

Potential risk of repeated nasal vaccination that induces allergic reaction with mucosal IgE and airway eosinophilic infiltration in cynomolgus macaques infected with H5N1 highly pathogenic avian influenza virus



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ABSTRACT

The efficacy and detrimental effect of mucosal vaccination with an inactivated influenza vaccine were examined in a macaque model by intranasal administration with small amounts of inactivated whole virus particles and challenge by a human-derived H5N1 highly pathogenic avian influenza virus infection. Repeated nasal inoculation with the whole particle vaccine of an inactivated virus, A/duck/Hokkaido/Vac-3/2007 (H5N1) (Vac-3), induced antigen-specific IgA and IgG antibody production in nasal swabs and plasma. Vac-3-specific IgE production was also found in the nasal swabs. Nasal vaccination with Vac-3 induced broader cross-clade neutralization activity than did subcutaneous vaccination. After challenge infection, repeated nasal vaccination almost completely prevented the propagation of virus in the upper and lower airways and protected cynomolgus macaques from viral pneumonia by induction of IgA-producing B cells in the lungs. On the other hand, eosinophil clusters were observed in the lungs of vaccinated macaques. Although Vac-3-specific IgE antibody and IL-13 levels were decreased after infection compared to those before infection and no anaphylaxis in vaccinated macaques was detected after challenge infection, our results suggest that we have to pay attention to potential allergic responses at repeated nasal vaccination, especially in people who have an airway allergy.

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1. Introduction

Vaccination against influenza virus infection is the only means to elicit acquired immune responses against influenza virus prior to actual infection, and these acquired immune responses ameliorate severe symptoms caused by the virus. A strategy to induce sufficient protective immunity depends on the property of the vaccine, route of vaccination and schedule of vaccination. Since the importance of mucosal IgA responses to protect against respiratory pathogens is widely recognized, nasal vaccination is expected to prevent influenza virus infection by induction of IgA

responses in nasal mucosa, which is an entry site of influenza virus in respiratory droplets [1–3].

It has been reported that nasal vaccination induced cross-reactive nasal IgA and local memory responses more effectively than did subcutaneous vaccination in murine models [4,5]. Nasal vaccination with a live influenza virus was found to be more effective than subcutaneous injection with live virus vaccines in our previous study using mice [6], and nasal vaccination using a live attenuated influenza virus is provided in some countries as seasonal influenza vaccination [7–10]. However, in our previous study using macaques, subcutaneous inoculation with inactivated whole influenza virus particles three times induced higher neutralizing antibody titers than did nasal inoculation of the same inactivated virus particles three times [11]. We have also reported that subcutaneous vaccination with inactivated whole influenza virus parti-

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cles was more effective than nasal vaccination for induction of immune responses and protection in mice [6]. Another study has also shown that nasal vaccination induced cross-protective CD8⁺ T cells less efficiently than did subcutaneous vaccination in a murine model [12].

Considering these conflicting results regarding the routes of vaccination that induces protective immunity, we selected inactivated whole virus particles of the vaccine strain A/duck/Hokkaido/Vac-3/2007 (H5N1) (Vac-3) to examine the efficacy of repeated nasal vaccination in our nonhuman primate model [13]. To improve the efficacy of nasal vaccination, we increased the number of administrations with whole virus particles. We also assessed undesirable reactions induced by nasal vaccination of inactivated whole virus particles since the safety of seasonal influenza vaccination in asthmatic patients has been a concern [14–16] and vaccine antigen-specific IgE in the serum of children with influenza vaccine-associated anaphylaxis has been reported [17]. We found that repeated nasal vaccination with a small amount of the inactivated whole virus particles induced protective immunity against H5N1 highly pathogenic avian influenza virus (HPAIV) and induced IgE and eosinophilic responses in the macaque model.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the Guidelines for the Husbandry and Management of Laboratory Animals of the Research Center for Animal Life Science at Shiga University of Medical Science and Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The protocols were approved by the Shiga University of Medical Science Animal Experiment Committee (Permit numbers: 2012-10-8, 2013-10-3). The animal experiments were conducted as described previously [13].

2.2. Animals

Six- to nine-year-old healthy female cynomolgus macaques (*Macaca fascicularis*) from Vietnam were used. The macaques were free from herpes B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp., and *Entamoeba histolytica*. Food, drinking water and housing were provided and all procedures were performed as described previously [13]. The macaques were taken care of under the following environmental conditions: humidity (51–61%), temperature (23.8–24.3 °C) and light (12-h light/12-h dark cycle, lights on at 8:00 a.m.). In the text and figures, individual macaques are distinguished by identification numbers (Supplementary Table 1). To monitor body temperature, a telemetry probe (TA10CTA-D70, Data Sciences International, St. Paul, MN) was implanted in the peritoneal cavity of each macaque. To collect swab samples, cotton sticks (TE8201, Eiken Chemical, Ltd., Tokyo, Japan) were held in nasal cavities and tracheas of macaques for 30 s, and the sticks were subsequently immersed in 1 mL of minimal essential medium containing 0.1% bovine serum albumin (BSA) and antibiotics [13]. A bronchoscope (MEV-2560, Machida Endoscope Co. Ltd., Tokyo, Japan) and cytology brushes (BC-203D-2006, Olympus Corporation, Tokyo, Japan) were used to obtain bronchial samples.

2.3. Viruses and vaccines

We used influenza virus A/duck/Hokkaido/Vac-3/2007 (H5N1) (Vac-3, National Center for Biotechnology Information (NCBI) taxonomy database ID: 463698) as a vaccine strain. This virus is a reassortant between A/duck/Hokkaido/101/2004 (H5N3) and A/duck/Hokkaido/262/2004 (H6N1) [18].

The Vac-3 virus was propagated in allantoic cavities of embryonated hen's eggs, and inactivated whole virus particles were prepared as described previously [13]. Virus particles in the allantoic fluid infected with Vac-3 were purified by high-speed centrifugation (112,500g for 90 min) through a 10–50% sucrose density gradient and then treated in 0.2% formalin at 4 °C for one week. The amount of vaccine is indicated as the amount of entire proteins including HA and other viral proteins. The inactivated whole virus particles (0.02 mg or 0.2 mg) in saline was inoculated intranasally (0.25 mL for each nostril) into macaques using MicroSprayer Aerosolizer Model 1A-1B-R (Penn-Century, Inc.) 8 times under ketamine/xylazine anesthesia.

The challenge virus strain A/Vietnam/UT3040/2004 (H5N1) (VN3040, NCBI taxonomy database ID: 755291) was isolated from a human patient [19,20]. VN3040 was propagated twice in Madin-Darby canine kidney (MDCK) cells at the University of Tokyo. The macaques were challenged with VN3040 (1×10^7 PFU₅₀/mL) by inoculation into the nostrils (0.5 mL for each nostril) with a pipette 28 weeks after the first vaccination under ketamine/xylazine anesthesia. The following virus strains were used in neutralization assays (Supplementary Table 2): A/crow/Kyoto/T1/2004 (H5N1) (KT1) [21], A/mountain hawk-eagle/Kumamoto/1/2007 (H5N1) (Kuma1, NCBI taxonomy ID: 682110) [22], A/whooper swan/Hokkaido/1/2008 (H5N1) (Hok1, NCBI taxonomy ID: 527168) [23], and A/peregrine falcon/Hong Kong/810/2009 (H5N1) (HK810, NCBI taxonomy ID: 673326). KT1 and Kuma1 were kindly provided by Dr. Toshihiro Ito (Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, Tottori, Japan). Experiments using the above viruses were performed in the biosafety level 3 facility of the Research Center for Animal Life Science, Shiga University of Medical Science. Virus replication in the swab samples was assessed as described previously [13].

2.4. Detection of antibody specific for virus antigen by enzyme-linked immunosorbent assay (ELISA)

Titers of IgA and IgG antibodies against Vac-3 antigens in the plasma and swab samples were determined using ELISA as described previously [13]. For detection of an antibody against ovalbumin (OVA), 96-well plates were coated with OVA (0.1 µg/well) (Nacalai Tesque, Inc., Kyoto, Japan). Antigen-specific IgE was detected using horseradish peroxidase (HRP)-conjugated anti-monkey IgE antibodies (Acris Antibodies GmbH, Herford, Germany) (1:2000 × 50 µL). Levels of total IgE were determined using the Monkey IgE ELISA kit (Life Diagnostics, Inc., West Chester, PA).

2.5. Virus neutralization assay

The plasma samples were pretreated with a receptor-destroying enzyme (RDEII, Denka Seiken, Tokyo, Japan) at 37 °C overnight and then inactivated at 56 °C for 1 h. One hundred PFU of viruses was mixed with diluted samples for 1 h. Then the mixture was added to MDCK cell monolayers in 6-well plates. After 1 h, MDCK cells were covered by Eagle's minimal essential medium containing 1% agar, 0.1% BSA, and 5 µg/mL trypsin (only for Vac-3). After incubation at 35 °C for 2 days, the number of plaques was counted. Neutralization titers were expressed as the dilution in which the numbers of plaques were reduced to 50% of plaques without plasma.

2.6. Histopathological examination and immunohistochemistry

For histopathological examination, one or two portions of the lungs from each lobe (bilateral upper, middle and lower lobes) were collected at autopsy and were immersed in 10% neutral buffered formalin for fixation. The lung tissues were embedded in paraffin and cut into 3- μ m-thick sections on glass slides. The sections were stained with hematoxylin and eosin (H & E) and observed under a light microscope. For immunohistochemistry, monoclonal antibodies against CD79a (DAKO, Glostrup, Denmark) (1:50, clone HM57) and IgA (DAKO) (1:200, polyclonal rabbit) were used after antigen retrieval with heat. The numbers of eosinophil clusters in H & E sections of each lobe were counted by two pathologists for histological scoring of eosinophils. The total numbers of eosinophil clusters of six lobes were summed up for each macaque. The average of total numbers of eosinophil clusters determined by the two pathologists was defined as an eosinophil score.

2.7. Cytokine measurement

Levels of cytokines were measured using the Milliplex MAP nonhuman primate cytokine panel and Luminex200 (Millipore Corp., Billerica, MA) according to the manufacturer's instructions. Levels of histamine were measured using the Histamine ELISA kit (Enzo Life Sciences Inc., Farmingdale, NY).

3. Results

3.1. Antigen-specific IgA, IgG and IgE responses after nasal vaccination with Vac-3

To examine the immunogenicity of inactivated Vac-3 via nasal vaccination in macaques, macaques were intranasally inoculated with saline or with 0.02 mg (low dose) or 0.2 mg (high dose) of inactivated whole virus particles of Vac-3 using a microsprayer (Supplementary Table 1). The macaques received vaccination eight times at 0, 2, 4, 6, 8, 10, 12 and 26 weeks (first vaccination at week 0) to determine the effects of boost vaccination on and plateau responses of neutralizing antibodies (Supplementary Fig. 1). The total amounts of Vac-3 after the 8th (last) vaccination were 0.16 mg and 1.6 mg in the low dose and high dose groups, respectively. IgA specific for Vac-3 antigen in nasal swab samples was detected 4 weeks after the first vaccination in the high dose group and 8 weeks after the first vaccination in the low dose group (Fig. 1A). Antigen-specific IgA secretion in the plasma was detected in all macaques of the high dose group and in one of the macaques in the low dose group (#706) (Fig. 1B). No IgA antibody responses specific for Vac-3 were detected in control macaques inoculated with saline solution (Fig. 1A and B).

Antigen-specific IgG secretion in nasal swab samples was detected 4 weeks after the first vaccination in the high dose vaccination group and 8 weeks after the first vaccination in the low dose vaccination group (Fig. 1C). IgG specific for Vac-3 antigen was detected in the plasma samples 4 weeks after the first vaccination in both the high dose and low dose vaccination groups (Fig. 1D). Thus, repeated nasal inoculation with two different doses of the inactivated Vac-3 induced antigen-specific IgA and IgG both nasally and systemically with early increase in the high dose group.

To evaluate allergic responses upon nasal vaccination, we examined IgE responses in vaccinated macaques. In the high dose group, IgE specific for Vac-3 antigen was detected in nasal swab samples of all three macaques at 4 weeks after the first vaccination (Fig. 1E). In nasal swab samples of the low dose group, IgE specific for Vac-3 antigen was detected in two macaques at 4 weeks and in

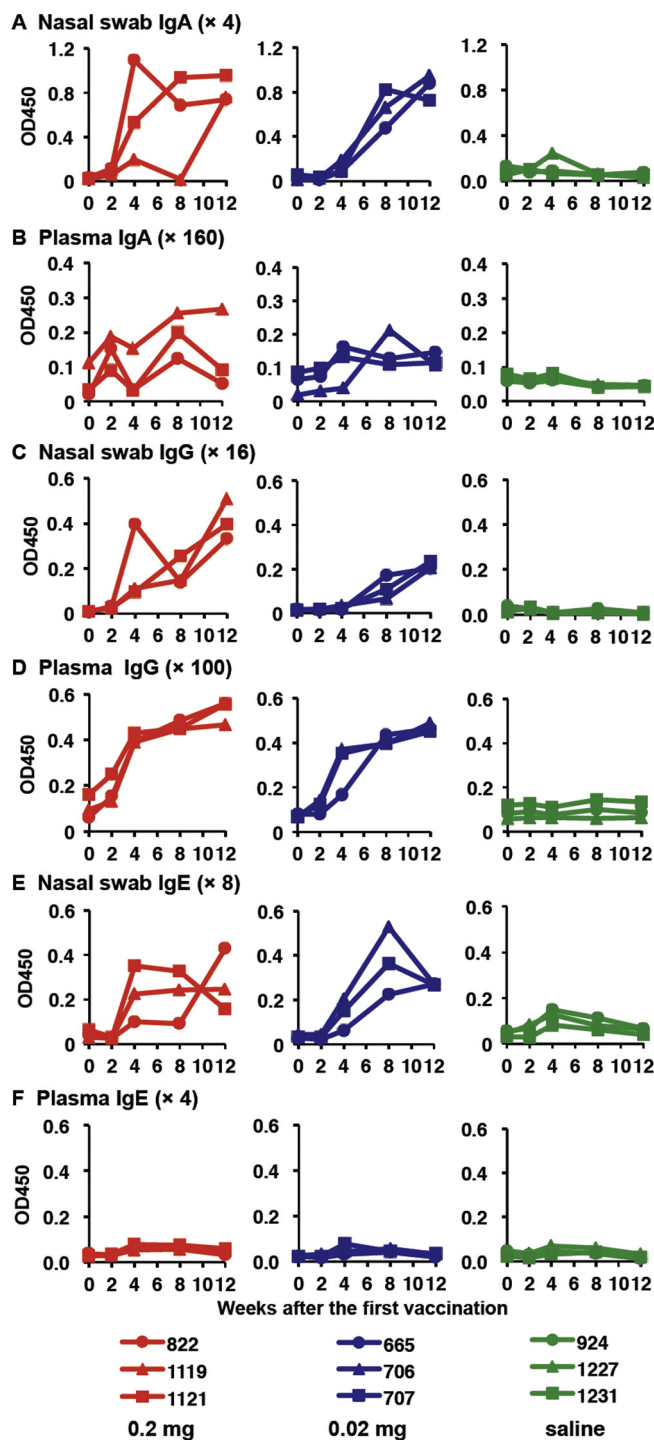


Fig. 1. Antibody responses specific for H5N1 vaccine antigens in cynomolgus macaques after nasal vaccination with inactivated whole virus particles. Cynomolgus macaques were intranasally inoculated with inactivated whole virus particles of Vac-3 (0.2 mg/dose (left column) or 0.02 mg/dose (middle column)) 5 times with 2-week intervals between vaccinations until week 12. Control macaques were intranasally inoculated with saline solution (right column). Nasal swabs (A, C, E) and plasma (B, D, F) were collected before (0 weeks) and after vaccination (2, 4, 8 and 12 weeks after the first vaccination). Samples were analyzed at indicated dilutions using ELISA. Antibodies bound to Vac-3 antigen were detected with HRP-conjugated anti-Ig isotype antibodies. Optical densities are shown. IgA (A, B), IgG (C, D) and IgE (E, F) antibodies specific for Vac-3 antigens in nasal swabs (A, C, E) and plasma (B, D, F).

one macaque at 8 weeks after the first vaccination. Antigen-specific IgE secretion in the plasma was barely detected (Fig. 1F).

Thus, repeated nasal vaccination induced not only IgA and IgG but also IgE specific for vaccine antigens in the macaque model. On the other hand, no IgE specific for egg antigen OVA was detected in the nasal swab samples (Supplementary Fig. 2).

Twenty-six weeks after the first vaccination (26w), levels of IgA and IgG specific for Vac-3 in nasal samples of the vaccinated macaques except for IgA of #707 were higher than those before vaccination (Fig. 2A and B). Additional vaccination 26 weeks after the first vaccination induced increases of IgA and IgG levels on day 0 before challenge infection (0d). IgE specific for Vac-3 was detected 26 weeks after the first vaccination in nasal samples of one of the macaques in each of the vaccination groups (#822 and #706), but the levels of IgE specific for Vac-3 were not increased after boost vaccination (Fig. 2C). No plasma IgE specific for Vac-3 was detected between week 26 and day 7 after challenge infection (Fig. 2D). Levels of total IgE in plasma varied in the macaques, though the saline group showed low levels of total plasma IgE (Fig. 2E). Macaque #706 vaccinated with the low dose of Vac-3 showed the highest levels of IgE in weeks 12 and 26 and on day 0 before challenge infection, whereas #822 did not (Fig. 2E). Therefore, levels of total IgE in plasma were not necessarily correlated with those IgE specific for vaccine antigens.

3.2. Neutralization activity of the plasma after nasal vaccination with Vac-3

Neutralization activity in plasma against the vaccine strain Vac-3 after nasal vaccination was measured since neutralization activity against Vac-3 in nasal samples was below the detection limit (data not shown). Neutralization activity in the plasma was increased 6 weeks and 8 weeks after the first vaccination (2 weeks after the 3rd and 4th vaccinations) in the high dose and low dose groups, respectively (Table 1). No significant increase in neutralization activity against Vac-3 was detected after the 6th vaccination (12 weeks after the first vaccination). To examine recall immune responses before challenge infection, the 8th vaccination was performed 26 weeks after the first vaccination. Neutralization activity of the plasma was increased 2 weeks after the boost vaccination in both groups (Table 1). Therefore, memory immune responses were maintained in macaques by repeated nasal vaccination.

Neutralization activity of the plasma against various clades of H5N1 viruses after nasal vaccination with Vac-3 was measured and compared with that of plasma of macaques vaccinated subcutaneously in the previous study [13] (Supplementary Tables 1 and 2). Neutralization activity against A/Vietnam/UT3040/2004 (H5N1) (VN3040) (clade 1) was detected in the plasma of all three macaques in the high dose group (Table 2). Neutralization activity against A/crow/Kyoto/T1/2004 (H5N1) (KT1) (clade 2.5) and A/mountain hawk-eagle/Kumamoto/1/2007 (H5N1) (Kuma1) (clade 2.2) was detected in the plasma of both intranasally and subcutaneously vaccinated macaques. Thus, the plasma antibody induced by nasal vaccination showed broad neutralization activity against three H5N1 viruses of different clades.

3.3. Protective effects of nasal vaccination with Vac-3 after challenge infection of macaques

To examine the protective effects of nasal vaccination with Vac-3, the highly pathogenic avian influenza virus VN3040, a virus strain isolated from a human patient who died of influenza virus infection [19], was inoculated into nasal cavities of the vaccinated and unvaccinated macaques. In macaques without vaccination, virus was detected until 7 days, 6 days and 6 days after infection in nasal, tracheal and bronchial swabs, respectively (Fig. 3A–C and Supplementary Table 3). In contrast, in macaques in the low dose and high dose vaccination groups, virus was not detected or

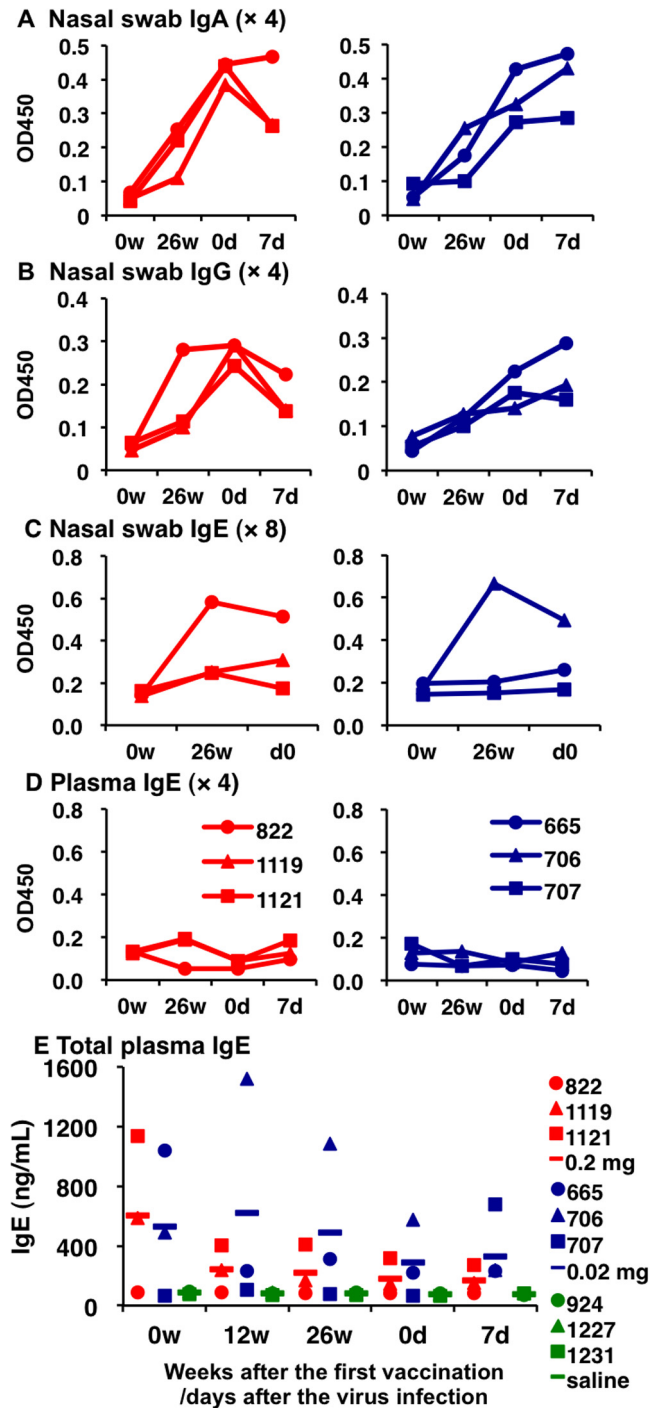


Fig. 2. Memory antibody responses against Vac-3 antigen in vaccinated macaques. Cynomolgus macaques were intranasally vaccinated with Vac-3 (0.2 mg/dose (left column) or 0.02 mg/dose (right column)) as described in the legend for Fig. 1. Twenty-six weeks after the first vaccination (26w), the eighth vaccination was done. Two weeks after the final vaccination, a challenge virus VN3040 was inoculated. Swab samples were collected before (0d) and 7 days after (7d) inoculation with the virus. Swab samples collected before the first vaccination (0w) were examined simultaneously for comparison. IgA (A), IgG (B) and IgE (C) antibodies specific for Vac-3 antigens in nasal swab samples and IgE in plasma specific for Vac-3 antigens (D) were analyzed at indicated dilutions using ELISA. Optical densities at 450 nm are shown. (E) Total IgE levels in plasma of individual macaques (circles, triangles and squares) were examined using ELISA and averages of each group (horizontal bars) were calculated.

was only detected one day or two days after infection in each sample. At autopsy, virus was detected in the oronasopharynx, tonsils

Table 1

Neutralization activity against the vaccine strain Vac-3 in plasma after repeated intranasal vaccination.

Weeks after the first nasal vaccination	50% neutralization titers ($\log_2$) ^a								
	2	4	6	8	10	12	14	26	28 (d0) ^b
Animal	Total amount of vaccine (mg) ^c								
	0.02	0.04	0.06	0.08	0.1	0.12	0.14	0.14	0.16
665	<4	<4	<4	5.00	6.92	4.95	5.21	4.65	6.93
706	<4	<4	<4	7.17	9.68	6.50	7.90	7.06	9.42
707	<4	<4	<4	4.82	4.90	4.96	5.27	<4	6.01
Animal	Total amount of vaccine (mg) ^c								
	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.4	1.6
822	<4	<4	5.54	5.71	8.54	6.98	7.03	7.09	9.57
1119	<4	4.27	4.34	4.84	7.42	5.01	5.17	4.48	8.59
1121	<4	<4	5.77	8.84	9.40	8.61	8.52	7.34	10.81

^a Dilution titers that show 50% neutralization activity are indicated as $\log_2$ dilution. Results are representative of at least three experiments.^b Samples at 28 weeks after the first vaccination were collected on day 0 before virus inoculation.^c Cynomolgus macaques were intranasally vaccinated (i.n.) with inactivated whole virus particles of Vac-3 (0.02 mg or 0.2 mg/dose) 8 times: 0, 2, 4, 6, 8, 10, 12 and 26 weeks after the first vaccination.**Table 2**

Cross-reactive neutralization activity against several clades of H5N1 viruses in plasma of the macaques after repeated intranasal vaccination.

Vaccine amount	Route ^b	Strain ^a Clade	Vac-3 –	VN3040 1	KT1 2.5	Kuma1 2.2	Hok1 2.3.2.1	HK810 2.3.4
		Animal	50% neutralization titers (log ₂) ^c					
0.1 mg (0.02 mg × 5)	i.n.	665	6.92 ^d	<3	<3	<3	<3	<3
		706	9.68	<3	4.86	5.93	<3	<3
		707	4.90	<3	<3	3.04	<3	<3
0.16 mg (0.02 mg × 8)	i.n.	665	6.93	<3	3.38	3.77	<3	<3 (66%)
		706	9.42	3.75	4.55	4.61	<3	<3 (78%)
		707	6.01	<3	<3	<3	<3	<3
1 mg (0.2 mg × 5)	i.n.	822	8.54	<3	3.11	5.32	<3	<3 (61%) ^e
		1119	7.42	<3	<3	3.26	<3	<3
		1121	9.40	<3	<3 (69%)	3.37	<3	<3
1.6 mg (0.2 mg × 8)	i.n.	822	9.57	3.34	3.46	4.71	<3	<3 (77%)
		1119	8.59	3.47	<3 (65%)	4.80	<3	<3
		1121	10.81	3.50	4.79	6.41	<3	<3 (75%)
1 mg (1 mg × 1)	s.c.	1229	5.06	<3	3.06	<3	<3	<3
		1232	5.02	<3	<3	6.61	<3	<3
		1233	5.72	<3	<3 (76%)	<3 (67%)	<3	<3
2 mg (1 mg × 2)	s.c.	1229	7.16	<3	<3 (62%)	3.21	<3 (71%)	<3 (65%)
		1232	4.65	<3 (62%)	4.87	6.70	<3 (81%)	<3
		1233	6.87	<3	3.10	5.85	<3 (73%)	<3

^a Names of H5N1 virus strains are listed in [Supplementary Table 2](#).^b Cynomolgus macaques were intranasally vaccinated (i.n.) with inactivated whole virus particles of Vac-3 (0.02 mg or 0.2 mg/dose) 8 times. Plasma from macaques subcutaneously vaccinated (s.c.) twice with a two-week interval with Vac-3 (1 mg/dose) was used for comparison [13].^c Dilution titers that show 50% neutralization activity are indicated as $\log_2$ dilution. Results are representative of three experiments.^d Fifty percent neutralization titers against Vac-3 in plasma obtained from macaques vaccinated intranasally are cited from [Table 1](#) for comparison.^e Percentages in parentheses indicate the percentages of plaques using plasma diluted at an 8-times concentration compared with plaques without sera (100%).

and bronchi of two unvaccinated macaques and in the oropharynx and a part of the lung of the other unvaccinated macaque ([Supplementary Table 4](#)). No virus was detected in any tissues of the six vaccinated macaques. These results indicate that repeated nasal inoculation of Vac-3 was effective for inhibiting virus propagation in both the high dose and low dose vaccination groups.

All three unvaccinated macaques showed higher body temperatures over 40 °C after virus infection than those before infection, and the higher body temperature continued until 7 days after infection (end point of this study) ([Fig. 3D](#) and [Supplementary Fig. 3](#)). Vaccinated macaques showed higher body temperatures 1 to 3 days after infection except for one macaque (#1119), which showed higher body temperature until 7 days after infection, probably due to an abscess around the implanted telemetry probe. Thus, repeated nasal vaccination shortened the duration of high body temperature.

Protection against viral pneumonia was histologically examined since the results of a previous study indicated that nasal vaccination induced IgA production in the upper airway [2]. In the lungs of the unvaccinated macaques, infiltration of lymphocytes admixed with macrophages was seen in the alveoli with thickening of the alveolar walls and partial hemorrhage ([Fig. 4A](#) and [C](#)), whereas inflammation was reduced in the vaccinated macaques ([Fig. 4B](#) and [D](#)). One lobe with the strongest inflammation from each macaque was chosen to quantify the percent areas of inflammation, which were approximately 90% (right upper lobe (RU) of #924), 75% (right lower lobe (RL) of #1227) and 60% (left lower lobe (LL) of #1231) in the unvaccinated macaques; 30% (RL of #665), 10% (RL of #706) and 30% (RL of #707) in the low dose group; and 20% (RL of #822), 30% (LL of #1119) and 20% (RL of #1121) in the high dose group. In the vaccinated macaques, formation of bronchus-associated lymphoid tissue (BALT) was prominent with accumulation of CD79a-positive B lymphocytes ([Fig. 4F](#)). In the peripheral zone of BALT, IgA-producing plasma cells were seen

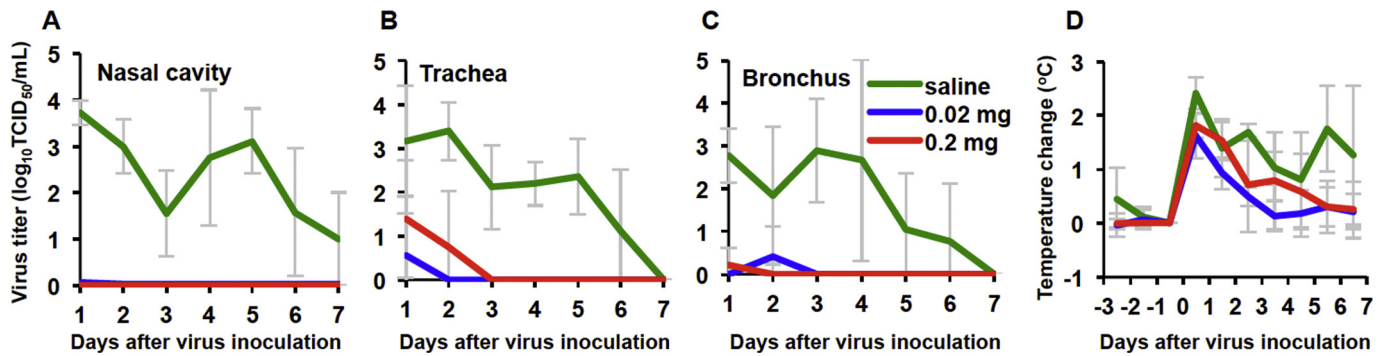


Fig. 3. Virus titers and body temperature changes of vaccinated macaques infected with H5N1 highly pathogenic avian influenza virus. H5N1 highly pathogenic avian influenza virus VN3040 was inoculated into the nostrils of macaques on day 0. (A–C) Averages of virus titers in nasal (A), tracheal (B) and bronchial (C) samples of three macaques were calculated on the basis of titers shown in [Supplementary Table 3](#). (D) Body temperature changes of cynomolgus macaques infected with VN3040. Average temperatures of individual macaques from 8 p.m. to 8 a.m. every night were calculated from temperatures recorded every 15 min as shown in [Supplementary Fig. 3](#) (e.g., averages on day -1 indicate average temperatures from 8 p.m. on day -1 to 8 a.m. on day 0 before virus inoculation and averages on day 0 indicate average temperatures from 8 p.m. on day 0 to 8 a.m. on day 1 after virus inoculation). Body temperature changes of individual macaques each day after virus inoculation were compared with average body temperature on day -1 before virus inoculation. Thereafter, averages and standard deviations of body temperature changes of three macaques are indicated in the graphs.

(Fig. 4H). In the unvaccinated macaques, on the other hand, IgA-producing cells were found in only small numbers without formation of BALT (Fig. 4A, E and G). Thus, repeated nasal vaccination with Vac-3 induced IgA antibody-producing responses in the lung to protect cynomolgus macaques from viral pneumonia.

3.4. Increase in eosinophils in the lungs of macaques with nasal vaccination

In histological observation, we found many peribronchial and intraepithelial eosinophils in the vaccinated macaques (Fig. 5A–D). Large clusters of eosinophils were seen beneath bronchial epithelia, and a single layer of immature respiratory epithelium with submucosal granulation tissue was often observed in proximity to eosinophil clusters (Fig. 5A and B). Eosinophilic infiltration was detected predominantly around the bronchi but not in the peripheral area. To quantitate the degree of eosinophil infiltration, the total numbers of eosinophil clusters in each lung section from all 6 lobes of individual macaques were counted as an eosinophil score. Many clusters of eosinophils were detected in two macaques of each vaccination group, whereas a small number of eosinophil clusters was detected in the unvaccinated group, though the differences in the number of clusters among groups were not statistically significant (averages $\pm$ standard deviations: high dose, 10.2 ± 7.5 ; low dose, 17.3 ± 14.4 ; control, 3.1 ± 4.1) (Fig. 5E).

Other responses related to allergy and Th2 cells after challenge infection were examined. Vac-3-specific IgE in nasal samples was decreased in all vaccinated macaques six days after challenge infection compared to that before infection (day 0) (Fig. 5F), indicating that Vac-3-specific IgE production induced by nasal vaccination was not activated in the vaccinated macaques after challenge infection. The level of IL-13 was decreased in all vaccinated macaques 7 days after challenge infection compared to that before infection (day 0) and was significantly lower than that in the unvaccinated macaques (Fig. 6A). The level of IL-5 was not significantly increased in the vaccinated macaques 7 days after challenge infection compared to that before infection (day 0) (Fig. 6B). In tracheal samples of the low dose group, average levels of the Th2 cytokines IL-13, IL-5 and IL-33, but not IL-4, were increased 7 days after challenge infection (7d) compared to those before infection (0d) (Fig. 6C–F). In tracheal samples of the saline group, the average level of IL-6 was increased 7 days after challenge infection (7d) compared to that before infection (0d) and compared to the levels in the vaccinated groups, probably due to inflammation

caused by viral propagation (Fig. 6G). Average levels of the Th1 cytokine IFN- γ in tracheal samples of the saline group and low dose group were increased 7 days after challenge infection compared to those before infection (Fig. 6H). Average levels of eotaxin and histamine in the blood of vaccinated macaques 7 days after challenge infection showed a tendency for dose-dependent increase by vaccination, though the differences were not statistically significant (Fig. 6I and J). These results suggest that allergic responses other than histamine release in blood and eosinophilic infiltration in the lungs of vaccinated macaques were not activated by challenge infection.

4. Discussion

In the present study, to determine the efficacy and detrimental effect of mucosal vaccination, we performed repeated nasal vaccination with inactivated whole virus particles of Vac-3 in a macaque model. Repeated nasal vaccination induced antigen-specific IgA and IgG in nasal swabs and broad reactive neutralizing antibody in plasma. Neutralization activity against Vac-3 was detected after 3 and 4 sprays of high dose and low dose vaccination, respectively. In addition, maximal responses of the neutralizing antibody were observed 2 weeks after the 5th or 6th vaccination. Therefore, multiple vaccinations are required, especially when using a low dose of vaccine. However, nasal vaccination also frequently induced vaccine antigen-specific IgE secretion, and eosinophilic infiltration was detected after challenge infection.

Broad neutralization activity in plasma against VN3040 (clade 1), KT1 (clade 2.5) and Kuma1 (clade 2.2) was induced by nasal vaccination with Vac-3. This is in accordance with results of previous studies showing strong cross-reactive immunity induced by nasal vaccination [4,24]. It has been reported that cross-clade neutralization activity is due to multivalent IgA in nasal mucosa with high avidity to virus antigen [25]. On the other hand, since IgA is secreted as monomers in plasma, the results of the present study indicate that IgA and IgG monomers in plasma show broad cross reactivity. Therefore, repeated nasal vaccination with inactivated whole virus particles is thought to induce effective affinity maturation in B cells secreting not only mucosal IgA but also blood IgA and IgG [26,27]. This is partly supported by the BALT formation in lung tissues of vaccinated macaques, in which somatic hypermutation occurs, but not in lung tissues of unvaccinated macaques [28,29]. Cross-clade neutralization activity is required for an H5N1 virus stockpile vaccine to provide protective immunity against expand-

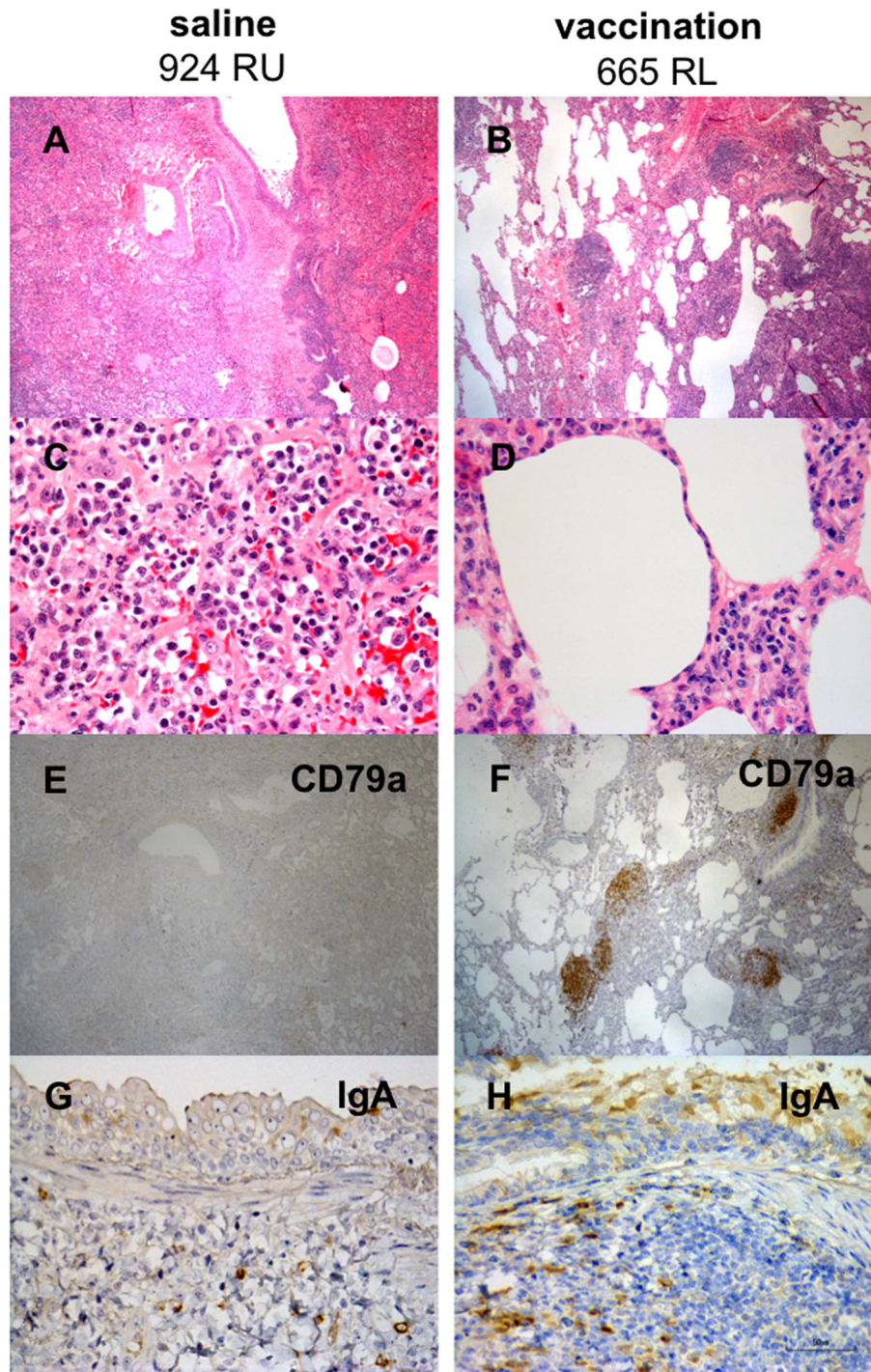


Fig. 4. BALT formation in vaccinated macaques infected with H5N1 highly pathogenic avian influenza virus. Cynomolgus macaques were intranasally vaccinated with inactivated whole virus particles of Vac-3 (0.02 mg/dose) or saline. Two weeks after the last vaccination, macaques were challenged with VN3040. Tissue sections of the right upper lobe (RU) of unvaccinated macaque #924 (A, C, E, G) and of the right lower lobe (RL) of vaccinated macaque #665 (B, D, F, H) are shown as representative tissue sections. Lung tissues were collected at autopsy 7 days after challenge infection and tissue sections were stained with H & E (A–D). Immunohistochemistry was performed with antibodies against the B cell marker CD79a (E, F) and IgA (G, H).

ing clades of H5N1 viruses due to the rapid evolution of the virus [30], suggesting that nasal vaccination with our formalin-inactivated whole virus particles of Vac-3 is one of the candidates for an H5N1 virus vaccination. However, a disadvantage of nasal vaccination is multiple inoculations to induce sufficient titers of the antibody for neutralization.

We detected Vac-3-specific IgE production in nasal swabs of all 6 vaccinated macaques, indicating the induction of allergic reac-

tions by repeated nasal vaccination. It has been reported that repeated exposure to a small amount of antigen in the airway causes elevation of antigen-specific IgE and type I hypersensitivity such as allergy against pollen [31], though no symptoms such as sneezing or nasal discharge were observed in macaques after repeated vaccination in the present study. Furthermore, Vac-3-specific IgE in nasal swabs was decreased shortly after challenge infection, indicating that B cells producing virus-specific IgE were

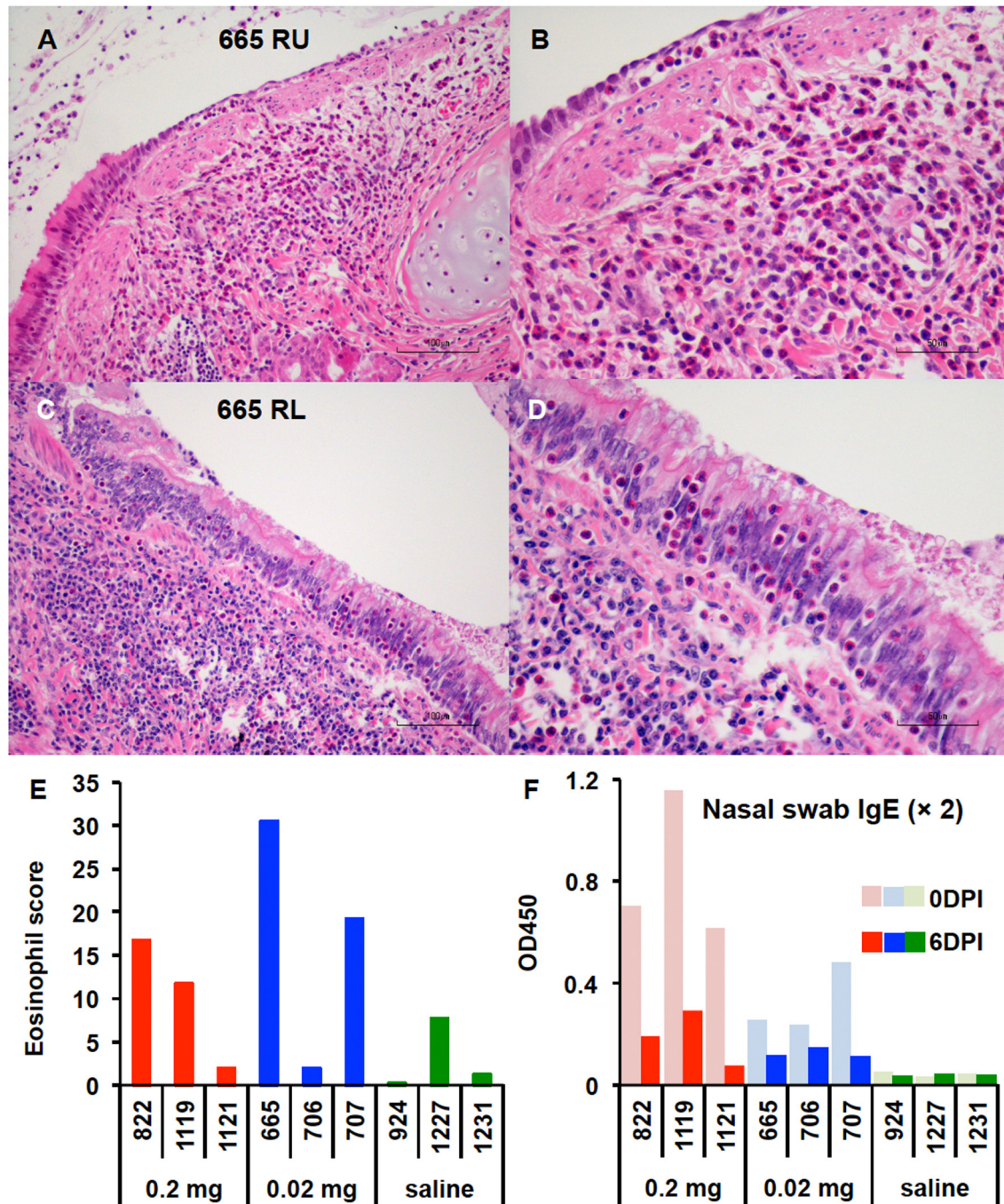


Fig. 5. Eosinophil infiltration in the lungs of vaccinated macaques infected with H5N1 highly pathogenic avian influenza virus. One macaque (#665) was intranasally vaccinated with inactivated whole virus particles of Vac-3 (0.02 mg/dose). Two weeks after the last vaccination, the macaque was challenged with VN3040. Lung tissues were collected at autopsy 7 days after challenge infection and sections were stained with H & E. (A, B) Peribronchial eosinophil clusters in the right upper lobe. (C, D) Intraepithelial eosinophils in the right lower lobe. (A, C) Low magnification. (B, D) high magnification. (E) Histological scoring of eosinophils in the lungs of macaques was determined by counting eosinophil clusters. (F) IgE antibodies in nasal swabs specific for Vac-3 antigens on day 0 before challenge infection (0DPI) and 6 days post infection (6DPI) were analyzed at the indicated dilution using ELISA. Optical densities are shown.

not activated by challenge infection. Therefore, IgE specific for vaccine antigen may not be harmful at present as it has been reported that influenza vaccine-specific IgE may play a protective role in adaptive immunity and allergy reaction [32,33]. However, since prevention of seasonal influenza virus requires annual vaccination, repeated nasal vaccination every year may cause the accumulation of antigen-specific IgE in the nasal mucosa and an increase in the frequency of allergy. In addition, IgE responses in children vaccinated with split vaccines were reported in seasonal influenza virus

vaccination [17,34], and split vaccines induced Th2 responses rather than Th1 responses that were preferentially induced by a whole particle vaccine in our previous studies [35,36]. Therefore, it is possible that nasal vaccination with a split vaccine induces antigen-specific IgE responses in the respiratory mucosa more effectively than does vaccination with a whole particle vaccine.

In addition to Vac-3-specific IgE production, increased eosinophil clusters in the lungs of vaccinated macaques compared to those in unvaccinated macaques after challenge infection further

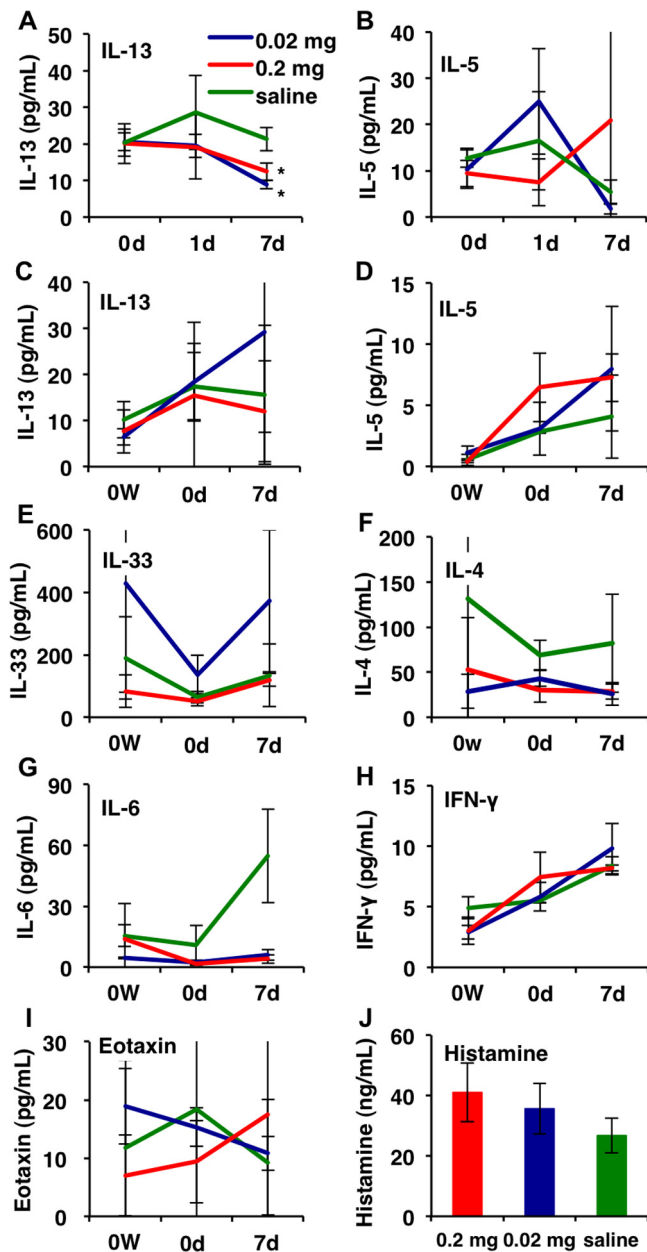


Fig. 6. Levels of cytokines and histamine in swab and blood samples of cynomolgus macaques infected with H5N1 highly pathogenic avian influenza virus VN3040. Cynomolgus macaques were intranasally vaccinated with Vac-3 (0.2 mg or 0.02 mg/dose) 8 times. Two weeks after the last vaccination, macaques were challenged with VN3040. Levels of IL-13 (A) and IL-5 (B) in nasal samples, levels of IL-13 (C), IL-5 (D), IL-33 (E), IL-4 (F), IL-6 (G) and IFN- γ (H) in tracheal samples, and levels of eotaxin (I) in plasma of macaques before vaccination (0w), on day 0 before infection (0d) and on day 7 post infection (7d) were measured by a bead array assay. Levels of histamine (J) in sera of macaques on day 7 post infection were measured by ELISA. Averages and standard deviations for three macaques in each group are shown. Asterisks indicate significant differences between the saline and 0.02 mg/0.2 mg groups ($P < 0.05$, Student's t -test).

indicated the possibility of induction of allergy-related reactions by nasal vaccination. Furthermore, histamine levels in the blood of vaccinated macaques were relatively high after challenge infection compared to those in unvaccinated macaques. It has been reported that in macaques vaccinated with formalin-inactivated whole particle respiratory syncytial virus (RSV), eosinophilic infiltration in the lungs was accompanied by a rapid boosting of T cells producing the asthma-related cytokine IL-13 after challenge with

RSV [37]. However, we did not detect any clinical allergic symptoms in the vaccinated macaques after challenge infection. This indicates that the levels of eosinophilic infiltration and histamine in the vaccinated macaques were not high enough to cause the symptoms and/or that eosinophils have functions other than allergic responses. Taken together with the frequent observation of eosinophil clusters with immature respiratory epithelia and sub-mucosal granulation tissue, it is possible that eosinophils appeared in the context of tissue repair in the vaccinated macaques [38,39].

The present study showed that repeated nasal vaccination with formalin-inactivated whole virus particles of Vac-3 with a micro-sprayer was effective against H5N1 HPAIV infection in macaques. The possibility of induction of allergic reactions by nasal vaccination of whole virus particles should be further studied by using different strains of viruses including seasonal influenza viruses. We will compare IgE and eosinophil responses induced by nasal vaccination with whole virus particles to those induced by vaccination with an ether-split vaccine using a macaque model in the future. Although we did not find any proof of clinical allergic symptoms and anaphylaxis after vaccination or challenge infection in macaques, we should pay attention to allergic reactions after nasal vaccination and infection, especially in people who have an airway allergy.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2017.01.008>.

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