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β-glucan microparticles targeted to epithelial APN as oral antigen delivery system

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ABSTRACT

Enteric diseases still have a devastating impact on global health. Oral vaccination is crucial to prevent intestinal infections, since only vaccines delivered to the intestinal tract elicit potent immune responses at the site of pathogen entry. However, oral vaccines encounter multiple barriers, including poor uptake and tolerance mechanisms, preventing the immune system to react to innocuous environmental antigens. Antigen delivery systems combined with selective targeting seem a promising strategy to overcome these obstacles. The current study evaluates the capacity of aminopeptidase N (APN)-targeted β-glucan microparticles (GPs) as antigen delivery system. Antibodies against APN, an intestinal epithelial receptor, are efficiently oriented conjugated to GPs via the biolinker protein G. The resultant microparticles were analyzed for their antigen load, adjuvanticity and interaction with enterocytes and dendritic cells (DCs). Functionalization of GPs with antibodies neither impedes antigen load nor adjuvanticity. In addition, targeting to APN increases the uptake of microparticles by enterocytes and DCs, leading to an enhanced maturation of the latter as evidenced by an upregulation of maturation markers and a strong pro-inflammatory cytokine response. Finally, oral administration of APN-targeted antigen-loaded particles to piglets elicits higher serum antigen-specific antibody responses as compared to control particles. Taken together, these data support the use of APN-targeted GPs for oral delivery of antigens.

1. Introduction

Gastrointestinal infections are still the main cause of enteric morbidity and mortality in man and animals. Since parenteral vaccines are unable to elicit potent immune response at the intestinal mucosa, the oral route seems to be the most favorable way to prevent intestinal infections. However, the few current oral vaccines, consisting of live attenuated or inactivated organisms, often do not completely prevent infection and have a high safety risk profile [1]. Therefore, several subunit vaccines with recombinant or purified antigens are currently being explored to avoid these problems. Until now no oral subunit vaccine affording protection against intestinal infections has been commercialized as their development encounters multiple challenges, such as instability and limited immunogenicity [2–5]. Antigen encapsulation in nano/microparticles is a promising approach to overcome these problems, as it can protect the antigens against degradation as well as carry potent adjuvants or immune modulators to enhance their immunogenicity [6–8]. Great advances have been made in the development of microparticle systems, of which particles based on poly(lactic-co-glycolic acid) (PLGA) are the most used one. However, low antigen encapsulation efficiency and the necessity to dissolve antigens in organic solvents limit their use in commercial vaccines [9].

β-glucan microparticles (GPs) represent an alternative antigen particle system for oral delivery. These hollow and porous GPs are known for their safety, immunogenicity and high antigen encapsulation efficiency [10–19]. These promising antigen carriers are derived from the cell wall of *Saccharomyces cerevisiae* (Baker's yeast) and are mainly composed of β-1,3-D-glucans, a 'microbe-associated molecular patterns' (MAMPs) with adjuvant ability [16,20,21]. β-glucan microparticles display a strong potency to elicit durable immune responses [10,11,15,22,23]. For instance, subcutaneous immunization of mice with GP-OVA induced strong humoral and Th1- and Th17-biased $CD4^+$ T cell responses [15], while oral administration of GP-OVA resulted in a

Abbreviations: GP, β-glucan microparticle; mAb, monoclonal antibodies; APN, aminopeptidase N; PLGA, poly(lactic-co-glycolic acid); MAMPs, microbe-associated molecular patterns; PP, Peyer's patches; ROS, reactive oxygen radicals; MoDCs, monocyte-derived dendritic cells.

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Th17-biased response and the production of OVA-specific secretory IgA in intestinal fluids [22]. This local immune response is obtained via the transepithelial transport of GPs by Peyer's patch M cells [11,22,24], resulting in the accumulation of GPs in $CD11c^+$ phagocytes situated in the Peyer's patch sub-epithelial dome (SED) regions [11]. However, M cells represent only a minor cell population in the intestinal tract and a huge amount of particles is necessary to induce immunity. Villous enterocytes vastly outnumber M cells and possess a transcytotic capability for macromolecules and inert particles, making them interesting target cells [25]. Selective targeting of these microparticles to a transcytotic receptor present on the apical surface of both intestinal villous cells and M cells may further enhance their passage through the epithelial barrier.

Although many approaches have been reported, selective targeting is still a challenge. A classical approach for selective targeting is mediated by recognition of the target via capture molecules, such as antibodies [25,26]. However, conventional methods to conjugate antibodies to particles often result in a loss of binding activity due to the random orientation and structural or conformational disruption of the antibodies [27]. Here, we took advantage of the antibody-binding properties of the biolinker protein G, which binds the Fc region of the antibody, to conjugate monoclonal antibodies in a proper orientation to the surface of β-glucan microparticles. To demonstrate the feasibility of this design to functionalize microparticles, aminopeptidase N (APN, CD13) was selected as a target. APN is a conserved membrane glycoprotein expressed by many cell types, including small intestinal enterocytes and dendritic cells. Although APN is mainly known to regulate the biological activity of various peptides by proteolysis, recent data from our lab revealed that antibody-mediated targeting to intestinal APN resulted in a strong immune response [28]. In addition, crosslinking APN on immune cells promotes phagocytosis [29], indicating that APN may represent a promising target for delivery of antigens across the epithelial barrier and simultaneously to boost uptake by phagocytes.

The present study aimed to evaluate APN-targeted GPs as candidate antigen delivery system for oral vaccination. We report on the characteristics and adjuvanticity of these microparticles and their functional surface decoration with anti-APN-specific antibodies. The interaction between APN-targeted GPs and intestinal epithelial cells was explored as well. Furthermore, we report on the intracellular uptake of the particles by porcine monocyte-derived dendritic cells (MoDCs) and their capacity to induce DC maturation. Finally, we loaded FedF, a clinically relevant antigen, inside the particles. FedF is the tipadhesin of F18 fimbriae, which are colonization factors of a porcine-specific enterotoxigenic *Escherichia coli* strain. These strains cause diarrhea in infected animals upon FedF-mediated binding of the bacteria to small intestinal epithelial fucosylated glycosphingolipids [30,31]. These APN-targeted FedF-loaded GPs were orally administered to piglets to investigate their capacity to trigger FedF-specific systemic immunity.

2. Materials and methods

2.1. Reagents

Protein G, 2-(N-morpholino)ethanesulfonic acid (MES), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (sulfo-NHS), O-(2-aminoethyl)polyethylene glycol ($PEG\text{-}NH_2$), glycine, sodium borate, dimethyl pimelimidate (DMP), triethanolamine, ethanolamine, luminol, accutase, sheep anti-mouse IgG/FITC N-acetyl-L-cysteine, cholera toxin (CT) and 3,3',5,5'-tetramethylbenzidine liquid substrate for membranes (TMB) were obtained from Sigma. Goat anti-mouse IgG-AlexaFluor647 antibody was purchased from Abd Serotec. Percoll gradient was purchased from GE healthcare. Lymphoprep, latex beads, Sytox red, Phalloïdin-Texas Red, isotype-matched irrelevant antibodies and secondary fluorescent antibodies were purchased from Life Technologies. Cell culture products and reagents, unless mentioned otherwise, were purchased from GIBCO (Life Technologies). Immunomagnetic beads (MACS) were purchased from Miltenyi Biotec. Phorbol myristate acetate (PMA) was obtained from Enzo Life Sciences. Sterile biopsy foam pads were obtained from Simport. Human CTLA4-muIgG2a fusion protein was purchased from Ancell. Porcine IL-4 and ELISA kits were purchased from R&D systems. Colistin, ProMycine® Pulvis, was obtained from VMD. Pariet® was purchased from Janssen-Cilag. Anti-pig-Ig (H+L), IgA/HRP and HRP-conjugated anti-mouse IgG antibodies were obtained from Bethyl Laboratories.

2.2. Preparation of protein-loaded β-glucan microparticles

Hollow β-glucan microparticles (GPs) were prepared from *S. cerevisiae* using a series of alkaline and acidic extraction steps and loaded with BSA-FITC as an antigen model as previously described [15,16]. The resulting BSA-FITC-loaded GPs were washed four times in 0.9% saline and stored at −20 °C (2.5 mg/ml). To calculate the amount of BSA-FITC trapped inside the GPs, the unbound BSA-FITC protein in the wash fractions was measured by fluorimetry against a BSA-FITC standard (3.125–1000 μg/ml). The incorporation of BSA-FITC into the GPs was 96.89% ± 0.31 (data not shown).

2.3. Functionalization of β-glucan microparticles

β-glucan microparticles were targeted to minopeptidase N (APN) by surface decorating the GPs with an in-house produced monoclonal antibody (mAb) against porcine APN (IMM013). An overview of all required steps to synthesize these antibody functionalized GPs is given in Fig. 1. First, protein G was conjugated to the particles via carbodiimide crosslinker chemistry [18,32,33]. Briefly, BSA-FITC GPs (500 μg/ml) were centrifuged (500 g, 5 min) and resuspended in 1 ml 2-(N-morpholino)ethanesulfonic acid (MES) buffer (0.1 M MES; pH 6.0). To avoid aggregation, GPs were sonicated for 2 min (230 V, 50 Hz). N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 1 mM) and N-hydroxysuccinimide (sulfo-NHS, 2.5 mM) were added to activate the surface carboxyl groups (15 min incubation, RT). Subsequently, the particles were centrifugated and resuspended in 1 ml PBS (pH 7.2). After another sonication period of 5 min (230 V, 50 Hz), 62.5 μg protein G was added to the GPs and incubated overnight on a shaker at 4 °C. To block the remaining activated carboxyl groups, the particles were PEGylated by mixing with O-(2-aminoethyl)polyethylene glycol (10 mg, 3000 MW) for 30 min, followed by 0.1 M glycine (30 min).

Next, these protein G-decorated particles were functionalized with APN-specific mAbs. The particles were first washed twice and resuspended in antibody binding buffer (50 mM sodium borate, pH 8.2). After sonication for 2 min, mAbs against APN (250 μg/ml; IgG1) or irrelevant isotype-matched control mAbs (250 μg/ml; IgG1) were added and gently rocked for 45 min at room temperature. Subsequently, the antibodies were crosslinked to the GPs by adding 20 mM dimethyl pimelimidate (DMP) dissolved in crosslinking buffer (0.2 M triethanolamine, pH 8.2). To block the remaining antibody binding spots, 0.1 M ethanolamine (pH 8.2) was added to the particles for 10 min. The particles were stored at −20 °C in PBS at a concentration of 0.5 mg/ml.

The conjugation efficiency was determined by flow cytometry and confocal microscopy. Goat anti-mouse IgG-Alexa647 antibody (1/250) was added to the uncoated or coated β-glucan microparticles (2.5 μg) and incubated for 30 min. These particles were examined by flow cytometry (FACSCanto, BD Biosciences) to determine the percentage of conjugated particles and by confocal microscopy (Leica DMI6000 B inverted microscope attached to an Andor DSD2 confocal scanner) to determine the position of the antibodies on the particles. FACS analysis was performed on 20,000 events within the $FITC^+$ gate with FACSDiva software 6.1.3. Confocal images were processed with Imaris software.