



Leukemia inhibitory factor in the neuroimmune communication pathways in allergic asthma

Min-Juan Lin^{a,*}, Xue-Jun Lao^b, Sheng-Ming Liu^a, Zhen-Hua Xu^a, Wei-Feng Zou^a

^a Department of Pulmonology, First Affiliated Hospital, Jinan University, Guangzhou, China

^b Department of General Surgery, First Affiliated Hospital, Jinan University, Guangzhou, China

HIGHLIGHTS

- Increased LIF and NGF were detected in lung, DRG, and brain stems of asthmatic rat.
- De novo synthesis of LIF/NGF only increased in lung, but not in DRG and brain stem.
- Pretreatment with LIF antibody decreased the level of LIF and NGF in asthmatic rat.
- LIF seems to be required for airway inflammation and central sensitization in asthma.

ARTICLE INFO

Article history:

Received 1 September 2013

Received in revised form 8 January 2014

Accepted 14 January 2014

Keywords:

Allergic asthma

Dorsal root ganglia

Brain stem

Leukemia inhibitory factor

Nerve growth factor

ABSTRACT

In the pathogenesis of asthma, central sensitization is suggested to be an important neural mechanism, and neurotrophins and cytokines are likely to be the major mediators in the neuroimmune communication pathways of asthma. However, their impact on the central nervous system in allergic asthma remains unclear. We hypothesize that central neurogenic inflammation develops in the pathogenesis of allergic asthma, and nerve growth factor (NGF) and leukemia inhibitory factor (LIF) are important mediators in its development. An asthma model of rats was established by sensitization and challenged with ovalbumin (OVA). For further confirmation of the role of LIF in neurogenic inflammation, a subgroup was pretreated with intraperitoneally (i.p.) LIF antibody before OVA challenge. The levels of LIF and NGF were measured with reverse transcription and polymerase chain reaction (RT-PCR), in situ hybridization (ISH) and immunohistochemistry stain in lung tissue, airway-specific dorsal root ganglia (DRG, C7–T5) and brain stem of asthmatic rats, anti-LIF pretreated rats and controls. A significantly increased number of LIF- and NGF-immunoreactive cells were detected in lung tissue, DRG and the brain stem of asthmatic rats. In the asthma group a significantly increase level of mRNA encoding LIF and NGF in lung tissue was detected, but not in DRG and the brain stem. Pretreatment with LIF antibody decreased the level of LIF and NGF in all tissues. LIF is an important mediator in the crosstalk between nerve and immune systems. Our study demonstrate that the increased level of LIF and NGF in DRG and brain stem may be not based on result from de novo synthesis, but rather on result from retrograde nerve transport or passage across the blood–brain-barrier.

© 2014 Elsevier Ireland Ltd. All rights reserved.

Asthma is an inflammatory disease of the airway. Although an altered immune response is generally accepted to be a major mechanism in the pathogenesis of asthma, some clinical phenomena cannot be explained by immunologic response alone, e.g. nocturnal asthma or stress-related worsening of asthma. A possible

explanation for these manifestations of asthma is an interaction between the nervous and immune system.

It is well known, that tachykinins induced by neurotrophic factors from ganglia are associated with the neurogenic inflammation in asthma [16]. And increased levels of pro-inflammatory mediators (e.g. TNF- α , IL-4, NO, and 5-lipoxygenase) are detected both in cerebral cortex and lung tissues of asthmatic rats [4]. Pinto et al. [15] have also demonstrated that ovalbumin (OVA) challenge induced the up-regulation of c-Fos protein in the central nerve system (CNS). These support our hypothesis, that not only peripheral nerve system but also CNS may be involved in the pathogenesis of asthma.

* Corresponding author at: Department of Pulmonology, First Affiliated Hospital, Jinan University, Guangzhou, Guangdong Province 510630, China.
Tel.: +86 2038688629; fax: +86 2038688306.

E-mail address: amnlmj94@hotmail.com (M.-J. Lin).

For a long time, the brain was considered to be a privileged organ from an immunological point of view. But there is compelling evidence showing that the immune system can communicate with the CNS [19]. The immune system might interact with the CNS primarily by some cytokines that can pass the blood–brain barrier (BBB). And leukemia inhibitory factor (LIF) was suggested to an important proinflammatory cytokine working as a mediator in the immuno-neural interaction in recent works. Lipopolysaccharide (LPS)-induced circulating LIF can cross the BBB by a saturable transport system [14]. High level of LIF has already been found in atopic patients and patients with diffuse pulmonary inflammation [9,21].

Our previous study has showed that increased LIF level was detected in the lung of a rat model of allergic asthma [13]. Also, Renz and Kiliç point out that nerve growth factor (NGF) is an important neurotrophic factor in neurogenic inflammation in asthma [16], which can be found in airways and ganglia in allergic asthma.

Thus, it can be concluded that LIF and NGF might play an important role in the immuno-neural genesis of asthma, but it remains unknown whether LIF and NGF up-regulate in the CNS as part of the immuno-neural communication of allergic asthma.

In this study, we investigated LIF and NGF levels and mRNA levels encoding LIF and NGF in the lungs, DRG and brain stems of rats sensitized for ovalbumin (OVA) in an allergic asthma model.

We used male Sprague Dawley rats (Guangdong Provincial Medical Laboratory Animal Center) weighing 200–250 g. Procedures were carried out according to the guidelines approved by the Animal Care and Use Committee of Jinan University and were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

On day 0 and day 7, rats in the asthma group were sensitized by an intraperitoneal (i.p.) injection of 100 mg OVA (Sigma), and 100 mg aluminum hydroxide ($\text{Al}(\text{OH})_3$) (Sigma) and 5×10^9 heat-killed *Bordetella pertussis* vaccine (Wuhan Institute of Mediators of Inflammation Biological Products) in 1 ml of 0.9% sterile pyrogen-free sodium-chloride (NaCl) solution. From days 14 to 21, the sensitized rats were challenged daily with an aerosol of 1% OVA/saline solution delivered by nebulizer for 20 min. From days 14 to 21, a subgroup of sensitized rats were pre-treated with 200 $\mu\text{g}/\text{kg}$ anti-mouse LIF antibody (R&D Systems, USA) i.p. before each challenge. The control group received vehicle injections of equal amounts i.p. and the challenge was carried out with saline. The animals were sacrificed 24 h after the last aerosol challenge by i.p. application of 40 mg/kg pentobarbital sodium (LEO Pharma, Denmark). Airway inflammation was evaluated by histological examination with hematoxylin and eosin (HE) staining. DRG (C7–T5) were evaluated by in situ hybridization (ISH) and immunohistochemistry, and Lung samples, brain stems were evaluated by RT-PCR and immunohistochemistry.

Total RNA was extracted from homogenized lung tissues and the brain stems by TRIzol Reagent solution (Invitrogen, USA). 1 μg of total RNA was reverse-transcribed to cDNA. 5 μl of the RT product was then added to 25 μl of PCR mixture that contained 2.5 μl of $10\times$ PCR buffer, 2 μl of 2.5 mM deoxynucleotide triphosphate mixture, 1.5 unit of Taq DNA polymerase (TaKaRa, Dalian, China), and 240 nM primers (Sangon, Shanghai, China). The β -actin was used as an internal standard. Amplification for LIF cDNA was initiated by 7 min of denaturation at a temperature of 94°C followed by 30 cycles at 94°C for 45 s, 58°C for 30 s, and 72°C for 1 min using a thermal cycler (GeneAmp PCR System 9600, Perkin-Elmer). The annealing time and temperature for NGF amplification was 55°C for 45 s. Amplified PCR products were size-fractionated by electrophoresis through 1.5% Tris–acetate–EDTA agarose gels and stained with ethidium bromide. The intensity of DNA bands was analyzed by computerized densitometry with Quantimet 970 image analyzer (Cambridge Instruments, Ltd., Cambridge, UK) and expressed as

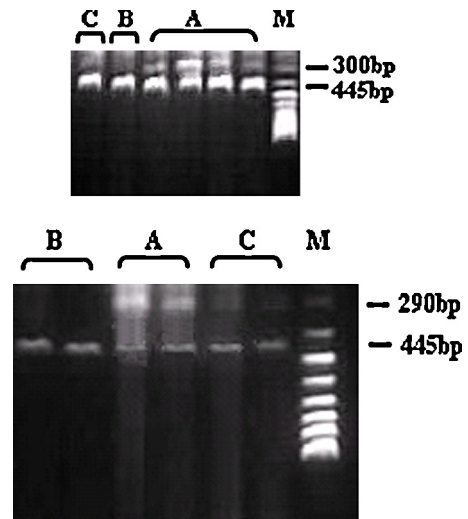


Fig. 1. RT-PCR amplification bands using primers against the LIF (300 bp), NGF (290 bp), beta-actin (445 bp) in rat lungs (M: 100 bp ladder, A: asthma group, B: control group, C: anti-LIF group). LIF: leukemia inhibitory factor, NGF: nerve growth factor.

the ratio of the densitometric score measured for each target gene normalized by the beta-actin control.

ISH kit was purchased from Boster Biological Technology Co., Wuhan, China. Ganglion sections were carried out by treatment with 0.2 M hydrochloric acid (HCl) for 20 min at room temperature,

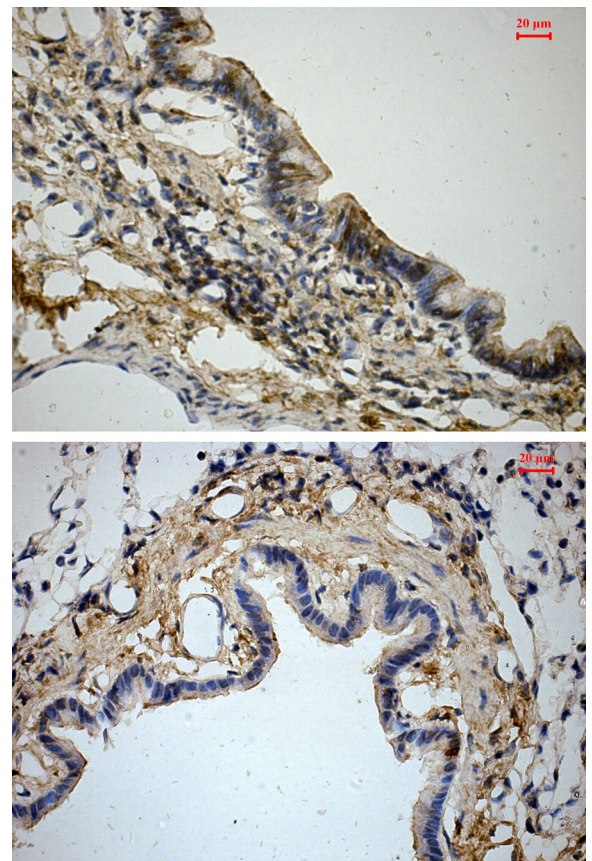


Fig. 2. LIF- and NGF-immunohistochemistry in airways. LIF- and NGF-immunoreactive cells mainly located in cytoplasm of airway epithelial cells, airway smooth muscle, vascular smooth muscle, and some interstitial cells in asthma rats. (A) Immunoreaction against NGF; (B) immunoreaction against LIF. Scale bar represents 20 μm .

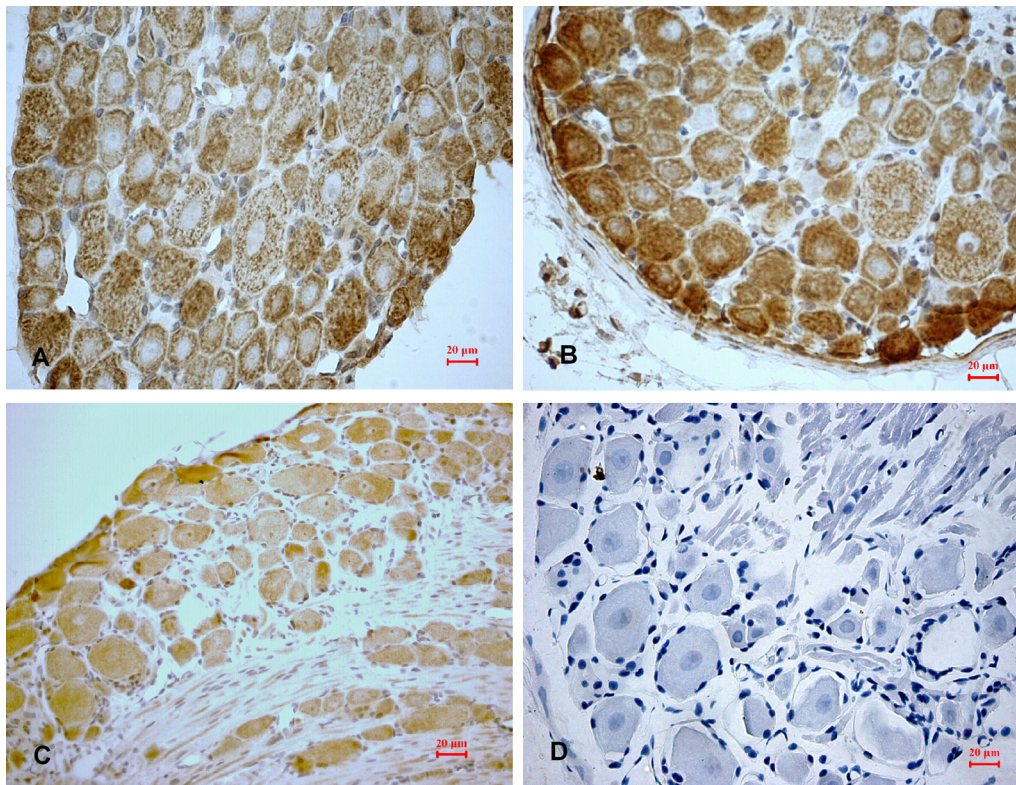


Fig. 3. LIF- and NGF-immunohistochemistry in airway-specific rat dorsal root ganglion (DRG, segmental levels C7–T5). (A) DRG in asthmal group with strong and diffuse NGF expression in the cytoplasm of neurons; (B) DRG in asthmal group with strong and diffuse LIF expression in the cytoplasm of neurons; (C) less immunolabeled neurons for NGF in DRG in control group compared with asthmal group; (D) no immunolabeled neurons in the DRG in negative control. Scale bar represents 20 µm.

and then with 3% H_2O_2 for 20 min. After digestion with proteinase K in phosphate buffered saline (PBS) for 20 min at 37 °C and sequential rinsing with 0.1 M PBS and 0.2× SSC, the slides were pre-hybridized at 42 °C for 4 h in a humidified chamber. After washing in 0.2× SSC, the sections were incubated overnight with hybridization solution at 42 °C. After rinsing with 2× SSC, 0.2× SSC and then 0.1 M PBS, the slides were sequentially incubated with biotin-labeled mouse-anti-digoxigenin antibody, biotin-labeled peroxidase. The signal was detected with 3,3'-diaminobenzidine (DAB).

The right-lower-lobe lungs, DRG (C7–T5) and brain stems were immersed in a 4% paraformaldehyde solution. After 12 h the ganglia and brain stems were subsequently placed in a 20% sucrose PBS overnight. Cross sections of the DRG and brain stems were cut on a cryostat (Leica CM850, Germany) at 10 µm. The lung samples were Paraffin embedded and cut at 4 µm for each section. The sections were incubated overnight at 4 °C with a polyclonal antibody against NGF beta (rabbit anti-rat 1:50, Boster Biological Technology Co., Wuhan, China), LIF (rabbit anti-rat 1:50, Santa Cruz Technology, USA). After washing 4 times in PBS, 50 µl of a Horseradish Peroxidase (HRP)-polymer-Goat anti-rabbit IgG (MaxVision™, Maixin.Bio, Fuzhou, China) was applied for 15 min. After washing an additional 3 times in PBS, sections were incubated with a mixture of DAB (Maixin.Bio, Fuzhou, China) for 3–5 min. Thereafter, the slides were counterstained with hematoxylin and aqueously mounted. For negative controls, separate sections were processed similarly, but the primary antibody was replaced with off-target IgG. Images were acquired using a Leica DMLBHC microscope in combination with a Leica DC 100 charge-coupled-device camera and analyzed by Leica Qwin V3.2.1 image manager software (Leica Microsystems, Germany). The distributions of the immunopositive cells were observed in 8 vision fields for each section. The positive area unit (PU) was introduced to describe the intensity of immunopositive expression: $PU = 100 \times (G1 - G2)/G_{max}$ [17]. In

this equation G1, G2, and G_{max} represent the average gray value of immunopositive deposition (G1), background (G2), and maximum (G_{max}) threshold gray value respectively.

All values were expressed as mean ± S.E.M. Differences between two groups were analyzed for statistical significance. The variance tests were performed on data. One way analysis of variance (ANOVA) test was used for data, which showed equal variances. Kruskal–Wallis test was used for data, which showed unequal variances. A *P* value of less than 0.05 was accepted as statistically significant.

The levels of mRNA encoding LIF and NGF were significantly increased in the lungs of rats in the asthmal group, but neither in the control group nor in the anti-LIF group (Fig. 1). In contrast to the results in lung tissue, no significant difference in the mRNA levels of LIF and NGF were found in DRG among the three groups (Fig. 4) and there were no detectable bands by RT-PCR in the brain stems among the three groups.

LIF- and NGF-immunoreactivity was mainly located in the cytoplasm of airway epithelial cells, airway smooth muscle, vascular smooth muscle, and some interstitial cells (Fig. 2). Compared to the asthmal group, the immunopositive deposition of LIF and NGF in the anti-LIF subgroup was clearly lower in airway epithelial cells and peribronchial tissue. In airways of rats in the control group, few positive cells could be found.

Fig. 3 shows representative images of immuno-labeled LIF and NGF in airway-specific rat DRG (C7–T5). Neurons in DRG of asthmatic rats were strongly immuno-stained with antibodies against LIF and NGF. The PU values for NGF and LIF in neurons were significantly higher in asthmatic group compared with control group and anti-LIF group (Fig. 4).

The distribution of NGF immuno-labeled neurons in the brain stem of rats in the asthmal group was widespread, but particularly concentrated in the region of the nucleus of the solitary tract

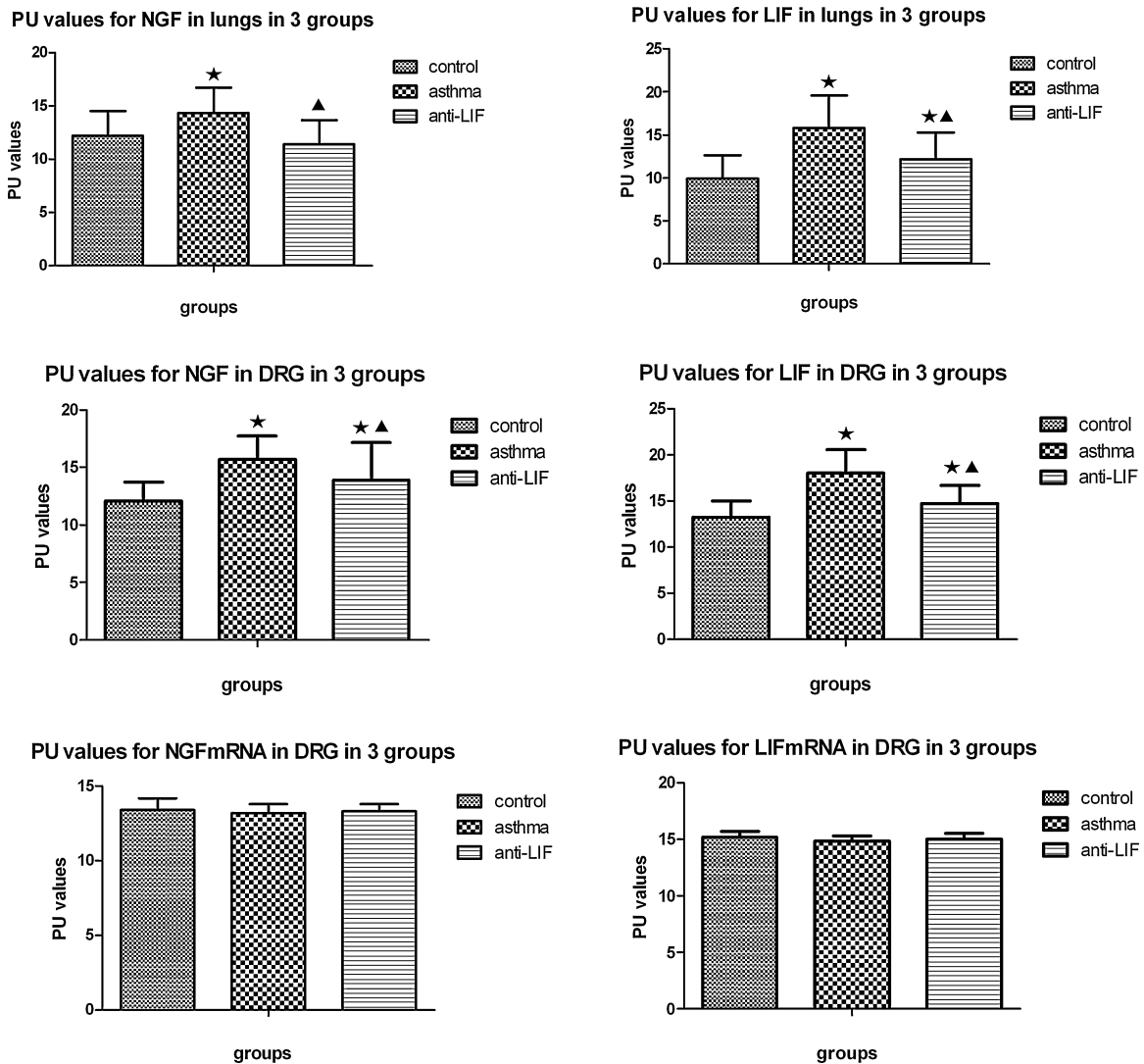


Fig. 4. Statistic graphs for comparison the PU value of NGF and LIF in lungs and DRG among the control, asthma and antibody groups (A–D). Statistic graphs for comparison the levels of NGFmRNA and LIFmRNA in DRG among the control, asthma and antibody groups (E and F). * $P < 0.05$ versus control group, ▲ $P < 0.05$ versus asthma group.

(NTS) (Fig. 5A and B), while LIF immuno-reactive neurons in the brain stem in the asthma group were distributed mainly in NTS (Fig. 5D). However, LIF and NGF were not detectable in the brain stem of rats either in the control group or in the anti-LIF group (Fig. 5C).

The major findings of our study are as follows: (1) Levels of LIF and NGF are significantly increased in lung tissue, airway-specific DRG, and the brain stems of rats in a model of allergic asthma; (2) Increased levels of mRNA encoding LIF and NGF detected exclusively in the lung tissue of asthmatic rats indicate that de novo synthesis of LIF and NGF only increases in lung tissue, but not in DRG and brain stem; and (3) Application of anti-LIF prior to OVA-challenge prevents the increase of LIF and NGF levels in the lung, DRG and brain stem.

Previous studies suggested that LIF and NGF play a pivotal role in neurogenic inflammation in allergic asthma [10,13,14]. Our present results confirm preceding observations [11,13] that LIF and NGF exist abundantly in lungs and airway-specific DRG in a rat model of allergic asthma. This elevated levels of LIF and NGF appears to be caused by de novo synthesis in the lungs, as indicated by the significantly increased both mRNA and protein level. Nevertheless, only increased LIF and NGF immunoreactivity, but not that of mRNA, is observed in DRG of asthmatic rats. This is in line with

previous reports showing that LIF and NGF will be retrogradely transported to the DRG following de novo synthesis at the site of lesion [1,18]. Elevated levels of LIF in the lungs of asthmatics can have an significant impact on the development of asthma, e.g. LIF can markedly up-regulate eosinophil migration in response to classical agonists such as platelet activating factor (PAF) as well as substance P [21]; LIF in airway smooth muscle cells can enhance ACh-induced $[Ca^{2+}]_i$ response and airway contractility [7]; furthermore, LIF can induce the expression of neurokinin-1 receptor in cultured human bronchial epithelial cells [8]. In the DRG, LIF and NGF lead to substance P expression with respect to upregulated neurotrophin-receptors and transient receptor potential vanilloid (TRPV1) expression [5,6]. Thus, LIF and NGF contribute to the airway inflammation and hyper-reactivity through regulating the function of both neural and immune systems.

So far, most of the studies about neurogenic inflammation in asthma focused on airway and ganglion. Our study showed for the first time an increased LIF and NGF immunoreactivity in the brain stem of rats in an asthma model, but not at the level of mRNA (RT-PCR), which were similar to the findings in DRG. Our experiments also revealed that removal of LIF by i.p. application of anti-LIF antibodies resulted in a significant loss of LIF- and NGF-immunoreactivity in neurons of DRG and the brain stem.

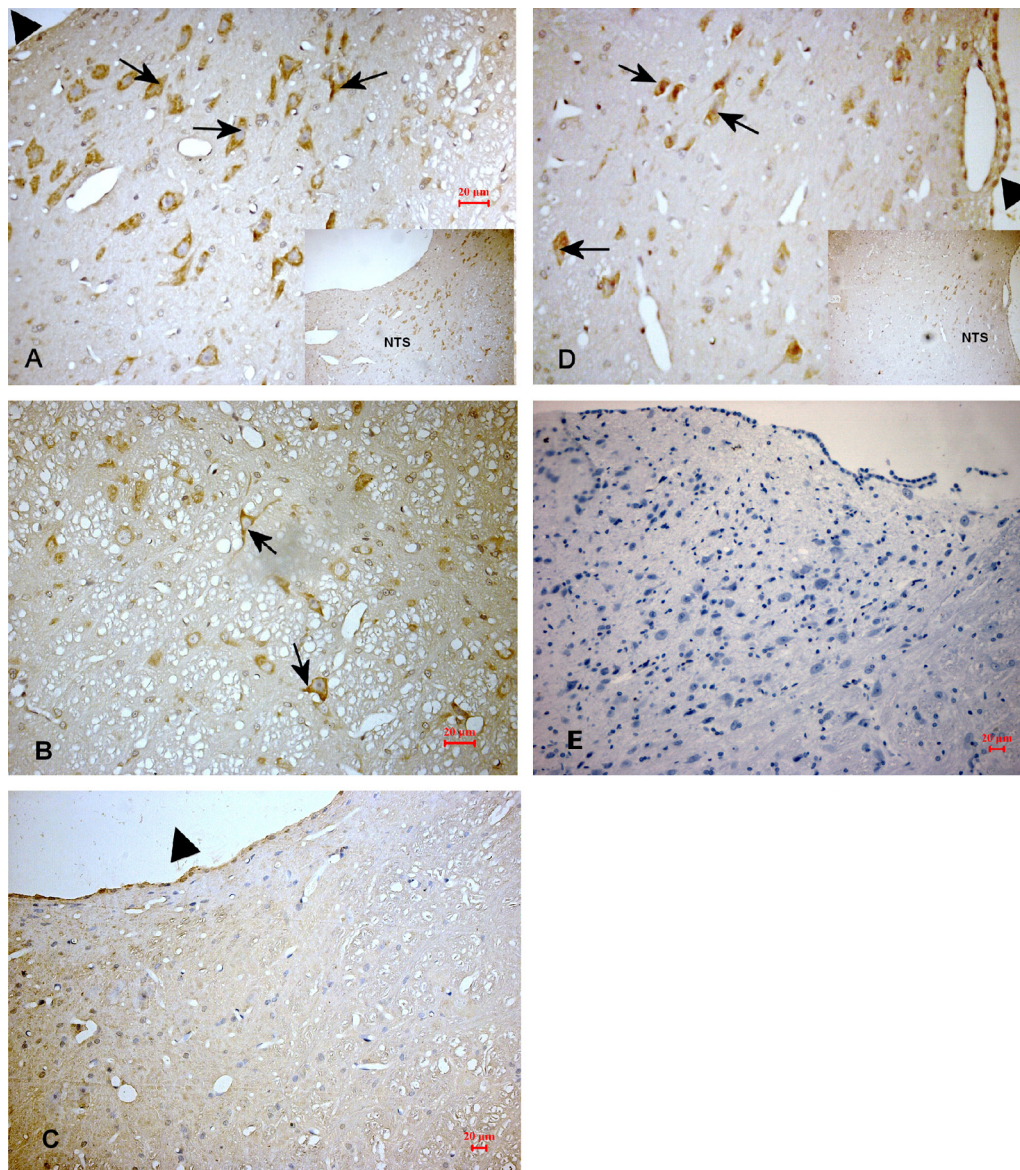


Fig. 5. LIF- and NGF-immunohistochemistry in brain stem on cross section. (A and B) Immunolabeled for NGF in brain stem of rat in asthma group (A, arrow indicates immunolabeled neurons in NTS; B, arrow indicates widespread immunolabeled neurons); (D) immunolabeled for LIF in the region of NTS of the brain stem in asthmatic rat (arrow indicates immunolabeled neurons in NTS); (C) no detectable immunolabeled signs of NGF in the brain stem of rats in the control group; (E) no detectable immunolabeled signs of NGF or LIF in the region of NTS in negative control. ▲ stands for the dorsal brain stem on cross section. NTS: nucleus of the solitary tract. Scale bar in (A) is also for (B and D). Scale bar represents 20 μ m.

Combining these findings with the data mentioned above [14], we put forward the hypothesis that LIF and NGF were not synthesized in the brain stem, but transported across the BBB from the lung tissue, possible by a saturable transport system, as shown by previous studies [14]. Furthermore, we demonstrate that LIF and NGF distribute concentrately in the region of NTS of the brain stem, which is the focus of the CNS innervation of airway-related parasympathetic preganglionic neurons. Chen et al. [2] have also shown in a rhesus monkey model that repeated exposure to an allergen depolarizes the resting membrane potential of NTS neurons and increases the number of action potentials. LIF and NGF can subsequently induce the c-Fos activity [3,20], which increases also in NTS following OVA challenge. This can be referred to as “central sensitization” in asthma. But it needs to be further delineated whether the functional significance of LIF and NGF level in NTS, found in our study, plays a role in the change of neuronal phenotypes or in the modulation of neurotransmitter receptors. This possibility was already proved by Lieu et al. [12] in nodose

ganglion in guinea pig asthma model. They showed that following OVA challenge neurotrophin-receptor, TRPV1 expression and capsaicin sensitive neurons increased in nodose tracheal A δ fibers, which are capsaicin insensitive and do not express TRPV1 in physiological condition. This phenotypic change was mimicked by neurotrophic factor. Further investigation about the role of NGF and LIF in NTS is to confirm the functional role of LIF and NGF.

In conclusion, the findings of our study strongly support the hypothesis that central neurogenic inflammation develops in the pathogenesis of allergic asthma, and NGF and LIF are important mediators in its development. We demonstrate that OVA challenge induces a significant increase in LIF and NGF level in the lungs of asthmatic rats, which can subsequently be retrogradely transported to airway-specific DRG and across the BBB to the brain stem. This process can be inhibited by the removal of LIF from the site of lesion (airways) and circulation, using anti-LIF antibodies. This indicates that LIF seems to be required for airway inflammation and central sensitization in asthma. Our study shows the possible

role of LIF in the pathogenesis of asthma as a mediator of crosstalk between neural and immune systems.

Acknowledgements

This study was supported by the National Natural Science Foundation (No. 30300146) and Guangdong Natural Science Foundation (No. 5006052).

References

- [1] R.B. Campenot, B.L. MacInnis, Retrograde transport of neurotrophins: fact and function, *J. Neurobiol.* 58 (2004) 217–229.
- [2] C.Y. Chen, A.C. Bonham, E.S. Schelegle, L.J. Gershwin, C.G. Plopper, J.P. Joad, Extended allergen exposure in asthmatic monkeys induces neuroplasticity in nucleus tractus solitarius, *J. Allergy Clin. Immunol.* 108 (2001) 557–562.
- [3] S.D. Chen, Z.H. Wen, W.K. Chang, K.H. Chan, M.T. Tsou, C.S. Sung, G.J. Tang, Acute effect of methylprednisolone on the brain in a rat model of allergic asthma, *Neurosci. Lett.* 440 (2008) 87–91.
- [4] Y.M. Deng, Q.M. Xie, S.J. Zhang, J.Q. Chen, Q.H. Yang, R.L. Bian, Changes of 5-lipoxygenase pathway and proinflammatory mediators in cerebral cortex and lung tissue of sensitized rats, *Acta Pharmacol. Sin.* 26 (2005) 353–358.
- [5] Q.T. Dinh, D.A. Groneberg, C. Peiser, E. Mingomataj, R.A. Joachim, C. Witt, P.C. Arck, B.F. Klapp, A. Fischer, P. Substance, expression in TRPV1 and trkA-positive dorsal root ganglion neurons innervating the mouse lung, *Respir. Physiol. Neurobiol.* 144 (2004) 15–24.
- [6] S. Engert, J.R. Wendland, A. Schwab, M. Petersen, Leukemia inhibitory factor differentially regulates capsaicin and heat sensitivity in cultured rat dorsal root ganglion neurons, *Neuropeptides* 42 (2008) 193–197.
- [7] M. Fayon, M. Rebola, P. Berger, S. Daburon, O. Ousova, F. Lavrand, B. Moukaïla, W. Pujol, J.L. Taupin, A. Labbé, M. Molimard, R. Marthan, Increased secretion of leukemia inhibitory factor by immature airway smooth muscle cells enhances intracellular signaling and airway contractility, *Am. J. Physiol. Lung Cell Mol. Physiol.* 291 (2006) L244–L251.
- [8] C.P. Hu, J.T. Feng, Y.L. Tang, J.Q. Zhu, M.J. Lin, M.E. Yu, LIF upregulates expression of NK-1R in NHBE cells, *Mediators Inflamm.* 5 (2006) 84829.
- [9] P.G. Jorens, R. De Jongh, L.L. Bossaert, W. De Backer, A.G. Herman, H. Pollet, E. Bosmans, J.L. Taupin, J.F. Moreau, High levels of leukemia inhibitory factor in ARDS, *Cytokine* 8 (1996) 873–876.
- [10] D. Knight, Leukemia inhibitory factor (LIF): a cytokine of emerging importance in chronic airway inflammation, *Pulm. Pharmacol. Ther.* 14 (2001) 169–176.
- [11] D.A. Knight, K. McKay, B. Wiggs, R.R. Schellenberg, T.R. Bai, Localization of leukemia inhibitory factor to airway epithelium and its amplification of contractile responses to tachykinins, *Br. J. Pharmacol.* 120 (1997) 883–891.
- [12] T.M. Lieu, A.C. Myers, S. Meeker, B.J. Undem, TRPV1 induction in airway vagal low-threshold mechanosensory neurons by allergen challenge and neurotrophic factors, *Am. J. Physiol. Lung Cell Mol. Physiol.* 302 (2012) L941–L948.
- [13] M.J. Lin, X.J. Lao, H.M. Ma, Y. Tang, The relationship between leukemia inhibitory factor and neurokinin receptors in a rat model of asthma, *Zhonghua Jie He He Hu Xi Za Zhi* 28 (2005) 820–824 (in Chinese).
- [14] W. Pan, A.J. Kastin, J.M. Brennan, Saturable entry of leukemia inhibitory factor from blood to the central nervous system, *J. Neuroimmunol.* 106 (2000) 172–180.
- [15] F.A. Pinto, A.S. Basso, L.C. de Sá-Rocha, L.R. Britto, M. Russo, J. Palermo-Neto, Neural correlates of IgE-mediated allergy, *Ann. N. Y. Acad. Sci.* 1088 (2006) 116–131.
- [16] H. Renz, A. Kiliç, Neurotrophins in chronic allergic airway inflammation and remodeling, *Chem. Immunol. Allergy* 98 (2012) 100–117.
- [17] Y.L. Tang, C.P. Hu, J.T. Feng, J.Q. Zhu, M.J. Lin, Regulation effect of nerve growth factor on Ras-MAPK signal transduction pathway in neurogenic inflammation of asthma, *Zhong Nan Da Xue Xue Bao Yi Xue Ban* 31 (2006) 319–325.
- [18] S.W. Thompson, A.B. Vernall, J.K. Heath, J.V. Priestley, Leukemia inhibitory factor is retrogradely transported by a distinct population of adult rat sensory neurons: co-localization with trkA and other neurochemical markers, *Eur. J. Neurosci.* 9 (1997) 1244–1251.
- [19] D. Wrona, Neural-immune interactions: An integrative view of the bidirectional relationship between the brain and immune systems, *J. Neuroimmunol.* 172 (2006) 38–58.
- [20] T. Yamamori, CDF/LIF selectively increases c-fos and jun-B transcripts in sympathetic neurons, *Neuroreport* 2 (1991) 173–176.
- [21] X. Zheng, D.A. Knight, D. Zhou, T. Weir, C. Peacock, R.R. Schellenberg, T.R. Bai, Leukemia inhibitory factor is synthesized and released by human eosinophils and modulates activation state and chemotaxis, *J. Allergy Clin. Immunol.* 104 (1999) 136–144.