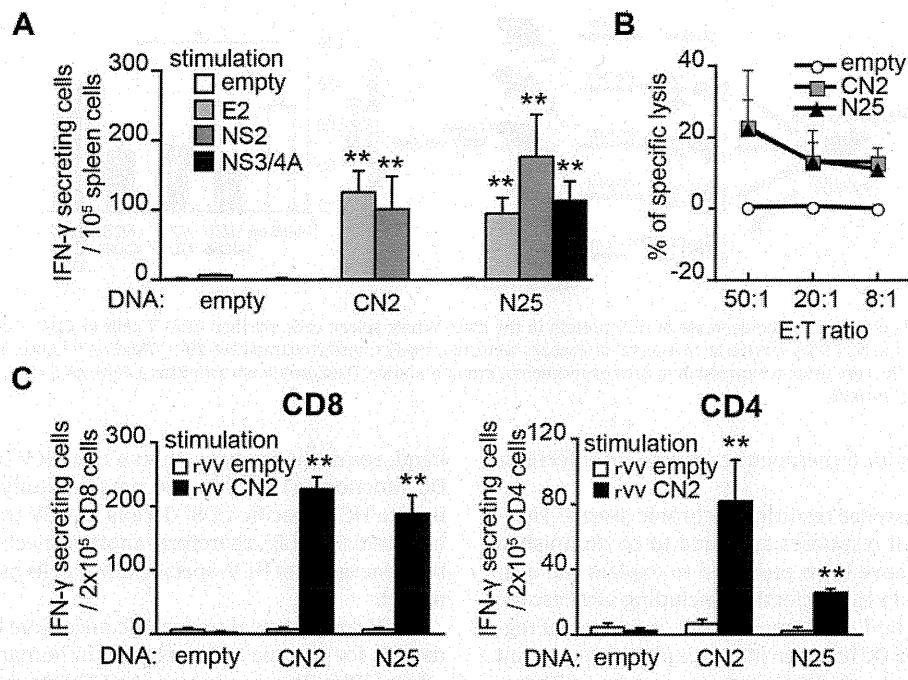

Fig. 2. Immunization of C57BL/6 mice with DNA vaccine induced strong HCV-specific cellular immune responses. C57BL/6 mice were immunized twice with the DNA vaccine at an interval of 2 weeks. At 2 weeks after the final immunization, spleen cells were isolated and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins (A), and ^{51}Cr release assay using EL-4 target cells expressing HCV NS2 protein at indicated ratios (B). CD8 and CD4 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using syngenic splenocytes infected with a recombinant vaccinia virus (rVV) carrying the cDNA of HCV Core-NS2 (rVV CN2) (C). Data are shown as means ± SEM, $n = 3$ (A and B); $n = 5$ (C). Data shown in A–C are representative of at least 3 repeated experiments. $^{**}P < 0.01$ vs stimulated with empty vector-transfected cells.

that use live-attenuated or killed pathogens, proteins, or synthetic peptides [35]. Moreover, DNA vaccine against HIV and malaria showed high levels of safety and good tolerability profile in human clinical trials [36,37]. DNA vaccines can induce cytoplasmic expression of encoded antigens (Ags) that more closely resemble native conformation of pathogens than can immunization with proteins. A DNA vaccine induced immunity against encoded Ags, whereas cytoplasmic expression using a viral vector delivery system induced immune responses to not only vaccine Ags but also vector organisms [38]. Infection with a vaccine vector virus might be an obstacle by induction of unneeded immune responses to vectors as side effects [38]. A DNA vaccine might be

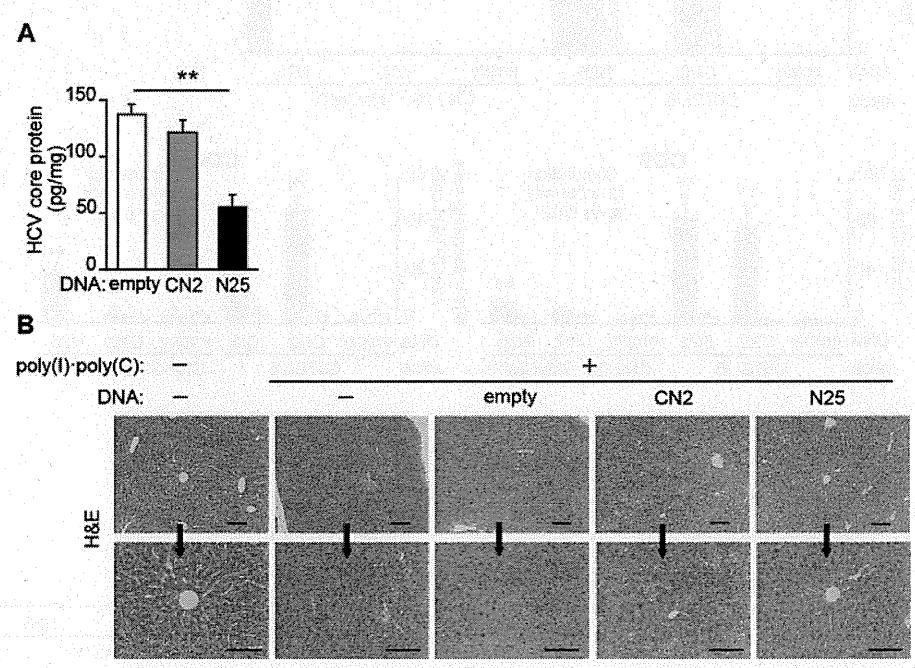

Fig. 3. Immunization with N25 DNA vaccine showed therapeutic effects in the liver of HCV transgenic mice. (A) Three months after induction of HCV protein by poly(I)·poly(C) injection, CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice were immunized twice with the DNA vaccine at an interval of 2 weeks. At 2 weeks after the final immunization, livers were isolated and examined for HCV core protein expression. The core protein expression in each experimental group is shown. Data are shown as means ± SEM, $n = 5$ each. $^{**}P < 0.01$. (B) Liver sections from CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice before (–) and after poly(I)·poly(C) injection (+). Three months after induction of HCV protein, mice were immunized three times with the DNA vaccine at intervals of 2 weeks. At 4 weeks after the final immunization, livers were isolated and stained with hematoxylin and eosin (H&E). Scale bars: 100 μm. Data shown in A and B are representative of at least 3 repeated experiments.

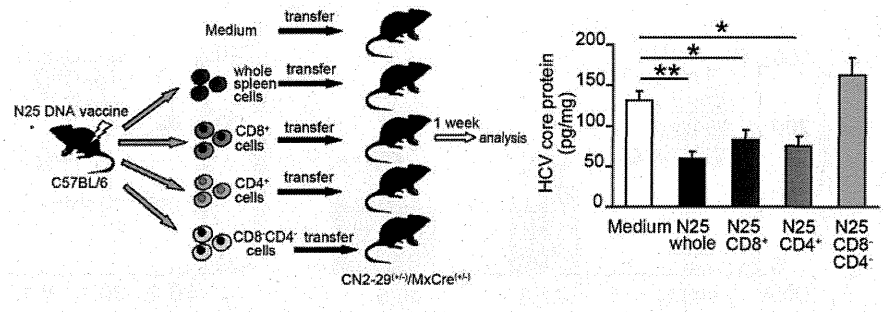

Fig. 4. $CD8^+$ and $CD4^+$ T cells are required for the decrease of HCV protein in the liver. Whole spleen cells, purified $CD8^+$ T cells or $CD4^+$ T cells from C57BL/6 mice that had been immunized twice with the N25 DNA vaccine at an interval of 2 weeks were adoptively transferred into CN2-$29^{(+/-)}$/MxCre$^{(+/-)}$ mice, and livers were isolated from the recipient mice 1 week later. The core protein expression in each experimental group is shown. Data are shown as means ± SEM, $n = 5$ each. Data are representative of 3 repeated experiments. $^{**}P < 0.01$; $^{*}P < 0.05$.

one of the best candidates for a therapeutic vaccine for infectious disease.

In the case of viral persistence resulting in chronic disease, HCV-specific CD4 and CD8 T cell responses appeared to be diminished [39]. Various hypotheses have been proposed to explain the dysfunctional T cell response in HCV infection, including viral escape mutations, exhaustion of the T cell compartment, induction of regulatory T cells and impaired DC function [40]. DCs play an important role in triggering the primary antiviral immune responses. Therefore, modulation of the function of DCs has been suggested as one of the mechanisms used by persistent viruses to evade the immune system. Several studies have demonstrated impairment of DC function in HCV-infected individuals [32,34]. On the other hand, some studies have shown that HCV proteins did not impair DC function [41–44]. In the present study, the ability of DCs to induce HCV-specific $CD8^+$ T cells in HCV transgenic mice was not impaired (Fig. 6B), therefore, another mechanism that suppressed the generation of HCV-specific CD8 T cells might exist in this mouse model.

HCV conventional transgenic mice have been used as surrogate models for chronic HCV infection in humans. In a previous study, when FVB/n Tg mice expressing HCV structural proteins (core, E1 and E2) and WT FVB/n mice were intramuscularly immunized with plasmid DNA encoding core/E1/E2, CTL activities against E2 were detected in WT mice but not in Tg mice [23], and either CD4 or CD8 T cell responses against the envelope proteins appeared to be

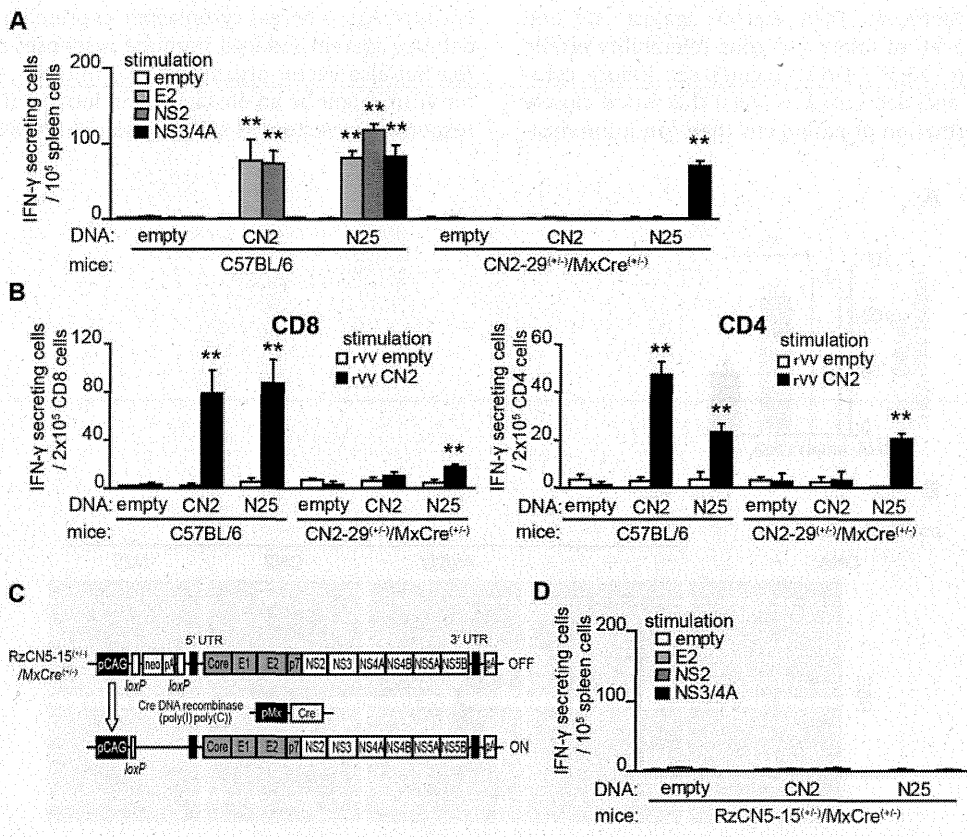

Fig. 5. Immunization of HCV-Tg mice with DNA vaccine failed to induce strong HCV-specific cellular immune responses. CN2-$29^{(+/-)}$/MxCre$^{(+/-)}$ and RzCN5-$15^{(+/-)}$/MxCre$^{(+/-)}$ mice were immunized with the DNA vaccine as in the experiment for which results are shown in Fig. 3A. (A and D) Spleen cells were isolated and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins. (B) CD8 and CD4 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using syngenic splenocytes infected with a recombinant vaccinia virus (rVV) carrying the cDNA of HCV Core-NS2 (rVV CN2). (C) HCV gene structure in RzCN5-$15^{(+/-)}$/MxCre$^{(+/-)}$ mice. RzCN5-$15^{(+/-)}$/MxCre$^{(+/-)}$ mice conditionally express full length of HCV genotype 1b cDNA. Data are shown as means ± SEM, $n = 3$ (A, B and D). Data shown in A, B and D are representative of at least 3 repeated experiments. $^{**}P < 0.01$ vs stimulated with empty vector-transfected cells.

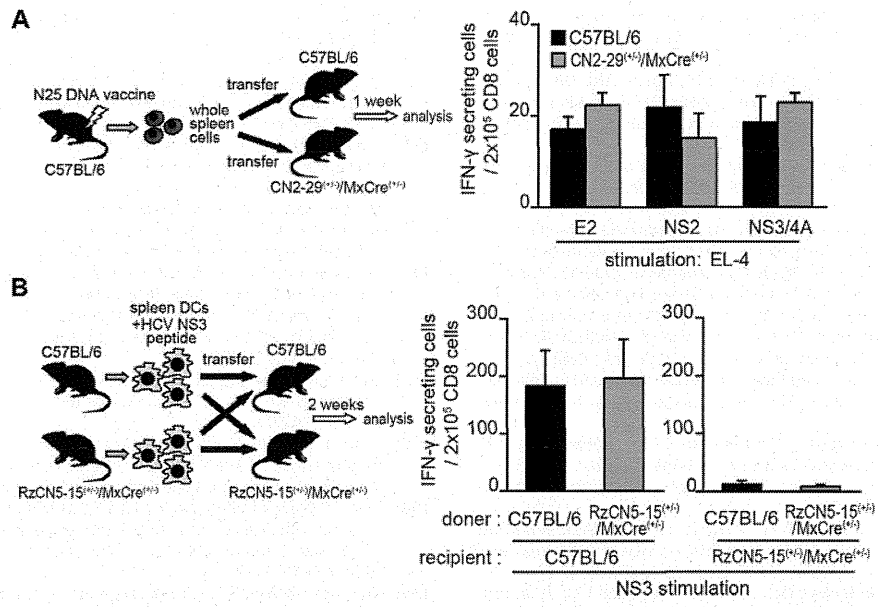

Fig. 6. Ability of DCs to induce HCV-specific CD8⁺ T cells in HCV transgenic mice was not impaired. (A) Whole spleen cells (1×10^8 cells) from C57BL/6 mice that had been immunized twice with the N25 DNA vaccine at an interval of 2 weeks were adoptively transferred into C57BL/6 or CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$ mice, and spleen cells were isolated from the recipient mice 1 week later. CD8 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using EL-4 cells expressing each of the HCV proteins. (B) HCV NS3 peptide-pulsed spleen DCs (2×10^5 cells) derived from C57BL/6 or RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice were adoptively transferred to C57BL/6 or RzCN5-15$^{(+/-)}$/MxCre$^{(+/-)}$ mice. Two weeks later, spleen cells were isolated from the recipient mice. CD8 T cells were purified from whole spleen cells and examined by the IFN-γ ELISPOT assay using HCV NS3 peptide. Data are shown as means ± SEM, $n = 3$ each. Data shown in A and B are representative of at least 3 repeated experiments.

immunologically tolerant and could not overcome this tolerance by DNA immunization in the Tg mice [23]. These observation are consistent with our findings that cellular immune responses to several HCV structural and non-structural proteins were abolished in the CN2 DNA vaccine-immunized HCV-Tg (CN2-29$^{(+/-)}$/MxCre$^{(+/-)}$) mice, unlike in WT mice (Fig. 5A and B). On the other hand, the N25 DNA vaccine induced HCV-specific CD4 and CD8 T cell responses in HCV-Tg mice, and the level of CD4 T cell responses were equivalent to those in WT mice. However, the activities of CD8 T cell responses were not high compared with those in WT mice (Fig. 5B). This difference in the efficacy of CN2 and that of N25 may be caused by the difference of the expression site of HCV construct. The CN2 DNA construct contained the HCV core protein-encoding region. Several studies have demonstrated that the HCV core protein has the immunomodulatory function of suppressing host immune responses [45]. It has been reported that HCV core protein could suppress host immune responses by inhibiting antiviral CTL activity in mice infected with recombinant vaccinia virus expressing the core protein [7], and it has also been reported that Tg mice in which the HCV core protein was expressed in T cells under the control of the CD2 promoter showed significantly reduced T cell responses, including the production of IFN-γ and IL-2, compared to those in non-Tg mice [46]. The N25 DNA construct did not contain the HCV core protein-encoding region, and the N25 DNA vaccine may therefore not be susceptible to these immunosuppressive factors derived from DNA vaccine's own self.

Immunization of HCV-Tg mice with N25 DNA vaccine resulted in improvement in pathological changes in the liver. Sekiguchi et al. [27] reported that immunization with recombinant vaccinia virus strain (rVV-N25), which encoded the same non-structural HCV proteins as those encoded by N25 DNA vaccine in this study, alleviated the symptoms of pathological changes in the liver of HCV-Tg mice. They showed that TNF-α and IL-6 are responsible for the pathological symptoms in HCV-Tg mice and that immunization with rVV-N25 rapidly suppressed the inflammatory responses. The mechanism of action of N25 DNA vaccine may be similar to that of rVV-N25, though further examination is required.

In the present study, using novel HCV conditional transgenic mice to overcome the problem of immune tolerance in HCV conventional transgenic mice, the efficacy of a candidate HCV vaccine was evaluated. The use of DNA vaccines, especially the N25 DNA vaccine, expressing a non-structural protein gene resulted in reduced expression of HCV protein and improved pathological changes in the liver. Our findings may provide new avenues toward the development of an alternative approach for the treatment of individuals chronically infected with HCV, although further studies are needed.

Acknowledgments

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PLOS ONE

Intranasally Administered Antigen 85B Gene Vaccine in Non-Replicating Human *Parainfluenza* Type 2 Virus Vector Ameliorates Mouse Atopic Dermatitis

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Abstract

Atopic dermatitis (AD) is a refractory and recurrent inflammatory skin disease. Various factors including heredity, environmental agent, innate and acquired immunity, and skin barrier function participate in the pathogenesis of AD. T-helper (Th) 2-dominant immunological milieu has been suggested in the acute phase of AD. Antigen 85B (Ag85B) is a 30-kDa secretory protein well conserved in *Mycobacterium* species. Ag85B has strong Th1-type cytokine inducing activity, and is expected to ameliorate Th2 condition in allergic disease. To perform Ag85B function in vivo, effective and less invasive vaccination method is required. Recently, we have established a novel functional virus vector; recombinant human *parainfluenza* type 2 virus vector (rhPIV2): highly expressive, replication-deficient, and very low-pathogenic vector. In this study, we investigated the efficacy of rhPIV2 engineered to express Ag85B (rhPIV2/Ag85B) in a mouse AD model induced by repeated oxazolone (OX) challenge. Ear swelling, dermal cell infiltrations and serum IgE level were significantly suppressed in the rhPIV2/Ag85B treated mouse group accompanied with elevated IFN-γ and IL-10 mRNA expressions, and suppressed IL-4, TNF-α and MIP-2 mRNA expressions. The treated mice showed no clinical symptom of croup or systemic adverse reactions. The respiratory tract epithelium captured rhPIV2 effectively without remarkable cytotoxic effects. These results suggested that rhPIV2/Ag85B might be a potent therapeutic tool to control allergic disorders.

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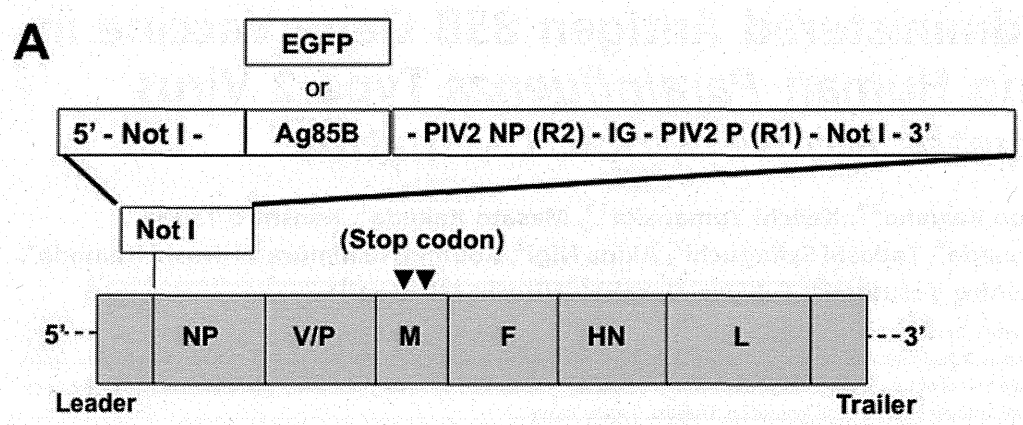
Introduction

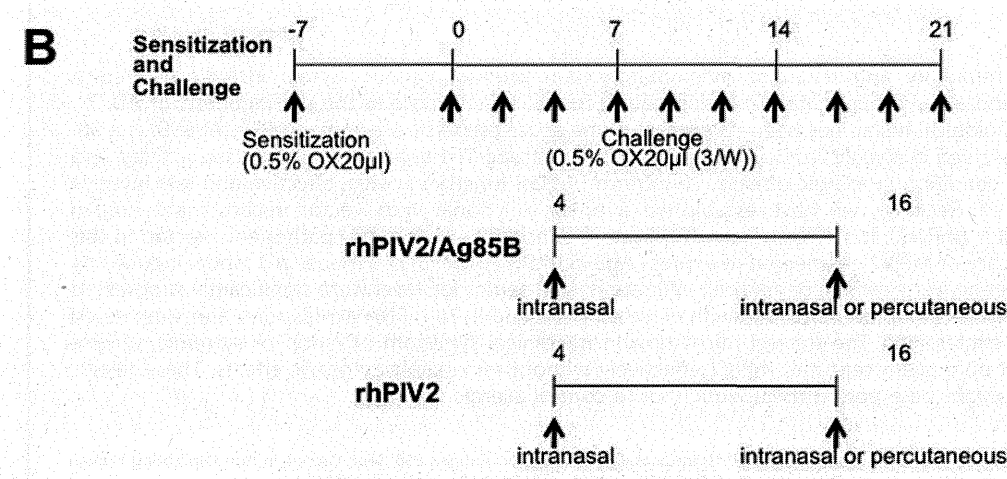
Atopic dermatitis (AD) is a refractory and recurrent inflammatory skin disease. Heredity, environmental agent, immunity, and skin barrier function participate in the pathogenesis of AD. AD symptoms are triggered by various non-specific or specific allergic reactions. The cytokine pattern of AD, especially in the acute phase skin lesion is Th2-type cytokine dominant [1]. The barrier disrupted skin in AD is easily permitted the percutaneous entry of environmental allergens that strongly promotes Th2 immunological responses [2]. Th2 cells as well as T regulatory cell (Treg) subsets play key roles in development of AD. Patients with AD have significantly increased numbers of peripheral blood Treg compared with healthy controls, which is correlated with disease activity in AD [3,4]. This suggests involvement of some self regulation system in immune responses in AD [5].

Repeated elicitation with hapten such as oxazolone (OX) on the ear of BALB/c mice develops immediate type responses with late phase reactions followed by delayed type hypersensitivity responses. This accompanied with balance shift of cytokines in the lesional skin from Th1 to Th2 type [6], and has been utilized as mouse AD.

Ag85B is 30-kDa major secretory protein well conserved in *Mycobacterium* species [7]. The studies for the tuberculosis vaccine revealed strong activities of Ag85B in priming naïve T cells for Th1 effector cells under the appropriate conditions, and induction of strong Th1-type immune responses in mice as well as in humans [8,9]. Recently we reported that plasmid DNA vaccination encoding Ag85B derived from M. *kansasii* inhibits immediate-type hypersensitivity responses with Treg induction in skin [10], and a combined vaccination with heat-killed BCG followed by Ag85B also suppressed skin eczematous reactions in AD model mice by inducing Treg [11].

Human parainfluenza type 2 virus (hPIV2) is one of the human respiratory pathogens and a member of the genus Rubulavirus of the family Paramyxoviridae in the order Mononegavirales,





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groups	Day4	Day16
PBS	PBS	PBS
Ag ear	rhPIV2/Ag85B	rhPIV2/Ag85B (percutaneous)
Ag nasl	rhPIV2/Ag85B	rhPIV2/Ag85B (intranasal)
PIV2 ear	rhPIV2	rhPIV2 (percutaneous)
PIV2 nasal	rhPIV2	rhPIV2 (intranasal)

Figure 1. Schematic diagram of constructs and strategy used in this study. A. The constructs of recombinant hPIV2/EGFP and hPIV2/Ag85B. The EGFP or Ag85B gene open reading frame was engineered to be flanked by hPIV2-specific gene end of NP gene (R2), intergenic sequence (IG), and gene start (R1) transcriptional signal of V/P gene. It was inserted into a cloned cDNA of the hPIV2 antigenome at a Not I site that had been engineered to be at 5′-noncoding region of NP gene. A genomic nucleotide length divisible by six (the rule of six) was maintained. For generating of replication-deficient virus, two stop codons (▼) were introduced on the M gene. **B.** Schedule for the development of a hapten-induced atopic dermatitis model and vaccination of rhPIV2/Ag85B. Mice were initially sensitized with 20 μl of 0.5% OX solution to their right ear 7 days prior to the first challenge (day -7) and then 20 μl of 0.5% OX solution was repeatedly applied on the right ear 3 times per week from day 0 until day 21. Mice were inoculated intranasally with 20 μl (5×10^6 $TCID_{50}$) of rhPIV2/Ag85B or rhPIV2 on day 4. rhPIV2 vector or phosphate buffered saline (PBS) were also applied as controls. On day 16, mice were vaccinated again intranasally or subcutaneously with PBS, rhPIV2 or rhPIV2/Ag85B. **C.** Summarized schedule of the experimental groups.
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possessing a non-segmented and negative-stranded RNA genome of 15,654 nucleotides. The genome of hPIV2 encodes 7 mRNAs [12–14] and has about 60-nt leader sequence at 3′ end and about 20-nt noncoding trailer sequence. The gene order is 3′ (leader)-NP-V/P-M-F-HN-L-(trailer)-5′. The coding proteins are the nucleocapsid (NP), the V (V) and phospho (P), the matrix (M), the fusion (F), the haemagglutinin-neuraminidase (HN), and the polymerase protein (L). The genomic RNA of the virus: viral RNA (vRNA) is encapsidated with the NP proteins, and the nucleocapsids are associated with the P and L proteins to form the

ribonucleoprotein complex. In paramyxovirus particles, vRNA is enclosed by the viral envelope composed of a cellular lipid bilayer and two envelope glycoproteins, HN and F, which are integral transmembrane proteins mediating virus attachment and cell fusion, respectively [15]. M protein underlies the lipid bilayer to ensure the structural integrity of the viral particles and is essential for interactions between the viral envelope and the RNP complex [15]. This association leads to the budding and release of viral particles from the cell surface [15].

Recently, as technology advances in reverse genetics [16], hPIVs offer several advantages as a vaccine vector. hPIVs efficiently infect the respiratory tract but don't spread far beyond it, which is an important safety factor. hPIV-based vectors have proven the effect in inducing local and systemic immunity against a number of foreign antigens [17]. hPIVs infect to various cell types and cause little cytopathic effects. Moreover, they replicate exclusively in the cytoplasm of infected cells, don't have a DNA phase during their life cycle and can thus avoid the possibility of integration of foreign genes into the host DNA genome [18].

In the present study, we utilized newly engineered rhPIV2: replication-deficient rhPIV2 vector. rhPIV2 lacks M gene that is an essential gene for virus particle formation by insertion of two stop codons. This alteration might support much safer application to animals than original proliferating virus vector. We first

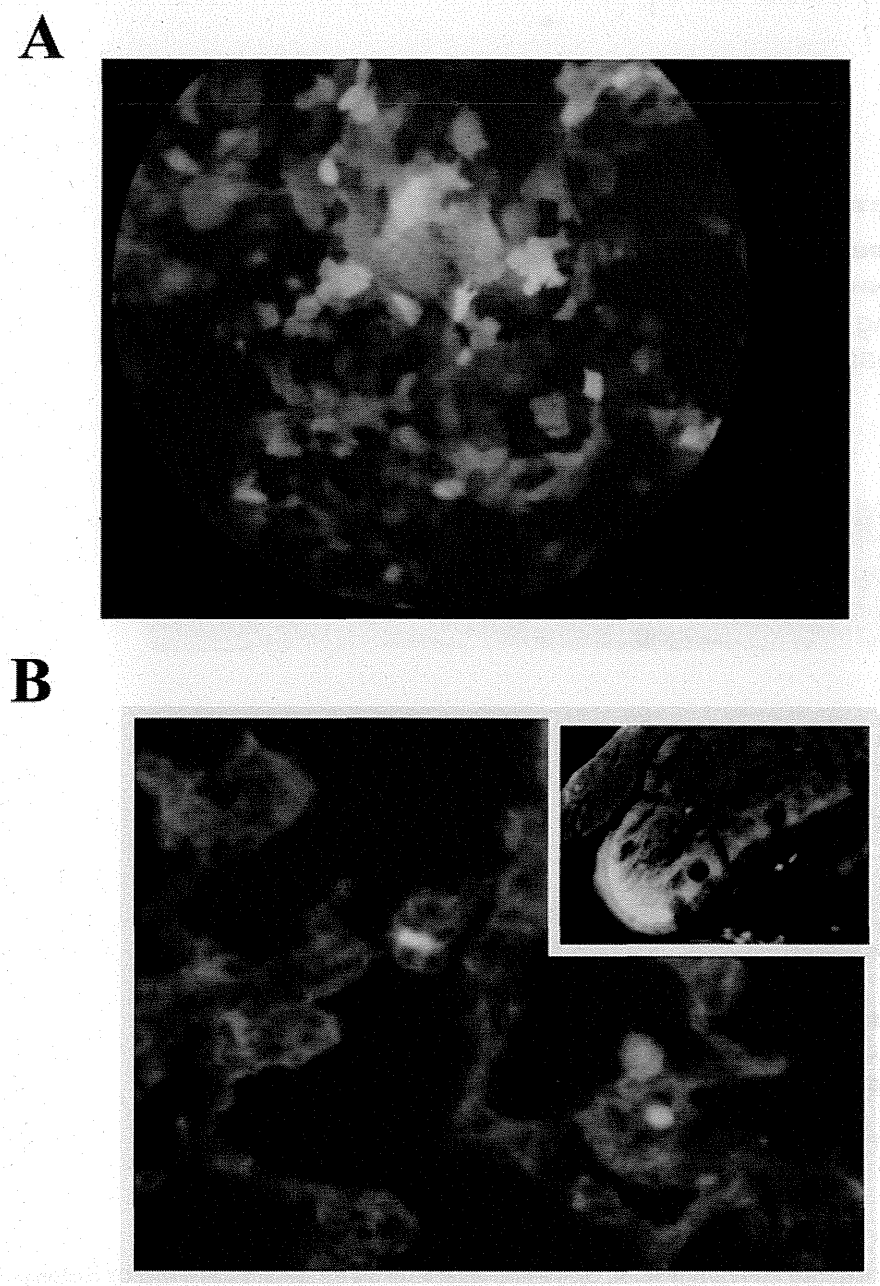

Figure 2. Expression of EGFP from rhPIV2/EGFP. A. HaCat cells were infected with rhPIV2/EGFP at an MOI of 0.5. Three days after, EGFP was clearly visualized using a fluorescence microscopy (x100). **B.** The rhPIV2/EGFP (5×10^6 $TCID_{50}$) were administered to a wild type BALB/c mice intranasally EGFP was visualized clearly in the airway epithelial cells 4 days after administration (x200, upper right box, x400).
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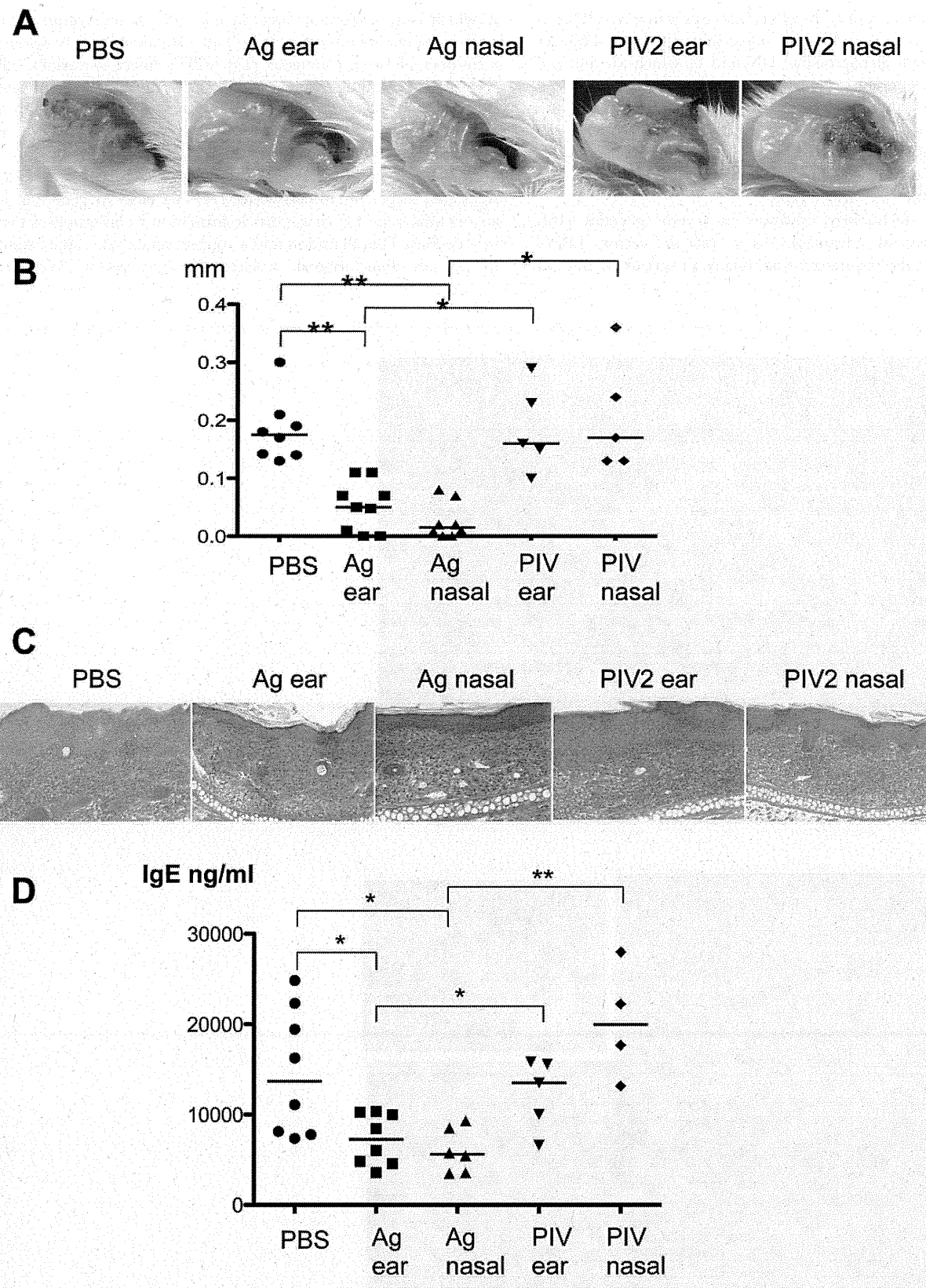

Figure 3. Anti-inflammatory effects of vaccination with rhPIV2/Ag85B. A. Clinical manifestation of the ear skin at 6 hours after OX challenge on day 21. The control groups (PBS, PIV2 ear, and PIV2 nasal on the panel) showed severe edema with erythema, however the intranasal and/or subcutaneous administration of the rhPIV2/Ag85B (Ag nasal and Ag ear on the panel, respectively) clearly reduced skin reactions in OX-sensitized mice. **B.** Ear thickness measured before and 6 hours after each OX application on day 21. The ear swelling was suppressed significantly in rhPIV2/Ag85B treated groups in two ways compared to those in the placebo treated groups. (*$P<0.05$, **$P<0.01$.) **C.** Histopathological changes of the ear skin obtained on day 21 in paraffin embedded sections stained with hematoxylin and eosin. The placebo treated groups (PBS, PIV2 ear and PIV2 nasal

on the panel) revealed marked inflammatory reactions with acanthosis and ulceration in epidermis, and marked edema with cellular infiltration including mononuclear cells and neutrophils in the dermis. The skin infiltration of inflammatory cells and epidermal thickness were decreased in rhPIV2/Ag85B treated group (Ag85B ear and Ag85B nasal on the panel). Original magnification × 100. **D.** Plasma IgE levels on day 21. Plasma IgE level was decreased in rhPIV2/Ag85B treated groups (Ag ear and Ag nasal). *P<0.05, **P<0.01.
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investigated efficiency of rhPIV2 vaccine vector expressing enhanced green fluorescence protein (EGFP) gene (rhPIV2/EGFP) in infection and expression in vitro and in vivo. Then, we evaluated effectiveness of the vaccination pathways: subcutaneous or intranasal administration of rhPIV2 expressing Ag85B gene (rhPIV2/Ag85B) in a mice AD model induced by repeated hapten challenge.

Materials and Methods

Animals

BALB/c 6-week old male mice were purchased from Japan SLC Co. (Shizuoka, Japan) and used at 7-week. Animal care was performed according to ethical guidelines, and approved by the Institutional Board Committee for Animal Care and Use of Mie University.

Construction of rhPIV2/Ag85B and rhPIV2/EGFP

rhPIV2/Ag85B and rhPIV2/EGFP was constructed according to the method reported previously, except for methods of the supply of T7 and hPIV2 RNA polymerases (NP, P, L). In brief, to generate replication-deficient rhPIV2 vector, two nucleotides change [ATG to TAG (position of 89aa) and AAG to TAG (259aa)] were introduced into the M frame of the plasmid pPIV2, a full-length cDNA copy of hPIV2 anti-genome [19] (Fig. 1A). Consequently, the 6 n length cDNA of Ag85B or EGFP, followed by transcriptional end sequence of NP gene (R2), intergenic sequence (IG), and transcriptional start signal of V/P gene (R1) ([20] was synthesized by PCR using appropriate primers), was inserted into a Not I site of the plasmid DNA encoding the replication-deficient rhPIV2 genome described above. Then, the viruses (rhPIV2/Ag85B and rhPIV2/EGFP) were recovered by co-transfection of each anti-genomic plasmid and plasmids expressing the NP, P, M and L, each cloned in a mammalian gene expression vector (pCAGGS) [21] into BSR7/5 cells expressing T7 RNA polymerase [22]. The cells were harvested, and then co-cultured with fresh Vero cells every 48 hr. Approximately 90% of the cells showed syncytia formation in the 10^{th} co-cultured cells, and its state was maintained in further co-culture. Furthermore, for virus propagation, Cos7 cells were transfected with the plasmid expressing M and co-cultured with above-mentioned 10^{th} cells. The supernatant was centrifuged at 9,000 g for 12 h at 4°C. The virus pellet was suspended in Opti-MEM (Invitrogen, Carisbad, CA, USA). The virus titers were determined by CPE method using Vero cells, and were expressed as 50% tissue culture infectious dose ($TCID_{50}$).

In vitro and in vivo Infection of rhPIV2 Vector Expressing EGFP

HaCat cells (Cell Line Service, Eppelheim, Germany) were cultured in Dulbecco's MEM supplemented with 5% (v/v) FBS, 2.0 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. HaCat cells were seeded one day before the transduction at 1×10^6 cells/ml (1 ml/well) in 6-well culture plates (Costar, NY, USA). The cells were incubated for 8 hours at 37°C in a 5% CO_2 atmosphere. The next day, the media was removed and 1 ml of the rhPIV2/EGFP viruses were added to the cells to be adjusted to 1×10^6 $TCID_{50}$. Two hours after infection, the media was removed and fresh culture media was supplemented to the cells. After 3 days culture, each well was observed by fluorescence microscopy.

At the next step, 20 μl of concentrated rhPIV2/EGFP (5×10^6 $TCID_{50}$) were administered into the cavity of the nose of the mice. Four days after infection, the respiratory tract and lung were sampled, embedded in Tissue-Tek OCT compound (Miles, Elkhart, USA), frozen in liquid nitrogen, and cut into 7 μm-thick sections. Sections were examined and recorded by fluorescence microscopy.

Sensitization and Challenge Schedule

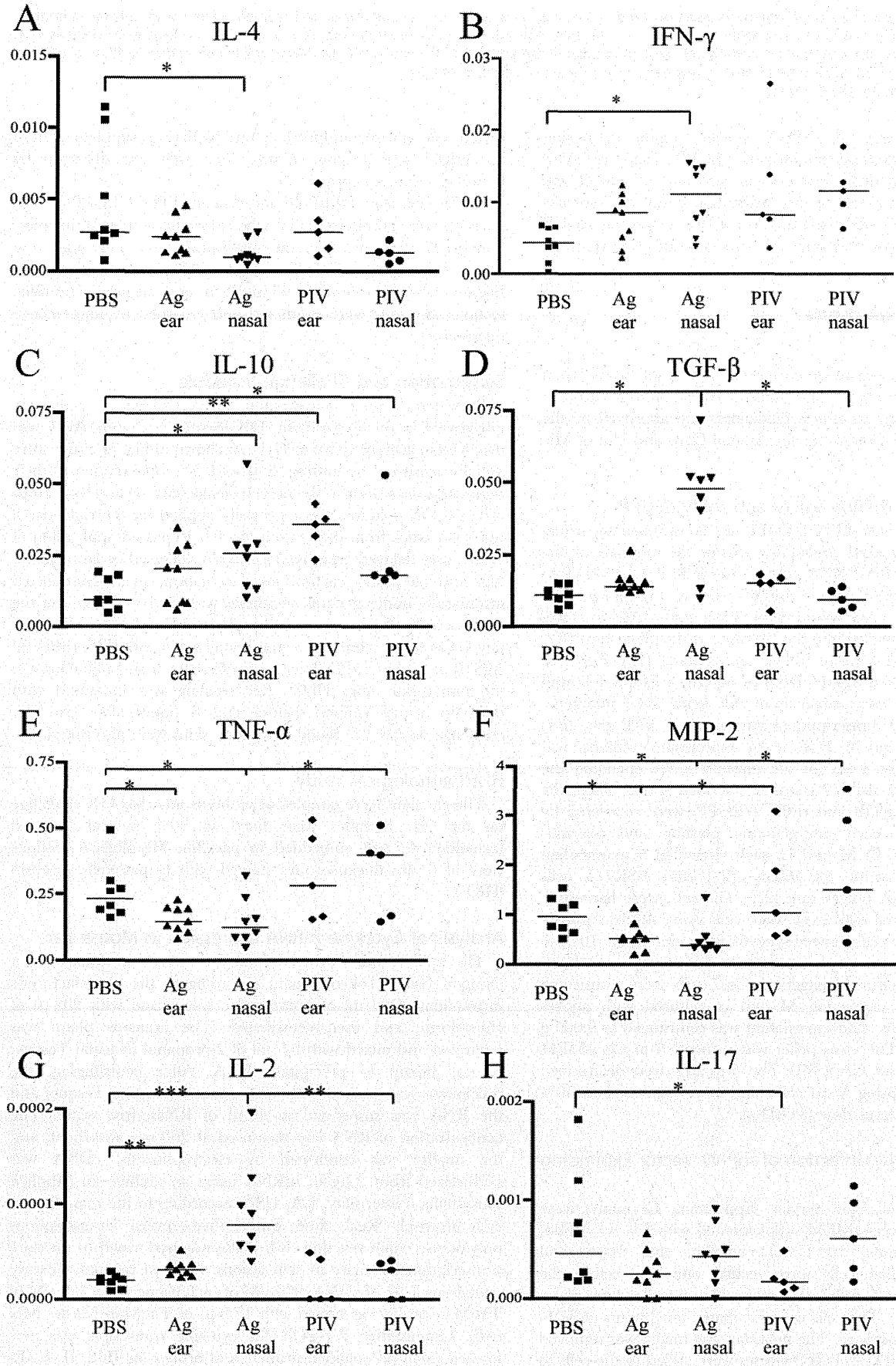
Repeated hapten sensitization and challenge system was introduced in this experiment. OX (Sigma, St. Louis, MO) was dissolved in acetone/olive oil (1:1). As shown in Fig. 1B, mice were initially sensitized by pasting 20 μl of 0.5% OX solution to their right ear 7 days prior to the first challenge (day -7) and then 20 μl of 0.5% OX solution was repeatedly applied on the right ear 3 times per week from day 0 until day 21. Repeated application of OX causes delayed type hypersensitivity followed by immediate-type and late phase reaction. For vaccination, mice were infected intranasally under general anesthesia with 5×10^6 $TCID_{50}$ of the virus in a 20 μl inoculum or phosphate-buffered saline (PBS) on day 4. On day 16, mice were vaccinated again with PBS, rhPIV2/Ag85B or control rhPIV2 vector intranasally or subcutaneously to the pinna skin (Fig. 1B,C). Ear swelling was measured with thickness gauge calipers before and 6 hours after last OX challenge on day 21. Blood and pinna skins were also sampled.

Histopathological Study

The ear skins were sampled at six hours after last OX challenge on day 21. Samples were fixed in 10% neutral buffered formaldehyde and embedded in paraffin. Histological sections were of 6 mm thickness and stained with hematoxylin & eosin (H&E).

Analysis of Cytokine mRNA Expression in Mouse Ear

The mRNA was extracted from the mouse ear using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions: One ml of homogenate was mixed with 200 μl of chloroform, and then centrifuged. The aqueous phase was separated and mixed with 0.5 ml of 2-propanol (Nacalai Tesque, Kyoto, Japan) to precipitate RNA. After centrifuging, the precipitate was washed with 70% ethanol (Nacalai Tesque) and the RNA was suspended in 40 μl of RNase-free water. The concentration of RNA was measured at 260 nm absorbent, and the quality was confirmed by electrophoresis. cDNA was synthesized from 2 μg of mRNA using an archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Real time quantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed to measure transcriptional activity in skin lesions. A 25 μl reaction mixture containing 1 μg of cDNA, 900 nM of each primer, and 250 nM of TaqMan probe was mixed with 12.5 μl of TaqMan Master Mix (AB). Quantitative RT-PCR for cytokine transcripts was performed using prequalified primers and probes for IL-2, IL-4, IL-10, IL-17A, MIP2, TNF-α, TGF-β, IFN-γ and GAPDH (AB). The


A
IL-4
0.015
0.010
0.005
0.000
B
IFN-γ
0.03
0.02
0.01
0.00
C
IL-10
0.075
0.050
0.025
0.000
D
TGF-β
0.075
0.050
0.025
0.000
E
TNF-α
0.75
0.50
0.25
0.00
F
MIP-2
4
3
2
1
0
G
IL-2
0.0002
0.0001
0.0000
H
IL-17
0.002
0.001
0.000
PBS
Ag ear
Ag nasal
PIV ear
PIV nasal