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Chimeric SV40 virus-like particles induce specific cytotoxicity and protective immunity against influenza A virus without the need of adjuvants

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ABSTRACT

Virus-like particles (VLPs) are a promising vaccine platform due to the safety and efficiency. However, it is still unclear whether polyomavirus-based VLPs are useful for this purpose. Here, we attempted to evaluate the potential of polyomavirus VLPs for the antiviral vaccine using simian virus 40 (SV40). We constructed chimeric SV40-VLPs carrying an HLA-A*02:01-restricted, cytotoxic T lymphocyte (CTL) epitope derived from influenza A virus. HLA-A*02:01-transgenic mice were then immunized with the chimeric SV40-VLPs. The chimeric SV40-VLPs effectively induced influenza-specific CTLs and hetero-subtypic protection against influenza A viruses without the need of adjuvants. Because DNase I treatment of the chimeric SV40-VLPs did not disrupt CTL induction, the intrinsic adjuvant property may not result from DNA contaminants in the VLP preparation. In addition, immunization with the chimeric SV40-VLPs generated long-lasting memory CTLs. We here propose that the chimeric SV40-VLPs harboring an epitope may be a promising CTL-based vaccine platform with self-adjuvant properties.

Introduction

Vaccination has significantly contributed to human health in the prevention and the control of various infectious diseases. However, there are still numerous challenges for making effective vaccines against a number of critical human pathogens such as HIV, HCV, and *Mycobacterium tuberculosis* (Houghton and Abrignani, 2005; McMichael et al., 2010; Skeiky and Sadoff, 2006). Most of these pathogens appear to cause persistent and latent infections. In order to develop successful vaccines against such diseases, it is essential for the vaccine not only to induce humoral immunity involving neutralizing antibodies but also to generate cellular immunity, in particular cytotoxic T lymphocytes (CTLs) (Koup and Douek, 2011). To eliminate virus-infected cells or tumor cells, CTLs are more essential than neutralizing antibodies.

Because viruses offer dramatic effectiveness to elicit both strong humoral and cellular immune responses, many scientists have attempted to use them in the attenuated form as a vaccine carrier for the delivery of foreign epitopes as well as a direct immunogen (Liu, 2010; Shiver and Emini, 2004; Sutter and Moss, 1992). However, they have a variety of potential problems such as reversed virulence and unwanted inflammatory responses. By contrast, subunit vaccines composed of antigenic proteins or peptides are relatively safe. At the same time, we know that they generally fail to stimulate protective cellular immunity. Due to the poor immunogenicity, they should often contain adjuvants for the enhancement of immune responses. A variety of adjuvants have been developed for experiments, but few adjuvants have been licensed for human use due to the risk of side effects. Furthermore, the widely used adjuvants for humans, alum (Marrack et al., 2009) and the oil-based formulation MF59 (O'Hagan et al., 2012) are useful for the augmentation of antibody responses, but not for cellular immune responses. Therefore, it is necessary to

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develop a new vaccine platform that is safe and highly effective for the induction of strong cellular immunity without the need of adjuvants.

Virus-like particles (VLPs) are comprised of multiple copies of viral surface structural proteins such as the capsid or envelope without including viral genomes. Since VLPs retain the efficient entry activity of the virus into host cells without replication, they are considered to be a much safer alternative than attenuated viruses as an efficient vaccine platform in the antigen delivery system (Roy and Noad, 2008). A variety of VLPs have been constructed from many different viruses (Roy and Noad, 2008), and it was demonstrated that they could induce neutralizing antibodies (Buonaguro et al., 2002; Herbst-Kralovetz et al., 2010; Kang et al., 2009). Some of these VLPs were shown to be a highly immunogenic antigen carrier to elicit CTLs against inserted foreign antigens (Ding et al., 2009; Lacasse et al., 2008; Mazeike et al., 2012). It is considered that VLPs are taken up and cross-presented to T cells by professional antigen presenting cells (APCs) such as dendritic cells (DCs) (Win et al., 2011). Although several VLPs were reported to require the co-administration of adjuvants (Qian et al., 2006; Storni et al., 2004; Young et al., 2006) for the induction of CTLs, many kinds of VLPs could induce potent cell-mediated immune responses even without adjuvants (Buonaguro et al., 2002; Lacasse et al., 2008). However, the adjuvant-like role of viral structural proteins is poorly understood.

Both papillomavirus and polyomavirus are non-enveloped DNA viruses, and their capsids are similarly formed by 72 pentameric capsomers composed of the major capsid proteins, L1 and VP1, respectively, which are arranged on a skewed icosahedral lattice. Papillomavirus L1-based VLPs are considered to be one of the most promising VLPs for a vaccination vehicle because a number of experiments have proven that they could induce potent cellular immune responses as well as humoral immunity even in the absence of adjuvants (Greenstone et al., 1998; Lenz et al., 2001; Pejawar-Gaddy et al., 2010). Chimeric papillomavirus VLPs incorporating a foreign epitope effectively induced epitope-specific CTLs and protected mice from tumor formation without the addition of adjuvants (Greenstone et al., 1998; Liu et al., 2000; Pejawar-Gaddy et al., 2010; Sadeyen et al., 2003). Human papillomavirus VLPs are already available as a prophylactic subunit vaccine against cervical cancers (Campo and Roden, 2010). Despite the morphological similarity, however, it is still unclear whether polyomavirus VP1-based VLPs are able to evolve into a self-adjuvanting vaccine platform that provides strong cellular immune responses. It was shown that hamster (Mazeike et al., 2012) and murine (Tegerstedt et al., 2005) polyomavirus chimeric VLPs carrying foreign epitopes could induce epitope-specific CTLs and reject epitope-expressing tumors in the absence of adjuvants. By contrast, other groups reported that human (Goldmann et al., 1999) and murine (Eriksson et al., 2011) polyomavirus chimeric VLPs failed to stimulate immune responses and protect against outgrowth of an epitope-expressing tumor unless administrated with adjuvants. In addition, although papillomavirus VLPs were capable of inducing phenotypic and functional maturation of DCs (Lenz et al., 2001), human (Lenz et al., 2001) and murine (Boura et al., 2005; Tegerstedt et al., 2007) polyomavirus VLPs could not activate DCs *in vitro* at all, suggesting that polyomavirus VLPs may not possess self-adjuvant properties.

In the current study, we evaluated the potential of polyomavirus VLPs for the antigen delivery system using simian virus 40 (SV40), which belongs to the *Polyomaviridae* family. We have previously developed the method to prepare SV40 VP1-based VLPs using the baculovirus expression system (Ishizu et al., 2001; Kawano et al., 2013; Kosukegawa et al., 1996), and found that there were two loops of VP1, called the DE and HI loops that could accommodate a foreign peptide on the surface of VLPs without disrupting the assembly of VLPs (Kawano et al., 2013; Takahashi et al., 2008). Here, we constructed SV40-VP1 with an HLA-A*02:01-restricted CTL epitope corresponding

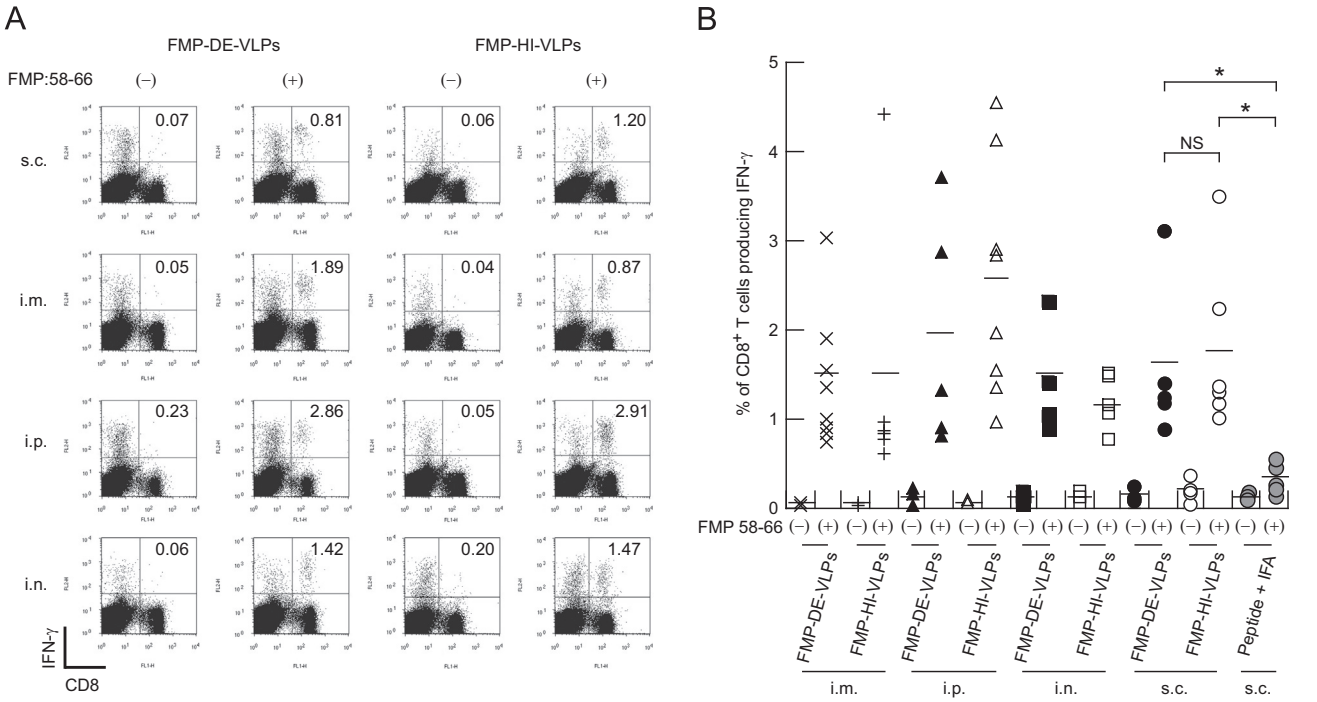

Fig. 1. Intracellular IFN-γ staining of FMP 58-66-specific CD8$^+$ T cells in HHD mice immunized with the chimeric SV40-VLPs. HHD mice were immunized once with either FMP-DE-VLPs or FMP-HI-VLPs without adjuvants via various immunization routs including s.c., i.m., i.p., and i.n. administrations. After one week, splenic lymphocytes were prepared, and stimulated with (+) or without (–) FMP 58-66 for 5 h. (A) Cells were then stained for their surface expression of CD8 (*x* axis) and their intracellular expression of IFN-γ (*y* axis). The numbers shown indicate the percentages of intracellular IFN-γ cells within CD8$^+$ T cells. The data are representative of two independent experiments. At least three mice per group were used in each experiment. (B) The data indicate the percentages of intracellular IFN-γ cells within CD8$^+$ T cells in mice immunized once with either the chimeric SV40-VLPs via various immunization routs or the FMP 58-66 peptide plus IFA via the s.c. route. Each symbol represents an individual mouse. Horizontal bars represent the mean. One-way ANOVA was performed for comparison of data. *, $P < 0.05$; NS, not significant.

to the influenza A virus matrix peptide 58-66 (FMP 58-66) (Gotch et al., 1987) inserted into the DE loop or the HI loop. We then investigated whether the chimeric SV40-VP1-based VLPs effectively induced protective cellular immunity in HLA-A*02:01 transgenic mice without the need of adjuvants, and attempted to demonstrate the presence of self-adjuvant properties in the chimeric SV40-VLPs.

Results

Induction of FMP-specific IFN-γ-producing CD8$^+$ T cells in mice immunized with the chimeric SV40-VLPs

To investigate whether SV40-VLPs carrying the FMP 58-66 epitope could elicit FMP-specific CTLs, HHD mice were immunized once with either FMP-DE-VLPs, FMP-HI-VLPs or WT-VLPs without adjuvants via various immunization routes. After one week following immunization, splenic lymphocytes were prepared and stimulated with FMP 58-66. Cells were then stained for their surface expression of CD8 and antigen-induced intracellular expression of IFN-γ. As shown in Fig. 1A and B, both FMP-DE-VLPs and FMP-HI-VLPs significantly induced IFN-γ-producing CD8$^+$ T cells in mice injected via any routes tested without the need of adjuvants. By contrast, WT-VLPs without an FMP 58-66 insert was not able to elicit IFN-γ-producing CD8$^+$ T cells (data not shown). Since i.p. injection was likely to generate high frequencies (Fig. 1A and B), i.p. immunization was commonly used for the following experiments.

We also performed comparative studies on the efficiency of FMP-specific CTL induction between the SV40-VLP vaccination and a conventional peptide vaccination (Fig. 1B). Mice were immunized s.c. once with 50 μg of FMP 58-66 peptide in incomplete Freund's adjuvant (IFA) or an equivalent amount of chimeric SV40-VLPs harboring only 1.2 μg of FMP 58-66 peptide, followed by intracellular IFN-γ staining. It was demonstrated that the frequency of IFN-γ-secreting CD8$^+$ T cells following the SV40-VLP vaccination was significantly higher than that following the peptide vaccination (Fig. 1B). These data imply that the SV40-VLP vaccination appeared to be superior to the conventional peptide vaccination in terms of CTL induction.

Detection of the FMP-specific killing activity in the chimeric SV40-VLPs-immunized mice

To address the FMP-specific cytotoxicity, ^{51}Cr release assays were carried out (Fig. 2A). Effector cells were prepared from spleens of mice that had been immunized with WT-VLPs, FMP-DE-VLPs or FMP-HI-VLPs in the absence of adjuvants. As shown in Fig. 2A, FMP-specific killing responses were clearly detected in FMP-DE-VLPs or FMP-HI-VLPs-immunized mice, but not in WT-VLPs-injected mice (Fig. 2A). There was no significant difference in the induction of FMP-specific CTL activities between FMP-DE-VLPs and FMP-HI-VLPs (Fig. 2A). To confirm these results, we next performed *in vivo* CTL assays. In accordance with the data in ^{51}Cr release assays (Fig. 2A), FMP-specific CTL activities were significantly observed in FMP-DE-VLPs- or FMP-HI-VLPs-immunized mice (Fig. 2B) but not in WT-VLPs-immunized mice (data not shown). These data demonstrate that both FMP-DE-VLPs and FMP-HI-VLPs are efficient to elicit CTLs that have the robust activity of FMP 58-66-specific killing without the need of adjuvants.

Heterosubtypic protection against influenza A virus infection in the chimeric SV40-VLPs-immunized mice

It was expected that FMP-specific CTLs induced by the chimeric SV40-VLPs could preferentially kill influenza A virus-infected cells,

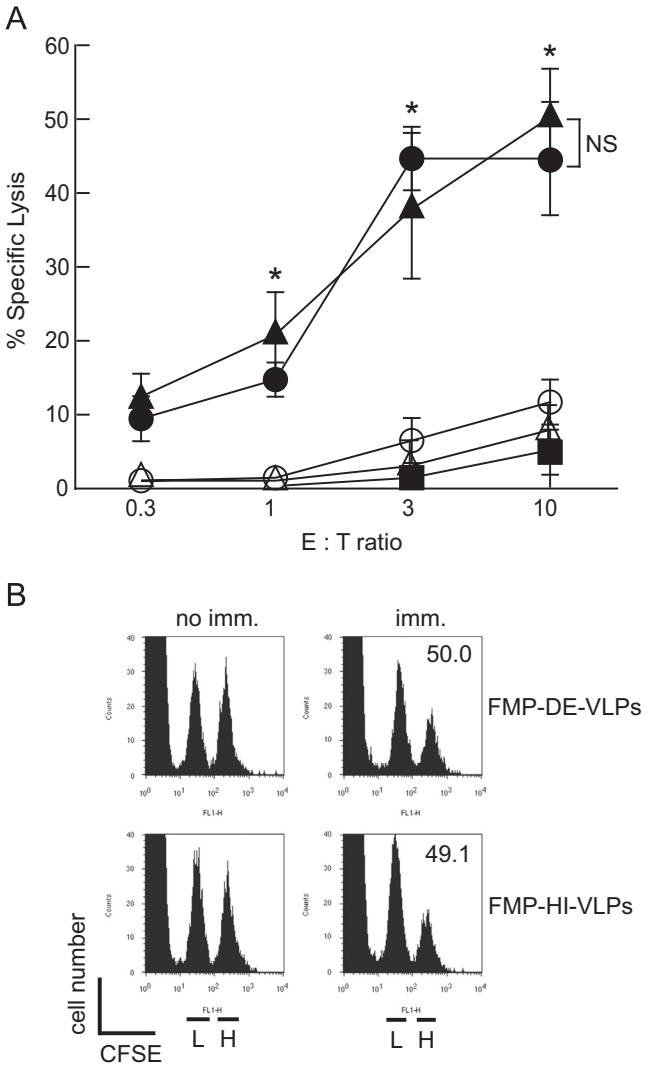

Fig. 2. Detection of FMP 58-66-specific CTL killing activities by *in vitro* (A) and *in vivo* (B) CTL assays. (A) HHD mice were immunized i.p. with either WT-VLPs (square), FMP-DE-VLPs (circle) or FMP-HI-VLPs (triangle) without adjuvants. After two weeks following immunization, spleen cells of the mice were stimulated twice with peptide-pulsed syngeneic spleen cells. ^{51}Cr-release assays were then performed at various *E*:*T* ratios, using RMA–HHD cells pulsed with (black symbols) or without (white symbols) FMP 58-66 as targets. Data are shown as the mean ± SD. Similar results were obtained in two independent experiments. Three mice per group were used in each experiment. One-way ANOVA was performed for comparison of data. *, $P < 0.01$ compared to WT-VLPs; NS, not significant between FMP-DE-VLPs and FMP-HI-VLPs. (B) Spleen cells of naive HHD mice were pulsed with FMP 58–66 and labeled with a high concentration (2.5 μM) of CFSE (H). As a control, unpulsed spleen cells were labeled with a lower concentration (0.25 μM of CFSE (L). A 1:1 mixture of each target cell population was injected i.v. into immunized (imm.) and unimmunized (no imm.) mice. The numbers shown indicate the percentage of antigen specific lysis. The data shown are representative of three independent experiments.

leading to the elimination of virus from the body. To verify this assumption, mice were immunized with either WT-VLPs, FMP-DE-VLPs or FMP-HI-VLPs without adjuvants, and then challenged intranasally with 1×10^2 $TCID_{50}$ influenza virus A/PR8/34 (H1N1) or A/Aichi/2/68 (H3N2). Firstly, we examined the body weight change of mice challenged with influenza virus A/PR8/34. As shown in Fig. 3A and B, mice immunized with FMP-DE-VLPs or FMP-HI-VLPs did not change much their body weights after virus challenge, whereas a significant loss in body weight was observed in the control mice inoculated with WT-VLPs (Fig. 3A and B). Furthermore, virus titers in the lung were determined after 4 days following the challenge with either influenza virus A/PR8/34

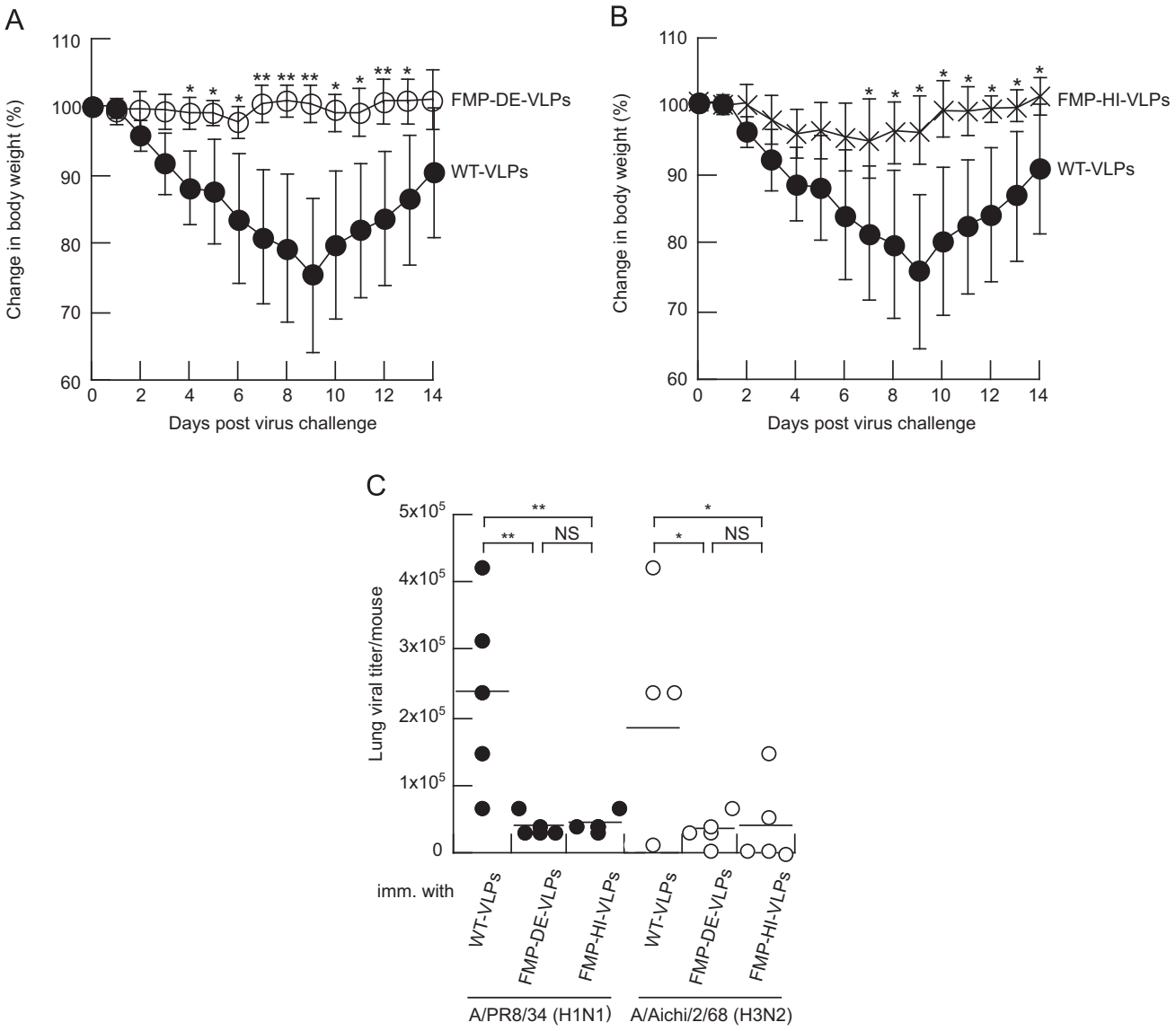

Fig. 3. Heterosubtypic protection against influenza A virus challenge. Mice were immunized with either WT-VLPs, FMP-DE-VLPs or FMP-HI-VLPs without adjuvants. After one week following immunization, mice were challenged with either H1N1 (A/PR/8/34) virus (A–C) or H3N2 (A/Aichi/2/68) virus (C). (A and B) Mice were weighted daily, and changes in body weight of mice were calculated as a percentage of the mean weight per group as compared with starting body weight. The data represent mean body weights and SD of 5 mice per group. One-way ANOVA was performed for comparison of data between vaccination groups. *, $P<0.05$; **, $P<0.01$, compared to mice immunized with WT-VLPs. There was no significant difference in body weight change between FMP-DE-VLPs-immunized mice (A) and FMP-HI-VLPs-immunized mice (B). (C) After 4 days following viral challenge, viral titers in the lungs were determined by calculating $TCID_{50}$ using MDCK cells. Each symbol represents an individual mouse and horizontal bars represent the mean. One-way ANOVA was performed for comparison of data between vaccination groups. *, $P<0.05$; **, $P<0.01$; NS, not significant.

(H1N1) or A/Aichi/2/68 (H3N2). It was demonstrated that immunization with FMP-DE-VLPs or FMP-HI-VLPs significantly inhibited the growth of both influenza PR8 and Aichi viruses in the lung (Fig. 3C). These data strongly suggest that vaccination with either FMP-DE-VLPs or FMP-HI-VLPs in the absence of adjuvants are sufficiently capable of inducing heterosubtypic protection in mice against influenza A infection presumably due to the substantial accumulation of FMP 58-66-specific CTLs in the lung.

DNase I treatment of the chimeric SV40-VLPs did not disrupt the induction of FMP-specific CTLs

During the purification process of SV40-VLPs in the baculovirus expression system, it was quite possible that baculoviral genome was contaminated in the purified SV40-VLP fraction. The contaminated DNA might play an adjuvant-like role to trigger innate immunity for the following acquired immunity. To exclude this possibility, the FMP-DE-VLP and FMP-HI-VLP fractions were treated with DNase I prior to the inoculation of them into mice. After immunization with DNase I-treated chimeric SV40-VLPs, spleen cells were prepared from mice, and intracellular cytokine staining assay and CTL assay were performed. As shown in Fig. 4, DNase I treatment of the FMP-DE-VLP or FMP-HI-VLP fraction did not disturb the induction of either IFN-γ^+ $CD8^+$ T cells (Fig. 4A and B) or FMP 58-66-specific CTLs (Fig. 4C) in mice. These results suggest that the chimeric SV40-VLPs themselves may possess intrinsic adjuvant-like properties. To discount the possible effect of the contaminated genomic DNA, SV40-VLP fractions were pretreated with DNase I in the following experiments.

To prove the self-adjuvant activity of SV40-VLPs, we investigated the expression of maturation markers on the cell surface of DCs exposed to SV40-VLPs. DCs of naive mice were incubated *in vitro* with either WT-VLPs, FMP-DE-VLPs or FMP-HI-VLPs for 24 h, and the expression of CD80, CD86 and CD40 molecules,

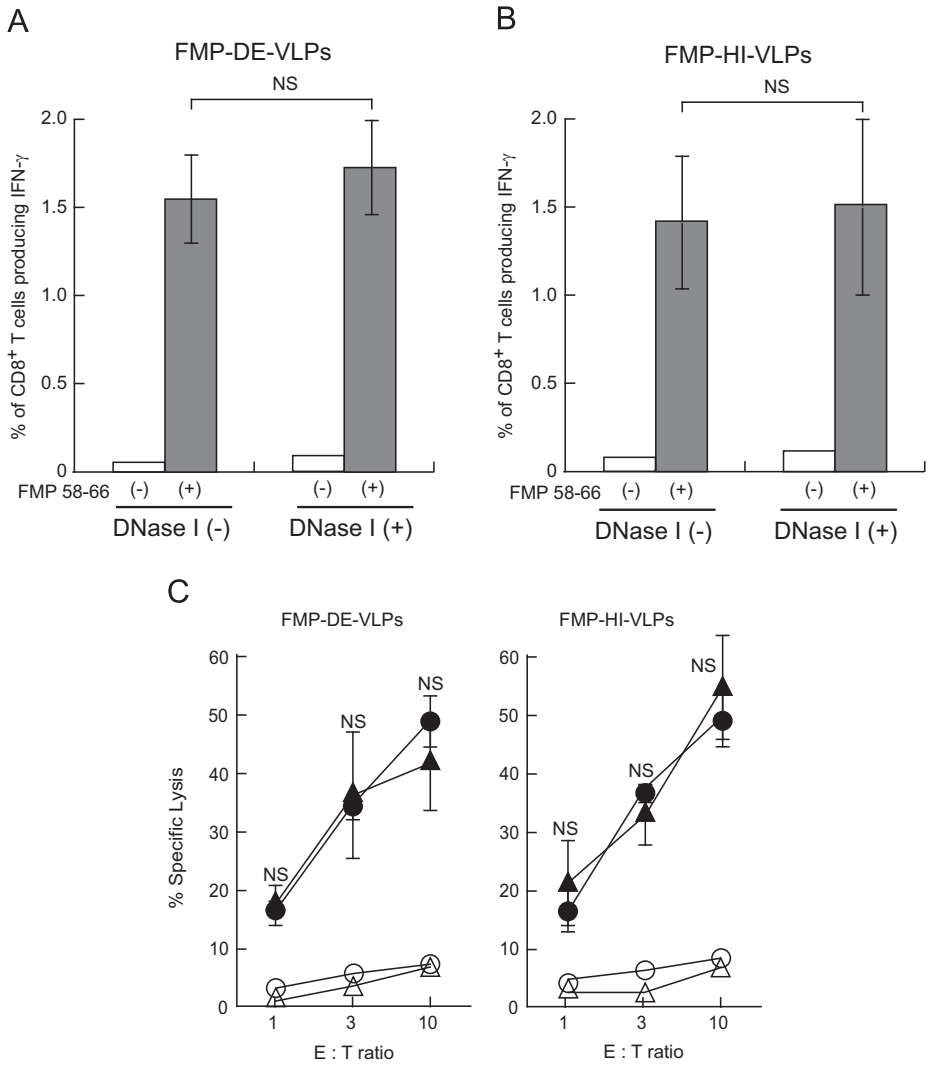

Fig. 4. Effect of DNase I treatment of the chimeric SV40-VLPs on the induction of FMP-specific CTLs. (A, B) Mice were immunized with FMP-DE-VLPs (A) or FMP-HI-VLPs (B) that had been treated with (DNase I (+)) or without (DNase I (−)) DNase I. After one week, splenic lymphocytes were prepared, and stimulated with (+) or without (−) FMP 58-66 for 5 h. Cells were stained for their surface expression of CD8 and their intracellular expression of IFN-γ. The data indicating the percentages of intracellular IFN-γ^+ cells within $CD8^+$ T cells are shown as the mean ± SD. At least four mice per group were used in each experiment. NS, not significant. (C) FMP-DE-VLPs and FMP-HI-VLPs were treated with (triangle) or without (circle) DNase I and were inoculated into mice. After two weeks following immunization, spleen cells were stimulated twice with peptide-pulsed syngeneic spleen cells. ^{51}Cr-release assays were then performed at various *E/T* ratios, using RMA–HHD cells pulsed with (black symbols) or without (white symbols) FMP 58-66 as targets. Data are shown as the mean ± SD. At least four mice per group were used in the experiments. NS, not significant between DNase I-treated and -untreated SV40-VLPs.

which provide co-stimulatory signals for T cell activation, was analyzed by flow cytometry. However, any substantial upregulation of these molecules was not observed in DCs pulsed with either of them (data not shown).

Induction of FMP-specific memory CTLs in the chimeric SV40-VLPs-immunized mice

We next examined whether long-lasting FMP-specific CTLs could be elicited in the chimeric SV40-VLPs-immunized mice. Mice were immunized once with either WT-VLPs, FMP-DE-VLPs or FMP-HI-VLPs. After 30 days following the immunization, spleen cells were prepared and stimulated *in vitro* with syngeneic spleen cells pulsed with FMP 58-66. ^{51}Cr-release assays were then performed at various *E*:*T* ratios. FMP 58-66-specific CTLs were detected in mice even on day 30 after immunization with the chimeric SV40-VLPs (Fig. 5), but not in mice immunized with WT-VLPs (data not shown). These data indicate that immunization with the chimeric SV40-VLPs can efficiently generate long-lasting memory CTLs in mice.

Discussion

It is well documented that papillomavirus L1-based VLPs are able to induce potent humoral and cellular immune responses in the absence of adjuvants (Greenstone et al., 1998; Liu et al., 2000; Pejawar-Gaddy et al., 2010; Sadeyen et al., 2003), and therefore, they are considered to be a promising vaccine vehicle in the antigen delivery system. Because of the striking resemblance in shape, polyomavirus VP1-based VLPs were also expected to develop into a self-adjuvanting vaccine platform. However, several reports have pointed out that they required the co-administration of an adjuvant for the induction of cell-mediated immunity (Eriksson et al., 2011; Goldmann et al., 1999) although others have taken the opposite view (Mazeike et al., 2012; Tegerstedt et al., 2005). In the current study, we evaluated the potential of polyomavirus VLPs as a CTL-based vaccine platform using SV40. Our

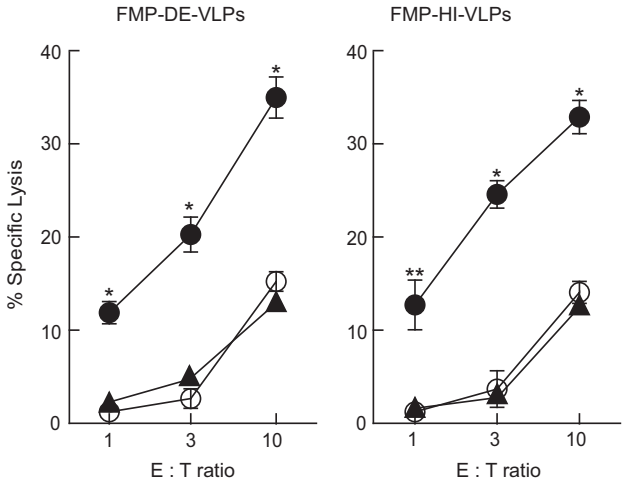

Fig. 5. Induction of long-lasting memory CTLs. HHD mice were immunized once with FMP-DE-VLPs or FMP-HI-VLPs. At day 30 after the immunization, spleen cells were prepared, and stimulated *in vitro* twice with peptide-pulsed syngeneic spleen cells. ^{51}Cr-release assays were then carried out at various *E*:*T* ratios, using RMA–HHD cells (circle) and RMA cells (triangle) pulsed with (black symbols) or without (white symbols) FMP 58-66 as targets. Data are shown as the mean ± SD. Six mice per group were used in the experiments. *, $P < 0.01$; **, $P < 0.05$ compared to RMA–HHD cells pulsed without FMP 58-66.

results have clearly revealed that the chimeric SV40-VLPs carrying the FMP 58-66 epitope efficiently induced FMP-specific CTLs and protective immunity against influenza A virus without the addition of adjuvants. As far as we know, this is the first report indicating that SV40-VLPs are able to serve as a CTL-based vaccine platform with self-adjuvant properties.

It is possible to invoke several explanations for the discrepancy in the requirement of adjuvants for CTL induction between SV40-VLPs and other polyomavirus VLPs (Eriksson et al., 2011; Goldmann et al., 1999). The key to the solution may be that the plasma membrane receptor for SV40 is different from those for other polyomaviruses (Ewers et al., 2010; Tsai et al., 2003). The model we favor is that the signal transduction pathway associated with the stimulation of SV40-VLPs may activate innate immunity more vigorously than others by an unknown mechanism. It is also possible to assume that chimeric SV40-VLPs carrying an epitope may be more efficiently internalized, processed and cross-presented by APCs such as DCs than other polyomavirus VLPs. If this is the case, several other polyomavirus VLPs may require the addition of adjuvants to improve their immune responses. These two hypotheses are not mutually exclusive, and both may occur simultaneously. Alternatively, the immunogenicity of inserted epitopes may account for the difference in the adjuvant requirement.

Papillomavirus VLPs were demonstrated to strongly elicit phenotypic maturation of DCs *in vitro* by upregulating maturation markers such as CD80, CD86, and CD40 (Lenz et al., 2001). The level of DC maturation was as high as that after exposure to a positive control, bacterial LPS (Lenz et al., 2001). On the contrary, several groups have shown that human (Lenz et al., 2001) or murine (Boura et al., 2005; Tegerstedt et al., 2007) polyomavirus VLPs did not induce phenotypic DC maturation *in vitro* at all in the similar experiments although they were internalized into cells. In agreement with these results, we also found that SV40-VLPs failed to induce upregulation of maturation markers on the surface of DCs (data not shown). In contrast to the *in vitro* experiments mentioned above, we (Figs. 1–3) and others (Mazeike et al., 2012; Tegerstedt et al., 2005) have shown that the *in vivo* immunization with polyomavirus VLPs generated potent CTL responses and protective immunity against viral infection and tumors. Boura et al. (2005) attempted to explain the difference in behavior between *in vivo* and *in vitro* treated cells with an alternative cross-presentation mechanism described by Neijssen et al. (2005). In this paper, it was demonstrated that peptides generated by proteasomal degradation could be transferred directly from the cytoplasm of one cell into the cytoplasm of its neighbor through gap junctions. Alternatively, a novel pathway of indirect antigen presentation, referred to as cross-dressing (Wakim and Bevan, 2011), may also be able to account for this issue, whereby an uninfected APC acquires pre-existing MHC class I/peptide complex molecules from a virus-infected cell. Taken together, it may be possible to explain that the gap-junction-mediated intracellular peptide coupling and/or the cross-dressing may play an important role in the *in vivo* induction of strong cellular immunity after immunization with the SV40-VLP vaccine platform although further experiments are required.

SV40 was discovered as a contaminated material in the polio vaccine (Butel and Lednicky, 1999; Hilleman, 1998), and hundreds of millions of people have been unknowingly exposed to it during immunization with polio vaccine. Fortunately, there is no evidence so far that SV40 is harmful to human who received polio vaccine. It appears unlikely that SV40 infection alone is sufficient to cause human malignancy but it seems possible that SV40 may act as a cofactor in the pathogenesis of some tumors (Qi et al., 2011). However, it is considered to be safe for humans to use SV40-VLPs as a vaccine material because there is no evidence that SV40 VP1 protein has acute or chronic cytotoxicity against human cells. In general, multiple immunizations are required for effective vaccines. Similar to other VLPs and viral vectors, however, SV40-VLPs may potentially limit the effectiveness of multiple administrations due to the induction of anti-SV40-VLP immunity including neutralizing antibodies. Although we showed that the SV40-VLP vaccine was superior to the synthetic peptide vaccine in the efficiency of CTL induction (Fig. 1B), multiple injections could allow the peptide vaccine to improve the efficiency. In addition, people who received the polio vaccine produced around 1960 were potentially sensitized with SV40 because SV40 had been contaminated in the polio vaccine (Butel and Lednicky, 1999; Hilleman, 1998). However, it was shown that the mucosal immunization with a recombinant vaccinia virus expressing HIV gp160 induced strong HIV-specific CTL responses even in mice preimmunized with wild type vaccine virus (Belyakov et al., 1999). Therefore, the mucosal vaccination may overcome the barrier caused by pre-existing immunity against SV40-VLPs. It should be noted that i.n. mucosal immunization was sufficient to induce detectable IFN-γ-producing $CD8^+$ T cell production in the current study (Fig. 1A and B). Furthermore, immunization with SV40-VLP-pulsed APCs may enable repeated application because it was reported that immunization with adenoviral vector-infected cells elicited strong cellular immune responses specific for an inserted antigen but induced very weak anti-adenovirus antibodies (Sun et al., 2012). The heterologous prime-boost immunization may also be able to circumvent this problem (Amara et al., 2001).

In the current study, we demonstrated the data of SV40-VLPs with only a single epitope, FMP 58-66. We also know that SV40-VLPs carrying a tumor-related epitope derived from Wilms' tumor protein, WT1 (Oka et al., 2008) could induce WT1-specific CTLs in mice (unpublished data), suggesting that our VLP system could be applied to a broad range of epitopes. However, it should be difficult to make a single chimeric SV40-VLP carrying many different kinds of epitopes inserted into the DE or HI loop. The epitope approach to vaccine development offers several potential advantages. For example, synthetic peptides are relatively safer than antigenic proteins of origin. However, the large degree of HLA polymorphism is a major obstacle in the development of epitope-based vaccines. Moreover, broad T-cell immune responses that are directed against multiple epitopes are desirable because

viruses are less likely to accumulate escape mutations under broad immune responses. We previously showed that SV40-VLPs could encapsulate heterologous proteins fused to the C-terminus of minor capsid proteins VP2/3, and deliver them into mammalian cells (Inoue et al., 2008). Therefore, it is possible to make SV40-VLPs that accommodate an antigenic protein containing multiple epitopes associated with a variety of HLA class I molecules.

In conclusion, we have shown that the chimeric SV40-VLPs harboring the FMP 58-66 epitope could effectively induced influenza-specific CTLs and protective immunity against influenza virus infection without the need of adjuvants. Because DNase I treatment of the chimeric SV40-VLPs did not disrupt the induction of CTLs, the intrinsic adjuvant property of the chimeras may result from VLP structural elements and not from DNA contaminants in the VLP preparation. Thus, these data may offer strong evidence that the chimeric SV40-VLPs harboring a CTL epitope is a promising CTL-based vaccine platform with self-adjuvant properties.

Materials and methods

Synthetic peptide

A synthetic peptide corresponding to the HLA-A*02:01-restricted FMP 58-66 epitope (sequence: GILGFVFTL) (Gotch et al., 1987) was synthesized by Operon Biotechnologies (Tokyo, Japan).

Mice

Mice express a transgenic HLA-A*02:01 monochain, designated as HHD, in which human β2-microglobuin (β2m) is covalently linked to a chimeric heavy chain composed of HLA-A*02:01 (α1 and α2 domains) and H-2D^b (α3, transmembrane, and cytoplasmic domains) (Pascolo et al., 1997). Eight- to twelve-week-old mice were used for all experiments. Mice were housed in appropriate animal care facilities at Saitama Medical University, and were handled according to the international guideline for experiments with animals. Experiments in the present study were approved by the Animal Research Committee of Saitama Medical University.

Cell lines

RMA–HHD (Pascolo et al., 1997) is a mouse lymphoma cell line, RMA transfected with the HHD gene. RMA–HHD cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, Lenexa, KS) (R-10) containing 500 μg/ml G418 (Sigma-Aldrich). The parental cell line, RMA was cultured in R-10. Madin-Darby canine kidney (MDCK) cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD), and was maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% FCS. Sf9 insect cells (Invitrogen, Carlsbad, CA) were cultured in supplemented Sf-900 II medium (Invitrogen) containing 10% FCS.

Generation of the chimeric SV40-VLPs

Chimeric SV40-VP1 genes containing a nucleotide sequence encoding the FMP 58-66 epitope were constructed by the overhanging PCR method as described previously (Takahashi et al., 2008). In brief, six nucleotides corresponding to the amino acid residues 137-138 in the DE loop or 273-274 in the HI loop of SV40 VP1 were replaced by a 45-nucleotide insertion encoding the 15 amino-acid-long peptide (GGG-GILGFVFTL-GGG) which is the FMP 58-66 epitope flanked by three tandem glycine spacers at both the N- and C-terminal ends. The PCR-amplified, chimeric SV40-VP1 genes were cloned into the pFastBac1 plasmid vector (Invitrogen), and the cloned pFastBac1 was transformed in DH10Bac (Invitrogen) competent cells which hold a baculovirus shuttle vector, bacmid. The recombinant bacmid DNA was then isolated and transfected into Sf9 insect cells using the transfection reagent, Lipofectin (Invitrogen), resulting in the production of recombinant baculovirus expressing the chimeric SV40-VP1. The culture medium containing virus was collected at 3 days after transfection, centrifuged to remove cells and cell debris, and stored as a viral stock. The viral titer in the stock was determined by the standard plaque assay using Sf9 cells.

To prepare the chimeric SV40-VLPs carrying the FMP 58-66 epitope in the DE loop (FMP-DE-VLPs) or HI loop (FMP-HI-VLPs), Sf9 cells were infected with the recombinant baculovirus in the stock, and incubated in TC-100 medium (AppliChem Inc., St. Louis, MO) containing 10% FCS and tryptose phosphate broth at 27 °C for 3 days. Sf9 cells were then harvested, washed, and resuspended in a buffer containing 20 mM Tris–HCl (pH 7.9) and 1% deoxycholic acid. After sonication, the cell lysate was centrifuged and the pellet was resuspended in the Optiprep buffer containing 20 mM Tris–HCl (pH 7.9) and 50% Optiprep (Axis-Shield plc, Dundee, Scotland). The presence of SV40-VLPs in the solution was confirmed under an electron microscope using H-7500 (Hitachi, Tokyo, Japan). It was found that the chimeric SV40-VLPs retained spherical structures of 40–45 nm in diameter. In some experiments, the Optiprep solution containing 2.5 μg SV40-VLPs was treated with 0.33 U of DNase I (Worthington, Lakewood, NJ) at 37 °C for 1 h. Endotoxin levels in the preparations were determined to be below the detection limit (*i.e.* < 0.06 EU/ml) by PYROGENT Plus (Lonza, Walkersville, MD)

Immunization

Mice were immunized once with 50 μg/mouse of the chimeric SV40-VLPs or wild-type SV40-VLPs (WT-VLPs) in 100 μl of the Optiprep buffer via various immunization routes, including subcutaneous (s.c.), intraperitoneal (i.p.), and intramuscular (i.m.) administrations without a booster immunization. For intranasal (i.n.) immunization, mice were anesthetized by an intraperitoneal injection of ketamine (175 mg/g weight) (Sigma-Aldrich) and xylazine (3.5 mg/g weight) (Bayer Holding Ltd., Tokyo, Japan), and inoculated intranasally once with 40 μl/mouse of solution containing 20 μg of SV40-VLPs. Peptide immunizations were performed by one-shot s.c. injection at the base of the tail with 50 μg/mouse of FMP 58-66 in 100 μl of IFA (Sigma-Aldrich) as previously described (Schell et al., 2001).

Intracellular cytokine staining

Intracellular cytokine staining was performed as described previously (Matsui et al., 2005). Briefly, after one week following immunization, 2×10^6 spleen cells of mice were incubated with 10 μM of FMP 58-66 for 5 h at 37 °C in the presence of brefeldin A (GoldiPlug™, BD Biosciences, San Jose, CA). After Fc receptors were blocked with the rat anti-mouse CD16/CD32 mAb (Fc Block™, BD Biosciences), cells were stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD8α monoclonal antibody (mAb) (BD Biosciences) for 30 min at 4 °C. The cells were then fixed, permeabilized, and stained with phycoerythrin (PE)-conjugated rat anti-mouse interferon-gamma (IFN-γ) mAb (BD Biosciences). After the cells were washed, flow cytometric analyses were performed.

In vivo CTL assay

In vivo CTL assay was carried out as described previously (Matsui et al., 2010). Briefly, spleen cells from naive HHD mice were split into two populations. One population was pulsed with 10 μM of FMP 58-66 and labeled with a high concentration (2.5 μM) of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR), and the other population was unpulsed and labeled with a lower concentration (0.25 μM) of CFSE. An equal number (1×10^7) of cells from each population was adoptively transferred into mice that had been immunized with the chimeric SV40-VLPs one week earlier. Twelve hours later, spleen cells were prepared and analyzed by flow cytometry. To calculate specific lysis, the following formula was used: % specific lysis = (1 − {(number of $CFSE^{low}$ cells in normal mice)/(number of $CFSE^{high}$ cells in normal mice)}/{(number of $CFSE^{low}$ cells in immunized mice)/(number of $CFSE^{high}$ cells in immunized mice)} × 100.

^{51}Cr-release assay

^{51}Cr-release assays were carried out as described before (Matsui et al., 2005). In brief, after two weeks following immunization, spleen cells of the immunized mice were stimulated twice with gamma-irradiated, syngeneic spleen cells pulsed with 10 μM of FMP 58-66 after one-week interval, and were used as effector cells. For the detection of memory CTLs, spleen cells were prepared on day 30 after the immunization, and stimulated twice with FMP 58-66 as described above. In the second peptide stimulation, human recombinant interleukin-2 (Cetus, Berkeley, CA) was added at a final concentration of 200 units/ml into the culture medium. RMA–HHD cells pulsed with or without 10 μM of FMP 58-66 were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ as target cells. After a 4-h incubation, supernatant of each well was harvested and the radioactivity was counted. Results were calculated as the means of triplicate assays. Percent specific lysis was calculated according to the formula: specific lysis = [($cpm_{sample} - cpm_{spontaneous}$)/($cpm_{maximum} - cpm_{spontaneous}$)] × 100. Spontaneous release represents the radioactivity released by target cells in the absence of effectors, and maximum release represents the radioactivity released by target cells lysed with 5% Triton X-100.

Influenza A virus

Two influenza A virus strains, H1N1 (A/PR8/34) and H3N2 (A/Aichi/2/68) were propagated in 10-day-old embryonated hen's eggs at 35 °C for 3 days. Egg allantoic fluid containing virus was then harvested and stocked at −80 °C. Virus titers were determined by calculating the 50% tissue culture infectious dose ($TCID_{50}$) using MDCK cells (Suda et al., 2011). H1N1 (A/PR/8/34) virus was purchased from ATCC, and H3N2 (A/Aichi/2/68) virus was kindly provided by Dr. H. Kida at Hokkaido University, Japan.

Viral challenge

After one week following immunization, mice were anesthetized by an i.p. injection of ketamine (Sigma-Aldrich) and xylazine (Bayer Holding Ltd.), and were challenged i.n. with 1×10^2 $TCID_{50}$ of either H1N1 (A/PR/8/34) virus or H3N2 (A/Aichi/2/68) virus resuspended in 40 μl of PBS per animal. Mice were weighted daily for two weeks. For virus titration, mice were sacrificed on day 4 after the virus challenge, and virus titers in their lungs were determined by calculating $TCID_{50}$ using MDCK cells as described previously (Suda et al., 2011).

Detection of maturation markers on the surface of DCs

DCs were generated from mouse bone marrow cells as described (Lutz et al., 1999; Matsui et al., 2002). To investigate maturation, 5×10^5 DCs of naive HHD mice were resuspended in 200 μl of R-10, and were incubated with 2.5 μg of DNase I-treated FMP-DE-VLPs, FMP-HI-VLPs, or WT-VLPs for 24 h at 37°C in each well of a round-bottomed 96-well plate. Cells were stained with FITC-conjugated rat anti-mouse CD80, CD86 or CD40 mAb (BD Biosciences) for 30 min at 4 °C, and were analyzed by flow cytometry.

Statistical analyses

Statistical analyses between two groups were performed with Student's *t*-test. One-way ANOVA followed by post hoc tests were carried out for statistical analyses between multiple groups using GraphPad Prism 5 software. A value of $P < 0.05$ was considered statistically significant.

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M.K., H.H., and M.M. conceived and designed the experiments. M.K., K.M., T.S., and M.M. performed the experiments. M.K., H.H., and M.M. analyzed the data. N.O., S.M., and T.A. contributed reagents/materials/analysis tools. M.K. and M.M. wrote the paper.

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