Vaccine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Monophosphoryl lipid A coating of hydroxyethyl starch nanocapsules drastically increases uptake and maturation by dendritic cells while minimizing the adjuvant dosage

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ARTICLE INFO

Article history:
Received 10 July 2014
Received in revised form
24 November 2014
Accepted 25 December 2014
Available online xxx

Keywords:
Nanocapsules
Dendritic cell targeting
Hydroxyethyl starch
Vaccine
Monophosphoryl lipid A
TLR4

ABSTRACT

Enhancing delivery of antigens to dendritic cells (DCs) is essential for the induction of vigorous antigen-specific cellular immune responses. Aim of the present study was to evaluate the properties of hydroxyethyl starch nanocapsules (HES-NCs) functionalized with anti-CD40, anti-DEC205, interferon-γ (IFNγ) and/or monophosphoryl lipid A (MPLA) with respect to the overall uptake, the released cytokine profile, and the influence on phenotypic maturation of human monocyte-derived DCs using flow cytometry, confocal microscopy and enzyme-linked immunosorbent assays.

NC uptake by DCs was significantly enhanced by functionalizing NCs with anti-CD40 or MPLA. With respect to the cytokine profile and the maturation status, coating with MPLA evoked a strong T_h1-type cytokine response and significantly increased CD80 and CD83 expression on DCs, contrasting the moderate effects of MPLA in solution. Notably, an at least 20 fold higher amount of MPLA in solution was needed compared to the dosage of MPLA attached to HES-NCs in order to induce comparable effects, evidencing the intense dose-sparing potential of particle-bound MPLA.

Reducing the amount of the vaccine adjuvant MPLA, while maintaining or even surpassing the effects on human DCs, reveals the potential of HES-NCs as a promising carrier system for the simultaneous delivery of antigen along with compounds promoting a T_h1-prone cellular immune response.

1. Introduction

Nanomedicine is a rapidly growing research area with multiple applications, including imaging [1], vaccination [2,3], biosensoring [4], and drug delivery systems [5–7]. Due to the potential of nanocarriers to promote cell-specific targeting and to protect drugs on its way to the desired cell/organ/tissue, nanocarriers play an important role as drug delivery systems. There are many different preparation techniques for nanocarriers. However, miniemulsion is the preparation method of choice for many applications, which is owed to its excellent properties, including the tuning of size and size distribution, surface functionalization opportunities and the high payload efficiency. In the present study hydroxyethyl starch (HES) was chosen as biopolymer for nanocapsule synthesis, based on its biocompatible properties and its established use in various clinical applications [8]. In previous investigations we observed, that HES nanocapsules without any surface functionalization display a diminished unspecific cell uptake [9], a liver specific deposition [10], and excellent release properties [11]. In particular, we could demonstrate in an *in vitro* murine model, that hydroxyethyl starch-based nanocapsules (HES-NCs) are efficiently ingested by resident liver macrophages (Kupffer cells) and that they are able to release their content (dexamethasone) followed by a significant suppression of cytokine release [11].

The latter observation prompted the present study focusing on the interaction of functionalized HES-NCs with human dendritic cells (DCs), which would potentially qualify HES-NCs as a vaccine platform. A critical obstacle in the development of vaccines against viral pathogens is the induction of vigorous and

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http://dx.doi.org/10.1016/j.vaccine.2014.12.072
0264-410X/

long-lasting, antigen-specific cellular immune responses [12]. DCs – professional antigen presenting cells – are a key cell population linking the innate with the adaptive immune system and are essential for the initiation of cellular immune responses [13]. Accordingly, DCs have become a primary target for novel vaccine formulations, such as virus-like particles, replicons, and nanoparticles [14].

Tailor-made nanocarriers for vaccination with encapsulated antigen have a number of advantages, including (a) prevention of proteolytic degradation [15]; (b) prolonged antigen presentation [16]; (c) enhanced phagocytosis by antigen presenting cells (APCs) [17]; (d) promotion of endosomal release of antigen, leading to enhanced cross-presentation [18]; (e) co-delivery of antigen and adjuvant, promoting T_h1 responses [19]; and (f) receptor-mediated targeting of DCs by immobilization of antibodies on the polymer surface [20].

Commonly targeted DC receptors [21] include CD205 (DEC205) [22], CD209 (DC-SIGN) [23], and CD40 [24]. Importantly, receptor mediated targeting not only enhances uptake but is also capable to induce activation of cells; *e.g.* in CD40-mediated phagocytosis.

In the present study we investigated the effects of HES-NCs functionalized with commonly used targeting receptors (anti-DEC205 and anti-CD40) and compare the latter with monophosphoryl lipid A-(MPLA) and interferon-γ-(IFNγ) coated HES-NCs. MPLA was chosen since it is a commonly used vaccine adjuvant, known to induce maturation of dendritic cells [25,26]. Notably, MPLA has been approved by US and European authorities as a vaccine adjuvant, *e.g.* in a Hepatitis B vaccine (Fendrix, GlaxoSmithKline). In addition, we have recently observed that coating of HES-NCs with MPLA promotes uptake by macrophages and DCs in a murine model [10]. Importantly, combining MPLA with IFNγ is a particular suitable approach to induce IL-12 secretion by human monocyte-derived DCs [27,28]. In summary, we aimed to investigate how MPLA and IFNγ immobilized on HES-NCs affects phagocytosis and maturation of human DCs, with the particular interest to induce a cytokine environment promoting T_h1 T cell responses. Anti-DEC205- and anti-CD40-coated HES-NCs served as a reference.

2. Material and methods

2.1. Nanocapsule synthesis and characterization

2.1.1. Materials used for the NCs synthesis

Materials purchased included: hydroxyethyl starch (HES, $M_w = 200{,}000\,g\,mol^{-1}$; Fresenius Kabi), 2,4-toluene diisocyanate (TDI) and cyclohexane (>99.9%; Sigma Aldrich), sodium dodecylsulfate (SDS; Fluka), *N*-ethyl-*N*′-(3-dimethylaminopropyl)-carbodiimide (EDC), monochloroacetic acid (MCA; Aldrich), and Cy5-labeled oligonucleotides (5′-Cy5-CCACTCCTTTCCAG-AAAACT-3′, Thermo Scientific). The surfactant poly((ethylene-*co*-butylene)-*b*-(ethylene oxide)), P(E/B-*b*-EO) [29] and 4-sulfotetrafluorophenyl (STP) were synthesized at the Max-Planck Institute for Polymer Research [30].

Antibodies and other materials for coupling onto the NCs surface were: anti-DEC205 (BD Pharmingen; clone MG38), anti-CD40 (eBioscience; clone 5C3), unspecific anti-human IgG (BD Pharmingen; clone 27–35), interferon-γ (IFNγ; Peprotech) or monophosphoryl lipid A (MPLA; Sigma Aldrich).

2.1.2. Preparation of nanocapsules

HES nanocapsules were synthesized by a polyaddition reaction performed at the miniemulsion droplet's interface according to previously published protocols [9,10] as shown in Fig. 1. Nanocapsules were labeled with Cy5-oligonucleotides in order to assess NC uptake by DCs *in vitro*. Afterwards, HES nanocapsules were functionalized by a carboxymethylation procedure as previously published [31]. Coupling of anti-DEC205, anti-CD40, IFNγ or IgG, adsorption of MPLA onto HES nanocapsules and their subsequent characterization were performed using a method previously described [10].

2.2. Biological analysis

2.2.1. Generation of human monocyte-derived dendritic cells

Adult peripheral blood mononuclear cells (PBMCs) were isolated from fresh buffy coats, obtained from healthy voluntary donors (blood bank of the University Medical Center Mainz), upon informed and signed consent, by centrifugation through Histopaque-1077 density gradient media (Sigma-Aldrich) for 20 min at $900 \times g$ and 20 °C. The interphase consisting of PBMCs were subsequently extracted and washed with Hank's balanced salt solution (Sigma-Aldrich). $CD14^+$ monocytes were isolated from the PBMC fraction by positive selection using CD14 MicroBeads, MACS LS columns and a magnetic cell separator (MACS; Miltenyi Biotec) in accordance with the manufacturer's instructions. CD14 positive monocytes were washed in X-Vivo 15 medium (Lonza). Subsequent flow cytometric analysis (LSR II; BD Biosciences) verified a high purity of $CD14^+$ monocytes (>98%). Purified monocytes were cultured at a concentration of 10^6 cells per ml in 6-well plates (Greiner Bio-One) in X-Vivo 15 medium supplemented with L-glutamine, $100\,U\,ml^{-1}$ penicillin and $100\,\mu g\,ml^{-1}$ streptomycin. Finally, GM-CSF ($200\,U\,ml^{-1}$) and IL-4 ($200\,U\,ml^{-1}$) was added to the medium following 6 days of culture at 37 °C and 5% CO_2 with addition of 1 ml fresh medium at days 2 and 4. Immature moDCs were obtained by harvesting the non- or loosely adhering cell population (approx. 30% of $CD14^+$ monocytes). Incubation of moDCs with different nanocapsule formulations was performed using X-Vivo 15 medium supplemented with antibiotics and cytokines as described above.

2.2.2. Confocal laser scanning microscopy (CLSM)

Nanocapsule uptake by moDCs was evaluated using a Zeiss LSM 710 NLO confocal laser scanning microscope. Immature DCs were harvested as described above and cultured with a density of $3 \times 10^5\,ml^{-1}$ in 8-well chamber slides (ibidi) at 37 °C for 4 h in the presence of $7.5\,\mu g\,ml^{-1}$ HES-IgG nanocapsules. Nuclei were stained with $2\,\mu g\,ml^{-1}$ Hoechst 33342 (Life Technologies) for 30 min. Immediately before analysis $2\,\mu g\,ml^{-1}$ CellMask Orange (Life Technologies) was added for plasma membrane staining.

2.2.3. In vitro loading of moDCs with nanocapsules and flow cytometric analysis

In vitro uptake of nanocapsules by moDCs and maturation analysis were performed by coincubation of immature moDCs in a density of $10^6\,ml^{-1}$ with $7.5\,\mu g\,ml^{-1}$ of Cy5-labeled nanocapsules (HES-IgG, HES-αCD40, HES-αDEC205, HES-IFNγ, HES-MPLA, HES-IgG-MPLA, HES-αCD40-MPLA, HES-αDEC205-MPLA, HES-IFNγ-MPLA) or without nanocapsules (control) for 4 and/or 24 h in 48-well plates (Nunclon Surface; Nunc—Thermo Scientific). Cell culture supernatants were collected after 24 h of incubation at 37 °C and stored at −20 °C for subsequent cytokine analysis. Uptake, maturation and toxicity were analyzed by flow cytometry using the multi-channel cytometer BD LSR II (BD Biosciences) equipped with FACSDiva software (BD Biosciences). Data analysis was performed with FlowJo software (Tree Star). Approximately 5×10^5 moDCs were incubated with excess human polyvalent IgG antibody (Sandoglobulin Liquid; CSL Behring) in order to block the Fc receptor and avoid unspecific binding of primary antibodies, followed by a 30 min incubation with fluorochrome-conjugated antibodies CD14 (PerCP; clone MφP9), CD40 (PE; clone 5C3), CD80 (FITC; clone BB1), CD83 (PE; clone HB15e), CD86 (FITC; clone

Please cite this article in press as: Fichter M, et al. Monophosphoryl lipid A coating of hydroxyethyl starch nanocapsules drastically increases uptake and maturation by dendritic cells while minimizing the adjuvant dosage. Vaccine (2015), http://dx.doi.org/10.1016/j.vaccine.2014.12.072