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Encapsulation of Nod1 and Nod2 receptor ligands into poly(lactic acid) nanoparticles potentiates their immune properties

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ABSTRACT

Most successful vaccines are able to induce persistent antibody responses that can last a lifetime. Emerging evidences indicate that activation of immune cells through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) or Nod-like receptors (NLRs) may be critical mechanisms. Among PRRs, the use of TLR ligands as adjuvants is already largely described whereas the use of NLRs ligands remains largely unexplored. As activation of intracytoplasmic NLRs is able to induce proinflammatory molecules, the added value of encapsulation of Nod1 and Nod2 receptor ligands into Poly(Lactic Acid) (PLA) biodegradable nanocarriers to modulate their immune properties on human dendritic cells (DCs) maturation has been evaluated. Their ability to induce systemic immune responses in mice was also measured and compared to free ligands and the Alum adjuvant. Nod ligands encapsulated into PLA NPs were efficiently taken up by DCs and subsequently induced a strong up-regulation of maturation markers and the enhancement of proinflammatory cytokine secretion by DCs. Furthermore, co-injection of encapsulated Nod-ligands with PLA particles carrying Gag p24 HIV-1 antigen allowed a 100 fold increase in antibody responses in comparison to Alum. These results suggest that encapsulation of Nod ligands into PLA-NPs could be an effective way to improve vaccine efficiency.

1. Introduction

Synthetic peptides and recombinant protein subunits are largely used as vaccine candidates, as they are less toxic than whole pathogens. Although these compounds offer advantages, they are often poorly immunogenic. Thus, it is necessary to enhance their immune responses by developing improved vaccine adjuvants [1], which are potent, safe and compatible with protein subunits and peptides [2]. Adjuvant may be defined as additives or vehicles that improve the adaptive immune response or stimulate the innate immune system in such a way that desired effectors or mediators are induced.

Over the last two decades, nanoparticle-based delivery systems have turned out to be promising vaccine vehicles. A variety of polymers exists from which nanoparticles (NPs) for drug delivery can be prepared. However, the most commonly studied polymers are poly(D,L-lactic-co-glycolic acid) (PLGA) and polylactide (PLA) [3]. These biodegradable, biocompatible polymers have been approved for human use (e.g., as sutures, bone implants and screws as well as implants for sustained drug delivery). They have been extensively studied for their use in the formulation of vaccine antigens (i.e., proteins, peptides, DNA) [4–7], a large body of literature being available that demonstrates the advantages of such nanoparticles for antigen delivery [8–10]. Indeed, these particles can be tailored to degrade over a range of rates and can act as a depot from which the encapsulated ligand is gradually released [3]. Additionally, PLA particles allow the encapsulation of hydrophobic molecules and may protect encapsulated antigens. Moreover, preclinical studies have shown that PLA nanoparticles can induce systemic antibody titers comparable to those of aluminium salts or MF59 [3,6,11].

The targeting of pattern recognition receptors (PRRs) by adjuvants is a very promising way to modulate immune responses. The use of Toll-like receptors (TLRs) ligands as adjuvant for vaccine administration is already largely described, but the use of nucleotide-binding and oligomerization domain (Nod)-like receptor (NLR) ligands is still under investigation [12,13]. Here we described the study of Nod1 and Nod2, two members of NLRs class. Whereas TLRs sense PAMPs (Pathogen-Associated Molecular Pattern) in the extracellular space and endosomes, NLRs function exclusively as pathogen intracytoplasmic sensors [14]. This family of receptors recognizes microbial structures, such peptidoglycan (PGN) and flagellin, but also danger-associated molecules released by dead or dying cells [15]. Nod2 detects muramyl dipeptide (MDP) which is a motif common to Gram-positive and Gram-negative bacterial PGN [16]. In contrast, Nod1 especially detects the γ-D-Glu-mDAP (iE-DAP), which

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is a dipeptide present in the PGN of all Gram-negative and certain Gram-positive bacteria [17,18]. iE-DAP is the minimal motif recognized by the intracellular receptor Nod1.

Both iE-DAP and MDP must be intracellularly delivered either by bacteria that invade the cell or through other cellular uptake mechanisms (pannexin, PepT1/PepT2 or endocytosis) to be detected by Nod1 and Nod2 respectively [19]. Signalling downstream of Nod1 and Nod2 mainly results in the activation of mitogen-activated protein kinase (MAPK) and NF-κB signalling [20] resulting in the production of proinflammatory cytokines (IL-1β, IL-6, TNF and IL-8) and chemokines (CCL5 and CXCL5) [21].

Nod1 and Nod2 are mainly expressed by antigen presenting cells (APCs) and epithelial cells that are highly exposed to and/or deal with microorganisms that express PGN. In both humans and mice, APCs such as macrophages and dendritic cells (DCs) highly express Nod1 and Nod2 [22], whereas other hematopoietic cells (such as T cells and B cells) do not express these PRRs [18].

In our study, a nanoparticle-based vaccine has been designed. A biodegradable synthetic polymer, Poly(Lactic Acid) (PLA), was used to prepare ~200 nm sized nanoparticles containing encapsulated Nod1 or Nod2 ligand with the possibility to adsorb HIV-1 Gag p24 antigen on their surface. Encapsulated Nod ligands have already been studied in different particulate models as liposomes or chitosan [23,24] but not so far, into PLA nanoparticles for tuning the maturation of DCs *in vitro* and for tuning immune response *in vivo*. We first characterized the physico-chemical properties of the particles and the encapsulated Nod ligands, and then we evaluated the *in vitro* ability of both encapsulated and free Nod ligands to induce dendritic cells maturation. Moreover, the mechanisms by which this maturation occurs have been studied. Finally, the immunogenicity of our adjuvanted vaccine has been evaluated in mice by measuring the specific humoral immune responses induced by subcutaneous co-administration of synthetic NPs coated with HIV-1 Gag p24 as antigen and Nod1 or Nod2 ligands encapsulated into different NPs, compared to standard Alum formulation.

2. Materials and methods

2.1. Nod receptor ligands

The Nod1 ligand CL235 [tetradecanoyl-δ-D-glutamyl-L)-meso-lanthionyl-(D)-alanine] is an acyl tripeptide containing meso-Lanthionine instead of meso-DAP amino acid. The Nod2 ligand CL365 [6-O-stearoyl-N-glycolyl-Murabutide] is an acyl Murabutide analog (Supplementary Fig. 1). These compounds, developed and manufactured by CAYLA-InvivoGen (Toulouse, France), have been selected for the ease of chemical synthesis and because the attachment to a hydrophobic acyl residue enhanced stimulation of Nod1 or Nod2 up to several hundred folds, as assessed by *in vitro* cell based assays.

2.2. PLA and HIV-1 Gag p24 antigen

Poly(D,L-lactic acid) (PLA50 Mn = 47,000 g/mol, molecular weight distribution Mw/Mn = 1.8) with a carboxylic end group was purchased from Anabior (Grenoble, France).

HIV-1 p24 antigen was produced and purified by PX'Therapeutics (Protein'eXpert, Grenoble, France) from *Escherichia coli* BL21 DE3 strain and endotoxins were removed as previously described [11]. The purity of p24 was higher than 97% as assessed by silver nitrate stained reducing SDS-PAGE; with an endotoxin content lower than 5 EU/mg of p24 protein, as determined using the QCL-1000 Quantitative Chromogenic Limulus Amebocyte Lysate (LAL) kit (BioWhittaker, Walkersville, Verviers, Belgique). The resulted recombinant p24 protein has a molecular weight of 26.9657 kDa and a theoretical pI of 5.92.

CellTrace™ BODIPY® TR methylester was obtained from Invitrogen (Invitrogen AG, Switzerland). All other reagents were of analytical grade and received from commercial sources.

2.3. Preparation of Nods- and fluorescent-PLA nanoparticles by nanoprecipitation and physico-chemical characterization

PLA nanoparticles were prepared by nanoprecipitation as previously described [7]. Briefly, the polymer was dissolved in acetone (polymer: acetone in a 2% w/v ratio), and this solution was added dropwise to an aqueous solution (ethanol/water; 40%/60%) under slow stirring. Organic solvents were then removed under reduced pressure at 30 °C.

For their encapsulation into PLA nanoparticles, Nod1, Nod2, or the fluorescent hydrophobic probe (CellTrace™ BODIPY®) were dissolved in acetone with the polymer (at a polymer:drug ratio of 1% w/w for the Nod ligands and 2% w/w for the fluorophore) and the nanoprecipitation was conducted in the same way.

The final PLA concentration was between 60 and 70 mg/ml, depending on the batch and was accurately measured by weighing the wet and dried materials.

Solvent was detected by gas chromatography from dissolved particle samples to the sensitivity limits of this method.

Nanoparticle average hydrodynamic diameter and size distribution (PSD or PI) were determined by photon correlation spectroscopy at 25 °C using a Zetasizer Nano ZS (Malvern, UK). Highly diluted (1/250) colloidal dispersions in 1 mM NaCl solution were used and each value was the mean of three to five measurements. The electrophoretic mobilities were measured with the Zetasizer Nano ZS using the same highly diluted samples, at 25 °C, and were converted to Zeta (ζ) potentials according to Smoluchowski's equation [25]. The values were the mean of five independent measurements.

Particles observation by Scanning Electron Microscopy (SEM) was performed by deposing a drop of particles diluted in water on a Formvar-coated copper grid that was dried by evaporation. The grid was then sputtered with 20 nm of gold with the metallizer BaltecMED020 under vacuum prior observation (Hitachi S800, Centre Technologique des Microstructures, Lyon 1, Villeurbanne, France) (data on Supplementary Fig. 2.).

2.4. Determination of encapsulation efficiency of Nod1 and Nod2 ligands into nanoparticles

Ligand concentrations were estimated by using the HEK-Blue™-hNod reporter cell lines (InvivoGen) by monitoring the activation of the NF-κB pathway using a secreted embryonic alkaline phosphatase (SEAP). In the HEK-Blue™-hNod reporter cell line, the SEAP reporter gene is placed under the control of the IFN-β minimal promoter fused to five NF-κB and AP-1 binding sites into HEK293 cells. Stimulation with Nod ligands activates NF-κB and AP-1, which induce the production of SEAP. Level of SEAP can be easily quantified with a detection medium that turns purple/blue in the presence of alkaline phosphatase.

Nod ligand encapsulation efficiencies were obtained through determination of the amount of remaining free ligand in the supernatant after two successive centrifugations of the nanoparticle solution (10 min at 10,000 × *g*). The supernatants were added in 96-microwell plates (50,000 cells/well in duplicate) for 16 h in presence of a specific SEAP colour substrate (HEK-Blue™ Detection, InvivoGen). The absorbance of the samples was measured at 650 nm using a microplate reader (BioRad). Nod1 and Nod2 ligand solutions (respectively 0–0.8 μg/ml and 0–4 μg/ml) were initially assayed with HEK-Blue™-hNod Cells for establishing a calibration curve. The calibration curves were found to be linear with a correlation coefficient of $R^2 = 0.9971$ and $R^2 = 0.9992$ respectively. The encapsulation efficiencies of Nod ligands were calculated by the ratio of the ligand mass in nanoparticles over the total mass of ligand in the solution.