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Targeting and activation of antigen-specific B-cells by calcium phosphate nanoparticles loaded with protein antigen

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ABSTRACT

Cross-linking of the B-cell receptors of an antigen-specific B-cell is the initial signal for B-cell activation, proliferation, and differentiation into antibody secreting plasma cells. Since multivalent particulate structures are efficient activators of antigen-specific B-cells, we developed biodegradable calcium phosphate nanoparticles displaying protein antigens on their surface and explored the efficacy of the B-cell activation after exposure to these nanoparticles. The calcium phosphate nanoparticles were functionalized with the model antigen Hen Egg Lysozyme (HEL) to take advantage of a HEL-specific B-cell receptor transgenic mouse model. The nanoparticles were characterized by scanning electron microscopy and dynamic light scattering. The functionalized calcium phosphate nanoparticles were preferentially bound and internalized by HEL-specific B-cells. Co-cultivation of HEL-specific B-cells with the functionalized nanoparticles also increased surface expression of B-cell activation markers. Functionalized nanoparticles were able to effectively cross-link B-cell receptors at the surface of antigen-matched B-cells and were 100-fold more efficient in the activation of B-cells than soluble HEL. Thus, calcium phosphate nanoparticles coated with protein antigens are promising vaccine candidates for induction humoral immunity.

1. Introduction

Antibodies as one arm of the adaptive immune response are essential for the efficacy of many vaccines. Antigen receptors on the surface of B-lymphocytes trigger adaptive immune responses after binding to the matched antigen (protein) in its native form. It is known that particulate antigens are better activators of the B-cell response in comparison to their monovalent forms [1,2]. Therefore, different immunisation platforms use biological nanostructures, such as exosomes, virus-like particles, attenuated viruses and others [3], to improve the antibody response. However, besides the protein of interest, these nanoparticles of biological origin can contain a multitude of other biomolecules partially derived from production systems which often could be undesirable [4].

Nanoparticles made of immunologically inert materials are widely used in biomedical research [5–10]. They can be loaded with all kinds of (bio-) molecules, e.g. plasmids for the transfection, siRNA for gene silencing, sensitive dyes for photodynamic therapy, or proteins for drug delivery [11–13]. Nanoparticles were also suggested as good candidates for vaccine systems, as they can be designed to carry antigens of interest and therefore serve as effective delivery system [14].

Different kinds of synthetic nanoparticles have been suggested for vaccination [14–16]. However, there are some advantages to use calcium phosphate nanoparticles for targeted delivery of biomolecules. Calcium phosphate nanoparticles occur in the body as mineral component of mammalian bone and are therefore not harmful to the body [17]. They can be prepared in a multi-shell way in order to protect biomolecules inside and after cellular uptake release their cargo inside the cell upon lysosomal dissolution at lower pH [18]. Finally, their surface can be covalently functionalized with biomolecules to an effective cell targeting [19]. Recently, we developed and tested biodegradable calcium phosphate (CaP) nanoparticles, functionalized with immunogenic peptides of the influenza virus, that efficiently activated dendritic cells and induced virus-specific CD4+ and CD8+ T-cell responses *in vitro* and *in vivo* [5,10,20].

In contrast to T-cells, the initiation of adoptive humoral immune response requires a direct encounter of the B-cell receptor (BCR) with a specific antigen in the form of a non-degraded protein.

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Cross-linking of B-cell receptors also plays an important role in the effective initiation of antigen-specific B-cell response. Therefore, a candidate protein has to be attached to the surface of nanoparticles firmly and repetitively. Our method of CaP nanoparticles production permits such a covalent attachment of proteins onto the particle surface [19].

The B-cells population of each individual carries a broad repertoire of B-cell receptors. However, since each B-cell only expresses a single member of the B-cell receptor repertoire, the frequency of B-cells recognising a particular antigen is very rare (down to one cell out of one million cells) [21,22]. This makes the analysis of antigen-specific B-cells in humans and wild type animal models rather complicated. To be able to study the direct impact of CaP on antigen-specific B-cells, we chose BCR-transgenic B-cells from SW-HEL mice (HEL^+B-cells) as a model to study B-cell responses to antigen-matched CaP [23]. Wild type C57Bl/6 mice (BL6) were used as a control for a BCR-unspecific action of B-cells by CaP.

In this study, we have performed a side-by-side comparison of CaP nanoparticles functionalized with HEL protein (CaP-HEL) on targeting, binding and internalisation by antigen-matched vs. non-matched B-cells *in vitro*. In addition, CaP nanoparticles functionalized with BSA (CaP-BSA) were used to prove the antigen-specificity of our transgenic model.

2. Materials and methods

2.1. Preparation of nanoparticles

Polyethylenimine-stabilised calcium phosphate nanoparticles (CaP/PEI) were prepared as previously described [19]. Aqueous solutions of calcium lactate (18 mM, pH = 10), $(NH_4)_2HPO_4$ (10.8 mM, pH = 10), and PEI (2 g L^{-1}; Sigma–Aldrich; branched; 25 kDa) were simultaneously pumped in a volume ratio of 5 mL: 5 mL: 7 mL into a stirred glass vessel containing 20 mL of ultrapure water during 1 min at room temperature. After 20 min stirring, 5 mL of the CaP/PEI-nanoparticle dispersion was added to a mixture of 20 mL ethanol, 25 μL tetraethylorthosilicate (TEOS; Sigma–Aldrich) and 13 μL aqueous ammonia solution (30–33%) for coating with a thin layer of silica. This reaction mixture was stirred for 16 h at room temperature. Then the particles were isolated by ultracentrifugation and redispersed in the original volume of water (5 mL) (UP50H, Hielscher, Ultrasound Technology; sonotrode 7, cycle 0.8, amplitude 70%, 15 s). The covalent functionalisation of CaP/PEI/SiO_2 nanoparticles with thiol groups was carried out as follows: 25 μL (3-thiolpropyl)trimethoxysilane (MPS; Sigma–Aldrich) were dissolved in 20 mL ethanol. 5 mL of the CaP/PEI/SiO_2 nanoparticle dispersion was added, and the mixture was stirred for 8–10 h at room temperature. Then the particles were dispersed in 3 mL of water under ultrasonication as described above.

For covalent attachment of HEL-Alexa488, we chemically activated 133 μL of a 1:1 vol% mixture of HEL-Alexa488 (0.5 mg mL^{-1}; Sigma; M = 14328.0 g mol^{-1}) and HEL (total concentration of HEL 0.75 mg mL^{-1}) with 50 μL of the cross-linker 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (sulfo-SMCC; 4 mM, p.a., Sigma–Aldrich) for 3–4 h at room temperature. 1 mL of CaP/PEI/SiO_2/SH nanoparticles were then added to the activated cross-linker and protein mixture and reacted at 4 °C for 24 h.

After the conjugation reaction, the nanoparticles were purified by centrifugation for 15 min at 14,500 g and redispersed in 1 mL of water (UP50H, Hielscher, Ultrasound Technology; sonotrode 2, cycle 0.8, amplitude 70%, 8 s).

The covalent attachment of the control protein BSA-FITC was carried out as described above for HEL-Alexa488 66.5 μL of a 1:4 vol% mixture of BSA-FITC (0.5 mg mL^{-1}, Sigma–Aldrich; M = 67,000 g mol^{-1}) and BSA (total concentration of BSA 0.90 mg mL^{-1}) were chemically activated with 50 μL of the cross-linker sulfo-SMCC (4 mM, p. a. Sigma–Aldrich) for 3–4 h at room temperature. 1 mL of CaP/PEI/SiO_2/SH nanoparticles were then added to the activated cross-linker and protein mixture and reacted at 4 °C for 24 h.

After the conjugation reaction, the nanoparticles were purified by centrifugation for 15 min at 14,500 g and redispersed in 1 mL of water (UP50H, Hielscher, Ultrasound Technology; sonotrode 2, cycle 0.8, amplitude 70%, 8 s).

2.2. Characterisation

Scanning electron microscopy was performed with an ESEM Quanta 400 instrument with gold/palladium-sputtered samples. Dynamic light scattering and zeta potential determinations were performed with a Zetasizer Nano series instrument (Malvern Nano-ZS, laser: λ = 532 nm) using the Smoluchowski approximation and taking the data from the Malvern software without further correction. The particle size data refer to scattering intensity distributions (z-average). Ultracentrifugation was performed at 25 °C with an Optima XL-I instrument (Beckman–Coulter). The cells were analysed by flow cytometry with an LSR II instrument using the DIVA software (BD Biosciences).

The *in-house* developed anti-HEL ELISA test was described previously [24].

2.3. Mice

Mice were housed at the animal facility of the Faculty of Medicine, Ruhr University Bochum, Germany, under pathogen-free conditions, and handled according to the Federation of European Laboratory Animal Science Association. Six to eight week-old C57BL/6J mice (BL6) (Janvier, France) and SW-HEL mice (in house breeding) were used. The BCR-transgenic SW-HEL mice breeding-pairs were kindly provided by Dr. A. Freitas, The Lymphocyte Population Biology Unit, Pasteur Institute, France.

2.4. Mouse cell purification and cultivation

A single-cell suspension of splenic cells was prepared as described before [24]. Naïve untouched $CD5^-$ B2 cells were isolated from single-cell suspension of splenic cells with the B-Cell Isolation Kit (Miltenyi Biotec GmbH, Germany) according to the manufacturer's instruction. The resulting cell purity was routinely >98%. Cells were seeded in U-bottom 96-well plates at a density of $0.5 \cdot 10^6$ cells per well for pure B-cells and $1 \cdot 10^6$ per well for total splenocytes and incubated in R10 medium (RPMI-1640 medium, supplemented with 10% FCS, penicillin, streptomycin) with different concentrations of calcium phosphate nanoparticles.

2.5. BCR Staining with fluorochrome-conjugated HEL

HEL protein (Sigma) was conjugated with Alexa488 and Alexa647 (protein labelling kits, Invitrogen) according the manufacturer's instructions. For BCR staining, B-cells were incubated with HEL-conjugates (0.5 μg mL^{-1}) in R10 medium for 10 min on ice, washed twice with R10 medium and used for further antibody staining.

2.6. Binding and uptake assay

To perform binding assays, B-cells were pre-chilled in R10 medium for 1 h on ice. Calcium phosphate nanoparticles were added to the cells in different concentrations. After incubation on ice, the cells were carefully washed twice with ice-cold R10 medium, stained with HEL-Alexa647, anti-CD3 and anti-B220 antibodies and analysed by flow cytometry. For the uptake assay, the cells were pre-warmed in R10 medium for 1 h at 37 °C. After adding of fluorescent calcium phosphate nanoparticles, cells were further incubated at 37 °C. After incubation, the cells were washed and stained as above, and split into two equal parts. One half was incubated with trypsin-EDTA solution (Biochrom AG, Germany) for 15 min at 37 °C and extensively washed with R10 to remove adsorbed calcium phosphate nanoparticles from the surface; the other half was incubated with PBS instead of trypsin-EDTA.

2.7. Shedding of CD62L from B-cell surface

Suspensions of splenic cells form SW-HEL RAG^+ and BL6 mice were incubated with calcium phosphate nanoparticles for 18 h. The cells were collected, stained with HEL-Alexa647, anti-CD3, anti-B220 and anti-CD62L antibodies (BD PharMingen, Heidelberg, Germany, eBioscience) and analysed by flow cytometry.

2.8. Activation assay

After 24 h of incubation, the cells were collected and (after BCR labelling with HEL) stained with anti-CD3, anti-B220, anti-CD69 and anti-CD86 antibodies (BD

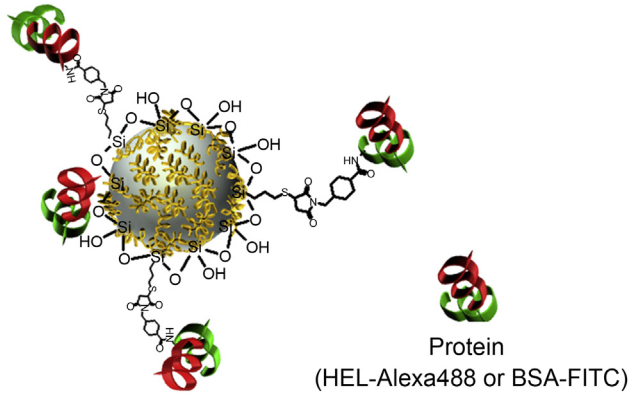

Fig. 1. Schematic representation of HEL-Alexa488- and BSA-FITC-conjugated calcium phosphate nanoparticles (molecules not drawn to scale).