의학석사 학위논문

The effect of rhinovirus infection on the expression of programmed cell death ligand and inflammatory cytokines in patients of allergic rhinitis

알레르기 비염 환자에서 Rhinovirus 감염이 Programmed cell death ligand 와 사이토카인 발현에 미치는 영향

2015년 8월

서울대학교 대학원

의학과 면역학 전공

고영경

Abstract

The effect of rhinovirus infection on the expression of programmed cell death ligand and inflammatory cytokines in patients of allergic rhinitis

Young Kyung Ko

Department of Immunology

The Graduate school of Medicine

Seoul National University

Background and Objectives: Rhinovirus (RV) is a common viral pathogen associated with upper respiratory infection (URI). There are complex interactions between allergic reaction and viral infection. Some studies suggest that patients with allergy appear to be more vulnerable to rhinovirus infections, however other studies

have contradicted this. Rhinovirus infection affects the expression of co-stimulatory molecules and inflammatory cytokines. However, it remains unclear that the relevance between allergic status and rhinovirus infection. Therefore, in this study, we aimed to find out the susceptibility to rhinovirus infection and its effects on the expressions of co-stimulatory molecules and cytokines in patients with allergic rhinitis.

Materials and Methods: Uncinate process tissues were obtained from 39 patients with allergy and 21 patients without allergy who underwent surgical management of endoscopic sinus surgery (ESS) due to chronic rhinosinusitis (CRS). The mucosa was infected with RVs, and RT-PCR and semi-nested PCR were used to confirm the rhinovirus infection. Real-time PCR was used to determine gene expression level of programmed cell death ligand 1 (PDL1), programmed cell death ligand 2 (PDL2), intracellular adhesion molecule 1 (ICAM-1), IFN-γ, IL-4, IL-5, and IL-10 in nasal mucosa tissues. Western blotting and immunohistochemistry were performed to evaluate the level of PDL1, PDL2, ICAM-1 and IL-10 protein in nasal mucosa tissues.

Results: The RV infection rates were not significantly different between allergy and non-allergy group.

In allergy group, compared to control tissues, RV infected tissues showed significantly elevated expression of PDL2, ICAM-1, IL-4

and IL-10. On the other hand, RV infection to tissues from non-allergy group increased levels of IL-4 and IL-5 compared to those of control tissues. In cases of PDL1 and IL-10, infected tissues from allergy group exhibit higher levels than those from non-allergy group. Interestingly, a high degree of correlation between PDL1 and IL-10 was seen in allergy group, but there was no definite correlation seen in non-allergy group. In cases of IL-4 and IL-5, infected tissues from non-allergy group exhibit higher levels than those from allergy group. There was no significant difference in expression of IFN-γ mRNA between two groups.

Conclusion: Allergy and non-allergy subjects tend to have a similar degree of susceptibility to RV infection. This study suggests that enhanced PDL1 and IL-10 can suppress the immune response to RV infection, and this suppression process is more likely induce severe symptoms in allergic patients with rhinovirus. Furthermore, elevated levels of IL-4 and IL-5 in infected tissue from non-allergy group suggest that repeated rhinovirus infection history may cause Th2 biased environment.

Key words: Allergy, CRS, rhinovirus infection, PDL1, PDL2, IL-10

Student Number: 2012-23632

Contents

List of Tables and Figures

List of Abbreviations

RV : Rhinovirus

ESS : Endoscopic sinus surgery

CRS : Chronic rhinosinusitis

PDL : Programmed cell death ligand

ICAM-1 : Intracellular adhesion molecule 1

ssRNA : single-stranded RNA

LDLR : Low density lipoprotein receptor

HRV : Human rhinovirus

IL : Interleukin

PD-1 : Programmed cell death 1

AHR : Airway hyperresponsiveness

iNKT : invariant Natural killer T cell

iTreg : induced T regulatory cell

TNF : Tumor necrosis factor

GM-CSF : Granulocyte-macrophage colony-stimulating factor

AR : Allergic rhinitis

SPT : Skin prick test

$TCID_{50}$: 50% tissue culture infective dose

FBS : Fetal bovine serum

MEM : Minimal essential medium

PBS : Phosphate buffered saline

RT-PCR : Reverse transcription polymerase chain reaction

IHC : Immunohistochemisty

DEPC : Diethylpyrocarbonate

cDNA : Complementary DNA

Tris : Tris(hydroxymethyl)aminomethane

EDTA : Ethylenediaminetetraacetic acid

DTT : Dithiothreitol

GAPDH : Glyceraldehyde 3-phosphate dehydrogenase

Ig : Immunoglobulin

HRP : Horseradish peroxidase

DAB : 3,3'-Diaminobenzidine

RIPA buffer : Radioimmunoprecipitation assay buffer

BCA assay : Bicinchoninic acid assay

SDS-PAGE : Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

PVDF : Polyvinylidene difluoride

TBST : Tris buffered saline-Tween20

SPSS : Statistical package for the social sciences

SEM : Standard error mean

ANOVA : One-way analysis of variance

Introduction

A common viral pathogen associated with upper respiratory infection is rhinovirus, which initially infects the nasal and nasopharyngeal epithelial cells (1). RV is a 30nm in diameter, non-enveloped, positive, and single-stranded RNA (ssRNA) virus belonging to the *Picornaviridae* family. Monocytes, macrophages and fibroblasts can be infected by RV, and airway epithelial cell is the major site of RV infection (2). Epithelial cells express the ICAM-1 or low density lipoprotein receptors (LDLR), which are the sites of RV attachment, on their surface (3). Human rhinovirus (HRV) induces expression of a wide range of pleiotropic cytokines, growth factors, and chemokines (4).

Viral modulation of inhibitory molecule B7 family on epithelial cells results in suppression or termination of immune responses. Programmed cell death ligand-1 (PDL1, B7-H1, CD274) and Programmed cell death ligand-2 (PDL2, B7-DC, CD273) belong to the B7 family and are widely expressed on cells such as activated T cells, B cells, monocytes (5, 6), dendritic cells (7), and macrophages (8) in order to modulate activation or inhibition (9). It is also noted that PDL1 and PDL2 were found on non-lymphoid tissues including the airway epithelial cells (10), suggesting that they may modulate local tissue T cell functions. Binding of PDL1

and PDL2 to their receptors, programmed cell death 1 (PD-1), inhibits regulates T cells, decreasing proliferation and production of effector cytokines, including IL-2 and IFN-γ (5, 7). Recently, PD-1 activity is required for the termination of the late phase of allergic inflammation (11). It is reported that IL-4 induces PDL2 more preferentially than IFN-γ, while IFN-γ induces PDL1 more preferentially than IL-4 on macrophage, suggesting that Th1 and Th2 responses mobilize PDL1 and PDL2 differentially (11). Opposing roles are shown between PDL1 and PDL2, regarding the regulation of airway hyperresponsiveness (AHR) and invariant natural killer T (iNKT)-cell-mediated activation. By Feedback loop, as PDL2, upregulated by IL-4, increases, it suppresses IL-4 production, and modulates the severity of asthma. Loss of PDL2 in this PDL2/IL-4 circuit results in increased IL-4 and AHR. On the other hand, the enhanced PDL1 expression inhibits IFN-γ by negative feedback loop and increases AHR. As a result, loss of PDL1 in this PDL1/IFN-γ circuit results in increased IFN-γ and diminished AHR (12).

Induction of PDL1 and PDL2 on epithelial cells stimulates the response (2). PDL1 promotes CD4 T cells to convert into the induced T regulatory cells (iTreg), which suppress the response of effector T cells (13). PDL1 Up-regulated by activated CD4 T cells, binds to PD-1, and triggers IL-10 production (14). IL-10 is

known to be the major anti-inflammatory cytokine with important function in controlling diverse immune responses such as preventing autoimmune and allergic inflammatory responses (15, 16). It down-regulates production of cytokines, expression of co-stimulating accessory molecules, and the processing and presentation of antigens (17, 18). Furthermore, it is primarily produced by monocytes, regulatory T cells, and a certain subsets of activated T cells and B cell. IL-10 can autoregulate its expression by negative feedback loop such as autocrine stimulation of the IL-10 receptor and inhibition of the p38 signaling pathway (19). IL-10 stimulates macrophages and regulatory T-cells to produce proinflammatory cytokines such as IFN-γ, IL-2, IL-3, tumor necrosis factor (TNF)-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). It also has an ability to suppress the functions of APC, to downregulate the function of dendritic cells, and to prevent the development of allergen-induced airway inflammation and AHR (17). However, it also stimulates Th2 cell, mast cells and B cell maturation and antibody production.

This study was conducted to examine whether nasal mucosa of patients with allergy might be more susceptible to RV infection. In addition, I investigated the effects of RV infection on the expression of PDL and inflammatory cytokines in nasal mucosa of patients of allergic and non-allergic patients.

Materials & Methods

Study subjects

Uncinate process mucosa were obtained from 39 patients with allergic rhinitis (AR) and 21 patients without allergic rhinitis who underwent surgical management of CRS at the Seoul National University Hospital and Seoul National University Bundang Hospital. The allergy group was consisted of 39 individuals (26 males and 13 females), ranging in age from 14 to 72 years (mean age, 43.5 years). The non-allergy group was consisted of 21 individuals (12 males and 9 females), ranging in age from 13 to 78 years (mean age, 37.5 years). The patients were diagnosed with CRS according to established criteria, and were admitted from December 2012 to January 2015. The presence of allergy was confirmed by skin prick test (SPT)(Allergopharma, Reinbek, Germany). Allergens used in skin prick test are listed in <Table 1>. Individuals who had viral URIs within six weeks and had been treated with medications, or had been visited hospital within six weeks, were excluded from the study.

Viral stock preparation

HRV-16, was bought ATCC. Fifty percent tissue culture infective dose ($TCID_{50}$) was performed for virus titration. The $TCID_{50}$ was determined by infection of HeLa cells in 96-well microtiter plates using serial dilution of virus samples using 50㎕ inoculum per well. And then, 2% fetal bovine serum (FBS) was added to a total of 500 ㎕ per well and the cells were incubated at 37℃, 5% CO2 for 5 days. The titer was calculated according to the Speraman-Karber method.

Experimental RV16 infection in the organ culture

Uncinate process tissues were washed three times in a normal saline solution to remove blood cells and mucus, divided into small pieces, placed in minimal essential medium(MEM) (GIBCO, Grand Island, NY, USA), and supplemented with 2mM L-glutamine, 20mM HEPES(GIBCO, Grand Island, NY, USA), penicillin(100U/ml), streptomycin(100㎍/ml) (GIBCO, Grand Island, NY), and amphotericin B(0.25㎍/ml) (GIBCO, Grand Island, NY) for overnight at 4℃.

Each tissue sample was incubated at the air-liquid interface organ culture system, with the epithelium up and the submucosa down, on a 6mm X 6mm support of muliticel (Kwang Woo medix Inc, Seoul, Korea), prehydrated for at least 1hr with culture medium.

Each tissue piece was placed on merocel in a well of a 24 well plate (Greenpia technology, Gyeonggi-do, South Korea) along with 1.25ml culture medium, so that the epithelium was above the liquid phase. Merocel soaked with culture medium can supply nutrients to nasal mucosa on the merocel.

One uncinate mucosa was cut into several pieces and the pieces were divided into three groups: treated with medium (control group), treated with avirulent RV16 that was exposed to UV, replication-deficient HRV (UV group) and treated with RV16 (experimental group). The surface of each tissue was applied with 50$\mu\ell$ of medium, avirulent RV16 and RV16(10^5 $TCID_{50}$U/ml) for 4h, and the samples were washed 6 times with phosphate buffered saline(PBS) to remove previously applied HRVs. In order to ascertain that no HRVs remain on the surface of tissue, HRVs were assayed in the 6^{th} washed PBS by using semi-nested reverse transcription polymerase chain reaction (RT-PCR). The mucosae were transferred to a second set of identical plates and incubated for 48h at 33℃ (21). The mucosae were transferred to a new plate, and were incubated for 48hr at 33℃

Determination of successful RV infection

When nasal mucosa tissues were infected with HRV, viral replication takes places, and HRVs are released to the cell surface

so that HRVs were detectable from the surface fluid. The tissues were moved to second plates, incubated for 48h and washed out with 280㎕ PBS to get a surface fluid. The surface fluid samples were centrifuged at 1800rpm at 4℃ for 15min. Then, the separated supernatant was kept frozen at −80℃ until measurement. HRV RNA was extracted from mucosal surface fluid using Trizol LS reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. On the other hand, a part of tissue was fixed in 10% formaldehyde for immunohistochemistry (IHC). The rest of the tissues were immediately frozen in liquid nitrogen and stored at −80℃ for extraction of RNA and protein.

Preparation of RNA and Detection of infection

Total RNA was extracted from surface fluids using Trizol LS reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. Extracted RNA was amplified by both RT−PCR and semi−nested PCR in a thermal cycler (Perkins Elmer 9600, Minneapolis, Minnesota, USA). In RT−PCR, extracted RNA was reverse−transcribed and amplified using the Superscript One−Step RT−PCR with Platinum Taq kit (Invitrogen, Gaithersburg, MD, USA). In a final volume of 20㎕, each PCR mixture contained 2X reaction Mix (a buffer containing 0.4mM of each dNTP, 2.4mM MgSO4), 1 ㎕ of

primer mix (OL26:OL27=2:1)<Table 1>, 0.4㎕ of Platinum Taq mix, 150ng of total RNA template and Diethylpyrocarbonate(DEPC) water. Amplification was performed in a thermal cycler (Perkins Elmer 9600, Minneapolis, Minnesota, USA) programmed for initial complementary DNA(cDNA) synthesis and pre-denaturation 50℃ for 1h and 94℃ 2min, followed by 36 cycles of denaturation for 30s at 94℃, annealing for 30s 50℃ and extension for 30s at 68℃. Final elongation time was 7min at 72℃. The PCR products were 388 base pair(bp), 5㎕ of the 20㎕ total PCR reaction was analyzed in 2% agarose gel.

For semi-nested PCR, the 388-bp RT-PCR product was subsequently amplified using the primers OL26 and JWA-1b which generated a 292-bp fragment within the original amplicon and the AmpliTaq 360 DNA polymerase kit (Applied Biosystems, Carlsbad, CA, USA). Each PCR mixture contained 10X AmpliTaq 360 buffer (a buffer containing 20mM trishydroxymethylaminomethane(Tris), pH8.0, 100mM KCl, 0.1mM ethylenediaminetetraacetic acid (EDTA), 1mM dithiothreitol(DTT), 0.5% Tween 20, 50% glycerol), 0.8㎕ of primer mix(OL26:JWA-1b=2:1)<Table.2>, 0.8㎕ of dNTP, 1.5㎕ of 25mM Magensium chloride, 2㎕ of GC enhancer, 0.2㎕ of AmpliTaq 360 DNA polymerase, 1㎕ of The RT-PCR product and DEPC water in a final volume of 20㎕. Amplification was performed in a thermal cycler (Perkins Elmer 9600, Minneapolis, Minnesota, USA)

and pre-denaturation for 2min at 95℃, followed by 25 cycles of denaturation for 30s at 94℃, annealing for 30s 55℃ and extension for 30s at 72℃. Final elongation time was 7min at 72℃. The PCR product was analyzed in 2% agarose gel.

RNA extract from tissue and cDNA synthesis

Total RNA was extracted and purified from human nasal mucosa using Trizol LS reagent (Invitrogen, Carlsbad, California) according to the manufacturer's protocol. 1.0㎍ of total RNA was reverse-transcribed into cDNA using amfiRivert Platinum cDNA synthesis master mix (GenDEPOT, Baker, USA). cDNA synthesis was performed in a thermal cycler (Perkins Elmer 9600, Minneapolis, Minnesota, USA).

Quantitative real-time PCR analysis

Real-time PCR was used to detect PDL1 (Hs01125301_m1), PDL2 (Hs01057777_m1), ICAM-1(CD54), IFN-γ, IL-4, IL-5 and IL-10. PDL1 and PDL2 were performed using a TaqMan Gene Expression Assay kit(Applied Biosystems, Foster City, CA, USA) with an ABI 7500 (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Hs99999905_m1) was an internal positive control. On the other

hand, ICAM-1, IFN-γ, IL-4, IL-5 and IL-10 were performed using a SYBR Green PCR master mix(Applied Biosystems, Warringron, UK) with an ABI 7500 (Applied Biosystems, CA, USA). Housekeeping gene GAPDH was an internal positive control. <Table 2> lists the genes that were analyzed and primers used. The comparative cycle of threshold (△△Ct) method was used to demonstrate the relative levels of target genes

Histology

To evaluate the histology of human nasal mucosal tissues, nasal mucosal tissues were fixed in 10% formalin at 4℃ with slow shaking and embedded in paraffin blocks. Paraffin-embedded tissue samples were cut into 4-㎛-thick section. The nasal tissues on slides were stained with immunohistochemistry (IHC) for PDL1. PDL1 IHC was performed by using Histostain Plus Broad Spectrum (Invitrogen, CA, USA) according to the manufacturer's protocol. Sectioned tissue was deparaffinized in xylene, and dehydrated through a graded series of ethanol solution. For antigen unmasking, sections were incubated at microwave for 15 minutes with low pH antigen unmasking solution (Vector laboratories.Inc, CA, USA). Endogenous peroxidase activity was inhibited by incubation for 10 min at room temperature with 3% hydrogen peroxide containing 100% methanol. Sections were incubated with blocking solution

(Invitrogen, CA, USA) for 1h at room temperature, and then incubated with 5㎍/㎕ of anti-CD274 antibody, polyclonal antibody PDL1 (Abcam, MA, USA) for overnight at room temperature. Negative control samples were incubated with blocking solution as primary antibody, and isotype control sample were incubated with rabbit immunoglobulin (Ig) G as a primary antibody. Slides were washed three times with PBS, labeled for 20 min with secondary antibody (Invitrogen, CA, USA), washed thrice with PBS, incubated with horseradish peroxidase (HRP)-streptavidin(Invitrogen, CA, USA) for 20min, and again washed with PBS. Reaction products were visualized with peroxidase substrate kit 3,3 ' - diaminobenzidine (DAB)(vector laboratories, CA, USA) and the slides were counter-stained with hematoxylin, dehydrated, and coverslipped. Histologic analyses were performed in a blinded manner by light microscopy linked to an image capture system (CX31 microscope, DP25 digital camera, and DP2-BSW software, version 2.1, Olympus).

Western blot analysis

PDL1, PDL2, ICAM-1 and IL-10 protein were measured by western blot analysis. The crushed tissue were suspended in radio immunoprecipitative assay buffer(RIPA buffer) (0.5ml of a 2X lysis buffer containing 1% Triton-X, 300mM NaCl, 100mM Tris HCl, 2mM EDTA, 10mM NaF, and bromophenyl blue), and protease

inhibitor for 30min at 4℃, and then centrifuged for 15min at 13000rpm. Protein concentraions were determined using Bicinchoninic acid(BCA) Protein Assay reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Samples were mixed with sample buffer and heated at 95℃ for 5min.

Equal amounts of protein samples(25㎍ protein per lane) were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto the polyvinylidene difluoride membrane(PVDF membrane) (Amersham Biosciences, Piscataway, NJ, USA). For blocking, membrane was incubated with 5% skim milk in 0.1% Tris buffered saline-tween20 (TBST) for 1hr at room temperature. And the membranes were incubated with anti-PDL1 antibody (Abcam, MA, USA), human PDL2 antibody(R&D systems, MN, USA), ICAM-1 antibody(Cell signaling technology, MA, USA), IL-10 antibody(Abcam, MA, USA), and anti-β-actin antibody(Cell signaling technology, MA, USA). The membranes were immunoblotted with a secondary anti-goat IgG-HRP, or secondary anti-rabbit IgG-HRP (Santa cruz Biotechnology, CA, USA). The blots were visualized using Super Signal West Pico Chemiluminescent Substrate (Pierce, IL, USA).

Densitometric analysis was performed using the Alpha View SA software(Protein simple, CA, USA).

Statistical analysis

The data was presented as mean ± standard error mean(SEM). If the data was not distributed normally, a Mann -Whitney U-test was used to compare results between allergy group and non-allergy group, and between control tissues and infection tissues. On the other hand, if the data was distributed normally, a Student's *t* test was used to compare results between allergy group and non-allergy group, and between control tissues and infection tissues. Pearson correlation tests were used to evaluate the correlations of PDL1 and IL-10 in allergy group and non-allergy group. A p-value < .05 was considered statistically significant. Statistical analysis was performed using SPSS 18.0 software.

Data & Result

RV16 detection from the mucosal surface fluid

Evidence of RV16 infection, viral replication and release of the virus into the mucosal surface was detected by RT-PCR and semi-nested PCR.

In this experiment, RV16 was applied to the mucosa surface of the tissues for 4hr and the tissues were washed out with PBS for six times to ensure no unattached RVs exist. Procedure of washing out unattached RVs is very important because unattached RVs can give false positive results during PCR. It is important to completely washed out unattached RV16 from the mucosa. If RVs are not washed out completely, RT-PCR performed after 48h in culture can give false positive data. It confirmed that extensive washing of the cells completely remove RV by conducting semi-nested RT-PCR on the sixth wash fluid. There was no positive band at the sixth wash fluid(result not shown) indicating that the washing process completely removed the remaining and unattached RV16. The PBS used to wash out the mucosal surface 48h later, it was used to assayed by both RT-PCR and semi-nested PCR for RVs detection. The RT-PCR product was 388 bp. There was no detectable semi-nested PCR product at 292bp in the negative control using distilled

water, and in the mucosal surface fluid of uninfected tissue. With the surface fluids of the experimental group, a 292bp was seen, indicating that these cultures had been successfully infected with RVs and subsequently released RVs to their mucosal surfaces <Figure 2>.

Infection rate of allergy group and non-allergy group

After an experiment, among the 21 uncinate process tissue from non-allergy group, 16 tissues (76.19%) showed the 292 bp RV specific band, indicating a successful RV infection. Of the 39 uncinate process tissue from allergy group, 29 mucosae (74.36%) were successfully infected with RVs. There was no significant difference in allergy and non-allergy group. These results suggest a similar degree of susceptibility to RV infection in the allergy and non-allergy group

Difference response to infection between allergy group and non-allergy group

PDL1 and PDL2 in the nasal mucosa

To determine the immune responses to RV infection in allergy group and non-allergy group, we investigated the expression of PDL1 and PDL2 in the nasal mucosa by real-time PCR, IHC and

western blotting. As shown in <Figure. 3A>, the level of PDL1 mRNA was slightly elevated in HRV infection tissue from allergy group, but it was not statistically significant. (p=0.068). In non-allergy group, PDL1 had no difference in mRNA level between control and infected tissues. When compared with the infected tissues from non-allergy group, the infected tissues from allergy group showed significantly elevated expression of protein level of PDL1 (p=0.049). As shown in <Figure. 3B>, PDL2 had no differences of mRNA levels in both allergy and non-allergy groups. However, in allergy group, the infected tissues showed significantly higher amount of protein expression of PDL2 than control tissues (p=0.034). These results demonstrate that the different responses to HRV infection between allergy and non-allergy group.

To confirm the presence of PDL1, immunohistochemical staining was performed on nasal mucosa samples using monoclonal antibodies against PDL1. Data in <Figure 3.C> show that samples from CRS patients expressed PDL1 despite of whether the patient has allergy or not. However, the intensity of staining varied among subjects. Samples depicted in upper panel were from a patient in allergy group and displayed intense staining in both control and infected tissue. Samples depicted in lower panel were from a patient in non-allergy group and displayed light and moderately light staining in each control and infected tissue.

ICAM-1 in the nasal mucosa

Major group of HRVs (including HRV16) binds to intracellular adhesion molecule-1(ICAM-1)(22) that is up-regulated in respiratory epithelial cells in allergic patients(23). To compare the susceptibility and reaction to viral infection in allergy group and non-allergy group, we investigated the expression of ICAM-1 on the nasal mucosa by real-time PCR and western blotting. In allergy group, as shown in < Figure 4 >, ICAM-1 mRNA was significantly increased in infected tissues (p= 0.026). The elevated expression of protein of ICAM-1 was also significant in infected tissues in allergy group (p=0.048). In non-allergy group itself, the amounts of expressed mRNA and protein of ICAM- 1 in control and infected tissues were not significantly different. Also, when comparing allergy and non-allergy groups, there was no significant difference in both mRNA and protein expressions of ICAM-1.

IFN-γ, IL-4 and IL-5 in the nasal mucosa

Earlier (in the introduction part), we figured out how the rhinovirus infection affects IFN-γ, IL-4, and IL-5 level in allergy group and non-allergy group. We investigated the expression of these cytokines on the nasal mucosa by real-time PCR. As shown in real-time PCR data in <Figure 5>, in both allergy and non-allergy groups, IFN-γ had no difference in mRNA levels. In cases

of IL-4, the amount of mRNA expression was significantly increased in infected tissues in both allergy and non-allergy groups (p=0.026, p=0.015). Furthermore, there was significant elevation of mRNA expression between infected tissues from non-allergy group and those from allergy group (p=.048). The differences of mRNA expression of IL-5 were more prominent in IL-5. The elevation was significant between infected tissues and control tissues in non-allergy group (p=0.001), and significant difference was also shown between both infected tissues from non-allergy group and allergy group (p=0.008).

IL-10 in the nasal mucosa

We also investigated the expression of IL-10 on the nasal mucosa by real-time PCR and western blotting. As shown in <Figure 6>, when the tissue was infected, the expression of IL-10 mRNA was significantly increased in allergy group (p=.039), but not in non-allergy group There also was significant difference between both infected tissues from each group (p=0.037). In the expression of IL-10 protein, the infected tissues showed significant difference between allergy and non-allergy group. (p=.035)

Association between PDL1 and IL-10

Pearson correlation tests were used to evaluate the correlations between PDL1 and IL-10 in allergy group (n=13) and non-allergy group (n=10). There was a strong correlation between PDL1 and IL-10 in allergy group (r = .839, p=.001). In contrast, no significant correlations were found between the degree of PDL1 and IL-10 in non-allergy group. (r =.032, p =.931).

Discussion

In this present study, we aimed to find out the susceptibility to rhinovirus infection and its different effects on the expression of co-stimulatory molecules, PDL1 and PDL2, and inflammatory cytokines between allergy and non-allergy subjects.

Uncinate process mucosal tissues were obtained from 39 patients with allergy and 22 patients without allergy who underwent surgical management of CRS.

To better understand the pathophysiology of RV infection, an ideal experimental model is essential. In this study, in attempting to overcome the, we used an air-liquid interface organ culture system (21) that much alike real situation of rhinovirus infection. To demonstrate successful viral infection and replication in epithelial cells, RT-PCR and semi-nested PCR was conducted with the surface wash fluid that was infected 48h ago. Semi-nested RT-PCR is a highly sensitive method for the detection of RV (21). When RVs infect epithelial, viral replication takes place, and RVs are released to the cell surface, and it detected in the surface fluid of epithelial cells (22).

Some studies suggest that patients with allergy appear to more vulnerable to HRV infection. Asthmatic patients have increased frequency and severity of lower respiratory tract infections,

compared to subjects without asthma (23-25). In addition, patients with atopic dermatitis are highly susceptible to cutaneous bacterial, viral, and fungal infections (26, 27). In contrast, asthmatic patients and those with atopic disease do not appear to have increased frequency, severity, or longer duration of upper respiratory tract infections(24, 25). In our experiment data, 74.36% tissues of allergy group and 76.19% tissues of non-allergy group from patients with CRS were successfully infected by HRV. According to this data, the susceptibility to RV infection did not differ significantly between allergy and non-allergy group. These results suggest that, under the same conditions, allergic and non-allergic subjects have a similar degree of susceptibility to RV infection. The results could not prove our hypothesis that patients with allergy might be more vulnerable to HRV infection.

A review of several studies has shown that HRV infection can be asymptomatic; it reveals that 12%-22% samples from asymptomatic children were positive for HRV (23), and similar findings have been reported for seronegative adult volunteers after experimental inoculation with HRV (24). In Our study, HRV infections have a relatively short latency period (1-3 days) between inoculation and the onset of symptoms. Most HRV illnesses last 3 to 7 days and the intensity of viral shedding roughly correlates with the intensity of symptoms (25). Term of infection in

our experiment data means that HRVs exist on the surface of nasal mucosa, while, clinical diagnosis of cold means that patients complains specific symptoms of cold. Despite of the fact whether people are infected by RVs or not, someone recognize that they have a cold but others do not. However, if the infection gets more severe or prolonged inflammatory responses, most of the people can recognize that they have a cold.

Various conditions and many molecules effect on rhinovirus infection, and HRV infection also regulate the expressions of a wide range of factors. To determine the response to infection in allergy group and non-allergy group, we investigated the expression of PDL1, PDL2, ICAM-1, IFN-γ, IL-4, Il-5, and IL-10 on the nasal mucosa by real-time PCR, western blotting and IHC.

Viral modulation of inhibitory molecules, PDL1 and PDL2, on the nasal mucosa results in suppression or termination of immune responses. Therefore, we investigated the expression of PDL1 and PDL2 on the nasal mucosa by real-time PCR, western blotting and IHC. As shown in real-time PCR data in <Figure 3>, there were no significant differences (P>.05) in RV-induced PDL1 and PDL2 mRNA levels in both allergy and non-allergy groups. However, expression of PDL1 protein was significantly elevated in infected tissues from allergy group, compared to those from non-allergy group.

These results demonstrate that the response to infection were different between allergy and non-allergy group.

< Figure 4 > shows that infected tissues from allergy group exhibits significantly higher levels of ICAM-1 expression than control tissues from the same group. Researches on which factors of allergic status are related with increased level of ICAM-1 should be further studied. Also in allergy group, as shown in <Figure 5>, IL-4 mRNA expression level was slightly increased in infected tissues than in control tissues. Meanwhile, in non-allergy group, infected tissues induced significantly higher expressions of IL-4 and IL-5 mRNA than control tissues. At the same time, increased expressions of IL-4 and IL-5 of infected tissues from non-allergy group were also significantly higher than those of infected tissues from allergy group.

Miscellaneous studies have been suggested diverse opinions about the relationship between HRV infection and allergic reaction. Viral infection has both positive and negative effects on the development of airway allergy: Viral infection as an inhibitor of allergic sensitization, and the alternative view that some viral infections can enhance allergic sensitization.(28) Our data suggests the possibility of non-allergic subjects to have an increased risk of becoming Th2 biased enviorment after repeated rhinovirus infection.

As shown in <Figure 6>, the infected tissues from allergy group

induced significantly higher expression of IL-10 than both control tissues from the same group and infected tissues from the non-allergy group.

In summary, when a nasal mucosa is infected with HRV, expression of PDL1 and IL-10 is elevated in allergy group, but not in non-allergy group. Besides, a high degree of correlation exists between the levels of PDL1 and IL-10 in allergy group. However, in contrast, no correlation was seen in non-allergy group. PDL1 binds to PD-1 on monocytes and triggers IL-10 production (14). Further researches will need to how allergy works in a pathway that PDL1 increases IL-10.

When HRV-16 enters the upper respiratory tract, it binds to ICAM-1 receptor on respiratory epithelial cells and then, virus begins to replicate and spread. The anti-viral immune responses in the host result in direct interference with viral replication apoptosis of infected cells and recruitment of not only primarily neutrophil granulocytes but also cytotoxic and NK cells which produces IFN-γ (29-31). Induction of the inhibitory costimulatory molecules PDL1 and PDL2 on epithelial cells occurred as a response to viral infection, a major trigger of inflammation (2). Upon IFN-γ stimulation, PDL1 is expressed on T cell, NK cell, macrophages, myeloid DCs, epithelial cells, and vascular endothelial cells (32). PDL1 binds to PD-1 on monocytes and triggers IL-10 production

(14). IL-10 is capable of inhibiting macrophages and regulatory T-cells to produce pro-inflammatory cytokines, such as IFN-r, IL-2, IL-3, TNFα, and GM-CSF. It has an ability to suppress the function of antigen presenting cells, and thereby down-regulating the function of dendritic cells.

These processes might enable more viral replications, more severe infections and more prolonged inflammatory responses in allergic patients, which, in turn, may result in more severe HRV infection symptoms.

References

1. Winther B, Gwaltney JM, Jr., Mygind N, Hendley JO. Viral-induced rhinitis. American journal of rhinology. 1998;12(1):17-20.

2. Heinecke L, Proud D, Sanders S, Schleimer RP, Kim J. Induction of B7-H1 and B7-DC expression on airway epithelial cells by the Toll-like receptor 3 agonist double-stranded RNA and human rhinovirus infection: In vivo and in vitro studies. The Journal of allergy and clinical immunology. 2008;121(5):1155-60.

3. Casasnovas JM, Springer TA. Pathway of rhinovirus disruption by soluble intercellular adhesion molecule 1 (ICAM-1): an intermediate in which ICAM-1 is bound and RNA is released. Journal of virology. 1994;68(9):5882-9.

4. Proud D. Role of rhinovirus infections in asthma. Asian Pacific journal of allergy and immunology / launched by the Allergy and Immunology Society of Thailand. 2011;29(3):201-8.

5. Freeman GJ, Long AJ, Iwai Y, Bourque K, Chernova T, Nishimura H, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. The Journal of experimental medicine. 2000;192(7):1027-34.

6. Dong H, Zhu G, Tamada K, Chen L. B7-H1, a third member of the B7 family, co-stimulates T-cell proliferation and

interleukin-10 secretion. Nature medicine. 1999;5(12):1365-9.

7. Carter L, Fouser LA, Jussif J, Fitz L, Deng B, Wood CR, et al. PD-1:PD-L inhibitory pathway affects both CD4(+) and CD8(+) T cells and is overcome by IL-2. European journal of immunology. 2002;32(3):634-43.

8. Yamazaki T, Akiba H, Iwai H, Matsuda H, Aoki M, Tanno Y, et al. Expression of programmed death 1 ligands by murine T cells and APC. Journal of immunology. 2002;169(10):5538-45.

9. Butte MJ, Pena-Cruz V, Kim MJ, Freeman GJ, Sharpe AH. Interaction of human PD-L1 and B7-1. Molecular immunology. 2008;45(13):3567-72.

10. Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annual review of immunology. 2002;20:29-53.

11. Deppong C, Juehne TI, Hurchla M, Friend LD, Shah DD, Rose CM, et al. Cutting edge: B and T lymphocyte attenuator and programmed death receptor-1 inhibitory receptors are required for termination of acute allergic airway inflammation. Journal of immunology. 2006;176(7):3909-13.

12. Akbari O, Stock P, Singh AK, Lombardi V, Lee WL, Freeman GJ, et al. PD-L1 and PD-L2 modulate airway inflammation and iNKT-cell-dependent airway hyperreactivity in opposing directions. Mucosal immunology. 2010;3(1):81-91.

13. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. The Journal of experimental medicine. 2009;206(13):3015-29.

14. Said EA, Dupuy FP, Trautmann L, Zhang Y, Shi Y, El-Far M, et al. Programmed death-1-induced interleukin-10 production by monocytes impairs CD4+ T cell activation during HIV infection. Nature medicine. 2010;16(4):452-9.

15. Fu CL, Ye YL, Lee YL, Chiang BL. Effects of overexpression of IL-10, IL-12, TGF-beta and IL-4 on allergen induced change in bronchial responsiveness. Respiratory research. 2006;7:72.

16. Kosaka S, Tamauchi H, Terashima M, Maruyama H, Habu S, Kitasato H. IL-10 controls Th2-type cytokine production and eosinophil infiltration in a mouse model of allergic airway inflammation. Immunobiology. 2011;216(7):811-20.

17. Koya T, Matsuda H, Takeda K, Matsubara S, Miyahara N, Balhorn A, et al. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. The Journal of allergy and clinical immunology. 2007;119(5):1241-50.

18. Banchereau J, Pascual V, O'Garra A. From IL-2 to IL-37: the expanding spectrum of anti-inflammatory cytokines. Nature immunology. 2012;13(10):925-31.

19. Hammer M, Mages J, Dietrich H, Schmitz F, Striebel F,

Murray PJ, et al. Control of dual-specificity phosphatase-1 expression in activated macrophages by IL-10. European journal of immunology. 2005;35(10):2991-3001.

20. Wang JH, Kwon HJ, Chung YS, Lee BJ, Jang YJ. Infection rate and virus-induced cytokine secretion in experimental rhinovirus infection in mucosal organ culture: comparison between specimens from patients with chronic rhinosinusitis with nasal polyps and those from normal subjects. Archives of otolaryngology--head & neck surgery. 2008;134(4):424-7.

21. Myatt TA, Johnston SL, Rudnick S, Milton DK. Airborne rhinovirus detection and effect of ultraviolet irradiation on detection by a semi-nested RT-PCR assay. BMC public health. 2003;3:5.

22. Jang YJ, Lee SH, Kwon HJ, Chung YS, Lee BJ. Development of rhinovirus study model using organ culture of turbinate mucosa. Journal of virological methods. 2005;125(1):41-7.

23. van Benten I, Koopman L, Niesters B, Hop W, van Middelkoop B, de Waal L, et al. Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants. Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology. 2003;14(5):363-70.

24. DeMore JP, Weisshaar EH, Vrtis RF, Swenson CA, Evans MD, Morin A, et al. Similar colds in subjects with allergic asthma and nonatopic subjects after inoculation with rhinovirus-16. The

Journal of allergy and clinical immunology. 2009;124(2):245-52, 52 e1-3.

25. Kim WK, Gern JE. Updates in the relationship between human rhinovirus and asthma. Allergy, asthma & immunology research. 2012;4(3):116-21.

26. Ring J, Abeck D, Neuber K. Atopic eczema: role of microorganisms on the skin surface. Allergy. 1992;47(4 Pt 1):265-9.

27. Lubbe J. Secondary infections in patients with atopic dermatitis. American journal of clinical dermatology. 2003;4(9):641-54.

28. Tantilipikorn, P. and P. Auewarakul . Airway allergy and viral infection. Asian Pac J Allergy Immunol. 2011;29(2): 113-119.

29. Padovan E, Spagnoli GC, Ferrantini M, Heberer M. IFN-alpha2a induces IP-10/CXCL10 and MIG/CXCL9 production in monocyte-derived dendritic cells and enhances their capacity to attract and stimulate CD8+ effector T cells. Journal of leukocyte biology. 2002;71(4):669-76.

30. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, et al. Role of deficient type III interferon-lambda production in asthma exacerbations. Nature medicine. 2006;12(9):1023-6.

31. Kohlmeier JE, Cookenham T, Roberts AD, Miller SC, Woodland DL. Type I interferons regulate cytolytic activity of memory CD8(+) T cells in the lung airways during respiratory virus challenge. Immunity. 2010;33(1):96-105.

32. Flies DB, Chen L. The new B7s: playing a pivotal role in tumor immunity. Journal of immunotherapy. 2007;30(3):251-60.

Table 1. Allergens used in skin prick test

Allergens used in skin prick test		
Histamine	Normal saline	D.farinae
D.pteronys	Tyrophagus	Moulds mixture
Alternaria	Cladosporium	Fusarium
Candida	Asper	Albicans
Fumigatus	Asper niger	Mucor mucedo
Penicillium	Cat	Dog
Rat	Chicken	Rabbit
American Cockroach	German Cockroach	Alder
Hazel	Popul	Elm
Willow tree	Birch	Beech
Oak	Plane tree	Japanese cedar
Ash	Elder	False Acacia
Pine	Velvet grass	Orchard grass
Rye grass	Timothy grass	Kentucky blue grass
Meadow fescue	Nettle	Bermuda grass
Ragweed	Mugwort	Hop japanese
Dandelion		

Table 2. Primer sequences for detection of RV16 infection

Target	Primer sequence
OL26	5’ -GCACTTCTGTTTCCCC-3’
OL27	5’ -CGGACACCCAAAGTAG-3’
JWA-b1	5’ -CATTCAGGGGCCGAGGA-3’

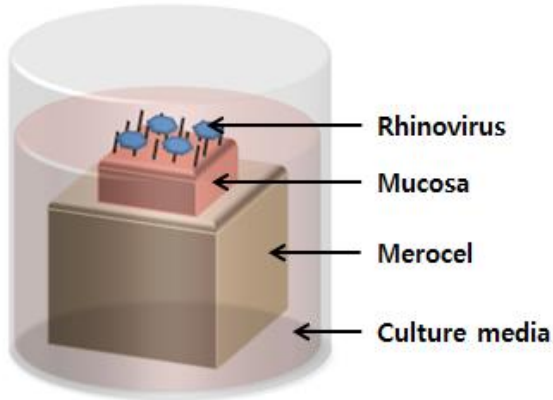

Figure 1. Schematic drawing of the organ culture system used in this study.

A mucosal piece was placed on merocel that had been soaked in culture medium, and epithelium was above the liquid phase. Rhinovirus was applied to the apical surface of mucosa.

Table 3. Primer sequences for real-time PCR

Target		Primer sequence
GAPDH	Forward	GAGAAGGCTGGGGCTCAT
	Reverse	TGCTGATGATCTTGAGGCTG
ICAM-1	Forward	TGTCCCCCTCAAAAGTCATC
	Reverse	TAGGCAACGGGGTCTCTATG
IFN-γ	Forward	AGGGAAGCGAAAAAGGAGTC
	Reverse	ATATTGCAGGCAGGACAACC
IL-4	Forward	AGCCACCATGAGAAGGACAC
	Reverse	GTTTCAGGAATCGGATCAGC
IL-5	Forward	CGTTTCAGAGCCATGAGGAT
	Reverse	GCAGTGCCAAGGTCTCTTTC
IL-10	Forward	ACGGCGCTGTCATCGATT
	Reverse	TTGGAGCTTATTAAAGGCATTCTTC

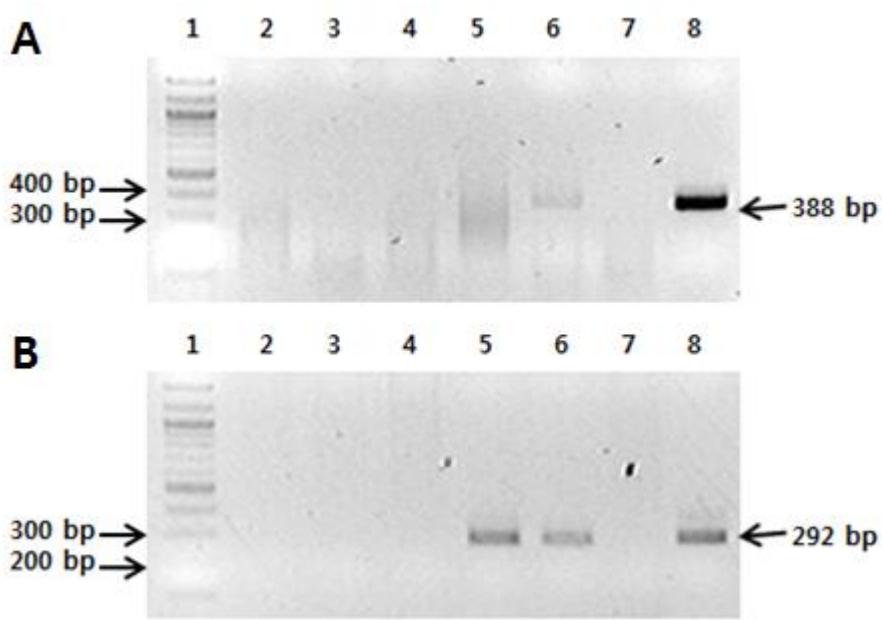

Figure 2. Detection of RV16.

(A) RT-PCR (B) Semi-nested PCR. Lane 1: molecular size marker, Lane 2: surface fluid of control group tissue, Lane 3: surface fluid of UV group tissue, Lane 4-8: surface fluid of experimental group tissue, Lane 9: negative control (distilled water), Lane 10: positive control (RV16). The 388 bp band is a product of RT-PCR and 292 bp band is a product of semi-nested PCR.

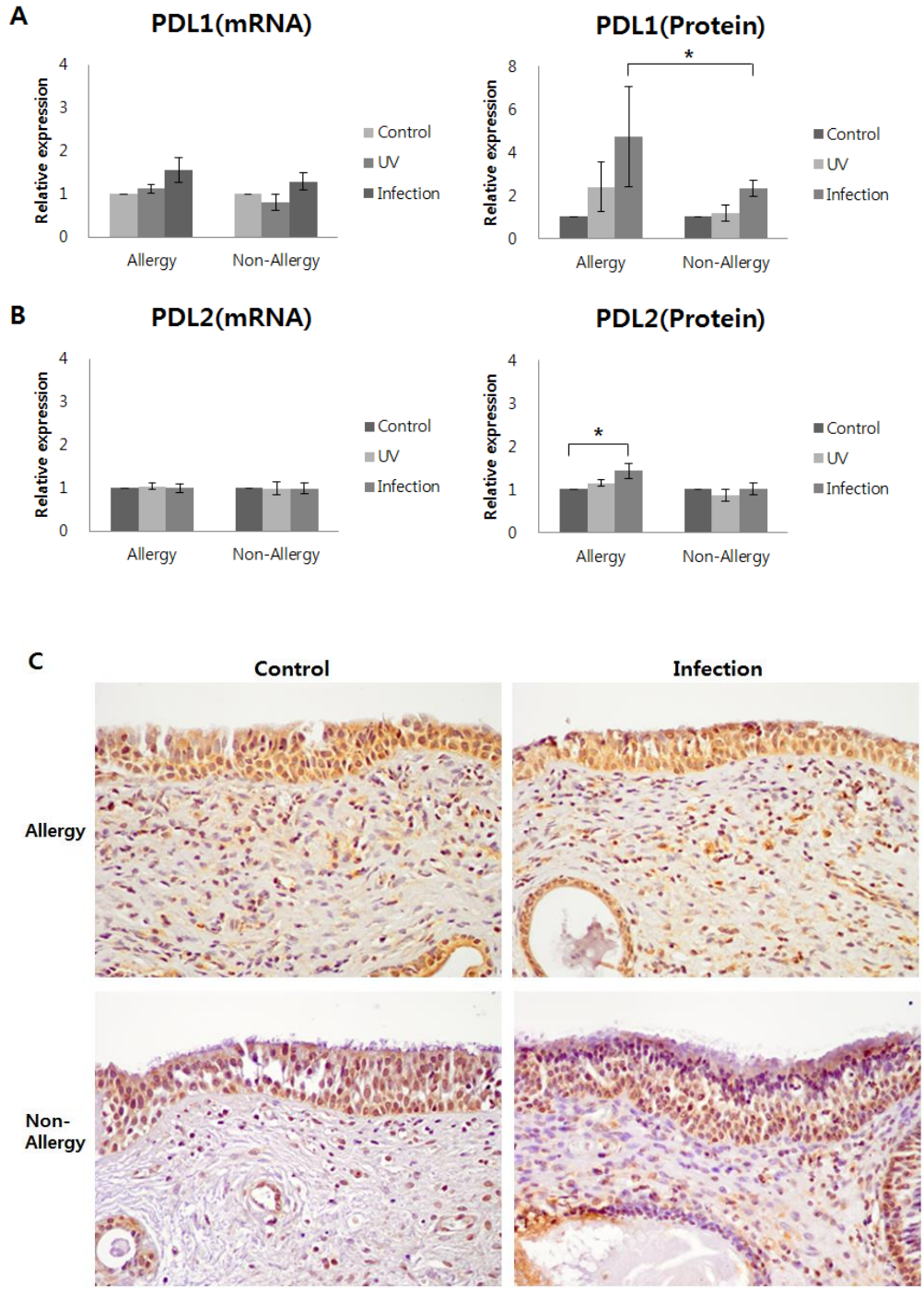

Figure 3. Response to RV16 infection in PDL1 and PDL2 level on the nasal mucosa.

(A) mRNA and protein level of PDL1. (B) mRNA and protein level of PDL2. Real-time PCR analysis of mRNA level in the nasal mucosa from allergy subjects (n=17) and non-allergy subjects (n=12). The relative mRNA amount in each sample was calculated based on the $\triangle\triangle C_T$ method using housekeeping gene GAPDH and each control tissues. Values shown are Mean±SEM from Taqman analysis and reflect target mRNA. Western blotting analysis of expression of PDL1 protein on the nasal mucosa from allergy subjects (n=16) and non-allergy subjects (n=12). Relative expression represents ratio of PDL1 or PDL2 /β-actin, and data are expressed as Mean±SEM. $*p<.05$. (C) Immunohistochemical results of PDL1 expression level in nasal mucosa from two subjects (upper: allergy patient, lower: non-allergy patient) as described in Materials and Methods. Original magnification, X400

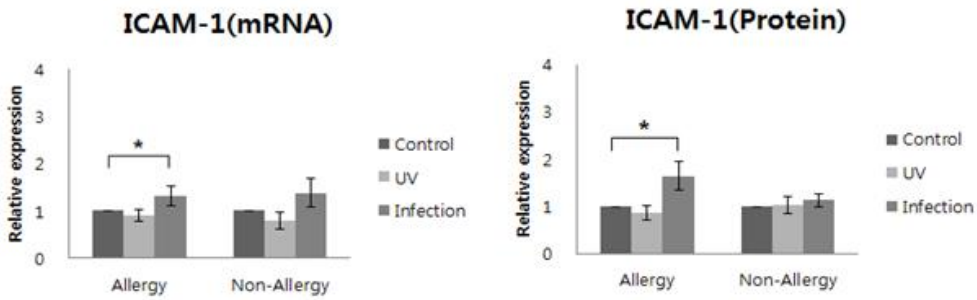

Figure 4. Response to RV16 infection in ICAM-1 level on the nasal mucosa.

Real-time PCR analysis of mRNA level of ICAM-1 in the nasal mucosae from allergy subjects (n=12) and non-allergy subjects (n=11). The relative mRNA amount in each sample was calculated based on the $\triangle\triangle C_T$ method using housekeeping gene GAPDH and each control tissues. Values shown are Mean±SEM from Taqman analysis and reflect target mRNA. Western blotting analysis of expression of ICAM-1 protein level on the nasal mucosa from allergy subjects (n=12) and non-allergy subjects (n=11). Relative expression represents ratio of ICAM-1/β-actin, and data are expressed as Mean±SEM. $*p<.05$.

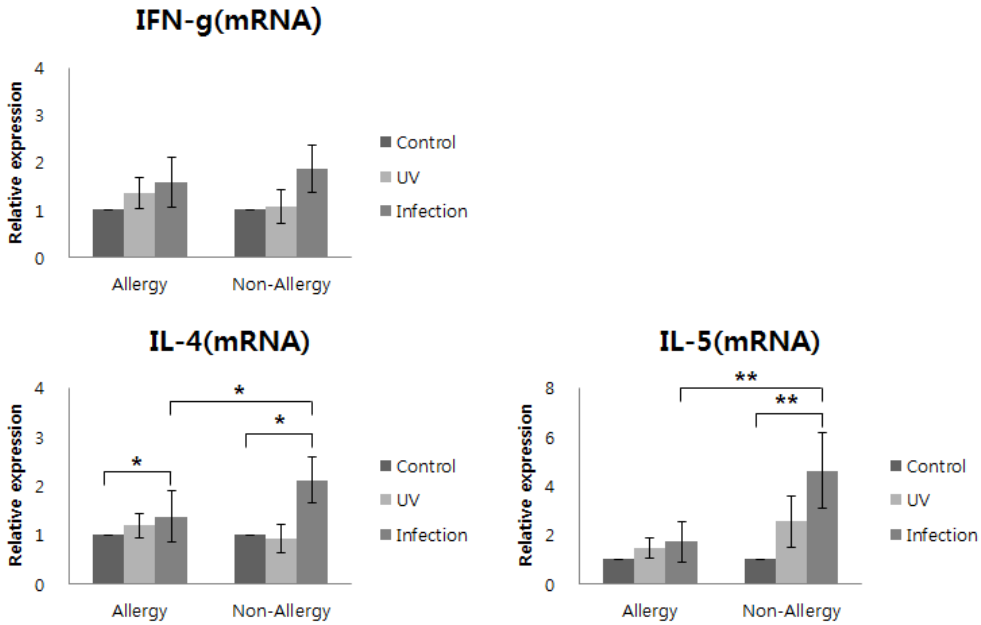

Figure 5. Response to RV16 infection in IFN- γ, IL-4 and IL-5 level on the nasal mucosa.

Real-time PCR quantification of IFN-γ, IL-4, and IL-5 in the nasal mucosa from allergy subjects (n=12) and non-allergy subjects (n=11). The relative mRNA amount in each sample was calculated based on the $\triangle\triangle C_T$ method using housekeeping gene GAPDH and each control tissues. Values shown are Mean±SEM. (*p<.05, **p<.01).

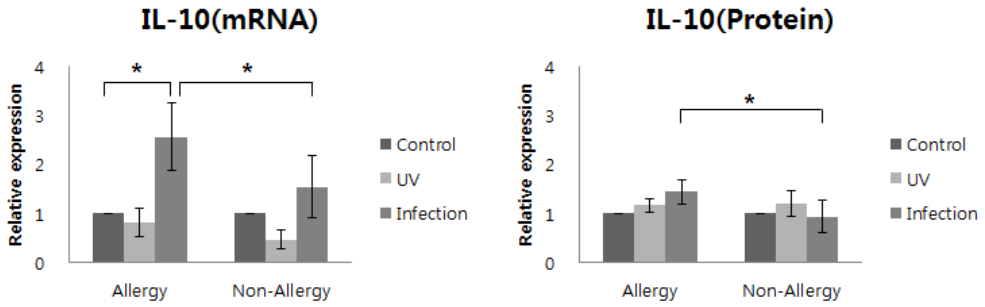

Figure 6. Response to RV-16 infection in IL-10 level on the nasal mucosa.

Real-time PCR analysis of mRNA level of IL-10 in the nasal mucosa from allergy subjects (n=15) and non-allergy subjects (n=11). The relative mRNA amount in each sample was calculated based on the $\triangle\triangle C_T$ method using housekeeping gene GAPDH and each control tissues. Values shown are Mean±SEM from Taqman analysis and reflect target mRNA. (B) Western blotting analysis of expression of IL-10 protein on the nasal mucosa from allergy subjects (n=11) and non-allergy subjects (n=9). Relative expression represents ratio of ICAM-1/β-actin, and data are expressed as Mean±SEM. *p<.05.

초 록

알레르기 비염 환자에서 Rhinovirus 감염이 Programmed cell death ligand 와 사이토카인 발현에 미치는 영향

고 영 경

의학과 면역학 전공

서울대학교 대학원

연구배경과 목적: Rhinovirus(RV)는 대표적인 상기도 감염 병원체로서, 알레르기와 RV 감염 및 면역반응의 상관관계에 대해 다양한 연구들이 진행되고 있으며, 그 사이에서는 복잡한 상호작용이 있을 거라 생각되지만, 아직 그 관련성은 명확하게 밝혀지지 않았다. 한편, RV 감염과 그로 인해 발생하는 면역반응은 다양한 공동자극분자와 염증 사이토카인에도 영향을 준다. 이에 본 연구에서는 알레르기 만성 부비동염과 비 알레르기 만성 부비동염 환자에서 RV 감염률 알아보고,

RV 에 감염된 후의 알레르기 군과 비 알레르기 군 사이에서의 공동자극분자와 사이토카인 발현 차이를 분석함으로써 알레르기가 RV 감염과 면역기전에 미치는 영향을 알아보고자 한다.

연구방법: 알레르기 만성 부비동염 환자(39명)와 비 알레르기 만성 부비동염 환자(21명)로부터 구상돌기조직을 얻어 air-liquid interface culture system하에서 RV 감염 실험을 시행하였다. 알레르기와 비알레르기 군의 RV 감염된 조직에서 공동자극분자(PDL1, PDL2), RV 수용체인 ICAM-1, 사이토카인(IFN-γ, IL-4, IL-5, IL-10) 발현차이를 분석하였다.

연구결과: 알레르기 군과 비 알레르기 군의 RV 감염률에 유의미한 차이는 보이지 않았다. 알레르기군 내에서 대조군 조직과 RV16에 감염된 조직을 비교했을 때, PDL2, ICAM-1, IL-4 , IL-10의 발현이 증가하였으며, 비 알레르기군내에서 대조군 조직과 RV16에 감염된 조직을 비교했을 때, IL-4, IL-5의 발현이 증가하였다. 알레르기 군과 비 알레르기 군에서 RV16에 감염된 조직을 비교하였을 때, 알레르기 군에서는 PDL1과 IL-10의 발현이 증가하였으며, PDL1과 IL-10는 높은 상관관계를 갖는 것으로 나타났다. 그러나 비 알레르기 군에서는 PDL1과 IL-10은 상관관계가 없는 것으로 나타났다. 두 군간에 IFN-γ 은 차이가 없었다.

결 론: 알레르기 군과 비 알레르기 군의 RV 감염률에 유의미한 차이는 보이지 않았다. RV 감염 시, 알레르기 군의 증가된 PDL1 과 IL-10은 RV에 대한 면역반응을 억제시킬 수 있으며, 이것은 알레르기 군과 비 알레르기 군간의 감염률은 차이가 없더라도, 알레르기 환자가 더 강한

감기 증상을 가질 수 있는 가능성 중에 하나로 볼 수 있다. 한편, 비알레르기 만성 부비동염 환자가 반복적으로 RV에 감염될 경우, Th2에 편향된 환경이 되는 가능성을 높일 수 있다.

주요어: 알레르기, 만성부비동염, RV감염, PDL1, PDL2, IL-10

학 번: 2012-23632