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International Immunopharmacology 101 (2021) 108280

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Role of interleukin-6 in antigen-specific mucosal immunoglobulin A induction by cationic liposomes

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ARTICLE INFO

Keywords:
Cationic liposome
Interleukin-6
Nasal vaccine
Mucosal adjuvant

ABSTRACT

The COVID-19 pandemic, caused by a highly virulent and transmissible pathogen, has proven to be devastating to society. Mucosal vaccines that can induce antigen-specific immune responses in both the systemic and mucosal compartments are considered an effective measure to overcome infectious diseases caused by pathogenic microbes. We have recently developed a nasal vaccine system using cationic liposomes composed of 1,2-dioleoyl-3-trimethylammonium-propane and cholesteryl 3β-*N*-(dimethylaminoethyl)carbamate in mice. However, the comprehensive molecular mechanism(s), especially the host soluble mediator involved in this process, by which cationic liposomes promote antigen-specific mucosal immune responses, remain to be elucidated. Herein, we show that intranasal administration of cationic liposomes elicited interleukin-6 (IL-6) expression at the site of administration. Additionally, both nasal passages and splenocytes from mice nasally immunized with cationic liposomes plus ovalbumin (OVA) were polarized to produce IL-6 when re-stimulated with OVA *in vitro*. Furthermore, pretreatment with anti-IL-6R antibody, which blocks the biological activities of IL-6, attenuated the production of OVA-specific nasal immunoglobulin A (IgA) but not OVA-specific serum immunoglobulin G (IgG) responses. In this study, we demonstrated that IL-6, exerted by nasally administered cationic liposomes, plays a crucial role in antigen-specific IgA induction.

1. Introduction

The highly virulent and transmissible virus that caused the coronavirus disease 2019 (COVID-19) pandemic demonstrated the extent of devastation that can be caused by such pathogens to society, even in highly developed countries with modern medicines. The COVID-19 pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has become a global disaster [1]. Human beings have continuously developed various antibiotics and vaccines to treat and/or prevent infectious diseases caused by pathogenic microbes. Infectious diseases remain a major risk factor for human life [2,3]. Therefore, the development of new mechanisms and modalities for antibiotic or anti-infective strategies is urgently needed.

Vaccination is considered the most fundamental approach to overcoming illness and death caused by infectious diseases. The SARS-CoV-2 pandemic has triggered the first generation of next-generation vaccines. Two RNA vaccines and a viral vector vaccine have been clinically approved for human use [4–6]. In addition, mucosal vaccines are promising next-generation candidates for preventing infectious diseases [7]. We have previously established that intranasal administration of cationic liposomes, composed of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and cholesteryl 3β-*N*-(dimethylaminoethyl)carbamate (DC-chol) (hereafter named as DOTAP/DC-chol liposomes), with antigen proteins can induce antigen-specific antibody responses in both

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https://doi.org/10.1016/j.intimp.2021.108280
Received 7 September 2021; Received in revised form 14 October 2021; Accepted 15 October 2021
Available online 29 October 2021

systemic and mucosal tissues, partly because of the promotion of antigen delivery to dendritic cells in the nasal mucosa [8]. One mechanism of mucosal adjuvant activity is the enhancement of antigen uptake and presentation by antigen-presenting cells (APCs). However, activation of the innate immune system, such as the migration of immune cells and induction of humoral factors, including cytokines, is considered to be more important for adjuvant activity [9–11]. In our study, to clarify the mechanism(s) behind mucosal adjuvant activities of cationic liposomes when administered via the nasal route, we have revealed that double-stranded DNA (dsDNA), which leaks upon mild nasal tissue damage induced by intranasal administration of cationic liposomes, is fundamental to mucosal adjuvant activity [12]. However, the involvement of host soluble mediators, such as cytokines, in the mucosal adjuvant activity of cationic liposomes is still unclear.

Cytokines play a pivotal role in the initiation and maintenance of various immune responses, including the induction of mucosal immune responses to soluble antigenic proteins. IL-6 is a host soluble mediator with a pleiotropic effect on various immune responses and inflammation [13]. Additionally, the induction of immunoglobulin A (IgA) class switch recombination (CSR), which produces IgA^+ B cells from IgM^+ B cells in the mucosa, is required to produce IgA in the mucosa. During this process, the activated $CD4^+$ T cells by APCs are involved in the presence of various cytokines, such as IL-6 [14]. Furthermore, Ramsey *et al.* reported that the number of IgA^+ B cells in the mucosa is significantly decreased, and mucosal antibody responses following mucosal vaccination with antigenic proteins are diminished in IL-6 knockout mice [15]. These reports strongly indicate that IL-6 play a pivotal role in the mucosal adjuvant activities induced by various stimuli. In the present study, we hypothesized that IL-6 might be associated with the induction of mucosal immune responses of cationic liposomes when administered nasally to mice. Thus, we investigated the role of IL-6 in cationic liposome-induced antigen-specific antibody responses in mice.

2. Materials and methods

2.1. Animals

Female BALB/cCrSlc mice (six weeks of age) were purchased from Japan SLC (Shizuoka, Japan). All mice used in this study were housed under specific pathogen-free (SPF) conditions. All mice were 7–10 weeks of age in all experiments. The animal experimental protocols were approved by the Committee for Laboratory Animal Experiments at the Tokyo University of Pharmacy and Life Sciences (approval numbers: P13–22, P14–31, P15–33, P16–12, P17–26, and P21–71).

2.2. Materials

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β-[N-(N',N'-dimethylaminoethane)-carbamoyl] (DC-chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Low endotoxin (<1 EU/mg) egg white ovalbumin (OVA) and endotoxin-free phosphate-buffered saline (PBS) were obtained from FUJIFILM Wako Pure Chemical Industries (Osaka, Japan). Rat anti-mouse interleukin 6 receptor (IL-6R) antibody (clone MR16-1) was provided by Chugai Pharmaceutical Co., Ltd. Rat IgG1 κ isotype control antibody (clone RTK2071) was purchased from BioLegend (San Diego, CA, USA).

2.3. Preparation of cationic liposomes

The cationic liposomes used in the experiments were prepared as previously described [16–19]. Briefly, 10 μmol of lipid (1:1 M ratio of DOTAP and DC-chol) dissolved in chloroform was mixed in a glass tube, and the solvent was removed with an evaporator, followed by drying in a desiccator for 1 h *in vacuo*. The resulting lipid film was hydrated with 250 μL of PBS and vortexed for 5 min at 25 °C. The obtained multilamellar vesicles were extruded 10 times by passing through a polycarbonate membrane (ADVANTEC, Tokyo, Japan) with a pore size of 100 nm and sterilized using a 0.2 μm cellulose acetate membrane (IWAKI, Tokyo, Japan). The particle size and ζ-potential of the cationic liposomes (termed as DOAP/DC-chol liposomes) were 137.9 ± 11.6 nm and 4.0 ± 2.1 mV, respectively, which were measured by NICOMP 380 ZLS (Particle Sizing Systems; Port Richey, FL, USA).

2.4. Intranasal immunization of OVA plus DOTAP/DC-chol liposomes and sampling schedule

The mice were nasally immunized with PBS (vehicle), OVA alone (5 μg/mouse), or OVA (5 μg/mouse) with DOTAP/DC-chol liposomes (400 nmol/mouse) at a volume of 13 μL on days 0 and 7. To assess systemic OVA-specific antibody responses, blood was collected and incubated at 25 °C for 30 min. The obtained samples were incubated for 1 h at 4 °C, and serum samples were collected after centrifugation at 1200 × g for 30 min. To monitor the induction of OVA-specific immunoglobulin A (IgA) in nasal washes, nasal wash samples (250 μL of cold PBS) were collected [20–22]. The samples were stored at − 80 °C until further analysis.

2.5. Enzyme-linked immunosorbent assay (ELISA) to assess OVA-specific antibody titers

A 96-well Nunc MaxiSorp plate (Thermo Scientific, MA, USA) was coated with 1.25 μg of OVA (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M carbonate buffer (pH 9.5) overnight at 4 °C. The plate was then washed with PBS containing 0.05% Tween 20 (PBST) and blocked with 1% bovine serum albumin (BSA; FUJIFILM Wako Pure Chemical Industries) containing PBST (BPBST) at 37 °C for 60 min. The plate was washed and incubated with the samples for 60 min at 37 °C. For the detection of anti-OVA immunoglobulin G (IgG) antibodies, plates were washed with PBST and treated with peroxidase-conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich) in BPBST. For the detection of other antibody classes, the plates were washed with PBST, treated with biotin-conjugated anti-mouse IgA, IgG1, or IgG2a secondary antibodies (BioLegend) in BPBST, followed by addition of avidin-HRP (BioLegend) in BPBST to each well. After washing with PBST, the plates were color-developed using a tetramethylbenzidine (TMB) substrate system (KPL, Maryland, USA). Color development was terminated using 1 N phosphoric acid, and the optical density was measured at 450 nm (reference filter 650 nm) using a Varioskan Flash Micro Plate Reader (Thermo Scientific). The endpoint titers of antibodies were calculated as the reciprocal of the last dilution, reaching cut-off values set to twice the mean absorbance value of the control, as reported earlier [23,24].

2.6. Quantitative real-time polymerase chain reaction (qRT-PCR)

Mice were nasally administered PBS or DOTAP/DC-chol liposomes (400 nmol/mouse) under anesthesia with an intraperitoneal injection of 0.2 mL of a mixture containing 0.75 mg/kg of medetomidine, midazolam (4 mg/kg), and butorphanol tartrate (5 mg/kg). Mice were sacrificed by cervical dislocation [25] 6 or 16 h after administration, and their nasal tissues and spleens were harvested. Changes in gene expression induced by intranasal administration of DOTAP/DC-chol liposomes were verified using qRT-PCR, as previously described. Briefly, total RNA was extracted from nasal mucosal tissue or spleen using a FavorPrep Tissue Total RNA Muni Kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan) followed by DNase I (Roche Life Science, Penzberg, Germany) treatment. RNA concentration in the extracted samples was quantified using spectrophotometry. Next, complementary DNA (cDNA) was synthesized from total RNA (0.5 μL) in 10 μL of the reaction mixture using ReverTra Ace qPCR RT Master Mix (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. The reaction conditions were as follows: 37 °C for 15 min, 50 °C for 5 min, and 98 °C for 5 min. Then, qRT-PCR was performed using the Thunderbird SYBR qPCR Mix

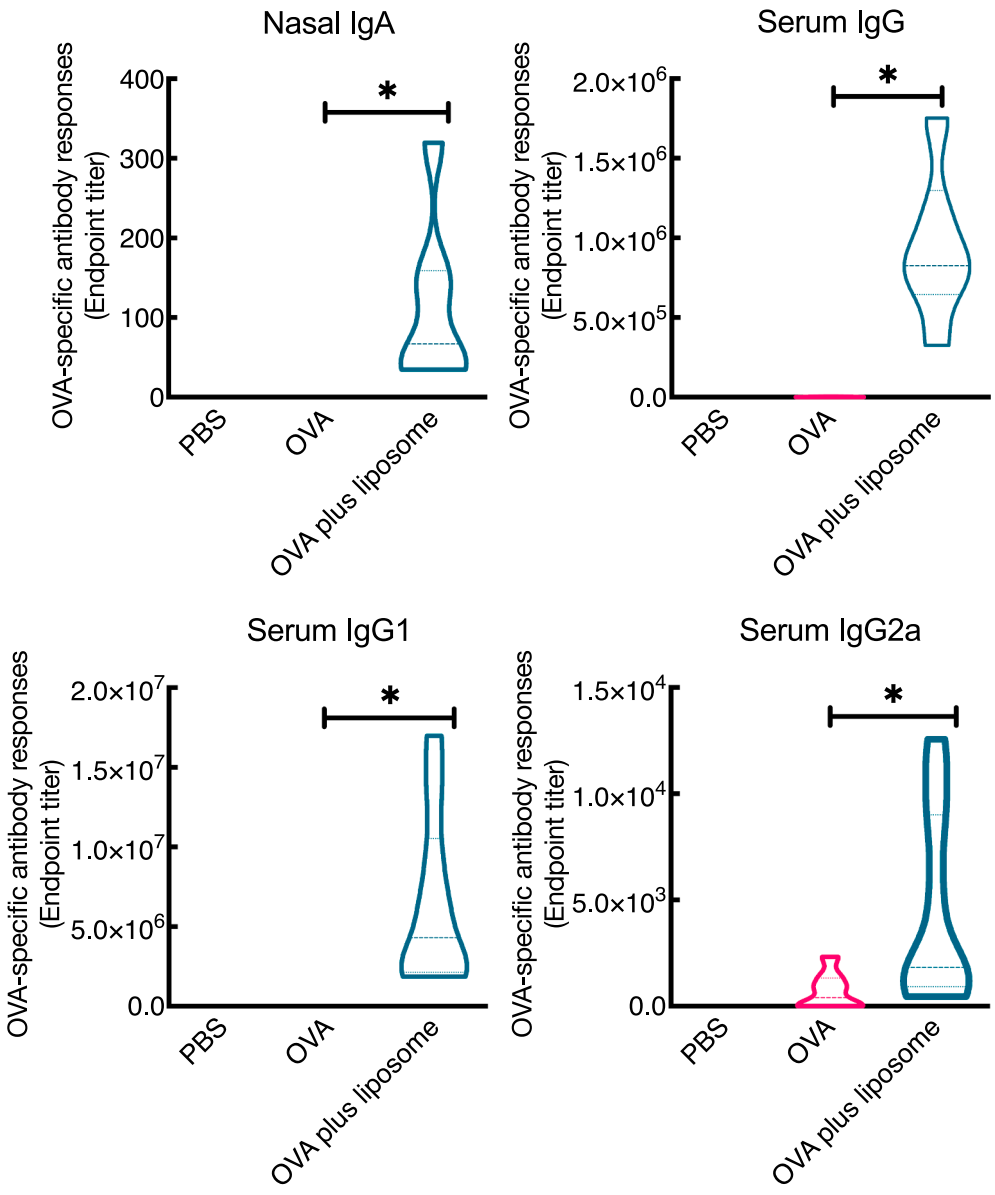

Fig. 1. Promotion of antigen-specific nasal immunoglobulin A (IgA) and serum immunoglobulin G (IgG) in mice nasally immunized with ovalbumin (OVA) plus DOTAP/DC-chol liposomes.
BALB/c female mice were nasally immunized with PBS, OVA (5 μg/mouse) alone, or OVA (5 μg/mouse) in combination with DOTAP/DC-chol liposomes (400 nmol/mouse) on days 0 and 7. Nasal fluid and serum samples were collected on Day 14. OVA-specific IgA in nasal fluids and OVA-specific serum IgG, IgG1, and IgG2a were examined by enzyme-linked immunosorbent assay (ELISA). Data were obtained from three independent biological experiments. Significant differences were evaluated using the Kruskal–Wallis test with Dunn's post-hoc test. * $p < 0.05$.

(Toyobo), according to the manufacturer's instructions, using a CFX Connect Real-Time PCR Detection System (BIO-RAD, Hercules, CA, USA). The qRT-PCR conditions were as follows: 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer pairs used in this experiment were synthesized by Eurofins Genomics (Tokyo, Japan): IL-6, 5′-GAAATGATGGATGCTACCAAACTG-3′ (forward) and 5′-CTCTCTGAAGGACTCTGGCTTTG-3′ (reverse); β2-microglobulin (B2M), 5′-TTCTGGTGCTTGTCTCACTGA-3′ (forward) and 5′-CAGTATGTTCGGCTTCCCATTC-3′ (reverse). Data were analyzed with CFX manager software version 3.1 (BIO-RAD), and cycle threshold (Ct) values were calculated. The values of IL-6 expression in the samples were normalized to that of the reference gene Beta-2 microglobulin (B2M). The specific amplification was confirmed by melting curve analysis. Finally, relative gene expression among samples was calculated using the comparative Ct (ΔΔCt) method.

2.7. *In vitro antigen re-stimulation of nasal passages and splenocytes*

Splenocytes were prepared as previously described for splenocyte culture [26–29]. Briefly, mice immunized intranasally with DOTAP/DC-chol liposomes and OVA were sacrificed by cervical dislocation [25], and their spleens were harvested. After treatment with red blood cell (RBC) lysis buffer (BioLegend) to remove red blood cells, splenocytes were suspended in Roswell Park Memorial Institute (RPMI) 1640 medium (FUJIFILM Wako Pure Chemical Industries) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest, Nuaillé, France), 100 U/mL of penicillin G potassium salt (Sigma-Aldrich), and 100 μg/mL of streptomycin sulfate salt (Sigma-Aldrich). The cells were seeded in 48-well flat-bottomed plates (Thermo Scientific) at a density of 2×10^6 cells/well in 0.5 mL of culture medium. The culture supernatants were collected after re-stimulation with OVA (FUJIFILM Wako Pure Chemical Industries) at 37 °C in 5% CO_2 for the indicated time.

For nasal passage culture, nasal passages were prepared as previously described [30,31]. Briefly, mice immunized intranasally with DOTAP/DC-chol liposomes and OVA were sacrificed by cervical dislocation [25], and their nasal passages were harvested and teased apart in RPMI 1640 medium using a stainless-steel mesh. After being suspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 100 U/mL of penicillin G potassium salt, and 100 μg/mL of streptomycin sulfate salt. The cells were seeded in 96-well flat-bottomed plates (Thermo Scientific) at a density of 1×10^5 cells/well in 0.1 mL of culture medium. The culture supernatants were collected after re-stimulation

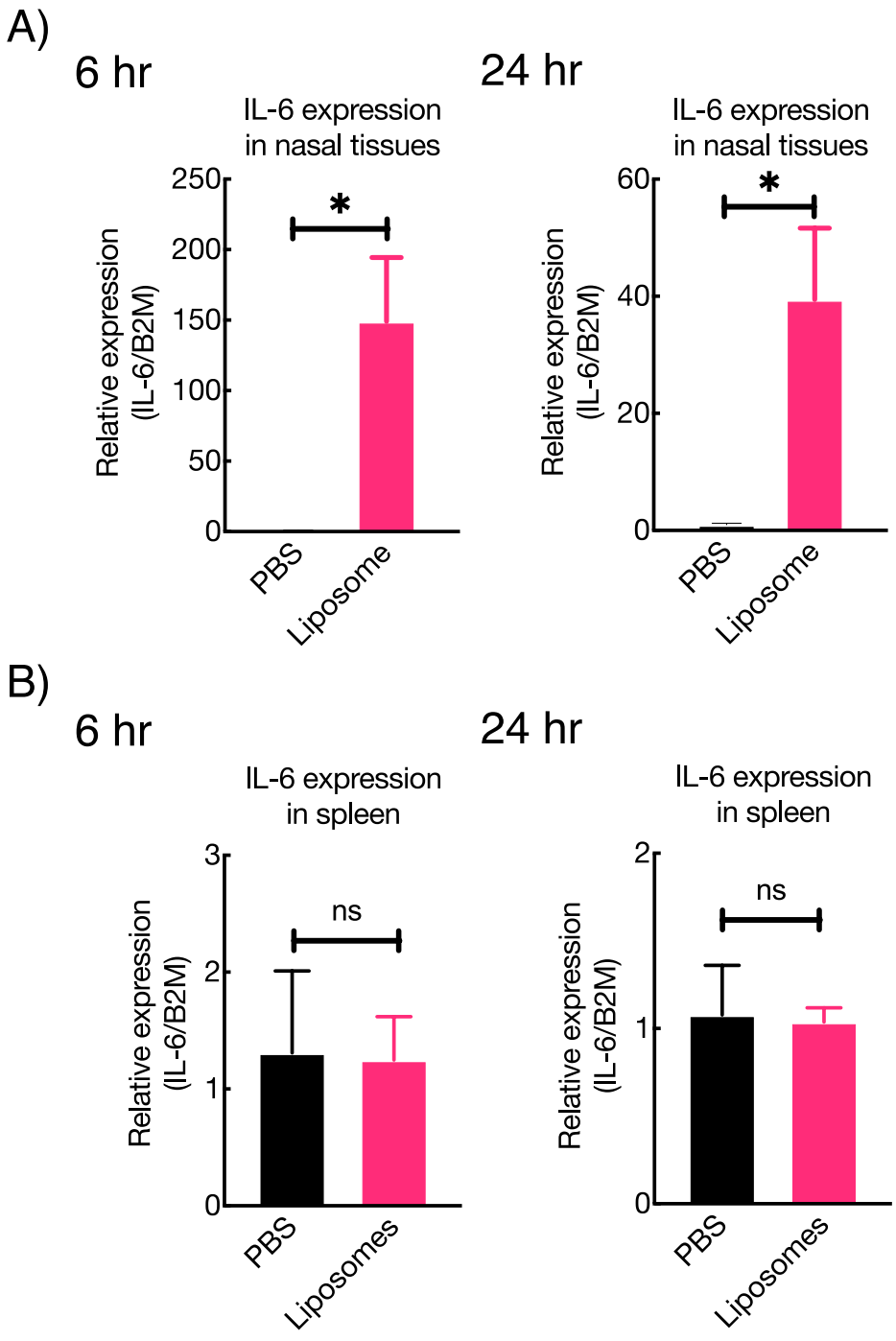

Fig. 2. Gene expression of interleukin-6 (IL-6) in nasal tissues and spleen from mice nasally administered with cationic liposomes.
Nasal tissues (a) and spleen (b) from the mice nasally administered with PBS or DOTAP/DC-chol liposomes (400 nmol/mouse), were collected 6 or 24 h after administration. After total RNA extraction and subsequent cDNA synthesis, changes in gene expression were measured using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis. Values represent the mean ± standard deviation (SD) of biological triplicates. The experiments were performed using three independent biological experiments. Significant differences were assessed using an unpaired *t*-test with Welch's correction. * $p < 0.05$.

with OVA at 37 °C in 5% CO_2 for the indicated time.

2.8. Cytokine assay

IL-6 concentration in the culture supernatant was measured using ELISA MAX™ Standard Sets (BioLegend), following the manufacturer's instructions. Data are presented as mean ± standard deviation from three independent biological experiments.

2.9. Blocking effect of anti-IL-6 receptor (IL-6R) antibody on mucosal adjuvant activity of cationic liposomes

BALB/cCrSlc female mice were pre-treated with anti-IL-6R antibody (250 μg/mouse) 2 days before (day −2) first immunization and 1 h before each immunization (days 0 and 7) as reported in the literature [32]. The mice were nasally immunized with PBS (vehicle), OVA alone (5 μg/mouse), or OVA (5 μg/mouse) with DOTAP/DC-chol liposomes (400 nmol/mouse) at a volume of 13 μL on days 0 and 7. After sacrifice, serum and nasal wash samples were collected on day 14, as previously described.

2.10. Statistics

Statistical differences were analyzed with the unpaired *t*-test with Welch's correction, the Kruskal–Wallis test with Dunn's post-hoc test, and the Mann–Whitney *U* test for IL-6 expression, antibody production, and the evaluation of anti-IL-6R effect, respectively, using GraphPad Prism 8 software (GraphPad Software, San Diego, CA, USA). *Statistical*

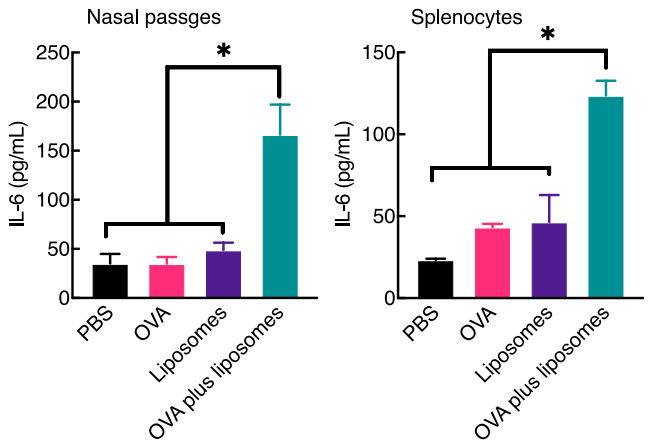

Fig. 3. Antigen-specific interleukin 6 (IL-6) production in nasal passages and splenocytes from mice nasally administered with ovalbumin (OVA) and cationic liposomes.
Nasal tissues and spleens from vaccinated mice were harvested a day after the last immunization. The obtained nasal passages and splenocytes were cultured for 72 h in the presence of OVA (100 μg/mL). After culture, the culture supernatants were collected, and the concentrations of IL-6 were determined using ELISA. Values are expressed as the mean ± SD of biological triplicates from three independent biological experiments. Significant differences were assessed using an unpaired *t*-test with Welch's correction (*$p < 0.05$).

significance was set at $P < 0.05$.

3. Results

3.1. Intranasal immunization of cationic liposomes promotes antigen-specific nasal and serum antibodies

We first tested ovalbumin (OVA)-specific antibody production by intranasal administration of OVA in combination with DOTAP/DC-chol liposomes in female BALB/c mice. As expected, we observed increased production of OVA-specific nasal immunoglobulin A (IgA) and serum immunoglobulin G (IgG) by intranasal immunization. In contrast, intranasal administration of OVA alone or PBS (as a vehicle) did not promote OVA-specific antibody production in the nasal mucosa or systemic compartments (Fig. 1).

3.2. Expression of interleukin 6 (IL-6) at mucosal and systemic sites with nasally administered cationic liposomes in mice

Immune responses to antigens are generally regulated by soluble factors, such as cytokines, secreted by immune and non-immune cells [9–11]. To explore the possible mode of action(s) of mucosal adjuvant activity of cationic liposomes, we screened for genes involved in innate immune responses that could enhance mucosal IgA responses induced by intranasal administration of DOTAP/DC-chol liposomes. During the course of our experiments, we found that intranasal administration of DOTAP/DC-chol liposomes strongly induced IL-6 expression in nasal tissues, as shown in Fig. 2a. Expression of IL-6 in nasal tissues was observed as early as 6 h after intranasal administration of cationic liposomes and declined after 24 h. In contrast, intranasal administration of cationic liposomes did not induce IL-6 expression in the spleen, a distal site of administration (Fig. 2b). Taken together, these results indicate that nasally administered cationic liposomes are potent early inducers of local IL-6.

3.3. Production of IL-6 in nasal passages and splenocytes of nasally vaccinated mice

Cytokines play an essential role in antigen-driven immune responses [33]. Among these cytokines, IL-6 is reported to be secreted by immune cells from immunized mice when re-exposed to antigens *in vitro* [34]. Therefore, we examined whether intranasal administration of cationic liposomes with OVA enhances antigen-specific IL-6 production via antigen re-stimulation in nasal passages or splenocytes *in vitro*. Fig. 3 shows that both nasal passages and splenocytes from mice that received OVA in combination with DOTAP/DC-chol liposomes secreted higher amounts of IL-6 compared to those from mice that were administered OVA only when re-stimulated with OVA *in vitro*. Collectively, these results indicate that intranasal inoculation of DOTAP/DC-chol liposomes was polarized to express IL-6 in mice.

3.4. Antigen-specific mucosal IgA, but not systemic IgG responses induced by cationic liposomes, relies upon IL-6

The data thus far suggest that intranasal administration of cationic liposomes induces the production of IL-6. To evaluate the importance of IL-6 in mucosal adjuvant activity of cationic liposomes, we immunized mice pre-treated with anti-IL-6R antibody with OVA in combination with cationic liposomes intranasally, as reported in the literature, to block the biological activities of IL-6 *in vivo* [35–37]. Compared to mice pre-treated with isotype control antibody, the production of OVA-specific nasal IgA was dramatically reduced in mice pre-treated with anti-IL-6R blocking antibody that were intranasally vaccinated with OVA and DOTAP/DC-chol liposomes (Fig. 4a). However, no statistical difference was observed in OVA-specific serum IgG responses (Fig. 4b). Cumulatively, antigen-specific IgA responses in the mucosa induced by cationic liposomes rely on IL-6, but not on antigen-specific IgG responses in systemic compartments.

4. Discussion

The demand for vaccine research and development has increased owing to the ongoing COVID-19 pandemic. Consequently, a novel vaccine modality, called the RNA vaccine, has been clinically approved and used in humans with tremendous efficacy. However, it is obvious that society will continue to demand research and develop other vaccine modalities. Among these, mucosal vaccine systems are expected to be the most promising and are under active study, since existing RNA vaccines are reported to be ineffective in inhibiting the transmission of pathogens. Therefore, we have long been exploring mucosal adjuvants for nasal vaccine formulations, which are essential for developing subunit types of mucosal vaccines. In our study, we discovered that cationic liposomes composed of DOTAP and DC-chol show potent mucosal adjuvant activities, resulting in mucosal and systemic antigen-specific antibody responses in mice when nasally administered in combination with an antigenic protein [8,19]. However, the comprehensive molecular mechanism(s) by which cationic liposomes promote antigen-specific mucosal immune responses remain to be elucidated, especially the host soluble mediator involved in this process. In the present study, we demonstrated that cationic liposomes elicited interleukin 6 (IL-6) expression at the site of administration *in vivo* and in nasal passages and splenocytes from immunized mice when re-stimulated with ovalbumin (OVA) *in vitro*. Furthermore, pretreatment with anti-IL-6R antibody attenuated the production of OVA-specific nasal immunoglobulin A (IgA) but not OVA-specific serum immunoglobulin G (IgG) responses, indicating that IL-6 plays a crucial role in antigen-specific IgA induction by cationic liposomes.

Developing safe and potent mucosal adjuvants is required for efficient mucosal vaccine systems to overcome fatal infectious diseases caused by deadly pathogens. To achieve this, understanding the comprehensive mechanism(s) by which mucosal adjuvants promote antigen-specific mucosal immune responses is important. Adjuvants partly show their activities via the depot effect. The depot effect retains the antigen at the site of administration while slowly releasing the antigen, promoting uptake by APCs, thus enhancing antigen-specific

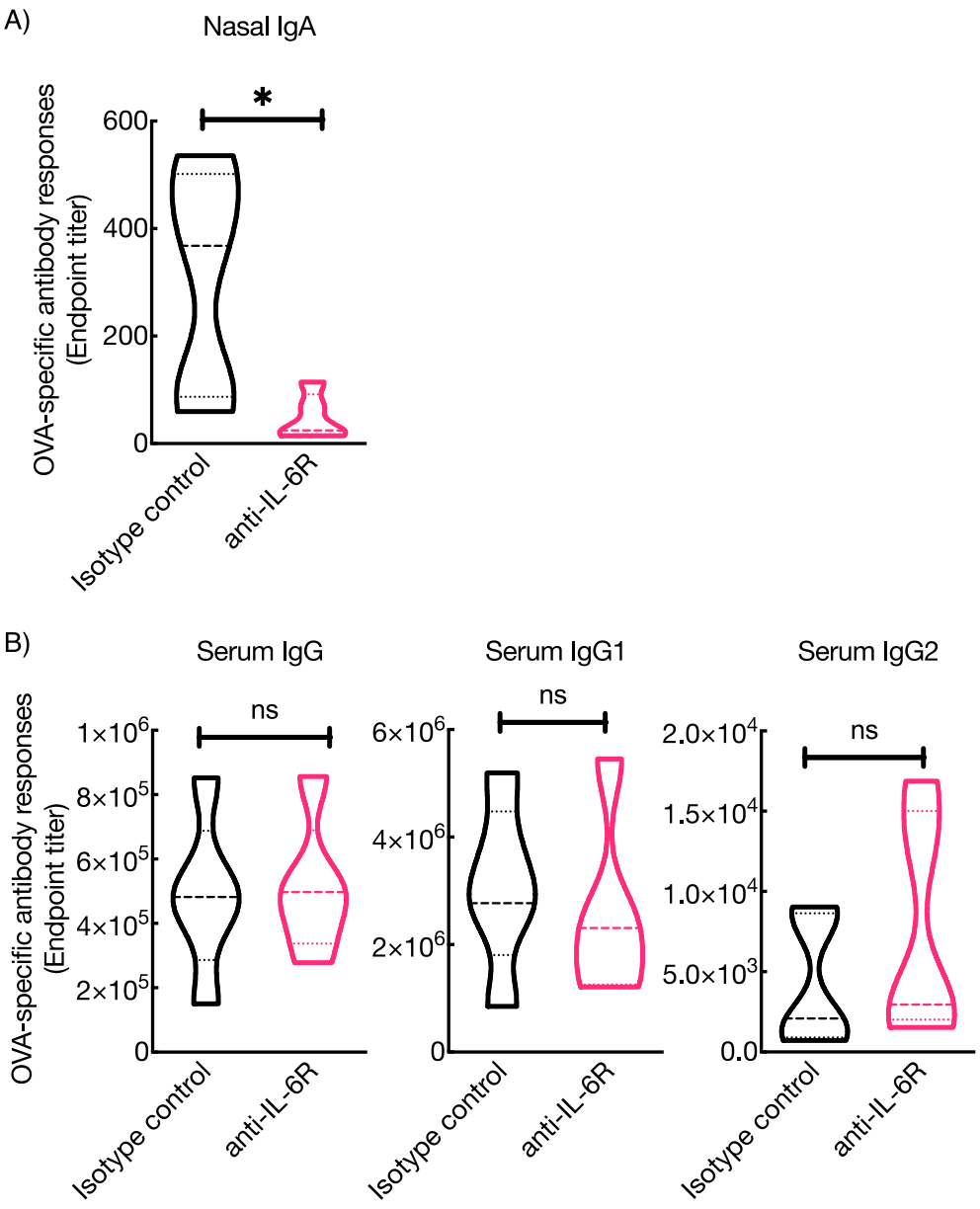

Fig. 4. Effect of anti-interleukin-6R (anti-IL-6R) blocking antibodies on ovalbumin (OVA)-specific antibody production enhanced by cationic liposomes.
Mice were pre-treated with anti-IL-6R antibody (250 μg/mouse) on day −2 and 1 h before each immunization (days 0 and 7) and then immunized intranasally with PBS (vehicle), OVA alone (5 μg/mouse), or OVA (5 μg/mouse) with DOTAP/DC-chol liposomes (400 nmol/mouse) at a volume of 13 μL on days 0 and 7. After sacrifice, serum and nasal wash samples were collected on day 14. OVA-specific nasal immunoglobulin A (IgA) and serum immunoglobulin G (IgG) levels were determined using ELISA. Data were obtained from two independent experiments. Significant differences were evaluated using the Mann–Whitney *U* test. * $p < 0.05$.

immune responses. In recent years, research has focused on the role of activating innate immunity in adjuvant effects [38]. In this regard, we have reported that extracellular host double-stranded DNA (dsDNA), called damage-associated molecular patterns (DAMPs) that stimulate innate immune responses of the host, released from dying cells in the nasal cavity induced by nasally administered cationic liposomes, plays a pivotal role in mucosal adjuvanticity. However, the immunological events that exert antigen-specific mucosal immune responses remain unknown [12].

The host soluble mediators, such as cytokines and chemokines, secreted by various stimuli, including adjuvants, are well-known determinants of the immune response to exogenous antigens [39–41]. For instance, IL-6 is a potent inflammatory cytokine that has been shown to be involved in shaping adaptive immune responses as a T helper 17 (Th17), inducing activity as well as being a B cell stimulating factor [13,42]. Additionally, IL-6 is involved in the induction of IgA CSR to produce IgA^+ B cells from IgM^+ B cells in the mucosa and is required to produce IgA in the mucosa [14]. Furthermore, increased mucosal IgA responses to antigens were observed when nasally administering IL-6 in combination with antigens [43]. In the present study, we found that intranasal immunization with cationic liposomes induced IL-6 expression in the nasal mucosa (Fig. 2), and immune cells from mice that received cationic liposomes were polarized to produce IL-6 stimulated with antigens (Fig. 3). IL-6 activity neutralized experiments using anti-IL-6 monoclonal antibody clearly showed that IL-6 plays an essential role in antigen-specific IgA enhancement induced by cationic liposomes (Fig. 4). Notably, the blocking of IL-6 signaling did not affect antigen-specific serum IgG responses; thus, further experiments are needed to clarify how cationic liposomes initiate adaptive immune responses toward soluble antigenic proteins. The limitation of our study is that although the cationic liposomes enhanced various antigen-specific immune responses, such as pneumococcal surface protein A [19] and lysozyme (unpublished results), the involvement of IL-6 in the induction of antigen-specific IgA responses toward such antigens was not tested in the present study. Therefore, further studies are needed to clarify this.

In conclusion, we offer insight into the molecular mechanisms of enhancing antigen-specific mucosal IgA antibody responses when nasally administered cationic liposomes in combination with antigens. Although further investigations are required to understand the comprehensive mechanism(s) of mucosal adjuvanticity of cationic

liposomes, we believe that nasal vaccine systems using cationic liposomes might be useful for the development of safe and efficient mucosal vaccine systems to combat infectious diseases.

Funding

This study was partly funded by JSPS KAKENHI Grant Number 18K06798 (Grant-in-Aid for Scientific Research C to R.T.); JSPS KAKENHI Grant Number 21K06715 (Grant-in-Aid for Scientific Research C to R.T.); 18H02150 (Grant-in-Aid for Scientific Research B to J.K.); and the Japan Agency for Medical Research and Development (grant numbers 20fk0108145h0001 and 20ak0101068h0004 to J.K.

CRediT authorship contribution statement

Rui Tada: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing – original draft. **Akira Hidaka:** Data curation, Investigation. **Yuya Tanazawa:** Data curation. **Akari Ohmi:** Data curation. **Shoko Muto:** Data curation. **Miki Ogasawara:** Data curation. **Momoko Saito:** Data curation. **Akihiro Ohshima:** Data curation. **Naoko Iwase:** Data curation. **Emi Honjo:** Data curation. **Hiroshi Kiyono:** Methodology, Resources, Supervision. **Jun Kunisawa:** Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing. **Yoichi Negishi:** Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We would like to thank Editage [www.editage.com] for English language editing.

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