$CD11b^{+}$ dendritic cells-mediated immune induction of inactivated eyedrop vaccine

Eun-Do Kim

Department of Medical Science

The Graduate School, Yonsei University

$CD11b^{+}$ dendritic cells-mediated immune induction of inactivated eyedrop vaccine

Eun-Do Kim

Department of Medical Science

The Graduate School, Yonsei University

$CD11b^{+}$ dendritic cells-mediated immune induction of inactivated eyedrop vaccine

Directed by Professor Kyoung Yul Seo

Doctoral Dissertation

submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Eun-Do Kim

December 2016

연세대학교
YONSEI UNIVERSITY

This certifies that the Doctoral Dissertation of Eun-Do Kim is approved.

Thesis Supervisor: Kyoung Yul Seo

Thesis Committee Member#1: Jeon-Soo Shin

Thesis Committee Member#2: Je-Wook Yu

Thesis Committee Member#3: Sung Jae Shin

Thesis Committee Member#4: Sun-Young Chang

The Graduate School

Yonsei University

December 2016

Acknowledgements

I deeply thanks to my God. He gives me wisdom, intelligence, cleverness, ideas, understanding and creativeness. He opened the life of research and has guided me all along my Ph.D. course.

I want to give thanks to Professor Kyoung Yul Seo who has guided, trusted and supported my all research and learning during my Ph.D. course. Importantly, I could learn how to investigate as an independent scientist. It is absolutely worth.

I am grateful to Prof. Jeon-Soo Shin, Prof. Je-Wook Yu, Prof. Sung Jae Shin and Prof. Sun-Young Chang for much efforts and patience to review my Ph.D. thesis. I wish to thank Soo Jung Han who has given many valuable helps and advices for my research during over a half-decade. I also want to thank special helps from Dae-Hui Kim and Soonjang Lee. Without their contribution it would be impossible to finish the work in this winter. I also appreciate to Dr. Kyuong Sub Choi and Sang Chul Yoon, who gave me supports, ideas and inspirations.

I want to express gratitude to my parents who always supports my dreams and vision. Based upon their supports, I could choose to be a scientist. I want to thanks to Keumdo, Youngdo and Yedo, who are best brothers and friends to me all the time. Finally, I want to dedicate this achievement to my beloved fiancée, Sunyoung Kim, for she waited for me with patience and trust. Her belief that I am an excellent scientist contributing to a better world is the most encouraging support.

Again, I give all of my honors and glory only to God, my Lord.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I

CHAPTER II

LIST OF TABLES

CHAPTER I

LIST OF ABBREVIATIONS

CHAPTER I

CT	cholera toxin
EDV	eyedrop vaccine
CNS	central nervous system
IN	intranasal
S-IgA	secretory-IgA
LAIV	live-attenuated influenza vaccines
OB	olfactory bulb
CALT	conjunctiva-associated lymphoid tissue
TALT	tear-associated lymphoid tissue
poly(I:C)	polyriboinosinic:polyribocytidylic acid
HA	hemagglutinin
ERG	electroretinalgram
OVA	ovalbumin
MPLA	monophosphoryl lipid A
PBS	phosphate-buffered saline
$TCID_{50}$	50% tissue culture infective doses
IP	intraperitoneal
BALF	bronchoalveolar lavage fluid
ELISA	enzyme-linked immunosorbent assay
dpi	days post infection
PFU	plaque forming units
HI	hemagglutination inhibition
Abs	antibodies
IM	intramuscular
TLR	toll-like receptor

CHAPTER II

EDV	eyedrop vaccine
APCs	antigen presenting cells
dLN	draining lymph nodes
MdLN	mandibular lymph nodes
SPLN	superficial parotid lymph nodes
SCJ	subconjunctival
DCs	dendritic cells
TLR	toll-like receptor
LNs	lymph nodes
CLNs	Cervical lymph nodes
cDCs	classical dendritic cells
OVA	ovalbumin
CT	cholera toxin
PBS	phosphate-buffered saline
MsLNs	mediastinal LNs
PE	phycoerythrin
FACS	fluorescence-activated cell sorting
Ags	antigens
dpi	days post injection

GENERAL ABSTRACT

CD11b+ dendritic cells-mediated immune induction of inactivated eyedrop vaccine

Eun-Do Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Kyoung Yul Seo)

The eye route has been evaluated as an efficient vaccine delivery routes. However, in order to induce sufficient antibody production with inactivated vaccine, testing of the safety and efficacy of the use of inactivated Ag plus adjuvant is needed. Here, I assessed various types of adjuvants in eyedrop as an anti-influenza serum and mucosal Ab production-enhancer in BALB/c mice. Among the adjuvants, poly (I:C) showed as much enhancement in Ag-specific serum IgG and mucosal IgA antibody production as cholera toxin (CT) after vaccinations with trivalent hemagglutinin-subunits or split H1N1 vaccine Ag in mice. Vaccination with split H1N1 eyedrop vaccine (EDV) Ag plus poly(I:C) showed a similar or slightly lower efficacy in inducing antibody production than intranasal vaccination; the EDV-induced immunity was enough to protect mice from lethal homologous influenza A/California/04/09 (H1N1) virus challenge. Additionally, ocular inoculation with poly(I:C) plus vaccine Ag generated no signs of inflammation within 24 hours: no increases in the mRNA expression levels of inflammatory cytokines nor in the infiltration of mononuclear cells to administration sites. In contrast, CT administration induced increased expression of IL-6 cytokine mRNA and mononuclear cell infiltration in the conjunctiva within 24 hours of vaccination. Moreover, inoculated visualizing materials by eyedrop did not contaminate the surface of the olfactory bulb in

mice; meanwhile, intranasally administered materials defiled the surface of the brain. On the basis of these findings, I propose that the use of inactivated influenza EDV plus poly(I:C) is a safe and effective mucosal vaccine strategy for inducing protective anti-influenza immunity.

Although the efficacy of inactivated EDV was confirmed, the type of antigen presenting cells (APCs) that mediates antigen-specific immune induction has not been reported. Moreover, how the EDV is delivered into the draining lymph nodes (dLN), which are mandibular lymph nodes (MdLN) and superficial parotid lymph nodes (SPLN), is not clarified. In here, I showed that the delivery of proteins or fluorescent beads into the dLN administered by eyedrop or subconjunctival (SCJ) injection is not dependent on the migration of dendritic cells (DCs) or the activation of DCs by TLR stimulation. Instead, the particulates were delivered by flow of lymphatic drainages into the dLN. Among two comprising parts of the dLN, cells in MdLN showed higher levels of percentages of PE-beads^{+} than SPLN. In MdLN, CD11b^{+} DCs were in significantly higher percentages of phycoerythrin (PE)-beads^{+} than other subsets of DCs do in both resident and migratory DCs. In CD11b knockout mice, the levels of antigen-specific serum IgG or mucosal IgA production were significantly decreased. Thus, it is expected that the strategy targeting resident CD11b^{+} DCs in MdLN utilizing lymphatic drainage can strengthen the development of effective EDV.

CHAPTER I

Eyedrop vaccines in mice and ferrets Induce protective immunity against viral challenges

ABSTRACT (CHAPTER I)

Eyedrop vaccines in mice and ferrets induce protective immunity against viral challenges

Eun-Do Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Kyoung Yul Seo)

The eye route has been evaluated as an efficient vaccine delivery routes. However, in order to induce sufficient antibody production with inactivated vaccine, testing of the safety and efficacy of the use of inactivated Ag plus adjuvant is needed. Here, I assessed various types of adjuvants in eyedrop as an anti-influenza serum and mucosal Ab production-enhancer in BALB/c mice. Among the adjuvants, poly(I:C) showed as much enhancement in Ag-specific serum IgG and mucosal IgA antibody production as cholera toxin (CT) after vaccinations with trivalent hemagglutinin-subunits or split H1N1 vaccine Ag in mice. Vaccination with split H1N1 eyedrop vaccine (EDV) Ag plus poly(I:C) showed a similar or slightly lower efficacy in inducing antibody production than intranasal vaccination; the EDV-induced immunity was enough to protect mice from lethal homologous influenza A/California/04/09 (H1N1) virus challenge. Additionally, ocular inoculation with poly(I:C) plus vaccine Ag generated no signs of inflammation within 24 hours: no increases in the mRNA expression levels of inflammatory cytokines nor in the infiltration of mononuclear cells to administration sites. In contrast, CT administration induced increased expression of IL-6 cytokine mRNA and mononuclear cell infiltration in the conjunctiva within 24 hours of vaccination. Moreover, inoculated visualizing materials by eyedrop did not contaminate the surface of the olfactory bulb in

mice; meanwhile, intranasally administered materials defiled the surface of the brain. On the basis of these findings, I propose that the use of inactivated influenza EDV plus poly(I:C) is a safe and effective mucosal vaccine strategy for inducing protective anti-influenza immunity.

Besides, I investigated EDV in pre-clinical development for immunological protection against influenza and for potential side effects involving ocular inflammation and the central nervous system (CNS). Live attenuated influenza EDV, CA07 (H1N1), PZ-4 (H1N2) and Uruguay (H3N2), induced both systemic and mucosal virus-specific antibody responses in ferrets. In addition, EDV resulted in a clinically significant protection against viral challenge, and suppression of viral replication in nasal secretion and lung tissue. Regarding safety, I found that administered EDV flow through the tear duct to reach the base of nasal cavity, and thus do not contact the olfactory bulb. All analyses for potential adverse effects due to EDV, including histological and functional examinations, did not reveal significant side effects. On the basis of these findings, I propose that EDV as effective, while being a safe administration route with minimum local side effects, CNS invasion, or visual function disturbance.

Key words: eyedrop, poly(I:C), influenza, inactivated-vaccine, ferret, live-attenuated influenza vaccines

Eyedrop vaccines in mice and ferrets induce protective immunity against viral challenges

Eun-Do Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Kyoung Yul Seo)

I. INTRODUCTION

For immunization against influenza, there are two major routes of vaccination: muscular injection and intranasal (IN) administration. Parenteral injection is the most widely and traditionally used method in almost all vaccine regimens; nevertheless, such injections mainly induce serum IgG antibody without inducting secretion of IgA to mucosal surfaces of the respiratory tract, which is the main infection route of the influenza virus. In contrast, intranasal administration induces both systemic IgG and mucosal secretory-IgA (S-IgA) production, initiating mucosal immunity; therefore, intranasal vaccination is more potent than parenteral injection for the prevention of influenza.[1,2] Moreover, IN vaccination is advantageous in that is does not require the use of syringes, enabling anyone to readily administer the vaccine without special training.

Recently, some nasal spray live-attenuated influenza vaccines (LAIV), such as FluMist®, were approved by the Food and Drug Administration (FDA) for human use in the United States. However, LAIV can cause some side effects such as sore throat, coryza, and febrile reactions.[3] As a result, it is not allowed for use in pregnant woman and immunodeficient patients, as well as in children under the age of 12 months[4] or adults over 50.[5] Therefore, two major high-risk

groups are excluded from vaccination with the live-virus vaccine. Meanwhile, studies showed that if the inactivated influenza vaccines are intranasally administered, it can induce nerve damage with olfactory bulb (OB)-mediated Ag and adjuvant diffusion into the brain, in the presence of cholera toxin (CT) adjuvant.[6,7] Moreover, introduction of inactivated intranasal influenza vaccine reportedly provoked Bell's palsy in human.[8] Thus, many studies have attempted to devise alternative ways of inducing mucosal immunity to circumvent the side effects of the intranasal influenza vaccines.

Lately, the eye mucosa has come to the forefront as a promising vaccination route. The eye mucosa, which exhibits the common immunological structures of mucosal tissues, including conjunctiva-associated lymphoid tissue (CALT)[6,9-11] and tear-associated lymphoid tissue (TALT).[12,13] is an inductive site for the acquirement of systemic and mucosal immunity. The early trials of eyedrop vaccination in avian and bovine models showed that eyedrop vaccination induces protective immunity against Newcastle disease virus and Brucella melitensis, respectively[14,15]. Additionally, our group was the first to report the feasibility of the use of eyedrop influenza and Salmonella vaccines in mice.[6] Furthermore, eyedrop vaccination does not redirect Ag with CT into the CNS as in intranasal vaccination.[6,7]

Polyriboinosinic:polyribocytidylic acid (poly(I:C)), a ligand of mammalian toll-like receptor 3, a known receptor for double-stranded RNA, induces interferon alpha/beta production via activation of NF-κB pathways.[16] Poly(I:C), therefore, exerts adjuvant effects by linking the gap between innate and adaptive immunity by enhancing primary antibody responses.[17] Additionally, the mucosal adjuvant effect of poly(I:C) against influenza virus has also been shown in intranasally vaccinated inactive-influenza virus hemagglutinin (HA) vaccine plus poly(I:C) in mice.[18] Recently, the activity of poly-L-lysine stabilized poly(I:C) analogue (poly ICLC) as a vaccine adjuvant and innate-immunity activator was assessed in non-human primates and human models, respectively.[19,20] Thus, clinical trials of the use of poly(I:C) and its safe analogue

may demonstrate the possibility of using potent adjuvant in influenza vaccine immunization.

In the previous study, it was reported that eyedrop vaccination with live-attenuated vaccines, such as influenza virus or Salmonella bacteria, can protect mice from lethal challenge of pathogens.[6] However, the vaccines used in the study were live-attenuated, which may cause unexpected side-effects. Therefore, examining the efficacy of inactivated vaccines immunized by the eye-route is necessary in order to circumvent the use of live-vaccine. However, since it is hard to induce a sufficient immune response by inactivated influenza vaccine-Ag alone, use of adequate adjuvants to induce complete protective immunity is critical to vaccination with inactivated-vaccine Ags.

Meanwhile, the eye mucosa has proven to be an effective Ag delivery route by previous studies in fowls, bovine, goats, and chicken models of immunization.[12,21-24] Importantly, a recent investigation in mice determined that animals immunized with an influenza eyedrop vaccine (EDV) were protected from lethal pathogen infection.[6] Nevertheless, the clinical significance of these results is inherently compromised since mice are not natural hosts of influenza.

Ferret is one of the most appropriate animal models for the study of influenza EDV for several reasons. First, ferrets are widely used in the study of visual system because their ocular anatomy and physiology are similar to those of humans.[25] Second, ferrets have shown to be good model to investigate the pathogenesis and transmission of influenza since they exhibit a similar level of susceptibility and clinical response to human influenza virus in terms of clinical presentation and respiratory physiology.[26,27] Lastly, compared to the nonhuman primates, ferrets are highly preferable in terms of availability, cost of caring, and regulations associated with procurement and maintenance.

Here, I show that poly(I:C) is a potent adjuvant, except for CT, for use in eye-route vaccination for the immunization of killed-influenza virus vaccine Ag. Administration of inactivated influenza vaccine plus poly(I:C) by eyedrops

exerted significantly enhanced production of Ag-specific Ab in both systemic and mucosal immunity, by which mice were protected from lethal influenza virus challenge. Also, there was no signs of inflammation in the eyes after poly(I:C) was administered. In addition, administration of vaccine materials by eyedrops did not contaminate the surface of the brain in contrast to IN, which defiled olfactory bulb regions of the mouse brain.

Moreover, this study aims to evaluate the safety and efficacy of EDV in a ferret model of influenza. Previous investigations detailing immune provocation and acquired protection in mouse models are extended with our analyses in ferrets by providing critical commentary data on clinical presentation and organ histology. Furthermore, I have also been able to address potential safety concerns relating to adverse effects on the CNS and ocular inflammation. On the basis of my findings, I propose that eyedrop vaccination of killed-influenza vaccine along with poly(I:C) or live EDV is an alternative effective and safe preventive mucosal vaccines to induce protective immunity against influenza virus infection in both mice and ferrets.

II. MATERIAL AND METHODS

1. Animals

All studies using mice were performed in strict accordance with the recommendations in the Guide for the Care and Use Committee of Yonsei University Health System. The committee has reviewed and approved the animal study protocol (Approval No: 2011–0137). Specific pathogen-free female BALB/c mice, aged 6-10 wks, were purchased from Charles River Laboratories (Orient Bio, Sungnam, Korea). All mice were maintained in the experimental animal facility under specific pathogen-free conditions at Yonsei College of Medicine (Seoul, Korea) and received sterilized food (Certified Diet MF; Oriental Yeast, Osaka, Japan) and filtered tap water ad libitum. All surgeries were performed after sacrificed by CO_2 narcosis and every effort was made to minimize suffering.

For ferret experiments, all experiments related to vaccination and virus infection of animal subjects along with the sample preparation of the ferrets were conducted in strict accordance and adherence to relevant Council of the Republic of Korea and international guidelines regarding animal handling approved by the Animal Use and Care by Laboratory Animal Research Centre (LARC; permit #BLS-ABSL-12-010) of Chungbuk National University (Cheong-ju, Korea), a member of the International Animal Care and Use Committee (IACUC). All experiments using electroretinalgram (ERG) to animal subjects were conducted in strict accordance and adherence to relevant national and international guidelines regarding animal handling approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System (Seoul, Korea; permit #2011-0137).

2. Vaccine Ags and adjuvants for mice

The influenza virus subunit vaccines were provided by Dr. Na Gyong Lee (Sejong University, Seoul, Korea). The trivalent HA vaccine comprised the HA subunits from three influenza virus strains: A/New Caledonia/20/99 (H1N1), A/Panama/2007/99 (H3N2) and B/Shandong/7/97. The split H1N1 influenza

virus vaccine was provided by Green Cross Co. (Yongin, Gyeonggi, Korea). The vaccine comprised the split influenza A/California/7/2009 (NYMCX-181) (H1N1) virus. The following commercial vaccines were purchased (all from SANOFI PASTEUR S.A., Lyon, France unless noted) : Avaxim® 160U Adult Inj., Imovax Polio Inj., Act-HIB®, BCG (Japan BCG Laboratory, Tokyo, Japan), M-M-R®II (Merck Sharp & Dohme Corp., PA), Hepavax-Gene®TF inj. (Green Cross Co., Yongin, Korea), Suduvax® (Green Cross Co., Yongin, Korea), DPT-3 VACCINE inj. (SK Chemical Co., Osan, Korea), and Cervarix® (GlaxoSmithKline, London, England). Various adjuvants were used including cholera toxin (CT; List Biological Laboratories, Campbell, CA), polyinosinic-polycytidylic acid (poly(I:C), monophosphoryl lipid A (MPLA) from Salmonella minnesota, CpG oligonucleotide (InvivoGen, San Diego, CA), and Imject® Alum (Thermo Scientific, Rockford, IL).

3. Immunization for mice

Prior to experimental manipulation, 6-10-wk-old female mice were anesthetized by intraperitoneal (IP) injection of zoletil (30 mg/kg body weight) and rompun (10 mg/kg body weight). For conjunctival immunization, 100 μg of ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) and various adjuvants (0.1 μg to 10 μg poly(I:C) or 10 μg monophosphoryl lipid A (MPLA) or 2 μg CT or 1 mg Imject® Alum) were suspended in 5 μl of phosphate-buffered saline (PBS) and dropped weekly for 3 consecutive wks onto both conjunctival sacs by a micropipette. In some experiments, mice were immunized with 1 μg of trivalent-HA vaccine or 1 μg of H1N1 split vaccine with various adjuvants resuspended in 15 μl or 10 μl of PBS, respectively, and subsequently dropped once more at a 2-wk interval. For HA Ag-dose dependent immunization experiments, 0.5 μg or 1 μg of HA Ags plus 10 μg poly(I:C) were resuspended in 10 μl of PBS, and 2 μg of HA Ags plus 10 μg poly(I:C) were resuspended in 15 μl of PBS. For IN immunization, mice were administered with 1 μg of trivalent-HA vaccine or 1 μg of H1N1 split vaccine with various adjuvants resuspended in 20 μl of PBS and subsequently vaccinated once more at a 2-wk interval. For long-term immunity induction experiments, mice were immunized

with 1 μg of H1N1 split vaccine plus 10 μg of poly(I:C) resuspended in 10 μl of PBS three times at 2-wk intervals.

4. Viruses for ferrets

The live-attenuated viruses (Table. 1) used in this study were adapted in egg at 27℃ (cold adaptation, ca) more than 10 times. The inefficient growth of the ca, live-attenuated viruses compared to wild type corresponding viruses (10-100 fold decreases) was confirmed at 37℃. The human and animal infectious influenza viruses (Table 1) used for challenge in this study were provided by Chungbuk National University (Cheongju, South Korea) and amplified in 10-day-old embryonated chicken eggs. Viruses were serially diluted (10-fold) prior to infection in Mardin-Darby canine kidney cells and the 50% tissue culture infective doses ($TCID_{50}$) was then calculated by the Reed-Muench method.[28] Stock viruses were kept at -82°C, and thawed right before use. Viral growth was determined by observing changes in cellular morphology (cytopathic effects) and hemagglutinin (HA) assay.

Table 1. List of influenza viruses used for vaccination and challenge in this study

Group (subtype)		Virus strain	Abbreviation	Challenge dose ($TCID_{50}$)	Similarity
H1N1	Vaccination	A/California/7/09 x PR8[§]	CA07 (H1N1)	-	-
	Challenge	A/California/04/09	CA04 (H1N1)	10^5	Homologous
H1N2	Vaccination	Sw/Korea/PZ4/06[†]	PZ-4 (H1N2)	-	-
	Challenge	A/Sw/Korea/1204/09	Sw09 (H1N2)	10^6	Heterologous
H3N2	Vaccination	A/Uruguay/716/07x PR8[§,‡]	Uruguay (H3N2)	-	-
	Challenge	A/Hongkong/68	HK68 (H3N2)	10^6	Heterologous

[§]A/Puerto Rico/8/34; this virus is used as a backbone strain. [†]Cross-reactive HI titer between Sw/Korea/PZ4/06 and Sw/Korea/1204/09 is 2 fold lower than the HI titer of homologous Sw/Korea/PZ4/06 virus (160 Vs 320 HI units). [‡]Cross-reactive HI titer between Uruguay/716/07xPR8 and Hongkong/68 is 0.

5. Vaccination and virus challenge for ferrets

15- to 16-wk-old ferrets were purchased from Marshall BioResources (North Rose, NY, USA). All animals were confirmed seronegative for all vaccine strains of influenza A viruses used in this study, including CA07 (H1N1), PZ-4 (H1N2), and Uruguay (H3N2) by serologic assay. For conjunctival immunizations, ferrets were anesthetized by inhalation of isoflurane, and then 10^5 $TCID_{50}$ of the CA07 (H1N1), PZ-4 (H1N2), and Uruguay (H3N2) LAIVs in 100 μL PBS were dropped to each eye (200 μL/head) twice with a two wk interval. Two wks after the second immunization, ferrets were intranasally instilled with 1.0 mL volume of 10^5 $TCID_{50}$/mL for the CA04 (H1N1) and 10^6 $TCID_{50}$/mL for the Sw09 (H1N2) and HK68 (H3N2) for virus challenge.[29] Body temperatures and body weights of infected ferrets were monitored daily. There was no death after the viral infection.

6. Sample collection from mice

At two wks or one year after the final immunization, serum was obtained by retro-orbital bleeding. Tear-wash samples were obtained by lavaging with 10 μl of PBS per eye. Saliva was obtained following IP injection of mice with pilocarpine (500 mg/kg body weight; Sigma-Aldrich). Fecal extract was obtained by adding weighed feces to PBS containing 0.1% sodium azide. The feces samples were mixed by vortexing and subsequently centrifuged, and the supernatants were collected for assay. Vaginal wash samples were collected by lavage with 100 μl of PBS. To obtain bronchoalveolar lavage fluid (BALF), tracheas were cannulated after exsanguination, and the lungs were washed with 1 ml of PBS. After the mice were sacrificed, nasal wash samples were obtained by flushing 100 μl of PBS through the anterior (oral) entrance of the nasal passages using a pipette.

7. Sample collection from ferrets

The ferrets were anesthetized with Zoletil 50® (125 mg zolazepam and 125 mg tiletamine hydrochloride [Vibrac, Carros, France]; 0.2 mg/kg of body weight) and Rompun® (2% xylazine hydrochloride [Bayer Animal Health, Leverkusen, Germany]; 5 mg/kg of body weight) administered intramuscularly 10 min

before sampling. Serum samples from ferrets were collected for HI assays two wks after each vaccination. Nasal secretion samples were collected in 1X PBS with antibiotics two wks after every vaccination for HI assay or at 1, 3, 5, 7, and 9 days post infection (dpi) after viral challenge for titer assays. For the preparation of lung tissue samples, ferrets were euthanatized by CO_2 inhalation. The lung tissues were collected at 5 dpi for histopathology or homogenized in 1X PBS containing antibiotics for virus titration. Tissue homogenates of both lobes were clarified by centrifugation at 12,000*g*, and supernatants were then transferred to fresh tubes. All samples were stored at -82°C until use.

8. ELISA for detection of Ag-specific Ab

Enzyme-linked immunosorbent assay (ELISA) plates (Nunc, Roskilde, Denmark) were coated with OVA (100 μg/ml) or HA vaccine Ag (2 μg/ml) or H1N1 split vaccine (1 μg/ml) in coating buffer and incubated overnight at 4°C. Blocking was done with 1% bovine serum albumin (Sigma-Aldrich) in PBS, and two-fold serially diluted samples were applied to plates. HRP-conjugated goat anti-mouse IgG or IgA Ab (Southern Biotechnology Associates, Birmingham, AL) was added to each well and incubated overnight at 4°C. For color development, tetra-methylbenzidine solution (Thermo Scientific, Rockford, IL) was used. Plates were then measured at 450 nm on an ELISA reader (Molecular Devices, Sunnyvale, CA) after addition of stopping solution (0.5 N HCl). Endpoint titers of Ag-specific Ab were expressed as reciprocal log2 titers of the last dilution that showed > 0.1 absorbance over background levels.

9. cDNA synthesis and real-time quantitative PCR

After a wash with nuclease-free water, I homogenized the whole conjunctival or corneal tissue samples at different time points after eye-drop immunization with HA vaccine alone (1 μg), HA plus poly(I:C) (10 μg), or HA plus CT (2 μg). Total RNA was extracted using TRIzol (Invitrogen), and cDNA was synthesized by Superscript II reverse transcriptase with oligo (dT) primer (Invitrogen). cDNA was amplified with HotStart-IT® SYBR® Green qPCR Master Mix (USB, Cleveland, OH) and gene-specific forward and reverse primers on an

ABI 7300 Real- Time PCR system (Applied Biosystems). Results are expressed as mean ± S.D. after normalizing to the expression of β-actin gene using the ΔΔCt method. Primer sequences are available upon request.

10. Protection assay against influenza virus A/California/04/09

At two wks or one year after the final eyedrop or intranasal immunization with 1 μg of H1N1 split vaccine alone or plus 10 μg of poly(I:C), five mice in each group were anesthetized and challenged with 50 μl of mouse-adapted live influenza A/California/04/09 (H1N1) virus suspension (10X LD_{50}; 0.75 $TCID_{50}$) via the IN route. Animals were monitored for weight loss and survival every day for 14 days, and there were 3 unexpected deaths in PBS or H1N1 Ag alone administered groups. The specific clinical signs I used to determine when the animals should be euthanized were the loss of 20% of initial bodyweights. Euthanasia was done by CO_2 inhalation with a fill rate of about 10% to 30% of the chamber volume per minute with carbon dioxide, added to the existing air in the chamber.

11. Plaque assay

The measurement of the viral titers in lung tissues in virus-challenged mice were measured as previously described.[30] Briefly, the viral titers used in this study were expressed as the plaque forming units (PFU) calculated by plaque assay. Monolayers of Madin-Darby Canine Kidney cells in 12-well-plates were infected with 10-fold serial dilutions of virus solutions for 45 min at room temperature. After the removal of the solutions, the cells were washed with PBS and overlaid with minimum essential medium, containing 1% low melting agarose (Lonza, ME, USA) and 10 μg/ml of trypsin (Life Technology, NY, USA). After the overlay turned solid, the plates were moved to an incubator with 5% CO_2. The plaques formed were fixed by formaldehyde solution and visualized by staining with crystal violet.

12. Serologic assays in ferrets

Hemagglutination inhibition (HI) assays were performed as previously described to determine the sero-prevalence of vaccinated and challenged

viruses.[31] Briefly, serum samples were heat inactivated at 56°C for 30 min and pretreated with receptor-destroying enzyme (RDE) from *Vibrio cholerae* (Denka Seiken; Tokyo, Japan) to remove non-specific serum inhibitors. Sera were then analyzed for the presence of virus-specific antibody by HI assays with 0.5% turkey red blood cells (RBC). The HI titer was determined by the reciprocal of the last dilution that contained turkey RBCs with no agglutination. Neutralization tests performed on selected HI-positive sera to confirm results and used to determine MDCK cell infection and expressed as the reciprocal of the highest dilution of serum that gave 50% neutralization of 100 $TCID_{50}$ of virus after incubation at 37°C for 72 h.[32,33] HI assays were performed according to WHO/World Organization for Animal Health (OIE) recommendations.[34]

13. Histology for mice

Eye tissues including the conjunctiva and eye balls from controls and 1 μg HA vaccine plus 10 μg poly(I:C) or 2 μg CT treated mice were washed with PBS and fixed in 4% formaldehyde for 24 hr at 4°C. The tissues were dehydrated by gradual soaking in alcohol and xylene and then embedded in paraffin. The paraffin-embedded specimens were cut into 5mm sections and stained with H&E.

14. Histology for ferrets

The lungs or eye tissue of ferrets from each group were harvested at 5 dpi or 24 hour, respectively. Tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned (4 μm), and examined in the pathology laboratory of Chungbuk National University Hospital or Yonsei University College of Medicine. Histological assessment was performed by standard hematoxylin and eosin staining and light microscopy at 200X magnification. In blind fashion, either left or right lobes of five lungs were examined with five to seven slides per each lobe. Semi-quantitative analysis of lung inflammation severity in influenza virus-challenged ferrets was performed with some modification as reported elsewhere[35] for the alveoli.

15. Micro-CT

Imaging was performed as previously described[36] with minor modification using a volumetric CT scanner (NFR-Polaris-G90MVC: NanoFocusRay, Iksan, Korea). Briefly, images were acquired at 65 kVp, 115 μA, and 142-millisecond per frame, and for 700 views. The estimated radiation dose was ~150 mGy using this image acquisition protocol. Images were reconstructed using the volumetric cone-beam reconstruction (FDK) off line mode. The size of reconstruction images was 1,204 x 1,024 pixels, and 512 slices were acquired. The final reconstructed data were converted to the Digital Imaging and Communications in Medicine (DICOM) format to generate 3D-rendered images using 3D-rendering software (Lucion, MeviSYS, Seoul, Korea). For ex vivo brain CT imaging, all BALB/c mice were sacrificed, and the brains of each ocularly or intranasally treated mouse were taken out and CT images thereof were acquired.

16. Electroretinalgram (ERG)

The ferrets were anesthetized with Zoletil 50® (125 mg zolazepam and 125 mg tiletamine hydrochloride [Vibrac, Carros, France]; 0.2 mg/kg of body weight) and Rompun® (2% xylazine hydrochloride [Bayer Animal Health, Leverkusen, Germany]; 5 mg/kg of body weight) administered intramuscularly 10 min before ERG examinations. Pupils were maximally dilated with achieved with 0.5% phenylephrine hydrochloride and 0.5% tropicamide (Mydrin-P®, Santen, Osaka, Japan). Animals were placed in a special holding system to prevent unfavorable movement during full-field ERG recording.

Full-field ERG recording was performed with RETIscan® (Roland Consult, Wiesbaden, Germany) in the same examination room. A Goldring electrode® with diameter of 3 mm (Roland Consult, Wiesbaden, Germany) was placed on the corneal surface as an active electrode using 0.3% hypromellos (GenTeal®, Novartis, Basel, Switzerland), while a second reference electrode was placed on the fornix of the same eye. Concentric subdermal needle electrodes (Roland Consult, Wiesbaden, Germany) were then used as a ground electrode after insertion into the tail of the animal.

Full-field ERG was recorded following the standards of International Society of Clinical Electrophysiology of Vision (ISCEV). Full-field stimulation was produced using Ganzfeld stimulator of the RETIscan unit (Roland Consult, Wiesbaden, Germany), which was positioned just in front of animal's face. To assess rod responses by ERG, the animals were dark-adapted for 12 hr before anesthesia. A dim white flash with a stimulus strength of 0.01 cd·s/m^2 was used for dark-adapted 0.01 ERGs with intervals of 2 sec between flashes. A white 3.0 cd·s/m^2 flashes were produced in dark-adapted eye for dark-adapted 3.0 ERGs and oscillatory potentials recordings. Subsequently, light-adapted ERGs recordings were performed after 15 min of light adaptation. Light-adapted 3.0 ERGs elicited by white flashes at an intensity of 3.0 cd·s/m^2 under white background of 30 cd/m^2. Furthermore, 30-Hz flicker responses were recorded using white light flashes at an intensity of 3.0 cd·s/m^2 and a rate of 30 stimuli per sec (30 Hz). All of responses were amplified at 10,000 times, and were filtered with a band pass between 1 and 300 Hz. Five waveforms for each response were averaged to reduce variability and background noise. ERG abnormality is reflected in alterations in A- and B-wave amplitude. A-wave amplitude measures the trough of the negative deflection as difference from the baseline value. B-wave amplitude measures the difference between the trough of A-wave and the peak of B-wave recording. Recordings of full-field ERG were performed for both eyes prior to the vaccination and 1 day after the treatment, respectively. Vaccination was applied only on right eyes, and left eyes were considered as a control eye.

For statistical analysis of the ERG parameters, the paired *t*-test was used to assess the difference between the values before and after the vaccination, respectively. The differences were considered to be significant when P-value was less than 0.05.

17. Data and statistical analyses

Data in mice experiments were expressed as the mean ± SD, and statistical analyses were conducted by the ANOVA test (Microsoft Office Excel program).

Data in ferret experiments were expressed as the mean ± SD, and statistical

analyses were conducted by the student's *t*-test (Microsoft Office Excel program). Number of animals in all groups was 3 or 4. For the data normality test, the Shapiro-Wilk test was used. All data was checked before I use the student's *t*-test and concluded that all data came from a normal distribution.

18. Ethics Statement

All experiments involving animal subjects were conducted in strict accordance and adherence to relevant national and international guidelines regarding animal handling as mandated by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University Health System (Seoul, Korea). Approval number is 2011-0137.

III. RESULTS

1. Significant enhancement of Ag-specific Ab production by eyedrop influenza vaccine adjuvanted by poly(I:C)

To evaluate the efficacy of various adjuvants in regards to whether they can enhance systemic and mucosal antibody production when used with protein Ag by eye-route vaccination, BALB/c mice were immunized with OVA (100 μg/head) protein plus several conventionally used adjuvants, including CT (2 μg/head), poly(I:C) (10 μg/head), MPLA (10 μg/head), or Imject® Alum (1 mg/head), which is a commercially used alum adjuvant, three times at one-wk intervals. One wk after final immunization, the levels of OVA-specific antibodies (Abs) were measured by ELISA. All mice given OVA plus adjuvant showed significantly higher levels of OVA-specific serum IgG Ab in there sera than those found in mice given PBS or OVA alone (Fig. 1A). Among the adjuvants, CT showed the highest serum IgG Ab production level. However, only mice given OVA plus CT or poly(I:C) showed significantly higher levels of IgA Ab in mucosal compartments (e.g., tear, nasal, fecal, and vaginal washes) than the other adjuvant-treated groups.

To examine whether the Ab production-enhancing effect adjuvanted by CT or poly(I:C) was retained in influenza subunit-vaccine immunized mice, as shown in Fig. 1A, BALB/c mice were immunized with 1 μg of the seasonal influenza vaccine, consisting of trivalent HAs of A/New Caledonia/20/99 (H1N1), A/Panama/2007/97 (H3N2), and B/Shandong/7/97 influenza (B) viruses, with the same adjuvants that were previously used with OVA protein. As shown in Fig. 1B, Ab production levels for serum IgG and mucosal IgA Ab in mice treated with CT or poly(I:C) were significantly higher than those for the other groups. The enhancing effects of MPLA and the Imject® adjuvants were not observed for either types of Abs, compared to mice given PBS or Ag alone. Unexpectedly, the efficacy of Abs-production enhancement of poly(I:C) was similar to that of CT. Moreover, poly(I:C) significantly improved the production of mucosal IgA Ab to as much as CT did in the respiratory passageways, in

nasal cavity wash and BALF samples, which is the main route of influenza infection, indicating that it could be of use as a front-line defense against influenza invasion.[37] While CT has been shown to cause CNS damage when it was administrated intranasally,[7,8] there is a report of a lack of a destructive effect on nasal or brain tissue after administration of poly(I:C).[18] Therefore, in the following experiments, I focused on assessing the adjuvanticity of poly(I:C) via eyedrop administration.

2. Dose-dependent Ab responses in eyedrop vaccines with various amounts of Ag and poly(I:C)

Next, I attempted to elucidate the minimum amount of Ag and poly(I:C) that could effectively induce the enhancement of systemic and mucosal Ab production via eyedrop vaccination. To do so, BALB/c mice were immunized with 10 μg of poly(I:C) and various amounts of HA vaccine, 0.1 μg, 1 μg or 2 μg, via eyedrop vaccination (Fig. 2A). Since the maximum volume of eyedrops per eye is 15 μl and the concentration stocks of HA vaccine and poly(I:C) were 1 μg per 11 μl and 10 μg per 4 μl, respectively, I could not examine the effect of Ag amounts over 2 μg HA vaccine plus 10 μg poly(I:C) in the eyedrop vaccination. As shown in Fig. 2A, 1 μg of HA Ag plus 10 μg poly(I:C) significantly enhanced serum IgG and mucosal IgA Ab production amounts greater than those for mice given doses of 0.1 μg or 2 μg Ag plus 10 μg poly(I:C). Unexpectedly, the enhancement from 2 μg HA eyedrops with 10 μg poly(I:C) was less effective than that from 1 μg HA vaccine Ag eyedrops. Since the total volume of 1 μg or 2 μg HA Ags plus 10 μg poly(I:C) were 10 μl or 15 μl, respectively, it was estimated that the concentration of poly(I:C) was diluted in the doubled volume of 2 μg HA Ag eyedrops and, accordingly, the effect of poly(I:C) was diminished, compared to that for 1 μg Ag plus 10 μg poly(I:C) eyedrops. Thus, for following experiments, I used 1 μg of HA vaccine Ag for eyedrop vaccination.

After the discovery of the effective minimum amount of HA vaccine Ag in the eyedrop vaccination, I checked the dose-dependent efficacy of poly(I:C) in the

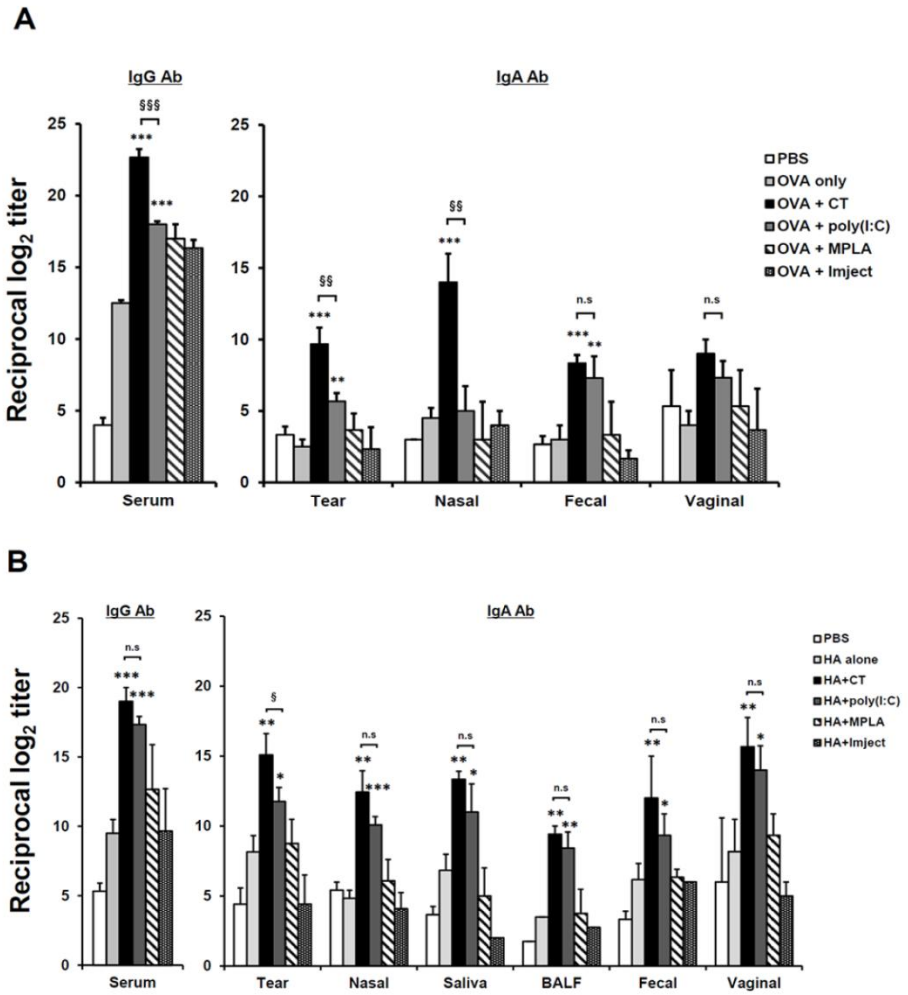

Figure 1. CT and poly(I:C) enhances systemic and mucosal Ab production by eyedrop vaccination of OVA or HA. Groups of female BALB/c mice received OVA (100 μg) (A) or HA (1 μg) (B) plus CT (2 μg), poly(I:C) (10 μg), MPLA (10 μg), Imject® Alum (320 μg) resolved in 5 μl of PBS, or 5 μl of PBS alone by drops on both eyes every wk (three times). Ag-specific Ab levels were measured in serum and in various mucosal fluids 1 wk after final vaccination by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus Ag alone; §§ $p < 0.01$, §§§ $p < 0.001$ versus poly(I:C) group; 'n.s.', non-significant. Results are representative of three independent experiments, with three mice in each group.

1μg HA EDV in mice. The levels of HA-specific Ab production in all groups of mice in which poly(I:C) was administered together with 1 μg HA-Ag EDV were significantly enhanced, compared to mice treated with 1 μg HA-Ag alone in both serum and the mucosal washes (Fig. 2B). Additionally, there was almost no difference in the adjuvant efficacies of all poly(I:C) administered groups; even administration of 1 μg of poly(I:C) significantly augmented Abs production levels in the 1 μg of HA-Ag eyedrop vaccinated mice to as much as those for mice treated with 30 μg of poly(I:C). Thus, these results indicate that the efficacy of 1 μg poly(I:C) was enough to induce significant Ag-specific immunity when it was vaccinated with HA-Ag via the eye route.

3. Comparison of the poly(I:C)-adjuvanted inactivated vaccine efficacy between eyedrop and intranasal vaccination

Reportedly, vaccination via the IN route is the most effective, among mucosal routes.[38] Thus, to examine whether the adjuvant efficacy of poly(I:C) in the eyedrop vaccination is more effective than that of poly(I:C) administration via other vaccination routes, I compared the efficacy of the immunization of HA vaccine plus poly(I:C) adjuvant via the eye route with that of intranasal vaccination in mice. In Fig. 3A, when the serum IgG Ab levels of the eyedrop and intranasal vaccination groups, which were administered the same amounts of poly(I:C) and HA vaccine, were compared, all of the intranasal vaccination groups showed significantly higher serum IgG Ab responses than their eyedrop vaccination counterparts. However, in the mucosal wash fluids, there was no significant difference in the levels of mucosal IgA Ab-production between the eyedrop and IN vaccination groups, except for saliva fluids. Additionally, the enhancement of IgA Ab production levels in all of the mucosal fluids from mice administered 1 μg poly(I:C) plus HA EDV was significantly similar to that in intra-nasally immunized mice.

Next, to evaluate whether the adjuvanticity of poly(I:C) in eyedrop vaccination is maintained when the influenza virus HA-vaccine Ag is replaced with commercially used inactivated split A/California/04/09 H1N1 (H1N1) influenza

virus vaccine Ag in eyedrops, BALB/c mice were immunized with 1 μg of split H1N1 influenza vaccine plus 10 μg poly(I:C) via eyedrops or intranasally twice at a 2-wk intervals. Two wks after the final immunization, as shown in Fig. 3B, H1N1 vaccine-specific serum IgG Ab production levels in intranasally immunized mice with poly(I:C) plus vaccine Ag were significantly higher than those for eyedrop vaccinated mice. In mucosal fluids, except tear and vaginal wash samples, Ab production levels in the intranasally vaccinated groups were significantly higher than those in the eyedrop groups. However, the levels of Ag-specific Ab production in the poly(I:C) adjuvanted eyedrop vaccinated group were still significantly higher than those in the eyedrop Ag alone treated groups. Therefore, these results indicate that the efficacy of poly(I:C) in eyedrop vaccination is sufficient for use in commercial influenza vaccine Ags, with an efficacy similar with or slightly weaker than that for IN vaccination.

4. Eyedrop split H1N1 influenza vaccine plus poly(I:C) vaccination can protect mice from lethal influenza virus challenge

To examine whether the immunity induced by vaccination of inactivated EDV plus poly(I:C) can protect mice from lethal influenza virus challenge, I vaccinated BALB/c mice with two doses of 1 μg of split H1N1 influenza vaccine plus 10 μg of poly(I:C) by eyedrops or IN as a positive control group at a 2-wk intervals. Two wks after the final vaccination, mice were challenged IN with mouse-adapted live influenza A/California/04/09 (H1N1) virus (10X LD_{50}; 0.75 $TCID_{50}$) and monitored for 2 wks (Fig. 4). Mice in the intranasally Ag + poly(I:C) treated group maintained their initial bodyweights throughout the monitoring period. Although eyedrop Ag + poly(I:C) treated mice lost about 10 percent of their initial bodyweight (Fig. 4A), all mice began to recover their bodyweights on day 6 and survived against the lethal influenza virus challenge (Fig. 4B). Meanwhile, PBS or Ag alone administered mice both by eyedrop and IN were critically affected by the influenza virus infection (Fig. 4A) and all mice of PBS group and both Ag alone treated groups were sacrificed at humane endpoints (Fig. 4B). Additionally, titers of the challenged influenza virus in lung tissue of mice were measured (Fig. 4C). Proliferating viruses on day 1 began to

reduce on day 4 in both the eyedrop and IN groups, and viruses were significantly cleared on day 7 in both vaccinated groups.

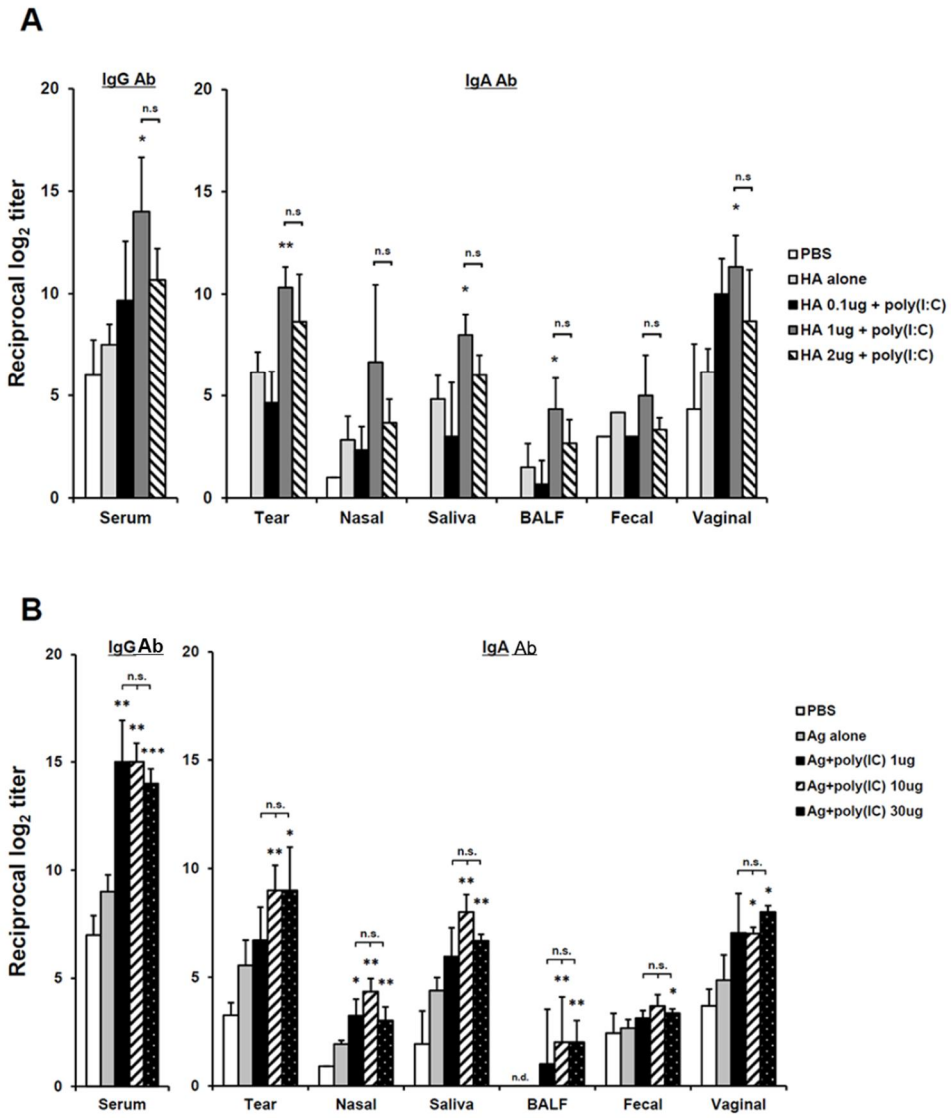

Figure 2. Comparison of antibody production by the amount of hemagglutinin (HA) or poly(I:C). (A). Dose-dependent HA vaccine Ags were administered with 10 μg poly(I:C) resolved in 5 μl of PBS by drops on both eyes two times at a 2-wk interval in female BALB/c mice. HA-specific Ab levels were measured in serum and in various mucosal fluids 2 wks after final vaccination by ELISA. (B) 1 μg of HA vaccine Ag plus dose-dependent

poly(I:C) was vaccinated by drops on both eyes two times at a 2-wk interval in female BALB/c mice. HA-specific Ab levels were measured in serum and in various mucosal secretions 2 wks after final vaccination by ELISA. * $p < 0.05$; ** $p < 0.01$ versus Ag alone group; 'n.s.', non-significant. Results are representative of three independent experiments, with three mice in each group.

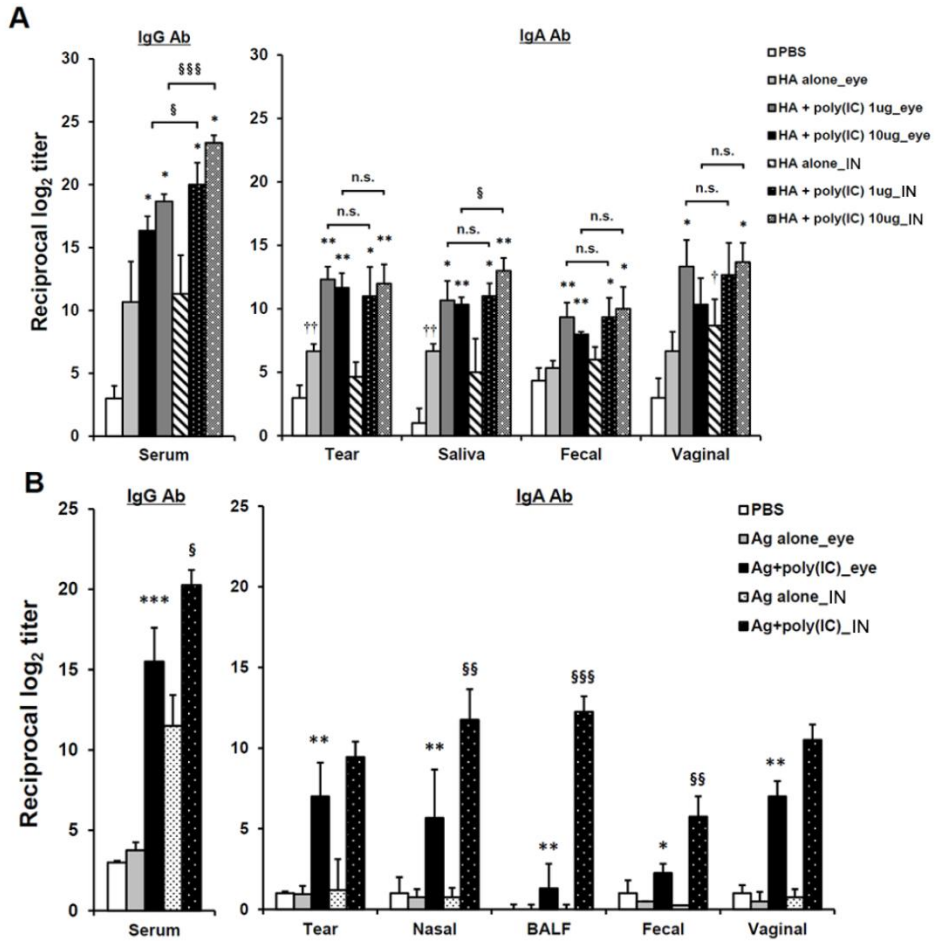

Figure 3. Comparison of Ab production between intranasal and eyedrop administration routes. (A) Dose-dependent HA vaccine Ags were administrated with 10 μg poly(I:C) resolved in 5 μl or 20 μl PBS by drops on both eyes (eye) or nostrils (IN), respectively, two times at a 2-wk interval in female BALB/c mice. HA-specific Ab levels were measured in serum and in various mucosal secretions 2 wks after final vaccination by ELISA. (B) 1 μg H1N1 split vaccine Ag plus dose-dependent poly(I:C) resolved in 5 μl or 20 μl

PBS were vaccinated by drops on both eyes (eye) or nostrils (IN), respectively, two times at a 2-wk interval in female BALB/c mice. Ag-specific Ab levels were measured in serum and in various mucosal secretions 2 wks after final vaccination by ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus Ag alone; § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ versus poly(I:C) group. Results are representative of three independent experiments, with four mice in each group. eye, eyedrop; IN, intranasal.

For an effective vaccine, the ability to induce long-lasting protection is an important requisite. To test whether the protective immunity induced by eyedrop vaccination with the split eyedrop H1N1 influenza vaccine is maintained for a long period of time, mice were vaccinated with the eyedrop H1N1 vaccine three times over a 1-wk interval. At one year after the final vaccination, immunized Ag-specific Ab titers were examined with ELISA without additional boosting. The levels of Ag-specific IgG and IgA Abs in all samples, including serum and

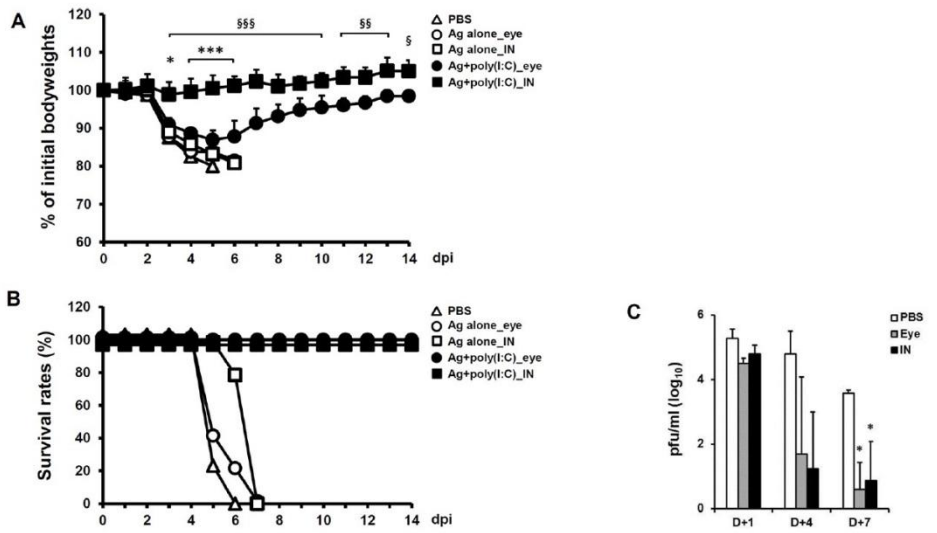

Figure 4. Eyedrop inactivated influenza vaccine plus poly(I:C) administration protects mice from lethal influenza virus challenge. Female BALB/c mice were given PBS (△) or H1N1 split vaccine Ag alone (eyedrop, ○; IN, □) or Ag plus 10 μg poly(I:C) (eyedrop, ●; IN, ■) by eyedrop or intranasal (IN) two times at a 2-wk interval. Eyedrop groups were vaccinated with 5 μl of

vaccine on both eyes and IN groups were received with 20 μl of vaccine. At 2 wks after the last administration, mice were challenged IN with 10X LD_{50} of homologous mouse-adapted H1N1 influenza virus. Body weights (A) and survival rates (B) were monitored daily. (C) Nine mice in each group were vaccinated by eyedrop or intranasal immunization with 1 μg of H1N1 split vaccine plus 10 μg of poly(I:C). At two wks after the final vaccination, mice were anesthetized and challenged with 50 μl of mouse-adapted live influenza A/California/04/09 (H1N1) virus suspension (10X LD_{50}; 0.75 $TCID_{50}$) via the IN route. To measure the viral titers in the lung organs, three mice per group were sacrificed on day +1, +4 and +7. After the lungs were removed, they were homogenized in 1 ml PBS using a small motor and upper respiratory tract was rinsed with 1ml PBS. Samples were centrifuged at 12,000×*g* and the supernatant fluids were removed, and then the supernatants were stored at −70◦C until assayed for viral titers. Viral titers were assayed by plaque assay with MDCK cells. * $p < 0.05$; *** $p < 0.001$ between Ag plus 10 μg poly(I:C)_eye and PBS; § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ between Ag plus poly(I:C) treated eye and IN groups. Results are representative of three independent experiments, with five mice in each group.

mucosal washes from mice immunized with eyedrop H1N1 Ag plus poly(I:C), were significantly higher than those of PBS or Ag alone treated groups (Fig 5). Unfortunately, I cannot show survival data in which those vaccinated mice were challenged with homologous A/California/04/09 H1N1 influenza virus (10X LD_{50}; 0.75 $TCID_{50}$) since all mice in the PBS groups survived, even with the lethal influenza virus infection. Thus, these results suggest that the immunization of eyedrop inactivated influenza vaccine Ag plus poly(I:C) can elicit long-lasting Ag-specific humoral immunity, which was maintained for a year and the memory immunity might be enough to protect mice from influenza virus infection.

Additionally, I briefly compared the efficacy of the eyedrop vaccination with that over intramuscular (IM) vaccination (Fig. 6) since IM vaccination is most

prevalent inoculation method. As shown in Figure 6A, Ag-specific serum IgG Ab levels in IM vaccinated groups were significantly higher than those of eyedrop group, but nasal washes or BALF IgA Ag levels were not significantly increased than those of PBS. However, unexpectedly, after the lethal influenza H1N1 challenge there were almost no body weight loss in IM group (Fig. 6B) and the differences in body weight changes between IM and poly(I:C) adjuvanted eyedrop group were significant (Fig. 6B). Nevertheless, loss of body weights in EDV group was significantly lesser than those of Ag alone group and adjuvanted eyedrop vaccination protected mice from lethal influenza virus infection (Fig. 6C). Through these results, although IM vaccine showed less body weight loss than EDV, EDV showed possibility that it can be alternatively used instead of IM vaccine with perfect protective efficacy of the protection of host from influenza virus infection.

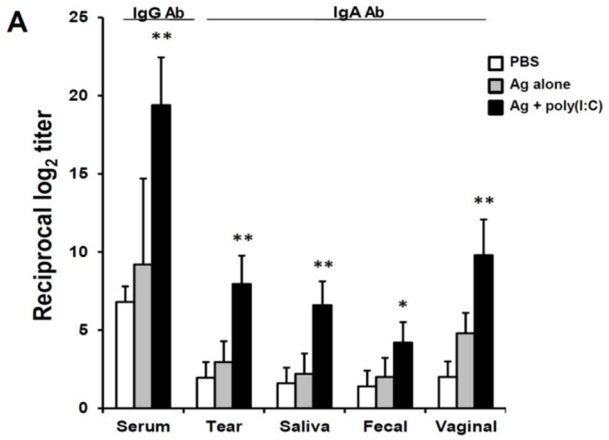

Figure 5. Long-term Ag-specific Ab production induction in eyedrop vaccinated mice. Female BALB/c mice were given PBS, H1N1 split vaccine Ag alone, or Ag plus 10 μg poly(I:C) by eyedrop three times at a 2-wk interval. At one year after the last immunization, Ag-specific Ab production levels were measured by ELISA. * $p < 0.05$; ** $p < 0.01$ versus PBS. Results are representative of two independent experiments, with five mice in each group.

5. Safety of the administration of eyedrop poly(I:C) plus inactivated influenza vaccines

To examine the safety of inactivated influenza vaccine plus poly(I:C) in EDVs, I checked whether the treatment with the EDV provoked inflammatory conditions in tissues of the conjunctiva in mice. For screening mRNA expression levels of inflammatory cytokines, BALB/c mice were given 1 μg HA vaccine Ag alone or plus 10 μg poly(I:C) or 2 μg CT for 1 hr, 6 hr, 12 hr, and 24 hr (Fig. 7A-7D). RNAs of the conjunctival tissues were extracted and mRNAs of several inflammatory cytokines, such as IL-1, IL-6, IFN-γ, and TNF-α, were quantified by qPCR. As shown in Fig. 7, in all groups of mice, except the HA plus CT treated group, the mRNA expression levels of inflammatory cytokines at all designated time points were not up-regulated, compared to those at 0 h. Unexpectedly, LPS treatment did not induce any significant increase in mRNA expression levels of inflammatory cytokines (Fig. 7B). Furthermore, I observed no histopathological changes in conjunctival and corneal tissues in LPS treated mice (data not shown). However, CT administration via eyedrops significantly induced about a 100-fold increase in IL-6 mRNA expression at 6 hr, which gradually decreased up to 24 hr (Fig. 7B). Also, IFN-γ mRNA was increased about 7-fold at 6 hr after CT treatment, although the difference compared to mRNA levels at 0 hr was not significant (Fig. 7C). Otherwise there were no increases in inflammatory cytokine mRNA expression levels in corneal tissues or those of IFN-α or IFN-β in corneal or conjunctival tissues at all designated time points (data not shown).

In histopathological assays of tissues of eyes after 24 hr of 1 μg HA alone or HA plus 10 μg poly(I:C) or 2 μg CT eyedrop treatment, epithelial and goblet cells from the bulbar conjunctival to the tarsal conjunctival areas were intact and there was no symptom of hyperplasia in all conditions, including HA plus poly(I:C) or plus CT treatment, in both conjunctival (Fig. 8A b-d) and corneal tissues (data not shown). When mononuclear cells in sub-epithelial areas in all the conjunctival tissues were counted, excluding stromal cells, which have a distinctive long and narrow shape , there were no increases therein in the tissues

of HA plus poly(I:C) treated eyes, compared to that of the PBS group (Fig. 8B). However, as shown in Fig. 8Ad, there were significantly more mononuclear cells in the tissues of HA plus CT administered eyes than those of PBS or poly(I:C) treated mice (Fig. 8B), and the conjunctival tissues were slightly swollen after 24 hr of CT treatment (data not shown). Since CT treatment on eyes provoked increased mRNA expression of IL-6 and IFN-γ (Fig. 7B and 7C),

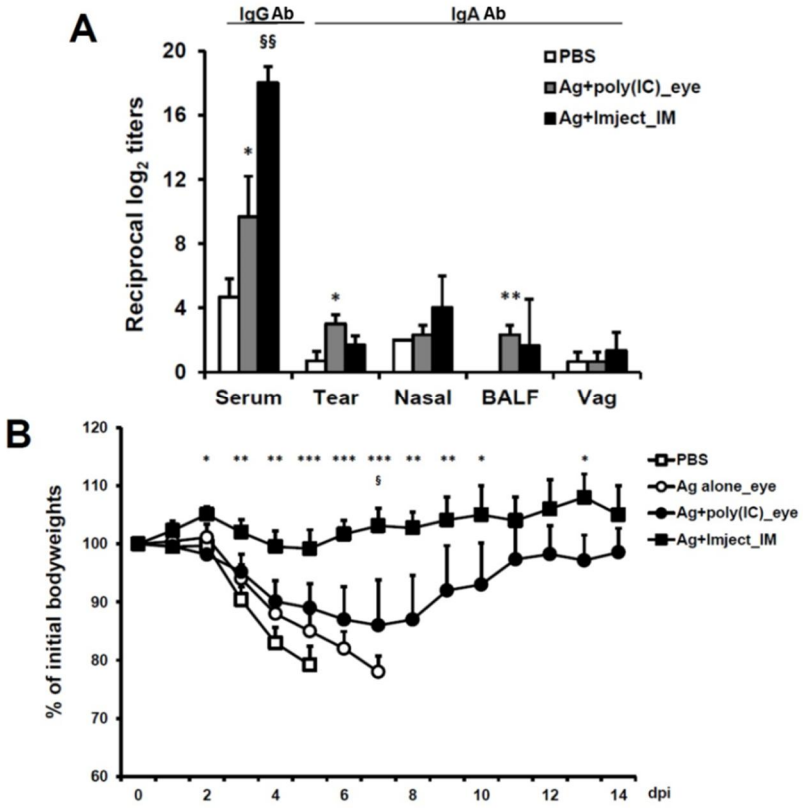

Figure 6. Comparison of the efficacy of immunity induction between IM and eyedrop vaccination. Female BALB/c mice were given PBS or 1 μg of H1N1 split vaccine Ag alone (eyedrop) or 1 μg Ag plus 10 μg poly(I:C) (eyedrop) or 1 μg Ag plus Imject® (IM) two times at a 2-wk interval. At 2 wks after the last immunization, Ag-specific Ab levels were measured in serum and in various mucosal secretions by ELISA, and mice were challenged IN with 10X LD_{50} of homologous mouse-adapted H1N1 influenza virus. Body weights (A) and survival rates (B) were monitored daily. * $p < 0.05$; ** $p < 0.005$ versus

PBS (A). * $p < 0.05$; ** $p < 0.005$; *** $p < 0.001$ between Ag+Imject_IM and Ag+poly(IC)_eye or § $p < 0.05$ between Ag+poly(IC)_eye and Ag alone (B). Results are representative of two independent experiments, with five mice in each group.

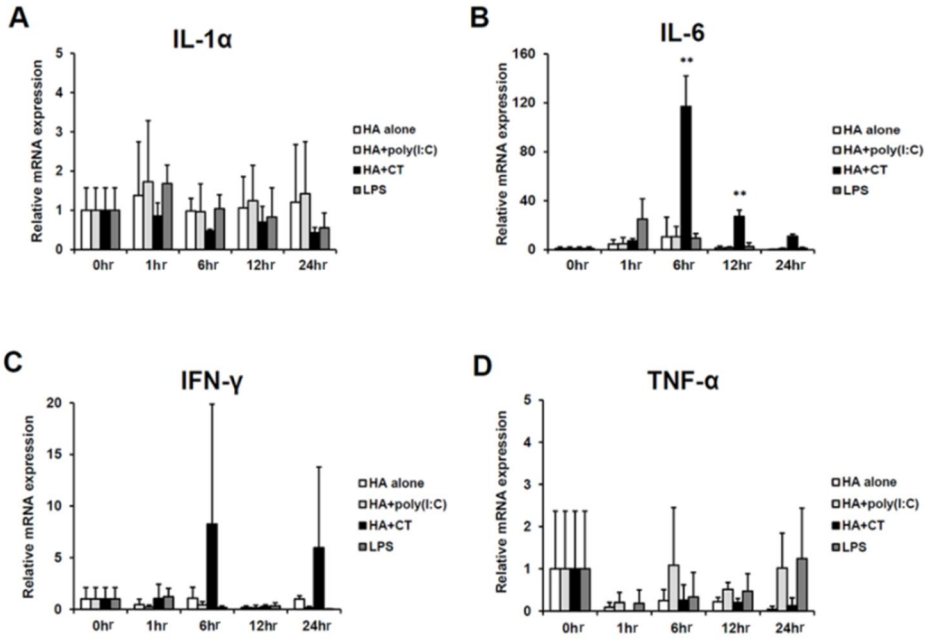

Figure 7. No inflammation in the eyes after administration of HA Ag plus poly(I:C) in mice. (A-D) Female BALB/c mice were administered 1 μg of HA vaccine alone, 1 μg HA plus 10 μg poly(I:C), or plus 2 μg CT resolved in 5 μl of PBS on the eyes for various time periods. Total RNA was extracted from homogenized conjunctival tissues with TRI reagents for reverse transcription and real-time PCR analysis. Gene expression levels were calculated as a relative ratio to the average value of house-keeping genes, β-actin. ** $p < 0.01$ versus 0 h. Data represent means ± S.D. of 3 independent experiments.

I discerned that increased mononuclear cells had infiltrated to the areas upon increased expression or secretion of the inflammatory cytokines. Meanwhile, poly(I:C) treatment on the eyes for 24 hr did not induce any cell infiltration in the conjunctival and corneal tissues.

Additionally, since I previously showed that vaccinated eyedrop Ag did not redirect to the brain in the presence of CT, I attempted to visualize the presence of topically inoculated solution on the surface of the mouse brain with micro-computerized tomography (CT) scanning. The iodinated contrast medium was inoculated via eyedrops or IN in mice, and after 30 min, brains of each group

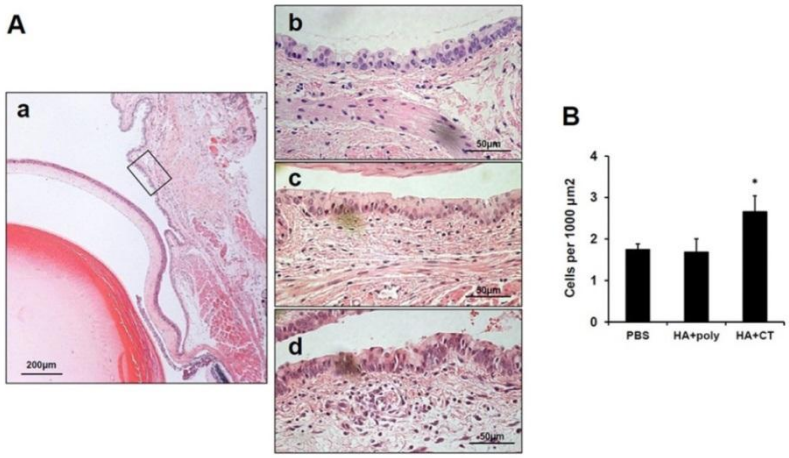

Figure 8. No induction of cellular infiltration in conjunctival tissues after administration of HA Ag plus poly(I:C). (A) Female BALB/c mice were administered PBS, 1 μg / 10 μl of HA vaccine Ags plus 10 μg /10 μl poly(I:C), or 2 μg/10μl CT on the eyes, and eye tissues were prepared and stained with H&E. Every representative figure of the same position of the rectangle (Aa) are shown (Aa-b, PBS; Ac, poly(I:C); Ad, CT). (B) Mononuclear cells were counted in sub-epithelial regions of tarsal conjunctival areas and plotted as a graph (cells per 1000 μm2). * $p < 0.05$; ** $p < 0.01$ versus Ag alone group. Results are representative of three independent experiments, with three mice in each group.

were removed for micro-CT scanning. As shown in figure 9, there were no spots of the contrast medium on the surface of brains from the naive or eyedrop groups. However, there were several spots (arrows) of the contrast medium on the OB region (dashed circles) of the brains from the IN treated mice. Thus, taken together, these results suggest that eyedrop inoculation is safe and

effective, and may of use as an alternative to intranasal vaccination for inactivated influenza vaccine administration.

6. Cervarix® administered by eyedrop could induce Ags-specific immunity in mice.

As adscititious experiments, I tested the efficacy of various commercially provided EDV Ags in mice (table 2): BCG, M-M-R®II, Avaxim® 160 U Adult Inj., Imovax Polio Inj., Hepavax-Gene®TF inj., Act-HIB®, Suduvax®, DPT-3 VACCINE inj., and Cervarix®. All vaccines were tested with CT adjuvant for the strongest mucosal immune response induction. Among many vaccines, only the levels of Ags-specific Abs of Cervarix® were significantly increased (Fig 10). Moreover, the vaccine showed higher efficacy when it was used with poly(I:C) than CT uses (data not shown). Since the vaccine Ags are inactivated forms, it is suggested that the vaccine could be used as eyedrop, and it is needed to evaluate in further study why other vaccine Ags are not enhanced in eyedrop vaccination.

7. Induction of systemic and mucosal immune responses in ferrets by eyedrop LAIV

To evaluate the capacity for the eyedrop influenza vaccine to elicit protective immunity in ferrets, I separately administered three different strains of LAIV (Table 1) to three groups of ferrets (n = 3 or 4) twice with two wks interval. Serum and nasal lavage samples were collected after each immunization to assess anti-LAIV HI titer levels. As shown in Figure 11A, all samples from influenza-naïve ferrets were negative for virus-specific Abs; however, serum HI titers were significantly increased in all immunized ferrets following the first vaccination as compared to those of the naïve group. CA07- and Uruguay-vaccinated groups exhibited a 40% and 50% increase in the serum HI levels after the second vaccination, respectively, when compared to the primary vaccination titers (Fig. 11A). In agreement, the HI titer levels of nasal lavage samples isolated from each group following the first vaccination (at two wks after the priming; 2 wk) were significantly increased compared to those

observed in naïve counterparts (Fig. 11B). The average HI titer in nasal lavage samples was lower than those of serum samples across all immunized animals at 24.4 and 45 HI, respectively. No significant increases in nasal lavage HI titers were present between the first (2 wk) and second (4 wk) vaccinations. Moreover, I tried to measure the increase of IgG Ab by ELISA, and, unfortunately, I failed to detect any significant increase due to the high level of non-specific binding of

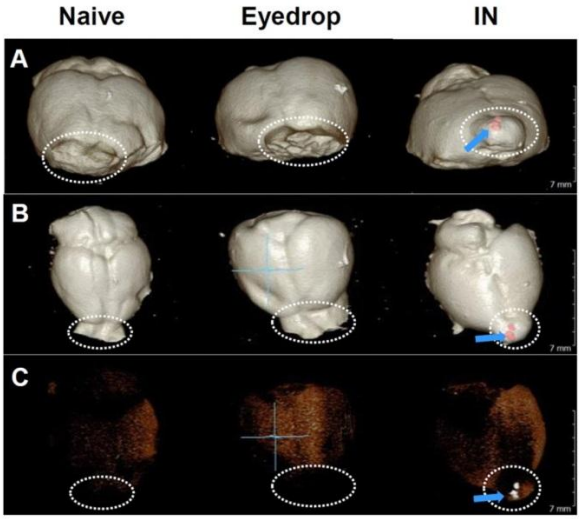

Figure 9. No detection of contrast medium on the olfactory bulbs from mice administered eyedrops. Female BALB/c mice were administered 10 μl or 50 μl of contrast medium by eyedrop or IN, respectively. After 30 min of contrast medium treatment, micro-CT pictures of the brains of each mouse were taken. Frontal view (A), top view (B), or dimmed top view (C). Inner dashed circle, olfactory bulbs; blue arrows and red dots, spots of the contrast medium.

Table 2. List of vaccines tested for the eyedrop vaccine immune response induction in mice

Commercial name	Pathogens or diseases to prevent	Immune response
Cervarix®	HPV	O
Hepavax-Gene®TF inj.	HBV	X
Avaxim® 160U Adult Inj.	HAV	X
M-M-R®$_{II}$	MMR	X

DPT-3 VACCINE inj.	DPT	X
Act-HIB®	HIB	X
Suduvax®	Chickenpox	X
BCG	BCG	X
Imovax Polio Inj.	Polio	X

secondary anti-ferret IgG or IgA Abs in PBS sample (data not shown). However, since nasal lavage and blood serum samples mainly contain secreted IgA and IgG, respectively, the observable increase in HI titers from nasal lavage samples suggest that an induction of LAIV-specific IgA Abs secretion occurred. Therefore, these results demonstrate that eyedrop LAIV vaccination is sufficient to provoke Ag-specific systemic and mucosal immune responses in ferrets.

8. EDV protects ferrets from intranasal influenza virus challenge

To evaluate whether LAIV administration could confer a protective immunity against influenza infection, four immunized ferrets in each EDV or PBS treated ferrets as control group were challenged with CA04 (H1N1), Sw09 (H1N2), and HK68 (H3N2) administered intranasally. Changes in body weight and temperature were then monitored to assess disease onset (Fig. 12). Mock (PBS)-immunized ferrets significantly began to lose about 10% of initial body weight compared to EDV vaccinated groups in CA04 (H1N1) and in HK69 (H3N2) infected ferrets or about 20% loss compared to EDV vaccinated groups in Sw09 (H1N2) infected ferrets (Fig. 12A-C). However, all ferrets of EDV groups showed no body weight loss. Notably, PZ-4 (H1N2)-vaccinated ferrets gained weight after Sw09 (H1N2) viral challenge, while the control group demonstrated significant weight loss until day 5 post-infection (Fig. 12B).

Likewise, all ferrets in the PBS treated control groups showed significantly higher rise of temperature compared to their counterparts that spiked to over 40°C in three days following influenza virus challenge (normal body temperature ranges from 38.5 to 39.5°C). Additionally, animals infected with Sw09 (H1N2) virus exhibited the highest temperature in control ferrets, concordant with the increased weight loss in shown in Figure 2B (Fig. 12E). In

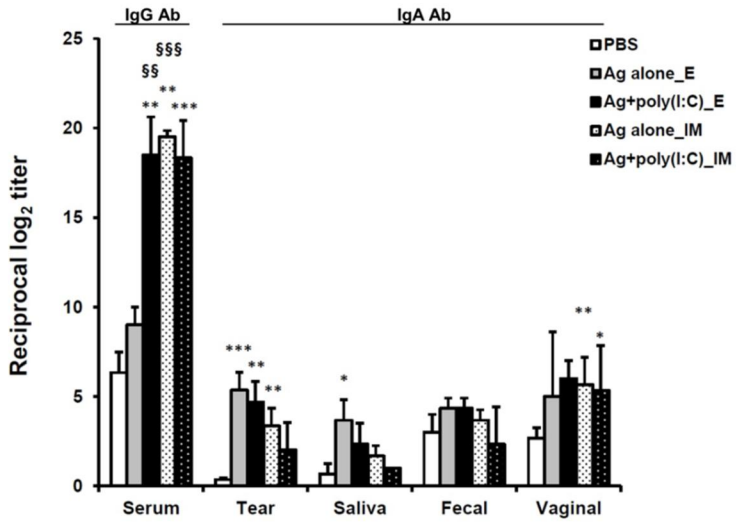

Figure 10. Comparison of Ags-specific Ab production induction in eyedrop Cervarix® vaccinated mice. Female BALB/c mice were given PBS, Cervarix® vaccine Ag alone, or Ag plus 10 μg poly(I:C) by eyedrop or intramuscular injection three times at a 2-wk interval. At two wk after the last immunization, Ag-specific Ab production levels were measured by ELISA (A). * $p < 0.05$; ** $p < 0.01$ versus PBS. §§ $p < 0.01$; §§§ $p < 0.001$ versus Ag alone_E. Results are representative of two independent experiments, with five mice in each group.

contrast, ferrets of all types of EDV groups showed no noticeable signs of fever throughout the monitoring period; although, their body temperature raised ≤ 0.5°C after 24 hr, but it were still within the normal temperature range (Fig. 12D-F). Importantly, the PZ-4 (H1N2) vaccinated ferrets showed absolutely no sign of fever and remained in healthy condition following challenge with Sw09 (H1N2) virus.

9. EDV effectively cleared influenza virus in respiratory organs

To assess the presence of live virus in the nasal passages of challenged ferrets, influenza virus titers were measured in nasal lavage samples at designated time points post-infection. Proliferating viruses were detectable until 7 dpi with maximum titers of 3.0-4.0 $TCID_{50}$/ml for CA04 (H1N1), 6.5-7.2 $TCID_{50}$/ml for Sw09 (H1N2), and 4.7-5.5 $TCID_{50}$/ml for HK69 (H3N2) in mock-immunized

animals (Fig. 13A). In contrast, viral propagation in EDV counterparts was significantly abrogated beginning at 3 dpi in CA04 (H1N1) and Sw09 (H1N2) challenged groups and from 5 dpi in the HK69 (H3N2) group (Fig. 13A). Furthermore, in order to confirm viral clearance in the lower respiratory organs, I also assessed the viral titers present in homogenized total lung tissues of control and vaccinated ferrets at 5 dpi. As shown in the Figure 13B, viruses were not detected in homogenized lung tissues of immunized animals, but present at levels demonstrative of a persistent infection in control animals.

Histopathological comparisons of lung tissues prepared from immunized ferrets at 5 dpi demonstrated the presence of protective immunity in EDV ferrets. Challenge with all infectious viral strains resulted in extensive immune cell

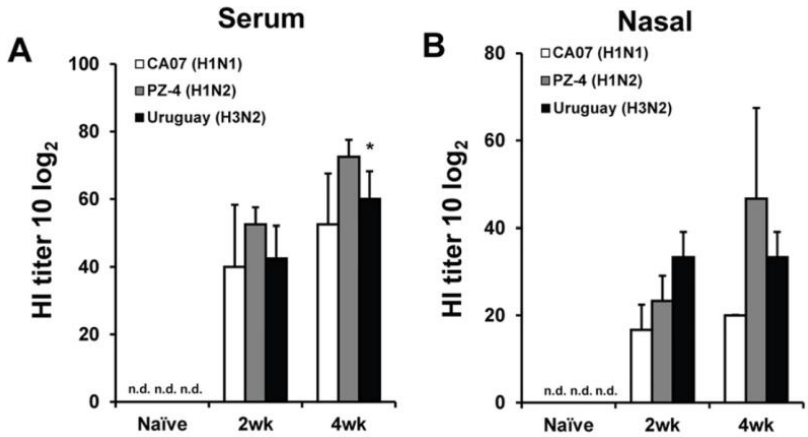

Figure 11. Eyedrop vaccination with live attenuated influenza vaccine (LAIV) elicits immunological responses in ferrets. Three groups of ferrets (n = 3) were administrated with two doses of CA07 (H1N1) or PZ-4 (H1N2) or Uruguay (H3N2) LAIV eyedrops, respectively. Levels of anti-LAIV HI titers were measured by HI assay in serum (A) and nasal lavage (B) samples, respectively, two wks after the first (2wk) and the second (4wk) vaccination. * $p < 0.05$ compared with the findings in the 2 wks group; n.d., non-detected. Statistical analyses were conducted by the student's *t*-test.

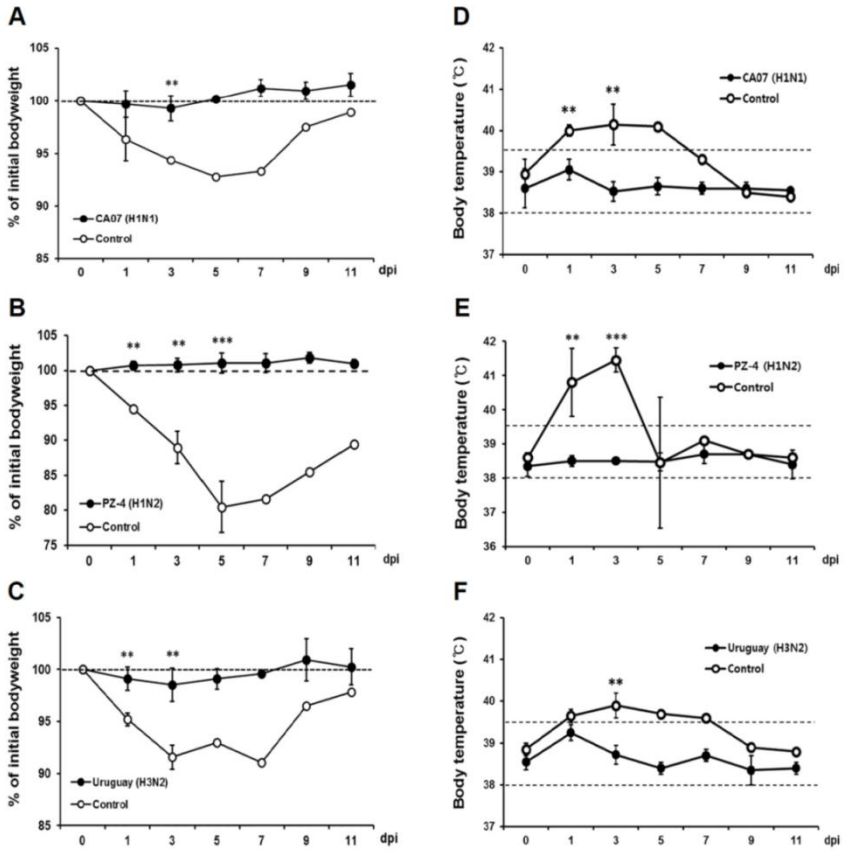

Figure 12. Eyedrop influenza vaccine protects ferrets from influenza virus challenge. At two wks after the last eyedrop vaccination, ferrets were challenged by IN with 1 × 10^5 $TCID_{50}$ of CA04 (H1N1) on CA07 (H1N1) LAIV-vaccinated group (A, D) or 1 × 10^6 $TCID_{50}$ of Sw09 (H1N2) or HK69 (H3N2) on PZ-4 (H1N2) LAIV vaccinated (B and E) or Uruguay (H3N2) LAIV-vaccinated (C and F) group, respectively. Body weight (A-C) and body temperatures (D-F) were monitored for 11 days with an interval of 2 days. Dotted lines in figures D-F: normal body temperature range. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with the findings in the PBS control group (n = 3 for each group). Statistical analyses were conducted by the student's *t*-test.

infiltration into the perivascular space and alveoli, accompanied by a marked reduction in the number of alveoli in unimmunized ferrets; whereas the lung tissue of eyedrop-vaccinated ferrets was nearly intact with regular alveolar

morphology and no interstitial pneumonia (Fig. 13C). Overall, these results demonstrate that LAIV eyedrop inoculation yields a sufficient immunological response to confer protective immunity against infectious respiratory challenge with either homologous or heterologous influenza infection in ferrets.

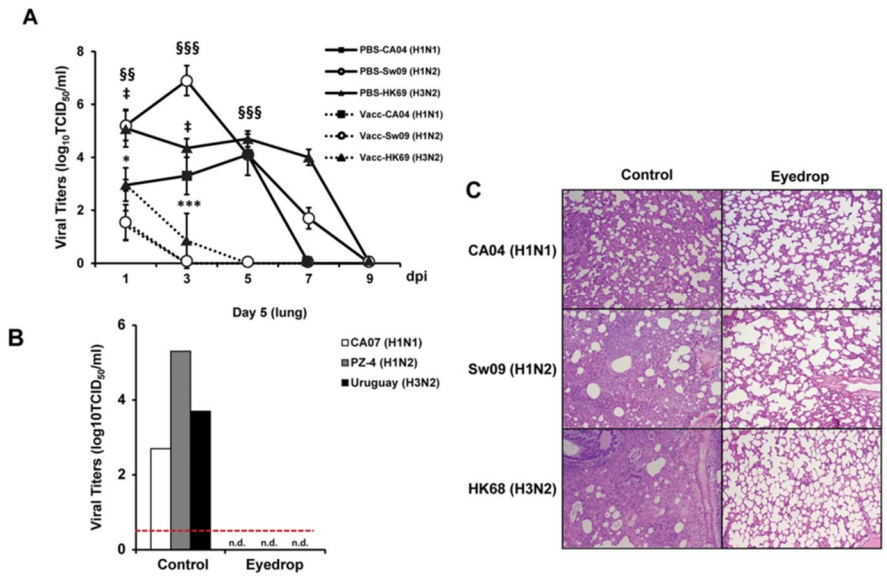

Fig 13. Successful viral clearance by eyedrop influenza vaccination. After IN challenge with CA04 (H1N1) or Sw09 (H1N2) or HK68 (H3N2), viral titers in nasal secretion samples taken at 9 dpi (A) or homogenized lung tissues at 5 dpi (B) were measured by plaque assay. Half of all mock or vaccinated ferrets in each group were sacrificed at 5 dpi for the lung tissue preparation (n = 3 for each group). (C) Hematoxylin and eosin-stained sections of lung tissue collected from: CA04 (H1N1; Top panels) or Sw09 (H1N2; Middle panels) or HK68 (H3N2; Bottom panels) challenged ferrets. Left panels, PBS administered ferrets (HI titer <20); right panels, eyedrop vaccinated ferrets (HI titer≤40). Magnification 200 ×. * $p < 0.05$, *** $p < 0.001$ compared with the findings in the CA07 (H1N1) LAIV-vaccinated and CA04 (H1N1) challenged group; §§ $p < 0.01$, §§§ $p < 0.001$ compared with findings in the PZ-4 (H1N2) LAIV-vaccinated and Sw09 (H1N2) challenged group; ‡ $p < 0.05$ compared with the findings in the Uruguay (H3N2) LAIV-vaccinated and HK69 (H3N2) challenged group. n.d., non-detected.

10. Influenza eyedrop vaccine is safe in ferret eyes

I next examined whether vaccinated LAIV materials were able to elicit acute eye inflammation. Eyes and the surrounding tissue were excised 24 hr after LAIV infection and stained with H&E. As shown in Figure 14A, conjunctiva tissues displayed normal histopathology in all eyes treated with LAIVs, with additional mononuclear cell infiltration in superior fornix and bulbar conjunctiva and normal number of goblet cells in tarsal conjunctiva areas. ERG was also used to evaluate the effect of vaccination on ocular function. ERG amplitudes remained consistent with no significant changes in A- and B-waves measured in scotopic or photopic conditions when comparing readings obtained before and one day after EDV. Within individual comparisons, no observable differences were found between the vaccinated right eye and non-treated left eye (Fig. 14B).

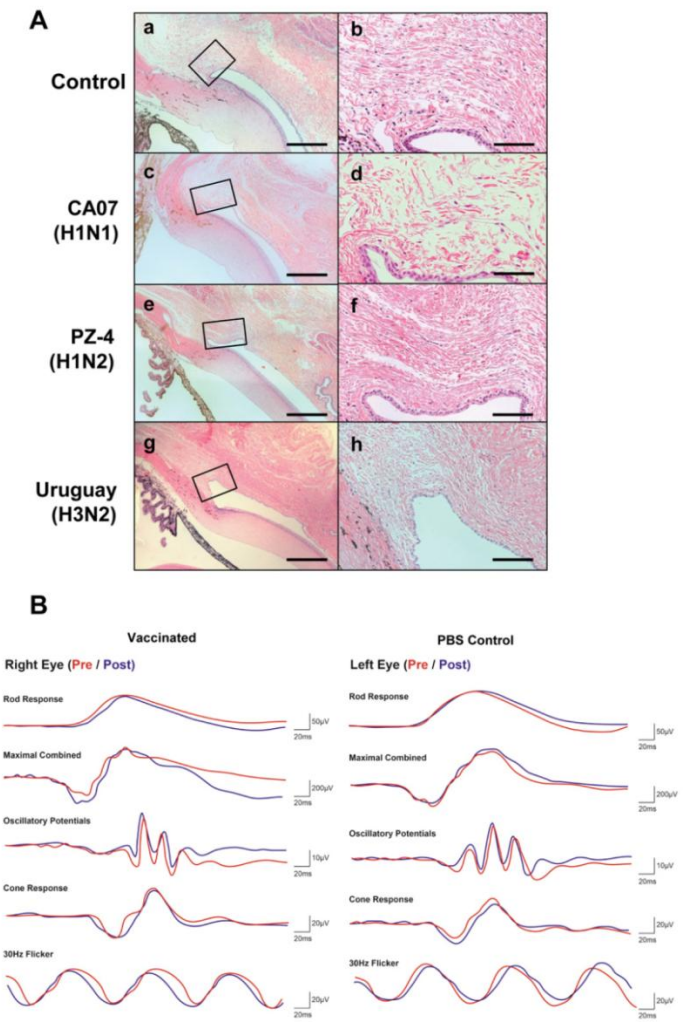

Figure 14. Eyedrop inoculation of vaccine-strain LAIVs does not induce inflammation in ferret eye tissues. (A) Histology of eye tissues 24 hr after PBS (a, b) and various LAIVs including CA07 (H1N1) (c, d), PZ-4 (H1N2) (e, f), Uruguay (H3N2) (g, h) on eyes of ferrets. b, d, f, and h are magnified ones of the rectangle area in their paired pictures. (B) After the ferrets were dark-adapted for 12 hr, full-field ERG recording was performed on both eyes of under the anesthesia (Pre), then retaining the ferrets in dark room for ferret's dark adaptation eyedrop CA07 (H1N1) LAIV was administered only on right eye. After 24 hr the eyedrop vaccination, full-field ERG recording was performed again on both eyes of under the anesthesia (Post).

IV. DISCUSSION

In this study, I showed that poly(I:C) is an effective eyedrop adjuvant of inactivated influenza vaccine in mice, eliciting enhanced immune response without adverse effects. Consistent with the previous study, in which the efficacy of EDV using live attenuated influenza virus was shown[6], inactivated influenza vaccine was enough to induce an enhanced immune response, and no local inflammation was observed in mice after eyedrop administration. My results provide evidence that eyedrop vaccination of killed influenza vaccine is enough to safely induce protective immunity against influenza virus infection, and poly(I:C) can be used as a potent EDV adjuvant alternative to CT.

Besides, I demonstrate that the eye mucosa serves as an effective route of vaccine administration against influenza virus in ferrets. While, previous data gathered in mouse models already established that EDV can provoke immunogenicity.[6] However, this study with ferrets is significant in that I evaluated the clinically meaningful level of protection through an actual post-vaccination challenge. Mice are not natural hosts of influenza and do not demonstrate the classical changes in body weight and temperature were in response to infection. This study confirmed that influenza-like clinical signs are evident in the non-vaccinated ferrets but not in those that received EDV. Regardless of the influenza subtype (H1N1, H1H2, or H3N2), EDV were shown to be effective. In addition, an actual post-vaccination challenge with viruses clearly demonstrates that animals in the EDV group were protected from both an acute rise in viral titer and histological damage within the lung. Unfortunately, I failed to directly compare the efficacy of different subtypes of EDV between other groups. Even though the maximum infection dose of the challenge viruses were inoculated to corroborate the protective efficacy of the eyedrop vaccine, the different infection doses (10^5.0 $TCID_{50}$ for CA04 and 10^6.0 $TCID_{50}$ for Sw09 and HK68) could limit the comparison of protective efficacy between groups.

Herein, I evaluated the potential of killed virus Ags as an effective EDV in mice

for the first time. Although the LAIV offers potentially greater immunity induction with smaller amounts of vaccine Ag than inactivated influenza vaccine by mucosa vaccination,[3,4] it can cause some adverse effects,[3] and it allows the use of LAIV in restricted aged peoples.[4,39] Thus, much research has been performed regarding the efficacy of killed influenza vaccine as an alternative to LAIV, along with studies on adjuvants that can be used to augment the activity of less efficient inactivated vaccines. Previously, I confirmed that eye mucosa is an alternative potent but safe mucosal vaccine administration route against influenza virus infection.[6] Thus, here, I attempted to evaluate the efficacy of inactivated vaccines adjuvanted by poly(I:C). HA subunit Ags and split H1N1 virus Ags were enough to induce strong immune response when poly(I:C) was used together (Fig. 3). Since I could not prepare homologous HA subunits and split virus Ags derived from one virus strain, comparison of the immunity-inducing effect between those Ags was impossible. However, the potency of split vaccine induced-immunity was enough to protect mice from lethal (10X LD_{50}) homologous influenza virus challenge (Fig. 4). Therefore, it can be suggested that eyedrop inactivated influenza vaccine can be used for influenza eyedrop vaccination as an alternative to LAIV, if it is adjuvanted with potent adjuvants, such as poly(I:C).

Along with the efficacy of killed virus Ags, poly(I:C) was examined as a potent adjuvant for eye mucosa. Although the efficacy of CT, the most effective mucosal adjuvant, is well reported, it causes severe adverse effect.[7] Thus, a study of alternative mucosal adjuvants other than CT, by which killed-influenza vaccine can be adjuvanted, is needed. Accordingly, I tested the efficacy of many conventionally used adjuvants for use in eye mucosa together with HA subunit Ag. When HA subunits and split influenza virus Ags were used with CT and poly(I:C), they induced significant enhancement of Ag-specific immune responses (Figs. 1B and 2). Interestingly, the adjuvanticity of poly(I:C) was better when it was used with HA subunit Ags than OVA protein Ag. Poly(I:C) has once been shown as an effective adjuvant for use in nasal-mucosal vaccination against influenza virus.[18] Therefore, since eyedrop vaccination

utilizes similar common cranial mucosal immune systems, poly(I:C) could also exert effective adjuvanticity along with inactivated influenza vaccine Ag in the eye mucosa (Fig. 3B). Although alum and MPLA are potential adjuvants when they are used intramuscularly,[40-42] their efficacy as an adjuvant in eye mucosa was as low as that of vaccine alone administration (Fig. 1). Thus, I suggest that poly(I:C), which was as effective as CT for influenza vaccine inoculation, may be useful in eye mucosa vaccination as a potent adjuvant.

In the present study, eyedrop inactivated influenza vaccine was as effective as IN influenza vaccines. In many studies, it has been shown that intranasal or sublingual mucosa is an effective route for influenza vaccine immunization, and the efficacy of intranasally administered vaccine was shown to be similar to or better than that of sublingual influenza vaccine.[43,44] However, intranasal administration of inactive influenza vaccine causes Bell's palsy.[8] Thus, evaluation of other safe and effective alternative vaccination routes is required. Since IN is the most potent mucosal vaccination route for influenza vaccines, I evaluated the efficacy of killed vaccine in eye mucosa compared to that of intranasal vaccine. In the case of human papilloma virus vaccine, with as Ag amounts as low as 30 μg, the immunity inducing activity of intranasally vaccinated was more effective than those of other mucosal vaccination routes, (i.e., s.l., oral, i.m., i.vag., or rectal).[45] In this study, I showed that the immunity-inducing effect of eyedrop influenza HA subunit vaccine was similar to that of the intranasal vaccine (Fig 3A). Although the efficacy of anti-H1N1 split vaccine antibody production tended to be better in IN vaccine than that of eyedrop, IgA Ab production levels in mucosal wash samples from both IN and eyedrop treated mice were similar (Fig. 3B). Since protection against influenza virus challenge is exerted via secretion of IgA in mucosal barriers,[46,47] I suggest that vaccination via eyedrop inactivated influenza vaccines adjuvanted with poly(I:C) induces protective immunity against influenza virus infection, as much as IN vaccinations do.

IM vaccination is considered the most prevalently used vaccination method for the immunity induction against both systemic and mucosal viral infection. In

our study, the efficacy of IM vaccine were confirmed again that even though mucosal IgA Ab levels in respiratory tract including nasal passage and lung airway of mice were lower than those of EDV treated group, IM vaccine showed less body weight loss than EDV and 100% protection from lethal influenza virus infection (Fig 5). Although a host in which low Ag-specific IgA Ab levels in mucosal compartments were induced by IM vaccine seems to be vulnerable to mucosal virus infection, it has been shown that systemic IM vaccination successively induced Ag-specific cytotoxic cellular immunity in both systemic and mucosal compartments in both mice and rhesus monkeys.[48,49] Nevertheless, it requires both needles to inject the vaccine and trained-health care provider to handle the needled syringes. Therefore, the requirements limit the range of the IM vaccine usage up to the area where medical workers can reach. In the context, EDV is suggested that it can be used as an alternative mucosal vaccine as it effectively protected hosts from lethal influenza virus challenge although impeding efficacy of body weight loss were better in IM vaccine group (Fig 5B).

Using low amounts of Ag in vaccines is an ideal goal of vaccine research, since it is correlated with less induction of unexpected side effects. Thus, several types of influenza vaccines have been evaluated in order to use fewer amounts of Ags. For intranasal HA subunit influenza vaccine, the effective minimum amount of Ag is 1 μg for one dose, when it is treated with 10 μg of poly(I:C) adjuvant.[18] In the EDV, likewise, the least effective amount of HA subunit or H1N1 split virus vaccine Ag was 1 μg, when it was immunized with 10 μg of poly(I:C) twice at a 2-wk interval, to significantly enhance Ag-specific Abs production (Fig. 2A). In regards to the use of poly(I:C), two doses of 1 μg poly(I:C) was enough to induce significantly enhanced Ag-specific Abs production together with 1 μg of HA subunit Ag. Moreover, eyedrop vaccination with 1 μg of split H1N1 influenza vaccine with 10 μg of poly(I:C) was enough to protect mice from lethal influenza virus challenge (Fig. 4). Therefore, I suggest that eyedrop vaccination of influenza vaccines is as effective as IN vaccination. Moreover, vaccination via eye mucosa requires no

greater amounts of Ag and adjuvant than those of IN route for inducing the same level of immunity.

With regard to safety, poly(I:C) could be suggested as a safe eyedrop adjuvant, with almost no possibility of evoking side effects on the eyes. Daily administration of poly(I:C) in nasal cavities, and even in the brains, of mice showed no adverse effects on the treated tissues, and mice maintained normal body weights and exhibited intact tissue histology.[18] Likewise, in this study, eyedrop influenza vaccine plus poly(I:C) administration induced neither inflammatory cytokine mRNA expression (Fig. 7) nor infiltration of mononuclear cells (Fig. 8) in conjunctivas after poly(I:C) treatment. Additionally, the safety of the use of polyICLC, an RNase-resistant analogue of poly(I:C) stabilized with poly-L-lysine, has been reported in several human clinical trials.[19,50,51] According to these studies, i.m. or s.c. administration of polyICLC was enough to elicit protective immunogenicity as much as poly(I:C) without adverse effects. Meanwhile, as shown in figures 7 and 8, the presence of CT human monocytes reportedly differentiated into $CD14^{high}CD1^{low}$ macrophage-like DCs, induced increases in IL-1, IL-6 and IL-10, as well as decreases in TNF-α and IL-12.[52] Therefore, it seems that in eye mucosa, poly(I:C) involves a mechanism by which protective-immunity induction is initiated without inducing increases in inflammatory cytokine mRNA expression and cellular infiltration in conjunctival tissues. Meanwhile, the mechanism of CT is different from that of poly(I:C) in that its adjuvanticity is exerted by recruiting mononuclear cells accompanied with IL-6 cytokine production.[52] Thus, based on previous studies and our results, I suggest that polyICLC, a safe derivative of poly(I:C), may be of use as an alternative to poly(I:C). Also, the efficacy of poly(I:C) as a safe EDV adjuvant should be further evaluated in clinical trials of inactive eyedrop influenza vaccines.

Furthermore, as an alternative to intranasal vaccination, eye mucosa is a safe vaccination route. Firstly, the mechanism of immune privilege in the eyes guarantees the use of EDV. In the eyes, the immune privilege suppresses sight-

damaging inflammation. Even if LPS is injected in vitreous cavities of the eyes in BALB/c mice to evoke intraocular inflammation, the intensity of inflammation declines after at 9 hr, followed by immune privilege; even the growth of injected tumor cells is not suppressed by intraocular immunity.[53] Consistent with these results, I showed that eyedrop LPS treatment on the eyes does not evoke increases in inflammatory cytokine mRNA expression (Fig. 7). Secondly, the eye route is an anatomically safe entry site for vaccine administration. The eye route comprises a tear drainage system, in which tears are constantly produced and drained away through tear ducts to protect the eyes from foreign particles that contaminate eye mucosa. Therefore, once vaccine solution is applied by eyedrops on the eyes, most of the solution drains into the nasal cavity via the punctum and nasolacrimal ducts with normal tear drainage, and then, finally, it is swallowed.[14] By virtue of the drainage system, deleterious effects provoked by topically applied materials on eye tissues, including the lens, retina, optic nerve, and so on, can be avoided.

The vaccination of inactivated Ags by nasal administration has a potential to induce side effects by which nerve cells can be damaged. Previously, it was shown that OB epithelial cells can uptake proteins that their sizes are up to approximately 66 kDa and accumulates it in nerve cells.[54,55] Additionally, our group showed that intranasally administered acridinium-labeled OVA was detected on OB[6] and the size of OVA is 45 kDa.[56] Since the size of inactivated influenza vaccine Ag molecules, such as HA or neuraminidase glycoproteins, or matrix protein, M1, ranges from 29 to 70 kDa, there is a possibility that intranasally administered inactivated influenza vaccine Ags or adjuvant molecules can be taken up by OB epithelial cells and it might exert damage effect on nerve cells which are connected with OB epithelial cells. In fact, it has been confirmed that EDV Ag is not redirected to the CNS in the presence of CT in mice. In contrast, intranasal administration of the acridinium-labeled OVA alone resulted in contamination of the OB in mice.[6] Thus, intranasal vaccination of inactivated Ags, as well as CT, has deleterious potential to induce unexpected side effects, especially on facial nerve cells.

Local side effects, such as tearing or red-eye, were anticipated with direct vaccine administration to the mucosa; however, I was unable to detect any histological signs of local adverse effects, owing to virus dilution by the circulation of tears. In addition, the distribution of sialic acid in the eye mucosa is not fitting for influenza virus tropism. In the ferret, sufficient amounts of α2,3-linked sialic acid (SA 2,3) is expressed in the conjunctiva, while α2,6-linked sialic acid (SA 2,6) is mainly expressed in nasal cavity and upper respiratory tract mucosa similar to humans.[57,58] Because influenza viruses mainly bind to SA 2,6, administering LAIV with eyedrop cannot cause conjunctival inflammation. Furthermore, our study used ERG to ascertain the effect of eyedrop vaccination on ocular function. ERG usually consist of two sequential waveforms: the first, termed the A-wave, is a negative wave, which reflects the photoreceptor function, whereas the second, or B-wave, is a positive wave originating from the main bipolar cells. Importantly, no changes reflective of retinal damage or vitreous haziness were observed in our readings, as interpreted from post-vaccination change in wave amplitude, and confirmed that eyedrop vaccination had no adverse effects on retina and vitreous.

No induction of immune responses against commercial EDV in mice was unexpected. Although inactivated vaccines, such as Hepavax-Gene®TF inj., Act-HIB®, DPT-3 VACCINE inj., Avaxim® 160 U Adult Inj. and Imovax Polio Inj., are not efficient unless they are used with appropriate adjuvants, it was unexpected that even some live-attenuated vaccines, such as Suduvax®, BCG and M-M-R®$_{II}$, did not show any Ab production response in mice with eyedrop vaccination. However, since the design of BCG vaccine has been guided to induce Th1 response,[59] no increase in the levels of BCG-specific Ab production in BCG-vaccinated mice was reasonable. Moreover, all inactivated vaccines that were tested in this study were adjuvanted with CT. Although CT shows most powerful mucosal immune response among many adjuvants, activation of toll-like receptors (TLRs) on APCs with appropriate adjuvant molecules is needed for some Ags. Therefore, further studies by which optimal adjuvant was tested for each vaccine Ags is needed. Meanwhile, BCG EDV showed side effects,

such as hair loss in facial region, slight bodyweight loss. This phenomenon might be due to the robust activation of BCG-specific $CD4^+$ T cells[60] and consequent induction of Th1 response and enhancement of IFN-γ production.[61] Therefore, BCG eyedrop vaccination of BCG is not recommended.

In conclusion, this study was the first to show that the eye mucosa is a safe and potent immunity inductive site, even for the use of inactivated influenza vaccine when it is adjuvanted by poly(I:C). Moreover, it was demonstrated that eyedrop vaccination elicits an immunological response sufficient to produce protective immunity in ferrets and poses no risk of CNS side effects. Since pathogen originated vaccine Ags show species-specific or tissue specific immune responses, strategies of eyedrop inactivated vaccine Ags of various pathogens could be established based upon this study.

V. CONCLUSION

Although intranasal influenza vaccine is the most effective mucosal vaccine for influenza prevention, inactivated vaccines should be given only by muscular injection, and LAIV are not allowed for use in vulnerable people. Recently, it was reported that eyedrop LAIV is effective in intranasal vaccination. This study aimed to examine the possibility of using eyedrop inactivated influenza vaccine in mice as an effective and safe alternative to mucosal influenza vaccine. The EDV adjuvanted with poly(I:C) induced strong immunity, enough to protect mice from lethal influenza challenge, eliciting no inflammation, unlike CT adjuvant. In addition, ferrets are superior animal models to mice for modified virus-based vaccine experiments because they are susceptible to a wide range of human influenza strains. Additionally, they are ideal for studying the efficacy of EDV as their visual system largely similar to that of humans. In here, I demonstrated that EDV can induce satisfactory immunity and protection against influenza in ferrets and confirmed the lack of any potential contraindications involving the central nervous or ocular systems through histologic and functional examination. This study accomplished an important step forward to the human application of EDV in humans by establishing their safety and effectiveness for influenza vaccine immunization.

REFERENCES

1. Brandtzaeg P, Pabst R. Let's go mucosal: communication on slippery ground. Trends Immunol 2004;25:570-7.
2. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005;11:S45-53.
3. Tamura S, Hasegawa H, Kurata T. Estimation of the effective doses of nasal-inactivated influenza vaccine in humans from mouse-model experiments. Jpn J Infect Dis 2010;63:8-15.
4. Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med 2007;356:685-96.
5. CDC. Influenza vaccine: live, intranasal 2012–2013 Atlanta: CDC. 2012.
6. Seo KY, Han SJ, Cha HR, Seo SU, Song JH, Chung SH, et al. Eye mucosa: an efficient vaccine delivery route for inducing protective immunity. J Immunol 2010;185:3610-9.
7. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. J Immunol 2000;165:4778-82.
8. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004;350:896-903.
9. Choi KS, Kim SH, Kim ED, Lee SH, Han SJ, Yoon S, et al. Protection from hemolytic uremic syndrome by eyedrop vaccination with modified enterohemorrhagic *E. coli* outer membrane vesicles. PLoS One 2014;9:e100229.
10. Malik B, Rath G, Goyal AK. Are the anatomical sites for vaccine administration selected judiciously? Int Immunopharmacol 2014;19:17-26.
11. Steven P, Rupp J, Huttmann G, Koop N, Lensing C, Laqua H, et al. Experimental induction and three-dimensional two-photon imaging of

conjunctiva-associated lymphoid tissue. Invest Ophthalmol Vis Sci 2008;49:1512-7.

12. Nagatake T, Fukuyama S, Kim DY, Goda K, Igarashi O, Sato S, et al. Id2-, RORgammat-, and LTbetaR-independent initiation of lymphoid organogenesis in ocular immunity. J Exp Med 2009;206:2351-64.
13. Okada K, Yamasoba T, Kiyono H. Craniofacial mucosal immune system: importance of its unique organogenesis and function in the development of a mucosal vaccine. Adv Otorhinolaryngol 2011;72:31-6.
14. Da Costa Martins R, Gamazo C, Sanchez-Martinez M, Barberan M, Penuelas I, Irache JM. Conjunctival vaccination against Brucella ovis in mice with mannosylated nanoparticles. J Control Release 2012; 162:553-60.
15. Hikono H, Mase M, Matsuu A, Nakayama M, Saito T. Intraocular vaccination with an inactivated highly pathogenic avian influenza virus induces protective antibody responses in chickens. Vet Immunol Immunopathol 2013;151:83-9.
16. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732-8.
17. Hafner AM, Corthesy B, Merkle HP. Particulate formulations for the delivery of poly(I:C) as vaccine adjuvant. Adv Drug Deliv Rev 2013;65:1386-99.
18. Ichinohe T, Watanabe I, Ito S, Fujii H, Moriyama M, Tamura S, et al. Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J Virol 2005;79:2910-9.
19. Caskey M, Lefebvre F, Filali-Mouhim A, Cameron MJ, Goulet JP, Haddad EK, et al. Synthetic double-stranded RNA induces innate immune responses similar to a live viral vaccine in humans. J Exp Med 2011;208:2357-66.
20. Stahl-Hennig C, Eisenblatter M, Jasny E, Rzehak T, Tenner-Racz K, Trumpfheller C, et al. Synthetic double-stranded RNAs are adjuvants

for the induction of T helper 1 and humoral immune responses to human papillomavirus in rhesus macaques. PLoS Pathog 2009;5:e1000373.

21. Fensterbank R, Pardon P, Marly J. Vaccination of ewes by a single conjunctival administration of *Brucella melitensis* Rev. 1 vaccine. Ann Rech Vet 1985;16:351-6.
22. Zundel E, Verger JM, Grayon M, Michel R. Conjunctival vaccination of pregnant ewes and goats with *Brucella melitensis* Rev 1 vaccine: safety and serological responses. Ann Rech Vet 1992;23:177-88.
23. Jayawardane GW, Spradbrow PB. Mucosal immunity in chickens vaccinated with the V4 strain of Newcastle disease virus. Vet Microbiol 1995;46:69-77.
24. Chentoufi AA, Dasgupta G, Nesburn AB, Bettahi I, Binder NR, Choudhury ZS, et al. Nasolacrimal duct closure modulates ocular mucosal and systemic CD4(+) T-cell responses induced following topical ocular or intranasal immunization. Clin Vaccine Immunol 2010;17:342-53.
25. Jackson CA, Hickey TL. Use of ferrets in studies of the visual system. Lab Anim Sci 1985;35:211-5.
26. Schulman JL. The use of an animal model to study transmission of influenza virus infection. Am J Public Health Nations Health 1968;58:2092-6.
27. Bouvier NM, Lowen AC. Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2010;2:1530-63.
28. Reed LJ MH. A simple method of estimating fifty per cent endpoints. Am J Epidemiol 1938;27:493-7.
29. Pascua PN, Song MS, Lee JH, Park KJ, Kwon HI, Baek YH, et al. Evaluation of the efficacy and cross-protectivity of recent human and swine vaccines against the pandemic (H1N1) 2009 virus infection. PLoS One 2009;4:e8431.
30. Jang YH, Lee EY, Byun YH, Jung EJ, Lee YJ, Lee YH, et al. Protective efficacy in mice of monovalent and trivalent live attenuated influenza

vaccines in the background of cold-adapted A/X-31 and B/Lee/40 donor strains. Vaccine 2014;32:535-43.

31. Song MS, Lee JH, Pascua PN, Baek YH, Kwon HI, Park KJ, et al. Evidence of human-to-swine transmission of the pandemic (H1N1) 2009 influenza virus in South Korea. J Clin Microbiol 2010;48:3204-11.
32. Kim JK, Seiler P, Forrest HL, Khalenkov AM, Franks J, Kumar M, et al. Pathogenicity and vaccine efficacy of different clades of Asian H5N1 avian influenza A viruses in domestic ducks. J Virol 2008;82:11374-82.
33. Lee JH, Pascua PN, Song MS, Baek YH, Kim CJ, Choi HW, et al. Isolation and genetic characterization of H5N2 influenza viruses from pigs in Korea. J Virol 2009;83:4205-15.
34. Webster R CNSK. World Health Organization Manual on Animal Influenza Diagnosis and Surveillance. WHO 2002.
35. van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, de Wit E, et al. Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. J Infect Dis 2010;201:993-9.
36. Cai QY, Kim SH, Choi KS, Kim SY, Byun SJ, Kim KW, et al. Colloidal gold nanoparticles as a blood-pool contrast agent for X-ray computed tomography in mice. Invest Radiol 2007;42:797-806.
37. Liew FY, Russell SM, Appleyard G, Brand CM, Beale J. Cross-protection in mice infected with influenza A virus by the respiratory route is correlated with local IgA antibody rather than serum antibody or cytotoxic T cell reactivity. Eur J Immunol 1984;14:350-6.
38. Ichinohe T, Tamura S, Kawaguchi A, Ninomiya A, Imai M, Itamura S, et al. Cross-protection against H5N1 influenza virus infection is afforded by intranasal inoculation with seasonal trivalent inactivated influenza vaccine. J Infect Dis 2007;196:1313-20.
39. CDC. Influenza Vaccine (Live, Attenuated). Vaccine information statement. 2012:p. p1–2.
40. Baz M, Samant M, Zekki H, Tribout-Jover P, Plante M, Lanteigne AM,

et al. Effects of different adjuvants in the context of intramuscular and intranasal routes on humoral and cellular immune responses induced by detergent-split A/H3N2 influenza vaccines in mice. Clin Vaccine Immunol 2012;19:209-18.

41. Miyaki C, Quintilio W, Miyaji EN, Botosso VF, Kubrusly FS, Santos FL, et al. Production of H5N1 (NIBRG-14) inactivated whole virus and split virion influenza vaccines and analysis of immunogenicity in mice using different adjuvant formulations. Vaccine 2010;28:2505-9.
42. Quintilio W, Kubrusly FS, Iourtov D, Miyaki C, Sakauchi MA, Lucio F, et al. *Bordetella pertussis* monophosphoryl lipid A as adjuvant for inactivated split virion influenza vaccine in mice. Vaccine 2009;27:4219-24.
43. Pedersen GK, Ebensen T, Gjeraker IH, Svindland S, Bredholt G, Guzman CA, et al. Evaluation of the sublingual route for administration of influenza H5N1 virosomes in combination with the bacterial second messenger c-di-GMP. PLoS One 2011;6:e26973.
44. Song JH, Nguyen HH, Cuburu N, Horimoto T, Ko SY, Park SH, et al. Sublingual vaccination with influenza virus protects mice against lethal viral infection. Proc Natl Acad Sci U S A 2008;105:1644-9.
45. Cho HJ, Kim JY, Lee Y, Kim JM, Kim YB, Chun T, et al. Enhanced humoral and cellular immune responses after sublingual immunization against human papillomavirus 16 L1 protein with adjuvants. Vaccine 2010;28:2598-606.
46. Mestecky J, McGhee JR. Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response. Adv Immunol 1987;40:153-245.
47. Underdown BJ, Schiff JM. Immunoglobulin A: strategic defense initiative at the mucosal surface. Annu Rev Immunol 1986;4:389-417.
48. Kaufman DR, Liu J, Carville A, Mansfield KG, Havenga MJ, Goudsmit J, et al. Trafficking of antigen-specific CD8+ T lymphocytes to mucosal surfaces following intramuscular vaccination. J Immunol 2008;181:4188-98.

49. Sircar P, Furr KL, Letvin NL. Systemic vaccination induces clonally diverse SIV-specific CD8+ T-cell populations in systemic and mucosal compartments. Mucosal Immunol 2013;6:93-103.

50. Okada H, Kalinski P, Ueda R, Hoji A, Kohanbash G, Donegan TE, et al. Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with {alpha}-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. J Clin Oncol 2011;29:330-6.

51. Rosenfeld MR, Chamberlain MC, Grossman SA, Peereboom DM, Lesser GJ, Batchelor TT, et al. A multi-institution phase II study of poly-ICLC and radiotherapy with concurrent and adjuvant temozolomide in adults with newly diagnosed glioblastoma. Neuro Oncol 2010;12:1071-7.

52. Veglia F, Sciaraffia E, Riccomi A, Pinto D, Negri DR, De Magistris MT, et al. Cholera toxin impairs the differentiation of monocytes into dendritic cells, inducing professional antigen-presenting myeloid cells. Infect Immun 2011;79:1300-10.

53. Mo JS, Streilein JW. Immune privilege persists in eyes with extreme inflammation induced by intravitreal LPS. Eur J Immunol 2001;31:3806-15.

54. Jansson B. Models for the transfer of drugs from the nasal cavity to the central nervous system. Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy 2004;305.

55. Kristensson K, Olsson Y. Uptake of exogenous proteins in mouse olfactory cells. Acta Neuropathol 1971;19:145-54.

56. Nisbet AD, Saundry RH, Moir AJ, Fothergill LA, Fothergill JE. The complete amino-acid sequence of hen ovalbumin. Eur J Biochem 1981;115:335-45.

57. Kumlin U, Olofsson S, Dimock K, Arnberg N. Sialic acid tissue distribution and influenza virus tropism. Influenza Other Respir Viruses 2008;2:147-54.

58. Paulsen F, Thale A, Kohla G, Schauer R, Rochels R, Parwaresch R, et al. Functional anatomy of human lacrimal duct epithelium. Anat Embryol (Berl) 1998;198:1-12.

59. Brennan MJ, Stone MR, Evans T. A rational vaccine pipeline for tuberculosis. Int J Tuberc Lung Dis 2012;16:1566-73.

60. Kaufmann E, Spohr C, Battenfeld S, De Paepe D, Holzhauser T, Balks E, et al. BCG Vaccination Induces Robust CD4+ T Cell Responses to *Mycobacterium tuberculosis* Complex-Specific Lipopeptides in Guinea Pigs. J Immunol 2016;196:2723-32.

61. Fletcher HA, Snowden MA, Landry B, Rida W, Satti I, Harris SA, et al. T-cell activation is an immune correlate of risk in BCG vaccinated infants. Nat Commun 2016;7:11290.

CHAPTER II

$CD11b^{+}$ dendritic cells in mandibular lymph nodes capture lymph borne ocular surface particulates to induce immune responses

ABSTRACT (CHAPTER II)

CD11b$^+$ dendritic cells in mandibular lymph nodes capture lymph borne ocular surface particulates to induce immune responses

Eun-Do Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Kyoung Yul Seo)

The eyedrop vaccine (EDV) is easy to use and safely exert effectiveness in animal models. However, the type of antigen presenting cells (APCs) that mediates antigen-specific immune induction has not been reported. Moreover, how the EDV is delivered into the draining lymph nodes (dLN), which are mandibular lymph nodes (MdLN) and superficial parotid lymph nodes (SPLN), is not clarified. In here, I showed that the delivery of proteins or fluorescent beads into the dLN administered by eyedrop or subconjunctival (SCJ) injection is not dependent on the migration of dendritic cells (DCs) or the activation of DCs by toll-like receptor (TLR) stimulation. Instead, the particulates were delivered by flow of lymphatic drainages into the dLN. Among two comprising parts of the dLN, cells in MdLN showed higher levels of percentages of PE-beads$^+$ than SPLN. In MdLN, CD11b$^+$ DCs were in significantly higher percentages of PE-beads$^+$ than other subsets of DCs do in both resident and migratory DCs. In CD11b knockout mice, the levels of antigen-specific serum IgG or mucosal IgA production were significantly decreased. Thus, it is expected that the strategy targeting resident CD11b$^+$ DCs in MdLN utilizing lymphatic drainage can strengthen the development of effective EDV.

Key words: eyedrop vaccine, resident DCs, CD11b$^+$DCs, lymphatic drainage, mandibular lymph nodes

CD11b$^+$ dendritic cells in mandibular lymph nodes capture lymph borne ocular surface particulates to induce immune responses

Eun-Do Kim

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Kyoung Yul Seo)

I. INTRODUCTION

Mucosal vaccine is a promising vaccine for easy use and induction of both systemic and mucosal immunity. Among many types of mucosal vaccine, eyedrop vaccine (EDV) is an emerging new type of alternative mucosal vaccine.[1-4] In previous studies, the inoculation of live-attenuated influenza or Salmonella vaccine induced competent anti-influenza or anti-salmonella immunity in mice[1] or ferret[4] against challenged pathogens. Moreover, eyedrop vaccination with inactivated Ags plus adjuvants induced protective immunity in mice.[2,3] Since eye is an organ in which immune privilege is developed to protect the structure from inflammation induced by foreign materials or pathogens, eye is considered as an optimal route for vaccine delivery.

Cervical lymph nodes (CLNs) are known as draining lymph nodes (dLNs) of EDV. However, it is composed with two distinct lymph nodes (LNs), mandibular LNs (MdLNs) and superficial parotid LNs (SPLNs). MdLNs are originally known as the eye tissue dLNs that take in solutions or antigens (Ags) from cornea, anterior chamber, conjunctiva, tears and etc.[5] SPLNs are known as a dLNs for skin of facial or head[5]. However, there are no reports that SPLN receives lymph or solutions from tear which are drained from eye drops. Therefore, it is needed to identify whether SPLNs roles as a dLNs of eyedrop

vaccination.

There are two major subsets of classical dendritic cells (cDCs) in LNs, resident DCs and migratory DCs, and they are distinguished by different expression levels of CD11c and MHCII; resident DCs express $CD11c^{hi}MHCII^{+}$ and migratory DCs express $CD11c^{+}MHCII^{hi}$.[6,7] Resident DCs are considered as lymphoid DCs since it is differentiated from blood derived cells[8-10] and there are two types of sub-sets that is distinguished by expression of CD11b ($CD11b^{+}CD8^{-}$ DCs) or CD8α ($CD8^{+}CD11b^{-}$ DCs) surface proteins.[6,9] Resident $CD11b^{+}$ DCs account for major DCs in peripheral LNs and are exclusive for CD8 expression.[9] Meanwhile, migratory DCs are originated from tissue resident DCs and locate in peripheral LNs,[9] and their migration into the dLNs is dependent of CCR7 chemokine receptors.[11-13]

In this study, I clarified that EDV Ag delivery into the dLNs needs no roles of migratory DCs but lymphatic drainage is enough. Among two subsets of resident DCs, $CD11b^{+}$ DCs are mainly responsible for capture of eyedrop-drained soluble Ags and immune induction of EDV Ag-specific immunity. Clarifying the type of DC subset that mediates EDV-induced immunity, efficacy and safety could be enhanced by designing the EDVs which targets $CD11b^{+}$ DCs in dLNs.

II. MATERIAL AND METHODS

1. Mice

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use Committee of Yonsei University Health System. Specific pathogen-free female BALB/c mice or C57BL/6 mice, aged 6–10 wks, were purchased from Charles River Laboratories (Orient Bio, Sungnam, Korea). All mice were maintained in the experimental animal facility under specific pathogen-free conditions at Yonsei College of Medicine (Seoul, Korea) and received sterilized food (Certified Diet MF; Oriental Yeast, Osaka, Japan) and filtered tap water ad libitum. Dr. Mi-Na Kweon (College of medicine, Asan University, Seoul, Korea) generously provided TLR3 KO mice. Dr. Hyunjung Ko (College of medicine, Kangwon University, Chuncheon, Korea) generously made available the CCR7 KO mice. Dr. Keunwook Lee (College of Natural Science, Chuncheon, Korea) generously made available the CD11b KO mice. All surgeries were performed after sacrificed by CO_2 narcosis and every effort was made to minimize suffering.

2. Immunization or administration of fluorescent molecules

Prior to all eyedrop or IN or SCJ administration, 6 to 10-wk-old female mice were anesthetized by i.p. injection of zoletil (30 mg/kg body weight) and rompun (10 mg/kg body weight). For eyedrop immunization, 100 μg of ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO) plus 2μg cholera toxin (CT) or 10 μg poly(I:C) were suspended in 10μl of phosphate-buffered saline (PBS) and then 5μl per conjunctival sac were dropped weekly for 3 consecutive wks by micropipette. For FITC-solution or fluorescent beads administration, three different methods were used. For eyedrop inoculation, 10μg of FITC or 10μg of FITC-beads or 10μg of PE-beads resolved in 10μl of PBS were treated with 5μl volumes per conjunctival sac by micropipette. For IN administration, 10μg of FITC resolved in 20μl of PBS were inoculated with 10 μl volumes per nasal cavity by micropipette. For SCJ injection, 10μg of PE-beads resolved in 20μl of PBS were injected with 10 μl volumes per sub-conjunctiva by 31G insulin

syringe.

3. Sample preparation

For ELISA sample preparation, at two wks after the final immunization, serum was obtained by tail vein bleeding. Tear-wash samples were obtained by lavaging with 10 μl of PBS per eye. Fecal extract was obtained by adding weighed feces to PBS containing 0.1% sodiumazide. The feces samples were mixed by vortexing and subsequently centrifuged and the supernatants were collected for assay. Vaginal wash samples were collected by lavage with 50 μl of PBS twice. To obtain broncho-alveolar lavage fluid (BALF), tracheas were cannulated after exsanguination, and the lungs were washed with 1 ml of PBS. After the mice were sacrificed, nasal wash samples were obtained by flushing 100 μl of PBS through the anterior (oral) entrance of the nasal passages using a pipette. For sample preparation of fluorescent microscopy observation, lymphoid tissues from naïve or FITC-beads inoculated mice were harvested and embedded in OCT compound (Sakura Finetec). After freezing the samples under -20℃, cryostat sections(20μm) were made.

4. Cell preparation from draining LN

DCs were isolated from CLN including MdLN and SPLN according to previously established methods.[14] Briefly, CLN were digested with 0.5 mg/ml collagenase D (5 mg/ml; Roche) plus 1 μg/ml DNase I (0.1 mg/ml; Sigma-Aldrich) enzyme solution in RPMI 1640 medium containing 10% FBS with continuous stirring at 37°C for 30 min. After EDTA was added at a final concentration of 10 mM, tissues were incubated for an additional 5 min at 37°C. Cells were filtered using a 100-μm cell strainer (BD Falcon) and recovered by centrifugation.

5. ELISA for Detection of Ag-Specific Ab

ELISA plates (Nunc, Roskilde, Denmark) were coated with OVA (100 μg/ml) in coating buffer and incubated overnight at 4°C. Blocking was done with 1% bovine serum albumin (Sigma-Aldrich) in PBS, and two-fold serially diluted

samples were applied to plates. HRP-conjugated goat anti-mouse IgG or IgA Ab (Southern Biotechnology Associates, Birmingham, AL) was added to each well and incubated overnight at 4°C. For color development, tetra-methyl benzidine solution (Thermo Scientific, Rockford, IL) was used. Plates were then measured at 450 nm on an ELISA reader (Molecular Devices, Sunnyvale, CA) after addition of stopping solution (0.5 N HCl). Endpoint titers of Ag-specific Ab were expressed as reciprocal $\log_2$ titers of the last dilution that showed > 0.2 absorbance over background levels.

6. Flow cytometric analysis

Phenotypic analyses of APC were performed using the following Abs (all from eBioscience unless noted): anti-CD45 (clone 30-F11), anti-CD3e (clone 145-2C11), anti-CD45R (B220) (clone RA3-6B2), anti-F4/80 (clone BM8), anti-Ly6G (Gr-1) (clone 1A8-Ly6g), anti-CD11c (clone N418), anti-MHCII (clone M5/114.15.2), anti-8a (clone 53-6.7), anti-CD11b (clone M1/70), anti-CD103 (clone M290; BD Pharmingen). Pertinent isotype-matched rat IgG2aκ, IgG2bκ, or rat IgG1 (clones R35-95, A95-1, and R3-34, respectively; BD Pharmingen) were used as controls. Cell surface FcR were blocked by incubation with purified anti-FcγRII/III Ab (clone 2.4G2; BD Pharmingen). Analyses were performed on the LSR Fortessa X-20 using Fluorescence-activated cell sorting (FACS) Diva™ software (both BD Biosciences).

7. Data and Statistical Analyses

Data were expressed as the mean ± SD, and statistical analyses were conducted by the ANOVA test (Microsoft Office Excel program).

III. RESULTS

1. Inoculated eyedrop antigens drains into lymph nodes through lymphatic drainage.

To determine in what extend eyedrop administered solutions can be reached, BALB/c mice were administered with FITC solution by eyedrop or IN. At 24 hr after treatment, LNs were harvested and single cells were analyzed with FACS. As shown in figure 1, including CLNs (MdLN and SPLN), spleen and inguinal LNs were FITC positive. Unlike IN group in which MdLN showed the highest FITC positive levels among all harvested LNs and spleen, FITC positive levels of all LNs in eyedrop treated mice were similar with each other (Fig 1). When FITC-labeled beads were inoculated by eyedrop or IN, fluorescent beads were detected within 2 hr of post treatment in CLNs and mediastinal LNs (MsLNs) and the phycoerythrin (PE)-labeled beads were detected until 24 hr later in CLNs (Fig 2). These data indicates that eyedrop or IN treated soluble or particle antigens (Ags) can be reached to all systemic secondary lymphoid organs, including spleen, through lymphatic drainage.

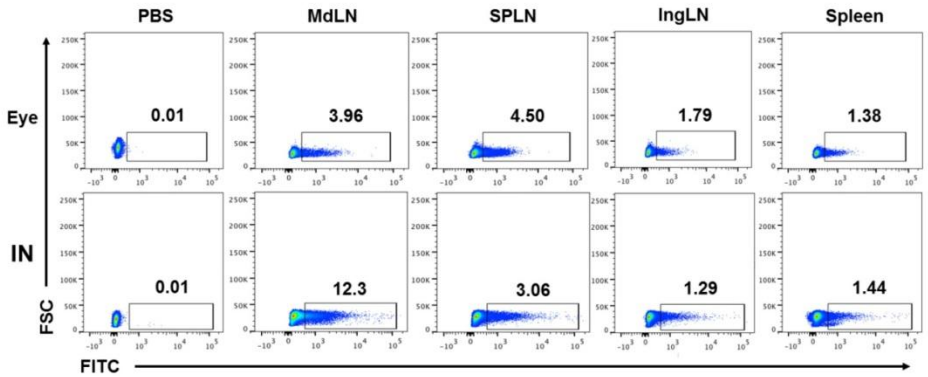

Figure 1. FITC positive cells in lymph nodes and spleen. 10 μg of FITC solution was administered by eyedrop (10μl volume (5μl/eye)) or intranasal (20μl volume) in five BALB/c mice. 24 hr later, LNs and spleen were harvested and $FITC^{+}$ cells were assayed with FACS. Data shown are representative of two independent experiments. PBS, navie MdLN; MdLN, mandibular LN; SPLN, superficial parotid LN; IngLN, inguinal LN.

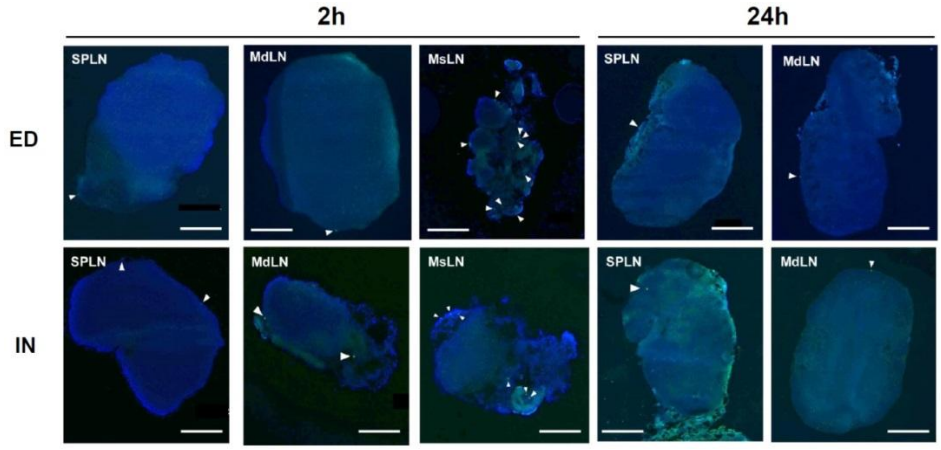

Figure 2. PE-labeled beads were detected 2 hr and 24 hur after the inoculation in draining lymph nodes. 1 μg of PE-labeled beads (45 nm) resolved in PBS were administered by eyedrop or IN in 10μl of volume (5μl/eye). Arrow heads, bead. Blue, DAPI staining. SPLN, superficial parotid LN; MdLN, madibular LN; MsLNs, mediastinal LNs. Scale bar, 100 μm.

2. $CD11b^{+}CD8^{+}$ or $CD11b^{+}CD8^{-}$ DCs are PE^{+} cells when PE-beads are injected by sub-conjunctival injection in draining lymph nodes.

To further evaluate which sub-types of DCs in dLNs are involved in eyedrop vaccinated Ags uptake, I utilized PE-beads as tracer molecules for eyedrop inoculated Ags. Since there are two types of conventional DCs in LNs, resident DCs and migratory DCs, I discriminated these two types of DCs by the expression levels of CD11c and MHCII molecules as in previously reported studies[6,7] after pre-gating the cells on $CD45^{+}CD3^{-}F4/80^{-}Ly6G^{-}B220^{-}$ (Fig 3); resident DCs as $CD11c^{hi}MHCII^{+}$ cells or migratory DCs as $CD11c^{+}MHCII^{hi}$ cells (Fig 4). However, too small amount of $PE\text{-}beads^{+}$ cells to analyze the types of subpopulation of DCs in dLNs were detected in mice that were administered by eyedrop (Fig 5). In previous study, it was shown that when even four nasolacrimal duct puncta of mouse were sutured, blocking the drainage from eye to nose, the levels of Ag-specific serum and mucosal Abs production were identical to those in the non-sutured mice.[1] Thus, I surmised that most inoculated eyedrop Ags are penetrated to conjunctival lymphatic vessels

through conjunctiva, and I used SCJ injection to maximize the amount of penetrated beads into conjunctival lymphatic vessels (Fig 5).

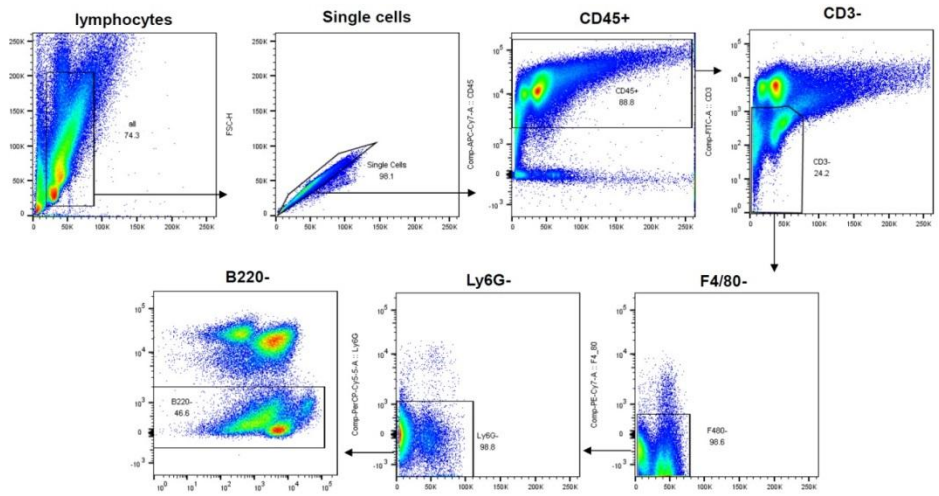

Figure 3. Gating strategy on DCs. After dLNs were harvested, single cells were prepared. The cells were gated on $CD45^{+}CD3^{-}F4/80^{-}Ly6G^{-}B220^{-}$.

At one day post injection (dpi), the majority of PE^{+} cells were $CD11b^{+}CD8^{+}$ DCs and $CD11b^{+}CD8^{-}$ DCs in both resident DCs and migratory DCs in MdLNs and SPLNs and the percentages of PE^{+} cells were higher in MdLNs then SPLNs (Fig 6). Interestingly, PE^{+} cells were detected in the beads alone treated mice as shown in the poly(I:C) adjuvant treated mice. It seemed that the uptake of beads by DCs was occurred in the absence of DC activation.

3. SCJ injected antigens can be taken by resident DCs without the activation of DCs in draining lymph nodes.

To further examine the role of APCs in SCJ injected vaccine antigen uptake and delivery to the dLNs, TLR3 KO mice were used for tracking of inoculated beads. Wild type or TLR3 KO BALB/c mice were administered with 0.5 μg of PE-beads plus 10 μg poly(I:C) by SCJ. At 24 hr of SCJ, MdLNs and SPLNs were harvested and resident or migratory DCs were analyzed with FACS. Although there were no activation of DCs through TLR3 receptors, similar or higher amount of cells were PE positive (Fig 7).

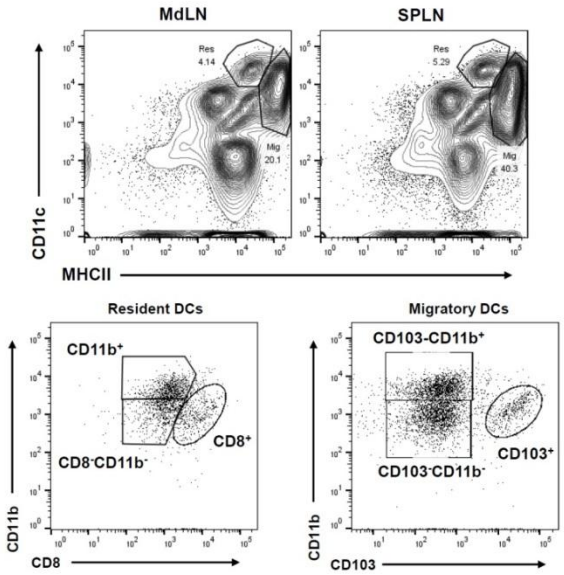

Figure 4. Gating strategy on resident or migratory DCs. After non-DCs were pre-excluded, resident or migratory DCs were distinguished with CD11c and MHCII. Resident DCs are $CD11c^{hi}MHCII^{+}$; Migratory DCs are $CD11c^{+}MHCII^{hi}$. Resident DCs were further gated with CD11b and CD8 and migratory DCs were with CD11b and CD103.

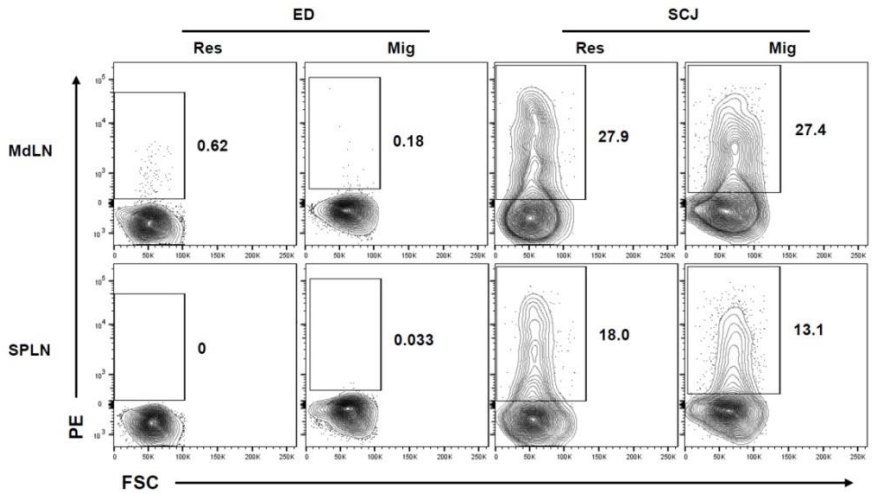

Figure 5. PE-beads were detected in dLNs administered by eyedrop and SCJ. 1 μg or 0.5 μg of PE-beads were administered by eyedrop or SCJ. At 24 hr later, dLNs were harvested and single cells were prepared. The cells were pre-gated as resident and migratory DCs and the percentages of PE-positive were

shown. Res, resident DCs; Mig, migratory DCs. Forty mice per group in eyedrop treated groups and five mice per group in SCJ treated mice.

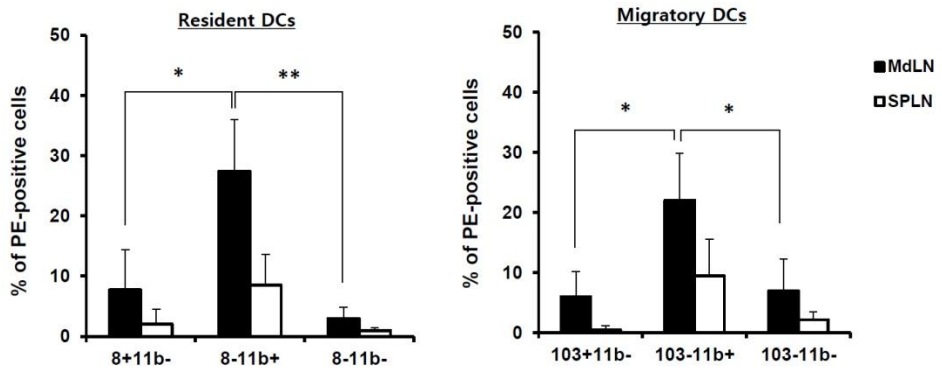

Figure 6. CD11b^{+} DCs are in highest percentages of PE-beads positive. 0.5 μg of beads were injected as SCJ, dLNs were harvested and DC cells were analyzed with FACS. The percentages of PE-positive cells were shown in resident DCs and migratory DCs from MdLN and SPLN. A representative experiment was shown among five independent experiments. *, $p < 0.5$; *, $p < 0.01$. ANOVA was used for statistical analysis.

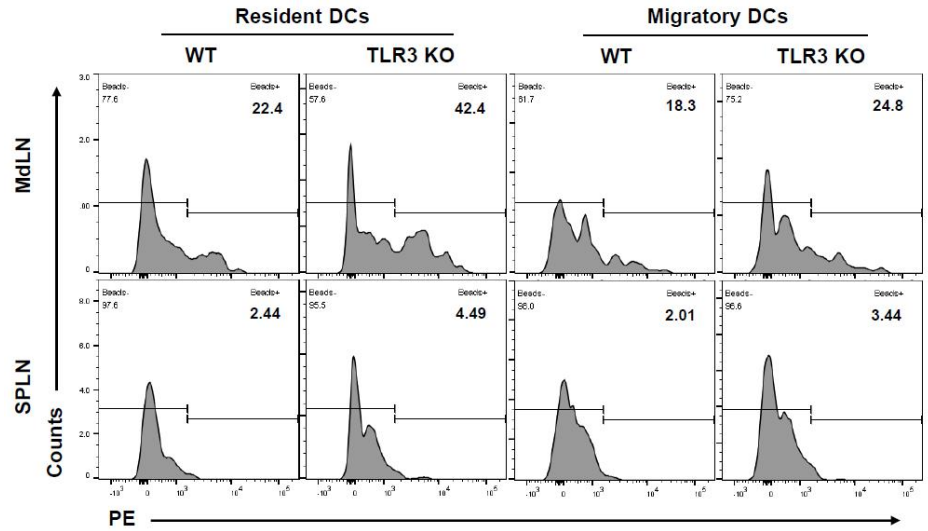

Figure 7. PE-beads positive DCs were detected in TLR3 KO mice. 0.5μg PE-beads plus 10 μg poly(I:C) was injected with sub-conjunctival injection in BALB/c WT or TLR3 KO mice. 24 hr later, MdLNs and SPLNs were harvested and DCs were prepared, then PE^{+} cells were analyzed with FACS. Data shown

are representative of three independent experiments (five mice per group).

Moreover, in the absence of TLR3 expression, the pattern that the percentages of PE^+ cells of $CD11b^+$ DCs were higher than the other two sub-types was not different from that of wild type mice (Fig 8). Therefore, it seems that the uptake of SCJ injected beads by DCs in eyedrop dLNs needs no activation of DCs by adjuvants, such as poly(I:C) or CT.

4. Migratory DCs are dispensable for eyedrop vaccinated Ag-specific immune induction in dLNs.

In previous study, it was shown that even in the absence of CCR7 Ag-specific Ab titers of serum or mucosal wash samples were not decreased in eyedrop vaccinated mice.[1] The result might mean that migratory DCs are not important in eyedrop vaccinated Ab-specific immune induction since CCR7 is needed for migration of migratory DCs from peripheral tissues to dLNs.[12,14,15] Thus, I

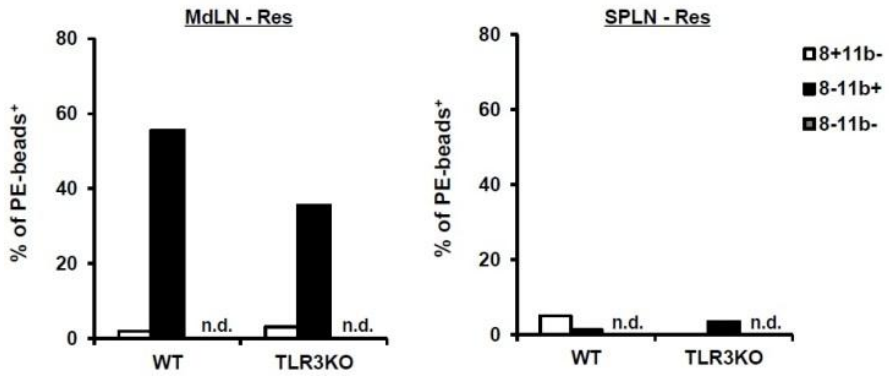

Figure 8. $CD11b^+$ DCs showed highest percentages of PE^+ in TLR3 KO mice. 0.5μg PE-beads plus 10 μg poly(I:C) was injected with sub-conjunctival injection in BALB/c WT or TLR3 KO mice. 24 hr later, MdLNs and SPLNs were harvested and resident DCs were prepared, then PE^+ cells were analyzed with FACS. Data shown are representative of three independent experiments (pooling of five mice per group). n.d., non-detected.

utilized CCR7 KO mice to check whether the role of migratory DCs in Ag delivery from eye to dLNs is critical. After the induction of Ag-specific

immunity in CCR7 KO mice, the levels of OVA-specific Ab titers of serum or mucosal wash samples were not decreased as in previous study and it were similar with those of wild type mice (Fig 9). However, as I assumed, the population of migratory DCs in both dLNs in CCR7 KO mice were almost disappeared (Fig 10). These suggest that the induction of eyedrop inoculated Ag-specific immunity is almost exclusively mediated by resident DCs in dLNs, not by migratory DCs which are newly immigrated from peripheral tissues into dLNs.

5. Resident CD11b$^+$ DCs are indispensable to immune induction for eyedrop vaccine antigens.

In previous results, CD11b$^+$ DCs accounts for large proportions of PE-beads$^+$ cells. Therefore, I assumed that the role of CD11b$^+$ DCs will be important for eyedrop vaccinated Ag-specific immunity induction. To check the influence of CD11b$^+$ DCs-mediated immune induction in EDV, I used CD11b KO mice for their *itgam* gene was deleted and lack integrin alpha M expression.[16] After

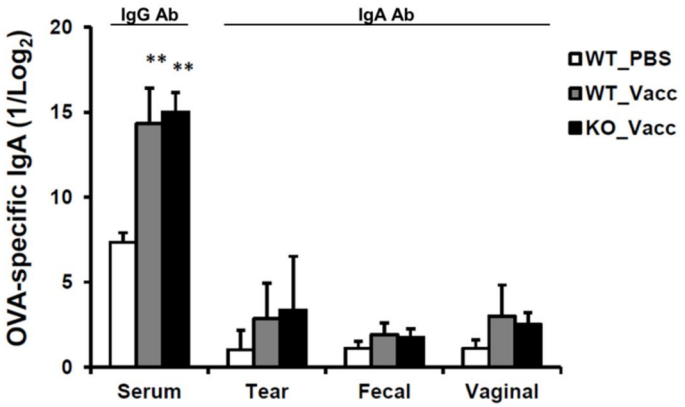

Figure 9. Anti-OVA Ab levels were not decreased in CCR7 KO mice. 100 μg OVA + 2μg CT or PBS were inoculated by eyedrop in WT or CCR7 KO B6 mice. After 3 consecutive vaccinations with one wk interval, serum and mucosal wash samples were harvested and OVA-specific Abs were detected by ELISA. Five mice per group. **, $p < 0.01$ vs. PBS group. ANOVA was used for statistical analysis.

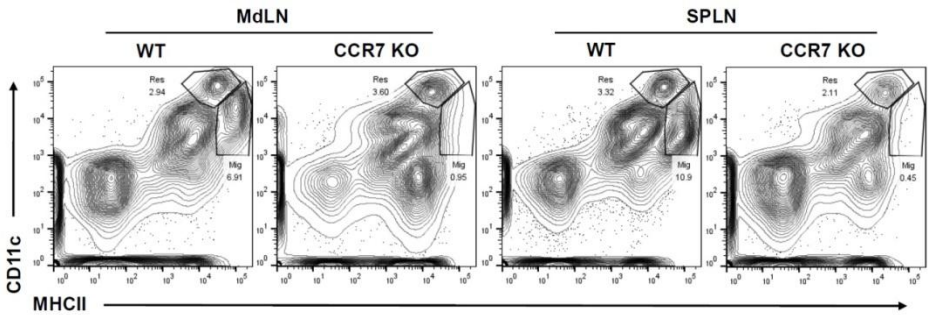

Figure 10. Migratory DCs in dLNs were depleted in OVA+CT eyedrop immunized CCR7 KO mice. 100μg OVA + 2μg CT was inoculated by eyedrop in B6 WT or CCR7 KO mice for three times with one wk interval. After seven days last inoculation, dLNs were harvested and DCs were prepared. Resident DCs or migratory DCs were analyzed with FACS. A representative experiment was shown among two independent experiments.

OVA+CT were vaccinated by eyedrop on the mice, the levels of OVA-specific serum and mucosal Abs were measured. In the CD11b KO mice, the level of Ags-specific serum Abs was significantly decreased and the levels of mucosal IgA Abs were not detected in tear and vaginal wash samples (Fig 11). When the two sub-types of resident or migratory DCs in dLNs were analyzed, there was no significant decrease of increase in the proportions of resident DCs or migratory DCs compared to those of wild type mice (Fig 12). Since even in the absence of the migratory DCs the eyedrop inoculated Ag-specific immunity was developed, it suggests that uptake and presentation of eyedrop inoculated Ags by resident $CD11b^{+}$ DCs in MdLNs are important for EDV Ag-specific immunity induction.

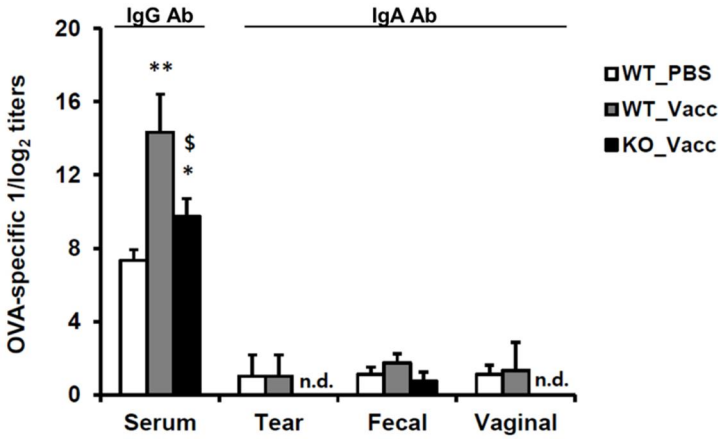

Figure 11. The levels of Ag-specific Ab production was decreased in CD11b KO mice. 100 μg OVA + 2μg CT or PBS were inoculated by eyedrop in WT or CCR7 KO B6 mice. After 3 consecutive vaccinations with one wk interval, serum and mucosal wash samples were harvested and OVA-specific Abs were detected by ELISA. Five mice per group. *, $p < 0.05$; **, $p < 0.01$ vs. PBS. $, $p < 0.05$ vs. WT_Vacc. ANOVA was used for statistical analysis.

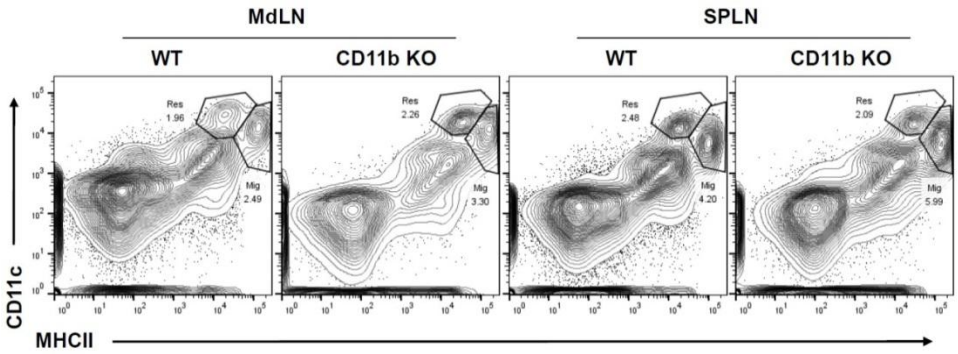

Figure 12. No changes in percentages of resident or migratory DCs in CD11b KO mice. 100μg OVA + 2μg CT was inoculated by eyedrop in B6 WT or CCR7 KO mice for three times with one wk interval. After seven days last inoculation, dLNs were harvested and DCs were prepared. Resident DCs or migratory DCs were analyzed with FACS. Five mice per group. A representative experiment was shown among two independent experiments.

IV. DISCUSSION

For an effective, safe and easy vaccination, many attempts to show the feasibility of mucosal route-specific efficient vaccines. Recently, among various mucosal vaccination strategies, eyedrop inoculation is suggested as an alternate method for vaccine delivery. However, the reports examining the identity or mechanism of Ag uptake and presentation of eyedrop vaccine Ags-mediating DCs are little. In this study, I investigated that which subtypes of DCs are involved in EDV Ag uptake and induction of Ag-specific immunity. Eyedrop inoculated Ags can be drained into dLNs in the absence of the uptake and transportation by migratory DCs. Moreover, migratory DCs are not involved in the process of eyedrop vaccinated Ag-specific immune induction. Although both resident and migratory DCs in dLNs are able to uptake eyedrop Ags, DC activation through adjuvant ligation is needed in EDV-induced immunity development. Among three types of resident DCs, $CD11b^{+}CD8^{-}$ or $CD11b^{+}CD8^{+}$ or $CD11b^{-}CD8^{+}$ DCs, it seems that $CD11b^{+}$DCs are most responsible for eyedrop inoculated Ags uptake and induction of Ag-specific immunity. These data suggest new insight into the drainage of EDV and identifying DC subtypes of EDV-specific immune induction in dLNs.

Understanding the mechanism of lymphatic drainage of vaccine molecules into draining lymph nodes is important for several reasons; high concentration of immature DCs in lymph nodes than peripheral tissues,[17] resident immature DCs are specialized for efficiently sampling and processing Ags provided by constant lymph flow when it is exposed to stimulating signals,[6,18] and vaccine molecules transported by lymph circulate can be repeatedly taken up by DCs from many different lymph nodes before it is being returned to the blood circulation.[18] Until now, it was unclear whether the EDV Ags are mainly transported by migratory DCs or lymph flow without the activity of migratory DCs. However, it was shown that Ag-specific immunity can be induced in the absent of migratory DCs in draining lymph nodes against eyedrop vaccine Ags. Moreover, I showed that Ag delivery to the dLNs needs no activation of DCs since pattern of PE-beads uptake of DCs in TLR3 KO mice was not different

from those in WT mice. If migratory DCs were used for transportation of SCJ injected beads, PE-beads were not detected in migratory DCs in dLNs in the absence of DC activation through TLR3 receptor. Thus, although it was previously reported that eyedrop inoculated Ag-specific immunity was developed even in CCR7 KO mice,[1] this study is the first report which shows the complete exclusion of the role of migratory DCs in the induction of eyedrop vaccine specific immunity in eyedrop draining lymph nodes, MdLNs and SPLNs, in CCR7 KO mice.

When vaccine complex, Ags plus adjuvants, are injected the complex moves faster by lymph flow than the transportation by migratory DCs into the draining lymph nodes. After the molecules arrive in the draining lymph nodes, these are taken up by dLNs-resident DCs. Therefore, antigen uptake by the resident DCs in the draining lymph node might be the initiation of the process in the adaptive immunity induction. However, the known sub-types of resident DCs which are involved in the uptake of lymph borne Ags are controversial among various studies. In one study, $CD8^+$ DCs are important for uptake and presentation of drained foreign pathogens, such as malaria sporozoites, to the $CD8^+$ OT-1 T cells.[19] In other study, $CD11b^+$ DCs are critical for lymph borne antigen-specific immune induction.[6] The main difference between these two studies is the type of antigen molecules; live pathogens (sporozoites) or particulate molecules (micro beads or OVA proteins), Moreover, OT-1 cells were used for proliferation assay in Radtke's study. Whereas, Gerner's study used 40 nm beads and OVA protein as injected tracing molecules and both OT-I and OT-II cells were used for proliferation study. In addition, $CD11b^+$ DCs account for highest population among lymph node resident DCs[20] and are specialized for CD4 T cell activation for its great capacity for MHCII molecules expression.[6,9] In this study, 40 nm PE-labeled beads were used as Gerner's study to detect APCs which uptake eyedrop or SCJ administered Ags in dLNs, and $CD11b^+$ DCs in both migratory DCs ($CD103^-CD11b^+$ DCs) and resident DCs ($CD11b^+CD8^-$ DCs) were in highest percentages of PE-beads positive. Thus, it seems that deciding the sub-type of mainly participating APCs is dependent of

antigen's nature: $CD8^+$ DCs for live Ags and $CD11b^+$ DCs for particulate Ags. However, it is remained to be fully elucidated which mechanism make these two different types of DCs as main participating APCs in dLNs.

For the evaluation of the role of $CD11b^+$ DCs in eyedrop vaccination, CD11b KO mice were used in this study. As expected, CD11b KO mice showed significantly decreased level of serum IgG and mucosal IgA production. However, the decrease of IgG or IgA in CD11b KO mice might be due to the following three possibilities: The regulation of Th17 responses by $CD11b^+$ DCs in mucosal tissues,[21-23] $CD11b^+$ DCs are specialized in presentation of soluble Ags to $CD4^+$ T cells,[6] and the necessity of CD11b expression in activation of AID and antibody class switching in activated B cells (unpublished data). Since the regulation of Th17 response by $CD11b^+$ DCs is mainly exerted in mucosal tissues, it could be excluded from the immune induction in the environment of dLNs. My data showed that the production of both serum IgG and mucosal IgA was down regulated in eyedrop vaccinated CD11b KO mice. It is possible that there was no activation of $CD4^+$ T cells by $CD11b^+$ DCs in dLNs and consequently failure of B cell activation. Therefore, it is needed to check stagy by stage in the process of EDV-specific immune induction in CD11b KO mice. Unfortunately, I could not discriminate which mechanism between $CD4^+$ T cell activation and B cell activation governs the defect in Ag-specific antibody production, and the detailed mechanism is remained to be clarified.

In conclusion, I unraveled the main APCs in EDV Ag-specific immunity induction in eyedrop dLNs. Unlike other mucosal vaccine, i.e., oral vaccine needs uptake of vaccine Ags from mucosal sites and migration of the Ag-captured DCs into the dLNs, EDV needs no capturing of vaccine Ags by peripheral migratory DCs. Instead, it needs the carry by the lymph flow from inoculated sites into the dLNs. However, in dLNs, the uptake of drained EDV Ags and mediation of immune induction by resident $CD11b^+$ DCs are indispensable for EDV Ag-specific immunity induction. Thus, it is expected that strategic design of $CD11b^+$ DCs targeting-EDVs which utilizes lymphatic drainage into dLNs could enhance the potency and safety of EDV.

V. CONCLUSION

Since investigating EDV is not just about a novel type of antigen or adjuvant, its potential will be countless once detailed mechanism of EDV-induced immunity is fully clarified. Therefore, although other types of immune cells participating EDV Ag capturing or presenting process are remained to be elucidated, identification of the type of main APC will be the first step forward to open the gate for a new feasible clinical EDV. Moreover, if a vaccine which is adapted for the utilization of lymphatic drainage other than Ag transportation by DCs is developed, it will accelerate the emergence of the first clinical EDV. Thus, following consecutive studies of EDV will be pilling up the steppingstone for the first clinical EDV study and the first commercial EDV development.

REFERENCES

1. Seo KY, Han SJ, Cha HR, Seo SU, Song JH, Chung SH, et al. Eye mucosa: an efficient vaccine delivery route for inducing protective immunity. J Immunol 2010;185:3610-9.
2. Choi KS, Kim SH, Kim ED, Lee SH, Han SJ, Yoon S, et al. Protection from hemolytic uremic syndrome by eyedrop vaccination with modified enterohemorrhagic E. coli outer membrane vesicles. PLoS One 2014;9:e100229.
3. Kim ED, Han SJ, Byun YH, Yoon SC, Choi KS, Seong BL, et al. Inactivated Eyedrop Influenza Vaccine Adjuvanted with Poly(I:C) Is Safe and Effective for Inducing Protective Systemic and Mucosal Immunity. PLoS One 2015;10:e0137608.
4. Yoon S, Kim ED, Song MS, Han SJ, Park TK, Choi KS, et al. Eyedrop Vaccination Induced Systemic and Mucosal Immunity against Influenza Virus in Ferrets. PLoS One 2016;11:e0157634.
5. Van den Broeck W, Derore A, Simoens P. Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrl mice. J Immunol Methods 2006;312:12-9.
6. Gerner MY, Torabi-Parizi P, Germain RN. Strategically localized dendritic cells promote rapid T cell responses to lymph-borne particulate antigens. Immunity 2015;42:172-85.
7. Miller JC, Brown BD, Shay T, Gautier EL, Jojic V, Cohain A, et al. Deciphering the transcriptional network of the dendritic cell lineage. Nat Immunol 2012;13:888-99.
8. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol 2005;5:617-28.
9. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. Annu Rev Immunol 2013;31:563-604.

10. Gerner MY, Kastenmuller W, Ifrim I, Kabat J, Germain RN. Histo-cytometry: a method for highly multiplex quantitative tissue imaging analysis applied to dendritic cell subset microanatomy in lymph nodes. Immunity 2012;37:364-76.
11. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 1999;99:23-33.
12. Forster R, Davalos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 2008;8:362-71.
13. Ohl L, Mohaupt M, Czeloth N, Hintzen G, Kiafard Z, Zwirner J, et al. CCR7 governs skin dendritic cell migration under inflammatory and steady-state conditions. Immunity 2004;21:279-88.
14. Song JH, Kim JI, Kwon HJ, Shim DH, Parajuli N, Cuburu N, et al. CCR7-CCL19/CCL21-regulated dendritic cells are responsible for effectiveness of sublingual vaccination. J Immunol 2009;182:6851-60.
15. Randolph GJ, Ochando J, Partida-Sanchez S. Migration of dendritic cell subsets and their precursors. Annu Rev Immunol 2008;26:293-316.
16. Coxon A, Rieu P, Barkalow FJ, Askari S, Sharpe AH, von Andrian UH, et al. A novel role for the beta 2 integrin CD11b/CD18 in neutrophil apoptosis: a homeostatic mechanism in inflammation. Immunity 1996;5:653-66.
17. Wilson NS, El-Sukkari D, Belz GT, Smith CM, Steptoe RJ, Heath WR, et al. Most lymphoid organ dendritic cell types are phenotypically and functionally immature. Blood 2003;102:2187-94.
18. Swartz MA, Hubbell JA, Reddy ST. Lymphatic drainage function and its immunological implications: from dendritic cell homing to vaccine design. Semin Immunol 2008;20:147-56.
19. Radtke AJ, Kastenmuller W, Espinosa DA, Gerner MY, Tse SW, Sinnis P, et al. Lymph-node resident CD8alpha+ dendritic cells capture antigens from migratory malaria sporozoites and induce CD8+ T cell responses. PLoS Pathog 2015;11:e1004637.

20. Mildner A, Jung S. Development and function of dendritic cell subsets. Immunity 2014;40:642-56.
21. Denning TL, Norris BA, Medina-Contreras O, Manicassamy S, Geem D, Madan R, et al. Functional specializations of intestinal dendritic cell and macrophage subsets that control Th17 and regulatory T cell responses are dependent on the T cell/APC ratio, source of mouse strain, and regional localization. J Immunol 2011;187:733-47.
22. Cao AT, Yao S, Gong B, Elson CO, Cong Y. Th17 cells upregulate polymeric Ig receptor and intestinal IgA and contribute to intestinal homeostasis. J Immunol 2012;189:4666-73.
23. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. Immunity 2013;38:970-83.

ABSTRACT (IN KOREAN)

CD11b+ 수지상세포에 의해 매개되는 점안형 불활성화 백신에 대한 면역반응 유발

<지도교수 서 경 률>

연세대학교 대학원 의과학과

김 은 도

약독화된 생백신을 마우스에 점안으로 접종한 점안백신의 효능이 발표된 이후, 점안백신은 전신 면역과 점막 면역을 동시에 일으키는 점막 백신으로서 새로운 형태의 점막 백신으로 제시되고 있다. 이에 따라, 본 연구에서 생백신보다 안전한 불활성화된 인플루엔자 백신 및 기타 상용화된 백신들의 효능을 점안 접종법에서 확인하였으며, 인플루엔자 백신 연구에 주로 사용되는 페렛에서 점안백신의 효능을 확인하였다. 또한 마우스에서 점안백신 항원을 전달하는 항원 제시세포의 종류와 그 아형을 동정하였다.

불활성화 인플루엔자 백신 항원을 점안 백신으로 사용하기 위하여 적합한 아주번트를 일 차적으로 확인해본 결과, 효과는 가장 좋지만 독성이 있는 콜레라톡신을 대체할 아주번트로 poly(I:C)가 적합함을 확인하였다. Poly(I:C)를 아주번트로 사용할 경우 서브 유닛 및 분할 인플루엔자 백신 항원을 점안으로 접종할 때 혈청 및 점막액에서 항원특이 항체가 유의하게 증가하였다. H1N1 바이러스 분할 백신을 poly(I:C)와 함께 점안으로 접종할 경우 백신에 접종된 마우스들은 치명적인 인플루엔자 바이러스 감염에 보호되었으며, 백신 접종 부위인 눈에서 염증반응이 유발되지 않고 백신 물질이 중추신경계로 흡수되지 않았다.

한 편, 페렛에서는 H1N1, H1N2, H3N2의 세 가지 다른 약독화 인플루엔자 생바이러스를 백신항원으로 점안접종 한 후 감염성이 있는 인플루엔자 바이러스를 감염시켰다. 그 결과 점안백신을 접종 받은 페렛들에서 대조군에 비해 바이러스 중화 항체가 유의하게 증가하였으며 체중감소와 체온상승이 일어나지 않았다. 또한 감염된 바이러스는 5일 안에 폐에서 모두 제거되었으며, 마우스와 마찬가지로 눈 조직에서의 염증반응이 일어나지 않았다. 따라서 점안으로 불활성화 인플루엔자 백신을 poly(I:C)와 함께 접종하는 것이 새로운 안전하고 효과적인 점막 인플루엔자 백신 접종법이 될 수 있으며, 페렛 모델에서 확인한 바를 바탕으로 임상 단계에서의 실험이 가능할 것으로 기대된다.

또한, 기존의 근육주사로 접종되고 있는 상용화된 백신들을 점안 접종법으로 대체하여 효능을 확인하기 위해 HPV, BCG, 수두, MMR, DTP, HIP, HBV 백신 등을 점안으로 접종해보았다. 그 결과 HPV만 점안 접종 시 항원 특이 항체가 전신 및 점막에서 유의하게 증가하였으며, 다른 백신 항원들은 면역반응을 유발하지 못함을 확인하였다.

한 편, 백신을 점안으로 접종할 경우 백신항원이 어떤 경로를 통해 배출 림프절로 이동하며 어떤 APC가 항원특이면역 유발에 관여하는지에 대해서는 아직 연구된 바가 없다. 이를 확인하기 위해 FITC 용액과 PE-labeled 비즈를 점안으로 투여한 결과, FITC 용액은 30분만에 배출 림프절 및 비장에서 확인됐으며, PE-beads는 2시간 후부터 배출 림프절에서 확인이 됐다. TLR3 결핍 마우스에 poly(I:C)를 PE-beads와 함께 점안 투여 시에 PE-양성 수지상세포가 배출림프절에서 발견되었다. 또한 CCR7 결핍 마우스에 점안백신을 접종하였을 때에는 이동성 수지상세포가 배출 림프절에서 결핍되어 있음에도 혈청 및 점막 항원 특이 항체 형성이 유도되었다. 이는 점안백신 항원이 APC에 의해 섭취되어 배출 림프절로 이동하는 것보다는 눈물길 및 림프관 배출에 의해 배출 림프절로 도달하며, 배출 림프절에서는 이동성 수지상세포

의 역할보다는 정착성 수지상세포의 역할이 크다는 것을 의미한다고 볼 수 있다. 한 편, PE-beads를 결막하주사로 접종 시 배출 림프절 중 MdLN에서 PE-양성 수지상세포가 많았으며, 그 중 주로 $CD11b^{+}$수지상세포에서 PE-양성을 나타냈다. 또한, CD11b 결핍 마우스에서는 배출 림프절의 이동성 수지상세포는 감소하지 않았지만 항원특이 항체 생성 유도가 정상적으로 일어나지 않았다. 이는 정착성 수지상세포 중 $CD11b^{+}$ 수지상세포가 림프선을 타고 배출림프절로 배출되는 백신 항원을 섭취하여 항원특이 면역을 유발하는 역할을 하는 것임을 나타낸다고 할 수 있을 것이다. 따라서 점안 접종법에서는 배출 림프절에 정착하고 있는 정착성 $CD11b^{+}$ 수지상세포가 점안백신의 항원특이 면역반응을 매개하는 주요 역할을 할 것으로 예상되며, 이에 따라 점안백신 개발에 있어서 림프선 배출을 활용하여 배출림프절에 상주하는 $CD11b^{+}$ 수지상세포를 표적으로 하는 백신 개발이 점안백신의 효능을 증강시켜주는 새로운 방법으로 제시될 수 있을 것이다.

핵심 되는 말: 점안, poly(I:C), 인플루엔자, 불활성화 백신, 페렛, 약독화생백신

PUBLICATION LIST

1. Yoon S*, Kim ED*, Song MS, Han SJ, Park TK, Choi KS, et al. Eyedrop vaccination induced systemic and mucosal immunity against influenza virus in ferrets. PLoS One 2016;11:e0157634.
2. Kim ED, Han SJ, Byun YH, Yoon SC, Choi KS, Seong BL, et al. Inactivated eyedrop influenza vaccine adjuvanted with poly(I:C) is safe and effective for inducing protective systemic and mucosal immunity. PLoS One 2015;10:e0137608.
3. Choi KS, Yoon SC, Rim TH, Han SJ, Kim ED, Seo KY. Effect of voriconazole and ultraviolet-A combination therapy compared to voriconazole single treatment on Fusarium solani fungal keratitis. J Ocul Pharmacol Ther 2014;30:381-6.
4. Choi KS, Kim SH, Kim ED, Lee SH, Han SJ, Yoon S, et al. Protection from hemolytic uremic syndrome by eyedrop vaccination with modified enterohemorrhagic *E. coli* outer membrane vesicles. PLoS One 2014;9:e100229.