
NGF/TrkA-mediated Kidins220/ARMS signaling activated in the allergic airway challenge in mice

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Background: Nerve growth factor (NGF), combined with its high-affinity receptor tyrosine kinase receptor A (TrkA), has been reported to be involved in the pathogenesis of asthma.

Objective: To investigate whether the downstream protein ankyrin-rich membrane spanning (ARMS), a novel transmembrane substrate of protein kinase D (Kidins220), is activated in the pathogenesis of asthma.

Methods: The asthmatic model was established by the inhalation of ovalbumin in BALB/c mice. The effects of NGF and TrkA on Kidins220/ARMS in an allergic airway challenge were assessed by administering anti-NGF or anti-TrkA antibody to the mice. Pathologic changes in the bronchi and lung tissues were examined by means of hematoxylin and eosin staining; the inflammatory cells in the bronchoalveolar lavage fluid (BALF) were counted; and co-expression of ARMS and TrkA in BALF cells was observed by means of immunofluorescence. In addition, Kidins220/ARMS, CrkL, NGF, TrkA protein, and Kidins220 messenger RNA levels were determined using Western blot or quantitative reverse transcription-polymerase chain reaction.

Results: Using fluorescence microscopy, we found that Kidins220 and TrkA were co-expressed on the membranes of the BALF cells of asthmatic mice. Compared with expression in control animals, Kidins220/ARMS, CrkL, NGF, and TrkA were overexpressed in the lungs after allergen challenge. Moreover, after the mice were treated with anti-NGF or anti-TrkA, the Kidins220/ARMS levels and allergen-induced airway inflammation decreased.

Conclusions: These results suggest that Kidins220/ARMS partly participates in the pathogenesis of asthma through the NGF-TrkA signaling pathway, possibly representing a new mechanism in asthma.

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INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways that involves many cells and cellular elements. Chronic airway inflammation and airway hyperresponsiveness are important pathophysiologic features of asthma. Nerve growth factor (NGF) exerts an important functional effect on the pathogenesis of allergic diseases.¹ Data from animal models of allergic bronchial asthma indicate that NGF alters sensory nerve function and promotes allergic inflammation, bronchial hyperreactivity, and airway obstruction.^{2,3} Growing evidence suggests that NGF acts as an important mediator in neuroimmune mechanisms of asthma by regulating neuronal plasticity and connecting neuroimmune mechanisms in immune cells and neurons, thereby mediating airway hyperresponsiveness.¹ NGF induces airway inflammation by causing neurogenic inflammation and amplifying the effects of immune cells.⁴ The diverse actions of NGF

are mediated by its binding to the tyrosine kinase receptor A (TrkA), a specific high-affinity receptor.^{5,6}

The ankyrin-rich membrane spanning protein (ARMS), originally identified as a substrate for protein kinase D (Kidins220), serves as a novel downstream target of Trk receptor tyrosine kinases.^{7,8} The Kidins220/ARMS complex is frequently co-expressed with Trk and p75 and represents the only membrane-associated protein known to interact with both receptors.⁹ An association between TrkA and ARMS was previously found to persist for many hours.⁷ Furthermore, the interaction of Trk and ARMS resulted in an increase in mitogen-activated protein kinase (MAPK) activation that persisted for hours.¹⁰ These data suggest that ARMS may regulate the generation of high-affinity NGF receptor binding sites. Therefore, ARMS may serve as a scaffold for key adaptor proteins that are directly involved in neurotrophin signaling.^{11,12} During NGF-induced neuronal differentiation, there is direct engagement of the TrkA receptor with ARMS, leading to sustained MAPK activation that regulates neurite outgrowth.¹⁰ The number of potential interactions with ARMS indicates that ARMS may represent a dynamic multifunctional protein responsible for the specialized effects of neurotrophins. Growing evidence suggests the importance of ARMS as a signaling molecule in the NGF pathway. Both NGF and TrkA have been reported to participate in the pathogenesis of asthma; however, whether ARMS is also involved in this process and whether it acts through the NGF-TrkA pathway have not been determined.

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In this study, we investigated whether Kidins220/ARMS participates in the pathogenesis of asthma through the NGF-TrkA signaling pathway. These findings demonstrate that Kidins220/ARMS was overexpressed in the lungs after allergen challenge, and the increased expression of Kidins220/ARMS was decreased by anti-NGF or anti-TrkA antibody treatment.

METHODS

Chemicals and Reagents

Goat polyclonal anti-ARMS and anti-NGF and rabbit polyclonal anti-TrkA primary antibodies and goat anti-rabbit and rabbit anti-goat IgG–horseradish peroxidase–conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, California). Tetramethyl rhodamine isothiocyanate–labeled monkey anti-rabbit and fluorescein isothiocyanate–labeled cow anti-goat IgG secondary antibodies were also obtained from Santa Cruz Biotechnology Inc. Rabbit polyclonal anti-CrkL antibodies were obtained from Bioworld Technology Inc (St Louis Park, Minnesota). The TRIzol reagent was purchased from Invitrogen Life Technologies (Carlsbad, California). The SYBR Green system was purchased from Bio-Rad Laboratories Ltd (Hercules, California). Unless otherwise noted, all the other reagents were purchased from Sigma-Aldrich Corp (St Louis, Missouri).

Experimental Animals

Pathogen-free female BALB/c mice, 6 to 8 weeks of age and weighing approximately 18 to 20 g, were provided by the Laboratory Animal Research Center in Beijing, China. These mice were maintained with 12 hour/12 hour light/dark cycles and free access to food and water. All animal experiments and manipulation procedures were approved by the Ethics Committee for Animal Use and Care at the Institute of Education of China Medical University.

Animal Groups and Protocol for Allergic Sensitization

Forty-six BALB/c mice were randomized into 4 groups: the phosphate-buffered saline (PBS) control group ($n = 10$), the asthmatic model group ($n = 12$), the anti-NGF group ($n = 12$), and the anti-TrkA group ($n = 12$). In the asthmatic model group, mice were sensitized to ovalbumin (20 μg per injection) absorbed to 2.0 mg per injection of aluminium hydroxide by intraperitoneal injection on day 1. On days 8, 15, and 22, mice were sensitized again to ovalbumin (10 μg per injection) absorbed to 1.0 mg per injection of aluminium hydroxide by intraperitoneal injection. Beginning on day 23, the mice were administered inhaled aerosols of 4% ovalbumin in PBS for 25 to 30 minutes (until the onset of bronchial obstruction) daily for 7 consecutive days as previously described^{13,14} with some modifications. The mice in the anti-NGF and anti-TrkA groups were also subjected to ovalbumin sensitization and asthma induction in the same manner. Intranasal application of 50 μL (1:50 dilution) of polyclonal goat anti-mouse NGF antibody (biological activity: 1:4,000 dilution blocks bioactivity of NGF at 5 ng/mL) (Santa Cruz

Biotechnology Inc) dissolved in sterile PBS was performed 3 hours before each airway allergen challenge in the anti-NGF group. Intranasal treatments with anti-NGF antibody were performed according to the methods of Braun et al,¹³ Glaab et al,¹⁵ and Nagai et al¹⁶ with minor modifications. The mice in the anti-TrkA group received 0.2-mmol/L anti-TrkA antibody (200 μL prepared with PBS) by intranasal application^{5,17} 3 hours before the induction of asthma. All the antibodies were administered daily for 5 consecutive days. Mice in the control group were administered PBS alone by injection and were challenged with PBS. All the animals were humanely killed within 24 hours after the last ovalbumin or PBS exposure.

Pathologic Examination of Bronchial and Lung Tissues

The left lungs of the BALB/c mice were perfused with 4% paraformaldehyde to allow the pleura to extend and flatten before fixation of the tissue in 4% paraformaldehyde. The tissues were routinely embedded in paraffin and sectioned (5 μm) for hematoxylin and eosin staining to assess the pathologic changes in the lung and bronchial tissues by means of microscopy.

Bronchoalveolar Lavage Fluid

Bronchoalveolar lavage fluid (BALF) samples were collected 24 hours after the last challenge according to the methods previously described.¹⁸ After humanely killing the mouse by cervical dislocation, the lungs were lavaged with 1.6 mL (0.8 mL per lavage) of ice-cold PBS via the tracheal cannula. The lavage fluid was recovered by gentle manual aspiration using a syringe. The retrieved volume, averaging 75% to 80% of the instilled PBS, was then centrifuged at 500g. After the supernatant was discarded, the resultant pellet was washed twice with PBS and was resuspended in 1 mL of PBS. The total cell numbers were counted using a hemocytometer. Smears of BALF cells prepared with Cytospin II (Shandon, Runcorn, United Kingdom) were stained with Wright and Giemsa staining fluid for differential cell counting. Using a microscope, 2 independent blinded investigators counted the cells. Approximately 200 cells were counted in each of 4 randomly selected locations.

Immunofluorescence Detection of ARMS and TrkA

Smears of BALF cells from the PBS of the control and asthmatic groups were fixed with cold acetone and were incubated with goat polyclonal anti-ARMS and rabbit polyclonal anti-TrkA primary antibodies (1:50 dilution, 0.01-mmol/L PBS, pH 7.4) at 4°C overnight. Tetramethyl rhodamine isothiocyanate–labeled monkey anti-rabbit and fluorescein isothiocyanate–labeled cow anti-goat IgG secondary antibodies (diluted 1:200) were added at 37°C for 30 minutes of incubation in the dark. Nuclei were counterstained with Hoechst 33258. Instead of primary antibodies, PBS was used for the negative control. The slides were then observed and photographed using a fluorescence microscope.

Western Blot for ARMS, TrkA, CrkL, and NGF

Western blot analysis for ARMS, TrkA, CrkL, and NGF was conducted in the right lung tissues freshly isolated from mice in each group. The lung fragments (0.05 g) were homogenized in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Protein concentrations of the tissue lysates were determined using the Enhanced BCA Protein Assay Kit (Beyotime), and the supernatants were boiled in sodium dodecyl sulfate sample buffer for 5 minutes. Equal amounts of lysate proteins were separated using 7.5% or 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then were transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). After blocking, the blots were incubated with specific primary antibody overnight at 4°C and then were further incubated for 1 hour with horseradish peroxidase–conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence kit with a Lumino image analyzer (Taitec Corp, Tokyo, Japan). Integrated density values were analyzed using a computerized image analysis system (Fluor Chen 2.0; Olympus, Yokohama, Japan) and were normalized to those of β -actin.

Quantitative Reverse Transcription–Polymerase Chain Reaction for ARMS Messenger RNA

Total RNA from lung tissues was isolated using a rapid extraction method (TRIzol reagent) according to the manu-

facturer's instructions. Reverse transcription polymerase chain reaction was performed on the complementary DNA samples using the SYBR Green system. The primers used were Kidins220 sense 5'-GGT GCC CCT CGT CAC AGT AAT-3' and Kidins220-3 antisense 5'-TTC CGC TGT GAC TGT CAT CTT-3'. Cycling conditions were 1 cycle at 95°C for 90 seconds and 40 cycles each corresponding to 5 seconds at 95°C and 30 seconds at 60°C. Analysis used the sequence detection software supplied with the instrument. The relative quantification value was expressed as $2 \pm \text{DCT}$, where DCT was the difference between the mean cycle threshold value of the sample duplicates and of the glyceraldehyde-3-phosphate dehydrogenase control.

Statistical Analysis

All the data were analyzed using statistical software (SPSS version 15.0; SPSS Inc, Chicago, Illinois). The data are expressed as mean (SEM). Differences between groups were analyzed, as appropriate, using *t* tests and 1-way analysis of variance followed by the Fisher least significant difference test. The differences were considered significant at $P < .05$.

RESULTS

Pathologic Changes in the Bronchi and Lung Tissues

We found that mice in the control group had normal smooth bronchial and alveolar structures and no inflammatory cell

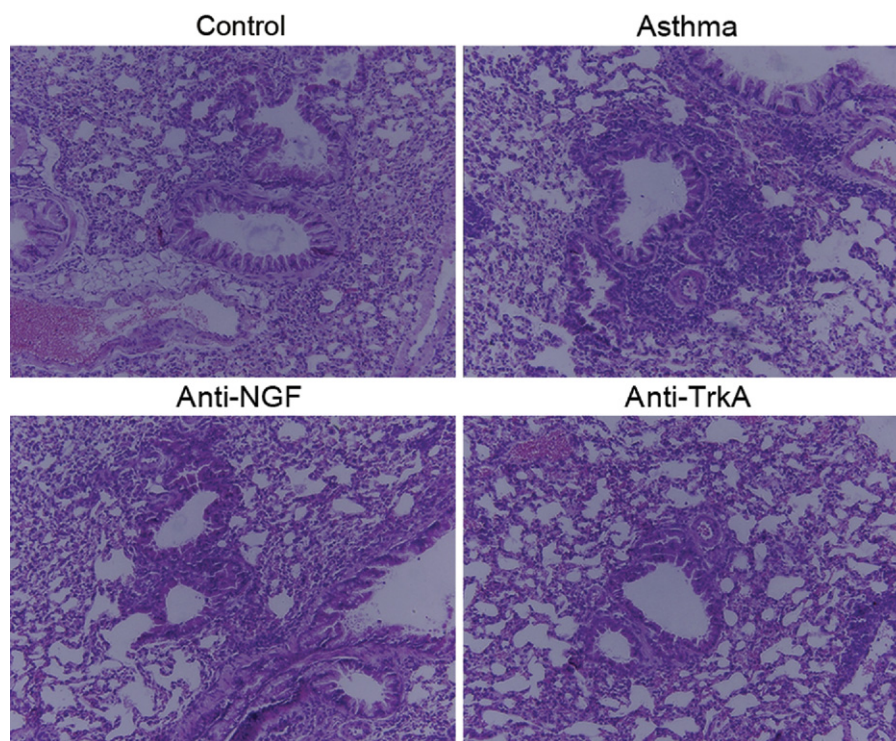


Figure 1. Pathologic changes in the bronchi and lung tissues of BALB/c mice in the different groups. Obvious bronchiolar stenosis can be seen in the asthmatic model group, and the bronchiolar and lung tissues show obvious inflammation and inflammatory cell infiltration. The bronchiolar and lung tissue inflammation and bronchiolar narrowing was alleviated significantly after intervention with the antibodies. Sections were stained by hematoxylin and eosin (original magnification $\times 200$). NGF indicates nerve growth factor; and TrkA, tyrosine kinase receptor A.

infiltration in the airway and lung tissues. Histologic results from mice in the asthma group showed extensive infiltration of inflammatory cells around the bronchioles, blood vessels, and alveoli. The airway mucosa had obvious edema, with bronchiolar wall thickening causing luminal stenosis, suggesting successful establishment of the asthmatic model. In asthmatic mice treated with anti-NGF and anti-TrkA antibodies, the extent of inflammation and cellular infiltration in the airway was reduced, and the pathologic changes of the bronchi and lung tissues were milder compared with the changes found in the asthma group without treatment. However, the extent of inflammation and cellular infiltration in the airways of asthmatic mice with anti-NGF and anti-TrkA treatment was still obvious compared with that in the airways of the control group (Fig 1).

Total Cell Numbers and Differential Cell Percentages in BALF

The total cell numbers (Fig 2A) and the percentages of macrophages, eosinophils, lymphocytes, and neutrophils (Fig 2B) in BALF from asthmatic mice increased significantly

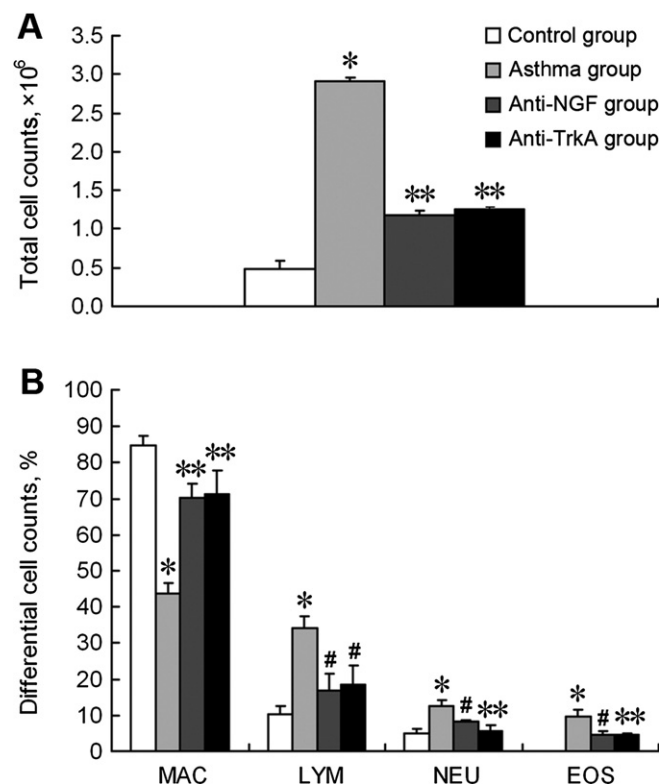


Figure 2. Mean total and differential cells in the bronchoalveolar lavage fluid from the different groups of mice. A, Total cell numbers. B, Percentages of macrophages (MAC), lymphocytes (LYM), neutrophils (NEU), and eosinophils (EOS). * $P < .01$ compared with the control group. ** $P < .01$ compared with the asthma group. # $P < .05$ compared with the asthma group. Error bars represent standard error of the mean ($n = 6$). NGF indicates nerve growth factor; and TrkA, tyrosine kinase receptor A.

compared with those in BALF from control mice ($P < .01$). The increased numbers of inflammatory cells, including eosinophils, were significantly reduced by the administration of anti-NGF and anti-TrkA antibodies in asthmatic mice compared with the numbers found in nontreated asthmatic mice ($P < .05$).

Localization of ARMS and TrkA in BALF Cells

Triple-labeling immunofluorescence of BALF cells was used to demonstrate the co-localization of ARMS and TrkA (Fig 3). Yellow regions in the combined fluorescence images corresponded to overlapping green and red images (ARMS was green, TrkA was red, and Hoechst 33258 was blue), and these regions suggest that the 2 stained proteins reside in the same intracellular compartment; we found that they were mainly expressed on the cell membrane and in the cytoplasm. The fluorescence intensities of BALF cells immunostained for ARMS and TrkA were different among the experimental groups. The BALF cells from asthmatic mice exhibited much greater fluorescence intensity than did those from control mice. These findings demonstrate the differential expression of ARMS/TrkA in BALF cells from normal and asthmatic mice, suggesting the involvement of ARMS together with TrkA in the pathogenesis of asthma. Whether the intracellular distribution of these proteins represents the cytoplasmic internalization of ARMS in response to inflammation during the course of asthma is not known and is the subject of ongoing investigations.

Expression of ARMS, TrkA, CrkL, and NGF Protein

Western blot analysis was conducted to study ARMS (Fig 4A), CrkL (Fig 5A), TrkA (Fig 6A), and NGF protein levels (Fig 7A) in each group. The results demonstrate that ARMS and CrkL were expressed by both the control and asthma groups. Moreover, the expression of ARMS and CrkL in asthmatic mice was significantly higher than that in control mice (ARMS: $P < .01$; CrkL: $P < .05$). The expression of ARMS and CrkL in asthmatic mice treated with anti-NGF or anti-TrkA antibodies was significantly lower than that in untreated asthmatic mice (ARMS: $P < .01$ [Fig 4B]; CrkL: $P < .05$ [Fig 5B]). Similarly, the expression of TrkA (Fig 6B) and NGF (Fig 7B) in asthmatic mice was significantly higher than that in control mice ($P < .01$). Intervention with anti-NGF antibody resulted in a significant reduction in TrkA expression in the lungs ($P < .01$) (Fig 6B). The expression of ARMS and CrkL as well as TrkA and NGF increased in allergic asthma, suggesting the involvement of ARMS and CrkL (the adaptor protein of ARMS) with TrkA and NGF in the pathogenesis of asthma.

Expression of ARMS Messenger RNA in Lung Tissues

Quantitative reverse transcription-polymerase chain reaction analysis revealed that the level of ARMS messenger RNA (mRNA) in lung tissues in the asthma group was significantly higher than the level in the control group ($P < .01$). The increased ARMS mRNA expression found in asthmatic mice

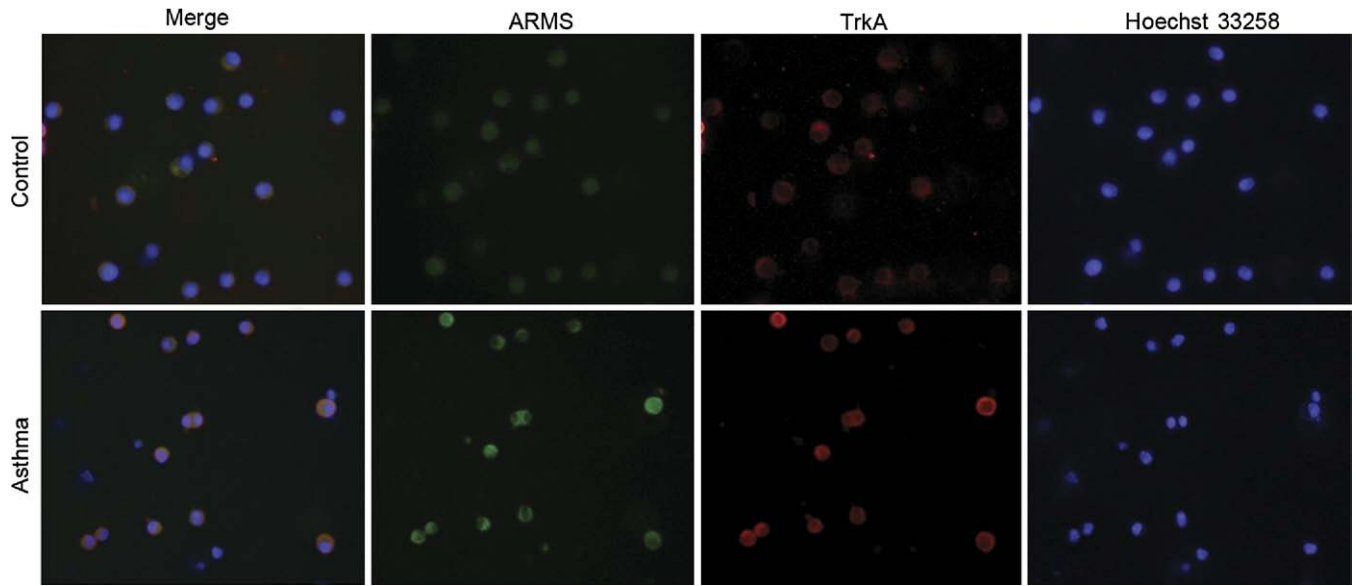


Figure 3. Co-localized immunofluorescence of tyrosine kinase receptor A (TrkA) and ankyrin-rich membrane spanning protein (ARMS) in cells of bronchoalveolar lavage fluid from control and asthmatic BALB/c mice. Cells were stained with anti-ARMS and anti-TrkA primary antibodies and with the corresponding secondary antibodies. Representative images ($n = 4$ experiments) are shown with ARMS staining (green), TrkA staining (red), nuclear staining by Hoechst 33258 (blue), and merged images. The green, red, and merged fluorescence were obviously more intense in cells of bronchoalveolar lavage fluid from asthmatic mice than from control mice (fluorescence microscopy, original magnification $\times 400$).

decreased with the administration of anti-NGF and anti-TrkA antibodies (Fig 8).

DISCUSSION

NGF acts as an important mediator in the neuroimmune mechanisms of asthma by regulating neuronal plasticity and

connecting neuroimmune mechanisms in immune cells and neurons, thereby mediating airway hyperresponsiveness. TrkA receptors, the high-affinity receptors for NGF, are involved in the hyperreactivity of afferent sensory nerve fibers, reflective broncho-obstruction to capsaicin, and airway inflammation.¹⁹ In the present study, NGF and TrkA

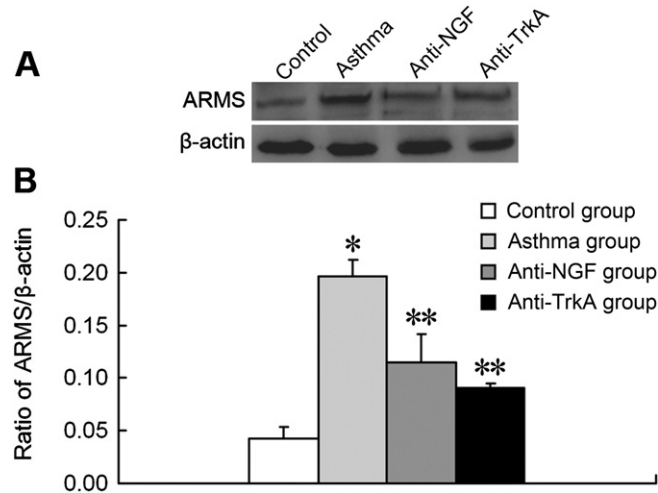


Figure 4. Mean expression of ankyrin-rich membrane spanning protein (ARMS) in lung tissues of BALB/c mice by means of Western blot. A, Expression of ARMS and β -actin as the control in asthmatic mice and in the anti-nerve growth factor (NGF) and anti-tyrosine kinase receptor A (TrkA) treatment groups. B, Optical density values for ARMS were relative to those for β -actin. * $P < .01$ compared with the control group. ** $P < .01$ compared with the asthma group. Error bars represent standard error of the mean ($n = 6$).

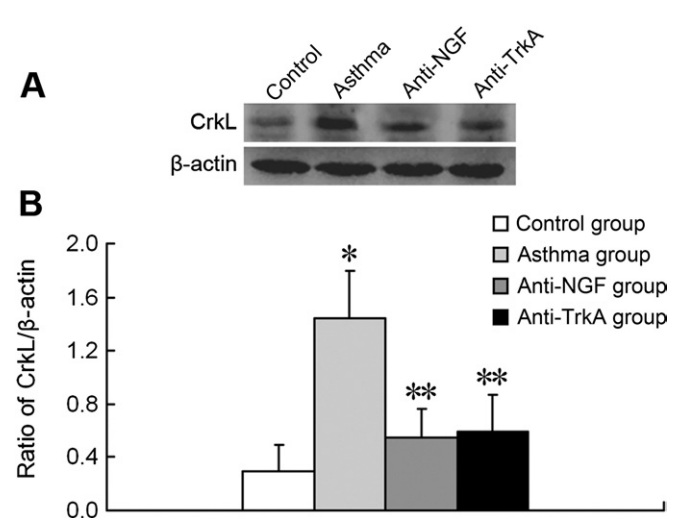


Figure 5. Mean expression of CrkL protein in lung tissues of BALB/c mice by means of Western blot. A, Expression of CrkL and β -actin in the control, asthmatic model, anti-nerve growth factor (NGF), and anti-tyrosine kinase receptor A (TrkA) groups. B, Optical density values for CrkL protein relative to β -actin. * $P < 0.05$ compared with the control group. ** $P < .05$ compared with the asthma group. Error bars represent standard error of the mean ($n = 6$).

expression in the lungs of asthmatic mice was significantly higher than that in the control group. Treatment with anti-NGF or anti-TrkA reduced the extent of inflammation and cellular infiltration in asthmatic mice. These results suggested that the NGF-TrkA pathway was activated in the asthma model. In addition, these results were consistent with those observed in human lung sections and with the levels of Trk receptor expression in various cells types in healthy individuals^{20,21} and asthmatic patients.²²

The ARMS, a novel transmembrane protein substrate of Trk receptors, is directly involved in the downstream signaling events for neurotrophins.⁷ The ARMS has been identified as an important molecule in the signaling pathway because it binds to NGF-TrkA and activates downstream enzymes.²³ Previously, an association between TrkA and ARMS was found to persist for many hours.^{7,24} The interaction between Trk and ARMS resulted in an increase in MAPK activation that persisted for hours. The ARMS also serves as a substrate for protein kinase D.²⁵ The specific tyrosine residue in ARMS allows the adaptor protein CrkL to engage C3G and activate Rap1. The constitutive interactions between Trk and ARMS and between ARMS and CrkL are critical for the initiation of Rap1-dependent signaling. Therefore, ARMS may serve as a scaffold for key adaptor proteins that are directly involved in neurotrophin signaling.²⁶ Increasing evidence suggests that ARMS is involved in a new mechanism that goes beyond the established models of neurotrophin signaling.^{27–29} However, whether ARMS is involved in asthma inflammation and its

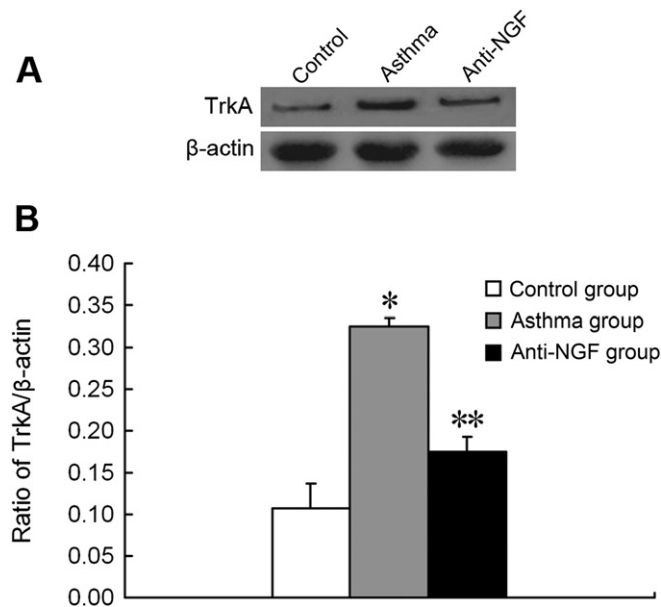


Figure 6. Mean expression of tyrosine kinase receptor A (TrkA) protein in the lung tissues of BALB/c mice by means of Western blot. A, Expression of TrkA and β -actin in the control, asthmatic model, and anti-nerve growth factor (NGF) groups. B, Optical density values for TrkA relative to β -actin. * $P < .01$ compared with the control group. ** $P < .01$ compared with the asthma group. Error bars represent standard error of the mean ($n = 6$).

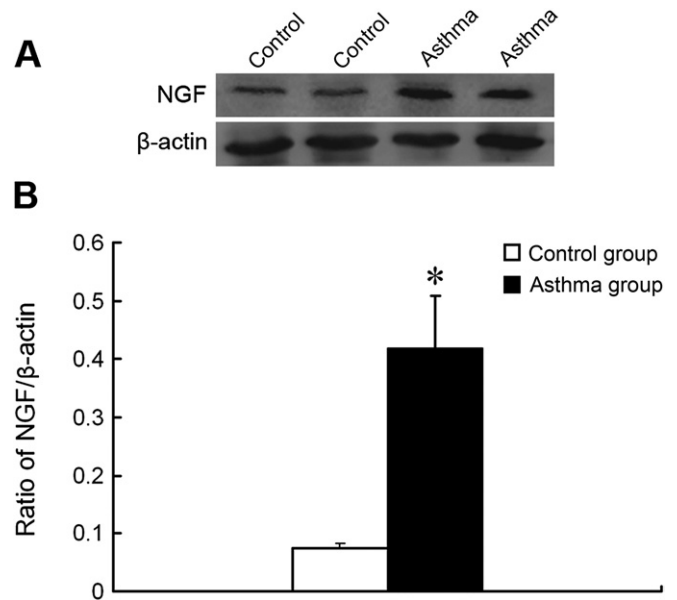


Figure 7. Mean expression of nerve growth factor (NGF) protein in the lung tissues of BALB/c mice by means of Western blot. A, Expression of NGF and β -actin in the control and asthmatic model groups. B, Optical density values for NGF relative to β -actin. * $P < .01$ compared with the control group. Error bars represent standard error of the mean ($n = 6$).

mechanisms have not yet been investigated. The present data show the co-localization of ARMS and TrkA in BALF cells of BALB/c mice by means of triple-labeled immunofluorescence staining, suggesting that the 2 proteins significantly co-expressed on the cell membrane. The immunofluorescence assay revealed enhanced ARMS and TrkA expression

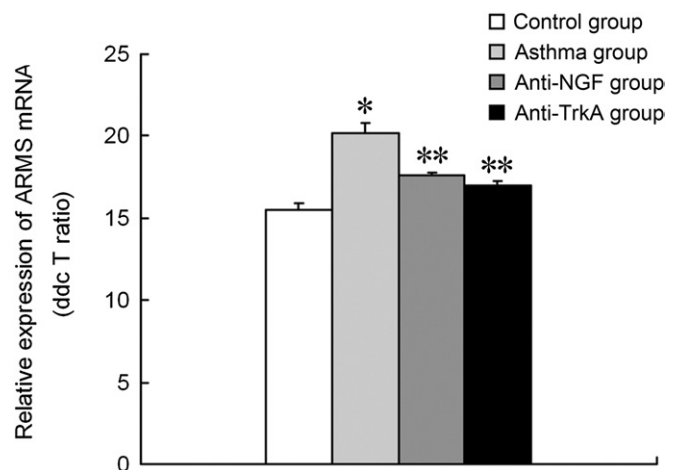


Figure 8. Quantification of mean ankyrin-rich membrane spanning protein (ARMS) messenger RNA (mRNA) expression in lung tissues by means of quantitative reverse transcription–polymerase chain reaction. * $P < .01$ compared with the control group. ** $P < .01$ compared with the asthma group. Error bars represent standard error of the mean ($n = 5$). NGF indicates nerve growth factor; and TrkA, tyrosine kinase receptor A.

in BALF cells of asthmatic mice compared with that of control mice. Moreover, ARMS, CrkL, TrkA, NGF protein, and ARMS mRNA levels in the lungs of sensitized mice were also significantly higher compared with those of control mice. Intervention with an anti-NGF or anti-TrkA antibody resulted in significant reductions in ARMS, CrkL, TrkA protein, and ARMS mRNA levels in the lungs of sensitized mice. These findings suggest that ARMS may participate in the pathogenesis of asthma with CrkL (adaptor protein of ARMS) through the NGF-TrkA signaling pathway. More detailed studies on the pathogenesis of asthma are required to elucidate the molecular mechanism by which Kidins220/ARMS plays a role and to gain a better understanding of the effect of ARMS on airway inflammation and responsiveness.

TrkA receptor signaling is currently thought to be mediated by 3 signaling pathways, which include p38 MAPK, PI3K/Akt, and phospholipase C γ .^{1,30,31} Through activation of its TrkA receptor, NGF plays a role in nerve differentiation and survival and in airway inflammatory diseases. The protective effect of NGF in neural stem cells against apoptotic stimuli is mediated by the PI3K/Akt and MAPK signaling pathway via Trk receptors.³² NGF is also an important factor in inflammatory or injury-induced hyperalgesia. The sensitizing effect of NGF on the capsaicin receptor TRPV1 is mediated by TrkA and requires its downstream PI3K and ERK pathways.³⁰ Moreover, NGF can enhance cough and airway obstruction via a mechanism that involves activation of the TrkA receptor and TRPV1 but not the p38 MAPK-dependent pathway.³¹ The ARMS facilitates the interaction between Trk receptors and CrkL and Rap-1 proteins, thereby promoting neurotrophin-dependent activation of MAPK.³³ PKD and Kinesin-1 are necessary for the role of Kidins220/ARMS mediated by NGF.³⁴ The ARMS might promote nuclear factor- κ B through a MAPK mechanism.³⁵ Nuclear factor- κ B may be a therapeutic target in the inflammatory aspects of asthma. In turn, recent studies have demonstrated that the activation of ARMS is mainly realized by the MAPK signaling pathway.³⁶ Further study on the effect of NGF-TrkA on the expression of ARMS may shed new light on the neuro-pathophysiologic mechanisms of asthma. The ARMS might, therefore, be evaluated as a potential therapeutic approach in the animal model of asthma and in airway-allergic diseases in humans.

In conclusion, we demonstrated the local upregulation of Kidins220/ARMS in allergic inflammation. These data support the hypothesis that the signaling complex Kidins220/ARMS is expressed not only in PC12 cells but also in the lung tissues of mice, and the signaling complex may play a role through the NGF-TrkA pathway to initiate airway inflammation and hyperresponsiveness in BALB/c mice.

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