Polyelectrolyte Capsules-containing HIV-1 p24 and Poly I:C Modulate Dendritic Cells to Stimulate HIV-1-specific Immune Responses

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Polyelectrolyte microcapsules (MCs) are potent protein delivery vehicles which can be tailored with ligands to stimulate maturation of dendritic cells (DCs). We investigated the immune stimulatory capacity of monocyte-derived DC (Mo-DC) loaded with these MCs, containing p24 antigen from human immunodeficiency virus type 1 (HIV-1) alone [p24-containing MC (MCp24)] or with the Toll-like receptor ligand 3 (TLR3) ligand poly I:C (MCp24pIC) as a maturation factor. MO-DC, loaded with MCp24pIC, upregulated CCR7, CD80, CD83, and CD86 and produced high amounts of interleukin-12 (IL-12) cytokine, to a similar extent as MCp24 in the presence of an optimized cytokine cocktail. MO-DC from HIV-infected patients under highly active antiretroviral therapy (HAART) exposed to MCp24 together with cytokine cocktail or to MCp24pIC expanded autologous p24-specific $CD4^+$ and $CD8^+$ T-cell responses as measured by interferon-γ (IFN-γ) and IL-2 cytokine production and secretion. *In vivo* relevance was shown by immunizing C57BL/6 mice with MCp24pIC, which induced both humoral and cellular p24-specific immune responses. Together these data provide a proof of principle that both antigen and DC maturation signal can be delivered as a complex with polyelectrolyte capsules to stimulate virus-specific T cells both *in vitro* and *in vivo*. Polyelectrolyte MCs could be useful for *in vivo* immunization in HIV-1 and other infections.

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INTRODUCTION

Worldwide 33 million people are infected with human immunodeficiency virus type 1 (HIV-1) causing 2 million casualties annually.[1] With no effective preventive vaccine available in the near future,[2] highly active antiretroviral therapy (HAART), which suppresses viral replication but fails to eradicate the virus,[3] remains the only treatment option. Life-time HAART is however very costly, and side effects emphasizing the need for new treatment strategies. Given the correlation between long-term nonprogressor HIV patients and the induction of multifunctional T-cell responses, the development of therapeutic vaccines capable of inducing such responses might allow the patient's immune system to control the virus and supplement or even replace HAART.

The recognition of the unique role of dendritic cells (DCs) in initiating T-cell responses has boosted interest in harnessing their potential as nature's own adjuvant.[4] Although DC-based vaccination has been explored mainly in the field of cancer immunotherapy, it has more recently also drawn the attention of HIV researchers. Up to now DC-based vaccination has relied on the *ex vivo* differentiation of immature DCs (iDCs) from blood precursors, which are subsequently loaded with antigen, matured with a cytokine cocktail and reinfused into the patient. In this context, DCs have been pulsed with inactivated virus,[5,6] recombinant HIV-1 proteins,[7,8] antigenic peptides,[9] or mRNA by transfection/electroporation.[10–13] After injection of those antigen-loaded DCs, HIV-specific immune responses could be induced *in vivo*. Nevertheless *ex vivo* loading of the DCs with antigens remains a very complex, labor-intensive and costly procedure. Developing strategies that directly target the antigen to DCs *in vivo* could allow a broader applicability of DC-based therapeutic vaccinations.

An interesting approach to achieve this goal is encapsulation of antigens in particles with a 0.1–10 μm range. A plethora of studies using a variety of particulate carriers in this size range has now demonstrated that particulate antigen delivery not only strongly enhances antigen uptake[14,15] but also promotes cross-presentation, allowing the induction of cytotoxic T cells.[16–19] Despite their effectiveness in enhancing immune responses, none of these polymeric carriers has however reached clinical practice, which is mainly due to the complex and multiple steps involved in their production, difficulties with reproducibility, low-antigen encapsulation efficiency, and antigen denaturation due to the use of chemical solvents.

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More recently, a new type of biodegradable microcapsules (MCs) composed of polyelectrolytes was developed, based on a layer-by-layer technique with opposite charges.[20–22] These polyelectrolyte MCs allow a much more efficient encapsulation of proteins under nondenaturing conditions.[23] Using ovalbumin as a model antigen, antigen encapsulation in these MCs has been demonstrated to strongly promote antigen presentation by DCs to both $CD4^+$ and $CD8^+$ T cells, making them attractive antigen delivery vehicles also in the field of HIV vaccination.[24] In the present study, we aim to evaluate the potential of these MCs to stimulate HIV-specific immune responses both *in vitro* and *in vivo* by encapsulating the HIV antigen p24. Although MCs might be interesting tools to enhance antigen presentation by DCs, DCs also need to become activated to induce potent effector T-cell responses. Thereby, we have tailored the MCs by electrostatic interaction with the negatively charged Toll-like receptor ligand 3 (TLR3) poly I:C (pIC), which strongly activates DCs to release high amounts of interleukin-12 (IL-12) and type I interferons (IFNs).[25] The latter factors are known to promote the induction of T helper 1 (Th1) and cytotoxic T lymphocyte responses, which are thought to be necessary for viral control in HIV. In this article, the effects of pIC tailoring on the MC was addressed.

RESULTS

Characterizing and visualizing polyelectrolyte MCs

Polyelectrolyte MCs with or without HIV-1 p24 were generated using the layer-by-layer method in three steps. First p24 was co-precipitate d by mixing with calcium chloride and sodium carbonate, resulting in 3-μm sized spherical calcium carbonate ($CaCO_3$) microparticles-containing p24 in its pores. Next, these microparticles were coated by alternated deposition of two bilayers of dextran sulfate and poly-L-arginine, using electrostatic interaction as driving force. These capsules will be further denoted as (dextran sulfate/poly-L-arginine)2. Finally the $CaCO_3$ was dissolved in an aqueous EDTA solution yielding hollow p24-containing capsules. When measuring the electrophoretic mobility of the (dextran sulfate/poly-L-arginine)2 coated $CaCO_3$, a value of +25 mV (±5.1 mV) was observed indicating a positive charge of the microparticles (**Figure 1a**). However, upon dissolution of the $CaCO_3$ core template, a reversal of the surface charge was observed as a ζ-potential (zeta-potential) of −50 mV (±4.5 mV) was measured. This phenomenon is attributed to a rearrangement of an excess of anionic dextran sulfate which is redistributed through the capsule membrane upon dissolution of the $CaCO_3$ core template, the latter is in accordance with previous observations. There was no influence of p24 on the ζ-potential of the MCs (data not shown).The capsules were visualized using poly-L-arginine, labeled with rhodamine isothiocyanate (RITC) (**Figure 1b**).

pIC is a polyanion and therefore should be easily deposited on a positively charged capsule surface. However, as the ζ-potential of the (dextran sulfate/poly-L-arginine)2 capsule is negative after dissolution of the core, pIC will be repulsed. Therefore the (dextran sulfate/poly-L-arginine)2 coated $CaCO_3$ microparticles were coated with a layer of pIC before $CaCO_3$ core dissolution, yielding microparticles with a ζ-potential of +5 mV. After core dissolution the ζ-potential of the pIC functionalized capsules further decreases to −52 mV (±4.5 mV). The presence of pIC on the capsule surface after core dissolution was confirmed by confocal microscopy using fluorescent (fluorescein isothiocyanate) labeled pIC (**Figure 1c**).

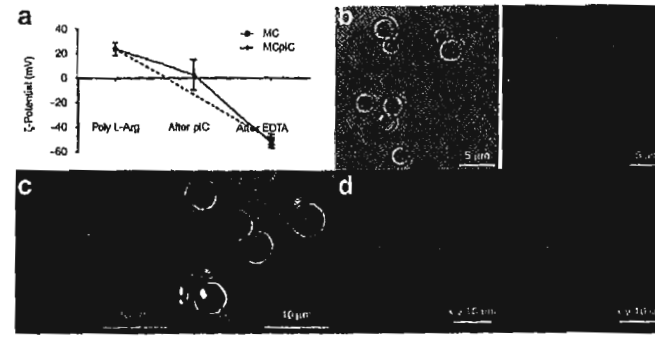

Figure 1 Characterization and visualization of the polyelectrolyte microcapsules. Polyelectrolytes microcapsules (MC) consist of two bilayers of dextran sulfate and poly-L-arginine. (**a**) Zeta-potential (ζ-potential) versus preparation step. Black line, empty polyelectrolyte capsules tailored with poly I:C (MCpIC). Dashed line: empty polyelectrolyte capsules (MC). (**b**) Transmission and confocal image of microcapsules stained with RITC (red). (**c**) Transmission and confocal image of microcapsules tailored with poly I:C stained with fluorescein (green). (**d**) Confocal microscopy of DC, microcapsules' shell was stained labeled with RITC (red)-poly-L-arginine, nucleus stained with DAPI (blue). DAPI, 4′-6-diamidino-2-phenylindole; DC, dendritic cell; MCpIC, MC with pIC; RITC, rhodamine isothiocyanate.

The uptake of the MCs by iDCs was also visualized with confocal microscopy (**Figure 1d**). In this case, the nucleus of DC was stained blue with 4′-6-diamidino-2-phenylindole and the MCs' shell was stained red by incorporation of RITC-poly-L-arginine. Clearly the capsules were dispersed in the cytoplasm. As reported earlier for murine bone marrow-derived DC, human monocyte (Mo)-derived DCs ware capable of endocytosing large amounts of polyelectrolyte MCs.[24]

Phenotypic maturation and IL-12 production of DCs after incubation with polyelectrolyte MCs-containing p24 and pIC

To address the capacity of MC to stimulate DC maturation, DCs derived from healthy donors, were either left untreated (iDC), preincubated with MC alone (iDC+MC), with MC tailored with pIC (iDC+MCpIC) with MC and soluble pIC (iDC+MC+pIC) or with MC and an optimized maturation cocktail consisting of prostaglandin E2, tumor necrosis factor-α, IFN-γ, and the TLR7/8 ligand R848 (iDC+MC+mat), as a positive control. The purpose was to assess whether preincubation of iDC with MC and soluble pIC or MC with surface bound pIC would induce a similar maturation profile as DCs matured with the cocktail.

As shown in **Figure 2a**, treatment of iDC with MC resulted in a selective increase of the costimulatory molecule CD86, but failed to upregulate the expression of other costimulatory markers, indicating MC on their own induce at best a very partial DC maturation. Next, we addressed whether surface modification of MC with pIC (MCpIC) could stimulate DC maturation to a similar extent as mixtures of MC and soluble pIC and of MC and an optimized maturation cocktail. As depicted in **Figure 2a**, surface modification of MCpIC resulted in a strong increase of the maturation markers CCR7 (marker for lymph node (LN) migratory

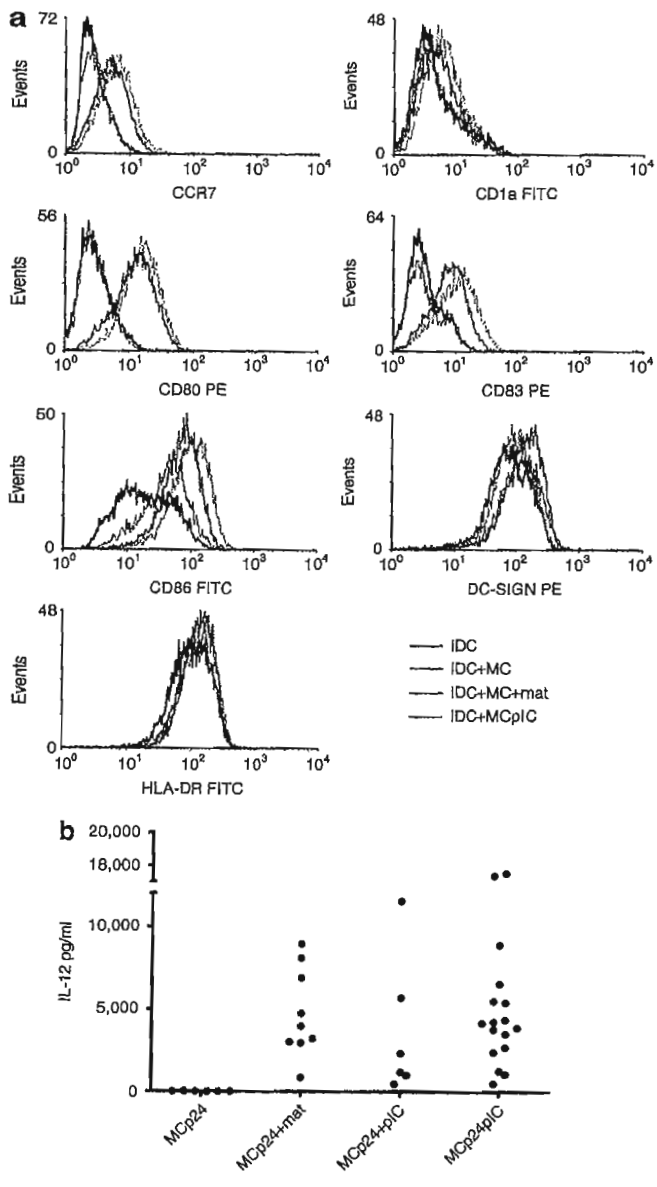

Figure 2 Maturation status and IL-12p70 production of DCs after incubation with polyelectrolyte microcapsule-containing p24 and poly I:C. (a) FACS dotplots of iDC from a healthy donor incubated without microcapsules (iDC, black line), with microcapsules (iDC+MC, red line), with microcapsules and optimized maturation cocktail (iDC+MC+mat, blue line), with microcapsules tailored with poly I:C (iDC+MCpIC, green line). Results depicted are from one representative experiment out of six. **(b)** IL-12p70 production of DC from HIV$^+$ blood donors (P11–P27) incubated with microcapsules-containing p24 (MCp24), MCp24, and optimized maturation cocktail (MCp24+mat), MCp24 and added soluble poly I:C (MCp24+pIC) or MCp24 tailored with poly I:C (MCp24pIC). FACS, fluorescence-activated cell sorting; HIV, human immunodeficiency virus; iDC, immature dendritic cell; IL, interleukin; MC, microcapsule; MCpIC, MC with pIC.

capacity), CD80 (costimulatory molecule), CD83 (marker for DC maturation) and CD86, with the MCpIC being equally potent to the mixture of MC with soluble pIC or with the optimized maturation cocktail. For the sake of clarity, data of iDC+MC+pIC are not depicted, because they completely overlapped with iDC+MCpIC.

Besides the maturation phenotype of DCs, the secretion of various cytokines orchestrates the nature of the T-cell response. Production of IL-12 p70 is important for a Th1 immune response.[26,27] **Figure 2b** shows that DCs from HIV$^+$ subjects under HAART secrete high amounts of IL-12p70 when incubated with p24-containing MC (MCp24) and additional maturation stimulus like cytokine cocktail or pIC (either soluble or fixed to the MC), as compared to iDC (data not shown) or DCs incubated with MCp24 alone. This result indicates that polyelectrolyte capsules tailored with pIC strongly stimulate IL-12 production (**Figure 2b**) which is of major importance for the development of an immunogenic vaccine.

In vitro T-cell stimulatory capacity of DCs with MCp24

Blood from HIV-1$^+$ individuals under HAART was used, to investigate the capacity of DCs, loaded with MCp24, to expand IFN-γ responses. Therefore, peripheral blood lymphocytes (PBL) from three HAART-treated patients were cocultured with autologous DCs that had been loaded with either empty MCs (MCmock) or MCp24 and subsequently matured with the optimized cytokine cocktail. After 10 days these DC-stimulated PBL were assayed in a restimulation (on day 10) enzyme-linked immunosorbent spot assay. As a baseline control, nonstimulated (frozen and freshly thawed on day 10) PBL from the same donor were simultaneously assayed with or without Gag peptides (representing day 0 responses). As shown in **Figure 3a**, PBL cocultured with DC MCp24 induced three to four times more IFN-γ spot-forming cells as compared to freshly thawed PBL or DC mock-stimulated PBL. Kinetic experiments indicated that the optimal response to MCp24 loaded DCs was always after 10–14 days. For consistency, we chose to coculture PBL with DCs for 10 days.

Next, the PBL stimulating capacities of DCs loaded with either MCp24 or soluble p24 protein (sp24) were compared. **Figure 3b,c** summarizes the results of five independent donors. PBL cocultured with antigen-loaded DCs induced significantly more IFN-γ and IL-2 spot-forming cells compared to day 0 responses, regardless whether soluble p24 or MCp24 was used, confirming that encapsulation of the protein has no negative influence on the induction of immune responses.

Coculture of PBL with DCs incubated with MCp24 tailored with pIC: stimulates both IL-2 and IFN-γ, predominantly, but not exclusively in CD4$^+$ T cells

In order to further characterize the quality of the induced responses, we investigated the production of IFN-γ and IL-2 in CD8$^+$ and CD8$^-$ (CD4$^+$) T cells. To this end, three conditions of stimulating PBL were compared (i) DCs loaded with MCp24 only, (ii) DCs loaded with MCp24 and stimulated with soluble pIC (MCp24+pIC), and (iii) DCs loaded with MCp24 and surface adsorbed pIC (MCp24pIC).

As shown in **Figure 4**, DCs loaded with MCp24+pIC or MCp24pIC similarly expanded IFN-γ (**P = 0.006 and **P = 0.002, respectively) and IL-2 (*P = 0.023 and **P = 0.006, respectively) secreting autologous PBL. In addition, both performed better than MCp24 alone (**Figure 4a,b**).

For the same donors as in **Figure 4a,b**, we also measured the intracellular cytokine production at day 0 and day 10 in the

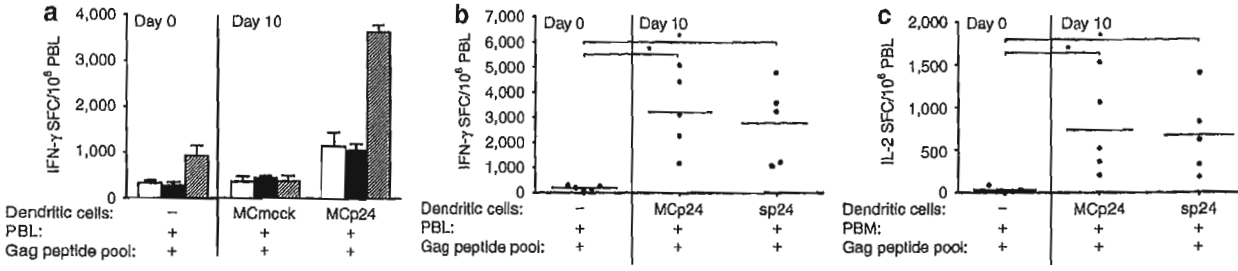

Figure 3 T-cell stimulatory capacity of DCs incubated with microcapsules-containing p24. (**a**) Functionality of DCs loaded with MCp24 versus empty MC. As a baseline control freshly thawed PBL were stimulated with HxB2 Gag peptide pool (day 0). After 10 days coculture of PBL with DC, loaded with empty microcapsules (MCmock) or with p24-containing microcapsules (MCp24) and matured with cytokine cocktail, PBL were restimulated in ELISPOT with HxB2 Gag peptides (Gag peptide pool$^+$). White bars, patient P1; black bars, P2; and the dashed bars, P3. (**b**,**c**) Functionality of DC loaded with MCp24 versus soluble p24,. The immunogenicity of these antigen-loaded and cytokine cocktail matured DC was measured in a restimulation (**b**) IFN-γ or (**c**) IL-2 ELISPOT for patients P4–P8. In the graphs, the amount of SFC per million PBL is shown. A two-way paired *t*-test was used to assess significance of differences between various conditions. $^*P < 0.05$. DC, dendritic cell; ELISPOT, enzyme-linked immunosorbent spot assay; MC, microcapsule; PBL, peripheral blood lymphocytes; IFN-γ, interferon-γ; IL, Interleukin; SFC, spot-forming cells.

$CD3^+CD8^+$ and $CD3^+CD8^-$ (= essentially $CD4^+$) fractions of PBL. A small, but significant proportion of $CD3^+CD8^+$ T cells incubated with DCs loaded with MCp24+pIC or MCp24pIC produced IFN-γ (about 1%) and IL-2 (about 0.15%) after restimulation with a p24 peptide pool. Interestingly, in the same experiment, MCp24+pIC or MCp24pIC potently stimulated $CD4^+$ T-cell responses, with the proportion of $CD4^+$ T cells producing IFN-γ reaching up to 10%, and with about 2% of the $CD4^+$ population producing IL-2. Summarizing, combining MCp24 with pIC, either surface adsorbed or in solution, strongly expanded the population of IFN-γ or IL-2 secreting HIV-1-specific $CD4^+$ and $CD8^+$ T cells.

Induction of cellular and humoral immune responses in mice

To assess whether immunogenicity of an antigen is increased after encapsulation and tailoring with TLR-ligand pIC, immunization experiments were performed in mice ($n = 6$ per group). Animals were immunized two times at a 4-week interval with MCp24, MCp24+pIC (soluble), MCp24pIC (complex), sp24, sp24+pIC, or phosphate-buffered saline (PBS) (negative control) and the induction of T cells and humoral immune responses was characterized.

As shown in **Figure 5a,b**, immunization with soluble p24 failed to elicit IFN-γ or IL-2 producing cells in the draining LNs. Although antigen encapsulation inside polyelectrolyte MCs resulted in a slight increase in the number of IFN-γ-secreting cells ($^*P = 0.026$), p24-loaded MC were clearly superior in eliciting IL-2 secreting PBL compared to soluble p24 ($^{**}P = 0.0005$). Adding the Th1 skewing adjuvant pIC had little impact on the number of IL-2 producing cells, but potently increased levels of IFN-γ producers, regardless whether antigen was delivered in soluble or encapsulated format (sp24+pIC $^*P = 0{,}023$; MCp24+pIC $^{**}P = 0.002$; MCp24pIC $^{**}P = 0.008$), the latter however still evoking the most potent response. Remarkably, mixture of MCs with soluble pIC were clearly more potent in stimulating T-cell responses compared to MCs tailored with pIC.

Finally, we also addressed the capacity of MC and pIC to enhance humoral immune responses. As depicted in **Figure 5c,d**, particulate antigen delivery strongly promoted the induction of immunoglobulin G1 (IgG1) compared to soluble antigen ($^{**}P < 0.01$). Regarding the IgG1 response, no additional benefit was observed by combining the MCpIC (either free or surface adsorbed). Adding pIC was however crucial for the induction of an IgG2c response, which was totally absent after immunization with p24-loaded MC or soluble p24. Although pIC promoted IgG2c responses when added to soluble p24 ($^*P < 0.01$), combining MCpIC was clearly superior in doing so ($^{**}P < 0.01$). Again, combinations of MC with soluble pIC performed better than MC with surface adsorbed pIC.

DISCUSSION

Polyelectrolyte MCs form a new type of MCs that have been demonstrated to strongly enhance antigen presentation by DCs to both $CD4^+$ and $CD8^+$ T cells *in vitro*. Given the paramount importance of the induction of strong effector T-cell responses in controlling HIV infection, these MCs might be interesting tools to boost immune responses against HIV in a therapeutic setting. In the presented work, we have evaluated the potential of MC to increase pre-existing immune responses against the HIV antigen p24 by *ex vivo* incubation of PBL from HAART-treated HIV patients with MCp24 loaded MO-DC. In addition, as the main goal of particulate antigen delivery is to directly target DCs *in vivo*, we have analyzed the capacity of MCs either alone or in combination with the TLR3 agonist pIC to induce cellular and humoral immune responses by immunizing C57BL/6 mice.

In agreement with earlier results obtained with murine bone marrow-derived DCs, MCs were efficiently taken up by human MO-DCs. MC uptake resulted in a moderate upregulation of the costimulatory ligand CD86, but failed to increase the expression of other maturation markers or to stimulate IL-12 secretion, indicating that MC uptake by itself is insufficient to trigger full DC activation. Similar observations were made using other particulate delivery vehicles including the extensively explored polymer of lactic and glycolic acid particles (PLGA).[18,28] Combining the MC with an optimized maturation cocktail or the TLR3 ligand pIC, resulted in a strong maturation of MC loaded DC. Due to their cationic nature MCs can be easily modified with negatively charged TLR ligands such as pIC. This characteristic is important to simultaneously deliver antigen and activation stimulus to the same DC, a feature which has been reported to strongly augment the strength of the induced adaptive

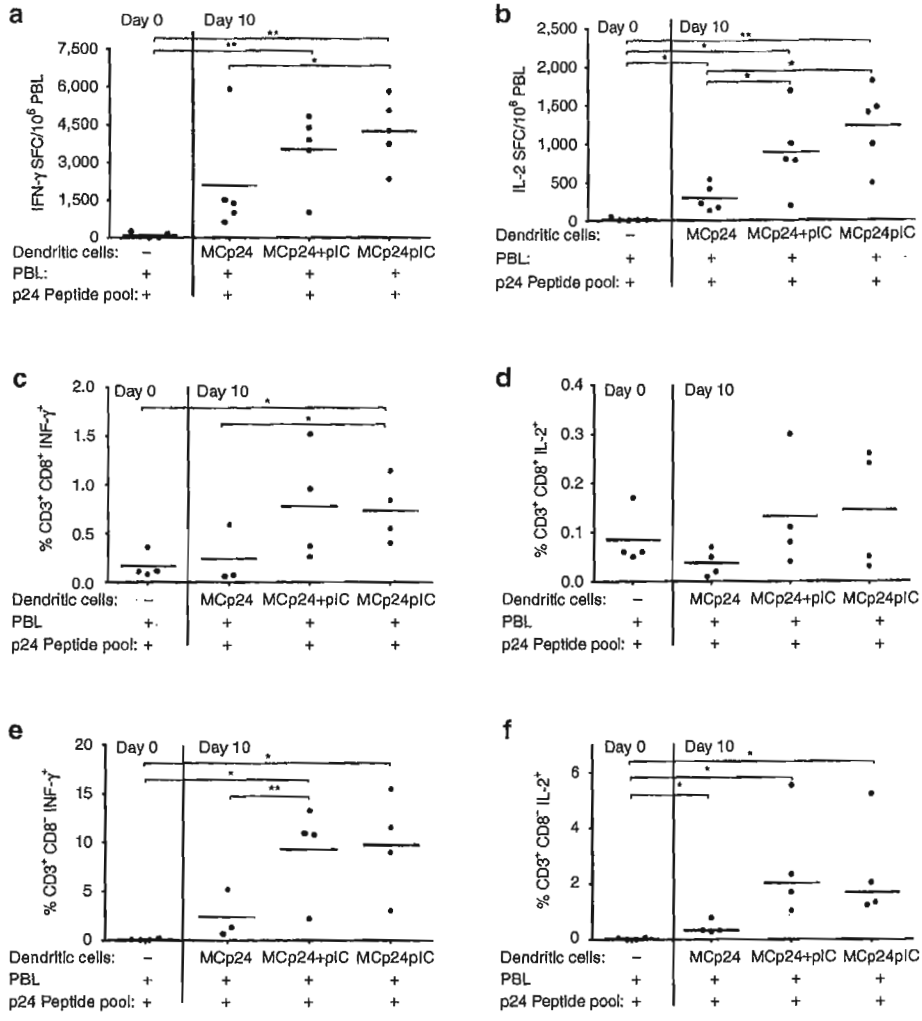

Figure 4 Immunostimulatory capacity of DCs loaded with p24-containing microcapsules tailored with poly I:C (MCp24pIC). (**a**,**b**) Antigen-loaded DCs were cocultured with autologous PBL for 10 days and afterwards restimulated with p24 peptide pool in the (**a**) IFN-γ or (**b**) IL-2 ELISPOT assay. As a baseline control freshly thawed PBL were assayed in parallel (day 0). In the graphs, the amount of SFC per million PBL is shown. Three conditions were used: DC loaded with p24-containing microcapsules without adding maturation cocktail (MCP24), DC loaded with p24-containing microcapsules and adding separately poly I:C (1.3 μg/ml) (MCp24+pIC), DC loaded with p24-containing microcapsules which are tailored with poly I:C (MCp24pIC). Five HIV$^+$ donors (P11–P15) are shown, horizontal line represents mean. (**c**–**f**) Intracellular staining of cytokines in the CD3$^+$CD8$^+$ and CD3$^+$CD8$^-$ population of PBL which were stimulated with p24 peptide pool after 10 days of coculture with DCs loaded with MCp24 alone, MCp24+pIC, and MCp24pIC. Patients P12–P15 are represented. A two-way paired *t*-test was used. $^*P < 0.05$, $^{**}P < 0.01$. DC, dendritic cell; ELISPOT, enzyme-linked immunosorbent spot assay; MC, microcapsule; MCp24, p24-containing MC; PBL, peripheral blood lymphocytes; IFN-γ, interferon-γ; IL, interleukin; SFC, spot-forming cells.

immune responses.[29,30] Incubation of DCs with pIC adsorbed MCs induced a similar DC activation status as DCs cultured with soluble pIC, clearly demonstrating that the adsorbed pIC is still available for receptor triggering in the endosomal compartments.

Following activation with the optimized maturation cocktail, DCs loaded with MC encapsulated p24 were able to induce IFN-γ and IL-2 secreting T cells after a 10 days coculture. These results indicate that following MC uptake encapsulated p24 becomes available for processing and is channeled into the antigen presentation pathway to stimulate T cells. Similar results have been reported by De Rose *et al.*, who observed an increase in the numbers of IFN-γ-secreting CD8 T cells following incubation of peptide-containing polymer capsules with blood from Simian immunodeficiency virus-infected macaques.[31] Antigen encapsulation did however not produce a significant benefit compared to soluble antigen, as DC loaded with soluble p24 were capable of evoking similar T-cell responses. These data are in contrast with earlier observations made by De Koker *et al.*,[24] who have demonstrated a clear benefit of antigen encapsulation in MCs in stimulating antigen presentation by murine bone marrow-derived DCs to T cells. These discrepancies might be attributed to the much lower amount of antigen applied during the encapsulation process in the current setting. In addition, also differences in antigen processing and presentation between human MO-DC and murine bone marrow-derived DC and the source of T cells used in the assays (PBL from HAART-treated HIV patients versus naive murine splenocytes) might underlie these different observations.

Next, we investigated whether CD4$^+$ and CD8$^+$ T cells would be differentially activated by MCp24 loaded DC with adsorbed or

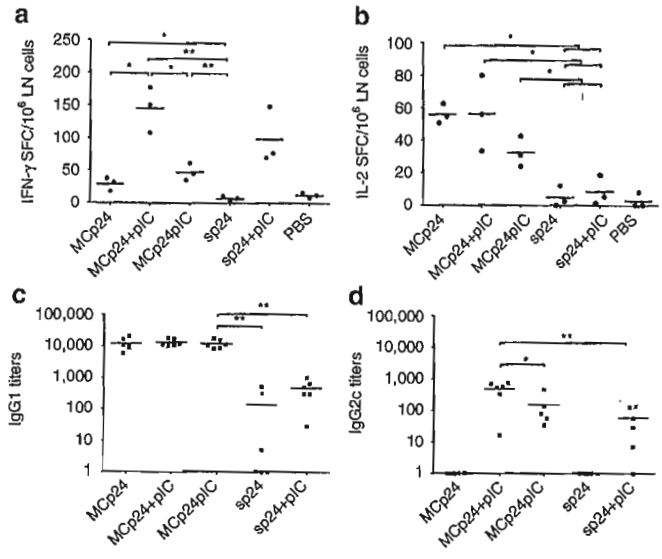

Figure 5 Induction of cellular and humoral immune responses by MCp24pIC in mice. Mice ($n = 6$ per group) were immunized two times subcutaneously with MCp24 alone, MCp24+pIC, MCp24pIC, sp24, sp24+pIC, or PBS at 4-week interval. Four weeks after the last immunization cells from inguinal LN were pooled for two mice (three pools per group) and restimulated with p24 peptide pool. The number of (**a**) IFN-γ and (**b**) IL-2 producing cells was measured by ELISPOT. At the same time (**c**) IgG1 and (**d**) IgG2c antibody titers against p24 were determined by ELISA in the serum of each individual mouse. ELISPOT, enzyme-linked immunosorbent spot assay; IFN-γ, interferon-γ; IgG, immunoglobulin G; IL, interleukin; LN, lymph nodel; MCp24, p24-containing MC; PBS, phosphate-buffered saline.

soluble pIC. In fact both forms of pIC increased levels of IFN-γ and IL-2 secreting cells to a similar extent. Although both $CD4^+$ and $CD8^+$ T cells responses were increased after addition of pIC, especially $CD4^+$ T-cell responses reached high levels, with ~10% of $CD4^+$ T cells producing IFN-γ and 2% producing IL-2, as assessed by intracellular staining. This is very promising, because chronic HIV-1 infection is characterized by dysfunction of $CD4^+$ T cells, especially the proliferation function which depends on IL-2.[32] Combinations of MCs with pIC also increased the levels of IFN-γ and IL-2 secreting $CD8^+$ T cells, which are also considered to be very important in controlling HIV infection. Nevertheless, levels of activated $CD8^+$ T cells remained about tenfold lower compared to those of $CD4^+$ T cells.

Although *ex vivo* studies using DCs and T cells can give a first indication about the potency of a certain formulation, such studies are a tremendous simplification of the *in vivo* situation, where much more cell types and tissue-derived factors interact. Illustrative for these important differences is that the fact that although MCs do not directly activate *in vitro* generated DC, their *in vivo* administration provokes an inflammatory response resulting in subsequent DC activation, pinpointing to important differences between *ex vivo* and *in vivo* situations.[33] As a result, the real potential of any vaccine formulation should be assessed *in vivo*. To explore the *in vivo* importance of particulate antigen delivery and to assess potential immune potentiating effects of adding pIC, we immunized mice with soluble p24 (either with or without pIC) or MC encapsulated p24 (either with or without pIC). Furthermore, as it has been proposed that targeting of an antigen and TLR ligand to the same DC and even to the same phagosomal compartment is required for optimal antigen presentation,[34,35] we compared immune responses induced by immunization with p24 MC and surface adsorbed pIC with a mere mixture of p24 MC and soluble pIC. Immunization with MC-containing p24 was clearly more potent in inducing IL-2 secreting T cells compared to immunization with soluble p24, which failed to elicit significant T-cell immunity. In contrast to IL-2 responses, IFN-γ responses were only slightly elevated following MC-based vaccination. Addition of pIC strongly increased the numbers of IFN-γ secreting T cells, but did not further augment IL-2 responses in case of MC-based immunization. These effects are likely due to the known capacity of pIC to promote the differentiation of T helper cells toward IFN-γ secreting Th1 by stimulating DCs to secrete IL-12 and type I IFNs.[25,36] Remarkably, surface adsorbed pIC was less potent in stimulating Th1 differentiation compared to soluble pIC, in contrast with observations made by others using different types of particles.[34,35,37–40] Why surface adsorbed pIC was less potent in stimulating T-cell responses currently remains unclear. One possible explanation could be that the pIC is so tightly associated to the MCs' surface it is no longer available for receptor recognition. *In vitro*, MCp24pIC were however fully capable of activating DCs, and MCp24pIC loaded DCs were at least equally potent arguing against such mechanism. Alternatively, DCs might become overactivated and exhausted by MCp24pIC, or pIC coated capsules might aggregate after subcutaneous injection, decreasing their uptake by DCs. On the level of the humoral immune response, antigen encapsulation resulted in a 100-fold increase in the levels of IgG1 compared to soluble antigen. Although adding pIC slightly increased IgG1 levels when added to soluble p24, it did not further increase IgG1 levels in combination with MCs. In contrast, pIC was crucial to induce IgG2c antibodies, with the combination of MCp24 and soluble pIC being clearly the most potent formulation. As isotype switching toward IgG2c is dependent on IFN-γ and Th1 help, these observations are in accordance with the earlier described induction of IFN-γ positive T cells following immunization with pIC as adjuvant.[41] Similar to the induction of Th1 responses, the combination of MCs with soluble pIC was superior in eliciting IgG2c responses compared to surface adsorbed pIC MCs.

In conclusion, we have demonstrated the potential of MCs as antigen delivery vehicles. Although DCs loaded with either soluble p24 or encapsulated p24 were equally potent in expanding T-cell responses from HAART patients *in vitro*, antigen encapsulation was clearly superior to soluble antigen *in vivo*, mice immunized with encapsulated p24 particularly showed stronger IL-2 T-cell responses and higher IgG1 titers. Combining p24 encapsulation with pIC significantly increased $CD4^+$ and $CD8^+$ T-cell responses *in vitro*, regardless whether pIC was attached to the MCs' surface or merely delivered in solution. *In vivo*, codelivery of pIC was crucial to evoke Th1 responses and concomitant antibody isotype switching toward IgG2c, with the combination of encapsulated p24 and soluble pIC being clearly more potent than soluble p24 and soluble pIC. Remarkably, surface attached pIC was less efficient in promoting Th1 responses compared to soluble pIC. Taken together, these data confirm the potential of MC, either alone or in combination with immune potentiators, in enhancing immune

responses and encourage their further exploration as antigen delivery vehicles for preventive and therapeutic vaccination.

MATERIALS AND METHODS

Study population. Peripheral blood samples (100 ml) were obtained from 17 HIV-1–seropositive patients (P1–P27) on HAART who had an undetectable viral load (viral load <50 copies/ml) for at least 1 year and CD4 T-cell counts above 300 cells/μl. They were recruited at the Clinical Department of the Institute of Tropical Medicine of Antwerp (Antwerp, Belgium) according to institutional guidelines and after obtaining informed consent. Peripheral blood samples (buffy coats) from healthy HIV-1-seronegative controls were provided by the Antwerp Blood Transfusion Center (Red Cross Flanders, Belgium).

Separation of mononuclear cell fractions and in vitro generation of Mo-derived DC. Peripheral blood mononuclear cells were isolated by Ficoll–Hypaque gradient separation (Amersham Biosciences, Freiburg, Germany). Mo were isolated from peripheral blood mononuclear cells by magnetic isolation using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Mo-depleted peripheral blood mononuclear cells, further referred to as PBL, were cryopreserved, according to our standard procedure.[42]

Mo-derived iDC were generated as previously described.[43] Briefly, Mo were cultured in RPMI (Cambrex, Verviers, Belgium) supplemented with 2.5% pooled human serum, 20 ng/ml granulocyte-macrophage colony-stimulating factor (Gentaur, Brussels, Belgium) and 20 ng/ml IL-4 (Biosource Europe, Nivelles, Belgium). In most experiments, maturation of iDC was induced on day 6 using a mixture of 250 ng/ml IFN-γ; Biosource Europe), 2 μg/ml R848 (Alexis, Zandhoven, Belgium), 2.5 ng/ml tumor necrosis factor–α (Roche Molecular Biochemicals, Mannheim, Germany), and 10^{-7} mol/l prostaglandin E_2 (Sigma, St Louis, MO).

Polyelectrolyte MCs. Polyelectrolyte MCs (**Figure 1**) were made as described elsewhere.[23,44] Briefly, 50 μg of p24 protein (obtained from NIBSC, Potters Bar Hertfordshire, UK), $CaCl_2$ and Na_2CO_3 solutions (0.33 mol/l; Merck; VWR, Leuven, Belgium) were mixed with vigorous stirring for 30 seconds followed by extensive washing with pure water to remove unreacted reagents. Spherically shaped $CaCO_3$ microparticles with an average diameter of 3 μm were obtained. The $CaCO_3$ particles were coated using the layer-by-layer technique as described.[20] pIC (100 mg/ml; Invivogen, San Diego, CA) was added before dissolving the $CaCO_3$ core with 10 ml of 0.2 mol/l EDTA solution (pH 5.2) for 10 minutes. The dissolved ions were then removed by three centrifugation and washings steps. Finally the capsules were resuspended in 1 ml PBS. The capsule concentration (700×10^6 capsules/ml) was determined by hemocytometry. The supernatant from each step was stored at −20 °C for p24 enzyme-linked immunosorbent assay to calculate the amount of encapsulated p24 by subtraction. The electrophoretic mobility of the polyelectrolyte MCs was measured in deionized water at room temperature using a Malvern Zetasizer 2000 (Malvern Instruments, Worcestershire, UK) and the ζ-potential was calculated as described elsewhere.[20]

Fluorescence microscopy. RITC (Sigma-Aldrich, Bornem, Belgium) labeled microspheres were made as described.[20] One million iDC were incubated with 10^7 RITC MCs for 24 hours. After washing the free particles, the RITC-labeled microspheres inside the iDC were imaged with a Leica SP5 AOBS confocal microscope (63 × 1.4 oil objective, HCX PL APO 63.0 × 1.40 OIL UV), using the 543 nm HeNe laser line. Nuclei of the DCs were stained with 4′-6-diamidino-2-phenylindole included in the mounting medium Vectashield, and excited with the 405 nm line of a UV diode laser.

pIC was fluorescently labeled with the Label IT fluorescein isothiocyanate Nucleic Acid labeling kit (Mirus, Madison, WI) as described by manufacturers instructions.

Peptides. Peptides corresponding to the Gag sequence of consensus HIV-1 HxB2 were kindly provided by the National Institutes of Health AIDS Research Reagent Program (NIH, Germantown, MD). These peptides consisted of 15-mers overlapping by 11 amino acids. For most experiments, all Gag peptides ($n = 121$) or p24 peptides ($n = 60$) were pooled together (HxB2 Gag or p24 peptide pool).

DCs loading with MCs. After 1 week of culture with granulocyte-macrophage colony-stimulating factor and IL-4, iDC were harvested and incubated at which 10^6 DC/ml with empty or MCp24 (10–20 capsules/cell; 1.4 μg p24/ml) in 6-well plates (cultures of × 3 ml). One hour later (without washing), the maturation cocktail was added to a part of the cultures. Twenty-four hours later DCs were harvested, washed, and used for phenotyping or as stimulators for autologous PBL.

Protein pulsing of DCs. Pulsing with MCp24 was performed as described previously.[42] Briefly, 10^6 iDC/ml were washed with RPMI 1640 medium (Lonza, Verviers, Belgium) and incubated for 1 hour with 1.4 μg/ml (corresponding to 20 capsules/cell) or 0.7 μg/ml p24 (corresponding to 10 capsules/cell) (NIBSC). Afterwards, DC were matured, washed, and used as stimulators for autologous PBL.

Phenotyping of DCs. iDC (10^6 iDC/ml) were cocultured with p24 MCs tailored with or without pIC. After 1 hour of incubation either maturation cocktail was added or not. Twenty-four hours later DCs were harvested, washed, aliquoted (10^5 DC/fluorescence-activated cell sorting tube) and stained for 15 minutes at 4 °C with the following antibodies anti-CD80 [phycoerythrin (PE)], anti-CD86 (fluorescein isothiocyanate), anti-CD83 (PE), anti-HLA-DR (fluorescein isothiocyanate), anti-CD1a (PE), anti-CCR7 (PE), and anti-DC-SIGN (PE) (all antibodies purchased from Becton Dickinson, Erembodegem, Belgium). As a control nontreated iDC were used. The samples were analyzed on a FACScan (BD Biosciences, Erembodegam, Belgium) and data analysis was performed using WinMDI version 2.9 (Phoenix, AZ).

Induction of IL-12. Supernatant was collected after 24 hours from DC cultures incubated with MCsIL-12p70 enzyme-linked immunosorbent assay (eBioscience, Frankfurt, Germany) was performed according manufacturer's instructions.

Induction of HIV-1-specific T cells using antigen-loaded DCs. For T-cell stimulation, antigen-loaded DCs [matured with either optimized cytokine cocktail or pIC (1.3 μg/ml)] were cocultured with autologous PBL (ratio 1:10) in RPMI supplemented with 2.5% pooled human serum. After 10 days of culture, cells were always analyzed in IFN-γ or IL-2 enzyme-linked immunosorbent spot assays (Diaclone, Besançon, France). Briefly, freshly thawed PBL as well as PBL already stimulated with antigen-loaded DC were incubated side by side at a concentration of 2×10^5 cells/well with the Gag or p24 peptide pool (2 μg/ml) in antihuman IFN-γ or IL-2 (Diaclone) antibody-coated 96-well plates. For the development of spots, biotin-labeled antihuman IFN-γ or IL-2 was used. Spots were counted using an automated enzyme-linked immunosorbent spot reader (AID, Strassberg, Germany).

Induction of cellular and humoral immune responses in mice. Female C57BL/6 mice were purchased from Janvier (Le Genest Saint Isle, France) and housed in a specified pathogen-free facility in microisolator units. Animals ($n = 6$/group) were immunized by subcutaneous injection near the inguinal LNs on weeks 0 and 4 with following formulations (100 μl): MCp24 alone (polyelectrolyte MCs-containing 5 μg p24), MCp24 and 5 μg pIC, MCp24pIC (MCs-containing 5 μg p24 and tailored with pIC), 5 μg soluble p24 (sp24) or sp24 and 4 μg pIC. Control mice were immunized with PBS. At week 8, blood samples were collected and animals were sacrificed by cervical dislocation. Cells (2.10^5/well) from inguinal LNs (two per mice) were pooled for two animals and stimulated for 24 hours at 37 °C

with 2 μg/ml p24 peptide pool (NIH) in a murine IFN-γ and IL-2 enzyme-linked immunosorbent spot (Diaclone). Specific antibodies (IgG1 and IgG2c) in animal sera were detected by enzyme-linked immunosorbent assay.[16] Briefly 96-well plates were coated overnight at 4°C with 100 μl of a 1 μg/ml p24 solution in PBS buffer. The plates were washed three times with PBS-Tween and blocked for 2 hours at room temperature with 50 μl of a 2% nonfat dry milk solution in PBS–0.1% Tween. After washing serial dilutions of the mouse serum were prepared in PBS–Tween–1% fetal calf serum; 50 μl were added in duplicate and incubated for 2 hours at room temperature. After three washes with PBS-Tween, bound mouse antibodies were detected with 50 μl of a dilution of horse radish peroxidase-conjugated goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL; 1/2,000 dilution) or IgG2c (Immunology Consultants Laboratory, Newberg, OR; 1/10,000 dilution) in PBS–0.1% Tween–1% fetal calf serum for 1 hour at room temperature. Following five washes, plates were developed with 50 μl BD OptEIA reagents A and B (BD Bioscience) for 7 minutes in the dark and reaction was stopped with 25 μl of a 1 mol/l H_2SO_4. The optical density was measured at 450 nm in an automated plate reader. Antibody titers were obtained after linear regression.

Statistical analysis. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA). Paired (for *in vitro* experiments) or unpaired (for *in vivo* experiments) variables were compared using a paired *t*-test, *P* values of ≤0.05 (*) were considered significant.

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