Enhanced Mucosal Immune Responses to HIV Virus-Like Particles Containing a Membrane-Anchored Adjuvant

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ABSTRACT **Previously, a modified HIV Env protein with a heterologous membrane anchor was found to be incorporated into HIV virus-like particles (VLPs) at 10-fold-higher levels than those of unmodified Env. To further improve the immunogenicity of such VLPs, membrane-anchored forms of bacterial flagellin (FliC) or a flagellin with a truncated variable region (tFliC) were constructed to be incorporated into the VLPs as adjuvants. HIV-specific immune responses induced by the resulting VLPs were determined in a guinea pig model. The VLPs induce enhanced systemic antibody responses by either systemic or mucosal vaccination and enhanced mucosal immunity by a mucosal immunization route, as demonstrated by high levels of HIV-specific serum IgG and mucosal IgG and IgA. The quality of the antibody responses was also improved, as shown by enhanced neutralization capacity. VLPs incorporating FliC were more effective in inducing systemic responses, while VLPs containing tFliC were more effective in inducing mucosal IgA responses. The IgG titers in sera were found to last for at least 5 months without a significant drop. These results indicate that HIV VLPs incorporating high levels of Env and a molecular adjuvant have excellent potential for further development as a prophylactic HIV vaccine.**

IMPORTANCE **A prophylactic vaccine is urgently needed to control the spread of HIV/AIDS. Antigens inducing strong systemic and mucosal immune responses are promising as vaccines for this mucosally transmitted disease. We found that novel HIV virus-like particles (VLPs) presenting a high level of Env in its native membrane-bound form and coincorporating an innate immune-signaling adjuvant in the same particles were effective in inducing enhanced systemic and mucosal immunity. As new HIV vaccine candidates, these VLPs bridge the gaps of the innate and adaptive, as well as systemic and mucosal, immune responses, providing a new approach for HIV vaccine development.**

Received 14 December 2010 **Accepted** 20 December 2010 **Published** 15 February 2011

Citation Vassilieva, E. V., B.-Z. Wang, A. N. Vzorov, L. Wang, Y.-C. Wang, et al. 2011. Enhanced mucosal immune responses to HIV virus-like particles containing a membrane-anchored adjuvant. mBio 2(1):e00328-10. doi:10.1128/mBio.00328-10.

Editor Vinayaka Prasad, Albert Einstein College of Medicine

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Human immunodeficiency virus type 1 (HIV-1) has infected over 60 million individuals, resulting in acquired immunodeficiency syndrome (AIDS), and has caused the death of over 20 million people worldwide since its discovery as the etiologic agent of AIDS (1). The development of a prophylactic vaccine would be the most effective measure to curb this fatal AIDS pandemic. Although previous clinical trials of HIV vaccines have failed, a recent trial conducted in Thailand reported a modest ability (around 30%) to protect from HIV by a combined vaccination strategy, indicating the possibility of a prophylactic vaccine and the importance of further studies (2).

A prophylactic HIV-1 vaccine should elicit effective antibody responses, including broadly cross-reactive neutralizing activity (3–5). The gp120-gp41 Env protein on the native virion is the primary target for eliciting such antibodies. Recent studies demonstrated that effective antibodies even at lower titers can protect against low-dose repeated mucosal simian/human immunodeficiency virus (SHIV) challenge in macaques, which mimics infection of humans by sexual contact (6). This finding supports the important potential role of mucosal antibody in protective immunity. Progress has been achieved in recent years in enhancing the immunogenicity of Env by modifying its structure, employing novel delivery platforms, and coadministering of adjuvants (7–9). The use of virus-like particles (VLPs) has shown great potential as a new vaccine platform (10–14). Such VLPs present the Env protein in its native membrane-bound form and have a high level of safety because they lack the viral genome.

Flagellin is the primary protein component of the highly complex flagellar structures that extend from the outer membrane of Gram-negative organisms. It is known that Toll-like receptor 5 (TLR5) recognizes a conserved site on flagellin (15). Acting as the natural agonist of TLR5, flagellin was found to be a highly potent and effective adjuvant in humans and nonhuman primates (16–18). The adjuvant effects of flagellin for antibody responses, as well as T cell responses, have been demonstrated for purified natural, recombinant, and truncated forms (19–22). Removal of the vari-

able region from flagellin has been shown to stimulate mucosal innate immunity while decreasing the antigenicity of flagellin (23). Here we investigated whether the immunogenicity of HIV VLPs can be enhanced by incorporation of a membrane-bound form of flagellin or a flagellin with a truncated variable region as an adjuvant for induction of both systemic and mucosal antibody responses.

RESULTS

Construction of membrane-anchored forms of full-length flagellin and flagellin with a truncated variable region. In a previous study, we found that a modified form of HIV-1 Env with heterologous transmembrane/cytoplasmic (TM/CT) domains derived from the mouse mammary tumor virus (MMTV) glycoprotein was incorporated into Gag-derived HIV-1 VLPs at 10- to 15-fold-higher levels than native Env (24). In the present study, we determine whether coincorporation of a membrane-anchored flagellin, the Toll-like receptor 5 (TLR5) ligand, into such VLPs will induce enhanced antibody responses and neutralization reactivity. Thus, we constructed genes encoding membrane-anchored flagellin (FliC), as depicted in Fig. 1A. We also constructed a membrane-anchored flagellin with a truncated variable region (tFliC) because the soluble truncated protein was reported to be more effective as a mucosal adjuvant (23). The N-terminal domain 1 and 2 (ND1-2; amino acids [aa] 1 to 176) and C-terminal domain 2 and 1 (CD2-1; aa 402 to 459) sequences corresponding to the full-length *Salmonella enterica* serovar Typhimurium flagellin-encoding DNA were fused by overlapping PCR with a hinge region to enhance the flexibility for the terminal sequences of flagellin to form the necessary conformation for TLR5 recognition (21). The mellitin signal peptide (SP) and MMTV TM/CT domain-encoding sequences were used in the membrane-anchored flagellin constructs because they were found to result in a higher incorporation of modified HIV Env protein into VLPs (24). The resulting chimeric flagellin genes were used to generate recombinant baculoviruses (rBVs) expressing membrane-anchored flagellin in insect cells.

Characterization of HIV-1 cVLPs containing high levels of Env and membrane-anchored flagellin. Standard and chimeric HIV VLPs (cVLPs) were produced by rBV expression as described in Materials and Methods. Our hypothesis is that incorporation of the antigen (Env) and the adjuvant into the same cVLP structure, as schematized in Fig. 1B, will deliver both components to the same cells and thus be advantageous for enhancing immunogenicity. The protein composition of VLPs was characterized by Western blotting, as shown in Fig. 1C. As described previously, the modified Con-S Env is a derivative of Con-S gp145ΔCFI (25), with heterologous TM/CT domains from the MMTV Env glycoprotein to enhance incorporation into VLPs at high levels (24). As shown in Fig. 1C, the modified Env incorporated into standard or cVLPs was observed to have a molecular mass of about 120 kDa. This is lower than expected for mammalian cell expression (145 kDa), probably because of a different glycosylation pattern in insect cells. Quantitative analysis showed that these VLPs contained up to 16 μg of Env per 100 μg of VLPs, which is similar to the previously observed incorporation level (24). The membrane-anchored flagellin is predicted to have a molecular mass of about 60 kDa. Western blotting showed protein bands around 60 kDa, but with isoforms, suggesting nonuniform glycosylation. These results are consistent with our previous observation that a

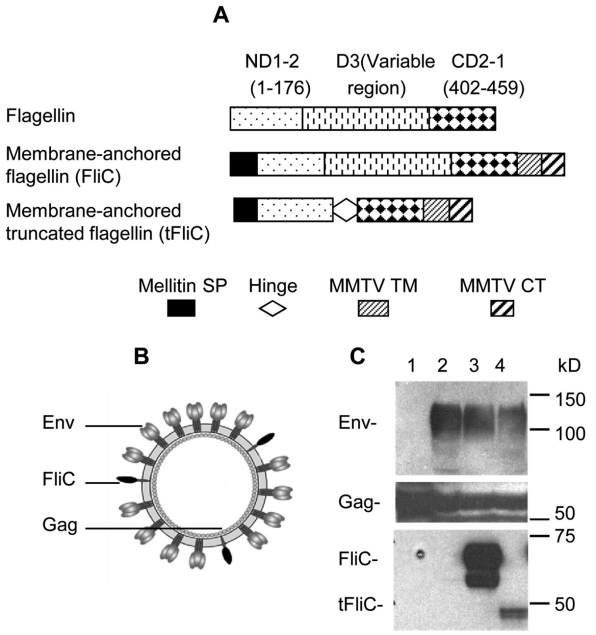

FIG 1 Schematic diagrams of flagellin constructs and characterization of HIV cVLP. (A) Flagellin constructs. Soluble monomeric flagellin contains 459 amino acid (aa) residues. The N-terminal (ND1-2; aa 1 to 176) and C-terminal (CD2-1; aa 402 to 459) domains contain motifs that are necessary for TLR5 recognition. The membrane-anchored flagellin (FliC) construct was generated by fusing the mellitin signal peptide, flagellin, and MMTV transmembrane/cytoplasmic domain-encoding sequences by overlapping PCR. The membrane-anchored, FliC construct with a truncated variable region (tFliC) was formed by replacement of the variable region sequence (corresponding to aa 177 to 401 in full-length flagellin) with a flexible hinge by using overlapping PCR. (B) Diagram of HIV-1 cVLP. Env and membrane-anchored flagellin (FliC) are both anchored in the envelope. The Gag protein is assembled as a core inside the VLPs during the budding process. (C) Western blot characterizing Env, Gag, and flagellin components in HIV VLPs. VLP samples (1 μg) were resolved by SDS-PAGE followed by Western blotting. Env bands were visualized by goat anti-gp120 polyclonal antibody (top); Gag bands were detected by HIV-positive human sera; flagellin bands were visualized with mouse anti-flagellin polyclonal antibody. Lane 1, Gag-only VLPs; lane 2, Env/Gag VLPs; lane 3, Env/Gag/FliC cVLPs; lane 4, Env/Gag/tFliC cVLPs.

membrane-anchored form of flagellin had a nonuniform glycosylation pattern in insect cells (12). Multiple bands also were observed for the membrane-anchored truncated flagellin (molecular mass of approximately 39 kDa), for which the Western blot showed two bands detected by flagellin-specific antibody. The incorporation levels of flagellin into VLPs (ranging from 1 to 2 μg/100 μg of VLPs in different preparations) can be regulated by varying the multiplicity of infection (MOI) of the rBV used for expression.

HIV-1 cVLPs induce high titers of Env-specific antibody. As shown in Fig. 2, cVLPs containing membrane-anchored flagellin administered either intramuscularly (i.m.) or intranasally (i.n.) in guinea pigs induced serum antibody responses with higher titers than those observed with standard VLPs. After three immunizations (bleed 3 in Fig. 2A), guinea pigs immunized i.m. with flagellin-containing VLPs (Env/Gag/FliC cVLPs) achieved 36-fold-higher IgG levels (endpoint titer, 1.8×10^5) than a standard VLP group (5×10^3). The tFliC-containing group (Env/Gag/tFliC cVLPs) also exhibited 11-fold-higher IgG levels (5.4×10^4) than the standard VLP group. Data in Fig. 2B demonstrate that HIV-1

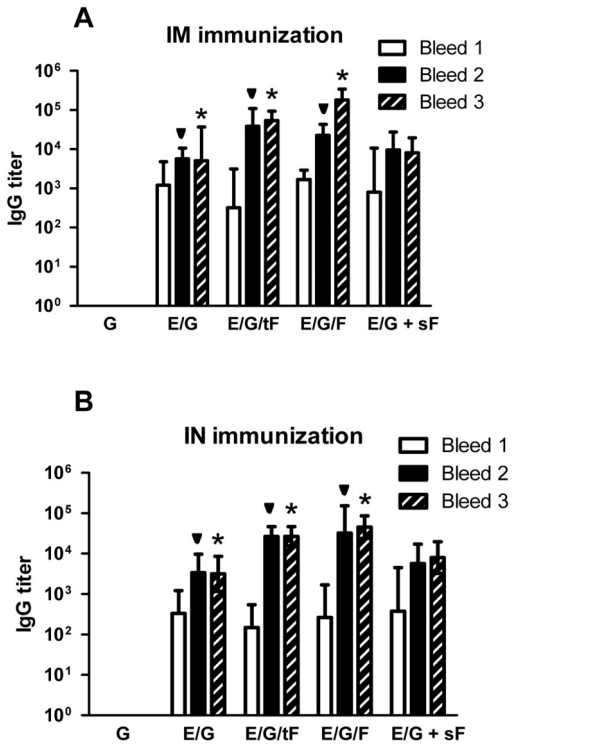

FIG 2 Flagellin-containing HIV-1 cVLPs induce enhanced systemic immune responses. Guinea pigs (four animals per group) were immunized with the indicated VLPs either intramuscularly (IM; A) or intranasally (IN; B) at weeks 0, 4, and 8, respectively. Serum IgG endpoint titers were assayed as described previously (12). Maxisorb 96-well plates were coated with purified His-tagged Env (Con-S gp120; 100 ng per well) purified from HeLa cells infected with a recombinant vaccinia virus (rVV) expressing Env gp120. Serum IgG endpoint titers were measured at week 2 (bleeding 1), week 6 (bleeding 2), and week 10 (bleeding 3). Results are expressed as geometric mean titers with 95% confidence intervals. Triangles and stars indicate statistically significant differences between the FliC- or tFliC-containing VLP groups and the standard VLP group ($P < 0.05$ by Student's t test) in bleeds 2 and 3, respectively. G, Gag VLPs; E/G, Env/Gag VLPs; E/G/tF, Env/Gag/tFliC cVLPs; E/G/F, Env/Gag/FliC cVLPs; E/G + sF, Env/Gag VLPs plus soluble flagellin.

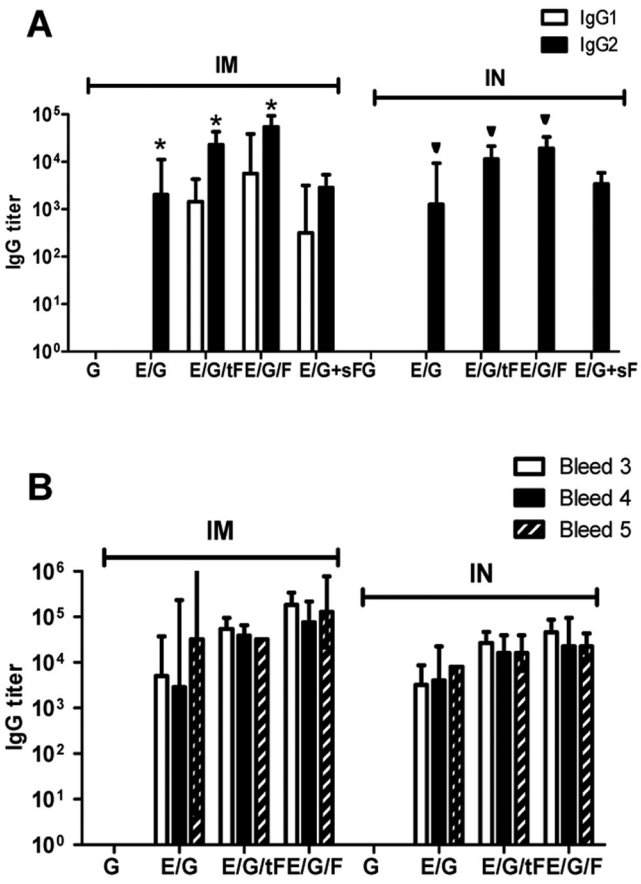

FIG 3 IgG subclasses and duration of antibody responses induced by flagellin-containing cVLPs. (A) Endpoint titers of IgG1 and IgG2. For IgG isotyping, the endpoint ELISA was performed as above, except that HRP-conjugated goat anti-guinea pig IgG1 or IgG2 secondary antibodies (Immunology Consultants Laboratory, Newburg, OR) were used. Asterisks indicate a statistically significant difference ($P < 0.05$) from the Env/Gag group. (B) Duration of antibody response. Serum IgG endpoint titers at week 10 (bleed 3), week 30 (bleed 4), and week 43 (bleed 5) were determined as in Fig. 2.

cVLPs also induce enhanced systemic responses by i.n. immunization. FliC- and tFliC-containing cVLPs elicited 14- and 8-fold-higher serum IgG titers (4.5×10^4 and 2.7×10^4, respectively) than a standard VLP group (3×10^3). In contrast, similar amounts of soluble flagellin did not show any adjuvant effect when mixed with standard VLPs prior to i.m. immunization, but induced slightly enhanced serum antibody responses by i.n. immunization. These results indicated that incorporation of the flagellin molecules into the VLPs is much more effective in enhancing anti-Env immune responses than addition of soluble flagellin to standard VLPs. Although flagellin-containing VLPs induced enhanced IgG responses, we did not detect IgA immune responses in the sera of immunized animals.

We also evaluated the serum IgG subclass profiles in bleed 3 and observed that standard VLPs induced IgG2-dominant immune responses, while the FliC-containing cVLPs enhanced both IgG1 and IgG2 production when administered i.m. (Fig. 3A), demonstrating that a balanced antibody profile is induced by cVLPs. In contrast, both full-length cVLPs and cVLPs containing flagellin with the variable region deleted as well as standard VLPs induced IgG2-dominant antibody responses (Fig. 3A), but IgG1 levels were undetectable when guinea pigs were vaccinated by the i.n. route, indicating different effects of flagellin when administered i.n. and i.m. It is desirable for vaccine-induced immune responses to persist for a prolonged period after immunization. As shown in Fig. 3B, serum IgG titers were high after three immunizations (bleed 3) and remained at similar levels for 5 months (bleed 4). An additional boost immunization did not raise the response levels any further (bleed 5). These data demonstrate that flagellin-containing cVLPs are effective in inducing long-lasting immune responses.

HIV-1 cVLPs induce enhanced neutralizing reactivity. The above data demonstrate that HIV-1 cVLPs induce significantly higher titers of serum IgG compared to standard VLPs by either the i.m. or i.n. immunization route. We further compared the resulting immune sera for neutralizing infection by a panel of HIV-1 pseudoviruses, using a high-throughput, pseudotype virus assay system. Five type B viruses, including two tier 1 and two tier 2 strains and one intermediate strain, were compared in the neutralizing antibody assay. Their coreceptors and neutralization sen-

TABLE 1 HIV isolates used for neutralization assays

Isolate[a]	Subtype	Coreceptor	Sensitivity/resistance to neutralization
HXB2	B	CXCR4	Sensitive (tier 1)
NL4-3	B	CXCR4	Sensitive (tier 1)
6535.3	B	CCR5	Intermediate
RHPA4259.7	B	CCR5	Intermediate
Du156.12	C	CCR5	Intermediate
Yu2	B	CCR5	Resistant (tier 2)
Du422.1	C	CCR5	Resistant (tier 2)

[a] The subtype B and C isolates were arranged in the order of the most-sensitive (tier 1), intermediate, and most-resistant (tier 2) strains to neutralization from the top to the bottom of the table.

sitivity/resistance are described in Table 1 (26–28). Data in Fig. 4 show that the neutralizing reactivity of the 80-fold-diluted immune sera (20-fold diluted for RHPA4259.7) obtained after the third immunization varied: standard VLP groups neutralized less than 20% of the virus infectivity for both i.m. and i.n. immunizations, except against HXB2 (27.5%) after i.n. immunization (Fig. 4A and B). FliC-containing cVLPs (Env/Gag/FliC cVLPs) by i.m. immunization induced the highest neutralization antibody responses against HXB2, NL4-3, 6535.3, or RHPA4259.7 (Fig. 4A). In contrast, tFliC-containing cVLPs (Env/Gag/tFliC cVLPs) were more effective in inducing neutralizing activity against tier 1 strains (HXB2 and NL4-3) when administered i.n. (Fig. 4B).

We also evaluated the capacity of the immune sera for neutralizing one tier 2 and one intermediate type C pseudovirus, Du422.1 and Du156.12, as listed in Table 1 (27). We found that immune sera at a 100-fold dilution after the third immunization (bleed 3) had no detectable neutralization of Du156.12; sera from groups immunized with FliC-conaining cVLPs (Env/Gag/FliC cVLPs) neutralized 45% of Du422.1, while other groups showed much lower neutralization (less than 20%) against this strain after i.m. immunization (Fig. 4C). The sera from guinea pigs immunized by the i.n. route with tFliC-containing cVLPs (Env/Gag/tFliC cVLPs) and FliC-containing cVLPs neutralized 47.5 and 15% of Du422.1 infectivity, respectively, while other groups did not show detectable neutralizing activity (Fig. 4D). These results indicate that the IgG levels in sera correlate with neutralization. We also found that when soluble flagellin was mixed with standard HIV-1 VLPs (Env/Gag VLPs plus sFliC), there was no significant increase in neutralizing antibody responses compared to that with the standard VLPs alone, demonstrating the importance of the physical association of flagellin with antigens.

HIV-1 cVLPs incorporating tFliC are more effective in inducing mucosal immunity. Mucosal immunization is known to induce mucosal responses in local, as well as distal mucosal sites (29, 30). To determine whether flagellin-containing cVLPs induce strong mucosal immune responses, we evaluated the vaginal mucosal secretory IgA (S-IgA) and IgG levels after i.n. immunization at week 18 (Fig. 5A and B) and after the last boost at week 38 (Fig. 5C and D). As observed in blood, the highest Env-specific IgG level in vaginal secretions was detected in the FliC-containing cVLP group (compare Fig. 5B and D and Fig. 2). S-IgA profiles in vaginal secretions were, however, different. Remarkably, tFliC-containing cVLP-immunized guinea pigs showed 20-fold-higher S-IgA levels at week 18 (Fig. 5A) and 60-fold-higher levels at week 38 (Fig. 5C) than standard VLP-immunized guinea pigs. FliC-containing VLP-immunized guinea pigs showed 2-fold-higher S-IgA at week 18 and 30-fold-higher S-IgA at week 38 than the standard VLP group (Fig. 5A versus C), thus demonstrating that the truncated form of flagellin is a more effective adjuvant for mucosal immunization. In mucosal samples, the data scattering is intrinsically larger than in sera due to the sample collection procedure and variations in the estrous cycle. There was a difference

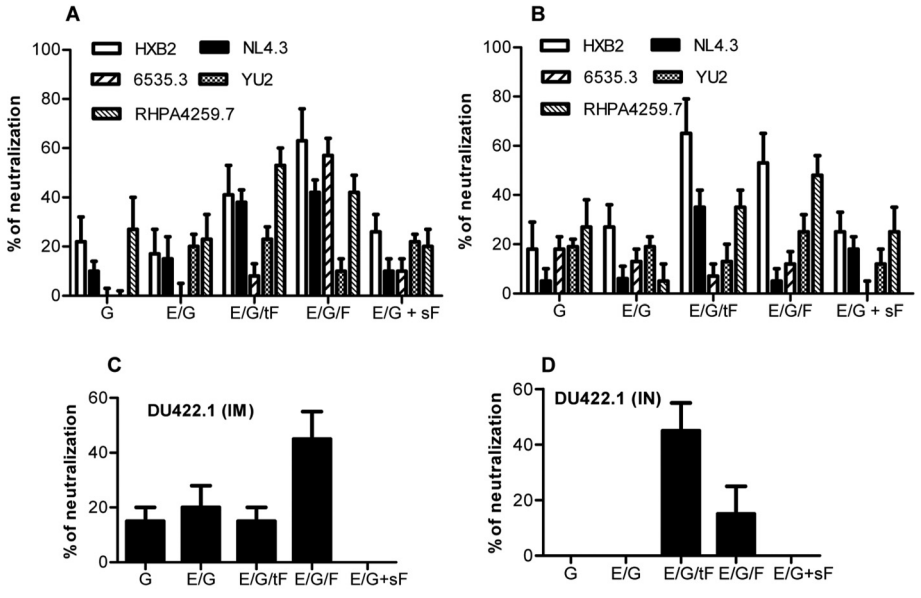

FIG 4 Neutralization of subtype B and C HIV-1 strains by sera from immunized guinea pigs. (A and B) Intramuscularly (IM) and intranasally (IN) immunized groups neutralizing subtype B pseudoviruses. Immune sera (bleed 3) were diluted to 80-fold for assays. For neutralization of RHPA4259.7, serum samples were diluted to 20-fold. Neutralization assays were performed on JC53-BL cells, a derivative of HeLa cells that express high levels of CD4 and the HIV-1 coreceptors CCR5 and CXCR4. Infected cells were detected by luciferase activity. (C and D) Intramuscularly and intranasally immunized groups neutralizing a subtype C pseudovirus, Du422.1. Results are expressed as means ± standard deviations (SD).

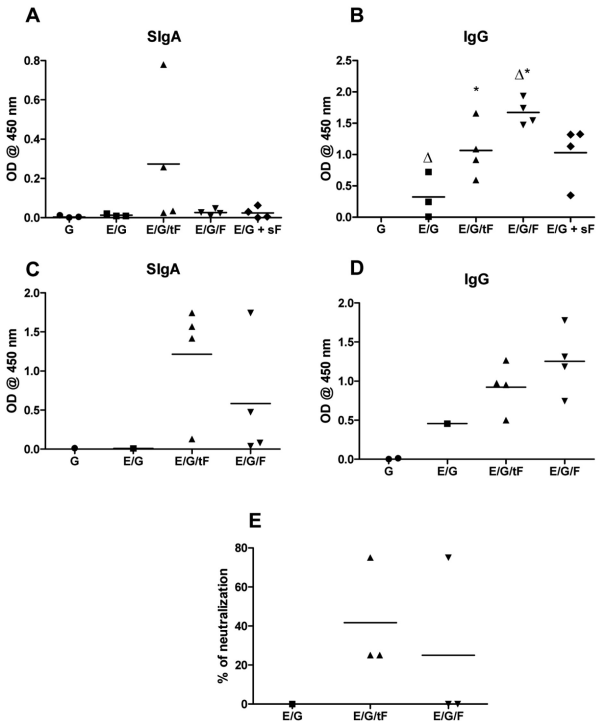

FIG 5 Mucosal responses. Chimeric VLPs induce enhanced mucosal immune responses measured at 18 weeks (A and B) or 38 weeks (C and D) in vaginal washes. The final dilutions of the vaginal secretion were 5-fold in panel A (S-IgA), 40-fold in panel B (IgG), 7-fold in panel C (S-IgA), and 150-fold in panel D (IgG). Env-specific S-IgA and IgG were detected by ELISA and expressed as mean OD_{450} ± standard deviation (SD). Asterisks and triangles indicate statistically significant differences between two cVLP groups ($P < 0.05$ by Student's *t* test). (E) Neutralization of HXB2 pseudovirus. Vaginal washes from i.n.-immunized guinea pigs were diluted 10-fold for the neutralization assay. The infected cells were detected by β-galactosidase expression.

between group mean values; however, this difference was not statistically significant. Importantly the same trend, namely the highest S-IgA levels in the truncated-flagellin group, was observed in samples collected at two different time points 143 days apart, despite variations introduced by sample collection and the estrous cycle phase. These data support the conclusion that the truncated form of flagellin is a more effective adjuvant for mucosal immunization.

We further determined whether mucosal antibodies conferred neutralization of infectivity. We used type B tier 1 HXB2 pseudovirus to evaluate the neutralizing activity of the vaginal samples collected at week 38. As shown in Fig. 5E, vaginal washes at low dilution from VLP-immunized guinea pigs partially neutralized HXB2. Samples from the standard VLP group had no detectable neutralization capacity. These results were consistent with the S-IgA levels in the same vaginal wash samples (Fig. 5C).

We also observed that cVLPs induced flagellin-specific serum IgG responses, and tFliC-containing VLPs induced lower responses than FliC-containing VLPs by either i.m. or i.n. immunization (data not shown), although it has been known that preexisting immunity to flagellin has no effect on its adjuvant function (12, 17, 31).

DISCUSSION

The design of antigens which are effective in inducing improved functional antibody responses is of high importance for AIDS vaccine development. Since VLPs are particulate structures which present trimeric Env proteins in their native membrane-bound form, they have attractive features as vaccine antigens. We observed previously that the Env incorporation levels into HIV VLPs can be improved significantly by engineering of constructs having heterologous TM/CT domains (24). Here we have shown that by attaching a membrane anchor sequence from MMTV Env, the TLR5 ligand flagellin can also be incorporated as an adjuvant into HIV VLPs. We observed that these flagellin-containing VLPs elicit high levels of systemic antibody responses by either i.m. or i.n. vaccination, as well as both systemic and mucosal immunity by i.n. vaccination. Chimeric VLPs containing full-length flagellin were more effective in inducing systemic responses, whereas cVLPs containing a flagellin with a truncated variable region were more effective in eliciting mucosal IgA responses. We also observed that these cVLPs induced antibody responses with broad subtypic reactivity.

High antibody responses are expected to be important for a prophylactic HIV vaccine. Our results demonstrated that antibody binding titers against gp120 were surprisingly high, reaching 36-fold higher levels than those of the standard VLPs after three i.m. immunizations with cVLPs containing FliC or 8-fold higher than those of the standard VLPs after three i.n. immunizations with cVLPs containing tFliC. Previous studies with simian immunodeficiency virus (SIV) have also shown that VLPs are highly immunogenic when antigens and adjuvants are coincorporated into the same VLP structure (11) or when adjuvant molecules are chemically conjugated with VLPs (32). The present data demonstrate the potential of HIV cVLPs incorporating membrane-anchored flagellin in inducing antibody responses. Flagellin binds to TLR5 and thus not only initiates innate signaling to secrete cytokines which can upregulate other immune cells, but also facilitates DCs to take up antigens and then present them to helper T cells in secondary lymph nodes (33, 34). These helper T cells form stable cognates with B cells and enhance the ability of B cells to proliferate and differentiate into plasma B cells (35). It is likely that the relationship of TLR5 signaling to antigen presentation could contribute to the enhancement of antibody responses to HIV VLPs. Because TLR5 is found to be expressed in antigen-presenting cells (APCs), in particular dendritic cells (DCs), we have hypothesized that flagellin-containing cVLPs can be taken-up by APCs after TLR5 binding and promote antigen presentation because of innate signaling in the same cell (12).

We observed that FliC-containing cVLPs induced higher titers of both IgG1 and IgG2 after i.m. immunization. In contrast, tFliC-containing cVLPs induced enhanced IgG2 titers and undetectable IgG1 levels after i.n. immunization. In guinea pigs, the connection between Th1/Th2 responses and IgG subclasses is not established, but there are indications that IgG2 may be related to a Th1 type of immunity. For instance, Gag-specific IgG2 levels correlated with gamma interferon (IFN-γ) production (36). Different systemic effects of full-length flagellin and flagellin with a truncated variable region have been reported previously upon systemic or mucosal immunization, but the underlying mechanisms are not clear (23). Studies with fusion proteins indicated that physical association of flagellin with antigens is necessary for flagellin to promote

antigen-specific immune responses (20). The present results showed that soluble flagellin has no adjuvant effect by i.m. immunization and has a low adjuvant effect by i.n. immunization when mixed with VLP antigens. Although the exact mechanisms involved in the different adjuvant effects of the bound and soluble flagellins are not completely understood, a different association pattern of flagellin with antigen could contribute to its ability to function as an adjuvant. For cVLPs, incorporated flagellin can bind to surface TLR5 on APCs and thus facilitate VLP uptake and consequent antigen processing and presentation. In contrast, if coadministered with VLPs as a mixture, flagellin and VLPs may interact with different APCs. Furthermore, independent of TLR5, a member of the NOD-like receptor (NLR) family, Ipaf, has been found to detect intracellular flagellin and initiate innate signaling by caspase 1 activation (37). VLPs are similar in their structure to viruses which the immune system has evolved to combat and thus can be taken up directly by APCs (38). Consequently, flagellin incorporated into cVLPs may also be sensed by its cytosolic receptor for independent innate signaling. In contrast, soluble flagellin mixed with VLP antigens cannot be transported into the cytosol without the help of bacterial virulence factors (39). These differences in interactions with the dual sensors for cVLPs containing flagellin may explain why the VLP-bound molecules are more effective in translating innate immune signaling into adaptive immunity.

It is of particular interest that HIV cVLPs incorporating membrane-anchored truncated flagellin induced enhanced mucosal antibody responses. Mucosal transmission is the predominant pathway for HIV infection in humans, and the intestinal mucosa is the major reservoir for virus replication and amplification (7). Effective HIV-specific mucosal antibody responses can be capable of reducing the occurrence of initial infection and possibly blocking the escape of virus from the genital or intestinal mucosa into systemic lymphoid organs (6, 7, 40). Recent studies demonstrate that effective antibody even at low titer can block repetitive low-dose SHIV infection, which simulates the natural sexual transmission process of HIV (6). Although our results show that the IgA levels in mucosal samples are consistent with the neutralizing activity, it has not been demonstrated that IgA is responsible for the increased neutralization capacity.

Most Env protein-based vaccine candidates are able to induce Env-binding IgG responses, but without enhancement of neutralization (41). The quality of antibody responses is more important than their quantity. The present data demonstrate that flagellin-containing cVLPs elicit broadened neutralization activity against five strains from both clades B and C. Two characteristics of these cVLPs may contribute to their ability to induce broadened neutralizing activity: (i) HIV Env is presented by VLPs in its natural trimeric structure in a membrane-bound microenvironment like that of the native protein (42); (ii) the adjuvant effect of flagellin can enhance HIV-specific adaptive immunity, in particular by increasing the efficiency of antigen presentation of epitopes shared by different subtypes. HIV cVLPs incorporating flagellin as an adjuvant therefore are promising as antigens for the development of a prophylactic HIV vaccine.

MATERIALS AND METHODS

Generation of rBVs expressing membrane-anchored full-length flagellin (FliC) or FliC with a truncated variable region (tFliC). To produce a gene encoding the membrane-anchored FliC, the coding DNA sequences for the mellitin signal peptide, full-length form of flagellin (*fliC*; GenBank accession no. D13689 [a gift from Alan Aderem]), and TM/CT domain of MMTV Env were fused in frame by overlapping PCR (12, 24). To produce the membrane-anchored tFliC-encoding gene, the DNA fragment coding the variable region (corresponding to aa 177 to 401 in the flagellin protein) was deleted from the flagellin gene, resulting in a gene encoding a truncated flagellin. The coding DNA sequences of the mellitin signal peptide, truncated flagellin, and TM/CT domain of MMTV Env were fused in frame by overlapping PCR to generate the membrane-anchored tFliC-encoding gene. The membrane-anchored FliC or tFliC gene was cloned into pFastBac-1 (Invitrogen, Carlsbad, CA), and the integrity of these genes was confirmed by DNA sequencing. Recombinant BVs expressing FliC or tFliC were generated by using the Bac-to-Bac insect cell protein expression kit (Invitrogen) following the manufacturer's instructions.

Production of standard or chimeric HIV VLPs. Standard HIV VLPs (Env/Gag VLPs) were produced by coinfection of insect cells (*Spodoptera frugiperda* Sf9 cells) with rBVs expressing Env and Gag as described previously (24). The cVLPs containing membrane-anchored FliC or tFliC (Env/Gag/FliC or Env/Gag/tFliC cVLPs) were produced by coinfection of Sf9 cells with rBVs expressing Env, Gag, FliC, or tFlic. VLPs containing Gag only used as a control were produced by infection of Sf9 cells with BVs expressing Gag. VLPs were concentrated from the supernatants of Sf9 cell culture 62 to 70 h postinfection by porous fiber filtration using the Quixstand benchtop system (GE Healthcare, Uppsala, Sweden) followed by sucrose density gradient ultracentrifugation, as described previously (12), and were characterized by protein assay, Western blotting, and sterility assay. Amounts of incorporated Env and flagellin were quantified by Western blotting using corresponding purified recombinant proteins as standards. The final preparations of VLPs were normalized by Env content.

Immunization of guinea pigs and sampling. Female guinea pigs were obtained from Charles River Laboratory (Wilmington, MA) and immunized (four animals in each group) four times with Gag-only VLP (100 μg total protein) or with Env-containing VLPs with doses containing 10 μg Env with or without incorporated flagellin, or with a mixture of standard VLPs (10 μg Env) with soluble flagellin (1.86 μg sFliC as an average), by the intramuscular (i.m.) or intranasal (i.n.) route. As averages, one dose of cVLP contained 1 to 2 μg FliC. For i.n. immunization, VLPs in 100 μl phosphate-buffered saline (PBS) were instilled into each nostril (50 μl plus 50 μl) of guinea pigs lightly anesthetized with ketamine. Four immunizations were performed at weeks 0, 4, 8, and 30. Serum samples were collected at weeks 0 (preimmune bleeding), 2 (bleed 1), 6 (bleed 2), 10 (bleed 3), 30 (bleed 4), and 43 (bleed 5) by vena cava bleeding of anesthetized guinea pigs. To collect vaginal samples, guinea pigs were anesthetized, and the vaginal membranes were wiped with medical swabs. Preweighed medical cotton Q-tips were inserted into the genital tract and left for 30 min and then weighed and extracted with PBS plus 0.02% Tween 20. Samples were stored at −80°C. Vaginal samples were collected at week 18 (10 weeks after the third immunization) and 38 (8 weeks after the last boost).

Serum and mucosal ELISA endpoint titers. Histidine-tagged Con-S Env gp120 protein was purified from supernatants of HeLa cells infected with a recombinant vaccinia virus expressing this protein, as described by Blasco and Moss (43), using a nickel bead column. The Env-specific antibody endpoint titers, including serum IgG and its subtypes (IgG1 and IgG2) and mucosal IgA and IgG, were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (44). In brief, 96-well microtiter plates (Nunc-Immuno Plate Maxisorp; Nunc Life Technologies, Basel, Switzerland) were coated with 100 μl/well of recombinant Con-S Env gp120 protein (5 μg/ml) in a carbonate coating buffer (pH 9.2) overnight at 4°C. The serum and mucosal samples were serially diluted in 2-fold steps. After washing and blocking with 1.5% bovine serum albumin (BSA), plates were used for assay of antibody binding with the diluted samples. The detection color was developed by binding horseradish peroxidase (HRP)-labeled goat anti-guinea pig IgG, IgG1, IgG2, or IgA anti-

bodies at 37°C for 1 h. After extensive washing, the substrate tetramethylbenzidine (TMB; Zymed, Invitrogen) was added. The optical density at 450 nm (OD_{450}) was read with an ELISA reader (model 680; Bio-Rad). The highest dilution which gave an OD_{450} 2 times higher than that of the naive group without dilution was designated as the antibody endpoint titer.

Antibody neutralization assays. Serum samples were heat inactivated at 56°C for 30 min, followed by neutralization assays. Neutralization was performed with a highly sensitive, single-round infectivity assay with Env-pseudotyped virions (45–48). Neutralization activity is measured as the percentage of reduction of viral infectivity in comparison to that in control wells infected with virus alone. The optical density reading in the control well is indicative of the number of cells being productively infected with virus. A reduction in luciferase production in wells infected with virus preincubated with plasma indicates neutralization activity.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institutes of Health (AI077406 and AI090840) and a pilot grant from the Yerkes National Primate Research Center (P51RR000165-50).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Erin-Joi Collins for valuable assistance with manuscript preparation and submission.

B.-Z.W., J.B., R.W.C., and Emory University have equity interests in Zetra Biologicals, which is developing virus-like particle technology under license from Emory University.

REFERENCES

1. **Sodora, D. L., J. S. Allan, C. Apetrei, J. M. Brenchley, D. C. Douek, J. G. Else, J. D. Estes, B. H. Hahn, V. M. Hirsch, A. Kaur, F. Kirchhoff, M. Muller-Trutwin, I. Pandrea, J. E. Schmitz, and G. Silvestri.** 2009. Toward an AIDS vaccine: lessons from natural simian immunodeficiency virus infections of African nonhuman primate hosts. Nat. Med. **15**: 861–865.
2. **Rerks-Ngarm, S., P. Pitisuttithum, S. Nitayaphan, J. Kaewkungwal, J. Chiu, R. Paris, N. Premsri, C. Namwat, M. de Souza, E. Adams, M. Benenson, S. Gurunathan, J. Tartaglia, J. G. McNeil, D. P. Francis, D. Stablein, D. L. Birx, S. Chunsuttiwat, C. Khamboonruang, P. Thongcharoen, M. L. Robb, N. L. Michael, P. Kunasol, and J. H. Kim.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. N. Engl. J. Med. **361**:2209–2220.
3. **Song, L., Z. Y. Sun, K. E. Coleman, M. B. Zwick, J. S. Gach, J. H. Wang, E. L. Reinherz, G. Wagner, and M. Kim.** 2009. Broadly neutralizing anti-HIV-1 antibodies disrupt a hinge-related function of gp41 at the membrane interface. Proc. Natl. Acad. Sci. U. S. A. **106**:9057–9062.
4. **Pantophlet, R., T. Wrin, L. A. Cavacini, J. E. Robinson, and D. R. Burton.** 2008. Neutralizing activity of antibodies to the V3 loop region of HIV-1 gp120 relative to their epitope fine specificity. Virology **381**: 251–260.
5. **Zwick, M. B., and D. R. Burton.** 2007. HIV-1 neutralization: mechanisms and relevance to vaccine design. Curr. HIV Res. **5**:608–624.
6. **Hessell, A. J., P. Poignard, M. Hunter, L. Hangartner, D. M. Tehrani, W. K. Bleeker, P. W. Parren, P. A. Marx, and D. R. Burton.** 2009. Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. Nat. Med. **15**:951–954.
7. **Ahlers, J. D., and I. M. Belyakov.** 2009. Strategies for optimizing targeting and delivery of mucosal HIV vaccines. Eur. J. Immunol. **39**: 2657–2669.
8. **Hu, S. L., and L. Stamatatos.** 2007. Prospects of HIV Env modification as an approach to HIV vaccine design. Curr. HIV Res. **5**:507–513.
9. **Manrique, M., P. A. Kozlowski, S. W. Wang, R. L. Wilson, E. Micewicz, D. C. Montefiori, K. G. Mansfield, A. Carville, and A. Aldovini.** 2009. Nasal DNA-MVA SIV vaccination provides more significant protection from progression to AIDS than a similar intramuscular vaccination. Mucosal Immunol. **2**:536–550.
10. **Quan, F. S., G. Sailaja, I. Skountzou, C. Huang, A. Vzorov, R. W. Compans, and S. M. Kang.** 2007. Immunogenicity of virus-like particles containing modified human immunodeficiency virus envelope proteins. Vaccine **25**:3841–3850.
11. **Sailaja, G., I. Skountzou, F. S. Quan, R. W. Compans, and S. M. Kang.** 2007. Human immunodeficiency virus-like particles activate multiple types of immune cells. Virology **362**:331–341.
12. **Wang, B. Z., F. S. Quan, S. M. Kang, J. Bozja, I. Skountzou, and R. W. Compans.** 2008. Incorporation of membrane-anchored flagellin into influenza virus-like particles enhances the breadth of immune responses. J. Virol. **82**:11813–11823.
13. **Doan, L. X., M. Li, C. Chen, and Q. Yao.** 2005. Virus-like particles as HIV-1 vaccines. Rev. Med. Virol. **15**:75–88.
14. **Yao, Q., V. Vuong, M. Li, and R. W. Compans.** 2002. Intranasal immunization with SIV virus-like particles (VLPs) elicits systemic and mucosal immunity. Vaccine **20**:2537–2545.
15. **Smith, K. D., E. Andersen-Nissen, F. Hayashi, K. Strobe, M. A. Bergman, S. L. Barrett, B. T. Cookson, and A. Aderem.** 2003. Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility. Nat. Immunol. **4**:1247–1253.
16. **Mizel, S. B., A. N. Honko, M. A. Moors, P. S. Smith, and A. P. West.** 2003. Induction of macrophage nitric oxide production by Gram-negative flagellin involves signaling via heteromeric Toll-like receptor 5/Toll-like receptor 4 complexes. J. Immunol. **170**:6217–6223.
17. **Honko, A. N., N. Sriranganathan, C. J. Lees, and S. B. Mizel.** 2006. Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with *Yersinia pestis*. Infect. Immun. **74**:1113–1120.
18. **Cuadros, C., F. J. Lopez-Hernandez, A. L. Dominguez, M. McClelland, and J. Lustgarten.** 2004. Flagellin fusion proteins as adjuvants or vaccines induce specific immune responses. Infect. Immun. **72**:2810–2816.
19. **Cunningham, A. F., M. Khan, J. Ball, K. M. Toellner, K. Serre, E. Mohr, and I. C. MacLennan.** 2004. Responses to the soluble flagellar protein FliC are Th2, while those to FliC on *Salmonella* are Th1. Eur. J. Immunol. **34**:2986–2995.
20. **Huleatt, J. W., A. R. Jacobs, J. Tang, P. Desai, E. B. Kopp, Y. Huang, L. Song, V. Nakaar, and T. J. Powell.** 2007. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine **25**:763–775.
21. **McDonald, W. F., J. W. Huleatt, H. G. Foellmer, D. Hewitt, J. Tang, P. Desai, A. Price, A. Jacobs, V. N. Takahashi, Y. Huang, V. Nakaar, L. Alexopoulou, E. Fikrig, and T. J. Powell.** 2007. A West Nile virus recombinant protein vaccine that coactivates innate and adaptive immunity. J. Infect. Dis. **195**:1607–1617.
22. **Skountzou, I., M. D. Martin, B. Wang, L. Ye, D. Koutsonanos, W. Weldon, J. Jacob, and R. W. Compans.** 2009. *Salmonella* flagellins are potent adjuvants for intranasally administered whole inactivated influenza vaccine. Vaccine **28**:4103–4112.
23. **Nempont, C., D. Cayet, M. Rumbo, C. Bompard, V. Villeret, and J. C. Sirard.** 2008. Deletion of flagellin's hypervariable region abrogates antibody-mediated neutralization and systemic activation of TLR5-dependent immunity. J. Immunol. **181**:2036–2043.
24. **Wang, B. Z., W. Liu, S. M. Kang, M. Alam, C. Huang, L. Ye, Y. Sun, Y. Li, D. L. Kothe, P. Pushko, T. Dokland, B. F. Haynes, G. Smith, B. H. Hahn, and R. W. Compans.** 2007. Incorporation of high levels of chimeric human immunodeficiency virus envelope glycoproteins into virus-like particles. J. Virol. **81**:10869–10878.
25. **Liao, H. X., L. L. Sutherland, S. M. Xia, M. E. Brock, R. M. Scearce, S. Vanleeuwen, S. M. Alam, M. McAdams, E. A. Weaver, Z. Camacho, B. J. Ma, Y. Li, J. M. Decker, G. J. Nabel, D. C. Montefiori, B. H. Hahn, B. T. Korber, F. Gao, and B. F. Haynes.** 2006. A group M consensus envelope glycoprotein induces antibodies that neutralize subsets of subtype B and C HIV-1 primary viruses. Virology **353**:268–282.
26. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59**:284–291.
27. **Binley, J. M., E. A. Lybarger, E. T. Crooks, M. S. Seaman, E. Gray, K. L. Davis, J. M. Decker, D. Wycuff, L. Harris, N. Hawkins, B. Wood, C. Nathe, D. Richman, G. D. Tomaras, F. Bibollet-Ruche, J. E. Robinson, L. Morris, G. M. Shaw, D. C. Montefiori, and J. R. Mascola.** 2008. Profiling the specificity of neutralizing antibodies in a large panel of plasmas from patients chronically infected with human immunodeficiency virus type 1 subtypes B and C. J. Virol. **82**:11651–11668.
28. **Li, Y., H. Hui, C. J. Burgess, R. W. Price, P. M. Sharp, B. H. Hahn, and G. M. Shaw.** 1992. Complete nucleotide sequence, genome organization, and biological properties of human immunodeficiency virus type 1 in

vivo: evidence for limited defectiveness and complementation. J. Virol. **66**:6587–6600.
29. **Fujihashi, K., T. Koga, F. W. van Ginkel, Y. Hagiwara, and J. R. McGhee.** 2002. A dilemma for mucosal vaccination: efficacy versus toxicity using enterotoxin-based adjuvants. Vaccine **20**:2431–2438.
30. **Mestecky, J., S. M. Michalek, Z. Moldoveanu, and M. W. Russell.** 1997. Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans. Behring Inst. Mitt. **98**:33–43.
31. **Ben-Yedidia, T., and R. Arnon.** 1998. Effect of pre-existing carrier immunity on the efficacy of synthetic influenza vaccine. Immunol. Lett. **64**: 9–15.
32. **Kang, S. M., Q. Yao, L. Guo, and R. W. Compans.** 2003. Mucosal immunization with virus-like particles of simian immunodeficiency virus conjugated with cholera toxin subunit B. J. Virol. **77**:9823–9830.
33. **McSorley, S. J., B. D. Ehst, Y. Yu, and A. T. Gewirtz.** 2002. Bacterial flagellin is an effective adjuvant for $CD4^+$ T cells in vivo. J. Immunol. **169**:3914–3919.
34. **Singh, M., and D. O'Hagan.** 1999. Advances in vaccine adjuvants. Nat. Biotechnol. **17**:1075–1081.
35. **LeBien, T. W., and T. F. Tedder.** 2008. B lymphocytes: how they develop and function. Blood **112**:1570–1580.
36. **Kawahara, M.** 2008. Recombinant *Mycobacterium bovis* BCG vector system expressing SIV Gag protein stably and persistently induces antigen-specific humoral immune response concomitant with IFN gamma response, even at three years after immunization. Clin. Immunol. **129**: 492–498.
37. **Miao, E. A., E. Andersen-Nissen, S. E. Warren, and A. Aderem.** 2007. TLR5 and Ipaf: dual sensors of bacterial flagellin in the innate immune system. Semin. Immunopathol. **29**:275–288.
38. **Vyas, S. P., and P. N. Gupta.** 2007. Implication of nanoparticles/microparticles in mucosal vaccine delivery. Expert Rev. Vaccines **6**:401–418.
39. **Sun, Y. H., H. G. Rolan, and R. M. Tsolis.** 2007. Injection of flagellin into the host cell cytosol by *Salmonella enterica* serotype Typhimurium. J. Biol. Chem. **282**:33897–33901.
40. **Kozlowski, P. A., and M. R. Neutra.** 2003. The role of mucosal immunity in prevention of HIV transmission. Curr. Mol. Med. **3**:217–228.
41. **Haynes, B. F., and D. C. Montefiori.** 2006. Aiming to induce broadly reactive neutralizing antibody responses with HIV-1 vaccine candidates. Expert Rev. Vaccines **5**:579–595.
42. **Crooks, E. T., P. L. Moore, M. Franti, C. S. Cayanan, P. Zhu, P. Jiang, R. P. de Vries, C. Wiley, I. Zharkikh, N. Schulke, K. H. Roux, D. C. Montefiori, D. R. Burton, and J. M. Binley.** 2007. A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120. Virology **366**:245–262.
43. **Blasco, R., and B. Moss.** 1995. Selection of recombinant vaccinia viruses on the basis of plaque formation. Gene **158**:157–162.
44. **Quan, F. S., C. Huang, R. W. Compans, and S. M. Kang.** 2007. Virus-like particle vaccine induces protective immunity against homologous and heterologous strains of influenza virus. J. Virol. **81**:3514–3524.
45. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw, and E. Hunter.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. **74**:8358–8367.
46. **Wei, X., J. M. Decker, S. Wang, H. Hui, J. C. Kappes, X. Wu, J. F. Salazar-Gonzalez, M. G. Salazar, J. M. Kilby, M. S. Saag, N. L. Komarova, M. A. Nowak, B. H. Hahn, P. D. Kwong, and G. M. Shaw.** 2003. Antibody neutralization and escape by HIV-1. Nature **422**:307–312.
47. **Montefiori, D. C.** 2009. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol. Biol. **485**:395–405.
48. **Montefiori, D. C.** 2005. Unit 12.11. Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. Curr. Protoc. Immunol. doi:10.1002/0471142735.im1211s64.