Nanostructured Lipid Carriers to Deliver HIV p24 Antigen and CpG Immunostimulant in immunogenicity studies

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ABSTRACT

HIV caused 35 million deaths around the world. Despite the huge efforts spent in R&D in the last decades to develop new therapeutics and vaccines, the virus spread remains uncontrolled. With the last clinical trials evidencing the challenges to develop an effective prophylactic HIV vaccine, smart and innovative solutions to design vaccine formulations which overcome these immunological obstacles are still highly anticipated. In this context, we used nanostructured lipid carriers (NLC) for the delivery of p24 antigen as an HIV model antigen. We have designed tuned formulations comprising NLC grafted with p24, together with cationic NLC optimized for the delivery of CpG immunostimulant. This tailored system strongly promoted specific immune responses in mice. More importantly, the potency of NLC for the induction of specific immune responses was further supported by a study on non-human primates (NHP). This work paves the way towards the development of future HIV vaccines.

Keywords: Vaccines- HIV- lipid nanoparticles- adjuvant delivery systems

1 INTRODUCTION

HIV still remain a crucial infectious disease to cure for worldwide health care agencies. According to UNAIDS statistics from 2016, HIV is responsible for 35 million deaths around the world, with 76 million people having been infected since the beginning of the epidemic in the 1970s. Progresses in drug development led to highly active antiretroviral therapy (HAART), which considerably improved life expectancy and living conditions of infected people [1]. However, HAARTs alone are insufficient today to control the epidemic because of a lack of efficacy at eradicating the virus in treated individuals and the limited access offered for a majority of patients. Today, all epidemiological models predict that an efficient control of HIV spread would require efficient prophylactic strategies like vaccines. Particulate systems, including viral vectors and synthetic carriers, have proven to be excellent tools for the delivery of antigens to antigen presenting cells (APC), promoting immune specific responses with the production of antibodies and activation of cytotoxic T lymphocytes. Notably, particles of less than 100 nm in diameter are of high interest as they are capable of entering lymphatic vessels to reach lymph nodes where they directly interact with APC such as dendritic cells (DC) and macrophages [2]. Lipid particles are particularly advantageous compared to polymeric particles, in regard to their smaller size. More specifically, nanostructured lipid carriers (NLC) are highly attractive due to their improved stability in biological media compared to liposomes. To further promote antigen-associated immune responses, immunostimulatory molecules could also be transported by such particulate systems, in the aim to deliver a danger signal jointly with the antigen to APC for an optimal activation of these immune cells [3]. Here, we examined p24-bearing NLC immunogenicity in mice and non-human primates (NHP), which is the most relevant animal model for assessing immunity against HIV [4].

2 MATERIALS AND METHODS

Preparation of basic NLC

NLC were prepared as described elsewhere [5], [6]. Briefly, a lipid phase containing triglycerides (Suppocire NC™, Gattefossé; super refined soybean oil, Croda Uniqema) and phospholipids (Lipoid S75; Lipoid) and an aqueous phase containing PEGylated surfactants (Myrj™ S40, Croda Uniqema) were separately prepared before being added together for ultrasonication. Resulting NLC were purified by dialysis in PBS. Different batches were formulated by adjusting the composition of either the lipid phase or aqueous phase: NLC-SH for OVA grafting, NLC-mal for p24 grafting and cationic NLC (NLC+). All final particles were filtered on a 0.22 μm cellulosic membrane (Millipore) and characterized by their hydrodynamic diameter and zeta potential using respectively dynamic light scattering (DLS) and electrophoretic light scattering (ELS) (Zeta Sizer Nano ZS Malvern Instrument).

Preparation of NLC-p24 via NLC-mal

For p24 grafting, maleimide-presenting NLC (NLC-mal) were prepared by addition of (N-maleimidomethyl) cyclohexane functionalized PEGylated surfactants in aqueous phase and then purified by dialysis and filtered on a 0.22 μm membrane. Thiol groups were introduced on p24 (p24-SH) by derivatization of p24 (PX'Therapeutics) with 2-iminothiolane (Thermo Scientific). Excess linker was removed by eluting the whole solution on a Sephadex G25 gel in PD10 desalting column (GE Healthcare). p24-SH was added dropwise to a solution of NLC-mal. Unreacted maleimide functions were capped with 2-mercaptoethanol (Sigma Aldrich) and resulting NLC-p24 purified by SEC. Quantification was performed using DiD-loaded NLC-mal and fluorescein-tagged p24.

Preparation of NLC+

Cationic NLC (NLC+) were prepared by adding cationic and fusogenic surfactants (Avanti Polar Lipids) in the lipid phase, i.e. respectively DOTAP (1,2-dioleoyl-3-trimethylammonium-propane chloride) and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine).
Complexation of NLC+ with negatively charged CpG oligodeoxynucleotides (ODN1826 for mice and M362 for macaques, Invivogen) occurred spontaneously by mixing them together. This was verified by electrophoretic mobility shift assay.

Animals and ethics statement

BALB/c and C57BL/6 female mice were purchased from Janvier. They were housed in the animal facility of the Institute for Advanced Biosciences (France) according to institutional guidelines. The protocol for experimentation on animals was approved by the ethical committee (reference 2015062612254918).

Adult cynomolgus macaques (Macaca fascicularis) were imported from Mauritius and housed in the facilities of the Infectious Disease Models and Innovative Therapies (IDMIT) Center (CEA, Fontenay-aux-Roses, France). Both male and female were included with a sex-ratio of 1:1 in each experimental group. Non-human primates were used at the CEA in accordance with French national regulations and under the supervision of national veterinary inspectors (CEA Permit Number A 92-032-02). The CEA complies with the Standards for Human Care and Use of Laboratory Animals, of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number #A5826-01. The use of NHP at the CEA is in conformity with the recommendations of European Directive (2010/63, recommendation N°9). The animals were used under the supervision of the veterinarians in charge of the animal facility. This study was approved and accredited under statement number A15-085 by the ethics committee "Comité d'Ethique en Expérimentation Animale du CEA" registered under number 44 by the French Ministry of Research. Animals were housed by pairs in modules allowing social interactions, under controlled conditions of humidity, temperature and light (12-hour light/12-hour dark cycles). Water was available ad libitum. Animals were monitored and fed 1-2 times daily with commercial monkey chow and fruits by trained personnel. Macaques were provided with environmental enrichment including toys, novel foodstuffs and music under the supervision of the CEA Animal Welfare Body. Experimental procedures (animal handling, immunizations and blood samplings) were conducted after animal sedation with ketamine chlorhydrate (Rhône-Mérieux, Lyon, France, 10 mg/kg).

Immunizations and sampling

At day 0, 8-week old female BALB/c mice were immunized intra-peritoneally with a prime injection containing the equivalent of OVA or p24, free or grafted to NLC and co-administered with CpG. At day 21, boost was performed in same conditions. At day 28, blood samples were collected from cave vein using a 25G needle and spleens were harvested. Macaques were immunized at weeks 0, 6, 12 and 25 by the intradermal route. Blood samples were collected before and after each immunization to analyze specific antibody response in serum and cellular response in peripheral blood mononuclear cells (PBMC).

Antibody Assays

The measurement of anti-OVA and anti-p24 antibodies in mice sera was carried out by ELISA. Briefly, 96- well microplates were coated with antigen (OVA or p24) and stored overnight at 4°C. A PBS blocking solution containing 1% BSA was used for preventing non-specific adsorption. Diluted mice sera were applied to the wells for 2h at room temperature under gentle agitation. Then, an anti-mouse-HRP antibody (Sigma Aldrich) was added for 1h, followed by TMB enzymatic reaction (BD Biosciences), stopped with sulfuric acid after 15 min. Absorbance was read at 450 nm thanks to a VICTOR microplate reader (Perkin Elmer). Standard curves were performed using serial dilutions of monoclonal mouse anti-OVA (Enzo Life Sciences) and anti-p24 (Abcam) antibodies for the expression of antibody titers in immunized mice. The same protocol was used to measure antibodies in macaque sera, using an anti-human-HRP antibody (MyBioSource) for detection and a monoclonal human anti-p24 antibody (MyBioSource) to perform a comparative standard curve.

T lymphocyte Assays

Mouse fresh splenocyte suspensions were prepared from mice spleens by mechanical grinding on a 100 μm cell strainer and erythrocytes removal with RBC lysis buffer. Cells were stimulated for 3 days with 50 μg/mL OVA or 10 μg/mL p24 in RPMI-1640 complete culture medium supplemented with 10% FCS (Life Technologies), 1% penicillin/streptomycin (Invitrogen), 1% non-essential amino acids (Life Technologies) and 1% sodium pyruvate (Life Technologies). The supernatants were then isolated for IFNγ measurement using mouse IFNγ ELISA Ready-

Set-Go (eBiosciences). Specific T-cell responses were analyzed in macaques by IFNγ ELISPOT assay (Monkey IFNγ ELISpot PRO kit (Mabtech). Macaque PBMC were isolated from whole blood using vacutainer sodium heparin CPT tubes (BD Biosciences). PBMC were stimulated for 18 hours with a pool of 15-mer overlapping peptides covering the entire sequence of HIV p24. Spots were counted with an Automated ELISpot Reader ELR08IFL (Autoimmun Diagnostika GmbH).

Statistical analysis

Data are expressed as mean ± sem of the different analyzed variables (cell viability, cytokine secretion). *In vivo* data are compared among groups by using 1 way ANOVA (Analysis Of Variance) test followed by Fisher's protected least significant difference method (mice immunogenicity studies) or 2 way ANOVA test followed by Holm-Sidak's method (NHP immunogenicity studies) for pairwise multiple comparison.

3 RESULTS

We first set up the optimal conditions for vaccine antigen delivery. Gag p24 antigen was selected as a well characterized HIV-1 capsid protein antigen widely used in NHP studies. Moreover, its highly conserved structure makes it a suitable model as a proof of concept for the design of an HIV vaccine. Ovalbumin was used jointly to p24 as a control antigen. NLC were decorated with either OVA or p24 proteins (NLC-OVA and NLC-p24), allowing multimeric presentation of the antigen with an optimized ratio of 50 proteins per nanoparticle. Their hydrodynamic diameter is around 80 nm, with a slightly anionic surface charge at neutral pH.

The potency of these new particulate formulations was investigated *in vivo* by immunizing mice with different combinations of p24 and CpG, while keeping OVA as a control (***Figure 1***).

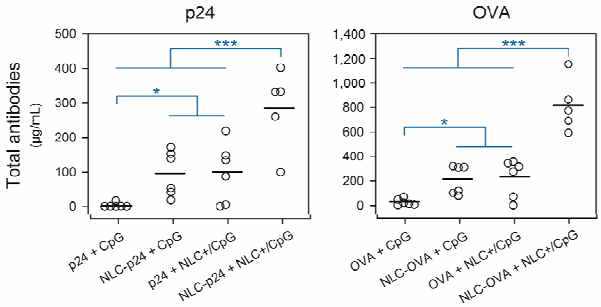

Figure 1: *Humoral response in mice after immunization with p24 and OVA formulations in co-administration with CpG formulations, expressed by total anti-p24 and anti-OVA antibody levels in mice sera. Each circle represents an individual mouse and bars represent the group mean. Data were compared among groups using 1-way ANOVA (Analysis Of Variance) test followed by Fisher's protected least significant difference test. *** p<0.001,; * p<0.05.*

Vectorization of p24 by NLC could enhance the production of specific antibodies by a factor 30 (*p24 + CpG: 3 ± 3 µg/mL; NLC-p24 + CpG: 94 ± 23 µg/mL; p=0.042*). Similarly, the delivery of CpG by NLC+ also increased antibody levels by a factor 30 (*p24 + NLC+/CpG: 99 ± 35 µg/mL; p=0.038 compared to p24 + CpG*). Therefore, the co-delivery of p24 and CpG on distinct carriers further significantly improved the humoral response as compared to all other groups (*NLC-p24 + NLC+/CpG: 285 ± 51 µg/mL; p<0.001 compared to all groups*). Similar trends were obtained with OVA formulations, as the combination of vectorized OVA and CpG significantly increased OVA-specific antibody titers, (*p<0.001 compared to all groups*).

The study of the cell-mediated immune response further highlighted the benefit from using NLC+ carrier, as it potentialized the production of IFNγ by p24-stimulated splenocytes (***Figure 2***).

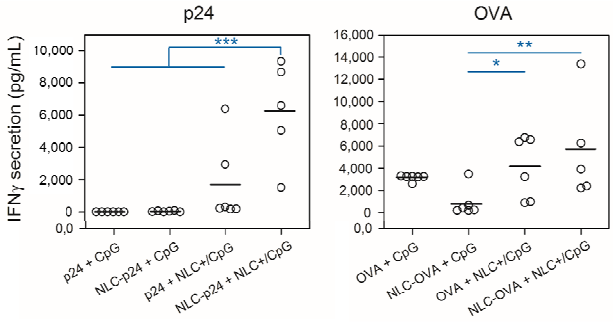

Figure 2*. T-cell response after mice immunization with p24 and OVA formulations in co-administration with CpG formulations, expressed by the secretion of IFNγ from splenocytes after 72h of antigen stimulation. Each circle represents an individual mouse and bars represent the group mean. Data were compared among groups using 1-way ANOVA test followed by Fisher's protected LSD test. *** p<0.001; ** p<0.01; * p<0.05.*

Besides, the combination of vectorized p24 and CpG significantly intensified IFNγ production as compared to all other groups (*P24 + CpG: 0 ± 0 pg/mL; NLC-p24 + CpG: 32 ± 11 pg/mL; p24 + NLC+/CpG: 1,696 ± 1,037 pg/mL; NLC-p24 + NLC+/CpG: 6,220 ± 1,403 pg/mL; p<0.001 compared to all groups*). The control with OVA formulations confirmed that the co-delivery of antigen and immunostimulant was the best formulation to stimulate T cells.

Our results obtained in mice undoubtedly demonstrated the significance of NLC as delivery systems. To further investigate the performance of NLC, p24 formulations were tested in non-human primates (NHP) taking cynomolgus macaques as a relevant model of human immunity. Three groups of four animals immunized by the intradermal route with vectorized p24 and CpG (*NLC-p24 + NLC+/CpG*), vectorized p24 plus free CpG (*NLC-p24 + CpG*) and free

molecules (*p24 + CpG*) were compared. The analysis of p24-binding antibodies revealed that the group immunized with NLC-p24 + NLC+/CpG stood out from the others in terms of significant levels of immunoglobulins.

In addition to the humoral response, specific T cell responses were evaluated by IFNγ ELISPOT. The analysis of IFNγ producing cells over time showed a very consistent trend with antibody results. The main difference was that all groups responded, although with different magnitudes. Since the first boost injection (week 8), the group NLC-p24 + NLC+/CpG was significantly more efficient at activating T cells than the other groups.

4 CONCLUSION

Altogether, these results especially highlighted the critical role of NLC+ to deliver CpG jointly to p24, as this was mandatory for promoting both humoral and T cell immune responses. The benefit from the use of NLC was demonstrated by its versatility, enabling to successfully vectorize p24 antigen as well as CpG immunostimulant, and the resulting enhancement of immune responses in NHP. To our knowledge, the description of p24-specific immune responses in NHP of such a high magnitude was rarely if ever reported with synthetic carriers.

In perspectives, NLC is intended to be further associated with other HIV antigens, in the aim to generate complex and multi-targeted immune responses necessary for protection against HIV/SIV challenge.

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