Biomaterials 35 (2014) 5862–5874

Contents lists available at ScienceDirect

Biomaterials

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

Molecular factors in dendritic cell responses to adsorbed glycoconjugates

Nathan A. Hotaling [a], Richard D. Cummings [b], Daniel M. Ratner [c], Julia E. Babensee [a,*]

[a] Wallace H. Coulter Dept. of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA 30332, USA
[b] Department of Biochemistry, Emory University, Atlanta, GA 30322, USA
[c] Dept. of Bioengineering, University of Washington, Seattle, WA 98195, USA

ARTICLE INFO

Article history:
Received 27 February 2014
Accepted 18 March 2014
Available online 16 April 2014

Keywords:
Glycoconjugates
Dendritic cells
High throughput
Immunomodulation

ABSTRACT

Carbohydrates and glycoconjugates have been shown to exert pro-inflammatory effects on the dendritic cells (DCs), supporting pathogen-induced innate immunity and antigen processing, as well as immunosuppressive effects in the tolerance to self-proteins. Additionally, the innate inflammatory response to implanted biomaterials has been hypothesized to be mediated by inflammatory cells interacting with adsorbed proteins, many of which are glycosylated. However, the molecular factors relevant for surface displayed glycoconjugate modulation of dendritic cell (DC) phenotype are unknown. Thus, in this study, a model system was developed to establish the role of glycan composition, density, and carrier cationization state on DC response. Thiol modified glycans were covalently bound to a model protein carrier, maleimide functionalized bovine serum albumin (BSA), and the number of glycans per BSA modulated. Additionally, the carrier isoelectric point was scaled from a pI of ~4.0 to ~10.0 using ethylenediamine (EDA). The DC response to the neoglycoconjugates adsorbed to wells of a 384-well plate was determined via a high throughput assay. The underlying trends in DC phenotype in relation to conjugate properties were elucidated via multivariate general linear models. It was found that glycoconjugates with more than 20 glycans per carrier had the greatest impact on the pro-inflammatory response from DCs, followed by conjugates having an isoelectric point above 9.5. Surfaces displaying terminal α1-2 linked mannose structures were able to increase the inflammatory DC response to a greater extent than did any other terminal glycan structure. The results herein can be applied to inform the design of the next generation of combination products and biomaterials for use in future vaccines and implanted materials.

1. Introduction

Dendritic cells play a critical role in the adaptive immune response and have been shown to promote tolerance, limit sepsis, and maintain immune cell homeostasis [1,2]. Dendritic cells have a variety of pattern recognition receptors (PRRs) that recognize and respond to a plethora of inter- and extra-cellular ligands. C-type lectin receptors (CLRs) are a class of PRRs that are known to bind to carbohydrates. Ligation of CLRs on DCs has shown immense potential for engineering of immune response and controlled immune cell phenotype modulation. Ligation of CLRs has been shown to be key to the regulation of pathogen-induced innate immunity, antigen processing for adaptive immune responses, immune system evasion by pathogens and tumors, and in recognition of self-proteins [3–7]. However, to modulate DC phenotype with CLRs, a more mechanistic understanding of how specific glycan structures and molecular environments affect DC phenotype is needed.

The molecular factors that influence the DC response to surface adsorbed glycoconjugates are unknown. However, charge, via the addition of protamine [8] (small, arginine-rich, nuclear proteins that are highly positive) or poly L-lysine (PLL) has been found to enhance the immunogenicity of vaccine conjugates. Enhanced immunogenicity has been found for a variety of vaccines and therapies including: potent anti-tumor vaccines [9], non-viral transduction of cells [10], enhanced siRNA delivery [11], allergy vaccines [12], etc. [13–15] Furthermore, several cationic glycan carriers have shown increased phagocytosis and DC internalization over that of non-cationic glycoconjugates [16]. Additionally, increased glycan density has been shown to be correlated to increased phagocytosis of glycan coated microparticles [17–19]. Also, Wattendorf et al. [20] found that the efficiency of phagocytosis by DCs increased with increasing amounts of mannose exposed

* Corresponding author.
E-mail address: julia.babensee@bme.gatech.edu (J.E. Babensee).

http://dx.doi.org/10.1016/j.biomaterials.2014.03.048
0142-9612/

from microspheres' surface [18]. Enhanced phagocytosis with increased glycan density also agreed with findings from other groups who used mannosylated emulsions [17] or liposomes [19]. Sugar structure has also been shown to cause differential binding specificity for CLRs [21–25]. Several labs have also shown the high specificity of lectins by taking recombinant forms of the receptors and incubating them with glycan structures of interest or with glycan microarrays [22–27].

Therefore, the above molecular parameters' (charge, glycan density, and glycan structure) were modulated for glycoconjugate presentation from well surfaces. A high throughput (HTP) assay was then used to assess the effect of the neoglycoconjugates on DC phenotype. An HTP assay was used in lieu of traditional cellular analysis techniques (flow cytometry, mixed lymphocyte reaction, etc.) due to the relatively large amounts of pure glycan needed for such techniques. Complex glycan structures are extremely precious. Thus, the greatest limiting factor to obtaining most immunologically relevant cellular readouts from glycoconjugates other than simple live/dead or adhesion/phagocytosis assays is the availability of these structures in sufficient quantities. New strategies in both synthetic carbohydrate chemistry and biological isolation have improved the speed and quantity of pure glycan able to be obtained; however, these methods still require current cell analysis techniques to be scaled down to volumes typical of high throughput (HTP) assays [21,28–31].

2. Materials and methods

2.1. Carrier functionalization and purification

Thiol-OEG2 functionalized glycans (Sussex Research) or OEG3-SH (A kind gift from Dr. Daniel Ratner, University of Washington) were reduced in TCEP reducing gel (Pierce) in sealed spin cups (Pierce) for 1 h in degassed buffer 1 (0.1 M EDTA, 0.15 M NaCl, 0.1 M NaH_2PO_4) at room temperature (RT) while shaking at 600 RPM. Glycans were then spun down at 100 RCF and the resultant effluent was immediately added to 1 mg/ml Maleimide functionalized BSA (Pierce) in the indicated molar ratio as compared to the maleimide functionalized carrier (0:1, 1:1, 25:1 or 100:1 sugar: carrier ratio). A glycan to protein molar conjugation ratio of 500:1 was used in preliminary studies but no increase in glycan functionalization was seen, thus for these studies, 100:1 was used as the highest molar ratio. Argon gas was passed over the solution and the tubes were sealed with paraffin and allowed to react for 16 h at RT. After conjugation the glycoconjugates were purified using purification protocol 1:10K Membrane Centrifugal Filter Unit (Millipore) using 9 rounds of 1:10 buffer exchanges against distilled, endotoxin free, water. An identical procedure was followed for the GlcNAc, Man3-Br, Man3-A2, Man4-A2, Man5-Br, and Man5-A2 conjugates except that only a molar ratio of 100:1 glycan:BSA was used. All glycan thiols were provided by Dr. Daniel Ratner, or produced as discussed below and thiolated via Traut's reagent (Pierce). Conjugates with permanently opened rings were created from these purified glycans via periodate oxidation and sodium borohydride reduction via a standard protocol [32].

2.2. Cationization of carrier

Three solutions of ethylenediamine (EDA) (0.1, 0.3, or1.8 M) were prepared and pH adjusted to pH 4.5 using 2 M NaOH and ultrapure endotoxin free water. Additionally, a 200 mg/ml 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC or EDAC; Pierce) was prepared using ultrapure endotoxin free water. Using a stock 1 mg/ml glycoprotein solution a 1:1 volume ratio of EDA was added to the glycoprotein making the final concentration of EDA 0.05, 0.15, or 0.9 M. 0.9 M EDA was used in all cases where excess EDA is specified. To these solutions EDC was added to a 7.5 mM concentration. The resultant solution was allowed to react for 2 h at RT while being shaken at 900 RPM. After conjugation the glycoconjugates were purified using purification protocol 1. Fig. 1 shows an overview of the process of creating each of the conjugates and gives a list of all 40 conjugates created.

2.3. Isolation and functionalization of Man5-Br

Isolation, functionalization, and quantification of Man5-Br were performed via an established method [21]. Briefly, Man5-Br was isolated from ribonuclease B (RNase B) via digestion of RNase B with Pronase. The digested protein then had its N-linked glycans removed by Peptide N-Glycosidase F (PNGase F). The free reduced glycans were then functionalized with a fluorescent linker, 2-Amino-N-(2-amino-ethyl)-benzamide, (AEAB; A gift from Dr. Richard Cummings, Emory University). The resultant fluorescently modified glycans were then purified via vacuum centrifugation. Finally, the pellet was then dissolved in 1 ml of water and glycan fractions were collected using reverse phase HPLC with an excitation of 330 nm and emission at 420 nm.

2.4. Preparation and assessment of ζ-potential, mass and endotoxin content of glycoconjugates

Mass spectra of the cationized and non-cationized glycoconjugates were determined using Matrix Assisted Laser Desorption Ionization (MALDI) Mass Spectrometry. The glycoconjugates were first dissolved in ultrapure, endotoxin free water, and then were spotted in a 1:1 vol. ratio with diammonium hydrogen citrate (DHC) onto a MALDI plate. A linear positive detection method was used for the conjugates. Mass profiles were then exported, plotted and the mean of each mass peak was determined.

To determine the isoelectric point, glycoconjugates were diluted to 500 ng/ml in ultrapure endotoxin free water and then each conjugate was divided between five different cuvettes. The pH in each cuvette was then adjusted to 3.0, 5.0, 7.0, 9.0, or 11.0 using 1 M sterile NaOH or 1 M sterile HCl. Using a Malvern Nano-Zetasizer (Malvern, Malvern, Worcestershire, UK.) in a cleanroom rated at ISO 6, the ζ-

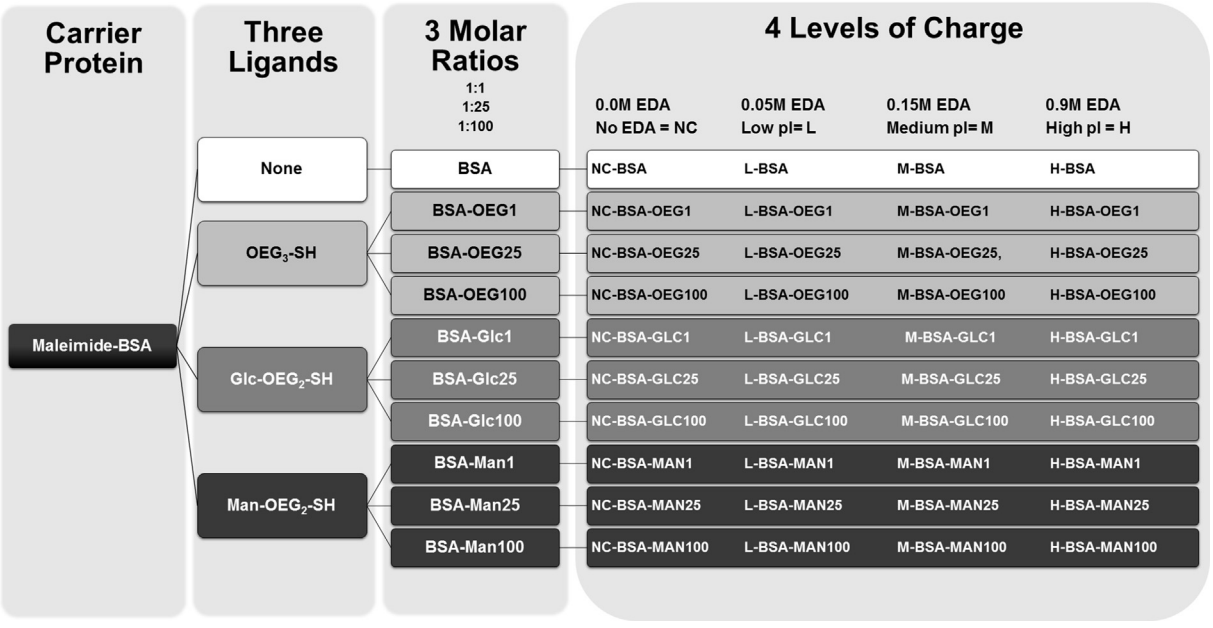

Fig. 1. Process and characterization methodology that was used to create the 40 conjugates tested in Fig. 4.