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Directing vaccine immune responses to mucosa by nanosized particulate carriers encapsulating NOD ligands

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ABSTRACT

Mucosal surfaces are a major portal of entry for many pathogens that are the cause of infectious diseases. Therefore, effective vaccines that induce a protective immune response at these sites are much needed. However, despite early success with the live attenuated oral polio vaccine over 50 years ago, only a few new mucosal vaccines have been subsequently licensed. Development of new adjuvants, comprising antigen delivery platforms and immunostimulatory molecules, are critical for the successful development of new mucosal vaccines. Among them, biodegradable nanoparticle delivery systems are promising and NOD-like receptors are considered as potential new targets for immunostimulatory molecules.

In this work, different NOD1 and NOD2 ligands were encapsulated in polylactic acid (PLA) nanoparticles, coated with HIV-1 gag p24 antigen. We showed that these new formulations are able to induce proliferation of HIV-specific T cells from HIV^+ individuals as well as autophagy. *In vivo*, these formulations highly enhanced p24-specific systemic and mucosal immune responses in mice not only after mucosal administration but also after immunization *via* the parenteral route.

Our results provide a rational approach for combining nanosized particulate carriers and encapsulated NOD receptor ligands as potent synergistic tools for induction of specific mucosal immunity.

1. Introduction

The vast majority of vaccines in use today are administered by subcutaneous or intramuscular injection and induce strong systemic immune responses which in some cases can protect against pathogens that enter through or infect a mucosal tissue (e.g. influenza virus, poliovirus, human papillomavirus, etc.) [1]. Indeed, for some mucosal pathogens, systemic induction of high titers of neutralizing antibodies (Abs) that enter tissue parenchyma or transude into the mucosal lumen are sufficient for clearing cell-free virus [2].

However, for protection at mucosal surfaces that are normally impermeable to serum Abs, as well as to protect against invasive pathogens that lead to chronic infection (e.g. hepatitis C virus, HIV and parasitic infections) systemic immune response generated by parenteral vaccination may be insufficient [3]. Induction of the mucosal innate and adaptive immune systems, including secretory IgA and IgG neutralizing Abs, T helper 1 (T_H1) and T_H2 $CD4^+$ T cells, T_H17 and high avidity $CD8^+$ CTL at the site of pathogen entry may be required for protection and could be generated by mucosal vaccination [4]. Mucosal vaccines also offer several other advantages over parenteral immunization such as, ease of administration, non-invasiveness, high-patient compliance and suitable for mass vaccination [4].

In the past decade the successful development of live attenuated mucosal vaccines against influenza virus (nasal route) and rotavirus (oral route) infections has boosted interest in this field, and great expectations for new mucosal vaccines lie ahead. However, despite early success with the live attenuated oral polio vaccine over 50 years ago, only a few vaccines targeting mucosal surfaces have been

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developed [5]. This is partly due to the lack of safe and effective mucosal adjuvants.

Major efforts are made to develop new vaccine candidates able to induce mucosal immune responses either by mucosal or parenteral administration by designing new mucosal routes of administration and selecting controlled release vehicles as well as immune-stimulatory adjuvant molecules able to redirect immune responses to mucosal sites [6,7].

Over the last two decades, nanoparticle (NP) based delivery systems have received a lot of attention in the fields of biology and medicine and appear promising as vaccine vehicles [8]. In specific, NPs have been shown to be capable of not only achieving optimal vaccine release, but also for targeting immune cells of interest and loading with immunostimulatory molecules [9]. NP formulations offer potential solutions to overcome biological barriers and target antigen presenting cells (APCs) at mucosal surface [10,11].

Nanoparticle-based synthetic vaccines including biomolecular NPs (liposomes, virus-like particles, micelles and immunostimulatory complexes), inorganic NPs and polymeric NPs represent a wide range of nanomaterials that have been utilized as vaccine delivery platforms [9]. Among polymeric NPs, a variety of biodegradable polymers exists from which NPs for vaccine delivery can be prepared but the most commonly studied are the poly(lactic-*co*-glycolic acid) (PLGA) and the poly(lactic acid) (PLA) [11–13]. These biodegradable and biocompatible polymers have been licensed for human use and are approved by US FDA [14]. PLA and PLGA polymers have been studied extensively for their use in the formulation of vaccine antigens (proteins, peptides, DNA) and there is a large body of literature demonstrating the advantages of such particles to increase Ab production following parenteral administration [12]. Additionally, these particles allow the encapsulation of hydrophobic molecules such as pattern recognition receptor (PRR) ligands which are able to specifically stimulate dendritic cells (DCs) which act at the crossroads between innate and adaptive immunity [15,16].

Recently, a family of cytosolic PRRs – named the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family – has received considerable attention [17–19]. NOD1 and NOD2 represent two well-characterized PRRs of the NLR family; they sense conserved fragments found in the cell wall of many types of bacteria and activate intracellular signaling pathways that drive proinflammatory and antimicrobial responses [17]. Specifically, NF-κB activation by NOD1 occurs in response to γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), primarily found in Gram-negative bacteria [20], whereas activation by NOD2 is triggered by muramyl dipeptide (MDP), a common peptidoglycan motif in both Gram-positive and Gram-negative bacteria [21].

NOD1 is a ubiquitous NLR that is expressed in most cell types and tissues tested [22]. Initially the expression of NOD2 was thought to be restricted to monocytes [23] however, further investigations revealed that NOD2 is also expressed in lymphocytes and Paneth cells of the intestine [24]. A wider expression pattern of NOD2 was confirmed further by several studies showing that NOD2 expression can be upregulated by stimulation with TNFα or IFNγ in a number of cell types [25].

In contrast to PRRs such as Toll-like receptors (TLRs), which recognize microbial ligands at the cell surface or within endosomes, NOD1 and NOD2 sense bacterial products in the host cytosol to provide another level of microbial surveillance. Research findings from us and others previously provided evidence that NOD ligands encapsulated into PLA particles were efficiently taken up by DCs and subsequently induced a strong up-regulation of maturation markers and enhancement of proinflammatory cytokine secretion by these cells compared to soluble ligands [26,27].

To date, no direct evaluation of NP carrying NOD ligands on mucosal immune responses has been performed. In order to assess this, synthetic PLA NPs coated with the HIV-1 gag p24 antigen and containing NOD1 or NOD2 ligands (PLA(NOD)-p24) were designed and compared using three routes of vaccination in mice: subcutaneous (s.c.), oral and intranasal (i.n.). The different PLA(NOD)-p24 formulations were compared to the gold standard alum adjuvant administered by the s.c. route and the heat-labile enterotoxin of *Escherichia coli* (LT) administered by the mucosal route [28,29].

The aim of our study was to identify the most potent NOD ligands and to determine whether their combination with biodegradable nanoparticulate carriers would be able to stimulate and redirect mucosal effector cells (both APCs and epithelial cells) to overcome the current challenge of designing vaccines able to induce mucosal immunity.

2. Materials and methods

2.1. PLA, HIV-1 gag p24 antigen and NOD receptor ligands

Poly(D,L-lactic acid) (PLA50 Mn = 30,000 g/mol, molecular weight distribution Mw/Mn = 1.7) with a carboxylic end group was purchased from Anabior (Grenoble, France).

HIV-1 gag p24 antigen was produced and purified from *E. coli*, and endotoxins were removed as previously described [30]. The purity of p24 was higher than 97% with an endotoxin content lower than 5 EU/mg of p24 protein, as determined using the Quantitative Chromogenic Limulus Amebocyte Lysate (LAL) kit (BioWhittaker, Walkersville, Verviers, Belgique).

The NOD1 ligand is an acyl tripeptide containing meso-Lanthionine instead of meso-DAP amino acid (molecule CL235 [tetradecanoyl-δ-D-glutamyl-(L)-meso-lanthionyl-(D)-alanine]).
The NOD2 ligand is an acyl Murabutide analog (molecule CL365 [6-O-stearoyl-N-glycolyl-Murabutide]). These compounds were selected for the ease of chemical synthesis and because the attachment to a hydrophobic acyl residue enhanced stimulation of the NOD receptors up to several hundred folds as assessed by cell based assays. These compounds were developed and manufactured by InvivoGen (Toulouse, France).

All other reagents were of analytical grade laboratory reagents and were procured from commercial sources.

2.2. Preparation of PLA nanoparticles with encapsulated NOD1 or NOD2 ligand

NOD ligands encapsulated into PLA nanoparticles were prepared by nanoprecipitation as previously described [26]. Briefly, the polymer and the NOD ligand (NOD1 or NOD2) were dissolved in acetone (polymer: acetone in a 2% w/v ratio and ligand: PLA concentration of 1% w/v), and this solution was added dropwise to an aqueous solution (ethanol/water) under slow stirring. Organic solvents were removed under reduced pressure at 30 °C.

No residual solvents were detected by gas chromatography from dissolved particle samples to the sensitivity limits of this method. Nanoparticle size and size distribution (PDI) were determined by photon correlation spectroscopy at 25 °C using a Zetasizer Nano ZS (Malvern, UK). Diluted colloidal dispersions in NaCl (1 mM) solution were used and each value was the mean of three to five measurements.

NOD ligand encapsulation efficiency was obtained through determination of the amount of remaining free ligand in the supernatant after centrifugations of the nanoparticle solution (10 min at 10,000×g). Supernatants were cultured with HEK-Blue™-hNOD reporter cell lines (InvivoGen) in 96-microwell plates (50,000 cells/well in duplicate) to study the stimulation of NOD receptors (NOD1 or NOD2) by monitoring the activation of NF-κB pathway thanks to