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CpG ODN G9.1 as a novel nasal ODN adjuvant elicits complete protection from influenza virus infection without causing inflammatory immune responses

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ABSTRACT

This study examined the protective efficacy of and immune response to a nasal influenza vaccine combined with a novel mucosal oligodeoxynucleotide (ODN) adjuvant, CpG ODN G9.1 (G9.1), in a model of infection limited to the upper respiratory tract (URT) and a model of infection in the lower respiratory tract (LRT). Mice were nasally primed with an A/California/7/2009 (Cal7) split vaccine (X179A) plus G9.1 and were then nasally given a booster with X179A alone. When mice were challenged with either a large (infection of the LRT) or small (infection limited to the URT) volume of live Cal7 influenza virus, mice nasally given G9.1 combined with X179A had a markedly higher rate of protection against infection limited to the URT. Moreover, this group of mice promptly recovered from an infection of the LRT. When mice were subcutaneously (s.c.) given X179A as a current form of vaccination, they had no protection from an infection limited to the URT but they did recover from an infection of the LRT. The patterns of protection were closely correlated with influenza virus-specific mucosal secretory IgA (SIgA) or serum IgG antibody (Ab) responses. Thus, SIgA Abs responses play an important role in protection from an infection limited to the URT while influenza virus-specific serum IgG Ab responses help to protect from an infection of the LRT. A finding of note is that lungs from mice nasally given G9.1 had low levels of type I IFN-associated protein- and transcription factor-specific mRNA expression. These results suggest that nasal G9.1 can be used as an effective and safe mucosal adjuvant for influenza vaccines since this nasal vaccine system elicits both mucosal SIgA and serum IgG Ab responses that provide complete protection without inducing potent inflammatory responses.

1. Introduction

Influenza virus infections occur worldwide every year, mainly during the winter months, and usually result in significant morbidity and deaths especially in infants and older adults [1–4]. In order to control influenza virus infection, a quadrivalent inactivated vaccine (flu shot) containing strains of the four influenza viruses circulating in humans, two type A influenza viruses (H1N1pdm09 and

Abbreviations: ODN, oligodeoxynucleotide; LD_{50}, median lethal dose; PBMC, peripheral blood mononuclear cell; pfu, plaque forming unit; BSA, bovine serum albumin; SEM, standard error of the mean; SD, standard deviation; SRD, single radial immunodiffusion.

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H3N2) and two type B influenza viruses (Yamagata and Victoria lineage), is currently used in various countries. However, current influenza vaccines fail to provide effective protection against mismatched virus strains. Indeed, amino acid substitutions in hemagglutinin (HA) occur in the field or during the process of vaccine manufacturing. Moreover, a flu shot induces only a systemic response but not a mucosal immune response, so it fails to provide protection against a virus undergoing antigenic drift [5]. In contrast, nasal delivery of inactivated vaccine has been advocated as being effective in providing mucosal immunity (secretory IgA antibodies) that can provide cross protection against a virus with antigenic substitution, in addition to systemic immunity against influenza [6]. However, mucosal adjuvants must be used when delivering a vaccine, and especially a recent split influenza vaccine, via the nasal route [7]. Previous studies have used a seasonal influenza split vaccine given nasally with a B subunit of either cholera toxin or heat-labile enterotoxin as a mucosal adjuvant in order to induce protective immunity [7–9]. However, these toxin-based nasal adjuvants carry the risk of causing a severe adverse reaction [10,11]. Polyinosinic:polycytidylic acid [poly(I:C)] is known to be an effective adjuvant, inducing innate immune responses by activating dendritic cell (DC) maturation and inflammatory cytokine secretion [12]. In a clinical study, however, toxic adverse reactions including shock, renal failure, coagulopathy, and hypersensitivity reactions occurred in some patients [13]. Based on these previous studies, mucosal adjuvants that can enhance immune responses but that cause no clinical complications are promising components of nasal influenza vaccines.

Synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs trigger cells that express Toll-like receptor (TLR) 9 (including human plasmacytoid dendritic cells and B cells), inducing an innate immune response characterized by the production of Th1- and proinflammatory-cytokines [14,15]. When CpG ODNs were used as vaccine adjuvants, they up-regulated the function of professional antigen-presenting cells (APCs) and subsequently enhanced the induction of vaccine-specific humoral and cellular immune responses [16,17]. Evidence from clinical trials has indicated that CpG ODNs are reasonably safe when administered as vaccine adjuvants. Thus, both conventional and CpG-adjuvanted vaccines result in similar safety profiles [18,19].

Four classes of CpG ODN - D(A), K(B), C, and P - have been described, each with distinct structural and biological properties [20]. A recent study indicated that an ODN containing the 10-mer palindromic sequence GACGATCGTC linked with a 9-mer oligo G at the 5′ end and a single G at the 3′ end possessed more potent activity at inducing IFN-α in human PBMCs than other ODNs. This novel ODN is classified as a D(A) class ODN and was named G9.1 (Patent US7,718,623B2, May 2010) [21]. G9.1 also displayed significant adjuvant activity. Indeed, mice nasally given diphtheria toxoid (DT) plus G9.1 had elevated levels of DT-specific mucosal secretory IgA (SIgA) and serum IgG antibody (Ab) responses [21]. However, whether G9.1 provides adjuvant activity when nasally administered with an influenza vaccine remains unclear. To that end, the current study assessed influenza virus-specific immune responses and protective efficacy in mice given a novel nasal influenza vaccine consisting of the X179A split vaccine plus G9.1.

2. Materials and methods

2.1. Antigen and adjuvant

The A/California/7/2009 (A/H1N1pdm09) split vaccine (X179A) was generously provided by the Handai-Biken Institute (Kannonji, Kagawa). The protein concentration of this vaccine was measured with the BCA Protein Assay Kit (Thermo Science, Rockford, IL). X179A contained an HA concentration of 50% (measured with SRD).

CpG ODN G9.1 (G9.1), ODN 1585 (1585), ODN 1826 (1826), and poly(I:C) were used in this study. G9.1 was synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). The type and the sequences of this ODN were as previously described [21]. G9.1 was diluted with a 0.5% BSA PBS buffer before vaccine concentration in order to stabilize G9.1 biological activity. 1585 (InvivoGen, San Diego, CA) 1826 (InvivoGen, San Diego, CA), and poly(I:C) (InvivoGen, San Diego, CA) were purchased and prepared according to the manufacturer's instructions.

2.2. Immunization

The experimental schedule for assessing protective efficacy and immune responses are shown in Supplemental Fig. 1. Female BALB/c mice (6–8 weeks old), were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka). Mice were anesthetized via intraperitoneal injection of ketamine and xylazine and were then given 4 μL of PBS (in each nostril, total of 8 μL/mouse) containing a required dose of X179A alone or with a mucosal adjuvant via the nasal route. Two weeks after initial nasal immunization, mice were nasally given a required dose of X179A alone. As controls, mice were subcutaneously (s.c.) immunized with 100 μL of PBS containing a required dose of X179A twice at a 2-week interval [22].

2.3. Viral challenge

To assess protective immunity, mice were nasally challenged with a small (4 μL) or large (50 μL) volume of a $10 \times LD_{50}$ (8×10^5 pfu) dose of the A/California/7/2009 (Cal7) influenza virus two weeks after the last vaccination [8,22–23]. Mice were nasally given 4 μL (2 μL in each nostril) of a Cal7 virus suspension as an upper respiratory tract (URT) challenge. This procedure causes viral replication predominantly in the URT without weight loss and recovery from influenza within 11 days (Fig. 1A and 1C). Other mice were nasally given 50 μL of a Cal7 virus suspension as a lower respiratory tract (LRT) challenge. This procedure induces severe pneumonia and subsequently leads to lethal damage in the LRT, including the lungs (Fig. 1B and 1C). The methods for assessment of the time course of the viral titer in the URT (nasal washes, NWs) and LRT (bronchoalveolar lavages, BALs) were as previously described [8,23]. In addition, LD_{50} values in mice were calculated (as pfu) using the Reed-Muench method [24] based on the survival rate of mice nasally given 50 μL of 10-fold serial dilutions of Cal7 in PBS containing 0.1% BSA. After inoculation of the virus into the lungs, mice were monitored every other day for weight loss of more than 25% (Supplemental Fig. 2).

All animal experiments were performed in accordance with the Guidelines for Animal Experiments Performed at the National Institute of Infectious Diseases (NIID) and were approved by the Animal Care and Use Committee of the NIID.

2.4. Sample collection and virus titration

Nasal washes (NWs) and serum were collected 3 days after the influenza virus challenge. Briefly, NWs were obtained by instillation of 1 mL of PBS on three occasions into the posterior opening of the nasopharynx with a 30-gauge hypodermic needle, and serum was obtained from immunized mice as described previously [23,25]. NW samples were centrifuged to remove cellular debris and subjected to a Cal7-specific ELISA as described below. The viral titers were determined with a plaque assay using Madin-Darby canine kidney (MDCK) cells as described previously [26].

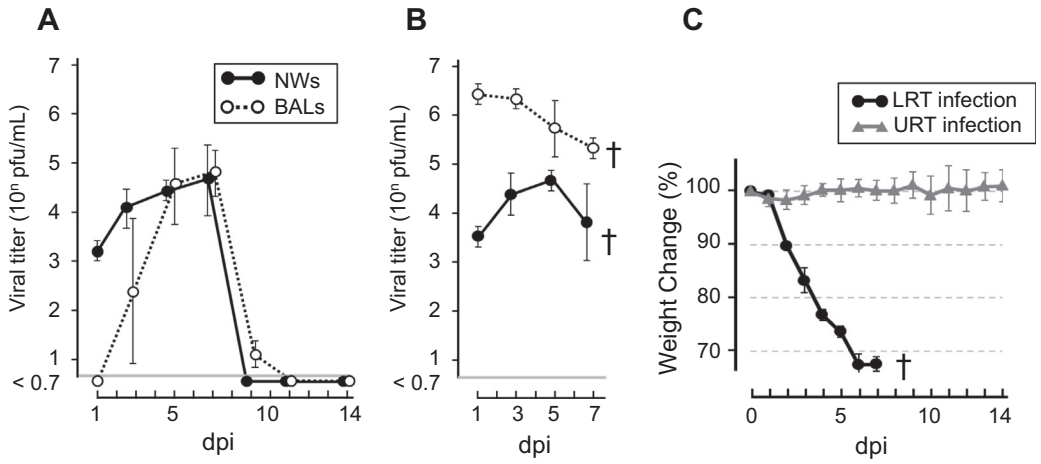

Fig. 1. Kinetics of viral titers and weight changes of mice given URT or LRT infection with the A/California/7/2009 (Cal7) virus. Mice were nasally given a small volume (2 μL in each nostril; total of 4 μL) or a large volume (50 μL) of a 10 × LD_{50} (8×10^5 pfu) dose of a Cal7 influenza virus suspension under light anesthesia. (A) A small volume of the virus served as an upper respiratory tract (URT) challenge whereas (B) A large volume of the virus served as a lower respiratory tract (LRT) challenge. At each time point, nasal washes (NWs) and lung washes (LWs) were collected and subjected to a plaque assay using Madin-Darby canine kidney cells in order to assess viral titers. The limit of viral detection was 5 pfu/mL (grey line). (C) Weight changes after URT or LRT infection. Each point represents the mean ± SD of viral titers and weight changes from five mice in each group.

2.5. Antibody titration

A hemagglutination inhibition (HAI) assay and ELISA were performed in order to determine Cal7-specific antibody titration. The hemagglutination assay and HAI assay were performed with 0.5% turkey red blood cells (TRBC) using standard methods [27]. Turkey whole blood was purchased from Nippon Bio-Test Laboratories, Inc. (Asaka, Saitama). Cal7-specific antibodies (Abs) in NWs and serum were determined with ELISA using isotypes of anti-Cal7 standard Abs as described previously [7,23,28,29]. Briefly, the EIA plate (Costar, Cambridge, MA) was coated with the X179A split vaccine (1 μg/mL). After blocking (1% BSA in PBS), 2-fold serial dilutions of samples and isotype standards were added and incubated overnight at 4 °C. Biotinylated goat anti-mouse IgA (α-chain specific) or IgG (γ-chain specific) was added, and this was followed by alkaline phosphatase-conjugated streptavidin. A color reaction was developed with *p*-nitrophenylphosphate. As a Cal7-specific SIgA standard, mouse NW samples were collected 28 days after infection with Cal7 and were then pooled and diluted to 1:5 (16 U/mL). Mouse serum samples were collected 28 days after infection with Cal7. These samples were pooled and diluted to 1:1000 for use as a Cal7-specific IgG standard (16 U/mL) [23,30]. The Ab titers of NWs and serum were determined using the standard regression curve obtained from optical density values of a serial 2-fold dilution of the standard IgA or IgG for each assay.

2.6. Expression of inflammatory cytokine-associated genes in the lungs

In order to determine potent inflammatory responses induced by nasal adjuvants, expression of inflammatory cytokine-associated genes was assessed. To that end, lung tissues were subjected to the Mouse Type I Interferon Response PCR Array (Qiagen, Crawley, UK) to determine the expression of 84 genes, including those mediating the inflammatory response, according to quantitative real-time RT-PCR (RT-qPCR). The 84 genes are listed on the manufacturer's website (http://www.sabiosciences.com/us/shop/pcr/primer-sets/rt2-profiler-pcr-arrays/?catno = PAMM-016Z# geneglobe). Total RNA was extracted from the lung tissue of mice 24 h after they were nasally given G9.1, 1826, poly(I:C) (20 μg/50 μL), or PBS (a negative control), and RNA was then treated with RNase-free DNase I (Qiagen). RNA was subjected to the RNeasy MinElute Cleanup Kit (Qiagen) according to the manufacturer's protocol to eliminate DNA contamination. Each RNA sample (0.8 μg) was suspended in RNase-free water and reverse-transcribed using the RT2 First Strand Kit (Qiagen). The resulting cDNA was amplified with PCR using specific primer sets according to the manufacturer's protocol. Data on expression were analyzed using a free online module from SABiosciences, and the relative expression of each target gene was normalized using five "housekeeping" genes. Primers of the "housekeeping" genes were supplied in the PCR array plate and calculated according to the online module's instructions.

2.7. Measurement of IFN-α concentrations

Cytokine concentrations in BALs from mice nasally given various adjuvants were measured using the Verikine™ Mouse IFN-α Multi-subtype ELISA Kit (PBL InterferonSource, Piscataway, NJ) according to the manufacturer's instructions. Mice were nasally given 50 μL of PBS, whole virion inactivated influenza vaccine (5 μg), poly(I:C) (10 or 20 μg) (Invivogen, San Diego, CA), 1585 (20 μg) (Invivogen, San Diego, CA), 1826 (10 or 20 μg) (Invivogen, San Diego, CA), G9.1 (20 μg), G9.1 (20 μg) in PBS with 0.5% BSA, or G9.1 (20 μg) combined with the X179A split vaccine (5 μg) in PBS with 0.5% BSA. Twenty-four hours after the nasal injection, BALs from mice were collected and assessed.

2.8. Statistical analysis

Viral titers were compared among groups using the Student's *t*-test. A p value < 0.05 was considered significant.

3. Results

3.1. An influenza vaccine with G9.1 as a nasal adjuvant provides complete protection

This study initially examined whether G9.1 as a mucosal adjuvant would improve the effectiveness of a nasal influenza vaccine in terms of the induction of protective influenza virus-specific antibody (Ab) responses. Mice were nasally immunized with X179A plus G9.1 or X179A alone. Both groups of mice were further given a booster immunization with X179A alone 2 weeks later. When a

large volume of the Cal7 influenza virus was given via the nasal route (an infection of the LRT), both groups of vaccinated mice survived; however, mice nasally given X179A plus G9.1 gained back weight earlier than mice nasally given X179A alone (Fig. 2A). Mice nasally given G9.1 as an adjuvant began to gain back weight 6 days after infection and attained their original weight 4 days later, which they subsequently maintained (Fig. 2A). However, mice nasally given X179A alone still lost weight 6 days after infection and never attained their original weight. As expected, all of the unimmunized mice died 6 days after infection. All groups of mice had high virus titers in the BAL at 3 dpi; however, the titers seen in the BAL of mice given X179A with and without G9.1 were lower than those seen in unimmunized mice (Fig. 2B left). A point worth noting is that when vaccinated mice were challenged with a small volume of the Cal7 influenza virus (infection limited to the URT), the influenza virus was essentially undetectable in NWs of mice given X179A plus G9.1 (Fig. 2B right). In contrast, significantly higher levels of virus titers were noted in NWs of mice given X179A alone when compared to those titers in mice given X179A plus G9.1 (Fig. 2B right). These findings are corroborated by the fact that higher levels of Cal7-specific SIgA Ab responses were noted in NWs of mice given G9.1 as nasal adjuvant than those responses seen in mice given nasal X179A alone (Fig. 2C left). Mass spectrometry analysis [31] revealed that >95% of IgA Abs in NWs were in polymeric form (data not shown). There were no significant differences in Cal7-specific IgG Ab responses in NWs from all of the groups (Fig. 2C right). Both groups of vaccinated mice given X179A plus G9.1 and X179A alone had essentially the same levels of anti-Cal7 serum IgG and HAI Ab responses, which were significantly higher than those seen in the unimmunized group (Fig. 2D). These results indicate that nasal delivery of G9.1 upregulates co-administered vaccine antigen-specific mucosal SIgA Ab responses and subsequently elicits protective immunity against an infection limited to the URT and an infection of the LRT. Moreover, the current findings suggest that the effectiveness of protection from an infection limited to the URT is closely correlated with Cal7-specific SIgA Ab responses in the URT.

3.2. Protective efficacy and Ab responses in s.c. Vaccinated mice

Since G9.1 as a nasal adjuvant provided complete protection from influenza virus in different models of infection, we next examined whether similar patterns of protection would be elicited by subcutaneous (s.c.) immunization with X179A since s.c. immunization represents a current form of vaccination. Mice were s.c. immunized with two different doses (0.5 or 2,0 μg/mouse) of the X179A vaccine and challenged with a large (an infection of the LRT) or small (an infection limited to the URT) volume of the Cal7 influenza virus. When the virus was used to produce an infection of the LRT, both groups of vaccinated mice survived; however, mice given a low dose of vaccine (0.5 μg/mouse) gained back weight slower than mice given 2.0 μg of the X179A vaccine (dose-dependent recovery) (Fig. 3A). Similarly, a greater vaccine dose-dependent reduction in the virus titer was noted in the BAL at 3 dpi (Fig. 3B left). When experiments involving an infection limited to the URT were performed, both groups of vaccinated mice lacked protective immunity. Significantly higher viral titers were evident in NWs that were comparable to titers in unimmunized control mice (Fig. 3B right). When Cal7-specific Ab responses were examined, there were essentially no Cal7-specific SIgA and IgG Ab in NWs from both groups of s.c. vaccinated mice (Fig. 3C). In contrast, mice given a high dose of X179A had significantly higher

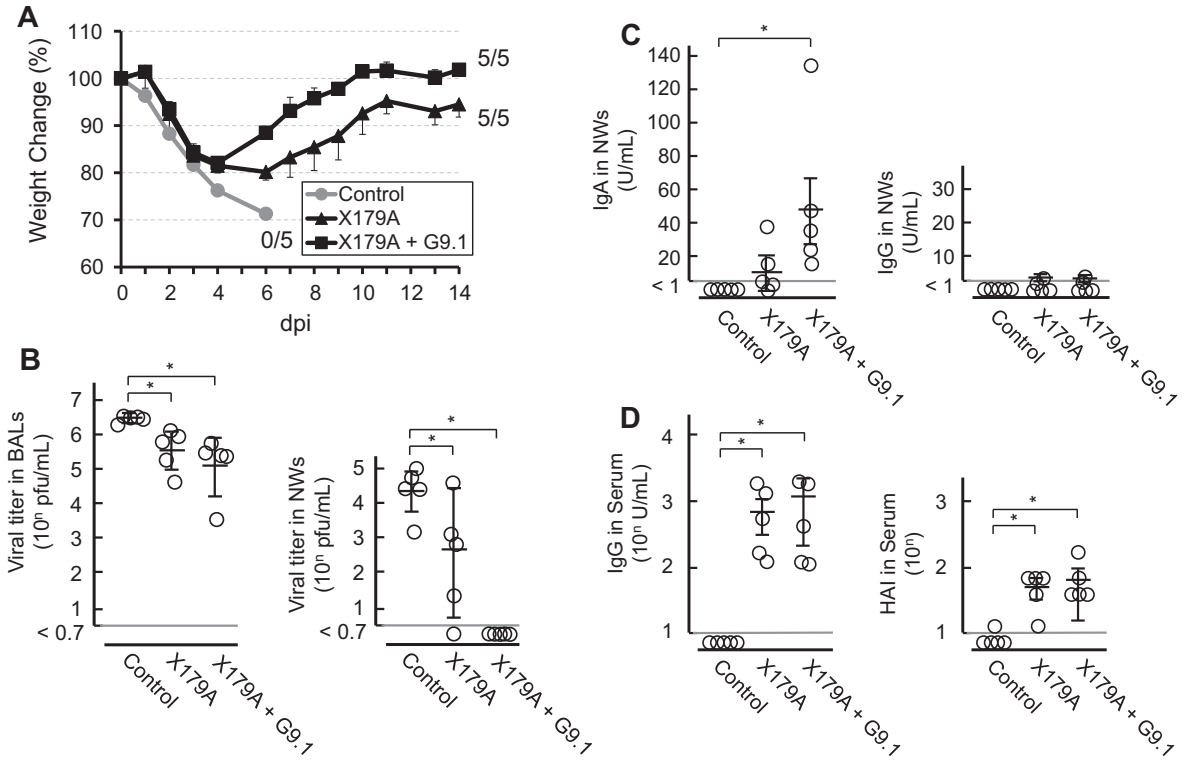

Fig. 2. Protection against Cal7 virus challenge and immune responses in mice nasally given X179A combined with G9.1. Mice were nasally immunized with 0.5 μg of X179A plus G9.1 or X179A alone. Two weeks after initial immunization, mice were nasally given X179A alone (0.5 μg/mouse). (A) Two weeks after the second vaccination, the mice were infected with a large volume of the Cal7 influenza virus (an infection of the LRT) and their weight loss was determined. (B) In other experiments, another group of vaccinated mice was challenged with a small or large volume of the Cal7 influenza virus. Three days after the LRT (B left panel) or URT (B right panel) challenge, BALs or NWs were collected and virus titers were assessed. (C) The Cal7-specific SIgA (left) and IgG (right) Ab responses in NWs from mice given nasal vaccine were assessed. (D) The Cal7-specific IgG (left) and HAI (right) Ab responses in serum from mice given nasal vaccine were determined. Virus titers are expressed as the mean ± SD and Abs titers are expressed as the mean ± SEM for five mice in each group.

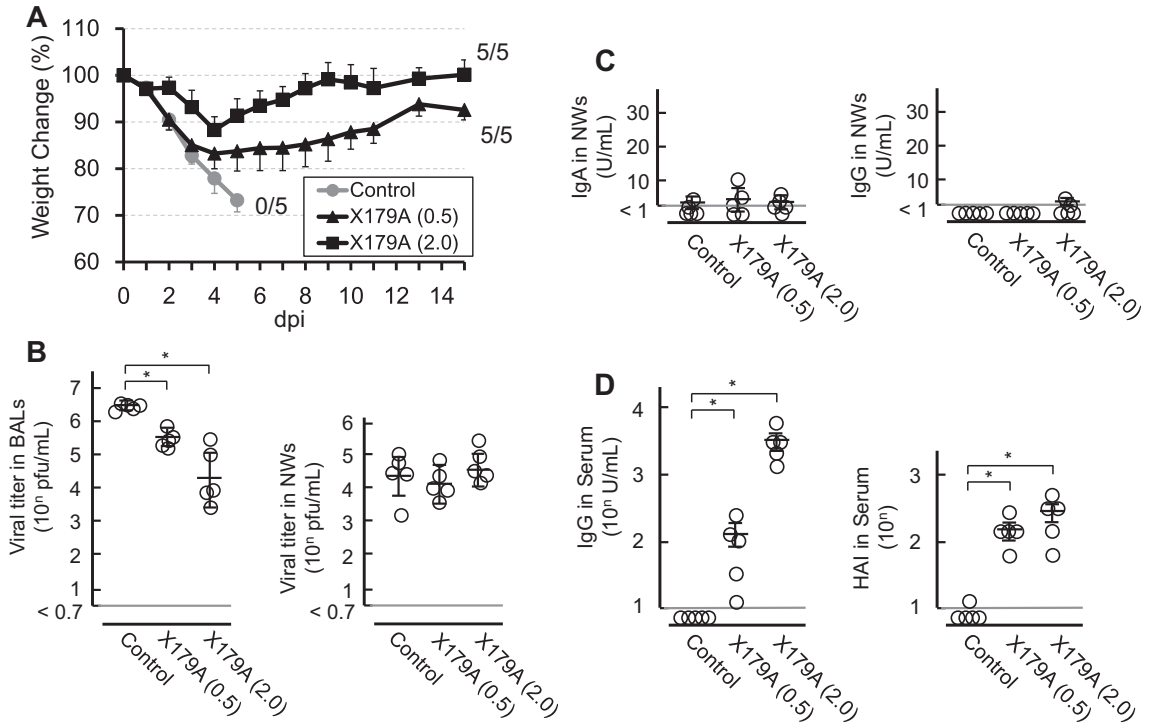

Fig. 3. Protection against Cal7 influenza virus challenge and immune responses in subcutaneously (s.c.) vaccinated mice. Mice were subcutaneously (s.c.) immunized with 0.5 or 2.0 μg of X179A. Two weeks after initial immunization, mice were given the same dose of X179A. (A) Two weeks after the second vaccination, the mice were infected with a large volume of the Cal7 influenza virus (an infection of the LRT) and their weight loss was determined. (B) In other experiments, another group of vaccinated mice was challenged with a small or large volume of the Cal7 influenza virus. Three days after the LRT (B left panel) or URT (B right panel) challenge, BALs or NWs were collected and virus titers were assessed. (C) The Cal7-specific SIgA (left) and IgG (right) Ab responses in NWs from mice given nasal vaccine were assessed. (D) The Cal7-specific IgG Ab (left) and HAI (right) responses were determined using serum from mice s.c. given the vaccine. Virus titers are expressed as the mean ± SD and Abs titers are expressed as the mean ± SEM for five mice in each group.

levels of Cal7-specific serum IgG and HAI Ab responses. Moreover, mice injected with a low dose of X179A had increased levels of anti-Cal7 serum IgG and of HAI Ab responses (Fig. 3D). These results indicate that the current systemic vaccination strategy induces influenza virus-specific serum IgG Abs that can facilitate recovery from an infection of the LRT in a vaccine dose-dependent manner. However, this strategy fails to induce influenza virus-specific mucosal SIgA Abs and protection from an infection limited to the URT [29,32]. Taken together, these findings indicate that a nasal vaccine containing G9.1 as a mucosal adjuvant is a superior strategy with which to elicit complete protective immunity against influenza virus infection, a goal that cannot be achieved with current injectable influenza vaccines.

3.3. Analyses of pro-inflammatory-associated gene expression in the lungs of mice nasally given G9.1

The safety of a vaccine adjuvant is a critical aspect of vaccine development in addition to the efficacy of the vaccine. The innate immune responses induced by G9.1 as a nasal adjuvant need to be understood since prolonged and hyper innate immunity could result in undesired inflammatory immune responses. In order to assess the safety of G9.1, the expression of 84 type I IFN-associated genes was examined in the lungs of mice nasally given G9.1, poly(I:C), or 1826. Lung tissue isolated from mice nasally given G9.1 had significantly lower levels of expression of type I IFN-associated genes when compared to tissue from the other two groups. Changes in levels of expression of all 84 genes are shown in Supplemental Fig. 3. Significant changes in levels of mRNA expression were noted for 12 (IFN-α2, IFN-β1, IL-6, CCL2, CCL4, IP10, IRF7, IFIT1, BST2, IL-10, MX1, and MX2) of the 84 genes studied (Fig. 4). The profile of expression of these 12 genes induced by 1826 was very similar to that induced by nasal poly(I:C). The fold changes in mRNA expression induced by nasal G9.1 were significantly lower than those associated with nasal delivery of 1826, which has been used as a potential nasal adjuvant. To verify that low levels of expression of type I IFN-associated genes corelated with nasal delivery of G9.1, IFN-α production was assessed in the lungs of mice given these nasal adjuvants. Increased levels of IFN-α production were seen in BALs of mice given 1826 or poly (I:C), whereas very limited levels of IFN-α production were noted in BALs of mice given G9.1 alone or of mice given a combination of G9.1 + X179A (Fig. 5). Interestingly, levels of IFN-α production induced by nasal G9.1 were lower than those induced by lower doses of 1826 or poly(I:C) as well as by the inactivated whole-virion influenza vaccine, which has been used as a toxicity reference in leukocyte toxicity testing [33]. These findings indicate that G9.1 does not elicit potentially harmful inflammatory immune responses when used as a nasal adjuvant.

4. Discussion

In general, an influenza infection initially develops in the URT and subsequently spreads into the LRT. The currently approved influenza vaccines can protect from severe infections of the LRT since parenteral vaccination induces specific serum IgG Abs that migrate into the LRT. However, this vaccination strategy fails to prevent the attachment and subsequent dissemination of pathogens at the mucosal surfaces of the host's URT since essentially no virus-specific mucosal SIgA Abs are induced [5]. Given these facts, different models of infection could presumably be used to properly assess the effectiveness of mucosal and injectable influenza vaccines. Two murine models of infection with the Cal7 influenza virus were created in the current study. A small volume

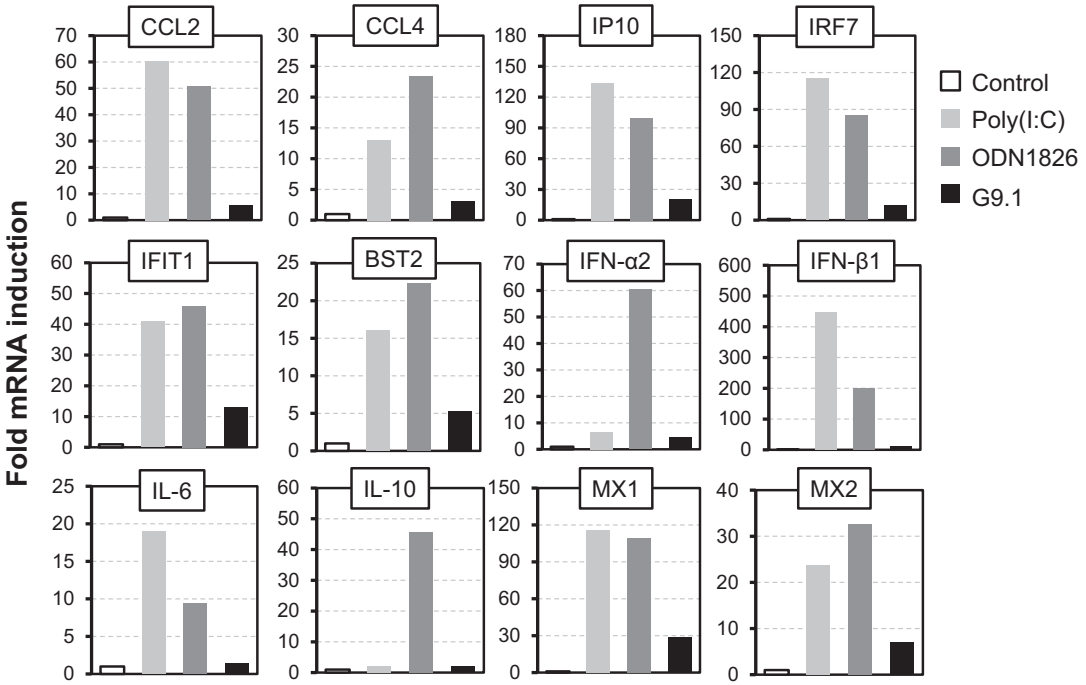

Fig. 4. Expression of inflammatory cytokine-associated genes in lungs of mice nasally given an adjuvant. Mice were nasally given 20 μg of G9.1, 1826, or poly (I:C) (50 μL). Twenty-four hours after nasal delivery, total RNA was extracted from lung tissue of mice, and expression of various type I IFN-associated genes was assessed with quantitative real time-PCR. Values represent the relative fold increase in expression of genes as described in the Materials and Methods (n = 2).

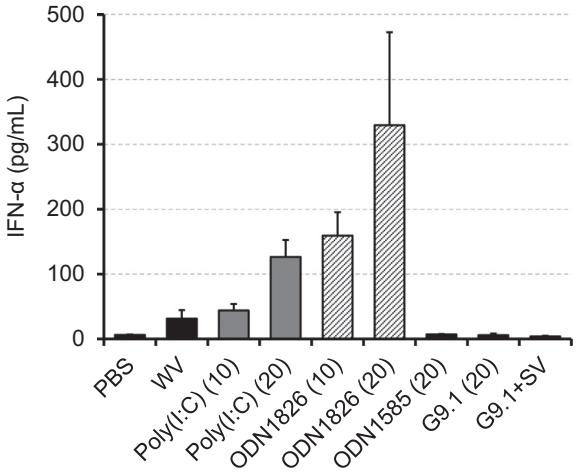

Fig. 5. Production of IFN-α in the lungs of mice nasally given adjuvants. Mice were nasally given 50 μL of various adjuvants or vaccines. Twenty-four hours after nasal delivery, BALs were collected from the lungs and then subjected to IFN-α-specific ELISA. Values are expressed as the mean ± SD for five mice in each group.

(2 μL in each nostril) of Cal7 given nasally served as a model of infection limited to the URT while a nasal challenge with a large volume (50 μL) served as a model of severe infection. The current results indicated that subcutaneous X179A vaccination resulted in recovery from severe infection, but it failed to provide protective immunity in the URT. In contrast, nasal immunization with G9.1 combined with X179A provided complete protection against an infection limited to the URT and it facilitated recovery from a severe infection. These findings suggest that a nasal vaccine consisting of G9.1 and X179A has an additional advantage of being able to prevent viral attachment to the mucosa of the URT in addition to providing protection from a severe infection on par with that provided by current vaccines. Accordingly, both models of infection should be used in order to evaluate the effectiveness of newly developed influenza vaccines.

Since safety is a critical concern when developing a vaccine, innate immune responses induced by nasal G9.1 were assessed. Mice given G9.1 had low levels of expression of type I IFN-associated genes and IFN-α production. In contrast, markedly higher levels of expression of type I IFN-associated genes and IFN-α production were noted in mice nasally given poly(I:C). Based on these findings, G9.1 is a safer nasal adjuvant than poly(I:C) and 1826. Indeed, studies have indicated that poly(I:C) causes immunotoxicity in humans and animals [13]. A recent study used the levels of expression of 18 biomarkers that could be potentially harmful to the host to assess the safety of influenza vaccines [34]. Lungs of mice given 20 μg of a nasal poly(I:C)-combined split influenza vaccine had essentially the same levels of biomarkers as those in mice given an inactivated whole-virion influenza vaccine (a reference vaccine) via the nasal route. Since studies have indicated that the reference vaccine was classified as a toxic vaccine based upon the occurrence of adverse reactions including a fever [13,35–37], poly(I:C) may possess potent toxicity when used as a nasal adjuvant. Similarly, the current results indicate that poly(I:C) given nasally resulted in significantly elevated levels of type I IFN-associated gene expression in the lungs of mice. In contrast, mice given G9.1 nasally as an adjuvant had markedly lower levels of type I IFN-associated gene expression when compared to mice given poly(I:C) or 1826 nasally as an adjuvant (Fig. 4). Taken together, these results indicate that G9.1 can be used as a safe nasal adjuvant for influenza vaccine development.

A study has indicated that the immunostimulatory activity of a certain CpG ODN differs across species [38]. Indeed, 1826 (Class B) effectively up-regulates Ag-specific immune responses in mice, whereas CpG ODN 10104 (Class B) is required for the activation of human as well as non-human primate immunity [39]. Several studies have indicated that species-specific properties are determined by the nucleotide context of the CpG motifs within the CpG ODN [40–43]. In order to use G9.1 as a nasal adjuvant in

clinical studies of potential influenza vaccines, G9.1 needs to be identically effective in both mice and humans. Previous studies and the current study have showed that G9.1 effectively activates Ag-specific immune responses as a systemic or nasal adjuvant. Moreover, a study has shown that G9.1 was able to up-regulate CD80 expression by human pDCs [44]. A recent study showed that G9.1 induced the highest level of IFN-α production by human PBMCs in comparison to other ODNs [21]. Similarly, stimulation of murine PBMCs with G9.1, resulted in increased levels of IFN-α production (Supplemental Fig. 4). In contrast, results of the current *in vivo* experiments revealed that BALs of mice nasally given G9.1 contained had essentially the same levels of IFN-α production as those seen in BALs of naive mice (Fig. 5). The difference was due to the experimental systems used in those studies. Previous studies used an *in vitro* system to directly stimulate human PBMCs. In contrast, nasal administration of G9.1 indirectly stimulates immune competent cells in the mucosal tissue, inducing IFN-α production. Accordingly, assessing IFN-α production in BAL is a more appropriate biological strategy with which to evaluate the safety of mucosal adjuvants. Interestingly, the current results also revealed lower levels of IFN-α induced by G9.1 than those induced by lower doses of poly(I:C) of ODN 1826 (Fig. 5). The doses of poly(I:C) and ODN 1826 were determined based on doses providing optimal adjuvant activity according to previous studies [34,45]. G9.1 can immediately be used in clinical studies to develop human nasal influenza vaccines since the current findings clearly indicated the effectiveness and safety of G9.1 as a nasal adjuvant in an animal study.

A crucial role of a nasal influenza vaccine is to induce mucosal SIgA Ab responses in the URT as a first line of defense. Nasal influenza vaccine-induced SIgA Abs provide effective cross-protection [29,32]. Since the current study showed that a nasal influenza vaccine combined with G9.1 successfully induced influenza-specific SIgA Abs in the URT, these SIgA Abs presumably have cross-protective activity. This contention is corroborated by a separate study indicating that mice given a nasal quadrivalent vaccine including the former H1N1 strain, A/Brisbane/59/2007 plus G9.1 had significantly lower levels of virus titers in NWs when challenged with a URT dose of the A/H1N1pdm09 strain (manuscript in preparation). In addition, the current authors are testing the cross-protective activity of SIgA Abs induced by a nasal influenza vaccine containing G9.1 as a mucosal adjuvant (manuscript in preparation).

In conclusion, G9.1 is a potent nasal adjuvant that effectively promotes mucosal immunity in the URT. Much like a toxin-based adjuvant or poly(I:C), G9.1 has a synergistic effect when co-administered with an influenza vaccine, making it a promising mucosal adjuvant. In contrast to those other adjuvants, however, G9.1 given nasally induces reduced inflammatory responses that result in less toxicity and fewer adverse reactions. G9.1 is an effective and safe mucosal adjuvant that can be used to develop nasal influenza vaccines for humans.

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Author contributions

Conceived and designed the experiments: KF, HA; performed the experiments: KT, AA, KS, NY, HA; analyzed the data: KT, KF, NY, HA; contributed reagents/materials/analysis tools: SI, SY, JM; wrote the manuscript: KF, HA; HH, TO: Management of some experiments.

Declaration of Competing Interest

The authors have declared that no competing interests exist.

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Appendix A. Supplementary material

Supplemental Fig. 1. Changes in body weight of mice after nasal infection with the Cal7 virus. Fifty μL of 10-fold serial dilutions (2.5×10^3–2.5×10^6 pfu) of Cal7 in PBS containing of 0.1% of BSA were nasally administered to each group of five mice. After nasal infection, mice were monitored every other day in order to detect weight loss of more than 25%. **Supplemental Fig. 2.** A flow diagram of vaccination and infection. X179A[1)], A/California/7/2009 split vaccine; G9.1[2)], CpG ODN G9.1; A/California[3)], A/California/7/2009 virus; LRT[4)], lower respiratory tract; URT[5)], upper respiratory tract. **Supplemental Fig. 3. Comparison of type I IFN-associated genes in radar chart.** Relative gene expression in the lungs of mice given nasally with each adjuvant were plotted. The highest gene expression of an individual gene induced by each adjuvant was indicated as 1.00. **Supplemental Fig. 4. IFN-α production by G9.1 stimulated murine PBMCs.** Mice PBMCs (1×10^6 cell/mL) were cultured with or without G9.1 (1 μg/mL) for 24 h. The culture supernatants were collected and subjected to IFN-α specific ELISA (VeriKine).

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