A Novel Human Anti-VCAM-1 Monoclonal Antibody Ameliorates Airway Inflammation and Remodeling in Murine Asthma Model

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Directed by Professor Chein-Soo Hong

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Jae-Hyun Lee

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the Doctoral Dissertation of
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Here, I dedicate this thesis to my precious family.

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ABSTRACT

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(Directed by Professor Chein-Soo Hong)

Background: Asthma is a chronic inflammatory disease induced by Type 2 helper T cells (Th2) and eosinophils. Vascular cell adhesion molecule-1 (VCAM-1) is the regulatory receptor implicated with recruiting eosinophils and lymphocytes to pathologic site in asthma. A monoclonal antibody (mAb) against VCAM-1 may attenuate allergic inflammation and pathophysiologic features of asthma.

Objective: To evaluate whether a recently developed human anti-VCAM-1 mAb can inhibit pathophysiologic features of asthma in a murine asthma model induced by ovalbumin (OVA).

Methods: We evaluated whether human anti-VCAM-1 mAb binds to human or mouse VCAM-1. Leukocyte adhesion inhibition assay was performed to evaluate the *in vitro* blocking activity of human anti-VCAM-1 mAb. OVA sensitized BALB/c mice were treated with human anti-VCAM-1 mAb or isotype control Ab before intranasal OVA challenge. We evaluated airway hyperresponsiveness (AHR) and cell counts in bronchoalveolar lavage (BAL) fluid, measured inflammatory cytokines, and examined histopathological features, including VCAM-1 immunohistochemistry.

Results: The human anti-VCAM-1 mAb bound to human and mouse VCAM-1 molecules and inhibited adhesion of human leukocytes *in vitro*. AHR and inflammatory cell counts in BAL fluid were reduced in mice treated with human anti-VCAM-1 mAb as compared to a control Ab. The levels of interleukin (IL)-5 and IL-13, and transforming growth factor-β in lung tissue were decreased in treated mice. Human anit-VCAM-1 mAb reduced goblet cell hyperplasia and peribronchial fibrosis. *In vivo* VCAM-1 expression decreased in treated group.

Conclusion: Human anti-VCAM-1 mAb can attenuate allergic inflammation and pathophysiological features of asthma in OVA induced murine asthma model. This data suggest that human anti-VCAM-1 mAb could be an additional anti-asthma therapeutic medicine.

Key words: VCAM-1, monoclonal antibody, allergic inflammation, asthma, cell adhesion molecule, anti-inflammatory effect

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I. INTRODUCTION

Asthma is a chronic respiratory disease and its prevalence is increasing rapidly in worldwide. The characteristic pathophysiological features of asthma are chronic eosinophilic inflammation of airways, airway hyperresponsiveness (AHR) and reversible airflow obstruction.[1] Many cytokines play important roles in developing chronic allergic inflammation of airway. Interleukin (IL)-4, IL-5 and IL-13 (the Th2 cytokines) are key mediators.[2] Especially IL-5 can recruit eosinophils to inflamed site and prolong their life span. Monitoring the number of eosinophils in induced sputa of asthmatics may be clinically relevant to diagnose and monitor the response to treatment.[3,4]

The first step in recruiting inflammatory cells in asthma, is cell adhesion to the vascular endothelial surface. Various cell adhesion molecules play critical roles in leukocytes contact endothelial cells. Vascular cell adhesion molecule-1 (VCAM-1), known as CD106, is the ligand of very late antigen-4 (VLA-4), which is expressed on the plasma membrane surface of eosinophils. Thus, VCAM-1 can have an important role in the development of eosinophilic inflammation.[5] Adhesion molecules, including intercellular cell adhesion molecule-1 (ICAM-1) and VCAM-1, are primarily expressed on the surface of vascular endothelial cells. Once inflammation begins, their extracellular expression increases in endothelial cells by inflammatory mediators.[6]

Allergic airway diseases such as asthma and allergic rhinitis are characterized by Th2 inflammation. IL-4 and IL-13 potentiate VCAM-1 expression in vascular endothelial cells, accelerating eosinophilic inflammation.[7,8] In regulating VCAM-1 expression, nuclear factor-kappaB (NF-κB) is important and can be restricted by Poly [ADP-ribose] polymerase 1 (PARP-1).[9] Medications that inhibit cysteinyl leukotriene-1 receptor such as montelukast can affect eosinophils adhering to VCAM-1.[10]

In ovalbumin (OVA) induced murine model of acute asthma, systemically administrated rat anti-murine VCAM-1 antibody (Ab) and rat anti-murine VLA-4 Ab could reduce eosinophil infiltration into lung tissue.[11] Thus, VCAM-1 could be a novel therapeutic target for several diseases characterized by eosinophilic inflammation such as asthma, allergic rhinitis and eosinophilic bronchitis. In atopic dermatitis mouse model, VCAM-1 blockade could delayed disease onset and its severity.[12] As well as these allergic diseases, left ventricular remodeling after various heart diseases also associated with VCAM-1 expression and its blockade could be important to reduce the myocardial fibrosis.[13]

Inhaled corticosteroids as a potent anti-inflammatory drug have been established as primary treatment for persistent allergic asthma. Recently, several biologic agents, including anti-immunoglobulin E (IgE) monoclonal Ab (mAb),[14] anti-IL-13 mAb,[15] and anti-IL-5 mAb[16] have been developed for difficult-to-treat or severe asthma. One potential pitfall of these biologic agents is safety issue. Therefore, human or humanized isoform antibodies should minimize unexpected auto-immune reactions in human body.

In this study, we tested a novel monoclonal antibody designed to bind human VCAM-1 molecule whether this antibody attenuated the allergic inflammation and ameliorated the pathophysiologic features of asthma in an OVA induced murine model.

II. MATERIALS AND METHODS

Human anti-VCAM-1 mAb

We used human anti-VCAM-1 mAb (HD101) Hanwha Chemical, Daegeon, Korea) that bound both human and mouse VCAM-1. HD101 designed to bind to domain 1 and 2 of VCAM-1 specifically. It has an immunoglobulin G4 (IgG4) backbone (molecular weight 150 kD). Thus, it has low antibody -dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity.

Cross-reactivity assay

A 96-well plate was coated with a recombinant human VCAM-1/Fc (862-VC, R&D systems, Minneapolis, MN, USA) or mouse VCAM-1/Fc (643-VM, R&D systems, MN, USA) at 4°C for overnight. The plate was washed with phosphate buffered saline (PBS) and blocked with 1% bovine serum albumin (BSA) in PBS at 37°C for 2 hours. Human anti-VCAM-1 mAb was added at 37°C for 2 hours. The binding affinity of human anti-VCAM-1 mAb to coated VCAM-1 molecule was observed with horseradish peroxidase (HRP)-conjugated anti-F(ab')$_2$ Ab using 3,3,5,5-tetramethylbenzidine (TMB) colorigenic substrate.

Adhesion inhibition assays for recombinant VCAM-1

Each well of a 96-well plate (446612, Nunc, Roskilde, Denmark) was coated with 100 μL of recombinant human VCAM-1 (2 μg/mL for U937 and $CD4^+$ T cell assay, 5 μg/mL for EoL-1 cell assay, 809-VR, R&D systems, MN, USA) at 4°C for 16 hours. The plate was blocked with 1% BSA in PBS for 2 hours at room temperature (RT). Then, human anti-VCAM-1 mAb was added to the VCAM-1-coated wells for antigen binding for 1 hour at RT. Meanwhile, human leukocytes – U937 cell (CRL-1593.2, ATCC, Manassas, VA, USA), EoL-1 cell (94042252, ECACC, Salisbury, UK) or $CD4^+$T cell (isolated from human

peripheral blood mononuclear cells, CC-2702, Lonza, Basel, Swiss) were stained with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (C34554, Invitrogen, Carlsbad, CA, USA). Fluorescence labeled cells were incubated at 37°C for 15 or 30 minutes to allow cell to interact with coated recombinant VCAM-1. Non-adherent cells were removed by centrifuging the sealed plate inverted at 200g for 5 minutes, and 150 μL of cell lysis buffer (50 mM Tris-HCl, pH 8.5, 0.1% SDS) was added to each well for 10 minutes to lyse the bound cells. Subsequently, fluorescence intensity was measured at 485 to 530 nm emission wavelength using a fluorometer (GeminiX, Molecular Devices, Sunnyvale, CA, USA). Values are the means of triplicate measurements for each condition. Finally, the reduction of fluorescence intensity in samples was analyzed as the inhibition percent compared to the isotype antibody-treated group.

Adhesion inhibition assays using HUVEC expressing VCAM-1

Human umbilical vein endothelial cells (HUVECs, CC2517A, Lonza, Basel, Swiss) were plated on a 96-well plate (353072, BD-Falcon, Franklin Lakes, NJ, USA) at a density of 2×10^4 cells per well, and cultured in EGM-2 media (CC-3162, Lonza, Basel, Swiss) for 3-4 days according to the manufacturer's instruction. HUVEC cells of passage 1-4 were stimulated with 20 ng/ml of human tumor necrosis factor (TNF)-α (ab9642, Abcam, Cambridge, UK) for 24 hours. Thereafter, each well was washed twice with 200 μL of EGM-2 medium, and human anti-VCAM-1 mAb was added. Subsequent steps were performed as above using fluorescently labeled U937 cells.

Internalization assay

TNF-α primed HUVECs were prepared as above. Human anti-VCAM-1 mAb (10 μg/mL) was added and cells were incubated at 4°C. After fixation on ice, the mixture was incubated at 37°C for 10 or 60 minutes for internalization

of VCAM-1/mAb complex then cells were washed with acidic PBS (pH 2.5) to remove VCAM-1/mAb complex on cell surface. FITC-conjugated goat anti-human IgG Ab (F9512, Sigma-Aldrich, St. Louis, MO, USA) was added to detect human anti-VCAM-1 mAb in permeabilized cells. Therefore internalized VCAM-1/mAb complex was analyzed by flow cytometry and mean fluorescence intensity (MFI) values were compared.

Animal

Female 6-8 weeks old BALB/c mice (Orient, Daegeon, Korea) were used for all experiments. All mice were kept under specific pathogen free conditions, according to the standard of American Association for the Accreditation of Laboratory Animal Care-approved facilities, and maintained on a 12 hour light-dark cycle with food and water *ad libitum*. All experiments described in this study were approved by Animal Research Ethics Board of Yonsei University (Seoul, Korea). All control and experimental groups were contained five mice.

OVA induced acute asthma model

On experimental day 1 and 14, mice were systemically sensitized with 10 μg of OVA (Sigma-Aldrich, St. Louis, MO, USA) mixed with 1% aluminum hydroxide (Resorptar, Indergen, New York, NY, USA) via intraperitoneal injection. One week after the second sensitization, mice were challenged with 3 intranasal inoculations of 1% OVA in 30 μL of PBS under inhalation anesthesia with 5% isoflurane. Antibody treatment (100 μg/mouse [about 4 mg/kg], intravenously) occurred once, 1 day before OVA challenge (Figure 1).

OVA induced chronic asthma model

As described above, mice were sensitized twice. One week after sensitization, intranasal inoculations (1% OVA 30 μL under anesthesia each time) occurred twice weekly for 4 weeks. A total of 4 intravenous (IV) Ab treatments (100 μg/mouse for each time) occurred at weekly intervals, beginning 1 day before OVA challenge (Figure 1).

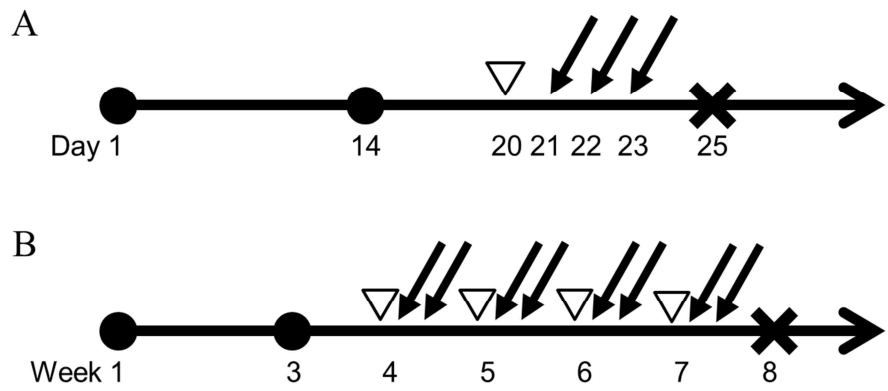

Figure 1. Schema of *in vivo* experiments. Closed circles represent intraperitoneal OVA sensitization with 1% alum. Black arrows represent intranasal (IN) inoculations of 1% OVA in PBS. Inverted open triangles represent Ab treatment (1 day before IN challenge). In acute asthma model (A), mice were sacrificed at day 25 for evaluation, besides in chronic asthma (B) mice were sacrificed at the first day on 8th week.

Measurement of airway hyperresponsiveness (AHR)

Forty-eight hours after the last OVA challenge, AHR to methacholine (MCh, Sigma-Aldrich, St. Louis, MO, USA) was measured. In each mouse, tracheostomy was done and an 18 gauge steel tube was inserted and ventilated with a small animal ventilator (Flexivent 5.1®, Scireq, Montreal, PQ, Canada) at a respiration rate 160 breaths per minute with 10 mL/kg tidal volume under appropriate anesthesia. A positive end expiratory pressure of 3 cmH_2O was applied. Mice were challenged with saline aerosol at baseline followed by a serially increasing concentration of MCh (3.1, 6.25, 12.5, 25.0, and 50.0 mg/mL). Each aerosol was delivered via nebulizer in the Flexivent® system using a full inspiration maneuver (respiration rate 60 breaths per minute, tidal volume 25 mL/kg) for 12 seconds (10 full inspirations).

Analysis of bronchoalveolar lavage fluid

A bronchoalveolar lavage (BAL) of each mouse was performed with 1 mL of Hank's balanced salt solution (HBSS) to collect BAL fluid via tracheostomy tube. The total inflammatory cell count was measured by hemocytometer. The BAL fluid was then centrifuged at 1,500 rpm for 3 minutes at 4°C to collect supernatant. After removing the supernatant, the cell pellet was re-suspended in HBSS. A BAL cell smear was made by cytocentifugation (cytospin3, Thermo, MA, USA) at 1,000 rpm for 3 minutes at RT. All cell smear slides were stained using the Diff Quick staining method. Differential inflammatory cell counts included at least 200 neutrophils, eosinophils, lymphocytes or macrophages.

Preparation of lung homogenate

After collecting BAL fluid, the right lung was resected from the bronchial tree and weighed. Then it was homogenized using a tissue homogenizer (Biospec Products, Bartlesville, OK, USA) in 3 mL of lysis buffer containing 0.5% Triton X-100, 150 mM NaCl, 15 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$,

and protease inhibitor solution (Sigma-Aldrich, St. Louis, MO, USA) at pH 7.4. After incubating for 30 minutes on ice, homogenates were centrifuged at 2,500 rpm for 10 minutes. Supernatants were harvested and passed through a 0.45–micron filter (Gelman Sciences, Ann Arbor, MI, USA). The final preparations were stored at -20°C for cytokine measurements

Cytokine level measurement

We used ELISA kits (R&D Systems, Minneapolis, MN, USA) to measure IL-5, IL-13, interferon (IFN)-γ, and transforming growth factor (TGF)-β in the lung homogenates.

Histopathological examination

After collecting BAL fluid, the left lung was filled with 10% formalin solution, embedded in paraffin and cut into 3-μm thick sections. We used hematoxylin and eosin staining (H&E) for general examination, periodic acid-Schiff staining (PAS) to measure goblet cell hyperplasia, and Masson's trichrome staining to measure fibrosis. Besides of light microscopic examination, quantification analysis was done using software (Metamorph®, Molecular Devices, Sunnyvale, CA, USA). Briefly, PAS stained slide placed light microscope under ×200 magnifying power. Circling 2 bronchi along basement membrane in each slide then count the number of goblet cells in selected bronchi. Finally goblet cell numbers per μm of basement membrane was calculated and analyzed statistically. Fibrosis area was measured by color pixel count over pre-set threshold color in whole field containing several bronchovascular bundles.

Immunohistochemical examination

Slides were prepared as described in "Histopathological examination," deparaffinized 3 times with xylene, and washed in ethanol. Hydrogen peroxide (3% in methanol) was used to inactivate endogenous peroxidases. Slides were incubated with anti-VCAM-1 Ab (Millipore, Billerica, MA, USA) for 1 hour at RT followed by 4°C for 24 hours. After washing in PBS, slides were treated with secondary antibody (DakoCytomation, Carpentaria, CA, USA) and incubated for 20 minutes at room temperature. Diaminobenzidine (DAB) was developed for 5 minutes. Slides were counterstained with hematoxylin and mounted using mounting medium.

Statistical analysis

Statistical differences among the means were analyzed using one-way ANOVA. In all cases, $p<0.05$ was considered statistically significant. We used SPSS 12.0 (SPSS Inc., Chicago, IL, USA) statistical software.

III. RESULTS

Cross-reactivity of human anti-VCAM-1 mAb against murine VCAM-1

The affinities of human anti-VCAM-1 mAb for human and mouse VCAM-1 were evaluated. The antibody bound both human and mouse VCAM-1. While the antibody was designed to bind human VCAM-1, it had good affinity for human VCAM-1 at concentrations over 10 ng/mL (OD 0.187±0.040 at 10 ng/mL, OD 0.880±0.132 at 100 ng/mL), and it also bound mouse VCAM-1 at 100 ng/mL (OD 0.170±0.009) (Figure 2).

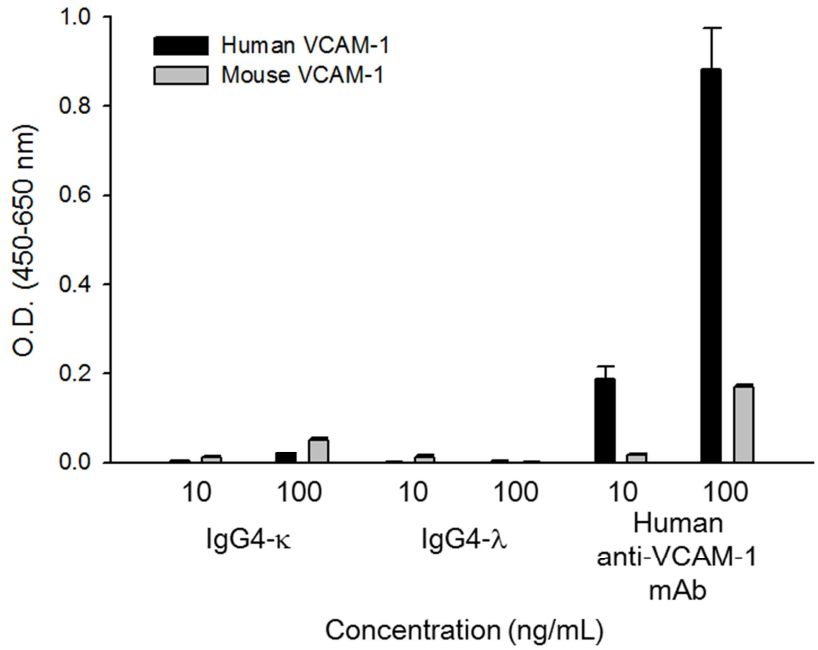

Figure 2. Cross-reactivity assay. After blocking with 1% BSA, human anti-VCAM-1 mAb was added at 37°C for 2 hours. The binding affinity was visualized with HRP-conjugated anti-F(ab')$_2$ Ab using TMB substrate. Human anti-VCAM-1 mAb have binding affinity to both recombinant human VCAM-1/Fc and mouse VCAM-1/Fc.

Leukocyte adhesion inhibition to recombinant human VCAM-1

Human anti-VCAM-1 mAb inhibited U937, EoL-1 and $CD4^+$ T cells from adhering to recombinant human VCAM-1 molecules. This interference was remarkable at concentrations over 0.1 μg/well (Figure 3). U937 and EoL-1 cell adhesion to recombinant human VCAM-1 were effectively blocked at 0.1 μg of human anti-VCAM-1 mAb/well (75.5±14.9% for U937, 88.4±0.9% for EoL-1)(Figure 3A, 3B). The adhesion inhibition assay for $CD4^+$ T cell showed up to 64.5±3.3% inhibition (at 10 μg/well) in a dose-dependent manner (Figure 3C).

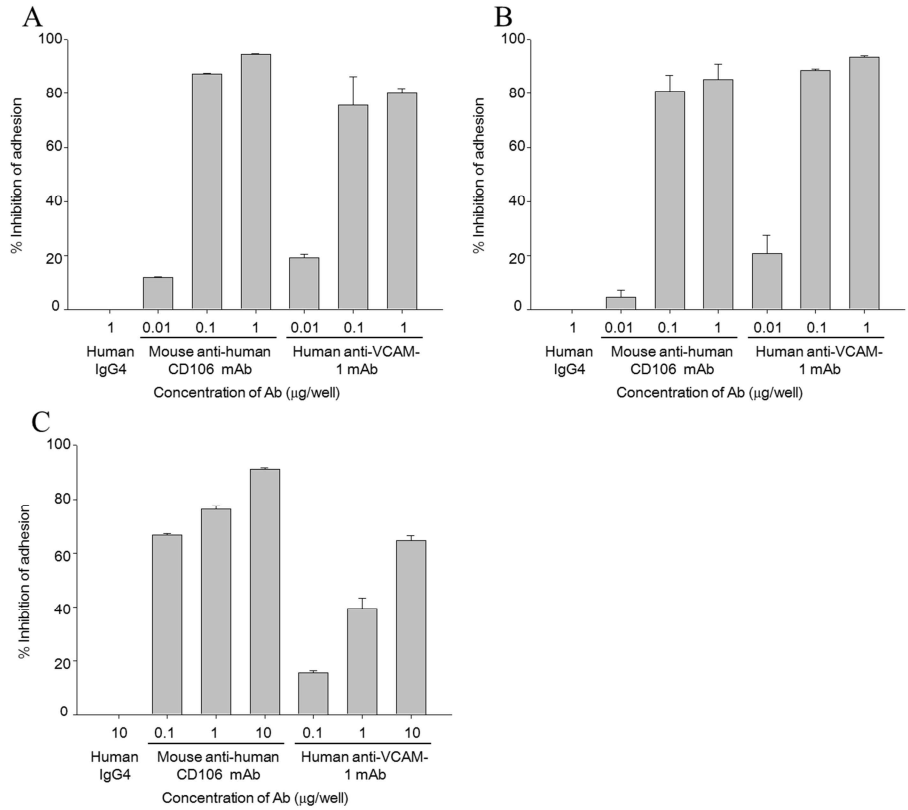

Figure 3. Adhesion inhibition assay. Recombinant human VCAM-1 was coated at plate. After blocking with 1% BSA, human anti-VCAM-1 mAb was added. Fluorescence labeled human leukocytes were added and incubated. After removal of non-adherent cells, lysis buffer was added to lyse bound cells. Reduction of fluorescence intensity was analyzed as inhibition percent. Human anti-VCAM-1 mAb effectively inhibited leukocytes from binding to recombinant VCAM-1. U937 cells (A) and EoL-1 cells (B) adhesion were inhibited from 0.1 μg/well concentration. In $CD4^+$ T cells (C), 64.5±3.3% inhibition was obtained at 10 μg/well concentration.

Adhesion inhibition of U937 cells to HUVECs expressing VCAM-1

Human anti-VCAM-1 mAb inhibited U937 cells from adhering to a HUVEC monolayer primed with TNF-α to express VCAM-1 in a dose-dependent manner. The maximum inhibition was 63.1±5.9% at 100 μg/mL (Figure 4).

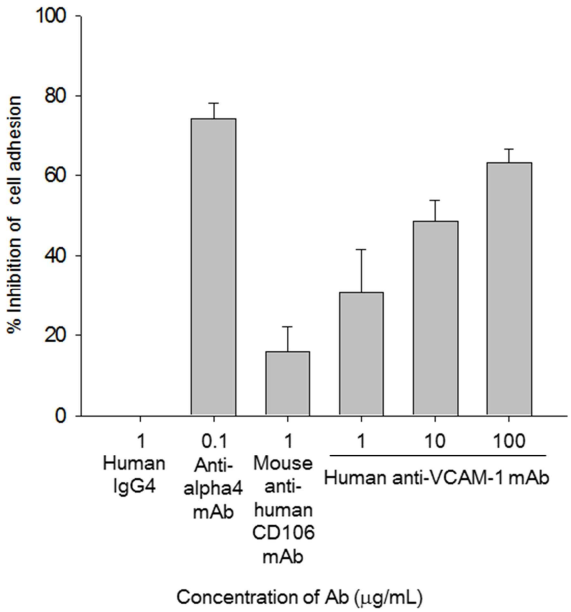

Figure 4. Adhesion inhibition assay using HUVEC. HUVECs were stimulated with 20 ng/ml of TNF-α for 24 hours to express VCAM-1, then human anti-VCAM-1 mAb was added. Therefore fluorescence labeled U937 cell was added for binding to VCAM-1 expressing HUVEC. After removal of non-adherent cells and lysing adherent cell, fluorescence intensity reduction was calculated. Human anti-VCAM-1 mAb effectively inhibited U937 cells from binding to VCAM-1 expressing HUVEC mono-layer in dose-dependent manner.

Internalization of VCAM-1 into cytosol

TNF-α primed HUVEC expressed VCAM-1 molecules on their surface. Treating human anti-VCAM-1 mAb induced internalization of VCAM-1 into cytosol. MFI of total expression was 62.4. MFI values of internalized VCAM-1 were 14.7 at 10 minutes and 32.8 at 60 minutes after human anti-VCAM-1 mAb treatment (Figure 5).

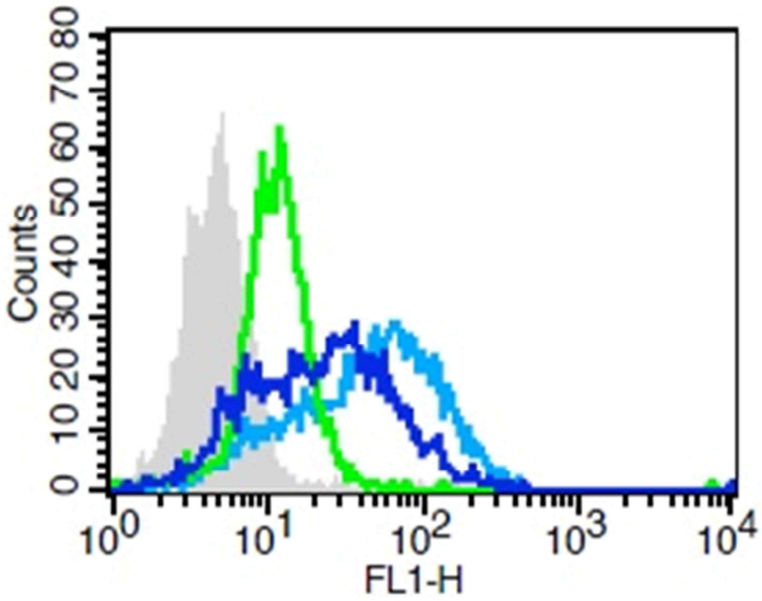

Figure 5. Internalization assay. TNF-α pretreated HUVECs were incubated with human anti-VCAM-1 mAb. After fixation, the mixture was incubated at 37°C for 10 or 60 minutes. To remove VCAM-1/mAb complex on the cell surface, acidic PBS washing was done. FITC-conjugated 2^{nd} antibody was added. Human anti-VCAM-1 mAb treatment induced internalization of VCAM-1 molecules. Internalized VCAM-1/mAb complex analyzed by flow cytometry. Grey area represents isotype control (MFI 5.2). Each colored lines represent; sky blue line total VCAM-1 expression (MFI 62.4), green line for 10 minutes (MFI 14.7) and blue line for 60 minutes (MFI 32.8), respectively.

The effects of human anti-VCAM-1 mAb on pathophysiological features in acute asthma murine model

We evaluated the anti-inflammatory effects of IV treatment with human anti-VCAM-1 mAb using OVA induced acute asthma model. Administering 100 μg of IV human anti-VCAM-1 mAb significantly reduced the MCh induced AHR at 12.5, 25.0, and 50.0 mg/mL concentrations (resistance at 12.5 mg/mL 4.81±1.40 (OVA/control Ab group) vs. 1.75±0.41 cmH_2O (OVA/human anti-VCAM-1 mAb group), $p<0.001$; at 25 mg/mL 5.83±1.16 vs. 2.14±0.34 cmH_2O, $p<0.001$; at 50 mg/mL 7.82±0.47 vs. 3.16±0.51 cmH_2O, $p<0.001$) (Figure 6A).

BAL fluid analysis showed significantly reduced eosinophilic and neutrophilic inflammation and fewer macrophages due to IV human anti-VCAM-1 mAb (total inflammatory cell number 98.4±19.4 (OVA/control Ab group) vs. 36.8±14.9 $\times 10^4/mm^3$ (OVA/human anti-VCAM-1 mAb group), $p<0.001$; macrophage 29.0±8.4 vs. 12.1±4.9 $\times 10^4/mm^3$, $p=0.001$; neutrophil 8.3±2.8 vs. 3.0±1.6 $\times 10^4/mm^3$, $p=0.002$; eosinophil 54.3±26.9 vs. 17.3±7.7 $\times 10^4/mm^3$, $p=0.009$) (Figure 6B).

The IL-5, IL-13, and TGF-β levels in lung homogenate were significantly lower in human anti-VCAM-1 mAb treated group (IL-5 level 208.8±56.7 (OVA/control Ab group) vs. 55.0±41.7 pg/mL (OVA/human anti-VCAM-1 mAb group), $p<0.001$; IL-13 428.6±143.6 vs. 259.0±53.5 pg/mL, $p=0.042$; TGF-β 139.8±14.6 vs. 103.4±9.8 pg/mL, $p=0.001$) (Figure 7A-B, 7D). IFN-γ levels did not differ between groups (Figure 7C).

On histopathological examination, we observed remarkable decrease in peribronchial and perivascular inflammatory cell infiltration. Goblet cell hyperplasia in the bronchial epithelia was also markedly decreased in mice treated with human anti-VCAM-1 mAb (Figure 8A-C). Quantification analysis also revealed decreased number of goblet cells in human anti-VCAM-1 mAb

treated group (Cell number 0.053±0.021 cells/μm (OVA/control Ab group) vs. 0.039±0.015 cells/μm (OVA/human anti-VCAM-1 mAb group), p=0.043) (Figure 8H). There was no significant difference in peribronchial fibrosis (Figure 8D-F, 8I).

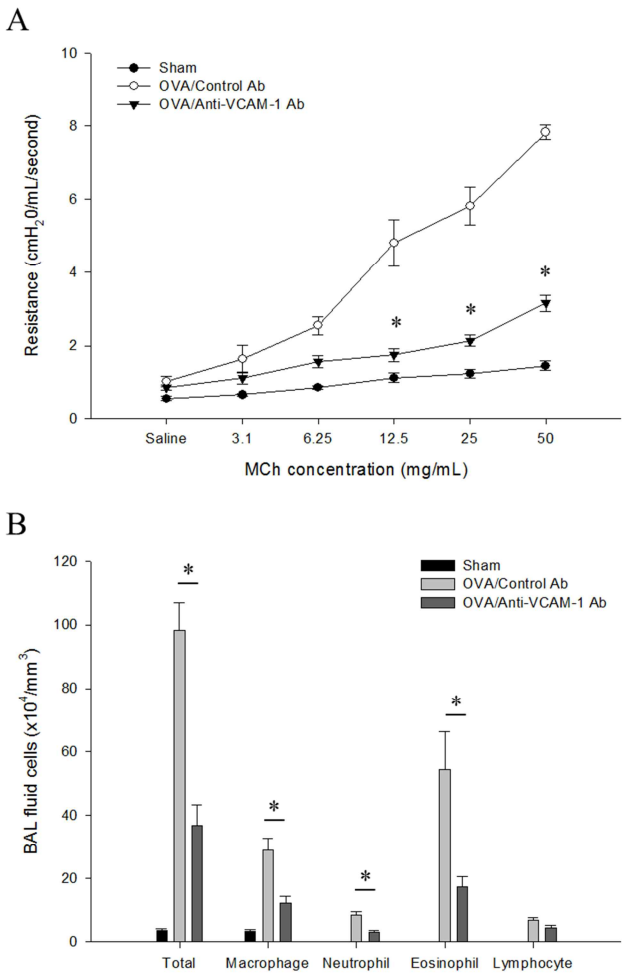

Figure 6. Changes of physiological feature in acute asthma model. Forty eight hours after the last OVA challenge, AHR to MCh was measured under anesthesia. BAL fluid was collected via tracheostomy tube. Cell numbers were analyzed by hemocytometer and differential count under light microscope. Human anti-VCAM-1 mAb treatment attenuated AHR at 12.5, 25.0, and 50.0 mg/mL concentration of MCh (A). Inflammatory cells were decreased in BAL fluid significantly in OVA/human anti-VCAM-1 mAb as treated mice (B). All data showed as mean±SEM. *:p<0.05 between OVA/control Ab and OVA/ human anti-VCAM-1 mAb group

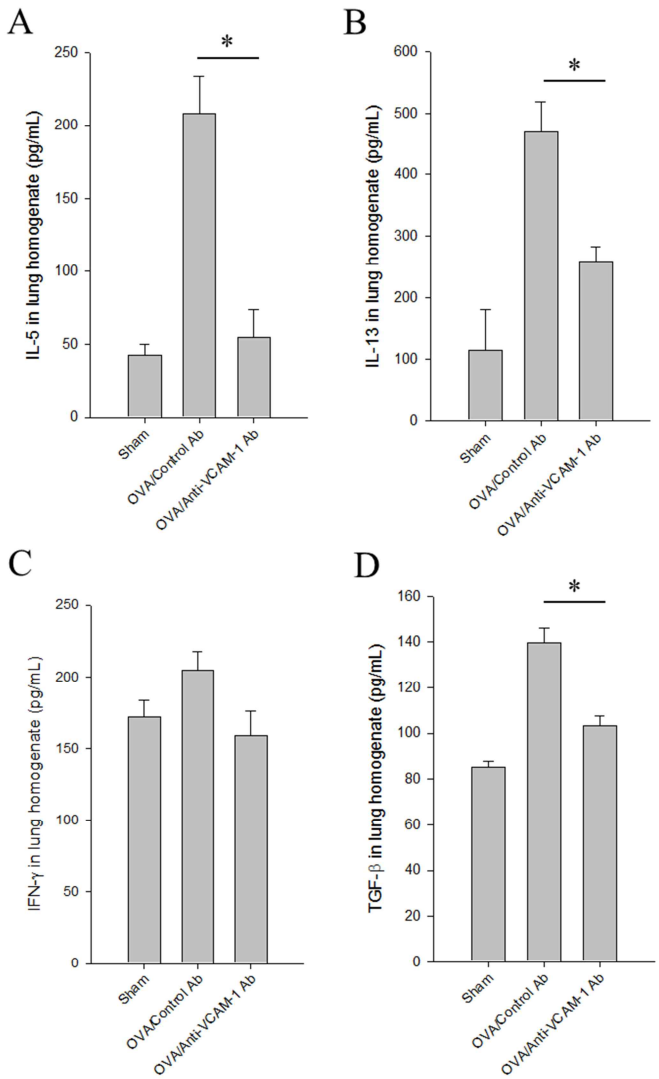

Figure 7. Changes of cytokine profile in acute asthma model. All cytokines were measured from homogenate of right lung of each mouse. IL-5 (A), IL-13 (B) and TGF-β (D) levels in the lung homogenate significantly reduced in the treated group. IFN-γ (C) level showed no difference. All data showed as mean±SEM. *:p<0.05 between OVA/control Ab and OVA/human anti-VCAM-1 mAb group

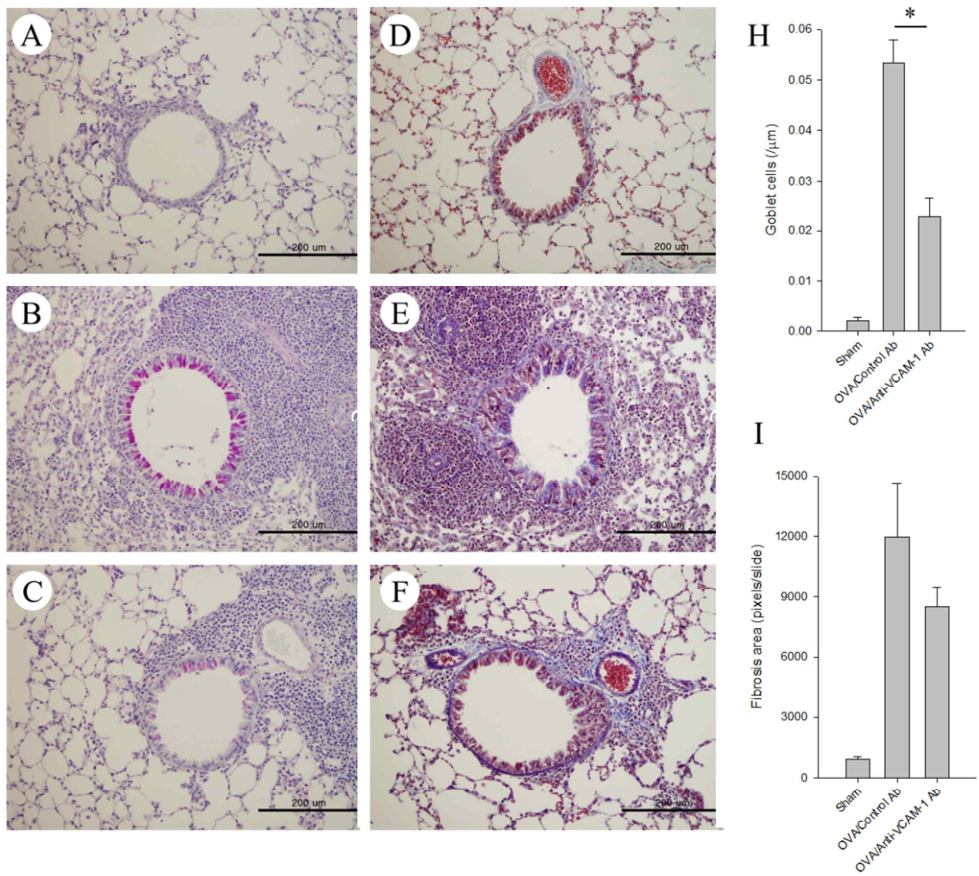

Figure 8. Changes of histopathological feature in acute asthma model. IV administration of human anti-VCAM-1 mAb attenuated peribronchial and perivascular inflammation and mucus metaplasia of respiratory epithelium compared to control Ab treated group (A: sham, B: OVA/control Ab, C: OVA/human anti-VCAM-1 mAb; PAS stain; H: goblet cell count). However lung fibrosis findings are not remarkable (D: sham, E: OVA/control Ab, F: OVA/human anti-VCAM-1 mAb; Masson's trichrome stain; I: calculated fibrosis area). All data showed as mean±SEM. *: $p<0.05$ between OVA/control Ab and OVA/human anti-VCAM-1 mAb group.

The effects of human anti-VCAM-1 mAb on pathophysiological features in chronic asthma murine model

In the chronic asthma mouse model, we observed similar anti-inflammatory and anti-asthma effects of IV human anti-VCAM-1 mAb as in acute asthma mice. Four times of treatments (100 μg each) significantly reduced MCh induced AHR at 25.0 and 50.0 mg/mL concentrations (resistance at 25.0 mg/mL 7.06±1.72 (OVA/control Ab group) vs. 4.09±0.26 cmH_2O (OVA/human anti-VCAM-1 mAb group), p=0.002; at 50.0 mg/mL 10.26±1.42 vs. 4.88±0.71 cmH_2O, p<0.001) (Figure 9A).

In the BAL fluid analysis, total inflammatory cells, eosinophils, neutrophils, macrophages, and lymphocytes were significantly decreased in human anti-VCAM-1 mAb treated group (total inflammatory cell number 120.4±29.2 (OVA/control Ab group) vs. 29.6±9.9 $\times 10^4/mm^3$ (OVA/human anti-VCAM-1 mAb group), p<0.001; macrophage 46.0±14.8 vs. 11.2±2.5 $\times 10^4/mm^3$, p<0.001; neutrophil 9.4±4.1 vs. 2.0±1.3 $\times 10^4/mm^3$, p=0.001; eosinophil 45.6±17.6 vs. 9.3±4.1 $\times 10^4/mm^3$, p<0.001; lymphocyte 19.4±10.5 vs. 7.2±4.8 $\times 10^4/mm^3$, p=0.033) (Figure 9B).

IL-5, IL-13, TGF-β, and IFN-γ levels were decreased in lung homogenates from the treated group (IL-5 level 182.6±42.9 (OVA/control Ab group) vs. 98.6±26.2 pg/mL (OVA/human anti-VCAM-1 mAb group), p=0.003; IL-13 944.4±169.9 vs. 631.0±173.3 pg/mL, p=0.029; IFN-γ 335.4±121.4 vs. 21.8±7.0 pg/mL, p=0.023; TGF-β 144.4±14.0 vs. 116.6±9.0 pg/mL, p=0.003) (Figure 10).

Histopathological examination showed remarkably reduced peribronchial and perivascular inflammatory cell infiltration and goblet cell hyperplasia in the bronchial epithelia (Figure 11A-C), Goblet cell count also decreased significantly (Cell number 0.042±0.015 cells/μm (OVA/control Ab group) vs. 0.026±0.013 cells/μm (OVA/human anti-VCAM-1 mAb group), p=0.041)

(Figure 11H). In terms of airway remodeling, perivascular and peribronchial fibrosis was also ameliorated in the treated group (Figure 11D-F). Calculated fibrosis area was significantly reduced in human VCAM-1 mAb treated group (fibrosis area 16,616±4374 (OVA/control Ab group) vs. 7350±1570 pixel/slide (OVA/human anti-VCAM-1 mAb), p=0.002) (Figure 11I).

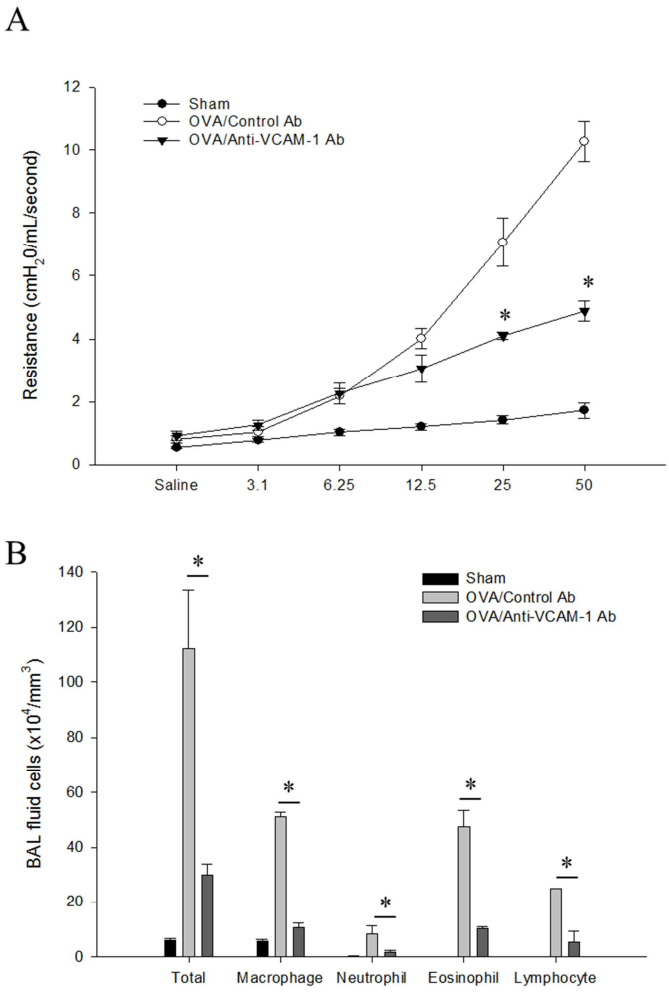

Figure 9. Changes of physiological feature in chronic asthma model. Forty eight hours after the last OVA challenge, AHR to Mch was measured under anesthesia. BAL fluid was collected via tracheostomy tube. Hemocytometric examination and differential counting were done. Human anti-VCAM-1 mAb treatment attenuated AHR at 25.0, and 50.0 mg/mL concentration of MCh (A). Inflammatory cells were decreased in BAL fluid significantly in OVA/human anti-VCAM-1 mAb as treated mice (B). All data showed as mean±SEM. *: $p<0.05$ between OVA/control Ab and OVA/human anti-VCAM-1 mAb group.

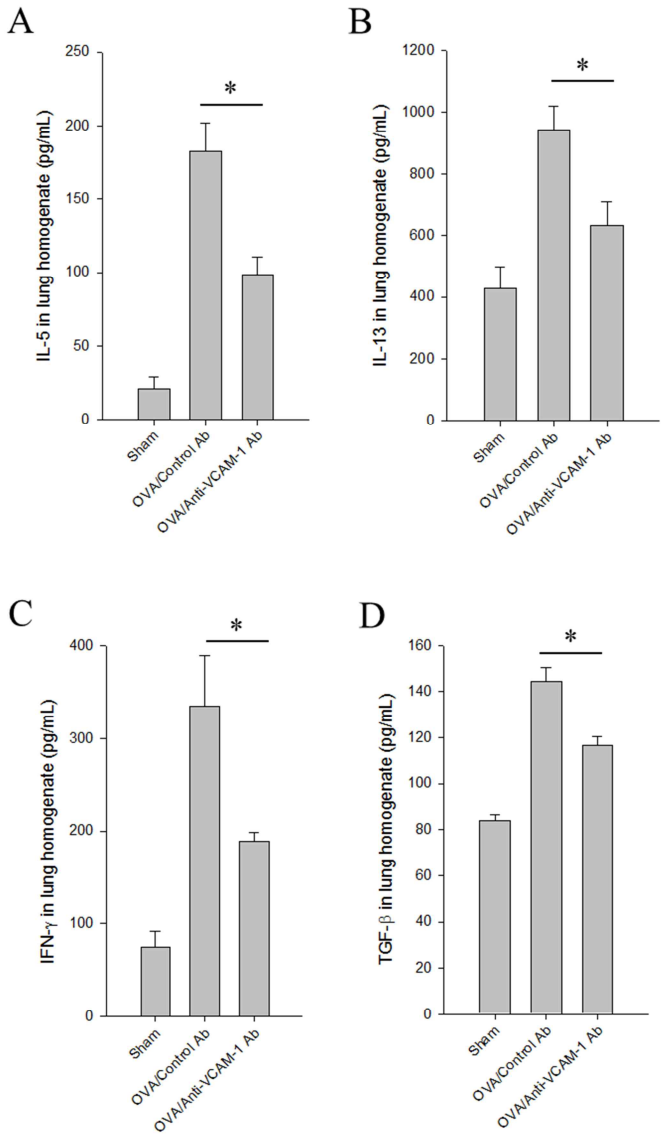

Figure 10. Changes of cytokine profile in chronic asthma model. All cytokines were measured from homogenate of right lung of each mouse. IL-5 (A), IL-13 (B), IFN-γ (C) and TGF-β (D) levels in the lung homogenate significantly reduced in the treated group. All data showed as mean±SEM. *: $p<0.05$ between OVA/control Ab and OVA/human anti-VCAM-1 mAb group.

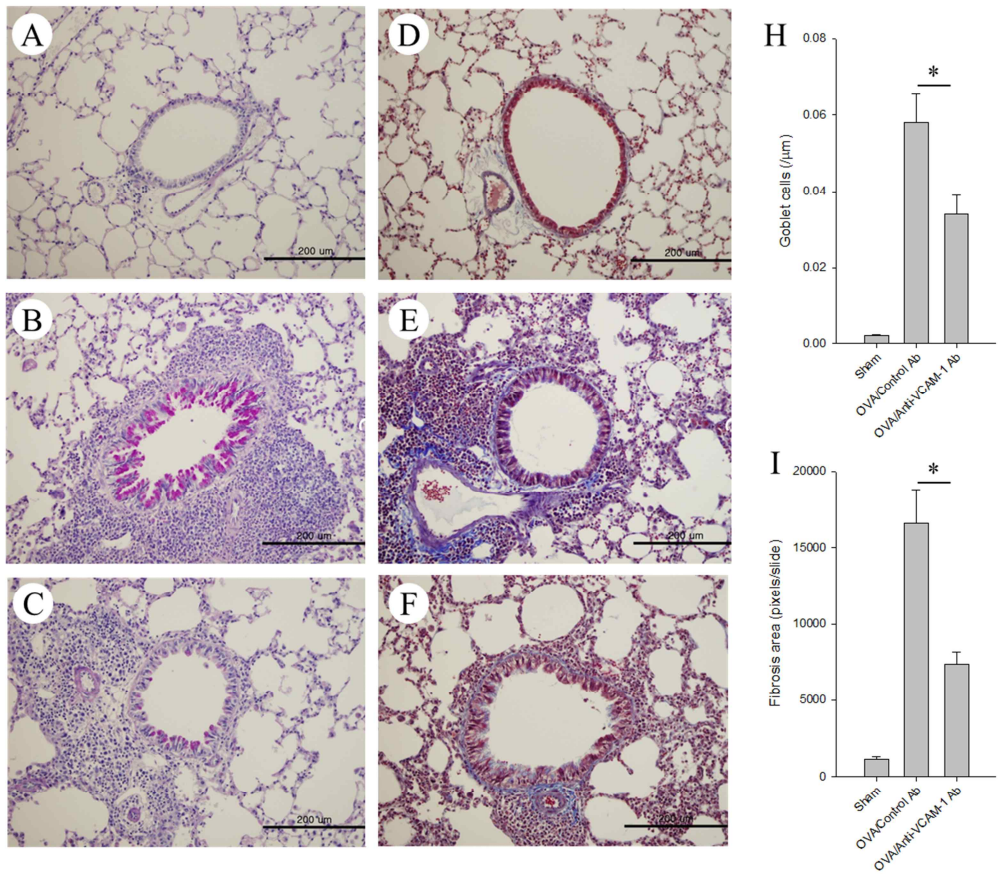

Figure 11. Changes of histopathological feature in chronic asthma model. four times of human anti-VCAM-1 mAb treatment attenuated peribronchial and perivascular inflammation and goblet cell hyperplasia of respiratory epithelium compared to control Ab treated group (A: sham, B: OVA/control Ab, C: OVA/human anti-VCAM-1 mAb; PAS stain; H: goblet cell count). Moreover, peribronchial and perivascular fibrosis was reduced in human anti-VCAM-1 mAb treated group (D: sham, E: OVA/control Ab, F: OVA/human anti-VCAM-1 mAb; Masson's trichrome stain; I: calculated fibrosis area). All data showed as mean±SEM. *: $p<0.05$ between OVA/control Ab and OVA/human anti-VCAM-1 mAb group.

The effects of human anti-VCAM-1 mAb on in vivo VCAM-1 expression

Immunohistochemistry revealed increased VCAM-1 expression in the lung tissue in both of acute and chronic OVA induced asthma mice. And we observed reduced expression of VCAM-1 in mice treated with human anti-VCAM-1 mAb in both of acute and chronic asthma model (Figure 12).

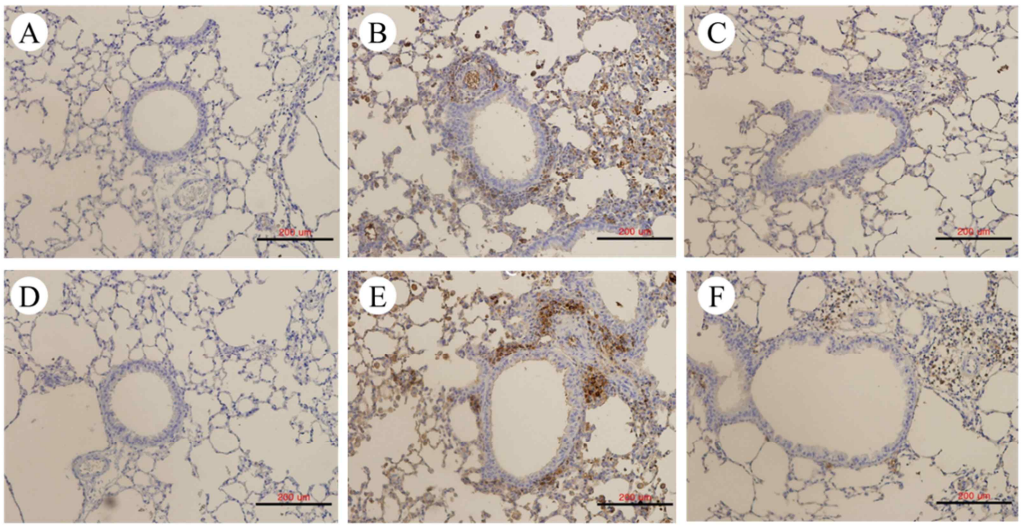

Figure 12. Changes of *in vivo* VCAM-1 expression pattern. After deparaffinizing 3 times with xylene, endogenous peroxidase activity was blocked. Slides were incubated with anti-VCAM-1 Ab and secondary Ab serially. Color development was done using DAB system. IV administration of human anti-VCAM-1 mAb attenuated endothelial and epithelial VCAM-1 expression(brownish color) in acute and chronic asthma mice (A: sham of acute model, B: OVA/control Ab of acute model, C: OVA/human anti-VCAM-1 mAb of acute model, D: sham of chronic model, E: OVA/control Ab of chronic model, F: OVA/human anti-VCAM-1 mAb of chronic model).

IV. DISCUSSION

In this study, we demonstrated that a novel human anti-VCAM-1 mAb had anti-inflammatory and anti-asthma effects in OVA induced murine asthma model. Intravenous treatment with human anti-VCAM-1 mAb reduced allergic inflammation in BAL fluid, airway hyperresponsiveness, inflammatory cell infiltration, and goblet cell hyperplasia in the lung tissue from acute and chronic asthma mice. Furthermore, mice with chronic asthma had less fibrosis in the lung tissue after treatment of human anti-VCAM-1 mAb. Therefore, we suggested that anti-VCAM-1 treatment could be a new therapeutic modality in allergic airway diseases.[17]

The cell that orchestrates allergic asthma is Th2 lymphocyte. Th2 cells produce the cytokines IL-4, IL-5 and IL-13 to influence B lymphocytes to differentiate into plasma cells and produce IgE. They also recruit eosinophils, the main effector cell, to inflammation sites.[18] At the initiation of allergic inflammation, a few inflammatory mediators (such as histamine) and pro-inflammatory cytokines (such as TNF-α) are released from primary site. These mediators stimulate endothelial cells to express cell adhesion molecules, such as ICAM-1 and VCAM-1. The cell adhesion molecules capture neutrophils, eosinophils, and Th2 lymphocytes from the bloodstream and help migration to the inflamed tissue. These recruited cells produce a large amount of cytokines and mediators, perpetuating the cycle.[19] In this study, intravenous human anti-VCAM-1 treatment reduced allergic inflammation in murine models of both acute and chronic asthma. We speculate that human anti-VCAM-1 mAb blocks VCAM-1 molecules expressed on the endothelial cell surface and inhibits recruitment of inflammatory effector cells.[20]

The influence of eosinophils upon AHR, a characteristic of asthma, remains under debate.[21-23] IL-5 is a crucial cytokine for AHR. IL-5 in allergic asthma pathogenesis primarily comes from Th2 lymphocytes and tissue-infiltrated

eosinophils. Therefore, the anti-inflammatory effects of VCAM-1 also decreased IL-5 in the inflamed lung tissue. In previous work, we observed a dose-response relationship of intravenous human anti-VCAM-1 mAb, and found that 100 μg/mice (approximately 4 mg/kg) had the best effect.

Clinically, some severe asthmatics having a long disease duration that can result in airway remodeling, which is characterized by peribronchial and perivascular fibrosis, fixed airflow obstruction, and permanent loss of lung function.[24,25] In this study, treating chronic asthma mice with IV human anti-VCAM-1 mAb reduced peribronchial and perivascular fibrosis. TGF-β is crucial for fibrosis and airway remodeling. The main source TGF-β is eosinophils recruited to the tissue. Therefore, VCAM-1 blockage for the long-term may protective against airway remodeling. Furthermore, airway epithelial cells are important in the development and propagation of allergic inflammation. VCAM-1 and ICAM-1expression increased in the bronchial epithelium of asthmatics, and these cell adhesion molecules were related to the release of eosinophil cationic protein (ECP).[26] In this study, we delivered the antibody intravenously. Further studies should test other delivery routes, such as intranasal or intratrachial inoculation, to determine their effects on airway epithelial cells.

A few studies have assessed soluble (not membrane bound) VCAM-1 in the blood. Soluble VCAM-1 was increased in the sputa[27] and sera[28,29] of asthmatic patients. Soluble VCAM-1 could increase chemotactic activity of eosinophils via Src and MAP kinase signaling,[30] but the pathophysiological roles of soluble VCAM-1 are not clear. Further study is needed to determine how human anti-VCAM-1 mAb affects soluble VCAM-1 to reduce inflammation.

VCAM-1 can activate downstream signaling through the Rho and Rac protein pathways. These pathways can reconstruct the actin cytoskeleton and produce radical oxygen species (ROS) in endothelial cells via p38 MAP kinase.[31] The molecular effects of down-regulating VCAM-1 in endothelial and

epithelial cells, such as effects on cytokine production and macrophage or lymphocyte activation, should be studied.

Interestingly, we observed human anti-VCAM-1 mAb treatment reduced *in vivo* expression of VCAM-1. This finding could be explained by our *in vitro* data for internalization assay of VCAM-1 molecules in human anti-VCAM-1 mAb treated HUVECs. From these results, VCAM-1 blockage therapy may have one important additional effect that target molecule down-regulation by targeted therapy although this phenomenon was well known in other monoclonal Ab[32] and adhesion molecule.[33] The mechanism of VCAM-1 internalization is known to be related with clathrin-associated pathway.[34]

We demonstrated decreased inflammation in allergic asthma one of the typical allergic airway diseases in this study. For further studies, we can suggest that human anti-VCAM-1 mAb may have anti-inflammatory effects in other eosinophilic diseases, such as eosinophilic gastroenteritis, Churg-Strauss syndrome, and idiopathic hypereosinophilic syndrome.[35] We also expect that human anti-VCAM-1 mAb may have positive effects on conditions related to VCAM-1 over-expression, like atherosclerosis.[36]

V. CONCLUSION

In conclusion, we demonstrated that a novel human anti-VCAM-1 mAb inhibited leukocytes from adhesion to VCAM-1 molecules *in vitro*. Furthermore, we observed anti-inflammatory effects of IV treatment of human anti-VCAM-1 mAb using acute and chronic asthma mouse models and it showed anti-fibrosis effect in chronic asthma mice. Further research on another specified mechanism of VCAM-1 blockade would be needed.

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ABSTRACT (IN KOREAN)

새로운 인간 항-VCAM-1 단클론 항체가 생쥐 천식 모델의 기도 염증과 개형에 미치는 영향

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이 재 현

연구 배경 및 목적: 천식은 제2형 T 세포(Th2)와 호산구에 의해 유발되는 기도의 만성 염증성 질환이다. Vascular cell adhesion molecule-1 (VCAM-1)은 천식의 병태생리가 일어나고 있는 조직으로 호산구와 림프구가 이동하는데 중요한 세포 부착 인자이다. 따라서 항-VCAM-1 단클론 항체의 투여는 알레르기 염증과 천식의 병태생리학적 변화를 감소시킬 수 있는 가능성이 있다. 이에 본 연구에서는 인간 항-VCAM-1 단클론 항체를 난황(ovalbumin, OVA)으로 유발시킨 생쥐 천식 모델에 투여하여 천식의 병태생리학적 특징에 미치는 영향을 연구하였다.

연구 재료 및 방법: 본 연구에서 사용한 인간 항-VCAM-1 단클론 항체가 인간 및 생쥐의 VCAM-1 분자에 결합하고 이를 통해 백혈구의 부착을 저해하는가를 평가하기 위하여 U937, EoL-1 및 $CD4^+$ T 세포를 이용하여 백혈구 부착 억제 시험을 실시하였다. 난황으로 전신 감작된 BALB/c 생쥐에 난황 유발 전, 인간 항-VCAM-1 단클론 항체를 투여하였다. 침습적 기도과민성 및

기관지 폐포 세척액 내의 염증세포의 분율을 관찰하였으며, 폐조직 내의 염증성 사이토카인 치를 측정하고 조직절편에서 VCAM-1에 대한 면역조직화학염색을 포함한 조직병리학적 소견을 관찰하였다.

연구 결과: 인간 항-VCAM-1 단클론 항체는 인간 및 생쥐의 VCAM-1 분자에 대한 결합능이 있었으며 실험실적 조건에서 효과적으로 백혈구의 부착을 억제하였으며 생체내 투여시 기도과민성 및 기관지 폐포 세척액 내의 염증세포 수를 의미있게 감소시켰다. 폐조직내 IL-5와 IL-13, 두 Th2 사이토카인치도 감소하였다. 인간 항-VCAM-1 단클론 항체는 기관지 상피의 술잔세포 화생 및 염증세포 유입을 감소시켰으며 혈관내피세포의 VCAM-1 분자의 발현 또한 감소시켰다. 만성 천식 생쥐에서는 기도 섬유화도 감소시켰다.

결론: 생쥐 천식 모델에 인간 항-VCAM-1 단클론 항체를 투여한 결과, 기도의 알레르기 염증 및 천식의 병태생리학적 특징을 감소시킴을 확인하였다. 이 결과는 인간 항-VCAM-1 단클론 항체가 효과적인 항천식 치료제로 사용될 수 있을 가능성을 시사한다.

핵심되는 말 : VCAM-1, 단클론항체, 천식, 알레르기 염증, 세포부착인자, 항염증 효과