removed from the bronchial tree, placed in the sterile container, and stored at -70°C until use. Nasopharyngeal lavage was performed introducing 3 to 7 mL sterile saline solution into the nasal fossa, maintaining the patient's head leant backwards, and then collecting the solution in a sterile container placed below the nasal fossae. The samples were stored at -70°C until use. Blood samples (5 mL) without anticoagulant were obtained from patients and controls for chemokines, SP, and CRP determinations. Serum was isolated by centrifugation and stored at -70°C until use.

Material analysis

Nasopharynx and bronchoalveolar samples from patients were sonicated and centrifuged, and supernatants were added to HEP-2 cell cultures. Previously, cells were grown to 50% confluence in Eagle's minimum essential medium containing 7% fetal bovine serum and 1% antibiotic/antimycotic. After 2 washes with phosphate-buffered saline, 200 μL supernatant was added to cellular cultures. Cultures were incubated for 1 hour, after which 300 μL minimum essential medium containing 10% fetal bovine serum and 1% antibiotic/antimycotic was added. Cultures were then incubated for 96 to 120 hours. Supernatants from cultures were centrifuged and stored as a source of virus. Viral cell culture infection (RSV) was determined by direct immunofluorescence using a commercial kit (Light Diagnostics SimulFluor Respiratory Screen Kit, Chemicon Internacional, Hayward, California).

Mononuclear leukocytes were obtained from heparinized venous blood from five healthy adult donors and three patients with asthma without RSV infection. Cells were isolated through density gradient centrifugation in Hystopaque 1.077 (Sigma Chemical Co,

St. Louis, Missouri) and cultured for 3 hours at 37°C and 5% CO_2 . Thereafter, adherent cells were enriched by washing away unattached cells twice. After washing, a mean of 3×10^5 adherent cells/well was obtained. Monocyte cultures were infected (MOI: 1) with RSV obtained from infected HEP-2 cell culture supernatants.¹¹ Monocytes cultured in HEP-2 cell culture supernatants without virus were used as negative controls. Viral monocyte infection was determined by direct immunofluorescence (Light Diagnostics SimulFluor Respiratory Screen Kit, Chemicon Internacional). After 24 hours, supernatants were collected, and chemokines and SP determinations were performed by enzyme-linked immunosorbent assay (ELISA). Results were expressed as picograms per milligram of cellular protein.

Mediators' measurements

Serum or culture supernatant obtained from patients or monocyte cultures were tested for the content of chemokines, SP, or CRP using sandwich ELISA. MCP-1, RANTES, and SP contents were measured using commercially available ELISA kits (MCP-1: Endogen, IL, USA; RANTES: Cayman Chemical, Ann Arbor, Michigan), and the results were expressed as pictograms per milliliter or pictograms per milligram cellular protein. CRP was measured with an ELISA (Hemagen Diagnostics, Inc., Columbia, Maryland), and the results were expressed as micrograms per milliliter.

Statistical analysis

Values were expressed as mean $\pm$ standard deviation (SD). The significance of differences was tested by analysis of variance and Bonferroni post hoc test. Two-tailed P values less than .05 were considered statistically significant.

Results

Eight of 15 patients with asthma and 8 of 16 patients without asthma were positive for RSV. The rest of the patients had other respiratory virus infections. The levels of serum MCP-1 and RANTES were higher in patients with asthma than in patients without asthma or healthy individuals ($P < .01$; Fig 1). Patients with asthma with RSV infection had higher levels of chemokines than in the other studied patients (RANTES: $P < .05$; MCP-1: $P < .01$) (Fig 1B, D). No significant differences were seen in SP and CRP levels between controls and acute respiratory infection patients (Fig 2).

Respiratory syncytial virus-infected monocyte cultures from healthy individuals showed similar findings to those observed in RSV-infected patients with asthma. Increased content of RANTES and MCP-1 were observed at 24 hours in supernatants from infected cultures ($P < .01$; Fig 3A, B). SP content remained similar to controls. The RSV-infected monocyte cultures from patients with asthma showed increased production of MCP-1 ($P < .01$; Fig 3B). Levels of RANTES and SP did not reach significant increase when compared with noninfected cultures.

Discussion

Infection of the respiratory tract by viruses can result in common cold, pharyngitis, tracheobronchitis, croup, bronchiolitis, or pneumonia. These infections may be superimposed on a background of preexisting chronic respiratory disease, such as asthma. In an asthmatic individual, exacerbation may occur because of the functional interaction between viral pathologic conditions and asthmatic pathologic conditions.¹ Our results showed that asthmatic condition was relevant in the serum chemokine levels induced by RSV infection, suggesting an asthma enhancer mechanism underlying the interaction with the virus. In addition, the persistence of cytokines and chemokines in fully recovered patients with RSV in the long term can provide a substratum for the development of subsequent asthma.¹² Regarding this, the Th1/Th2

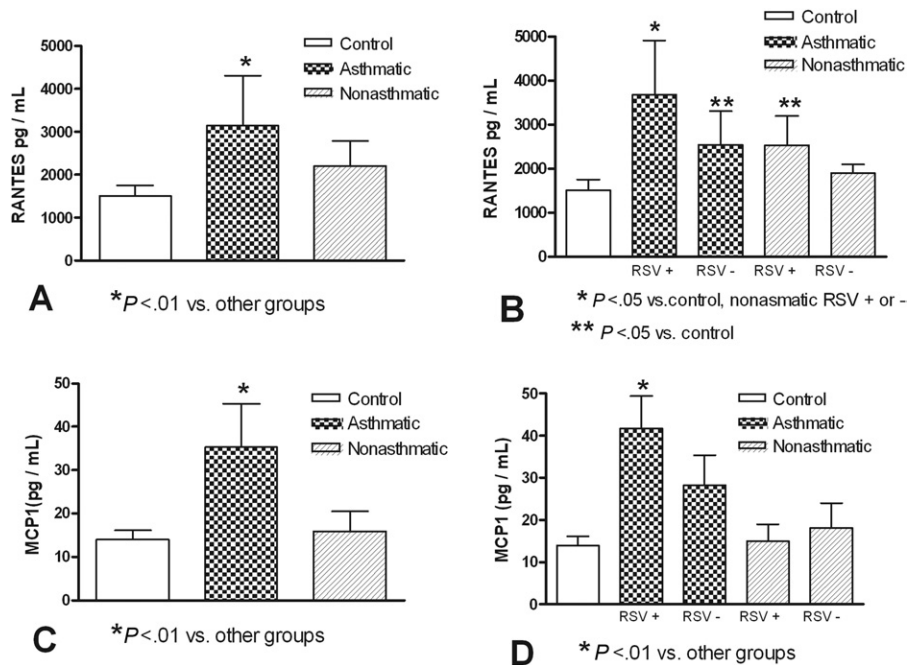


Fig. 1. Serum level of MCP-1 (monocyte chemotactic protein 1) and RANTES (regulated upon activation, normal T-cell expressed, and secreted) chemokines in controls and patients. Increased levels of MCP-1 and RANTES were observed in patients with asthma (A and C). These were related to respiratory syncytial virus (RSV) infection (B and D).

predisposition in the host regulates inflammatory reactions in the airways through alteration of gene expression, and MCP-1/CCL2 plays an important role in the pathogenesis of severe RSV infection and the subsequent development of asthma in Th2-predisposed hosts.¹³ In addition, reduction in the Th1, but not Th2, and CD25-positive lymphocyte populations associated with exaggerated interferon- γ inducible protein-10 production occurs in severe RSV bronchiolitis.¹⁴

C-reactive protein levels were studied because this molecule has stimulatory effect on MCP-1 production by endothelial cells and it has been associated with increased serum levels of MCP-1

and RANTES in uremic patients and in patients with juvenile rheumatoid arthritis.^{7,8,15} Because CRP levels were observed at the normal limits in the different studied groups, probably CRP had no role in chemokine production.

Increased production of monocyte chemotactic proteins and RANTES have been found in nasal aspirates from children with infection by RSV^{2, 5}; however, few studies have been performed on the systemic chemokine response during RSV infection. In this regard, systemic increased cytokines and chemokine levels have been reported in children with acute RSV infections.^{16,17} To our knowledge, this is the first report showing increased serum chemo-

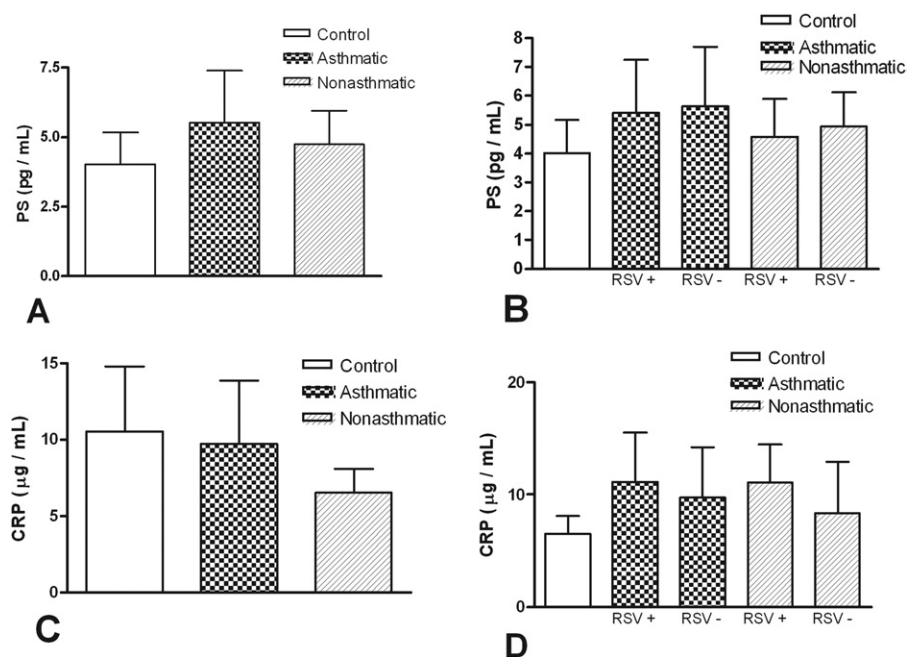


Fig. 2. Serum levels of substance P (SP) and C-reactive protein (CRP) in controls and patients. The SP and CRP serum levels remained similar in the studied groups (A and C). This situation was not altered by respiratory syncytial virus (RSV) infection (B and D).

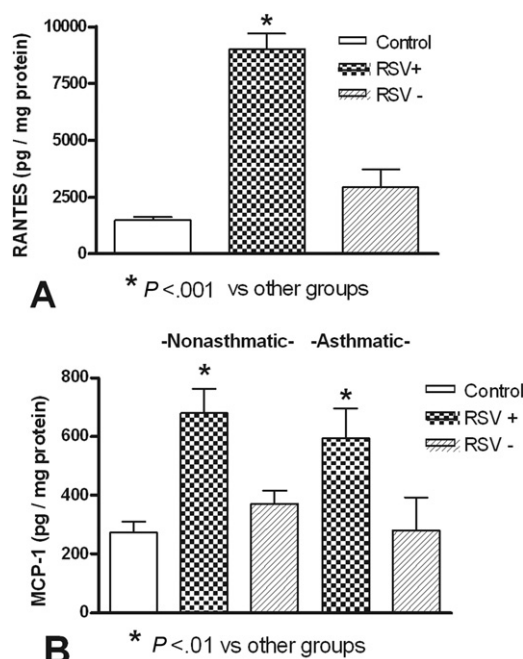


Fig. 3. Chemokine production by respiratory syncytial virus (RSV)-infected monocytes. (A), Increased production of RANTES (regulated upon activation, normal T-cell expressed, and secreted) was observed in RSV-infected monocyte cultures from healthy individuals. (B), Increased MCP-1 (monocyte chemoattractant protein 1) production was observed in RSV-infected monocyte cultures from healthy individuals and from patients with asthma.

kines in adult patients with asthma compared with patients without asthma.

Human monocytes from healthy individuals and from patients with asthma were capable of responding to RSV infection by producing increased levels of chemokines. These results suggest that monocytes may be in part responsible for the serum chemokines levels observed in patients infected by RSV. Similarly, rhinovirus entry into human monocytes does result in the activation and production of both interleukin-8¹⁸ and tumor necrosis factor alpha.¹⁹ The capacity of circulating monocytes to produce chemokines after RSV infection could have an important role in the airways. Human alveolar macrophages could make up 90% of the cells in the lower airway. They are ideally placed for early phagocytosis of virus particles and are likely to play an important role in the immune response through antigen presentation to T cells and through the production of cytokines and other mediators.⁶ Accordingly, RANTES and MCP-1 were found to play an important role in the lung allergic inflammation in the pathogenesis of asthma.²⁰ Because human circulating monocytes can be infected by RSV^{21,22} and induced to produce RANTES and MCP-1, these cells can represent an important source of chemokines in airways and could explain, in part, the severe respiratory symptoms observed in asthma after RSV infection.²¹ However, the contribution of circulating monocytes to the increased levels of serum MCP-1 and RANTES could be partial, because increased airway production by macrophages and epithelial cells of those chemokines have been reported.⁵

As an interesting point in this study, RSV did not induce increased production of RANTES in monocytes cultures from patients with asthma; however, monocytes from healthy individuals were capable of producing both RANTES and MCP-1 after virus infection. These results suggest a possible inhibitory mechanism for RANTES production in monocytes from patients with asthma, and this condition could have a deleterious effect in patients with asthma, because RANTES has been shown to have an inhibitory effect on RSV infection.²³ Because RSV is among the most potent biological

stimuli that are able to induce chemokine and cytokine mediators by activation of the transcription factor nuclear factor kappa-B,²⁴ alterations in the activation of this factor in monocytes from patients with asthma could be involved. We have no clear explanation for increased levels of RANTES found in patients with asthma observed in this study, but probably other types of cells^{5,25} are involved in the in vivo production of RANTES by patients with asthma during RSV infection. However, the small number ($n = 3$) studied in monocyte cultures from patients with asthma could be related to lack of significant statistical values in monocyte chemokine production.

Neuronal mechanisms are important in RSV infection and subsequent airway hyperreactivity. Stimulation of sensory nerve fibers below the epithelial surface of airways can induce the release of the proinflammatory neuropeptide SP. During RSV infection, increased pulmonary upregulation of SP and its receptor have been reported, and this may represent an important link between early life viral infections and childhood asthma.^{26,27} We did not find increased levels of SP in the serum of patients with and without asthma or in RSV-infected monocytes from both types of patients. These data support the local production of SP during RSV airway infection and a marginal role of monocyte/macrophages in its production.

In conclusion, this study shows that increased serum chemokines is an important feature associated with asthmatic condition and RSV infection. The increased levels of chemokines in serum of RSV-infected patients could be in part attributable to circulating monocytes. Further work is needed to elucidate the functional relationship between airway monocyte/macrophage and circulating monocytes.

References

- [1] Message SD, Johnston SL. The immunology of virus infection in asthma. *Eur Respir J*. 2001;18:1013–1025.
- [2] Santiago J, Hernández-Cruz JL, Manjarrez-Zavala ME, et al. Role of monocyte chemoattractant protein-3 and -4 in children with virus exacerbation of asthma. *Eur Respir J*. 2008;32:1243–1249.
- [3] Dakham A, Park JW, Taube C, et al. Alteration of airway neuropeptide expression and development of airway hyperresponsiveness following respiratory syncytial virus infection. *Am J Physiol Lung Cell Mol Physiol*. 2005;288:L761–L770.
- [4] Ogra PL. Respiratory syncytial virus: the virus, the disease and the immune response. *Paediatr Respir Rev*. 2004;5:S119–S126.
- [5] Becker S, Soukup JM. Airway epithelial cell-induced activation of monocytes and eosinophils in respiratory syncytial viral infection. *Immunobiology*. 1999;201:88–106.
- [6] Calhoun WJ, Dick EC, Schwartz LB, Busse WW. A common cold virus, rhinovirus 16, potentiates airway inflammation after segmental antigen bronchoprovocation in allergic subjects. *J Clin Invest*. 1994;94: 2200–2208.
- [7] Pawlak K, Pawlak D, Myśliwiec M. Inflammation but not oxidative stress is associated with beta-chemokine levels and prevalence of cardiovascular disease in uraemic patients. *Cytokine*. 2006;35:258–262.
- [8] Yao TC, Kuo ML, See LC, et al. RANTES and monocyte chemoattractant protein 1 as sensitive markers of disease activity in patients with juvenile rheumatoid arthritis: a six-year longitudinal study. *Arthritis Rheum*. 2006;54:2585–2593.
- [9] Bousquet J. Global initiative for asthma (GINA) and its objectives. *Clin Exp Allergy*. 2000;1:2–5.
- [10] Masoli M, Fabian D, Holt S, Beasley R. Global Initiative for Asthma (GINA) Program. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy*. 2004;59:469–478.
- [11] Arnold R, König W. Respiratory syncytial virus infection of human lung endothelial cells enhances selectively intercellular adhesion molecule-1 expression. *J Immunol*. 2005;174:7359–7367.
- [12] Zeng R, Li C, Li N, Wei L, Cui Y. The role of cytokines and chemokines in severe respiratory syncytial virus infection and subsequent asthma. *Cytokine*. 2011; 53:1–7.
- [13] Yamada Y, Matsumoto K, Hashimoto N, et al. Effect of Th1/Th2 cytokine pretreatment on RSV-induced gene expression in airway epithelial cells. *Int Arch Allergy Immunol*. 2011;154:185–194.
- [14] Roe MF, Bloxham DM, Cowburn AS, O'Donnell DR. Changes in helper lymphocyte chemokine receptor expression and elevation of interferon- γ inducible protein-10 P-10 during acute respiratory syncytial virus infection in infants. *Pediatr Allergy Immunol*. 2011;22:229–234.
- [15] Pasceri V, Cheng JS, Willerson JT, Yeh ET. Modulation of C-reactive protein-mediated monocyte chemoattractant protein-1 induction in human endothelial cells by anti-atherosclerosis drugs. *Circulation*. 2001;29:2531–2540.
- [16] Sung RY, Hui SH, Wong CK, Lam CW, Yin J. A comparison of cytokine responses in respiratory syncytial virus and influenza A infections in infants. *Eur J Pediatr*. 2001;160:117–122.

- [17] Patel JA, Nair S, Grady J, et al. Systemic cytokine response profile associated with respiratory virus induced acute otitis media. *Pediatric Infect Dis*. 2009;28:407–411.
- [18] Johnston SL, Papi A, Monick MM, Hunninghake GW. Rhinoviruses induce interleukin-8 mRNA and protein production in human monocytes. *J Infect Dis*. 1997;175:323–329.
- [19] Gern JE, Dick EC, Lee WM, et al. Rhinovirus enters but does not replicate inside monocytes and airway macrophages. *J Immunol*. 1996;156:621–627.
- [20] Conti P, DiGioacchino M. MCP-1 and RANTES are mediators of acute and chronic inflammation. *Allergy Asthma Proc*. 2001;22:133–137.
- [21] Kallal LE, Lukacs NW. The role of chemokines in virus-associated asthma exacerbations. *Curr Allergy Asthma Rep*. 2008;8:443–450.
- [22] Midulla F, Huang YT, Gilbert IA, Cirino NM, McFadden ER Jr, Panuska JR. Respiratory syncytial virus infection of human cord and adult blood monocytes and alveolar macrophages. *Am Rev Respir Dis*. 1989;140:771–777.
- [23] Elliott MB, Tebbey PW, Pryharski KS, Scheuer CA, Laughlin TS, Hancock GE. Inhibition of respiratory syncytial virus infection with the CC chemokine RANTES (CCL5). *J Med Virol*. 2004;73:300–308.
- [24] Haeberle HA, Casola A, Gatalica Z, et al. IkappaB kinase is a critical regulator of chemokine expression and lung inflammation in respiratory syncytial virus infection. *J Virol*. 2004;78:2232–2241.
- [25] Oshansky CM, Barber JP, Crabtree J, Tripp RA. Respiratory syncytial virus F and G proteins induce interleukin 1alpha, CC, and CXC chemokine responses by normal human bronchoepithelial cells. *J Infect Dis*. 2010;201:1201–1207.
- [26] Piedimonte G. Contribution of neuroimmune mechanisms to airway inflammation and remodeling during and after respiratory syncytial virus infection. *Pediatr Infect Dis J*. 2003;22:S66–S74.
- [27] Tan YR, Yang T, Liu SP, et al. Pulmonary peptidergic innervation remodeling and development of airway hyperresponsiveness induced by RSV persistent infection. *Peptides*. 2008;29:47–56.