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Exploring mucosal immunization with a recombinant influenza virus carrying an HIV-polyepitope in mice with pre-existing immunity to influenza

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ABSTRACT

HIV-1 vaccines based on recombinant vectors have been developed to elicit immune responses; however, the failure of the STEP HIV-1 vaccine trial has caused concern regarding the impact on vaccine efficacy of pre-existing vector seropositivity in humans. By using a mouse model of infection, we evaluated the immune responses elicited by intranasal and vaginal immunization with the recombinant influenza virus WSN/CKG carrying the PCLUS3-P18 peptide and a Gag epitope in its hemagglutinin, and the impact of pre-existing vector immunity on protection against recombinant vaccinia virus challenge. We found that despite the protective immunity induced in naïve mice by the WSN/CKG virus via either route, the vaginal immunization of mice with pre-existing influenza immunity restricted vPE16 replication more significantly in the ovaries than intranasal immunization. Thus, successful vaccination strategies under limiting conditions, such as pre-existing vector immunity, require the local induction of mucosal immunity at the site of virus infection.

1. Introduction

A successful HIV vaccine still represents a promising strategy for the prevention of AIDS [1,2]. Various vaccine vectors expressing different HIV-1 antigens have been assayed to determine the magnitude of the immune response, and the correlation with vaccine-induced protection against challenge in animal models [3,4]. Among them, recombinant adenovirus vectors, used for this purpose in clinical trials, have raised some concerns regarding reduced immunogenicity and vaccine failure that appears to correlate with pre-existing vector-specific immunity [5]. This has led to a better understand as to what extent pre-existing immunity against vaccine vectors could influence the immunogenicity of the vector-delivered HIV-1 antigens [6–8].

Recombinant influenza viruses engineered to express foreign antigens have been shown to induce effective immune responses in mice [9,10]. Their ability to infect and induce full maturation of dendritic cells is critical for effective antigen-presentation and induction of immunity [11,12]. Influenza viruses continue to cause epidemics in human populations, and the cross-reactive immune responses to the conserved influenza proteins detectable in healthy adults might be responsible for the poor efficacy of this virus as a vector of foreign antigens in humans [13,14]. Indeed, in a recent study performed in mice, pre-existing immunity to influenza was shown to considerably compromise the immunogenicity of a foreign antigen carried by a recombinant influenza virus [15]. We previously found that vaginal (i.vag.) immunization of progesterone-treated female mice with a recombinant influenza virus bearing an HIV CTL epitope in the neuraminidase (NA) stalk of A/WSN/33 (H1N1) (WSN) virus induced a vigorous and durable HIV-1-specific CD8+ T cell response in both mucosal and systemic compartments [16]. Thus, this mouse model of infection and the use of recombinant influenza viruses bearing foreign antigens could be useful to dissect further the influence of anti-vector immunity to heterologous influenza viruses on the development of antigen-specific immune responses.

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Here, we examined the characteristics of the immune responses elicited by infection of the respiratory or genital tracts of mice with a recombinant influenza virus (WSN/CKG) bearing a synthetic HIV polyepitope in its hemagglutinin (HA), and the extent of protection against viral challenge with recombinant vaccinia viruses in the ovarian tissue of mice with prior immunity to an heterologous influenza virus with antigenically distinct surface proteins but conserved internal antigens.

2. Materials and methods

2.1. Generation of recombinant WSN/CKG virus

The synthetic DNA construct (named CKG) containing the coding sequences for the cluster peptide PCLUS3 and the peptide 18 (P18) from the envelope glycoprotein gp160 of the HIV-1 IIl isolate [17,18], and the HIV-1 Gag-residues 192–208, containing the CTL epitope Gag_{197} [19], was introduced into the cloning cassette previously generated at the 3′ end of the signal peptide coding sequence of the pPolI–WSN HA plasmid [20].

The recombinant WSN/CKG (H1N1) virus bearing the CKG polyepitope at the N terminus of the HA protein was generated by using plasmid-based reverse genetics [21]. WSN/CKG virus was growing in MDCK cells with average titers of 6.3–7 log plaque-forming units (PFU)/ml. Stable expression of the 67 amino acid-long insert was confirmed by direct sequencing of RT-PCR products derived from plaques after several passages of growth in MDCK cells. RT-PCR amplification and sequence reactions were performed using primers for the HA gene [5′-GGCAAAACTACTGGTCCTGT-3′ (forward), 5′-TACTGAGCTCAATTGCTCCC-3′ (reverse)].

2.2. Immunization of mice

All animal work was performed in compliance with institutional guidelines and approved protocols. Female BALB/c mice were anesthetized with Avertin before being intranasally (i.n.) infected with 45 μl of PBS containing 10^6 PFU of WSN/CKG virus. For i.vag. infection, groups of mice were subcutaneously injected with 3 mg of progesterone (Pharmacia & Upjohn) [16]. Five days later, they were i.vag. infected with 10^6 PFU of WSN/CKG virus in a 10 μl volume.

For experiments involving animals with pre-existing immunity to influenza, mice were i.n. infected six weeks earlier with 10^5 PFU of the H3N2 reassortant virus X31 (A/Aichi/2/1968 (H3N2) HA and NA and A/PR/8/34 (H1N1) internal genes), and then immunized either i.n. or i.vag. with 10^6 PFU of WSN/CKG virus.

2.3. Viral replication in murine respiratory and genital tracts

To determine viral spread and replication in mouse respiratory tract, mice were anesthetized and infected i.n. with 10^6 PFU of WSN/CKG virus, or with 10^3 PFU of WSN virus. Four days after infection, mice were sacrificed, and virus titers were determined in lung homogenates and nasal turbinates by titration in MDCK cells.

Vaginal lavages were obtained at various times post-infection (p.i.) by rinsing the vaginal cavity with sterile PBS, and virus titers were determined by titration in MDCK cells.

2.4. IFN-γ-specific ELISPOT assay

Spleens and lymph nodes draining the respiratory tracts (mediastinal lymph nodes, MLN) and the vaginal tracts (iliac lymph nodes, ILN) of immunized mice were collected at the indicated time points p.i. and assayed for antigen-specific IFN-γ-producing cells by using an ELISPOT assay. A single-cell suspension from lymphoid tissues was cultured with the indicated synthetic peptides in anti-IFN-γ-coated plates at 37 °C for 36 h. Colored spots representing IFN-γ-releasing cells are reported as the number of spot-forming cells (SFC) per 10^6 cells.

2.5. ELISA

The presence of P18IIIB-specific IgG antibodies in serum from mice immunized with WSN or WSN/CKG virus was determined by means of an ELISA (18). Briefly, 96-well plates were coated overnight with 10 μM P18 peptide in 0.1 M carbonate buffer pH 9.6. Plates were blocked with 1% BSA in PBS, and serial two-fold dilutions of serum samples in PBS were added to the wells. After a 2-h incubation, plates were washed with PBS containing 0.05% Tween 20, and incubated with an HRP-goat anti-mouse IgG antibody. Bound antibodies were detected by the addition of TMB (Vector), and absorbance was read at a wavelength of 450 nm.

2.6. Protection against challenge infection with vaccinia viruses

The virus challenge experiments were performed by using recombinant vaccinia viruses expressing the full-length HIV-1IIIB gp160 (vPE16), and the HIV-1IIIB Gag gene (vDK1) [22,23]. Vaccinated mice were intraperitoneally (i.p.) challenged with 10^6 PFU of vaccinia viruses diluted in 300 μl of PBS. Six days after the virus challenge, ovaries from individual mice were homogenized in MEM/2% FCS and lysed by repeated freeze/thaw cycles. Titers were determined on infected CV-1 cells after 48 h by staining with 0.1% crystal violet. The limit of viral detection was 2 $\log_{10}$ PFU/ml. Statistical significance was determined by using the Student's *t*-test.

3. Results

3.1. Generation of a chimeric influenza virus expressing the CKG polyepitope and its replication in mice

A peptide construct containing the multideterminant Th peptide PCLUS3 from the gp160 of HIV-1, and P18 of the V3 loop, corresponding to the principal neutralizing determinant of HIV-IIIB and to the major immunodominant cytotoxic T cell epitope (P18–I10) in mice, as well as being recognized by human CTLs, was previously described [17,18]. Several studies have examined this HIV-1 peptide vaccine in mice [24,25]. Therefore, the CKG construct containing the PCLUS3–P18 domain-coding sequences, together with the CTL epitope Gag_{197} of HIV-1, were inserted into the HA of WSN virus, and the WSN/CKG virus was generated and used in our studies (Table 1).

The growth kinetics of the WSN/CKG virus in MDCK cells were slightly reduced compared with those of WSN virus (Fig. 1A). To evaluate the replication of the WSN/CKG virus in vivo, mice were inoculated i.n. with the recombinant virus and its growth kinetics were compared with that of the parental WSN virus. The viral loads in the nasal turbinates and lungs measured on day 4 p.i. in mice infected i.n. with 10^6 PFU of WSN/CKG virus were about two logs lower than those observed for mice infected with 10^3 PFU of WSN virus (Fig. 1B). Furthermore, viral replication in the vaginal mucosa was evident with the highest titers of WSN virus for up to day 6 p.i. being measured in the vaginal washes of progesterone-treated mice that had been infected via the i.vag. route, whereas WSN/CKG virus replicated less efficiently than did WSN virus and was completely cleared by day 5, even though a higher virus inoculum was used (Fig. 1C). Given that WSN/CKG virus was highly attenuated in vivo, an inoculum containing 10^6 PFU of virus was subsequently used for all experiments involving immunizing mice.

Table 1
HIV-1-derived epitopes contained in the CKG construct.

Foreign epitopes	Protein	Amino acid position	Sequence	Cell specificity
PCLUS3	gp120	421–444	KQIINMWQEVGKAMYAPPISGQIR	CD4+ T
P18	gp120	308–322	RIQ**RGPGRAFVTI**GK	CD8+ T[a], B
Gag197	Gag	192–208	GGHQA**AMQMLKETI**NEE	CD8+ T[a]

[a] CD8+ T epitopes are shown in boldface.

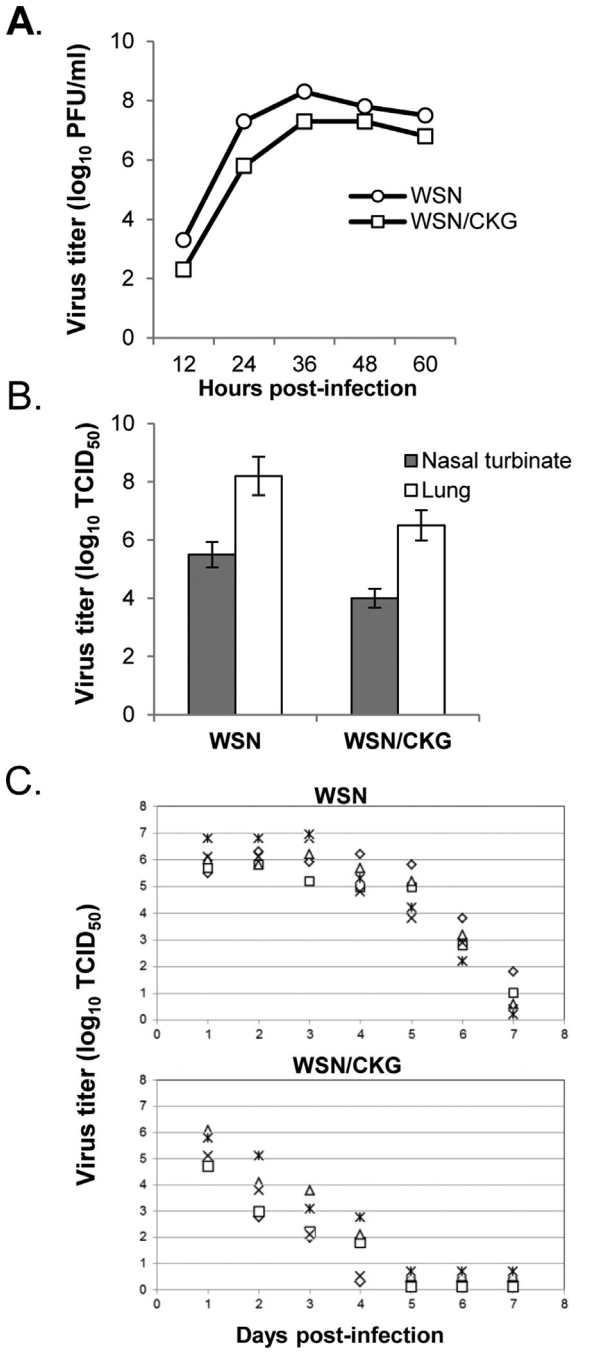

Fig. 1. In vitro and in vivo replication of WSN/CKG virus.
(A) Growth properties of WSN and WSN/CKG viruses in MDCK cells. The viruses were used to inoculate MDCK cells at a multiplicity of infection of 0.001, and at the indicated times after infection virus titers in the supernatants were determined. The values are means from duplicate experiments. (B) Female BALB/c mice ($n = 4$) were immunized i.n. under anesthesia with 10^3 PFU of WSN virus or 10^6 PFU of WSN/CKG virus, and sacrificed on day 4 p.i. for virus titration in lungs and nasal turbinates by calculating the fifty percent tissue culture infectious dose ($TCID_{50}$) in MDCK cells. (C) Progesterone-treated mice ($n = 5$) were i.vag. infected with the above viruses and virus titers in vaginal washes were determined at various times p.i.

3.2. Induction of mucosal and systemic immune responses by a single immunization with WSN/CKG virus

The primary CD8+ T cell responses detected in regional lymph nodes and spleens of mice during the acute phase of WSN/CKG virus infection via each route are shown in Fig. 2A. Although HIV-specific CD8+ T cell responses were slightly reduced in mice infected intravaginally compared with those infected intranasally, local and systemic CD8+ T cell responses were considerable high for the P18–I10 epitope (H-2D^d) and even higher than those specific for the influenza nucleoprotein immunodominant NP_{147} epitope (H-2K^d). In contrast, low levels of Gag_{197} (H-2K^d)-specific IFN-γ production were measured in lymphoid organs of mice infected via either route, revealing the poor immunogenicity of this foreign epitope.

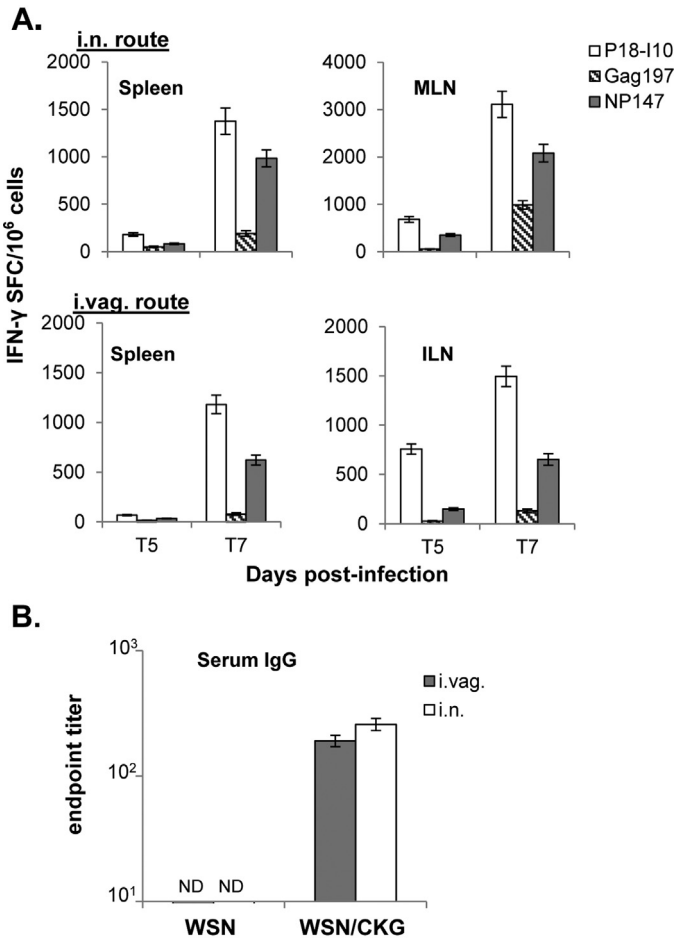

Fig. 2. Immune responses elicited in mice after immunization with WSN/CKG virus.
(A) Groups of naïve mice ($n = 5$) were immunized via the i.n. or i.vag. route with 10^6 PFU of WSN/CKG virus and the evolution of CD8+ T cell responses was monitored at 5 and 7 days p.i. in the spleens of individual mice and in the pool of draining LNs by using an IFNγ-ELISPOT assay with the indicated peptides. Results are expressed as SFC ± SD/10^6 cells. (B) Mice were immunized as described above. HIV IIIB P18-specific IgG antibodies were measured in serum samples collected on day 28 post-immunization by using an ELISA. Mean reciprocal endpoint titers for duplicate assays are shown. ND = none detected.

To investigate whether WSN/CKG virus infection could elicit a P18IIIB-specific antibody response, mice immunized with a single dose of WSN/CKG virus were bled 28 days post-immunization. Similar levels of P18-specific serum IgG antibodies were induced after either i.n. or i.vag. infection with WSN/CKG virus (Fig. 2B), suggesting that the V3 loop region was well exposed on the viral HA.

3.3. Protection against recombinant vaccinia virus challenge in WSN/CKG-immunized mice

Rectal immunization of mice with the synthetic PCLUS3–P18 peptide vaccine induces protective immunity against challenge with the recombinant vaccinia virus vPE16 [24–26]. Therefore, in this study, mice primed with WSN/CKG virus were challenged 6 weeks later with 10^6 PFU of VP16 or vDK1. Because the vaccinia virus replicates most efficiently in ovaries, they were removed 6 days after i.p. challenge and tested for vaccinia titers on a monolayer of CV1 cells. Mice primed i.n. with WSN/CKG virus were completely protected against challenge with vPE16, as no virus was found in ovaries (Fig. 3A). Mice primed i.vag. with WSN/CKG virus were also protected against virus challenge, and a reduced level of virus replication was detected in only one of seven mice. The protection against vPE16 infection observed in mice primed with WSN/CKG virus correlated with the presence of a vigorous P18-I10-specific recall response measured in their spleens on day 6 post-challenge with vPE6 (Fig. 3B). The suboptimal Gag-specific immune response elicited by WSN/CKG priming, particularly after i.vag. immunization, was ineffective in protecting mice against vDK1 challenge. These mice showed less than a 1 $\log_{10}$ reduction in viral titers in the ovaries compared with unprimed mice (Fig. 3A). Despite this lack of protection, it is important to note that the small number of Gag_{197}-specific CD8+ T cells elicited by i.n. or i.vag. priming with WSN/CKG virus was considerably boosted by vDK1 virus infection (Fig. 3B).

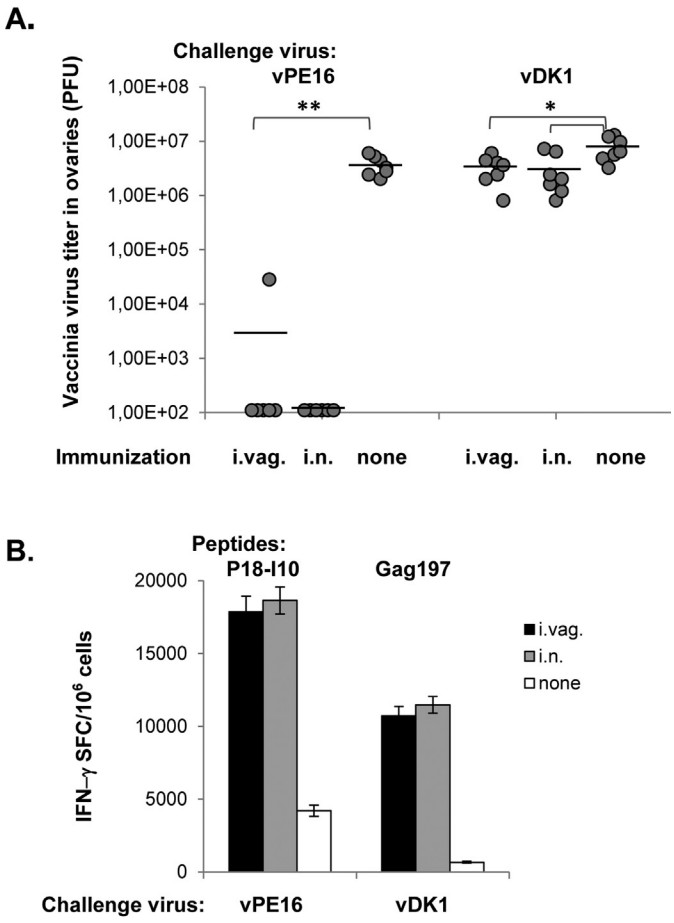

Fig. 3. Protection against recombinant HIV-1 vaccinia challenge of WSN/CKG-immunized mice.
WSN/CKG-vaccinated mice (7/group) were challenged with 10^6 PFU of vPE16 or vDK1 viruses, 6 weeks after immunization. Control mice received PBS only on the same schedule. Six days after challenge, mice were sacrificed and their ovaries and spleens were removed. (A) Ovaries were homogenized, sonicated, and the vaccinia virus titers of the individual mice were determined from serial 10-fold dilutions on CV1 cells. (B) The frequencies of P18–I10- and Gag_{197}-specific IFN-γ-producing T cells were measured by using the ELISPOT assay on spleen-derived lymphocytes from individual mice. Bars represent means ± SD for seven mice per group. The data are representative of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ compared to control mice.

3.4. Induction of immune responses by WSN/CKG immunization of mice with pre-existing immunity to influenza

We next sought to determine whether prior exposure of mice to a heterologous influenza A virus affected the WSN-CKG-mediated induction of protective immunity against vPE16 challenge. Groups of mice were i.n. primed with influenza X31 virus, and six weeks later were infected with the heterologous WSN/CKG virus via the i.n. or i.vag. route. WSN/CKG virus replication was severely inhibited in X31-preimmune mice, with virus titers barely detectable and at only early times after infection (data not shown). Although X31 is heterologous to WSN/CKG virus for surface glycoproteins, both viruses express conserved internal antigens, including the NP_{147} epitope. Thus, a vigorous early recall response of NP_{147}-specific CD8+ T cells was measured on day 5 in the lymphoid tissues of both groups of mice vaccinated in the presence of pre-existing immunity to X31 (Fig. 4A). Notably, considerable numbers of P18-I10-specific CD8+ T cells were still measurable in these mice, thus showing that the primary CD8+ T cell response specific to the P18–I10 epitope was reduced but not completely abrogated by prior infection with X31 virus. By contrast, the K^d restricted Gag_{197}-specific CD8+ T cells were unable to minimally compete with the recall of the NP_{147}-specific CD8+ T cells, and thus were undetectable in the IFN-γ-ELISPOT assay (data not shown). As expected, the presence of pre-existing immunity to X31 virus reduced the levels of P18IIIB-specific serum IgG antibodies in mice immunized via either the i.n. or i.vag. route, relative to naïve mice (Fig. 4B), which is consistent with previous observations [15]. Background IFN-γ production to HIV-specific peptides ($\leq$30 SFC/10^6 cells) was detected by using lymphoid cells from both naïve mice and mice immunized with the WSN virus.

3.5. Protection against VPE16 challenge in WSN/CKG-immunized mice with pre-existing immunity to influenza

Lastly, we determined the potential impact of X31-pre-existing immunity on protection against vPE16 challenge of WSN/CKG-immunized mice. The beneficial effect of WSN/CKG immunization via the i.n. route, as described above in mice not previously exposed to influenza virus, was notably affected in mice with pre-existing immunity to heterologous influenza virus strains (Fig. 5A). In particular, all of the X31-preimmune mice that were intranasally infected with WSN/CKG virus showed around a 1 $\log_{10}$ reduction in vPE16 virus replication in the ovaries relative to vPE16 replication in mice that did not receive WSN/CKG virus. In contrast, we consistently found statistically significant differences in X31-preimmune mice that were i.vag. infected with WSN/CKG virus. In this case, about two out of seven (28.5%) of the vaccinated mice showed an almost 2 $\log_{10}$ reduction in vPE16 virus titer, yet complete protection from infection was achieved in most of the mice. The presence of higher

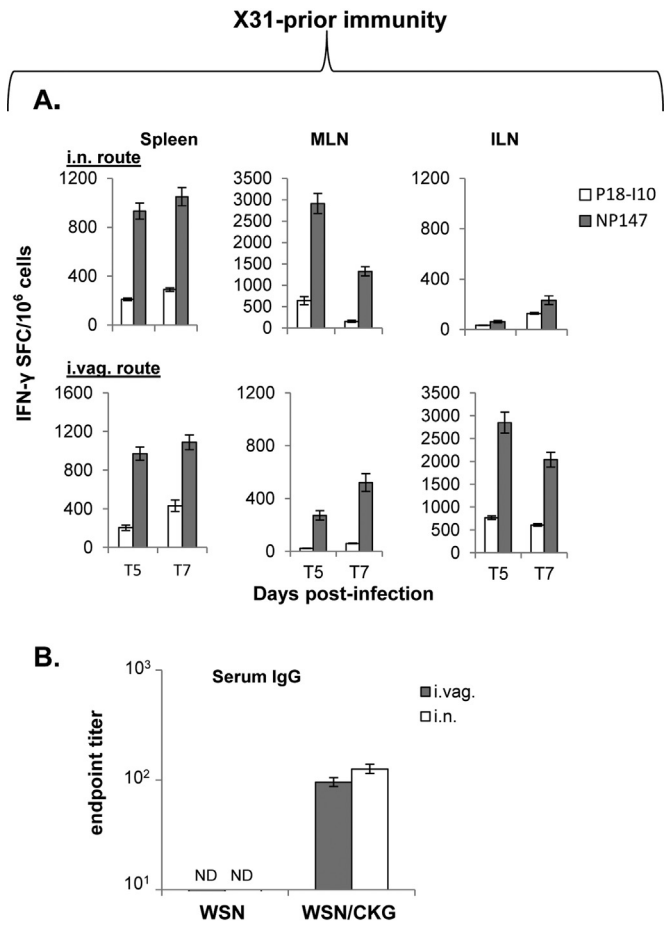

Fig. 4. The effect of previous exposure to influenza virus on the immunogenicity of WSN/CKG virus in mice.
Groups of mice previously infected with X31 virus were immunized i.n. or i.vag. with 10^6 PFU of WSN/CKG virus and the evolution of CD8+ T cell responses was monitored at 5 and 7 days p.i. among the spleen-derived lymphocytes of individual mice and in the pool of LNs by using an IFNγ-ELISPOT assay with the indicated peptides. Results are expressed as SFC ± SD/10^6 cells. (B) X31-preimmune mice were infected either i.vag. or i.n. with WSN/CKG virus or WSN virus. HIV-1 P18IIIB-specific IgG antibodies were measured in serum samples collected on day 28 after immunization by using an ELISA. Mean reciprocal endpoint titers for duplicate assays are shown. ND = none detected.

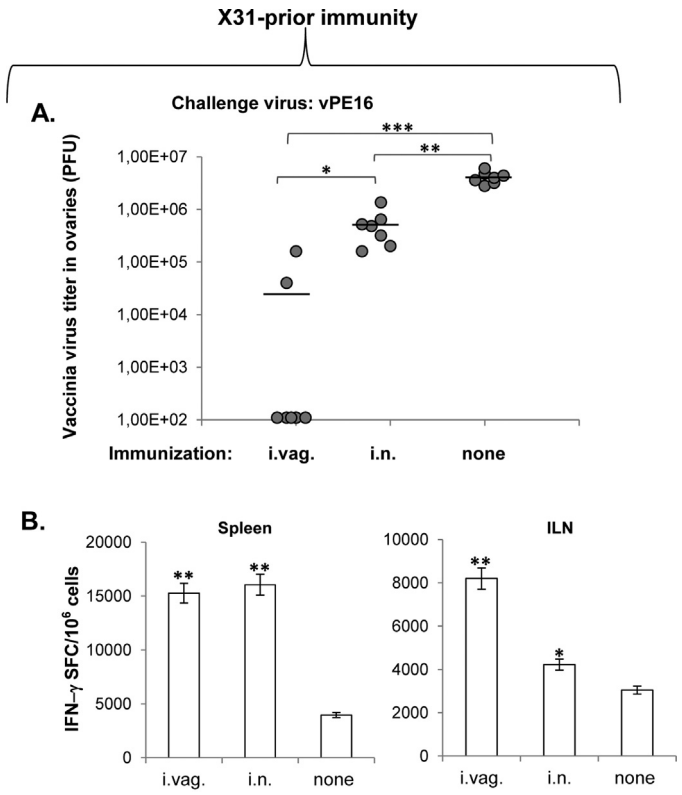

Fig. 5. Protection induced by WSN/CKG virus immunization in the presence of pre-existing immunity to influenza virus.
Mice previously infected with X31 virus (7/group) were vaccinated 6 weeks later with WSN/CKG virus. Four weeks later, they were challenged with 10^6 PFU of vPE16. Six days after challenge, the mice were sacrificed and their ovaries and lymphoid organs were removed. (A) Ovaries were homogenized, sonicated, and the vaccinia virus titer was determined from serial 10-fold dilutions on CV1 cells. (B) The frequency of P18–I10-specific IFN-γ-producing T cells was measured by using the ELISPOT assay on spleen-derived lymphocytes from individual mice and pools of ILN. Results are expressed as SFC ± SD/10^6 cells. The data are representative of three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$, $^{***}P < 0.0001$.

numbers of P18–I10-specific T cell effectors in the ILN of mice after i.vag. immunization with WSN/CKG virus compared with i.n. immunization likely accounts for the higher protection from vPE16 challenge (Fig. 4A). Accordingly, higher recall responses of antigen-specific CD8+ T cells were detected on day 6 post vPE16 challenge in the ILN of these mice, compared with similar levels measured in the spleens of both groups of immunized mice (Fig. 5B).

4. Discussion

In the present study, we evaluated the immune responses elicited by i.n. and i.vag. immunization with the recombinant influenza virus WSN/CKG carrying the PCLUS3–P18 peptide and a short region of Gag in its HA, and the impact of a pre-existing immunity to the viral vector on the immunogenicity of the foreign epitopes.

Reduced viral growth in vivo is usually observed with recombinant influenza viruses bearing foreign antigens inserted in either their NA or HA. Here, the WSN/CKG virus replicated at low levels in the respiratory and vaginal tract of mice infected via the i.n. and i.vag. route, respectively. Despite this, high numbers of P18–I10-specific CD8+ T cells were induced in the lymphoid organs of these mice, and were able to provide protection against massive viral replication in the ovaries following vPE16 challenge. In contrast to previous studies showing high levels of Gag_{197}-specific CD8+ T cells induced by a single administration of vaccine vectors encoding the HIV-1 Gag protein [27–29], the delivery of the Gag_{197} epitope by the WSN/CKG virus induced poor Gag-specific T cell responses likely because of binding to the same allele of MHC class I molecule as the immunodominant NP_{147} epitope. Despite the boosting of Gag_{197}-specific CD8+ T cell numbers that was observed in the spleens of these mice following challenge with vDK1 virus, effector CD8+ T cells were unable to control vDK1 in the early stages of the infection. Overall, the poor response to the Gag_{197} epitope elicited during the primary infection with the WSN/CKG virus, as well as with vDK1, and the vigorous expansion of Gag_{197}-specific CD8+ T cells after vDK1 challenge clearly indicate a subdominant hierarchy of the Gag_{197} epitope in these viral vectors, thus highlighting the influence that vector antigenicity may have on the immunogenicity of foreign epitopes.

Pre-existing immunity in the human population to candidate viral vectors may substantially reduce their immunogenicity and clinical utility, and thus hamper their use. Indeed, attempts to circumvent the problem, such as employing the mucosal versus the systemic route of immunization, or using vaccine vectors capable of inducing antigen-specific CD8+ T cells even under pre-existing vector immunity, have been proposed [30–33]. Recently, Langley et al. [15] found that mice previously infected with influenza viruses,

even those expressing HA and NA proteins of completely different subtypes, were severely compromised in their ability to mount an immune response against the inserted epitopes. Our results show that heterosubtypic immunity induced by previous i.n. infection with X31 virus substantially reduces the levels of P18-I10-specific CD8+ T cells, and only the local vaccine delivery, via the i.vag. route, is able to confer protection to the ovaries from vPE16 challenge. In particular, the higher numbers of P18-I10-specific CD8+ T cells in the ILN of WSN/CKG-vaginally-immunized mice relative to those immunized via the i.n. route, but not the similar levels of effectors measured in the spleens of both groups, were correlated with the vaccine-induced protective efficacy. Furthermore, the low levels of P18IIIB-specific antibodies detected in the serum of mice immunized via either the i.n. or i.vag. route suggest that the antibody response may not be responsible for the different degrees of protection observed for these two groups of mice. Although we did not quantitate the PCLUS3-specific CD4+ T cells or the P18-I10-specific memory T cells in the vPE16 target tissues, the correlation between the superior protection of mice i.vag. immunized with WSN/CKG virus and the higher numbers of effector T cells in ILN implies that the anatomical location of these memory T cells at the early time of infection was essential to successfully control virus replication with minimal delay.

Consistent with previous studies showing the importance of delivering HIV antigens to genito-rectal mucosa [34–36], our data further highlight its particular relevance in situations, such as the presence of pre-existing vector immunity, that can affect the efficacy of vaccine candidates. Vaccination strategies aimed at inducing immune responses at potential sites of infection would facilitate the establishment of lymphoid and tissue-resident memory cells, and thus mediate local virus control with prompt effector functions. Although the effects of pre-existing immunity to the vector would differ depending on the antigen and system utilized, these findings may provide useful information for successful vaccination strategies against sexual-transmitted pathogens.

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