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Toll-like receptor 2 promiscuity is responsible for the immunostimulatory activity of nucleic acid nanocarriers

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ABSTRACT

Lipopolyamines (LPAs) are cationic lipids; they interact spontaneously with nucleic acids to form lipoplexes used for gene delivery. The main hurdle to using lipoplexes in gene therapy lies in their immunostimulatory properties, so far attributed to the nucleic acid cargo, while cationic lipids were considered as inert to the immune system. Here we demonstrate for the first time that di-C18 LPAs trigger pro-inflammatory responses through Toll-like receptor 2 (TLR2) activation, and this whether they are bound to nucleic acids or not. Molecular docking experiments suggest potential TLR2 binding modes reminiscent of bacterial lipopeptide sensing. The di-C18 LPAs share the ability of burying their lipid chains in the hydrophobic cavity of TLR2 and, in some cases, TLR1, at the vicinity of the dimerization interface; the cationic headgroups form multiple hydrogen bonds, thus crosslinking TLRs into functional complexes. Unravelling the molecular basis of TLR1 and TLR6-driven heterodimerization upon LPA binding underlines the highly collaborative and promiscuous ligand binding mechanism. The prevalence of non-specific main chain-mediated interactions demonstrates that potentially any saturated LPA currently used or proposed as transfection agent is likely to activate TLR2 during transfection. Hence our study emphasizes the urgent need to test the inflammatory properties of transfection agents and proposes the use of docking analysis as a preliminary screening tool for the synthesis of new non-immunostimulatory nanocarriers.

1. Introduction

Gene therapy, a technique that aims to replace a defective or missing gene with its normal allele at its natural location, emerged in the 70's [1], with the first successful somatic treatment to leave permanent DNA modification performed in the 90's. Nonetheless the technique is still in its infancy, and remains experimental in treating most diseases that can be traced back to gene disorders. Recently, the European Commission has approved treatment for adult patients diagnosed with familial lipoprotein lipase deficiency (LPLD) and for children with severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) [2–4].

The success of gene therapy is conditioned by the development of vectors able to transfect cells efficiently with minimal adverse effects [5–9]. The most widespread technique of transfection involves viral vectors [10,11]. Viral vectors exhibit a high efficiency of transfection, but because of their inherent immunogenicity, the risk of gene transmission and/or recombination with germline cells, the limited space for foreign therapeutic genes and the important limitations with respect to scale-up procedures and costs, synthetic alternatives have been proposed [7, 12–15].

Among available synthetic vectors, cationic lipids, introduced by Felgner in 1987 [16], have been widely studied and commonly used as a result of their relatively high effectiveness, ease of production, lower toxicity and immunogenicity and the possibility to confer tissue specificity [14,17–21]. Nevertheless, it was further demonstrated that transfection with cationic lipids/nucleic acids complexes, called lipoplexes, causes inflammatory responses in vitro and in vivo [22–25]. In the last decade it became apparent that delivery of foreign nucleic acids using

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cationic lipids maximizes their exposure to pattern recognition receptors (PRRs) located in the endosomal compartment and cytosol with a significant risk of triggering a dangerous immune response and decreasing the transfection efficiency [8,26]. In particular several endosomal Toll-like receptors (TLRs) are dedicated to nucleic acid recognition: TLR9 recognizes unmethylated CpG motifs of plasmid DNA (pDNA) [27–30] and TLR7/8 and TLR3 recognize single (ssRNA) and double stranded (dsRNA) RNA, respectively [31,32]. TLR engagement by nucleic acids activates a signalling cascade leading to translocation of the nuclear factor -κB (NF- κB) into the nucleus, followed by transcription and production of several pro-inflammatory cytokines such as Tumor Necrosis Factor α (TNF-α), Interleukin 6 and 12 (IL-6 and IL-12), which were all reported after administration of lipoplexes [22,23,25].

Despite these cytokines being shared by multiple signalling pathways [33,34] the immuno-stimulatory properties of lipoplexes were generally attributed to activation of TLR3, TLR7/8 and TLR9 by foreign nucleic acids [22,23,35]. New approaches to minimize nucleic acid-dependent immune responses were developed. Among them, minicircle DNA, in which bacterial sequences required for production in bacteria but not for gene expression have been removed [36,37], and CpG-free technologies, that avoid TLR9 activation by using pDNA completely devoid of unmethylated CpG [38], are the most advanced methods. Also several RNA chemical modifications were performed to avoid interaction with PRRs and prevent activation of an immune response [39]. Despite all these efforts, the results were not as successful as expected: although preventing nucleic acids from triggering an immune response does contribute to reducing the inflammation associated with lipoplexes, cytokine secretion has not been eliminated [40–44]. This suggests that there are other mechanisms responsible for the innate immune responses of lipoplexes, which might be linked to their second component, the cationic lipids.

Cationic lipid nanocarriers were typically considered as inert to the immune system. Recent studies have shown that they are instead involved in several cell-signalling mechanisms either with inflammatory or anti-inflammatory properties [45–49]. Because of the wide number of structures and variety of cellular effectors that can be involved, the number of cationic lipids that have been investigated so far in terms of their immunostimulatory properties is quite limited [49]. Among cationic lipids, the number of lipopolyamines (LPAs) available is increasing due to ease of synthesis, high transfection efficiency and low toxicity. Indeed spreading the cationic charge with primary and secondary amines improves interaction with nucleic acid and reduces the toxicity associated with the localized cationic charge of tertiary amines and the ether linkage, while the amide linker improves serum compatibility and biodegradability [50–55].

In this paper, we investigate the role of three LPAs (Fig. 1) on the inflammatory processes induced during transfection with lipoplexes. These cationic lipids were previously developed in the context of an intracellular delivery program and successfully used as DNA and siRNA delivery vectors [56–61]. Our results demonstrate that they all activate TLR2 and confer inflammatory properties to the corresponding lipoplexes. We elucidated the structural parameters that cause TLR2 recognition and compared the LPAs mode of binding to known TLR2 ligands.

2. Materials and methods

2.1. Reagents and cell lines

Human embryogenic kidney cells were purchased from the American Type Culture Collection (293 [HEK293] (ATCC® CRL1573™)) and human acute monocytic leukemia cell line (THP1 ECACC 88081201) were obtained from European Collection of Authenticated Cell Cultures. RPMI 1640 (Roswell Park Memorial Institute) and DMEM (Dulbecco's Modified Eagle's Medium) media, L-glutamine, sodium pyruvate, penicillin and streptomycin were from Lonza. Phorbol 12-myristate 13-acetate (PMA) was from Sigma Aldrich. Fetal bovine serum (FBS) from South America was from Lonza and FBS from North America was purchased from Sigma-Aldrich. Ultrapure standard lipopolysaccharide (LPS) from *E. coli* 0111:B4, Pam_3CSK_4, and Pam_2CSK_4 were from InvivoGen. Human TLR2, TLR1, TLR6 and TLR4 neutralizing antibody were purchase from InvivoGen (Cat. Code pab-hstlr2, pab-hstlr1, pab-hstlr6 and pab-hstlr4).

2.2. Liposome preparation

RPR120525, RPR120535 and RPR128506 were synthesized as described earlier [45] and stored as powder at −20 °C. Lipid films were formed by dissolving powder in chloroform, followed by solvent evaporation under nitrogen stream and vacuum drying overnight, and kept at −20 °C. Liposomes were freshly formed by resuspending lipid films into filtered Hepes 10 mM heated at 56 °C and sonicated for 5 min (BioRuptor, Diagenode) before each experiment.

2.3. Lipoplex preparation

The protocol used for lipoplex formation was one described earlier for transfection experiment (6 nmol of cationic lipid per μg of nucleic acids) [59–61]. Briefly, small interfering RNA (MISSION® siRNA Universal Negative Control from SigmaAldrich Cat. N. SIC001) and plasmid DNA (pcDNA3.1 from Invitrogen Cat. N. V79020) were suspended in 150 mM NaCl and mixed with an equivalent volume of liposomes, then incubated for 20 min at room temperature.

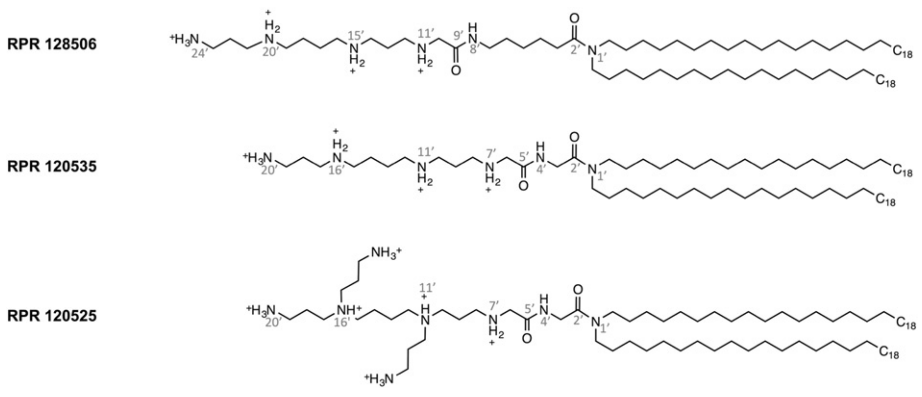

Fig. 1. Structures of cationic LPAs tested in this study.