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# 1 Poly(lactic acid) Nanoparticles and Cell-Penetrating 2 Peptide potentiate mRNA-based vaccine expression in 3 Dendritic cells triggering their activation

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## 9 ABSTRACT

10 Messenger RNA-based vaccines have the potential to trigger robust cytotoxic immune  
11 responses, which are essential for fighting cancer and infectious diseases like HIV. Dendritic  
12 Cells (DCs) are choice targets for mRNA-based vaccine strategies, as they link innate and  
13 adaptive immune responses and are major regulators of cytotoxic and humoral adaptive  
14 responses. However, efficient delivery of antigen-coding mRNAs into DC cytosol has been  
15 highly challenging. In this study, we developed an alternative to lipid-based mRNA delivery  
16 systems, using poly(lactic acid) nanoparticles (PLA-NPs) and cationic cell-penetrating  
17 peptides as mRNA condensing agent. The formulations are assembled in two steps: (1)  
18 formation of a polyplex between mRNAs and amphipathic cationic peptides (RALA, LAH4  
19 or LAH4-L1), and (2) adsorption of polyplexes onto PLA-NPs. LAH4-L1/mRNA polyplexes  
20 and PLA-NP/LAH4-L1/mRNA nanocomplexes are taken up by DCs *via* phagocytosis and  
21 clathrin-dependent endocytosis, and induce strong protein expression in DCs *in vitro*. They  
22 modulate DC innate immune response by activating both endosome and cytosolic Pattern  
23 Recognition Receptors (PRRs), and induce markers of adaptive responses in primary human  
24 DCs *in vitro*, with prevalent Th1 signature. Thus, LAH4-L1/mRNA and PLA-NP/LAH4-  
25 L1/mRNA represent a promising platform for *ex vivo* treatment and mRNA vaccine  
26 development.

27

28 **Keywords:** mRNA-vaccine, nanoparticle (NP), poly(lactic acid) (PLA), LAH4-L1, Cell-  
29 Penetrating Peptide (CPPs), dendritic cells

30

## 31           **1. Introduction**

32           Over the past decades, traditional vaccines consisting of killed, inactivated or subunit  
33 pathogens have been successfully used against infectious diseases allowing an efficient and  
34 protective humoral immune response [1]. Despite their major successes in modern medicine,  
35 these approaches have limitations and are not applicable for chronic infections or cancers  
36 where cellular immune responses are mostly required [2]. These diseases require vaccine  
37 formulations that promote robust cell-mediated (Th1-biased) immune response instead of  
38 humoral (Th2-biased) immune response. Cellular immune responses with CD8<sup>+</sup> cytotoxic T  
39 cells (CTLs) have the unique potential to eliminate infected or altered cells, and may have a  
40 central role in host responses to viral infection like HIV and cancers [3]. CTLs are elicited  
41 against intracellular antigens produced in the cytosol, processed by the proteasome and loaded  
42 onto major histocompatibility class I (MHC-I) complexes for cell surface presentation [4].  
43 This requires that vaccine proteins have to be delivered to the cytosol of antigenic presenting  
44 cells (APCs), like dendritic cells (DCs), which play a central function in immunity by  
45 orchestrating innate and adaptive immune responses, through antigen presentation [5].

46           In recent years, interest in using *in vitro* transcribed (IVT) messenger RNA (mRNA)  
47 to elicit CTLs and/or Th1-biased immune responses has exponentially increased [6–9].  
48 mRNA vaccines have several advantages over other vaccine approaches, like a high safety  
49 profile, a flexibility to encode any protein as antigen and a simple, inexpensive and rapid  
50 production, which is of great importance in case of pandemic crisis [6,9,10]. Moreover,  
51 mRNA is transiently translated in cells and is degraded in a relatively short but controllable  
52 amount of time [7,9]. This capacity allows a more controlled antigen exposure and minimizes  
53 the risk of tolerance induction that can be associated with long-term antigen exposure [10].  
54 One other important advantage is the higher capacity of mRNA-based vaccines to transfect  
55 non-dividing cells, as DCs, where mRNA is translated into functional protein [10]. This  
56 intracellular production of antigenic protein is responsible for antigen presentation and T-cell  
57 activation, with both CD4<sup>+</sup> and CD8<sup>+</sup> T aspects. That is why, mRNA have shown  
58 considerable promises for both prophylaxis and the treatment of various diseases, including  
59 HIV [9,11] and more than ten types of cancers [9,12].

60           However, to induce mRNA expression in DC, their efficient delivery into the cytosol  
61 before degradation must be achieved. In fact, exogenous mRNA must penetrate the barrier of  
62 the lipid membrane in order to reach the cytoplasm to be translated into a functional protein.

63 Two major strategies have been described to target DCs with mRNA [9]. The first strategy is  
64 based on *ex vivo* mRNA loading into DCs and re-infusion of the transfected cells [13]. *Ex vivo*  
65 DC loading allows an optimal control of the cellular target with high transfection efficiency.  
66 However, this is an expensive and constrained approach. The second strategy consists in a  
67 direct mRNA injection in a more rapid and cost-effective approach, but without cell type  
68 specificity. Both approaches have been explored with a variety of formulations to protect the  
69 antigenic mRNA from RNases, facilitate its uptake into cells and improve their potency [9].  
70 Promising results have been obtained with several types of delivery system (reviewed in [9]),  
71 including polyplexes [14–18] or lipoplexes [19–22]. But the most efficient approach currently  
72 used is based on mRNA vectorization of cationic lipid-based nanoparticles, and particularly in  
73 lipid nanoparticles (LNP) [23–27]. Some formulations have already entered clinical trials in  
74 the field of cancer immunotherapy and infectious diseases like *influenza* virus and HIV [9]. In  
75 contrast, reports on the use of polymeric nanoparticle carriers for mRNA delivery are scarce,  
76 even if they have shown great potentials in vaccination field [28–31].

77 Biodegradable nanoparticles based on poly(lactic acid) (PLA) backbone have gained  
78 recent interest in vaccine development. Indeed, PLA is a biodegradable and biocompatible  
79 polymer which presents a high safety profile [31] and has been approved by the Food and  
80 Drug Administration [32]. PLA-nanoparticles (PLA-NPs) constitute a versatile vectorization  
81 platform for their capacity to encapsulate and/or adsorb various antigens and  
82 immunostimulant molecules [29,30,33,34]. PLA-NPs have shown advantages in the  
83 vaccinology field for the induction of immune responses against various model antigens after  
84 parenteral administration in mammals [30,33,35]. Moreover, PLA-NPs are efficiently taken  
85 up by DCs *in vitro* and *in vivo* [33,36,37]. In fact, the nanoparticle format presents advantages  
86 for the delivery of mRNA to DCs and could improve their uptake by these cells [38].  
87 However, both the PLA-NP surface and mRNA biomolecules are negatively charged. To  
88 adsorb mRNAs onto the surface of PLA-NPs, it is necessary to reverse the net surface charge  
89 of the NP or to design a mRNA intermediate complex which is positively charged. Thus, the  
90 use of cationic intermediates can be a promising approach. Indeed, in a pioneering work,  
91 Bettinger *et al.* demonstrated that low molecular weight cationic polymers form complexes  
92 with mRNAs [18]. Moreover, the addition of a membrane-active peptide is required for the  
93 endosomal escape of these complexes, and efficient mRNA translation in the cytosol. Recent  
94 studies have proposed that cationic cell-penetrating peptides (CPPs), small peptides composed  
95 of 8 to 30 amino acids with a net positive charge, might constitute an excellent vehicle for

96 mRNA delivery [39]. They combine low charge densities with an ability to induce membrane  
97 disruption for endosomal escape. In fact, this process is essential to allow cytosol delivery of  
98 mRNA where it can be recognized by protein synthesis apparatus and translated into protein  
99 [10].

100 In this study, we have developed and compared new mRNA platforms using three  
101 different CPPs (RALA, LAH4 and LAH4-L1) as cationic intermediates for vectoring mRNA  
102 onto PLA-NPs. We have evaluated the interest of these peptide/mRNA polyplexes and PLA-  
103 NP/peptide/mRNA nanocomplexes to transfect DCs and activate innate and immune signaling  
104 responses through transcriptomic analysis. The highest protein expression in DCs was  
105 observed with LAH4-L1 and PLA-NP/LAH4-L1 formulations. These formulations trigger  
106 innate immunity through PRR activation and activate adaptive immune responses, including a  
107 prevalent Th1 aspect. To understand the mechanisms of each formulation involved in these *in*  
108 *vitro* activations, we also have investigated the uptake mechanism and the intracellular traffic  
109 of particles in DCs, indicating a clathrin-mediated endocytosis and phagocytosis pathways.  
110 Thus, these data show that LAH4-L1/mRNA polyplexes and PLA-NP/LAH4-L1/mRNA  
111 nanocomplexes are promising platforms for mRNA vaccine delivery to DCs.

## 112 **2. Materials and methods**

### 113 ***a. Production of in vitro transcribed mRNA***

114 pGEM4z-HxB2gag-64A encoding Gag antigen was kindly given by Guido Vanham  
115 (University of Antwerp, Antwerp, Belgium). The HxB2 gag gene is under the control of a T7  
116 promoter and a unique *EheI* restriction site is located just downstream of the (A)<sub>64</sub> tail.  
117 pGEM4z-HxB2gag-64A was propagated in NEB® Stable *Escherichia coli* bacteria (New  
118 England Biolabs (NEB), France) and purified using NucleoBond Xtra Maxi Endotoxin Free  
119 kit (Macherey-Nagel, France). The plasmid was linearized with *EheI* restriction enzyme  
120 (NEB), purified using a Wizard Genomic DNA Purification Kit (Promega, France), and used  
121 as a DNA template for *in vitro* transcription. Capped mRNA transcripts (IVT) were produced  
122 using the T7 MessageMachine Kit (Ambion, Life technologies, France). mRNA purification  
123 was performed by DNase I digestion, followed by LiCl precipitation and mRNA pellet was  
124 solubilized in RNase-free water.

125 eGFP mRNA encoding green fluorescent protein was provided by Guido Vanham  
126 (University of Antwerp, Antwerp, Belgium). RNA was quantified by spectrophotometric

127 analysis at 260 nm and analyzed by standard agarose gel electrophoresis to confirm the  
128 integrity of this full-length mRNA. All mRNAs were stored in RNase free water at  $-80^{\circ}\text{C}$ .

### 129 ***b. Production of fluorescent labeled mRNA***

130 Gag mRNA was labeled with MF488 using the Label IT Nucleic Acid Labeling  
131 Reagent kit (Mirus, Euromedex, France) according to the manufacturer's instructions. Briefly,  
132 5  $\mu\text{g}$  of mRNA was stained with kit reagents for 1 h at  $37^{\circ}\text{C}$ , and fluorescent mRNA was  
133 purified using ethanol precipitation. The pellet was resuspended with 10  $\mu\text{L}$  of nuclease-free  
134 water and mRNA labeled was quantified by spectrophotometric analysis, while mRNA  
135 integrity was checked through agarose gel electrophoresis.

### 136 ***c. Polyplexes and nanocomplexes preparation and characterization***

137 To form polyplex, mRNA was diluted in RNA-free water at 40  $\text{ng}/\mu\text{L}$  and mixed  
138 (V/V) with 800  $\text{ng}/\mu\text{L}$  in RNA-free water of LAH4-L1  
139 (KKALLAHALHLLALLALHLAHALKKA), LAH4  
140 (KKALLALALHHLAHLALHLALALKKA) or RALA  
141 (WEARLARALARALARHLARALARALRACEA) peptides (GeneScript, China). Then,  
142 equal volumes of those polyplexes and PLA-NP (at 0.5% in water; Adjuvatis, France) were  
143 mixed to form PLA-NP/Peptide/mRNA nanocomplexes.

144 The hydrodynamic diameter and zeta potential (surface charge) of mRNA  
145 formulations were determined by dynamic light scattering (DLS) using the Zetasizer Nano ZS  
146 (Malvern Instrument, U.K.).

147 Complexes were also analyzed using an electrophoretic mobility shift assay (ESMA).  
148 In some samples, a heparin/proteinase K treatment was performed in order to separate mRNA  
149 from the complexes. Briefly, complexes were first treated or not with heparin (Sigma, France)  
150 during 1 h at RT, and secondly with proteinase K (NEB, France) at  $56^{\circ}\text{C}$  during 30 min. Each  
151 sample (treated or not) containing 200  $\text{ng}$  mRNA was loaded into one well and analyzed by  
152 electrophoresis in a standard 1% agarose gel in presence of ethidium bromide (Genesee  
153 Scientific).

154 To highlight the association between mRNA and PLA-NP, a Fluorescence Resonance  
155 Energy Transfer (FRET) assay was performed. Components were associated as described  
156 above, with a Ribogreen fluorescent mRNA (Quant-IT Ribogreen<sup>TM</sup> RNA assay kit,  
157 Invitrogen) and CellTrace<sup>TM</sup> BODIPY-TR Methyl Ester fluorescent PLA-NP (Adjuvatis,  
158 France). 100  $\mu\text{L}$  of each complex was deposited in triplicate in black 96-plate well (flat-  
159 bottom). Fluorescence was recorded on Tecan i-control Infinite M1000) (Tecan, Swiss) for

160 Ribogreen<sup>TM</sup> (460nm/520nm), BODIPY-TR (561nm/625nm) and FRET (460nm/625nm).  
161 Fluorescence was determined as the mean of three independent experiments and results were  
162 normalized to remove the contribution of free PLA-NPs.

#### 163 *d. Expression efficiency analysis*

164 Immortalized DC2.4 (a murine bone marrow derived dendritic cell lines [40] and  
165 kindly provided by Jacqueline Marvel group (CIRI, France)) was propagated in complete  
166 RPMI-1640 medium with 10 % heat-inactivated fetal bovine serum (FBS), 10 mM HEPES and  
167 50  $\mu$ M  $\beta$ -mercaptoethanol (Gibco, Life Technologies, France) at 37° and 5 % CO<sub>2</sub>. HeLa  
168 (ATCC<sup>®</sup> CCL-2<sup>™</sup>) and HEK293(ATCC<sup>®</sup> CRL-1573<sup>™</sup>) were cultured in DMEM containing  
169 10% heat-inactivated FBS (Gibco, Life Technologies, France) at 37°C and 5 % CO<sub>2</sub>. Cells  
170 were used with a low passage number (less than 12).

171 One day before transfection, cells were seeded in a 96-well plate at the density of  
172 25 000 cells (in 100  $\mu$ L of complete medium) per well. After 24 h, complete medium was  
173 replaced by 100  $\mu$ L of serum-free medium. Formulations were added to cells with a volume  
174 allowing the transfection of 20 ng of eGFP mRNA or equivalent volume for controls. Four  
175 hours after transfection, supernatants were removed and 100  $\mu$ L of complete medium was  
176 added. Then, cells were incubated at 37°C and 5% CO<sub>2</sub> until the analysis (0 to 48 h). Positive  
177 control of transfection was performed with Trans IT<sup>®</sup>-mRNA Transfection Kit (Mirus,  
178 Euromedex, France) using the manufacturer's recommendations. Briefly, 0.5  $\mu$ g of mRNA  
179 was added to 50  $\mu$ L of serum-free medium, and 1  $\mu$ L of Trans IT mRNA reagent and 1  $\mu$ L of  
180 mRNA Boost Reagent were then added. After 5 min at room temperature, complex mixture  
181 was added to cell at the desired concentration. Negative controls were performed with peptide  
182 alone, PLA-NP/Peptide complex, naked PLA-NP and naked mRNA at equivalent  
183 concentrations that nanocomplexes.

184 To quantify the percentage of eGFP fluorescent-positive cells and Mean Fluorescence  
185 Intensity (MFI), transfected DCs were harvested, stained with propidium iodide (Invitrogen)  
186 and analyzed by AccuriC6 flow cytometer (BD Biosciences) on the platform "AniRA  
187 cytométrie" (SFR BioSciences, UMS3444/CNRS). Dead cells were excluded using propidium  
188 iodide, and then living DCs were gated based on their large forward scatter (FSC) and large  
189 side scatter (SSC) profile, to avoid cell debris. Data analysis was performed with BD Accuri  
190 C6 Software CFlow Plus (BD Biosciences). Data were determined as the mean of three  
191 independent experiments.

192 ***e. Stability of formulations***

193 To verify formulation stability, polyplexes and nanocomplexes were prepared and  
194 conserved at 4°C during 21 days. At each time-point, the hydrodynamic diameter and zeta  
195 potential were determined by DLS using the Zetasizer Nano ZS (Malvern Instrument, U.K.)  
196 and the expression efficiency was determined as described in the precedent part by flow  
197 cytometry.

198 ***f. Cytotoxicity of formulations***

199 Cytotoxicity of complexes was evaluated by Presto Blue Assay (Thermo Fisher  
200 Scientific) according to the manufacturer's instructions. Briefly, DC2.4 cells were seeded at a  
201 density of 25 000 cells/well into 96-well plates a day prior to the transfection. Transfection  
202 with complexes was performed, as previously described, with a volume allowing the  
203 transfection of 20 ng of eGFP mRNA or equivalent volume for controls, and cytotoxicity was  
204 measured 24 h later. After incubation, 10 µL of Presto Blue Reagent was added and plates  
205 were incubated 10 min at 37°C. Fluorescence was detected on Tecan i-control Infinite M1000  
206 (560nm/590nm; Bandwidth 10 nm; Gain 91) (Tecan, Swiss). Fluorescence was determined as  
207 the mean of two replicates and three independent experiments.

208 ***g. Endocytosis mechanisms analysis***

209 DC2.4 cells were seeded into 96-well plates at the density of 25000 cells/well a day  
210 prior to the transfection. Medium was removed 15 min before transfection and 100 µL of  
211 serum-free medium in presence of one molecule inhibitor was added. Used concentrations and  
212 mode of action of genistein, chlorpromazine, dynasore, nocodazole, amiloride, pimozide and  
213 cytochalasine inhibitors were indicated in supplementary data (**Table I**). After 15 min, cells  
214 were treated with formulations (with a volume allowing the transfection of 20 ng of eGFP  
215 mRNA or equivalent volume for controls) during 2 h. To analyze uptake efficiency, cells  
216 were incubated with an MFP488 fluorescent mRNA alone or in complex. After 2 h, cells were  
217 harvested, treated with 0.2% trypan blue and analyzed by flow cytometry, as described  
218 previously.

219 ***h. Endosome escape evaluation***

220 DC2.4 were seeded into 96-well plates at the density of 25000 cells/well a day prior to  
221 the transfection. Cells were treated 15 min before transfection with 175 mM of Bafilomycine  
222 A1 (Invivogen, France) in 100 µL of serum-free media. Complexes and associate controls  
223 were added (with a volume allowing the transfection of 20 ng of eGFP mRNA or equivalent



224 volume for controls) for 4 h. After medium replacement, cells were incubated 12 h at 37°C  
225 and 5% CO<sub>2</sub>, and then they were harvested and analyzed by flow cytometry as described  
226 previously.

#### 227 *i. Immunostaining of moDCs*

228 To verify gag expression in moDCs, p17 protein (a component of gag polyprotein)  
229 was stained in moDCs. Cells were seeded at a seeding density of 50 000 cells/well into μSlide  
230 12 well Chamber (Ibidi, Germany) and transfected as previously described with a volume  
231 allowing the transfection of 40 ng of mRNA or equivalent volume for controls in 200 μL of  
232 serum-free medium for 2 h. Then, cells were rinsed with PBS 1X, fixed with 4%  
233 paraformaldehyde for 20 min at RT, permeabilized in 0.1% Triton-PBS 1X at RT for 15 min  
234 and saturated with 1% BSA-PBS 1X for 1 h at RT. After saturation, cells were incubated 2 h  
235 with anti-p17 (5 μg/mL; kindly given by Christophe Guillon, CNRS/University of Lyon,  
236 France), washed and stained with secondary antibody FITC anti Mouse IgG (5 μg/mL;  
237 Jackson Immuno Research) in the dark for 1 h at RT. Then, cells were washed and mounted  
238 on glass slides using Vectashield mounting medium with DAPI (Vector laboratories). Slides  
239 were analyzed using a SP5 upward confocal microscope (Leica) with 63×/1.4NA objective  
240 and data were analyzed using ImageJ Software.

#### 241 *j. Determination of innate immune-stimulatory activity*

242 HEK-Blue hTLR7 and HEK-Blue hTLR73 reporter cell lines (InvivoGen) were used  
243 as specified by the manufacturer to detect TLR7 and TLR3 stimulation. Briefly, 50 000  
244 cells/well were seeded into 96-well plates and stimulated during 24 h with the different  
245 formulations. TLR7 or TLR3 activation was monitored by quantifying the amount of the  
246 secreted embryonic alkaline phosphatase (SEAP) which is placed under the control of IFN-β  
247 minimal promoter fused to five NF-κB and AP-1-binding sites. The level of alkaline  
248 phosphatase can be determined with a detection medium that turns blue in its presence.  
249 Sample absorbance was measured at 620 nm using a microplate reader (Bio-Rad).

#### 250 *k. Preparation and stimulation of human monocyte-derived DCs*

251 Monocytes were purified from human peripheral blood and differentiated into moDCs  
252 as previously described [33]. Briefly, PBMCs were isolated from venous blood samples (from  
253 Etablissement Français du Sang, Lyon) by density gradient centrifugations using Ficoll-  
254 Paque™ plus and Percoll (GE Healthcare). Remaining erythrocytes, NK, B- and T- cells were  
255 then depleted by anti-glycophorin A, anti-CD56, anti-CD19 and anti-CD3 antibodies

256 (Beckman Coulter) respectively, using Dynabeads® Pan Goat antimouse IgG (Invitrogen). To  
257 differentiate purified monocytes ( $0.5 \times 10^6$  cell/ml) into MoDCs, cells were cultured in RPMI  
258 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), gentamycin (50  
259 U/ml), 100 U/ml penicillin and 100 µg/ml streptomycin in the presence of 62.5 ng/ml of  
260 human interleukin-4 (IL-4) (Miltenyi Biotec, France) and 75 ng/ml of human granulocyte  
261 macrophage colony stimulating factor (GM-CSF) (Miltenyi Biotec, France). After 6 days,  
262 complexes were added to  $1 \times 10^6$  differentiated moDCs in similar culture medium depleted of  
263 serum for 2 h (volume allowing the transfection of 660 ng of gag mRNA or equivalent  
264 volume for controls). After addition of FCS, cells were incubated 24 h or 48 h at 37 °C, 5%  
265 CO<sub>2</sub>. DCs only cultured in complete RPMI medium were used as a negative control of  
266 stimulation, and those cultured in the presence of 2.5 µg/ml of LPS (Invivogen, France) were  
267 used as a positive control.

268 DC maturation was assessed by cell surface immunostaining using monoclonal  
269 antibodies against lineage markers and co-stimulatory molecules. Cells were then stained with  
270 anti-CD1a-FITC, anti-CD14-FITC, anti-HLA DR-FITC, anti HLA-ABC-FITC, anti-CD40-  
271 PE, anti-CD80-PE, and anti-CD86-PE mAbs (BD Pharmingen). Data were acquired using a  
272 FACSCanto flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree  
273 Star).

#### 274 *l. Real-time qPCR analyses*

275 The different complexes were incubated 2 h to  $4 \times 10^6$  of differentiated moDCs  
276 without serum (volume allowing the transfection of 2640 ng of gag mRNA or equivalent  
277 volume for controls). FBS was added and cells were incubated at 37 °C, 5% CO<sub>2</sub>. After 6h of  
278 incubation, RNA was extracted from moDCs using QIamp RNA Blood Mini Kit (Qiagen).  
279 Using 0.5 ng of mRNA per conditions, the expression of 84 genes was quantified using an  
280 RT<sup>2</sup> Profiler PCR Array Human Innate and Adaptive Immune Response plate (PAHS-052Z,  
281 Qiagen). Data were acquired using a AriaMx Real-time PCR System (Agilent) and analyzed  
282 with GeneGlobe Data Analysis Center (Qiagen).

#### 283 *m. Quantification of cytokines*

284 The experimental procedure is similar to the one used for moDC stimulation. After  
285 stimulation by complexes during 24 h and 48 h, supernatant was harvested and cytokine and  
286 chemokine quantifications were performed using a Luminex Magpix instrument (Bio-Rad) in  
287 combination with human Miliplex cytokine/chemokine immunoassay kit (HCYTMAG-60K-  
288 PX29, Merck). Data were analyzed with BioPlex Manager (BioRad). For each condition,

289 concentrations were determined as the mean of three replicates for each independent  
290 experiment (N=3). We excluded from the standard curve any points with accuracy outside of  
291 70-130 %. The coefficient of variation of the three assayed were shown to be less than 20%  
292 CV for all data.

### 293 *n. Statistical Analysis*

294 Statistical analysis was performed using GraphPad Prism Version 6.0 software. All of  
295 the data are presented as the mean  $\pm$  SEM. Difference between groups was analyzed as  
296 described in figure legends. Statistical significances were indicated on the figures.

## 297 **3. Results:**

### 298 *a. mRNAs can be associated onto PLA-NPs via cationic peptides*

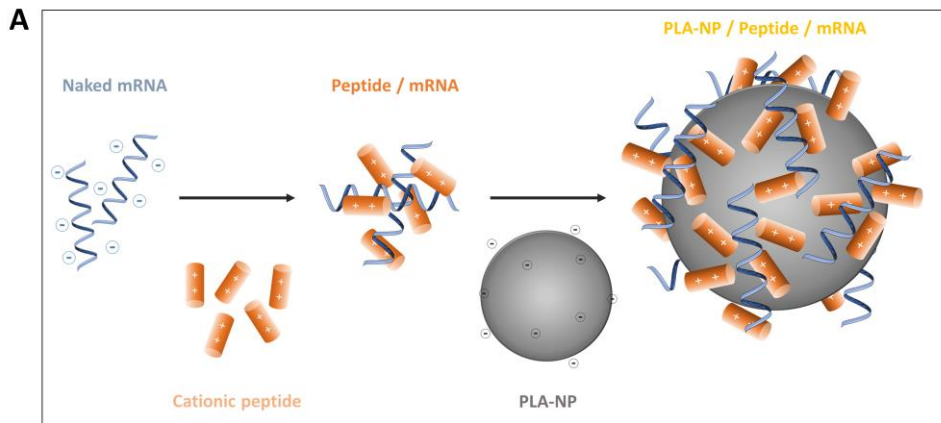
299 In order to vectorize mRNAs, we designed novel formulations based on polymeric  
300 nanoparticles, PLA-NPs, which have demonstrated several advantages in the vaccination  
301 field. Whereas hydrophobic molecules can be efficiently encapsulated in the core of PLA-  
302 NPs, the hydrophilic nature of mRNAs prevents their simple encapsulation into PLA-NPs.  
303 Instead, we developed strategies to adsorb mRNAs at the surface of PLA-NPs. Because the  
304 PLA-NP surface and mRNAs are both negatively charged, we used cationic peptides as an  
305 intermediate between mRNA and PLA-NPs. We compared three positively charged cell-  
306 penetrating peptides (CPPs) with nucleic acid condensation properties: RALA, LAH4 and  
307 LAH4-L1. Those cationic peptides are predicted to have a helical structure with amphipathic  
308 profile, which presents advantages for cellular uptake and membrane disruption (**Fig. S1**). In  
309 this strategy, mRNAs were first complexed with cationic peptides to form polyplexes, which  
310 were subsequently adsorbed onto PLA-NPs to produce nanocomplexes, as described in  
311 Materials and Methods section (**Fig. 1A**). The PLA-NP doses used in the formulations were  
312 shown to be non-toxic in a previous work from our laboratory [41].

313 The hydrodynamic diameter and zeta potential of nanocomplexes formed with the 3  
314 cationic peptides were analyzed by DLS (**Fig. 1B**). The addition of Peptide/mRNA polyplexes  
315 onto PLA-NPs (~ 188 nm; -50 mV) induced an increase in size and an inversion of the surface  
316 charge, as reflected by the zeta potential of ~ 30mV, suggesting that the 3 types of polyplexes  
317 are efficiently adsorbed onto PLA-NPs. The LAH4 peptide induced the formation of larger  
318 nanocomplexes (~ 275.8 nm) than RALA and LAH4-L1 (~ 237.8 nm and ~ 220.1 nm,  
319 respectively) with the highest polydispersity index (PdI). For RALA and LAH4-L1 peptides,  
320 the polydispersity indexes in the final formulations were close to 0.1 (PdI=0.134 and 0.129,

321 respectively), reflecting that the nanocomplexes were homogenous in size. As shown for  
322 LAH4-L1 in Fig. S2, the pre-formulation peptide/mRNA polyplexes presented a high  
323 polydispersity index (PdI=0.3), the size distribution graph indicating the presence of  
324 aggregates. Yet, the addition of PLA-NP, which are highly monodisperse and negatively  
325 charged, allowed a modification of the equilibrium of charges to form a monodisperse final  
326 product (PdI=0.129) with a rearrangement of peptide/mRNA polyplexes at the surface of  
327 PLA-NP. Finally, we showed that the colloidal formulations were stable for 14 days at 4°C, as  
328 indicated by hydrodynamic diameter and zeta potential measurements (results obtained with  
329 LAH4-L1 peptide are shown in **Fig. S3**).

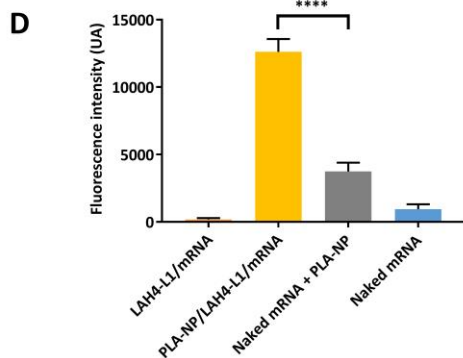
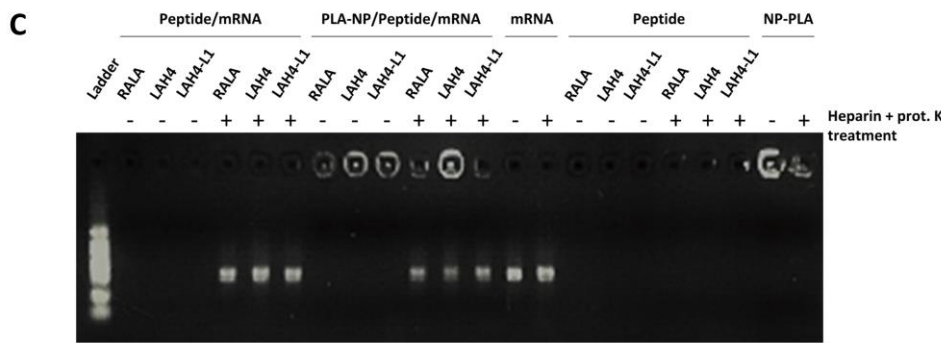
330 To verify the ability of peptides and PLA-NPs to bind mRNAs, an electrophoretic  
331 mobility shift assay (EMSA) was applied (**Fig. 1C**). To detach mRNA from the complexes, a  
332 treatment with heparin (for PLA-NP detachment) and proteinase K (for peptide degradation)  
333 was performed. The same results were obtained for RALA, LAH4 and LAH4-L1 peptides:  
334 mRNA complexed with one of these peptides or in PLA-NP nanocomplexes were retained in  
335 the wells, while their electrophoretic mobility was identical to that of naked mRNA after  
336 heparin/proteinase K treatment. To confirm the binding of Peptide/mRNA polyplexes onto the  
337 surface of PLA-NPs, another test based on Fluorescence Resonance Energy Transfer (FRET)  
338 was developed. In this test, we used red fluorescent PLA-NPs (containing BODIPY-TR  
339 Methyl Ester dye) and a green fluorescent mRNA (labelled with Ribogreen<sup>TM</sup>). The close  
340 proximity of green fluorescent mRNAs to red fluorescent PLA-NPs causes red fluorescence  
341 emission (625 nm) upon Ribogreen<sup>TM</sup> excitation (460 nm). PLA-NP/LAH4-L1/mRNA  
342 nanocomplexes displayed significantly higher 625 nm emission than that obtained in samples  
343 containing a simple mix of mRNAs and PLA-NPs (**Fig. 1D**), confirming the presence of  
344 mRNA nearby the core of PLA particles (distance < 10 nm).

345 Altogether, these results confirm the ability to form stable nanocomplexes of mRNAs and  
346 PLA-NPs using RALA, LAH4 or LAH4-L1 peptides. mRNA condensation capacity of CPPs  
347 was conserved after polyplex adsorption onto PLA-NP.



**B**

Nanocomplex	Hydrodynamic diameter (nm)	PdI	Zeta potential (mV)
PLA-NP	188.0 ( $\pm$ 1.80)	0.053 ( $\pm$ 0.046)	- 51.8 ( $\pm$ 0.6)
PLA-NP / RALA / mRNA	237.8 ( $\pm$ 29.2)	0.134 ( $\pm$ 0.057)	+ 34.8 ( $\pm$ 5.2)
PLA-NP / LAH4 / mRNA	275.8 ( $\pm$ 43.2)	0.244 ( $\pm$ 0.051)	+ 29.0 ( $\pm$ 4.4)
PLA-NP / LAH4-L1 / mRNA	220.1 ( $\pm$ 22.9)	0.129 ( $\pm$ 0.058)	+ 37.3 ( $\pm$ 4.4)



348

349 **Fig 1. mRNA can be vectorized by PLA-NPs using cationic peptides as intermediates.** (A)  
 350 Schematic representation of the vectorization strategy of mRNAs onto PLA-NPs. Negatively charged mRNA was  
 351 associated with cationic peptides (RALA, LAH4 or LAH4-L1) to form Peptide/mRNA polyplexes. Then, this  
 352 complex was adsorbed onto PLA-NPs to form PLA-NP/Peptide/mRNA nanocomplexes. (B) Comparison of  
 353 thermodynamic diameter, polydispersity index (PdI) and zeta potential of nanocomplexes formed between the 3  
 354 peptide/mRNA combinations and naked PLA-NPs. Each value provided is the average of four series of  
 355 measurements and four independent experiments (N=4). (C) Electrophoretic mobility shift assay showing eGFP  
 356 mRNAs complexed with peptides or in PLA-NP/Peptide/mRNA nanocomplexes. A heparin and proteinase K  
 357 (prot. K) treatment (+) was applied to the samples in order to detach the mRNA from the complexes. (D)  
 358 Fluorescence Resonance Energy Transfer (FRET) analysis. Red fluorescence emitted by different formulations  
 359 was evaluated after green fluorescent mRNA (RiboGreen) excitation (n=3). Statistical significance between two  
 360 groups was determined using a one-way Anova: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

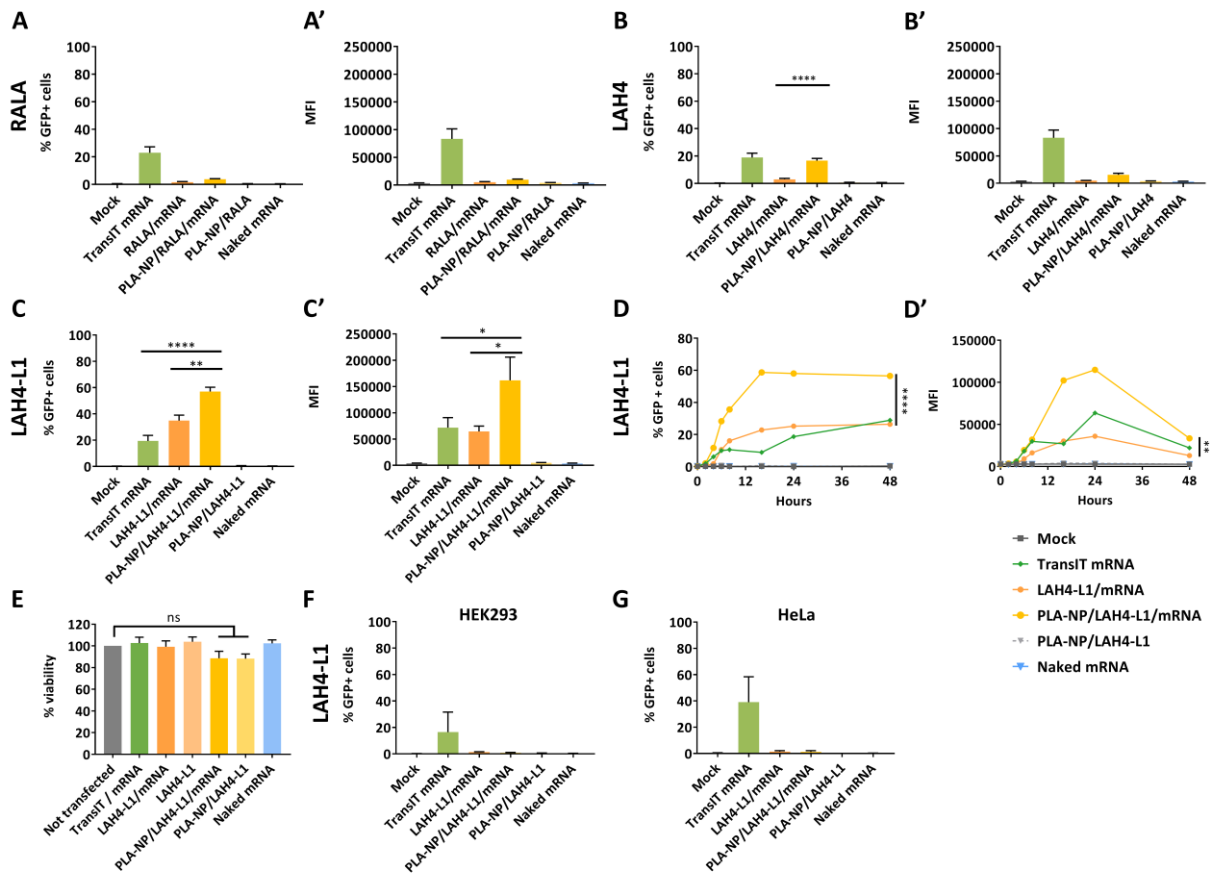
361 ***b. LAH4-L1-based formulations allow efficient mRNA uptake and protein expression***  
362 ***in dendritic cells***

363 The ability of mRNA complexes to transfect DCs was evaluated *in vitro* using the  
364 mouse DC2.4 cell line as a model (**Fig. 2A-C**). To that end, each complex containing an  
365 equivalent of 20 ng of eGFP mRNA was added to DC2.4 cells in serum-free conditions, and  
366 the percentage of GFP positive cells (**A-B-C**) and the Mean Fluorescence Intensity (MFI) (**A'-**  
367 **B'-C'**) were measured after 16h by flow cytometry. As a control, the same amount of naked  
368 eGFP mRNA was transfected into DC2.4 cells using the commercial TransIT-mRNA  
369 transfection kit (Mirus Bio). As shown in **Fig. 2A-A'**, mRNA complexes formed with RALA  
370 peptides failed to efficiently transfect DCs, while a low transfection capacity was observed in  
371 LAH4 peptide assays (**Fig. 2B-B'**). However, LAH4-L1-based formulations induced an  
372 efficient transfection of DC2.4. With LAH4-L1/mRNA polyplexes or PLA-NP/LAH4-  
373 L1/mRNA nanocomplexes, about 35% or 57% of DC2.4 cells expressed eGFP mRNA,  
374 respectively, with a significantly higher transfection efficiency than TransIT control assay  
375 (**Fig. 2C**). Moreover, the intensity of expression was significantly more important using PLA-  
376 NP/LAH4-L1/mRNA nanocomplexes than TransIT or LAH4-L1/mRNA assays (p-value <  
377 0.05) (**Fig. 2C'**). These results indicate that transfection efficiency is potentiated by the  
378 presence of PLA-NPs in the formulations.

379 A time-course study of eGFP expression in DCs was performed to compare  
380 mRNA/LAH4-L1 polyplexes and PLA-NP/LAH4-L1/mRNA nanocomplexes. At 4 h post  
381 transfection, eGFP-expressing DCs were already detectable, and the number of positive cells  
382 reached a plateau in all assays at 16-24 h post transfection (**Fig. 2D**). Interestingly, a bi-phasic  
383 curve was observed for the intensity of fluorescence, with a rapid increase until 16 h and a  
384 gradual decrease after 24 h (**Fig. 2D'**). The amount of GFP positive cells and the MFI were  
385 higher with PLA-NP/LAH4-L1/mRNA than with LAH4-L1/mRNA or TransIT-mRNA  
386 complexes, up to 48 h post transfection (**Fig. 2D-D'**, **Fig. S4**). These nanocomplexes showed  
387 stable expression efficiency after storage at 4°C for up to 7 days (results obtained with LAH4-  
388 L1 peptide were shown in **Fig. S4**). We did not observe any toxicity of the formulations in  
389 DCs under the conditions of the experiments (**Fig. 2E**).

390 The uptake of LAH4-L1-based formulations by different cell types was investigated.  
391 LAH4-L1 polyplexes and nanocomplexes failed to transfect HEK293 and HeLa cells, two  
392 epithelial cell lines, in the same experimental conditions (**Fig. 2F-G**). These results indicate

393 that the transfection capacity of LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA complexes  
 394 depends on cell type and seem to be particularly efficient to target DCs.



395 Thus, LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA formulations constitute  
 396 efficient methods for DC transfection with low doses of mRNA. We focused on these two  
 397 formulations for further analysis.

398 **Fig. 2. LAH4-L1-based formulations allow efficient mRNA expression in DC2.4 cells in**  
 399 **vitro.** Untreated DCs (Mock), TransIT eGFP mRNA (positive control), eGFP mRNA complexes and negative  
 400 controls (PLA-NP/LAH4-L1 and naked mRNA) were transfected in serum-free medium for 4 h (20 ng  
 401 mRNA/well). The transfection efficiency was measured 16h after the addition of mRNA complexes to the cells.  
 402 (A-C') Percentage of green fluorescent protein (eGFP) positive DC2.4 cells (A-B-C) and mean fluorescence  
 403 intensity (MFI) (A'-B'-C') were quantified after transfection in presence of RALA-, LAH4- and LAH4-L1-based  
 404 complexes (n=3). (D-D') Kinetics of expression efficiency after transfection with LAH4-L1-based formulations.  
 405 The percentage of eGFP positive DC2.4 cells (D) and the MFI (D') are presented. (E) Cytotoxicity of LAH4-L1-  
 406 based nanocomplexes on DC2.4 cells. (F-G) Percentage of eGFP positive DC2.4 cells after LAH4-L1-based  
 407 formulations transfections in (F) HEK293 and in (G) HeLa cells (n=3). Statistical significance of differences  
 408 between two groups was determined using one-way or two-way Anova: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p <$   
 409  $0.001$ ; \*\*\*\*,  $p < 0.0001$ .

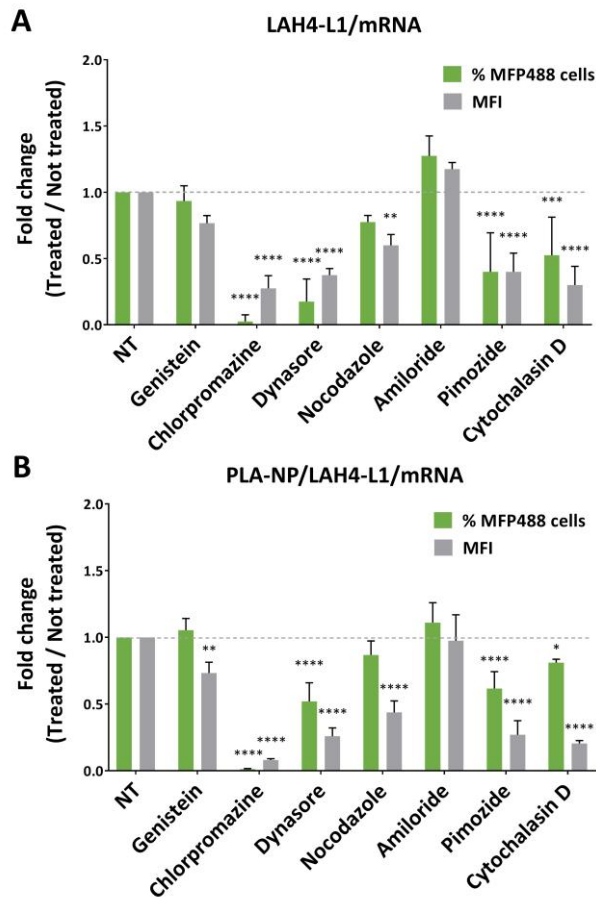
410 **c. mRNA vaccine formulations enter in dendritic cells through clathrin-mediated**  
 411 **endocytosis and phagocytosis**

412 To understand the intracellular mechanisms allowing mRNA expression, we  
 413 monitored the cellular uptake of LAH4-L1-based formulations by DCs. We studied their

414 mechanisms of internalization by treating DC2.4 cells with various endocytosis inhibitors.  
415 Targets and action mechanisms of each inhibitor were described in supplementary **Table I**.  
416 To visualize the complexes, we used MFP488-labelled fluorescent mRNAs. The mRNAs  
417 were complexed with LAH4-L1 or PLA-NP/LAH4-L1 and incubated for 2 h with inhibitor  
418 treated DC2.4 cells. To distinguish between complexes located inside the cells or at cell  
419 surface, extracellular fluorescence was quenched with trypan blue [42]. The percentage of  
420 cells taking up MFP488 mRNAs and the MFI were measured by flow cytometry (**Fig. 3A-B**).  
421 Surprisingly, the same profiles were obtained for LAH4-L1/mRNA polyplexes and PLA-  
422 NP/LAH4-L1/mRNA nanocomplexes. Aggregation presence with LAH4-L1/mRNA  
423 polyplexes can explained this result. Cells treated with chlorpromazine or dynasore, two  
424 clathrin-mediated endocytosis inhibitors, and with pimozide, a phagocytosis inhibitor, showed  
425 a significant decrease of MFP488 fluorescence (p-value < 0.0001). In agreement with a role  
426 of the clathrin-dependent and phagocytosis pathways, inhibition of microtubule and actin  
427 polymerization with nocodazole and cytochalasin D, respectively, also induced an important  
428 decrease of complexes uptake by DCs.

429         These results suggest that clathrin-dependent endocytosis and phagocytosis are  
430 important mechanisms involved in the uptake of LAH4-L1/mRNA polyplexes and PLA-  
431 NP/LAH4-L1/mRNA nanocomplexes by DCs. On the contrary, the lack of inhibition by  
432 genistein and amiloride suggests that caveolae-dependent endocytosis and macropinocytosis  
433 are not essential for this uptake.





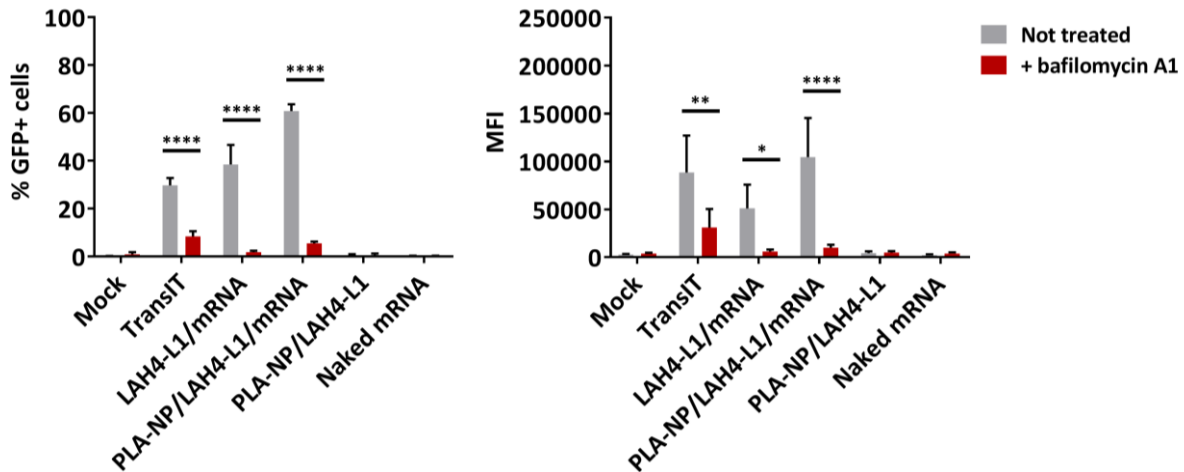
434

435 **Fig 3. Clathrin-mediated endocytosis and phagocytosis are involved in complexes uptake by**  
 436 **DC2.4 cells.** Uptake efficiency of MFP488-labelled mRNA complexed with LAH4-L1 (A) or PLA-NP/LAH4-  
 437 L1 (B). MFP488 mRNA was incubated for 2 h with DC2.4 cells in presence of various inhibitors, and uptake  
 438 efficiency was quantified by flow cytometry (n=3). Green bars: percentage of cells which present intracellular  
 439 MFP488 fluorescence; White bars: Mean of fluorescence (MFI). Green bars: percentage of GFP positive cells;  
 440 Grey bars: MFI. The value of not treated cells (NT) were set as 1. Statistical significance between two groups  
 441 was determined using a two-way Anova test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

442 **d. Endosomal escape is needed to induce efficient translation**

443 The previous results suggest that clathrin-mediated endocytosis and phagocytosis are  
 444 involved in the cellular uptake of formulations. These pathways induce the formation of  
 445 vesicular structures that eventually fuse with lysosomes. To allow mRNA expression,  
 446 nanocomplexes must escape the endosomes before lysosome fusion. Positive charges of  
 447 amphipathic cationic LAH4-L1 have been involved in this endosomal escape through the  
 448 proton sponge effect [43]. To evaluate the importance of this proton sponge mechanism, we  
 449 performed a transfection in presence of Bafilomycin A1, a specific inhibitor of vacuolar  
 450 proton ATPases, which is essential for proton regulation in endosome. The percentage of  
 451 eGFP positive cells and the MFI were measured after 16 h by flow cytometry (Fig. 4). In  
 452 presence of Bafilomycin A1, the percentage of eGFP positive cells and the MFI were  
 453 drastically reduced.

454 These results show the importance of endosome acidification for eGFP mRNA  
 455 expression, in agreement with a model whereby a proton sponge effect allows mRNA escape  
 456 from the endosomes to the cytosol.



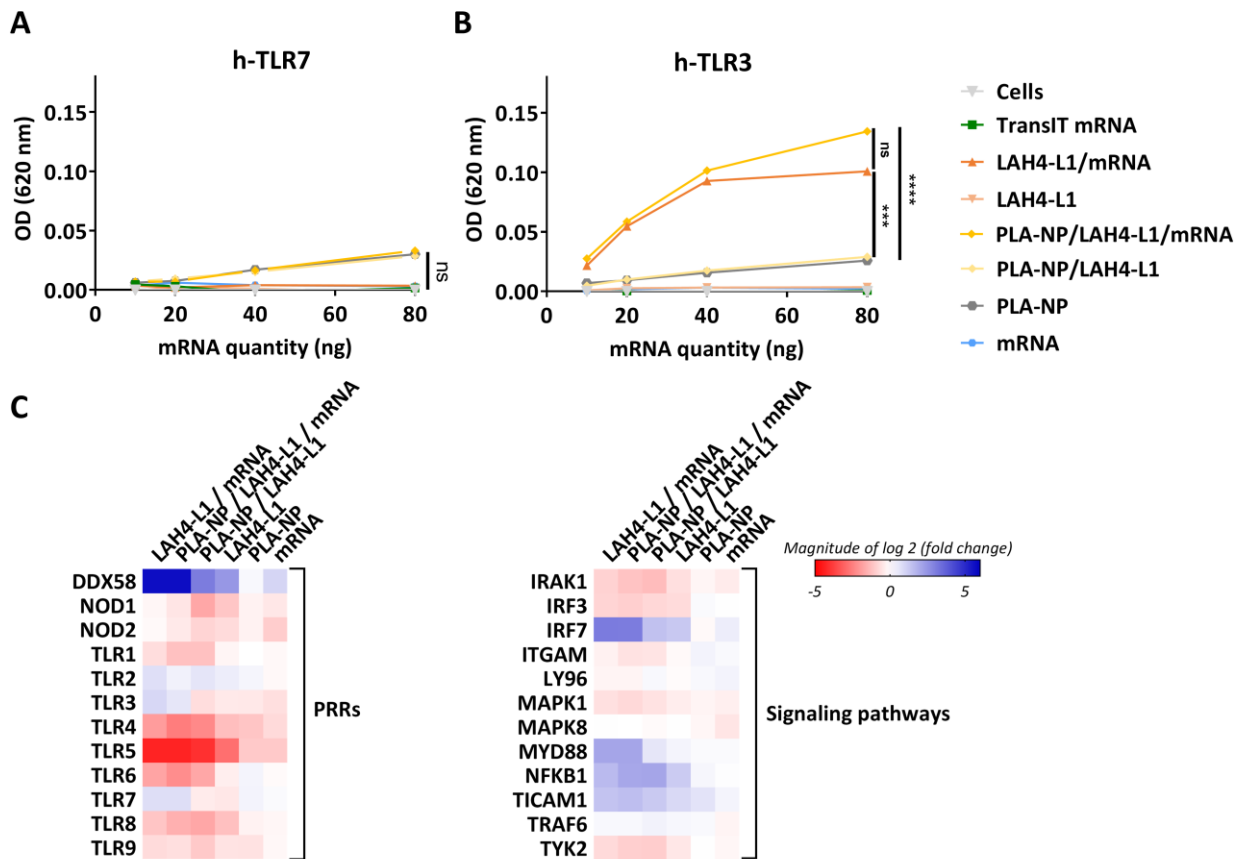
457 **Fig 4. Endosomal escape is essential for mRNA expression in DC2.4 cells.** Percentage of eGFP  
 458 positive DC2.4 cells and mean fluorescence intensity (MFI) were quantified after 16 h of transfection by LAH4-  
 459 L1-based formulations in absence (grey bars) or in presence (red bars) of bafilomycin A1 (n=3). Statistical  
 460 significance between two groups was determined using a two-way Anova test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p$   
 461  $< 0.001$ ; \*\*\*\*,  $p < 0.0001$ .

462 ***e. mRNAs in LAH4-L1-based formulations induce an innate immune-stimulatory***  
 463 ***activity through PRR activation***

464 We then analyzed the immune-stimulatory properties of LAH4-L1-based  
 465 formulations, using mRNAs coding for the Gag protein of HIV-1, a model antigen protein  
 466 relevant for vaccine development. DCs express a repertoire of PRRs allowing the recognition  
 467 of a large range of pathogen molecules, including some RNA sequences. The intrinsic  
 468 adjuvant activity of formulations was evaluated *in vitro* using cell lines which overexpress  
 469 only one specific PRR (h-TLR7 or h-TLR3). These cell lines were transfected with different  
 470 nanocomplex concentrations and PRR activation was evaluated (**Fig. 5A-B**). LAH4-  
 471 L1/mRNA and PLA-NP/LAH4-L1/mRNA complexes induced a significant h-TLR3  
 472 activation in a dose-dependent manner, while the same complexes devoid of gag mRNA only  
 473 activated weakly h-TLR3 cells while a non-significant h-TLR7 activation was observed.  
 474 These results show the specific TLR3 immune-stimulation activity of PLA-NP/LAH4-  
 475 L1/mRNA nanocomplexes. In this experiment, the mRNA complexed onto LAH4-L1-based  
 476 formulations seemed to be taken up by modified HEK293 cell lines to activate endosomal  
 477 PRR, whereas **Fig. 2F** showed that mRNAs are not translated into functional protein in  
 478 HEK293. Those observations suggest an alteration of RNA escape from endosome or of  
 479 translation in this cell type.

480 To further analyze the immune-stimulatory properties of LAH4-L1-based  
481 formulations, subsequent experiments were performed using primary DCs derived from  
482 human monocytes (moDCs), a cellular model particularly suitable for activation studies. A  
483 comparative gene expression analysis was carried out on moDCs treated with the different  
484 formulations, using the Human Innate and Adaptive Immune Responses RT2 Profiler™ PCR  
485 Array. After 6 h of stimulation, the formulations induced a modification in the expression  
486 pattern of multiple genes encoding TLR and signaling proteins associated to their pathways  
487 (**Fig. 5C**). Main effects were observed for formulations containing the LAH4-L1 peptide.  
488 PLA-NPs and mRNAs alone did not have the ability to modify the gene expression profile.  
489 However, the presence of mRNA in formulations (see LAH4-L1/mRNA vs LAH4-L1 and  
490 PLA-NP/LAH4-L1/mRNA vs PLA-NP/LAH4-L1) upregulated the expression of TLR3,  
491 TLR7 and DDX58 (also called RIG-I), three PRR receptors involved in RNA recognition  
492 [44]. Their target genes (IRFF7, MYD88, NFKB1, and TICAM1) were also upregulated after  
493 LAH4-L1/mRNA stimulation. Interestingly, these formulations decreased the expression of  
494 other TLR genes, suggesting that the presence of mRNAs favors a signaling through

495 TLR3/TLR7/DDX58. However, it should be noted that the data shown in **Fig.5C** only



496 represent a signature of moDCs 6h after transfection.

497 **Fig 5. mRNA complexes stimulate the innate immunity through PRR activation.** (A-B)  
 498 Evaluation of PRR activation via the TLR7 and TLR3 pathways in HEK-Blue hTLR7 and HEK-Blue hTLR3 cells  
 499 with mRNA formulations ( $n=2$ ;  $N=2$  and  $n=2$ ;  $N=3$  for h-TLR7 and h-TLR3 respectively). Statistical  
 500 significance between two groups was determined using a two-way Anova test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p$   
 501  $< 0.001$ ; \*\*\*\*,  $p < 0.0001$ . (C) Expression profile of TLR pathway genes in moDCs after 6 h stimulation with  
 502 mRNA formulations. This heat map represents the mean of three independent experiments performed with the  
 503 supernatant of moDCs derived from three different patients, duplicate points being used in each experimental  
 504 sample.

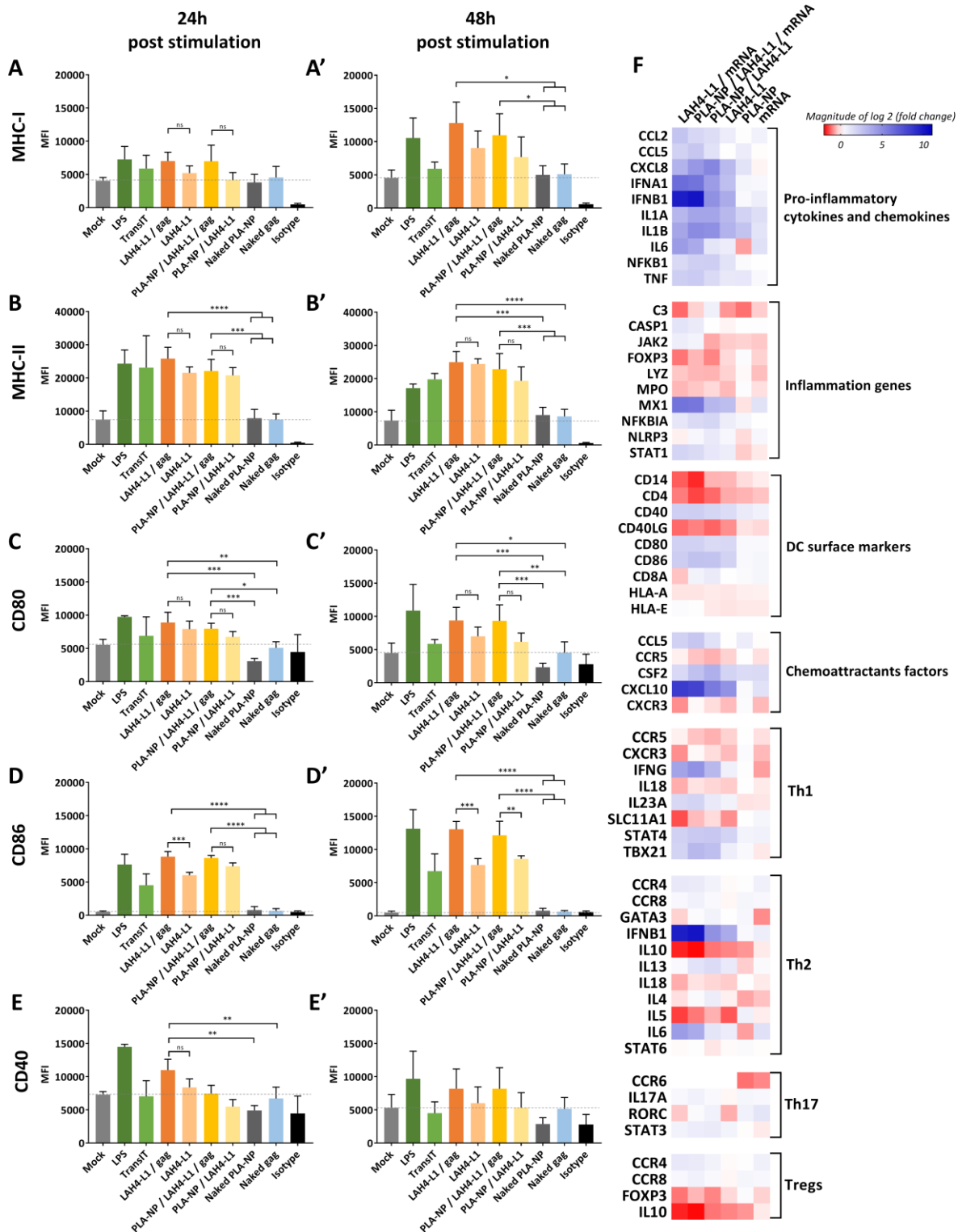
505 **a. LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA complexes promote moDC**  
 506 **maturation**

507 DCs are central effectors of immunity, acting as messengers between the innate and  
 508 adaptive immune systems. Their stimulation induces their maturation, antigen presentation  
 509 and the production of cytokines and chemokines leading to the immune response.

510 In order to evaluate the impact of our formulations on moDC maturation, we first  
 511 verified the translational efficiency of gag mRNAs in moDCs. As shown in **Fig. S5** moDCs  
 512 transfected with gag mRNA formulations express the HIV-1 gag protein.

513 Then, moDCs were stimulated with formulations containing an equal amount of gag  
514 mRNAs for 24 h and 48 h, and the expression of different markers of maturation was  
515 analyzed by flow cytometry (**Fig. 6A-E'**). LAH4-L1/gag-mRNA and PLA-NP/LAH4-L1/gag-  
516 mRNA formulations induced a moDC maturation similar to that observed with a positive  
517 control using lipopolysaccharide (LPS). More precisely, these formulations significantly  
518 increased MHC-I, MHC-II, CD80 and CD86 expression after 24 h and 48 h of stimulation, in  
519 comparison to naked gag mRNAs or formulations devoid of mRNAs. Comparable but less  
520 significant results were obtained for the cluster of differentiation CD40. Altogether, these  
521 results suggest that mRNA-based formulations promote moDC maturation in our  
522 experimental model.

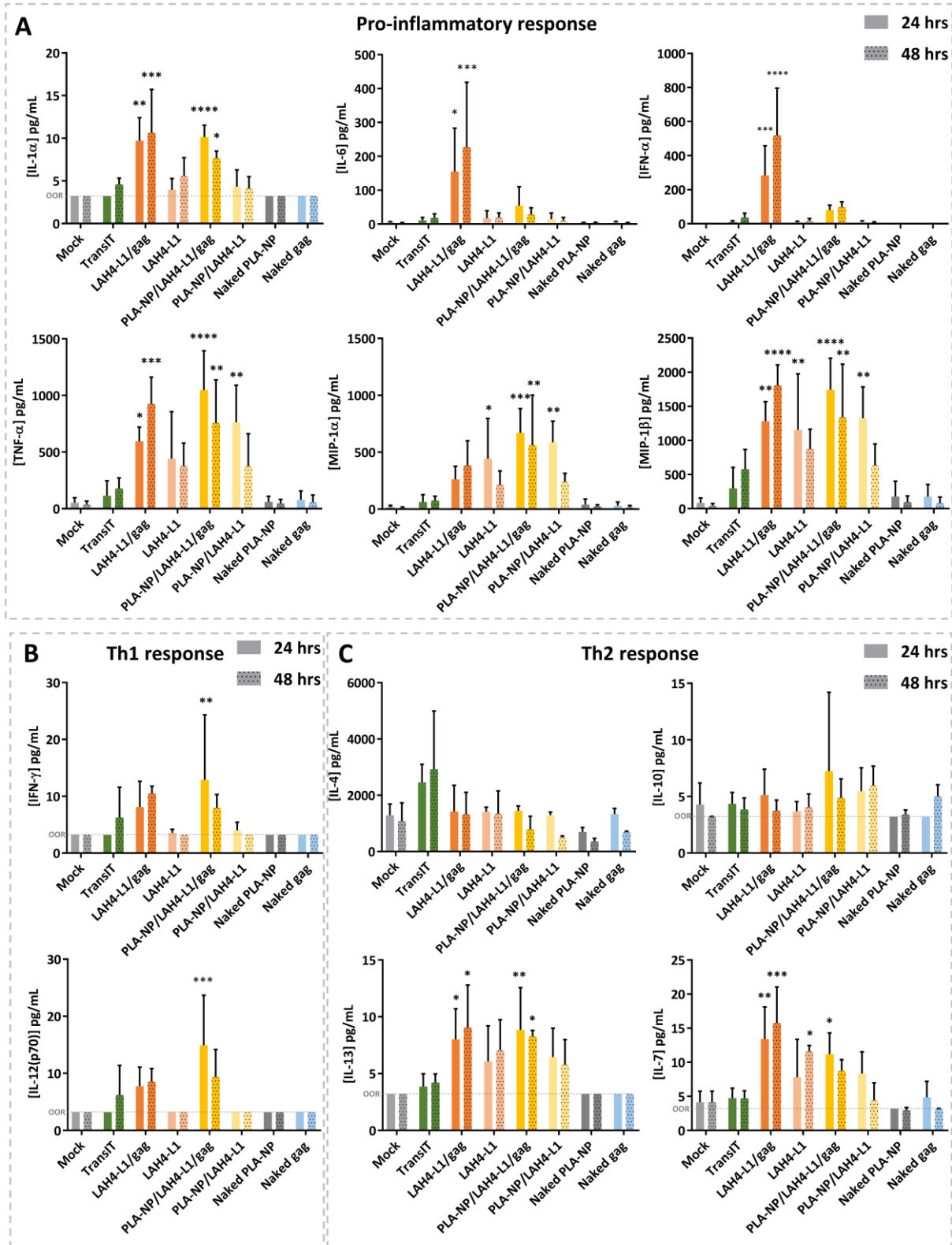
523 In addition, the expression of 84 genes associated with innate and adaptive immune  
524 responses was evaluated in moDCs after 6 h of stimulation with the different formulations. As  
525 shown in **Fig. 6F**, LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA complexes induced the  
526 overexpression of most of the tested genes encoding pro-inflammatory cytokines and  
527 chemokines, such as IFN- $\alpha$ , IFN- $\beta$ , IL-6 and proteins implicated in inflammation, like MX1.  
528 This overexpression was induced by LAH4-L1 peptide and amplified by the presence of  
529 mRNA. In agreement to the previous expression data, genes involved in DC maturation  
530 (CD40, CD80 and CD86) were also overexpressed, validating the precedent results. As  
531 represented in the heat map, immune cell chemoattractant factors (CCL5 and CXCL10) and  
532 Th1-related genes (IFNG, IL23A, STAT4 and TBX21) were overexpressed whereas Th2-  
533 related (IL-10, IL-4, IL5 or Stat6), Th17-related (IL17A, STAT3) and Tregs genes were  
534 downregulated or stable. These results indicate that mRNAs complexed in LAH4-L1-based  
535 formulations induce an inflammatory response and seem to activate a Th1-biased immune  
536 response in moDCs.



537 **Fig. 6: LAH4-L1/gag-mRNA based formulations induce moDC activation and maturation.**  
538 (A-E') Expression of the co-stimulatory molecules MHC-I, MHC-II, CD80, CD86 and CD40 on the surface of  
539 CD1a<sup>+</sup> CD14<sup>-</sup> moDCs was determined by flow cytometry 24 h and 48 h after stimulation with volume allowing  
540 the transfection of 660 ng of gag mRNA or equivalent volume for controls. The data represent four independent  
541 experiments. Statistical significance between two groups was determined using one-way Anova test: \*,  $p < 0.05$ ;  
542 \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , \*\*\*\*,  $p < 0.0001$ . (F) Expression profile of genes involved in innate and adaptive  
543 immune response 6 h after stimulation with mRNA formulations (volume allowing the transfection of 2640 ng of  
544 gag mRNA or equivalent volume for controls). This heat map represents the mean of three independent  
545 experiments performed with the mRNAs of cells from three different patients, duplicate points being used in each  
546 experimental sample.

547           The maturation of DCs is associated with the expression of cytokines and chemokines  
548 that induce and direct adaptive immune responses. The secretion of those mediators  
549 implicated in immune response was characterized by Luminex assay 24 h or 48 h after moDC  
550 transfection by formulations (**Fig. 7**). Similar profiles were obtained after 24 h and 48 h of  
551 stimulation. The presence of gag mRNA in LAH4-L1-based formulations significantly  
552 increased the secretion of pro-inflammatory cytokines and chemokines, suggesting a direct  
553 impact of mRNA in the inflammatory response. More precisely, LAH4-L1/mRNA  
554 formulations induced the secretion of pro-inflammatory cytokines (IL-1 $\alpha$ , IL6, IFN- $\alpha$ , TNF-  
555  $\alpha$ ), chemokines (MIP-1 $\beta$ ), Th1-related (IFN- $\gamma$ , IL-12 (p70)) and Th2-related (IL-13, IL-7)  
556 cytokines. However, there was no significant increase in the amount of IL-4 and no secretion  
557 of IL-5 (not shown), two well-known markers of the Th2 response. In comparison, PLA-  
558 NP/LAH4-L1/mRNA nanocomplexes gave a similar result but with a reduced inflammatory  
559 induction (lack of IL-6 and IFN- $\alpha$ ) and a higher Th1 induction (IFN- $\gamma$ , p-value < 0,01; IL-12  
560 (p70), p-value < 0,001).

561           Overall, these results indicate that LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA  
562 formulations are potential activators of pro-inflammatory responses and Th1-oriented immune  
563 responses, although a subset of Th2-specific markers are also induced.



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**Fig. 7: LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA promote cytokine and chemokine secretion by moDCs.** moDCs were stimulated by volume allowing the transfection of 660 ng of gag mRNA or equivalent volume for controls for 24 h or 48 h. The secretion of cytokines and chemokines involved in inflammatory processes (A), Th1 (B) or Th2 (C) immune responses was quantified by Luminex assay on CD1a+ CD14- moDCs supernatants. Data represent the mean of three independent experiments performed with the supernatant of cells from three different patients, triplicate points being used in each experimental sample. OOR; Limit of detection (3.2 pg/mL). Statistical significance between two groups was determined using two-way Anova test: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$ .



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## 4. Discussion

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In recent years, antigen-encoding mRNAs have emerged as promising alternatives to classical vaccine approaches [9]. The highest challenge with mRNA vaccine is the development of a safe and effective delivery system. Indeed, mRNAs are large polyanionic biomolecules that do not cross efficiently cell membrane barriers in their naked format and are generally unstable in blood circulation, at the site of injection and in the extracellular compartment due to the presence of RNases [45]. While most studies in the mRNA vaccine field have focused on lipid nanoparticles (LNP) [6,9,10,46], we present here a novel platform to vectorize mRNA, using poly(lactic acid) nanoparticles (PLA-NPs) and the amphipathic cationic peptide LAH4-L1, as an intermediate. These formulations allow effective *in vitro* uptake of mRNA by DCs, *via* clathrin-mediated endocytosis and phagocytosis, and efficient mRNA expression, which depends on vacuolar proton ATPase activity and mRNA escape from endosomes. Moreover, these novel mRNA formulations induce DC activation and maturation, and trigger pro-inflammatory and Th1-biased immune responses.

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PLA-NPs are versatile biodegradable platform, able to codeliver antigens and/or immunostimulatory molecules to DCs either *in vitro* or *in vivo* [29,30]. However, due to their physicochemical properties, i.e. they are negatively charged, the vectorization of mRNAs using PLA-NPs has been a technological challenge. Thus, we have chosen to use cationic peptides as intermediates. RALA, LAH4 and LAH4-L1 are three cell penetrating peptides (CPPs) with amphipathic cationic properties, which have the ability to condensate nucleic acids [43,47]. However, only few studies have used CPPs to deliver mRNAs [48–50]. In this study, we have developed a strategy based on a two steps process (1) the formation of polyplexes between a cationic peptide and mRNAs and (2) their adsorption onto PLA-NPs (**Fig. 1A**). The three peptides have shown an ability to condensate mRNAs in polyplexes (Peptide/mRNA), which can be adsorbed onto PLA-NPs to form stable nanocomplexes (PLA-NP/Peptide/mRNA) (**Fig. 1B-D and S3**). However, only the LAH4-L1-based formulations have shown promising results for the induction of mRNA expression (**Fig. 2**) and the potentiation of immune responses (**Fig 5-7**). The difference obtained between the three peptides might be partly explained by their structure (**Fig. S1**). Indeed, LAH4-L1, which is a LAH4 derivative, is a histidine-rich, cationic amphipathic peptide with pH-responsive properties. Sequence alignment of these two peptides reveals that two leucine and two histidine residues of LAH4 have been replaced by two histidines and two leucines in LAH4-L1, respectively. According to *in silico* modeling, the new location of the two histidine

606 residues in the LAH4-L1 peptide narrows the angle subtended by the helix hydrophilic face to  
607 80 degrees, instead of 100 degrees in LAH4. This may give this peptide an enhanced capacity  
608 in membrane disruption [43,51].

609 In this study, we also provided *in vitro* evidence that a low dose of mRNA present in  
610 LAH4-L1 and PLA-NP/LAH4-L1-based complexes can induce a strong protein expression in  
611 dendritic cells (DCs) (**Fig. 2A-C'**). Moreover, *in vitro* efficiency of mRNA transfection and  
612 translation in DCs is significantly more important using these formulations than with TransIT  
613 (a commercially reagent) for the same mRNA doses. Although, we found that LAH4- and  
614 RALA-based mRNA complexes are unable to transfect DCs, others works have shown  
615 efficient transfection of DNA, siRNA and/or recently mRNA transfection using RALA,  
616 LAH4 and GALA CPPs in DCs and/or different cell lines [39,43,47,50,52]. These differences  
617 may be explained by variations in the experimental conditions, especially regarding the  
618 amount of transfected mRNA. Indeed, for RALA-[39] and GALA-[50] based polyplexes, the  
619 formulations contain 26- and 10-fold more RNA/cell, respectively, than in our study.  
620 Although LAH4-L1- and PLA-NP/LAH4-L1-based formulations were efficient for DC  
621 transfection and expression, they failed to induce mRNA expression in HEK293 and HeLa  
622 cells (**Fig. 2C and F-G**). Since DCs are specialized in antigen capture and processing [5], the  
623 hydrodynamic diameter of LAH4-L1-based complexes might induce DC endocytosis and  
624 activation, in comparison to the two epithelial cell lines with a low endocytosis capacities.  
625 Hence, a low dose of transfected mRNA could be sufficient to detect its translation in DCs but  
626 not in epithelial cells in our assays.

627 The addition of PLA-NPs to LAH4-L1/mRNA polyplexes induced mRNA expression  
628 in a higher number of DCs and at a stronger level (**Fig. 2D and S4**). We showed that in  
629 presence of PLA-NPs, the eGFP intensity curve presents a bi-phasic profile with an increase  
630 from 4 h to 16-24 h post transfection and a decrease after 24 h (**Fig. 2D**). Using lipid-based  
631 formulations to transfect DC lines, Phua *et al.* have shown a biphasic expression kinetics with  
632 a rapid peak at 5-7 h, then a rapid decrease of transgene expression until 9 h, followed by  
633 gradual loss of expression [53]. Moreover, with a low polydispersity index, nanocomplexes  
634 are monodisperse and present a zeta potential of +37,3 mV. Polyplexes present a higher  
635 polydispersity index testifying the presence of aggregates and a lower zeta potential (+25,0  
636 mV). Nanocomplexes could force the membrane to bend around the vector and promote its  
637 internalization by endocytosis or phagocytosis: this is the wrapping phenomenon described  
638 and illustrated by Nel *et al.* [38]. This model implies that the surface charge value, but also  
639 the size and shape of the material could directly influence the interaction of the material with

640 the membranes and thus the uptake of NPs. This could in part explain why nanocomplexes  
641 that have a larger surface charge and a more homogeneous size than polyplexes are  
642 internalized more efficiently, leading to a more sustained expression in cells. A sustained  
643 mRNA expression obtained with PLA-NP/LAH4-L1-based formulations could be  
644 advantageous for vaccine applications as it could increase the amount of antigen processed by  
645 DCs and consequently immune responses.

646 Here, we report that LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA are taken up by  
647 DCs through clathrin-mediated endocytosis and phagocytosis (**Fig. 3**). However, most lipid-  
648 based mRNA vaccines are predominantly internalized by micropinocytosis [10,22,54]. It is  
649 now well-described that physicochemical properties of delivery systems (such as size,  
650 geometry and polydispersity index), but also target cells properties, influence immune  
651 recognition and uptake of vaccine by APCs [30,55–57]. The particulate nature and the size of  
652 PLA-NP/LAH4-L1/mRNA nanocomplexes, similar to many viruses, could enhance  
653 interaction of the vaccine formulations with cell membranes, promoting antigen delivery into  
654 cells. LAH4-L1/mRNA polyplexes seem to follow the same internalization mechanisms than  
655 PLA-NP/LAH4-L1/mRNA nanocomplexes, which could partly be due to the presence of  
656 some aggregates in LAH4-L1/mRNA formulations (**Fig. S2**).

657 After endocytosis, a pH gradient is established inside the endocytic vesicles, ranging  
658 from the least acid early endosomes (pH 6.5 - 6.8) to acidic lysosomes (pH 4.5). mRNAs need  
659 to reach the cytosolic compartment to be translated. Protonation of the histidine imidazole  
660 groups in LAH4-L1 plays an important role in the transfection process and more importantly  
661 for endosomal escape [43]. Indeed, the presence of the pH-responsive cell-penetrating peptide  
662 LAH4-L1 in vaccine formulations enhanced mRNA expression in a vacuolar proton ATPase-  
663 dependent mechanism (**Fig. 4**), suggesting that LAH4-L1 promoted endosome membrane  
664 disruption in DCs for cytosolic delivery of nanocomplexes [58]. Following its release into the  
665 cytosol, mRNAs can be translated into antigenic protein. Both endosomal and cytosolic  
666 localization of mRNA may participate to their effect on immune responses, as we found that  
667 mRNA activates both endosomal (TLR3) and cytosolic (RIG-I) PRRs (**Fig. 4**). mRNAs act as  
668 an adjuvant, stimulating the secretion of immune-stimulatory factors. For vaccination, the  
669 immune-stimulatory effect and the intrinsic-adjuvant activity of IVT mRNAs could be  
670 beneficial, but must be balanced and controlled in time, so that DC activation does not prevent  
671 the expression of the antigenic mRNAs supposed to stimulate adaptive immune responses.  
672 The activation of both innate and adaptive immune responses is needed for efficient  
673 vaccination. The results presented here suggest an ability of LAH4-L1/mRNA and PLA-

674 NP/LAH4-L1/mRNA nanocomplexes to activate both innate immunity, and a wide spectrum  
675 of adaptive immune responses markers (**Fig. 6-7**). The adaptive response signature observed  
676 *in vitro* 6 h post transfection suggests that both MHC-I and MHC-II antigen presentation  
677 mechanisms are activated, with a prevalent Th1-oriented response and a lower Th2  
678 counterpart (**Fig. 6-7**). This indicates that LAH4-L1/mRNA polyplexes and PLA-NP/LAH4-  
679 L1/mRNA nanocomplexes may be suitable mRNA delivery vectors to induce cytotoxic  
680 responses in addition to humoral, which remains to be explored *in vivo*. We could observe that  
681 gene expression analysis and luminex assays were performed at specific time-points, which  
682 can influence some results of specific chemokines. Next step will be to perform the same type  
683 of analysis with a more complete kinetic for a longer period of time.

684 A hypothetical model explaining the different steps leading to cytotoxic and humoral  
685 responses after endocytosis of LAH4-L1-gag mRNA-based formulations into DCs is  
686 presented in **Fig. 8**. In this scenario, after clathrin-mediated endocytosis or phagocytosis,  
687 endosome escape and mRNA translation, the gag protein can be processed and presented  
688 through MHC-I allowing T-CD8<sup>+</sup> activation and CTL activation. During this period, mRNAs  
689 induce the activation of PRRs. The induction of CTLs is potentiated by DC activation and the  
690 production of Th1-associated cytokines. Gag expression could also induce vesicles-like  
691 particle (VLPs) formation, as described elsewhere [59], which can be later released by DCs  
692 and taken up by immature DCs. Secreted VLPs which are recognized like extracellular  
693 pathogens, are then processed and presented onto MHC-II, allowing T-CD4<sup>+</sup> activation. The  
694 generation of an antigen-specific CTL response is important for the development of effective  
695 antiviral or antitumor immune responses. Moreover, humoral response is essential to elicit  
696 antibodies and memory B cells. The signature of immune responses triggered in primary DCs  
697 *in vitro* by LAH4-L1-based gag mRNA formulations, suggests this strategy could be used to  
698 transfect human DCs in the case of *ex vivo* therapy, and especially in HIV-1 vaccine-based  
699 therapy of chronically infected patients [60,61]. Indeed, by inducing a wide spectrum of both  
700 innate and adaptive immune response, this mRNA vaccine formulation would permit clearing  
701 the viral reservoir by eliminating infected cells leading to a potential HIV cure[62].

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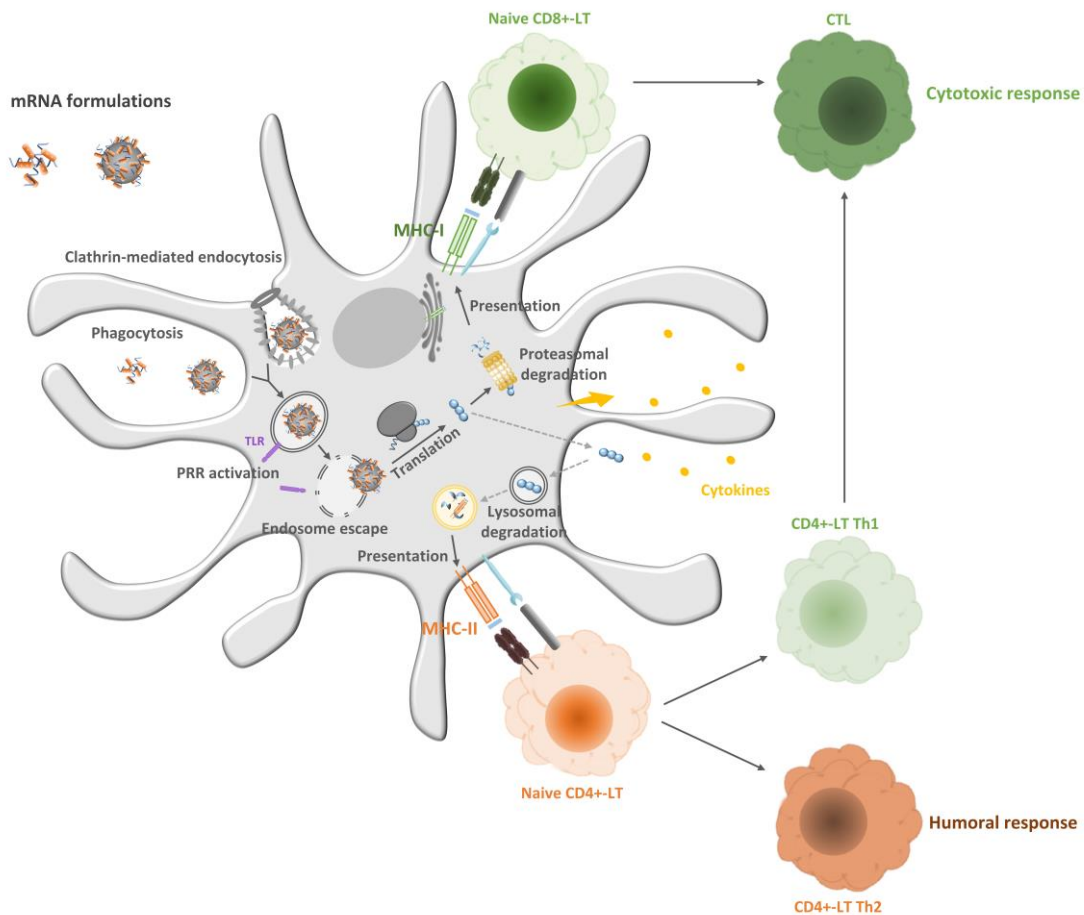
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710 **Fig. 8: Model for trafficking of LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA into DCs**  
 711 **to induce immune responses.** Formulations are taken up by DCs via clathrin-mediated endocytosis or  
 712 phagocytosis. In the endosomal compartment, LAH4-L1 peptides induce endosome escape and release of mRNA  
 713 into the cytosol. The presence of mRNA in both endosome and cytosol trigger innate immune stimulation via  
 714 PRRs. In the cytosol, mRNA is translated into an antigenic protein, which is subsequently processed by the  
 715 proteasome, and presented onto Major Histocompatibility Complex-type I (MHC-I). This intracellular pathway  
 716 leads to CD8<sup>+</sup> T lymphocyte (LT) activation. Intracellular protein (and especially VLPs formed by gag antigen)  
 717 can be released by DCs and taken up by novel immature DCs, degraded by lysosomes and presented onto MHC-  
 718 II. This presentation leads to CD4<sup>+</sup> T lymphocyte (LT) activation to form Th1 and Th2 LTs, involed in the  
 719 activation of cytotoxic and humoral responses, respectively.

720 **5. Conclusions**

721 In this study, we have designed and developed new strategies to vectorize mRNA  
 722 using PLA-NPs platform and LAH4-L1 amphipathic cationic peptides. We demonstrated a  
 723 high capability of LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA to transfect DCs and to  
 724 induce strong protein expression. The presence of PLA-NPs in the formulations enhances this  
 725 effect. mRNA nanoformulations trigger PRR activation and promote innate immunity  
 726 activation. Moreover, these complexes could potentiate the humoral immune response, about  
 727 also a prevalent Th1-biased aspect. Thus, PLA-NP/LAH4-L1/mRNA formulations are

728 versatile platforms for efficient *in vitro* mRNA delivery to DCs, which could be applied to the  
729 *ex vivo* treatment of a wide variety of chronic infectious diseases, like HIV-1.

### 730 **CONFLICT OF INTEREST**

731 We declare no competing financial interest

### 732 **AUTHORS CONTRIBUTIONS**

733 ALC participated in the conception and design of the study, performed experiments, analyzed  
734 data, and wrote the manuscript. CLG selected peptides and designed formulations. BV  
735 participated in the conception and design of the study. JYE, ED and BV assisted in data  
736 analysis, and wrote the manuscript. PMG performed uptake analysis and assisted in the moDC  
737 purification. CM performed FRET analysis. All the authors read, critiqued, and approved the  
738 final manuscript.

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### 750 **REFERENCES**

- 751 [1] S. Plotkin, History of vaccination, PNAS. 111 (2014) 12283–12287 SPECIAL.  
752 doi:10.1073/pnas.1400472111.
- 753 [2] C.M.C. Rodrigues, M. V. Pinto, M. Sadarangani, S.A. Plotkin, Whither vaccines?, J.  
754 Infect. 74 (2017) S2–S9. doi:10.1016/S0163-4453(17)30184-6.
- 755 [3] V. Appay, D.C. Douek, D.A. Price, CD8+ T cell efficacy in vaccination and disease,  
756 Nat. Med. 14 (2008) 623–628. doi:10.1038/nm.f.1774.
- 757 [4] M. Moser, O. Leo, Key concepts in immunology, Vaccine 28S. 28 (2010) 2–13.  
758 doi:10.1016/j.vaccine.2010.07.022.
- 759 [5] R.M. Steinman, J. Banchereau, Taking dendritic cells into medicine, Nature. 449  
760 (2007) 419–426. doi:10.1038/nature06175.
- 761 [6] A.J. Geall, C.W. Mandl, J.B. Ulmer, RNA: the new revolution in nucleic acid vaccines,  
762 Semin. Immunol. 25 (2013) 152–9. doi:10.1016/j.smim.2013.05.001.

- 763 [7] U. Sahin, K. Karikó, Ö. Türeci, mRNA-based therapeutics — developing a new class  
764 of drugs, *Nat. Rev. Drug Discov.* 13 (2014) 759–780. doi:10.1038/nrd4278.
- 765 [8] S. Pascolo, The messenger’s great message for vaccination, *Expert Rev. Vaccines.* 14  
766 (2015) 153–6. doi:10.1586/14760584.2015.1000871.
- 767 [9] N. Pardi, M.J. Hogan, F.W. Porter, D. Weissman, mRNA vaccines - a new era in  
768 vaccinology., *Nat. Rev. Drug Discov.* 17 (2018) 261–279. doi:10.1038/nrd.2017.243.
- 769 [10] C. Pollard, S. De Koker, X. Saelens, G. Vanham, J. Grooten, Challenges and advances  
770 towards the rational design of mRNA vaccines, *Trends Mol. Med.* 19 (2013) 705–13.  
771 doi:10.1016/j.molmed.2013.09.002.
- 772 [11] M. Zhao, M. Li, Z. Zhang, T. Gong, X. Sun, Induction of HIV-1 gag specific immune  
773 responses by cationic micelles mediated delivery of gag mRNA, *Drug Deliv.* 23 (2015)  
774 2596–2607. doi:10.3109/10717544.2015.1038856.
- 775 [12] S. Uchida, H. Kinoh, T. Ishii, A. Matsui, T.A. Tockary, K.M. Takeda, H. Uchida, K.  
776 Osada, K. Itaka, K. Kataoka, Systemic delivery of messenger RNA for the treatment of  
777 pancreatic cancer using polyplex nanomicelles with a cholesterol moiety, *Biomaterials.*  
778 82 (2016) 221–228. doi:10.1016/J.BIOMATERIALS.2015.12.031.
- 779 [13] D. Benteyn, C. Heirman, A. Bonehill, K. Thielemans, K. Breckpot, mRNA-based  
780 dendritic cell vaccines, *Expert Rev. Vaccines.* 14 (2015) 161–176.  
781 doi:10.1586/14760584.2014.957684.
- 782 [14] H. Debus, P. Baumhof, J. Probst, T. Kissel, Delivery of messenger RNA using  
783 poly(ethylene imine)-poly(ethylene glycol)-copolymer blends for polyplex formation:  
784 biophysical characterization and in vitro transfection properties, *J. Control. Release.*  
785 148 (2010) 334–43. doi:10.1016/j.jconrel.2010.09.007.
- 786 [15] T. Démoulin, P. Milona, P.C. Englezou, T. Ebensen, K. Schulze, R. Suter, C. Pichon,  
787 P. Midoux, C.A. Guzmán, N. Ruggli, K.C. McCullough, Polyethylenimine-based  
788 polyplex delivery of self-replicating RNA vaccines, *Nanomedicine Nanotechnology,*  
789 *Biol. Med.* 12 (2016) 711–722. doi:10.1016/j.nano.2015.11.001.
- 790 [16] S. Li, M.A. Rizzo, S. Bhattacharya, L. Huang, Characterization of cationic lipid-  
791 protamine-DNA (LPD) complexes for intravenous gene delivery, *Gene Ther.* 5 (1998)  
792 930–7. doi:10.1038/sj.gt.3300683.
- 793 [17] B. Petsch, M. Schnee, A.B. Vogel, E. Lange, B. Hoffmann, D. Voss, T. Schlake, A.  
794 Thess, K.-J. Kallen, L. Stütz, T. Kramps, Protective efficacy of in vitro synthesized,  
795 specific mRNA vaccines against influenza A virus infection., *Nat. Biotechnol.* 30  
796 (2012) 1210–6. doi:10.1038/nbt.2436.
- 797 [18] T. Bettinger, R.C. Carlisle, M.L. Read, M. Ogris, L.W. Seymour, Peptide-mediated  
798 RNA delivery: a novel approach for enhanced transfection of primary and post-mitotic  
799 cells, *Nucleic Acids Res.* 29 (2001) 3882–91.  
800 <http://www.ncbi.nlm.nih.gov/pubmed/11557821> (accessed August 10, 2018).
- 801 [19] F.T. Zohra, E.H. Chowdhury, S. Tada, T. Hoshiba, T. Akaike, Effective delivery with  
802 enhanced translational activity synergistically accelerates mRNA-based transfection,  
803 *Biochem. Biophys. Res. Commun.* 358 (2007) 373–378.  
804 doi:10.1016/J.BBRC.2007.04.059.
- 805 [20] F.T. Zohra, E.H. Chowdhury, T. Akaike, High performance mRNA transfection  
806 through carbonate apatite–cationic liposome conjugates, *Biomaterials.* 30 (2009) 4006–  
807 4013. doi:10.1016/j.biomaterials.2009.02.050.
- 808 [21] C. Pollard, J. Rejman, W. De Haes, B. Verrier, E. Van Gulck, T. Naessens, S. De  
809 Smedt, P. Bogaert, J. Grooten, G. Vanham, S. De Koker, Type I IFN Counteracts the  
810 Induction of Antigen-Specific Immune Responses by Lipid-Based Delivery of mRNA  
811 Vaccines, *Mol. Ther.* 21 (2013) 251–259. doi:10.1038/mt.2012.202.
- 812 [22] L.M. Kranz, M. Diken, H. Haas, S. Kreiter, C. Loquai, K.C. Reuter, M. Meng, D. Fritz,

- 813 F. Vascotto, H. Hefesha, C. Grunwitz, M. Vormehr, Y. Hüsemann, A. Selmi, A.N.  
814 Kuhn, J. Buck, E. Derhovanessian, R. Rae, S. Attig, J. Diekmann, R.A. Jabulowsky, S.  
815 Heesch, J. Hassel, P. Langguth, S. Grabbe, C. Huber, Ö. Türeci, U. Sahin, Systemic  
816 RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy,  
817 *Nature*. 534 (2016) 396–401. doi:10.1038/nature18300.
- [23] 818 A.J. Geall, A. Verma, G.R. Otten, C.A. Shaw, A. Hekele, K. Banerjee, Y. Cu, C.W.  
819 Beard, L.A. Brito, T. Krucker, D.T. O'Hagan, M. Singh, P.W. Mason, N.M. Valiante,  
820 P.R. Dormitzer, S.W. Barnett, R. Rappuoli, J.B. Ulmer, C.W. Mandl, Nonviral delivery  
821 of self-amplifying RNA vaccines, *Proc. Natl. Acad. Sci. U. S. A.* 109 (2012) 14604–9.  
822 doi:10.1073/pnas.1209367109.
- [24] 823 N. Pardi, S. Tuyishime, H. Muramatsu, K. Kariko, B.L. Mui, Y.K. Tam, T.D. Madden,  
824 M.J. Hope, D. Weissman, Expression kinetics of nucleoside-modified mRNA delivered  
825 in lipid nanoparticles to mice by various routes, *J. Control. Release*. 217 (2015) 345–  
826 351. doi:10.1016/j.jconrel.2015.08.007.
- [25] 827 K. Bahl, J.J. Senn, O. Yuzhakov, A. Bulychev, L.A. Brito, K.J. Hassett, M.E. Laska,  
828 M. Smith, Ö. Almarsson, J. Thompson, A.M. Ribeiro, M. Watson, T. Zaks, G.  
829 Ciaramella, Preclinical and Clinical Demonstration of Immunogenicity by mRNA  
830 Vaccines against H10N8 and H7N9 Influenza Viruses, *Mol. Ther.* 25 (2017) 1316–  
831 1327. doi:10.1016/j.ymthe.2017.03.035.
- [26] 832 N. Pardi, M.J. Hogan, R.S. Pelc, H. Muramatsu, H. Andersen, C.R. DeMaso, K.A.  
833 Dowd, L.L. Sutherland, R.M. Scarce, R. Parks, W. Wagner, A. Granados, J.  
834 Greenhouse, M. Walker, E. Willis, J.-S. Yu, C.E. McGee, G.D. Sempowski, B.L. Mui,  
835 Y.K. Tam, Y.-J. Huang, D. Vanlandingham, V.M. Holmes, H. Balachandran, S. Sahu,  
836 M. Lifton, S. Higgs, S.E. Hensley, T.D. Madden, M.J. Hope, K. Karikó, S. Santra, B.S.  
837 Graham, M.G. Lewis, T.C. Pierson, B.F. Haynes, D. Weissman, Zika virus protection  
838 by a single low-dose nucleoside-modified mRNA vaccination, *Nature*. 543 (2017) 248–  
839 251. doi:10.1038/nature21428.
- [27] 840 J.M. Richner, B.W. Jagger, C. Shan, C.R. Fontes, K.A. Dowd, B. Cao, S. Himansu,  
841 E.A. Caine, B.T.D. Nunes, D.B.A. Medeiros, A.E. Muruato, B.M. Foreman, H. Luo, T.  
842 Wang, A.D. Barrett, S.C. Weaver, P.F.C. Vasconcelos, S.L. Rossi, G. Ciaramella, I.U.  
843 Mysorekar, T.C. Pierson, P.-Y. Shi, M.S. Diamond, Vaccine Mediated Protection  
844 Against Zika Virus-Induced Congenital Disease, *Cell*. 170 (2017) 273–283.e12.  
845 doi:10.1016/j.cell.2017.06.040.
- [28] 846 V.B. Joshi, S.M. Geary, A.K. Salem, Biodegradable particles as vaccine antigen  
847 delivery systems for stimulating cellular immune responses, *Hum. Vaccin.*  
848 *Immunother.* 9 (2013) 2584–90. doi:10.4161/hv.26136.
- [29] 849 V. Pavot, M. Berthet, J. Ressaygues, S. Legaz, N. Handké, S.C. Gilbert, S. Paul, B.  
850 Verrier, Poly(lactic acid) and poly(lactic- co-glycolic acid) particles as versatile carrier  
851 platforms for vaccine delivery, *Nanomedicine*. 9 (2014) 2703–2718.  
852 doi:10.2217/nmm.14.156.
- [30] 853 A. Gutjahr, C. Phelip, A.-L. Coolen, C. Monge, A.-S. Boisgard, S. Paul, B. Verrier,  
854 Biodegradable Polymeric Nanoparticles-Based Vaccine Adjuvants for Lymph Nodes  
855 Targeting., *Vaccines*. 4 (2016). doi:10.3390/vaccines4040034.
- [31] 856 A. Mahapatro, D.K. Singh, Biodegradable nanoparticles are excellent vehicle for site  
857 directed in-vivo delivery of drugs and vaccines, *J. Nanobiotechnology*. 9 (2011) 55.  
858 doi:10.1186/1477-3155-9-55.
- [32] 859 B. Tyler, D. Gullotti, A. Mangraviti, T. Utsuki, H. Brem, Polylactic acid (PLA)  
860 controlled delivery carriers for biomedical applications, *Adv. Drug Deliv. Rev.* 107  
861 (2016) 163–175. doi:10.1016/j.addr.2016.06.018.
- [33] 862 V. Pavot, N. Rochereau, C. Primard, C. Genin, E. Perouzel, T. Lioux, S. Paul, B.



- 863 Verrier, Encapsulation of Nod1 and Nod2 receptor ligands into poly(lactic acid)  
864 nanoparticles potentiates their immune properties, *J. Control. Release.* 167 (2013) 60–  
865 7. doi:10.1016/j.jconrel.2013.01.015.
- 866 [34] A. Gutjahr, L. Papagno, F. Nicoli, A. Lamoureux, F. Vernejoul, T. Lioux, E. Gostick,  
867 D.A. Price, G. Tiraby, E. Perouzel, V. Appay, B. Verrier, S. Paul, Cutting Edge: A  
868 Dual TLR2 and TLR7 Ligand Induces Highly Potent Humoral and Cell-Mediated  
869 Immune Responses, *J. Immunol.* 198 (2017) 4205–4209.  
870 doi:10.4049/jimmunol.1602131.
- 871 [35] N. Climent, S. Munier, N. Piqué, F. García, V. Pavot, C. Primard, V. Casanova, J.M.  
872 Gatell, B. Verrier, T. Gallart, Loading dendritic cells with PLA-p24 nanoparticles or  
873 MVA expressing HIV genes induces HIV-1-specific T cell responses, *Vaccine.* 32  
874 (2014) 6266–6276. doi:10.1016/j.vaccine.2014.09.010.
- 875 [36] C. Primard, N. Rochereau, E. Luciani, C. Genin, T. Delair, S. Paul, B. Verrier, Traffic  
876 of poly(lactic acid) nanoparticulate vaccine vehicle from intestinal mucus to sub-  
877 epithelial immune competent cells, *Biomaterials.* 31 (2010) 6060–8.  
878 doi:10.1016/j.biomaterials.2010.04.021.
- 879 [37] J. Rességuier, E. Delaune, A.-L. Coolen, J.-P. Levraud, P. Boudinot, D. Le Guellec, B.  
880 Verrier, Specific and Efficient Uptake of Surfactant-Free Poly(Lactic Acid)  
881 Nanovaccine Vehicles by Mucosal Dendritic Cells in Adult Zebrafish after Bath  
882 Immersion., *Front. Immunol.* 8 (2017) 190. doi:10.3389/fimmu.2017.00190.
- 883 [38] A.E. Nel, L. Mädler, D. Velegol, T. Xia, E.M. V Hoek, P. Somasundaran, F. Klaessig,  
884 V. Castranova, M. Thompson, Understanding biophysicochemical interactions at the  
885 nano-bio interface., *Nat. Mater.* 8 (2009) 543–57. doi:10.1038/nmat2442.
- 886 [39] V.K. Udhayakumar, A. De Beuckelaer, J. McCaffrey, C.M. McCrudden, J.L.  
887 Kirschman, D. Vanover, L. Van Hoecke, K. Roose, K. Deswarte, B.G. De Geest, S.  
888 Lienenklaus, P.J. Santangelo, J. Grooten, H.O. McCarthy, S. De Koker, Arginine-Rich  
889 Peptide-Based mRNA Nanocomplexes Efficiently Instigate Cytotoxic T Cell Immunity  
890 Dependent on the Amphipathic Organization of the Peptide, *Adv. Healthc. Mater.* 6  
891 (2017) 1601412. doi:10.1002/adhm.201601412.
- 892 [40] Z. Shen, G. Reznikoff, G. Dranoff, K.L. Rock, Cloned dendritic cells can present  
893 exogenous antigens on both MHC class I and class II molecules., *J. Immunol.* 158  
894 (1997) 2723–30. <http://www.ncbi.nlm.nih.gov/pubmed/9058806> (accessed August 29,  
895 2018).
- 896 [41] S. Legaz, J.-Y. Exposito, C. Lethias, B. Viginier, C. Terzian, B. Verrier, Evaluation of  
897 polylactic acid nanoparticles safety using *Drosophila* model, *Nanotoxicology.* 10  
898 (2016) 1136–1143. doi:10.1080/17435390.2016.1181806.
- 899 [42] J. Rejman, V. Oberle, I.S. Zuhorn, D. Hoekstra, Size-dependent internalization of  
900 particles via the pathways of clathrin- and caveolae-mediated endocytosis., *Biochem. J.*  
901 377 (2004) 159–69. doi:10.1042/BJ20031253.
- 902 [43] B. Langlet-Bertin, C. Leborgne, D. Scherman, B. Bechinger, A. James Mason, A.  
903 Kichler, Design and Evaluation of Histidine-Rich Amphipathic Peptides for siRNA  
904 Delivery, *Pharm. Res.* 27 (2010) 1426–1436. doi:10.1007/s11095-010-0138-2.
- 905 [44] A. Gutjahr, G. Tiraby, E. Perouzel, B. Verrier, S. Paul, Triggering Intracellular  
906 Receptors for Vaccine Adjuvantation, *Trends Immunol.* 37 (2016) 716.  
907 doi:10.1016/j.it.2016.08.005.
- 908 [45] J.E. Pérez-Ortín, P. Alepuz, S. Chávez, M. Choder, Eukaryotic mRNA Decay:  
909 Methodologies, Pathways, and Links to Other Stages of Gene Expression, *J. Mol. Biol.*  
910 425 (2013) 3750–3775. doi:10.1016/j.jmb.2013.02.029.
- 911 [46] P. Midoux, C. Pichon, Lipid-based mRNA vaccine delivery systems, *Expert Rev.*  
912 *Vaccines.* 14 (2015) 221–234. doi:10.1586/14760584.2015.986104.

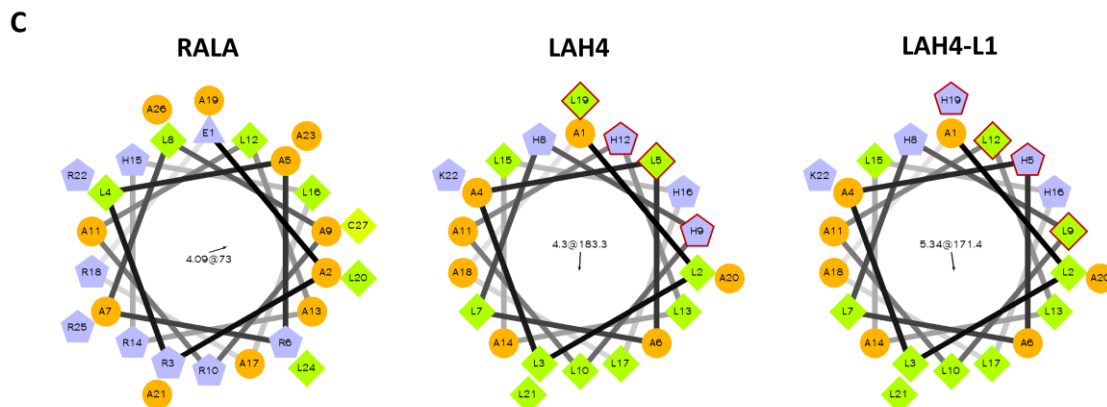
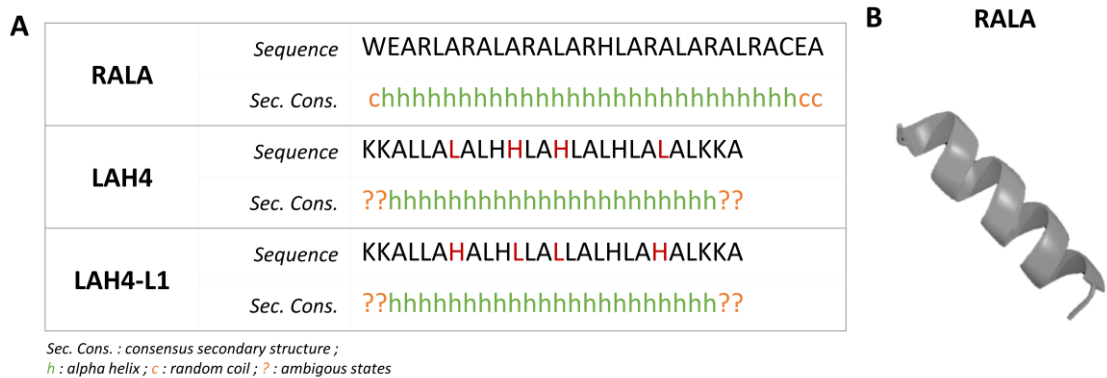
- 913 [47] H.O. McCarthy, J. McCaffrey, C.M. McCrudden, A. Zholobenko, A.A. Ali, J.W.  
914 McBride, A.S. Massey, S. Pentlavalli, K.-H. Chen, G. Cole, S.P. Loughran, N.J.  
915 Dunne, R.F. Donnelly, V.L. Kett, T. Robson, Development and characterization of  
916 self-assembling nanoparticles using a bio-inspired amphipathic peptide for gene  
917 delivery, *J. Control. Release.* 189 (2014) 141–149. doi:10.1016/j.jconrel.2014.06.048.
- 918 [48] G.D. Bell, Y. Yang, E. Leung, G.W. Krissansen, mRNA transfection by a Xentry-  
919 protamine cell-penetrating peptide is enhanced by TLR antagonist E6446, *PLoS One.*  
920 13 (2018) e0201464. doi:10.1371/journal.pone.0201464.
- 921 [49] I.R. de Figueiredo, J.M. Freire, L. Flores, A.S. Veiga, M.A.R.B. Castanho, Cell-  
922 penetrating peptides: A tool for effective delivery in gene-targeted therapies, *IUBMB*  
923 *Life.* 66 (2014) 182–194. doi:10.1002/iub.1257.
- 924 [50] B. Lou, S. De Koker, C.Y.J. Lau, W.E. Hennink, E. Mastrobattista, mRNA polyplexes  
925 with post-conjugated GALA peptides efficiently target, transfect and activate antigen  
926 presenting cells, *Bioconjug. Chem.* (2018). doi:10.1021/acs.bioconjchem.8b00524.
- 927 [51] P. Midoux, C. Pichon, J.-J. Yaouanc, P.-A. Jaffrès, Chemical vectors for gene delivery:  
928 a current review on polymers, peptides and lipids containing histidine or imidazole as  
929 nucleic acids carriers, *Br. J. Pharmacol.* 157 (2009) 166–78. doi:10.1111/j.1476-  
930 5381.2009.00288.x.
- 931 [52] R. Bennett, A. Yakkundi, H.D. McKeen, L. McClements, T.J. McKeogh, C.M.  
932 McCrudden, K. Arthur, T. Robson, H.O. McCarthy, RALA-mediated delivery of  
933 FKBPL nucleic acid therapeutics, *Nanomedicine (Lond).* 10 (2015) 2989–3001.  
934 doi:10.2217/nnm.15.115.
- 935 [53] K.K.L. Phua, K.W. Leong, S.K. Nair, Transfection efficiency and transgene expression  
936 kinetics of mRNA delivered in naked and nanoparticle format, *J. Control. Release.* 166  
937 (2013) 227–33. doi:10.1016/j.jconrel.2012.12.029.
- 938 [54] S. Persano, M.L. Guevara, Z. Li, J. Mai, M. Ferrari, P.P. Pompa, H. Shen,  
939 Lipopolyplex potentiates anti-tumor immunity of mRNA-based vaccination,  
940 *Biomaterials.* 125 (2017) 81–89. doi:10.1016/j.biomaterials.2017.02.019.
- 941 [55] V. Manolova, A. Flace, M. Bauer, K. Schwarz, P. Saudan, M.F. Bachmann,  
942 Nanoparticles target distinct dendritic cell populations according to their size, *Eur. J.*  
943 *Immunol.* 38 (2008) 1404–13. doi:10.1002/eji.200737984.
- 944 [56] M.F. Bachmann, G.T. Jennings, Vaccine delivery: a matter of size, geometry, kinetics  
945 and molecular patterns, *Nat. Rev. Immunol.* 10 (2010) 787–96. doi:10.1038/nri2868.
- 946 [57] K.L. Douglas, C.A. Piccirillo, M. Tabrizian, Cell line-dependent internalization  
947 pathways and intracellular trafficking determine transfection efficiency of nanoparticle  
948 vectors, *Eur. J. Pharm. Biopharm.* 68 (2008) 676–687.  
949 doi:10.1016/J.EJPB.2007.09.002.
- 950 [58] S.M. Farkhani, A. Valizadeh, H. Karami, S. Mohammadi, N. Sohrabi, F. Badrzadeh,  
951 Cell penetrating peptides: Efficient vectors for delivery of nanoparticles, nanocarriers,  
952 therapeutic and diagnostic molecules, *Peptides.* 57 (2014) 78–94.  
953 doi:10.1016/J.PEPTIDES.2014.04.015.
- 954 [59] E.O. Freed, HIV-1 assembly, release and maturation, *Nat. Rev. Microbiol.* 13 (2015)  
955 484–496. doi:10.1038/nrmicro3490.
- 956 [60] L. Leal, C. Lucero, J.M. Gatell, T. Gallart, M. Plana, F. García, New challenges in  
957 therapeutic vaccines against HIV infection, *Expert Rev. Vaccines.* 16 (2017) 587–600.  
958 doi:10.1080/14760584.2017.1322513.
- 959 [61] L. Leal, A.C. Guardo, S. Morón-López, M. Salgado, B. Mothe, C. Heirman, P. Pannus,  
960 G. Vanham, H.J. van den Ham, R. Gruters, A. Andeweg, S. Van Meirvenne, J. Pich,  
961 J.A. Arnaiz, J.M. Gatell, C. Brander, K. Thielemans, J. Martínez-Picado, M. Plana, F.  
962 García, iHIVARNA consortium, Phase I clinical trial of an intranodally administered

963 mRNA based therapeutic vaccine against HIV-1 infection, AIDS. (2018) 1.  
 964 doi:10.1097/QAD.0000000000002026.  
 965 [62] B. Autran, Toward a cure for HIV-Seeking effective therapeutic vaccine strategies,  
 966 Eur. J. Immunol. 45 (2015) 3215–3221. doi:10.1002/eji.201545513.  
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971 **SUPPLEMENTARY DATA**

972 **Table I: Mode of action and working concentration of chemical inhibitor used**

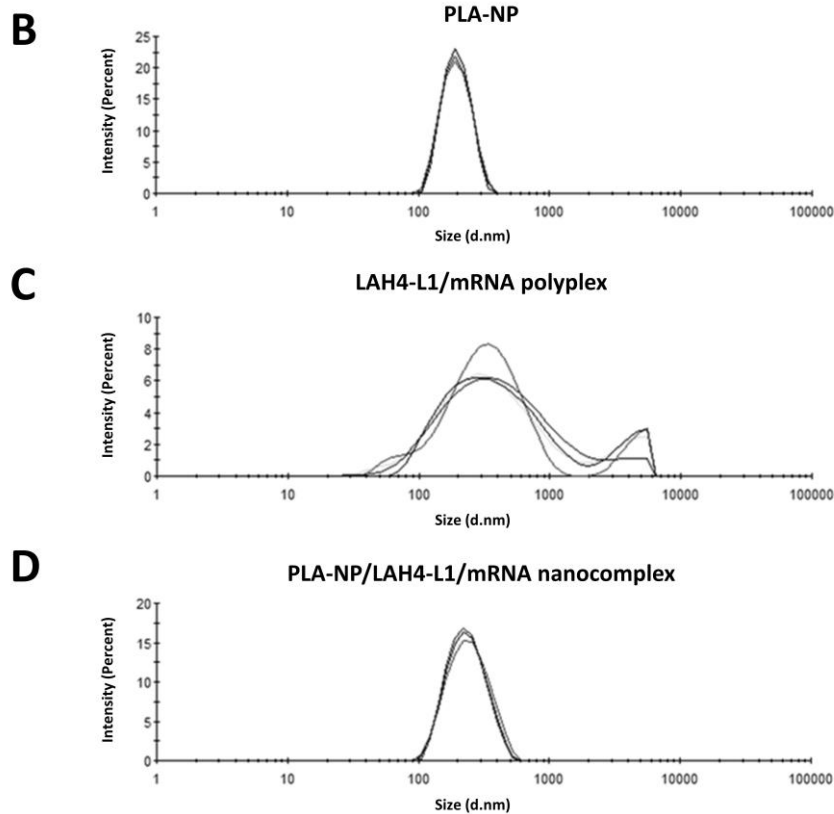
Inhibitors	Endocytosis pathways inhibited	Mode of action	Working concentration	Ref
<b>Genistein (Sigma-aldrich)</b>	Caveolae-dependent endocytosis	Inhibits actin cytoskeleton	50 µM	[1,2]
<b>Chlorpromazine (Sigma-aldrich)</b>	Clathrin-mediated endocytosis	Dissociation of clathrin and adaptator from plasma membrane	15 µg/mL	[2]
<b>Dynasore (Sigma-aldrich)</b>	Caveolae and clathrin-dependent endocytosis	Inhibits dynamin	50 µM	[3]
<b>Nocodazole (Sigma-aldrich)</b>	Caveolae and clathrin-dependent endocytosis	Blocks transport from early to late endosomes through inhibition of microtubules polymerization	1.25 µg/mL	[4]
<b>Amiloride (Sigma-aldrich)</b>	Macropinocytosis	Inhibits the Na <sup>+</sup> /H <sup>+</sup> exchange protein	100 µM	[2,5]
<b>Pimozide (Sigma-aldrich)</b>	Phagocytosis	Specific Ca <sup>2+</sup> /calmodulin inhibitor	10 µM	[6]
<b>Cytochalasin D (Sigma-aldrich)</b>	Macropinocytosis and phagocytosis	Potent inhibitor of actin polymerization	10 µM	[2]



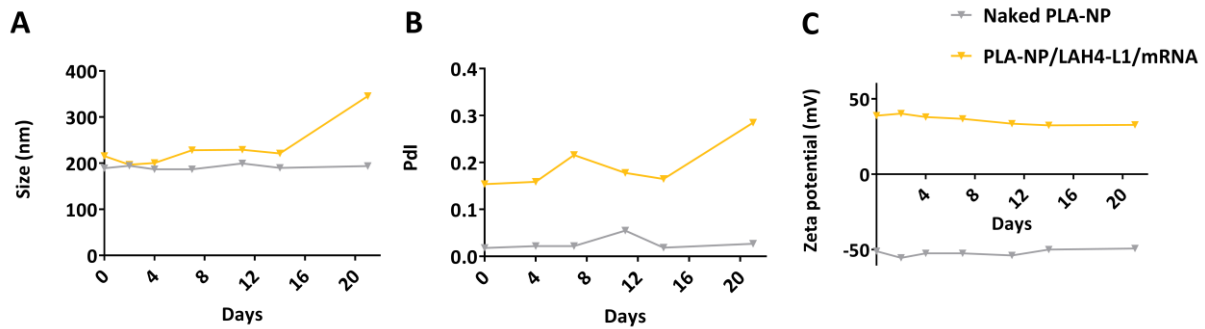
973 **Figure S1: Sequence and predictive structure of RALA, LAH4 and LAH4-L1 peptides.** (A)  
 974 Sequence and secondary structure of RALA, LAH4 and LAH4-L1, based on NPS@ (Network Protein Sequence  
 975 Analysis) interactive web server using 9 predictions[7]. In green (h) was represented the predicted alpha helix  
 976 structure and in orange (c) the random coil. Question mark (?) represent ambiguous state. Modified amino acids  
 977 between LAH4 and LAH4-L1 sequence are red. (B) Representation of RALA structure in alpha helix (rzlab.com)  
 978 (C) Helical wheel projection of RALA, LAH4 and LAH4-L1. The output represents the hydrophilic residues as  
 979 circles, hydrophobic residues as diamonds, potentially negatively charged amino acids as triangles, and  
 980 potentially positively charged residues as pentagons. Hydrophobicity is color coded as well: hydrophobic  
 981 residue is green and hydrophilic residues are coded orange. The potentially charged residues are light blue.  
 982 Modified amino acids between LAH4 and LAH4-L1 sequence are outlined in red.

**A**

Nanocomplex	Hydrodynamic diameter (nm)	PdI	Zeta potential (mV)
PLA-NP	188.0 ( $\pm$ 1.80)	0.053 ( $\pm$ 0.046)	- 51.8 ( $\pm$ 0.6)
LAH4-L1 / mRNA	262.0 ( $\pm$ 24.1)	0.300 ( $\pm$ 0.49)	+ 25.0 ( $\pm$ 2.8)
PLA-NP / LAH4-L1 / mRNA	220.1 ( $\pm$ 22.9)	0.129 ( $\pm$ 0.058)	+ 37.3 ( $\pm$ 4.4)

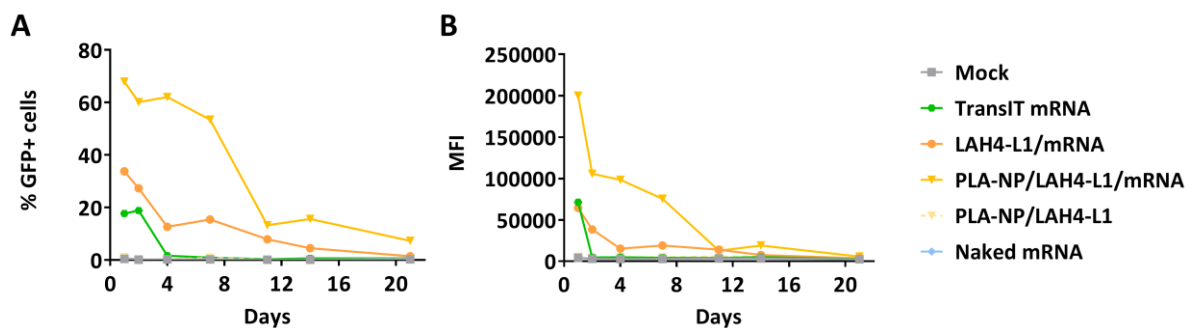


983 **Figure S2: Characterization of LAH4-L1/mRNA and PLA-NP/LAH4-L1/mRNA by DLS.**  
 984 (A) Evaluation of PLA-NP (in grey), LAH4-L1/mRNA (in orange) and PLA-NP/LAH4-L1/mRNA (in yellow)  
 985 hydrodynamic diameter, polydispersity index (PdI) and zeta potential. Each value provided is the average of  
 986 four series of measurements in five independent experiments (N=5). (B-D) Graphs represent the size distribution  
 987 by intensity of the four measurements obtained in one representative experiment for Naked PLA-NP (B), LAH4-  
 988 L1/mRNA polyplex (C) and PLA-NP/LAH4-L1/mRNA nanocomplex (D), respectively.



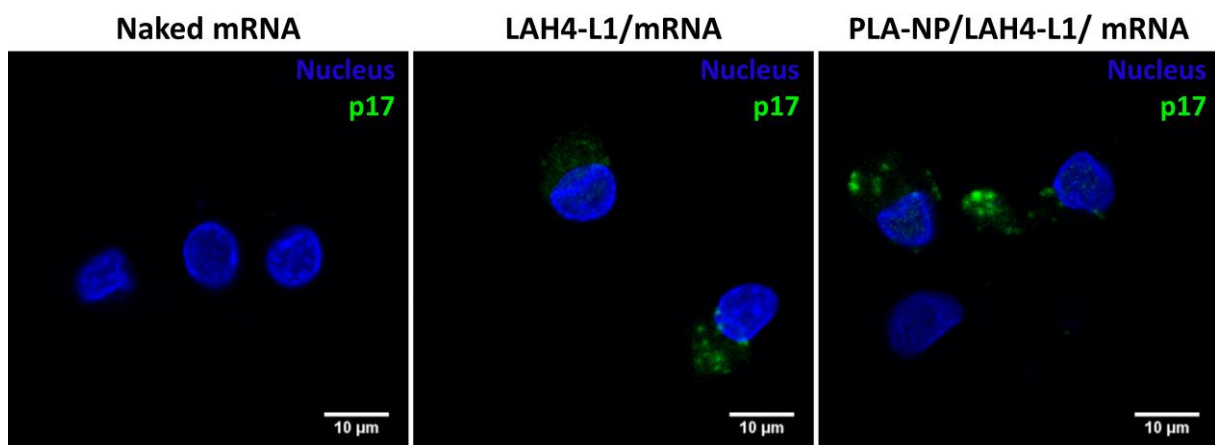
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990 **Figure S3: Stability of PLA-NP/LAH4-L1/mRNA formulation at 4°C over 21 days.**  
 991 Evaluation of PLA-NP (in grey) and PLA-NP/LAH4-L1/mRNA (in yellow) hydrodynamic diameter (A),  
 992 polydispersity index (PDI) (B) and surface charge (C) over 21 days at 4°C. Each value provided is the average  
 993 of four series of measurements.  
 994



995

996 **Figure S4: LAH4-L1 formulations retain ability to induce efficient mRNA expression**  
 997 **in DCs after conservation at 4°C for up to 7 days.** Formulations were performed at day 0 and  
 998 conserved at 4°C during 21 days. At each time point, untreated DCs (Mock), TransIT mRNA (positive control),  
 999 mRNA complexes and negative controls (PLA-NP/LAH4-L1 and naked mRNA) were transfected in serum-free  
 1000 medium for 4 hours (20 ng mRNA/well), and the transfection efficiency was measured after 16h. (A) Percentage  
 1001 of green fluorescent protein (eGFP) positive DC2.4 cells (B) mean fluorescence intensity (MFI).  
 1002  
 1003



1004

1005 **Figure S5: Expression of gag mRNA in moDCs.** Gag mRNAs, either naked or complexed in LAH4-  
 1006 L1-based formulations, were transfected into moDCs in serum-free medium during 4 hours (20 ng mRNA/well).  
 1007 An immunostaining of p17 protein, a component of gag polyprotein, was performed after 16 h. p17 is in green  
 1008 (FITC) and nuclei, in blue (DAPI).  
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1010 **Supplementary references**

- 1011  
1012 [1] L.H. Wang, K.G. Rothberg, R.G. Anderson, Mis-assembly of clathrin lattices on  
1013 endosomes reveals a regulatory switch for coated pit formation, *J. Cell Biol.* 123  
1014 (1993) 1107–17. <http://www.ncbi.nlm.nih.gov/pubmed/8245121>.
- 1015 [2] I.A. Khalil, K. Kogure, H. Akita, H. Harashima, Uptake Pathways and Subsequent  
1016 Intracellular Trafficking in Nonviral Gene Delivery, *Pharmacol. Rev.* 58 (2006) 32–45.  
1017 doi:10.1124/pr.58.1.8.
- 1018 [3] E. Macia, M. Ehrlich, R. Massol, E. Boucrot, C. Brunner, T. Kirchhausen, Dynasore, a  
1019 Cell-Permeable Inhibitor of Dynamin, *Dev. Cell.* 10 (2006) 839–850.  
1020 doi:10.1016/j.devcel.2006.04.002.
- 1021 [4] A.I. Idilli, P. Morandini, E. Onelli, S. Rodighiero, M. Caccianiga, A. Moscatelli,  
1022 Microtubule Depolymerization Affects Endocytosis and Exocytosis in the Tip and  
1023 Influences Endosome Movement in Tobacco Pollen Tubes, *Mol. Plant.* 6 (2013) 1109–  
1024 1130. doi:10.1093/mp/sst099.
- 1025 [5] J.P. Lim, P.A. Gleeson, Macropinocytosis: an endocytic pathway for internalising large  
1026 gulps, *Immunol. Cell Biol.* 89 (2011) 836–843. doi:10.1038/icb.2011.20.
- 1027 [6] K.A. Key, A.C. Oakley, S. V Pizzo, Effects of pimozide and penfluridol on the binding  
1028 and endocytosis of alpha 2-macroglobulin-CH<sub>3</sub>NH<sub>2</sub> by mouse peritoneal macrophages,  
1029 *Biochem. Pharmacol.* 33 (1984) 3712–4.  
1030 <http://www.ncbi.nlm.nih.gov/pubmed/6210087>.
- 1031 [7] C. Combet, C. Blanchet, C. Geourjon, G. Deléage, NPS@: network protein sequence  
1032 analysis., *Trends Biochem. Sci.* 25 (2000) 147–50. doi:[https://doi.org/10.1016/S0968-](https://doi.org/10.1016/S0968-0004(99)01540-6)  
1033 [0004\(99\)01540-6](https://doi.org/10.1016/S0968-0004(99)01540-6).
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