Abstract

Role of Vitamin D3 Up-regulated Protein 1 (VDUP1) in Mouse Asthma Model

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Vitamin D3 upregulated protein 1 (VDUP1) is a multifunctional protein that is mainly distributed in immune organs and can exacerbate tumorigenesis and chronic inflammation, it may be related to an increase of immune-mediated materials such as proinflammatory cytokines, lymphokines, and transcription factors. In asthma, several cytokines have been reported to be involved in the inflammatory process. Here, we evaluated the effect of a lack of VDUP1 in ovalbumin (OVA)-induced airway inflammation in a mouse model of asthma. VDUP1 -/- (KO) and wild type (WT) mice were sensitized to OVA by intraperitoneal injection,

and challenged with repeated exposure to OVA using an ultrasonic nebulizer. To investigate the difference between the groups, airway hyperresponsiveness (AHR), inflammatory cell number, and immunoglobulin E levels in bronchoalveolar lavage fluid (BALF), histopathological lesions (H&E stain), immunohistochemistry using inducible nitric oxide synthase (iNOS) antibody. And real-time polymerase chain reaction (PCR) using messenger RNA (mRNA) from lung tissue was analyzed. AHR, infiltration of leukocytes into the lung, total number of leukocytes recovered in BALF, and mucus secretion by goblet cells in bronchus were aggravated in KO/OVA mice compared with WT/OVA mice. The IgE level of BALF from KO/OVA treated mice was higher than that of WT/OVA mice. In lung sections, there was higher iNOS staining intensity in KO/OVA mice than WT/OVA mice. In addition, the mRNA of tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-4, IL-1β, and iNOS increased in KO/OVA treated mice compared with WT/OVA mice. Depletion of VDUP1 aggravated the development of allergic airway inflammation and hyperresponsiveness possibly through various cytokines involved in VDUP1.

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Key words: VDUP1, asthma, airway hyperresponsiveness, iNOS

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List of Abbreviations

VDUP1	vitamin D3 upregulated protein 1
OVA	ovalbumin
AHR	airway hyperresponsiveness
BALF	bronchoalveolar lavage fluid
TNF-α	tumour necrosis factor-alpha
IFN-γ	interferon-gamma
IL	interleukin
Trx	thioredoxin
NF-κB	nuclear factor kappa b
COX-2	cyclooxygenase-2
iNOS	inducible nitric oxide synthase
SPF	specific pathogen free
Mech	methacholine
P_{enh}	enhanced pause
PEF	peak expiratory flow
PIF	peak inspiratory flow
PAS	periodic acid-schiff stain

ELISA	enzyme linked immunosorbent assay
RT-PCR	reverse transcription polymerase chain reaction
WT	wild type
NS	normal saline

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Introduction

Overview of asthma

Asthma is an inflammatory disease of the airways, characterized by mucus hypersecretion by goblet cells, lung inflammation in response to inhaled allergens and airway hyperresponsiveness (AHR)(Oh, Cha et al. 2011). Asthma is perceived as a T-helper type 2 (Th2) disease with a particular profile of cytokine release, which is thought to include IL-4 and IL-5. Increasing evidence indicates that other cytokines such as TNF-α also associated with the inflammatory response (Thomas 2011). The process for the pathogenesis of the disease can be regarded as a two-step phenomenon. The first step consists of sensitization to an aeroallergen, which involves the preferential development of T-helper type 2 (Th2) cells. The second consists of targeting the Th2-driven allergic inflammation to the lower airways. This inflammatory process is regulated by a complex network of cytokines and growth factors, secreted by a range of inflammatory cells and from structural tissue components, including epithelial cells, fibroblasts and smooth muscle cells (Kips 2001).

Cytokines involved in asthma

Several cytokines including tumor necrosis factor (TNF)-α, interleukin (IL), inducible nitric oxide synthase (iNOS), and interferon (IFN)-γ have been reported to be involved in the inflammatory process in asthma (Chung and Barnes 1999). Asthma is perceived as a Th2 disease with a particular profile of cytokine release, which is thought to include IL-4 and IL-5. Increasing evidence indicates that other cytokines such as TNF-α are also associated with the inflammatory response that characterizes human asthma (Thomas 2011).

Vitamin D3-upregulated protein 1 (VDUP1)

Vitamin D3 up-regulated protein 1 (VDUP1), a stress-induced multifunctional 46 kDa protein, was originally identified in HL60 cells stimulated with 1,25-dihydroxyvitamin D3 (1,25[OH]$_2$D$_3$) (Kwon, Won et al. 2010). VDUP1 interacts with thioredoxin (Trx), which acts as an antioxidant to inhibit the reducing activity of Trx. In addition, to increase the susceptibility of cells to oxidative stress, it blocks the interactions of Trx with other factors, such as ASK-1 and PAG (Junn, Han et al. 2000). Clinically, VDUP1 has also been related to proliferation, apoptosis, tumorigenesis, metastasis, diabetes and immunological regulation (Kim,

Suh et al. 2007). In the immune system, VDUP1 is mainly distributed in the thymus and spleen (Junn, Han et al. 2000), and has been shown to plays a critical role in the development and function of natural killer (NK) cells (Lee, Kang et al. 2005). VDUP1 −/− mice are more sensitive to carcinogenesis than wild−type mice and are defective when it comes to establishing their immune system (Kim, Suh et al. 2007). According to a recent study, proinflammatory factors such as TNF−a, IL−6, and IL−1β are increased under stimuli like chemicals, infections, and carcinogens in a VDUP1−deficient condition (Kim, Suh et al. 2007, Kwon, Won et al. 2010). Furthermore, the lack of VDUP1 can exacerbate tumorigenesis and chronic inflammation, which could be related to an increase in TNF−α, NF−κB, and COX−2 (Kwon, Won et al. 2010, Kwon, Won et al. 2011).

In asthma, it has been shown that the expression of VDUP1 was decreased in eosinophils isolated from asthma (CAI, GAO et al. 2007); thus, VDUP1 correlates to eosinophil activation, and may influence the disease severity of asthma patients (Gao, Cai et al. 2006). But, still the exact function of VDUP1 in asthma remains to be elucidated. In this study, we examined effect on lack of VDUP1 in ovalbumin (OVA)−induced airway inflammation in a mouse model of asthma.

Purpose of the present study

Few studies have been conducted regarding VDUP1 itself or using the mice deficient in VDUP1 which focus on inflammation. According to several recent repots, it was found that VDUP1 affects tumorigenesis, chronic inflammation, and immune regulation. Here, we conducted the experiment described below using mice deficient in VDUP1 with the concept that VDUP1 would play a role in the immune regulation pathogenesis related to asthma.

Materials and Methods

Mice

Female VDUP1-knockout (KO) mice were generated as described previously and were bred onto C57BL/6 for more than 10generations (Kwon, Won et al. 2011). The specific pathogen-free (SPF), female C57BL/6 mice (8-10 weeks of age) were purchased from Orientbio Inc. (Seongnam, Korea). All animal study protocols were approved by the Animal Care Committee of Seoul National University. The mice were divided into four groups: (1) Alum-sensitized/saline-challenged C57BL/6 mice (WT/CON); (2) Alum-sensitized/saline-challenged VDUP1 KO mice (KO/CON); (3) OVA-sensitized/OVA-challenged C57BL/6 mice (WT/OVA); and (4) OVA-sensitized/OVA-challenged VDUP1 KO mice (KO/OVA) (Fig. 1).

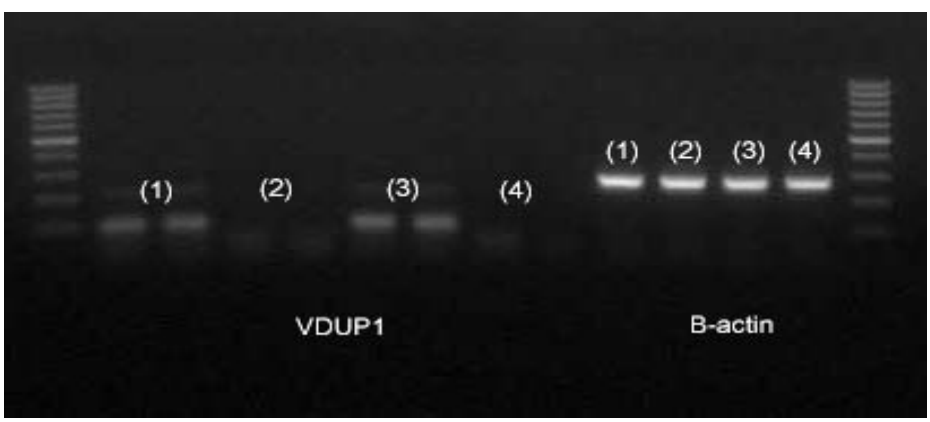

Fig. 1. Confirmed VDUP1 KO mouse. To confirm knock out of the VDUP1

gene, we performed polymerase chain reaction (PCR) using VDUP1 primer (101bp). (1) Alum-sensitized/saline-challenged C57BL/6 mice (WT/CON); (2) Alum-sensitized/saline-challenged VDUP1 KO mice (KO/CON); (3) OVA-sensitized/OVA-challenged C57BL/6 mice (WT/OVA); and (4) OVA-sensitized/OVA-challenged VDUP1 KO mice (KO/OVA).

Allergen sensitization and airway challenge

Mice (Groups (3 and 4)) underwent OVA sensitization and challenge as previously described with slight modifications (Cho, Seo et al. 2010). Mice received an intraperitoneal injection of 200 μg OVA (Sigma Aldrich, St. Louis, MO, USA) emulsified in 2.5mg of adjuvant aluminum hydroperoxide gel (alum; Thermo Scientific, Rockford, IL, USA). A second intraperitoneal injection of 20 μg OVA adsorbed onto alum (2.5mg) was administered 10 days later. After an additional 10 days, mice were exposed to an aerosol of 1% OVA in saline for 30 minutes daily on three consecutive days using an Ultrasonic Atomizer (Model 402A; YuYue Company, Jiangsu, China). On day 25, mice were finally challenged by provocation with 8% OVA aerosol.

The control mice (Groups (1 and 2)) received an intra-peritoneal

injection of saline with alum on the first day and 10 day later. After an additional 10 days, mice were exposed to an aerosol of saline on day 21, 22, 23, and 25(Fig. 2).

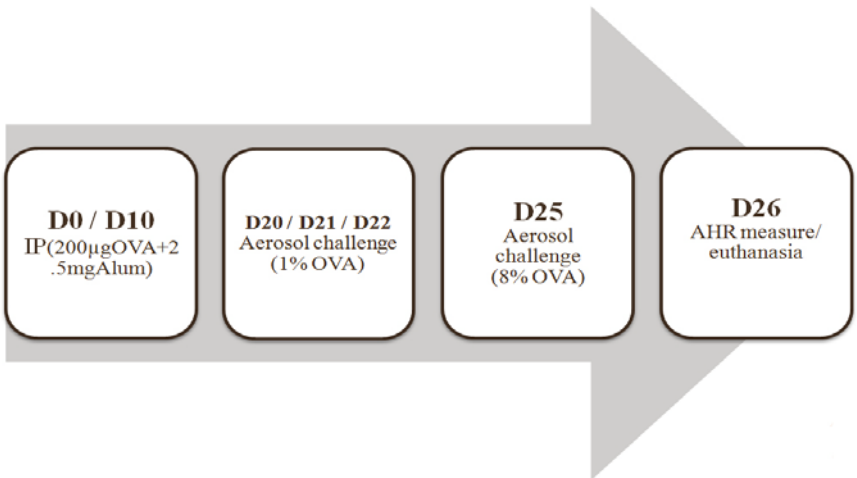

Fig. 2. Schedule of allergen sensitization and challenge for 26 days.

Airway hyperresponsiveness (AHR)

In vivo airway responsiveness to methacholine was measured 24 hours after the last OVA challenge in conscious, freely moving, spontaneously breathing mice, using whole-body plethysmography (Model PLY 3211; Buxco Electronics, Sharon, CT, USA). The mice were challenged with aerosolized saline or methacholine (3, 10, and 30 mg/ml), generated by an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA, USA) for 1 minute. The degree of bronchoconstriction was expressed as enhanced

pause (P_{enh}), a calculated dimensionless value that correlates with measurements of airway resistance, impedance, and intrapleural pressure in the same mouse. P_{enh} values, based on readings collected over 4 minutes and averaged after each nebulization challenge, were calculated as follows: $P_{enh} = [(T_e/T_r - 1) \times (PEF/PIF)]$, where T_e is the expiration time, T_r is the relaxation time, PEF is the peak expiratory flow, and PIF is the peak inspiratory flow × 0.67 (a coefficient).

Bronchoalveolar lavage fluid (BALF)

After measurement of AHR, mice were euthanized using CO_2 gas. Bronchoalveolar lavage fluid (BALF) was performed on the right lung (1.2 ml saline) after tying off the left lung at the main stem bronchus. BALF was centrifuged at 200g and 4℃ for 10 minutes, and the supernatant collected and stored at −70℃. Total BALF cell numbers were assessed by counting cells in at least five squares of a hemocytometer after exclusion of dead cells stained with trypan blue. The cell pellets were resuspended in saline on a slide, and centrifuged (200g, 4℃, 10 min) for fixation using a cytospin machine (Model Shandon Cytospin 4; Thermo Fishcer Scientific, Waltham, MA, USA). After slides were dried, cells were fixed and stained using Diff-Quik®

stain reagents (B4132-1A; Dade Behring Inc., Deerfield, IL, USA) according to the manufacturer's instructions.

IgE level in BAL fluid

Levels of IgE in BALF were measured using enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's protocols (BioLegend, San Diego, CA, USA).

Histopathology

After BALF sampling was completed, the trachea and upper and lower lobes of the left lung were removed, and fixed for 24 hours in 10% neutral buffered formalin solution. The tissues were embedded in paraffin, cut into 3 μm sections, and stained with hematoxylin and eosin (H&E) or periodic-acid stain (PAS). The degree of mucus occlusion of the airway diameter, and airway edema were determined by morphometry. Morphometric analyses were performed by individuals blinded to the protocol design, as previously described with minor modification (Oh, Cha et al. 2011). Briefly, for each mouse, eight airway sections, randomly distributed throughout the left lung, were analyzed, and the average score of each point was calculated. The occlusion of the

airway diameter by mucus was assessed on a semiquantitative 0–to–4+ scale based on the following criteria: 0, no mucus; 1, ~25% occlusion, 2, ~50% occlusion; 3, ~75% occlusion; 4, ~100% occlusion. The degree of edema was assessed according to the following criteria: 0, no edema; 1, ~25% airway involved; 2, ~50% airway involved; 3, ~75% airway involved; 4, ~100% airway involved. Total lung inflammation was defined as the sum of the peribronchial and perivascular scores (McKay, Leung et al. 2004). Peribronchial and perivascular inflammation was quantified and graded on the following scale; 0, none; 1, mild; 2, moderate; 3, marked; and 4, severe.

Immunohistochemistry

iNOS expression in mouse lung tissues was detected by immunohistochemistry using rabbit polyclonal iNOS antibody (Abcam, Cambridge, MA; 1:400). Briefly, the slides were deparaffinized, and rehydrated, and then antigen retrieval was performed by heating at 100℃ for 15 minutes in citrate buffer(pH 6.0). Three percent hydrogen peroxide and serum–free protein block were used to block endogenous peroxidases and prevent non–specific protein binding. The slides were incubated with the antibodies of iNOS at room temperature for 1 hour.

The sections were incubated with the secondary biotinylated anti-rabbit IgG (1:100) for 30 minutes followed by incubation with the Vector Elite ABC kit for 30 minutes. Specific antibody binding sites were visualized by incubation for 2 minutes with a diaminobenzidine (DAB) substrate kit. The slides were counterstained with hematoxylin for 1minutes. Negative controls were performed by omitting the primary antibody and substitution with diluent. Morphometric analyses were performed by individuals blinded to the protocol design, as previously described with modification (Xiang-feng, Shuang et al. 2010). The intensity of staining was graded as follows: 0. absent, 1. mild, 2. Moderate, or 3. Intense. For each slide, 20 peribronchial and perivascular areas were evaluated and a total score was assigned to each animal.

RNA isolation and real-time quantitative PCR

Total RNA was extracted from lung tissue using an RNA extraction kit (RNeasy Mini Kit, QIAGEN), according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed with and analyzed by real-time PCR using Rotor-Gene SYBR Green RT-PCR kit (QIAGEN, Hilden, Germany) with specific primers for TNF- α, IL-1β, IL-4, IL-5, IL-6, IFN-γ), iNOS, and β-actin (Table 1). Reaction was

amplified and quantified using Rotor-Gene Q and the manufacturer's software (QIAGEN, Hilden, Germany). The cycling condition included an initial step at 95℃ for 5 miniutes, then 45 cycles of the following steps: 95℃ for 10 seconds, 60℃ (TNF-α), 56℃ (IL-6, IL-4), 59℃ (iNOS, IL-1β), 58℃ (IFN-γ) for 30 seconds. The relative messenger RNA (mRNA) expression level was normalized by β-actin. Melting curves also showed a single sharp peak indicating one PCR product.

Table 1. Oligonucleotide primers for real-time quantitative PCR

Primer	Accession No.	Oligonucleotide
TNF-α	NM_013693	
Forward		5'-AGCCCCCACTCTGACCCCTTTAC-3'
Reverse		5'-TGTCCCAGCATCTTGTGTTTCT-3'
IL-6	NM_031168	
Forward		5'-GAGGATACCACTCCCAACAGACC-3'
Reverse		5'-AAGTGCATCATCGTTGTTCATACA-3'
IL-4	NM_021283	
Forward		5'-ACAGGAGAAGGGACGCCAT-3'
Reverse		5'-GAAGCCCTACAGACGAGCTCA-3'
iNOS	NM_010927	
Forward		5'-GGCAGCCTGTGAGACCTTTG-3'
Reverse		5'-CGTTTCGGGATCTGAATGTGA-3'
IL-1β	NM_008361	
Forward		5'-TGGAGAGTGTGGATCCCAAGCAAT-3'
Reverse		5'-TGTCCTGACCACTGTTGTTTCCCA-3'
IFN-γ	NM_008337	
Forward		5'-AGCGGCTGACTGAACTCAGATTGTAG-3'
Reverse		5'-GTCACAGTTTTCAGCTGTATAGGG-3
β-actin	NM_007393	
Forward		5'-CAGGAGATGGCCACTGCCGCA-3'
Reverse		5'-TCCTTCTGCATCCTGTCAGCA-3'

Statistical analysis

Statistical analyses were performed using SPSS Statistics (version 20.0; SPSS Inc., Chicago, IL, USA) All data are expressed as means ± standard error of the mean (SEM). Data were compared using an unpaired two-tailed Student's *t*-test. Differences with calculated values of less than 0.05 were considered statistically significant.

Results

Loss of VDUP1 effect on AHR

AHR to inhaled methacholine at 10mg/ml was significantly increased in the OVA-challenged knockout mice group (KO/OVA) compared with that in the OVA-challenged wild mice group (WT/OVA) ($P<0.01$). Furthermore there was a notable significant increase of AHR in KO/OVA compared with WT/OVA at a methacholine concentration of 30mg/ml ($P<0.005$) (Fig. 3).

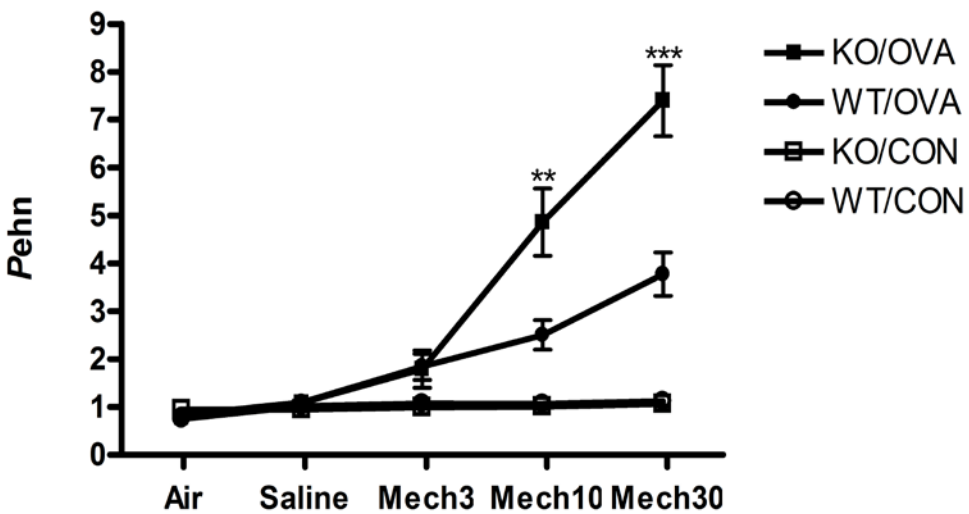

Fig. 3. Effect on AHR to methacholine in OVA-challenged VDUP1 KO mice. The degree of bronchoconstriction, expressed as P_{enh}, to inhaled methacholine (3, 10, and 30 mg/ml) was determined in the saline-

treated group (WT/CON, KO/CON; n =5), OVA-challenged wild mice group (WT/OVA; n=7), and OVA-challenged knockout mice group (KO/OVA; n=6). **P<0.01, ***P<0.005 compared with WT/OVA.

Loss of VDUP1 effect on inflammatory cells in BALF

In saline challenged groups both of WT/CON and KO/CON, the number of total leukocytes and eosinophils in the BALF remained essentially unchanged. The OVA-sensitized and OVA-challenged mouse groups displayed a significant increase both in total cells and eosinophils. In particular, VDUP1 KO/OVA caused a marked infiltration of leukocytes into the lung, increasing the total number of leukocytes recovered in BALF by 2.19-fold compared with that in the WT/OVA mice group (P<0.05) (Fig. 4a). In addition, there was a 2.49-fold increase in the total number of eosinophils in the KO/OVA group compared to WT/OVA (P<0.05) (Fig. 4b).

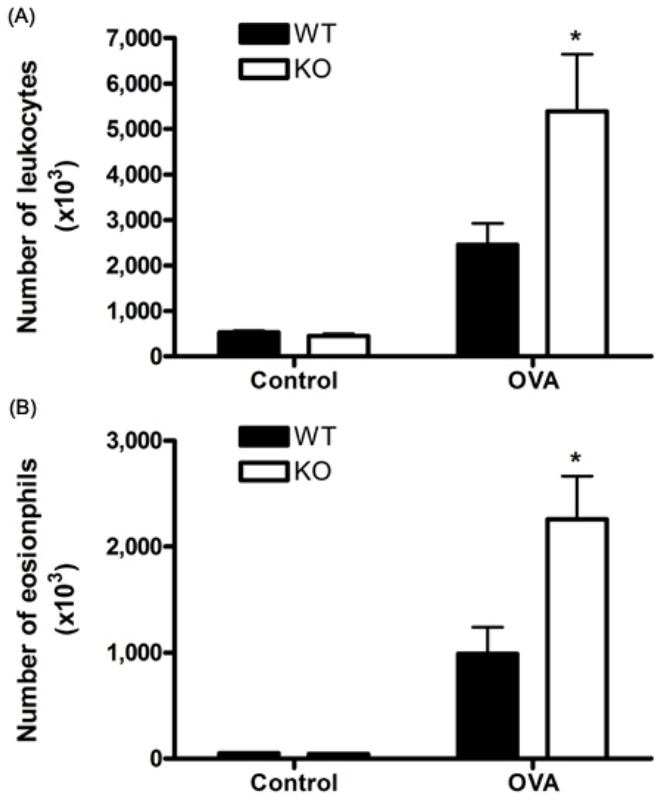

Fig. 4. Differences in inflammatory cells in BALF; Total leukocytes in BALF. BALF was obtained from saline treated wild type mice(WT/CON, n=5), saline-treated VDUP1/KO mice (KO/CON, n=5), OVA-challenged wild-type mice (WT/OVA, n=5), and OVA-challenged VDUP1/KO mice (KO/OVA, n=4). The numbers of total leukocytes (a) and eosinophils (b) present in the BALF from each group were determined. The results shown are the means ± SEM. $*p < 0.05$ compared with WT/OVA

Loss of VDUP1 effect on airway edema and airway goblet-cell hyperplasia and mucus production

Light microscopy revealed that the lungs of saline-challenged mice (WT/CON, KO/CON) were normal in appearance. In OVA-challenged mice (WT/OVA, KO/OVA), there were edema lesions in lung section. We stained lung sections with PAS stain to observe mucus secretion. Mucus hypersecretion was significantly detected in the airway (mucus observed as a violet color) in OVA-challenged mice compared with saline-challenged mice. We also found that there was a trend toward increased edema in KO/OVA by 1.75-fold compared with WT/OVA (Fig. 5i $P<0.05$). Mucus occlusions were also marked in KO/OVA (Fig. 5h) by 2.4-fold compared with WT/OVA (Fig. 5f, Fig. 5i, $P<0.05$).

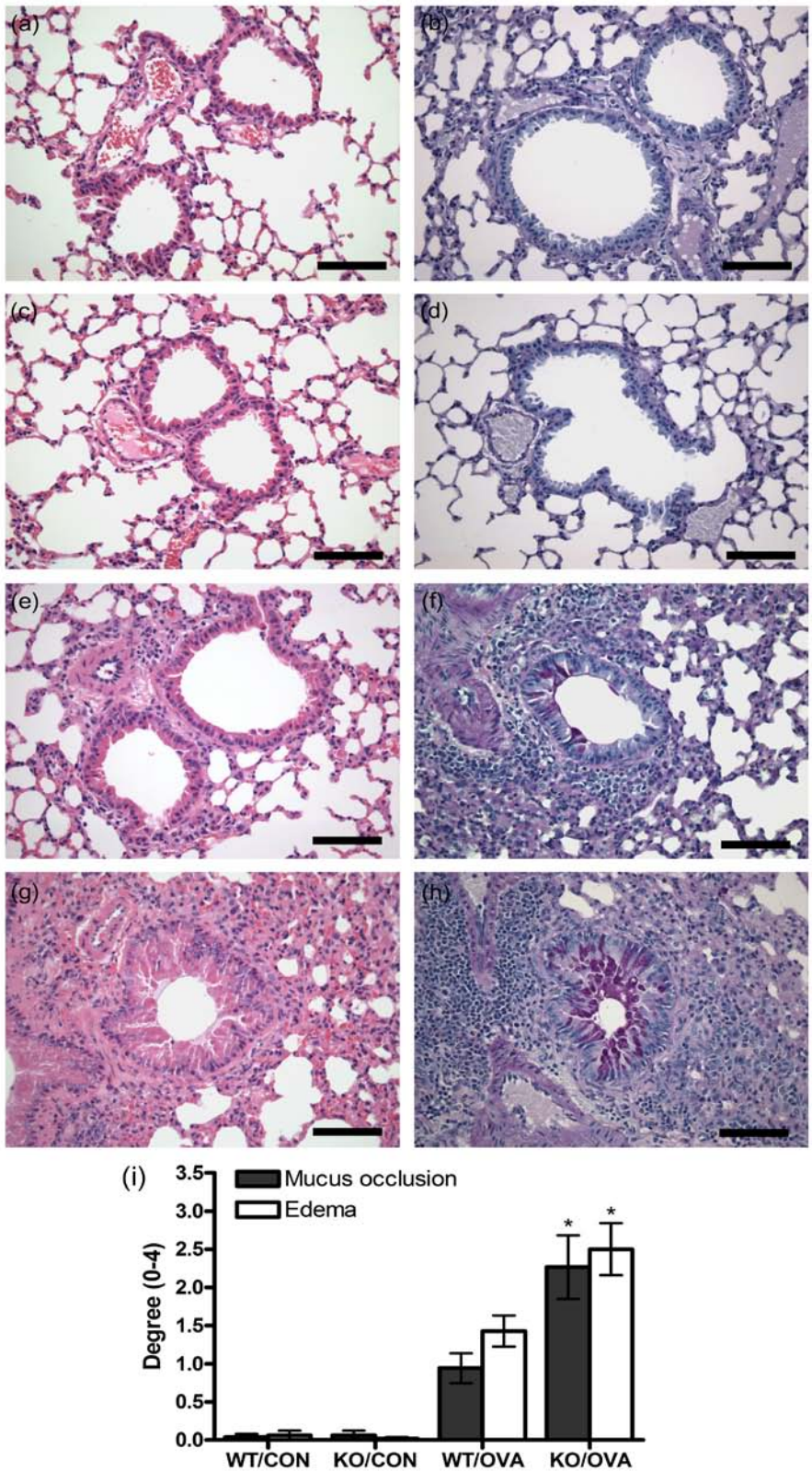
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Mucus occlusion
Edema
Degree (0-4)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
WT/CON
KO/CON
WT/OVA
KO/OVA

Fig. 5. Microscopic appearance of lung sections; (a, c, e, g) H&E stain, (b, d, f, h) PAS stain. Representative images of lung sections from WT/CON (a, b), KO/CON (c, d), WT/OVA (e, f) and KO/OVA (g, h) groups are shown. The lung sections were stained with H&E (a, c, e, g) or PAS stain (b, d, f, h). (i) The grades of intensity of mucus occlusion and airway edema. The results shown are the means ± SEM. $*p < 0.05$ compared with WT/OVA.

Loss of VDUP1 effect on inflammatory cell accumulation in lung tissue

Inflammatory cells were not detected in lungs of WT/CON mice or KO/CON mice, whereas the OVA-challenged mouse groups showed a marked infiltration of inflammatory cells, including eosinophils and mononuclear cells around the peribronchial and perivascular space. Infiltration of leukocytes into the lung was aggravated in KO/OVA mice (Fig. 6b, 6d) compared with WT/OVA mice (Fig. 6a, 6b). In particular, there was marked perivascular infiltration of inflammatory cells in KO/OVA mice (Fig. 6d).

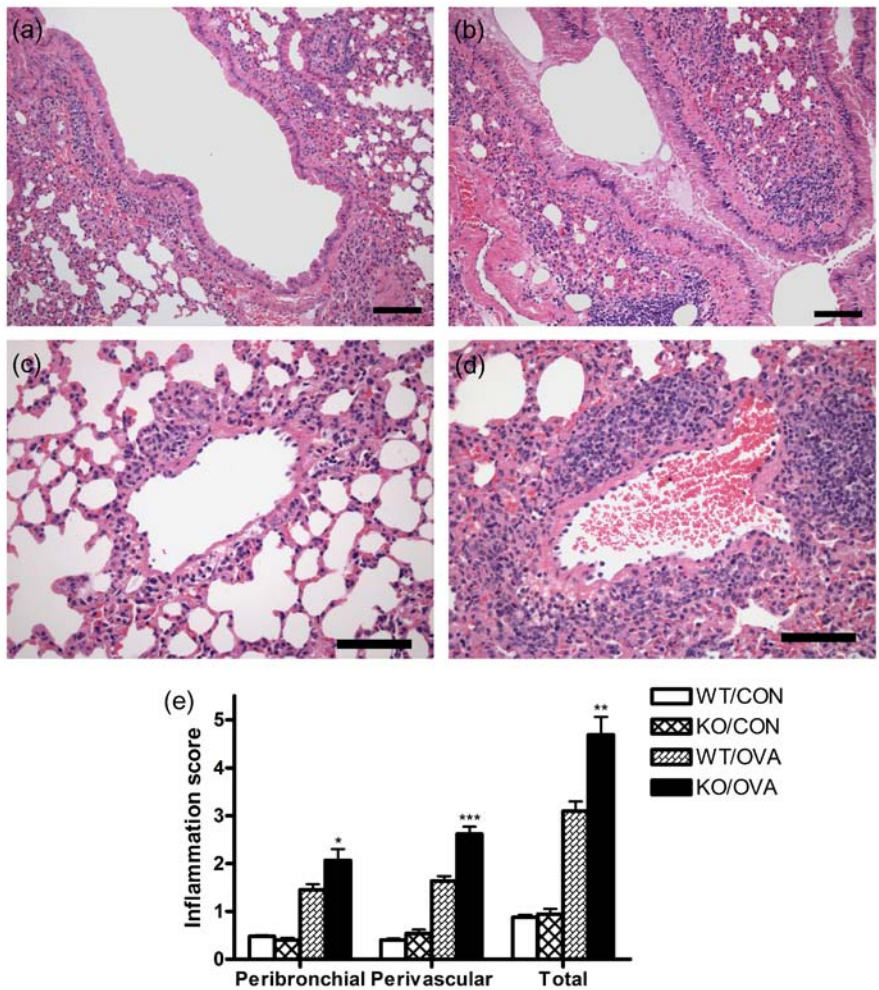

Fig. 6. Histological analysis demonstrated that lung inflammation increased in KO/OVA mice. WT/OVA; peribronchial(a) and perivascular(c) section. KO/OVA; perbronchial(b) and perivascular(d) section. In KO/OVA mice, an increase in inflammatory infiltrate was found compared with WT/OVA. Inflammation scores for the presence of peribronchial and perivascualr inflammation (e); these scores were added to total lung inflammation

score. The results shown are the means ± SEM. $*p < 0.05$ compared with WT/OVA.

Loss of VDUP1 effect on expression of iNOS protein in mouse lung tissue

iNOS protein was expressed in inflammatory cells mainly around vessels(Fig 7a, 7b). A number of iNOS-positive immunostaining cells were semiquantitatively higher in KO/OVA than WT/OVA mice, and these were mice mainly located in the cytoplasm (Fig. 7c).

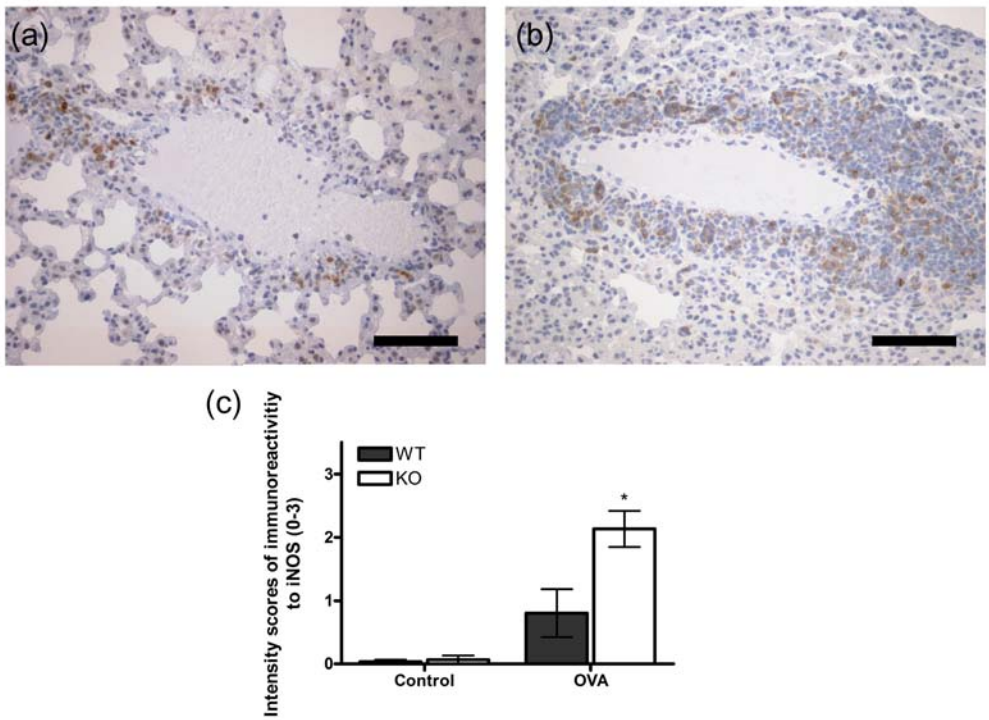

Fig. 7. Immunohistochemical expression of iNOS in the mouse lung. The level of iNOS protein in inflammatory cells around vessels was higher in the KO/OVA mice ((b), n=4) than in the WT/OVA mice ((a), n=3); intensity scores of immunoreactivity to iNOS (c), *p<0.05.

Loss of VDUP1 effect on expression of cytokines

To evaluate the difference in cytokine expression in lung tissue, we examined the levels of cytokines associated with asthma in BALF (Fig. 7). The mRNA levels of TNF-α, IL-4, and iNOS in lungs were markedly increased in KO/OVA, compared with WT/OVA. In addition, the expressed mRNA of IL-6, and IL-1β in the lung tissue of KO/OVA mice was higher than that of WT/OVA mice. There was no significant difference among KO/OVA mice and WT/OVA mice in term of the mRNA level of IFN-γ.

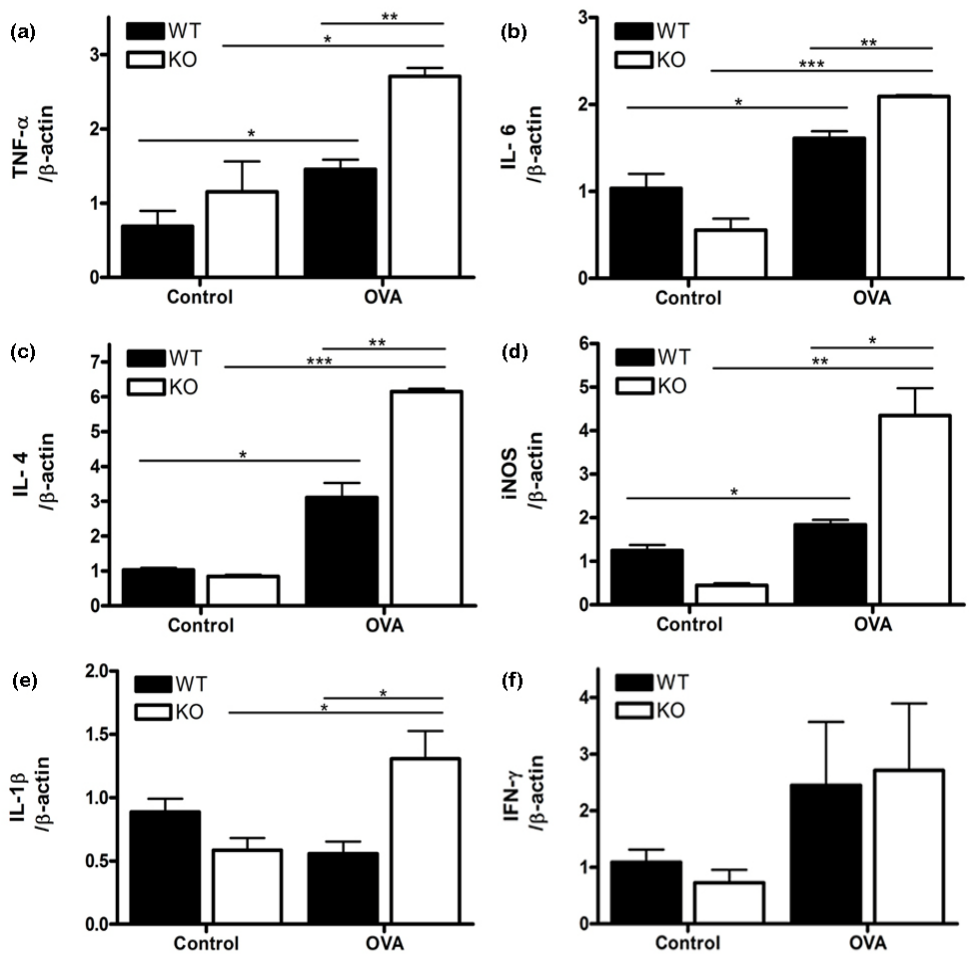

Fig. 8. Cytokine levels in lung tissue. Lung tissue was collected 48 h after the last OVA challenge. Quantitative real-time PCR was performed for each sample. TNF-a (a), IL-6 (b), IL-4 (c), iNOS (d) IL-1β (e) and IFN-γ level expression (f) in lung sections of various groups of mice were determined using quantitative real-time PCR. Values are presented as means±SEM (n=3-4 for each group). *p<0.05, **p<0.005, ***p<0.001.

Total IgE into BAL Fluid

IgE levels in BALF were markedly increased in KO/OVA mice compared with WT/OVA mice (7.505 ± 1.373 ng/ml vs. 19.979 ± 5.372 ng/ml; $p<0.05$) (Fig. 8).

Fig. 9. Level of total IgE in BAL fluid. BALF was collected 48 hours after the final OVA challenge in mice. Each sample was analyzed using ELISA (n=3−4/group). The results shown are the means ± SEM. $*p < 0.05$, compared with WT/OVA.

Discussion

To the best of our knowledge, this is the first report demonstrating a role of VDUP1 in regulating airway responsiveness and allergic airway inflammation using mice with targeted deletion of the VDUP1 gene in an animal model. In OVA-sensitized and exposed to VDUP1 KO mice, we observed; 1) significant aggravation in the severity of allergic airway inflammation, as evidenced by high inflammatory scores, edema, and increased mucus production; 2) significantly increased AHR; 3) increased number of total leukocytes and eosinophils and increased level of IgE in BALF. Therefore, VDUP1 may have a direct or indirect relation in the infiltration of inflammatory cells associated with the asthmatic response, and further, may affect the development of AHR.

In this study, we also demonstrated that deficiency of VDUP1 aggravated asthmatic lesion in association with several inflammatory molecules such as TNF-α and iNOS. We found that the mRNA level of TNF-α in VDUP1 deficiency increased in OVA- challenged asthmatic lung tissue. TNF-α plays a role in the initiation of allergic asthmatic airway inflammation and generation of airway hyperreactivity, and is released in allergic responses; elevated levels of TNF-α have been demonstrated in BALF (Thomas 2011). TNF-α is considered a

potentially important cytokine in asthma, in particular in those with severe refractory disease. In asthmatic patients, the mRNA and protein level of TNF-α are elevated compared with nonasthmatic people (Brightling, Berry et al. 2008). AHR is a key component of the asthma mouse model, caused by a direct effect of TNF-α on airway smooth muscle (ASM) (Huber, Beutler et al. 1988). Furthermore, TNF-α affect the activation of neutrophils, eosinophils and T-lymphocytes, which can release various cytokines concerned in typical lesions of asthma. In addition TNF-α affects the expression of NF-κB, which is the one of the transcription factor conducting a crucial role in asthma (Epstein, Barnes et al. 1997). Thus, anti-TNF-α has been considered as a therapeutic agent for asthma (Brightling, Berry et al. 2008). According to a recent study on the role of VDUP1 in a tumor formation model, a lack of VDUP1 results in increased tumorigenesis caused by increasing TNF-α.

We also found that there is increased of iNOS protein and iNOS mRNA expression in KO/OVA mice compared with WT/OVA mice. Although iNOS found in normal airway epithelium, it may be induced in several types of cells such as T cells and macrophages in response to cytokines, endotoxin, or reactive oxygen species(Dweik 2005, Batra, Pratap Singh et al. 2007). According to the literature, lung sections of OVA-

challenged asthmatic mice were found to be positive for iNOS immunostaining, which was found to be present mainly in the cells in the inflammatory infiltrate; in contrast in PBS-challenged mice, no such staining was observed (Batra, Chatterjee et al. 2007). In this study, we found that the expression of iNOS protein mainly occurs in inflammatory cells around perivascular spaces. Moreovere, the intensity of iNOS was higher in KO/OVA compared with WT/OVA.

iNOS gene expression is regulated by complex mechanisms including TNF-α, IL-1β, and NF-κB (Kwon and George 1999). Nitric oxide produced by iNOS, which requires a larger amount of substrate, also produces epithelial injury by forming peroxynitrite, which causes airway hyperresponsiveness (Duguet, Iijima et al. 2001). It was suggested that increasing iNOS may affect AHR. Moreover, as discussed above, TNF-α can also influence AHR, increasing eosinophils and several cytokines that effect mucus production, as well as Ig E levels.

In the current study, we confirmed the increase in the levels of cytokines such as IL-1β, IL-6 and IL-4(data not shown). Together with TNF-α, IL-1β can induce or up-regulate the expression of endothelial cells, as well as respiratory epithelial cells, which may lead to increased adhesion of eosinophils to the vascular endothelium and respiratory epithelium (Pober, Gimbrone Jr et al. 1986, Gosset,

Tsicopoulos et al. 1992). Moreover, there is increased release of IL-6 from alveolar macrophages from asthmatic patients after allergen challenge (Gosset, Tsicopoulos et al. 1991) and IgE-dependent triggering stimulates the secretion of IL-6 in both blood monocytes and alveolar macrophages *in vitro* (Gosset, Tsicopoulos et al. 1992). Furthermore, inhalation of IL-4 by asthmatics causes an increase in bronchial responsiveness. IL-4 overexpression in mouse airways induces mucin hypersecretion, indicating a potential role for IL-4 in mucus hypersecretion(Chung and Barnes 1999).

According to a recent study, proinflammatory factors such as TNF-α, IL-6, and IL-1β are increased under stimuli like chemicals, infections and carcinogens in VDUP1-deficient condition(Kim, Suh et al. 2007, Kwon, Won et al. 2010). Further, VDUP1 deficiency can exacerbate tumorigenesis and chronic inflammation, and could be related to the increase in TNF-α discussed above (Kwon, Won et al. 2010, Kwon, Won et al. 2011). In asthma, it has been shown that the expression of VDUP1 decreases in eosinophils isolated from asthma patients (CAI, GAO et al. 2007). It has also been reported that VDUP1 correlates to eosinophil activation (Gao, Cai et al. 2006) and we confirmed the increase of eosinophils in BALF collected from KO/OVA mice. We suppose that it could be loss of VDUP1 effect on TNF-α increases.

Based on the over-expression of several cytokines in VDUP1 KO mice, we assumed that VDUP1 depletion affects the release of cytokines, mainly TNF-α, and consequently inflammatory molecules such as iNOS and interleukin species. In particular, we found high expression of iNOS protein in inflammatory cells through immunohistochemistry in VDUP1 KO OVA-challenged mice. An increase in these inflammatory molecules affects asthmatic histopathological lesions and AHR, as well as IgE levels. This might be correlated with a recent study on decreasing VDUP1 in asthma patients (CAI, GAO et al. 2007). Provided that asthma is a complicated disease that involves complicated immunological communication between many kinds of cells, several cytokines, oxidative stress, and inflammatory-mediated materials. Therefore, a significant amount of work is needed both experimental and theoretical to fully elucidate the complex mechanism underlying VDUP1 and several cytokines related to asthmatic lesions. We investigated the role of VDUP1 in asthma focusing on TNF-α and TNF-α induced inflammatory molecules, that influence by depletion of VDUP1 and the difference in the severity of lesions compared to WT mice. Our findings suggest that VDUP1 may be a helpful therapeutic target for the treatment of asthmatic inflammation.

Conclusion

AHR, infiltration of leukocytes into the lung, total number of leukocytes recovered in BALF, and mucus secretion by goblet cells in the bronchus were aggravated in VDUP1 KO/OVA mice compared with WT/OVA mice. The IgE level of BALF in VDUP1 KO/OVA mice was higher than that of WT/OVA mice. In addition, the mRNA of TNF-a, IL-1β, IL-6, IL-4 and iNOS increased in VDUP1 KO/OVA-treated mice compared with WT/OVA treated mice. Depletion of VDUP1 aggravated the development of allergic airway inflammation and hyperresponsiveness, possibly through the overexpression several cytokines. TNF-α not only contributed to allergic inflammation and AHR, but was also enhanced by lymphocyte recruitment and Th2 cytokine production. It has been found that TNF-α and IL-1β can costimulate secretion of iNOS from human alveolar epithelial cells and influence asthmatic lesions. Our findings suggest that VDUP1 may be a helpful therapeutic target for the treatment of asthmatic inflammation.

References

Batra, J., et al. (2007). "Inducible Nitric Oxide Synthase (iNOS): Role in Asthma Pathogenesis." Indian Journal of Biochemistry & Biophysics **44**: 303-309.

Batra, J., et al. (2007). "Association of inducible nitric oxide synthase with asthma severity, total serum immunoglobulin E and blood eosinophil levels." Thorax **62**(1): 16-22.

Brightling, C., et al. (2008). "Targeting TNF-α: A novel therapeutic approach for asthma." Journal of Allergy and Clinical Immunology **121**(1): 5-10.

CAI, S., et al. (2007). "Expression of VDUP1 in peripheral eosinophil of asthmatic patients and its relation with eosinophil activation [J]." Chinese Journal of Pathophysiology **6**: 019.

Cho, K. J., et al. (2010). "Blockade of airway inflammation and hyperresponsiveness by inhibition of BLT2, a low-affinity leukotriene B4 receptor." American Journal of Respiratory Cell and Molecular Biology **42**(3): 294.

Chung, K. F. and P. J. Barnes (1999). "Cytokines in asthma." Thorax **54**: 825– 857.

Duguet, A., et al. (2001). "Eosinophil peroxidase mediates protein nitration in allergic airway inflammation in mice." American journal of respiratory and critical care medicine **164**(7): 1119-1126.

Dweik, R. (2005). "Nitric oxide, hypoxia, and superoxide: the good, the bad, and the ugly!" Thorax **60**(4): 265-267.

Epstein, F. H., et al. (1997). "Nuclear factor-κB—a pivotal transcription factor in chronic inflammatory diseases." New England Journal of Medicine **336**(15): 1066-1071.

Gao, F., et al. (2006). "Expression of thioredoxin-binding protein-2/vitamin D3 upregulated protein-1 in peripheral blood eosinophils of asthma patients." Nan fang yi ke da xue xue bao= Journal of Southern

Medical University 26(4): 371.

Gosset, P., et al. (1992). "Tumor necrosis factor alpha and interleukin-6 production by human mononuclear phagocytes from allergic asthmatics after IgE-dependent stimulation." American journal of respiratory and critical care medicine 146(3): 768-774.

Gosset, P., et al. (1991). "Increased secretion of tumor necrosis factor α and interleukin-6 by alveolar macrophages consecutive to the development of the late asthmatic reaction." Journal of Allergy and Clinical Immunology 88(4): 561-571.

Huber, M., et al. (1988). "Tumor necrosis factor α stimulates leukotriene production in vivo." European journal of immunology 18(12): 2085-2088.

Junn, E., et al. (2000). "Vitamin D3 up-regulated protein 1 mediates oxidative stress via suppressing the thioredoxin function." The Journal of Immunology 164(12): 6287-6295.

Kim, S. Y., et al. (2007). "Diverse Functions of VDUP1 in Cell Proliferation, Differentiation, and Diseases." Cellular & Molecular Immunology 4(5): 345-351.

Kips, J. C. (2001). "Cytokines in asthma." European Respiratory Journal 18(1): 24-33.

Kwon, H. J., et al. (2011). "Vitamin D3 upregulated protein 1 deficiency promotes N-methyl-N-nitrosourea and Helicobacter pylori-induced gastric carcinogenesis in mice." Gut 61(1): 53-63.

Kwon, H. J., et al. (2010). "Vitamin D3 Upregulated Protein 1 Suppresses TNF- -Induced NF- B Activation in Hepatocarcinogenesis." The Journal of Immunology 185(7): 3980-3989.

Kwon, H. J., et al. (2010). "Vitamin D3 upregulated protein 1 suppresses TNF-alpha-induced NF-kappaB activation in hepatocarcinogenesis." J Immunol 185(7): 3980-3989.

Kwon, S. J. and S. C. George (1999). "Synergistic Cytokine-Induced Nitric Oxide Production in Human Alveolar Epithelial Cells." NITRIC OXIDE: Biology and Chemistry 3(4): 348– 357.

Lee, K. N., et al. (2005). "VDUP1 is Required for the Development of Natural Killer Cells." Immunity **22**(2): 195-208.

McKay, A., et al. (2004). "A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma." The Journal of Immunology **172**(5): 2903-2908.

Oh, S.-W., et al. (2011). "Curcumin attenuates allergic airway inflammation and hyper-responsiveness in mice through NF-κB inhibition." Journal of Ethnopharmacology **136**(3): 414-421.

Pober, J. S., et al. (1986). "Overlapping patterns of activation of human endothelial cells by interleukin 1, tumor necrosis factor, and immune interferon." The Journal of Immunology **137**(6): 1893-1896.

Thomas, P. S. (2011). "Tumour necrosis factor-α: The role of this multifunctional cytokine in asthma." Immunology and Cell Biology **79**: 132-140.

Xiang-feng, Z., et al. (2010). "Osteopontin protects against hyperoxia-induced lung injury by inhibiting nitric oxide synthases." Chinese medical journal **123**(7): 929-935.

국 문 초 록

마우스 천식 모델에서 Vitamin D3 Up-regulated Protein 1(VDUP1)의 역할

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수의병리학 전공

천식은 사람에서 비교적 흔한 호흡기 질환으로 다양한 세포 및 싸이토카인들이 병변의 진행에 관여하여, 기도 내 점액 과분비와 폐 조직 내부의 염증 및 기도 과민성 증가 등을 유발한다. VDUP1 은 1,25[OH]$_2$D$_3$를 처치한 HL-60 leukemia 세포에서 처음으로 발견되었으며, 세포의 성장과 사멸 등에 영향을 미치는 것으로 알려져 있다. 이는 주로 가슴샘이나 비장 등에 분포하여 면역 조절에도 영향을 줄 수 있는 것으로 알려져 있으며 발암이나 만성 염증 등에 영향을 미칠 수 있다. 이러한 과정에는 싸이토카인, 림포카인, 전사인

자와 같은 면역 매개 인자들의 역할이 큰 것으로 알려져 있다. 현 연구에서 VDUP1의 결핍이 천식 유발에 어떠한 영향을 미치는지 알아보기 위하여 VDUP1 이 결핍된 마우스로 천식을 유발하여 기도저항 및 기관지세척액 분석, 폐의 조직학적 분석 및 면역염색, 싸이토카인 발현 정도 등을 확인해 보았다. 이 결과, VDUP1 이 결핍된 마우스에서 기도저항의 증가 및 조직학적으로 천식 병변의 악화가 관찰되었고 여기에는 TNF-α, iNOS 를 포함한 다양한 싸이토카인의 증가가 연관된 것을 확인하였다. 따라서 VDUP1과 천식과의 연관뿐 아니라 나아가 VDUP1이 천식 병변의 치료적 표적으로 연구 될 수 있음을 확인할 수 있었다.

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주요어 : VDUP1, 천식, 기도 과민성, iNOS

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