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A Versatile Bifunctional Dendritic Cell Targeting Vaccine Vector

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Abstract

We have developed an efficient versatile *in vivo* dendritic cell (DC) targeting vector for delivering different classes of antigens such as proteins, peptide, glycolipids and naked DNA for vaccine applications. A single chain antibody (scFv) that recognizes DEC-205 receptor of DC was fused with a core-streptavidin domain and expressed in *Escherichia coli* using the T7 expression system. The bifunctional fusion protein (bfFp) was expressed as a periplasmic soluble protein and affinity-purified in its monomeric form. The bifunctional activity against DEC-205 and biotin was characterized by ELISA and Western blot. *In vivo* DC targeting of a diverse group of biotinylated antigens such as viral and bacterial proteins, a cancer peptide, gangliosides and DNA of certain infectious diseases was conducted in mice. Results show that in the presence of bfFp and costimulatory anti-CD40 mAb, both humoral and cell-mediated responses were augmented in either the single antigen or multiple antigen targeting strategy. Lastly, bfFp based DC targeting of antigens in low doses may be a useful strategy for the design of monovalent or polyvalent vaccines for the masses.

Keywords

Bifunctional antibody; recombinant antibody; dendritic cells; DEC-205; antigen targeting; immunotargeting; bioterrorism; cancer

1. Introduction

Dendritic cells (DC) are the most specialized and potent antigen presenting cells in the immune system. DC plays a critical role in innate and adaptive immune responses, especially in priming and activating T cell and B cell immunity. Several clinical trials have been initiated for human tumor immunotherapy based on *ex vivo* stimulation of DC; however, the clinical responses have been observed in a minority of patients and the procedure is very costly and laborious. *In vivo* targeting of DC via surface receptors may present an alternative simpler route for targeted immunotherapy.[1] DC utilize several surface receptors (Gb3/CD77,2 CD40,[3] β2 integrins,[4] Fc receptors,[5] and C-type lectin receptors[6]) to internalize, process and present antigens to MHC class I and MHC class II pathways.[7] A number of DC receptors have been studied extensively for antigen targeting.[1,7] DEC-205 is a C-type lectin receptor that is present on both immature and mature DC in lymphoid tissues, lymph nodes and spleen.[8–12] DEC-205 targeting of antigens by an antibody along with costimulatory molecules is an efficient strategy to achieve protection of the host against tumor growth,[4] rejection of an existing tumor,[13] protection against a virus airway challenge,[14] as well as enhanced resistance to an established rapidly growing tumor or viral infection.[15] Moreover, DEC-205 targeting of a single protein enables cross-presentation of several peptides more efficiently than CD206 and CD209[16] and

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induces stronger T cell immunity at much lower doses of protein antigen, plasmid DNA or recombinant adenovirus.[14] In the absence of costimulation, DEC-205 targeting of antigen can be used to suppress the development of autoimmunity, such as type 1 diabetes.[17] Generation of the antibody-mediated antigen targeting system is often complex, costly and a time-consuming process. In addition, it requires either liposomal encapsulation of the antigen,[4] chemical cross-linking[13,15,17,18] or construction of a new hybrid antibody.[14,16,19] These designs may not be suitable for comparison studies with different antigens such as glycolipids and DNA and also *in vivo* targeting of multiple antigens.

We have previously reported the development of a quadroma (hybrid-hybridoma) based full length bispecific monoclonal antibody (bsmAb) for targeting of biotinylated protein antigens to DEC-205.[20] The successful targeting of biotinylated proteins to DC augmented immune responses against the antigen at a ~500-fold lower dose; however, quadromas express the bsmAb along with parental and unwanted heavy and light chain combinations.[21] Furthermore, the antibody-based biotin binding is several orders weaker than streptavidin. Consequently, we have now designed a truncated recombinant construct to demonstrate the versatility of this DC targeting vehicle to deliver four categories of antigens: proteins, a peptide, glycolipids and DNA.

A scFv that recognizes DEC-205 receptor of DC was successfully cloned from the HB290 hybridoma, fused with a truncated core-streptavidin domain and expressed in *Escherichia coli* using the T7 expression system. The monomeric form of the fusion protein was affinity purified and the bifunctional activity was demonstrated by ELISA and Western blot. *In vivo* DC targeting and immune response studies in mice were initiated with a variety of biotinylated antigens (Table 2). In the presence of bfFp and costimulatory anti-CD40 mAb, both humoral and cell-mediated responses were estimated.

2. Experimental Section

2.1. Materials

DC 2.4 is a DEC-205 expressing mouse bone marrow DC cell-line transduced with GM-CSF, *myc* and *raf* oncogenes.[22] HB290, a rat antimouse DEC-205 hybridoma, was obtained from ATCC. BSA (bovine serum albumin), streptavidin-HRPO (horseradish peroxidase), NHS-LC-biotin (biotinamidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester), photobiotin acetate, OVA and goat antimouse-HRPO (GAM-HRPO) were from Sigma (Oakville, Canada). Biotinylated MUC-1 peptide with amino acid sequence of B-GVTSAPDTRGVTSAPDTR (N-terminal biotinylated) was kindly provided by Biomira, Inc. (Edmonton, Alberta, Canada). The streptavidin gene was kindly provided by Dr. T. Sano, Center for Molecular Imaging, Diagnosis and Therapy and Basic Science Laboratory, Boston, MA. HSF (hybridoma serum free media), DMEM (Dulbecco's Modified Eagle Medium), PSG (penicillin, streptomycin and L-glutamine) and FBS (fetal bovine serum) were purchased from Gibco BRL (Burlington, Canada). B-BSA [$(biotin)_n$ labeled BSA] was prepared by biotinylation of BSA with NHS-LC-biotin (Sigma) as per vendor's protocol. TMB (3,3′,5,5′-tetramethylbenzidine) peroxidase substrate was purchased from Kirkegaard & Perry Laboratory Inc. (Gaithersburg, MD). Hybond ECL (enhanced chemiluminiscent) nitrocellulose membrane and the ECL Western blotting kit were from Amersham Pharmacia Biotech (BaiedUrfe, Canada). The *E. coli* strain BL21-CodonPlus (DE3)-RIPL was purchased from Stratagene (Cedar Creek, TX). pVAX1 mammalian expression vector and molecular cloning materials (modifying and restriction enzymes, mRNA isolation kit) were from Invitrogen (Burlington, Canada). Protein assay reagent was purchased from Bio-Rad (Mississauga, Canada). Ni-NTA agarose was purchased from Qiagen (Mississauga, Canada). Anti-His_6 mAb (monoclonal antibody) was purchased from Novagen (Madison, WI). Mouse IFN-γ (Interferon gamma) ELISA (enzyme-linked immunosorbent assay) Ready-SET-Go was purchased from eBioscience (San Diego, CA). LAL (limulus

amebocyte lysate) PYROGENT Plus Single Test Vials was purchased from Cambrex (Walkersville, MD). Rat antimouse CD40 mAb was prepared from the hybridoma IC10, kindly provided by Dr. M. Gold (University of British Columbia, Canada). pVHX-6, WEEV DNA encoding E1 and E2 proteins was provided by Dr. L. Nagata (Chemical Biological Defense Section, Defense R&D Canada).[23] SARS-CoV membrane codon optimized DNA was purchased from GENEART. EBOV GP1,2 DNA,[24] EBOV GP1,2 mammalian expressed protein, EBOV GP1,2 DNA (encoding EBOV glycoprotein 1 and 2) and SARS-CoV spike DNA (encoding SARS spike RBD, S1, S2 and transmembrane domain) were from National Microbiology Laboratory, Winnipeg, Canada. Biotinylated gangliosides (GM2 and GM3) and BSA conjugates were courtesy of Dr. D. Bundle (University of Alberta, Canada, unpublished data). Anthrax Protective Antigen (PA), extracted from the S-layer of recombinant *Caulobacter crescentus*, was gift of Dr. J. Smit (University of British Columbia, Canada).

2.2. Cells and Antigen Preparation

DC 2.4 was cultured at 37 °C with 5% CO_2 in DMEM-10 medium [10% (v/v) FBS and 1% (v/v) PSG]. HB290 hybridoma was cultured under the same condition in HSF medium [1% (v/v) FBS and 1% (v/v) PSG]. The various primers and antigens used for the experiments are listed in Tables 1 and 2. SARS-CoV spike DNA, SARS-CoV membrane DNA, EBOV GP1,2 DNA were PCR (polymerase chain reaction) amplified using primers WP011 and WP012, WP013 and WP014, or WP015 and WP016 respectively (Table 1). These primers inserted Kozak translation initiation sequence and initiation codon (ATG) into the restriction sites *Bam*HI and *Eco*RI. The PCR fragments were gel-purified, double digested with *Bam*HI and *Eco*RI, ligated to pVAX1 mammalian expression vector and transformed into TOP10 cells. The positive clones were screened by restriction digestion fragment mapping (*Bam*HI and *Eco*RI) and then biotinylated using photobiotin acetate.[25] Core-streptavidin, WEEV E1, WEEV E2 and EBOV GP1 (subfragment D) proteins were prepared based on our published work.[26–29] EBOV GP2, SARS-CoV spike S1, S2, RBD and membrane proteins were produced from *E. coli* expression system (unpublished data). All antigens for immunization were labeled with commercial biotin derivatives. DNA vectors were biotinylated using photobiotin acetate (B-pVHX-6, B-pEBOV GP1,2, B-pSARS-CoV spike, B-pSARS-CoV membrane) by UV photoconjugation.[25] All proteins were labeled with NHS-LC-biotin (B-OVA, B-SARS-CoV spike RBD, B-EBOV GP1, B-anthrax PA). Synthetic biotinylated glycolipids (B-GM2, B-GM3, B-BSA-GM2, B-BSA-GM3) and the biotinylated cancer peptide (B-MUC-1) were synthetically labeled with biotin. Biotinylation of all antigens was confirmed by dot blot assay probed with streptavidin-HRPO.

2.3. Cloning of HB290 scFv and bfFp

The HB290 scFv was generated from the hybridoma cell lines and DNA sequencing was done using established protocol.[30] The various cloning designs of HB290 scFv and bfFp are diagrammatically shown in Figure 1. The genes were cloned in different orientations to determine the best expression in *E. coli*. The heavy and light chain variable regions with or without partial constant regions of anti-DEC205 antibody were amplified from plasmids containing HB290 Fab DNA by PCR and cloned into pET-22b (+) plasmids. The heavy chain variable region gene of HB290 was fused to the 3′ end of the light chain variable region gene with a linker of 15 amino acids from the constant heavy chain region 1 sequence using PCR (PCR primers: WP005 to WP008). Subsequent restriction digest/ligation methods were employed to generate the V_H-V_L scFv gene (Figure 1A). The PCR primers WP005 and WP006 were inserted into the restriction sites *Eco*RI and *Sal*I; PCR primers WP007 and WP008 were inserted into the *Sal*I and *Not*I sites. The PCR fragments were gel-purified, double digested to the respective inserted restriction sites, and ligated to pET-22b (+) plasmids. The V_L-V_H scFv gene was generated by the same methods utilizing PCR primers WP001 to WP004. WP001 and WP002 were inserted into the *Eco*RI and *Sal*I and WP003 and WP004 were inserted into

the *Sal*I and *Not*I sites. The light chain variable region gene of HB290 was fused to the 3′ end of the heavy chain variable region gene via 15 amino acids of the constant light chain sequence (Figure 1B). Both scFv orientations were inserted into pET-22b (+) containing the core-streptavidin gene[31] in 3′ orientation fusion with the core-streptavidin gene (Figures 1C and 1D). The V_L-V_H scFv gene was fused to the 5′ terminus with the core-streptavidin gene by the same method described above (Figure 1E). In brief, the core-streptavidin gene was PCR amplified using WP009 and WP010 primers. These primers were inserted into restriction sites *Not*I and *Xho*I. The PCR fragment was gel-purified, double digested with *Not*I and *Xho*I, and ligated to pET-22b (+) containing the V_L-V_H scFv gene (Figure 1E). All clones were screened and characterized by both PCR and restriction digestion fragment mapping. The positive cloned fragment was sequenced using T7 promoter and terminator primers employing CEQ2000. The positive clones were designated as follows: (a) pWET5 encoding core-streptavidin-V_H-V_L, (b) pWET6 encoding core-streptavidin-V_L-V_H and (c) pWET7 encoding V_L-V_H-core-streptavidin.

2.4. Expression, Purification and Characterization of HB290 Recombinant Proteins

2.4.1. Expression of HB290 scFv and bfFp—Expressions of scFv and bfFp were done according to the previously published methods.[31] The pET-22b (+)-scFv or bfFp genes (pWET5–7) were chemically transformed into BL21-CodonPlus (DE3)-RIPL. The *E. coli* transformants were cultured, induced for protein expression and the whole-cell bacterial pellets were analyzed by Western blot. The pellets were resuspended in reducing SDS dye (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5 mM 2-mercaptoethanol) and heated at 95 °C for 10 min prior to SDS–PAGE. The pellets were electrophoresed and transferred to a Hybond ECL nitrocellulose membrane using the Trans blot apparatus (Bio-Rad). The membrane was then blocked with 5% skim milk, probed with mouse anti-His_6 mAb and GAM-HRPO, and revealed by ECL according to the manufacturer's protocol. The bfFp gene with the highest protein expression level was selected for medium scale expression in culture flask and affinity purification was performed from *E. coli* periplasmic extracts using IMAC purification protocol as described previously.[31] The induced periplasm and IMAC purified fractions were analyzed by SDS–PAGE using 10% polyacrylamide gels under reducing conditions following staining with Coomassie brilliant blue. The fractions were heated at 95 °C for 10 min prior to loading on the polyacrylamide gel.

2.4.2. Western Blot: Biotin Binding and Heat Stability of bfFp—IMAC purified bfFp was heated at either 60 or 95 °C for 10 min under reducing conditions and resolved in 10% SDS–PAGE. The resolved proteins were electrophoretically transferred onto a nitrocellulose membrane and probed with B-BSA followed by streptavidin-HRPO.

2.4.3. ELISA: bfFp Bispecificity and Binding to DC 2.4 Cells—DC 2.4 cells were seeded on a 96-well V-bottomed plate (Nunc, Denmark) in quadruplicate (1.0×10^5 cells/well). The plates were washed with PBS (phosphate buffer saline) and blocked with 1% PBS dialyzed BSA (to remove traces of biotin) for 3 h at 4 °C. After incubation, the plates were washed with PBS, and 100 μL of bfFp at 40 μg/mL was added to bind DEC-205 receptors. The plates were incubated at 4 °C for 3 h, then washed with PBS and B-OVA (20 μg/mL in 100 μL volume) was added to each well and incubated for 1 h at 4 °C. After the incubation, the plates were washed and then incubated with streptavidin-HRPO (10 μg/mL in 100 μL volume) for 1 h at 4 °C. Finally, the plates were washed with PBS, and TMB substrate was then added. The $OD_{650\ nm}$ was taken after 15 min using an ELISA V_{max} kinetic microplate reader (Molecular Devices Corp, CA) and subtracted with control (DC with only streptavidin-HRPO incubated at 4 °C for 1 h). The DEC-205 receptor binding specificity of bfFp was confirmed by competition study with full-length HB290 mAb. Various concentrations of HB290 mAb (50, 100, 200, 300 μg/mL in 100 μL volume) was added after bfFp binding to DEC-205 at 4 °C.

The full length HB290 mAb was incubated for 2 h at 4 °C to allow for displacement of bfFp from DC. Following a wash with PBS the bfFp was detected using B-OVA, streptavidin-HRPO and TMB as mentioned above.

2.5. In Vivo Targeting of DC in Mice

2.5.1. Mice and Immunization Protocol—Fifteen groups of female BALB/c mice (5 mice per group, average 6–8 weeks old) were obtained from Health Sciences Laboratory Animals Services of the University of Alberta, Edmonton, Canada. Animal treatment, care and euthanasia were carried out according to the Canadian Council of Animal Care guidelines. Mice were injected subcutaneously near the inguinal lymph node area with 0.1 mL of various formulations of antigens and bfFp in saline. The immunization protocol is listed in Table 3. Three separate animal experiments were conducted and each design has its own control group. The strategy was to test the versatility of bfFp based delivery of protein, peptide, glycolipids and DNA to DC in generating immune responses. Immunization of B-pVHX-6, B-OVA, B-MUC-1, B-GM2 and GM3 were performed in the first experiment (Groups 2–7). Animals in Group 2–4 were injected with various biotinylated DNA formulations to verify the essential requirement for DC targeting vehicle and costimulatory molecule. Group 2 mice were immunized with B-pVHX-6 and bfFp without the presence of anti-CD40 mAb to verify the essential role of anti-CD40 mAb costimulation. Group 3 mice were coimmunized with anti-CD40 mAb without the bfFp targeting vector to determine the nontargeted immune responses. Group 4 mice were coimmunized with both bfFp and anti-CD40 mAb. Protein antigen delivery was studied in Group 5 mice and peptide delivery was focused in Group 6 mice. Lastly, glycolipid antigens were investigated in Group 7. The second experiment was designed to confirm the versatility of the naked DNA delivery strategy. A variety of infectious disease viral DNA were targeted as a single antigen to DC using the bfFp mediated delivery system (Group 9, EBOV GP1,2 DNA; Group 10, SARS-CoV spike DNA; Group 11, SARS-CoV membrane DNA). Multivalent immune responses against a mixture of different class antigens were studied in the third experiment. Groups 13–15 were designed to evaluate the multivalent immune responses against proteins and peptide, B-OVA, B-EBOV GP1, B-SARS-CoV spike RBD, B-MUC-1 and B-anthrax PA immunized as a mixture in saline. Group 13 mice were coimmunized with anti-CD40 mAb and core-streptavidin without the bfFp to determine the level of nontargeted immune responses. Group 14 mice were coimmunized with bfFp without the presence of anti-CD40 mAb to verify the essential role of anti-CD40 mAb costimulation. Group 15 mice were immunized with antigens, bfFp and anti-CD40 mAb. Groups 1, 8 and 12 were the control groups for the three separate animal experiments. All mice were boosted with the same concentration of antigen(s) in each category in PBS 12 days following primary immunization. Prior to immunization, every reagent (antigens, bfFp and mAb) was checked using LAL PYROGENT Plus Single Test Vials kits to identify lipopolysaccharide (LPS) contamination if any (endotoxin sensitivity at 0.125 EU/mL). The immunization reagents were premixed for 30 min at RT prior to immunization. The mice were sacrificed after 9 days of boost, and the blood and the spleen were collected. The serum was isolated using standard procedures[32] and used to evaluate humoral immune responses. Spleens were used to study IFN-gamma immune responses. The spleens from the 15 different groups of immunized mice were aseptically removed and each group was pooled. The responder cells were isolated using nylon wool columns and the stimulator cells from a separate group of naïve mouse spleens and were treated with mitomycin C as previously described.[20]

2.5.2. Evaluation of Humoral Immune Responses and IFN-*γ*—Antibody titers were measured individually in each mouse by ELISA following immunization of the respective antigen (Table 2). The ELISA method was done by overnight coating of the specific antigen in the Nunc 96-well ELISA microplates (10 μg/mL in 100 μL volume). After overnight coating, the plates were washed with PBST (0.1% Tween 20 in PBS, pH 7.3) and the plates were blocked

with BSA for 3 h at RT. After incubation, the plates were washed with PBST and the 1:1000 diluted serum from each mouse in quadruplicate was added, incubated for 2 h at RT. Plates were then washed with PBST and incubated with GAM-HRPO for 1 h at RT. After 1 h, the plates were washed again with PBST and TMB was then added to each well and $OD_{650\,nm}$ was taken after 10 min using the microplate reader. IFN-γ activity was determined by the IFN-γ concentration generated after 3 days of incubation of the responder cells (2.5×10^5 cells) and/or stimulator cells (3×10^5 cells) with the respective antigens: OVA, SARS-CoV spike RBD, EBOV GP1, EBOV GP2, EBOV GP1,2, SARS-CoV spike S1, SARS-CoV spike S2, SARS-CoV spike RBD, SARS-CoV membrane antigen, anthrax PA, B-MUC-1, B-GM2, B-GM3, WEEV E1 and WEEV E2 (10 μg of each antigen was separately incubated). IFN-γ ELISA Ready-SET-Go kit was used to determine the IFN-γ concentration.

2.5.3. Immune Responses toward Biotin, bfFp and Core-Streptavidin—The 15 groups of mice serum were tested against B-BSA, bfFp and core-streptavidin using ELISA method. The serum reactivity was determined by the same method as described in section 2.5.2. The plates were coated with either B-BSA, bfFp or core-streptavidin (10 μg/well). The rest of the procedures followed the preceding sections.

3. Results

3.1. Construction, Expression and Purification of HB290 scFv and bfFp

The genes encoding HB290 Fab were generated by RT-PCR and TD-PCR. The sequence generated was compared with the known mAb sequences in the NCBI BLAST database indicating the sequence is antibody related. The HB290 V_L amino acid sequence from our recombinant clone was 100% identical to a previous report;[33] however, HB290 V_H amino acid sequence (EVKLVESGGGLVQPGGSLRLSCAASGFTFNDFYMNWIRQPPGQAPEWLGVIRNKG NGYTTEVNTSVKGRFTISRDNTQNILYLQMNSLRAEDTAIYYCARGGPYYYSGDDA PYWGQGVMVTVSS) shared only 46% homology. The V_H amino acid sequence appears to be unique from other published amino acid sequences in the NCBI protein database; whereas, the amino acid sequence published by Demangel et al. is 99% identical (only a two amino acid difference in the 5′ terminal) with the single chain antibody against rice stripe virus protein P20 (accession: AAG28706). The HB290 V_L-V_H and V_H-V_L genes were successfully cloned into separate pET-22b (+) plasmids by PCR, restriction digest and DNA ligation methods. The plasmid vectors pWET5 and pWET6 were constructed by inserting the sequence encoding the HB290 single-chain anti-DEC205 antibody (V_L-V_H or V_H-V_L respectively) next to the pelB leader sequence of core-streptavidin containing pET22b (+) plasmids (Figure 1A–D). The plasmid vector pWET7 was constructed by inserting the core-streptavidin sequence into HB290 V_L-V_H scFv containing pET22b (+) plasmids (Figure 1B and 1E). The core-streptavidin sequence is at the N-terminus of the scFv in pWET5 and pWET6 constructs; whereas, in pWET7 the core-streptavidin is at the C-terminal. The scFv and bfFp vectors were transformed into *E. coli*, cultured, induced and the whole-cell bacterial pellets were analyzed by Western blot using the anti-His_6 mAb (Figure 2A). The optimal scFv and bfFp orientation for expression were determined by Western blot. HB290 V_L-V_H scFv, pWET6 and pWET7 were successfully expressed and the proteins are shown at the desired molecular weight band either at ~30 kDa or ~46 kDa (Figure 2A). pWET7 had a higher level of protein expression compared to pWET6; therefore, pWET7 was subjected to medium scale expression. The bfFp in the periplasmic space was extracted and affinity purified by IMAC column. Both the periplasmic fraction and the affinity purified fraction were analyzed on SDS–PAGE (Figure 2B). IMAC purification of the pWET7 bfFp was successful; a clear band was seen at 46 kD in the affinity purified fraction (Figure 2B). Approximately 1.0 mg of pWET7 bfFp was affinity purified from a 2 L culture.

3.2. Characterization of bfFp

E. coli expressed bfFp appeared in 3 isoforms: monomeric, dimeric and tetrameric form.[34] Following affinity purification by either IMAC or iminobiotin column, the fusion protein was subjected to size exclusion chromatography for isolation of the tetrameric fusion protein.[34, 35] Fusion proteins are known to be heat sensitive and heating at 95 °C dissociates the tetramer completely into monomeric forms. The tetrameric form remains stable at temperature below 60 °C.[34] The bfFp isoforms and the biotin binding activity of the bfFp were analyzed by Western blot probed with B-BSA. pWET7 bfFp was detected using B-BSA and streptavidin-HRPO in Western blot and the bfFp appear predominantly in monomeric form after heating at either 60 or 95 °C (Figure 3A). The predominant monomeric form may be due to the difference in the linker between core-streptavidin and scFv.[31]

The bispecificity of the bfFp was confirmed by cell ELISA. The anti-DEC-205 activity was demonstrated on DC 2.4 cells employing B-OVA with streptavidin-HRPO for detection. In addition, specific DEC-205 receptor binding activity was also confirmed by competitive displacement of bfFp with increasing concentrations of HB290 mAb (Figure 3B). In addition confocal study was done to demonstrate DC binding (unpublished data).

3.3. bfFp Mediated Immune Responses: Humoral and IFN-γ

Four different classes of antigens were chosen to demonstrate the versatility of *in vivo* antigen targeting. Both humoral and cell-mediated responses were investigated using a variety of antigens [proteins,[24,36–39] peptide,[40,41] glycolipid[42,43] and DNA[24,36,37,39,44–47]] listed in Table 2. The immunization protocol is described in Table 3, and the results of humoral and cell-mediated responses are shown in Figures 4 and 5. Humoral responses were measured by the antibody titers against the immunized antigens or its respective proteins from the DNA vectors in each mouse by ELISA method. The magnitude of cell-mediated immune responses was determined by the amount of IFN-γ secreted from spleen T cells in response to the antigens. The spleen cells from each group were pooled to average the quality of data for IFN-γ secretion. Responder cells from both immunized and nonimmunized mice without stimulator cells had minimal IFN-γ secretion (data not shown). The 15 groups of mice were divided into three different experiments to demonstrate the ability and efficacy of DC targeting strategies. Group 1, 8 and 12 were the control groups for the three separate animal experiments and these groups were immunized and boosted with PBS only. In these groups both humoral and cell-mediated immune responses were minimal (Figure 4 and 5).

3.3.1. bfFp Mediated Protein, Peptide, Ganglioside and DNA Targeting to DC—

Groups 2–7 were designed to demonstrate the versatility of bfFp based delivery of distinct classes of antigens to DC in generating immune responses. Group 2–4 mice focused on the naked DNA delivery to DC and to verify the essential requirement of bfFp and anti-CD40 mAb. Group 2 mice were immunized with B-pVHX-6 and bfFp without the presence of anti-CD40 mAb. Group 3 mice were coimmunized with anti-CD40 mAb without the bfFp based targeting. Group 4 mice were coimmunized with both bfFp and anti-CD40 mAb. The results indicate that Group 4 had the highest antibody titer and augmented IFN-γ secretion against the WEEV E1 and WEEV E2 proteins compared to Groups 1–3 (Figures 4A and 5A). Immune responses against WEEV E2 appeared to be higher than WEEV E1 in Group 4. Immune responses in Groups 2 and 3 were minimal toward WEEV E1 and E2 protein antigens, and were probably due to LPS, anti-CD40 mAb or bfFp effect (Figures 4A and 5A). Anti-CD40 mAb and bfFp appeared to be essential for DNA targeting strategy. The similar immunization strategy was applied to the synthetic biotinylated peptide MUC-1 and glycolipids (GM2 and GM3). Ovalbumin was also included in our experiments as a model antigen and control. Humoral responses and cell-mediated immune responses were shown in mice immunized with protein (Group 5), peptide (Group 6) and glycolipids (Group 7) in the presence of bfFp and

anti-CD40 mAb (Figures 4A and 5A). In summary, the results show that anti-CD40 mAb and bfFp are required to achieve good immune responses based on DC delivery of DNA, protein, peptide and even gangliosides.

Direct biotinylated DNA targeting to DC also generated strong immune responses against the respective DNA encoding proteins. The same targeting strategy was applied for a variety of infectious disease DNA. Groups 9–11 were immunized with different naked DNA vectors encoding genes for different viral proteins (Group 9, EBOV GP1,2 DNA; Group 10, SARS-CoV spike DNA; Group 11, SARS-CoV membrane DNA). pEBOV GP1,2 encoded the EBOV GP1 and GP2 proteins, pSARS-CoV spike encoded the SARS-CoV S1, S2, RBD and the transmembrane domain, and pSARS-CoV membrane encoded the SARS-CoV membrane protein. Strong humoral and cell-mediated responses were achieved against the encoded viral proteins by targeting the viral DNA to DC (Figures 4B and 5B). EBOV GP1 generated the highest immune responses compared to GP2 and GP1,2. The immune responses against mammalian expressed GP1,2 protein were lower than the *E. coli* expressed fragments (GP1 and GP2) (Figures 4B and 5B). The immune response against EBOV GP1,2 was relatively lower than its *E. coli* fragment. This may be due to the glycosylation masking of the epitopes.[48] There was no significant difference in serum titer between SARS-CoV spike proteins, but higher IFN-γ concentrations were found in SARS-CoV RBD.

3.3.2. bfFp Mediated Multiple Antigen Targeting Strategy for Combo-Vaccines

—Groups 13–15, were designed to evaluate multivalent immune responses by simultaneously targeting a mixture of biotinylated proteins and peptide antigen in nanogram concentrations. Group 13 reflects the possible involvement of core-streptavidin targeting of biotinylated proteins and peptide to DC. Groups 14 and 15 demonstrated the efficacy of the bfFp in the absence and presence of the anti-CD40 mAb costimulator respectively. The results show that Group 15 has the highest antibody titer and augmented IFN-γ secretion against OVA, SARS-CoV RBD, anthrax PA and MUC-1 compared to Groups 12, 13 and 14 (Figures 4C and 5C). Groups 13–15 showed minimal immune responses against EBOV GP1 (Figures 4C and 5C). In Group 15, the highest antibody titer was found against OVA and the lowest against anthrax PA (Figure 4C); however, the highest IFN-γ secretion was found against anthrax PA (Figure 5C). Minimal humoral or cell-mediated immune responses were generated from Groups 12–14 (Figures 4C and 5C). MUC-1 and OVA immune responses were compared between the single antigen and multiple antigen targeting strategies. Higher IFN-γ secretion and serum titer against MUC-1 were achieved in the single antigen targeting strategy of MUC-1 peptide antigen (Group 6) compared to the multiple antigen targeting strategy (Group 15) (Figures 4A, 4C, 5A, 5C). Although the serum titer against OVA was not different between Groups 5 and 15 (Figures 4A and 4C), Group 5 had higher IFN-γ secretion compared to Group 15 (Figures 5A and 5C). To summarize, both bfFp and anti-CD40 mAb are required to achieve strong immune responses against protein and peptide delivered to DC. Single antigen targeting appears to achieve strong humoral and cell-mediated immune responses in comparison to multiple antigen targeting strategy. Immune responses were shifted or selective in the multiantigen targeting method, possibly toward immunodominant antigens.

3.4. Immunogenicity of bfFp and Biotin Hapten

Serum titer against biotin, bfFp and core-streptavidin were analyzed from every group to evaluate the immunogenicity of the bfFp targeting vehicle. Identical concentrations of bfFp and core-streptavidin were coated onto the plate. Serum reactivity was found to some extent against core-streptavidin (OD ~0.25) and bfFp (OD ~0.15). However, this level of serum reactivity is not different compare to the control groups (Figure 6). The serum reactivity toward B-BSA was minimal in all groups (OD ~0.01).

4. Discussion

In vivo targeting of the DC DEC-205 receptor has been shown to be effective in generating immune responses to protect the host against cancer, viral infection and autoimmune disease. Four types of DEC-205 targeting systems have been reported thus far. These include (a) HB290 scFv coated liposome,[4] (b) chemical cross-linking of HB290 with antigen,[13,15,17,18] (c) HB290 hybrid antibody[14,16,19] and (d) bsmAb targeting system.[20] Several limitations are seen in these systems. Currently, the above strategies have only demonstrated efficient delivery of peptides or proteins to DC. These delivery systems may not be versatile or flexible enough for clinical applications, comparison studies with different antigens, and *in vivo* targeting of multiple antigens such as DNA and glycolipids. Limitations of the scFv coated liposome targeting strategy include instability of the scFv on the liposome surface (scFv could be lost during circulation), liposome instability, scale-up issues, relative complexity in the encapsulation process and only certain class of antigens can be encapsulated efficiently. Additional issues include batch to batch variation (cross-linking and purification) and Fc domain mediated effects, which are limitations of the chemical cross-linking of the whole mAb with antigen. Hybrid antibody or recombinant antigen fusion protein generation using a mammalian expression system is labor intensive requiring careful monitoring of cell productivity, post-translational chemical modifications, degradation and aggregation of the final product.[49] Furthermore, a new antibody fusion protein is required for each antigen (protein or peptide) and the process is often time-consuming and costly. The bsmAb produced from a quadroma is not ideal for clinical application and the limitations include the yield and purity.[20] The major disadvantage for these systems could be dose-limiting toxicity (prolonged circulation time) and immunogencity against the targeting system, which can alter pharmacokinetics (biodistribution and clearance), block receptor-antigen interactions, and induce hypersensitivity reactions and injection site reactions.[49] Consequently, a truncated monomeric single-chain antibody-core-streptavidin fusion protein (bfFp) that can form a complex with any biotinylated antigen ($K_d \sim 10^{-15}$ M) with one paratope and deliver the antigen to the DC *via* the DEC-205 receptor with its second paratope may be a desired alternative for *in vivo* DC targeting. This system has several unique properties over the other targeting systems. Almost any class of antigen can be biotinylated by chemical conjugation (for instance, NHS-LC-biotin), photoactivation (photobiotin acetate) or incorporation by synthetic strategies. Biotinylation avoids the need of encapsulation, chemical cross-linking, or construction of a new hybrid fusion antibody. The bfFp lacks the Fc domain and the *E. coli* based production is consistent and economical. Faster clearance rate (kidney glomerular filtration cutoff is 70 kDa) and lower immunogenicity is expected due to the smaller molecular weight (~46 kDa) of the vector. If required, bfFp stability and half-life can be increased by polyethylene glycol linkage[50] or by isolation of the tetrameric form of the bfFp. Moreover, such a targeting vehicle can be translated into clinical applications.[51,52]

In this communication, we have developed a versatile bfFp that can bind any biotinylated antigen and target them to DC DEC-205 receptors using recombinant antibody technology. DEC-205 receptor binding specificity was also confirmed *via* competitive binding replacement of bfFp with the full length HB290 mAb study. *In vivo* studies in mice with biotinylated DNA (WEEV, EBOV GP1,2, SARS-CoV spike, SARS-CoV membrane), protein (OVA), peptide (MUC-1) or glycolipid (GM2 and GM3) have shown that in the presence of bfFp and anti-CD40 mAb as costimulator, both humoral and cell-mediated responses can be augmented. In the multiple antigen targeting strategy, we have also achieved humoral and cell-mediated responses for OVA, SARS-CoV spike RBD, MUC-1 and anthrax PA. Most antigens were administered at the very low doses of 200 ng by our DC targeting strategy. With respect to the above data, an *in vivo* fluorescence imaging would decisively establish the specificity for *in vivo* targeting and we propose to do this in the future. Such antigen sparing strategies are important to pandemic disease management if large populations need vaccination. We have

made theoretical calculations that suggest that in the event of a SARS or H5N1 avian influenza outbreak in Canada, the DC targeted vaccine needed to cover 33 M subjects require only 7 g (at 200 ng dose) of the vaccine antigen. In comparison, a recent commercial influenza vaccine clinical trial used 90 μg/patient with modest immune responses requires 3 kg of antigen (450-fold more). Our speculation is that DC targeting more efficiently delivers the antigen to the DC than the conventional subcutaneous vaccine that could encounter prolonged exposure to enzymes and uptake by other none DC cells. Another important application is the delivery of multiple pathogen antigens for combination vaccines that could provide sufficient protection for first emergency responders and biodefense applications.

In summary, our DC targeting approach achieves strong immune responses against all classes of antigen. Single antigen targeting exhibited strong humoral and cell-mediated immune responses. In comparison, multiantigen targeting strategy likely exhibited immune responses that are shifted toward the most immunogenic antigen. Higher immune responses were achieved for weak immunogens (MUC-1 and OVA) in single antigen targeting strategy compared to multivalent strategy. We have also demonstrated that DEC-205 targeting of bioterrorism agent DNA's (EBOV GP1,2, SARS-CoV spike, WEEV structural DNA) induces strong humoral and cell-mediated immune responses against the DNA encoded proteins. In this targeting formulation, low concentrations of biotinylated DNA (500 ng) in saline were adequate to achieve a strong immune response in mice. In contrast, conventional DNA vaccine or therapy strategy often requires high concentration and multiple immunizations, and cannot efficiently induce immune responses. More importantly, the widely used experimental viral vector based gene targeting strategies have caused severe adverse side-effects including death. We speculate that in the absence of such viral vector the naked biotinylated DNA targeting could be a safer alternative. *In vitro* and *in vivo* neutralization study of SARS-CoV and EBOV and challenge study of WEEV are in progress. The immune responses toward either the hapten biotin (B-BSA) or the carrier bfFp is minimal and can be further mitigated by deimmunization strategies. The slight reactivity toward bfFp appears to be against the core-streptavidin portion of the fusion protein. The bfFp targeting of biotinylated antigens to DC could be a convenient, versatile method to deliver single or multiple antigens to DC. Such formulations could induce immune responses toward peptide, protein, glycolipid, and DNA vaccine based expression products. bfFp targeting of DC may be a potential candidate for the development of vaccine or therapy for cancer (GM3, GM2, MUC-1) and bioterrorism agents (WEEV, EBOV, SARS-CoV, anthrax).

Translation of the *in vivo* targeting strategy to humans is likely to be more complex since DEC-205 receptors in humans may also target B cells, NK cells, T cells, monocytes and thymic epithelial cells. In addition, multiple immunizations may be required to achieve adequate immune responses in human. In fact even in our mouse model, the bfFp is only injected once followed by nontargeted booster dose.

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Abbreviations Used

bsmAb
: bispecific or bifunctional monoclonal antibody

scFv
: single chain antibody

bfFp
: bifunctional fusion protein

EBOV

Ebola virus

SARS-CoV
severe acute respiratory syndrome-coronavirus

GP
glycoprotein

RBD
receptor binding domain

PA
protective antigen

MUC-1
epithelial mucin 1

ATCC
American type culture collection

B-BSA
$(biotin)_n$ labeled BSA

B-OVA
$(biotin)_n$ labeled OVA

B-MUC-1
synthetically biotin labeled MUC-1

B-SARS-CoV spike RBD
$(biotin)_n$ labeled SARS-CoV spike protein

B-EBOV GP1
$(biotin)_n$ labeled EBOV GP1

B-anthrax PA
$(biotin)_n$ labeled anthrax PA

B-GM2
synthetically biotin labeled GM2

B-BSA-GM2
synthetically biotin labeled BSA and GM2

B-GM3
synthetically biotin labeled GM3

B-BSA-GM3
synthetically biotin labeled BSA and GM3

B-pVHX-6
photoactiviated biotin labeled WEEV DNA vector

B-pEBOV GP1,2
photoactiviated biotin labeled EBOV GP1,2 DNA vector

B-pSARS-CoV spike
photoactiviated biotin labeled SARS-CoV spike DNA vector

B-pSARS-CoV membrane
: photoactiviated biotin labeled SARS-CoV membrane DNA vector

C_L
: constant light chain region

C_{H2}
: constant heavy chain region 2

ECL
: enhanced chemiluminescence

GAM-HRPO
: goat antimouse-HRPO

His
: histidine

HRPO
: horseradish peroxidase

HSF
: hybridoma serum free media

IMAC
: immobilized metal affinity chromatography

LAL
: limulus amebocyte lysate

MMR
: macrophage mannose receptor

MHC
: major histocompatibility complex

Ni
: nickel

NCBI
: National Center for Biotechnology Information

ng
: nanogram

NHS-LC-biotin
: bioti-namidohexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester

PBST
: PBS with 0.1% Tween 20

PSG
: penicillin, streptomycin and L-glutamine

RT
: room temperature

RT-PCR
: reverse transcription polymerase chain reaction

TD-PCR
touch down polymerase chain reaction

V_L
variable light chain

V_H
variable heavy chain

WEEV
Western equine encephalitis virus

E
envelope protein

TMB
3,3′,5,5′-tetramethylbenzidine

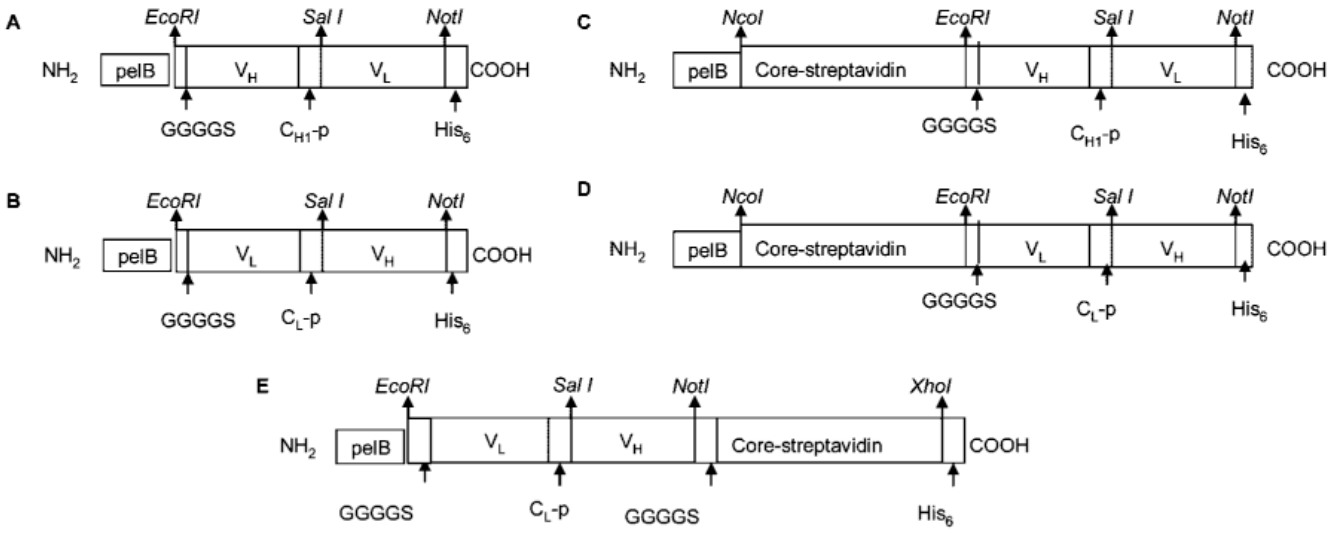

Figure 1.

Various constructs of HB290 scFv [(A) V_H-V_L and (B) V_L-V_H] and bfFp [(C) pWET5, (D) pWET6 and (E) pWET7]. The genes were cloned in different orientations to select the best production in *E. coli*. Abbreviations: pelB, bacterial leader sequence; V_L, variable domain light chain; V_H, variable domain heavy chain; $C_{H1\text{-}p}$, partial constant heavy chain region 1, 15 amino acid linker; $C_{L\text{-}p}$, partial constant light chain region, 15 amino acid linker; G, glycine; S, serine; His_6, hexa histidine amino acid tag.

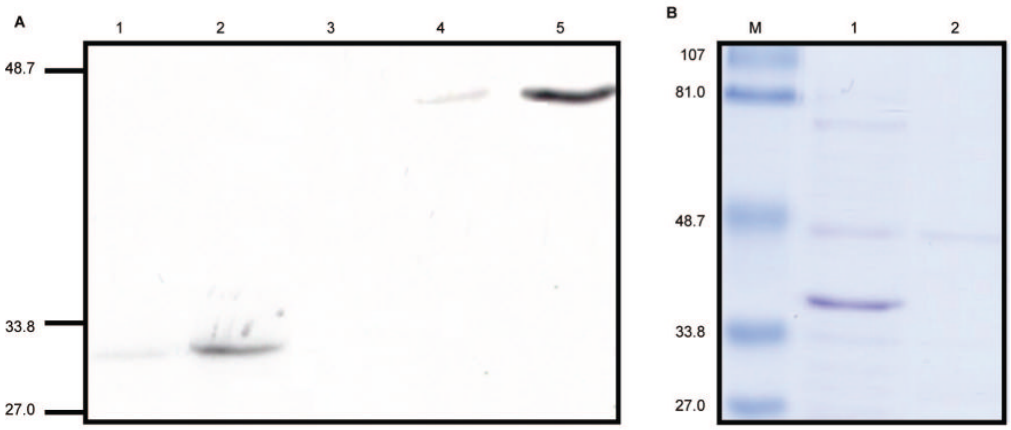

Figure 2.

Expression and purification of recombinant proteins. The scFv and bfFp genes were chemically transformed, cultured, induced and the whole-cell bacterial pellets were analyzed by Western blot. (A) Western blot probed with anti-His_6 mAb for analysis of scFv and bfFp expression. Lane 1: HB290 scFv V_H-V_L. Lane 2: scFv V_L-V_H. Lane 3: bfFp pWET5, core-streptavidin-V_H-V_L. Lane 4: bfFp pWET6, core-streptavidin-V_L-V_H. Lane 5: bfFp pWET7, V_L-V_H-core-streptavidin. pWET7 had the highest protein expression level. (B) SDS–PAGE of pWET7 before and after IMAC purification. Lane 1: pWET7 periplasmic protein. Lane 2: IMAC-purified pWET7. M stands for molecular weight marker in kDa.

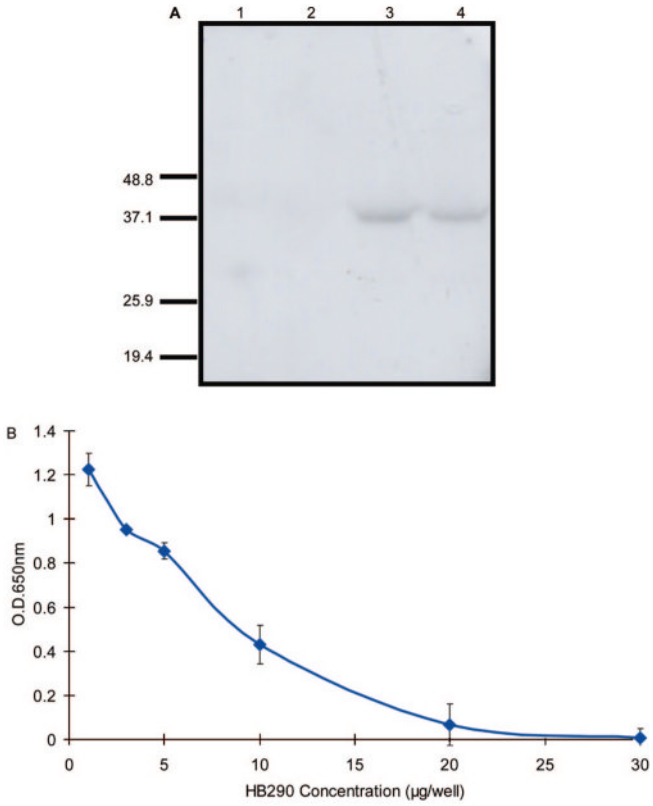

Figure 3.

Demonstration of IMAC-purified bfFp bifunctional activity and thermal stability. (A) IMAC purified bfFp was incubated either at 60 °C (lane 3) or 95 °C (lane 4) for 10 min and probed with B-BSA followed by streptavidin-HRPO. Lanes 1 (HB290 scFv V_H-V_L) and 2 (HB290 scFv V_L-V_H) are controls. The IMAC-purified bfFp bifunctional activity was tested against DC 2.4 cells and B-OVA by ELISA. (B) Competition binding studies of HB290 full length mAb and bfFp for binding to DC. The binding of bfFp is confirmed by B-OVA and streptavidin-HRPO. The error is depicted by the standard deviations.

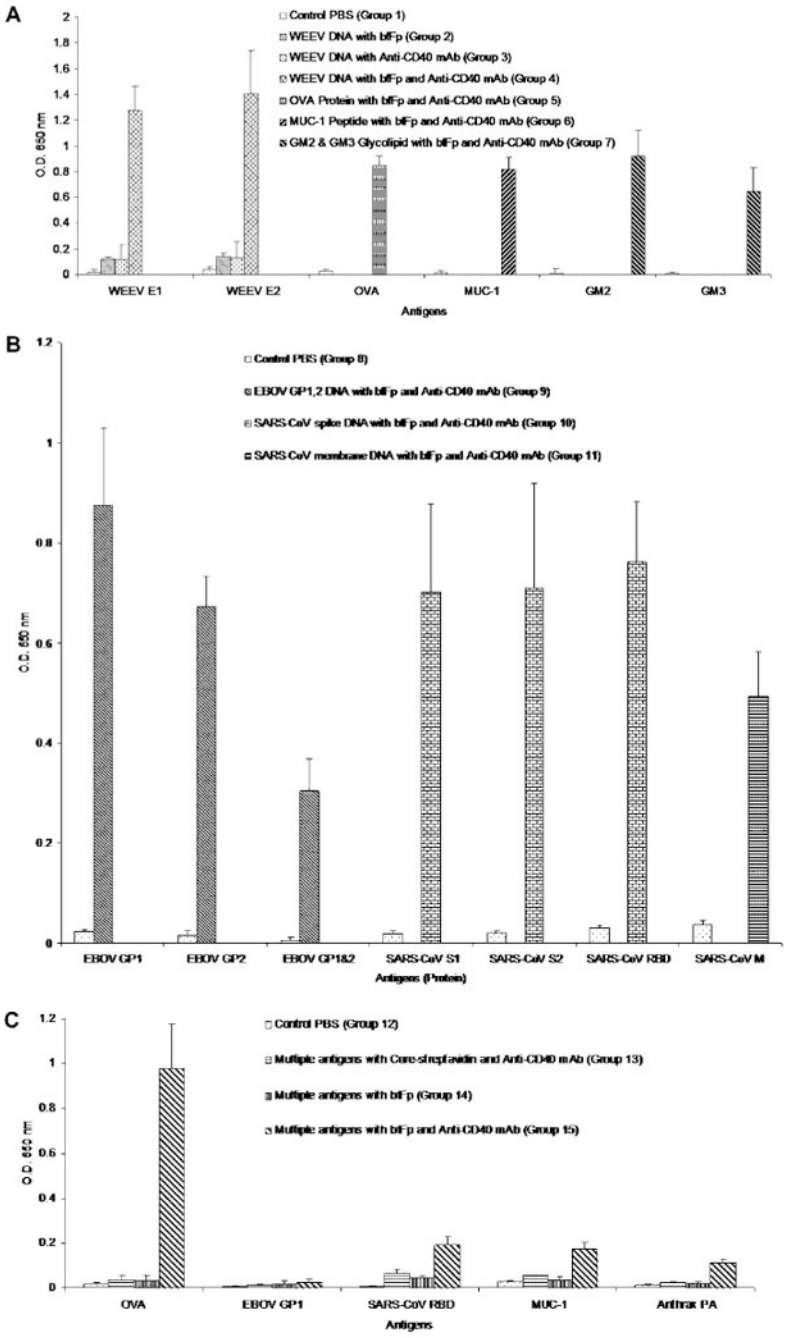

Figure 4.

Analysis of humoral immune responses to biotinylated antigens *in vivo*. Groups of five mice were immunized with different antigen combinations along with a PBS control. The specific components, amounts, and the schedule of immunizations are outlined in Table 3. A simplified form of the immunization components is listed in the figure legend. (A) Versatility of bfFp based delivery of protein, peptide, glycolipids and DNA to DC. (B) bfFp mediated delivery of other infectious diseases viral DNA to DC. (C) Multiple antigen delivery to DC using bfFp. The mice were analyzed individually, and the data was pooled. The humoral response, as quantified by serum antibody titers on day 21 post immunization, was measured by ELISA method against the respective antigens shown in *X*-axis (listed in Table 2). The method involved

coating of 100 μL of 10 μg/mL antigen in microtiter plates followed by addition of 1:1000 diluted serum antibody, and detection using GAM-HRPO. The ELISA measurements were done in quadruplicate for each mouse. The mean ELISA values obtained for each individual mouse were further averaged. The error bars represent the standard deviation in a group of 5 mice.

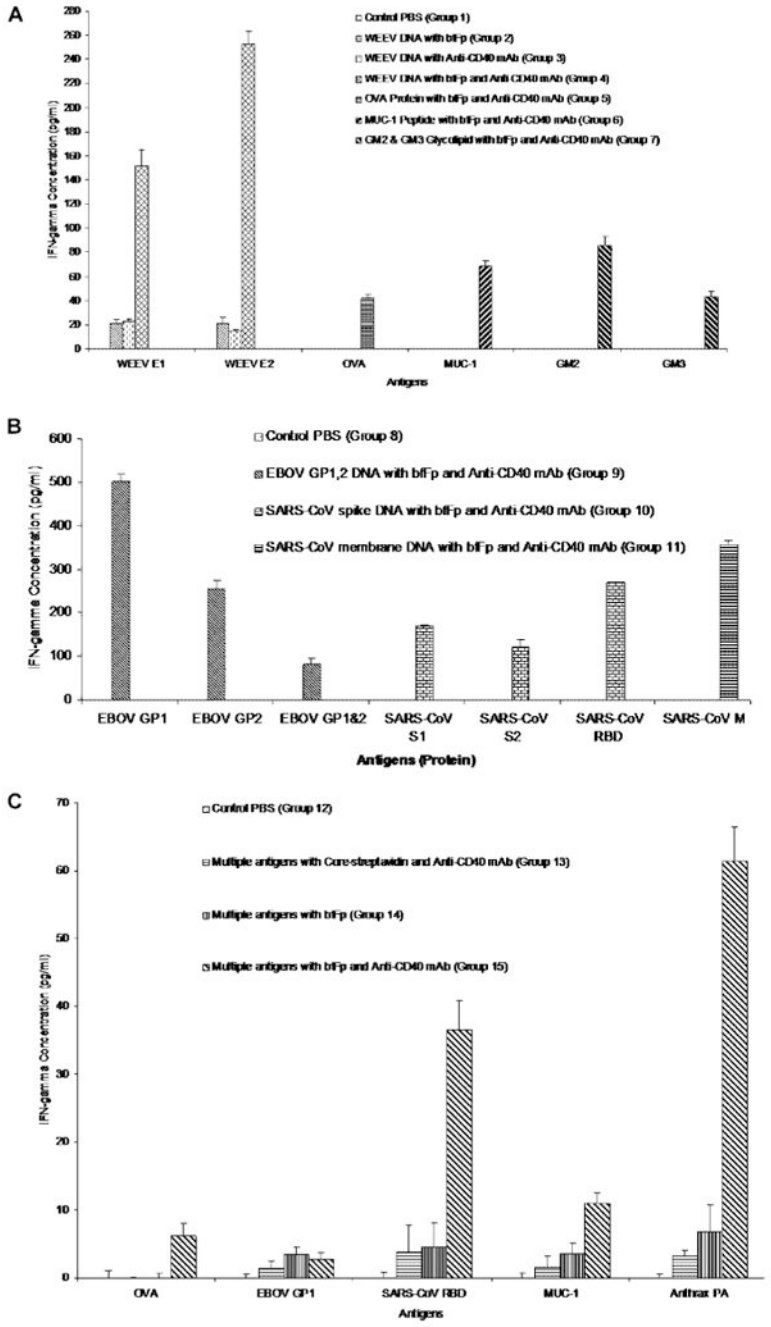

Figure 5.

Analysis of cell-mediated immune responses based on IFN-γ estimation. (A) Versatility of bfFp based delivery of protein, peptide, glycolipids and DNA to DC. (B) bfFp mediated delivery of other infectious diseases viral DNA to DC. (C) Multiple antigen delivery to DC using bfFp. Spleen T cells (responder cells) from the groups of mice immunized with different antigens (a simplified form of the immunization components is listed in the figure legend, for details see Table 2 and 3) were isolated and purified using a nylon wool column. The five spleens in each group were pooled and mixed in DMEM prior to nylon wool purification. Stimulator cells prepared from naïve mice spleen cells were isolated and treated with mitomycin C. Purified responder cells from both immunized and nonimmunized mice were

aliquoted in quadruplicate with or without stimulator cells. The cells were then incubated with 10 μg of antigen (shown in *X*-axis) for 3 days at 37 °C in a CO_2 atmosphere. After incubation, the IFN-γ concentration in the supernatant was determined using mouse IFN-γ ELISA Ready-SET-Go kit. Each data set is shown following subtraction of the corresponding ELISA values obtained without stimulator cells. The error bars are the standard deviations.

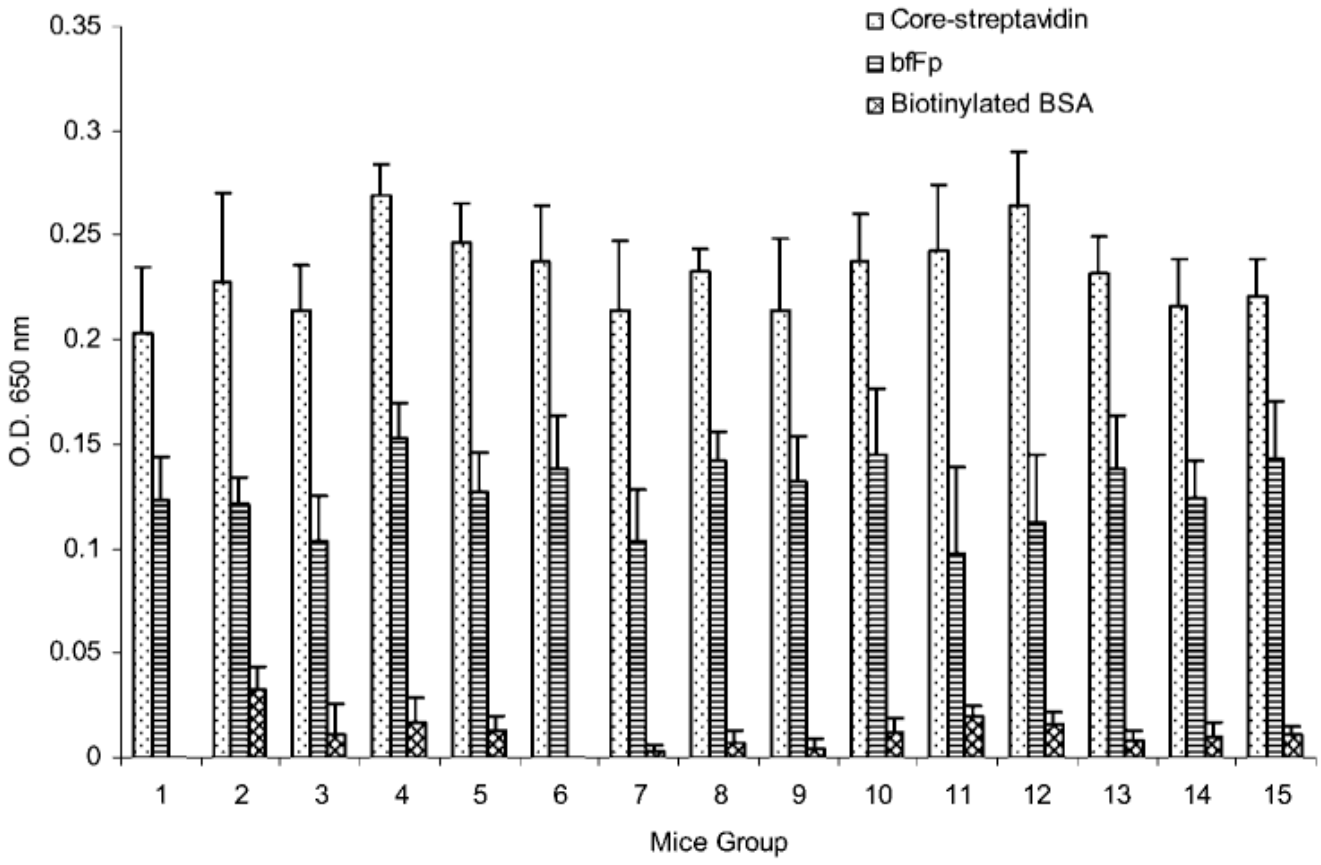

Figure 6.

Analysis of serum reactivity toward biotin, bfFp and core-streptavidin. The mice (5 mice per group) were analyzed individually, and the data was pooled. The mouse groups are listed on the *X*-axis and correspond to Table 3. Groups 1, 8 and 12 were the control groups with only PBS immunization. Other groups have been immunized in the presence or absence of the bfFp (according to Table 3). The serum reactivity, as quantified by serum antibody titers on day 21 post immunization, was measured by ELISA method against B-BSA, bfFp and core-streptavidin (shown in the figure legend). The method involved coating of 100 μL of 10 μg/mL antigen (shown in the figure legend) in microtiter plates followed by addition of 1:1000 diluted serum antibody, and detection using GAM-HRPO. The ELISA measurements were done in quadruplicate for each mouse. The mean ELISA values obtained for each individual mouse were further averaged. The error bars represent the standard deviation in a group of 5 mice.

Table 1

PCR Primers for Cloning of HB290 scFv (V_L-V_H and V_H-V_L) and bfFp (Core-Streptavidin-V_H-V_L, Core-Streptavidin-V_L-V_H and V_L-V_H-Core-Streptavidin Orientations) into pET-22b (+) *E. coli* Periplasmic Expression Vector[a]

primer	function	primer sequence (5′–3′)
WP001	5′PCR primer, HB290 V_L-$C_{L\text{-}p}$ (strep-V_L-V_H)	ATC AGT GAA TTC GGG AGG TGG CGG ATC AGA CAT CCA GAT GAC ACA GTC T
WP002	3′PCR primer, HB290 V_L-$C_{L\text{-}p}$ (strep-V_L-V_H)	TGG TTT CGC TCA TGC TAG GTC GAC CGT GGA TGG TGG GAA GAT AGA
WP003	5′PCR primer, HB290 V_H (strep-V_L-V_H)	GTT AAT GTC GAC GAA GTG AAG CTG GTG GAA TCT
WP004	3′PCR primer, HB290 V_H (strep-V_L-V_H)	TAC TAA GCG GCC GCA AGC TGA GGA GAC TGT GAC
WP005	5′PCR primer, HB290 V_H-$C_{H1\text{-}p}$ (strep-V_H-V_L)	ATC AGT GAA TTC GGG AGG TGG CGG ATC AGA AGT GAA GCT GGT GGA ATC T
WP006	3′PCR primer, HB290 V_H-$C_{H1\text{-}p}$ (strep-V_H-V_L)	TGG TTT CGG TCA TGA TAG GTC GAC AGC AGT TCC AGG AGC CAG T
WP007	5′PCR primer, HB290 V_L (strep-V_H-V_L)	GTT AGG GTC GAC GAC ATC CAG ATG ACA CAG TCT
WP008	3′PCR primer, HB290 V_L (strep-V_H-V_L)	TAC TAA GCG GCC GCA AGC CCG TTT CAA TTC CAG C
WP009	5′PCR primer, core-streptavidin (V_L-V_H-strep)	TACTAATGCGGCCGCGGAGGTGGCGGATCAGAGGCCGGCATCACCGGCA
WP010	3′PCR primer, core-streptavidin (V_L-V_H-strep)	ATTACTCTCGAGGGAGGCGGCGGACGGCTTC
WP011	5′PCR primer, pSARS-CoV spike	AAGAGGGGGATCCTACCATGGGTAGTGACCTTGACCGGTGCACCACT
WP012	3′PCR primer,	CTCGCTCGAGAGAATTCTATTATGTGTAATGTAATTTGACACCCTTGAG

primer	function	primer sequence (5′–3′)
	pSARS-CoV spike	
WP013	5′PCR primer, pSARS-CoV membrane	AAGAGGGTCTTCATATGGGGGATCCTACCATGGCAGACAACGGTACTATTACCGTTGAG
WP014	3′PCR primer, pSARS-CoV membrane	CTCGCTCGAGAGAATTCTAGTGATGATGGTGGTGATGCTGTACTAGCAAAGCAATATTGT CGTT
WP015	5′PCR primer, pEBOV GP1,2	AAGAGGGGGATCCTACCATGGGCGTTACAGGAATATTGCAGTTACCT

[a]These primers contain the following restriction inserts: *Eco*RI and *Sal*I for WP001, 002, 005 and 006; *Sal*I and *Not*I for WP003, 004, 007 and 008; *Not*I and *Xho*I for WP009 and WP010. WP011 to 16 are the PCR primers for cloning of viral DNA into the pVAX1 mammalian expression vector. These primers contain the Kozak translation initiation sequence, initiation codon, and *Bam*HI and *Eco*RI restriction sites.

Table 2

Summary Table of the Antigens Used for Testing anSD Targeting in the *in Vivo* Study[a]

classification	immunized antigens	testing antigens	references
protein	B-OVA	OVA	24, 36–39
	B-SARS-CoV spike RBD	SARS-CoV spike RBD	
	B-EBOV GP1	EBOV GP1	
	B-anthrax PA	anthrax PA	
peptide	B-MUC-1	B-MUC-1	40, 41
glycolipid	B-GM2	B-GM2 (IFN-γ assay)	42, 43
	B-GM3	B-GM3 (IFN-γ assay)	
		B-BSA-GM2 (humoral study)	
		B-BSA-GM3 (humoral study)	
DNA	B-pVHX-6 (DNA)	WEEV E1 (protein)	24, 36, 37, 39 44–47
	B-pEBOV GP1,2 (DNA)	WEEV E2 (protein)	
	B-pSARS-CoV spike (DNA)	EBOV GP1 (protein)	
	B-pSARS-CoV membrane (DNA)	EBOV GP2 (protein)	
		EBOV GP1,2 (protein)	
		SARS-CoV spike S1 (protein)	
		SARS-CoV spike S2 (protein)	
		SARS-CoV spike RBD (protein)	
		SARS-CoV membrane (protein)	

[a]The antigens are divided into four categories, and the antigens immunized into mice are listed in the table. Immunized and unimmunized mice were tested with the antigens for both IFN-γ secretion and serum immune responses.

Table 3

Immunization Protocol for Evaluating Humoral and Cell-Mediated Immune Responses to Biotinylated Antigens in Mice (n = 5 for Each Group)[a]

	groups	day 0	day 12	day 21	day 24
1	control	PBS	PBS	spleen (T cell)	IFN-γ assay
				serum (humoral)	
2	B-pVHX-6	500 ng	500 ng	spleen (T cell)	IFN-γ assay
	bfFp	20 μg	0	serum (humoral)	
3	B-pVHX-6	500 ng	500 ng	spleen (T cell)	IFN-γ assay
	anti-CD40 mAb	25 μg	0	serum (humoral)	
4	B-pVHX-6	500 ng	500 ng	spleen (T cell)	IFN-γ assay
	bfFp	20 μg	0	serum (humoral)	
	anti-CD40 mAb	25 μg	0		
5	B-OVA	200 ng	200 ng	spleen (T cell)	IFN-γ assay
	bfFp	20 μg	0	serum (humoral)	
	anti-CD40 mAb	25 μg	0		
6	B-MUC-1	200 ng	200 ng	spleen (T cell)	IFN-γ assay
	bfFp	20 μg	0	serum (humoral)	
	anti-CD40 mAb	25 μg	0		
7	B-GM3	1 μg	1 μg	spleen (T cell)	IFN-γ assay
	B-GM2	1 μg	1 μg	serum (humoral)	
	bfFp	20 μg	0		
	anti-CD40 mAb	25 μg	0		
	groups	day 0	day 12	day 21	day 24
8	control	PBS	PBS	spleen (T cell)	IFN-γ assay
				serum (humoral)	
9	B-pEBOV GP1,2	500 ng	500 ng	spleen (T cell)	IFN-γ assay
	bfFp	20 μg	0	serum (humoral)	
	anti-CD40 mAb	25 μg	0		
10	B-pSARS-CoV spike	500 ng	500 ng	spleen (T cell)	IFN-γ assay
	bfFp	20 μg	0	serum (humoral)	

	groups	day 0	day 12	day 21	day 24
	anti-CD40 mAb	25 μg	0		
11	B-pSARS-CoV membrane	500 ng	500 ng	spleen (T cell)	IFN-γ assay
	bfFp	20 μg	0	serum (humoral)	
	anti-CD40 mAb	25 μg	0		
	groups	day 0	day 12	day 21	day 24
12	control	PBS	PBS	spleen (T cell)	IFN-γ assay
				serum (humoral)	
13	B-OVA	200 ng	200 ng	spleen (T cell)	IFN-γ assay
	B-EBOV GP1	200 ng	200 ng	serum (humoral)	
	B-SARS-CoV spike RBD	200 ng	200 ng		
	B-MUC-1	200 ng	200 ng		
	B-anthrax PA	200 ng	200 ng		
	anti-CD40 mAb	25 μg	0		
	core-streptavidin	10 μg	0		
14	B-OVA	200 ng	200 ng	spleen (T cell)	IFN-γ assay
	B-EBOV GP1	200 ng	200 ng	serum (humoral)	
	B-SARS-CoV spike RBD	200 ng	200 ng		
	B-MUC-1	200 ng	200 ng		
	B-anthrax PA	200 ng	200 ng		
	bfFp	20 μg	0		
15	B-OVA	200 ng	200 ng	spleen (T cell)	IFN-γ assay
	B-EBOV GP1	200 ng	200 ng	serum (humoral)	
	B-SARS-CoV spike RBD	200 ng	200 ng		
	B-MUC-1	200 ng	200 ng		
	B-anthrax PA	200 ng	200 ng		
	bfFp	20 μg	0		
	anti-CD40 mAb	25 μg	0		

[a]The amounts of antigens, antibodies and bfFp are either in μg or ng per mouse. All mice were injected subcutaneously near the inguinal lymph node. Three separate experiments were conducted in 15 groups of mice and each experiment had its own control group. Groups 1, 8 and 12 were the control groups. The first experiment demonstrated the versatility of the bfFp based delivery of protein, peptide,

glycolipids and DNA to DC in generating immune responses (Group 2–7 mice). The second experiment was designed to confirm the DNA delivery strategy of targeting varies infectious disease viral DNA to DC (Groups 9–11). Multivalent immune responses against a mixture of antigens (proteins and peptide) were studied in the third set of experiments (Groups 13–15). All mice were boosted with the same concentrations of antigen(s) in PBS 12 days following primary immunization. The mice were sacrificed after 9 days of boost.