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Humoral and cellular immune responses to airway immunization of mice with human papillomavirus type 16 virus-like particles and mucosal adjuvants

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Abstract

Cervical cancer results from cervical infection by human papillomaviruses (HPV), especially HPV16. Intramuscular administrations of HPV16 virus-like particle (VLP) vaccines have been shown to induce strong neutralizing antibody responses and protect women against genital HPV16 infection and associated lesions. However, an alternative route of administration that avoids parenteral injection might facilitate vaccine implementation, particularly in developing countries which account for the majority of the worldwide cases of cervical cancer. In addition, inducing mucosal immunity could partially overcome the substantial variation in HPV16 antibodies at the cervix seen in ovulating women. Aerosol vaccination with HPV16 VLPs was previously shown to be immunogenic in mice and in women. Here, we examine whether exposure to other respiratory viral antigens may interfere with the HPV16 VLP-specific humoral response and whether two known mucosal adjuvants, CpG oligodeoxynucleotides and a natural non-toxic *Escherichia coli* heat-labile enterotoxin (HLT), can enhance the immunogenicity of airway immunization (nasal or aerosol-like) of mice with HPV16 VLPs. Our data show that HLT can significantly improve anti-VLP humoral responses in serum and mucosal secretions, as well as VLP-specific proliferative responses and IFN-γ production by CD8 T cells, and that recent exposure to influenza surface antigens can diminish mucosal, but not systemic, antibody responses to the VLPs.

Keywords: Human papillomavirus type 16; Virus-like particles; HLT; CpG ODN; Mucosal immunization; Antibody and cell-mediated immune responses

1. Introduction

Cervical cancer is the second leading cause of cancer mortality in women worldwide. The causal role of a subset of human papillomaviruses (HPV) in cervical carcinogenesis has now been clearly established, since high-risk HPV DNA has been found in virtually all cervical cancer samples, HPV16 being the most prevalent type (Bosch et al., 2002; Walboomers et al., 1999). A prophylactic vaccine that targets these HPV types might therefore substantially reduce the incidence of this cancer and its precursor lesions. The leading candidate is a subunit HPV virus-like particle (VLP) vaccine (reviewed in Lowy and Frazer, 2003; Schiller and Davies, 2004). Phase II efficacy trials have shown that three intramuscular (i.m.) vaccinations with HPV VLP vaccines comprising two high-risk types, HPV16 and 18, is very efficient in preventing both persistent genital HPV infection and associated lesions (Harper et al., 2004; Koutsky et al., 2002; Mao et al., 2006; Villa et al., 2005). However, the requirement for multiple i.m. injections may represent a substantial hurdle for widespread implementation, particularly in the developing world, which accounts for more than three quarters of the worldwide cases of cervical cancer (Bosch et al., 2002). In addition, although i.m. VLP vaccination induces readily detectable IgG at the cervix, the level of antibody decreases several fold during ovulation, which might impair the long term protective effects of the vaccine (Nardelli-Haefliger et al., 2003). Mucosal vaccination

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could represent a potential approach to overcome these difficulties. Indeed, the mucosal route has the theoretical advantage of not requiring injection and of inducing, in addition to serum-derived Igs, locally produced specific secretory IgA (SIgA) in mucosal secretions (Balmelli et al., 1998; Decroix et al., 2001; McDermott and Bienenstock, 1979; Nardelli-Haefliger et al., 1999, 2003).

We have recently shown in a small pilot study that half of the women receiving two doses of 50 μg and all the women receiving two doses of 250 μg of HPV16 L1 VLPs without adjuvant by aerosol vaccination produced neutralizing antibody responses that were similar to those elicited by two i.m. injections of 50 μg (Nardelli-Haefliger et al., 2005). In contrast, nasal administration induced weak responses. These data paralleled our previous findings in mice where lower respiratory tract VLP immunization ("aerosol-like" protocol) induced high antibody levels in the genital tract and was more efficient than the nasal route (Balmelli et al., 2002; Nardelli-Haefliger et al., 1999).

Adding a mucosal adjuvant to the vaccine might further enhance its immunogenicity (Harandi et al., 2003). In this study, we investigated in mice whether combination of VLPs with mucosal adjuvants, either *Escherichia coli* heat-labile enterotoxin (LT) derived from a natural non-toxic *E. coli* strain (HLT) (Glueck, 2001)) or synthetic oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN; Freytag and Clements, 2005), can improve VLP airway immunogenicity. These adjuvants differ in their mechanisms of action. LT has been shown to induce mixed $CD4^+$ Th1- and Th2-type cells with concomitant SIgA and serum IgG1, IgG2a and IgA responses (reviewed by Freytag and Clements, 2005). In mice CpG ODN motifs are known to trigger an immunostimulatory cascade that culminates in the maturation, differentiation and proliferation of multiple immune cells, including B and T lymphocytes, NK cells, monocytes, macrophages and dendritic cells (DCs). Together, these cells secrete cytokines that create a pro-inflammatory and Th1-biased immune microenvironment (reviewed by Klinman et al., 2004). We have therefore determined the effect of these adjuvants on both humoral and cell-mediated HPV16 VLP-specific immune responses after airway immunization. Since humans are frequently exposed to respiratory tract viruses, we wondered whether this may have an impact on the antibody responses to the VLPs. As exposure of mice to respiratory viral antigens is minimized in an animal facility, the VLP-specific antibody responses were compared in mice unprimed or recently exposed to influenza antigens.

2. Materials and methods

2.1. VLPs formulation and adjuvants

Purified baculovirus-expressed HPV16 VLPs were produced as described previously (Harro et al., 2001; Kirnbauer et al., 1992). The heat-labile enterotoxin (HLT) was derived from a natural non-toxic variant of *E. coli* as previously described (Gluck et al., 1999, 2000; Uesaka et al., 1994). CpG ODN 1826 optimized for stimulation of the mouse immune system was purchased from Coley Pharmaceutical Group, Wellesley, MA.

2.2. Immunizations

Six-week-old female BALB/c (analysis of humoral responses) and C57BL/6 (analysis of cell-mediated immune responses) were purchased from Iffa Credo, France. The inoculum consisted in various concentrations of HPV16 VLPs (1, 5, or 8.3 μg depending on the experiments) alone or in combination with 0.4 μg HLT or 10 μg CpG ODN and was diluted in phosphate-buffered saline (PBS) to 20 μl for administration in BALB/c mice or 10 μl for C57BL/6 mice. Intranasal vaccination of conscious (nasal) or anaesthetized (aerosol-like) mice was performed three times weekly as previously described (Balmelli et al., 1998). When indicated, mice received an intranasal priming (without anesthesia) 1 week before the first VLP administration with a monovalent virosomal influenza vaccine (20 μl containing 0.1 μg A/Sing. hemagglutinin and 40 ng HLT). Blood, saliva, and genital samples were taken as previously described (Hopkins et al., 1995) and were stored at −70 °C.

2.3. ELISA

The amounts of total IgA and IgG as well as anti-HPV16 VLPs antibodies were determined by enzyme-linked immunosorbent assay (ELISA) with biotinylated goat anti-mouse IgA (Kirkegaard & Perry Laboratories) or IgG (Amersham Pharmacia) as secondary antibodies, respectively, as described previously (Hopkins et al., 1995; Nardelli-Haefliger et al., 1997). For determination of anti-HPV16 VLPs antibodies, the ELISA plates were coated with 50 ng of HPV16 VLPs in PBS, and for the measurement of total IgG or IgA, plates were coated with 100 ng of sheep anti-mouse Ig (Boehringer) in carbonate buffer pH 9.6. For serum anti-HPV16 VLPs IgG isotype ELISA, biotinylated goat anti-mouse IgG1 and IgG2a (Amersham Pharmacia) were used as secondary antibodies. The specific IgA or IgG titers were expressed as the reciprocal of the highest dilutions that yielded an optical density at 492 nm four times that of preimmune samples. These titers were normalized to the amount of total IgA or IgG in saliva and vaginal washes (Hopkins et al., 1995).

2.4. Preparation of splenocyte suspensions

Mice were sacrificed by inhalation of CO_2 and spleens were harvested. Single-cell suspensions were obtained by pressing the organ onto a 70 μm filter (Falcon) using a syringe piston and subsequently passing the splenocytes through a 40 μm filter (Falcon). Dissociated cells were resuspended in high-glucose Dulbecco's modified Eagle medium containing glutamax-1 and sodium pyruvate supplemented with 10 mM HEPES, 1× non-essential amino acids, 100 U of penicillin–streptomycin/ml, 10% fetal calf serum (FCS) (all from Invitrogen) and 20 μM 2-mercaptoethanol (Sigma). Cell viability was determined by trypan blue exclusion.

2.5. Proliferation assay

2×10^5 splenocytes were incubated in triplicates in 96-well U-bottom plates (Nunc) in the presence of 5 μg/ml HPV16 VLPs or with medium alone. The quality of the splenocytes preparation was assessed by incubating them in the presence of 2 μg/ml Concanavalin A (Sigma). After 4 days incubation at 37 °C, cells were pulsed with 0.5 μCi of ^{3}H-thymidine (Amersham Pharmacia) for the final 16 h of incubation and then harvested onto Unifilter plates (Packard Instruments). Incorporated radioactivity was measured using a β-scintillation counter (Topcount, Packard Instruments). Results from triplicate wells were averaged and used to calculate a stimulation index as follows: mean counts per minute of VLP-stimulated cells divided by mean counts per minute of cells incubated in medium alone.

2.6. INF-γ ELISPOT assay

Multiscreen-HA 96-well plates (MAHA S4510, Millipore) were coated overnight at 4 °C with a monoclonal anti-IFN-γ antibody (R4-6A2, Pharmingen) at a concentration of 10 μg/ml in PBS. Plates were then blocked during 2 h at 37 °C with PBS/BSA 1%. Two dilutions (450,000 and 150,000 splenocytes/well) were incubated in triplicates with 5 μg/ml of the $L1_{165–173}$ peptide (Ohlschlager et al., 2003) or medium alone (control wells) during 16–24 h in the 96-well ELISPOT plates. Then, a biotinylated monoclonal anti-IFN-γ antibody (AN 18.03.C12; Slade and Langhorne, 1989) was added at a concentration of 2 μg/ml in PBS/BSA 1% and plates were incubated for 2 h at 37 °C: between each incubation step, plates were washed three times with PBS/Tween-20 0.1% (PBT) and three times with PBS. After 1 h incubation with streptavidin–alkaline phosphatase conjugate (1/2000 in PBT, Boehringer), plates were developed with a solution of BCIP/NBT (Roche) until apparition of blue spots. Tap water was used to stop the reaction and the plates were dried in air overnight. Individual spots were counted under a dissecting microscope. Counts of the control wells were subtracted from the samples stimulated with peptide.

3. Results

3.1. Influence of the influenza antigens priming on the VLP-specific antibody response induced after vaccination with HPV16 VLPs

Following airway vaccination of mice with three weekly doses of 5 μg HPV16 VLPs, we have previously shown that interaction of the antigen with the lower respiratory tract was necessary to induce high titers of neutralizing antibodies in genital secretions (Balmelli et al., 1998). This is achieved by immunizing anaesthetized mice (hereafter referred to as aerosol-like vaccination), while with nasal vaccination of conscious mice (hereafter referred to as nasal vaccination), the VLPs are not inhaled and no or few VLP-specific antibodies were induced. An ongoing response to a respiratory virus might have an impact on the antibody response to the VLPs when they are delivered through the aerosol-like route. To mimic the situation in humans, where antibodies against influenza antigens are present, we compared in the following experiment the VLP-specific antibody responses in mice unprimed or recently exposed to a monovalent virosomal influenza vaccine. Such vaccines have been shown to efficiently induce influenza-specific antibody responses after intranasal administration (Cusi et al., 2000; Gluck et al., 1999). Subsequently, primed and unprimed mice received three weekly doses of 5 μg HPV16 VLPs by the aerosol-like route. Serum, oral and genital secretions were sampled 1, 4 and 21 weeks after the last immunization and VLP-specific Igs titers were measured by ELISA (Fig. 1). The systemic and mucosal antibody responses measured after vaccination of the unprimed mice were similar to our previously published data (Balmelli et al., 1998). Interestingly, in influenza vaccine-primed mice, systemic VLP-specific IgG responses were not affected, while mucosal IgG and IgA responses were diminished (5-fold lower IgA response in vaginal secretions, although not statistically significant; 10-fold lower IgG response in vaginal secretions, $p < 0.05$ at week 7; 5–10-fold lower IgA response in saliva, $p < 0.05$ at weeks 3 and 7). Mounting an immune response against influenza antigens nasally administered seems therefore to negatively affect the development of antibody responses to HPV16 VLPs, at least in the mucosal compartment, when the VLP vaccine is delivered soon after the influenza priming.

3.2. Antibody responses after vaccination with HPV16 VLPs + HLT

We had previously shown that combination of HPV16 VLPs with cholera toxin (CT), a potent mucosal adjuvant, greatly improved the systemic and mucosal VLP-specific antibody response after aerosol-like vaccination (10-fold higher IgG titers in serum, vaginal secretions and saliva, and 5-fold higher IgA titers in vaginal secretions and saliva; Balmelli et al., 1998). As the significant toxicity of CT precludes its use in humans, we investigated here whether combination of HPV16 VLPs with a non-toxic HLT, another mucosal adjuvant of the same family of CT, could improve the VLP-specific humoral immune response after aerosol-like vaccination. We also examined whether the use of HLT could overcome the low efficiency of the nasal protocol of vaccination with HPV16 VLPs (Balmelli et al., 1998). The adjuvant effect of HLT was analyzed under the more stringent conditions, i.e. after recent exposure to influenza antigens as in the above experiment. Mice were primed by nasal vaccination with the influenza vaccine 1 week before the first VLP administration. Suboptimal (1 μg) doses of VLPs were given three times weekly and serum, oral and genital secretions were sampled 1, 4 and 21 weeks after the last immunization. VLP-specific Igs titers were measured by ELISA as described in Section 2. Similar differences between immunization groups were consistently observed throughout the experiment, therefore only titers obtained 4 weeks after the last immunization are shown in Fig. 2. After 1 μg dose aerosol-like immunizations, serum IgG titers were only slightly lower than after the optimal 5 μg dose ($p < 0.05$, compare group A in Fig. 2 and group "influenza-primed" in Fig. 1) while the IgG and IgA titers in the mucosal secretions were not significantly different. As expected, mice

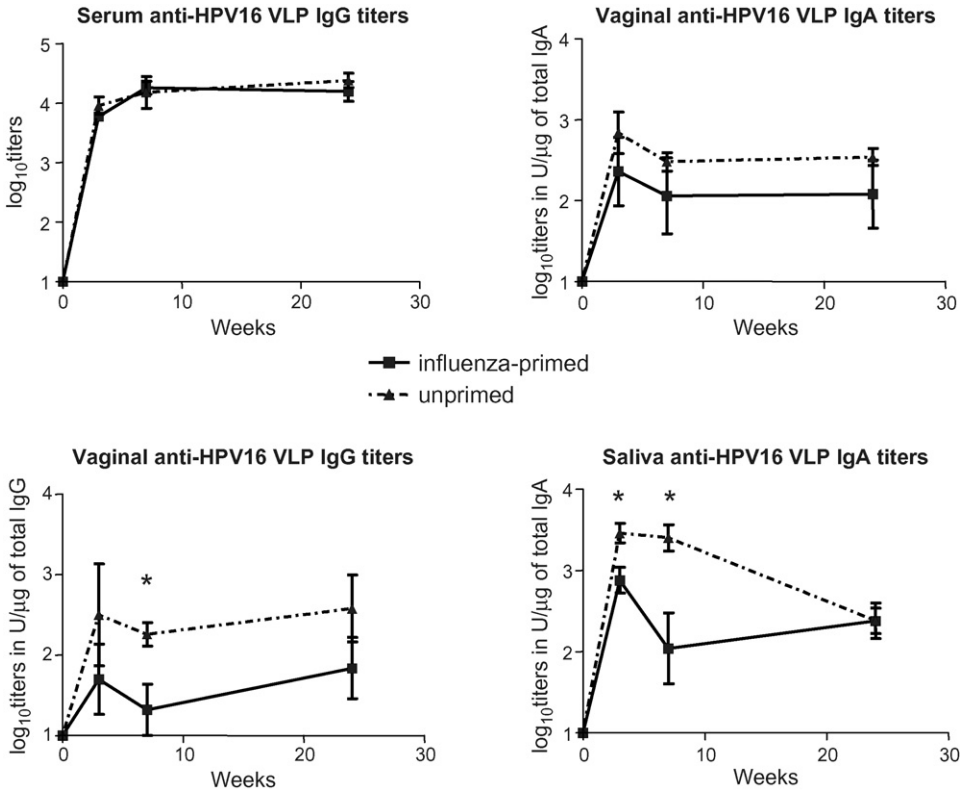
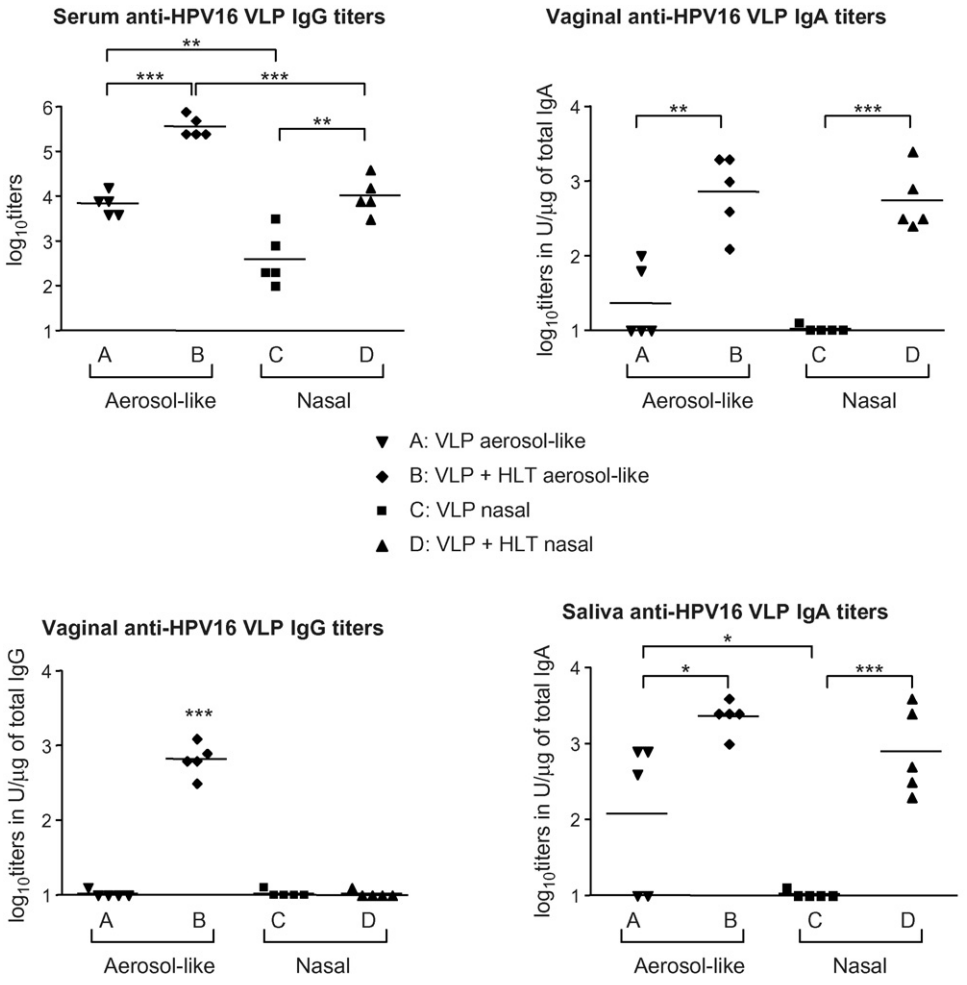


Fig. 1. Anti-VLP systemic and mucosal antibody responses after aerosol-like immunization of influenza vaccine-primed or -unprimed mice with purified HPV16 VLPs. Mice (five per group) were immunized three times weekly (at weeks 0, 1 and 2) with 5 μg of HPV16 VLPs. One group of mice was previously primed intranasally 7 days before the first VLP administration with an influenza vaccine (see Section 2). The VLP-specific antibody responses were analyzed at weeks 3, 7 and 24. Data are expressed as the geometric mean titers (GMT) ± standard error of the mean (S.E.M.). Statistical comparisons of the means were carried out using Student's *t*-test. *$p < 0.05$.

vaccinated under anesthesia developed higher VLP-specific Ig titers than mice immunized while conscious ($p < 0.01$ for serum IgG and $p < 0.05$ for salivary IgA in group A versus C). Administration of HLT with VLPs in both protocols of immunization greatly enhanced the antibody response ($p < 0.001$ for serum and vaginal IgG, $p < 0.01$ for vaginal IgA and $p < 0.05$ for salivary IgA in group B versus A; $p < 0.01$ for serum IgG, $p < 0.001$ for vaginal IgA and $p < 0.001$ for salivary IgA in group D versus C). Although the aerosol-like protocol with VLPs + HLT is more efficient than the nasal protocol ($p < 0.001$ for serum IgG and $p < 0.001$ for vaginal IgG in group B versus D), nasal vaccination with VLP + HLT is similarly efficient to aerosol-like vaccination with VLP (compare group A and D). Interestingly, high titers of anti-HPV16 VLPs IgA but not IgG were induced in mucosal secretions after nasal vaccination with VLP in combination with HLT. In conclusion, after airway vaccination the highest VLP-specific antibody titers were achieved in both serum and mucosal secretions when VLPs were administered together with HLT in anaesthetized mice.

3.3. Antibody responses after vaccination with HPV16 VLPs + CpG ODN

CpG ODN have also been reported to be an effective adjuvant after mucosal vaccination (Gallichan et al., 2001; McCluskie et al., 2000; McCluskie and Davis, 1998; Moldoveanu et al., 1998). We therefore investigated whether administration of CpG ODN with HPV16 VLPs could also enhance the systemic and mucosal VLP-specific antibody response. Conscious or anaesthetized mice were immunized intranasally three times weekly with the suboptimal 1 μg HPV16 VLP dose mixed or not with 10 μg CpG ODN. Sampling and ELISA were performed as described in the first experiments. When mice were immunized without CpG ODN, the serum and vaginal VLP-specific IgG titers were similar to the titers measured in the previous experiment, but in contrast, the VLP-specific IgA titers detected in mucosal secretions were substantially higher (statistically significant for vaginal IgA between group A in Fig. 2 and group A in Fig. 3, $p < 0.001$, and for saliva IgA between group C in Fig. 2 and group C in Fig. 3, $p < 0.001$). This may be linked to priming with the influenza vaccine in the previous experiment. We did not examine the ability of CpG ODN to enhance the humoral response of VLPs after priming with the influenza vaccine, since no enhancement of antibody induction was observed under the less stringent conditions. In conclusion, immunization of mice with HPV16 VLPs in combination with CpG ODN did not improve the IgG and IgA titers measured in serum and mucosal secretions, with either protocol of airway administration, suggesting that this adjuvant is of poor value for augmenting HPV16 VLP-specific humoral responses. Because the poor adjuvancy of CpG ODN was unexpected, we further evaluated the functionality of this adjuvant by examining the isotypes of VLP-specific IgG (IgG1



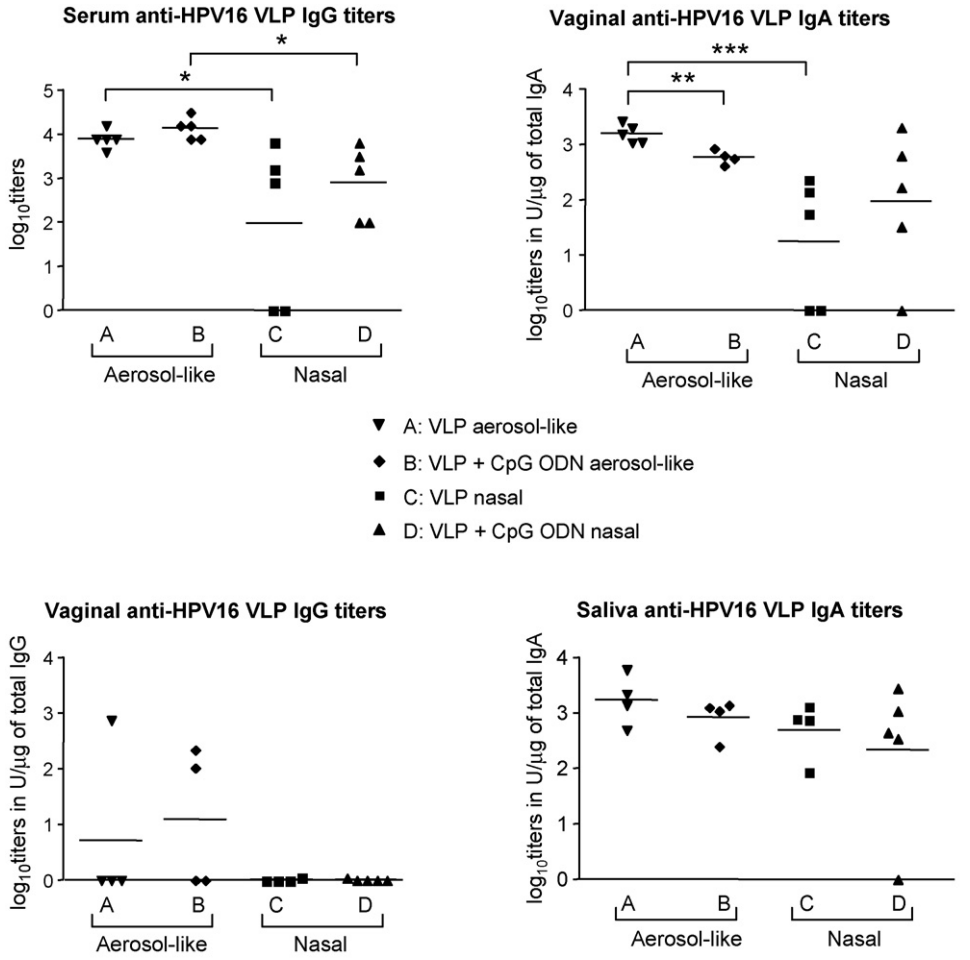
Protocole	GMT values +/- SEM of HPV16 VLP-specific antibodies			
	Serum IgG	Vaginal IgA	Vaginal IgG	Saliva IgA
A: VLP aerosol-like	3.84 +/- 0.11	1.36 +/- 0.22	1.02 +/- 0.02	2.08 +/- 0.44
B: VLP + HLT aerosol-like	5.56 +/- 0.10	2.86 +/- 0.23	2.82 +/- 0.10	3.36 +/- 0.10
C: VLP nasal	2.60 +/- 0.27	1.02 +/- 0.02	1.02 +/- 0.02	1.02 +/- 0.02
D: VLP + HLT nasal	4.02 +/- 0.18	2.74 +/- 0.19	1.02 +/- 0.02	2.90 +/- 0.26

Fig. 2. Anti-VLP systemic and mucosal antibody responses after airway immunization with purified HPV16 VLPs alone or in combination with HLT. Groups of five mice were intranasally immunized either conscious (nasal) or anaesthetized (aerosol-like) three times weekly (at weeks 0, 1 and 2) with 1 μg of VLPs in combination or not with HLT. All mice were previously primed with an influenza vaccine 7 days before the first VLP administration (see Section 2). The anti-VLP antibody responses at week 7 are represented. Data are expressed as the $\log_{10}$ IgG or IgA titers of individual mice. Geometric mean titers (GMT) of each group are shown by horizontal bars. GMT values ±S.E.M. for all groups are indicated below the graphs. Statistical comparisons of the means were carried out using Student's *t*-test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

and IgG2a) induced and compared them to those induced by HLT. Sera from mice immunized while anaesthetized with 1 μg HPV16 VLPs + HLT or CpG ODN and that were positive for VLP-specific IgG were analyzed (see Table 1). In accordance with the well-reported Th1-like pattern induced after immunization with CpG ODN, mice immunized with this adjuvant developed predominantly VLP-specific IgG2a. On the contrary, use of HLT adjuvant allowed the induction of both anti-VLP IgG1 and IgG2a, although with a greater ability to elicit specific IgG1 antibodies, suggesting the emergence of a more Th2-like pattern response.

3.4. Proliferative responses after immunization with HPV16 VLP + HLT or CpG ODN

The surprising inability of CpG ODN to improve HPV16 VLP-specific humoral immune responses via airway immunization led us to further investigate whether this is also the case for the induction of cell-mediated immune responses, i.e. proliferative responses and IFN-γ production by CD8 T cells. For that purpose, we decided to choose C57BL/6 mice, since a CTL epitope of the HPV16 L1 protein has been defined in this background (H-2D^{b}, L1$_{165–173}$) and already used to ana-



Protocole	GMT values +/- SEM of HPV16 VLP-specific antibodies			
	Serum IgG	Vaginal IgA	Vaginal IgG	Saliva IgA
A. VLP aerosol-like	3.90 +/- 0.10	3.20 +/-0.07	1.47 +/- 0.47	3.24 +/- 0.42
B. VLP + CpG ODN aerosol-like	4.14 +/- 0.11	2.78 +/- 0.06	1.59 +/- 0.35	2.92 +/- 0.18
C. VLP nasal	2.38 +/- 0.58	1.65 +/- 0.28	1.03 +/- 0.03	2.70 +/- 0.26
D. VLP + CpG ODN nasal	2.90 +/- 0.38	2.17 +/- 0.42	1.02 +/- 0.02	2.54 +/- 0.42

Fig. 3. Anti-VLP systemic and mucosal antibody responses after airway immunization with purified HPV16 VLPs alone or in combination with CpG ODN. Groups of four to five mice were intranasally immunized either conscious (nasal) or anaesthetized (aerosol-like) three times weekly with 1 μg of VLPs in combination or not with CpG ODN. The anti-VLP antibody responses 7 weeks after the first immunization are represented. Data are expressed as the $\log_{10}$ IgG or IgA titers of individual mice. GMT of each group are shown by horizontal bars. GMT values ±S.E.M. for all groups are indicated below the graphs. Statistical comparisons of the means were carried out using Student's *t*-test. $*p<0.05$, $**p<0.01$, $***p<0.001$.

Table 1
Serum HPV16 VLP-specific IgG isotype analysis[a]

Adjuvant	Geometric mean titer ± S.E.M.		IgG1/IgG2a ratio
	IgG1	IgG2a	
None	2,183 ± 1,199	2,733 ± 1,170	0.798
CpG ODN	70 ± 12	12,000 ± 3,836	0.006
HLT	83,200 ± 21,105	11,600 ± 2,561	7.172

[a] Six mice vaccinated with HPV16 VLPs without adjuvant (three from Fig. 2 and three from Fig. 3), three mice vaccinated with HPV16 VLPs + HLT and three mice vaccinated with HPV16 VLPs + CpG ODN were analyzed for their IgG1 and IgG2a titers.

lyze HPV16 L1-specific cellular immune responses (Da Silva et al., 2003; Dell et al., 2006; Ohlschlager et al., 2003). In addition, aerosol-like vaccination of BALB/c and C57BL/6 mice with HPV16 VLPs leads to very similar serum IgG responses (data not shown). As a first step, the ability of HLT or CpG ODN to enhance the proliferative response in splenocytes of C57BL/6 mice immunized with HPV16 VLPs by the aerosol-like route was analyzed. This route was chosen, because as seen in Figs. 2 and 3, it appears to be the most efficient to induce humoral immune responses. The highest dose of VLP that we can achieve given the concentration of VLP in our stocks and the restricted inoculum volume that can be administered in the

nostril of mice is 8.3 μg. Such a dose turned out to be inefficient at inducing VLP-specific proliferative responses in our preliminary experiments (data not shown). It has been previously reported that repetitive vaccination with chimeric VLPs incorporating the E7 protein for induction of cellular immune responses against E7 is of limited effectiveness due to the presence of neutralizing antibodies and/or T regulatory responses against the capsid proteins induced after the first administration (Da Silva et al., 2001; Liu et al., 2003). We therefore hypothesized that boosting with VLPs a primary response induced after vaccination with the same VLPs would similarly not increase the VLP-specific proliferative response. For these reasons, mice were immunized three times at 1 day interval with 8.3 μg of VLPs, allowing to reach a total dose of 25 μg. Eleven to 12 days after the last immunization, mice were sacrificed and the splenocytes were incubated with 5 μg/ml HPV16 VLPs for 4 days. Proliferation was then evaluated by ^{3}H-thymidine incorporation. A VLP-specific proliferation was measured in mice that were immunized with HPV16 VLPs in absence of adjuvant, as compared to naïve mice (mean stimulation indexes (SI) of 5.54 ± 1.05 versus 2.27 ± 0.16, $p < 0.05$, see Fig. 4). Combination of CpG ODN with VLPs did not significantly improve the proliferative response (mean SI of 8.85 ± 0.81, $p < 0.001$ as compared to naïve mice, but not statistically different from mice immunized with VLPs alone). In contrast, the SI was considerably enhanced when aerosol-like immunization was performed in presence of HLT (mean SI of 15.27 ± 1.07, $p < 0.001$ as compared to VLPs alone and $p < 0.05$ as compared to VLPs + CpG).

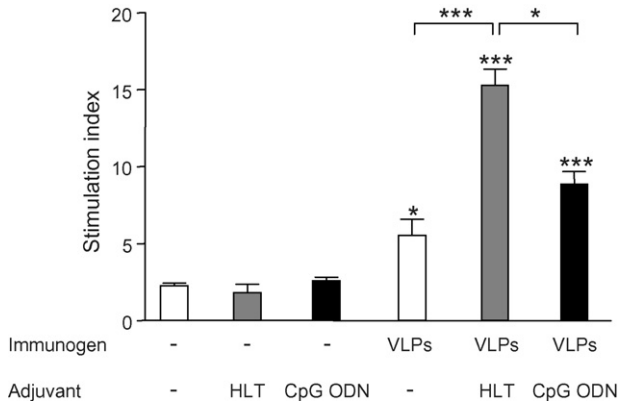


Fig. 4. VLP-specific proliferation in splenocytes of mice immunized via the aerosol-like route with HPV16 VLPs in combination with HLT or CpG ODN. Splenocytes were cultured in presence of 5 μg/ml VLPs or medium (control wells) for 4 days. Stimulation indexes are defined as the ^{3}H-thymidine incorporation measured in the VLP-simulated wells divided by the ^{3}H-thymidine incorporation measured in the control wells. The mean ± S.E.M. response was then calculated for each immunization group and is reported in the figure. Statistical comparisons of the means were carried out using Student's *t*-test. Unless mentioned with parentheses, the group of naïve mice was considered as the reference group for calculation of *p* values. $*p < 0.05$, $***p < 0.001$.

3.5. IFN-γ secretion of CD8 T cells after immunization with HPV16 VLP + HLT or CpG ODN

As a second way to analyze the ability of HLT versus CpG ODN to affect the cell-mediated immune response against HPV16 VLPs, we compared their ability to improve the L1-specific CD8 T cell response. C57BL/6 mice were immunized intranasally under anesthesia either once or three times at 1 day interval with 8.3 μg HPV16 VLPs (total doses of 8.3 or 25 μg, respectively) in presence or not of HLT or CpG ODN. Ten to 12 days after the last immunization, mice were sacrificed and the splenocytes were incubated in presence or not of the H-2D^{b} L1$_{165–173}$ CTL epitope in an *ex vivo* IFN-γ ELISPOT assay (Fig. 5). When mice were immunized with the low dose of VLPs, L1-specific IFN-γ secretion was detected only when the VLPs were mixed with HLT (mean IFN-γ spot numbers/10^{6} cells ± S.E.M. of 47.0 ± 12.0 as compared to 1.5 ± 0.8 in naïve mice, $p < 0.01$; 6.7 ± 5.1 in mice immunized with VLPs alone, $p < 0.05$; 3.4 ± 3.4 in mice immunized with VLPs + CpG ODN, $p < 0.05$). This better adjuvant effect of HLT was confirmed in mice immunized with 25 μg VLPs, since the number of L1-specific IFN-γ secreting CD8 T cells was significantly higher when the VLPs were administered in combination with HLT (mean IFN-γ spot numbers/10^{6} cells ± S.E.M. of 185.8 ± 17.9) than with CpG ODN (53.3 ± 27.3, $p < 0.05$) or alone (51.9 ± 26.4, $p < 0.05$). These results confirmed the superior adjuvant effect of HLT over CpG ODN after aerosol-like immunization of mice with HPV16 VLPs.

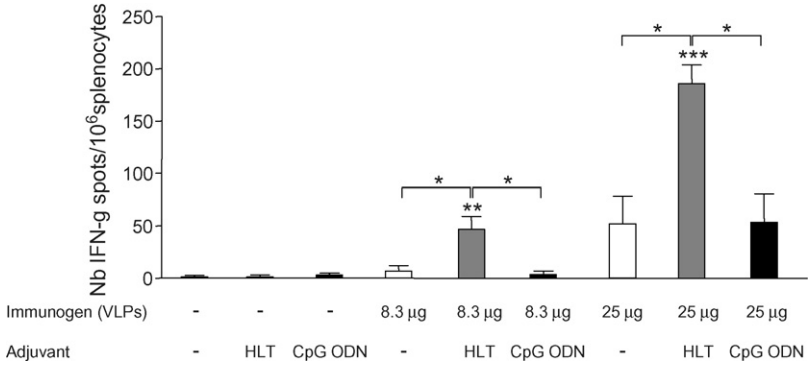


Fig. 5. L1-specific IFN-γ ELISPOT in splenocytes of mice immunized via the aerosol-like route with HPV16 VLPs in combination with HLT or CpG ODN. Splenocytes were cultured in presence of 5 μg/ml L1$_{165–173}$ or medium (control wells) for 16–24 h. Responses are defined for each individual mouse as the number of IFN-γ spots/10^{6} splenocytes in the L1-stimulated wells − the number of IFN-γ spots/10^{6} splenocytes in the control wells. The mean ± S.E.M. response was then calculated for each immunization group and is reported in the figure. Statistical comparisons of the means were carried out using Student's *t*-test. Unless mentioned with parentheses, the group of naïve mice was considered as the reference group for calculation of *p* values. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

4. Discussion

We have previously shown that nasal vaccination of anaesthetized mice with HPV16 VLPs without adjuvant is able to induce high titers of HPV16-neutralizing antibodies in the serum and genital secretions (Balmelli et al., 1998), and that mucosal delivery is necessary to induce HPV16-neutralizing antibodies in genital secretions throughout the estrous cycle of mice (Nardelli-Haefliger et al., 1999). Aerosol vaccination of women with HPV16 VLPs appeared to be promising although not optimal to induce high titers of anti-VLPs antibodies in serum and a local production of IgA in cervical secretions in some volunteers (Nardelli-Haefliger et al., 2005). In this study, we were interested in exploring in mice the aerosol protocol of vaccination with HPV16 VLPs in conditions that mimic the situation in humans, where the respiratory tract is frequently confronted to viral antigens. Interestingly, when aerosol-like vaccination with HPV16 VLPs was compared in unprimed and influenza antigens exposed mice, the anti-VLPs systemic antibody response was not affected while mucosal responses were diminished. We have previously shown that the aerosol-like protocol allows the VLPs to interact with the lower respiratory tract (Balmelli et al., 1998). In these conditions, the trachea, the lungs and the tracheobronchial lymph nodes are the major immune inductive sites, which is probably crucial for the induction of the systemic VLP-specific humoral response, while the nasal-associated lymphoid tissue (NALT) probably plays a minor although maybe not negligible role for the induction of the mucosal response (Balmelli et al., 2002). It has to be pointed out that the influenza priming was performed nasally in conscious mice. In these conditions, the inoculum primarily interacts with the NALT and does not efficiently reach the deeper respiratory tract tissues (Balmelli et al., 1998). We may therefore hypothesize that mounting an immune response against influenza antigens in the NALT affects the presentation of HPV16 VLPs to the immune cells in this tissue but not in the lower respiratory tract to explain why mucosal but not systemic humoral responses to the HPV16 VLPs were reduced. More investigations are needed to reveal whether the schedule of influenza antigens and VLP administrations as well as the nature of the priming (influenza vaccine versus influenza virus) are important. Furthermore, understanding the mechanisms responsible for the reduction of the anti-VLP mucosal response may provide useful information on the parameters that have to be considered for future clinical trials involving aerosol vaccinations in humans.

In an attempt to improve HPV16 VLP immunogenicity via a mucosal route, we also compared in this study the airway administration of VLPs with two known mucosal adjuvants, HLT and CpG ODN. HLT was the more potent adjuvant, enhancing more than 10-fold the VLP-specific serum IgG as well as mucosal IgA titers in the aerosol-like protocol of vaccination. Interestingly, even when mice were primed with influenza antigens, HLT could restore the systemic and mucosal IgG and IgA titers we previously obtained after aerosol-like vaccination with HPV16 VLPs + CT without the influenza priming (Balmelli et al., 1998), showing that HLT is a very efficient adjuvant even under more stringent conditions. The improvement was even more apparent in the case of anti-VLP mucosal IgA titers after nasal vaccination of conscious mice, with high titers of anti-VLP IgA but not IgG induced in secretions. This suggests that the HLT adjuvant specifically increases the mucosal immune response, possibly by enhancing the uptake or processing of VLPs through the NALT. As specific IgG in vaginal secretions represent most probably antibodies transudating from serum, their level reflects the lower IgG titer in serum. Surprisingly, co-administration of CpG ODN with VLPs did not enhance the specific antibody titers in either protocol of airway vaccination. VLP-specific IgG isotype analysis revealed that HLT predominantly induced a Th2-like response, whereas a clear Th1-like pattern emerged with the CpG ODN adjuvant. This is in agreement with previously published data (Freytag and Clements, 2005) and confirms that the CpG ODN did function in stimulating the immune system. Consistent with a greater ability to enhance humoral responses, the use of HLT was also associated with increased VLP-specific proliferative responses in the spleen of immunized mice, while the combination of VLPs with CpG ODN did not improve cell proliferation. More importantly, even if CpG ODN predominantly elicited a Th1-like response, known to preferentially support development of cytotoxic CD8 T cell (CTL) responses, HLT was still more efficient in inducing L1-specific CD8 T cell-mediated IFN-γ secretion. These cell-mediated immune responses were not detected with the suboptimal vaccine doses used to critically evaluate adjuvant effect on B cell responses. However, in vivo effects on B cell proliferation and isotype switching consistent with the high dose *in vitro* results might well occur at levels of T cell activity too low to be measured by the insensitive *in vitro* assays. Activation of specific CD8 T cells after mucosal vaccination of mice with VLPs has only been rarely investigated, and the responses measured were overall low and required *in vitro* restimulation (Da Silva et al., 2003; Dupuy et al., 1999; Liu et al., 1998). Here we report for the first time the *ex vivo* ability of CD8 T cells that have been primed by aerosol-like vaccination with HPV16 VLPs alone or in combination with adjuvants to secrete IFN-γ in response to L1 stimulation. L1-specific CD8 T cells are generally not considered to be of particular relevance in prevention or therapy of HPV-related diseases since HPV L1 proteins are detectably expressed only in the more superficial layers of the epithelium (De Bruijn et al., 1998). However, antiviral cytokines released by antigen-specific T cells, such as IFN-γ and TNF-α, may contribute to inhibition and control of infection (Guidotti and Chisari, 2001).

Our data contrast with several recent reports emphasizing the interesting potent effects of CpG ODN as a mucosal adjuvant (Choi et al., 2002; Freidag et al., 2000; Gallichan et al., 2001; Jiang et al., 2003; Mariotti et al., 2002; McCluskie and Davis, 1998, 2000; Moldoveanu et al., 1998). CpG ODN are known to exert their immunostimulatory function through interaction with Toll-like receptor 9 (TLR-9), leading to the engagement of the myeloid differentiation factor 88 (MyD88), which in turn triggers downstream cellular targets involved in the regulation of immune effector functions (reviewed in Wagner, 2002). Interestingly, the ability of papillomavirus VLPs to induce maturation of dendritic cells (DCs) (Lenz et al., 2001; Rudolf et al., 2001) has been recently shown to be dependent upon MyD88

(Yang et al., 2004). One might thus speculate that HPV VLPs by themselves are able to efficiently activate the MyD88 signaling pathway in interacting immunocytes and therefore exert immunostimulatory effects. Therefore it might not be surprising that combination with CpG ODN, whose main adjuvant property also involves triggering of MyD88, failed to further improve VLP immunogenicity. In contrast, the adjuvant property of LT seems to utilize a signal transduction pathway different from the TLR/MyD88 signaling cascade (reviewed in Freytag and Clements, 2005), which may allow synergy between HLT and HPV VLPs. An alternative explanation for the low adjuvancy of CpG ODN is that these readily diffusible small molecules may not frequently contact DC or B cells that have encountered the large and very cell adhesive HPV16 particles. A way to improve immunogenicity of VLPs might be to physically link them with CpG ODN. Incorporation of CpG ODN into the interior of phage capsids has been shown to be more effective than simply mixing the two to allow repeated boosting and maintenance of high cell-mediated immune responses to a CTL epitope fused to the capsid (Storni et al., 2004). Interestingly, although CpG ODN did not enhance VLP immunogenicity, it nevertheless efficiently promoted class switching to Th1-like Ig isotypes. HPV VLPs have recently been shown to directly activate B cells and induce class switching to IgG1, IgG2a, IgG2b and IgG3 through a MyD88-dependent pathway (Yang et al., 2005). CpG ODN are known to support Th1-like Ig isotypes secretion by B cells upon TLR-9 and MyD88, but to suppress Th2-like isotypes (such as IgG1) through an independent mechanism (Lin et al., 2004). Integration of the signals induced by VLPs and CpG ODN in B cells therefore might promote IgG2a production, while inhibiting IgG1 production. Alternatively, immunocytes responding to CpG ODN at a distance and thus secreting Th1 cytokines could indirectly influence the antibody isotype response to VLPs, which could also explain the marked IgG2a polarization of the serum IgG response to VLPs.

Combination of CpG ODN with HPV VLPs has previously been reported (Gerber et al., 2001). In that study, oral immunization of mice with HPV16 VLPs resulted in moderate serum antibody titers that could be enhanced by co-administration of CpG ODN, while in our experiments airway administration of VLPs alone was able to induce considerably higher antibody titers. This discrepancy between the two mucosal routes of vaccination may be explained in part by the degradation of the VLPs in the stomach and intestine or by a more limited exposure to immunocytes after oral vaccination. In the suboptimal situation of oral administration, combination of CpG ODN with VLPs may provide synergistic immunostimulatory properties, and thereby improve VLP immunogenicity. Interestingly, Gerber et al. (2001) also reported that oral immunization of mice with HPV16 VLPs and a non-toxic mutant of LT induced higher serum antibody titers than after co-administration with CpG ODN.

Antibody titers in genital secretions may be crucial for cervical cancer prevention, since the inducing HPV infections are strictly localized in the genital mucosa. Although the aerosol route of administration has been successfully used in mass vaccination against measles in Mexico (Valdespino-Gomez et al., 2006), use of the nasal route of administration might raise fewer safety concerns, such as induction of allergy or lung inflammation. Taken together, our data suggest that airway vaccination with HPV VLPs and HLT may represent a promising alternative to parenteral administration to prevent infection with HPV and associated diseases. The very first nasal vaccine to be approved for human use was a HLT-adjuvanted influenza vaccine (Glueck, 2001). However, very rare cases of Bell's palsy were reported following its introduction on the market. Despite the impossibility to differentiate between a potential link and temporal coincidence, due to the low incidence of the reported cases of facial paralysis, and the conclusions of extensive toxicological studies performed in several animal models that do not support such a link (Zurbriggen et al., 2003), the vaccine was withdrawn from the market. Uncertainty therefore remains for nasal administration of LT-based mucosal adjuvants in humans, which will need further investigation and development of relevant standard non-clinical models to evaluate the safety of nasal vaccines.

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