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Immune response to intranasal and intraperitoneal immunization with Kaposi's sarcoma-associated herpesvirus in mice

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ABSTRACT

A vaccine for Kaposi's sarcoma-associated herpesvirus (KSHV) is not currently available. To obtain the fundamental data in animals for vaccine development, KSHV particles were immunized to Balb/c mice through intraperitoneal and intranasal routes in the present study. Intranasal immunization with KSHV induced IgA to KSHV in not only serum, but also nasal wash fluid and saliva. A neutralization assay using recombinant KSHV that expressed green fluorescent protein revealed that nasal wash fluid and saliva from the KSHV-immunized mice neutralized KSHV infection to human embryonic kidney 293 cells in vitro in a dose-dependent manner to KSHV copies immunized. The serum and nasal wash fluid of KSHV-encoded K8.1 protein-immunized mice neutralized KSHV infection to 293 cells in vitro. These data suggest a possibility of mucosal vaccine for prophylaxis of KSHV infection.

1. Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) was identified as a causative agent of Kaposi's sarcoma (KS) in 1994 [1]. Since KSHV has been detected in all cases of KS, there is no doubt about the association between KS pathogenesis and KSHV infection [2]. More than 15 years after the discovery of KSHV, KS is still an important complication in AIDS patients. KS occurs frequently among human immunodeficiency virus (HIV)-infected men who have had sex with men (MSM), suggesting that homosexual behavior in males is an important risk factor for KS and KSHV infection [3]. Although vaccine is available for other herpes viruses, such as varicella zoster virus, KSHV vaccine is not available so far. There are several reasons why KSHV vaccine has not yet been developed.

First, most HIV-infected MSM are already infected with KSHV [3]. For example, an epidemiological study revealed that about 60% of HIV-infected MSM were positive for serum antibody to KSHV in Japan, suggesting widespread KSHV infection among MSM [4]. Immunodeficiency condition may cause some problems for vaccine to work in HIV-infected individuals [5]. However, vaccination of influenza vaccine to asymptomatic HIV-infected patients showed similar antibody production to uninfected group [6], suggesting possibility of vaccine strategy for KSHV in HIV-infected adults.

Second, immunohistochemical studies revealed that, while almost all KS cells express a latent protein, latency-associated nuclear antigen 1 (LANA-1), only a very small number (less than 1%) of KS cells express lytic proteins encoded by KSHV. This implies that replication of KSHV is very rare in KS regions, and latent KSHV infection is predominant and important in the pathogenesis of KS [7]. Generally, vaccine can prevent *de novo* infection or reactivation of virus in human bodies, but will not suppress function of latently infected virus. However, it is demonstrated that some lytic proteins encoded by KSHV such as K1, vGPCR, and vIL-6, promote KS development and angiogenesis. Condition with immunodeficiency is also required for KS pathogenesis. Thus, while LANA-1 may become a target of anti-tumor drug [8], KSHV vaccine may play a certain role in the suppression of lytic protein expression.

Third, it is difficult to evaluate a newly developed KSHV vaccine. Although it was recently demonstrated that common marmosets can be infected with KSHV [9], there is no convenient animal model in which KSHV can infect and replicate.

However, the occurrence of KS among MSM may still be prevented using a vaccine strategy. Although the details of infectious routes of KSHV are unknown, the mucosae in the oral cavity and rectum are possible entrances for KSHV, because saliva contains high copy numbers of KSHV, and because epidemiological studies have shown that KSHV infection is associated with homosexual behaviors [3,10]. Many studies have demonstrated that mucosal vaccine is a promising tool for prevention for viral and bacterial infections [11–16]. Those studies showed that the secreted form of IgA plays an important role in the mucosal immunity, and that

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mucosal immunity from IgA is more effective and cross-protective against viral infections than systemic immunity induced by serum IgG [17,18]. If the mucosae are main routes of KSHV infection, mucosal vaccine could become a tool to prevent the spread of KSHV among MSM.

Another reason for using vaccines for KSHV infection is that KS occurs frequently in HIV-infected MSM [19]. About 40% of HIV-infected MSM may be serologically negative for KSHV; they could be the target group for a KSHV vaccine [4]. Limiting use of an efficacious KSHV vaccine to $KSHV^{-}HIV^{+}$ MSM patients or $KSHV^{-}HIV^{-}$ MSM could prevent KS efficiently.

However, for vaccine development, there is little information about immune responses to KSHV infection in human and animals. KSHV infection in humans induces the production of serum antibodies to KSHV-encoded proteins [4,20]. Such serum antibodies recognize K8.1, ORF59, ORF65, and ORF73 (LANA-1) proteins encoded by KSHV as immunogens [4]. KSHV infection also induces CD8 T cells in the region of KS, which play an important role in the regression of KS in AIDS patients receiving highly active anti-retroviral therapy [21]. This information suggests that KSHV induces similar immune responses in human as do other herpes viruses. Nevertheless, KSHV does not infect normal mice or macaques [22–25]. There are few reports offering detailed descriptions of immune responses against KSHV in KSHV-immunized animals [9].

The aim of this study was to obtain fundamental data in animal experiments for KSHV vaccine development. To estimate immune responses against KSHV in animals, Balb/c mice were immunized intranasally or intraperitoneally with KSHV particles, and their immunoreactions were investigated. In addition, an *in vitro* neutralization assay was performed using green fluorescent protein-expressing recombinant KSHV and the serum, nasal wash fluid (NW), and saliva from the KSHV-immunized mice.

2. Materials and methods

2.1. Viruses

KSHV particles were prepared from BCBL-1 cells stimulated with phorbol 12-myristate-13 acetate (PMA; Sigma, St. Louis, MO) as described previously [26]. Briefly, BCBL-1 cells were stimulated with PMA at 20 ng/mL for 72 h. The supernatant of BCBL-1 cells was collected and filtered through a 0.8-μm-pored membrane. Filtered supernatant was ultracentrifuged at $20{,}000 \times g$ for 2 h. The pellet was dissolved in one-fiftieth volume of RPMI 1640. Virus copy number was measured with a real-time PCR as described previously [27]. A green and red fluorescent protein (GFP/RFP)-expressing recombinant KSHV, rKSHV.219 (kindly provided by Dr. Jeffrey Vieira, Washington University), was collected for the neutralization assay as described previously [28].

2.2. Immunization with virus or proteins

Female 8-week-old Balb/c mice were purchased from Clea Japan (Tokyo, Japan) and were kept under specific-pathogen-free conditions. 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 NIID (approvals No. 108056 and 209072). Five mice for each experimental group were anesthetized with isoflurane and immunized primarily by dropping 5 μl of phosphate buffered saline (PBS) containing 10^6–10^8 copies of KSHV or 10 ng of KSHV-encoded proteins with 10 μg of poly(I:C) (Sigma) into each nostril [29]. For immunization to the peritoneal cavity, 100-μl aliquots of PBS containing the viruses (10^6–10^8 copies) or proteins (100 ng) with poly(I:C) were immunized to the mice's peritoneal cavities. Additional immunizations were performed twice, 2 and 3 weeks later. Samples of blood, spleen, and NW were obtained from mice that were sacrificed under anesthesia with isoflurane 1 week after the final immunization. NW samples were taken as previously described [17]. Saliva samples were obtained using intraperitoneal administration of pilocarpine (150 μL of 1 mg/ml in PBS per mouse, P-6503, Sigma).

2.3. Real-time RT-PCR

Copy numbers of mouse IFN-γ, CD8 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined with real-time RT-PCR using probe-primer sets described previously [30]. Total RNA was extracted from 1×10^7 spleen cells of each mouse with Isogen RNA isolation kit (Nippon Gene, Toyama, Japan). Real-time RT-PCR was performed with one-step Quantitect probe RT-PCR kit (Qiagen, Hilden, Germany).

2.4. Detection of IFN-γ by ELISA

Spleen cells were cultured in RPMI 1640 for 8 h and mouse IFN-γ in supernatant was measured with ELISA (Quantikine Mouse IFN-γ Immunoassay Kit, R&D systems, Minneapolis, MN).

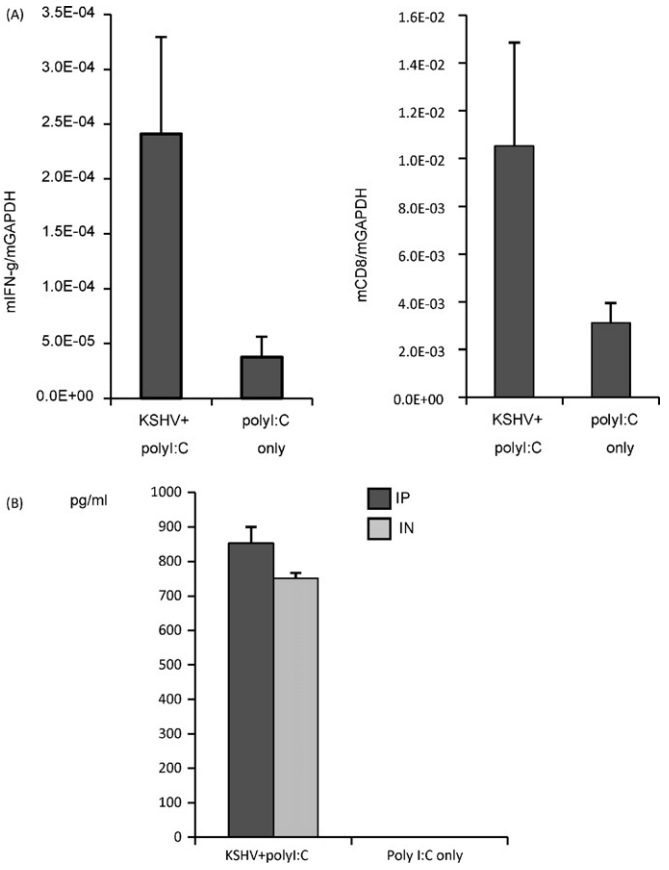

Fig. 1. Induction of cellular immune response in KSHV-immunized mouse. (A) Real-time RT-PCR for IFN-γ and CD8 mRNA. Copy numbers of IFN-γ, CD8 and GAPDH were measured with real-time RT-PCR in spleen cells. The ratio to GAPDH copy number is indicated on the *y*-axis. (B) IFN-γ production from spleen cells of KSHV-immunized mice. Error bars indicate standard deviations of triplicate experiments. IP: Intraperitoneal immunization. IN: Intranasal immunization.

2.5. Immunofluorescence assay

Titers of antibody to KSHV were determined by immunofluorescence assay (IFA) using PMA-stimulated TY-1, a KSHV-infected primary effusion lymphoma cell line [31]. TY-1 cells were stimulated with PMA for 48 h and smeared on slides. After acetone fixation, the smear slides were stored at −25 °C. Serum, NW, or saliva were diluted by dilution factors 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024 for IgA, and 50, 100, 200, 400, 800, 1600, 3200, 6400, 12,800, and 25,600 for IgG in Block Ace (Snow-Brand, Tokyo, Japan). Diluted samples were applied on the smear slides, and incubated at room temperature for 1 h. After washing with PBS, the slides were reacted with FITC-conjugated anti-mouse IgG or IgA antibody (BD Bioscience) for 30 min. Followed by washing and mounting, the slides were observed with a fluorescence microscope. Antibody titers were determined at the dilution of positive signals. For identification of immunogens in KSHV-immunized mice, dual-labeled IFA was performed. The mouse serum and anti-KSHV ORF K8, K8.1, ORF26, ORF59, ORF65, or ORF73 (LANA-1) rabbit polyclonal antibodies were reacted with the smear slides as the primary antibodies [7]. After washing, the slides were reacted with Alexa 488-conjugated anti-mouse IgG antibody and Alexa 568-conjugated anti-rabbit IgG antibody (Molecular Probe, Eugene, OR) as the secondary antibodies. After washing and mounting, the slides were observed with a confocal microscope (FV-1000, Olympus, Tokyo, Japan).

2.6. Neutralization assay

One hundred μl of 1000× diluted serum or 10× diluted NW or saliva were incubated with 10^6 copies of rKSHV.219, which contained about 100 infectious units, in DMEM in tubes at 37 °C for 2 h [28]. After the incubation, 100 μl of the virus solution was added to human embryonic kidney 293 cells (293 cells) in a 96-well plate. The plate was centrifuged for a short time at a low speed, and incubated for 2 h in a CO_2 incubator. After removing the supernatant, fresh media was added, and the cells were cultured at 37 °C. Five days after infection, the number of GFP^+ cells in each well was counted under a fluorescence microscope.

2.7. Recombinant proteins and western blotting analysis

Glutathione S-transferase (GST)-fusion proteins of K8, K8.1, ORF26, ORF59, ORF65, and ORF73 were synthesized as described previously [4]. Fifty nanograms of each GST-fusion protein was applied to western blotting. Since molecular sizes of these GST-fusion proteins range 41–60 kDa, 50 ng protein is corresponding to 0.8–1.2 pmol. The serum from mice and anti-GST rabbit polyclonal antibody were used as the primary antibodies. Anti-mouse or rabbit IgG antibodies (BD Bioscience) were used as the secondary antibodies; signals were detected with a chemiluminescence solution (Westdura, Pierce Biotechnology, Rockford, IL).

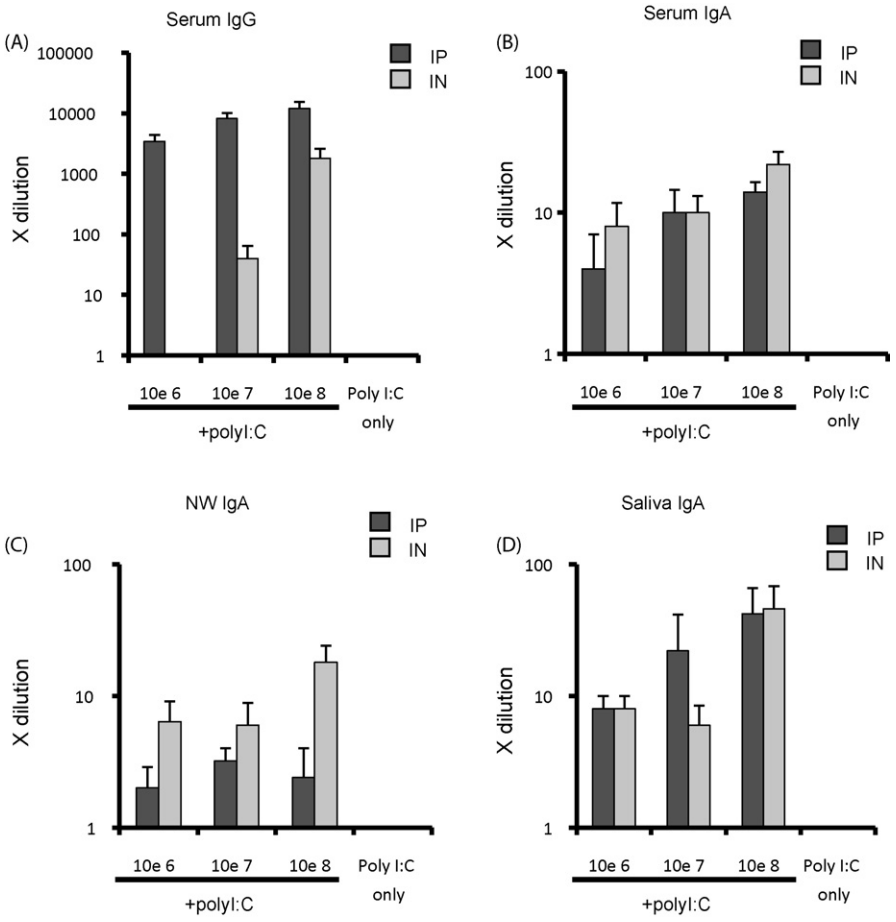

Fig. 2. Induction of humoral immunity to KSHV in KSHV-immunized mice. (A) Titers of serum IgG (A), serum IgA (B), nasal wash fluid (NW) IgA (C), and saliva IgA (D) were determined by IFA using TPA-stimulated TY-1. Copy numbers of immunized KSHV are shown in the *x*-axis. Error bars indicate standard deviations of five mice in each group. IP: Intraperitoneal immunization. IN: Intranasal immunization.

2.8. Statistical analysis

Student's *t*-test was applied for the comparison of mRNA levels and the KSHV neutralization assay.

3. Results

3.1. IFN-γ production in KSHV-immunized mice

To know whether KSHV induces cellular immune responses in mice, 10^8 copies of KSHV particles were immunized intranasally or intraperitoneally to Balb/c mice with poly(I:C) as an adjuvant. Poly(I:C) is a synthetic double-stranded RNA; it has been demonstrated to be an effective mucosal adjuvant for not only RNA viruses such as influenza virus, but also DNA viruses such as herpes virus and human papillomavirus [29,32]. Real-time RT-PCR showed that KSHV immunization to the peritoneal cavity increased mRNA levels of IFN-γ and CD8 in the spleen cells compared with poly(I:C)-immunized control mice (Fig. 1A). Similar data were obtained from the spleen cells of mice immunized intranasally with KSHV (data not shown). An ELISA to detect IFN-γ showed that both intranasal and intraperitoneal immunizations induced release of IFN-γ in the supernatant of the spleen cells after 8 h of culture (Fig. 1B). Release of IFN-γ was not observed in the spleen cells from poly(I:C)-immunized mice. These data suggest that both intranasal and intraperitoneal immunization with KSHV induced IFN-γ production in mice as a cellular immune response to KSHV.

3.2. Humoral response in KSHV-immunized mice

IgA plays an important role in protection from virus in the mucosae [33]. To know whether KSHV immunization induces humoral responses, including IgA expression, in mice, IgA and IgG titers in body fluids were measured in KSHV-immunized mice. There is currently no gold standard to measure the antibodies to KSHV, because the immunogens of KSHV are not constant in KSHV-infected individuals [34]. Therefore, IgA and IgG titers were determined with IFA using KSHV-infected lymphoma cells. IFA revealed that both intranasal and intraperitoneal immunization induced IgG and IgA to KSHV in serum (Fig. 2A and B). Titers of serum IgG and IgA increased in a dose-dependent man-

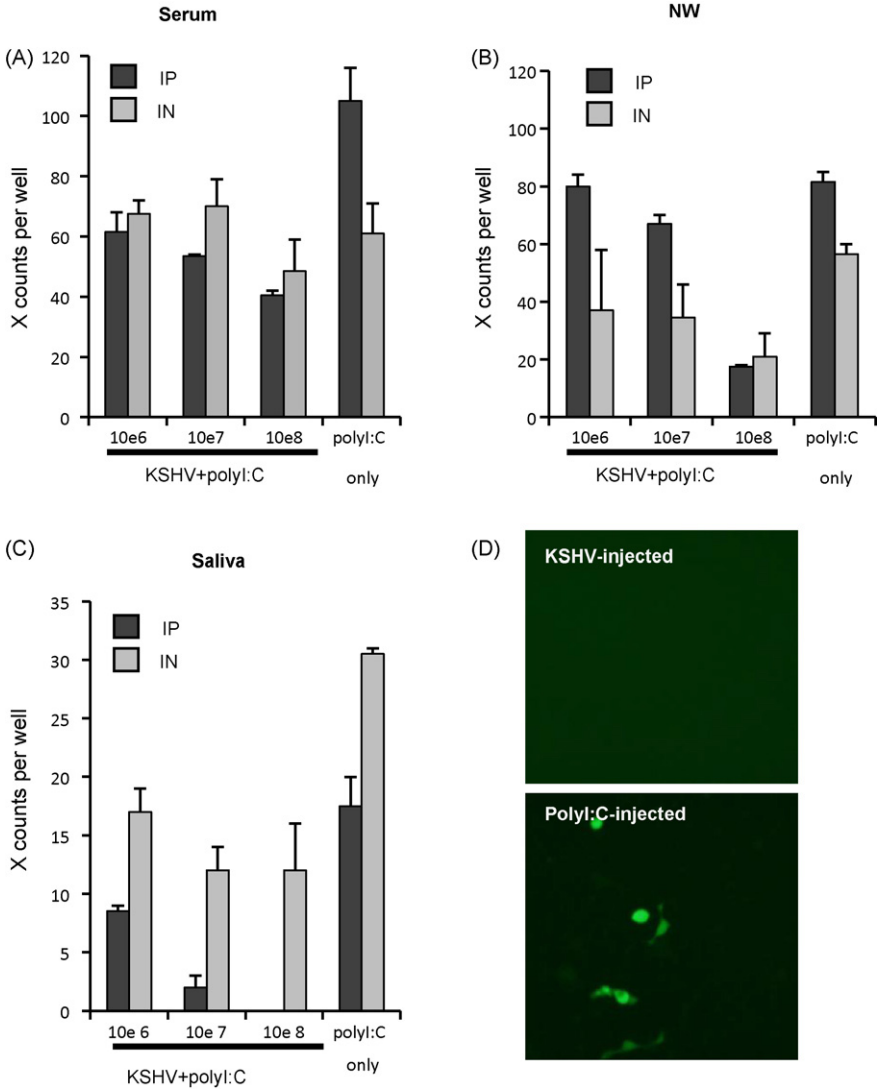

Fig. 3. Neutralization assay for KSHV using body fluids of KSHV-immunized mice. Serum (A), nasal wash fluid (NW) (B), and saliva (C) were used to neutralize GFP-expressing-KSHV infection to 293 cells. Numbers in the *y*-axis indicate counts of GFP^+ cells in each well. Error bars indicate standard deviations of triplicate experiments. IP: Intraperitoneal immunization. IN: Intranasal immunization. (D) Fluorescent images of neutralization assay using NW from 10^8 copies of KSHV-immunized (upper panel) and poly(I:C)-immunized (lower panel) mice.

ner to KSHV copies. In addition, IgA was detected in NW and saliva in mice immunized with KSHV intranasally (Fig. 2C and D), whereas the IgA titer in NW from intraperitoneally immunized mice with was low ($P<0.01$, in 10^8 copies of KSHV-immunized mice). These data indicate that both intranasal and intraperitoneal immunization with KSHV induced humoral response in mice, and IgA in the NW was induced effectively through the intranasal immunization.

3.3. Neutralization of KSHV infection with the body fluids of KSHV-immunized mice

To estimate the neutralization activity to KSHV of the serum, NW, and saliva, neutralization assay was performed using GFP-expressing recombinant KSHV, rKSHV.219, and 293 cells [28]. The serum of mice immunized intraperitoneally with 10^8 copies of KSHV showed reduced numbers of GFP^+ cells in 293 cells compared with serum of poly(I:C)-immunized mice ($P<0.05$, Fig. 3A). However, incubation with serum of intranasally immunized mice did not statistical significantly reduce the number of GFP^+ cells. The NW and saliva of mice immunized intraperitoneally or intranasally with 10^8 copies of KSHV showed reduced numbers of GFP^+ cells in a dose-dependent manner to KSHV copies immunized, compared with poly(I:C)-immunized mice ($P<0.05$, Fig. 3B–D). The numbers of GFP+ cells in saliva from poly(I:C)-immunized mice (Fig. 3C) was smaller than those in serum from poly(I:C)-immunized mice (Fig. 3A), implying that general humoral components in saliva reduced KSHV infection to 293 cells. Consequently, these data suggest that the body fluids from KSHV-immunized mice are able to reduce the efficacy of *in vitro* KSHV infection to 293 cells.

3.4. Immunogens encoded by KSHV in mice

Some of the KSHV-encoded proteins were identified as immunogens in human so far [4,34]. Among them, six KSHV-encoded proteins, K8, K8.1, ORF26, ORF59, ORF65, and ORF73 (LANA-1) were synthesized in *E. coli* as GST-fusion proteins to ascertain immunogens in KSHV-immunized mice [4]. Western blot revealed that GST-K8.1 and ORF59 proteins reacted more strongly with the serum from KSHV-intraperitoneally immunized mice than did other proteins (Fig. 4A). The serum also produced faint bands in the lanes of K8, ORF26, and ORF65 proteins, but not of ORF73C and ORF73N. Immunofluorescence assays using the serum and anti-KSHV-encoded protein antibodies demonstrated that the stain of the serum overlapped with those of K8.1 and ORF 59 frequently, of ORF26 and ORF65 partially, but not of K8 and ORF73. These data suggest that the serum of KSHV-immunized mice recognized mainly K8.1 and ORF59 protein, partially ORF26 and ORF65, but not K8 and ORF73.

3.5. Neutralization with the body fluids of KSHV-encoded protein-immunized mice

To know whether the KSHV-encoded proteins induce humoral immunity in mice, these proteins with poly(I:C) were immunized intranasally and intraperitoneally to mice. IFA using KSHV-infected cells revealed that intranasal and intraperitoneal immunization with the protein induced serum IgG and IgA to KSHV in the mice (Fig. 5A and B). Intranasal immunizations with the proteins also induced IgA to KSHV in the NW and saliva, as effectively as immunization with KSHV particles and ORF73 protein (Fig. 5C and D). The neutralization assay revealed that the serum from mice intraperitoneally immunized with GST-K8.1 reduced the numbers of KSHV-infected 293 cells in this assay ($P<0.05$), whereas the serum from mice intraperitoneally immunized with ORF59 and ORF73 proteins did not reduce them significantly ($P=0.55$, Fig. 6A). Neutralization activity of body fluid of K8.1-immunized mice was also shown in the NW of mice intranasally immunized with K8.1 protein ($P<0.01$, Fig. 6B). These data suggest the neutralization activity of the antibodies to K8.1 *in vitro*.

4. Discussion

In the present study, we demonstrated that KSHV immunization resulted in cellular and humoral immune response in mice. Spleen cells from KSHV-immunized mice produced IFN-γ, and the serum, NW and saliva of KSHV-immunized mice neutralized KSHV infection to 293 cells *in vitro*. The serum of KSHV-immunized mice recognized KSHV-encoded K8.1 and ORF59 proteins. The serum and NW from K8.1-immunized mice neutralized KSHV infection to 293 cells *in vitro* as effectively as the serum from KSHV-immunized mice. These results suggest a possibility of mucosal vaccine using inactivated KSHV particles or recombinant K8.1 protein for the prophylaxis of KS.

The animal experiments in the present study suggest that intranasal immunization of KSHV induced similar immune responses to intraperitoneal immunization in the production of serum IgA and saliva IgA (Fig. 2B and D). IgA level in NW of intranasally immunized mice is higher than those of intraperi-

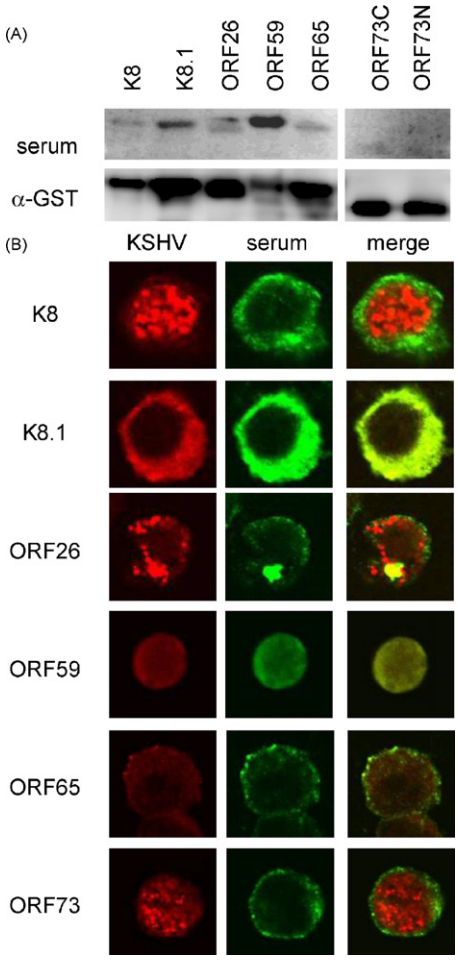

Fig. 4. Immunogens recognized by serum of KSHV-immunized mice. (A) Western blot. Seven KSHV-encoded proteins were synthesized as GST-fusion proteins and applied for western blot using serum of KSHV-immunized mice (upper panel) or anti-GST antibody (lower panel). (B) Dual-labeled immunofluorescence assay. KSHV-encoded proteins were labeled with Alexa-568 (red, left panels), and the mouse serum was labeled with Alexa-488 (green, middle panels). Merged images are shown in the right panels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

toneally immunized mice (Fig. 2C). Considering that KSHV infects humans through the mucosae in the oral cavity or rectum, vaccination to the mucosae seems effectively to induce cellular and humoral immunity in human. Although it is unknown if intranasal immunization would induce similar immunity to a route using the rectum or oral cavity, the nasal or oral cavity is a promising candidate as a route of KSHV vaccination.

Immunogens of KSHV are important for development of KSHV vaccine. In this study, we identified the KSHV-encoded proteins, K8.1 and ORF59, as immunogens to which mouse serum reacted (Fig. 4A). K8.1 protein, a glycoprotein composing of virion membrane, was contained by virion, while ORF59 protein, a processivity factor for viral DNA polymerase, is not detected in KSHV virions [35]. Recognition of the serum to ORF59 protein suggests a possibility that KSHV entered in mouse cells and expressed the protein for a short period. In this study, several mice immunized with KSHV were autopsied, and all organs were investigated histopathologically. However, there was no specific disease to KSHV like KS or lymphoma, and immunohistochemistry for LANA-1 or ORF59 did not detect any positive signal in any organ, suggesting that ORF59 protein expression occurred for a very short period or at a very low rate in mice. In any case, serum from mice immunized with

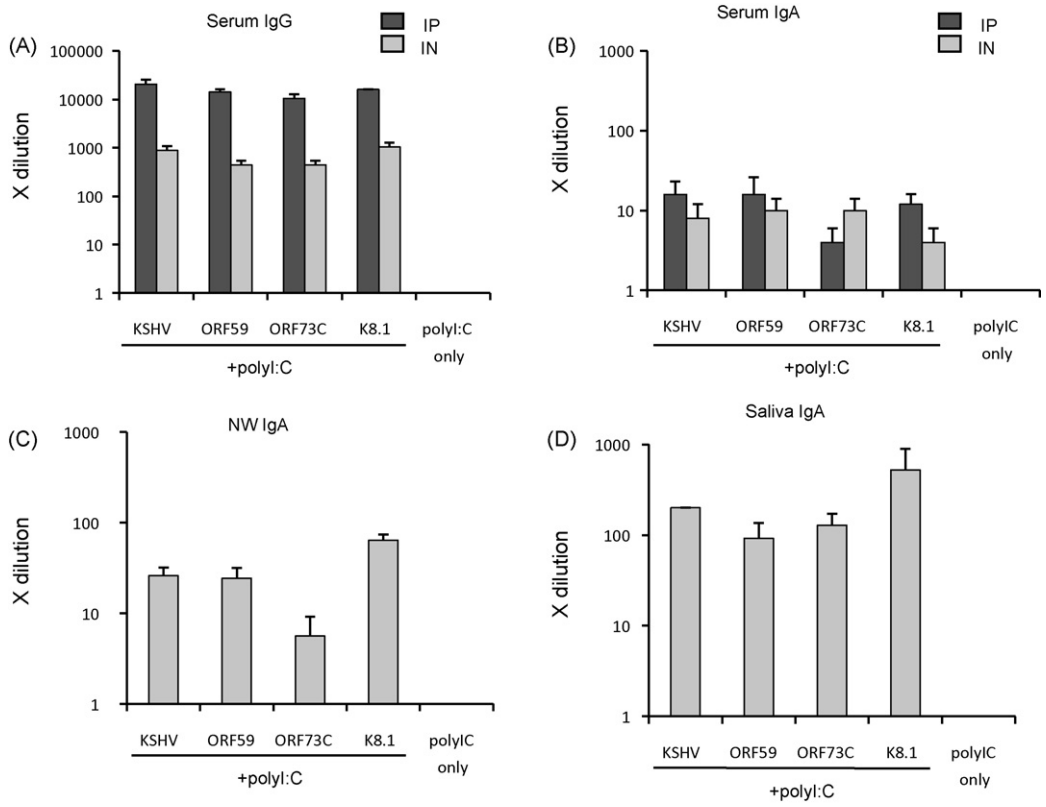

Fig. 5. Serum antibody titers of KSHV or KSHV-encoded protein-immunized mice by IFA. Titers of serum IgG (A), serum IgA (B), nasal wash fluid (NW) IgA (C), and saliva IgA (D) were determined by IFA using TPA-stimulated TY-1. Immunogens (10^8 copies of KSHV particles, and 10 ng of GST-ORF59, ORF73c, and K8.1) are shown in the *x*-axis. Error bars indicate standard deviations of five mice in each group. IP: Intraperitoneal immunization. IN: Intranasal immunization.

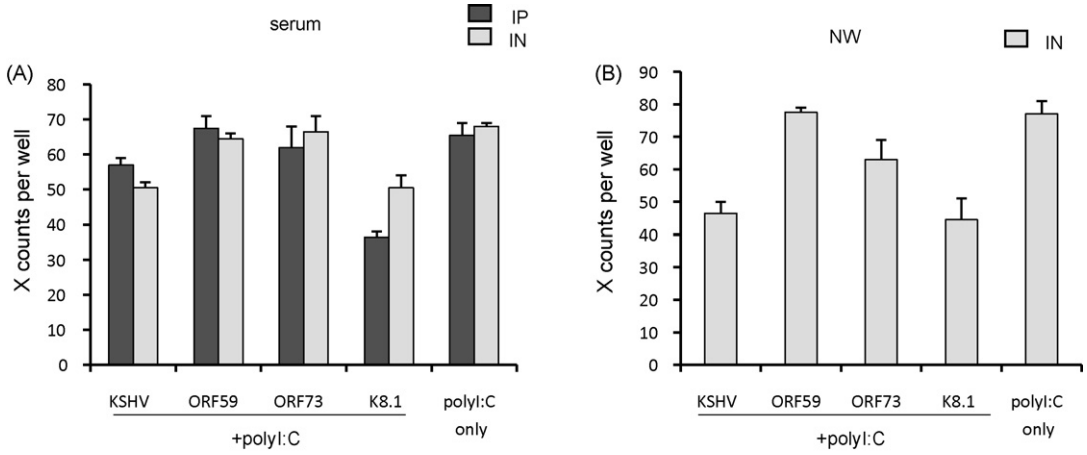

Fig. 6. Neutralization assay for KSHV using body fluids of KSHV or KSHV-encoded protein-immunized mice. Serum (A) and nasal wash fluid (NW) (B) were used to neutralize GFP-expressing-KSHV infection to 293 cells. Numbers in the *y*-axis indicate cell counts of GFP^+ cells in each well. Error bars indicate standard deviations of triplicate experiments. The serum was obtained from mice with intraperitoneal immunization; nasal wash fluid was obtained from mice with intranasal immunization.

the K8.1 protein, but not ORF59 protein, showed some effects for prevention of KSHV infection *in vitro* (Fig. 6). It is already shown that K8.1 protein interacts with cellular heparin sulfate, suggesting that K8.1 protein plays an important role in the attachment of KSHV to cell surfaces [36]. Like the serum from KSHV-immunized mice, the serum from K8.1-immunized mice reduced the number of KSHV$^+$ 293 cells partially, but not completely. The GST-fusion system cannot produce glycosylation modification, which may be one of the reasons why the serum protected 293 cells from KSHV infection partially. In addition, some previous studies demonstrated that one or a few proteins encoded by KSHV are not sufficient to detect serum antibodies to KSHV in humans, implying that single or a few recombinant viral proteins may not be sufficient for vaccine [4,34]. Although it is possible that some KSHV-encoded proteins may become vaccine targets [37,38], our data suggest that K8.1 may be one of suitable vaccine targets.

The selection of adjuvant is another issue for development of KSHV vaccine. Although poly(I:C) worked well in this study, the adjuvant should be selected considering the route of vaccination, volume of vaccine, and characterization of vaccine product. In addition, an animal model able to contract KSHV infection is required for development of KSHV vaccine. KSHV infects only humans, but no other species, including mice [22–25]. One study demonstrated that repeated intravenous immunizations of KSHV to NOD/SCID mice resulted in the establishment of latent KSHV infection; LANA-1 was immunohistochemically detected in the spleen of the mice in that report [24]. A recent study showed KSHV infected common marmosets [9]. However, there is currently no report describing successful KSHV infection in immunocompetent small animals. Thus, development of a new animal model is an important issue to estimate the efficacy of KSHV vaccine.

The seroprevalence of KSHV among the general population is extremely low compared with other herpes viruses [4,20]. Seropositivity of KSHV among the Japanese general population is about 1%, whereas many adults have antibodies to herpes simplex virus-1 (55–63%), varicella zoster virus (almost 100%), Epstein-Barr virus (>90%), cytomegalovirus (95% in pregnant women), and HHV-6 (79%) in Japan [4,39–43]. Since vaccine is generally effective for prevention of *de novo* infection of virus, a vaccine strategy could be effective for the prevention of KSHV infection in KSHV-uninfected individuals. Epidemiological data revealed that KSHV is widespread among MSM [3]. However, 40% of HIV-infected MSM were KSHV-uninfected in Japan [4]. In addition, vaccine should have some effect on the prevention of virus reactivation. In that sense, KSHV vaccine may have some effects on KSHV-infected individuals to prevent occurrence of KS. Thus, KSHV vaccine should be a promising tool for prophylaxis of KS. The present study provides a part of the fundamental data of animal experiments on KSHV. Further studies are required to develop the KSHV vaccine.

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