# Evaluation of cell-penetrating peptides as mucosal immune enhancers for nasal vaccination.

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**Abstract:**

Cell-penetrating peptides (CPPs) have been evaluated as enhancers in drug delivery, their addition in medical formulations favors absorption allowing obtaining the pharmacological effect with lower drug doses. In vaccine formulations their inclusion has been also explored with interesting results. Currently mucosal vaccination constitutes a promising alternative with the main advantage of inducing both systemic and mucosal immune responses, which are crucial for control tumors and infections at mucosal tissues. The known CPP Penetratin was recently evaluated in vaccine formulations designed for nasal administration. The authors demonstrated that this non-covalent linked CPP could improve the antigen-specific systemic and mucosal antibody responses. In the present work we evaluate in Balb/C mice the nasal immune-enhancing effect of four CPPs. Animals were intranasally immunized with CPP and the recombinant hepatitis B surface protein (HBsAg) as model antigen. The IgG antibody response in sera and the mucosal IgA response were measured by ELISA. The IFN-γ secretion response at spleen was also evaluated by ELISPOT and ELISA. Among the CPPs studied one novel peptide stand out by its ability to potentiate the humoral and cellular immune response against the co-administered antigen. Considering that the use of mucosal routes is a promising strategy in vaccination against infectious diseases and cancer, which are gaining special relevance nowadays in the development of novel candidates against SARS-CoV-2 and other potential emerging respiratory virus, the searching and development of safe mucosal adjuvants constitute a current need.

**Keywords:** cell-penetrating peptides, nasal vaccination, mucosal, immune enhancer, adjuvant

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## 1. Introduction

Currently, mucosal vaccination constitutes a compelling strategy for infectious diseases and cancer due to its several advantages: is able to induce both systemic and mucosal immune responses, differing from parenteral immunization that mainly induces a systemic response (**1**), mucosal tissues cover a wide surface area (~400m$^2$ in a human adult) (**1**), and approximately 80% of total immune cells in a healthy adult are associated to mucosal surfaces (**2**). In addition to other advantages related with needle-free administrations. Although, only one commercialized nasal vaccine has been described so far (**3**), there are several clinical trials and research applying this route to vaccine delivery (**4-9**). Furthermore, nasal administration seems to be a promising strategy to improve the performance of cancer vaccines (**10-12**).

The search for effective and safe vaccine adjuvants remains as a research focus worldwide. The general characteristics of the mucosal tissues, and specifically nasal mucosa (**13**), limit the use of certain established adjuvants like aluminum salts; and favor the employment of other variants like bacterial toxins and viral vectors (**1, 14, 15**). Currently, with the boom of nanotechnology, several nano-carrier systems have emerged as promising options (**16**). Otherwise, cell penetrating peptides (CPPs), described more than twenty years ago as peptide sequences with the ability to potentiate the entrance to cells, constitute a novel alternative. Due to its intrinsic characteristics these peptides are able to enhance drug delivery and improve cellular uptake of other molecules, conjugated or not (**17**). CPPs are mostly peptides of less than 30 amino acids, polycationic (basically comprising clusters of polyarginine), or amphipathic (**17**).

The first CPP was discovered in 1994 and called penetratin (RQIKIYFQNRRMKWKK), a 16-mer-peptide coming from the homeodomain of *Antennapedia* (**18**). A few years later the CPP Tat (YGRKKRRQRRR) was identified as a derived sequence from an HIV-1 protein (**19**). Many other CPPs able to activate the movement of a cargo through the cell membrane have been identified or designed since then (**17**). In line with this, the cell-penetrating capacity of the $LALF_{32-51}$ -derived peptides, comprising the amino acids 35 to 51 of the *Limulus* antilipopolysaccharide (anti-LPS) factor (LALF), was recently reported (**20**). Previously the $LALF_{32-51}$ peptide (HYRIKPTFRRLKWKYKGKFW) was described to bind and neutralize LPS-mediated activities, showing anti-inflammatory properties and also exhibiting antiviral activity mediated by the induction of α and γ interferons (**21**). Currently, a second generation of $LALF_{32-51}$ -derived peptides has been studied by its antitumor effect (**20, 22**). Furthermore, based on its CPP ability, a recombinant fusion protein containing the $LALF_{32-51}$ peptide conjugated to E7 antigen from HPV-16 has been evaluated, by parenteral route, as a novel vaccine candidate for the treatment of HPV related malignancies (**23, 24**).

In spite of the increasing interest on CPPs research for medical applications, mainly in cancer topics, currently there are no FDA approved CPP-conjugated drugs (**25, 26**). However, more than 25 CPP-conjugated drugs are under clinical evaluation for multiple uses and others are on preclinical studies (**25**). Although, its employment in vaccine formulations, with the aim to booster the immune response, constitute a more recently strategy (**27**). So far, there are some promising results using CPPs as vaccine adjuvants (**23, 28-31**). In the present work we comparatively evaluate the adjuvant effect of four CPPs (Penetratin, modified- Penetratin, Tat and $LALF_{32-51}$) formulated with the hepatitis B surface antigen as model antigen, and administered by intranasal route. The antigen-specific antibody and cellular immune response elicited by each preparation was measured.

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## 2. Materials and Methods

### 2.1 Peptides and Antigen

The general description of the evaluated CPPs is shown on Table 1. Peptides were synthesized on solid phase and purified using reverse-phase-HPLC to >99% purity on an acetonitrile/$H_2O$ trifuoracetic acid gradient (Peptide Synthesis Lab, CIGB, Cuba). The peptide identity was confirmed by ion-spray mass spectrometry (Micromass, Manchester, UK). All CPPs were C-terminal amidated to increase its stability against *in vivo* proteolytic degradation. Apirogenic water (Biochrom AG, Alemania) with 1% dimethyl sulphoxide (DMSO) (Sigma, USA) was employed to reconstitute the lyophilized peptides.

The HBsAg, subtype *adw2* was expressed and purified from *Pichia pastoris* yeast at >95% purity (CIGB, Havana, Cuba) as ingredient of the commercial anti-hepatitis B prophylactic vaccine Heberbiovac-HB (**32**).

**Table 1. Main characteristics of the CPPs evaluated.**

| CPP | Amino acid sequences (n) | Original source | Nature |
|---|---|---|---|
| Tat | GRKKRRQRRRPPQ (13 aas) | HIV virus | nonamphipathic (highly cationic) |
| Penetratin | RQIKIWFQNRRMKWKK (16 aas) | *Drosophila Antennapedia* homeodomain | secondary amphipathic |
| mod-Penetratin | RQI**k**IWFQNRRM**k**W**k**K (16 aas) | " | " |
| $LALF_{32-51}$ | HYRIKPTFRRLKWKYKGKFW (20 aas) | *Limulus polyphemus* | primary amphipathic |

The aas represented in bold lower case correspond to D-aas. The sites of substitutions correspond to theoretical trypsin susceptibility, according to an analysis at Peptide Cutter – Expasy database.

### 2.2 Mice and Immunization schedule

BalbC female mice (H-2d haplotype) of 8 to 12 weeks' old were employed (CENPALAB, Havana, Cuba). Groups of six animals each were immunized with four doses administered weekly by intranasal route. Each dose contains 2mM of CPP + 5µg HBsAg in a final volume of 25µL. As controls groups we used mice immunized with, 5µg HBsAg in PBS intranasally (group 5), 5µg HBsAg adjuvated with alum (0,5mg/mL) by intramuscular route (group 6), and saline solution (group 7). All the immunogens were dissolved in sterile PBS. For nasal administrations, mice were anesthetized by the intraperitoneal injection using 30 µL ketamine (50 mg/mL), placed in a supine position and the immunogens (25µL) were dispensed slowly, alternating the nostril, using a pipette tip. For intramuscular route the immunogen was administered in a final volume of 100 µL.

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Mice were maintained at animal facilities under specific-pathogen-free conditions (CIGB, Cuba). All protocols have been reviewed and approved by the institutional animal care committees in compliance with Regulation No. 39/04 of the Cuban Regulatory Agency (CECMED) and the European regulations on Animal Welfare.

2.3 Biological Fluids

The blood was collected by retro-orbital puncture. Samples were centrifuged at 7 800 g for 10 min (centrifuge 5415C, Eppendorf, Hamburg, Germany). The obtained sera were stored at -20°C until evaluation. Vaginal washes were obtained by a skilled technician to avoid trauma and blood contamination. They were obtained by reflushing 100 µL of a sterile PBS solution with a micropipette. The collected lavages were centrifuged as above, and the supernatant stored at –20°C.

2.4 ELISAs

Anti -IgG, -IgA ELISAs were carried out as previously described (**33**). Briefly, the plates were coating with 5µg/mL of HBsAg and blocked with 2% skim milk solution. Sera samples were evaluated using different dilution starting from 1/50 and vaginal lavages were assayed directly (w/o dilution). Specific horseradish peroxidase conjugates (Sigma, USA) were employed and an OPD (Sigma, USA) / hydrogen peroxide substrate solution was used. After 15 min of incubation, the reaction was stopped at using 2N sulfuric acid and the optical density (O.D) was read at 492nm in a multiscan (SUMA, Cuba).

2.5 IFN-γ ELISPOT and ELISA

IFN-γ ELISPOT assay was performed using a Mouse IFN-γ ELISpot kit (Mabtech, Sweden). Ten days after the last immunization ($4^{th}$ dose) the splenocytes were isolated in RPMI culture medium (Gibco, EU). The samples (five mice per group) were processed individualized, with the exception of control groups (HBs in alum and Placebo) which were processed as pooled samples of three randomly selected mice. Duplicates cultures ($2x10^5$ and $5x10^5$ splenocytes per well) were settled, for 48 h at 37°C, 5% $CO_2$, in a 96 well round-bottom plate with 10 µg/mL of $S_{28-39}$ CTL peptide (IPQSLDSWWTSL**)** from HBsAg (**34**), 10 µg/mL of ConA, and medium. Then, the whole content of this plate was transferred to an ELISPOT pre-coated plate and incubated for 16-20 h at 37°C, 5% $CO_2$. The incubations with the detection conjugated antibodies and following steps were done as recommended by the producers. An AELVIS ELISPOT reader were used for spots count.
In parallel, a similar 96 well round-bottom plate were incubated for measure by ELISA the IFN-γ secretion at culture supernatant. With this aim an IFN-γ standard and the same antibody pair (Mabtech, Sweden) were used.

2.6 Statistical analysis

For statistical analyses the GraphPad Prism version 5.00 statistical software (Graph-Pad Software, San Diego, CA, USA) was used. Antibody titers were transformed into log10 for normalization. The One-way Anova test followed by a Newman Keuls post-test was used as parametric tests for multiple groups comparisons. In case of non- parametric multiple comparisons, the Kruskal Wallis test and Dunns post-tests was employed. A *P* value of $<0.05$ was considered to be statistically significant.

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## 3 Results and Discussion:

### 3.1 Systemic IgG antibody response

To study the immune enhancer capacity of each evaluated CPPs we intranasally immunized mice with a mix of HBsAg, as model antigen, and each CPP described in Table 1. Immunogens were administered in four doses, weekly, and the sera were collected five days after each dose to IgG response evaluation.

After the second dose, for the evaluated intranasal immunized groups, we only detected anti-HBs positive IgG response in 1/6 animals from the group that received Penetratin, and 2/6 in the group immunized with LALF peptide (Fig 1a). On the other hand, in the positive control group, that received HBsAg in alum by intramuscular route, all animals show positive responses with titers $\geq 10^3$. The results obtained after third dose (Fig 1b), show that in the groups immunized with Penetratin and LALF all animals seroconverted. The IgG titers detected for these both groups were statistically similar, and significantly superior to the response generated by the other nasally immunized groups (Tat, mod-Pen and HBsAg in PBS). The IgG response obtained for the last three groups did not statistically differ from the placebo group. Later on, after the fourth dose, the groups immunized with Penetratin and LALF showed the higher IgG response, which resulted significantly superior to the generated by the group immunized with Tat and also to that one from the placebo group (Fig 1c). Otherwise, the IgG responses generated by the groups receiving Tat and mod-Penetratin were similar to each other, and did not differ from the placebo.

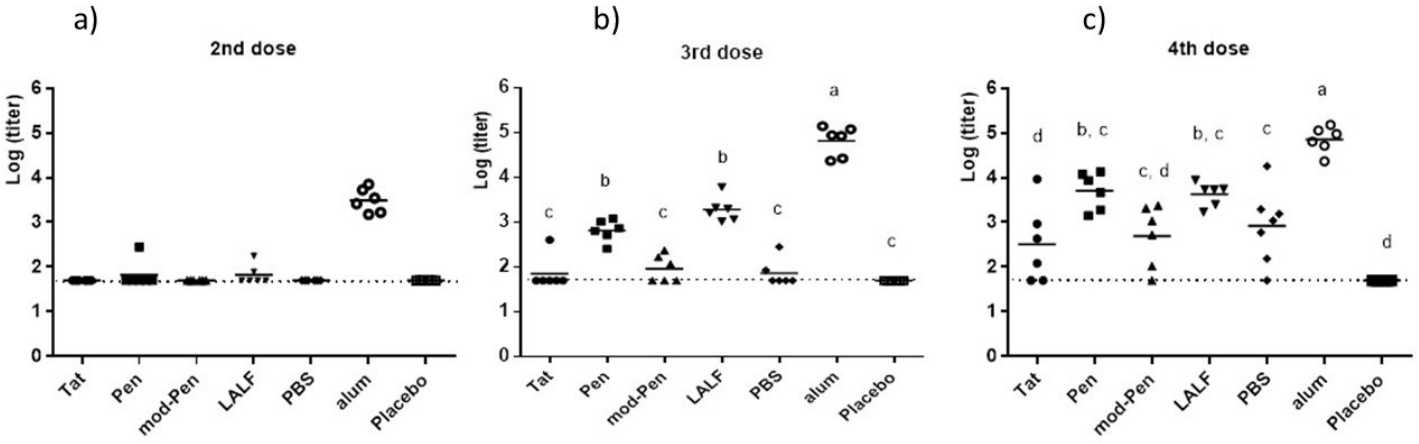


**Figure 1. Anti-HBsAg IgG antibody measured in sera following a) two, b) three, and c) four doses.** Six mice per group where nasally immunized with 5µg HBsAg plus 2mM of each individual CPP (Tat, Penetratin (Pen), modified-Penetratin (mod-Pen) and $LALF_{32-51}$) or PBS. Two controls group were included, 5µg HBsAg plus alum by intramuscular route, and nasally PBS (Placebo). Statistical differences were represented by different letters.

Taking into account that the generation of a Th1 pattern of immune response would be of interest for several vaccine candidate and the fact that the induction of IgG2a subclass in mice correlates with this kind of response (**35**) we evaluate this subclass after the last dose (Fig 2). The higher anti-HBs IgG2a response obtained correspond to the groups immunized with Penetratin and LALF,

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without significant differences between them, neither with the group immunized by intramuscular route in alum, or with the group receiving the HBsAg in PBS by intranasal route. The IgG2a response induced by the groups immunized with Tat and mod-Penetratin was similar to that one from the placebo group, and significantly lower to the generated by the first three groups mentioned above.

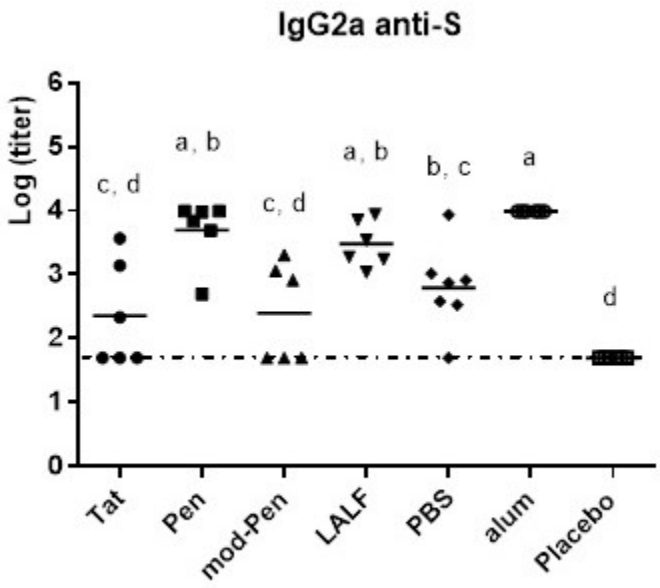


**Figure 2. Anti-HBsAg IgG2a antibody response measured in sera after four doses.** Groups of six mice where nasally immunized with 5µg HBsAg plus 2mM of each individual CPP (Tat, Penetratin (Pen), modified-Penetratin (mod-Pen) and $LALF_{32-51}$) or PBS. Two controls group were included, 5µg HBsAg plus alum by intramuscular route, and nasally PBS (Placebo). Statistical differences were represented by different letters.

The IgG results after three doses demonstrate a clear immunoenhancing capacity by nasal route for two out of four CPPs evaluated, Penetratin and LALF. This result is even more relevant if we consider that the antigen selected for the study is *per se* highly immunogenic by nasal route (**36**), which increases the threshold to overcome. This fact was evident at the IgG response obtained after the fourth dose, where the groups immunized with Penetratin and LALF still show a higher response compared with the antigen in PBS, but without statistical differences between them.

The results obtained using Penetratin as immune enhancer by nasal route is in line with a previously reported work, where this CPP shows a similar capacity to enhance the antibody immune response when administered with to different antigenic models, OVA and Influenza A (**31**). However, the results obtained here for the modified-Penetratin variant were unexpected. Based on the results reported by Muto and cols (**31**), the complete D-aas variant of Penetratrin shows a much better immune-enhancer capacity compared with its L- variant. In our case, considering the production cost that represents the complete D-aas variant, we decided to introduced D-aminoacids only in the positions theoretical predicted as more susceptible to trypsin cleavage. Nevertheless, our results with the modified Penetratin variant were worse than the

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obtained for its L-counterpart. For future studies we suggest to study the *in vivo* stability of Penetratin in nasal mucus, to determine which specific aas substitute by D-aas to improve the enhancing capacity, and if it is possible to do that without an all-D peptide variant.

3.2 IgA antibody response at vaginal mucosa

It is recognized that immune stimulation through the nasal mucosa is able to induce an IgA antibody response at distal mucosal tissues, being significant the response elicited at vaginal mucosa (**37**). On this base, in the present work we studied the anti-HBsAg IgA response at vaginal lavages after four doses. As show in figure 3, a trend to elicited a higher IgA response were observed for the group's immunized whit Penetratin and LALF, although no statistical differences were detected among all evaluated treatments. As expected, the positive control group immunized by intramuscular route was unable to induce a detectable IgA response at vaginal mucosa. This behavior is in line with the current knowledge indicating that to generate an immune response at mucosal surfaces a mucosal delivery route should be used, otherwise parenteral administration routes are not capable to effectively induce this kind of responses (**1**).

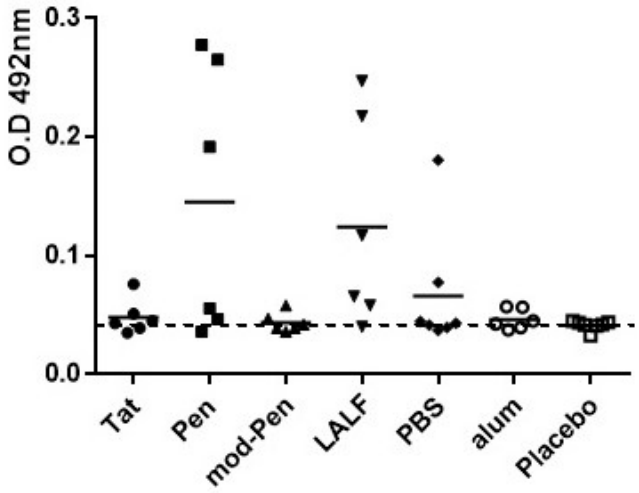


**Figure 3. Anti-HBsAg IgA antibody response measured at vaginal washes after four doses.** Groups of six mice where nasally immunized with 5µg HBsAg plus 2mM of each individual CPP (Tat, Penetratin (Pen), modified-Penetratin (mod-Pen) and $LALF_{32-51}$) or PBS. Two controls group were included, 5µg HBsAg plus alum by intramuscular route, and nasally PBS (Placebo).

3.3 IFN-γ secretion response by splenic CD8 T cells

In this study we also explore the effect of the evaluated CPPs in terms of improve the cellular immune response against co-administered antigens. With this aim we measured the HBs-specific IFN-γ secretion in spleen cells by ELISPOT after the fourth dose (Fig 4). Surprisingly, the group immunized with Tat, who shows a weak antibody response in sera, generated a clearly positive response in all the evaluated animals (5/5). The second better response was obtained for the group immunized with LALF where 4/5 mice respond positively, following by mod-Penetratin with 2/5, and Penetratin 1/5. On the other hand, the control group immunized with HBsAg in PBS intranasally shows positive response in 3/5 mice. This last result correlates with previous data reported showing that the HBsAg in PBS by intranasal route is capable to elicit a positive IFN-γ

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response at spleen of a magnitude that trends to be lower than the generated by the HBsAg in alum administered intramuscularly (**38**). Overall, although in statistics terms we did not detected significant differences among the geometric means for each evaluated group, a trend to induce a higher IFN-γ secretion response was observed for the groups receiving Tat and LALF jointly with the HBsAg.

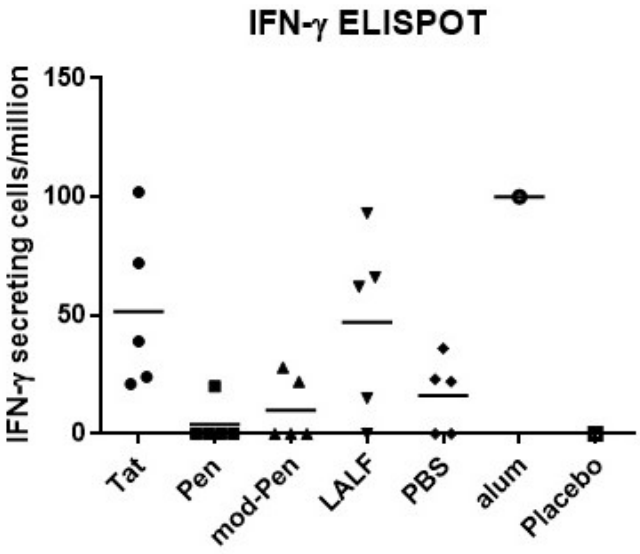


**Figure 4. HBsAg-specific IFN-γ secretion response at spleen.** The assay was carried out by ELISPOT after four doses. Six mice per group where nasally immunized with 5µg HBsAg plus 2mM of each individual CPP (Tat, Penetratin (Pen), modified-Penetratin (mod-Pen) and $LALF_{32-51}$) or PBS. Two controls group were included, 5µg HBsAg plus alum by intramuscular route, and nasally PBS (Placebo).

Furthermore, with the aim to corroborate the cellular immune response measured by ELISPOT we analyzed by ELISA the IFN-γ concentration in splenocyte's culture supernatant (Fig 5). Considering practical issues, in this assay we only evaluate the groups immunized with Penetratin and LALF, based on their better performance at humoral immunity, as well as the required control groups (HBsAg in PBS and placebo). The obtained results were consistent with the ELISPOT data. The group immunized with LALF shows IFN-γ response levels of variable magnitude in 4/5 animals (three of them >500 pg/ml), while in the group immunized with Penetratin respond only 2/5 animals (both with marginal values).

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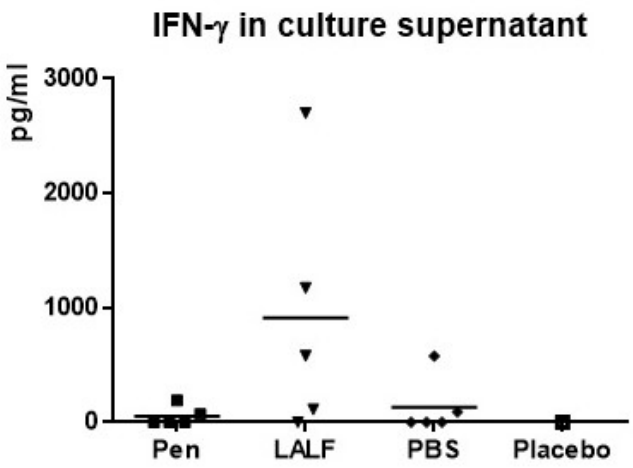


**Figure 5. HBsAg-specific IFN-γ secretion response at spleen.** The determination was carried out by ELISA in culture supernatant, after four doses. Six mice per group of where nasally immunized with 5µg HBsAg plus 2mM of each individual CPP (Penetratin (Pen) and $LALF_{32-51}$) or PBS. A Placebo control group (PBS nasally) was included.

In general, the present work extends the knowledge regarding the use of CPPs as immune-enhancer by nasal route. As far as we know, there are only very few previous publications on that line. One of them evaluates Penetratin peptide, in its L- and D-aas variants, and is limited only to the evaluation of IgG response in sera and IgA at nasal mucosa (**31**). Another published work evaluates the N-vinylacetamide-co-acrylic acid polymer linked to D-octa-arginine as mucosal delivery system (**39**). In both above mentioned works (**31, 39**) the same model antigens, OVA and Influenza A, and the same response parameters readout were employed. Here, we reproduced, with a different antigen (a VLP); the immune response already reported for L-Penetratin peptide in terms of humoral response (**31**) and we also expanded the data to the IFN-γ secretion response generated by splenic CD8+ T cells. In addition, interesting information regarding a partial D-Penetratin variant (mod-Penetratin) was obtained, posing new questions for future studies.
On the other hand, Tat peptide, one of the classic described CPPs; due to its origin has a closer relation with vaccines. Since its identification, Tat was employed for multiple developments in biomedicine field as a tool to potentiate the transportation of cargoes into cells. Currently there are some drug and vaccine candidates, some of them in clinical phases (like the CIGB300 for cancer therapy), that employ Tat as cell penetrating enhancer (**40-44**). Considering the present results obtained for the evaluation of Tat in a simple mix with HBsAg by nasal route, this CPP show the capacity to improve only the cellular immune response, without an impact on humoral response. This behavior, that should be evaluate using other model antigens, could be interesting in the case of infections where the development of cellular immune response correlates with protection and the generation of an antibody response results immunopathogenic.

In our point of view, the most exciting result emerging from this work is the data obtained for the LALF peptide. The results of humoral and cellular immune responses found with LALF peptide are promising. This peptide had been previously described as immune-modulator (**21, 23**), but the present work constitutes the first report of its use by nasal route as vaccine adjuvant. Previously, based on its CPPs ability, LALF peptide had been evaluated in conjugation with E7 antigen from

 doi:10.20944/preprints202106.0431.v1

HPV-16 by subcutaneous route as a vaccine candidate for HPV associated malignancies treatment (**23, 24**). In line with our results, the inclusion of LALF in the preparation favors the generation of cellular immune response in mice (**23**). Altogether with the relevance of elicit a mucosal immune response at vagina for efficiently deal with HPV infection and related malignancies, we preliminary evaluate this vaccine candidate by nasal route with promising results. This primary unpublished data with a second model antigen (E7 protein) supports the ability of LALF as immune-enhancer by nasal route.

We think that the behavior of each CPP as immune-enhancer may be influenced by the nature of the co-administered antigen, and should be probe case by case for the selection of the more suitable candidate. However, the present work reproduced the results in the case of L-Penetratin as an enhancer of humoral immune response, so far evaluated with three different model antigens (**31**). Other possible explanation to the different behavior of each CPP as immune-enhancer is the specific internalization mechanisms they could followed. It is well recognized that CPPs can be internalized by endocytic and nonendocytic paths, arriving to endosomes and cytoplasm, respectively. The internalization pathway may impact directly in the type of processing and presentation to the immune system, rendering different pattern of responses (**44**). In general, CPPs internalization mechanisms are influenced by different parameters like sequence and concentration of the peptide, and specific features of the cargo (**45**). Primary and secondary amphiphatic CPPs, at low micromolar concentrations, can directly enter through the cell membrane; while non-amphiphatic CPPs mostly uses endocytosis (**46**). However, for Penetratin cellular uptake endocytosis is the major mechanism suggested in the literature, both in the absence or presence of cargoes (**45**). On the other hand, the fact that LALF peptide sequence and extend is more similar to Penetratin than to Tat could support the similar behavior of the two former as humoral immune response enhancers. Otherwise, direct penetration is most probable for primary amphiphatic CPPs (**45**) (like LALF); this pathway could explain the high cellular immune response generated by the group immunized with LALF. However, direct penetration has been also suggested for arginine rich CPPs like Tat (**47, 48**). We hypothesized, that the dual ability of LALF to enhance humoral and cellular immunity against the co-administered antigen could also be explained by its capacity to penetrate cells by endocytosis and direct membrane transduction, using both pathways with appreciable rates. Without diminished the contribution of the immunomodulatory intrinsic properties previously described for LALF (**21, 23**). Indeed, the use of both internalization mechanisms has been previously described for CIGB-552 (a $LALF_{32-51}$ derived peptide), although the contribution of each pathway vary among different cell lines (**22**).

Regarding the concentration, we used in our experiments 2mM considering the findings reported by Muto and cols (**31**), who described it as the best evaluated for Penetratin nasal administration. This concentration is not so high if we considered that mucosal administration has different requirements, compared with parenteral, related with the several particular barriers to overcome. However, taking into account the different CPPs performance, in future studies lower concentrations should be evaluated by nasal route.

Another issue of concern in the development of vaccines and immune-enhancers is the safety of the formulation's components. Although has been widely reported that CPPs are usually nontoxic (**44**), a detailed study should be done case by case, exploring the intended administration route and evaluating different concentrations. The present study was not designed with this aim, however animals were visual observed post-immunizations and along the study and none concern signals

were reported. In favor, nonamphipathic CPPs like Tat and secondary amphipathic like Penentratin are reported as of low toxicity (**45**). On the other hand, LALF peptide has been showed its safety in several mice studies by subcutaneous route (**20, 23, 24**).

## 4 Conclusions:

The present work extends the knowledge regarding the use of CPPs as immune-enhancer by nasal route. In fact, the results described here for Penetratin, LALF and Tat peptides show potentialities for their future inclusion in nasal vaccine formulations. Considering the few vaccines adjuvants approved for human's use, CPPs seem to be a promising option for the future, in view of their safety, easy production and low cost. Certainly, deeper studies regarding the use of CPPs as vaccine adjuvants by nasal and parenteral routes are need.

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## Declaration of interest

The authors have no conflict of interests.

## 5 References

1. Kraehenbuhl JP and Neutra MR. Mucosal Vaccines: Where Do We Stand? Current Topics in Medicinal Chemistry 2013; 13: 2609-28.
2. Czerkinsky C and Holmgren J. Topical immunization strategies. Mucosal Immunology 2010; 3 (6): 545-55.
3. FDA Information Regarding FluMist Quadrivalent Vaccine. URL:https://www.fda.gov/biologicsbloodvaccines/vaccines/approvedproducts/ucm508761.htm Accesed on March 2019.
4. Al Mahtab M, Akbar SMF, Aguilar JC, Guillen G, Penton E, Tuero A, Yoshida O, Hiasa Y, Onji M. Treatment of chronic hepatitis B naïve patients with a therapeutic vaccine containing HBs and HBc antigens (a randomized, open and treatment controlled phase III clinical trial). PLoS ONE 2018, 13(8): e0201236.
5. Lobaina Y, Michel ML. Chronic hepatitis B: Immunological profile and current therapeutic vaccines in clinical trials. Vaccine (2017), 35: 2308 – 2314.
6. Bomsel M, Tudor D, Drillet AS, Alfsen A, Ganor Y, et al. Immunization with HIV-1 gp41 subunit virosomes induces mucosal antibodies protecting nonhuman primates against vaginal SHIV challenges. Immun 2011; 34: 269–80.
7. Brekke K, Lind A, Holm-Hansen C, Haugen IL, Sørensen B, et al. Intranasal Administration of a Therapeutic HIV Vaccine (Vacc-4x) Induces Dose-Dependent

Systemic and Mucosal Immune Responses in a Randomized Controlled Trial. PLoS ONE 2014; 9(11): e112556.

8. Iglesias E. Safety assessment of the vaccine candidate TERAVAC-HIV-1 in a phase I therapeutic clinical trial. Oral Presentation at IV International Workshop Infectious Diseases 2018. Varadero, Cuba, October 2018.
9. Thorstensson R, Trollfors B, Al-Tawil N, Jahnmatz M, Bergström J, Ljungman M, et al. A Phase I Clinical Study of a Live Attenuated Bordetella pertussis Vaccine - BPZE1; A Single Centre, Double-Blind, Placebo-Controlled, Dose-Escalating Study of BPZE1 Given Intranasally to Healthy Adult Male Volunteers. PLoS ONE 2014; 9 (1): e83449.
10. Nizard M, Diniz MO, Roussel H, Tran T, Ferreira LCS, Badoual C, Tartour E. Novel strategies and applications for the control of pathogens and tumors at mucosal sites. Hum Vacc & Immunother 2014; 10 (8): 2175-87.
11. Sandoval, F. et al. Mucosal imprinting of vaccine-induced CD8+ T Cells is crucial to inhibit the growth of mucosal tumors. Sci Transl Med 2013; 5: 172.
12. Nizard M, Roussel H, Diniz MO, Karaki S, Tran T, Voron T, Dransart E, Sandoval F, et al. Induction of resident memory T cells enhances the efficacy of cancer vaccine. Nat Commun 2017; 8, 15221 doi: 10.1038/ncomms15221.
13. Lobaina Mato Y. Nasal route for vaccine and drug delivery: Features and current opportunities. International Journal of Pharmaceutics 572 (2019) 118813.
14. Rosenthal KL, Jeyanathan M, Xing Z. Chapter 66 - Filling the Immunological Gap: Recombinant Viral Vectors for Mucosal Vaccines. Mucosal Immunology (Fourth Edition) 2015, Vol 2: 1291-1306.
15. Nizard M, Diniz MO, Roussel H, Tran T, Ferreira LCS, Badoual C, Tartour E. Mucosal vaccines. Human Vaccines & Immunotherapeutics (2014), 10:8, 2175-87.
16. Marasini N, Skwarczynski M, Toth I. Intranasal delivery of nanoparticle-based vaccines. Ther. Deliv. (2017), 8(3): 151–167.
17. Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. British Journal of Pharmacology (2009), 157: 195–206.
18. Derossi D, Joliot AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem (1994), 269: 10444–50.
19. Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J Biol Chem (1997), 272: 16010–17.
20. Guerra-Vallespi M, Fernández-Massó JR, Oliva-Argüelles B, Reyes-Acosta O, Garay-Pérez HE, Delgado-Roche L, Cabrales-Rico A, et al. CIGB-552: A new penetrating peptide with antitumor action mediated by the increased levels of the COMMD1 protein in cancer cell lines. Biotecnología Aplicada 2015, 32:3501-5.
21. Vallespi MG, Glaria LA, Reyes O, Garay HE, Ferrero J, Araña MJ. A *Limulus* Antilipopolysaccharide Factor-Derived Peptide Exhibits a New Immunological Activity with Potential Applicability in Infectious Diseases. Clinical and Diagnostic Laboratory Immunology, 2000, 7 (4): 669–675.

22. Astrada S, Fernández Massó JR, Vallespí MG, Bollati-Fogolín M. Cell Penetrating Capacity and Internalization Mechanisms Used by the Synthetic Peptide CIGB-552 and Its Relationship with Tumor Cell Line Sensitivity. Molecules 2018, 23, 801.
23. Granadillo M, Vallespi MG, Batte A, Mendoza O, Soria Y, Lugo VM, Torrens I. A novel fusion protein-based vaccine comprising a cell penetrating and immunostimulatory peptide linked to human papillomavirus (HPV) type 16 E7 antigen generates potent immunologic and anti-tumor responses in mice. Vaccine 29 (2011) 920–930.
24. Granadillo M, Batte A, Blanco A, Alfonso AB, Suárez J, Merino N, Carballo R, González BO, et al. LALF32-51-E7 therapeutic vaccine induces antitumor immunity against human papillomavirus type 16 E7-expressing murine tumor metastases in the lungs. Clin Exp Metastasis (2017) 34:241–249.
25. Habault J and Poyet JL. Recent Advances in Cell Penetrating Peptide-Based Anticancer Therapies. Molecules (2019), 24: 927.
26. Tripathi PP, Arami H, Banga I, Gupta J, Gandhi S. Cell penetrating peptides in preclinical and clinical cancer diagnosis and therapy. Oncotarget (2018), 9 (98): 37252-67.
27. Brooks NA, Pouniotis DS, Tang C-K, Apostolopoulos V, Pietersz GA. Cell-penetrating peptides: Application in vaccine delivery. Biochimica et Biophysica Acta (BBA) - Reviews on Cancer (2010), 1805(1): 25-34.
28. Belnoue E, Di Berardino-Besson W, Gaertner H, Carboni S, Dunand-Sauthier I, Cerini F, et al. Enhancing Antitumor Immune Responses by Optimized Combinations of Cell-penetrating Peptide-based Vaccines and Adjuvants. Molecular Therapy (2016), 24(9): 1675–85.
29. Brooks N, Hsu J, Esparon S, Pouniotis D, Pietersz GA. Immunogenicity of a Tripartite Cell Penetrating Peptide Containing a MUC1 Variable Number of Tandem Repeat (VNTR) and A T Helper Epitope. Molecules (2018), 23: 2233.
30. Rostami B, Irani S, Bolhassani A, Cohan RA. M918: A Novel Cell Penetrating Peptide for Effective Delivery of HIV-1 Nef and Hsp20-Nef Proteins into Eukaryotic Cell Lines. Current HIV Research (2018), 16: 280-287.
31. Muto K, Kamei N, Yoshida M, Takayama K, Takeda-Morishita M. Cell-Penetrating Peptide Penetratin as a Potential Tool for Developing Effective Nasal Vaccination Systems. Journal of Pharmaceutical Sciences (2016), 105: 2014-17.
32. Hardy E, Martínez E, Diago D, Díaz R, González D, Herrera L. Large-scale production of recombinant hepatitis B surface antigen from Pichia pastoris. Biotechnol. (2000) 77(2-3):157-67.
33. Lobaina Y, Trujillo H, Garcia D, Gambe A, Chacon Y, Blanco A, *et al*. The effect of the parenteral route of administration on the immune response to simultaneous nasal – parenteral immunizations using a new HBV therapeutic vaccine candidate. Viral Immunology (2010), 23(5): 521-529.
34. Schirmbeck R, Wild J, Reimann J. Similar as well as distinct MHC class I-binding peptides are generated by exogenous and endogenous processing of hepatitis B virus surface antigen. Eur.J. Immunol (1998), 28: 4149–61.

35. Snapper CM, and Paul WE: Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science (1987) 236: 944–947.
36. Lobaina Y, Palenzuela D, Pichardo D, Muzio V, Guillen G, Aguilar JC. Immunological characterization of two hepatitis B core antigen variants and their immunoenhancing effect on co-delivered hepatitis B surface antigen. Mol Immunol. 2005; 42: 289-94.
37. Johansson EL, Wassen L, Holmgren J, Jertborn M, Rudin A. Nasal and vaginal vaccinations have differential effects on antibody responses in vaginal and cervical secretions in humans. Infect Immun (2001) 69: 7481-86.
38. Aguilar JC, Lobaina Y, Muzio V, Garcia D, Penton E, Iglesias E, *et al*. Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen. Immunol Cell Biol. 2004; 82: 539-46.
39. Sakuma S, Suita M, Inoue S, Marui Y, Nishida K, Masaoka Y, Kataoka M, Yamashita S, et al. Cell-Penetrating Peptide-Linked Polymers as Carriers for Mucosal Vaccine Delivery. Mol. Pharmaceutics 2012, 9, 10, 2933-2941.
40. Tripathi PP, Arami H, Banga I, Gupta J, Gandhi S. Cell penetrating peptides in preclinical and clinical cancer diagnosis and therapy. Oncotarget 9(98), 37252–37267 (2018).
41. Sarduy MR, García I, Coca MA, Perera A, Torres LA, Valenzuela CM, Baladrón I, Solares M, Reyes V, Hernández I, Perera Y, et al. Optimizing CIGB-300 intralesional delivery in locally advanced cervical cancer. Br J Cancer, 2015, 112, 1636- 1643.
42. Sahay B, Aranyos AM, Mishra M, McAvoy AC, Martin MM, Pu R, Shiomitsu S, Shiomitsu K, Dark MJ, Sanou MP, Roff SR, Rathore MH, Yamamoto JK. Immunogenicity and Efficacy of a Novel Multi-Antigenic Peptide Vaccine Based on Cross-Reactivity between Feline and Human Immunodeficiency Viruses. Viruses 2019, 11, 136; doi: 10.3390/v11020136
43. Tang J, Yin R, Tian Y et al. A novel self-assembled nanoparticle vaccine with HIV-1 tat(49 57)/HPV e7(49-57) fusion peptide and GM-CSF DNA elicits potent and prolonged CD8(+) t cell-dependent anti-tumor immunity in mice. Vaccine (2012), 30(6): 1071–1082.
44. Grau M, Walker PR, Derouazi M. Mechanistic insights into the efficacy of cell penetrating peptide-based cancer vaccines. Cellular and Molecular Life Sciences (2018) 75:2887–2896.
45. Madani F, Lindberg S, Langel U, Futaki S, Graslund A. Mechanisms of Cellular Uptake of Cell-Penetrating Peptides. Journal of Biophysics, 2011, doi:10.1155/2011/414729.
46. Ziegler A. Thermodynamic studies and binding mechanisms of cell-penetrating peptides with lipids and glycosaminoglycans. Advanced Drug Delivery Reviews, 2008, vol. 60, no. 4-5, pp. 580–597.
47. Guterstam P, Madani F, Hirose H, et al. Elucidating cellpenetrating peptide mechanisms of action for membrane interaction, cellular uptake, and translocation utilizing the hydrophobic counter-anion pyrenebutyrate. Biochimica et Biophysica Acta, 2009, vol. 1788, no. 12, pp. 2509–2517.
48. Thoren PEG, Persson D, Isakson P, Goksor M, Onfelt A, Norden B. Uptake of analogs of penetratin, Tat (48-60) and oligoarginine in live cells. Biochemical and Biophysical Research Communications, 2003, vol. 307, no. 1, pp. 100–107.