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Multivalent glycopeptide dendrimers for the targeted delivery of antigens to dendritic cells

Juan J. García-Vallejo[a,*], Martino Ambrosini[a], Annemieke Overbeek[a], Wilhelmina E. van Riel[a,1], Karien Bloem[a], Wendy W.J. Unger[a], Fabrizio Chiodo[b], Jan G. Bolscher[c], Kamran Nazmi[c], Hakan Kalay[a], Yvette van Kooyk[a]

[a] Department of Molecular Cell Biology & Immunology, Faculty of Medicine, VU University Medical Center, Amsterdam, The Netherlands
[b] Laboratory of GlycoNanotechnology, Biofunctional Nanomaterials Unit, CIC biomaGUNE, San Sebastián, Spain
[c] Department of Oral Biochemistry, Academic Centre for Dentistry Amsterdam, University of Amsterdam and VU Amsterdam, Amsterdam, The Netherlands

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ABSTRACT

Dendritic cells are the most powerful type of antigen presenting cells. Current immunotherapies targeting dendritic cells have shown a relative degree of success but still require further improvement. One of the most important issues to solve is the efficiency of antigen delivery to dendritic cells in order to achieve an appropriate uptake, processing, and presentation to Ag-specific T cells. C-type lectins have shown to be ideal receptors for the targeting of antigens to dendritic cells and allow the use of their natural ligands – glycans – instead of antibodies. Amongst them, dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) is an interesting candidate due to its biological properties and the availability of its natural carbohydrate ligands. Using Le^b-conjugated poly(amido amine) (PAMAM) dendrimers we aimed to characterize the optimal level of multivalency necessary to achieve the desired internalization, lysosomal delivery, Ag-specific T cell proliferation, and cytokine response. Increasing DC-SIGN ligand multivalency directly translated in an enhanced binding, which might also be interesting for blocking purposes. Internalization, routing to lysosomal compartments, antigen presentation and cytokine response could be optimally achieved with glycopeptide dendrimers carrying 16–32 glycan units. This report provides the basis for the design of efficient targeting of peptide antigens for the immunotherapy of cancer, autoimmunity and infectious diseases.

1. Introduction

Dendritic cells (DCs) are the most powerful antigen-presenting cells in the induction and modulation of antigen-specific immune responses, which makes them ideal candidates for the development of immunotherapies against cancer, autoimmunity, and infectious diseases. DCs reside in peripheral tissues in an immature state. This state is characterized by an enhanced capacity to sample the environment for pathogens. In the steady state DCs migrate continuously to the primary lymphoid tissue and, upon pathogen recognition, their migration rate increases (Carbone et al., 2004). Another effect of pathogen recognition is the activation of receptors such as TLRs, which initiate signaling events that results in the maturation of DCs (Joffre et al., 2009). The maturation process ensures that DCs up-regulate the expression of co-stimulatory molecules (signal 2) and cytokines (signal 3) that are required for T-cell activation (Steinman and Banchereau, 2007). In parallel with this process, the captured antigen is taken through a maze of endosomes until it reaches acidic lysosomal compartments were it can be conveniently processed for presentation on MHC-I and MHC-II. Antigen processing for presentation on MHC-II is a relatively well-characterized process (Steinman and Banchereau, 2007; Neefjes et al., 2011; Paul et al., 2011). Besides classical MHC-II antigen presentation, DCs also process antigens for presentation on MHC-I, a phenomenon known as cross-presentation. This phenomenon remains poorly defined and attracts a great interest, since it has

Abbreviations: CLR, C-type lectin receptor; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3 grabbing non-integrin; $Le^{X, Y, a, or\ b}$, $Lewis^{X, Y, a, or\ b}$; MR, mannose receptor; PAMAM, poly(amido amine).

* Corresponding author at: Department of Molecular Cell Biology & Immunology, VU University Medical Center, FdG, B242, PO Box 7057, 1007 MB Amsterdam, The Netherlands. Tel.: +31 20 4448529/48080; fax: +31 20 4448081.
E-mail addresses: jj.garciavallejo@vumc.nl (J.J. García-Vallejo), m.ambrosini@vumc.nl (M. Ambrosini), annemiekeoverbeek@gmail.com (A. Overbeek), w.e.vanriel@uu.nl (W.E. van Riel), k.bloem@vumc.nl (K. Bloem), w.unger@vumc.nl (W.W.J. Unger), fchiodo@cicbiomagune.es (F. Chiodo), j.bolscher@acta.nl (J.G. Bolscher), k.nazmi@acta.nl (K. Nazmi), h.kalay@vumc.nl (H. Kalay), y.vankooyk@vumc.nl (Y. van Kooyk).
[1] Current address: Department of Cell Biology, Faculty of Science, Utrecht University Kruytgebouw, Padualaan 8, Utrecht, The Netherlands.

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important implication in the understanding but also the manipulation of cytotoxic anti-tumor T cell responses (Kurts et al., 2010; Heath and Carbone, 2001).

Preferred strategies for the targeting of the antigens to DCs in immunotherapy are based on the direct delivery of the antigen to DCs in vivo. This approach requires that the antigen vehicle recognize a specific marker on DCs that is able to induce internalization of the antigenic cargo. Previous research has focused on C-type lectin receptors (CLRs), such as DEC-205 (Hawiger et al., 2001; Bonifaz et al., 2002; Boscardin et al., 2006; Kretschmer et al., 2005), the DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) (Singh et al., 2009; Unger et al., 2012), mannose receptor (MR) (Burgdorf et al., 2007), CLEC9A (Sancho et al., 2008; Caminschi et al., 2008) and Langerin (Idoyaga et al., 2011). Since these receptors often show different expression profiles amongst DC subsets (Cao et al., 2007; Dudziak et al., 2007; Schreibelt et al., 2012; Robinson et al., 2006; Kanazawa, 2007), targeting CLRs provides the opportunity to specifically target a single or multiple DC subsets (Dudziak et al., 2007). Additionally, multiple CLRs have been shown to mediate efficient internalization and routing of their ligands into acidic compartments involved in antigen processing. Probably the most complete set of studies have been performed on DEC-205 using the monoclonal antibody NLDC145 (Kraal et al., 1986). Injection of mice with NLDC-145/antigen complexes resulted in draining lymph nodes filled with antigen-loaded DCs, which efficiently presented to antigen-specific T cells leading to the development of regulatory T cells (eight days post-challenge) and the elimination of effector T cells (three weeks post-challenge) (Mahnke et al., 2003; Bonifaz et al., 2002; Hawiger et al., 2001). A disadvantage of the use of monoclonal antibodies for DC targeting is that, even when humanized, they still elicit adverse immune reactions that can decrease the efficiency of the immunotherapy but also induce severe autoimmune side effects (Wang et al., 2012). An alternative to the use of CLR-specific antibodies is the use of CLR ligands, which lack the problem of immunogenicity and can be synthesized or purified in large amounts at a relatively low cost. A CLR with known glycan specificity and capable of mediating T cell responses is DC-SIGN (Unger and van Kooyk, 2011). The natural ligands of DC-SIGN comprise high-mannose oligosaccharides and Lewis-type epitopes, such as Lewis^X (Le^X), Lewis^Y (Le^Y), Lewis^a (Le^a) and Lewis^b (Le^b) (Appelmelk et al., 2003; van Liempt et al., 2006). In humans, DC-SIGN is predominantly expressed on DCs at mucosal sites, as well as in skin and lymph nodes (Engering et al., 2004). Glycan recognition by DC-SIGN occurs through its carbohydrate recognition domain, it is a Ca^{2+}-dependent event, and results in fast and efficient receptor internalization and trafficking to the lysosomes (Engering et al., 2002; Geijtenbeek et al., 2000b).

Previous work has demonstrated that the preparation of DC-SIGN ligands in multivalent systems increases affinity for the receptor and competes for the recognition of several DC-SIGN-specific pathogens (Berzi et al., 2012; Sattin et al., 2010; Luczkowiak et al., 2011). These data, besides a promising strategy in the prevention of HIV and other DC-SIGN-specific pathogens infections, raises the question of whether multimeric glycan-based compounds that target DC-SIGN provide efficient platforms for the delivery of antigen to DCs. One such type of multivalent platform for antigen targeting is PAMAM dendrimers. PAMAM dendrimers are symmetric highly branched monodisperse polymers with a compact spherical structure and are commercially supplied with their functional groups in an activated form, allowing the design and development of complex multivalent structures by simple chemical reactions (Biricova and Laznickova, 2009). Using DC-ligand conjugated PAMAM dendrimers we aimed to characterize the optimal level of multivalency to achieve the desired internalization, lysosomal delivery, Ag-specific T cell proliferation and cytokine response.

2. Materials and methods

2.1. Synthesis of glycopeptide dendrimers

tert-Butoxycarbonyl tert-butyl carbonate, triethylamine, cystamine dihydrochloride, NaH_2PO_4, NaOH, succinic anhydride, dimethylaminopyridine, trifluoroacetic acid, acetic acid, phenol, triisopropylsilane, picoline borane, PAMAM-generation 3.0 amino dendrimer, Tris(2-carboxyethyl)phosphine hydrochloride, 7-mercapto-4-methylcoumarine, Alexa 488-NHS ester were purchased from Sigma–Aldrich. Sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate and 4-(4-N-maleimidophenyl)butyric acid hydrazide hydrochloride, were purchased from Pierce (Thermo Fisher Scientific). L602 (lacto-N-difucohexaose) and maltohexaose were purchased from Dextralab. Methanol, diethyl ether, ethyl acetate, dichloromethane, dimethylformamide, 2-propanol, acetonitrile, DMSO and the Fmoc-Protected amino acids were purchased from Biosolve. HATU was purchased from IRIS Biotech.

4-[2-[2-(tert-Butoxycarbonylamino)ethyldisulfanyl]ethylamino]-4-oxo-butanoic acid (compound **1**) was prepared in a gram scale as previously described (Li and Zeng, 2007). The compound structure and purity was confirmed by NMR and ESI-MS analysis.

The peptide sequences SIINFEKL (Barnden et al., 1998) (compound **2** or CKOT1) and ISQAVHAAHAEINEAGR (Hogquist et al., 1994) (compound **2bis** or CKOT2) were prepared on an automated peptide synthesizer (Applied Biosystems) in a 50 μmol scale, using a double-coupling Fmoc-strategy of solid-phase peptide synthesis. An additional Cys, at the start of the sequence and Lys, flanking the epitopes, were added to facilitate conjugation to the dendrimer and release of the antigenic epitope during peptide processing, respectively. A 4-fold excess of Fmoc-protected amino acids were coupled to the chemmatrix resin using HATU as the activating agents. After the final coupling and the subsequent removal of the Fmoc group the resin was washed with DMF, isopropyl alcohol and DCM and then dried under a flux of nitrogen. The dried resin containing the protected amino acid sequence was used in the coupling reaction with (**1**). Peptidyl resin (50 μmol) was swelled in DMF for 10 min, and then washed with DMF (2 × 5 ml). To the peptidyl resin were added 3 ml of a solution containing 200 μmol of (**1**), 1.2 mmol of DIPEA, 1.2 mmol of HATU previously stirred for 30 min. The reaction mixture was shaken during 90 min at room temperature and then filtered to give a dark yellow resin. The resin was washed to remove unreacted compounds, first with DMF until the filtrate was colorless, then with isopropanol and DCM, before being dried under a flux of nitrogen for 10 min. Cleavage and deprotection was carried out by treatment of the dried resin with 3 ml of a mixture of TFA/phenol/TIS/H_2O, 92/3/2/3 (w/w) at room temperature for 3 h. The solution was collected, the resin was washed with TFA (2 × 2 ml), and the combined filtrates, after partial concentration under a flux of nitrogen to approx. 4–5 ml, were added to ice-cold diethyl ether (35 ml). The white-yellow precipitate was washed with diethyl ether (3 × 20 ml) and dried under a flux of nitrogen. Water was added (7 ml) and then the solution was lyophilized. The purification of the compounds **3** and **3bis** (CKOT1/2-Linker) was achieved by reverse-phase HPLC on a Vydac HPLC C18 Mass Spec column (10 mm × 250 mm, 5 μm) (Grace Division Discovery Sciences) using a solvent system of water/acetonitrile both containing 0.1% TFA, with a linear gradient from 5% to 60% in 60 min. The fraction containing the peptide was collected and lyophilized (70% yield for **compound 3** and 55% for **compound 3bis**).

A solution of 4-(4-N-maleimidophenyl)butyric acid hydrazide hydrochloride (3 eq.) and PicolineBorane (10 eq.), dissolved in DMSO/AcOH (8:2; 100 μl), was added to Lewis^b (L602: lacto-N-difucohexaose; 1 mg, 1 eq., Dextra Laboratories), or maltohexaose (G602, 1 mg, 1 eq., Dextra Laboratories). The resulting mixture was