Vaccine 35 (2017) 774–781

Contents lists available at ScienceDirect

Vaccine

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

Nanoparticles decorated with viral antigens are more immunogenic at low surface density

Matthew G. Brewer [a], Anthony DiPiazza [a,b], Joshua Acklin [a], Changyong Feng [c], Andrea J. Sant [a,b], Stephen Dewhurst [a,*]

[a] Department of Microbiology and Immunology, University of Rochester, Rochester, NY 14642, United States
[b] David H. Smith Center for Vaccine Biology and Immunology, University of Rochester, Rochester, NY 14642, United States
[c] Department of Biostatistics and Computational Biology, University of Rochester, Rochester, NY 14642, United States

ARTICLE INFO

Article history:
Received 8 June 2016
Received in revised form 10 November 2016
Accepted 19 December 2016
Available online 3 January 2017

Keywords:
HIV
Envelope
Influenza
Hemagglutinin
Nanoparticle
Humoral immunity
Antigen display
Antigen density

ABSTRACT

There is an urgent need to develop protective vaccines for high priority viral pathogens. One approach known to enhance immune responses to viral proteins is to display them on a nanoparticle (NP) scaffold. However, little is known about the effect of protein density on the B cell response to antigens displayed on NPs. To address this question HIV-1 Envelope (Env) and influenza hemagglutinin (HA) were displayed on a polystyrene-based NP scaffold at various densities - corresponding to mean antigen distances that span the range encountered on naturally occurring virions. Our studies revealed that NPs displaying lower densities of Env or HA more efficiently stimulated antigen-specific B cells *in vitro*, as measured by calcium flux, than did NPs displaying higher antigen densities. Similarly, NPs displaying a low density of Env or HA also elicited higher titers of antigen-specific serum IgG in immunized BALB/c mice (including elevated titers of hemagglutination-inhibiting antibodies), as well as an increased frequency of antigen-specific antibody secreting cells in the lymph node, spleen and bone marrow. Importantly, our studies showed that the enhanced B cell response elicited by the lower density NPs is likely secondary to more efficient development of follicular helper CD4 T cells and germinal center B cells. These findings demonstrate that the density of antigen on a NP scaffold is a critical determinant of the humoral immune response elicited, and that high density display does not always result in an optimal response.

1. Introduction

The development of effective vaccines for viral pathogens such as HIV-1 and pandemic influenza represents a major unmet public health need [1–3]. Unfortunately, recent vaccination approaches, such as adjuvanted protein and viral delivery vectors, have met with limited success, particularly in the case of HIV-1 [4,5]. One approach which has been shown to enhance the immune response against pathogens is to display their antigens on the surface of a nanoparticle (NP) scaffold [6–10]. However, little is known about the effect of antigen density on the magnitude and quality of the immune response to antigens displayed on NPs. This is an important question because the density of antigen display on human viruses – which represent naturally occurring biological NPs - varies considerably. For example, the number of Envelope glycoprotein (Env) spikes on the surface of the HIV-1 virion is very low (only ~14 copies/virion) [11], whereas hemagglutinin (HA) is very densely displayed on the influenza A virus particle (~500 copies/virion) [12]. As a result, the mean distance between individual antigens on the surface of different viruses varies greatly. In the case of HIV-1, this exceeds the separation between the two antigen-binding sites present on IgG antibodies (10–15 nm), thereby ensuring monovalent binding of Env-specific IgGs, and potentially contributing to viral immune evasion [11,13,14].

To better understand how antigen density affects the developing immune response we generated NPs on which viral glycoproteins (Env and HA) were displayed at densities that span the range of antigen densities encountered on HIV-1 and influenza virus particles. We then tested the effect of antigen density on the generation of antigen-specific humoral and cellular immune responses using both *in vitro* and *in vivo* approaches. Antigen-decorated NPs were evaluated for their ability to stimulate the activation of antigen specific B cell lines *in vitro* (as assessed by calcium flux) [15,16]. Additionally, BALB/c mice were immunized

* Corresponding author at: Department of Microbiology and Immunology, University of Rochester Medical Center, 601 Elmwood Avenue, Box 672, Rochester, NY 14642, United States.
E-mail address: stephen_dewhurst@urmc.rochester.edu (S. Dewhurst).

http://dx.doi.org/10.1016/j.vaccine.2016.12.049
0264-410X/

with antigen-decorated NPs at multiple densities and the resulting serologic and cellular response was evaluated using a number of approaches that evaluate B cell signaling, priming of follicular helper cell and germinal responses, production of serum antibody and development of antibody secreting cells. Collectively, these data suggest that the density of antigen display on NPs is an important factor for controlling the magnitude and quality of the immune response elicited, and that increased antigen density does not always result in a more robust response.

2. Materials and methods

2.1. Production of recombinant HIV-1 Env and influenza HA

Trimeric recombinant HIV-1 Env (strain YU2/426c) [17–19] and Influenza HA (strain A/*New Caledonia*/20/1999) [20] containing a C-terminal hexahistidine tag and Avitag sequence were produced by transient transfection of human 293F cells, as described [21]. After protein purification using metal ion chromatography [21], Bradford reagent (BioRad) was used to quantify recovered protein (in conjunction with a bovine serum albumin standard curve). Protein was quality controlled by analysis using a 7.5% SDS-PAGE gel to observe trimeric structure and purity of the antigens.

2.2. Biotinylation of recombinant Env and HA

Site-specific biotinylation of Env and HA was accomplished using the C-terminal Avitag motif and a BirA enzyme kit (Avidity). Proteins were then concentrated and depleted of biotin and BirA enzyme through use of a 50 K molecular weight cutoff centrifugal filter (Millipore).

2.3. NP decoration with Env and HA

Polystyrene nanoparticles of multiple sizes; 200, 400, 500 nm, functionalized with streptavidin (Bangs Laboratories), were used for all decorations. Nanoparticles were washed twice in PBS and then combined with biotinylated antigens. This mixture was rotated for 1 h at room temperature, spun down at 9000*g* and washed once. Supernatant and wash were collected and analyzed with the o-Phthaldialdehyde assay (Anaspec) to measure the amount of unbound protein, and thereby infer the protein decoration efficiency. Results were confirmed by using an antigen-specific ELISA to measure Env and HA levels in the supernatants of decorated NPs (see below).

2.4. Cell lines and single cell suspension protocol

Parental or transduced DG75 (ATCC CRL-2625) cells were maintained in RPMI 1640 supplemented with L-glutamine and 10% FBS [17]. Single cell suspensions were generated from spleen via mechanical disruption through a 40 μm filter, and bone marrow suspensions were generated as described [22]. Lymph nodes were processed into single cell suspensions using frosted glass tissue disruptors and then passed through a 40 μm filter. Samples were then treated with red blood cell lysis buffer (Biolegend), washed and counted via trypan blue dye exclusion. All cells were plated in IMDM medium (Invitrogen) with L-glutamine/10% FBS/streptomycin and penicillin.

2.5. Transfection of cells with antigen-specific B cell receptors

DG75 cells were transiently transfected with mammalian expression plasmids encoding human B cell receptors (BCR) specific for HIV-1 Env (germline NIH45-46 and NIH45-46) and influenza HA (FI6), as described [17]. To confirm expression of desired BCRs, aliquots of DG75 cells (corresponding to both transfected and untransfected cells) were stained with anti-human IgG antibody conjugated to APC (BD clone G18-145) at a 1:100 dilution in RPMI medium for 30 min on ice. Cells were then washed with 1 ml of RPMI, resuspended in 350 μl of fresh media and analyzed on a BD LSR 12-color flow cytometer.

2.6. Analysis of BCR-mediated intracellular signaling (Calcium Flux)

Aliquots of DG75 cells (corresponding to both transfected and untransfected cells) were loaded with 1 μM of Fura-Red AM (ThermoFisher), according to the manufacturer's instructions. Samples were then analyzed for 30 s to measure baseline signaling, prior to ligand treatment (i.e., addition of undecorated NPs and HA/Env-decorated NPs) and subsequent analysis for an additional 270–330 s. In the final 30 s, cells were treated with ionomycin at 10 μM to determine maximum calcium release. Samples were analyzed using kinetic analysis on FlowJo v9.8.5, where samples were adjusted to baseline signaling levels. The resulting stimulation curve was then used to determine area under the curve, which was normalized to cells exposed to undecorated NPs.

2.7. Mouse immunization

In vivo studies were approved by the University of Rochester's Committee on Animal Research (UCAR), and conducted in compliance with local, state and federal regulations. Female BALB/c mice (Charles River) were housed in the UR vivarium prior to use, and were immunized at 6–8 weeks of age in the right calf muscle and then boosted 21 days later at the same site. All mice received an equal mass of antigen, delivered on NP bearing different densities of protein. Serial bleeds were collected via the submandibular vein at day 14 and 28, and animals were sacrificed at day 35; a terminal blood sample was collected via cardiac puncture and immune organs of interest were harvested for subsequent analysis.

2.8. ELISA

Assays for antigen (Env, HA) specific serum IgG antibodies were conducted as published [21].

2.9. B Cell ELISpot

Antigen-specific antibody secreting cells (IgG) from spleen, lymph node and bone marrow were enumerated via ELISpot assay as reported [23]. Briefly, wells were coated with 1 μg/well of recombinant antigen (Env, HA) or 1 μg/well of anti-mouse IgG antibody. Cells from immune organs were plated based on expected frequency of antigen specific cells (1×10^6 for spleen/bone marrow and 2×10^5 for lymph node) and total IgG producing cells (2×10^5 for spleen/bone marrow and 2×10^4 for lymph node) and incubated for 6 h. Anti-mouse IgG alkaline phosphatase was used at a 1:1500 dilution to detect antibody foci (ThermoFisher). Spots were visualized using the Vector Blue Substrate Kit (Vectorlabs), which was used according to the manufacturer's instructions, and enumerated using Cellular Technologies LTD ELISpot counting software.

2.10. Flow cytometry

Single-cell suspensions were plated in a 96-well microtiter plate and stained with purified rat anti-mouse CD16/32 (clone 2.4G2) Fc Block (BD Biosciences) for 20 min at 4 °C, followed by addition of antibody cocktails to detect Tfh and B cell subsets. Cells were incubated for an additional 30 min at 4 °C and protected from