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Surface-assembled poly(I:C) on PEGylated PLGA microspheres as vaccine adjuvant: APC activation and bystander cell stimulation

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ABSTRACT

Biodegradable poly(lactic-*co*-glycolic acid) (PLGA) microspheres are potential vehicles to deliver antigens for vaccination. Because they lack the full capacity to activate professional antigen presenting cells (APCs), combination with an immunostimulatory adjuvant may be considered. A candidate is the synthetic TLR3 ligand polyriboinosinic acid-polyribocytidylic acid, poly(I:C), which drives cell-mediated immunity. However, poly(I:C) has also been linked to the pathogenesis of autoimmunity, as affected by widespread stimulation of non-hematopoietic bystander cells. To address this aspect, we propose to minimize the poly(I:C) dose as well as to control the stimulation of non-immune bystander cells by poly (I:C). To facilitate the maturation of APCs with minimal poly(I:C) doses, we surface-assembled poly(I:C) onto PLGA microspheres. The microspheres' surface was further modified by poly(ethylene glycol) (PEG) coronas with varying PEG-densities. PLGA microspheres loaded with tetanus toxoid (tt) as model antigen were manufactured by microextrusion-based solvent extraction. The negatively charged PLGA(tt) microspheres were coated with polycationic poly(L-lysine) (PLL) polymers, either PLL itself or PEG-grafted PLL (PLL-*g*-PEG) with varying grafting ratios ($g = 2.2$ and $g = 10.1$). Stable surface assembly of poly (I:C) was achieved by subsequent incubation of polymer-coated PLGA microspheres with aqueous poly(I:C) solutions. We evaluated the immunostimulatory potential of such PLGA(tt) microsphere formulations on monocyte-derived dendritic cells (MoDCs) as well as human foreskin fibroblasts (HFFs) as model for non-hematopoietic bystander cells. Formulations with surface-assembled poly(I:C) readily activated MoDCs with respect to the expression of maturation-related surface markers, proinflammatory cytokine secretion and directed migration. When surface-assembled, poly(I:C) enhanced its immunostimulatory activity by more than one order of magnitude as compared to free poly(I:C). On fibroblasts, surface-assembled poly(I:C) upregulated class I MHC but not class II MHC. Phagocytosis of PLGA(tt) microsphere formulations by MoDCs and HFFs remained mostly unaffected by PEG-grafted PLL coatings. In contrast, high concentrations of free poly(I:C) led to a marked drop of microsphere phagocytosis by HFFs. Overall, surface assembly on PEGylated PLGA microspheres holds promise to improve both efficacy and safety of poly(I:C) as vaccine adjuvant.

Abbreviations: APC, antigen presenting cell; CCL21, chemokine (C—C motif) ligand 21; CCR7, chemokine (C—C motif) receptor 7; CBA, cytometric bead array; FSC, forward scattering (measured by flow cytometry); GM-CSF, granulocyte macrophage colony-stimulating factor; HFF, human foreskin fibroblast; IL, interleukin; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; MoDC, monocyte-derived dendritic cell; MS, microsphere; PEG, poly (ethylene glycol); PLGA, poly(lactic-*co*-glycolic acid); PLGA(tt), PLGA microsphere formulation loaded with tetanus toxoid; PLL, poly(L-lysine); PLL-*g*-PEG, poly(L-lysine)-*graft*-poly(ethylene glycol); poly(I:C), polyriboinosinic acid-polyribocytidylic acid; PS, polystyrene; SSC, side scattering (measured by flow cytometry); TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; tt, tetanus toxoid.

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1. Introduction

Biodegradable poly(lactic-*co*-glycolic acid) (PLGA) microspheres represent an established vehicle for drug and vaccine delivery with a documented safety record (Hanes et al., 1997; Johansen et al., 2000a,b). In the optimal particle size range of about 1–10 μm PLGA microspheres are readily phagocytosed by professional antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages. Previous studies showed that microencapsulation of an antigen in PLGA microspheres reduced the antigen load necessary to achieve immunity, markedly prolonged antigen presentation and favoured cross-presentation of the encapsulated antigen (Men et al., 1997; Evans et al., 2004; Waeckerle-Men and Groettrup, 2005). A comprehensive review on the immunogenicity of PLGA particulates for the delivery of subunit vaccines has been recently published (Silva et al., 2016). Nevertheless, PLGA microspheres alone lack the full capacity to activate APCs to induce a potent immune response. Hence, complementary combination with an immunostimulatory adjuvant may be promising. To this end, formulation development has been claimed to play a key role (O'Hagan and Fox, 2015).

A promising immunostimulant is the synthetic TLR3 ligand polyriboinosinic acid-polyribocytidylic acid, poly(I:C) (Jin et al., 2010; Salvador et al., 2012; Hafner et al., 2013; Martins et al., 2015; Ammi et al., 2015). It is a mimic of viral dsRNA which drives cell-mediated immunity. On the down side, poly(I:C) has also been linked to the pathogenesis of autoimmunity, as affected by a wide spread stimulation of non-hematopoietic bystander cells (Lang et al., 2005). For instance, an aberrant class II major histocompatibility complex (MHC) molecule expression was reported in a variety of non-hematopoietic cells after the translocation of poly(I:C) into the cytoplasm by means of the transfection reagent lipofectamine (Suzuki et al., 1999). Such an upregulation of class II MHC molecule expression was associated with the danger of developing autoimmune diseases (Bottazzo et al., 1985; Jackson et al., 1988; Kohn et al., 2000). Furthermore, parenteral administration of free poly(I:C) in mice and rats has been identified as a trigger towards autoimmunity (Ewel et al., 1992; Sobel et al., 1992; Okada et al., 2005; Patole et al., 2005). To address this safety aspect we propose to minimize the dose of poly(I:C) necessary for the activation of APCs, as well as to control the adverse stimulation of non-hematopoietic bystander cells.

For more than a decade the coating of negatively charged PLGA microspheres with cationic polymers as well as the subsequent surface assembly of DNA or RNA were explored for gene as well as nucleic acid adjuvant delivery (Denis-Mize et al., 2000; Singh et al., 2000; Singh et al., 2001; Walter and Merkle, 2002; Jilek et al., 2005; Wischke et al., 2009). Furthermore, surface assembly of poly(I:C) onto microspheres was reported to result in a largely potentiated efficacy to induce maturation of monocyte-derived dendritic cells (MoDCs) as compared to free poly(I:C), allowing to minimize doses (Wischke et al., 2009; Hafner et al., 2011).

Dermal fibroblasts represent an abundant non-hematopoietic bystander cell type at the injection site of a subcutaneously injected vaccine. They express a wide array of pattern recognition receptors (PRRs) including TLR3 and play an important role in stimulating and modulating the response of the innate immune system to invading pathogens (Matsumoto et al., 2002; Ospelt et al., 2008). Thus human foreskin fibroblasts (HFFs) represent a meaningful *in vitro* model for evaluating their activation by poly(I:C). In a proof-of-concept study with negatively charged carboxylated polystyrene (PS) microspheres of 5 μm diameter we demonstrated the potential of poly(ethylene glycol) (PEG) coronas to impede the activation of HFFs by surface-assembled poly(I:C), as reflected by restrained IL-6 secretion and MHC I expression (Hafner et al., 2012). PEG coronas were formed via coating of PS microspheres with polycationic polymers via electrostatic interactions, using a small library of poly(L-lysine)-*graft*-poly(ethylene glycol) (PLL-*g*-PEG) copolymers with various degrees of PEGylation. PEGylated surfaces efficiently protected microspheres from aggregation and clotting.

As an expansion of our previous studies (Hafner et al., 2011, 2012) here we report on PLGA microspheres loaded with encapsulated tetanus toxoid (tt) as model antigen, further on denoted as PLGA(tt), tt being a frequently applied model antigen for studies with PLGA microparticles, both *in vitro* and *in vivo* (Men et al., 1995; Umetsu et al., 1985; Audran et al., 1998; Men et al., 1999; Johansen et al., 2000a,b; Waeckerle-Men et al., 2006). PLGA (tt) microspheres were manufactured by microextrusion-based solvent extraction, using a static micromixer as previously introduced by our group (Freitas et al., 2005a,b). The resulting negatively charged PLGA(tt) microspheres were either coated with PLL or with one of two PLL-*g*-PEG copolymers, PLL-*10.1*-PEG and PLL-*2.2*-PEG, respectively. Surface assembly of poly(I:C) was by electrostatically driven adsorption through exposure of the coated PLGA(tt) microspheres to an aqueous poly(I:C) solution.

We evaluated the immunostimulatory potential of such formulations on MoDCs as well as human foreskin fibroblasts (HFFs) as a model for non-hemopoietic bystander cells. The focus for MoDCs was on (i) microsphere phagocytosis, (ii) expression of maturation-related surface markers, (iii) cytokine secretion pattern and (iv) migration capacity of MoDCs after phagocytosis of PLGA(tt) microspheres loaded with surface-assembled poly(I:C). HFFs were studied for microsphere phagocytosis, and for class I and class II MHC molecule expression.

Surface-assembly of poly(I:C) allowed a dose reduction by more than one order of magnitude as compared to free poly(I:C). The formulations readily activated MoDCs with respect to the expression of maturation-related surface markers, proinflammatory cytokine secretion as well as directed migration. Phagocytosis of the PLGA(tt) microsphere formulations by MoDCs remained mostly unaffected. In contrast, phagocytosis by HFFs was markedly reduced by free poly(I:C), and upregulation of MHC molecules only occurred for class I but not for class II MHC. Taken together, surface assembly on PEGylated PLGA microspheres holds promise to improve both efficacy and safety of poly(I:C) as a vaccine adjuvant.

2. Materials and methods

2.1. Polymers

PLL (20 kDa, as hydrobromide salt; Sigma-Aldrich, Buchs, Switzerland); Cy3-labelled PLL (PLL-Cy3): Fluorescently labelled PLL of 20 kDa was made by conjugation of PLL with the orange fluorescing cyanine dye, Cy3 (ex/em 552 nm/570 nm), using the Cy3 mono-reactive dye pack (GE Healthcare Europe, Germany) according to an optimized manufacturer's protocol. In brief, 5 mg PLL were dissolved in 0.5 ml RNA grade 0.1 M Na_2CO_3 (pH 9.3; Sigma-Aldrich, Buchs, Switzerland), sterile filtered and added to the dye vial provided by the manufacturer. Dye and protein solution were thoroughly mixed and incubated during 2 h at RT under additional mixing every 10 min. Cy3-labelled PLL was separated from excess unconjugated dye using an Amicon Ultra PL-10 centrifugal filter device (Millipore, Zug, Switzerland) with a nominal molecular weight limit of 10 kDa, according to the manufacturer's protocol, using sterile-filtered RNA grade 10 mM HEPES buffer (pH 7.4; HEPES buffer) as washing buffer. The purified Cy3-labelled PLL was sterile filtrated and stored at −24 °C at a concentration of 1 mg ml^{-1} in HEPES buffer.

The PLL-*g*-PEG copolymers used in this study were synthesized as previously outlined (Pasche et al., 2003) and were kindly provided by the Laboratory for Surface Science and Technology of