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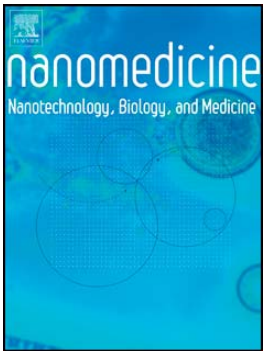

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ABSTRACT

Gold nanoparticles (GNPs) decorated with glycans ameliorate dendritic cells (DC) uptake, antigen-presentation and T-cells cross-talk, which are important aspects in vaccine design. GNPs allow for high antigen loading, DC targeting, lack of toxicity and are straightforward prepared and easy to handle. The present study aimed to assess the capacity of DC to process and present HIV-1-peptides loaded onto GNPs bearing high-mannoside-type oligosaccharides (P1@HM) to autologous T-cells from HIV-1 patients. The results showed that P1@HM increased HIV-specific $CD4^+$ and $CD8^+$ T-cell proliferation and induced highly functional cytokine secretion compared with HIV-peptides alone. P1@HM elicits a highly efficient secretion of pro-T_H1 cytokines and chemokines, a moderate production of pro-T_H2 and significant higher secretion of pro-inflammatory cytokines such as TNFα and IL-1β. Thus, co-delivery of HIV-1 antigens

and HM by GNPs is an excellent vaccine delivery system inducing HIV-specific cellular immune responses in HIV+ patients, being a promising approach to improve anti-HIV-1 vaccines.

KEYWORDS: Vaccine, gold nanoparticles (GNPs), HIV-1, CMV, high-mannoside-type oligosaccharides, cellular immunity.

Abbreviations

AIDS: acquired immune deficiency syndrome; CFSE: Carboxyfluorescein Diacetate Succinimidyl Ester; CMV: Cytomegalovirus; CTL: Citolytic T Lymphocyte; DC-SIGN: Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin; EDTA: Ethylenediaminetetraacetic acid; HIV: Human immunodeficiency virus; HLA: Human leukocyte antigens; IL-: Interleukin-; MHC: Major histocompatibility complex; MVA: Modified vaccinia Ankara; OVA: Ovalbumin; PGE_2: Prostaglandin E_2; SEA: Staphylococcus aureus enterotoxin A; SIV: Simian immunodeficiency virus; T_H1 and T_H2: T helper 1 and 2; TF: Thomsen–Friedenreich antigen; HM: high-mannosides derivatives; P1: KKWK-SLYNTVATL (HIV Gag p17) peptide

1. Background

The present paradigm for an optimal HIV-1 vaccine is to develop immunogens and vaccination protocols that permit the stimulation of a robust mucosal and systemic immunity, comprising both helper and cytotoxic T-lymphocyte mediated responses, in order to act at infection sites [1]. Until now, preventive and therapeutic vaccines strategies have been investigated against HIV. Preventive HIV vaccines are designed to protect from acquisition of HIV-infection, while therapeutic HIV vaccines are developed to control and eliminate the virus in order to achieve a 'functional cure' in individuals who have previously been infected by HIV [2–5].

Dendritic cells (DC) are potent antigen presenting cells (APC) widely spread in the mucosal tissues and play a critical role in the initiation and control of protective immune responses against pathogens [6]. For this reason, targeting DC or, particularly, monocyte-derived dendritic cells (MDDCs) with selected antigens is a widely accepted strategy to induce and potentiate antigen-specific T-cell responses and an effective protective immunity [7–11]. The use of MDDCs is reinforced by recent findings that show how DC can be generated *in vivo* from monocytes in tissues under inflammatory conditions [12,13]. Accordingly, our group is testing at both clinical and non-clinical levels different therapeutic HIV-1 vaccines based on the use of MDDC and different immunogens such as heat-inactivated HIV-1 [8] and MVA vectors [14] with promising results.

Recent studies suggested that nanotechnology could play a pivotal role in HIV-1 therapeutics and vaccine design [5,7,10,15–21]. Nanoparticles have been proposed as new carriers to ameliorate DC antigen loading, which is important in DC-based vaccine

approaches [5,10]. Modified Gold nanorods promote cellular and humoral immunity, through activating APC compared to naked HIV envelope plasmid DNA treatment *in vivo* [22]. Gold nanoparticles decorated with antigenic carbohydrate ligands have been validated as potential prophylactic vaccine candidates against *Streptococcus pnemoniae* [23,24] and *Burkolderia mallei* [25]. Brilliant examples related to GNPs loaded with multiples copies of tumor associated TF carbohydrate antigen have been reported as potential anti-cancer vaccines [26,27]. The success of these nanoplatforms stems from their dispersion in water, resistance to enzymatic degradations, biocompatibility and ability to incorporate different ligands [28]. Current knowledge suggests that a primary vaccination (inducing humoral and CD4+ T-cell immune responses) combined by a subsequent boost with agents inducing CD8+ T- cell immune responses provides the most efficient response with the elicitation of both cellular and humoral immune responses [29,30].

The C-type lectin called Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) constitute a major receptor for HIV-1 in dendritic cells [31]. DC-SIGN recognizes *N*-linked high-mannose glycan clusters on HIV gp120 [32,33]. Consequently, mimicking the cluster presentation of oligomannosides from the virus surface is a strategic approach not only for carbohydrate-based antiviral compounds [34] but also for targeting antigens to DC [35]. Glycan-coated dendrimers and liposomes loading ovalbumin (OVA) peptides have been described to enhance antigen presentation in human DC. It has been shown that modification of liposome and dendrimers modification with the DC-SIGN-binding glycans induce efficient MHC Class I and MHC Class II presentation to CD8+ and CD4+ T cells, respectively [36]. DC-SIGN targeting strategies have also been studied in the design of anticancer vaccines [37]. In the field of HIV, gold nanoparticles presenting multiple copies of structural motifs of

the *N*-linked high-mannose glycan (manno GNPs) are effective inhibitors of DC-SIGN-mediated trans-infection of human T cells [38]. Furthermore, manno GNPs are captured by immature MDDC without inducing DC maturation in a DC-SIGN-dependent and -independent manner and part of them are co-localized with DC-SIGN in early endosomes [39].

HIV-peptides corresponding to the sequence of HIV Gag epitopes have been previously described as highly immunogenic and able of inducing citolytic T lymphocyte (CTL) response [40,41]. However, soluble peptides alone may not be an efficient platform for HIV vaccines because they are not as immunogenic as those which are co-delivered with other vehicles or adjuvants [40]. Multiple copies of these antigens on the surface of nanoparticles could induce robust immune responses.

In this context, we hypothesized that GNPs bearing both high-mannosides (HM) derivatives and selected HIV peptides potentiated with pro-inflammatory cytokines could be an improved design to enhance antigen capture by stimulating DC presentation able to break the immunotolerance promoted by high-mannose type glycans [42]. Such engineered compounds could induce effective HIV specific T cell immune responses. Due to that neither animal model perfectly recapitulates HIV infection in humans [43], a good approach to validate such engineered compounds as prophylactic or therapeutic vaccines is to work *ex vivo* with freshly isolated human MDDCs and human T cells from HIV-infected patients, which represent the natural targets cells of HIV infection [43]. This *ex vivo* model is considered a more realistic scenario to support the efficacy of therapeutic vaccines (where HIV could have influenced on MDDC biology), in comparison with other models using MDDC from healthy volunteers [44,45].We therefore assessed *ex vivo* the interaction of GNPs bearing a dimannoside conjugate and peptides

with MDDCs of HIV-1 infected patients and the capacity of MDDC to process and present the HIV-1 antigens to autologous human T-cells.

2. Material and methods

2.1 Ethics statement

This study received the approval of the Committee of Ethics and Clinical Investigation of the Hospital Clinic Universitari (Barcelona, Spain). All the subjects participating in the study were recruited at the Service of Infectious Diseases & AIDS Unit of this Hospital and gave their informed written consent.

2.2 Study individuals

Samples of EDTA-anticoagulated venous blood samples were obtained from 9 chronic asymptomatic HIV-1-infected patients HLA A*0201 positives with baseline $CD4^+$ T cell counts >450 cells/mm^3, and plasma viral load <50 HIV-1 RNA copies/ml who were on antiretroviral therapy.

2.3 Reagents

Peptides of HIV and CMV epitopes of Gagp17 and pp65 proteins respectively were supplied by Gene Script. Peptide sequences are: KKWK-SLYNTVATL (p17 $_{77\text{-}85}$ HLA A*0201) and KKWK-NLVPMVATV (CMV pp65 $_{495\text{-}503}$ HLA A*0201). All chemicals were purchased as reagent grade from Sigma-Aldrich unless otherwise stated and were used without further purification (Supplementary methods).

Preparation of dimannose and glucose GNPs

For the preparation of HIV Gag p17 and CMV pp65 functionalized GNPs, gold glyconanoparticles carrying approximately 50% glucose or 50% dimannose (Manα1-2Man) and 50% of a carboxylated linker were prepared by reduction in situ of Au(III) salt with sodium borohydride in the presence of the ligands, as described [34,46–48].

Glucose, dimannose and carboxylated linker were derivatised with thiol-terminated linkers as previously reported [34] for binding onto gold nanoparticles core (Figure 2). 5-Mercaptopentyl-D-glucopyranoside ($GlcC_5SH$), dimannoside and carboxylic acid-containing ligand were used as thiol-ending ligands and prepared as previously reported [34].

Briefly, an aqueous solution of tetrachloroauric (Strem Chemicals) (0.025 M, 1 eq.) was added to a solution of a mixture of appropriate thiol ending ligands (0.012 M, 6 eq.) in $MeOH/H_2O/CH_3COOH$ (3:3:1). An aqueous solution of $NaBH_4$ (1 M, 22 eq.) was then slowly added and the mixture was stirred for 2 hours at 25 ºC. The solvent was subsequently evaporated at reduced pressure and the residue was washed with ethanol, re-dissolved in the minimum quantity of milliQ water, loaded into 5-10 cm segments of SnakeSkin® pleated dialysis tubing (Pierce, 3500 MWCO) and purified by dialysis against distilled water (3 l of water, recharging with fresh water every 6 hours over the course of 72 hours). The nanoparticles were obtained as brown powder after lyophilization (Supplementary methods).

2.5 Preparation of co-formulated GNPs including HIV-1- or CMV-peptides and dimannose

For the preparation of HIV Gag p17 and CMV pp65 functionalized GNPs, the previously prepared GNPs (GNP1 and GNP2) were coupled to KKWK tagged HIV Gag

p17 (P1) and CMV pp65 (P2) peptides (Gene Script) through a peptidic bond between the carboxylic group of the GNP1 and GNP2 and the amine group of the lysine present in the tagged peptide. 1-Ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 100 µl, 19 mg/ml) and sulfo-*N*-hydroxysuccinimide (sulfo-NHS, 100 µl, 43 mg/ml) were added to 60 µl of GNPs solution (sodium borate buffer, pH=9.2; 0.01 M ClNa at 4 mg/ml). The mixtures were left under shaking 1 h and a solution of P1 and P2 peptides (415 µl, 2 mg/ml in borate buffer) was added. Coupling reactions were left under shaking 24 h at RT. HIV Gag p17 (P1@glc and P1@HM) and CMV pp65 (P1@glc and P2@HM) GNPs were filtered and washed with HCl (5 mM) and H_2O on Amicon filters (10 KDa MWCO) until no peptide was detected by Bradford analysis and were finally dialyzed (Snakeskin, cut-off 3000 Dalton).

2.6 Characterization of co-formulated GNPs

After coupling, the presence of HIV-Gag p17 and CMV pp65 onto the GNPs were assessed by ^{1}H-NMR. GNPs were also characterized by transmission electron microscopy (TEM) to determinate the average gold diameter. 2.7 Generation of MDDCs

The immunophenotype of immature MDDCs (imMDDCs) derived from 9 HIV-1 individuals using a well-established method [14] using IL-4 and GM-CSF both at a concentration of 1000 IU/ml was: CD86+, CD11c+, CD40+ and HLA-DR+. The purity of imMDDCs obtained was higher than 95% (Supplementary methods).

2.8 Pulsing of MDDCs with GNP's and maturation induction

MDDCs were incubated for 48 h with GNP compounds containing 10 μg/ml of each peptide or with Gag p17 HIV or CMV pp65 peptides alone at 10 μg/ml each or with the HIV p24 antigen at 5 μg/ml and SEA as positive control of activation. To obtain mature MDDCs (mMDDCs), a maturation cocktail of recombinant human cytokines containing TNF-α, IL-6 (1000 IU/ml each, Strathmann Biotec AG), IL-1β (300 UI/ml, Strathmann Biotec AG) and PGE_2 (1 μg/ml, Pfizer, Madrid, Spain) was added at 2 h post-pulsing, and the mixture was incubated for a whole period of 48 h. Where indicated, pulsed MDDCs were not treated with the maturation cocktail.

2.9 Co-cultures of pulsed MDDCs with autologous lymphocytes: proliferation.

As a source of enriched T cells, we used 9 samples of fresh PBMCs depleted of monocytes after plastic adherence, as indicated above for the generation of MDDCs. These monocyte-depleted lymphocytes were washed (3×) and re-suspended in serum-free XVIVO-10 medium and labelled with CFSE in order to analyse proliferation following the instructions of the manufacturer (CellTrace CFSE cell proliferation kit, Molecular Probes, Paisley, UK). Autologous matured MDDCs (6×10^4 MDDC/well) were washed (4×) and re-suspended in XVIVO-10 and co-cultured with autologous fresh CFSE-labelled lymphocytes (2×10^5 T-lymphocytes/well) in a final volume of 200 μL in XVIVO-10 medium supplemented with 1 mM zidovudine to avoid possible replication of endogenous HIV-1. The contribution of MDDCs and monocyte depleted PBMCs (lymphocytes) alone were determined as negative controls of proliferation.

The co-cultures were done in triplicates at 37 °C in a humidified atmosphere with 5% CO_2. After 6–7 days, proliferating $CD4^+$ ($CD3^+$ $CD8^-$) and $CD8^+$ ($CD3^+$ $CD8^+$)

T cells were determined by direct staining with monoclonal Abs (mAbs) conjugated with α-CD3-Per-CP and α-CD8-PE. Mouse immunoglobulin (Ig) isotypes mAbs (from BD Biosciences) of unknown antigen specificity conjugated with PerCP or PE were used as negative control mAbs. The stained cells were analysed on a FACS Calibur flow cytometer (BD Biosciences). T cell populations were selected by forward and side light-scatter parameters and sub-gated for CD8- or CD8+ expression. Cells that proliferated after the co-culture had lower intensity of CFSE ($CFSE^{low}$) in comparison with basal conditions. Proliferation was expressed as the percentage of $CFSE^{low}$ cells after 6 days of co-culture.

2.10 Co-cultures of pulsed MDDCs with autologous lymphocytes: cytokine expression

In addition, the co-culture supernatants were collected after 6 days and stored at −80 °C for further cytokine quantification of 25 cytokines[14] (supplementary information).

2.11 Statistical analysis

Data were analysed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Comparisons between two groups were performed by paired parametric test (Student's *t*-tests) depending on normal distribution (Kolmogorov–Smirnov normality test). Non-parametric paired values were analysed by Wilcoxon signed rank test. For unpaired or paired non-parametric multiple comparisons Kruskal–Wallis test or Friedman's test both followed by Dunn's post-test was used, respectively. For all tests used, two sided *p*-values <0.05 were considered to indicate statistical significance.

3. Results

3.1 Preparation of GNPs bearing the HIV Gag p17 and CMV pp65 immunogenic peptides and mannosides or glucosides

In this work we have loaded gold nanoparticles (GNPs) with HIV-peptides or CMV-peptides and mannosides and challenged them through *ex vivo* assays with monocyte derived dendritic cells (MDDCs) and autologous lymphocytes from HIV+-infected patients in search for specific T cell responses (Figure 1A).

GNPs compound were prepared using a well-established methodology. Briefly, KKWK tagged P1 and P2 HLA-A*0201-restricted peptides, from HIV and CMV, respectively (Figure 1B) were covalently linked to GNP1 and GNP2 by amide chemistry and were characterized by TEM (Figure 1C and Figure 1D).

Precursors GNPs (GNP1 and GNP2) were obtained by reduction of an aqueous solution (0.025 M, 1 eq.) of tetrachoroauric Au(III) with $NaBH_4$ in the presence of an excess of a mixture of the corresponding thiol-derivatized glucose or dimannose saccharides and carboxylic acid-containing molecules (Figure 2 and Figure S1). After purification by dialysis, the GNPs were characterized by ^{1}H NMR spectroscopy and transmission electron microscopy (TEM) (Figure S2-S4). The amino group of lysines located on the KKWK sequence react with gold nanoparticles (GNP1 and GNP2 in Figure 2 and Figure S5) previously activated with 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-sulfo-hydroxysuccinimide (sulfo-NHS). After coupling, all P1@glc, P1@HM, P2@glc and P2@HM GNPs were characterized by ^{1}HNMR to check the presence of HIV-Gagp17 and CMV pp65 peptides on GNPs (Figure S6-S9). The ^{1}H-NMR of conjugates show a broad signal between 0.7-0.9 ppm

indicating the presence of peptides on the GNP (C*H3* of leucine residues). Less intense signals related to the peptides were also detected around 3 ppm and between 6.8-7.4 ppm (aromatic region). Water solutions of GNPs bearing the HIV Gagp17 and CMV pp65 immunogenic peptides and mannosides were stable for weeks. In addition, TEM micrographs showed no aggregations and uniform particle size (Figure 1D, and Figure S10-12). The mean diameter of the GNPs was determined to be 2.3nm (SD = 0.7 nm) for HIV Gagp17@manno GNPs and 1.6 nm (SD = 0.6nm) for CMV pp65@manno GNPs (Figure S11). Soluble free peptides, GNPs (GNP1 and GNP2), P1@glc and P2@glc GNPs were synthesized as comparative compounds. Zeta potential measurements (Figure S13 and S14) and UV-Vis scan were also determined of all GNPs (Figure S15).

3.2 T-cell proliferation induced by autologous MDDC pulsed with GNPs bearing the HIV Gag p17 and CMV pp65 immunogenic peptides

To determine the capacity of GNPs carrying the HIV Gag p17 peptide (P1) or the CMV pp65 peptide (P2) specific for HLA-A*02 and mannosides (HM) with the addition or without adding a maturation cocktail of cytokines to promote antigen specific T cell proliferation, the interaction of GNPs with MDDCs from 9 HIV+ patients in triplicates and their potency to induce subsequent stimulation of autologous T cells was studied.

Analysis and results of T cell proliferation of a representative individual is shown when maturation cocktail was added after the pulsing of MDDC (Figure 3A). In terms of T cell proliferation, HIV Gagp17@manno GNPs (P1@HM) induced a

significant better $CD8^+$ T cells response than negative control or manno GNPs or soluble peptide P1 or P1@glc or even recombinant soluble HIV Gag p24 antigen, reaching statistical significance in all cases ($p<0.05$)(Figure 3B). Similarly, a better response was obtained by CMV pp65@manno GNPs (P2@HM), the compound bearing CMV peptide and mannosides, than the compounds without HM, such as the soluble peptide (P2) or the CMV pp65@glucose GNPs (P2@glc) ($p=0.0992$ and $p<0.05$, respectively, Figure 3B).

In addition, the proliferation of $CD4^+$ T cells using HIV Gagp17@manno GNPs (P1@HM) had a tendency ($p<0.1$) to be higher than the induced by manno GNPs (GNP2), soluble peptide P1 or soluble recombinant HIV Gag p24 antigen (Figure 3C). In accordance with these results, an increased proliferation was induced by CMV pp65@manno GNPs (P2@HM) than by the soluble peptide P2 ($p<0.05$, Figure 3C).

Furthermore, GNP2 without peptide coating was unable to increase either $CD8^+$ or $CD4^+$ T cell proliferation (Figure 3B-C).

Surprisingly, the proliferation induced by the P1@HM was higher than the induced by whole HIV Gag p24 antigen (Figure 3B-C). In contrast, no increase of proliferation was found when any maturation agent was added after the pulsing of MDDCs with P1@HM or P2@HM GNPs (Figure 3D-E).

3.3 HIV Gagp17@manno GNPs increased the secretion of pro-T_H1 cytokines and chemokines

To determine the capacity of GNPs carrying HM and the P1 or P2 to promote cytokine secretion, the interaction of each batch of P1@HM and P2@HM with MDDCs from HIV+ patients, after the addition of maturation cocktail was studied in the co-cultures from 9 HIV-1 patients in duplicates.

P1@HM significantly increased more than 3-folds the secretion of pro-T_H1 cytokines including IFN-γ, IL-2R, IL-12 and IL-15 comparing with the soluble peptides ($p<0.01$). Similarly, P2@HM also promote the mentioned T_H1 cytokines comparing with the soluble peptides, ($p<0.05$, $p<0.1$, $p<0.01$, and $p<0.01$, respectively, Figure 4A) and with P2@glc ($p<0.05$, $p<0.01$, $p<0.01$, and $p<0.01$, respectively, Figure 4A). The T_H1 chemokines MIP-1α (CCL3), MIP-1β (CCL4), CCL5 (RANTES), MIG and IP-10 also increase with P1@HM compared with the corresponding soluble peptides ($p<0.05$, Figure 4B).

Accordingly to the proliferation results, P1@HM also induced a higher T_H1 cytokine and chemokine profile (IL2-R, IL-12, MIP-1α (CCL4), $p<0.05$) than soluble recombinant HIV Gag p24 antigen, suggesting that a unique peptide bound to manno GNPs could have a better immunogenic capacity.

Likewise, P2@HM also promote the mentioned cytokines profile of P1@HM comparing with the soluble peptides ($p<0.05$, Figure 4B), but the highest secretion of MIG and IP-10 by P2@HM reaches only significance when compared to P2@glc ($p<0.01$, Figure 4 B).

3.4 HIV Gagp17@manno GNPs increased the secretion of some pro-T_H2 cytokines

The secreted pro-T_H2 cytokines were also quantified in the co-cultures of GNPs-pulsed-MDDC, after the addition of a maturation cocktail, with autologous T cells from HIV+ patients.

Both P1@HM and P2@HM significantly increased more than 2 folds the secretion of pro-T_H2 cytokines such as IL-13, IL-5 and Eotaxin, however no significant differences were found for two pro-T_H2 cytokines such as IL-4, MCP-1 and IL-6 compared with the peptide alone (Figure 5). Only a trend to a higher increase of IL-4

was found between P1@HM and the peptide alone (Figure 5). Concerning the P2@HM compared with P2@glc, a rise was found in IL-4, and IL-6 secretion ($p<0.01$). Nevertheless, both P1@HM **a**nd P2@HM induced higher levels of IL-4, MCP-1 and IL-6 than GNP2 ($p<0.01$, $p<0.05$ and $p<0.01$, respectively).

3.5 HIV Gagp17@manno GNPs increased the secretion of pro-inflammatory cytokines

The pro-inflammatory cytokine secretion was also quantified in the co-cultures of MDDCs, after the addition of a maturation cocktail, with autologous T cells from HIV+ patients.

The pro-inflammatory cytokines TNFα, IL-1β, IL-8, GM-CSF, and IL-17 also increase with P1@HM compared with the corresponding soluble peptides ($p<0.05$, Figure 6). In contrast, IL1-RA, which is an anti-inflammatory protein, inhibiting IL-1β signalling, was decreased not only by P1@HM but also by any treatment compared to the negative control ($p<0.01$, Figure 6).

No significant differences were found between the secretion of IFN-α, IL-7 and IL-10, which is associated with suppressive responses, induced by P1@HM compounds compared with the controls (data not shown).

Likewise, CMV pp65@manno GNPs (P2@HM) also promote pro-inflammatory cytokines comparing with the soluble peptides P2 ($p<0.05$, Figure 6). Furthermore, GNP2, without any peptide coating, were able to enhance IL-12, IL-15 ($p<0.01$ and $p<0.05$, respectively, Figure 4A), MIG and TNFα ($p<0.01$, Figure 4B and $p=0.0578$, Figure 6, respectively). In contrast, the amount of pro-T_H2 cytokines such as IL-6 and MCP-1 (CCL2) and the anti-inflammatory cytokine IL1-RA were decreased with GNP2

compared with negative control ($p=0.0322$ and $p<0.005$ and $p<0.005$ respectively, Figure 5 and 6).

These results indicate the potency of the mannosides covalently linked to GNPs able to induce cytokines and chemokines as important immune factors that potentiate higher immune responses.

4. Discussion

We found that MDDCs pulsed with nanoparticles P1@HM or P2@HM exhibit enhanced capacities in stimulating virus antigen-specific CD8+ and CD4+ T-cell proliferation. These results suggest that co-delivery of high mannose ligands (HM) with the HIV peptides antigen coated on GNPs has an enhanced adjuvant effect, inducing strong T cell proliferative responses following *ex vivo* pulsing and after full maturation of autologous DC, whereas no addition of maturation agents or GNPs without P1 or P2 peptides fail to do so. The enhancement of T-cell proliferation by P1@HM or P2@HM is in agreement with the results of a previous work, where we observed an increased MDDC-capture of GNPs containing HM, which is mediated by both DC-SIGN-dependent and -independent pathways and suggesting that a higher antigen capture could induce higher inmunoresponses [39]. Although it appears that the stimulatory effect of P1@HM on CD4 T cell proliferation is less significant than that of CD8 T cell proliferation, in both cases the proliferation was higher than the p24 alone, suggesting that P1@HM had an adjuvant effect induced by HM incorporation to the GNPs. Vaccines based on HM conjugates are advantageous, because they may also target the C-type lectins (such as DC-SIGN) expressed by both macrophages and DCs and thus facilitate antigen capture and presentation [49–52]. Internalization of GNPs mediated by C-type lectin- like receptors in to DC, including DC residing in peripheral lymph nodes, promote antigen presentation to T cells both in an *ex vivo* and in an *in vivo* mouse model

and there is a good association between both *in vivo* experiments and in *ex vivo* DC–T cell co-cultures [53]. Such results suggest that targeting DCs by HM could be a good approach for targeting not only DC *ex vivo* but also DC *in vivo*. Alternatively, it is possible that GNPs, in addition of being captured by specific C type lectin receptors and/or macropinocytosis [52] they could trigger complement activation, not only mediated by MBL but also by the nonspecific adsorption of particular complement protein on GNPs contributing to the phagocytosis of GNPs by human APC. Consequently, GNPs could enhance innate immunity and cellular up-take by a complement dependent mechanism which could potentiate immunological responses [54].

In those second set of functional experiments with our *ex vivo* model, we also noted that P1@HM and P2@HM significantly increased the secretion of pro-T_H1 cytokines, including IFN-γ, IL-2R, IL-12 and IL-15, respect to free peptides. In addition, P2@HM significantly increased the secretion of pro-T_H1 cytokines compared to P2@glc. These results are consistent with the proliferation responses and suggested that the GNPs containing both HM and peptide induced a higher T_H1 immunoresponse suggesting that a higher antigen capture mediated by HM could induce higher inmunoresponses [39]. IL-12 secretion from APC potentiates the differentiation of T_H1 cells. We showed that all the compounds tested containing HM such as P1@HM, P2@HM and GNP2, after fully maturation, promoted high IL-12 secretion. In agreement with these results, alternative studies found that multifunctional NPs including both mannose and adjuvants have high cancer immunotherapeutic potential in terms of inducing an effective T_H1 immune response [55].

Moreover, it has been reported that HM alone are unable to induce maturation and secretion of pro-inflammatory cytokines by human MDDCs [42]. In accordance with

those results, no increase of proliferation was found when any maturation agent was added after the pulsing of MDDCs with P1@HM or P2@HM. As expected, maturating agents are required to process, present the antigens and maturate MDDC in order to activate proliferative T cell responses. These results suggest that a secondary signal such as the addition of a pro-T_H1 adjuvant as the maturation cocktail, which mimics a pro-inflammatory environment, is necessary to induce antigen presentation and full maturation. Consistent with our results, *in vivo* studies performed with compounds including mannose, antigen and adjuvants, demonstrated a superior pro-T_H1 immune response than strategies lacking both the adjuvant or the mannose compound [50,55].

Concerning the IL-15 up regulation, it has also been reported that IL-15 enhances effector memory $CD8^+$ T cells in primates immunized with a HIV-1 DNA vaccine, suggesting that this cytokine could be an adjuvant for HIV-1 vaccines [56]. The presence of HIV Gagp17@manno GNPs may produce the release of IL-15 that in turn could promote the expansion and survival of antigen-specific $CD8^+$ T cells [57].

In addition, HIV Gagp17@manno GNPs also significantly increased the T_H1 chemokine: the β-chemokines MIP-1α (CCL3), MIP-1β (CCL4) and RANTES (CCL5), but also MIG (CXCL9) and IP-10 (CXCL10). These chemokines are also known to induce migration of cells to both mucosal and systemic sites and specially attract DCs, monocytes, NK cells, and activated CD4+ and CD8+ T cells to sites of antigen processing to further mount the immune response [58–60]. The P1@HM-mediated increase on these β-chemokines is appropriate against HIV-1 infection since strong HIV-1 inhibition of R5 HIV-1 strains are related with these mediators [61]. Moreover, those chemokines contribute to initiate and expand antigen-specific mucosal immune responses through the activation of helper T cells, which enhance B cell responses and the function of APCs [62].

Furthermore, manno GNPs (HM) without any coated peptide, were able to enhance the secretion of pro-T_H1 mediators such as IL-12, IL-15, MIG and TNFα in the cocultures, suggesting that both HM coated on GNP and fully maturation, could polarize towards a T_H1 response mediated by IL-12 [55,63]. A more pronounced T_H1 versus T_H2 response is desired for successful control of HIV-1 infection [64]. Our findings suggest that P1@HM and P2@HM could play a role in polarization towards a T_H1 response, as suggested by the increased release of pro-inflammatory/T_H1 cytokines and chemokines observed in the presence of P1@HM.

Moreover, we found that HIV Gagp17@manno GNPs significantly increased the production of some pro-T_H2 cytokines such as IL-13, IL-5 [65] and Eotaxin [66], though a weak amount of IL-4 was detected. T_H2 cytokines are important in humoral immunity [67]. It has been reported that mannosylated compounds such as mannans induced a pattern of T_H1/ T_H2 cytokines including both IFN-γ and IL-12, but also with IL-13 and some degree of IL-4 secreted by murine dendritic cells [51]. IFN-γ, IL-2, and in a weak extent IL-4 and IL-5 were also produced by T cells in a murine model after immunization with OVA linked to HM compounds such as *O*-glycan oligomannoses when a potent adjuvant was added [50]. IL-10 is a T_H2 cytokine secreted by DC that promote immunosuppression and has been increased with HM compounds from the HIV-1 gp120 [42]. However, HIV Gagp17@manno GNPs under our conditions are unable to significantly increase IL-10, suggesting that P1@HM could not induce immunosuppression. This result coincides with our previous work where production of IL-10 was not enhanced by human MDDC exposed to manno GNPs [39] and results obtained by others sowing that HM compound such as mannans did not induce IL-10 secretion by murine DC [51].

It is noteworthy, that the amount of pro-T_H2 cytokines such as IL-6 and MCP-1 (CCL2) were decreased with manno GNPs alone compared to the negative control with medium alone. These results suggest that HM compounds on GNPs, when combined with a potent T_H1 activation agent which induce fully maturation of DC such as a pro-inflammatory environment are able to stimulate a T_H1 profile suitable, not only against HIV infection, but also against CMV.

Apart from T_H1 cytokines, our results indicated that P1@HM also increased the secretion of the proinflammatory cytokines TNF-α, IL-1β, GM-CSF, and IL-17, however, consistently reduced the anti-inflammatory cytokine IL1-RA that antagonized IL-1 function. These results are in agreement with reports by other authors, who found that HM related compounds such as mannans stimulated murine DC to express TNF-α, IL-1β, and GM-CSF [51]. Nevertheless, our previous results suggest that HM compounds alone were not able to induce human MDDC maturation [39]. For that reason and in order to bypass this inconvenient, the DC were fully maturated adding a pro-T_H1 maturation cocktail. As expected, addition of these maturating agents enhanced the co-stimulatory capacity of DC promoting DC maturation and a pro-inflammatory environment able to activate T cells. Accordingly, it has been reported that a high immune response was induced by immunization with OVA linked to oligomannose compounds when a potent pro T_H1-adjuvant named AbISCOH-100 was added [50]. Altogether, these results suggest that both a pro-inflammatory environment and a high capture of APC mediated by HM compounds are necessary to induce a potent antigen specific immune response.

Altogether, our results suggested that these formulations not only induce a T_H1 response but also promote a combined T_H1/T_H2 profile which could activate B cells enhancing the humoral arm of the immune system. We could speculate that our GNPs likely could induce not only cellular response but also antibodies against the loaded

antigens *in vivo*. In that scenario, the immunization with compounds containing HM epitopes and peptides could hypothetically induce not only a HIV-specific cellular immune response but also an antibody response, similar to that exerted by the broadly neutralizing 2G12 antibody [15,68] and could have an inherent capacity to block dendritic cell mediated HIV trans infection [38,69].

Summarizing, HIV Gagp17@manno GNPs formulation enhanced antigen presentation by DC, able to activate a highly functional CD4+ and CD8+ T cells immune responses. Those formulations based on multifunctional GNPs could promote multiple arms of the immune system inducing a high adjuvant effect in an HIV vaccine context.

In this study, GNPs were used as a vaccine delivery system after functionalization with mannosides and HIV-1 or CMV virus-derived T-cell epitopes. This formulations resulted in increased T-cell mediated immune response even in chronically infected HIV-1 positive humans in an *ex vivo* model, providing a promising approach to the development of clinically useful anti-HIV or CMV prophylactic and therapeutic vaccination strategies.

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Figure legends

Figure 1. Schematic representation of the new *ex vivo* assays performed with formulations based on peptide conjugated GNPs. (A) Schematic diagram of HIV-GNPs interaction, process and antigen presentation in APC (DC) to T cells (B) Schematic diagram of HIV Gag p17 and CMV pp65 peptides with a linker sequence KKWK used to manufacture the Peptide@manno GNPs**.** (C) Diagram of HIV Gagp17@manno GNPs bearing both mannose conjugate and the HIV Gag p17 immunogenic peptide SLYNTVATL (SL9), and CMV pp65@manno GNPs bearing both mannose conjugate and CMV pp65 peptide NLVPMVATV. (D) Characterization by Transmission Electron Microscopy (TEM) of GNPs.

Figure 2. Gold nanoparticles prepared and used in this work and their synthetic strategy. The starting GNPs have been functionalized with a glucose or a dimannose conjugate (GNP1 and GNP2) and a carboxylic acid-containing molecule as ligands. HIV-1 Gag p17 (P1) and CMV pp65 (P2) peptides were then introduced in a subsequent step by amide chemistry to obtain P1@glc, P1@HM, P2@glc and P2@HM GNPs.

Figure 3. T-cell proliferation induced by autologous MDDC pulsed with Peptide@man no GNPs. T cell proliferation in response to autologous MDDCs pulsed with P1, P1@glc, P1@HM P2, P2@glc, and P2@HM at a dose of 10 μg/ml of peptide and SEA as a positive control (1ng/ml) was assessed in co-cultures (n = 9). MDDCs were also untreated (NC) and pulsed with GNP2 and soluble p24 at 5 μg/ml. (A) Density plots of gating and results of T cell proliferation of a representative individual is shown when maturation cocktail was added after the pulsing of MDDC. Cells were gated by $CD3^+$ lymphocytes and then $CFSE^{low}$ was quantified in $CD8^+$ T cells and

$CD4^+$ T cells ($CD3^+$ $CD8^-$) by flow cytometry. Box and whisker plots of the percentage of proliferation from (B) $CD8^+$ T cells and (C) $CD4^+$ T cells by $CFSE^{low}$ are shown when maturation cocktail was added after the pulsing of MDDC (+). (D) $CD8^+$ T cells and (E) $CD4^+$ T cells by $CFSE^{low}$ are shown when any maturation cocktail was added after the pulsing of MDDC (-). Boxes represent interquartile ranges; the horizontal bar within each box is the median. Whiskers indicate the 10th and 90th percentiles. Wilcoxon signed rank test, $*P < 0.05$, $**P < 0.01$. GNP2: manno GNPs; NC: Negative Control; P1: HIV Gagp17; P1@glc: HIV Gagp17@glucose GNPs; P1@HM: HIV Gagp17@manno GNPs; P2: CMV pp65; P2@glc: CMV pp65@glucose GNPs; P2@HM: CMV pp65@manno GNPs; p24: HIV p24 antigen; SEA: Staphylococcus aureus enterotoxin A.

Figure 4. Secretion of pro-T_H1 cytokines and pro-T_H1 chemokines by autologous MDDC pulsed with Peptide@manno GNPs. Quantification by Cytokine Human 25-Plex Panel™ assay of cytokine secretion pattern from co-culture of MDDCs treated with P1, P1@HM, P2, P2@glc, and P2@HM at a dose of 10 μg/ml and p24 (5 μg/ml) with autologous T cells. The supernatants were collected and the concentrations of cytokines and chemokines were analysed (9 individuals in duplicates). (A) T_H1 cytokines, (B) T_H1 chemokines are shown. Boxes represent interquartile ranges; the horizontal bar within each box is the median. Whiskers indicate the 10th and 90th percentiles. Wilcoxon signed rank test, $*P < 0.05$, $**P < 0.01$, $***P <0.005$.

Figure 5. Secretion of pro-T_H2 cytokines by autologous MDDC pulsed with Peptide@manno GNPs. Quantification of cytokine secretion after co-culture of treated MDDCs with autologous T cells. The supernatants were collected and the

concentrations of cytokines and chemokines were analysed (9 individuals in duplicates). Th2 cytokines are shown: IL-13, IL-5, Eotaxin, IL-4, MCP-1 and IL-6. Wilcoxon signed rank test, $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

Figure 6. Peptide@manno GNPs activate T cells and promote pro-inflammatory cytokine secretion. Quantification of cytokine secretion after co-culture of treated MDDCs with autologous T cells. The supernatants were collected and the concentrations of cytokines and chemokines were analysed (9 individuals in duplicates). Pro-inflammatory cytokines are shown: TNF-α. IL1-β, IL-8, GM-CSF, IL-17 and the anti-inflammatory cytokine IL1-RA. Wilcoxon signed rank test, $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

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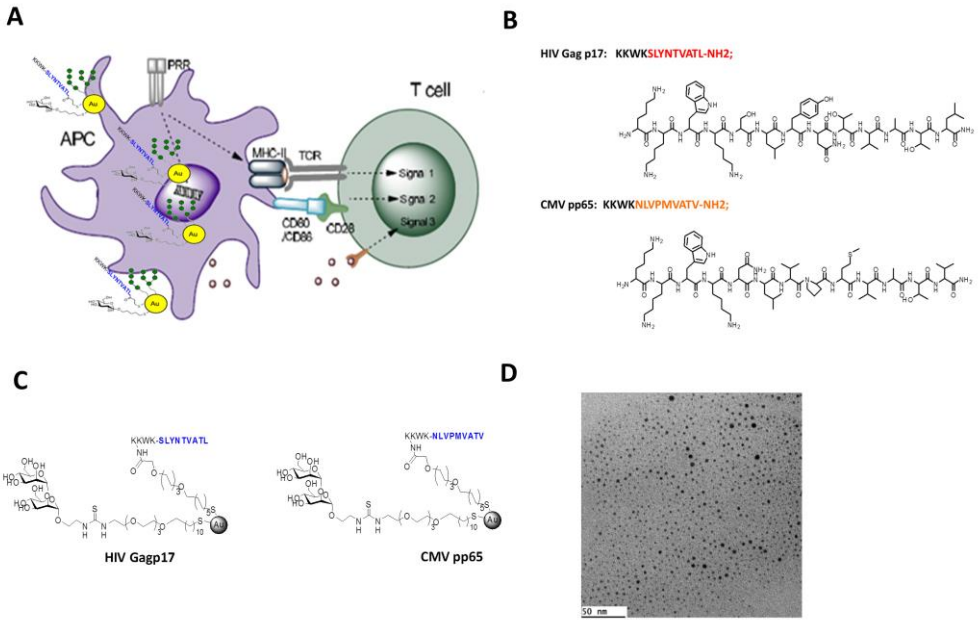


Figure 1

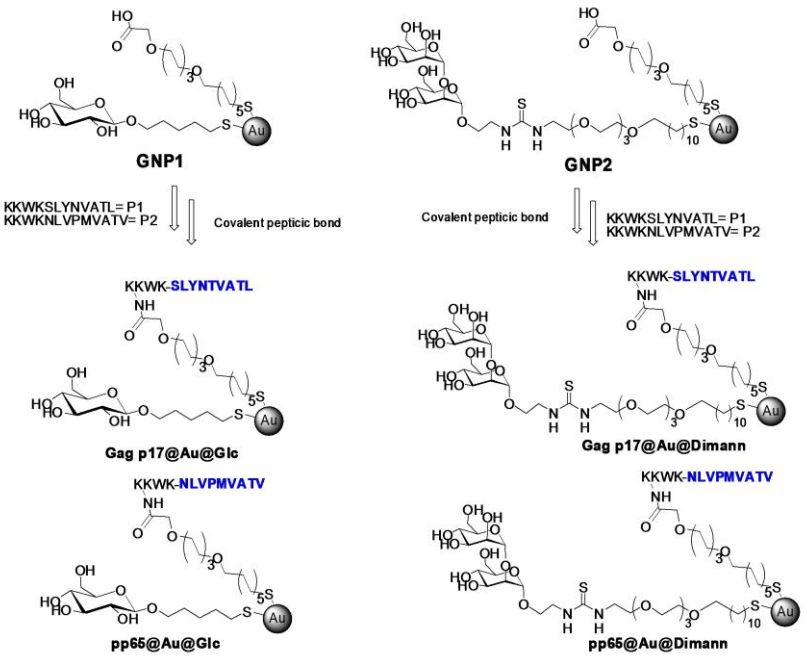


Figure 2

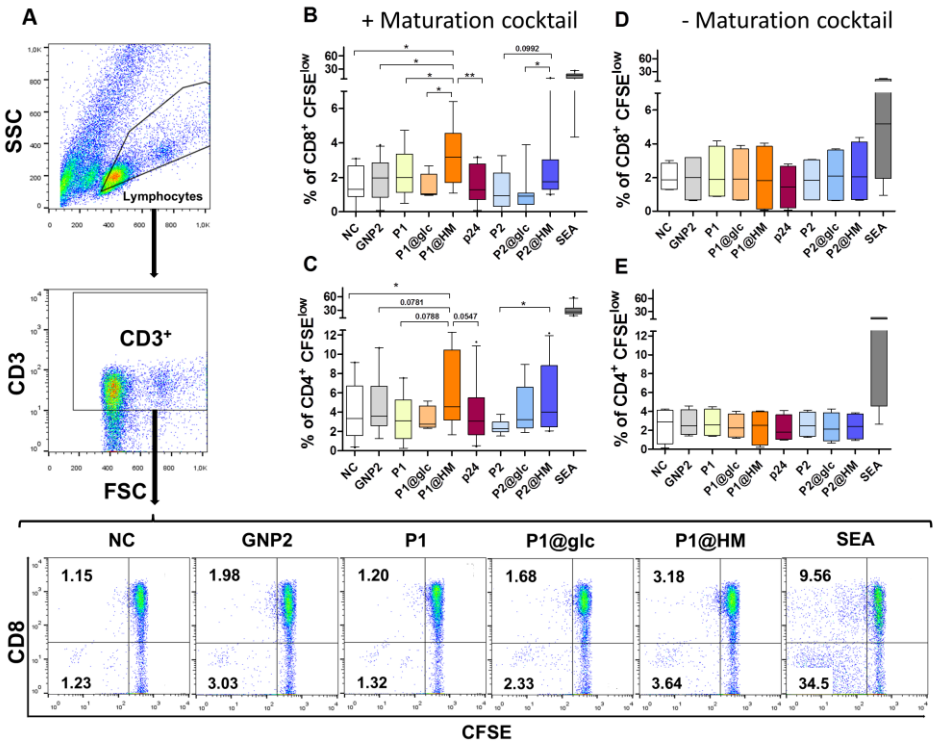


Figure 3

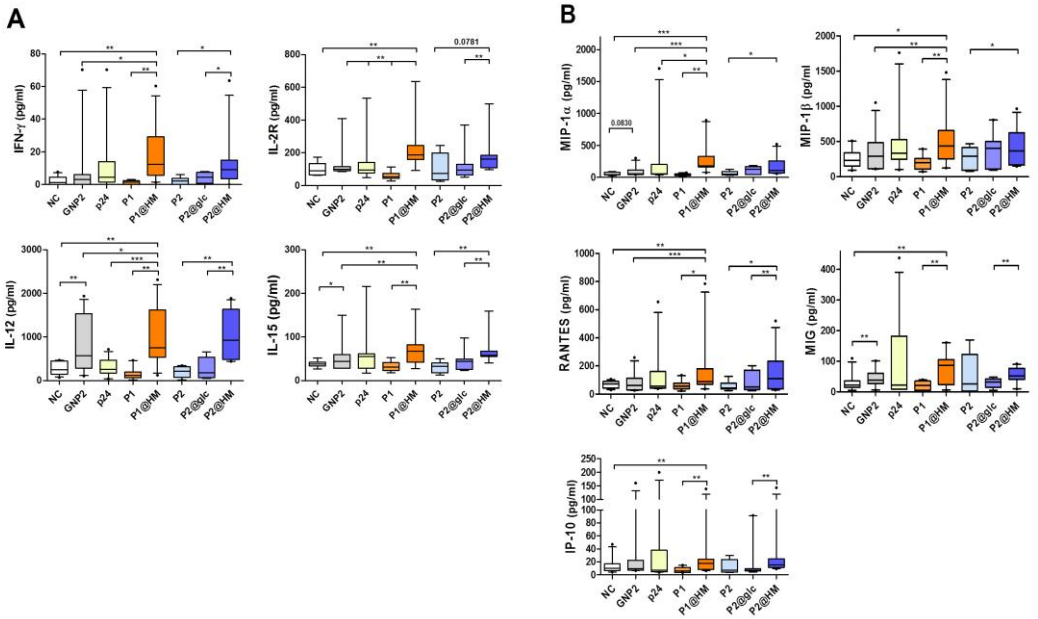


Figure 4

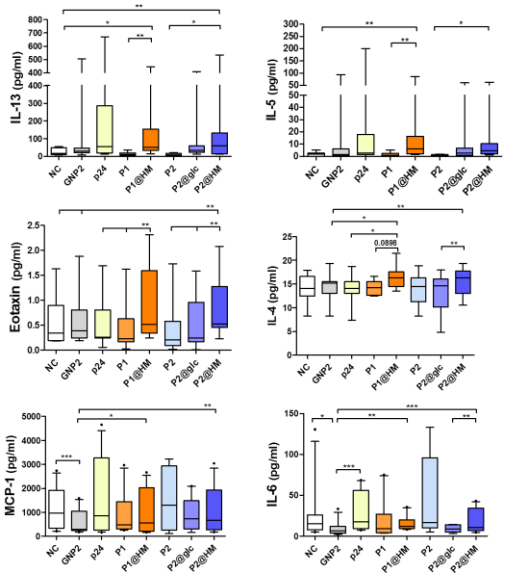


Figure 5

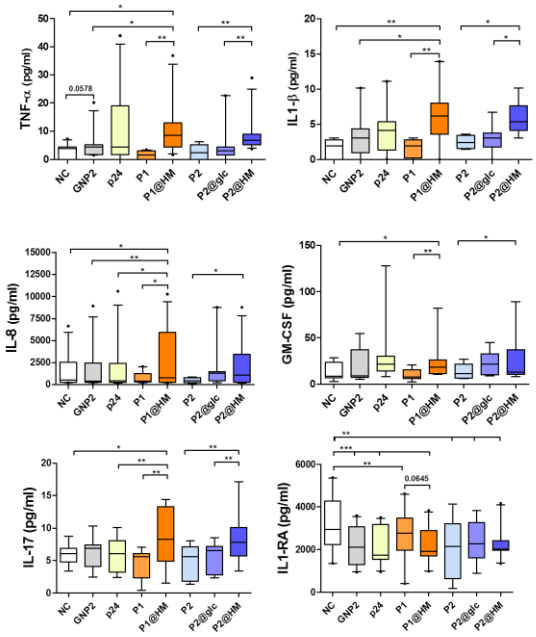
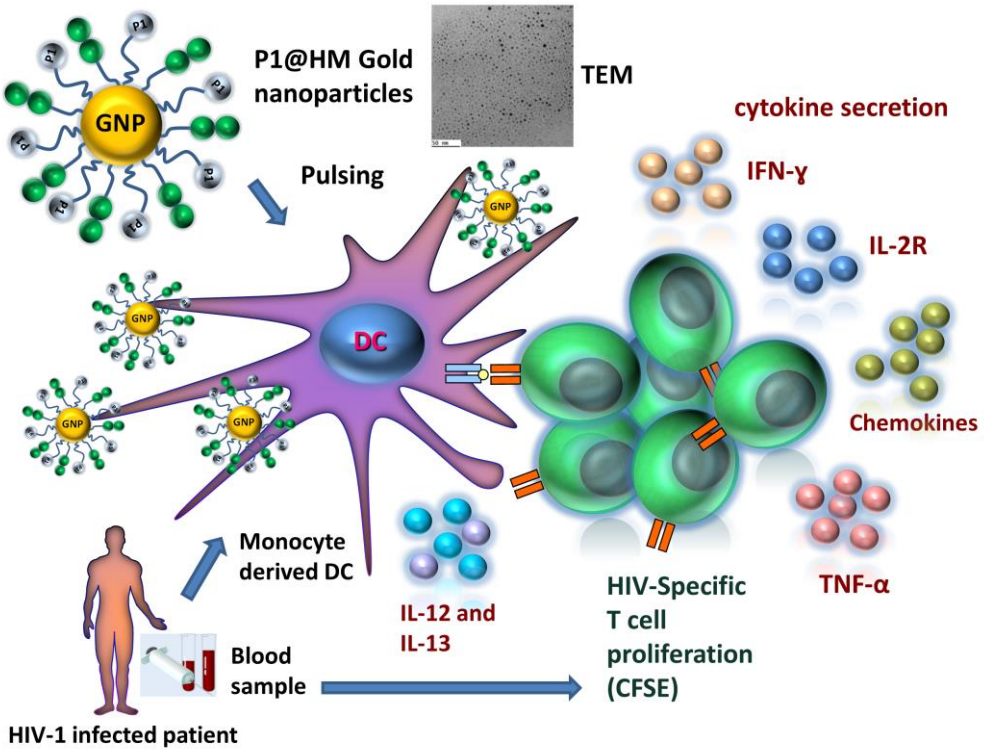


Figure 6



Graphical abstract

Schematic representation of *ex vivo* assays performed with formulations based on HLA-A*0201-restricted HIV-peptides and Mannosides co-conjugated to GNPs (P1@HM)**.** P1@HM interaction, process and antigen presentation by DC derived from blood monocytes of HIV+ individuals, promotes T-cell proliferation of autologous lymphocytes obtained from the same HLA-A*0201 HIV-1^{+} individuals. P1@HM treatment consistently enhances highly functional pro-T_H1 cytokines, pro-T_H1 chemokines**,** pro-inflammatory cytokine and several pro-T_H2 cytokines as important immune factors that could potentiate a high adjuvant effect in an HIV vaccine context.