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Research Article

# IMS 4112 and VLP of HBV as Th1 Adjuvants for a Recombinant Protein of HIV-1

## Abstract

**Background**: Current thinking suggests that vaccination approaches against the HIV-1 should be directed to elicit a Th1 cell-mediated immunity, neutralizing antibodies and/or ADCC mediating antibody responses. Also, experimental evidences suggest that both the systemic and mucosal compartments of the immune system must be stimulated to achieve protection. In this regard, effective adjuvants are necessary. Thus, we developed the multiantigenic vaccine candidate TERAVAC that comprise the recombinant protein CR3 of HIV-1 and the surface (S) and core (C) virus like particles (VLP) of Hepatitis B virus (HBV) as adjuvant to promote a Th1 immune response. On the other side, the new Th1 adjuvant Montanide® IMS 4112 (IMS 4112), an oil-in-water micro-emulsion was developed by SEPPIC.

**Purpose of study**: The aim of this work was to compare the adjuvant effect of IMS 4112 versus HBV VLP on the CR3(HIV-1)-specific immune response in Balb/c mice using schedules that combine nasal and/or subcutaneous inoculations.

**Results**: We found a better Th1 response with HBV VLP but a higher IgG response in vagina with IMS 4112 after i.n. inoculation. In s.c. immunization similar immune responses were detected using both adjuvants. When combining i.n. and s.c. coadministration, the HBV VLP seemed to require less immunization doses to achieve detectable proliferation of CD4+ and CD8+ lymphocytes in the systemic compartment.

**Conclusion**: Overall, HBV VLP seem to be a better adjuvant for the recombinant CR3 protein of HIV-1 than the IMS 4112 at the concentration it was used in this study.

## Abbreviations

VLP: Virus Like Particles; HBV: Hepatitis B virus; HBsAg (S): Surface Antigen of the HBV; HBcAg (C): Core Antigen of the HBV; i.n. Intranasal; s.c.: Subcutaneous; AlOOH: Aluminum Hydroxide Adjuvant.

## Introduction

Numerous evidences suggest that long-lasting cell immunity would be necessary to eliminate cells infected with the human immunodeficiency virus type 1 (HIV-1). For instance, the protective role of $CD8^+$ CTL responses in the elimination of such cells during primary infection and in controlling virus replication during the chronic phase is well documented [1]. Depletion of $CD8^+$ lymphocytes in simian immunodeficiency virus (SIV)-infected rhesus monkeys during chronic infection resulted in a rapid and marked increase in viremia. In this animal model, viral control was achieved after reappearance of SIV-specific $CD8^+$ T cells, supporting the importance of these cells in vaccination approaches against HIV-1 [2,3].

Considering that HIV-1 is mainly a mucosal transmitted infectious pathogen, vaccination efforts are re-focused on mucosal routes [4]. Belyakov and colleges reported the induction of HIV-1 specific interferon gamma (IFN-$\gamma$) secreting $CD8^+$ T cells in mice intestinal mucosa following mucosal, parenteral or heterologous mucosal-parenteral coadministration [5,6]. Additionally, they showed that $CD8^+$ T cells mediated preservation of mucosal $CD4^+$ T cells from depletion in macaques after mucosal challenge with pathogenic Simian/Human Immunodeficiency Virus (SHIV) [6].

Other evidences suggest the significant role of the humoral response (i.e.; antibody-dependent cell-mediated cytotoxicity (ADCC) or serum neutralizing antibodies (Nabs)) in controlling HIV infection [7-9]. Moreover, some authors have suggested that secretion of HIV-specific IgA and IgG in the vaginal



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mucosa might also play an important role in the prevention of HIV-1 transmission through sexual intercourse [10-14]. Therefore, immunogens to induce HIV-1-specific IgA and IgG antibodies in the genital tract might be considered for prophylactic vaccination against HIV-1. All together, these elements support the development of novel vaccine candidates for parenteral and mucosal inoculation in order to elicit anti-HIV-1 cellular and humoral responses in the systemic and mucosal compartment [15]. In this regard, it is known that simultaneous administration through a mucosal and parenteral route of HIV native proteins [16] or a recombinant chimeric antigen [17] enhanced the overall immune response in comparison with single route immunizations.

Another important piece in the vaccination puzzle is the choice of the appropriate adjuvant. Although during the last years a new generation of compounds has emerged, aluminum salts have been extensively used for more than 60 years for veterinary and human vaccines due to their safety, low production cost and verified adjuvant effect with many antigens [18,19]. In the past decade, SEPPIC, one of the leader enterprises for adjuvant engineering worldwide, developed a new generation of products, registered under the trade name Montanide® IMS, to obtain vaccine formulations with a good balance between safety and efficiency. These substances ready to dilute oil-in-water micro-emulsions based on the blend of undisclosed *GRAS* (Generally Recognized as Safe) immuno-stimulatory compounds and excipients. Some of these adjuvants have been administered to animals in veterinary vaccines with promising results [20-22] but their experimental use for human vaccines is almost unexplored. In this regard, IMS 4112 was developed for human vaccines production to elicit a Th1 biased immune response against intracellular pathogens (i.e.; viruses).

A vaccine candidate against HIV-1 must satisfy three requirements: first, HIV-related antigen(s) (recombinant or not) to stimulate a specific B and/or T cell immunity; second, a safe adjuvant formulation to modulate and enhance the immune response to a particular Th profile and third, stimulation of the mucosal compartment. According to these requirements, we produced the recombinant protein CR3. It comprises B-cell, Th and CTL epitopic rich regions from several subtypes B HIV-1 isolates. This recombinant antigen was coadministered with the Hepatitis B Virus (HBV) surface (HBsAg) and nucleocapsid (HBcAg) antigens through the nasal or subcutaneous route to Balb/c mice and we observed the induction of anti-CR3 (HIV) cellular and humoral immune responses. For subcutaneous immunization the antigens were adjuvated in aluminum hydroxide (AlOOH) and nasal administration were carried out with physiological buffers [23]. Furthermore, simultaneous administration of the above mentioned multiantigenic mixture (named TERAVAC) through the nasal and subcutaneous routes to Balb/c mice promoted a Th1-biased CR3 (HIV)-specific cellular and humoral immune responses [17,24]. In this regard, the VLP of HBV behaves as Th1 adjuvants enhancing the specific anti-CR3 (HIV) immune response and as delivery antigens for effective nasal inoculation [23,25]

Considering the high HBV-HIV co-infection rate in some geographic areas, the multiantigenic formulation TERAVAC might elicit protective immunity against both pathogens. However, the production cost of the three antigens associated to such anti-HIV vaccine will increase its price in the market. Because of that reason, we have considered the possibility to replace the HBV antigens with another adjuvant formulation to avoid the extra cost associated with their production. Thus, the aim of the present work was to compare the adjuvant effect of HBV VLP and IMS 4112 on the CR3 (HIV)-specific immune response in schedules through the nasal, subcutaneous and simultaneous nasal-subcutaneous administration.

## Materials and Methods

### Antigens

The recombinant HIV-1 antigen CR3 is a multiepitopic protein which consist of B cell, Th and CTL epitopic regions comprising T1 and T2 from gp120, an epitope from gp41, another from Vpr, a fragment of p66/p55 (reverse transcriptase [RT]) protein (positions 2663-3109, HIV-1 SF2 provirus), a part of Nef (positions 8516-8818, HIV-1 LAI isolate), and a part of Gag (positions 1451-1696, HIV-1 SF2) [23]. It was purified from *E.coli* as a pyrogen free product with more than 95% purity (Technological Development Unit, CIGB, Havana, Cuba) [26]. The entire recombinant HBcAg of 183 aminoacids (hereafter referred as C) was expressed in *E.coli* and purified as described [27]. Recombinant HBsAg subtype *Adw2* (hereafter referred as S) was taken from the production process of the Cuban hepatitis B vaccine Heberbiovac HB (CIGB, Havana, Cuba). This protein was expressed in the yeast *Pichia pastoris* and its purification procedure has been previously reported [28].

### Immunizations

Female Balb/c mice, 6-8 weeks old were purchased from CENPALAB (Habana, Cuba). Three different immunization schedules through the intranasal (i.n.), subcutaneous (s.c) or simultaneous (s.c./i.n.) inoculations were carried out as described in Table 1. In all schedules, the animals received 5 µg of each antigen (CR3, C and S) per route diluted in phosphate buffer saline (PBS). For the intranasal inoculation, they were anesthetized by intraperitoneal administration of 10 µL ketamine (50 mg/mL), placed in a supine position and the immunogen dispensed slowly in 50 L (25 µL/nostril) using a pipette tip. Montanide® IMS 4112 VG PR (SEPPIC, France) can be used in a range between 5 to 50% v/v in aquous phase. It was decided to use at 10% v/v ratio based on the mixed routes of inoculation (s.c./i.n.) targeted in this study. For subcutaneous immunizations, antigens were adjuvated in 1 mg/mL AlOOH (Superfos Biosector A/S, Vedbaek, Denmark) or 10% v/v IMS 4112.

Negative control groups were formulated without CR3 (Table 1; Schedule #1 and #2, groups 1 and 2; Schedule #3, groups 1-3). In all cases, immunogens were prepared the day before inoculations and stored at 4° C. All experiments were conducted according to institutional guidelines.



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**Table 1:** Immunization schedules used in this study.

| Schedule: Route | Group (mice/ group) | Inoculum_Adjuvant | Delivery (days) | Bledding (days) |
|---|---|---|---|---|
| Schedule #1: i.n. | 1 (n=10) | C+S | 0, 7, 14, 35, 56 | -2, 66 |
| | 2 (n=10) | PBS_IMS4112 | | |
| | 3 (n=10) | CR3+C+S (TERAVAC) | | |
| | 4 (n=10) | CR3_IMS4112 | | |
| Schedule #2: s.c. | 1 (n=6) | C+S _AlOOH | 0, 7, 21 | -2, 31 |
| | 2 (n=6) | PBS_IMS4112 | | |
| | 3 (n=10) | CR3+C+S (TERAVAC)_ AlOOH | | |
| | 4 (n=10) | CR3_IMS4112 | | |
| Schedule #3: s.c./i.n. | 1 (n=10) | C+S_AlOOH/C+S | 0, 14, 35, 56, 77, 99 | -2, 87, 109 |
| | 2 (n=10) | PBS_IMS4112/PBS_ IMS4112 | | |
| | 3 (n=10) | C+S_AlOOH/PBS_ IMS4112 | | |
| | 4 (n=15) | CR3+C+S (TERAVAC)_ AlOOH/ CR3+C+S | | |
| | 5 (n=15) | CR3_IMS41v12/CR3_ IMS4112 | | |
| | 6 (n=15) | CR3+C+S (TERAVAC)_ AlOOH/ CR3_IMS4112 | | |

i.n.: intranasal; s.c.: subcutaneous; s.c./i.n.: simultaneous s.c. and i.n. administration.

## Biological fluids

Animals were bled by retro-orbital puncture according to Table 1. Sera were collected by centrifugation at 7 800g for 10 minutes (centrifuge 5415C, Eppendorff, Germany)

Vaginal washes were obtained 10 days after the final dose by reflushing 100 µL of sterile PBS with a micropipette. Supernatants were collected by centrifugation as described above and properly stored at -20°C until antibody detection.

## Quantification of IFN-γ secreting cells

Ten days after the last doses, the frequency of CR3 (HIV)-specific IFN-γ secreting cells was detected in spleen of mice using an enzyme-linked immunospot (ELISPOT) assay as described [24]. Briefly, nitrocellulose-backed 96-well plates (MultiScreen-IP filter plates MAIPN4550; Millipore, USA) were coated with 100 µL of murine IFN-γ specific mAb AN18 (5 µg/mL; Mabtech Inc., USA) overnight at 4°C. Two dilutions (2 x $10^5$ and 1 x $10^5$) of freshly isolated splenocytes were incubated for 48 h at 37°C in 5% $CO_2$ in complete medium (RPMI 1640 [Invitrogen GIBCO, UK] supplemented with 10% fetal bovine serum [FBS], 2 mM glutamine, 2 mM sodium pyruvate, and antibiotics) with sterile and non-pyrogenic CR3, at 2.5µg/mL. Wells that contained cells in complete medium or 5 µg/mL Concanavalin A (Con A; Sigma, St. Louis, MO, USA) were used as negative and positive controls respectively. Secondary biotin-conjugated antibody R4-6A2 (0.5 µg/mL; Mabtech) was then added and incubated for 2 h at room temperature. Detection of CR3 (HIV)-specific IFN-γ secreting cells was achieved under a dissection microscope. Results were expressed as the number of spot-forming cells (SFC) per $10^6$ splenocytes after subtracting the spots of negative wells. Values above the mean number of spots in the placebo or negative group plus 3 standard deviations were considered positive.

In some experiments 20x$10^6$ of splenocytes at 2 x$10^6$ cells/mL where re-stimulated *ex vivo* with 2.5µg/mL CR3 protein at 37°C in 5% $CO_2$. After five days, half of the medium was removed and replaced with fresh medium and human recombinant IL-2 (BD Biosciences, USA) at 10 U/mL final concentration. At day seven, the ELISPOT assay was conducted as described above.

## CR3 (HIV)-specific T Cells Proliferation

The assay to assess CR3 (HIV)-specific CD4+ and CR8 + T cell proliferation was described previously [17]. Briefly, ten days after the fifth co-administration, 20x$10^6$ splenocytes were labelled with 5 µM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, OR, USA) and cultured in the presence of 2.5 µg/mL CR3 and 5 µg/mL Con A for 5 days at 37 °C in 5% $CO_2$. After incubation, one million cells were stained with 0.2 µg of the CD8a-APC antibody (clone Ly-2) or the CD4-APC antibody (clone L3T4) (eBiosciences, San Diego, CA, USA). The events were acquired using a PAS III flow cytometer (Partec GmbH, Münster, Germany) and the analysis was performed with Flomax v2.4f software (Partec GmbH). One-hundred thousand viable lymphocytes including blasts were gated (gate R1) for the analyses. For each experimental condition we first calculate the percent of $CD8^+$ or $CD4^+$ T cells that divided after *ex vivo* stimulation (Q1) relative to the bulk of $CD8^+$ or $CD4^+$ T cells gated (Q2+Q1). For each group, results are presented as percent of CR3 (HIV)-specific T cell population that divided after *ex vivo* stimulation with CR3 ($CFSE_{low}$) after substracting the percent corresponding to unstimulated cells. We considered a positive response in experimental groups when the percent values were at least twofold higher compared to the corresponding negative control group.

## Cytokine secretion in culture supernatants

Cytokine secretion was assessed by a sandwich ELISA in culture supernatants of splenocytes collected after five days re-stimulation with 2.5µg/mL CR3 protein. High binding capacity 96-well plates (Corning Life Sciences, USA) were coated with 50 µL anti-mouse IFN-γ clone AN18 (5 µg/mL; Mabtech Inc., USA) or anti-mouse IL-4 clone 11B11 (5 µg/mL; BD Pharmingen USA) overnight at 4°C in coating buffer (11 mM $Na_2CO_3$ and 35 mM $NaHCO_3$, pH 9.6). Then, plates were blocked with 100 µL of working solution (10% FBS in PBS) during 1 h at 37°C. Undiluted samples and the standard curve of recombinant mouse IFN-γ (0.39 ng/mL-50 ng/mL) or recombinant mouse IL-4 (0.95 pg/mL-30 pg/mL) were incubated 2 h at 37°C. Secondary biotin-conjugated anti-mouse IFN-γ, clone R4-6A2 (0.5 µg/mL; Mabtech) or biotin-conjugated anti-mouse IL-4, clone BVD6-24G2 (0.5 µg/mL, BD Pharmingen) was then added and incubated for additional 2 h at 37°C. Peroxidase-labeled streptavidin (Sigma, St. Louis, MO) was added at a 1:1000 dilution for 1 h at 37°C. Finally, colour reaction was developed with the substrate solution (52 mM $Na_2HPO_4$, 25 mM sodium citrate, o-phenylenediamine [OPD, 1 mg/mL] [Sigma-Aldrich,



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USA], and 0.1% $H_2O_2$) for 10 minutes at room temperature. The reaction was stopped with 25 µL of 3 M $H_2SO_4$. Then, the plates were read at 492 nm in a microplate reader (Sensident Scan 352; Labsystems, Helsinki, Finland). Washes (at least five) with 0.05% Tween 20 in PBS were carried out between each step. Standard curve and all reagents were diluted with working solution. To determine the cytokine concentration in the samples, we calculated the mean absorbance for each set of duplicate standards, controls and samples and subtracted the mean zero standard absorbance from each. Then, the logarithm of the absorbance values were interpolated into a linear log-log regression analysis plotting concentration of cytokine standards versus $A_{492}$. Finally, cytokine concentration was calculated as the antilog of the resulting value. The limit of detection (LoD) was calculated from the standard curve as described by Armbruster and Pry [29] using $A_{492}$ and the formula $A_{492}$ LoD = meanblank + 1.645 × (SDblank + SDlow concentration sample). Then, to obtain the LoD the same procedure as for the samples was followed.

### Antibody response

The CR3 (HIV)-specific antibody responses in serum and vaginal fluid were measured by enzyme-linked immunosorbent assay (ELISA) [17]. Plates were coated overnight with 5 µg/mL of CR3 at 4°C in sodium acetate buffer, pH 5.2. After blockage with 2% skim milk in PBS for 1 h at 37°C, vaginal washes or serum samples diluted in 1% skim milk, and 1% Tween 20 in PBS were added for 2 h at 37°C. Rabbit anti-mouse total IgG, $IgG_1$, $IgG_{2a}$ and IgA peroxidase conjugates (MP Biomedicals, USA) were incubated for 1 h at 37°C at appropriate dilutions. The reactions were then developed with the OPD substrate solution for 10 min at room temperature. The reaction was stopped with 50 µL of 3 M $H_2SO_4$. Finally, the plates were read at 492 nm in a microplate reader (Sensident Scan 352; Labsystems, Helsinki, Finland). Washes (at least five) with 0.05% Tween 20 in distilled water were carried out between each step. IgG titer was referred as standard units (SU) calculated by the interpolation of absorbance values at a fixed serum dilution into a linear regression analysis, plotting dilution versus $ABS_{492}$ of the standard curve from CR3-specific monoclonal antibody (CBSSCR.2, CIGB Santi Spiritus, Cuba). The titer was defined as the highest dilution that gave twice the absorbance of the negative control sera diluted 1:100. Titers are given as geometric mean with 95% CI from individual sera.

IgA and IgG responses from vaginal washes corresponded to 1:10 diluted samples. For IgG subclasses, results were expressed as mean $ABS_{492}$ at a fixed serum dilution. Threshold values were calculated as the average plus three standard deviations of the absorbance in the negative control group.

### Statistical procedures

GraphPad Prism version 5.0 software (Graph- Pad Software, San Diego, CA, USA) was used to carry out statistical analyses. All titers were transformed to log10 for a normal distribution. For the non-seroconverting sera, an arbitrary titer of 10 was assigned for statistical processing. A $p$ <0.05 was considered statistically significant.

## Results

### VLP of HBV induced a better CR3 (HIV)-specific Th1 immunomodulation than IMS 4112 adjuvant after nasal inoculation

As previously reported, immunization with the CR3 antigen alone did not induce a detectable Th1 response after i.n. administration [23]. In contrast, immunization of CR3 in a multiantigenic formulation with the VLP of HBV (C and S) through the nasal (in PBS) or subcutaneous route induced a CR3-specific Th1 pattern [23]. In an attempt to evaluate the new adjuvant IMS 4112, we examined the differential effects on the CR3 (HIV)-specific immune response of this compound versus the hepatitis B antigens after i.n. inoculation. After the fifth dose (Table 1; Schedule #1) the spleen of five Balb/c mice per group was aseptically removed and the secretion of IFN-γ and IL-4 and the frequency of IFN-γ secreting cells anti-CR3 (HIV) were determined after several days of *in vitro* re-stimulation with the protein. We found a higher median of IFN-γ secretion in culture supernatants in the group immunized with CR3+C+S (TERAVAC) (median, 37.6 ng/mL; [range,17-53]) compared to mice immunized with CR3_IMS 4112 (6.7 ng/mL; [2-13]) (p=0.0286; two-tailed Mann-Whitney test). CR3 (HIV)-specific IL-4 secretion was below the cut off value in all experimental groups (Figure 1A).

To further characterize the Th1 response, the frequency of IFN-γ secreting cells was also assessed in splenocytes of individual mice (Figure 1B). Although there was a trend in the group CR3_IMS 4112 to elicit a lower frequency of CR3-specific IFN-γ secreting cells compared to the group CR3+C+S (3 832 *vs* 6 905 SFC/$10^6$ splenocytes, respectively), this difference did not achieve statistical significance (p=0.1143; two-tailed Mann-Whitney test). In any case, this result was in line with previous results of IFN-γ secretion.

### Only IMS 4112 adjuvant induce CR3 (HIV)-specific IgG response in vagina after nasal inoculation

It is documented that antigen delivery by the intranasal route induces antigen-specific response at distal mucosal sites. Our group has previously reported that intranasal inoculation with CR3+C+S (TERAVAC) promote anti-C and anti-S, but not a detectable anti-CR3 IgA response in vaginal washes [23]. Here, we analyzed the effect of IMS 4112 on the anti-CR3 (HIV) IgA and IgG response in this organ ten days after the last immunization. Figure 2A shows that approximately 44% of mice immunized with CR3_IMS 4112 were positive for IgG secretion. This response was no detectable in the remaining groups. Furthermore, neither IMS 4112, nor the VLP of HBV could induce a detectable anti-CR3 (HIV) specific IgA response in vagina (Figure 2A). In the systemic compartment, after i.n immunization, none of the experimental groups achieved 100% IgG seroconvertion rate in sera and no statistical differences between CR3+C+S and CR3_IMS 4112 were observed (p=0.4267; two-tailed Mann-Whitney test) (Figure 2B).

### Similar Th1 adjuvant effect of IMS 4112 and VLP of HBV after subcutaneous administration

To further evaluate the adjuvant effect after parenteral immunization, we immunized similar experimental groups



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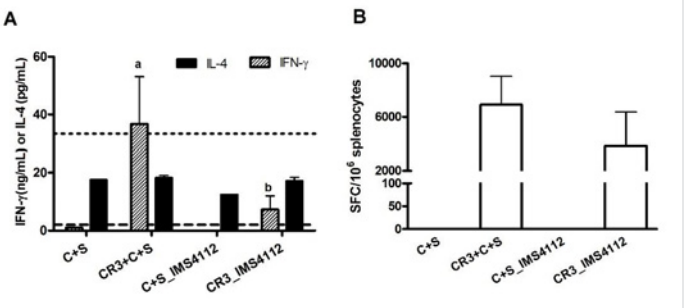


**Figure 1:** CR3 (HIV)-specific IFN-γ and IL-4 response induced by intranasal antigen delivery. Ten days after the fifth immunization the spleens of five mice per group were aseptically removed and cultured individually during 5 days with 2.5 µg/mL of CR3. IFN-γ and IL-4 secretion in culture supernatant was determined by ELISA (A) and the frequency of IFN-γ secreting cells was evaluated after re-stimulation by ELISPOT (B). Data represents mean + SD. The dotted (...) and dashed (---) lines represent the cut off value for IL-4 and IFN-γ, respectively. Different letters indicate statistical significance (p=0.0286; two-tailed Mann-Whitney test).

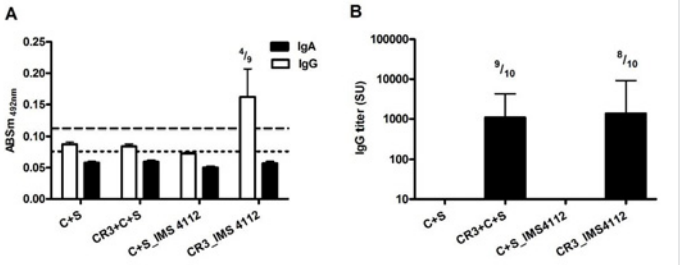


**Figure 2:** Anti-CR3 (HIV) antibody responses after intranasal antigen delivery. Ten days after the fifth dose, vaginal washes and sera were collected. Mean ABS $_{492nm}$ + SD of tested animals represent the CR3 (HIV)-specific IgA and IgG antibody levels in vagina (A). Serum anti-CR3 (HIV) IgG titers were also determined. Data represent geometric mean + 95 % CI (B). The dotted (...) and dashed (—) lines represent the cut off value for IgA and IgG, respectively. The seroconvertion frequency is indicated above the bars.

through the subcutaneous route (Table 1; Schedule #2). In these experiments the multiantigenic formulation CR3+C+S (TERAVAC) was also adjuvated in AlOOH as previously reported [25]. At the end of the immunizations, we measured the CR3 (HIV)-specific cellular immune response.

After three doses, the level of IFN-γ secretion in culture supernatants of CR3-stimulated splenocytes as well as the frequency of positive response was similar among experimental groups (p=0.2403; two-tailed Mann-Whitney test) (Figure 3A). Regarding the frequency of IFN-γ secreting cells measured by ELISPOT after re-stimulation, we performed a meta-analysis of two independent experiments. Figure 3B shows similar frequency of CR3 (HIV)-specific IFN-γ-secreting cells among groups immunized with CR3+C+S_AlOOH or CR3_IMS 4112 (1 130 vs 754 SFC/$10^6$ splenocytes, respectively; p=0.7173; two-tailed Mann-Whitney test) (Figure 3B). To additionally characterize the immune response, we examined the CR3 (HIV)-specific $IgG_1$ and $IgG_{2a}$ antibody production in sera of mice as a useful parameter to discriminate Th1-Th2 profiles [27,30]. A predominant $IgG_1$ response was found in all animal groups regardless of the immunogen. A lower $IgG_{2a}$ secretion was also detected. No statistical differences were found between the CR3-IMS 4112 and the CR3+C+S_AlOOH group in the antibody response ($IgG_1$ or $IgG_{2a}$) (Figure 3C).

Additionally, we determined the anti-CR3 (HIV) whole IgG titer in mice sera. A 100% seroconvertion and higher IgG geometric mean titers (8 801 SU) were achieved in the CR3+C+S_AlOOH group. The 90% of CR3_IMS 4112 immunized mice achieved a geometric mean titer of 423 SU, suggesting a better immunoenhancing effect of VLP of HBV on the overall CR3 (HIV)-specific IgG response after subcutaneous administration (p=0.0003; two-tailed unpaired t test with Welch's correction) (Figure 4).

## Subcutaneous/intranasal co-administration of the CR3 protein with IMS 4112 and HBV antigens in AlOOH

Mucosal and parenteral co-administration usually enhances the response against a given antigen in comparison with single route schedules [16,31]. According to those reports, we decided to compare the adjuvant effect of hepatitis B VLP and IMS 4112 on the anti-CR3 (HIV) immune response using such schedules as well as the combined effect of both adjuvants (Table 1; Schedule #3). Thus, a group of mice was inoculated with HBV VLP subcutaneously and IMS 4112 by the nasal route. In this case adjuvants were selected considering previous results where HBV VLP provided a similar Th1 adjuvant effect to IMS 4112 but higher IgG levels in sera via s.c. and for the intranasal inoculation the IMS 4112 adjuvant because of the induction of IgG antibodies in vagina.

After five immunizations, only the CR3+C+S_AlOOH / CR3+C+S group showed detectable levels of anti-CR3 (HIV) IFN-γ secreting cells in mice spleen measured by ELISPOT assay. An additional sixth dose was required to induce a similar response when CR3 was adjuvated with IMS 4112 or when the combination of adjuvants was tested in the CR3+C+S_AlOOH/ CR3_IMS 4112 group (Figure 5). In accordance with the above mentioned results, a detectable proliferative response of 16.5% $CD4^+$ and 19.6% $CD8^+$ T lymphocytes was only achieved in CR3+C+S_AlOOH/CR3+C+S immunized animals after five doses (Figure 6).

To further characterize the adjuvant effect on the humoral response, we analyzed the whole anti-CR3 (HIV) IgG titers and subclasses in animal's sera ten days after the fifth and the sixth dose (Figure 7). Overall, a predominant $IgG_1$ over an $IgG_{2a}$ response was detected and no statistical differences were found among groups in relation with IgG subclasses or even doses (Figure 7A,B). Additionally, no statistical differences were observed in whole IgG titers among groups after the 5$^{th}$ (p=0.1070, one-way ANOVA) or the 6$^{th}$ dose (p=0.9189, one-way ANOVA) (Figure 7C).

Humoral CR3 (HIV)-specific IgA and IgG response were also assessed in vagina after the fifth and the sixth dose. Levels of IgA were undetectable in all the experimental groups (data not shown). A low IgG response and similar frequency of responder mice per group were observed without any significant statistical



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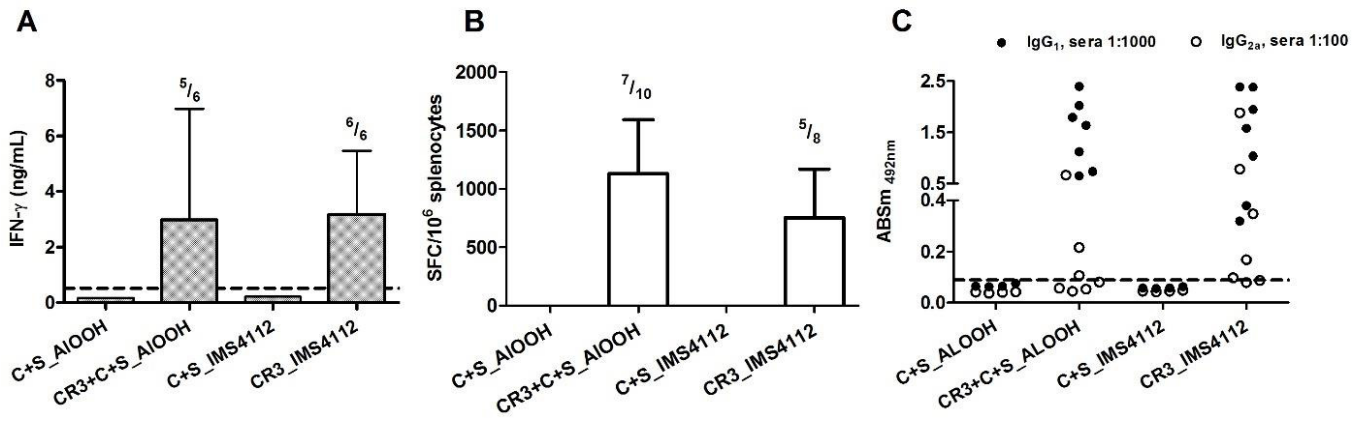


**Figure 3:** Th1 response after subcutaneous delivery of different antigen formulations. Ten days after the 3rd immunization mice spleens were aseptically removed and cultured individually during 5 days with 2.5 µg/mL of CR3. IFN-γ secretion in culture supernatant was determined by ELISA (A) and the frequency of IFN-γ secreting cells was evaluated after re-stimulation by ELISPOT (B). Both experiments are represented as mean + SD. The number of positive response is indicated above the bar. Anti-CR3 (HIV) IgG subclass pattern was analyzed in 7 mice per group at fixed serum dilution of 1:1 000 for $IgG_1$ and 1:100 for $IgG_{2a}$ (C). The dashed line represents the cut off values.

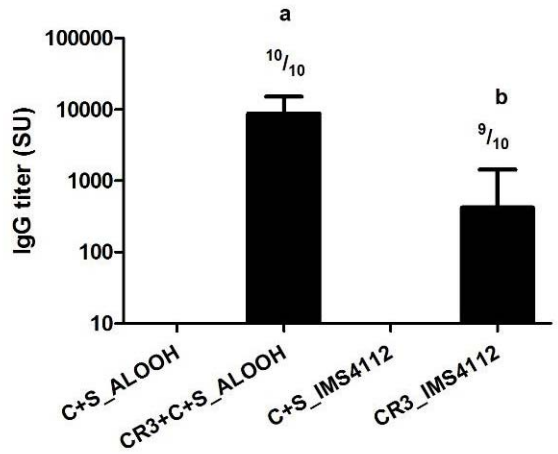


**Figure 4:** Whole anti-CR3 (HIV) IgG titer in mice sera 10 days after the 3rd dose of subcutaneous immunization. Data represents geometric mean with 95 % CI from 10 mice per group. The frequency of positive results is indicated above the bars. Letters are associated with statistical differences (p=0.0003; two-tailed unpaired t test with Welch's correction).

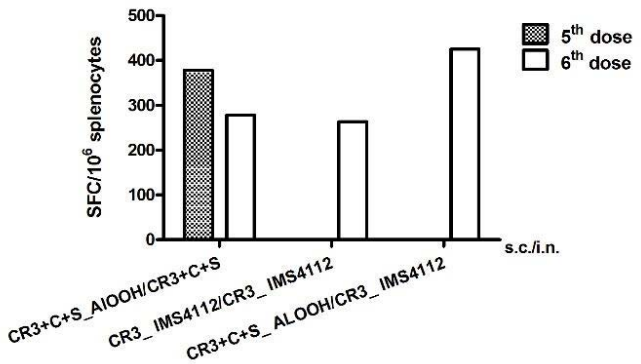


**Figure 5:** Frequency of CR3 (HIV)-specific IFN-γ secreting cells induced by the s.c./i.n coadministration regime. Ten days after 5th and 6th immunizations, splenocytes from 3 mice/group were stimulated *ex vivo* with 2.5 µg/mL of CR3 during 48 hours. Bars represent the frequency of IFN-γ secreting cells quantified in pooled samples.

differences after the 5th (p=0.9081, one-way ANOVA) or the 6th dose (p=0.6030, one-way ANOVA) (Figure 7D).

## Discussion

The purpose of this work was to compare the adjuvant effect of IMS 4112 versus the VLP of HBV after mucosal, parenteral and mucosal/parenteral immunizations of the recombinant multiantigenic protein CR3 of the HIV-1.

Previous studies demonstrate the Th1 adjuvant effect of the mixture of HBcAg and HBsAg on the CR3 (HIV)-specific cellular and humoral immune response after i.n. [23], s.c. [25] and even s.c./i.n. coadministration [24]. In this regard, the experimental evidence pointed out to the nucleic acid (double-stranded RNA, dsRNA) content inside the HBcAg as the primary cause [32]. Additionally, the nanoparticle size of both VLP of HBV, their spontaneous aggregation with CR3 [33] and the lipids content of HBsAg from the *P. pastoris* host [34], may all together further contribute to the adjuvant effect and effective nasal immunization.

During the last decade, SEPPIC developed a new generation of adjuvants registered under the name of Montanide® IMS, which comprise a range of ready-to-dilute oil-in-water micro-emulsions. These adjuvants are dispersions of liquid particles, varying in size between 50 to 500 nm, in an aqueous phase containing an immunostimulating compound listed as GRAS substances. One of them, IMS 3012, has proved its advantage for a veterinary vaccine against *Rhodococcus equi*, inducing $IgG_{2b}$ subclass prevalence and the IFN-γ, IL-2 and IL-10 mRNA expression in vaccinated mares [35]. In a different set of experiments, IMS 3012 was the adjuvant of choice for hyperimmunization of equines for the production of polyvalent snake antivenom sera. In that experiment, 100% of horses in IMS 3012 immunized group responded against different snake toxins and showed minimum local reactions at injection site, demonstrating the safety of this adjuvant in veterinary vaccines [22]. Another group of researchers have studied the



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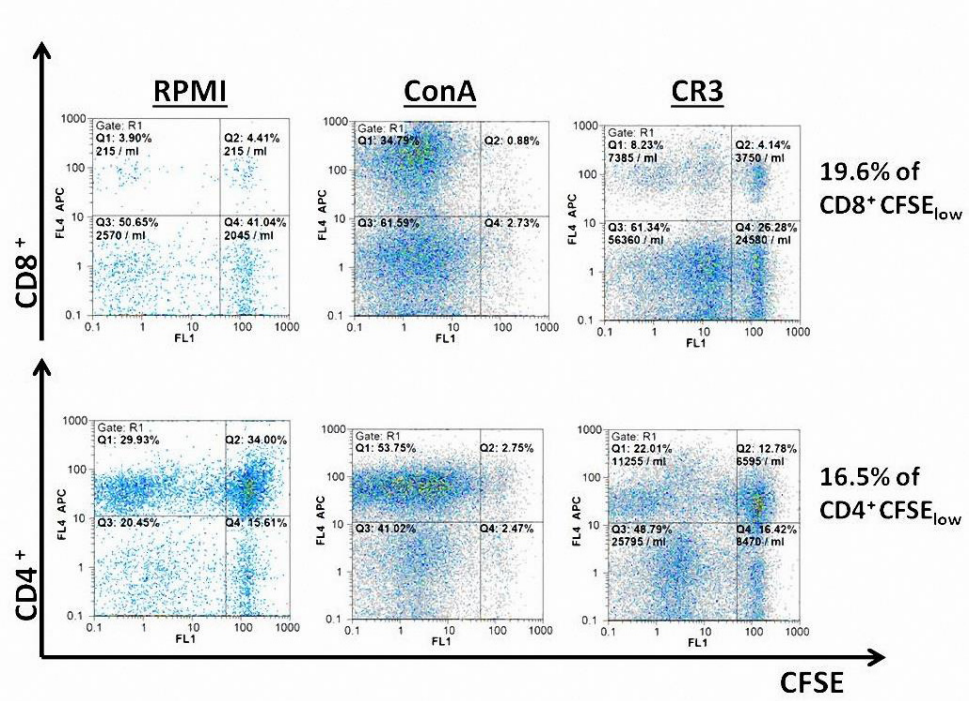


**Figure 6:** CR3 (HIV)-specific T cell proliferation induced after CR3+C+S_AlOOH/CR3+C+S s.c./i.n. coadministration. Ten days after the fifth dose, the splenocytes from three mice drawn randomly were isolated, stained with CFSE and re-stimulated *ex vivo* with CR3, concanavalin A (Con A) or complete medium (RPMI) during 5 days. Then, $CD4^+$ and $CD8^+$ lymphocyte labeling was performed in order to quantify proliferation by flow cytometry. One-hundred thousand viable lymphocytes including blasts (gate R1) were gated for the analyses and represented as dot-plot of $CD4^+$APC or $CD8^+$APC *vs* CFSE. For each experimental condition we first calculated the percent of $CD8^+$ or $CD4^+$ T cells that divided after *ex vivo* stimulation (Q1) relative to the bulk of $CD8^+$ or $CD4^+$ T cells gated (Q2+Q1). Then, the percent in RPMI medium was subtracted from the CR3 stimulation to obtain the CR3 (HIV)-specific proliferation (data at the right). In the negative control group C+S_AlOOH/C+S s.c./i.n. background values of 0% and 6.28% were observed for $CD8^+CFSE_{low}$ and $CD4^+CFSE_{low}$, respectively.

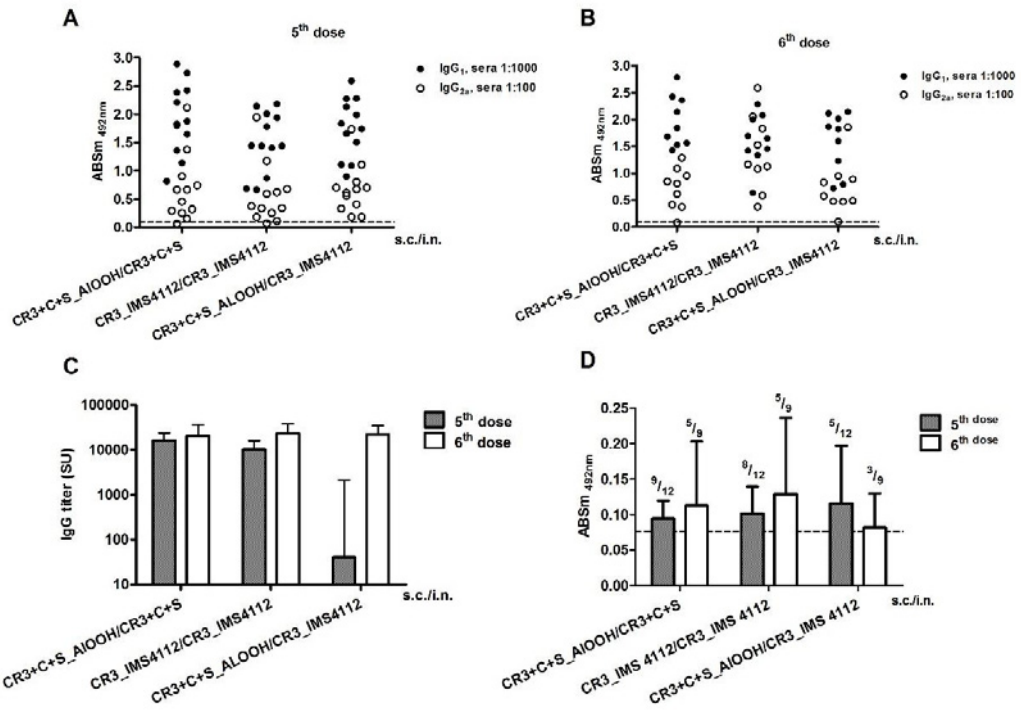


**Figure 7:** Anti-CR3 (HIV) antibody response in sera and vagina after the 5th and the 6th dose of s.c/i.n. administration. Twelve and nine mice were screened ten days after the 5th and the 6th dose, respectiely. Individual $IgG_1$ (•) and $IgG_{2a}$ (0) mean $ABS_{492\ nm}$ were assessed after the 5th (A) and the 6th dose (B) at fixed serum dilution (1:1 000 for $IgG_1$ and 1:100 for $IgG_{2a}$). Total IgG titers are represented as geometric mean with 95% CI (C). The level of CR3 (HIV)-specific IgG response was assessed in vaginal washes. Frequency of responders is indicated above the bar (D). The dashed line represents the cut off value. No statistical differences were observed neither between doses nor between groups.



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immunogenicity of a multi-allele and multi-antigenic vaccine against malaria using IMS 4112 as adjuvant. Their results showed the lowest serum IgG titers in rabbits immunized in the presence of IMS 4112 in comparison with other three experimental adjuvants. This was accompanied by the lowest inhibition of *P. falciparum* growth by the antibodies elicited in this group [36].

The results obtained in the present work showed a better Th1 adjuvant effect of the VLP of HBV than IMS 4112 after intranasal inoculation. It was evidenced by a significant higher IFN-γ secretion of splenocytes after *ex vivo* restimulation and a trend to a higher frequency of IFN-γ-secreting cells. However, IMS 4112 seems to be a better inducer of IgG response in vagina. IgA antibodies were not detectable in this organ. There are several explanations for that result. First of all, it is known that IgG antibody production is usually higher than IgA in this organ [37]. Additionally, it is also important to consider that samples of vaginal washes were collected in 0.1 mL and further diluted 1:10 for evaluation and such dilution factor might decrease the IgA concentration below the threshold value of detection of the ELISA assay. It is noteworthy that immunization of TERAVAC stimulates the production of gp120-specific IgG [23,38]. While the physiological significance of such IgG antibodies awaits further analysis, one can speculate that such Ig in the vaginal mucosa might contribute at some level of protection from infection as previously suggested by some authors [13,14]. A potential role of such IgG might be to trap HIV viral particles in the mucus as reported for herpes simplex virus serotype 1 (HSV-1) [39] and HSV-2 [39,40]. On the other hand, after intranasal immunization both adjuvants, IMS 4112 and the VLP of HBV, induced a similar level of IgG production and seroconvertion in the systemic compartment.

In contrast, after parenteral immunization (via s.c) we did not observed any statistical significant differences in relation to the secretion of IFN-γ in culture supernatant, the frequency of IFN-γ secreting cell or the production of $IgG_{2a}$ antibodies in serum. All these parameters suggest a similar Th1 adjuvant effect in the systemic compartment. But, it is worth of remark that HBV-VLP per se are not able to induce a detectable frequency of IFN-γ secreting cell in the spleen of mice after several doses and AlOOH must be added to achieved a good response (E. Iglesias, unpublished results). Considering that fact, it is possible to speculate that IMS 4112 would have a better Th1 adjuvant effect after parenteral immunization than the mixture of HBV-VLP per se without AlOOH. The combined adjuvant effect of AlOOH and the HBV-VLP explain the significant higher whole IgG titers observed in sera of immunized animals compared to IMS 4112. Previous studies have shown that AlOOH do not stimulate any known Toll-like receptor (TLR) [41,42]. It is well-known that the adjuvant effect of AlOOH is mediated by slow release of the antigens (the so call "depot effect"), better uptake by antigen presenting cells (APC) and Nod-like receptor pyrin containing 3 (NLRP3) inflammasome-dependent and independent signaling pathways [43]. In this regard, AlOOH does not induce a strong commitment of the immune system to either a Th1 or Th2 pathway but it enhances the Th1 effect provided by the HBV VLP in TERAVAC [25].

The results obtained with the subcutaneous immunization seem contradictory with those obtained after intranasal inoculation when a better Th1 effect was reported for the HBV-VLP. Nevertheless, it might be influenced by qualitative/quantitative expression pattern of TLRs between the nasal mucosa and the systemic compartment and at the cellular level depending also on the differentiation stage of APCs [44] among other parameters still under investigation. For instance, a recent report evidenced different outcomes of the immune response elicited by dendritic cells (DC) when a specific TLR stimulation is produced or not at the same time with another TLR on the same APC [45]. In that sense, the immunological outcome promoted by a particular adjuvant formulation (that may stimulate an array of TLRs) will be the result of a complex mesh of interactions influenced by the anatomical localization and cellular state of differentiation. Then, all these features of the innate immunity could have played a role.

Although there are few reports on this subject, it is clear that TLR3 expression in nasal epithelial cells is abundant in humans and mice [46-48]. Additionally, as Asahi-Ozaki and coworkers have shown, intranasal administration in mice of the synthetic dsRNA, poly (I:C) stimulates the up-regulation of TLR3 and TLR7 in the nasal-associated lymphoid tissues (NALT) which in turn promotes activation of the NF-kB pathway and production of type I IFNs [49] which eventually leads to a strong immune response. They did not find a similar stimulation effect in the spleen. This differential up-regulation of TLR3 and 7 between the mucosal and systemic compartment after i.n. administration might explain why the HBcAg (i.e. the mixture of HBV VLP) induced a better Th1 immunodeviation than IMS 4112 after intranasal administration with a similar level of IgG production in sera. Unfortunately, the chemical composition of IMS 4112 has not been disclosed and because of that our analysis are very limited. Additionally, concentration of use of IMS 4112 was arbitrarily fixed at 10% v/v and we consider that a dose escalation of the adjuvant could have also brought further elements for analysis.

When considering the results obtained in the schedule for combined parenteral/mucosal coadministration, it is not possible to conclude superiority of some group over any other. A possible explanation for the fact that only in the CR3+C+S_AlOOH/CR3+C+S was possible to detect anti-CR3 (HIV) IFN-γ-secreting cells and proliferation of CD4+ and CD8+ lymphocytes when whole IgG titers, $IgG_1$, $IgG_{2a}$ in sera or vaginal washes were not different among the groups, might be the low sensitivity of the assays. To reconcile such discrepancy, it is possible to speculate that frequency of CR3 (HIV) IFN-γ-secreting cells and proliferation of $CD4^+$ and $CD8^+$ lymphocytes in the group of mice immunized with IMS 4112 s.c./i.n. and HBV VLP in alum via s.c. and with IMS 4112 i.n. were near but below the threshold of detection of the assays and the values reported in the group of HBV-VLP (CR3+C+S_AlOOH/CR3+C+S) were above but near the same threshold. In any case, these results suggest a stronger adjuvant effect of the coadministration of VLP than any other combination tested. It is worth of remark that VLP for subcutaneous immunization were formulated with AlOOH.



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## Conclusion

In summary, our results suggest that HBV-VLP induce a better Th1 adjuvant effect than IMS 4112 at the concentration of 10% v/v after nasal immunization although the last adjuvant seems to be a better inducer of IgG in vagina. In the parenteral inoculation the adjuvant effect of IMS4112 and HBV VLP formulated with AlOOH behave in a very similar way. When using schedules of nasal and subcutaneous coadministration it seems that less inoculation doses are needed to achieve a detectable Th1 response with HBV-VLP. Taken together, these results suggest that HBV VLP are a better adjuvant for the recombinant CR3 protein of HIV-1 than the IMS 4112.

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