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# Interleukin-1 Family Cytokines as Mucosal Vaccine Adjuvants for Induction of Protective Immunity against Influenza Virus[▽]

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**A safe and potent adjuvant is needed for development of mucosal vaccines against etiological agents, such as influenza virus, that enter the host at mucosal surfaces. Cytokines are potential adjuvants for mucosal vaccines because they can enhance primary and memory immune responses enough to protect against some infectious agents. For this study, we tested 26 interleukin (IL) cytokines as mucosal vaccine adjuvants and compared their abilities to induce antigen (Ag)-specific immune responses against influenza virus. In mice intranasally immunized with recombinant influenza virus hemagglutinin (rHA) plus one of the IL cytokines, IL-1 family cytokines (i.e., IL-1α, IL-1β, IL-18, and IL-33) were found to increase Ag-specific immunoglobulin G (IgG) in plasma and IgA in mucosal secretions compared to those after immunization with rHA alone. In addition, high levels of both Th1- and Th2-type cytokines were observed in mice immunized with rHA plus an IL-1 family cytokine. Furthermore, mice intranasally immunized with rHA plus an IL-1 family cytokine had significant protection against a lethal influenza virus infection. Interestingly, the adjuvant effects of IL-18 and IL-33 were significantly decreased in mast cell-deficient *W/W$^v$* mice, indicating that mast cells have an important role in induction of Ag-specific mucosal immune responses induced by IL-1 family cytokines. In summary, our results demonstrate that IL-1 family cytokines are potential mucosal vaccine adjuvants and can induce Ag-specific immune responses for protection against pathogens like influenza virus.**

Because most pathogenic viruses, including influenza virus, enter through a mucosal surface (18), preventing infection at the viral entry site by inducing mucosal immunity should be an effective strategy for combating such pathogens. A key aspect of mucosal immunity is production of secretory immunoglobulin A (sIgA), as well as induction of cytolytic T lymphocytes (CTLs) against epithelium-transmitted pathogens (5, 21). Therefore, it is important to develop mucosal vaccines that induce effective immune responses at mucosal surfaces (31).

However, protein subunit antigens (Ags) generally evoke only a weak or undetectable adaptive immune response when administered intramucosally (1). Therefore, to produce effective mucosal vaccines, it is necessary to develop an appropriate mucosal vaccine adjuvant (34). Cholera toxin (CT) and *Escherichia coli* heat-labile enterotoxin are known potent mucosal vaccine adjuvants and have been used in nonclinical experimental systems (9, 27). However, their clinical application as nasal adjuvants had to be discontinued because of side effects such as Bell's palsy (29). Therefore, mucosal vaccine adjuvants with high efficacy and safety for clinical application continue to be urgently required.

Cytokines are key molecules that trigger the innate and adaptive immune responses (including maturation of Ag-presenting cells, differentiation of Th1 and Th2 cells, and induction of cytotoxic natural killer [NK] cells and CTLs), resulting in protective layers against virus infection (11, 41, 43). Therefore, cytokines are promising vaccine adjuvants for enhancing the immune response against infectious pathogens. At present, more than 30 members of the interleukin (IL) cytokine/IL receptor family have been identified and found to be involved

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in regulating and maintaining homeostasis of the immune system (3, 14). Specific IL cytokines have been used as vaccine adjuvants to enhance primary and memory immune responses against some cancers and infectious diseases (2, 6). However, there has been no comparative study of IL cytokines as mucosal vaccine adjuvants.

Recently, it was pointed out that identification of the cellular targets of vaccine adjuvants is an important issue (12). Dendritic cells (DCs) are responsible for Ag uptake and presentation to naive T cells and represent a key target for adjuvant activity (22, 33). Recent reports have demonstrated that other accessory cells, such as mast cells (MCs) and NKT cells, act as immunosensors to initiate and modulate innate and adaptive immune responses (16, 40). It has been reported that MCs contribute to the induction of an adaptive immune response or accessory function and that the synthetic Toll-like receptor 7 ligand imiquimod acts as a mucosal vaccine adjuvant in an MC-dependent manner (19). However, it is still not clear whether MCs are promising cellular targets for cytokine adjuvants in mucosal vaccines.

In this study to develop effective and safe mucosal vaccine adjuvants, we identified promising cytokines with mucosal vaccine adjuvant activity by screening 26 different IL cytokines. We also investigated the mucosal and systemic immune responses induced by these cytokines in normal and MC-deficient mice. The IL-1 family cytokines (IL-1α, IL-1β, IL-18, and IL-33) were found to be effective mucosal vaccine adjuvants for induction of protective sIgA and CTL immunity against influenza virus. In addition, the adjuvant activities of IL-18 and IL-33 were MC dependent.

## MATERIALS AND METHODS

**Cytokines and Ags.** CT was purchased from List Biological Laboratories (Campbell, CA). Twenty-six types of mouse recombinant IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-28B, IL-31, and IL-33) were purchased from R&D Systems (Minneapolis, MN). Baculovirus-expressed recombinant influenza virus hemagglutinin (rHA) derived from influenza virus A/New Caledonia/20/1999 (Protein Sciences, Meriden, CT) was used as the vaccine Ag.

**Mice and immunization protocols.** Female BALB/c mice and MC-deficient (WBB6F1 *W/W^v^*) and congenic littermate control (WBB6F1 WT) mice were purchased from Japan SLC (Hamamatsu, Japan) and used at 6 weeks of age. All animal experimental procedures used in this study were performed in accordance with our institutional guidelines for animal experiments. Mice were immunized intranasally with rHA alone (1 μg/mouse), rHA (1 μg/mouse) plus CT (1 μg/mouse), or rHA (1 μg/mouse) plus one of the IL cytokines (0.1 μg, 0.3 μg, or 1.0 μg/mouse) on days 0 and 28.

**Sample collection.** Fourteen days after the final immunization, plasma and mucosal secretions (nasal washes, saliva, vaginal washes, and fecal extracts) were obtained as previously described (24).

**Detection of Ab responses by ELISA.** rHA-specific antibody (Ab) levels in plasma and mucosal secretions were determined by enzyme-linked immunosorbent assay (ELISA) as previously described (24). Briefly, ELISA plates were coated with 2 μg rHA/ml of 0.1 M carbonate buffer and incubated overnight at 4°C. The plates were then incubated with blocking solution (Block Ace; DS Pharma Biomedical, Osaka, Japan) at 37°C for 2 h. Diluted plasma or mucosal secretions were added. After incubation at 37°C for 2 h, the coated plates were washed with phosphate-buffered saline (PBS)–polyoxyethylene sorbitan monolaurate (Tween 20; Wako Pure Chemical, Tokyo, Japan) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG solution to detect IgG in plasma or with a biotin-conjugated goat anti-mouse IgA detection Ab (Southern Biotechnology Associates, Birmingham, AL) solution to detect sIgA in mucosal secretions, at 37°C for 2 h. For detection of sIgA, the plates were incubated with HRP-coupled streptavidin (Zymed Laboratories, South San Francisco, CA) for 1 h at room temperature. After incubation, a color reaction was developed with tetramethylbenzidine (Moss, Inc., Pasadena, MD), stopped with 2 N $H_2SO_4$, and measured as the optical density at 450 to 655 nm ($OD_{450-655}$) in a microplate reader.

**Multiplex cytokine assay.** Splenocytes from immunized BALB/c, WBB6F1 *W/W^v^*, or WBB6F1 WT mice were harvested 14 days after the final immunization and stimulated *in vitro* with 10 μg rHA/ml. After 72 h, culture supernatants from *in vitro* unstimulated and rHA-stimulated cells were analyzed by a Bio-Plex multiplex cytokine assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Samples were analyzed on a Luminex 100 analyzer (Luminex, Austin, TX). The mean concentrations of cytokines in supernatants from rHA-stimulated cells were calculated relative to those in unstimulated cells.

**IFN-γ ELISPOT assay.** Splenocytes from immunized mice were harvested 14 days after the final immunization and stimulated at a cell density of $1 \times 10^7$ cells/ml with a mixture of two $H\text{-}2K^d$-restricted class I HA peptides, $HA_{240-248}$ (IYSTVASSL) and $HA_{462-470}$ (LYEKVKSQL) (MBL, Nagoya, Japan), at a final concentration of 10 μg total peptide/ml complete RPMI (25). After 24 h of incubation at 37°C, plates were washed, and gamma interferon (IFN-γ)-producing cells were measured by use of an enzyme-linked immunospot (ELISPOT) assay kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions.

**Tetramer assay.** Splenocytes from immunized mice were harvested 14 days after the final immunization and used as effector cells to determine $HA_{240-248}$-specific CTL responses. Splenocytes ($7 \times 10^6$ cells) were added to wells in a 24-well plate, followed by addition of 1 ml of medium containing a CTL epitope peptide ($HA_{240-248}$; IYSTVASSL) at a final concentration of 1 μg/ml. After incubation at 37°C for 2 days, medium containing human recombinant IL-2 (rIL-2) (Shionogi Co., Osaka, Japan) was added to each well of CTL effector cells, to a final concentration of 10 U human rIL-2/ml. Effector cells were stained for tetramers after restimulation for 7 days. For analysis, $1 \times 10^6$ cells were treated with purified anti-mouse CD16/CD32 Ab (Fc-γ III/II receptor Ab; BD Biosciences Pharmingen, San Diego, CA) and then stained with phycoerythrin (PE)-conjugated $H\text{-}2K^d\text{-}HA_{240-248}$ peptide tetramer (MBL, Nagoya, Japan) for 20 min at room temperature. Fluorescein isothiocyanate (FITC)-conjugated CD8α (clone KT15; MBL, Nagoya, Japan) was added for an additional 20 min. Cells were analyzed with a FACS Canto flow cytometer (BD Biosciences Pharmingen). Data analysis was done with FlowJo (TreeStar, Eugene, OR) software.

**Histopathological analysis.** BALB/c mice were immunized intranasally with rHA (1 μg/mouse), with or without IL-1α, IL-1β, IL-18, or IL-33 (1 μg/mouse), on days 0 and 28. Fourteen days after the final immunization, the heads of the mice were severed from the bodies and placed in fixative solution (4% paraformaldehyde). The samples then were sectioned and stained with hematoxylin and eosin (H&E) or Luna stain and examined for pathological changes under a light microscope. Histopathological examination was performed by the Applied Medical Research Laboratory (Osaka, Japan).

**Influenza virus infection *in vivo*.** To examine the prophylactic effect of IL cytokine treatment against influenza virus, mice were immunized intranasally on days 0 and 28 with 1 μg PR8 HA vaccine (inactivated-product vaccine with influenza virus A/Puerto Rico/8/34) (Charles River, North Franklin, CT)/mouse plus 1 μg CT or IL-1 family cytokine/mouse. Fourteen days after the final immunization, mice were fully anesthetized by intraperitoneal injection of pentobarbital, and each was infected by intranasal application of 25 μl PBS containing 256 hemagglutinating units (HAU) of influenza virus A/PR/8/34 (H1N1) (kindly provided by the Research Institute for Microbial Diseases of Osaka University, Osaka, Japan) per mouse. This procedure produced upper and lower respiratory tract infections.

**Statistical analysis.** All results are expressed as means ± standard errors of the means (SEM). Differences were compared using Bonferroni analysis of variance (ANOVA).

## RESULTS

**Comparative analysis of rHA-specific Ab responses induced by 26 different IL cytokines.** One potential advantage of successful mucosal immunization is the possibility of eliciting both systemic IgG and mucosal sIgA Ab responses against invading pathogens. Therefore, in this study, we tested 26 different IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-27, IL-28A, IL-28B, IL-31, and IL-33)

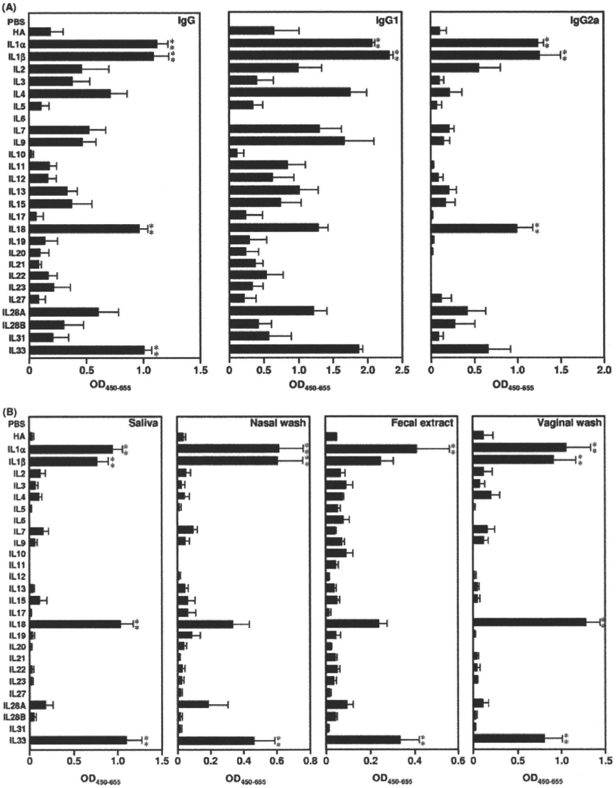

FIG. 1. Ab responses induced by IL-1 family cytokines. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone or rHA plus each interleukin. (A) Plasma was collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG, IgG1, and IgG2a. (B) Saliva, nasal washes, fecal extracts, and vaginal washes were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific sIgA. Data are presented as means ± SEM ($n = 5$). **, $P < 0.01$ compared to the value for the rHA-treated group.

as mucosal vaccine adjuvants. To examine the potential of these IL cytokines as mucosal vaccine adjuvants, BALB/c mice were immunized intranasally with 1 μg rHA plus 1 μg of an IL cytokine on days 0 and 28. Fourteen days after the final immunization, we examined the level of anti-rHA IgG in plasma by ELISA (Fig. 1A). Intranasal immunization with rHA plus 11 of the IL cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-7, IL-9, IL-13, IL-15, IL-18, IL-28A, and IL-33) induced higher rHA-specific IgG responses in plasma than those for mice immunized with rHA alone (Fig. 1A). In particular, immunization with rHA plus IL-1α, IL-1β, IL-18, or IL-33, referred to as IL-1 family cytokines, resulted in the highest rHA-specific IgG responses among the IL cytokines. The IgG subclass of the rHA-specific responses was then examined to assess the type of immune response induced by the 26 IL cytokines (Fig. 1A). Plasma Ag-specific IgG subclasses reflect the subset of $CD4^+$ T-helper cells induced by vaccination, with IgG1 and IgG2a corresponding to Th2 and Th1 responses, respectively. Consistent with the rHA-specific IgG responses, intranasal immunization with rHA plus IL-2, IL-3, IL-4, IL-7, IL-9, IL-13, IL-15, or IL-28A generally produced a greater rHA-specific IgG1 subclass response than immunization with rHA alone but a similar IgG2a response to that with rHA alone. In contrast, mice immunized with rHA plus IL-1 family cytokines showed

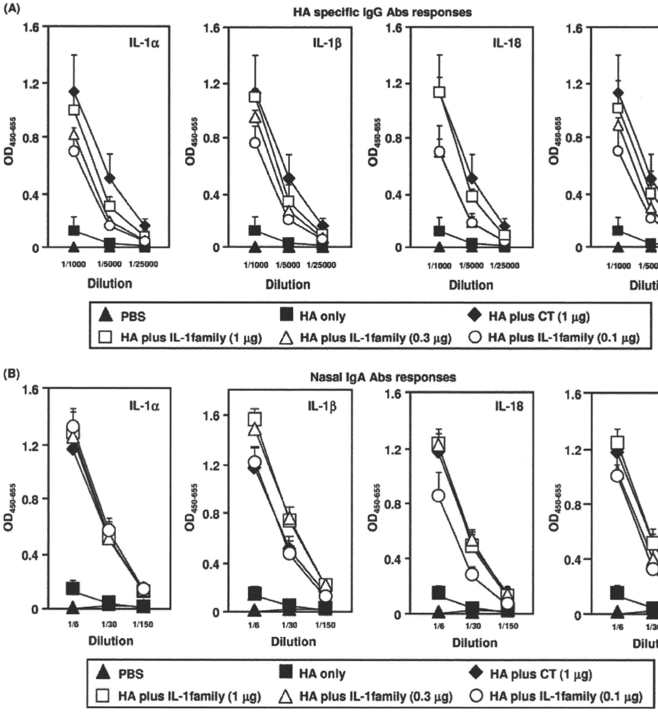

FIG. 2. Dose-response relationship for induction of rHA-specific Ab responses by nasal immunization with rHA plus an IL-1 family cytokine. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus an IL-1 family cytokine (0.1, 0.3, or 1 μg/mouse). (A) Plasma was collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG, at dilutions of 1/1,000, 1/5,000, and 1/250,000. (B) Nasal washes were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific sIgA, at dilutions of 1/6, 1/30, and 1/150. Data are presented as means ± SEM ($n$ = 5).

significantly higher IgG1 and IgG2a Ab responses than those immunized with rHA alone. These results indicate that nasal administration of IL-1 family cytokines has the potential to induce potent rHA-specific systemic IgG Abs, as well as IgG1 and IgG2a Ab responses. We then studied the rHA-specific sIgA response in mucosal secretions (i.e., in saliva, nasal washes, fecal extracts, and vaginal washes) induced by the 26 IL cytokines (Fig. 1B). For these 26 IL cytokines, IL-1 family cytokines induced the highest mucosal sIgA Ab responses in salivary, nasal, fecal, and vaginal mucosal secretions (Fig. 1B). Taken together, these results indicate that nasal immunization with IL-1 family cytokines effectively induced rHA-specific Ab responses in both systemic and mucosal immune compartments, suggesting that IL-1 family cytokines might be effective mucosal vaccine adjuvants.

**Dose-response relationship of IL-1 family cytokines as mucosal vaccine adjuvants for induction of rHA-specific Ab responses.** To determine the dose-response relationship of IL-1 family cytokines as mucosal vaccine adjuvants to induce rHA-specific IgG and sIgA Ab responses, mice were immunized intranasally with rHA plus 0.1, 0.3, or 1 μg of each IL-1 family cytokine (Fig. 2). Immunization with rHA plus the IL-1 family cytokines induced rHA-specific IgG in plasma in a dose-dependent manner. Even rHA plus the lowest dose (0.1 μg) of IL-1 family cytokines induced IgG to levels significantly higher than those induced by rHA alone (Fig. 2A). Importantly, the use of 1 μg of IL-1 family cytokines as an adjuvant resulted in

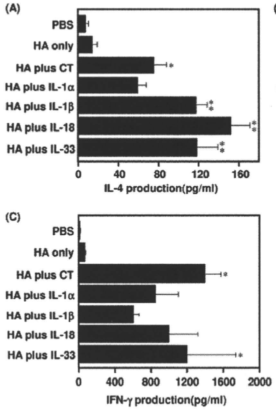

FIG. 3. Cytokine responses induced by nasal immunization with rHA plus IL-1 family cytokines. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine. Fourteen days after the final immunization, splenocytes from each group were cultured with 10 μg rHA/ml. Culture supernatants were harvested after a 3-day incubation and then assayed for rHA-specific IL-4 (A), IL-5 (B), and IFN-γ (C), using a Bio-Plex multiplex cytokine assay. Data are presented as means ± SEM ($n = 5$). *, $P < 0.05$; **, $P < 0.01$ compared to the value for the rHA-treated group. N.D., not done.

strong rHA-specific IgG Ab responses equivalent to those elicited by CT, which is one of the most potent mucosal vaccine adjuvants (Fig. 2A). Furthermore, the level of rHA-specific sIgA induced by rHA plus 0.1 μg of each IL-1 family cytokine in nasal secretions was significantly higher than that induced by rHA alone (Fig. 2B). The level of rHA-specific nasal sIgA induced in mice immunized intranasally with rHA plus 0.3 μg of each IL-1 family cytokine was equivalent to that observed in mice treated with 1 μg CT. Taken together, these results clearly indicate that nasal immunization with an IL-1 family cytokine as a mucosal vaccine adjuvant induced dose-dependent levels of both rHA-specific IgG and sIgA Abs in the mucosal and systemic immune compartments.

**Induction of rHA-specific Th1- and Th2-type responses after nasal administration of IL-1 family cytokines as mucosal vaccine adjuvants.** To evaluate the ability of IL-1 family cytokines to boost rHA-specific cytokine responses induced by mucosal immunization, splenocytes from mice that had been immunized intranasally with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine were restimulated *in vitro* with rHA and then assayed for Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines (Fig. 3). Splenocytes from mice immunized with rHA alone did not show significant cytokine production compared to those from PBS-treated mice. Consistent with the IgG subclass results (Fig. 1A), mice immunized with IL-1 family cytokines had higher levels of IL-4 and IL-5 (Th2-associated sIgA-enhancing cytokines) than mice given rHA alone. In particular, the highest levels of IL-4 and IL-5 were detected in splenocytes of mice immunized with rHA plus IL-18 or IL-33, and these responses were significantly higher than those in splenocytes of mice immunized with CT. It was also noteworthy that IFN-γ, a Th1 cytokine, was induced in mice immunized intranasally with rHA plus an IL-1 family cytokine. Thus, IL-1 family cytokines might induce CTL responses when administered nasally. These results show that as mucosal vaccine adjuvants, IL-1α, IL-1β, IL-18, and IL-33 elicit both Th1- and Th2-type cytokine responses.

***In vivo* CTL induction by nasal immunization with rHA plus IL-1 family cytokines as mucosal vaccine adjuvants.** Virus clearance is known to require strong Th1-polarized immune responses characterized by IFN-γ production and CTL responses in the systemic compartment. To investigate the ability of IL-1 family cytokines to act as mucosal vaccine adjuvants and to induce rHA-specific Th1/CTL immune responses, we measured $H\text{-}2K^d/HA_{240-248}$ tetramer$^+$ CD8$^+$ T cells (Fig. 4A) and $H\text{-}2K^d/HA_{240-248}$-specific IFN-γ-secreting cells (Fig. 4B) in splenocytes from mice that had been immunized intranasally with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine. The level of $H\text{-}2K^d/HA_{240-248}$ tetramer$^+$ CD8$^+$ T cells induced by rHA plus IL-1β was found to be similar to that induced by rHA alone, but the level induced by rHA plus IL-1α, IL-18, or IL-33 was significantly greater than that induced by rHA alone (Fig. 4A). Furthermore, the level of functionally active $H\text{-}2K^d/HA_{240-248}$-specific IFN-γ-secreting cells induced by rHA plus IL-1α, IL-18, or IL-33 was the same as or greater than that in mice intranasally immunized with rHA plus CT (Fig. 4B). Taken together, these results indicate that the IL-1 family cytokines IL-1α, IL-18 and IL-33 induce high-avidity CD8$^+$ CTLs. Therefore, intranasally administered IL-1α, IL-18, and IL-33 might be useful adjuvants for development of an effective mucosal influenza vaccine.

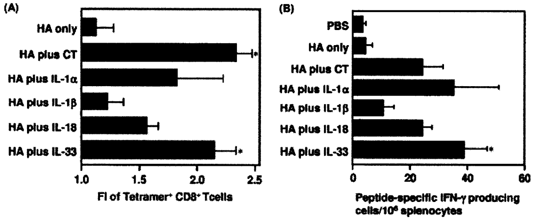

FIG. 4. Measurement of H-$2K^d/HA_{240-248}$ tetramer$^+$ CD8$^+$ T cells and H-$2K^d/HA_{240-248}$-specific IFN-γ-secreting cells in the spleen after nasal immunization with rHA plus an IL-1 family cytokine. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT, or rHA plus an IL-1 family cytokine. Fourteen days after the final immunization, splenocytes from immunized mice were harvested and stimulated with H-$2K^d$-restricted class I HA peptide at a final concentration of 10 μg total peptide/ml. (A) For detection of H-$2K^d/HA_{240-248}$ tetramer$^+$ CD8$^+$ T cells, splenocytes from immunized mice were cultured in medium containing a CTL epitope peptide ($HA_{240-248}$; IYSTVASSL) plus 10 U human IL-2/ml for 7 days, stained for CD8, and analyzed for tetramer-binding cells by flow cytometry. FI, fluorescence intensity. (B) After 24 h of incubation, IFN-γ-producing cells were measured by an ELISPOT assay. Data are presented as means ± SEM ($n = 5$). *, $P < 0.05$ compared to the value for the rHA-treated group.

**Histopathological changes due to IL-1 family cytokines administered intranasally as mucosal vaccine adjuvants.** Although enterotoxin-based adjuvants show strong mucosal immunity-inducing ability, they have significant toxic side effects on the central nervous system due to the presence of a specific receptor, GM1 ganglioside, which is highly expressed in neuronal tissue (39). To evaluate the *in vivo* toxicity of IL-1 family cytokines, histopathological changes in nasal tissues of mice given 1 μg of IL-1 family cytokines were investigated. No histological changes indicative of severe inflammation or membrane barrier disruption were observed in the nasal cavities of mice nasally administrated 1 μg of an IL-1 family cytokine (Fig. 5A). In particular, there was no evidence of massive accumulations of mononuclear cells around the airways and blood vessels or of infiltrates in the nasal tissues for all mice examined. Importantly, mice immunized intranasally with IL-1 family cytokines did not induce the goblet cell hyperplasia observed in patients with asthma and chronic obstructive pulmonary disease. Furthermore, Luna staining revealed that IL-1 family cytokine-treated mice did not develop infiltration of Luna-stained eosinophils into the nasal septum (Fig. 5B). Although further evaluation is required, these results indicate that the toxicity of IL-1 family cytokines is likely to be relatively low.

**Antiviral immune response to influenza virus infection in mice after nasal immunization with IL-1 family cytokines as mucosal vaccine adjuvants.** To determine the level of protection against viral infection provided by IL-1 family cytokines, BALB/c mice were immunized intranasally with 1 μg PR8 HA alone or with 1 μg of an IL-1 family cytokine on days 0 and 28. The immunized mice were then challenged with 256 HAU of mouse-adapted PR8 virus 14 days after the final immunization. The survival and weight of the infected mice were observed every other day (Fig. 6). All mice in the group receiving PBS alone and 86% of the mice immunized with PR8 HA alone died within 7 days of infection. In contrast, mice immunized intranasally with PR8 HA plus an IL-1 family cytokine showed a marked increase in survival (Fig. 6A). Notably, mice immunized with PR8 HA plus IL-1β or IL-18 had 100% survival 14 days after challenge, though with a slight loss of body weight (Fig. 6B). These results indicate that IL-1 family cytokines are potent nasal vaccine adjuvants for providing protection against viral infection.

**Role of MCs in rHA-specific immune responses induced by nasal immunization with rHA plus IL-1 family cytokines.** MCs are localized predominantly at the interface between the host and the environment (i.e., skin and mucosal surfaces). Recent reports have demonstrated the importance of IL-18-mediated MC activation for host defense, including innate sensing of pathogens (35) and recruitment of DCs and T lymphocytes to sites of inflammation. These findings prompted us to investigate whether MCs have a significant role in the immune response induced by IL-1 family cytokines as mucosal vaccine adjuvants. Hence, we examined MC-dependent rHA-specific systemic IgG and mucosal sIgA Ab responses induced by IL-1 family cytokine adjuvants. For this study, we compared the induction of specific Ab responses in MC-deficient (*W/W^v*) and WT mice immunized intranasally with rHA plus an IL-1 family cytokine (Fig. 7A and B). Both WT and *W/W^v* mice immunized with rHA had only minimal rHA-specific IgG Ab responses. However, rHA plus an IL-1 family cytokine induced significant rHA-specific IgG Ab responses in WT mice. *W/W^v* mice immunized with rHA plus IL-1α, IL-1β, or IL-33 also had significant rHA-specific IgG Ab responses (Fig. 7A), suggesting that IL-1α, IL-1β, and IL-33 act in an MC-independent manner. In contrast, the rHA-specific IgG Ab response induced in *W/W^v* mice by IL-18 was considerably lower than that in WT mice (Fig. 7A). Similar results were found for mucosal sIgA Ab responses: a significant response was seen with rHA plus IL-1α, IL-1β, or IL-33 in both WT and *W/W^v* mice, and a decreased response was seen with rHA plus IL-18 in *W/W^v* mice compared to WT mice (Fig. 7B). We then compared IL-4, IL-5, IL-2, and IFN-γ production in WT and *W/W^v* mice immunized with rHA plus IL-1 family cytokines (Fig. 7C). WT mice immunized with rHA plus IL-1 family cytokines showed significantly more rHA-specific IL-4, IL-5, IL-2, and IFN-γ production than did WT mice immunized with rHA alone. In contrast, the responses induced by rHA plus IL-18 were sig-

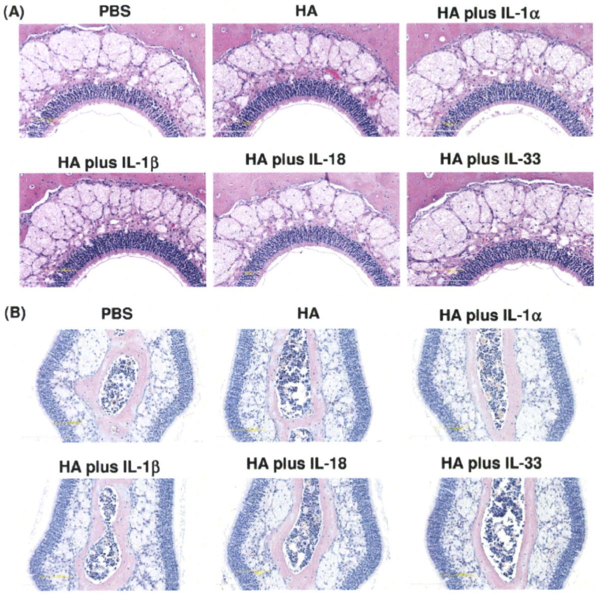

FIG. 5. Histopathological analysis of the nasal cavities of mice immunized intranasally with IL-1 family cytokines. Frontal cross sections of the nasal cavities of mice were taken after two administrations of PBS, rHA alone, or rHA plus an IL-1 family cytokine. Sections were prepared and stained with H&E (A) or Luna stain (B) to assess pathological changes. Overall views of the nasal epithelium (A) and of Luna-stained eosinophils in the nasal septum (B) are shown.

nificantly reduced in $W/W^v$ mice. In addition, although rHA-specific IL-2, IL-4, and IL-5 production in $W/W^v$ mice immunized with rHA plus IL-33 was comparable to that in WT mice, the rHA-specific IFN-γ response was significantly reduced in $W/W^v$ mice. Collectively, these results indicate that MCs have a crucial role in the rHA-specific immune response induced by nasal immunization with rHA plus IL-18. In particular, MCs appear to have an important role in regulating rHA-specific

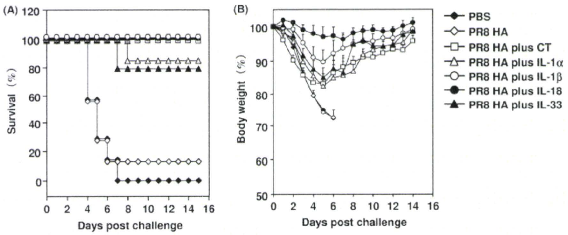

FIG. 6. Protection of BALB/c mice against lethal influenza virus infection by IL-1 family cytokine adjuvants. BALB/c mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus an IL-1 family cytokine (1 μg/mouse). Fourteen days after the final immunization, mice were intranasally infected with 256 HAU of influenza virus A/PR/8/34. Mice were monitored for survival (A) and weight loss (B) for 14 days after infection. The results are expressed as percent survival (A) and percent initial body weight (B). Data are presented as means ± SEM ($n$ = 4 to 7).

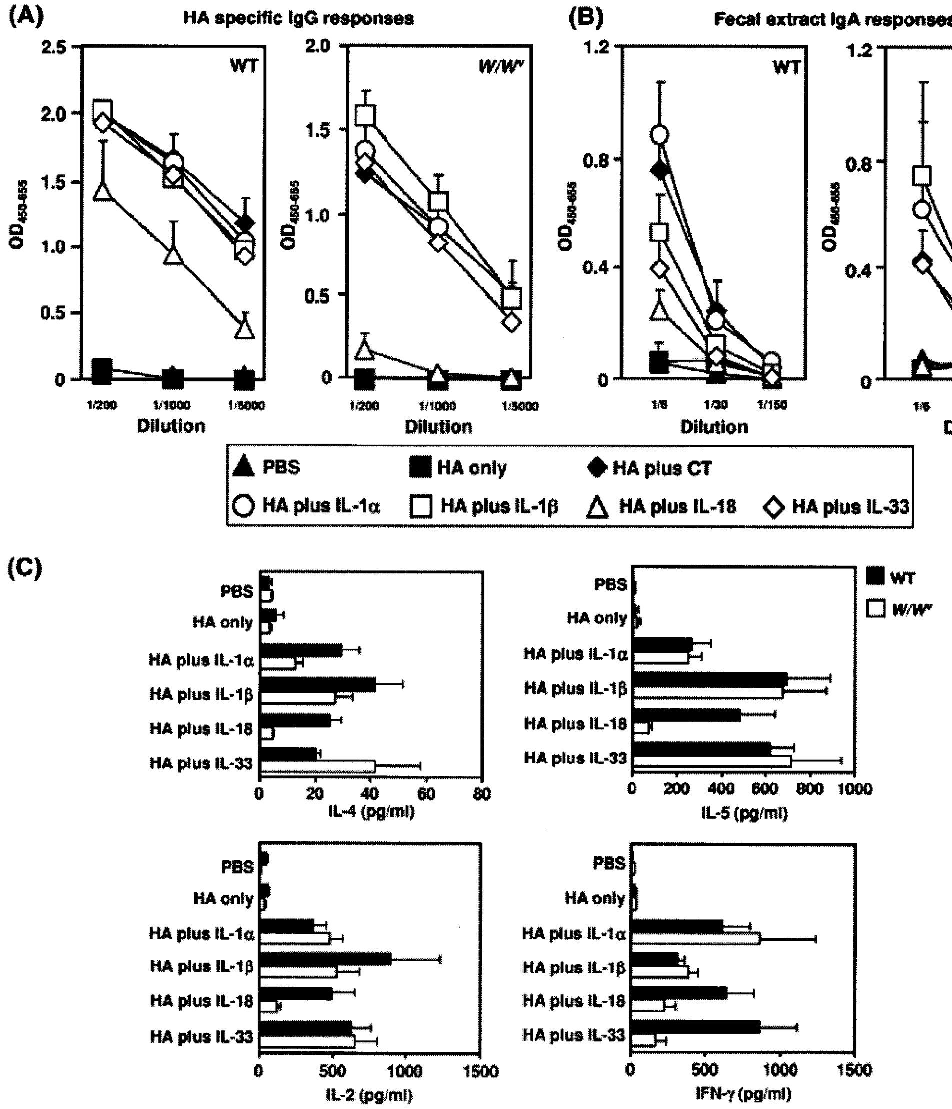

FIG. 7. Role of MCs in induction of rHA-specific immune responses by nasal immunization with rHA plus IL-1 family cytokines. WBB6F1 *W/W^v* and WT mice were immunized intranasally at 0 and 28 days with rHA alone, rHA plus CT (1 μg/mouse), or rHA plus an IL-1 family cytokine (1 μg/mouse). Plasma and fecal extracts were collected 14 days after the final immunization and analyzed by ELISA for rHA-specific IgG in plasma (A) and rHA-specific sIgA in fecal extracts (B). (C) Also, 14 days after immunization, splenocytes from each group of WBB6F1 *W/W^v* and WT mice were cultured with 10 μg rHA/ml. Culture supernatants were harvested after a 3-day incubation, and rHA-specific cytokine production (IL-4, IL-5, IL-2, and IFN-γ) in the culture supernatants was analyzed using a Bio-Plex multiplex cytokine assay. Data are presented as means ± SEM (*n* = 5).

IFN-γ-mediated Th1-type immunity in mice immunized with rHA plus IL-33 as a mucosal vaccine adjuvant.

## DISCUSSION

Of the 26 different IL cytokines studied here, intranasal immunization with rHA plus an IL-1 family cytokine (IL-1α, IL-1β, IL-18, and IL-33) induced the highest levels of rHA-specific systemic IgG. High levels of sIgA were also observed in the mucosa of IL-1 family cytokine-treated mice. However, IL-12 and IL-15 have been reported to promote systemic and mucosal immunity to intramucosally coadministered protein Ags (8, 45), although more frequent immunization was required to produce adjuvant activity. The apparent discrepancy concerning the adjuvant activity of IL-12 and IL-15 in this study and previous reports may be due to differences in immunization regimens and vaccine doses.

For IL-1 family cytokines, we showed that intranasal administration of rHA plus IL-1α, IL-18, or IL-33 induced higher levels of CD8$^+$ CTLs than intranasal administration of rHA alone, whereas the level induced by rHA plus IL-1β was similar to that induced by rHA alone. In agreement with these results, IL-1β has been reported to have a pivotal role in development of Th2-type immune responses (20). A previous report by

Shibuya et al. (36) showed that IL-1α is necessary for optimal Th1 development and IFN-γ secretion in BALB/c mice. In addition, Karupiah et al. (46) showed that IL-18 and IL-12p40 regulate cellular immune responses through $CD8^+$ T-cell activation. Thus, our data are in agreement with previous reports that IL-1α and IL-18 play a pivotal role in inducing Th1-type immune responses. Furthermore, there have been a few reports on the potential of IL-33 to induce a Th1-type immune response (37). In the present study, we showed that of the IL-1 family cytokines, IL-33 induced the highest levels of CTL and IFN-$\gamma^+$ cells. We are currently investigating the mechanism of IL-33 in Th1/CTL immunity.

We found that intranasal coadministration of influenza vaccine with IL-1 family cytokines provided protection against influenza viral infection, with IL-1β and IL-18 providing complete protection. It is known that nasal secretions containing locally produced sIgA and serum-derived IgG Abs contribute to forming a first line of defense for combating influenza viral infections (42, 44). Therefore, the prophylactic effects of IL-1 family cytokines may be due mainly to Ab-mediated immunity against influenza virus. Furthermore, previous studies have pointed out the importance of influenza-specific $CD8^+$ CTLs for host recovery from lethal influenza virus infections and protection against further infection (7, 15). Although the mechanism by which IL-18 provided complete protection against influenza remains to be elucidated, high-avidity $CD8^+$ CTLs induced by IL-1α, IL-18, or IL-33 probably confer protection against influenza viral infection. Recently, a requirement for NK cells or NKT cells for control of influenza virus infections was identified (10, 13). Since IL-18 is known to regulate NK and NKT cell activity (4, 38), it is possible that restimulation of these cells may have resulted in the reduction in virus replication and morbidity observed after viral challenge. We are currently investigating the involvement of these cell subsets in the induction of protection against influenza virus by IL-18.

Unfortunately, potent adjuvant action is often correlated with increased toxicity, as exemplified by CT adjuvant, which although it is potent is too toxic for human use. Therefore, one of the major challenges in adjuvant research is to gain potency while minimizing toxicity (17). Intranasal administration of 1 μg of an IL-1 family cytokine for four consecutive days has been shown to induce asthma-like symptoms, including airway hyperresponsiveness and goblet cell hyperplasia in the lungs (26). In contrast, in this study, we found that mice immunized intranasally with IL-1 family cytokines did not exhibit acute toxicity, i.e., there was no cytokine-induced mortality, no obvious weight loss, no abnormal behavior, and no histopathological changes. In addition, use of 0.1 μg of an IL-1 family cytokine as a nasal vaccine adjuvant was still effective at inducing systemic IgG and nasal sIgA Ab responses. Thus, although further safety evaluation is needed, our findings indicate a broad therapeutic utility for IL-1 family cytokines when used as adjuvants for mucosal vaccination.

To develop optimal vaccines for clinical applications, it is important to understand their mechanism of action on the immune system in terms of efficacy as well as safety (23). The present study demonstrates that the enhanced mucosal vaccine adjuvant effect of IL-18 operates via an MC-dependent mechanism. The rHA-specific immune response induced by intranasally administered rHA plus IL-18 in WT mice was significantly reduced in *W/W^v^* mice. In addition, the level of the rHA-specific IFN-γ response in mice intranasally immunized with rHA plus IL-33 was minimal in *W/W^v^* mice. Although studies are needed on the role of MCs in generation of Ag-specific immunity, the studies reported here show that MCs have a role in the effect of IL-18 as an adjuvant and in augmentation of the CTL response induced by IL-33 as a nasal vaccine adjuvant. MC activators (e.g., compound 48/80) have been reported to stimulate protective immune responses against infections (28, 32). In addition, these immune responses are correlated with DC trafficking and lymphocyte recruitment to draining lymph nodes (DLN). Nakae et al. (30) suggested that MC-derived tumor necrosis factor alpha (TNF-α) is required for enhanced recruitment of lymphocytes and DCs to DLN. MC-dependent induction of IL-18 mucosal vaccine adjuvant activity may involve these types of processes. In agreement with this possibility, the IL-18 receptor was highly expressed on the surfaces of MCs but not in nasal passage-associated lymphoid tissue CD11$c^+$ DCs, and IL-18 induced robust TNF-α and IL-6 production from MCs in a concentration-dependent manner *in vitro* (unpublished data). Although further studies are required, IL-18 appeared to exhibit MC-dependent adjuvant activity that was not directly regulated by DC functions, such as DC migration and DC activation.

In summary, IL-1 family cytokines used as mucosal vaccine adjuvants induced two layers of protective immunity when administered intranasally with an influenza virus vaccine Ag, indicating that they may be suitable for use in antiviral nasal vaccines.

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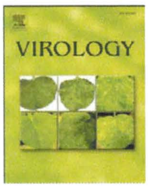

# Rapid and efficient introduction of a foreign gene into bacterial artificial chromosome-cloned varicella vaccine by Tn7-mediated site-specific transposition

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ABSTRACT

Using a rapid and reliable system based on Tn7-mediated site-specific transposition, we have successfully constructed a recombinant Oka varicella vaccine (vOka) expressing the mumps virus (MuV) fusion protein (F). The backbone of the vector was our previously reported vOka-BAC (bacterial artificial chromosome) genome. We inserted the transposon Tn7 attachment sequence, LacZα–mini-*att*Tn7, into the region between ORF12 and ORF13 to generate a vOka-BAC-Tn genome. The MuV-F expressing cassette was transposed into the vOka-BAC genome at the mini-*att*Tn7 transposition site. MuV-F protein was expressed in recombinant virus, rvOka-F infected cells. In addition, the MuV-F protein was cleaved in the rvOka-F infected cells as in MuV-infected cells. The growth of rvOka-F was similar to that of the original recombinant vOka without the F gene. Thus, we show that Tn7-mediated transposition is an efficient method for introducing a foreign gene expression cassette into the vOka-BAC genome as a live virus vector.

## Introduction

Varicella-zoster virus (VZV) is a human herpesvirus that causes varicella (chicken pox) and zoster (shingles). Varicella is characterized by viremia and skin lesions as a primary infection in children (Cohen et al., 2007). Herpes zoster is caused by the reactivation of VZV from sensory ganglia, where VZV establishes latency. The incidence of herpes zoster increases with age or immunosuppression (Arvin, 1996).

To control VZV infections, a live attenuated varicella vaccine, the Oka vaccine strain (vOka), was developed from the Oka parental strain (pOka) (Takahashi et al., 1974). The vOka was attenuated by multiple passages in human and guinea pig cells, and the resulting varicella vaccine became the first herpesvirus vaccine licensed for use in humans. This vaccine is efficient and causes few adverse events, and consequently is the only strain to be used worldwide (Arvin, 2001; Chaves et al., 2008).

Because this vaccine has been studied for more than 20 years, its immunogenicity and safety are well documented (Arvin and Greenberg, 2006; Galea et al., 2008; Takahashi et al., 1992), which provides a distinct advantage in choosing it as a vector over other attenuated vaccines. In addition, because of the size of its genome, it can accommodate and maintain large DNA inserts. vOka has been used as a vector to express the Epstein–Barr virus membrane glycoprotein (gp350/220) (Lowe et al., 1987), hepatitis B surface antigen (Shiraki et al., 1991), human immunodeficiency virus Env (Shiraki et al., 2001), and herpes simplex virus type 2 glycoprotein D (Heineman et al., 1995).

We recently succeeded in cloning the vOka genome into a bacterial artificial chromosome (BAC) (Yoshii et al., 2007). This vOka-BAC system allows the stable maintenance of the vOka genome in *E. coli*, and is useful for inserting foreign genes into the vOka genome (Wagner et al., 2002). Using the vOka-BAC system, we previously constructed a recombinant vOka containing the mumps virus (MuV) hemagglutinin–neuraminidase (HN) gene, rvOka-HN (Somboonthum et al., 2007), and showed that the recombinant vOka is potentially useful as a polyvalent vaccine providing protection for both VZV and mumps virus infections. Since the MuV-F protein has also been reported to play an important role in protection (Hishiyama et al., 1988; Sakata et al., 1984), the role of the F protein should also be considered.

Previously, we used RecA recombinase to introduce the HN gene into the vOka-BAC genome (Somboonthum et al., 2007). However, it takes 4–5 weeks to accomplish this kind of RecA recombinase-mediated recombination in *E. coli*, and the method is complicated.

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Transposons are DNA segments that can translocate from place to place in the genome. The bacterial transposon Tn7 targets a specific site in *E. coli* genome called *att*Tn7 (Barth et al., 1976; Lichtenstein and Brenner, 1981), which contains a 68-base-pair DNA segment that is essential for targeting by Tn7 (McKown et al., 1988). Four Tn7-encoded genes, tnsABC and tnsD are required for the target site-specific transposition of Tn7 (Kubo and Craig, 1990; Rogers et al., 1986; Waddell and Craig, 1988). TnsA, TnsB and TnsC form a core recombination machine that recognizes the transposon ends and execute DNA breakage and joining, and they are specifically targeted to *att*Tn7 by the function of TnsD.

Previously, Luckow et al. (1993) have described a rapid and efficient method for generating a recombinant baculovirus bacmid using *Autographa californica* nuclear polyhedrosis virus; this method is based on the site-specific transposition of Tn7 in *E. coli*. This baculovirus bacmid contains a mini-F replicon, a kanamycin-resistance marker, and a segment of DNA carrying the LacZα gene with a mini-*att*Tn7, which is the attachment site for Tn7. Because the foreign gene that is inserted into the mini-*att*Tn7 on the bacmid disrupts the expression of the LacZα peptide, colonies containing the recombinant bacmid are white when grown on medium containing X-Gal. This system has been developed into the commercially available AcNPV Bac-to-Bac™ Baculovirus Expression System (Invitrogen). It has also been applied to a herpesvirus, mouse cytomegalovirus and this technology should facilitate detailed mutagenic studies of herpesvirus genes and *cis* elements (Hahn et al., 2003).

In this report, we adapted this use of site-specific transposition by Tn7 to introduce a gene expression cassette into the vOka-BAC genome. Once a selectable marker (LacZα gene with a mini-*att*Tn7) is inserted into a specific site into the vOka-BAC genome with homologous recombination using RecA, the Tn7-mediated site-specific transposition can be done easily to rapidly introduce genes into the vOka-BAC genome. Finally, here we have succeeded in making a recombinant vOka expressing MuV-F gene by using this system. This system may be clinically applicable to the construction of a live polyvalent vaccine that is based on the current varicella vaccine.

## Results

### *Construction of a vOka-BAC-Tn and insertion of a foreign gene expression cassette*

The Tn7 attachment sequence, LacZα-mini-*att*Tn7, was inserted into the intergenic region between ORF12 and ORF13 of the vOka-BAC genome by RecA-mediated recombination to generate vOka-BAC-Tn (Figs. 1A–C), as described in Materials and methods. The insertion of the LacZα-mini-*att*Tn7 sequence into the BAC genome was confirmed by blue-white selection, PCR and Southern blotting.

The MuV-F donor plasmid, pFASTBac-MuV-F (Fig. 1D) was introduced into *E. coli* DH10B harboring the vOka-BAC-Tn genome and the helper plasmid, pMON7124, which expresses the Tn7 proteins necessary for the transposition of mini-Tn7. The insertion of the MuV-F expression cassette into the mini-*att*Tn7 attachment site on the vOka-BAC-Tn disrupted the expression of the LacZα peptide. Therefore, the *E. coli* colonies containing the vOka-BAC-F were distinguished by blue-white selection. The transposition of the MuV-F expression cassette into the vOka-BAC genome was confirmed by PCR (data not shown). The completed schematic flow of the construction of the vOka-BAC-F genome is shown (Fig. 1E).

The insertion of LacZα-mini-*att*Tn7 and MuV-F at the correct site of the vOka-BAC genome was verified by restriction enzyme digestion and Southern blot analyses using BamHI-digested BAC genome probed with either ORF12 or MuV-F gene (Fig. 2B). The 7.8 kb fragment of vOka-BAC genome disappeared in the vOka-BAC-Tn and vOka-BAC-F genome by the insertion of LacZα-mini-*att*Tn7 (Figs. 2B and C). In vOka-BAC-Tn and vOka-BAC-F genome, the 3.3 kb fragment was detected by an ORF12 probe (Fig. 2C. lanes 2, 3). The transposed MuV-F gene was detected as a 2.9 kb fragment with MuV-F probe (Fig. 2D lane 3). These results indicate that these fragments were successfully inserted into the vOka-BAC genome.

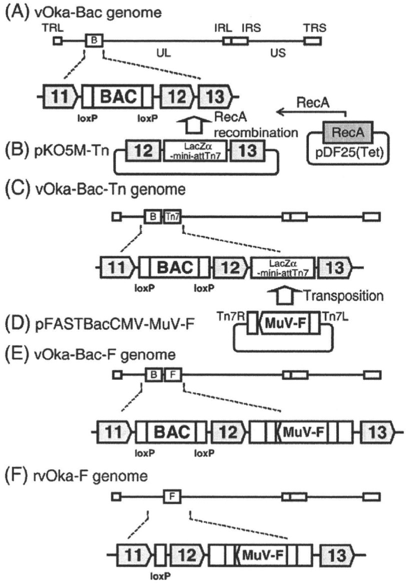

**Fig. 1.** Construction of the rvOka-F genome. The vOka-BAC genome (A) is about 125-kbp long and includes terminal repeats (TRL and TRS), a unique long (UL) domain, internal repeats (IRL and IRS), and a unique short domain (US). Note that the BAC vector sequence is flanked by Cre–lox recombination sites (loxP). The LacZα-mini-*att*Tn7 sequence in pKO5M-Tn (B) was inserted between ORF12 and ORF13 of the vOka-BAC genome by RecA-mediated recombination. RecA was encoded by pDF25 (tet). The LacZα-mini-*att*Tn7 sequence in the vOka-BAC-Tn (C) genome permitted site-specific insertion of the MuV-F expression cassette of pFASTBacCMV-MuV-F plasmid (D) by Tn7-mediated transposition. The BAC sequence was removed from vOka-BAC-F (E) by Cre-mediated recombination. The resulting bacmid, rvOka-F (F), was used for reconstitution of the recombinant virus.

### *Reconstitution of infectious virus from the vOka-F-BAC DNA*

To examine whether infectious viruses could be reconstituted from the vOka-F-BAC DNA, MeWo cells were transfected with vOka-F-BAC using Lipofectamine 2000 (Invitrogen). Five days after the transfection, typical cytopathic effects along with green fluorescence, were observed by fluorescence microscopy, indicating that vOka-F-BAC had been reconstituted as an infectious recombinant virus.

Because herpesviruses containing the additional BAC sequence might undergo mutations caused by the insertion of long non-VZV sequences, the BAC sequence, including the gpt and gfp genes, was excised from the rvOka-F-BAC genome, using the Cre-loxP system (Fig. 1F), as described previously (Somboonthum et al., 2007), to generate the recombinant virus, rvOka-F. Correct excision of the BAC sequence from the recombinant virus was confirmed by sequencing

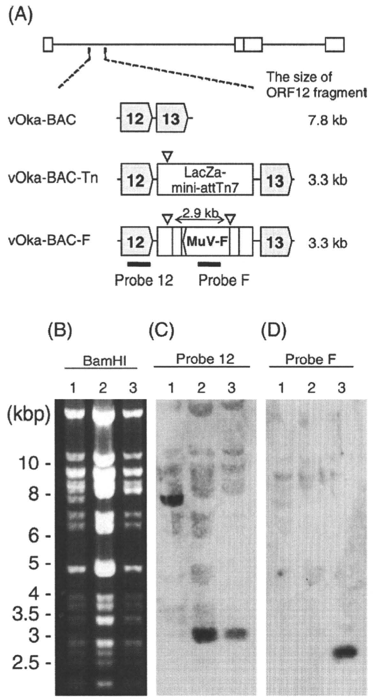

**Fig. 2.** Restriction enzyme digestion and Southern blot analyses. (A) Schematic diagram of the inserts at the ORF12–13 loci. Location of BamHI sites are indicated as arrowheads. The size of ORF12 fragment by BamHI digestion was indicated at the right. The locations of probes were indicated at below. (B–D) vOka-BAC, vOka-BAC-Tn and vOka-BAC-F were digested with BamHI and separated on 0.5% agarose gel. Molecular size markers are shown at the left. Southern blot analysis of BamHI-digested vOka-BAC (lanes 1), vOka-BAC-Tn (lanes 2) and vOka-BAC-F (lanes 3) were performed. Probes used were indicated.

(data not shown). The recombinant virus was amplified and used for further experiments.

### *The expression of a foreign gene from recombinant virus*

To determine the expression of the MuV-F protein, Western blotting analysis was performed (Figs. 3A and B). Recombinant vOka-infected MeWo cell lysates and MuV-infected Vero cell lysates were separated by SDS-PAGE and analyzed by Western blotting with anti-VZV glycoprotein B (gB) or anti-MuV-F polyclonal antibody. As shown in Fig. 3B, VZV-gB was detected in lysates from cells infected with both the control rvOka (Fig. 3B lane 2) and rvOka-F (Fig. 3B lane 3). On Western blots of rvOka-F-infected cell lysates, the anti-MuV-F antiserum specifically reacted with 60-kDa and 15-kDa bands (Fig. 3A, lane 3). Both bands were also detected in MuV-infected Vero cell lysates (Fig. 3A lane 5).

MuV-F is first translated as a 70-kDa F0 protein, which is cleaved to produce the mature protein (von Messling et al., 2004). Because the anti-MuV-F polyclonal antibody reacted with the C-terminal region of the MuV-F protein, it could detect the 60-kDa and 15-kDa bands, which are the predicted sizes of MuV-F1 and F1b, respectively. The detection of the 15-kDa species indicated that the foreign antigen was processed into its natural, mature form in the rvOka-F-infected cells. We also confirmed the expression of the MuV-F protein by immunofluorescence (Fig. 3C). As shown in Fig. 3C, MuV-F protein was detected in VZV-gB-positive cells, indicating that MuV-F protein was expressed in rvOka-F-infected cells, as expected. However, MuV-F did not co-localize with VZV-gB within the cells. These results indicate that the MuV-F protein was expressed and maturated normally in the rvOka-F infected cells. The localization of MuV-F protein in rvOka-F infected cells was the same as that of MuV-infected Vero cells (Fig. 3C).

### *Growth of the recombinant virus*

To compare the growth between rvOka and rvOka-F, we compared their plaque sizes and growth curves using MRC-5 cells. As shown in Fig. 4, plaque size (Fig. 4A) and growth curve (Fig. 4B) observed with rvOka-F infection were similar to those observed with rvOka, indicating that the normal growth characteristics of rvOka-F were retained.

### *Immunization of guinea pigs with rvOka-F induces neutralizing antibodies against VZV, but not MuV infection*

All the guinea pigs inoculated with rvOka-F-infected cells, showed antibody responses against both VZV (1:3200 ± 924) and MuV (1:1066 ± 462 ), but none of those inoculated with the control did. Neutralizing anti-VZV and anti-MuV antibody titers were also determined by a plaque reduction assay. The serum obtained from all the guinea pigs inoculated with rvOka-F-infected cells contained neutralizing antibodies against VZV (Table 1), but not MuV (Table 2).

## Discussion

In this report, we succeeded in constructing a rapid and handy VZV recombination system that utilized the Tn7 transposon to integrate gene(s) of interest into the vOka-BAC genome. The site-specific insertion of the foreign gene expression cassette was straightforward, and the selection of colonies bearing the recombinant BAC was easy, via the LacZα-based blue/white selection. This system permits the rapid construction of a recombinant vOka genome that harbors foreign gene(s) and the reconstitution of the infectious, recombinant virus from the genome.

The vOka-BAC genome has several advantages as a candidate for a live viral vector. First is its long history as a safe and efficient live vaccine (Chaves et al., 2008; Galea et al., 2008). vOka is the only live attenuated vaccine permitted for preventing VZV infection, and the only live DNA vaccine that is acceptable for use in immunocompromised individuals (Gershon et al., 1984; Kamiya et al., 1984).

Next, the vOka-BAC system is an efficient method for generating the recombinant vector in *E. coli*. Furthermore, the use of the transposon system, established here, makes the insertion of a foreign gene into vOka-BAC genome quick and easy. Thus, the vOka-BAC-transposon system may be useful as a tool for the construction of live varicella vaccines that also express specific genes of interest.

Finally, unlike other recombinant vaccines, a live recombinant vaccine has the potential to induce both humoral and cell-mediated immunity. Varicella vaccine induces both immunities, including delayed-type hypersensitivity. We showed here that the foreign antigen harbored by rvOka-F was expressed in rvOka-F-infected cells and posttranslationally modified as in a natural MuV infection. Their

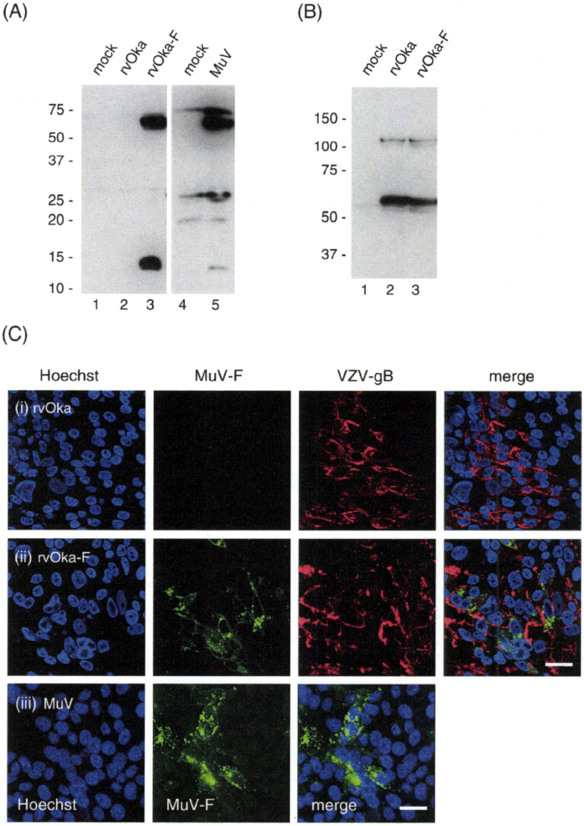

**Fig. 3.** The expression of MuV-F protein in rvOka-F infected cells. (A) MeWo cells were mock infected (lane 1; mock) or infected with rvOka (lane 2) or rvOka-F (lane 3) by cell-to-cell spread. Vero cells were mock infected (lane 4) or infected with MuV strain Urabe (lane 5). Samples were separated by 13% SDS-PAGE and analyzed by Western blotting with anti-MuV-F polyclonal antibody. (B) MeWo cells were mock infected or infected with rvOka or rvOka-F by cell-to-cell spread. Samples were separated by 8% SDS-PAGE and analyzed by Western blotting with anti-VZV-gB polyclonal antibody. (C) MeWo cells infected with cell-free rvOka (i) or rvOka-F (ii) were fixed at 72 hpi and analyzed by confocal microscopy. Cells were double-labeled with MuV-F polyclonal antibody and VZV-gB monoclonal antibody. Vero cells infected with MuV (iii) were fixed at 24 hpi and analyzed by confocal microscopy. Cells were labeled with MuV-F polyclonal antibody. Scale bar: 30 μm.

native tertiary structure may be important for eliciting protective humoral immunity. Expression of foreign antigen in the context of the major histcompatibility complex antigen would result in enhanced induction of cytotoxic T lymphocytes. Therefore, we expect that our recombinant vOka vaccine will be able to induce cell-mediated immunity as well as humoral immunity also against foreign protein(s).

However, the expression of foreign gene encoded in vOka-BAC genome may affect the cell tropism and/or pathogenesis of the recombinant viruses. Especially if the foreign proteins are expressed on the viral envelope, the tropism may be changed. Thus, the insertion of foreign genes into vOka which affect cell tropism and viral pathogenesis might be excluded in future vaccine candidates. Therefore, the development of the polyvalent vaccine based on VZV should be done carefully considering these points. If the vaccine is safe, the reactivation of VZV would be advantageous because it could induce the immunity against foreign proteins as well as VZV-encoded proteins. In addition, to consider with the attenuated property of vOka,

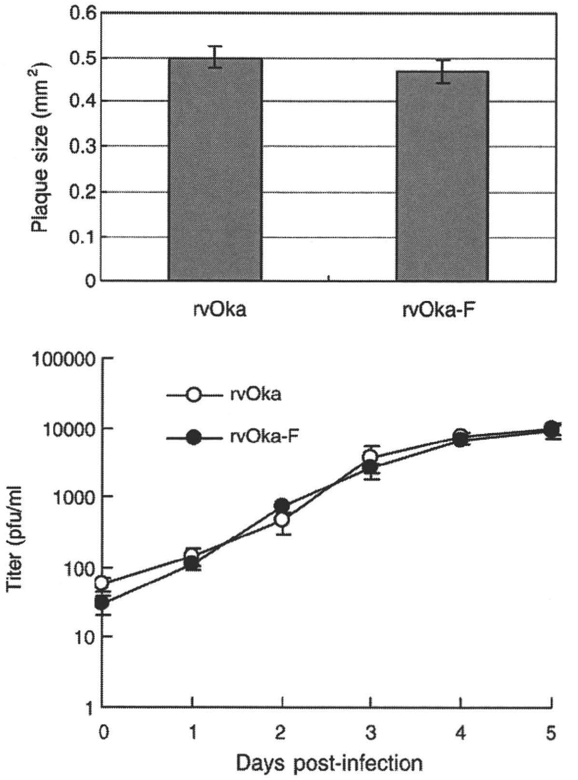

**Fig. 4.** Growth of the recombinant virus. (A) Plaque sizes of rvOka or rvOka-F. MRC-5 cells infected with the indicated virus were cultured for 2 weeks, fixed, and stained with 1% crystal violet. The areas of the plaques were calculated with NIH-image. (B) Infectious center assay of rvOka and rvOka-F. MRC-5 cells in 35-mm dishes were infected independently with similar titers of the indicated cell-free viruses, and then the cells were washed and trypsinized on days 0 to 5 pi. The trypsin-treated, infected cells were diluted and transferred onto a monolayer of MRC-5 cells, and the number of infected cells was assessed by counting the number of VZV plaques appearing after 7 days. The result shown is representative of three independent experiments.

the recombinant viruses produced from the vOka-BAC genome appear to be safe as well as original vOka. However, since the molecular mechanism of attenuation remains unknown, further study will be required to elucidate the mechanism of the vaccine attenuation. In the case of the recombinant virus used here, the commercially available antiviral drugs are able to be used effectively to control the infection of the recombinant virus, because TK gene of VZV remains in the genome.

The HN and F proteins are presented on the surface of mumps virus particles. Anti-F protein antibody contained in sera from patients and vaccinees contributes to the defense against MuV through activation of complement (Hishiyama et al., 1988; Sakata et al., 1984). Therefore the F protein was also thought to be a possible target in the host immune system. Here the MuV-F gene was successfully introduced into rvOka genome and the protein was expressed in rvOka-F infected cells in a similar manner to that seen in MuV-infected cells. However, the rvOka harboring F gene-immunized guinea pigs did not induce the production of neutralizing antibody against MuV, although it induced against VZV infection. The co-expression of both HN- and F proteins at the same cells might be required for the induction of the protective immunity against F protein. The construction of rvOka expressing both HN and F genes may be needed to compare the protective effect with that of rvOka expressing HN gene alone.

**Table 1**
Neutralizing antibody titers for VZV in guinea pigs immunized three times with rvOka-F.

| Immunization | Neutralizing antibody titers* | | | Mean |
|---|---|---|---|---|
| rvOka-F | 128 | 64 | 128 | 106.7 |
| Mock control | <8 | <8 | <8 | |

Two independent experiments were performed and three guinea pigs were used in one experiment.
One of two experiments is shown.
* Neutralizing antibody titer was defined as the reciprocal of the highest serum dilution that reduced the number of plaques by 90%.

**Table 2**
Neutralizing antibody titers for mumps virus in guinea pigs immunized three times with rvOka-F.

| Immunization | Neutralizing antibody titers* | | |
|---|---|---|---|
| rvOka | <10 | <10 | <10 |
| rvOka-F | <10 | <10 | <10 |
| Mock control | <10 | <10 | <10 |

Two independent experiments in triplicate were performed and three guinea pigs were used in one experiment.
One of two experiments is shown.
* Neutralizing antibody titer was defined as the reciprocal of the highest serum dilution that reduced the number of plaques by 50%.

Previous work (Houard et al., 1995) has shown that the recombinant vaccinia virus expressing F protein was able to induce neutralizing antibody in hamsters. The discrepancy found between this and ours, may be due to difference of animal model used or structure of F protein expressed. Further studies will be needed for the vaccine application of the recombinant virus.

## Materials and methods

### *Cells and viruses*

MRC-5 cells were cultured in modified minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). MeWo cells and Vero cells were cultured in Dulbecco's modified Eagle's medium supplemented with 8% FBS. A recombinant vOka-BAC clone (Yoshii et al., 2007), was used as the backbone for the recombination. rvOka and rvOka-F were propagated in MRC-5 cells. VZV-infected cells were cultured in DMEM supplemented with 3% FBS. The MuV Urabe strain was propagated in Vero cells.

### *Antibodies*

An anti-MuV-F rabbit polyclonal antibody was raised against an MuV-F peptide, NTISSSVDDLIRY, corresponding to the 13 carboxy-terminal residues. The anti-VZV-gB polyclonal (Sadaoka et al., 2007) and monoclonal (Okuno et al., 1983) antibodies were described previously. Secondary antibodies conjugated to fluorophores, anti-mouse IgG-Alexa Fluor 594 and anti-rabbit IgG-Alexa Fluor 488 (Invitrogen, Carlsbad, CA), were used for immunofluorescence assays. HRP-conjugated anti-rabbit IgG (GE Healthcare Bio-Science, Piscataway, NJ) was used for Western blotting.

### *Plasmids*

The gentamicin-resistance gene and the polyhedrin-promoter region of the pFASTBac1 plasmid (Invitrogen) were replaced with the CMV promoter to generate the pFASTBacCMV plasmid. The CMV promoter sequence was amplified by PCR from pcDNA3.1(+) and inserted into the SacII and BamHI sites of the pFASTBac1 plasmid. The MuV-F cDNA was inserted into the multi-cloning site of pFASTBacCMV to generate the pFASTBacCMV-MuV-F plasmid. The MuV-F cDNA was obtained as described previously (Somboonthum et al., 2007).

The VZV ORF 12 DNA fragment from the genomic position (in base pairs; bp) 16,689–18,352 was amplified using a primer pair that introduced Xho I and BamH I sites (underlined) at the 5′ and 3′ ends, respectively: 5′-TATCTCGAGAGGTACCGGTGACTTCAGAG-3′ (12TnFw) and 5′-CGAGGATCCAATCAACCAATCAGACCT-3′ (12TnRv). The VZV ORF 13 DNA fragment from bp 18,390 to 19,618 was amplified using a primer pair that introduced BamH I and Xho I sites (underlined) at the 5′ and 3′ ends, respectively: 5′-GAGGATCCGTACCCACAATATCAAGTGGT-3′ (13TnFw) and 5′-GACTCGAGCCTATTCGTGTCATCTAGATGG-3′ (13TnRv). The resulting fragments were digested with BamH I and Xho I and then ligated into the Xho I site of pBluescript SK(−) to generate pB1213Tn. pB1213Tn was digested with Xho I to release the 1213Tn fragment, which was then cloned into the Sal I site of pKO5M which encodes zeocin resistant gene (a kind gift from Dr. Y. Kawaguchi) (Tanaka et al., 2003), from which the BamH I site had been previously eliminated, to generate pKO5M-1213Tn. The LacZα–mini-*att*Tn7 region, which contains the attachment site for Tn7, of bMON14272 (Invitrogen) was amplified using the following primer pair: 5′-GAAGATCTGAACCAATACGCAAACCGCCTCTCCCCGCGCG-3′ (lacZα-PR1) and 5′-GAGAGTCTGAAGCGCGTAACCACCACACCCGCCGCGC-3′ (lacZα-PR2). The resulting fragment was digested with Bgl II and then cloned into the BamH I site of pKO5M-1213Tn to produce pKO5M-Tn (Fig. 1).

### *RecA-mediated recombination to generate the vOka–BAC–Tn*

The insertion of the LacZα–mini-*att*Tn7 cassette to produce vOka–BAC–Tn was performed using RecA-mediated recombination, essentially as described previously (Somboonthum et al., 2007). In brief, *E. coli* DH10B electrocompetent cells harboring circular vOka–BAC DNA genome, which encodes chloramphenicol resistant gene, were co-transformed with the targeting vector, pKO5M-Tn and pDF25(Tet) which encodes RecA gene and tetracycline resistant gene (a kind gift from Dr. J. Heath) (Lalioti and Heath, 2001) by electroporation, using a Gene Pulser II (Bio-Rad, Hercules, CA). The surviving co-integrant colonies, selected by their resistance to chloramphenicol and zeocin and a Lac+ phenotype on an LB plate containing X-Gal and IPTG, were made electrocompetent and transformed with pDF25(Tet). The desirable *E. coli* DH10B colonies containing the correct survival recombination were then selected upon by the following criteria: resistance to chloramphenicol, sensitivity to zeocin, and a Lac+ phenotype on LB containing X-Gal and IPTG. The correct integration of the insert was confirmed by restriction enzyme digestion.

*E. coli* DH10B harboring the vOka–BAC–Tn genome was transformed with pFASTBacCMV-MuV-F and the helper plasmid expressing the transposase, TnsABCD, pMON7124 (Invitrogen). The pMON7124 plasmid was isolated from DH10Bac cells (Invitrogen). The transformed *E. coli* was cultured on LB containing X-gal and IPTG for blue/white selection. White colonies were analyzed by PCR to verify the insertion of the MuV–F expression cassette.

### *Southern blotting*

vOka–BAC DNA, vOka–BAC–Tn DNA, or vOka–BAC–F DNA was digested with BamHI and separated by electrophoresis in 0.5% agarose gel. After transfer to Hybond-N+ nylon membranes (GE Healthcare Biosciences), the blots were hybridized with the appropriate DNA probe labeled with horseradish peroxidase using enhanced chemiluminescence direct nucleic acid labeling and detection systems (GE Healthcare Biosciences) according to the manufacturer's instructions.

### *Reconstitution of infectious virus from vOka–BAC DNA*

Reconstitution of the recombinant virus, named rvOka–F, was performed, with modifications, as described previously (Nagaike et al., 2004). Briefly, the recombinant bacmid was introduced into MeWo cells with Lipofectamine 2000 (Invitrogen). To remove the BAC sequence, which was flanked by loxP sequences and contained the gene for guanine phosphoribosyl transferase gene (gpt) and green fluorescence protein gene (gfp), MRC-5 cells were first infected with a recombinant adenovirus, AxCANCre (a kind gift from Dr. Y. Kawaguchi) (Tanaka et al., 2003), that expresses Cre recombinase. Twenty-four hours later, the cells were super-infected with the recombinant virus and cultured until plaques without GFP appeared. The GFP-negative plaques were isolated using glass isolation cups and transferred onto newly prepared MRC-5 cells. The successful excision of the BAC sequence was verified by PCR and the sequencing of cell-free viral DNA.

### *Immunofluorescence assays*

Immunofluorescence assays were performed, with modifications, as described previously (Koshizuka et al., 2008). Briefly, rvOka- or rvOka–F-infected MeWo cells, or MuV-infected Vero cells were fixed with cold acetone–methanol, and reacted with the anti-gB monoclonal and anti-MuV-F polyclonal antibodies. After the secondary antibody reaction, the cells were rinsed with PBS, mounted on glass slides, and observed with the Leica confocal microscope system.

### *Growth analysis of rvOka–F*

MRC-5 cells were infected with approximately 25 pfu of cell-free rvOka or rvOka–F virus, and incubated for 2 weeks at 37 °C followed by staining with 1% crystal violet/70% ethanol. The plaque sizes were calculated using NIH-image (NIH, USA). Infectious center assay was performed as described previously (Somboonthum et al., 2007).

### *VZV inoculation to animals*

VZV inoculation to guinea pigs was performed as described previously with slight modification (Somboonthum et al., 2007). The VZV-infected MRC-5 cells ($3 \times 10^6$ cells) suspended in PBS were administered to animals subcutaneously. The uninfected MRC-5 cells ($5 \times 10^6$ cells) in PBS were used as negative control. Three weeks after the inoculation, the same antigens were immunized to the animals again. Blood was collected by cardiac puncture 7 days after the second inoculation. Sera were inactivated at 56 °C for 30 min for the serological and Neutralization tests.

### *Plaque reduction neutralization assay for VZV and MuV*

The neutralizing anti-VZV antibody titers were also determined by a plaque reduction assay. Stock virus solution (0.1 mL containing 100 pfu of vOka strain) was mixed with 0.1 mL of serially diluted serum at the appropriate dilution (rvOka–F immunized serum; 1:16 to 1:512, or mock immunized serum; 1:8 to 1:256) and 0.1 mL of fresh guinea pig serum as a source of complement, and the mixtures were incubated at 37 °C for 30 min. Next, MeWo cells were inoculated with 0.2 mL of each mixture in six-well plates, and incubated at 37 °C for 1 h to allow adsorption. The inoculum was then removed, and the cells were washed and cultured for 5 days, followed by staining with 1% crystal violet/70% ethanol. The stained cells were then washed, and the plaques were counted. The neutralizing antibody titer was determined as the highest dilution of serum capable of reducing the number of virus plaques by 90% or more, compared with the control values, and expressed as the reciprocal of the dilution. Anti-MuV neutralizing antibody titers were determined by a plaque reduction assay as described previously (Somboonthum et al., 2007).

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