

Anti-tumor necrosis factor- α treatment reduces allergic responses in an allergic rhinitis mouse model

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Abstract

Background: Tumor necrosis factor (TNF)- α is a principal mediator of the acute inflammatory response, including allergic rhinitis. TNF- α inhibitors are widely used for the treatment of inflammatory conditions such as rheumatoid arthritis and inflammatory bowel diseases; however, the effects of TNF- α inhibitors on allergic rhinitis are not well established. We aimed to investigate the effects of infliximab, a TNF- α inhibitor, on allergic rhinitis in a mouse model.

Methods: BALB/c mice were sensitized with ovalbumin (OVA) and alum, and challenged intranasally with OVA. The TNF- α inhibitor, infliximab was administered intraperitoneally, and multiple parameters of allergic responses were evaluated to determine the effects of infliximab.

Results: Infliximab reduced allergic symptoms and eosinophilic infiltration into the nasal mucosa. It also suppressed total and OVA-specific IgE levels, and inhibited local Th2 cytokine transcription in the nasal mucosa and systemic Th2 cytokine production by splenocytes. Furthermore, the expression of E-selectin, neither intercellular adhesion molecule 1 (ICAM-1) nor vascular cell adhesion molecule 1 (VCAM-1), in the nasal mucosa was suppressed in the infliximab-treated group when compared to the nontreated group.

Conclusion: This study shows that the TNF- α inhibitor infliximab induces anti-allergic effects by decreasing local and systemic Th2 cytokine (IL-4) production, total and OVA-specific IgE levels, adhesion molecule (E-selectin) expression, and eosinophil infiltration into the nasal mucosa in an allergic rhinitis model. Therefore, infliximab should be considered as a potential agent in treating allergic rhinitis.

Tumor necrosis factor (TNF)- α , a principal mediator of the acute inflammatory response against various microbes, is responsible for many of the systemic complications associated with severe infections. The major source of TNF- α is activated mononuclear phagocytes. T cells, natural killer (NK) cells, and mast cells can also secrete this protein. TNF- α is also known to play an important role in allergic inflammation and is required for both the production of Th2 cytokines and the migration of Th2 cells to the sites of allergic inflammation (1, 2).

Abbreviations

ICAM-1, Intercellular adhesion molecule 1; IFN- γ , Interferon-gamma; OVA, Ovalbumin; TNF- α , Tumor necrosis factor- α ; VCAM-1, Vascular cell adhesion molecule 1.

Mast cells and macrophages release TNF- α through IgE-dependent mechanisms in allergic disease. Usually, IgE is produced in an IL-4-dependent manner, and TNF- α has been demonstrated to increase the effects of IL-4 on IgE production. In addition, in TNF- α knockout mice, the production of specific IgE and the expression of Th2 cytokines, endothelial-leukocyte adhesion molecule 1, and vascular cell adhesion molecule 1 are severely impaired, and the induction of allergic rhinitis is inhibited, thus demonstrating an indispensable role of TNF- α in allergic rhinitis.

TNF- α inhibitors are used clinically in the treatment of inflammatory conditions such as rheumatoid arthritis and inflammatory bowel diseases (3). Three kinds of TNF- α inhibitors are commercially available and used in humans: adalimumab, etanercept, and infliximab. Although the relationship between allergic inflammation and TNF- α is becoming more

accepted, the application of TNF- α inhibitors to the treatment of allergic rhinitis has not been previously studied.

In this study, we used a TNF- α inhibitor, infliximab, to evaluate its effects on allergic rhinitis and to elucidate the underlying mechanisms regulating its anti-allergic effects in a mouse model.

Methods

Animals

Four-week-old female BALB/c mice were obtained from Korea Biolink Co. (Eumsung, Korea). All animal experiments conducted in this study followed the guidelines and ethics of Institutional Animal Care at the Clinical Research Institute of Seoul National University Hospital.

Murine allergic rhinitis model and treatment

Mice were divided into four groups and each group consisted of ten mice (group A: negative control group, group B: positive control group, group C: split treatment group, group D: single treatment group; Fig. 1, Table 1). The procedures for allergen sensitization and treatment are summarized in Fig. 1. Briefly, mice were sensitized with an intraperitoneal injection of 25 μ g of ovalbumin (OVA; grade V; Sigma, St. Louis, MO, USA) and 1 mg of aluminum hydroxide gel on days 0, 7, and 14. After general sensitization, mice were locally challenged with 500 μ g of OVA into their nostrils from day 21 to day 27. Along with sensitization and challenge, selected groups of mice were treated with an intraperitoneal injection of the TNF- α inhibitor infliximab (group C: 20 μ g/day for 1 week, group D: 1 mg of single injection) 3 h before intranasal OVA challenge.

Symptom score and tissue preparation

Fifteen minutes after final OVA challenge on day 27, a blinded observer recorded the frequencies of sneezing and

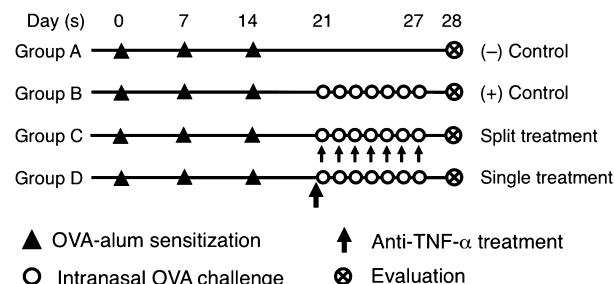


Figure 1 Experimental protocol. BALB/c mice were sensitized with ovalbumin (OVA) and 1 mg of aluminum hydroxide gel (Alum) on days 0, 7, and 14 (general sensitization). All groups except for group A received intranasal OVA from day 21 to day 27. In addition to sensitization and challenge, selected groups of mice were treated with intraperitoneal injections of the Tumor necrosis factor- α inhibitor, infliximab (group C: 20 μ g/day for 1 week, group D: 1 mg of single injection), 3 h before intranasal OVA challenge.

nasal rubbing over a 15-min interval. Mice were then killed 24 h after the last OVA challenge. After perfusion with 4% paraformaldehyde, the heads of five mice from each group were removed *en bloc* and then fixed in 4% paraformaldehyde. After exposing the nasal cavity out of the head of the other five mice, the nasal mucosa was taken out meticulously using a small curette. The samples of the same group were mixed together, because the nasal mucosa in each mouse we can obtain was too small. Nasal mucosa was immediately immersed in liquid nitrogen and stored at -70°C until use for reverse transcription-polymerase chain reaction (RT-PCR) and Western blot.

Eosinophils in the nasal septal mucosa

For evaluation of nasal histology, nasal tissues were decalcified, embedded in paraffin, and sectioned coronally (4 μ m thick) approximately 5 mm from the nasal vestibule. Each section was stained with hematoxylin and eosin, and the number of eosinophils on both sides of the septal mucosa was counted. The number of eosinophils in the submucosal area of the whole nasal septum was counted under a light microscope ($\times 400$ magnification).

Real-time RT-PCR for IL-4, IL-10, and IFN- γ in the nasal mucosa

Total RNA was prepared from the nasal mucosa with TriZol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using Superscript reverse transcriptase (Invitrogen) and oligo(dT) primers (Fermentas, Burlington, ON, Canada). For analysis of IL-4 (Mm00445258_g1), IL-10 (Mm00439616_m1), IFN- γ (Mm99999071_m1), and GAPDH (Mm03302249_g1), PDAR (Pre-Developed Assay Reagent) kits of primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). Amplification of IL-4, IL-10, IFN- γ , and GAPDH cDNA was carried out in MicroAmp optical 96-well reaction plates (Applied Biosystems). The reaction was performed using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The average transcript levels of genes were then normalized to GAPDH.

Measurement of cytokines (IL-4, IL-5, IL-10, and IFN- γ) in the spleen cell culture

Spleen single-cell suspensions were plated in 96-well tissue culture plates at a final concentration of 10^5 cells/ml using AIM-V media (Gibco, Grand Island, NY, USA). The cells were incubated in a CO_2 incubator at 37°C for 72 h, stimulated with OVA for 72 h, and stored at -70°C until cytokines were measured. Cytokines were assayed in culture supernatant using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. After measuring the optical density (OD) at 450 nm, the concentrations of IL-4, IL-5, IL-10, and IFN- γ were determined by interpolation from a standard curve, and all data were expressed as nanograms per milliliter.

Table 1 Experimental groups and characteristics

Group (n)		Systemic sensitization	Local sensitization	Treatment
A (5)	(-) control	PBS	PBS	50 μ l of PBS
B (5)	(+) control	25 μ g OVA	500 μ g OVA	50 μ l of PBS
C (5)	Split therapy group	25 μ g OVA	500 μ g OVA	20 μ g i.p. for 1 week
D (5)	Single therapy group	25 μ g OVA	500 μ g OVA	1 mg single i.p.

PBS, Phosphate-buffered saline; OVA, Ovalbumin.

Serum levels of total and OVA-specific immunoglobulin (Ig) E

Serum levels of total and OVA-specific IgE were measured by solid-phase ELISA. Serum samples collected from mice at the time of death were serially diluted and added to 96-well plates coated with purified anti-mouse IgE mAb (clone R35-72; BD Pharmingen, San Jose, CA, USA). A purified mouse IgE isotype (27-74; BD Pharmingen) was used as a standard for total IgE. Nonspecific antigen-antibody reactions were blocked with 3% bovine serum albumin. To detect total IgE, HRP-conjugated anti-mouse IgE (23G3; Southern Biotechnology, Birmingham, AL, USA) was added to the plate. To detect OVA-specific IgE, biotin-labeled OVA was added, followed by HRP-labeled anti-biotin (Vector Laboratories, Burlingame, CA, USA). The reactions were developed using 3,3',5,5'-tetramethylbenzidine (Moss Inc., Belfast, ME, USA) and terminated by adding 2 N H₂SO₄. The OD was recorded by a luminometer (iEMS Reader; Labsystems, Helsinki, Finland) set at 450 nm. The endpoint titers of OVA-specific IgE are expressed as the reciprocal log₂ of the last dilution of a sample that resulted in an OD value that was 0.1 higher than background.

Western blot for E-selectin, ICAM-1, and VCAM-1 in the nasal mucosa

Protein was obtained from the nasal mucosa of each mouse 24 h after the final nasal challenge using lysing buffer (0.5% Triton X-100, 150 mM NaCl, 15 mM Tris (pH 7.4) 1 mM CaCl₂, 1 mM MgCl₂). Protein concentrations were determined using BCA protein assay reagent (Thermo Fisher Scientific, Waltham, MA, USA). Samples (95 μ g protein per lane) were separated on 8% to 16% Tris-Glycine mini gels (NOVEX, San Diego, CA, USA) and transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA). E-selectin, ICAM-1, VCAM-1, and actin were immunoblotted with a primary chicken polyclonal anti-E-selectin Ab (R&D Systems), primary goat polyclonal anti-ICAM-1 Ab (R&D Systems), primary goat polyclonal anti-VCAM-1 Ab (R&D Systems), and anti-actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The membrane was then immunoblotted with a secondary anti-chicken IgY-HRP, secondary anti-goat IgG-HRP, or secondary anti-mouse IgG-HRP (Santa Cruz Biotechnology). The blots were visualized using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific). Quantification of Western blots was performed

using the ImageJ software from the National Institutes of Health.

Expression of TNF- α in the nasal mucosa

The TNF- α gene and protein expression levels were measured by real-time PCR and Western blot analyses, respectively, as described earlier. For real-time PCR analysis of TNF- α (Mm 00443259-g1), primer and probe were purchased from Applied Biosystems. TNF- α was immunoblotted with an anti-TNF- α Ab (Millipore, Billerica, MA, USA).

Statistical analysis

Results are shown as the mean $\pm$ SEM. A Mann-Whitney *U*-test was used to compare results between negative and positive controls, and treatment groups and positive control. A *P*-value < 0.05 was considered statistically significant.

Results

Symptom score (Fig. 2)

The nasal rubbing and sneezing symptom scores in the positive control group (group B) were 4.4 ± 1.28 and 5.8 ± 1.68 , respectively, and were significantly elevated when compared to those in the negative control group (group A). The nasal rubbing symptom score was significantly lower in both treatment groups (group C and D) when compared to the positive control group (*P* < 0.05), and group D showed a greater reduction in the nasal rubbing score compared to group C. The nasal sneezing symptom score significantly decreased in group D, but not in group C, when compared to the positive control group.

Histology of nasal mucosa and eosinophil infiltration (Fig. 3)

Eosinophil number in the positive control group was 35.4 ± 4.87 , which was significantly different from that of the negative control group (1.0 ± 0.5 , *P* = 0.079). Eosinophil counts in groups C and D were 38.8 ± 5.89 and 9.6 ± 64.43 , respectively, and were significantly reduced in group D (*P* = 0.016), but not in group C, when compared to the positive control (group B). These results show that the TNF- α inhibitor decreased eosinophil migration in the nasal mucosa. Fig. 3B shows histology from each group. This figure demonstrates decreased eosinophil infiltration in group D

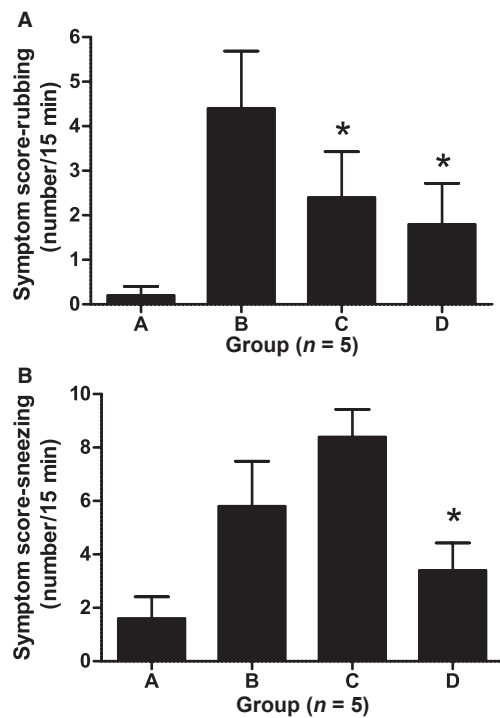


Figure 2 Systemic treatment of a Tumor necrosis factor- α inhibitor suppressed allergic symptoms. (A) Rubbing symptom score. (B) Sneezing symptom score. * $P < 0.05$.

and decreased epithelial layer disruption in groups C and D when compared to group B.

Cytokines in nasal mucosa and splenocytes culture (Fig. 4)

mRNA expression of the Th2 cytokine, IL-4, significantly decreased in the nasal mucosa of treatment groups C and D (Fig. 4A). Single treatment group D showed a greater decrease in IL-4 than split treatment group C, which is consistent with the observed symptom scores and histology. For

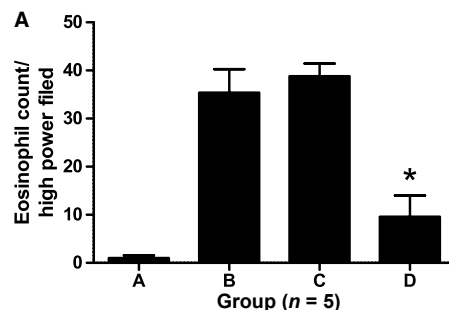


Figure 3 Systemic treatment of Tumor necrosis factor- α inhibitor suppressed eosinophil infiltration in the nasal mucosa ($\times 400$ magnification). (A) Eosinophil counts in the nasal mucosa. Eosinophil count was significantly reduced in treatment group D when com-

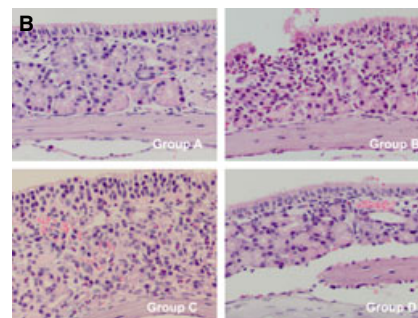
pared to the positive control (group B). (B) Histological findings of the nasal mucosa in each group. Eosinophil infiltration markedly decreased in treatment group D when compared to the positive control (group B). * $P < 0.05$.

Total and OVA-specific serum IgE levels (Fig. 5)

We also evaluated the effects of the TNF- α inhibitor, infliximab, on humoral immunity. Total IgE levels significantly decreased in both treatment groups C and D ($P < 0.05$) and OVA-specific IgE levels also decreased in both treatment groups, but significantly only in group D when compared to the positive control group B ($P < 0.05$). These results might suggest that infliximab reduces the IgE isotype switch in an allergic rhinitis mouse model. The single treatment group showed a greater decrease than the split treatment group for both total and OVA-specific IgE levels.

E-selectin, ICAM-1, and VCAM-1 expression in the nasal mucosa (Fig. 6)

Adhesion molecules are necessary for the migration of effector cells into tissues. To evaluate the mechanisms underlying the decrease in eosinophil infiltration by infliximab, the expression of adhesion molecules such as E-selectin, ICAM-1, and VCAM-1 was measured in the nasal mucosa of each group using an immunoblot. The expression of E-selectin significantly decreased in treatment groups C and D ($P < 0.05$, Fig. 6A,B). In contrast, the expression of ICAM-1 and VCAM-1 was not induced in positive control group B and was not affected significantly in treatment groups



pared to the positive control (group B). (B) Histological findings of the nasal mucosa in each group. Eosinophil infiltration markedly decreased in treatment group D when compared to the positive control (group B). * $P < 0.05$.

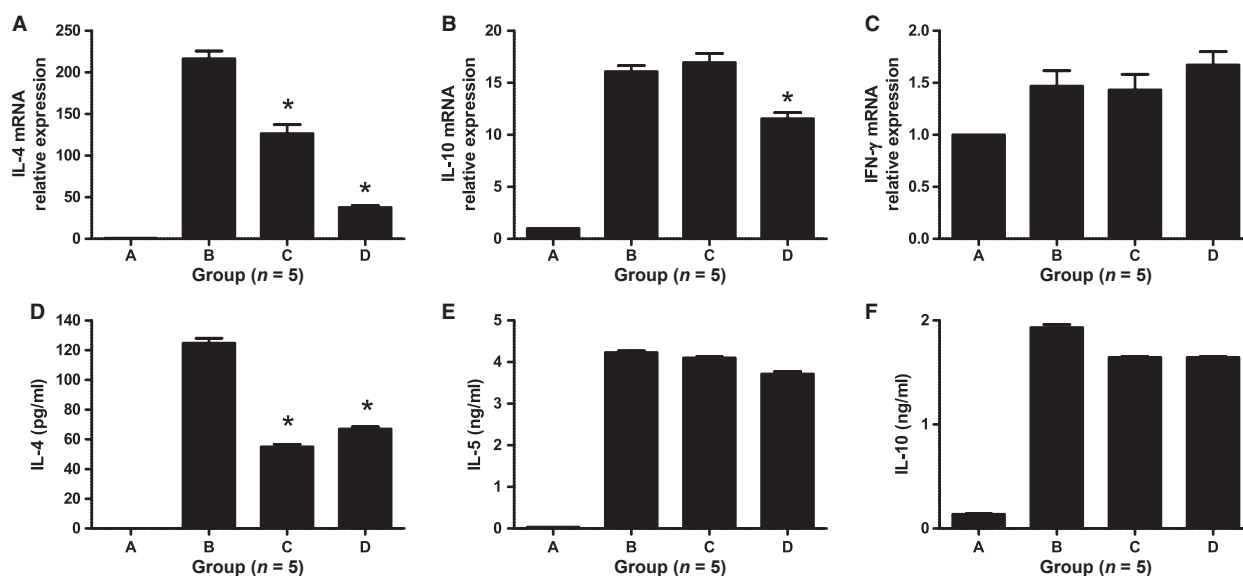


Figure 4 Local and systemic Th2 cytokines decreased after Tumor necrosis factor- α inhibitor administration. (A–C) IL-4 (A), IL-10 (B), and IFN- γ (C) mRNA expression levels in the nasal mucosa. Transcriptional activity of IL-4 was significantly reduced in treatment

groups C and D and that of IL-10 was significantly decreased in group D. D–F, in splenocyte culture, IL-4 (D) cytokine levels were significantly suppressed after treatment; however, decreases in IL-5 (E) and IL-10 (F) were not as significant as in IL-4. * $P < 0.05$.

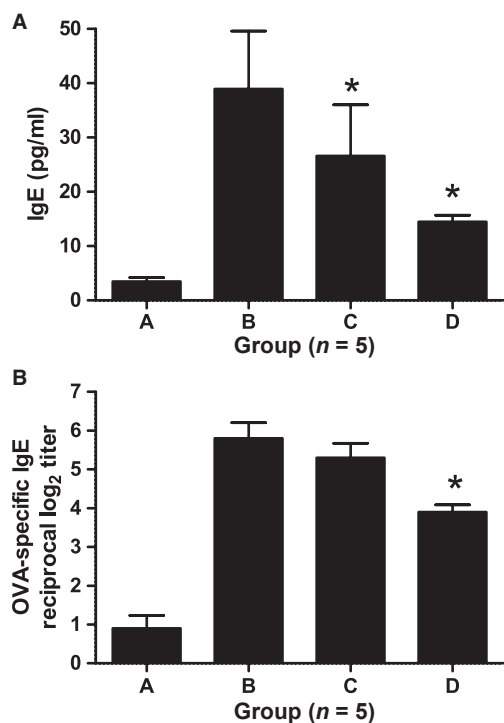


Figure 5 Systemic treatment with infliximab suppressed total and ovalbumin (OVA)-specific Immunoglobulin (Ig) E levels. (A) Serum total IgE levels. (B) Serum OVA-specific IgE levels. * $P < 0.05$.

(Fig. 6A,C,D). Collectively, these results show decreased expression of E-selectin on epithelial cells of the nasal mucosa.

Expression of TNF- α in the nasal mucosa (Fig. 7)

We evaluated the level of TNF- α transcriptional activity in the nasal mucosa of each group (Fig. 7A,B). Transcriptional levels of TNF- α significantly decreased in both treatment groups C and D when compared to the positive control group ($P < 0.05$). The protein level of TNF- α in the nasal mucosa also significantly decreased in treatment groups C and D ($P < 0.05$, Fig. 7C,D).

Discussion

The present study clarified the effects of the TNF- α inhibitor, infliximab, on allergic rhinitis in a mouse model and demonstrated an important role for TNF- α in Th2 responses during allergic rhinitis. Infliximab decreased the allergic symptom score, and this anti-allergic effect could be associated with not only suppression of Th2 cytokines and total and OVA-specific IgE levels but also decreased infiltration of eosinophil in the nasal mucosa.

Clinically available TNF- α inhibitors are often monoclonal antibodies against TNF- α . These types of inhibitors became commercialized in 1999 and have been used in the treatment of rheumatoid arthritis, psoriasis, and Crohn's disease. Infliximab is a chimeric monoclonal antibody against TNF- α that binds to both the soluble and transmembrane forms of TNF- α and antagonizes the proinflammatory actions of TNF- α (4). Although TNF- α inhibitors were also tested empirically in the treatment of severe bronchial asthma, their clinical use in allergic disease has not been established yet (5–7), and a few preclinical studies on allergic diseases have been published.

A few preclinical studies have shown the anti-allergic effect of TNF- α antibody in acute asthma model (8, 9). Deveci

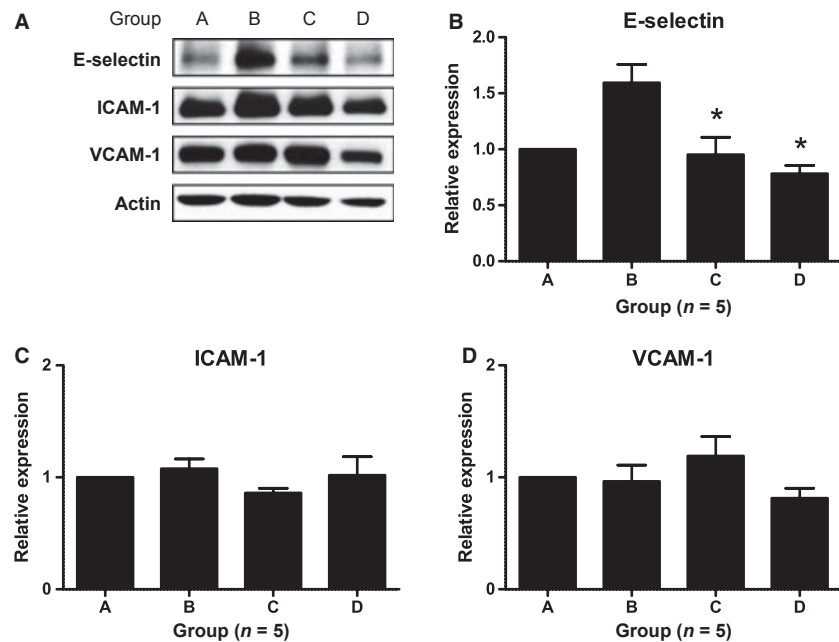


Figure 6 Systemic treatment of Tumor necrosis factor- α inhibitor significantly reduced the expression of E-selectin, but neither ICAM-1 nor VCAM-1 in the nasal mucosa. (A) Immunoblot of adhe-

sion molecules. (B–D) Quantitative expression levels of E-selectin (B), ICAM-1 (C), and VCAM-1 (D) in the nasal mucosa. * $P < 0.05$.

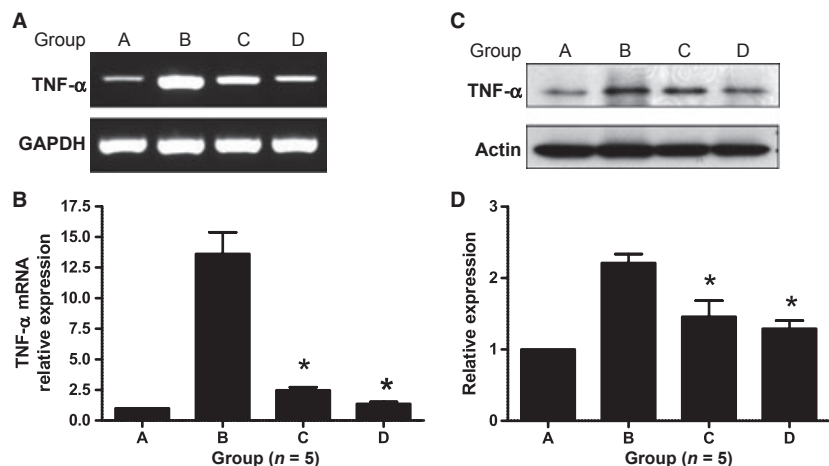


Figure 7 Expression of Tumor necrosis factor (TNF)- α in the nasal mucosa. (A, B) Transcriptional levels of TNF- α significantly decreased in groups C and D when compared to the positive con-

trol (group B). (C, D) The protein level of TNF- α in the nasal mucosa also significantly decreased in both treatment groups (group C and D) when compared to the positive control. * $P < 0.05$.

et al. (8) reported that infliximab exerted anti-inflammatory effects in a mouse model of OVA-induced acute asthma and showed that RANTES, IL-4, GM-CSF, TNF- α , IL-6, and MIP-2 levels in lung tissue were significantly decreased. Kim et al. (9) also showed anti-allergic effect of anti-TNF- α antibody in a murine asthma model induced by house dust, suggesting decreased level of eotaxin as a key mechanism for its anti-allergic effect. The role of TNF- α in a murine model of OVA-sensitized allergic rhinitis was also investigated using

TNF- α knockout mice (10). They showed that TNF- α is necessary for antigen-specific IgE production and for the induction of Th2-type cytokines and chemokines. Broide et al. (11) also reported that TNF receptor p55/p75-deficient and TNF receptor p55-deficient mice had reduced eosinophil infiltration after OVA challenge, proving the role of TNF- α in allergic inflammation.

However, some studies showed contradictory results (12, 13). A study using TNF- α receptor knockout mice showed

that knockout mice had equal or accentuated allergic responses than control mice (12). Another study using TNF- α knockout mice reported that TNF- α knockout mice had increased airway hyper-responsiveness, eosinophil infiltration, and levels of IL-5 and IL-10, showing conflicting results with above-mentioned studies (13). The discrepancy between the two TNF- α knockout mice studies could not be explained clearly and needs further clarification. However, most of recent publications showed pro-inflammatory effect of TNF- α or anti-inflammatory effect of TNF- α antibody.

In this study, we used two types of treatment modalities, multiple small-dose injection (20 μ g i.p. for 1 week, group C) and single large-dose injection (1 mg i.p. single, group D), and compared the efficacy of both modalities using several parameters. Generally, single bolus therapy showed better efficacy than multiple split therapy for almost every parameter. All the parameters were consistent with anti-allergic effect of infliximab in a single bolus treatment group, however, in group C, although TNF- α mRNA and protein expressions, local and systemic IL-4 levels, and total IgE level decreased, eosinophil infiltration and OVA-specific IgE level did not decrease when compared to those in positive control group. In the study of infliximab in acute asthma model, they also divided treatment groups into low-dose and high-dose groups and demonstrated decreased allergic inflammations even in a low-dose group (8). They used total dose of 0.5 mg/mouse of infliximab in a low-dose group and 1.25 mg/mouse in a high-dose group, which are much higher than total doses in our study. We could assume that the dose administered in split treatment group in our study might be not enough for inhibiting all allergic immune responses such as eosinophil recruitment and OVA-specific IgE isotype switch. In addition, the elimination half-life for infliximab is known to be about 9.0–12.3 days based on human studies (14). The total dosage administered might be more important than the duration of administration.

Infliximab significantly decreased mRNA expression of the Th2 cytokine IL-4 in the nasal mucosa (Fig. 4A); however, the effect on IL-10 was significant only in a single large-dose group (Fig. 4B). In addition to local Th2 cytokine mRNA expression, infliximab also suppressed systemic IL-4 production (Fig. 4D), as shown in splenocyte cultures with OVA incubation. IL-4 is well known to be an important cytokine not only for the Th2 phenotype but also for IgE production

(15, 16). These findings are consistent with our data in that infliximab inhibited IL-4 secretion, resulting in a decreased release of total and OVA-specific serum IgEs (Fig. 5). Although a local decrease occurred in IL-10 secretion, systemic IL-10 was not affected by infliximab treatment (Fig. 4B,F). IL-10 is an inhibitory cytokine of inflammation, and it was first identified as a Th2 cytokine and later revealed to be produced by Th1, Th2, Th17, and regulatory T cells (17). Considering that IL-10 mRNA expression was completely abolished in the nasal mucosa of TNF- α knockout mice (10), we could assume that TNF- α inhibitors might not completely abolish TNF- α activity.

Adhesion molecules, such as E-selectin, ICAM-1, and VCAM-1, are important for eosinophilic infiltration. In this study, the expression levels of ICAM-1 and VCAM-1 did not decrease in both treatment groups (group C and D) when compared to positive control group. Only E-selectin was markedly induced in positive control (group B) and significantly reduced in both treatment groups. TNF- α has been reported to induce the expression of E-selectin, ICAM-1, and VCAM-1, and the expression of such adhesion molecules in TNF- α knockout mice is reduced (10, 18). The observed decreased expression of E-selectin might suggest that inhibition of TNF- α prevents adhesion of eosinophils on endothelial cells, thus inhibiting eosinophil infiltration in the nasal mucosa.

In summary, the TNF- α inhibitor infliximab showed inhibitory effects on symptoms and other allergic parameters in a mouse model of allergic rhinitis. Infliximab inhibited Th2 cytokine release from T cells and total and OVA-specific IgEs secretion from B cells. Additionally, infliximab downregulated the expression of adhesion molecule, E-selectin, resulting in decreased effector cell migration in the nasal mucosa. These multiple effects might synergize to reduce allergic symptoms. Thus, TNF- α inhibitors should be considered as a treatment regimen for allergic rhinitis.

Acknowledgments

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Disclosure of potential conflict of interest

The authors declare that they have no conflicts of interest.

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