University of Alberta

Antigen Targeting and Drug Delivery Employing Bifunctional Fusion Protein and Bispecific Antibodies

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Pharmaceutical Sciences

Faculty of Pharmacy and Pharmaceutical Sciences

Edmonton, Alberta
Fall 2008

Your file Votre référence
ISBN: 978-0-494-46447-2
Our file Notre référence
ISBN: 978-0-494-46447-2

Abstract

Bispecific antibodies and bifunctional fusion proteins incorporate different binding specificities in one molecule. As a unique molecular design, they can bind two different antigens for numerous applications such as drug delivery and immune system targeting. Hypothesis: most of the protein based drug and immune system targeting vehicles are not versatile to deliver multiple molecules, and thus limits potential applications. Development of bispecific antibodies and bifunctional fusion proteins that can deliver distinct classes of molecules to the target sites is a promising strategy. Objectives: three strategies were tested using bispecific antibodies and bifunctional fusion proteins for targeting dendritic cells (DC) and ovarian cancer. The first study investigated the ability to construct full length bispecific monoclonal antibodies for targeting biotinylated antigens to DC. A quadroma secreting bispecific monoclonal antibodies (bsmAb), with one paratope specific for mouse DC DEC-205 receptor and another paratope specific for biotin, was developed. Studies have shown enhanced binding and internalization of biotinylated antigen to DC. *In vivo* studies in mice with biotinylated ovalbumin (OVA) have shown that in the presence of bsmAb and co-stimulator anti-CD40 mAb the immune responses were augmented. Refinement of the bispecific construct incorporated a truncated streptavidin and a single chain antibody (scFv) against the DC DEC-205 receptor. The versatile delivery of biotin labeled protein, peptide, glycolipids and DNA to DC was demonstrated. The bifunctional activity against DEC-205 and biotin were characterized. *In vivo* targeted immune response studies in mice with several proteins, cancer peptide, gangliosides and DNA were performed. In the presence of bifunctional fusion protein and anti-CD40 mAb, immune responses were augmented in single or

multiple antigen targeting. The third study investigated the development of a universal ovarian cancer cell targeting vehicle that can deliver biotinylated therapeutic drugs. A scFv that recognizes the CA125 antigen of ovarian cancer cells was fused with a core-streptavidin domain. The two distinct activities of the fusion protein were demonstrated. In the presence of fusion protein, there was enhanced binding of biotinylated antigen and liposome to ovarian cancer cells. Conclusion: these studies demonstrated the versatility of bispecific targeting systems for vaccine or therapy applications.

Acknowledgments

I would like to thank my supervisor Dr. M. R. Suresh and committee members Dr. R. Bundle and Dr. R. Loebenberg for their supervision, encouragement and support. I also like to thank Dr. D. Das, Dr. X. Tang, Dr. F. Jacobs, Dr. W. Budzynski and Dr. S. McQuarrie for their suggestions in my studies. I thank my friends and colleagues for their support over the years.

I dedicate this thesis to my parents, Su-Chien Wang and Jui-Chang Wang, and to my brother Wen-Ching Wang for their love and support.

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List of Abbreviations

%	percent
μg	micro gram
μl	micro liter
°C	degrees centigrade
ATCC	American type culture collection
BCCP	biotin carboxyl carrier protein
BsmAb	bispecific monoclonal antibody
BfFp	bifunctional fusion protein
BSA	bovine serum albumin
B-BSA	(biotin)n labeled BSA
B-M13	(biotin)n labeled M13
B-OVA	(biotin)n labeled OVA
B-MUC-1	biotin labeled MUC-1 peptide
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	synthetic biotin labeled GM2
B-BSA-GM2	synthetic biotin labeled BSA and GM2
B-GM3	synthetic biotin labeled GM3
B-BSA-GM3	synthetic biotin labeled BSA and GM3
B-pVHX-6	photoactivated biotin labeled WEEV DNA vector
B-pEBOV GP1,2	photoactivated biotin labeled EBOV GP1,2 DNA vector
B-pSARS-CoV spike	photoactivated biotin labeled SARS-CoV spike DNA vector
B-pSARS-CoV membrane	photoactivated biotin labeled SARS-CoV membrane DNA vector
B-liposome	(biotin)n labeled liposome
CD	cluster of differentiation
cDNA	complementary DNA

CFA	complete Freund's adjuvant
C_L	constant light chain region
C_{H2}	constant heavy chain region 2
CO_2	carbon dioxide
CS	core-streptavidin
CLSM	confocal laser-scanning microscopy
DC	dendritic cells
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EBOV	Ebola virus
EBOV GP1	Ebola virus glycoprotein 1
EBOV GP2	Ebola virus glycoprotein 2
EBOV GP1,2	Ebola virus glycoprotein 1 and 2
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
FACS	fluorescent-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
FPLC	fast protein liquid chromatography
g	gram
GAM-HRPO	goat anti-mouse-HRPO
GM-CSF	granulocyte-monocyte colony simulating factor
HAMA	human anti-mouse antibody responses
HIV	human immunodeficiency virus
His	histidine
h	hour
HPLC	high performance liquid chromatography
HRPO	horseradish peroxidase

HSLAS	Health Sciences Laboratory Animals Services
HSF	hybridoma serum free media
IgG	immunoglobulin class G
ID	internal diameter
IFA	incomplete Freund's adjuvant
IFN-γ	interferon gamma
IMAC	immobilized metal affinity chromatography
IPTG	isopropyl β-$_D$-thiogalactoside
Kd	dissociation constant
kDa	kilo Dalton
L	liter
LAL	limulus amebocyte lysate
LC/MS	liquid chromatography/mass spectrometry
LPS	lipopolysaccharide
M	mole
MUC-1	epithelial mucin 1
MMR	macrophage mannose receptor
MW	molecular weight
mAb	monoclonal antibodies
mg	milligram
MHC-I	major histocompatibility complex-class I
min	minute
ml	milliliter
mM	millimolar
Ni	nickel
NCBI	National Center for Biotechnology Information
ng	nanogram
NHS-LC-Biotin	biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester
nm	nanometer
OD	optical density

OVA	ovalbumin
PA	protective antigen
PBS	phosphate buffer saline
PBST	PBS with 0.1% Tween 20
PEG	polyethylene glycol
PCR	polymerase chain reaction
PSG	penicillin, streptomycin and L-glutamine
PKS	pBlueScript II KS vector
pVHX-6	WEEV DNA vector encoding E1 and E2 proteins
RBD	receptor binding domain
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
S. avidinii	*Streptomyces avidinii*
SARS-CoV	severe acute respiratory syndrome-coronavirus
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
rpm	rounds per minute
scFv	single chain antibody
T	Tween-20
TD-PCR	touch down polymerase chain reaction
TMB	3, 3', 5, 5'-tetramethylbenzidine
TRITC	tetramethyl rhodamine isothiocyanate
VL or V_L	variable light chain
VH or V_H	variable heavy chain
v/v	volume by volume
w/v	weight by volume
WEEV	Western equine encephalitis virus

CHAPTER 1
Introduction

1.1. Bispecific Monoclonal Antibodies

Bispecific monoclonal antibody (bsmAb) is a construct with two different paratope specificities in one molecule. The concept of bsmAb was first introduced by Nisonoff and Rivers by chemical methods [55]. The advent of monoclonal antibody technology (Kohler and Milstein) set the stage for the emergence of bispecific monoclonal antibodies and bifunctional antibodies. Bispecific antibodies are defined as second generation antibodies with two dissimilar antigen binding sites. Bifunctional antibodies are designs incorporating one antibody combining site fused with a non-antibody functionality.

1.1.1. Production of Bispecific Monoclonal Antibodies

Bispecific antibodies can be generated by either chemical, biological or by recombinant DNA methods. Early development of bsmAb was either by chemical cross-linking or cell fusion methods. Chemical cross-linking method is by far the easiest and fastest method to generate pure bsmAb. In general, bsmAb are generated by first dissociation of two different antibodies and re-associating them *via* introduction of reactive functional groups to the dissociated fractions. However, the chemical cross-linking method suffers from significant batch-to-batch variation and could denature the antibody [1]. Second derivative hybrid-hybrdomas generated from fusion of two different hybridomas (quadroma) produce antibodies of two desired populations. Fusion of one hybridoma with a lymphocyte derived from a mouse immunized with a different antigen generates a trioma secreting bsmAb [2, 3]. Cells can be fused by electrical current (electrofusion and microelectrofusion) or by a chemical method utilizing polyethylene glycol (PEG). Selection of fused cells can be done by drug selection or by fluorescence-activated cell sorter (FACS). The FACS method is less time-consuming compared to drug selection (a drug resistance gene has to be inserted into the cells). FACS sorting of fused cells is done by first labeling the two different cells with different fluorochromes on its membrane and then collect the cells stained with both fluorochromes. The bsmAb generated by biological methods are always functional and naturally generated.

However, the major drawbacks with biological methods are batch to batch variation, time consuming purification process and low yield. DNA recombinant technology is the latest method to generate bsmAb. In comparison to biological and chemical methods, genetic engineering is the best method to generate pure, functional and clinically applicable bsmAb. In short, the antibodies of different specificity genes are cloned and expressed as distinct polypeptides from the same transcript or from separate plasmid constructs. The major drawback of such technology is high cost in creating recombinant full-length bsmAb.

1.1.2. Applications of Bispecific Monoclonal Antibodies

Bispecific monoclonal antibodies are commonly used in diagnostic, therapeutic and in delivery applications. The concept of bsmAb in diagnostics was first introduced by Milstein and Cuello [2]. A bsmAb that can bind to the specific antigen and peroxidase are useful in immunohistochemistry and enzyme immunoassays for better sensitivity and clearer backgrounds compare to mAb. The main advantage of bsmAb over mAb is the ability to bind to the antigen and detector enzyme at the same time without the need to use secondary molecules, thus offering better sensitivity and assay time efficiency. BsmAb can be applied to pretargeted therapy and immunotherapy applications. Radioimmunotherapy of solid tumors has been largely ineffective due to suboptimal tumor targeting, slow serum elimination, and bone marrow toxicity. Unlike the direct radiolabeled antibodies, in pretargeted therapy, a radioactive and tumor-reactive bsmAb is first administered. After maximal accumulation of the bsmAb in the tumor, the radioisotope is administered for targeting and irradiation of the tumor. The pretargeted therapy enables the delivery of high doses of radioactivity to tumors resulting in a significantly increased therapeutic index as compared to the traditional radioimmunotherapy approaches [4]. In immunotherapy applications, bsmAb is used to activate and target the cellular immune system to kill tumor cells or pathogens. Immunotherapy of tumor cells and pathogens using traditional mAb are often ineffective due to the inability to trigger a cytotoxicity response (Fc receptors are often blocked by the serum antibodies) and unable to target the immune cells to the cancer cells or pathogens. Lastly, bsmAb can be applied as a universal delivery system for delivering of

drugs, toxins, cytokines, proteins and genes to the target sites. BsmAb mediated delivery has been applied in gene therapy, cancer treatment and immune system stimulation/activation. The delivery system offers lower toxicity and higher efficiency compared to other traditional methods [1, 5].

1.2. Recombinant Antibodies

Currently, there are about 20 recombinant antibodies products on the market and more than 100 are in clinical trials and even more (400) are in pre-approval clinical trials. Engineered antibodies are accounted for more than 30% of all revenues in the biotechnology market. Recombinant antibodies have been applied clinically for the treatment of cancer, inflammatory diseases, myocardial infraction, autoimmune disorder, infections and transplantation.

Despite advances in the applications of bsmAb technology, it is restricted within *in vitro* and animal studies. The clinical limitations include undesirable Fc domain (Fc mediated cytotoxicity and massive cytokine release), immunogenicity (human anti-mouse antibody response) and pharmacokinetic profiles (prolonged serum circulation, inefficient tumor penetration and kidney toxicity). New strategies and techniques have evolved to redesign the bsmAb for clinical applications. These include the diabody, bispecific Fv, bispecific scFv, bispecific Fab and bifunctional fusion protein by recombinant DNA technology. These recombinant antibodies lack Fc domains and they can be humanized to reduce the immunogenicity. In addition, pharmacokinetics profiles can be manipulated for the desired applications by chemical modification (PEGlyation of proteins) or by genetic alternation (point mutation) [6-8].

Recombinant antibody is also generated by phage display or by hybridoma cloning methods. The cloning method involves culturing of the hybridoma, extraction of the total RNA and purification of mRNA using oligo dT purification system. The first strand cDNA is synthesized using mRNA as template with specific antibody primers or degenerate primers for specific antibody subtype or isotype. For example, first-strand cDNA derived from the hybridoma mRNA VH (variable heavy chain) and VL (variable light chain) genes can be amplified using VH and VL specific primers for the construction of scFv (single chain) antibody. The full length scFv is generated by

combining equal amounts of VH and VL PCR products with 15 amino acid linkers and assembly reaction is performed by PCR.

1.2.1. Recombinant Fusion Protein

Fusion constructs comprising antibodies or antibody fragments with proteins or peptides have been generated by either recombinant DNA technology or by chemical conjugation for various therapeutic and vaccine applications [9]. Fusion constructs are generally used to direct therapeutics agents, such as toxins, cytokines, radioisotopes and drug-activating enzymes for cancer treatment. Fusion constructs can also efficiently deliver the antigen to the immune cells to generate efficient protective immune responses against cancer and infections in comparison to non-targeting methods. The fusion constructs are generally antibodies or their fragments fused with the therapeutic agents or antigens. Fusion constructs such as bispecific Fab, diabody, bispecific scFv and bifunctional fusion proteins can also be used to direct immune cells or therapeutics agents to cancer cells.

1.2.2. Streptavidin Engineering

Streptavidin, a tetrameric protein (60 kDa) naturally secreted by actinobacterium *Streptomyces avidinii*, has exceptionally high affinity (Kd of 10^{-13}-10^{-16} M) for biotin *via* non-covalent interaction. Avidin from chicken egg white is structurally and functionally analogous to streptavidin for its binding affinity towards biotin. One molecule of streptavidin and avidin binds to four molecules of biotin. In addition, these molecules are known for high stability against heat, denaturants, proteolytic enzymes and high or low pH. Biotin, also known as vitamin H, present in every living cell, acts as a co-factor of carboxylating enzymes. Streptavidin and avidin have been used in a variety of applications: immunoassays, hybridization assays, lymphocyte activation, affinity chromatography, localization of antigen, and purification of nucleic acids [10]. Avidin is a glycoprotein containing a disulfide-linkage as well as two methionine residues per subunit whereas streptavidin has no carbohydrate chain, no sulfur containing amino acids and has a lower isoeletric point (5 to 6). Avidin contains both mannose and N-acetylglucosamine and has an isoelectric point of ~10. The major problem of using avidin

is the high nonspecific binding, which is attributed to the presence of sugars and the high isoelectric point. Thus streptavidin is more useful for most applications compared to avidin for its specific binding profile [11].

Streptavidin and avidin can be made by recombinant DNA technology. Expression of streptavidin protein is often restricted to in *E.coli* expression system; whereas, avidin is in mammalian expression system. One major disadvantage of mammalian expression system is the need for biotin free culture environment [12].

Streptavidin produced by *Streptomyces avidinii* has many disadvantages including susceptibility to proteolysis near the N-terminus region of the molecule [13]. Core-streptavidin (CS) derived by proteolytic digestion of streptavidin exhibits higher solubility, reduced tendency towards oligomerization and resistance towards subsequent proteolytic degradation [14, 15]. In *E. coli* system, CS has been expressed either as fusion protein or alone, both as an insoluble or soluble protein [11, 16]. The CS and its fusion proteins have been exploited extensively for a variety of *in vitro* and *in vivo* applications [17, 18]. Conditions for optimal production of soluble CS and refolding of insoluble CS in *Escherichia coli* have been reported [16, 19]. Domain modification of CS for reduced *in vivo* immunogenicity and kidney retention has also been studied [20, 21]. Mutation of core-streptavidin residues E51, Y83, R53, and E116 can reduce patient sera reactivity less than 10% of the streptavidin. The fusion proteins of CS such as scFv-CS fusion proteins have been utilized for applications in cancer immunotherapy and pretargeted radioimmunotherapy therapy [24, 25].

Single-chain antibody-core-streptavidin fusion protein or bifunctional fusion protein (bffp) was first reported by Dubel and Kipriyanov in 1995 [22, 23]. The construction involves cloning of scFv gene in N-terminus and the CS gene in the C-terminus followed by His_5 tag (Fig. 1.1).

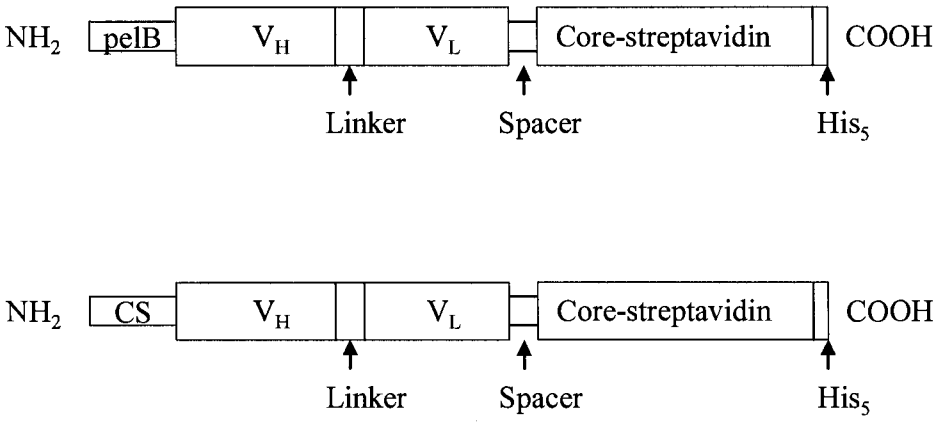

Figure 1.1. Single-chain antibody-core-streptavidin fusion protein designs. The construction involves cloning of scFv gene in the N-terminus and the core-streptavidin gene in C-terminus follow by His_5 tag. The scFv gene is followed by either pelB or CS leader sequence. A spacer of 5 amino acids is placed in between the scFv gene and CS gene. Abbreviations: pelB, bacterial leader sequence; CS, core-streptavidin leader sequence; V_L, variable domain light chain; V_H, variable domain heavy chain; His_5, five histidine amino acid tag.

The scFv gene is usually followed by either pelB or CS leader sequence. A spacer of 5 amino acids (Glycine-Serine-Glycine-Alanine-Alanine or Glycine-Serine-Glycine-Serine-Alanine) is placed in between the scFv gene and CS gene (Fig. 1.1). The fusion protein has only been produced in *E.coli* system as either soluble protein or insoluble protein. The fusion protein is found in 3 isoforms: monomeric, dimeric and tetrameric form due to the tendency of CS forming oligomers. Different isoforms of the proteins can be purified using IMAC (immobilized metal affinity chromatography) or iminobiotin purification strategies followed by HPLC. Tetrameric fusion protein is the main isoform of interest for pretargeting radioimmunotherapy for its tetravalent binding ability to biotin and antigen of interest.

Pretargeting radioimmunotherapy of a single-chain antibody-streptavidin fusion protein has been applied in clinical studies for treatment of cancer and the clinical results have demonstrated the superiority of pretargeting radioimmunotherapy compared to the traditional radiolabeled antibody therapy [24, 25]. Radioimmunotherapy of solid tumors has been largely ineffective due to suboptimal tumor uptakes, slow serum elimination,

and bone marrow toxicity. Unlike the direct radiolabeled antibodies, in pretargeted therapy, a non-radioactive tumor-reactive fusion antibody is first administered. After maximal accumulation of the fusion protein in the tumor, the radiolabeled biotin is administered for targeting and irradiation of the tumor. The disadvantage is that streptavidin is known to be taken up by the kidney and several studies have described renal radiotoxicity using the pretargeting approach [26-29]. A recent study has shown that succinylation of the fusion construct greatly reduces kidney uptake of the subsequently administered radiolabeled biotin, which greatly improves the therapeutic index associated with multistep immune targeting approaches to radioimmunotherapy [29]. An alternative strategy is the development of a smaller single-chain antibody-core-streptavidin fusion protein. The smaller fusion protein could potentially be applicable for the pretargeted radioimmunotherapy or tumor targeting with minimal side-effects.

1.3.Dendritic Cells

DC are the most specialized and potent antigen presenting cells in the immune system. DC express high levels of MHC class I, MHC class II and co-stimulatory molecules and have the unique ability to stimulate and prime naïve T cells. DC play a critical role in innate and adaptive immune responses, especially in priming and activating T cells and B cells, thus present as a potential candidate in vaccine and therapy development. DC can be targeted *ex vivo* or *in vivo* to induce antigen specific effector and memory cells for cancer therapy and infection prevention.

1.3.1. Dendritic Cell Targeting: *Ex vivo* and *In vivo*

Several clinical trials have been conducted on human tumor immunotherapy by *ex vivo* stimulation of DC [30]. In general, monocytes or CD34 positive precursors are isolated from patient blood and then cultured in the presence of various cytokines to produce immature DC, and loaded with antigen before or after DC maturation. The mature antigen-loaded autologous DC are then injected back to the patients to induce antigen specific immune response. The clinical responses have only been observed in a minority of patients. Only a small percentage of DC migrate from the injection site into the draining lymph node to present the antigen to T cells [30], and the procedure is very

costly and laborious. Hence this experimental approach is not feasible for the general population. Despite the recent disappointment of *ex vivo* clinical trials, *in vivo* targeting of DC surface receptors may present as a potential alternative for ex vivo targeting of DC [30]. *In vivo* targeting of DC surface receptor has been shown to be effective in generating immune responses to protect the host against cancer, viral infection and autoimmune disease. However, transition of *in vivo* targeting strategy to human still remains difficult since human equivalents of various mouse DC subsets are yet to be identified and may not be representative [30]. For instance, DC DEC-205 expression in humans is less DC restricted than in mice. Targeting DEC-205 receptors in humans may also target B cells, NK cells, T cells, monocytes and thymic epithelial cells which could cause unwanted adverse effects. Moreover, co-administration of maturation or activation stimulus is often required for *in vivo* targeting strategy [30].

1.3.2. Dendritic Cell Surface Receptors

DC utilizes surface receptors (Gb3/CD77, CD40, β2 integrins, Fc receptors, and C-type lectin receptors) to process and present antigens in context to MHC class I and MHC class II pathways [31]. The DC surface receptors that have been used for the targeting of antigens to DC are listed in Table 1.1.

Surface Receptors	Ligand	Distribution	Co-Stimulation Requirement	Applications
Gb3 (glycosphingolipid)	Shiga toxin	Monocytes, dendritic, endothelial, epithelial, and B cells	No	Vaccine vector
β2 integrins (CD11c/CD18)	CyaA	Myeloid dendritic cells, macrophages, monocytes, activated B cells, natural killer cells, granulocytes	Yes	Vaccine vector Tumor protection
CD40 receptors	CD40 ligand	Dendritic cells, B cells, macrophages, endothelial cells, keratinocytes, fibroblasts, thymic epithelial cells, CD34 hematopoietic cell progenitors	No	Vaccine vector Tumor protection Tumor therapy Adenovirus delivery
C-type lectin receptors: DEC-205 (CD205)	Unknown	Dendritic cells, Langerhans cells, monocytes, B cells, natural killer cells, T cells, respiratory tracts, thymic and gut epithelial cells	Yes	Vaccine vector Liposome delivery Tumor protection Tumor therapy Infection protection Suppress autoimmunity
C-type lectin receptors: LOX-I (lectin-like oxidized low-density lipoprotein receptor-I	Hsp70	Immature dendritic cells, macrophages, fibroblasts, smooth muscle cells and endothelial cells	No	Vaccine vector Tumor therapy Myocardial ischemia Atherosclerosis
C-type lectin receptors: DC-SIGN (CD209)	Mannose	Immature dendritic cells, macrophages and megakaryocytes	Unknown	Vaccine vector Liposome delivery Adenovirus delivery
C-type lectin receptors: mannose receptor (CD206)	Mannose Mannan	Immature dendritic cells, macrophages, interstitial dendritic cells, dermal dendritic cells, lymphatic endothelium, tracheal smooth muscle cells, kidney mesangial cells	Yes	Vaccine vector Immunosuppression Immunoactivation Cancer therapy
Fc receptors: FcγRI (CD64)	Fc	Dendritic cells, monocytes, macrophages, and activated neutrophils	Unknown	Vaccine vector Tumor protection Tumor therapy

Table 1.1. Summary of dendritic cell (DC) surface receptors for antigen targeting. The table shows the expression pattern of the receptors and also indicates whether antigen targeting to the receptor required co-stimulation for induction of immune responses.

The table also shows the expression pattern of the receptors and also indicates whether antigen targeting to the receptor required co-stimulation for induction of immune responses. To summarize, antigen targeting to Gb3, LOX-I and CD40 receptors does not require co-stimulation for induction of immune responses. DC-SIGN expression is restricted to DC and macrophages; whereas other receptors are distributed on a variety of cells. DEC-205 targeting strategies appear to be extensively studied and applied for a variety of applications. DEC-205 is a type 1 C-type lectin receptor that is present in both immature and mature DC in lymphoid tissues, lymph nodes and spleen [32-36]. In the presence of co-stimulatory molecules, DEC-205 targeting of antigens by an antibody is sufficient to protect the host against tumor growth [37], with rejection of existing tumor [38], protection against airway challenge of virus [39], and can enhance resistance to an

established rapidly growing tumor, as well as viral infection [40]. Moreover, DEC-205 targeting of a protein enables cross-presentation of several peptides more efficiently than CD206 and CD209 [41] and induces stronger T cell immunity at much lower doses of protein antigen, plasmid DNA or recombinant adenovirus [39]. In the absence of co-stimulation, DEC-205 targeting of antigen can be used to suppress the development of autoimmunity, such as type 1 diabetes [42]. Generation of antibody-mediated antigen targeting system is often a complex, costly and time consuming process, which requires either liposomal encapsulation of antigen [37], chemical cross-linking with the antigen [38, 40, 42, 43] or construction of a new hybrid antibody expressed in mammalian system [39, 41, 44]. Thus, these targeting systems may not be desirable for comparison studies with different antigens and *in vivo* targeting of multiple antigens such as DNA and glycolipids.

1.4. Ovarian Cancer

Ovarian cancer is divided into 3 categories namely: epithelial cancer, stromal tumors and germ cell tumors. Epithelial cancer accounts for 90% of ovarian cancers. Stromal and germ cell tumors account for the remaining 10% of the cancer. Thus ovarian cancer is often referred to ovarian cancers arise from cells that make up the epithelial layer that covers the surface of the ovaries [45]. Ovarian cancer is the fourth most common cause of death affecting women in Canada and the United States. The National Ovarian Cancer Association reports that 1 in 70 Canadian women are affected by ovarian cancer. Patients are often diagnosed at advanced stages with low overall cure rates due to the lack of specific symptoms and signs of the disease and lack of reliable screening strategies [45]. Patients diagnosed with ovarian cancer are usually treated with surgical resection follow by taxane- or platinum-based chemotherapy. The taxanes are a class of anticancer drugs (Paclitaxel and Docetaxel) that bind to and stabilize microtubules causing cell-cycle arrest and apoptosis. The platinums (Cisplatin and Oxaliplatin) are a class of anticancer metal that cross-link subunits of DNA impairing DNA synthesis, transcription, and function. Despite the aggressive treatment using either surgery or chemotherapy or both, the prognosis for the majority of patients is poor. Metastatic recurrences are common, often in the peritoneal cavity, which ultimately leads to death

from the progression of the disease. Newer therapeutic strategies have been developed and are promising for *in vitro* and *in vivo* application and in clinical trials [45].

1.4.1. Risk Factors and Diagnostics

The main cause of ovarian cancer is still unknown. There are many reports on the risk factors causing ovarian cancer and these can be divided into 3 categories: environmental, hormonal and hereditary predisposition. Conflicting reports regarding environmental factors include exposures to carcinogens, asbestos and the use of talcum powder. Hormonal factors are often associated with pregnancy and menstrual cycle. For instance, nulliparity, early menarche, and late menopause can increase the risk of developing ovarian cancer. Approximately 10-15% of all epithelial ovarian cancers have a hereditary predisposition from a strong family history of either breast or ovarian cancer. The usual finding of hereditary predisposition is the mutation in either *BRCA1* or *BRCA2* genes. Other cancers history including nonpolyposis colon cancer, colorectal cancer and endometrial cancers also increases the risk of ovarian cancer development [45].

The symptoms of ovarian cancer are usually nonspecific and often associated with metastasis (abdomen) and late stage of cancer. The frequent presenting symptoms are urinary urgency, bowel irritability, fatigue, painful intercourse and abdominal bloating [46]. Early detection of the disease generally increases the rate of cure. However, there is no effective diagnostic method for early stage detection of ovarian cancer. Ovarian cancer is often diagnosed by either tumor marker CA125 or ultrasonography. Abdominal and transvaginal ultrasonography are often performed to screen for ovarian cancer. The positive predictive value ranges from 1.5% to 10% [45]. CA125 testing by far is the best single tool to identify and follow ovarian cancer and to determine the prognosis of treatment. A rapid fall in serum CA125 during chemotherapy predicts a favorable prognosis [47]. However, only 20% of ovarian cancers have little or no expression of CA125 and more than 80% of women have CA125 levels that are often not elevated until cancer metastasis [46, 47].

1.4.2. Treatment

Ovarian cancer patients usually undergo surgery and/or chemotherapy as part of their primary treatment; however, metastatic disease and/or drug resistant variants manifest within 18 months [48]. The overall 5-year survival rate is approximately 45% and 75% of cancers recur within the first 2 years after diagnosis [49, 50]. Newer strategies such as targeting the CA125 antigen with B43.13 mouse monoclonal antibody was shown to be effective [51, 52]. CA125 is a circulating ovarian tumor antigen that is present in the majority of ovarian cancer, especially in those with advanced-stages of the cancer. B43.13 promotes both humoral and cell-mediated immune responses by forming immune complexes with CA125 [51, 53]. In addition, B43.13 may initiate a classical idiotype network of immune responses. A human anti-mouse antibody response (HAMA effect) to B43.13 mAb bound to the CA125 antigen, would in turn stimulate an anti-idiotype cascade response resulting in a humoral polyclonal immune response against CA125 [53]. HAMA and antibody responses are frequently observed in such antibody targeting strategies, and are not associated with limiting toxicities related to hypersensitivity [53, 54]. Although, it has been recently reported that antibody targeting treatment is well tolerated without serious adverse events or discontinuations due to therapy [52], CA125 targeting with monoclonal antibody has limitations. Antibody targeting strategies could not consistently activate immune responses and overcome immune escape mechanisms, especially in patients with a significant disease burden [52]. Consequently, utilizing multiple therapeutic strategies could be the most effective treatment to overcome immune escape mechanisms and drug resistant.

1.5. Development of Bispecific Monoclonal Antibodies and Bifunctional Fusion Protein – Rationale

Bispecific antibodies and bifunctional fusion proteins incorporate different binding specificities in one molecule. As a unique molecular design, they can bind two different antigens for numerous applications such as in drug delivery and immune system targeting. Most of the protein based drug and immune system targeting vehicles are not versatile to deliver multiple molecules, thus limits its potential applications. Development of bispecific antibodies and bifunctional fusion proteins that can be versatile to deliver

distinct classes of molecules towards the target sites is a promising strategy. Three strategies are described in this thesis using bispecific antibodies and bifunctional fusion proteins for targeting dendritic cells (DC) and ovarian cancer. Chapter 2 describes the construction of full length bispecific monoclonal antibodies for targeting biotinylated antigens to DC *in vitro* and *in vivo*. The contents of this chapter was published in Journal of Immunological Methods 2005;306(1-2):80-92. Refinement of the bispecific construct incorporated a truncated streptavidin (Chapter 3, published in Molecular Biotechnology 2005;31(1):29-40) and a single chain antibody (scFv) against the DC DEC-205 receptor is described in Chapter 4. Chapter 4 describes the versatile delivery and *in vivo* targeted immune response studies in mice with several proteins, cancer peptide, gangliosides and DNA. The contents of this chapter have been submitted as a manuscript for publication. Chapter 5 investigates the development of a universal ovarian cancer cell targeting vehicle that can deliver biotinylated antigen and liposome to ovarian cancer cells in an *in vitro* model system. The contents of this chapter was published in European Journal of Pharmaceutics and Biopharmaceutics 2007;65(3):398-405. Lastly, Chapter 6 concludes the above thesis chapters with a brief summary, general discussion and future studies.

1.5.1. Hypothesis

The central hypothesis of this research is that a bifunctional fusion protein design can be developed as a versatile delivery vehicle for biomedical applications. The above concept was tested by designing two bifunctional recombinant targeting molecules for a) dendritic cells for vaccine applications and b) ovarian cancer cell targeting.

The subhypotheses of this project are: a) bifunctional fusion protein can target several classes of antigens (protein, peptide, glycolipids and DNA) to DC and successfully generate humoral and cell mediated immune responses, and b) similar design with ovarian cancer antigen specificity can be constructed and tested for successful targeting in an *in vitro* model system.

1.5.2. Specific Aims

1. To construct a full length bispecific monoclonal antibodies for targeting biotinylated antigens to DC *in vitro* and *in vivo*.

a. To generate quadroma and screen for bsmAb
b. To affinity purify the bsmAb with biotin-agarose column
c. To test bispecific antibody activities
d. To demonstrate uptake and enhanced binding of biotinylated M13 with bsmAb to DC
e. To evaluate immune responses in immunized mice with biotinylated OVA in the presence or absence of bsmAb

2. To develop a recombinant bifunctional construct incorporating a truncated streptavidin with a scFv against the DC.
   a. To construct, express and affinity purify the core-streptavidin
   b. To characterize core-streptavidin
   c. To construct, express and affinity purify the scFv and bfFp
   d. To characterize bfFp
   e. To test bfFp activities
   f. To evaluate immune responses in immunized mice with biotinylated protein, peptide, DNA and glycolipid in the presence or absence of bfFp
   g. To evaluate the serum reactivity of the biotin, bfFp and core-streptavidin
3. To develop a universal ovarian cancer cell targeting vehicle that can deliver biotinylated materials (such as biotinylated liposome and protein) to ovarian cancer cells
   a. To construct, express and affinity purify the bfFp
   b. To characterize bfFp
   c. To test bfFp activities
   d. To demonstrate enhanced binding of biotinylated liposome and protein with bfFp to DC

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CHAPTER 2

Antigen Targeting to Dendritic Cells with Bispecific Antibodies

The contents of this chapter was published in Journal of Immunological Methods 2005;306(1-2):80-92.

2.1.Introduction

Dendritic cells (DC) play a critical role in antigen capture, processing and presentation in the immune system. DC constitutively express high levels of MHC class I, MHC class II, B7-1, B7-2 and other molecules. Moreover, DC are able to prime and activate naïve T cells, effector T cells and memory T cells. DC use the macrophage mannose receptor (MMR), the DEC-205 receptor, and macropinocytosis to concentrate the majority of foreign materials into the MHC I/II and CD1 compartments for processing and presentation to T cells. DEC-205 is an integral membrane protein, homologous to the MMR, which binds carbohydrates and mediates endocytosis [21]. DC expressing DEC-205 are widely distributed in the body [20, 44] and are present in both immature and mature DC populations [20]. In addition, DEC-205 is highly expressed by DC within the T cell areas of lymphoid tissues, especially on CD8+ DC [15] and in other T cell areas such as lymph nodes [44]. It has been shown that DEC-205 is involved in both MHC class I and MHC class II presentation of antigens [5, 27]. DEC-205 targeting of antigens by an antibody is efficient and leads to T cell activation [15, 21, 27]. Moreover, DEC-205 route of targeting could protect the host against tumor growth [40] and enhance resistance to an established rapidly growing tumor, as well as viral infection [6].

Antibody-mediated antigen targeting *via* the DEC-205 receptor increases the efficiency of vaccination for T cell immunity, including systemic and mucosal resistance in disease models [6]. Hence, targeting of disease specific antigens to the DC DEC-205 receptor could be a potential route for a variety of therapeutic applications. However, generation of antibody-mediated antigen targeting system is a complex and time consuming process, which requires either the encapsulation of the antigen [40], chemical cross-linking with the antigen [5, 6] or construction of a new hybrid antibody [19]. A bsmAb has two non-identical paratopes [31] and can be used in diagnostic and therapeutic applications [4, 7-9, 22, 24, 37, 41-43]. Previous studies in our laboratory

resulted in the development of an anti-HRPO x anti-biotin bsmAb as a universal probe for detection of biotinylated antigens [7].

In this communication, we report the successful generation of bsmAb bearing two distinct paratopes specific to DEC-205 and biotin. We demonstrated that biotinylated antigens can be successfully targeted to DC by ELISA and confocoal microscopy studies *in vitro*. Strong humoral and cell-mediated immune responses were also obtained *in vivo* with bsmAb mediated targeting of antigens to DC in the presence of co-stimulatory anti-CD40 mAb. BsmAb targeting of biotinylated antigens to DC as a common universal vector could be a convenient, alternative method for targeting any biotinylated antigen to DC.

2.2. Materials and Methods

2.2.1. Materials

Mouse DC were prepared from the bone marrow of C57BL/6 utilizing published methods [26]. JAWSII, p53 deficient immature bone marrow DC, were obtained from ATCC No. CRL-11904. DMEM, hybridoma serum free media, PSG and FBS were purchased from Gibco BRL (Burlington, Canada). CFA, IFA, BSA, OVA, mouse anti-rat IgG_{2a}-HRPO, NHS-LC-Biotin, TRITC, FITC, mitomycin C, streptavidin-HRPO, GAM-HRPO, PEG solution, biotin-Agarose column, and ammonium sulfate were purchased from Sigma (Oakville, Canada). Filamentous phage M13 was prepared, purified and quantified as described in our previous report [25]. B-BSA, B-OVA, and B-M13 were prepared by biotinylation of BSA, OVA, or M13 respectively with NHS-LC-Biotin as per vendor's protocol (Sigma). TMB peroxidase substrate was purchased from Kirkegaard & Perry Laboratory Inc (Gaithersburg, USA). Mouse IFN-γ ELISA Ready-SET-Go was purchased from eBioscience (San Diego, USA). GM-CSF was purchased from R&D Systems (Minneapolis, MN, USA). LAL PYROGENT® Plus Single Test Vials was purchased from Cambrex (Walkersville, USA). Rat anti-mouse CD40 mAb was prepared from the hybridoma (IC10) kindly provided by Dr. Michael Gold (University of British Columbia, Canada).

2.2.2. Antibodies and Hybridomas

P54, a mouse hybridoma that secretes anti-biotin IgG_1 mAb, was previously established in our laboratory [7]. HB290, a rat hybridoma that secretes anti-DC DEC-205 IgG_{2a} mAb, was obtained from ATCC, USA. Hybridoma serum free medium [1% (v/v) FBS and 1% (v/v) PSG] was used to culture P54, HB290, and quadroma P125.

2.2.3. Mice

6-8 week old female C57BL/6 mice were obtained from Health Sciences Laboratory Animals Services (HSLAS) of the University of Alberta, Edmonton, Canada. These mice were originally purchased from The Jackson Laboratory, USA. Animal treatment, care and euthanasia were carried out according to the Canadian Council of Animal Care guidelines.

2.2.4. Quadroma Generation and bsmAb Screening

Quadroma generation was accomplished as described in our previous reports [14, 38]. Briefly, parental hybridoma HB290 was labeled with TRITC and the second hybridoma P54 with FITC. TRITC-labeled HB290 hybridoma (4.5×10^6 cells) was fused with FITC-labeled P54 hybridoma (5×10^6 cells) by using 50% PEG solution (500 µl). After 2 min of incubation at 37°C, the cells were pelleted by centrifugation (500 x g for 5 min at RT), and then re-suspended in 15 ml of DMEM-20 media (20% (v/v) FBS and 1% (v/v) PSG). The cells were then washed three times, resuspended in 5 ml of DMEM-20, and transferred to a tissue culture flask. The flask was incubated for 1 h at 37°C with 5% CO_2. Flow cytometry was performed with an Epics Elite Cell Sorter from Coulter Corporation (Hialeah, FL, USA). Cells with dual fluorescence were sorted at two cells per well into a 96-well tissue culture plate, which was then cultured at 37°C with 5% CO_2 in DMEM-20 for ten days. Quadroma (P125) was obtained after three successive steps of recloning of the high responders. Screening to select quadromas was done by a bridge ELISA which only detects bsmAb [37]. Microtiter plates (Nunc, Denmark) were coated with 100 µl of B-BSA (10 µg/ml) at 4°C overnight. The plates were blocked with 200 µl of 1% (w/v) BSA in PBS at 37°C for 1 h, and then washed three times with PBST (0.1% Tween 20 in PBS, pH 7.3). Aliquots (100 µl) of various quadroma cell culture

supernatants were serially diluted (1:10, 1:100) and added to each well. The plates were incubated for 1 h at 37°C, washed three times with PBST, and incubated with mouse anti-rat IgG_{2a}-HRPO (1:10,000 dilution in 1% dialyzed BSA) for 1 h at room temperature. The plates were then washed with PBST, 100 µl/well of TMB was then added and OD_{650} was taken after 10 min using an ELISA V_{max} kinetic microplate reader (Molecular Devices Corp, California, USA). The quadroma that provided the highest OD value was cloned and recloned to obtain the P125 hybrid hybridoma.

2.2.5. Affinity Purification of bsmAb with Biotin-Agarose Column

One liter of the bsmAb supernatant from the quadroma cell-line P125 was precipitated with 50% saturated ammonium sulfate, dialyzed against PBS, and loaded onto a column containing 15 ml of biotin-Agarose matrix. Following an extensive wash step, the bound biotin paratope bearing antibodies (monospecific P54 and bispecific P125) were eluted with glycine-HCl pH 2.8, neutralized with Tris-HCl pH 8.4, and dialyzed against PBS pH 7.2. Measurement of the activities of affinity purified P125 bsmAb was done by bridge ELISA as described above. Additional ELISA was performed using GAM-HRPO as the detection antibody to detect P54 anti-biotin antibody in the biotin-column purified P125 solution. GAM-HRPO does not cross-react with HB290 mAb.

2.2.6. Bispecific Antibody Activities

The DC were harvested from the bone marrow of C57BL/6 mice. We have followed published protocols for isolating and culturing mouse DC [26]. DC were coated on a 96-well V-bottom plate (Nunc, Denmark) in quadruplicate (1.0 x 10^5cells/well). The plates were washed four times with PBS and blocked with 1% PBS dialyzed BSA for 2 h at 4°C. Washing involved suspension and centrifugation of DC. After incubation, the plates were washed with PBS, and bsmAb diluted in various concentrations in PBS were added. The plates were incubated for 2 h at 4°C, and then washed with PBS. To detect anti-DEC 205 activities, the plates were incubated with mouse anti-rat IgG_{2a}-HRPO (1:10,000 dilution in 1% dialyzed BSA) for 1 h at 4°C. For analysis of anti-biotin activity, plates with bsmAb bound to DC were incubated with B-BSA (100 µg/ml in 100 µl volume) for 1 h at 4°C, washed, and then incubated with streptavidin-HRPO (10 µg/ml in 100 µl volume)

for 1 h at 4°C. The plates were then washed with PBS, TMB was then added at 100 µl/well and OD_{650} was taken after 10 min using an ELISA V_{max} kinetic microplate reader. Additional ELISA was also performed using GAM-HRPO as detection antibody to detect possible anti-biotin monospecific mAb binding to DC in the presence or absence of B-BSA.

2.2.7. Confocal Microscopy: Uptake and Enhanced Binding of Biotinylated M13 with bsmAb

The transformed JAWSII cell line was used in the confocal microscopy uptake study. The cells were grown in DMEM-10 [10% (v/v) FBS and 1% (v/v) PSG] with 5 ng/ml recombinant mouse GM-CSF in a tissue culture plate (Nunc, Denmark). The biotinylated and FITC-labeled-M13 (10^7 phages/ml in 100 µl volume) with and without of P125 bsmAb (10 µg/ml in 100 µl volume) was added to the tissue culture plate (1 x 10^6 DC/well) and incubated for 1 h at 4°C and then 10 min at 37°C. A 10 µl aliquot of the culture was washed with PBS and then fixed with 1% (w/v) paraformaldehyde on a microscope slide. Coverslips were then mounted on slides and viewed with a Zeiss LSM 510 confocal laser microscope. The optical sections of a single JAWS II cell (32 sections span the entire cell) were followed to demonstrate the internalization of biontinylated and FITC-labeled-M13 virus. Images were edited using the Adobe Photoshop software program (Adobe Systems, Mountain View, CA).

2.2.8. Bispecific Antibody Targeting of Biotinylated M13 Phage to Dendritic Cells

The DC was plated on a 96-well V-bottom plate in quadruplicate (1.0 x 10^5cells/well). The plates were washed four times with PBS and blocked with 1% PBS dialyzed BSA. After incubation, the plates were washed with PBS. BsmAb premixed (for 10 min) with serially diluted B-M13 or B-M13 alone without the targeting agent were then added to the plates. The plates were incubated for 2 h at 4°C, and then washed with PBS. Streptavidin-HRPO (10 µg/ml in 100 µl volume) was added to each well and incubated for 1 h at 4°C. The plates were washed with PBS. TMB (100 µl) was added to each well and OD_{650} was taken after 10 min using a microplate reader. Statistical analyses were performed using Sigma 2000 and Minitab 13.30 statistical software to determine the

significance between binding in the presence and absence of bsmAb. Values were expressed as a mean of the quadruplicate. The analysis for each of the detection methods was done using the student t-test of significance and P value with 95% confidence interval. In all experiments, a value of $P<0.05$ indicated a significance difference.

2.2.9. Immunizations of Mice with Biotinylated OVA with or without bsmAb

5 groups of female C57BL/6 mice (5 mice per group, minimum 2 months old) were injected subcutaneously near the inguinal lymph node with 0.1 ml of various formulations of OVA and bsmAb. The composition of this 0.1 ml solution was different depending on the experimental group. The immunization protocol is shown in Table 2.2.

	Groups	Day 0	Day 12	Day 22	Day 25
1	Control	PBS	PBS	Spleen (T cell) Serum (Humoral)	IFN-γAssay
2	B-OVA	100 μg CFA	100 μg IFA	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
3	B-OVA Anti-biotin mAb Anti-CD40 mAb	200 ng 20 μg 25 μg	100 μg IFA	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
4	B-OVA BsmAb	200 ng 20 μg	100 μg IFA	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
5	B-OVA BsmAb Anti-CD40 mAb	200 ng 20 μg 25 μg	100 μg IFA	Spleen (T cell) Serum (Humoral)	IFN-γ Assay

Table 2.2. Immunization protocol for evaluating humoral and cell-mediated immune responses to B-OVA in mice (n=5 for each group). The amounts of antigens and antibodies are either in μg or ng per mouse. All mice were injected subcutaneously near inguinal lymph node. There is a 500 fold difference in B-OVA concentration between Group 2 (100 μg) and Group 3-5 (200 ng). Group 1 is the control group, immunized with PBS. Group 2 follows the standard CFA based protocol in immunization of mice. The third group of mice were immunized with B-OVA, P54 monospecific anti-biotin mAb

along with the anti-CD40 mAb since anti-biotin mAb was co-purified with bsmAb. Group 3 immune responses reflect the possible involvement of P54 mAb targeting of B-OVA to DC *via* the Fcγ receptor based uptake. Group 4 and 5 experiments compare the efficacy of the bsmAb in the presence or absence of the co-stimulator anti-CD40 mAb.

For Group 1, the control group, the five mice were immunized and boosted subcutaneously with PBS. For Group 2 mice, 100 μg of B-OVA emulsified in an equal volume of CFA was used to immunize each mouse. For Group 3, 200 ng of B-OVA, 20 μg of P54 mAb (control monospecific mAb) and 25 μg of IC10 (anti-CD40 mAb) were administered to each mouse. For Group 4, 200 ng of B-OVA and 20 μg of bsmAb were administered to each mouse. For Group 5, 200 ng of B-OVA, 20 μg of bsmAb and 25 μg of IC10 were injected into each mouse. There is a 500-fold difference in B-OVA concentration between Group 2 (100 μg) and Group 3-5 (200 ng). Two weeks following primary immunization, each mouse (excluding the control mice) was boosted with 100 μg of B-OVA emulsified in IFA. The purity of every reagent was checked by SDS-PAGE, and LPS contamination (endotoxin sensitivity at 0.125 EU/ml) was checked prior to administration using LAL PYROGENT® Plus Single Test Vials kits.

2.2.10. Evaluation of Serum Antibody Responses

Ten days following the boost, the mice were sacrificed and blood was collected by cardiac puncture. Serum was isolated using standard protocols [12]. Humoral responses in terms of antibody titres were measured individually in each mouse by ELISA method to measure anti-OVA, anti-biotin and anti-P125 bsmAb activities respectively. The ELISA method used involves overnight coating the Nunc 96-well ELISA microplates with the desired antigen: OVA, B-BSA, or P125 (10 μg/ml in 100 μl volume). The ELISA measurements were done in quadruplicate for each mouse for each serum dilution. The antigens were coated to the wells in 100 μl of carbonate buffer, pH 9.6 containing 0.02% (w/v) sodium azide (10 μg/ml) at 4°C. The plates were then washed four times with PBST and blocked with 1% PBS dialyzed BSA (with 3 changes to remove traces of free biotin) for 2 h at 37°C. After incubation, the plates were washed with PBST. Serum samples serially diluted in PBS were then added and plates were incubated for 2 h at

room temperature. Plates were washed with PBST and incubated with GAM-HRPO (1:10,000 dilution in 1% dialyzed BSA) for 1 h at room temperature. The plates were washed with PBST and 100 µl of TMB was then added to each well and OD_{650} was taken after 10 min using an ELISA V_{max} kinetic microplate reader. Statistical analyses were performed as described above to determine the significance between the anti-OVA antibody response in the different groups.

2.2.11. Evaluation of IFN-γ Response

Ten days following the final immunization (Table 2.2), spleens from the 5 different groups of immunized mice (section 2.9) were aseptically removed and pooled. Cell suspensions were prepared by mincing the spleens in DMEM medium [1% (v/v) PSG]. The cell suspensions were then passed through nylon wool columns (kindly provided by Biomira Inc., Edmonton). The columns were pre-equilibrated with 20-30 ml of warm DMEM-10 media and placed in a 37°C incubator for 1 h. After incubation, splenocytes from the control and the immunized mice were added to each column. Warm DMEM-10 medium was then added to each column which was then covered with tin foil and incubated for 45 min. After incubation, non-adherent responder cells were collected from the nylon wool column as the unbound fraction. Cells collected were counted using a haemocytometer. Stimulator cells were prepared from naïve mouse spleens. Approximately 2.5 x 10^7 stimulator cells were isolated and incubated with 50 µg/ml mitomycin C for 20 min at 37°C in a CO_2 environment. The cells were then washed with DMEM media three times, and suspended in DMEM-10 (10^6 cell/ml). The responder cells were counted using a haemocytometer. Stimulator cells (0.5 x 10^6) with or without 2.5 x 10^5 responder cells from both immunized and non-immunized mice were aliquoted to a 96-well tissue cultures plate in quadruplicate. Cells were incubated with or without 10 µg of various antigens: OVA, biotin, and P125 bsmAb for 3 days at 37°C in a 5% CO_2 atmosphere. After incubation, the cells were gently pelleted and the supernatant from each well was collected and analyzed for IFN-γ activity. IFN-γ concentration was determined using mouse IFN-γ ELISA Ready-SET-Go kit using the protocol from eBioscience.

2.3. Results

2.3.1. Development, Purification, and Characterization of bsmAb P125

A rapid method to prepare hybrid-hybridomas or quadromas is by PEG-fusion of two hybridomas labeled with different fluorescent markers and sorting only the fused cells bearing both markers [8, 25]. Approximately 89.1% of the HB290 and 80.3% of the P54 cells were labeled with TRITC and FITC, respectively. Dot plot FACS analysis was carried out after PEG fusion of the two hybridomas (Fig. 2.1).

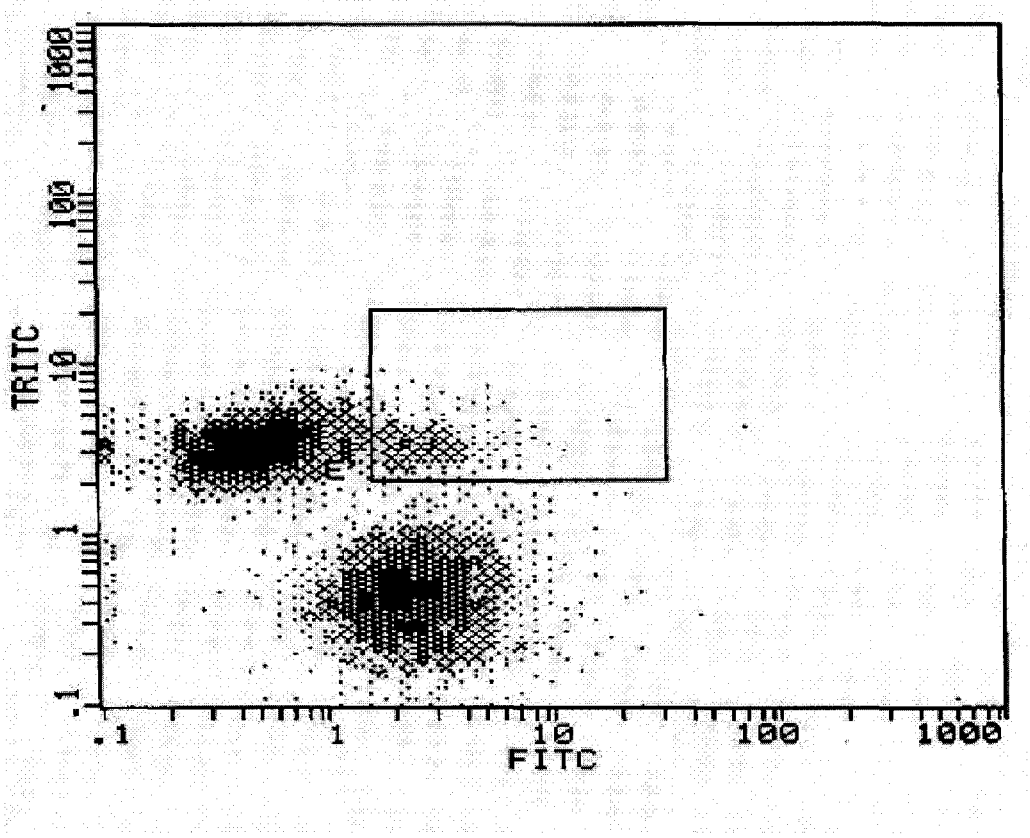

Figure 2.1. FACS analysis of the quadroma fusion: approximately 89.1% of the HB290 and 80.3% of the P54 cells were labeled with TRITC and FITC respectively. Dot plot FACS analysis was carried out after PEG fusion of HB290 and P54. The squared area contains the hybrid cells exhibiting dual fluorescence.

The squared area contains putative quadroma cells exhibiting dual fluorescence. These cells were sorted and seeded in a 96-well tissue culture plate at two cells per well in

DMEM-20. After 10 days of incubation, 110 quadroma clones were screened for bsmAb positivity using bridge ELISA. The best five clones were selected and recloned three times to select the P125 quadroma and cell banks were made. A bulk culture of the quadroma was prepared and 1 liter of supernatant was collected. Affinity purification of the quadroma supernatant was performed using a biotin-Agarose column. The unbound proteins were washed away with PBS, while the bound proteins (which includes the monospecific P54 and the bispecific P125) were eluted using glycine-HCl, neutralized with Tris-HCl, and dialyzed against PBS. The purification of bsmAb P125 was incomplete since antibodies with monospecific anti-biotin activity (detected in ELISA using GAM-HRPO) are also co-eluted with P125. We were unable to determine the proportion of P54 anti-biotin antibody in P125 purified antibody solution. ELISA was performed to demonstrate the bsmAb activity, including both the anti-DEC-205 activity to mouse bone marrow DC and the anti-biotin activity to B-BSA by employing different secondary detection reagents. The ELISA data shows that the biotin-Agarose column purified P125 bispecific binding activity (Fig. 2.2).

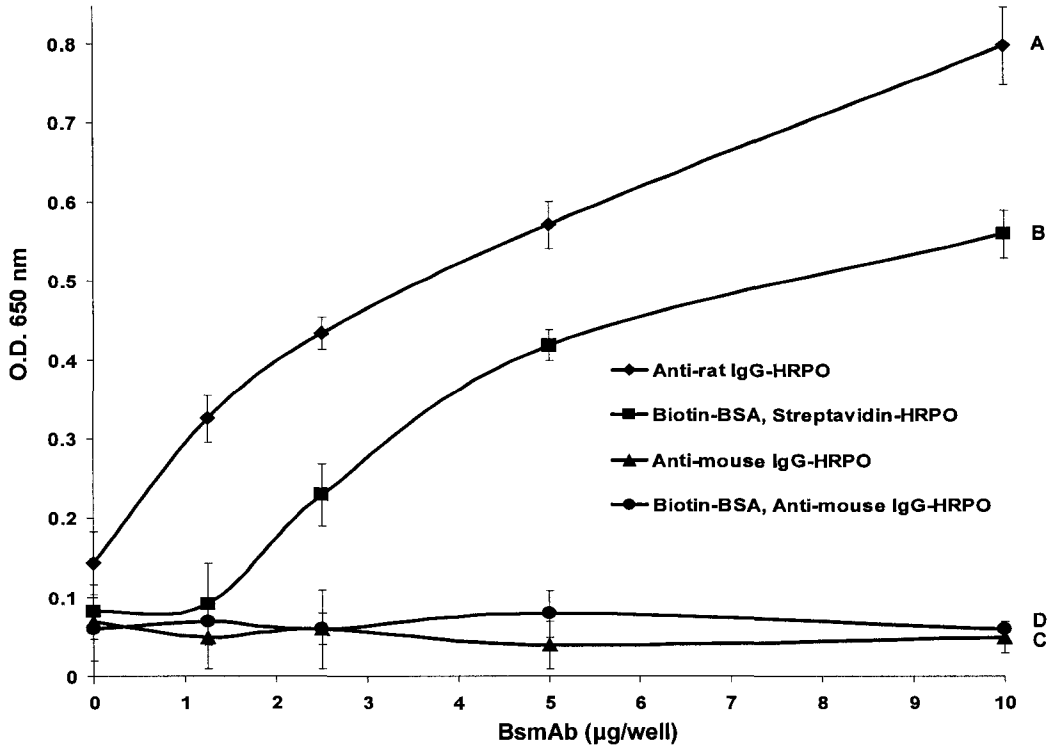

Figure 2.2. Demonstration of monospecific and bsmAb activity on DC: The activities of the biotin-Agarose column purified P125 bsmAb was tested against C57BL/6 bone marrow DC *via* ELISA method. This method involved coating of DC, adding various concentrations of P125 bsmAb, and detection by different reagents. Anti-DEC-205 activity was detected by mouse anti-rat IgG_{2a}-HRPO (Curve A). Anti-biotin activity in the free distal arm of bsmAb was detected by B-BSA & streptavidin-HRPO (Curve B). Additional ELISA was also performed using GAM-HRPO as detection antibody to detect possible anti-biotin monospecific mAb P54 binding to DC in the presence (Curve D) or absence (Curve C) of B-BSA. The error bars represent the standard deviations. (n = 4)

The monospecific P54 anti-biotin antibody from the P125 purified antibody fraction does not bind to DC *in vitro*, in the presence or absence of biotinylated antigen (Fig. 2.2).

2.3.2. BsmAb Mediated Uptake of Biotinylated Antigen by Dendritic Cells

A confocal fluorescence based demonstration of the enhanced uptake of a model antigen was done with the bsmAb. The DC used in the confocal microscopy uptake study

was JAWSII. JAWSII is an immortal immature bone marrow DC cell line. The M13 bacteriophage served as a model antigen/virus and it was labeled with both biotin and FITC. This dual labeled probe was added to the DC culture, with or without bsmAb. The culture was incubated for 1 h at 4°C to allow binding of B-M13 to DC, and then 10 min at 37°C for DC to internalize the antigen. The culture was washed with PBS and then fixed with 1% (w/v) paraformaldehyde. The slides were viewed with a confocal laser microscope (Fig. 2.3A and 2.3B).

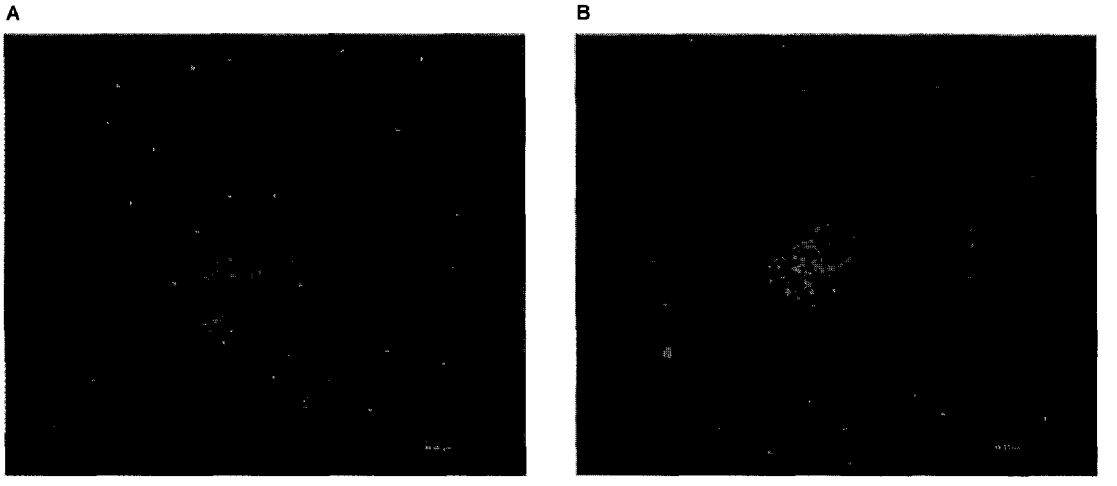

Figure 2.3. Confocal microscopy study. The transformed JAWSII cell line was used in the confocal microscopy uptake study. The biotinylated and FITC-labeled-M13 bacteriophages, with and without the presence of P125 bsmAb, was incubated for 1 h at 4°C and then 10 min at 37°C. An aliquot was washed with PBS and then fixed with 1% (w/v) paraformaldehyde on a microscope slide. A, picture of JAWSII with biotinylated and FITC labeled M13. B, picture of JAWSII with biotin/FITC labeled M13 in the presence of bsmAb. Bar, 40 μm.

Large quantities of B-M13 were bound and internalized to DC in the presence of bsmAb (Fig. 2.3B), in comparison to the control (Fig. 2.3A). Furthermore, serial optical sections analyzed by confocal microscopy confirmed the intracellular localization. The majority of fluorescence was detected to be accumulated below the DC surface indicating that biotin/FITC labelled M13 was actively internalized by DC.

BsmAb with two non-identical paratopes has the ability to bind to two different antigens. The magnitude of *in vitro* binding with and without bsmAb to target biotinylated antigens to mouse DC was also determined using the ELISA method. BsmAb targeting of serially diluted B-M13 to DC was studied. The activity was determined using streptavidin-HRPO and TMB. The optical density differences of B-M13 binding to DC, with and without bsmAb values were done in quadruplicate. The concentration of DC and bsmAb were fixed; whereas, the concentration of B-M13 was serially diluted. The analysis for each of the detection methods was determined by the student t-test. With a 95% confidence interval, a *P* value being <0.05 indicated a significant difference. The *P* value indicates that there was a significant higher binding of the B-M13 to DC in the presence of bsmAb compared to its absence. In the *in vitro* 96-well microtitre assay the background binding was higher in all the dilutions; nevertheless, increased binding in the presence of bsmAb was demonstrable (Table 2.1). The high background was not seen in the confocal study described above.

Log of B-M13 Concentration	Without bsmAb	With bsmAb	*P*-value
4	0.64±0.04	0.94±0.03	0.02
5	0.69±0.01	0.97±0.01	0
6	0.75±0.05	1.15±0.09	0.03
7	0.95±0.03	1.26±0.06	0
8	0.96±0.02	1.26±0.07	0.01

Table 2.1. Demonstration of bsmAb targeting of biotinylated M13 phage to DC: Average activities of the biotin-Agarose column purified P125 bsmAb against C57BL/6 bone marrow DC. The activities were measured by ELISA method. This method involved coating of DC, adding various concentrations of B-M13 in the presence or absence of bsmAb, and detection by streptavidin-HRPO. Statistical analyses of Student *t*-test of significance and *P* value with 95% confidence interval were performed using Sigma 2000 and Minitab 13.30 statistical software to determine the significance between binding in

the presence and absence of bsmAb. A value of $P < 0.05$ indicated a significant difference. ± represents the standard deviation. (n = 4)

2.3.3. P125 bsmAb Mediated Enhanced Immunity Towards Biotinylated Antigen *In Vivo*

In vivo studies were carried out to investigate the ability and efficacy of bsmAb targeting of antigens to DC. The proteins OVA, B-OVA, P54 mAb, anti-CD40 mAb and P125 bsmAb were found to be pure in SDS-PAGE; however, LPS contamination was found in OVA & B-OVA. Both humoral and cell-mediated responses were investigated using B-OVA. All mice were injected subcutaneously near the inguinal lymph node to enhance the immune response. The immunization protocol is described in detail in Table 2.2 and the results of humoral and cell-mediated responses are shown in Figure 2.4 and 2.5.

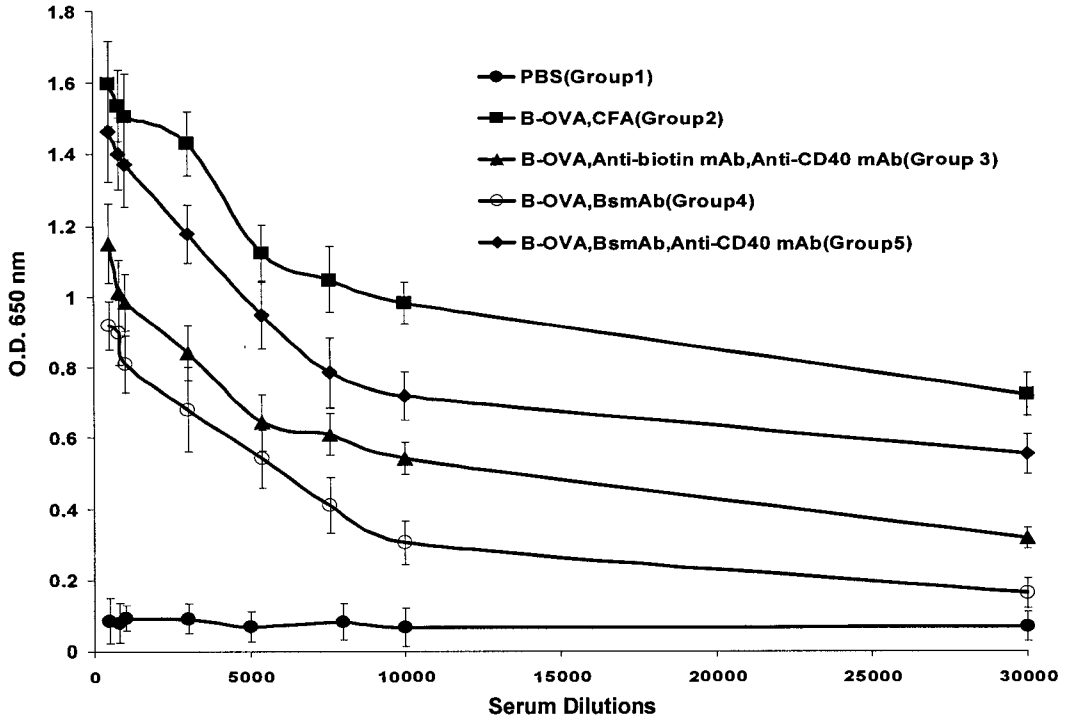

Figure 2.4. Analysis of humoral immune responses to biotin-OVA *in vivo*. Groups of five C57BL/6 mice were immunized with different antigen combinations along with a PBS control. The specific components, amounts, and the schedule of immunizations are

outlined in Table 2.1. The mice were analyzed individually and the data was pooled. The humoral response, as quantified by serum antibody titres on day 22 post immunization, was measured by ELISA method against OVA. The method involved coating of OVA in microtiter plates followed by addition of serially diluted serum antibody, and detection using GAM-HRPO. The ELISA measurements were done in quadruplicate for each mouse. The mean ELISA values at various serum dilutions obtained for each individual mouse were further averaged in each group to generate the curve. The error bars represent the standard deviation in a group of 5 mice.

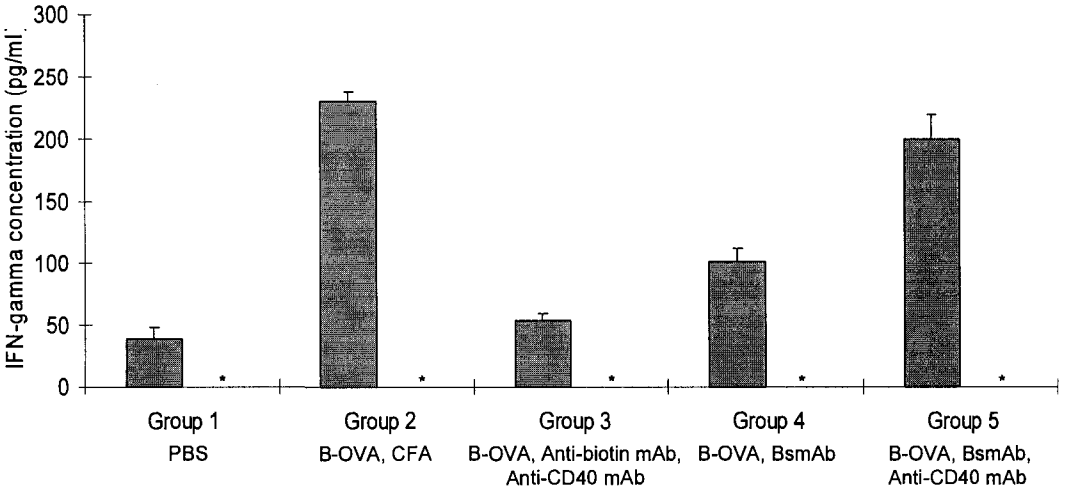

Figure 2.5. Analysis of cell-mediated immune responses based on IFN-γ estimation. Spleen T cells (responder cells) from the same five groups of mice immunized with different antigens (shown in X-axis and for details see Table 1) were isolated and purified using a nylon wool column. The five spleens in each group were pooled, mixed in DMEM prior to nylon wool purification. Stimulator cells prepared from naïve mice spleen cells were isolated and treated with 50 μg/ml mitomycin C. Purified responder cells (2.5×10^5) from both immunized and non-immunized mice were aliquoted to a tissue culture plate in quadruplicate with or without stimulator cells (0.5×10^6). The cells were then incubated with or without (*) 10 μg of OVA 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 using the protocol from eBioscience. Each data set is shown following subtraction of the corresponding ELISA values obtained without stimulator cells. The error bars are the standard deviations. (n = 4)

There is a 500 fold difference in B-OVA concentration used between Group 2 (100 μg) and Group 3-5 (200 ng). Group 1 is the control group, being only immunized with PBS. The anti-OVA and cell-mediated responses in this group are very low as expected. Group 2 follows the standard CFA based protocol in immunization of mice exhibiting strong humoral and cell-mediated responses. Mice were immunized with B-OVA in CFA and boosted with B-OVA in IFA which is a widely used protocol. The third group of mice were immunized with B-OVA, P54 monospecific anti-biotin mAb (instead of P125 bsmAb), along with the anti-CD40 mAb. Since a mixed population of antibodies were co-purified using the biotin-Agarose column the immune responses in Group 3 reflect the possible involvement of P54 mAb targeting of B-OVA to DC *via* the Fcγ receptor based uptake. Group 4 and 5 experiments compare the efficacy of the bsmAb in the presence or absence of the co-stimulator anti-CD40 mAb. The results show that Group 2 has the highest serum antibody titre against OVA while Group 1 has the lowest serum titre (Fig. 2.4). The second highest serum titre is Group 5 which approaches the classical CFA based immunization protocol (Group 2). There is no significant difference in OD between Group 2 and 5, but a significantly lower serum titre against OVA is seen in Group 3. In a comparison between Groups 3-5 serum titre activity to OVA, Group 3 serum reactivity to OVA may be due to the anti-CD40 mAb and/or LPS effect. The OD of Group 3 with Group 4 could be added together to equal Group 5 OD. The titre between Group 3 and 4 is statistically significant. A comparison of Group 3 and 5 demonstrates that the bsmAb generates a higher humoral response approaching the CFA levels with 500 fold lower amount of B-OVA (200 ng in group 5 vs 100 μg in group 2) compared to the purified P54 monospecific anti-biotin mAb. There is a significant difference in OD between Group 4 and 5. The co-stimulant anti-CD40 may be responsible for the heightening of antibody titre against OVA. BsmAb targeting of B-OVA to DC without the presence of anti-CD40 only produces a minimal humoral response against OVA (Fig. 2.4, Group 4).

The magnitude of cell-mediated immune response was determined by the amount of IFN-γ secreted from T cells in response to antigens. We did not use individual mice for cellular immune response experiments since our humoral response data is reproducible from five individual mice. The spleen cells were pooled to improve the quality of the data.

IFN-γ secretion was minimal in the absence of stimulator cells (data omitted) or OVA (Fig. 2.5) in all mice. Figure 2.5 shows the response of different groups of immunized mice to OVA, in terms of IFN-γ secretion. Group 2 and Group 5 are the highest; whereas, Group 1 and 3 are the lowest. Co-immunization of anti-CD40 mAb produces 2 fold increases in IFN-γ secretion in bsmAb targeting of B-OVA to DC (Fig. 2.5, Group 4 and 5). BsmAb targeting of B-OVA to DC with or without anti-CD40 produces a significant cell-mediated response against OVA (Fig. 2.5, Group 4 and 5). The lack of significant IFN-γ secretion in Group 3 is noteworthy compared to Group 5. In summary, the results show strong humoral and cell-mediated immunity can be achieved against B-OVA in mice immunized with B-OVA, bsmAb, and anti-CD40 mAb. Strong immune responses can be achieved approaching the CFA levels without administrating high concentrations of antigen. Immunization of bsmAb with B-OVA only produces moderate cell-mediated response, whereas mice immunized with P54 monospecific anti-biotin mAb, anti-CD40 mAb, and B-OVA produced little or no cell-mediated response. The immune responses (both humoral and cell-mediated response) towards either biotin or P125 bsmAb were minimal. Only Group 5 showed a low level of immune response towards both biotin and P125.

2.4. Discussion

The *in vivo* study of targeting antigens to DC stems from our hypothesis that efficient targeting of the antigen to the desired cell population enhances the immune response. In disease states such as cancer and infection, DC may exhibit inefficient migration to the site of infection or impaired antigen uptake/processing. Targeting strategies with co-stimulation may help overcome some aspects of migratory deficiencies and facilitate the antigen uptake processes. Impaired DC function has been recorded in patients with several different tumour and/or cancer types including head and neck, colorectal and renal cell cancer, hepatocellular carcinoma, malignant melanoma, chronic myeloid leukemia, and breast cancer [1, 11, 16, 17, 18, 30, 34, 36, 39]. However, the mechanisms of such impairment remain poorly defined for most of these cancers and appear to be heterogeneous in different cancers [11, 18, 30, 36, 39]. Targeting defective DC with antigens may overcome potential inhibitory or suppressive effects of cancer. For

instance, DC loaded with killed breast cancer cells can induce differentiation of tumor-specific cytotoxic T cells [28]. In addition, defective T-cell function in patients with advanced breast cancer can be overcome by stimulation with DC generated from precursors [17]. These studies involved culturing DC with antigens *ex vivo* as a substitute for targeting. *Ex vivo* stimulation of DC with antigens have been studied in several clinical fields. Administration of *ex vivo* antigen-loaded DC into mice stimulates protective T cell responses against pathogens and tumors [2, 33]. This approach is now applied to human tumor immunotherapy [3, 29, 35]. The disadvantage is that, isolation of DC from individual patients is very laborious and costly. As an alternate strategy, direct delivery of antigens to DC may be a desirable strategy [10]. Injection of engineered viral vectors to express antigens in activated DC may provide effective vaccines for priming an immune response [13]; however, more *in vivo* studies are required to address the efficiency and safety issues.

Priming an immune response to a single antigen may not be sufficient for an effective immune response. Different antigens during the boosting phase for independent presentation by DC can induce potent polyvalent T cell response and reverse the effect of immunodominance [32]. Cancerous cells usually express more than one tumor antigen, and some antigens may or may not be recognized by the immune system. Targeting of multiple tumor marker antigens to DC may provide strong immunity against tumors. Thus, a universal DC targeting vehicle such as the bsmAb that can bind to a mixture of biotinylated antigens may be useful to induce potent polyvalent T cell responses and overcome the potential inhibitory or suppressive effects of cancer on DC. Currently, three types of DEC-205 targeting systems have been reported and all of them are antibody-mediated antigen targeting systems. These systems are HB290 (anti-DEC-205 mAb) scFv coated liposome targeting system [40], chemical cross-linking of HB290 with antigen [5, 6], and HB290 hybrid antibody (fused with antigen) system [19]. There are some limitations for using these systems. For example, liposomal encapsulation of an antigen is a complicated and time consuming process. Chemical cross-linking with an antigen also has variation in each batch of cross-linking, and purification of the conjugate remains an issue. The construction of a new hybrid antibody is time consuming, costly and needs to be designed for each antigen as a fusion protein. Furthermore, comparison studies with

different antigens and the use of multiple antigens targeting to DC is often difficult. Hence, we have developed a bsmAb that can bind any biotinylated antigen and target them to DC DEC-205 receptors. Numerous biotinylating reagents are commercially available that can derivatize almost any antigen: protein, nucleic acid, carbohydrate or glycolipid. Examples of such reagents include NHS-LC-biotin (NH2-linkage), biotin hydrazide (carbohydrate linkage), maleimidobiotin (thiol linkage) and photobiotin (almost any C-C or C-N bonds). We have successfully generated a quadroma that secretes the bsmAb which forms a complex with a biotinylated antigen with one paratope and targets the antigen to the DC *via* the DEC-205 receptor with its second paratope. A one step affinity chromatography procedure has also been used to purify the bsmAb; however, purification of bsmAb free of the monospecific mAb is not complete and alternative designs have been developed described in Chapter 4. Attempts using a pH gradient and a biotin gradient elution of bsmAb bound on biotin-Agarose column were not successful. Further efforts to resolve the bsmAb from potential contaminants were partially successful [23]. Although the P54 monospecific mAb is present in our preparation, it does not bind to DC *in vitro*, with or without biotinylated antigen even though it has an intact Fc domain. It also does not exert any *in vivo* immune response. In the presence of bsmAb *in vitro*, enhanced binding and uptake of B-M13 virus to DC were observed both in ELISA and confocal microscopy. Strong humoral and cell-mediated responses (IFN-γ) against OVA were achieved in mice injected with biotinylated OVA, bsmAb and anti-CD40 mAb. Moreover, a very low concentration of antigen (500 fold difference) was adequate to achieve a similar immune response obtained in mice following a CFA based immunization protocol. The immune responses (both humoral and cell-mediated response) towards either the hapten biotin or the carrier bsmAb were minimal. In conclusion, this bsmAb may be used as a universal vector to deliver any biotinylated antigen to DC. In addition, it could be used to generate potent polyvalent T cell responses employing polyvalent antigens.

2.5. References

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CHAPTER 3

Biotin Carboxyl Carrier Protein Co-Purifies as a Contaminant in Core-Streptavidin Preparations

The contents of this chapter was published in Molecular Biotechnology 2005;31(1):29-40.

3.1. Introduction

Streptavidin, a tertrameric protein (60 kDa) naturally secreted by actinobacterium *Streptomyces avidinii*, has exceptionally high affinity (Kd of 10^{-13}-10^{-16} M) to biotin *via* non-covalent interaction [1]. It has been established that, one molecule of streptavidin binds to four molecules of biotin. Streptavidin has been used in a variety of applications: immunoassays, hybridization assays, lymphocyte activation, affinity chromatorgraphy, localization of antigen, and purification of nucleic acids [1-4]. Streptavidin produced by *Streptomyces avidinii* has many disadvantages including susceptibility to proteolysis near the N-terminus region of the molecule [2]. Core-streptavidin (CS) derived by proteolytic digestion of streptavidin exhibits higher solubility, reduced tendency towards oligomerization and resistance towards subsequent proteolytic degradation [3, 4].

In *E. coli* system, CS has been expressed either as fusion protein or alone, both as an insoluble or soluble protein [5-11]. The CS and its fusion proteins have been exploited extensively for a variety of *in vitro* and *in vivo* applications [10, 16]. Conditions for optimal production of soluble CS and refolding of insoluble CS in *Escherichia coli* have been reported [11-13]. Domain modification of CS for reduced *in vivo* immunogenicity and kidney retention has also been studied [14, 15]. The fusion proteins of CS have been utilized for applications in cancer immunotherapy and pretargeted lymphoma therapy [10, 16]. However, a potential contaminant while expressing and purifying CS in *Escherichia coli* has thus far merited limited attention. BCCP is the only naturally biotinylated proteins in *Escherichia coil,* and it is an essential component of acetyl-CoA carboxylase to catalyze the first committed and rate-limiting step of fatty acid synthesis [17, 18].

In this chapter, we report the successful cloning, expression and purification of CS in *E. coli* using T7 expression system as a prelude to the construction of the bifunction targeting molecule. The IMAC (immobilized metal affinity chromatography) purified CS

had a major contaminant which was identified as BCCP. We also demonstrated that in one step using CS immobilized magnetic beads, BCCP can be removed from the IMAC purified fraction to recover pure CS.

3.2. Materials and Methods

3.2.1. Materials

IPTG (isopropyl β-$_D$-thiogalactoside), BSA (bovine serum albumin), streptavidin-HRPO, streptavidin (*E.coli* produced), anti-His_6 mAb, NHS-LC-Biotin and goat-anti-mouse-HRPO were from Sigma (Oakville, Canada). Hybond ECL nitrocellulose membrane and ECL Western blotting kit were from Amersham Pharmacia Biotech (BaiedUrfe, Canada). Goat-anti-streptavidin antibody conjugated with HRPO was from Zymed Laboratories (South San Francisco, USA). Ni-NTA agarose, T7 promoter and terminator primers, *E. coli* strain BL21 (DE3) and expression vector pET-22b (+) were from Novagen (Madison, USA). Plasmid pUC8-SZ was kindly provided by Dr. T. Sano, Center for Molecular Imaging, Diagnosis and Therapy and Basic Science Laboratory, Boston, USA [5]. Molecular cloning materials were from Invitrogen (Burlington, Canada). Dynabeads^R MyOne^{TM} Streptavidin and Dynal MPC^R were from Dynal Biotech Inc (Brown Deer, USA). Biotin-HRPO was from Pierce (Canada). Protein Assay reagent was purchased from Bio-Rad (Mississauga, Canada).

3.2.2. Cloning of Core-Streptavidin

The CS coding region was amplified by polymerase chain reaction from pUC8-SZ [19] using primers 5'-ACT ATC GCC ATG GAT GAG GCC GGC ATC ACC GGCA 3' and 5'-ATT ACT TGA ATT CGT GGA GGC GGC GGA CGG CTTC 3'. These primers also inserted the restriction sites *Nco*I and *EcoR*I. The PCR fragment was gel-purified and double digested with *Nco*I and *EcoR*I, and ligated to pET-22b (+) generating pET-22b (+)-CS. The positive clone was screened and characterized by both PCR and restriction digestion (*Nco*I and *EcoR*I). The positive cloned fragment was sequenced using T7 promoter and terminator primers by CEQ^{TM}2000 (Beckman Coulter USA).

3.2.3. Expression and IMAC Purification of Core-Streptavidin

The pET-22b (+)-CS plasmid was chemically transformed into BL21 (DE3), and colonies were grown overnight and selected from 2x YT plate (16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl, pH 7.5) containing 100 μg/ml ampicillin. An ampicillin resistant clone was picked and grown at 37°C in 2x YT medium containing 100 μg/ml ampicillin. Once the OD_{600nm} reached ~0.6, 100 μM IPTG and 0.4 M Sucrose [20] were added, and incubated at 26°C for 5 h. Bacteria were harvested by centrifugation and resuspended in 5% of the original culture volume in periplasmic extraction buffer [200 mM Tris-HCl, 20% sucrose (w/v) and 1 mM EDTA, pH 8.0] and left on ice for 45 min with occasional shaking. The cell extract was centrifuged at 12000x g for 30 min at 4°C and the supernatant (soluble periplasmic extract) was separated and dialyzed against 50 mM Tris-HCl, 1 M NaCl, pH 7.0 at 4°C. The periplasmic extract containing recombinant streptavidin was then loaded on an IMAC column. The IMAC column was prepared by loading 2 ml of Ni-NTA agarose per liter of original bacterial culture and equilibrated with 3 bed volumes of 50 mM Tris-HCl, 1 M NaCl, pH 7.0. Following loading of the periplasmic extract, the IMAC column was washed with 10 bed volumes of 50 mM Tris-HCl, 1 M NaCl, pH 7.0 and followed by 20 bed volumes of washing buffer (50 mM Tris-HCl, 1 M NaCl, 20 mM immidazole, pH 7.0). Finally the column was washed with 20 bed volumes of 50 mM Tris-HCl, 1 M NaCl, 40 mM immidazole, pH 7.0. The bound proteins were eluted with elution buffer (50 mM Tris-HCl, 1 M NaCl, 300 mM immidazole, pH 7.0) and dialyzed against PBS pH 7.3. The various fractions such as the induced and uninduced culture cytoplasm, periplasm, IMAC washings of CS, and the eluted material were analyzed by SDS-PAGE using 15% polyacrylamide gels under reducing conditions (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5mM 2-mercaptoethanol) and following staining with Coomassie Brilliant Blue. The samples were incubated at 95°C for 10 min prior to loading on the electrophoretic gel. Western blot was performed on the IMAC purified CS under either reducing or non-reducing conditions (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and probed with the following reagents: a) streptavidin-HRPO, b) $(biotin)_n$ labeled BSA followed by streptavidin-HRPO or c) mouse anti-His_6 mAb with goat anti-mouse-HRPO. $(Biotin)_n$ labeled BSA was prepared by biotinylation of BSA with NHS-

LC-Biotin as per vendor's biotinylation protocol. Proteins were electrophoresed on SDS-PAGE using 15% polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane using the Trans blot apparatus (Bio-Rad) as per manufacturer's instructions. The membrane was blocked with 5% skim milk in PBST (0.1% Tween 20 in phosphate buffer saline, pH 7.3) for 2 h at RT. The membrane was washed three times with PBST and incubated with either streptavidin-HRPO, $(biotin)_n$ labeled BSA or mouse anti-His_6 mAb. After washing three times with PBST, the membrane incubated with $(biotin)_n$ labeled BSA was further incubated with streptavidin-HRPO. The membrane reacted with anti-His_6 mAb was subsequently incubated with goat anti-mouse-HRPO for 1 h at RT. Finally, the membrane was washed four times with PBST and ECL-based detection was performed according to the manufacturer's instructions.

3.2.4. Characterization of Core-Streptavidin and Oligotetramers

IMAC purified CS was incubated either at 60, 70, 80, or 95°C for 10 min under reducing conditions prior to analysis in 15% SDS-PAGE. Gel resolved proteins were electrophoretically transferred onto nitrocellulose membrane and probed with either streptavidin-HRPO or mouse anti-His_6 mAb with goat anti-mouse-HRPO.

3.2.5. BCCP Identification

BCCP identification was done at the Institute for Biomolecular Design, University of Alberta, Edmonton, Alberta, Canada. The BCCP spots (confirmed by Western Blot probed against streptavidin-HRPO) were excised and an automated in-gel trypic digestion was performed on a Mass Prep Station (Micromass, UK). The excised spots were destained, reduced with DTT, alkylated with iodoacetamide and digested with trypsin (Promega Sequencing Grade). The resulting peptides were extracted from the gel and analyzed via LC/MS on a CapLC HPLC (Waters, USA) coupled with a Q-ToF-2 mass spectrometer (Micromass, UK). The tryptic peptides were separated using a linear water/acetonitrile gradient (0.2% formic acid) on a Picofrit reversed-phase capillary column (5 μm BioBasic C18, 300 Å pore size, 75 μm ID x 10 cm, and 15 um tip) (New Objectives, MA, USA) with an in-line PepMap column (C18, 300 μm ID x 5 mm) (LC Packings, CA, USA) used as loading/desalting column.

3.2.6. Removal of BCCP from IMAC-Purified Core-Streptavidin

10 μg (0.1 mg/ml) of IMAC purified CS in PBS was incubated with 1 mg of MyOne™ Streptavidin (CS immobilized magnetic beads). Approximately 1 mg of the beads can bind 500 – 3,000 pmoles of biotin or biotinylated molecules, in 100 μl volume at room temperature in 1 h with gentle shaking. After incubation, the beads were placed on the magnet (Dynal MPC®) for 5 min, and the supernatant containing purified CS was collected. The streptavidin bead bound materials were eluted with 5 ml of 6 M guanidine HCl, pH 2.0 and dialyzed against PBS. The elutants were concentrated by precipitating with 50% saturated ammonium sulfate and dialyzed against PBS. IMAC purified CS, IMAC plus streptavidin bead purified CS, and the streptavidin bead elutants were analyzed by SDS-PAGE and Western blot. The samples were heated either at 55°C or at 95°C for 10 min under reducing conditions before loading on 10% polyacrylamide gels. Gels were either stained with Coomassie Brilliant Blue or electrophoretically transferred onto nitrocellulose membranes. The membrane containing samples heated at 55°C, were probed against different detecting reagents: goat anti-streptavidin HRPO, mouse anti-His_6 mAb with goat anti-mouse-HRPO, or $(biotin)_n$ labeled BSA & streptavidin-HRPO. The samples heated at 95°C were subjected to Western blot analysis with streptavidin-HRPO.

3.2.7. Biotin-Binding Activity

The biotin binding activities of commercial streptavidin (Sigma, produced from *E.coli*), IMAC purified CS and IMAC & streptavidin bead purified CS were compared. Streptavidin and CS were plated on a 96-well plate in quadruplicate in various concentrations overnight at 4°C. The plates were washed with PBS and blocked with 1% PBS dialyzed BSA. After incubation, the plates were washed with PBS. Biotin-HRPO was added and plates were incubated at 37°C for 1 h. The plates were then washed with PBS and TMB substrate was then added and OD_{650} was measured after 10 min using a microplate reader. Statistical analyses were performed using Sigma 2000 and Minitab 13.30 statistical software to determine the significance of biotin binding activities. Values were expressed as mean of quadruplicate. The analysis for each of the detection methods was determined by the student t-test of significance and *P* value with 95% confidence

interval. The theoretical limit of detection was calculated form the mean of 20 blanks + 2SD. In all calculations, $P<0.05$ indicated a significant difference.

3.3. Results

3.3.1. Construction, Expression, and IMAC Purification of Core-Streptavidin

The plasmid vector pET22b (+)-CS (Fig. 3.1) was constructed by inserting the sequence encoding CS [19] into pET22b (+) next to pelB leader sequence.

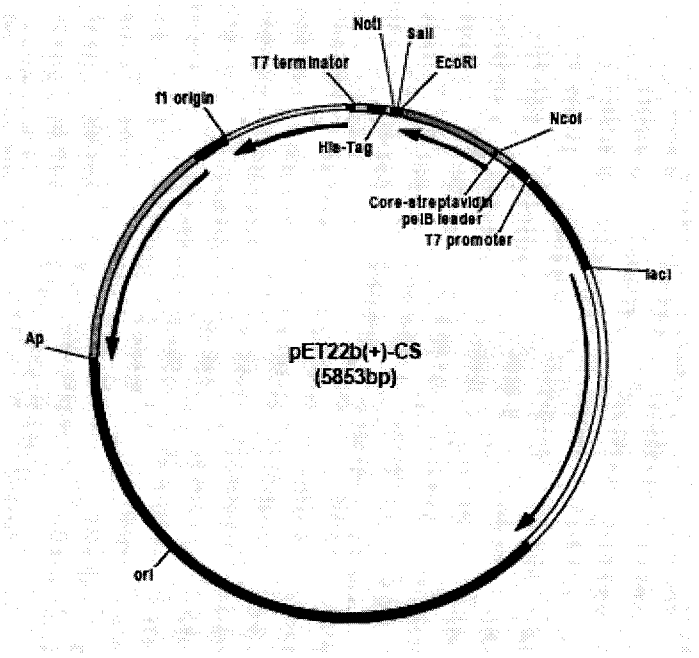


Figure 3.1. The expression vector pET22b (+)-CS. Restriction map showing the cloning sites for core-streptavidin.

E .coli transformed with this plasmid expressed recombinant protein into the periplasmic space, and the protein was conveniently purified by IMAC column. In the periplasm of uninduced non-transfected *E.coli* culture, BCCP could not be demonstrated by SDS-PAGE and Western blot. However in CS plasmid transfected *E.coli* induced cultures, the periplasm had both the CS as well as BCCP. The SDS-PAGE analysis of the various fractions is shown in Fig. 3.2A.

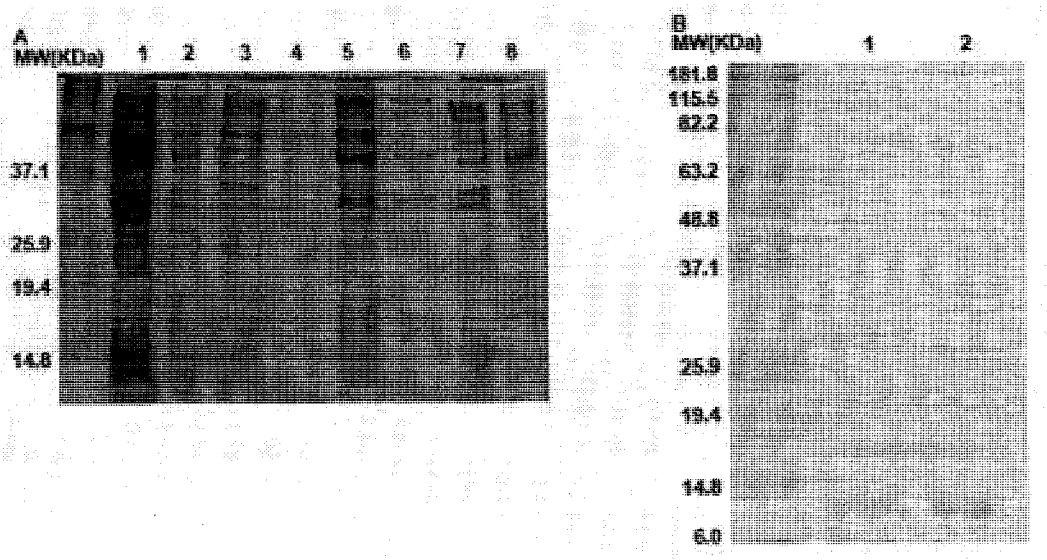


Figure 3.2. Expression and purification of core-streptavidin. (A) SDS-PAGE analysis of expression and purification. Lane 1, induced culture's cytoplasmic extract; lane 2, induced culture's periplasmic extract; lane 3, IMAC unbound fraction ; lane 4, IMAC column first wash; lane 5, IMAC second wash with 20 mM immidazole; lane 6, IMAC third wash with 40 mM immidazole; lane 7, uninduced culture's cytoplasmic extract; lane 8, uninduced culture's periplasmic extract. (B) SDS-PAGE analysis of IMAC purified core-streptavidin. Lane 1, is core-streptavidin heated at 95°C under reducing condition, while lane 2 was heated at 95°C under non-reducing condition.

The cytoplasmic (Lane 1) & periplasmic (Lane 2) extracts of induced cultures show a band at 14.5 kDa (Fig. 3.2A). This is not apparent in the uninduced cultures (Lane 7 & 8). The induced periplasmic protein of interest appeared in the appropriate position after 95°C of heating at ~13.5 kDa [5]. The periplasmic fraction containing CS was then purified by IMAC column. IMAC purified CS appears to be a pure, single band at the appropriate position after 95°C of heating, on SDS-PAGE and Western blot when detected by anti-His_6 mAb, under both reducing (Lane 1) and non-reducing (Lane 2) conditions (Fig. 3.2B and 3.3A).

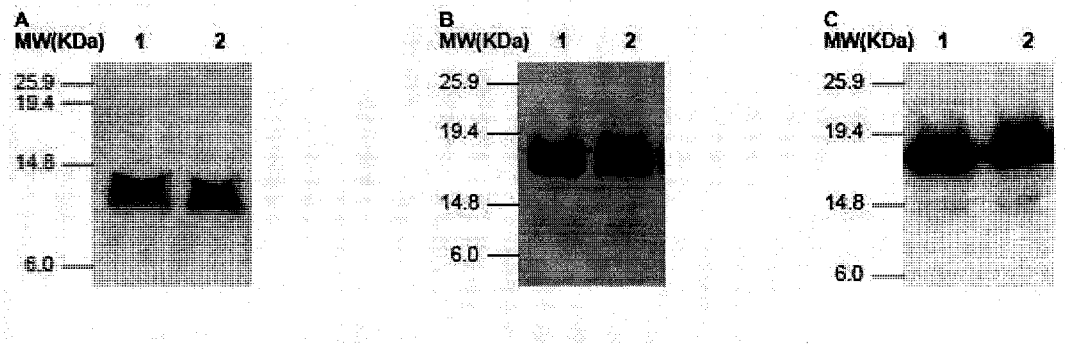


Figure 3.3. Characterization of IMAC purified core-streptavidin by Western blot. (A) Probed with mouse anti-His_6 monoclonal antibody and goat anti-mouse-HRPO. (B) Probed with $(biotin)_n$ labeled BSA, washed and subsequently with streptavidin-HRPO. (C) Probed with streptavidin-HRPO. Lane 1, IMAC purified core-streptavidin was heated at 95°C for 10 min under reducing condition; lane 2, sample was heated at 95°C under non-reducing condition.

Moreover, it appears to bind to biotin under both of the conditions, since addition of $(biotin)_n$-labeled BSA with streptavidin-HRPO to form a sandwich complex on the Western blot demonstrates the 13.5 kDa band (Fig. 3.3B). Interestingly, an extra band ~16 kDa was also detected and subsequently identified to be BCCP (Fig. 3.3B). BCCP (~16 kDa) was found to be co-purified with CS, detected in Western blot when probed with streptavidin-HRPO alone in the absence of $(biotin)_n$ labeled BSA (Fig. 3.3C). BCCP band shown in Western blot is a massive band (Fig. 3.3B and 3.3C) which however could not be detected in SDS-PAGE stained with Coomassie Brilliant Blue (Fig. 3.2B) and is consistent with other previous findings [26]. The anti-His_6 mAb however does not bind to the BCCP as shown in Fig. 3.3A.

3.3.2. Characterization of Core-Streptavidin

At 60°C, CS and native streptavidin do not dissociate into monomers [19, 21, 32]. The CS, CS fusion protein, and native streptavidin starts to dissociate into monomeric form at 70°C, and dissociates completely into monomeric form at 95-100°C [7-10, 21, 32]. Moreover, streptavidin, CS, and CS fusion protein have the tendency to aggregate into higher-order oligomeric forms [7-10, 21, 32]. The IMAC purified CS was incubated at

various temperatures, and when analyzed by Western blot with anti-His_6 mAb, exhibits the tetramer and oligotetramer (Fig. 3.4A, Lane 4) and correspond to the observations of several previous reports [7-10, 19, 21, 32].

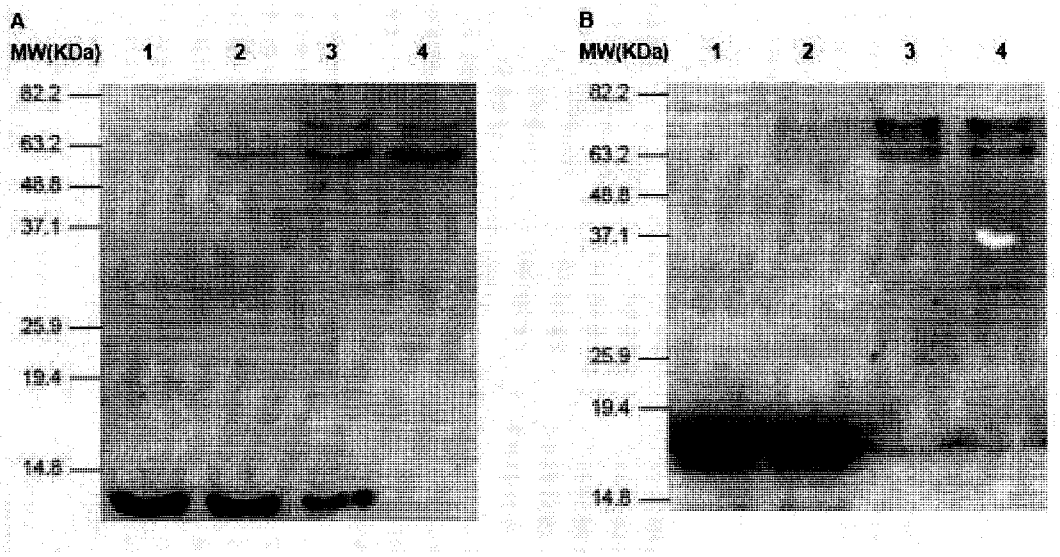


Figure 3.4. Thermal stability analysis of core-streptavidin. IMAC purified core-streptavidin was incubated either at 60, 70, 80, or 95°C for 10 min under reducing condition prior to loading in a 15% polyacrylamide gel. The gels were electrophoretically transferred onto nitrocellulose paper, and probed with streptavidin-HRPO (B) or mouse anti-His_6 mAb & goat anti-mouse HRPO (A). Lane 1, sample incubated at 95°C; lane 2, at 80°C; lane 3 at 70°C; and lane 4 at 60°C.

The higher ordered form of CS is not a streptavidin oligomer. We believed it is a by-product of one BCCP bound to one tetramer core-streptavidin. The higher ordered form of CS (~70 kDa) was undetectable when heated above 80°C in Western blot with anti-His_6 mAb (Fig. 3.4A, Lane 1 and 2). The BCCP was detectable only above 80°C of heating in Western blot probed with streptavidin-HRPO (Fig. 3.4B, Lane 1 and 2).

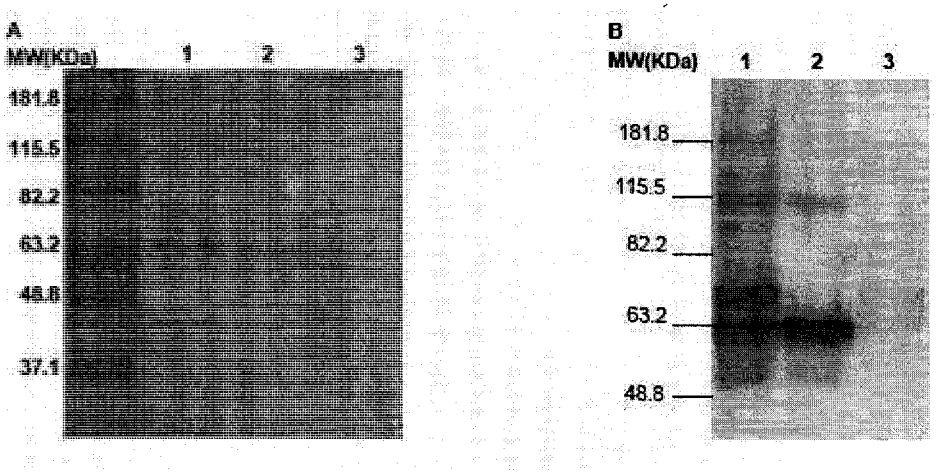
3.3.3. LC/MS Identification of BCCP

Protein identification was attempted by LC/MS analysis of the putative BCCP band and by searching the NCBI (National Center for Biotechnology Information) non-redundant database [Database: NCBInr (2329303 sequences; 789829196 residues)] using

Mascot Daemon search methodology (Matrix Science, UK, http://www.matrixscience.com). Search was restricted to *E.coli* (27165 sequences) Taxonomy. The parameters included carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide. Mascot search results showed hits for BCCP, the top hit was gil3892005 [structure of the Apo-Biotin Carboxyl Carrier Protein (Apo-Bccp87) of *Escherichia coli*], confirming that the protein is indeed BCCP.

3.3.4. Separation of Core-Streptavidin From BCCP

Dynabeads^{R} MyOne^{TM} Streptavidin is generally used for purification of biotinylated nucleic acids as well as small and large biotinylated molecules. We have exploited streptavidin-coupled Dynabeads to demonstrate that BCCP can be resolved from IMAC purified fractions as well as competitively displace the BCCP from binding to soluble CS. The purification was successful since no BCCP was detectable after streptavidin magnetic bead purification in Western blot when probed with streptavidin-HRPO (Fig. 3.5E, Lane 2).



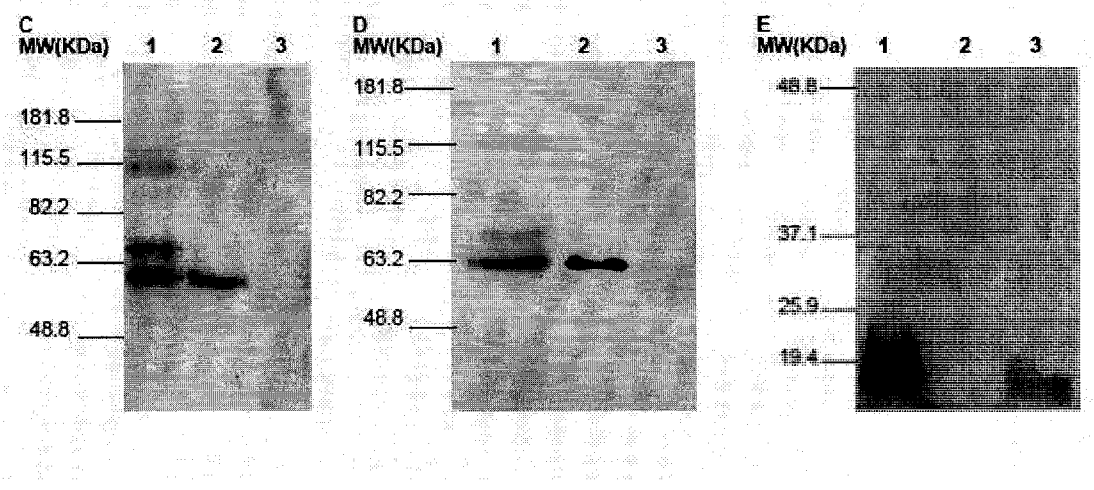


Figure 3.5. Purification of IMAC purified core-streptavidin from BCCP using streptavidin immobilized magnetic beads. Lanes: lane 1, 1 μg of IMAC purified core-streptavidin; lane 2, 0.5 μg of IMAC and streptavidin bead purified core-streptavidin; and lane 3, 10 μl of 1 ml streptavidin bead elutant. (A) SDS-PAGE of the samples heated at 55°C for 10 min under reducing condition. The samples heated at 55°C were probed with goat anti-streptavidin-HRPO (B), mouse anti-His_6 mAb with goat anti-mouse-HRPO (C), or $(biotin)_n$ labeled BSA with streptavidin-HRPO (D). (E) Western blot of samples heated at 95°C for 10 min in reducing dye probed with streptavidin-HRPO.

As further confirmation of this separation, only BCCP was recovered in the elutant of CS bead (Fig. 3.5A-E, Lane 3). Core-streaptavidin beads could be reutilized for an additional cycles of purification. Moreover, the enriched fractions of CS oligotetramers (Fig. 3.5A-D, Lane 1) were also removed (Fig. 3.5A-D, Lane 2), which we believe are the by-products of various mole ratios of BCCP binding to one tetramer CS. One molecule of streptavidin binds to four molecules of biotin. Four additional bands above CS are apparent in IMAC purified CS, and these bands correspond to the size of the possible mole ratios (1 to 4) of BCCPs bound to one CS (69.7 kDa, 86.4 kDa, 103.1 kDa, 119.8 kDa) (Fig. 3.5A-D, Lane 1). Furthermore, this method of purification could increase the recovery of CS; 0.5 μg of bead purified CS (Fig. 3.5A-D, Lane 2) produces similar band intensity as 1 μg of IMAC purified CS (Fig. 3.5A-D, Lane 1) and retains biotin binding activity of CS (Fig. 3.5D, Lane 2) without affecting to its tetrameric structure (Fig. 3.5A-D, Lane 2). The band above 181 kDa (top band in Fig. 3.5B, Lane 1) we believe is the

aggregate of BCCP bound streptavidin; nevertheless, this aggregate was also removed by streptavidin-coupled Dynabeads (Fig. 3.5B, Lane 2). Although complete removal was not achieved (Fig. 3.5B, Lane 2); nevertheless, the BCCP released at 95°C from heating of IMAC purified CS were completely removed (Fig. 3.5E, Lane 2).

3.3.5. Biotin-Binding Activity of Core-Streptavidin

Streptavidin and CS have the ability to bind to biotin. The relative magnitude of biotin binding activity of the various preparations was determined using the ELISA method. Commercial streptavidin, IMAC purified CS and IMAC & streptavidin Dynabead purified CS was serially diluted to test for activity. The activity was determined using Biotin-HRPO and TMB substrate and the data in Figure 3.6 shows the relative biotin binding activity.

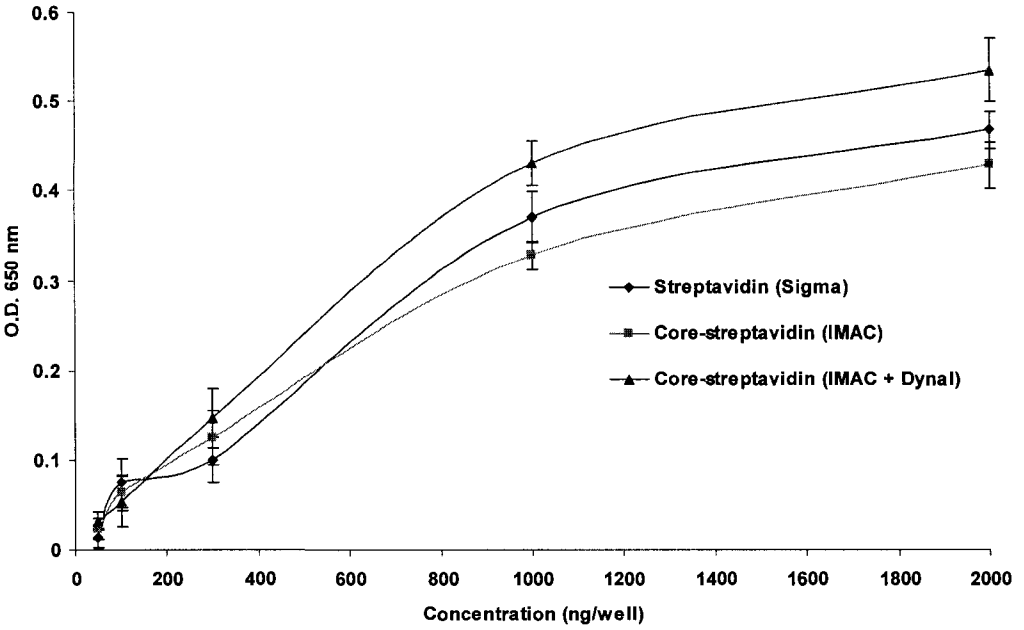


Figure 3.6. Demonstration of biotin binding activity: The activities of streptavidin (Sigma, produced from *E.coli*), IMAC purified core-streptavidin, and IMAC & streptavidin bead purified core-streptavidin were tested against biotin-HRPO. Streptavidin and core-streptavidin were plated on a 96-well plate in quadruplicate in various concentrations overnight at 4°C. The plates were washed with PBS and blocked with 1% PBS dialyzed

BSA. After incubation, the plates were washed with PBS, biotin-HRPO was added and plates were incubated at 37°C. The plates were then washed with PBS and TMB was then added and OD_{650} was taken after 10 min using microplate reader. Values were expressed as mean±standard deviation of quadruplicates.

The statistical analysis for each of the detection methods was determined by the student t-test of significance and *P* value with 95% confidence interval ($P<0.05$) indicated a significant difference. The concentration of Biotin-HRPO was fixed; whereas, the concentration of proteins (streptavidin, CS) are serially diluted. At 2 μg/well, the *P* value between commercial *E.coli* produced streptavidin and IMAC purified CS is 0.03. The *P* value between commercial streptavidin and IMAC & streptavidin-coupled beads purified CS is 0.02. The *P* value indicates that there is a significant difference in biotin binding activity between the commercial preparations compared to IMAC purified CS and IMAC & streptavidin-coupled Dynabead purified CS.

3.4. Discussion

In *Escherichia coli*, BCCP is the only protein that becomes biotinylated. BCCP is a homodimeric protein of 16.7 kDa subunits in which the C-terminal half of each comprises the biotin carrier domain [22, 23, 24]. Moreover, the full-length BCCP is a recalcitrant protein due to its tendency to aggregate [18, 25]. Due to its instability, Coomassie Brilliant Blue staining of BCCP is weak compared to typical proteins and the concentration is hard to determine [26]. Streptavidin, on the other hand, is made up of four identical subunits, known to interact with biotin in an almost irreversible binding manner due to its high affinity to the vitamin. Dissociation of biotin-streptavidin interaction requires the use of harsh or denaturing condition such as the use of 6 M guanidine HCl, pH 1.5-2 or by boiling in 2% SDS with 0.4 M urea [27, 28]. Hence, the introduction and use of 2-iminobiotin allows streptavidin binding (pH >9.5) and dissociation (pH 4) at milder conditions [29, 30]. Until now, streptavidin, CS, and CS fusion proteins have been purified using either 2-iminobiotin, HPLC (high performance liquid chromatography), FPLC (fast protein liquid chromatography), or tag specific separation strategies. Procedures involving HPLC and FPLC are relatively complex and

time consuming; whereas, purification of streptavidin using 2-iminobiotin requires pH changes which may affect the streptavidin biotin-binding activity. The biotin binding activity is stable at neutral pH and incubation of CS fusion protein at acidic pH or basic pH causes irreversible decrease in the biotin-binding activity [7].

The notion of BCCP or a biotin contaminant in CS product expressed in *E. coil* was recognized by Sano and Cantor [5], based on the 20% increase of streptavidin biotin-binding activity when *Escherichia coli* produced streptavidin is dialyzed against 6 M guanidine HCl (pH 1.5). They suspected that the expressed streptavidin had bound endogenous biotin in the cell or during purification [5]. Guan and Wurtele were the first group to report expression of soluble chimeric streptavidin protein bound biotin *in vivo* [33]. In our report, we have expressed the CS in *E. coli* as a soluble protein, and during IMAC purification we have identified that BCCP was the contaminant that co-purifies and binds to CS. CS was expressed and collected from the periplasm of *E. coli*. BCCP was identified in Western blot with streptavidin-HRPO as the probe and when the IMAC purified CS was heated above 80°C. BCCP is naturally bound upon production of CS. BCCP may bind to CS *in vivo* [33]. BCCP can also be detected in periplasm of induced cultures, but not in the uninduced culture. We have demonstrated that BCCP was released from CS in a temperature dependent manner when heated at 60, 70, 80 and 95°C (Fig. 3.4A & B). The thermal stability of streptavidin upon biotin binding is reported to be approximately 112°C [31]. However, in our study the thermal stability of CS upon BCCP binding is approximately 80°C, probably due to the instability of BCCP. The identity of BCCP was confirmed by *in vitro* gel digestion, LC/MS, and matching the fragments in the NCBI non-redundant database Mascot search. Nevertheless, BCCP can be removed from soluble CS *via* competitive binding to CS immobilized magnetic bead under mild conditions. The amount of BCCP removal is dependent upon the concentration of CS-coupled magnetic bead and the beads could be reutilized after elution of BCCP. The excess amount of CS-coupled magnetic bead binds to the BCCP dissociated from soluble CS. It has been reported that streptavidin, CS, and CS fusion proteins have the tendency to aggregate into higher-order oligomeric forms [7-10, 21, 32]. In our study, we found that the higher-order oligomeric forms of CS were likely the by-products of various mole ratios of BCCP binding to CS. The size of tetrameric CS is approximately 53 kDa;

whereas, BCCP is 16.7 kDa. In Western blot and SDS-PAGE, the multiple bands above CS (approximately 53 kDa) do correspond to the size of the number of possible BCCPs bound (one to four) to one tetrameric CS. One BCCP bound to one CS has the size of 69.7 kDa, two BCCPs bound has 86.4 kDa, three BCCPs bound has 103.1 kDa, and four BCCPs bound has 119.8 kDa. In addition, an aggregate form of BCCP-CS complex was also detected. Nearly all BCCPs bound to CS were removed and isolated by our method which included heating at 95°C. As a result, more functional CS was recovered using our method. In order to achieve absolute removal of BCCP bound complex, higher concentration of streptavidin immobilized magnetic bead may be required. Nevertheless, higher biotin binding activity can be achieved using our purification method compared to commercially available streptavidin produced from *E.coli*. In conclusion, BCCP is naturally bound upon production of CS and it can bind to the four biotin binding pockets of CS. BCCP can however be removed and purified from CS using streptavidin immobilized magnetic beads and as a consequence the CS biotin binding activity was enhanced. This method can be used for small-scale purification and recovery of pure CS, CS fusion protein, and BCCP expressed in *Escherichia coli* expression system.

3.5. References

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CHAPTER 4

Bifunctional Fusion Protein Targeting of Antigens to Dendritic Cells

The contents of this chapter have been submitted as a manuscript for publication.

4.1. Introduction

Dendritic cells (DC) are the most specialized and potent antigen presenting cells in the immune system. DC play a critical role in innate and adaptive immune responses, especially in priming and activating T cell and B cell immunity. Several clinical trials on human tumor immunotherapy based on *ex vivo* stimulation of DC have been initiated. However, the clinical responses have only been observed in a minority of patients and the procedure is very costly and laborious. Despite the recent disappointment of *ex vivo* clinical trials, *in vivo* targeting of DC surface receptors may present an alternative route for targeted immunotherapy [1]. DC utilizes surface receptors (Gb3/CD77, CD40, β2 integrins, Fc receptors, and C-type lectin receptors) to internalize, process and present antigens to MHC class I and MHC class II pathways [2]. DEC-205 is a C-type lectin receptor that is present in both immature and mature DC in lymphoid tissues, lymph nodes and spleen [3-7]. In the presence of co-stimulatory molecules, DEC-205 targeting of antigens by an antibody is an efficient strategy to protect the host against tumor growth [8], rejection of existing tumor [9], protection against airway challenge of virus [10], and enhance resistance to an established rapidly growing tumor, as well as viral infection [11].

DEC-205 targeting of a single protein enables cross-presentation of several peptides more efficiently than CD206 and CD209 [12] and induces stronger T cell immunity at much lower doses of protein antigen, plasmid DNA or recombinant adenovirus [10]. In the absence of co-stimulation, DEC-205 targeting of antigen can be used to suppress the development of autoimmunity, such as type 1 diabetes [13]. Generation of antibody-mediated antigen targeting system is often complex, costly and a time consuming process. In addition it requires either liposomal encapsulation of antigen [8], chemical cross-linking with the antigen [9, 11, 13, 14] or construction of a new hybrid antibody [10, 12, 15]. These targeting systems may not be desirable for comparison studies with different antigens and *in vivo* targeting of multiple antigens such as DNA and glycolipids.

We have demonstrated in Chapter 2 the development of a quadroma (hybrid-hybridoma) based full length bispecific monoclonal antibody (bsmAb) for targeting of biotinylated protein antigens to DEC-205 [16]. The successful targeting of biotinylated proteins to DC augmented immune responses against the antigen at ~500 fold lower dose. However, the quadroma secretes the bsmAb along with parental and unwanted heavy and light chain combinations [48]. Hence we designed in this chapter a truncated recombinant construct and to demonstrate the versatility of this DC targeting vehicle to delivery proteins [ovalbumin (OVA), Ebola virus glycoprotein 1 (EBOV GP1), Severe acute respiratory syndrome-coronavirus (SARS-CoV) spike Receptor Binding Domain (RBD), Anthrax Protective Antigen (PA)], peptide [epithelial mucin 1 (MUC-1)], glycolipids (GM2 and GM3) and DNA [Western equine encephalitis virus (WEEV) E1, E2, EBOV GP1,2, SARS-CoV spike and SARS-CoV membrane]. Cancer antigen MUC-1 is a well-known breast tumor marker. MUC-1 peptides as cancer vaccines have been studied extensively in clinical trials [17, 18]. Gangliosides GM2 and GM3 have been used as targets for therapy and vaccine for cancer. Gangliosides are often over expressed on human melanoma cells or other cancer cell types and the expression level correlates with clinical outcomes [19, 20]. Ebola virus can cause severe hemorrhagic disease in humans with high mortality rates and currently there is no cure, vaccine or treatment that is commercially available. The pathogenicity of EBOV is associated with its multifunctional glycoprotein (GP), mainly GP1 and GP2. GP1 is responsible for receptor binding whereas GP2 mediates fusion of the viral and cellular membranes [21-23]. Protection against EBOV GP1 may also provide protection against Marburg virus infection [21]. *Bacillus anthracis* is the causative agent of anthrax. Anthrax PA, secreted by the bacteria, is required for pathogenicity of the bacteria (forms a pore in the endosomal membrane and promotes translation of lethal factor and edema factor). Effective generation of immune responses against Anthrax PA will provide protective immunity against anthrax [24].

WEEV is endemic in Western Canada and can be easily transmitted as aerosols with high mortality rate. Pathogenicity of WEEV is mediated by glycoprotein E1 (fusion of viral and cellular membrane) and E2 (host cell receptor attachment) [25]. Efficient generation of immune responses against WEEV glycoprotein E1 and E2 can completely

protect the host from WEEV infection [26]. Lastly, SARS-CoV is a new infectious disease that recently caused an outbreak of atypical pneumonia worldwide. SARS-CoV pathogensis requires glycoprotein S1, S2 and membrane protein. The S1 incorporates the receptor binding domain and is responsible for virus binding to the cellular receptor. The S2 glycoprotein mediates membrane fusion and the membrane (M) protein induces host cell apoptosis [27, 28]. Protective immune responses can be achieved by inducing immune responses against RBD domain [29].

A scFv that recognizes DEC-205 receptor of DC was successfully cloned from HB290 hybridoma and fused with a core-streptavidin domain and expressed in *E.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 were conducted in mice with a variety of biotinylated antigens such as viral and bacterial proteins, cancer peptide, gangliosides and DNA. In the presence of bfFp and co-stimulatory anti-CD40 mAb, both humoral and cell-mediated responses were augmented in either single antigen or multiple antigens targeting strategy. In the absence of an efficient delivery system, bfFp targeting of biotinylated antigens to DC could be developed as a monovalent or polyvalent vaccine or therapy for cancer and infectious diseases.

4.2. Materials and Methods

4.2.1. Materials

DC 2.4 is a DEC-205 expressing mouse bone marrow DC cell-line transduced with GM-CSF, *myc* and *raf* oncogenes [30]. HB290, a rat anti-mouse DEC-205 hybridoma, was obtained from ATCC. BSA (bovine serum albumin), streptavidin-HRPO (horseradish peroxidase), anti-His_6 mAb (monoclonal antibody), NHS-LC-Biotin (biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester), photobiotin acetate, OVA and goat-anti-mouse-HRPO (GAM-HRPO) were from Sigma (Oakville, Canada). Biotinylated MUC-1 peptide with amino 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, USA. HSF

(hybridoma serum free media), DMEM, 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 as per vendor's protocol. TMB (3, 3', 5, 5'-tetramethylbenzidine) peroxidase substrate was purchased from Kirkegaard & Perry Laboratory Inc (Gaithersburg, USA). 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 and pBlueScript II KS-(PKS) were purchased from Stratagene (Cedar Creek, USA). T7 promoter and terminator primers and the expression vector pET-22b (+) were from Novagen (Madison, USA). pVAX1 mammalian expression vector and molecular cloning materials (TOP10 cells, FastTrack mRNA isolation kit, modifying and restriction enzymes) were from Invitrogen (Burlington, Canada). Protein assay reagent was purchased from Bio-Rad (Mississauga, Canada). Ni-NTA agarose was purchased from Qiagen (Mississauga, Canada). Mouse IFN-γ (Interferon gamma) ELISA Ready-SET-Go was purchased from eBioscience (San Diego, USA). LAL (limulus amebocyte lysate) PYROGENT® Plus Single Test Vials was purchased from Cambrex (Walkersville, USA).

Rat anti-mouse 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, Defence R&D Canada) [31]. SARS-CoV membrane codon optimized DNA was purchased from GENEART. EBOV GP1,2 DNA [23], EBOV GP1,2 mammalian expressed protein and SARS-CoV spike DNA were from Dr. S. Jones and Dr. T. Booth (Special Pathogens Program, National Microbiology Laboratory, Canada). Gangliosides and ganglioside conjugates were from Sandra Jacques and Dr. D. Bundle (University of Alberta, Canada). Gangliosides GM2 and GM3 antigens were biotinylated (B-GM2, B-GM3) and conjugated with BSA (B-BSA-GM2, B-BSA-GM3). Anthrax Protective Antigen (PA), extracted from the S-layer of recombinant *Caulobacter crescentus*, was provided by Dr. J. Smit (University of British Columbia, Canada).

4.2.2. 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 all the experiments are listed in Table 3.1A and 1B.

A

Primer	Function	Primer Sequence (5'-3')
WP001	5'PCR primer, HB290 V_L-C_{L-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-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-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-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, pSARS-CoV spike	CTCGCTCGAGAGAATTCTATTATGTGTAATGTAATTTGACACCCTTGAG
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
WP016	3'PCR primer, pEBOV GP1,2	CTCGCTCGAGAGAATTCTAAAAGACAAATTTGCATATACAGAATAAAGC

B

Classification	Immunized antigens	Testing antigens
Protein	B-OVA B-SARS-CoV spike RBD B-EBOV GP1 B-Anthrax PA	OVA SARS-CoV spike RBD EBOV GP1 Anthrax PA
Peptide	B-MUC-1	B-MUC-1
Glycolipid	B-GM2 B-GM3	B-GM2 (IFN-γ Assay) B-GM3 (IFN-γ Assay) B-BSA-GM2 (Humoral study) B-BSA-GM3 (Humoral study)
DNA	B-pVHX-6 B-pEBOV GP1,2 B-pSARS-CoV spike B-pSARS-CoV membrane	WEEV E1 WEEV E2 EBOV GP1 EBOV GP2 EBOV GP1,2 SARS-CoV spike S1 SARS-CoV spike S2 SARS-CoV spike RBD SARS-CoV membrane

Table 3.1. (A) 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* perplasmic expression vector. 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. WP011-16 are the PCR primers for cloning of viral DNA into pVAX1 mammalian expression vector. These primers contain Kozak translation initiation sequence, initiation codon, and *Bam*HI and *Eco*RI restriction sites. (B) Summary table for the antigen categories used for testing and targeted for *in vivo* study.

SARS-CoV spike DNA (encoding S1, S2, RBD and transmembrane domain), 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 3.1A). These primers inserted Kozak translation initiation sequence and initiation codon (ATG) into the restriction sites *Bam*HI, *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 [32]. Core-streptavidin, WEEV E1, WEEV E2 and EBOV GP1 (Subfragment D) were prepared based on our previous work in our laboratory [33-36]. 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 labelled with biotin. DNA vectors were biotin labelled using photobiotin acetate (B-pVHX-6, B-pEBOV GP1,2, B-pSARS-CoV spike, B-pSARS-CoV membrane), all proteins were labelled with NHS-LC-Biotin (B-OVA, B-SARS-CoV spike RBD, B-EBOV GP1, B-Anthrax PA), glycolipids (B-GM2, B-GM3, B-BSA-GM2, B-BSA-GM3) and peptide (B-MUC-1) were synthetically labelled with biotin. Biotinylation of all antigens was confirmed by dot blot assay probed with streptavidin-HRPO.

4.2.3. Sequencing and Cloning of HB290 Gene

4.2.3.1. Sequencing of HB290 Fab

1 x 10^9 HB290 cells were harvested and the mRNA was isolated using FastTrack® mRNA isolation kit according to vendor's protocol. PCR primers were designed based on protocols in Methods in Molecular Biology volume 178 [37]. cDNA of mAb from the variable regions (V_L and V_H) to the constant light chain region (C_L) and heavy chain

region (CH_2) were generated by RT-PCR (reverse transcription polymerase chain reaction) and TD-PCR (touch down polymerase chain reaction) using rat specific PCR primers and inserted in PKS plasmid. Positive clones were selected by restriction digestion mapping and DNA sequencing. The positive cloned fragment was sequenced using M13 primers by CEQ^{TM}2000 (Beckman Coulter, USA). DNA alignment between the forward sequence and backward sequence was done using DS Gene 1.1 software (Discovery Studio). The sequence generated after DNA alignment was compared with the known mAb sequences in NCBI BLAST databank. The consensus sequence of 2 individual clones was selected from two independent PCR resulted in 100% match in both nucleotide sequence and amino acid sequence.

4.2.3.2. Cloning of HB290 scFv and bfFp

The various cloning constructs of HB290 scFv and bfFp are listed in Figure 4.1.

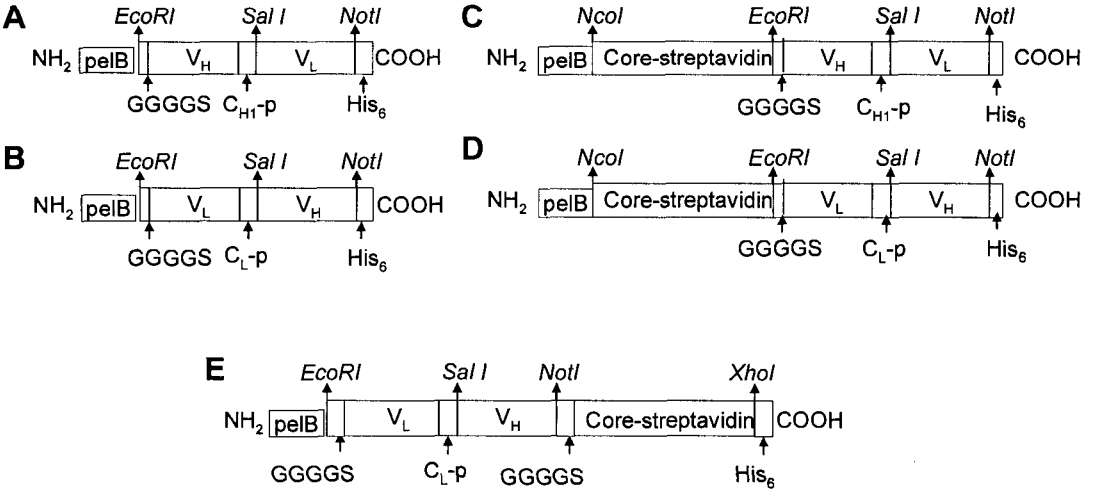


Figure 4.1. The construct of the HB290 scFv (A) V_H-V_L, (B) V_L-V_H; and bfFp (C) WET5, (D) WET6 and (E) WET7. The genes were cloned in different orientation to maximize the production in *E.coli* system. Abbreviations: pelB, bacterial leader sequence; V_L, variable domain light chain; V_H, variable domain heavy chain; $C_{H1\text{-}p}$, partial constant heavy chain region 1, 15 amino acids linker; $C_{L\text{-}p}$, partial constant light chain region, 15 amino acids linker; G, glycine; S, serine; His_6, six histidine amino acid tag.

The genes were cloned in different orientations to determine the best expression in *E.coli*. The heavy and light chain variable regions with/without partial constant regions of anti-DEC205 antibody were amplified from PKS plasmid containing HB290 Fab DNA by PCR and cloned into pET-22b (+) plasmid. The heavy chain variable region gene of HB290 was fused to the 3′ end of light chain variable region gene with a linker of 15 amino acids from constant heavy chain region 1 sequence using the PCR (PCR primers: WP005-WP008). Subsequently restriction digest/ligation methods were employed to generate V_H-V_L scFv gene (Fig. 4.1A). 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. The PCR fragments were gel-purified, double digested to the respective inserted restriction sites, and ligated to pET-22b (+) plasmid. V_L-V_H scFv gene was generated also by the same methods utilizing PCR primers WP001-WP004 (WP001 and WP002 were inserted into the *Eco*RI and *Sal*I; WP003 and WP004 were inserted into the *Sal*I and *Not*I). The light chain variable region gene of HB290 was fused to the 3′ end of heavy chain variable region gene *via* 15 amino acids of constant light chain sequence (Fig. 4.1B). Both scFv orientations were inserted into pET-22b (+) containing the core-streptavidin gene [38] in 3' orientation fusion with the core-streptavidin gene (Fig. 4.1C and 1D). V_L-V_H scFv gene was fused in 5' terminus with core-streptavidin gene by the same method described above (Fig. 4.1E). In short, 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 V_L-V_H scFv gene (Fig. 4.1E). 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 by CEQ^{TM}2000. The positive clones were named as follows: WET5 encoding core-streptavidin-V_H-V_L, WET6 encoding core-streptavidin-V_L-V_H and WET7 encoding V_L-V_H-core-streptavidin.

4.2.4. Expression, Purification and Characterization of HB290 Recombinant Proteins

4.2.4.1. Expression of HB290 scFv and bfFp

Expression of scFv and bfFp methods and conditions are enumerated in Chapter 5 [38]. Briefly, the pET-22b (+)-scFv or bfFp genes (WET5-7) were chemically transformed into BL21-CodonPlus® (DE3)-RIPL. *E.coli* transformants were cultured and induced 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. Affinity purification from *E.coli* periplasm using IMAC purification protocol was performed as described previously [38]. 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.

4.2.4.2. Western Blot: bfFp Biotin Binding and Heat Stability

IMAC purified bfFp was heated at either 60°C 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.

4.2.4.3. 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 bfFp (40 µg/ml in 100 µl volume) 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, California, USA) and subtracted with control (DC with only streptavidin-HRPO incubated at 4°C for 1 h). The bfFp DEC-205 receptor binding specificity 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. The mAb was incubated for 2 h at 4°C then washed with PBS and the bfFp was detected using B-OVA, streptavidin-HRPO and TMB as mentioned above.

4.2.5. *In Vivo* Targeting of DC in Mice

4.2.5.1. Mice and Immunization Protocol

15 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 3.2.

	Groups	Day 0	Day 12	Day 21	Day 24
1	Control	PBS	PBS	Spleen (T cell) Serum (Humoral)	IFN-γAssay
2	B-pVHX-6 bfFp	500 ng 20 μg	500 ng 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
3	B-pVHX-6 Anti-CD40 mAb	500 ng 25 μg	500 ng 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
4	B-pVHX-6 bfFp Anti-CD40 mAb	500 ng 20 μg 25 μg	500 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
5	B-OVA bfFp Anti-CD40 mAb	200 ng 20 μg 25 μg	200 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
6	B-MUC-1 bfFp Anti-CD40 mAb	200 ng 20 μg 25 μg	200 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
7	B-GM3 B-GM2 bfFp Anti-CD40 mAb	1 μg 1 μg 20 μg 25 μg	1 μg 1 μg 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay

	Groups	Day 0	Day 12	Day 21	Day 24
8	Control	PBS	PBS	Spleen (T cell) Serum (Humoral)	IFN-γAssay
9	B-pEBOV GP1,2 bfFp Anti-CD40 mAb	500 ng 20 μg 25 μg	500 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
10	B-pSARS-CoV spike bfFp Anti-CD40 mAb	500 ng 20 μg 25 μg	500 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
11	B-pSARS-CoV membrane bfFp Anti-CD40 mAb	500 ng 20 μg 25 μg	500 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay

	Groups	Day 0	Day 12	Day 21	Day 24
12	Control	PBS	PBS	Spleen (T cell) Serum (Humoral)	IFN-γAssay
13	B-OVA B-EBOV GP1 B-SARS-CoV spike RBD B-MUC-1 B-Anthrax PA Anti-CD40 mAb Core-streptavidin	200 ng 200 ng 200 ng 200 ng 200 ng 25 μg 10 μg	200 ng 200 ng 200 ng 200 ng 200 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
14	B-OVA B-EBOV GP1 B-SARS-CoV spike RBD B-MUC-1 B-Anthrax PA bfFp	200 ng 200 ng 200 ng 200 ng 200 ng 20 μg	200 ng 200 ng 200 ng 200 ng 200 ng 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay
15	B-OVA B-EBOV GP1 B-SARS-CoV spike RBD B-MUC-1 B-Anthrax PA bfFp Anti-CD40 mAb	200 ng 200 ng 200 ng 200 ng 200 ng 20 μg 25 μg	200 ng 200 ng 200 ng 200 ng 200 ng 0 0	Spleen (T cell) Serum (Humoral)	IFN-γ Assay

Table 3.2. Immunization protocol for evaluating humoral and cell-mediated immune responses to biotinylated antigens in mice (n=5 for each group). The amounts of antigens, antibodies and bfFp are either in μg or ng per mouse. All mice were injected subcutaneously near inguinal lymph node. Group 1, 8 and 12 are the control groups and each group of the control mice were immunized and boosted subcutaneously with PBS. The first experiment demonstrated the versatility of bfFp based delivery of protein, peptide, glycolipids and DNA to DC in generating immune responses (Groups 2-7 mice). Group 2-4 mice focused on the DNA delivery to DC and to verify the essential requirement of DC targeting vehicle and co-stimulatory molecule. Protein antigen delivery is studied in Group 5 mice, peptide delivery is focused in Group 6 mice, and glycolipids were investigated in Group 7 mice. A variety of infectious diseases viral DNA were targeted to DC using 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 antigens were studied in the third set of experiments. Groups 13-15 designed were to evaluate multivalent immune responses against proteins and peptide. All mice were boosted with the same concentration of antigen(s) in PBS 12 days following primary immunization. The mice were sacrificed after 9 days of boost.

Three separate experiments were conducted and each design has its own control group. All groups had n = 5 mice. The first set of experiments demonstrated the versatility of bfFp based delivery of protein, peptide, glycolipids and DNA to DC in generating immune responses. B-pVHX-6, B-OVA, B-MUC-1, B-GM2 & GM3 were studied (Groups 2-7 mice). Group 2-4 mice focused on the DNA delivery to DC and to verify the essential requirement of DC targeting vehicle and co-stimulatory 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 co-stimulation on DC. Group 3 mice were co-immunized with anti-CD40 mAb without the bfFp to determine the non-targeted immune responses. Group 4 mice were co-immunized with both bfFp and anti-CD40 mAb. Protein antigen delivery is studied in Group 5 mice, peptide delivery is focused in Group 6 mice, and glycolipids were investigated in Group 7 mice. The second group of experiments was designed to confirm the DNA delivery strategy. A variety of infectious diseases viral DNA were targeted to DC using 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 antigens were studied in the third set of experiments. Groups 13-15 were designed to evaluate multivalent immune responses against proteins and peptide. B-OVA, B-EBOV GP1, B-SARS-CoV spike RBD, B-MUC-1, B-Anthrax PA were immunized as a mixture in saline in Groups 13-15 mice. Group 13 mice were co-immunized with anti-CD40 mAb and core-streptavidin without the bfFp to determine the non-targeted immune responses. Group 14 mice were co-immunized with bfFp without the presence of anti-CD40 mAb to verify the essential role of anti-CD40 mAb co-stimulation. Group 15 mice were immunized with antigens, bfFp and anti-CD40 mAb. Group 1, 8 and 12 are the control groups for the three separate animal experiments. All mice were boosted with the same concentration of antigen(s) 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 (endotoxin sensitivity at 0.125 EU/ml). The mice were sacrificed after 9 days of boost. The blood and the spleen were collected. The serum was isolated using

standard procedure [39] and used to evaluate humoral immune responses. Spleens were used to study IFN-γ 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 the naïve mouse spleens were treated with mitomycin C as previously described [16].

4.2.5.2. Evaluation of Humoral Immune Responses and IFN-γ

Antibody titres were measured individually in each mouse by ELISA following immunization of the respective antigen (Table 3.1B). 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 serially diluted serum from each mouse in quadruplicate was added and 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 microplate reader. Statistical analyses were performed as described in Chapter 2 [16] to determine the significance between antibody responses in different groups. IFN-γ activity was determined by the IFN-γ concentration generated after 3 days of incubation of 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, Anthrax PA, B-MUC-1, B-GM2, B-GM3, WEEV E1 and WEEV E2 (10 μg per antigen, each antigen is separately incubated). IFN-γ ELISA Ready-SET-Go kit was used to determine the IFN-γ concentration.

4.2.5.3. Immune responses towards biotin, bfFp and core-streptavidin

All 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 above. The plates were coated with either B-BSA, bfFp or core-

streptavidin (10 μg/well). The rest of procedures and statistical analyses were as enumerated in the above section.

4.3. Results

4.3.1. 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 NCBI BLAST database indicating the sequence is antibody related. HB290 V_L amino acid sequence from our recombinant clone was 100% identical to a previous publication on cloning of NLDC-145 (HB290) scFv [40]. However, HB290 V_H deduced amino acid sequence(EVKLVESGGGLVQPGGSLRLSCAASGFTFNDFYMNWIRQPPGQAPEWL GVIRNKGNGYTTEVNTSVKGRFTISRDNTQNILYLQMNSLRAEDTAIYYCARGGP YYYSGDDAPYWGQGVMVTVSS) 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 and co-workers is 99% identical (only 2 amino acid difference in the 5' terminal) with single chain antibody against rice stripe virus protein P20 (Accession: AAG28706). HB290 V_L-V_H and V_H-V_L genes were successfully cloned into separate pET-22b (+) plasmid by PCR, restriction digest and DNA ligation methods. The plasmid vectors WET5 and WET6 were successfully constructed by inserting the sequence encoding the HB290 single-chain anti-DEC205 antibody (V_L-V_H or V_H-V_L respectively) into a core-streptavidin containing pET22b (+) plasmid next to the pelB leader sequence (Fig. 4.1A-D). The plasmid vector WET7 was constructed by inserting the core-streptavidin sequence into HB290 V_L-V_H scFv containing pET22b (+) plasmid (Fig. 4.1B and 1E). The core-streptavidin sequence is at the N-terminus of the scFv in WET5 and WET6 constructs; whereas, in WET7 the core-streptavidin is in 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 (Fig. 4.2A).

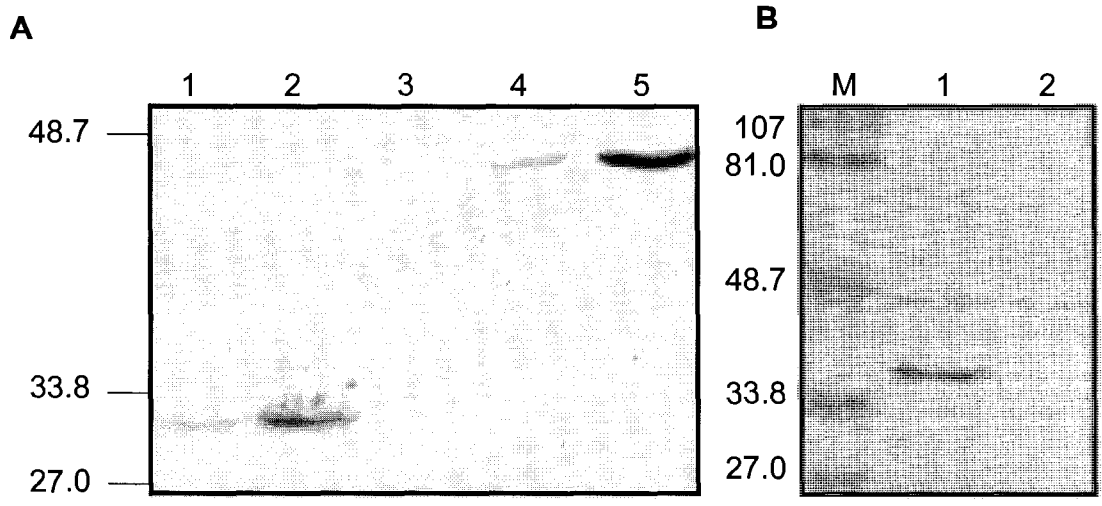


Figure 4.2. Expression and purification of recombinant proteins. The scFv and bfFp genes were chemically transformed, cultured, induced and the whole-cell bacterial pellets were analyzed by Western blot. (A) Western blot probed with anti-His_6 mAb for analysis of scFv and bfFp expression. Lane 1: HB290 scFv V_H-V_L; lane 2: scFv V_L-V_H; lane 3: bfFp WET5, core-streptavidin-V_H-V_L; lane 4: bfFp WET6, core-streptavidin-V_L-V_H; lane 5: bfFp WET7, V_L-V_H-core-streptavidin. WET7 has the highest protein expression level. (B) SDS-PAGE of WET7 before and after IMAC purification. Lane 1: WET7 periplasmic protein; lane 2: IMAC-purified WET7.

The optimal scFv and bfFp orientation for expression were determined by the Western blot. HB290 V_L-V_H scFv, WET6 and WET7 were successfully expressed and the proteins are shown at the desired MW (molecular weight) band either at ~30 kDa or ~46 kDa (Fig. 4.2A). WET7 had higher level of protein expression compare to WET6. WET7 was subjected to medium scale expression, and the bfFp in the periplasmic space was extracted and affinity purified by IMAC column. Both periplasmic fraction and the affinity purified fraction were analyzed on SDS-PAGE (Fig. 4.2B). IMAC purification of the WET7 bfFp was successful; a clear band is shown at 46 kDa in affinity purified fraction (Fig. 4.2B). Approximately 1.0 mg of WET7 bfFp was affinity purified from a 2 L culture.

4.3.2. Characterization of bfFp

E.coli expressed bfFp appears in 3 isoforms: monomeric, dimeric and tetrameric form similar to a published report [41]. 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 [41, 42]. Fusion protein is 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 [41]. The bfFp isoforms and the biotin binding activity of the bfFp were analyzed by Western blot probed with B-BSA (Fig. 4.3A).

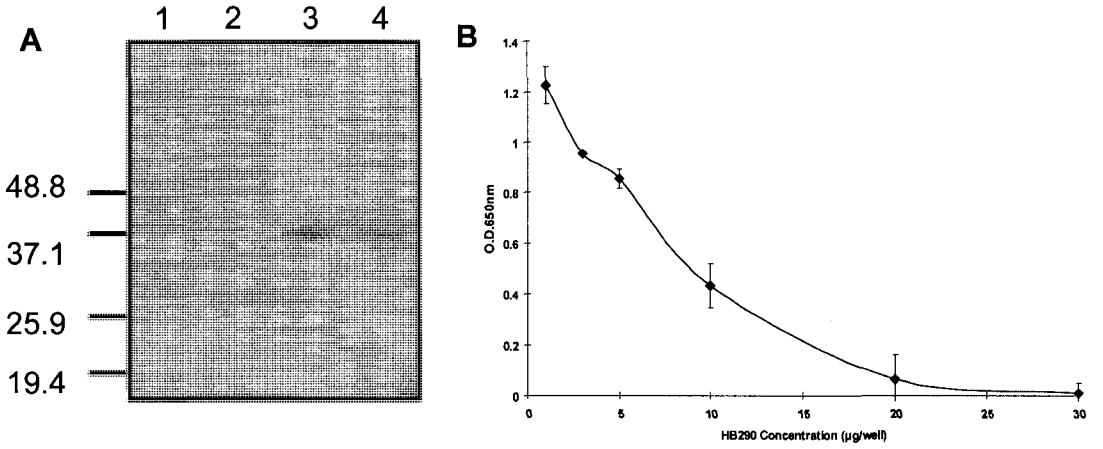


Figure 4.3. Demonstration of IMAC-purified bfFp bifunctional activity and thermal stability. (A) IMAC purified bfFp was incubated either at 60°C (lane 3) or 95°C (lane 4) for 10 min and probed with B-BSA followed by streptavidin-HRPO. Lanes 1 (HB290 scFv V_H-V_L) and 2 (HB290 scFv V_L-V_H) are controls. The IMAC-purified bfFp bifunctional activity was tested against DC 2.4 cells and B-OVA *via* ELISA method (B). Competition binding studies of HB290 mAb and bfFp for binding to DC. The binding of bfFp is confirmed by B-OVA and streptavidin-HRPO. The error bars are the standard deviations (n = 4).

WET7 bfFp was detected using B-BSA and streptavidin-HRPO in Western blot. The bfFp appears predominantly in monomeric form after heating at either 60°C or 95°C (Fig.

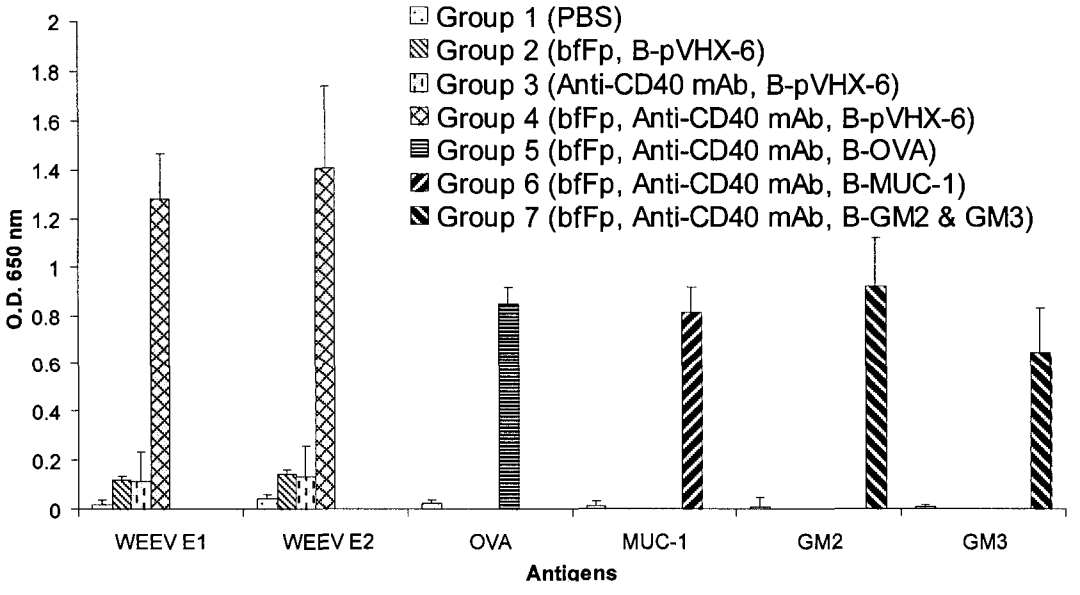
4.3A). The predominant monomeric form may be due to the difference in the linker between core-streptavidin and scFv [38].

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 (Fig. 4.3B).

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

In vivo studies were carried out to investigate the ability and efficacy of bfFp targeting of antigens to DC. Four classes of antigens were chosen to demonstrate the versatility and diversity of antigen delivery. These antigens were either made by recombinant DNA technology or obtained from several sources. LPS is identified in *E.coli* expressed proteins (SARS-CoV proteins, EBOV proteins, WEEV proteins, bfFp), OVA and Anthrax PA. DNA vectors, MUC-1 peptide, anti-CD40 mAb and mammalian expressed GP1,2 were LPS free. Both humoral and cell-mediated responses were investigated using a variety of antigens listed in Table 3.1B. The immunization protocol is described in detail in Table 3.2 and the results of humoral and cell-mediated responses are shown in Figure 4.4 and 4.5.

A



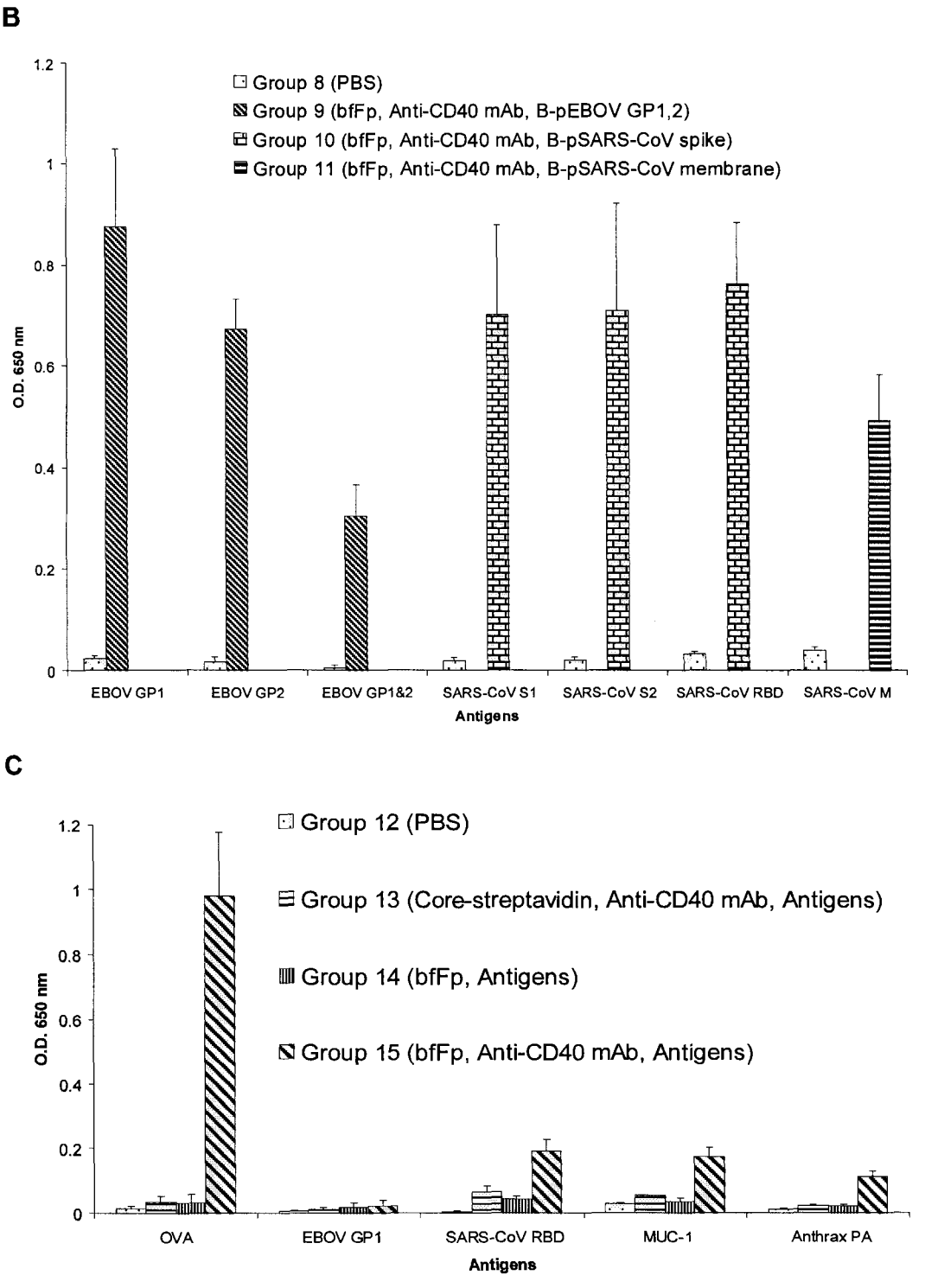
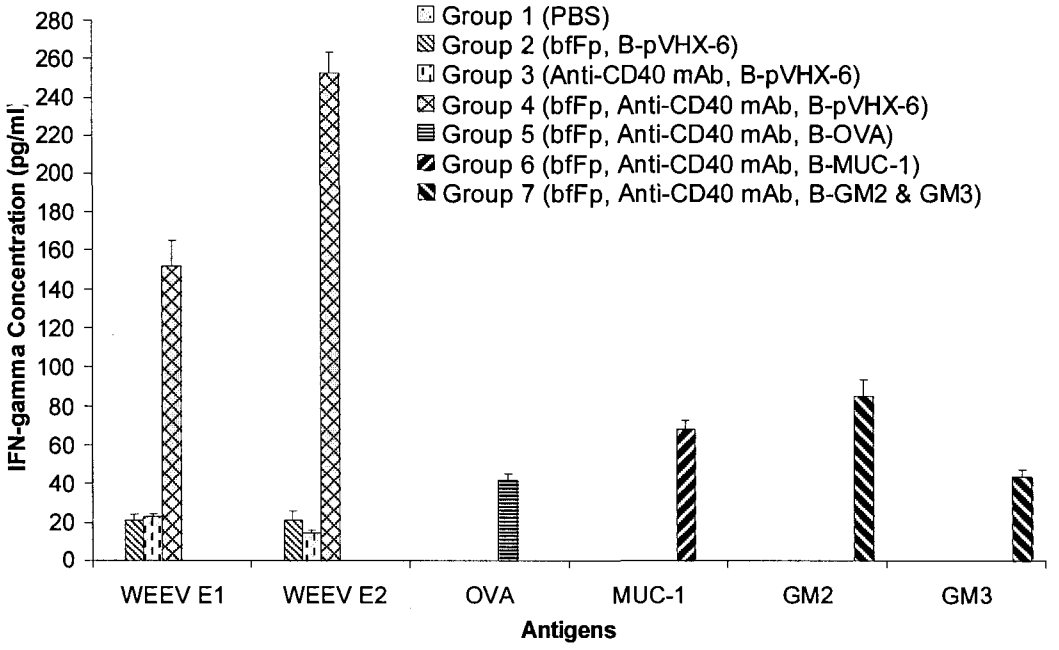


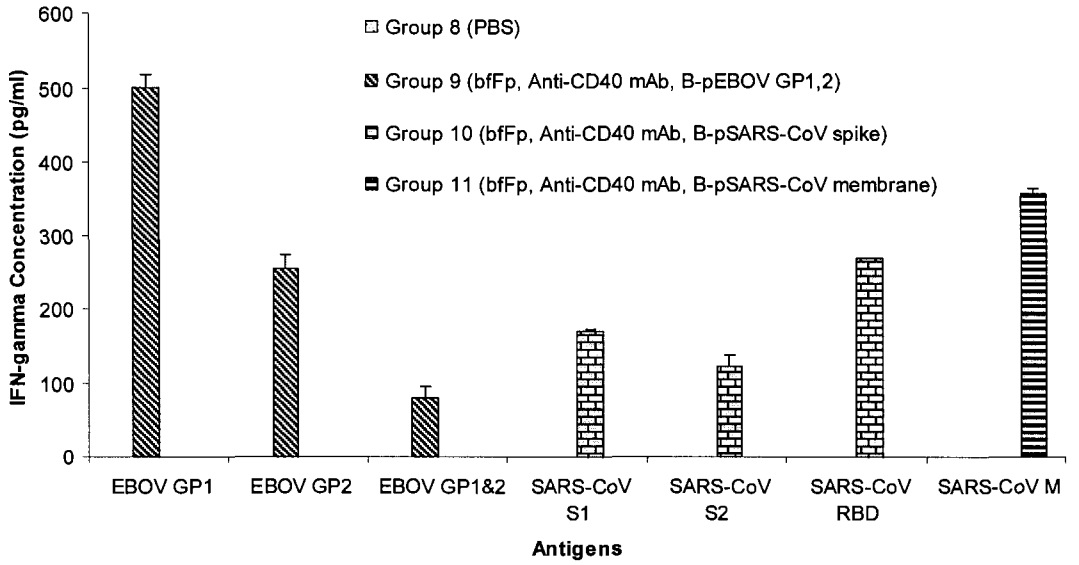
Figure 4.4. Analysis of humoral immune responses to biotinylated antigens *in vivo*. Groups of five mice were immunized with different antigen combinations along with a PBS control. The specific components, amounts, and the schedule of immunizations are

outlined in Table 3.2. The mice were analyzed individually and the data was pooled. The humoral response, as quantified by serum antibody titres on day 21 post immunization, was measured by ELISA method against the respective antigens listed in Table 3.1B. The method involved coating of 10 μg 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.

A



B



C

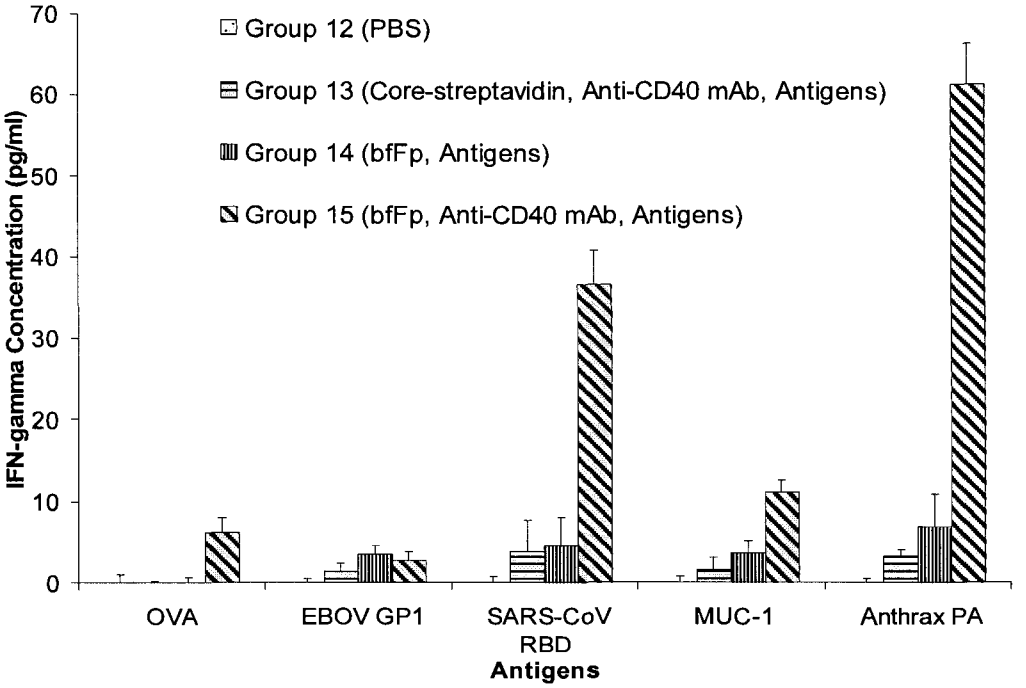


Figure 4.5. Analysis of cell-mediated immune responses based on IFN-γ estimation. Spleen T cells (responder cells) from the groups of mice immunized with different antigens (shown on *X*-axis and for details see Table 3.1B and 3.2) were isolated and

purified using a nylon wool column. The five spleens in each group were pooled, mixed in DMEM prior to nylon wool purification. Stimulator cells prepared from naïve mice spleen cells were isolated and treated with 50 μg/ml mitomycin C. Purified responder cells from both immunized and non-immunized mice were aliquoted to a tissue culture plate in quadruplicate with or without stimulator cells. The cells were then incubated with 10 μg of antigen 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 using the protocol from eBioscience. Each data set is shown following subtraction of the corresponding ELISA values obtained without stimulator cells. The error bars are the standard deviations.

Humoral responses were measured by the antibody titres against the immunized antigens or its respective proteins from DNA vectors individually in each mouse by ELISA method. The magnitude of cell-mediated immune response is determined by the amount of IFN-γ secreted from spleen T cells in response to the antigens. Individual mouse spleen was not studied since the humoral response data was reproducible from five individual mice. The spleen cells were pooled to average the quality of data. Responder cells from both immunized and non-immunized mice without stimulator cells had minimal IFN-γ secretion. 15 groups of mice were divided into three different experiments to demonstrate the diversity and efficacy of DC targeting strategies. Group 1, 8 and 12 are the control groups for the three separate animal experiments, being only immunized and boosted with PBS. Both humoral and cell-mediated immune responses are minimal (Fig. 4.4 and 4.5).

4.3.3.1. bfFp mediated protein, peptide, gangliosides and DNA targeting

Groups 2-7 were designed to demonstrate the versatility of bfFp based delivery of protein, peptide, glycolipids and DNA to DC in generating immune responses. Group 2-4 mice focused on the 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 in the absence of anti-CD40 mAb. Group 3 mice were co-immunized with anti-CD40 mAb without the bfFp. Group 4 mice were co-immunized with both bfFp and anti-CD40 mAb.

The results indicate that Group 4 has highest antibody titre (statistically significant, $P = 0.01$) and augmented IFN-γ secretion against WEEV E1 and WEEV E2 proteins compare to Group 1-3 (Fig. 4.4A and 4.5A). Immune responses against WEEV E2 antigen appear to be higher than WEEV E1 in Group 4 (Fig. 4.4A and 4.5A). Immune responses in Group 2 and 3 are minimal towards WEEV E1 and E2 protein antigens, probably due to LPS, anti-CD40 mAb or bfFp effect (Fig. 4.4A and 4.5A). Anti-CD40 mAb and bfFp appeared to be essential for DNA targeting strategy. The strategy was applied to protein (OVA), peptide (MUC-1) and glycolipids (GM2 and GM3). Essentially similar humoral responses and cell-mediated immune responses are shown in mice immunized with protein (Group 5), peptide (Group 6) and glycolipids (Group 7) in the presence of bfFp and anti-CD40 mAb (Fig. 4.4A and 4.5A). In summary, anti-CD40 mAb and bfFp are required to achieve immune responses in DC delivery of DNA.

We have demonstrated the efficiency of targeting different classes of antigens such as protein, peptide, glycolipid and DNA to DC. However, one DNA targeting model may not be sufficient to document the successful DNA targeting to DC. Thus, we have tested the same targeting strategy for a variety of infectious diseases DNA. Groups 9-11 were immunized with different 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 encodes EBOV GP1 and GP2 protein, pSARS-CoV spike encodes SARS-CoV S1, S2, RBD and transmembrane domain, and pSARS-CoV membrane encodes SARS-CoV membrane protein. Strong humoral and cell-mediated responses were achieved against the encoded viral proteins by targeting viral DNA to DC (Fig. 4.4B and 4.5B). EBOV GP1 generates highest immune responses compare to GP2 and GP1,2. The immune responses against mammalian expressed GP1,2 protein are lower than *E.coli* expressed fragments (GP1 and GP2) (Fig. 4.4B and 4.5B). Immune response against EBOV GP1,2 is relatively lower than it's fragment may be due to the glycosylation masking of the epitopes for serum or T cell reactivity [47]. There is no significant difference in serum titer activity between SARS-CoV spike proteins, but higher IFN-γ concentration is found in SARS-CoV RBD. Thus the naked DNA delivery strategy was successful in several infectious diseases models.

4.3.3.2. bfFp mediated multiple antigen targeting strategy

Groups 13-15, were designed to evaluate multivalent immune responses by targeting multiple biotinylated proteins and peptide antigen at the same time in nanogram concentrations. Group 13 reflects the possible involvement of core-streptavidin targeting of a mixture of biotinylated proteins and peptide to DC. Group 14 and 15 demonstrated the efficacy of the bfFp in the absence and presence of the anti-CD40 mAb co-stimulator respectively. The results show that Group 15 has the highest antibody titre (statistically significant, $P = 0.005$) and augmented IFN-γ secretion against OVA, SARS-CoV RBD, Anthrax PA and MUC-1 compare to Group 12-14 (Fig. 4.4C and 4.5C). Group 13-15 have shown minimal immune responses against EBOV GP1 (Fig. 4.4C and 4.5C). In Group 15, highest antibody titre was found against OVA and lowest against Anthrax PA (Fig. 4.4C); whereas, highest IFN-γ secretion was found against Anthrax PA (Fig. 4.5C). Minimal humoral or cell-mediated immune responses generated from Group 12-14 may be due to the anti-CD40 mAb, LPS and/or core-streptavidin effects (Fig. 4.4C and 4.5C). MUC-1 and OVA immune responses were compared between single antigen and multiple antigen targeting strategies. Higher IFN-γ secretion and serum titre against MUC-1 were achieved in single antigen targeting strategy of MUC-1 peptide antigen (Group 6) in comparison to multiple antigens targeting strategy (Group 15) (Fig. 4.4A, 4.4C, 4.5A, 4.5C). The serum titre against OVA is not different between Group 5 and 15 (Fig. 4.4A and C). However, Group 5 has higher IFN-γ secretion compare to Group 15 (Fig. 4.5A and C). 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 multi-antigen targeting strategy. Immune responses are variably shifted in multi-antigen targeting method, possibly towards immunodominant antigens.

4.3.4. Immunogenicity of bfFp

Serum titre against biotin, bfFp and core-streptavidin were analyzed from every one of the 15 groups to evaluate the immunogenicity of the bfFp targeting vehicle. The bfFp, B-BSA and core-streptavidin proteins were coated on the ELISA plate. Serum reactivity

was found to a minor extent against core-streptavidin (OD ~0.25) and bfFp (OD ~0.15). This level of serum reactivity is not significant compare to the control groups (Fig. 4.6).

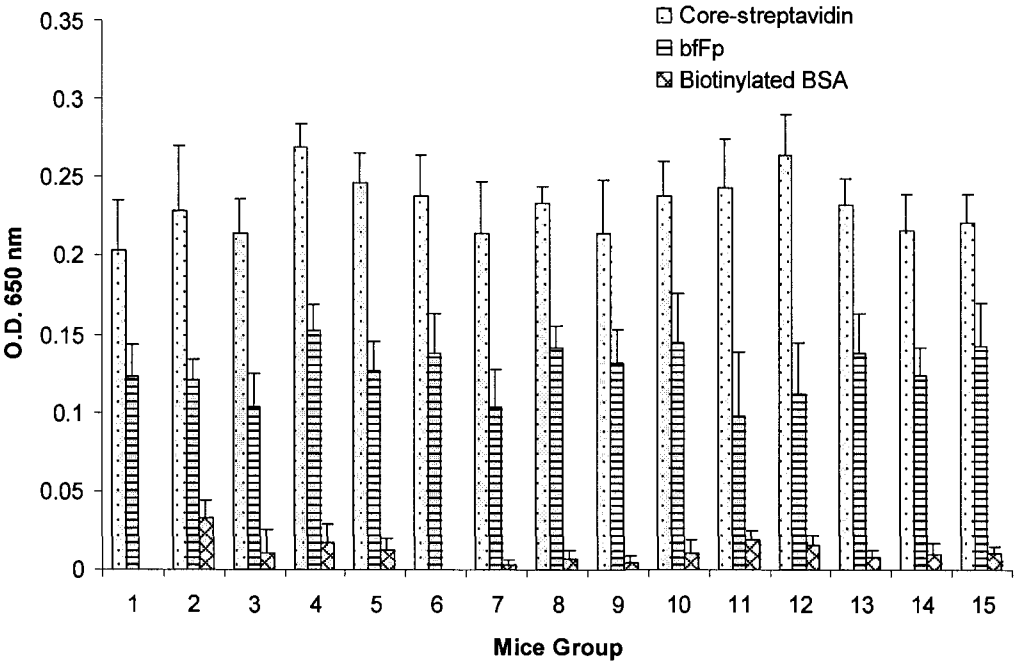


Figure 4.6. Analysis of serum reactivity towards biotin, bfFp and core-streptavidin. The mice (5 mice per group) were analyzed individually and the data was pooled. The mouse group is listed on the *X*-axis. The serum reactivity, as quantified by serum antibody titres on day 21 post immunization, was measured by ELISA method against B-BSA, bfFp and core-streptavidin. The method involved coating of 10 μg 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.

The serum reactivity towards B-BSA was minimal and not statistically significant between all groups (Fig. 4.6).

4.4. Discussion

In vivo targeting of 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 to date. These include a) HB290 scFv coated liposome [8], b) chemical cross-linking of HB290 with antigen [9, 11, 13, 14], c) HB290 hybrid antibody [10, 12, 15] and d) bsmAb targeting system [16]. Several limitations are found in these systems. Currently, the above strategies have only demonstrated the efficient delivery of protein or peptide to DC. These delivery systems may not be versatile and flexible for clinical applications, comparison studies with different antigens and *in vivo* targeting of multiple antigens such as DNA and glycolipids. Limitation in scFv coated liposome targeting strategy includes instability of the scFv on the liposome surface (scFv could be lost during circulation), liposome instability, scale-up issues, relative complexity in 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 are some of the limitations in chemical cross-linking of the whole mAb with antigen. Hybrid antibody or recombinant antigen fusion protein generation using mammalian expression system requires careful monitoring in cell productivity, post-translational chemical modifications, degradation and aggregation of the final product [43]. Moreover, a new antibody fusion protein is required for each antigen (protein or peptide) and the process is often time consuming and costly. A bsmAb produced from a quadroma is also not suitable for clinical applications and the limitations are the yields and purity [16]. Major drawback for these systems could be dose-limiting toxicity (prolonged circulation time) and immunogenicity against the targeting system that can alter pharmacokinetics (biodistribution and clearance), block receptor-antigen interactions, induce hypersensitivity reactions and injection site reactions [43].

Hence, a monomeric single-chain antibody-core-streptavidin fusion protein (bfFp) that can form a complex with any biotinylated antigen with one arm and targets the antigen to the DC *via* the DEC-205 receptor with its second paratope may be a desired alternative for *in vivo* DC targeting. Such a system has several unique properties over the other targeting systems including DC targeting with a variety of antigens. Almost any

antigen can be biotinylated by chemical conjugation (for instance, NHS-LC-Biotin), photoactivation (photobiotin acetate) or incorporated by synthetic strategies. This simple strategy avoids the need of encapsulation, chemical cross-linking and construction of a new hybrid fusion antibody. The bfFp in addition lacks Fc domain and the *E.coli* based production is consistent and economical. Faster clearance rate (kidney glomerular filtration cut-off is 70 kDa) and lower immunogenicities are expected due to smaller molecular weight (~46 kDa) of the vector. If required, bfFp stability and half-life can be increased by polyethylene glycol linkage [44] or by isolation of tetrameric form of bfFp. Moreover, such a targeting vehicle can be translated into clinical applications; several bfFp (tetrameric form) have been applied in clinical studies for pretargeting radioimmunotherapy [45, 46].

In this chapter, we have developed a versatile bfFp that can bind any biotinylated antigen and target them to DC DEC-205 receptors using recombinant antibody technology. A scFv that recognizes DEC-205 receptor of DC was successfully cloned from HB290 hybridoma in either V_L-V_H or V_L-V_H orientation and fused with a core-streptavidin domain (core-streptavidin-V_L-V_H, core-streptavidin-V_H-V_L and V_L-V_H-core-streptavidin orientations) and expressed in *E.coli* using T7 expression system. HB290 scFv in the V_L-V_H orientation and the bfFp in V_L-V_H-core-streptavidin orientation appear to be the optimal orientation for expression in *E.coli* system. The mechanism of increased expression is yet to be determined. The bfFp appears to be expressed predominantly as monomeric soluble protein and can be affinity purified from *E.coli* periplasm. ELISA and Western blot were performed using different secondary reagents to demonstrate the bifunctional activity. DC in culture and anti-DEC205 mAb were used to demonstrate the bfFp binding to DC DEC-205 receptors. Biotinylated OVA or biotinylated BSA was used to confirm anti-biotin activity. Both ELISA and Western blot results have shown the bifunctional activity and specificity of the fusion protein. DEC-205 receptor binding was 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) has shown that in the presence of bfFb and anti-CD40 mAb as co-stimulator, both humoral and cell-mediated responses can be augmented. In the multiple antigens

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 very low doses of 200 ng by our DC targeting strategy. Such antigen sparing strategies are important to pandemic 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 33M subjects require only 7 g (at 200 ng dose) of the vaccine antigen. In comparison, a recent commercial influenza vaccine clinical trial used 90 micrograms/patient with modest immune responses requires 3 kg of antigen (450 fold more). Our speculation is that DC targeting efficiently delivers the antigen to the DC than the conventional subcutaneous adjuvant depot that could encounter prolonged exposure to enzymes and uptake by other none DC cells. Another important application of our strategy is data showing the delivery of multiple pathogen antigens for combination vaccines including anthrax protective antigen. The first emergency responders and the defense personnel could use such low dose multiple vaccines in fewer injections.

In summary, both bfFp and anti-CD40 mAb are required to achieve strong immune responses against protein, peptide, glycolipid and naked DNA targeting. Single antigen targeting exhibited strong humoral and cell-mediated immune responses. In comparison, multi-antigen targeting strategy likely exhibited immune responses that are shifted towards the most immunogenic antigen. Higher immune responses were achieved for weak immunogens (MUC-1) in the absence of strong immunogen. 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 concentration of biotinylated DNA (500 ng) in saline is adequate to achieve a strong immune response in mice. Conventional DNA vaccine or therapy strategy often requires high concentration, multiple immunizations, and can not efficiently induce immune responses. More importantly the widely used experimental viral vector based gene targeting strategies may cause severe adverse side-effects. Unique advantages of naked DNA as an antigen are robust, relatively easy to sterilize compared to proteins, cost-effective to make and does not contain viral components. The serum reactivity towards

either the hapten biotin (B-BSA) or the carrier bfFp is minimal. The reactivity towards bfFp appears to be against the core-streptavidin portion of the fusion protein. Immunized and unimmunized mice had immunity (either adaptive or innate immunity towards *streptomyces avidinii*) against the core-streptavidin protein. bfFp targeting of biotinylated antigens to DC could thus be a convenient method to deliver single or multiple antigens to DC. Such formulations can induce immune responses towards any class of antigen including peptide, protein, glycolipids and DNA (DNA vaccine). Lastly, bfFp targeting of DC may present as the potential candidate for development of vaccine or therapy for cancer (GM3, GM2, MUC-1) and bioterrorism agents (WEEV, EBOV, SARS-CoV, Anthrax).

4.5. References

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CHAPTER 5

Design of a Bifunctional Fusion Protein for Ovarian Cancer Drug Delivery: Single-Chain Anti-CA125 Core-Streptavidin Fusion Protein

The contents of this chapter was published in European Journal of Pharmaceutics and Biopharmaceutics 2007;65(3):398-405.

5.1. Introduction

Ovarian cancer is one of the most common malignancies and causes of death affecting women in Canada and the United States. The National Ovarian Cancer Association reports that 1 in 70 Canadian women are affected by ovarian cancer. Despite the aggressive treatment using either surgery or chemotherapy or both, the prognosis for the majority of patients is poor. Metastatic recurrences are common, often in the peritoneal cavity, which ultimately leads to death from the progression of the disease. Newer therapeutic strategies have been developed and are promising for *in vitro* and *in vivo* application and in clinical trials [1-6]. One strategy is targeting the CA125 cancer antigen with B43.13 mouse monoclonal antibody [4, 7]. CA125 is both a membrane-bound and circulating ovarian tumor antigen that is present in the majority of ovarian cancers, especially in those with advanced-stages of the cancer. Our strategy is to design a bifunctional ovarian cancer-specific vector that can target any biotinylated agent to the disease tissue.

In this chapter, we report the successful cloning, expression and purification of a soluble bfFp in *E. coli* using the T7 expression system. The bfFp has two distinct binding sites specific to CA125 and biotin. We have shown that the IMAC purified bfFp can be successfully targeted to human OVCAR-3 cells using ELISA and Western blot studies. BfFp based targeting of biotinylated therapeutic materials or vesicles (liposomes or nanoparticles) to sites of ovarian cancer could be an alternative and convenient method to deliver multiple effective therapies for the treatment of ovarian cancer.

5.2. Materials and Methods

5.2.1. Materials

IPTG (isopropyl ß-D-thiogalactoside), BSA (bovine serum albumin), streptavidin-HRPO (horseradish peroxidise), anti-His_6 mAb (monoclonal antibody), ampicillin, chloramphenicol, FITC (fluorescein isothiocyanate), NHS-LC-Biotin (biotinamidohexanoic acid 3-sulfo-N-hydroxysuccinimide ester), and goat-anti-mouse-HRPO were from Sigma (Oakville, Canada). OVCAR-3 is a CA125 expressing human ovarian adenocarcinoma cell line from ATCC, USA. The plasmid containing the B43.13 single-chain antibody was kindly provided by Biomira, Inc. (Edmonton, Alberta, Canada). The streptavidin gene was provided by Dr. T. Sano, Center for Molecular Imaging, Diagnosis and Therapy and Basic Science Laboratory, Boston, MA, USA. DMEM, 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 as per vendor's protocol. B-liposome [$(biotin)_n$ labeled liposome] was prepared as per previously published protocols [5] using biotin phosphotidyl ethanolamine (Avanti Lipids Inc.). FITC labeling of bifunctional fusion protein was prepared as per vendor's protocol. TMB peroxidase substrate was purchased from Kirkegaard & Perry Laboratory Inc (Gaithersburg, USA). 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, USA). Anti-His_6 in mAb (monoclonal antibody), T7 promoter and terminator primers, and the expression vector pET-22b (+) were from Novagen (Madison, USA). Molecular cloning materials were from Invitrogen (Burlington, Canada). Protein Assay reagent was purchased from Bio-Rad (Mississauga, Canada). Ni-NTA agarose was purchased from Qiagen (Mississauga, Canada).

5.2.2. Cloning of bfFp

The two bfFps were constructed and expressed in different orientations as noted in Fig. 5.1A and B.

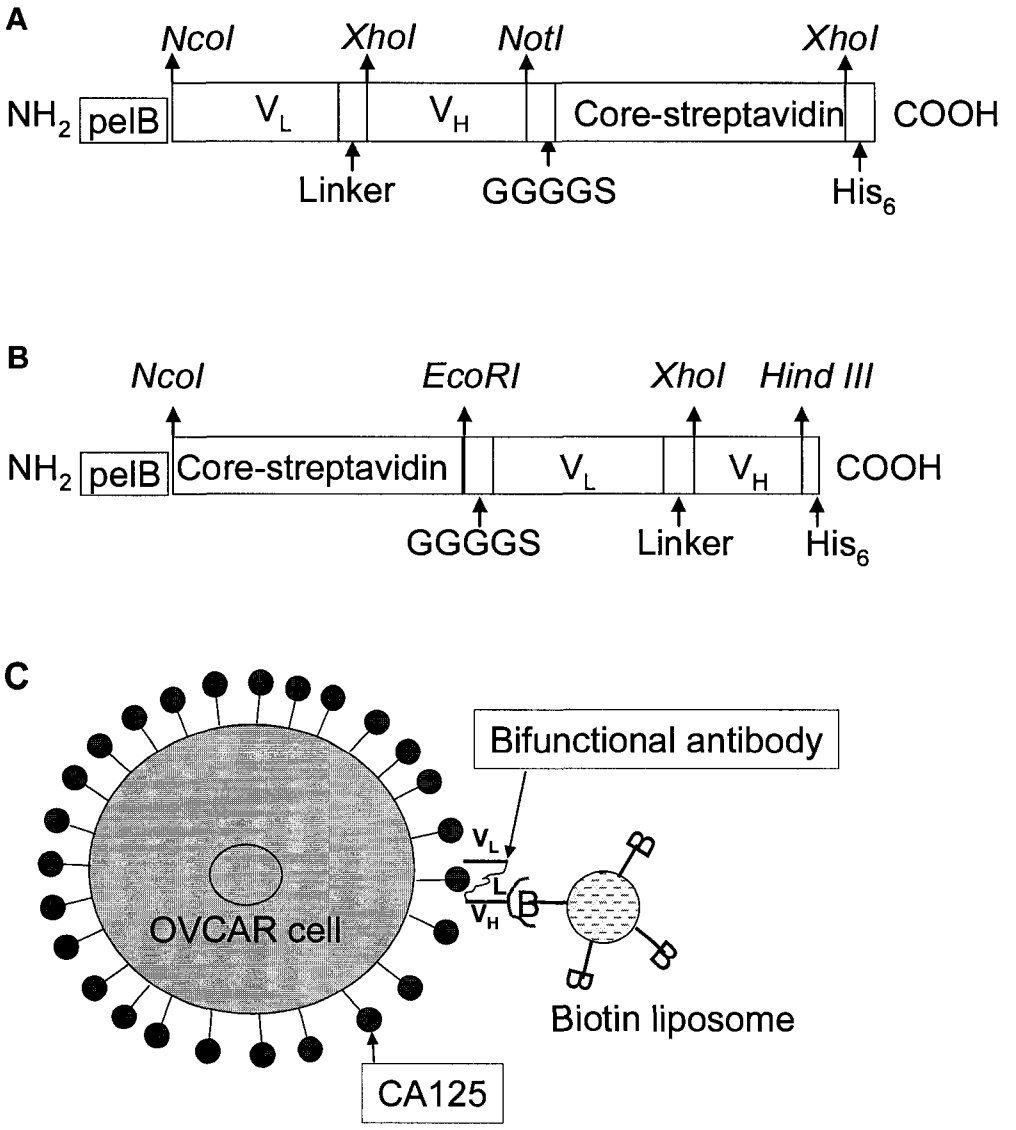

Figure 5.1. The construct of the fusion protein (A) WET8 and (B) WET9. The linker amino acid sequence was $(GGGGS)_5$. Abbreviations: pelB, bacterial leader sequence pelB; V_L, variable domain light chain; V_H, variable domain heavy chain; G, Glycine; S, Serine; His_6, six histidine amino acid tag. (C) Schematic diagram of Biotin labeled liposome loaded with drug targeted to CA125 antigen in OVCAR-3 cells via bifunctional antibody nanoprobes.

The WET8 single-chain antibody coding region was amplified by polymerase chain reaction (PCR) from the plasmid containing the B43.13 single-chain [8] using primers 5'-

ACT ATC GCC ATG GAT GAT ATC GTG ATG TCA CAGT 3' and 5'- TAC TAA GCG GCC GCA GGA GGA GAC GGT GAC TGA 3'. These primers inserted into the restriction sites *Nco*I and *Not*I. The WET9 single-chain antibody coding region was amplified by PCR from the B43.13 plasmid using primers 5'- ATC AGT GAA TTC GGG AGG TGG CGG ATC AGA TAT CGT GAT GTC ACA GTCT 3' and 5' ATT ACT AAG CTT GGA GGA GAC GGT GAC TGAG 3'. These primers inserted into the restriction sites *Eco*RI and *Hind*III. The PCR fragment was gel-purified, double digested with *Nco*I and *Eco*RI or *Eco*RI and *Hind*III, and ligated to pET-22b (+) containing the core-streptavidin gene [9]. The positive clones were screened and characterized by both PCR and restriction digestion fragment mapping (*Nco*I and *Eco*RI or *Eco*RI and *Hind*III). The positive cloned fragment was sequenced using T7 promoter and terminator primers by CEQ^{TM}2000 (Beckman Coulter USA).

5.2.3. Analysis of Recombinant Functional Clones

The recombinant plasmid containing the correctly oriented fusion scFv gene was used to transform *E. coli* BL21-CodonPlus® (DE3)-RIPL for recombinant protein expression. *E. coli* transformants were cultured in 10 ml 2x YT medium (16 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl, pH 7.5) containing amipicllin 100 μg/ml and chloramphenicol 50 μg/ml and incubated at 37°C with shaking at 250 rpm until an OD_{600} of ~0.4-0.5 was reached. The bacterial culture was induced with 1 mM IPTG and allowed to grow 5 hrs at 30°C. The culture was then harvested by centrifugation at 4,000xg for 10 min at 4°C. The total cell lysate was prepared by addition of Laemmli sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5mM 2-mercaptoethanol) to the pellet and heating at 95°C for 5 min. Total cell protein of induced and uninduced culture was analyzed by SDS-PAGE using 10% polyacrylamide gel performed according to the published method with a Biorad Mini Protean II apparatus. The protein gel was stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 10% acetic acid and 45% methanol and destained with 10% acetic acid and 30% methanol.

5.2.4. Medium Scale Expression and IMAC Purification of Fusion Protein

The pWET8 and pWET9 plasmids were chemically transformed into BL21-CodonPlus® (DE3)-RIPL, and colonies were grown overnight and selected from 2x YT plates containing ampicillin 100 μg/ml and chloramphenicol 50 μg/ml. An ampicillin resistant clone was picked and grown at 37°C in liquid 2x YT medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. Once the OD_{600nm} reached ~0.6, 500 μM IPTG and 0.4 M Sucrose were added, and the culture incubated at 26°C for 5 hrs. The IMAC purification protocol is described in chapter 3 [9]. The various fractions such as the induced and uninduced culture cytoplasm, periplasm, IMAC washings of fusion proteins, and the eluted material were analyzed by SDS-PAGE using 10% polyacrylamide gels under reducing conditions (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 5mM 2-mercaptoethanol), following staining with Coomassie Brilliant Blue. The samples were incubated at 95°C for 10 min prior to loading on the polyacrylamide gel.

5.2.5. Western Blot: Fusion Protein Bispecificity and Binding to OVCAR-3 Cells

The OVCAR-3 cell line used was grown in DMEM-10 [10% (v/v) FBS and 1% (v/v) PSG] in a tissue culture plate and CA125 expression was confirmed using B43.13 antibody. Western blot was performed on the OVCAR-3 cells under reducing or non-reducing conditions (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and probed with the following reagents: a) IMAC purified WET9, $(biotin)_n$ labeled BSA followed by streptavidin-HRPO, or b) IMAC purified WET9, mouse anti-His_6 mAb and goat anti-mouse-HRPO. The OVCAR-3 cells (1×10^5) were first electrophoresed on SDS-PAGE using 10% polyacrylamide gel and transferred to a Hybond ECL nitrocellulose membrane using the Trans blot apparatus (Bio-Rad) as per manufacturer's instructions. The membrane was blocked with 5% skim milk in PBST (0.1% Tween 20 in phosphate buffered saline, pH 7.3) for 2 h at RT. The membrane was washed three times with PBST and incubated with WET9 for 2 h at RT. After washing three times with PBST, the membrane was incubated with $(biotin)_n$ labeled BSA or mouse anti-His_6 mAb for 1 h at RT. After incubation, the membrane was washed three times with PBST and further incubated with streptavidin-HRPO or goat anti-mouse-

HRPO, respectively, for 1 h at RT. Finally, the membrane was washed four times with PBST and ECL-based detection was performed according to the manufacturer's instructions.

5.2.6. Characterization of Fusion Proteins

IMAC purified WET9 and WET8 were incubated either at 60, 70, 80, or 95°C for 10 min under reducing conditions prior to analysis in 10% SDS-PAGE. Gel resolved proteins were electrophoretically transferred onto a nitrocellulose membrane and probed with mouse anti-His_6 mAb with goat anti-mouse-HRPO.

5.2.7. ELISA: Fusion Protein Bispecificity and Binding to OVCAR-3 Cells

Cell lines used in this study followed the same culture conditions as the OVCAR-3 cells. OVCAR-3 or EMT6 mammary carcinoma cells were coated on a 96-well V-bottom plate (Nunc, Denmark) in quadruplicate (1.0×10^6 cells/well). The plates were washed four times with PBS by suspension and centrifugation of the cell lines and blocked with 1% PBS dialyzed BSA for 2 h at 4°C. After incubation, the plates were washed with PBS, and bfFp (0 or 10 μg/ml in 100 μl volume) were added. The plates were incubated for 2 h at 4°C, and then washed with PBS. B-BSA (100 μg/ml in 100 μl volume) or B-liposome (10 μg/ml in 100 μl volume) was added to each well and incubated for 1 h at 4°C. After incubation, the plates were washed, and then incubated with streptavidin-HRPO (10 μg/ml in 100 μl volume) for 1 h at 4°C. The plates were then washed with PBS, and TMB substrate was then added at 100 μl/well. The OD_{650nm} was taken after 10 min using an ELISA V_{max} kinetic microplate reader (Molecular Devices Corp, California, USA). Statistical analyses were performed using Sigma 2000 and Minitab 13.30 statistical software to determine the significance between binding in the presence and absence of bfFp. Values were expressed as a mean of the quadruplicate. The analysis for each of the detection methods was done using the student t-test of significance and P value with 95% confidence interval. In all experiments, a value of $P<0.05$ indicated a significant difference.

5.2.8. Affinity Analysis of bfFp

The bfFp affinity constant was determined by indirect ELISA method described above using B-BSA for detection of the bifunctional activity to OVCAR-3 except the bfFp concentration used were 100, 60, 40, 20 and 1 nM.

5.2.9. Confocal Microscopy: Enhanced Binding of bfFp to OVCAR-3

OVCAR-3 (1×10^4) were transferred from a tissue culture flask to a chambered cover-glass slide and incubated overnight to allow adherence of the ovarian cancer cells. The cells were washed three times with PBS and then mixed and incubated with FITC labeled bfFp (2 μg/ml) for 1 h at 4°C. After incubation, the cells were washed three times with PBS and then fixed with 4% (w/v) paraformaldehyde. Coversglass slides were then mounted and viewed with a Zeiss LSM 510 confocal laser microscope. Images were edited using the Adobe Photoshop software program (Adobe Systems, Mountain View, CA). A human osteosarcoma cell-line (143B) was used as a control.

5.3. Results

5.3.1. Construction, Expression, and Purification of bfFp

The plasmid vectors pWET8 and pWET9 were constructed by inserting the sequence encoding the B43.13 single-chain anti-CA125 antibody into a core-streptavidin containing pET22b (+) plasmid [9] next to pelB leader sequence (Fig. 5.1A and B). *E .coli* transformed with this plasmid should generally express the recombinant protein into the periplasmic space, which allows convenient purification of the protein by IMAC column. To verify that the transformed cells expressed WET8 and WET9 protein, cells were induced with and without IPTG, and then total cell lysates were prepared and separated on SDS-PAGE. It was observed from SDS-PAGE that the different clones expressed different levels of fusion protein at the expected MW band at ~43 kDa (Fig. 5.2A).

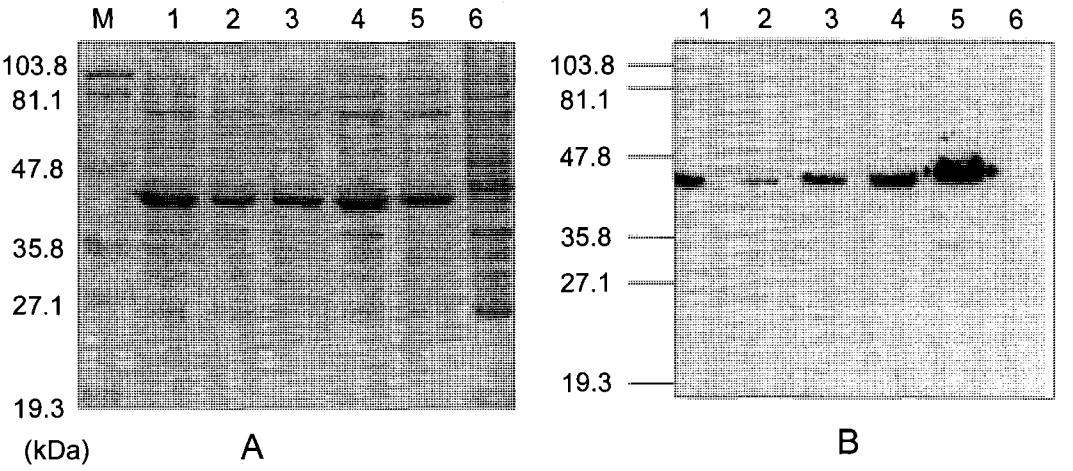

Figure 5.2. Expression of WET9 and WET8 clones. (A) SDS-PAGE of WET9 and WET8 clones. Lane M: Mol. Wt. markers; Lanes 1-4: different clones of WET9; Lane 5: WET8 clone; Lanes 6: Uninduced culture. (B) Western blot analysis probed with anti-His_6 monoclonal antibody. Lanes 1-4: different clones of WET9; Lane 5: WET8 clone; Lanes 6: Uninduced culture

Expression of the fusion protein was confirmed by Western blot analysis using the anti-His_6 mAb (Fig. 5.2B). The highest producing clones were selected for medium scale (2 L) expression in culture flask and IMAC purification. The SDS-PAGE analysis of the various fractions (total cell protein, cytoplasmic and periplasmic fractions) showed the expression of the fusion protein in the periplasmic fraction. The periplasmic fraction containing bfFp was then purified by an IMAC column. The IMAC pure periplasmic extracts of WET9 induced cultures showed a band at 43.5 kDa (Fig. 5.3A, Lanes 1-3).

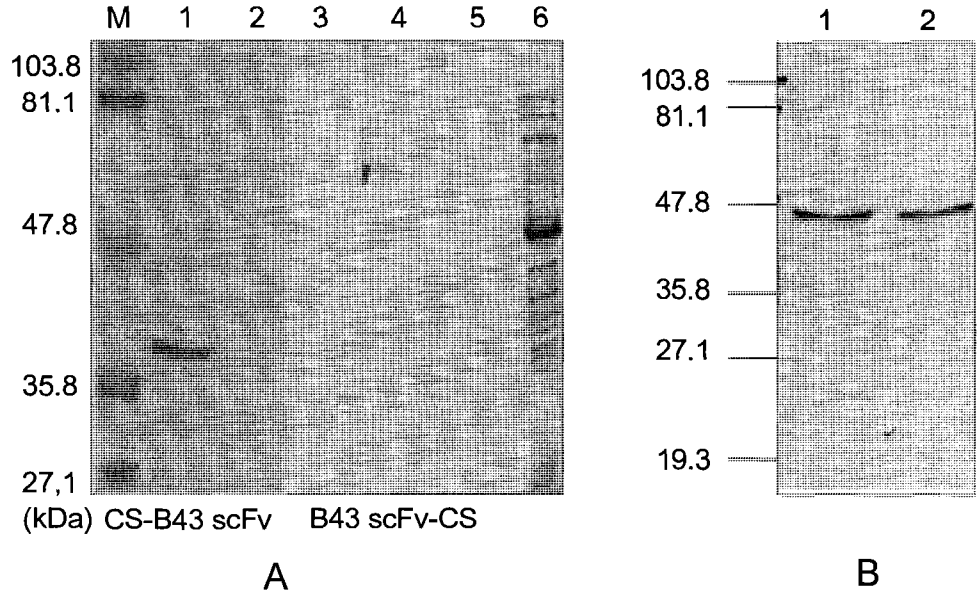

Figure 5.3. Expression, IMAC purification and characterization of WET9 and WET8. (A) Lane 1: WET9 Periplasmic protein; Lanes 2: IMAC purified WET9; Lane M: Mol. Wt. markers; Lanes 3-5: IMAC purified WET8; Lane 6: WET8 Total cell protein. (B) Western blot analysis of IMAC purified WET9 probed with anti-His_6 monoclonal antibody at 95°C and 65°C. Lane 1: WET9 at 95°C; Lane 2: WET9 at 65°C

This was not apparent in the WET8 induced culture with the alternate orientation of the recombinant construct (Fig. 5.1A). However, the expression level of the total cell protein of both the constructs were similar. In the WET9 construct, the amount of soluble scFv protein was higher than WET8 construct (Fig. 5.3A). The SDS-PAGE for the WET9 IMAC-purified bfFp appeared to coelute with a few minor bands after 95°C of heating, on SDS-PAGE for WET9. Approximately 1.5 mg of WET9 bfFp was affinity purified from a 2 L culture. No apparent band was seen in the WET8 IMAC purified fraction even after affinity purification (Fig. 5.3A). The WET8 fusion protein appeared to be in inclusion bodies. By inverting the core-streptavidin and single-chain antibody gene arrangement (Fig. 5.1B), we were able to generate soluble bfFp without the need to refold proteins from inclusion bodies.

5.3.2. Characterization of bfFp

The bfFp and native streptavidin starts to dissociate into monomeric forms at 70°C, and dissociates completely into monomeric forms at 95-100°C [9-13]. When analyzing the IMAC-purified bfFp (WET9) after incubation at 65°C and 95°C by SDS-PAGE and Western blot with anti-His_6 mAb, the bfFp in monomeric form was detected (Fig. 5.3B). The expression of the predominant monomeric form may be due to the difference in the linker between core-streptavidin and single-chain antibody.

Western blot and ELISA was performed to demonstrate the bfFp activity, including both the anti-CA125 activity on OVCAR-3 cells and the anti-biotin activity employing B-BSA or B liposome preparations with streptavidin-HRPO for detection. In Western blot, the OVCAR-3 cell extract was loaded on a SDS-PAGE under reducing and non-reducing conditions (Fig. 5.4A, Lane 1 and Lane 2) and transferred to a nitrocellulose membrane and probed with WET9, mouse anti-His_6 monoclonal antibody and goat anti-mouse-HRPO (Fig. 5.4B).

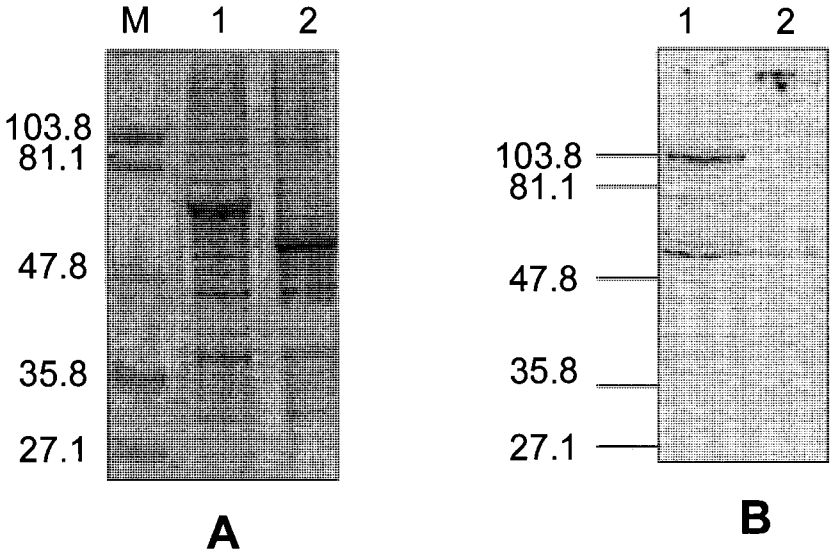

Figure 5.4. Demonstration of IMAC purified WET9 binding to OVCAR-3 by Western blot. (A) SDS-PAGE analysis of OVCAR-3 cell line. (B) Western blot probed with WET9, mouse anti-His_6 monoclonal antibody and goat anti-mouse-horseradish peroxidase. Lane 1, OVCAR-3 was heated at 95°C for 10 min under reducing condition; lane 2, cells were heated at 95°C under non-reducing condition.

Detection using the recombinant monoclonal antibody showed the high MW band of CA125 at the very top of the SDS-PAGE under non-reducing conditions. This is characteristic of large glycoproteins such as CA125 as confirmed by other groups [30]. The data showed that the WET9 bispecific fusion protein binds to OVCAR-3 cells under both reducing and non-reducing conditions as detected by anti-His_6 mAb (Fig. 5.4B, Lane 1 and Lane 2). In the Western blot study, the bfFp demonstrated binding to OVCAR-3 CA125 antigen. Under reducing conditions, binding was observed at lower MW bands. The difference in the binding pattern might be due to the presence of 2-mercaptoethanol which causes the CA-125 antigen to dissociate into polypeptide chains [32]. The CA-125 antigen has amino acid repeat domains which encompass the epitope binding sites [35].

The bispecific binding property of the WET9 protein employing various preparations was determined using the ELISA method (Fig. 5.5).

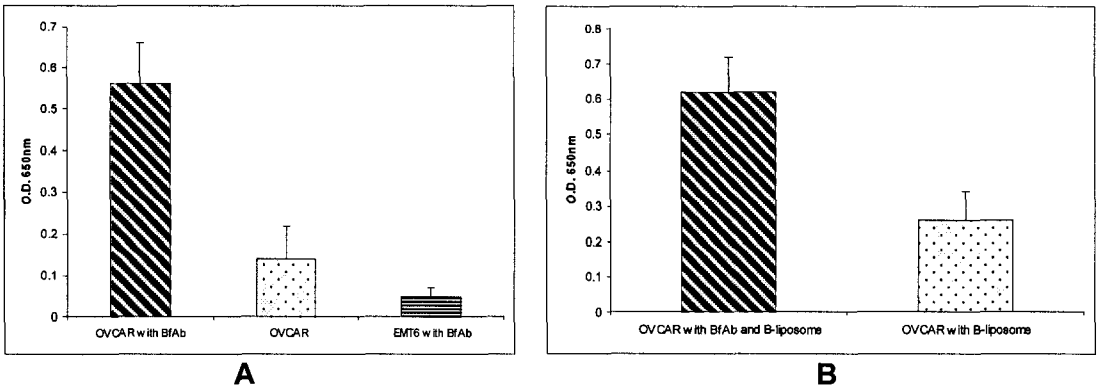

Figure 5.5. Enhanced binding of bfFp to OVCAR-3 cells and biotinylated liposome by ELISA. OVCAR-3 cells were plated on a 96-well V bottom well plate in quadruplicate at 1 x 10^6 cells/well. The plates were blocked with 1% PBS dialyzed BSA. The plates were washed and bfFp was added. The plates were again washed, (biotin)n-labeled BSA (A) or (biotin)n-labeled liposome (B) was added along with streptavidin-HRPO in a second step. After incubation the plates were washed and TMB was added, and OD_{650} was taken after 10 min using a microplate reader. Bars show the relative binding of WET9 to the different cell lines. The error bars are the standard deviations.

The OVCAR-3 cells and EMT6 mouse mammary cells, used as controls, were tested for the bfFp reactivity. B-BSA and B-liposomes were used to confirm the bispecificity of bfFp bound to OVCAR-3 cells. Streptavidin-HRPO and TMB substrate detected the relative bfFp binding activity (Fig. 5.5). Values are expressed as mean ± standard deviation of quadruplicates. The statistical analysis for each of the detection methods was determined by the student t-test of significance; a p value with 95% confidence interval ($p<0.05$) was used to indicate a significant difference. The concentrations of all reagents were fixed. The results show that in the absence of bfFp, B-BSA does not bind significantly to OVCAR-3 cells. The control EMT6 mouse mammary carcinoma cells, which do not express CA125 antigen, show negligible binding of B-BSA in the presence of bfFp (Fig. 5.5A). A similar pattern was seen when B-liposomes were used (Fig. 5.5B). The OD value, in the absence and in the presence, of the bfFp was compared and indicated that in the presence of bfFp, B-liposomes bind to OVCAR-3 cells. An additional ELISA was performed to determine the affinity constant of the fusion antibody WET9; 2.6×10^7 L/mol.

CLSM was carried to demonstrate bfFp binding to OVCAR-3. The slides were incubated for 1 h at 4°C to allow binding of FITC labeled bfFp to OVCAR-3. The cells were washed with PBS and then fixed with paraformaldehyde. Processing by CLSM showed that the FITC labeled bfFps were bound to OVCAR-3 (Fig. 5.6A), in comparison to the control 143B cells (Fig. 5.6B).

Figure 5.6. Confocal microscopy study: enhanced binding of bfFp to OVCAR-3. The OVCAR-3 and 143B cells were used in the confocal microscopy binding study. The FITC labeled bfFp was incubated with OVCAR-3 or 143B cells for 1 h at 4°C. Cells were washed with PBS and then fixed with paraformaldehyde. A, picture of OVCAR-3 with FITC labeled bfFp. B, picture of 143B cells with FITC labeled bfFp.

The majority of fluorescence was detected appeared to accumulate on the OVCAR-3 surface indicating that FITC labeled bfFp specifically binds to OVCAR-3.

5.4. Discussion

Ovarian cancer patients usually undergo surgery and/or chemotherapy as part of their primary treatment; however, metastatic disease and/or drug resistant variants manifest within 18 months [14]. Newer strategies such as targeting the CA125 antigen with B43.13 mouse monoclonal antibody has shown early promise [4, 7]. B43.13 promotes both humoral and cell-mediated immune responses by forming immune complexes with CA125 [4, 6]. In addition, B43.13 may initiate a classical idiotype network of immune responses. A human anti-mouse antibody response (HAMA effect) to B43.13 bound to the CA125 antigen, would in turn stimulate an anti-idiotype cascade response resulting in a humoral polyclonal immune response against CA125 [6, 15]. HAMA and antibody responses are frequently observed in such antibody targeting

strategies, and are not associated with limiting toxicities related to hypersensitivity [4, 16-17]. Although, it has been recently reported that antibody targeted treatment is well tolerated without serious adverse events or discontinuations due to therapy [7], CA125 targeting with monoclonal antibody has limitations. Antibody targeting strategies could not consistently activate immune responses and overcome immune escape mechanisms, especially in patients with a significant disease burden [7]. Consequently, utilizing multiple therapeutic strategies could add value as an effective treatment. However, multiple therapies are costly and therapeutic complications are likely; therefore, the development of a universal ovarian cancer cell targeting vehicle that could deliver multiple therapies might be desirable.

Previously, a strategy has been developed in our laboratory for targeting drug carriers (biotinylated liposome) to human ovarian cancer and squamous cancers cells *in vitro* and *in vivo* [5, 31, 33]. The *in vivo* strategy consisted of sequential intraperitoneal administration of the bispecific monoclonal antibody (with anti-CA125 and anti-biotin epitopes) coupled to biotinylated and radiolabeled liposomes to ovarian cancer bearing mice [31]. Employing the same construct we have shown the targeting of liposome loaded with fluorescent hapten (as a surrogate drug) to ovarian cancer cells [5]. Liposomes are well-known for their ability to reduce toxicity, enhance antitumor efficacy, and improve the therapeutic index of certain anti-cancer drugs [18]. Furthermore, immunoliposomes (antibody coated liposomes) encapsulated with drugs have been applied for tumor therapy and have shown to be more immunologically recognized than the free liposome [19-20]; however, this targeting strategy has yet to be applied in clinical studies. Pretargeting radioimmunotherapy of a single-chain antibody-streptavidin fusion protein has been applied in clinical studies and is very promising as a therapy [21-24, 34]. Radioimmunotherapy of solid tumors has been largely ineffective due to suboptimal tumor uptakes, slow serum elimination, and bone marrow toxicity. Unlike the direct radiolabeled antibodies, in pretargeted therapy, a non-radioactive tumor-reactive fusion antibody is first administered. After maximal accumulation of the fusion protein in the tumor, the radiolabeled biotin is administered for targeting and irradiation of the tumor. The pretargeted therapy enables the delivery of high doses of radioactivity to tumors resulting in a significantly increased therapeutic index as compared to the traditional

radioimmunotherapy approaches [25]. The disadvantage is that streptavidin is known to be taken up by the kidney and several studies have described renal radiotoxicity using the pretargeting approach [26-29]. A recent study has shown that succinylation of the fusion construct greatly reduces kidney uptake of the subsequently administered radiolabeled biotin, which greatly improves the therapeutic index associated with multistep immune targeting approaches to radioimmunotherapy [29]. As a result, development of a smaller single-chain antibody-core-streptavidin fusion protein would be desirable. The smaller fusion protein could potentially be applicable for the pretargeted radioimmunotherapy of ovarian cancer.

In our study, we have designed a newer targeting strategy that could be tested in clinical studies (Fig. 5.1C). One of the limitations of our previous chemical biotinylation of full length antibodies (>200 kDa) is the heterogeniety of the conjugates and potential immunogenicities of the full length streptavidin [5, 31]. A bfFp (~44 kDa) that can bind to both CA125 antigen and biotin was developed using recombinant antibody technology. The bfFp is expressed predominantly as monomeric soluble protein and can be affinity purified from *E. coli* periplasm. In addition, by inverting the core-streptavidin and single-chain antibody gene arrangement (Fig. 5.1), the production of the periplasmic soluble bfFp is increased. The mechanism of increased production is yet to be explained, but we suspect the gene inversion caused proper folding of the protein, which allowed the leader peptide (pel B) to transport the protein into the periplasm. The bfFp developed and applied from other groups is in a tetrameric form, whereas our bfFp was predominantly monomeric. The difference in isoform may be due to the variations in the linker between core-streptavidin and the single-chain antibody. The potential advantages of the monomeric form over the tetrameric form include: less immunogenicity, rapid blood clearance, and better tumor penetration due to the smaller molecular weight of the fusion protein. The bfFp activity was demonstrated using ELISA and Western blot studies. The ELISA method used human OVCAR-3 cells and biotinylated BSA or biotinylated liposomes, both of which bear multiple biotin moieties. The bfFp bound the OVCAR-3 cells with the B43.13 scFv antigen, while the second core-streptavidin antigen bound to $(biotin)_n$-BSA or $(biotin)_n$–liposome. As a result of the multiplicity of biotin haptens in the complex, cross linking and specific targeting could be demonstrated as shown

diagrammatically in Fig. 5.1C. ELISA and Western blot studies have shown and confirmed the bifunctional activity and its specificity to both CA125 and biotin (Fig. 5.4, 5.5). In addition, Confocal microscopy study has also confirmed the specificity in binding to CA125 (Fig. 5.6). In summary, in the presence of bfFp, there was enhanced binding of biotinylated antigen and liposome to OVCAR-3 cells. Further characterization of the bfFp including an extensive biodistribution study in an *in vivo* murine model of ovarian cancer that will include subsequent immune responses and an immuogenicity study is in progress. The bfFp could be tested *in vivo* to deliver any biotinylated therapeutic-biomaterials to ovarian cancer cells including biotinylated drugs, toxins, therapeutic proteins, biotin liposomal encapsulated vesicles or biotinylated nanoparticles with drugs or radioisotopes [33-34]. Furthermore, the bfFp could be applied in pretargeting radioimmunotherapy of ovarian cancer. The bfFp targeting of biotinylated therapeutic materials or clinical relevant pretargeting therapy to ovarian cancer could be an alternative and convenient method to deliver effective therapy in an adjuvant setting for ovarian cancer patients.

5.5. References

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CHAPTER 6
Conclusion

6.1. Summary of the Bispecific and Bifunctional Versatile Targeting Systems

Bispecific antibodies and bifunctional fusion proteins can be designed to bind any two different antigens for numerous applications in drug delivery and immune system targeting. Most of the protein based drug delivery and immune system targeting vehicles are not versatile enough to deliver multiple and distinct classes of molecules, thus limiting its potential applications. Development of bispecific antibodies and bifunctional fusion proteins that can bring multiple molecules towards the target sites is a promising strategy to overcome the current limitations. To validate the concept, three strategies were explored to investigate the feasibility of using bispecific antibodies and bifunctional fusion proteins for targeting dendritic cells and ovarian cancer.

The first strategy was to develop a universal DC targeting vehicle that could be a convenient method to deliver any type of antigen. A quadroma (hybrid-hybridoma) P125 secreting bispecific monoclonal antibodies (bsmAb), with one paratope specific for mouse DC DEC-205 and another paratope specific for biotin, was developed by PEG-fusion of the two parental hybridomas and selected by a fluorescence activated cell sorter. The bsmAb was purified using a biotin-Agarose affinity column and the bsmAb activity was demonstrated using ELISA method employing mouse bone marrow DC and biotinylated BSA. Both confocal microscopy and ELISA studies have shown enhanced binding and internalization of biotinylated and FITC-labelled M13 bacteriophage to DC cell in the presence of bsmAb. *In vivo* studies in mice with biotinylated OVA has shown that in the presence of bsmAb and co-stimulatory anti-CD40 mAb, both humoral and cell-mediated responses can be augmented. In addition, only a low concentration of antigen (500 fold less) is needed using bsmAb targeting. A similar immune response in mice without targeting requires higher amounts and complete Freund's adjuvant. In the absence of traditional adjuvants, bsmAb targeting of biotinylated antigens to DC could be an alternative, convenient method to deliver antigens to DC and other diseases. Moreover, this method could be an alternative method to *ex vivo* stimulation of DC to overcome DC defects and for treatment of cancer.

The *in vivo* study of targeting antigens to DC stems from the hypothesis that efficient targeting of the antigen to the desired cell population enhances the immune response. In disease states such as cancer and infection, DC may exhibit inefficient migration to the site of infection or impaired antigen uptake/processing. Targeting strategies with co-stimulation may help overcome some aspects of migratory deficiencies and facilitate the antigen uptake processes. For instance, DC loaded with killed breast cancer cells can induce differentiation of tumor-specific cytotoxic T cells. In addition, defective T-cell function in patients with advanced breast cancer can be overcome by stimulation with DC generated from precursors. These studies involved culturing DC with antigens *ex vivo* as a substitute for targeting. *Ex vivo* stimulation of DC with antigens have been studied in several clinical fields. The disadvantage is that, isolation of DC from individual patients is very laborious and costly. As an alternate strategy, direct delivery of antigens to DC may be a desirable strategy. Injection of engineered viral vectors to express antigens in activated DC may provide effective vaccines for priming an immune response; however, more *in vivo* studies are required to address the efficiency and safety issues.

DEC-205 targeting of antigens by an antibody is efficient and leads to T cell activation. Moreover, DEC-205 route of targeting could protect the host against tumor growth and enhance resistance to an established rapidly growing tumor, as well as viral infection. Antibody-mediated antigen targeting *via* the DEC-205 receptor increases the efficiency of vaccination for T cell immunity, including systemic and mucosal resistance in disease models. Hence, targeting of disease specific antigens to the DC DEC-205 receptor could be a potential route for a variety of therapeutic applications. However, generation of antibody-mediated antigen targeting system is a complex and time consuming process, which requires either the encapsulation of the antigen, chemical cross-linking with the antigen or construction of a new hybrid antibody. Furthermore, comparison studies with different antigens and the use of multiple antigens targeting to DC is often difficult. Hence, a bsmAb that can bind any biotinylated antigen and target them to DC DEC-205 receptors would be desirable. Numerous biotinylating reagents are commercially available that can derivatize almost any antigen be it a protein, nucleic acid,

carbohydrate or a glycolipid. However, quadroma technology has several limitations; application is limited to animals studies.

In the second strategy, a recombinant bifunctional fusion protein (scFv-CS fusion protein) that can target both biotin and DC DEC-205 was designed. CS was successfully cloned and expressed in *E. coli*. Core-streptavidin was expressed in shaker flask culture as a soluble protein, isolated by periplasmic extraction, purified by IMAC column, and analyzed for its size, thermal stability and biotin-binding activity. In Western blots using streptavidin-HRPO as a probe, a contaminant that co-purified with core-streptavidin was found and identified as biotin carboxyl carrier protein (BCCP). Although BCCP can not be detected on SDS-PAGE, it appears as a prominent band in Western blot when probed with streptavidin peroxidase conjugate. Based on the results from *in vitro* gel digestion, mass spectrometry and Mascot database search results, we confirmed the presence of BCCP. It was found that BCCP can complex with core-streptavidin and can dissociate when heated above 80°C. BCCP could be successfully removed and recovered by employing core-streptavidin immobilized magnetic beads under mild conditions. In addition, the enriched fractions of core-streptavidin oligotetramers were also separated, which may be the by-products of BCCP binding to core-streptavidin in various ratios. Finally, ELISA results have shown that the amount of biotin-horseradish peroxidase binding to core-streptavidin was higher compared to commercially available streptavidin. After the successful development and characterization of the CS, a bifunctional fusion protein that targets dendritic cell and biotin labeled compounds was developed.

In *E. coli* system, CS has been expressed either as fusion protein or alone, both as an insoluble or soluble protein. The CS and its fusion proteins have been exploited extensively for a variety of *in vitro* and *in vivo* applications. The fusion proteins of CS have been utilized for applications in cancer immunotherapy and pretargeted lymphoma therapy. However, a potential contaminant while expressing and purifying CS in *Escherichia coli* has thus far merited limited attention. BCCP is the only naturally biotinylated proteins in *Escherichia coil,* and it is an essential component of acetyl-CoA carboxylase to catalyze the first committed and rate-limiting step of fatty acid synthesis. In Chapter 3, the CS was successfully expressed in *E. coli* as a soluble protein, and during IMAC purification BCCP was identified as the contaminant that co-purifies and binds to

CS. Nevertheless, BCCP can be removed from soluble CS *via* competitive binding to CS immobilized magnetic bead under mild conditions. Higher biotin binding activity can be achieved using my purification method compared to commercially available streptavidin produced from *E.coli*. This method can be used for small-scale purification and recovery of pure CS, CS fusion protein, and BCCP expressed in *Escherichia coli* expression system.

In Chapter 4, a single chain antibody that recognizes DEC-205 receptor of DC was fused with a core-streptavidin domain and expressed in *E.coli* using T7 expression system. The bifunctional fusion protein was expressed as periplasmic soluble protein and IMAC affinity purified in its monomeric form. The bifunctional activity against DEC-205 and biotin were characterized by ELISA and Western blot. *In vivo* immune response studies in mice with biotinylated ovalbumin, Ebola virus glycoprotein 1, Severe acute respiratory syndrome-coronavirus spike Receptor Binding Domain, epithelial mucin 1 cancer peptide, Anthrax Protective Antigen, gangliosides GM3, GM2, Western equine encephalitis virus structural DNA, EBOV GP1,2 DNA, SARS-CoV spike DNA and SARS-CoV membrane DNA were conducted. In the presence of bfFb and anti-CD40 mAb, both humoral and cell-mediated responses were augmented in either single antigen or multiple antigens targeting strategy of protein, DNA, glycolipids and peptide.

In vivo targeting of 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. A bsmAb produced from a quadroma is not suitable for clinical applications and the limitations are the yields and purity. Major drawback for this systems could also be dose-limiting toxicity (prolonged circulation time) and immunogenicity against the targeting system that can alter pharmacokinetics (biodistribution and clearance), block receptor-antigen interactions, induce hypersensitivity reactions and injection site reactions. Hence, a monomeric single-chain antibody-core-streptavidin fusion protein (bfFp) that can form a complex with any biotinylated antigen with one arm and targets the antigen to the DC *via* the DEC-205 receptor with its second paratope may be a desired alternative for *in vivo* DC targeting. Such a system has several unique properties over the other targeting systems mentioned above including DC targeting with a variety of antigens. The bfFp in addition lacks Fc

domain and the *E.coli* based production is consistent and economical. Faster clearance rate and lower immunogenicities are expected due to smaller molecular weight of the vector. If required, bfFp stability and half-life can be increased by polyethylene glycol linkage or by isolation of tetrameric form of bfFp. Moreover, such a targeting vehicle can be translated into clinical applications; several bfFp (tetrameric form) have been applied in clinical studies for pretargeting radioimmunotherapy.

Most antigens were administered at very low doses of 200 ng by the DC targeting strategy. Such antigen sparing strategies are important to pandemic management if large populations need vaccination. A theoretical calculation suggest that in the event of a SARS-CoV or H5N1 avian influenza outbreak in Canada, the DC targeted vaccine needed to cover 33 million subjects require only 7 g (at 200 ng dose) of the vaccine antigen. In comparison, a recent commercial influenza vaccine clinical trial used 90 micrograms/patient with modest immune responses requires 3 kg of antigen (450 fold more). The speculation is that DC targeting efficiently delivers the antigen to the DC than the conventional subcutaneous adjuvant depot that could encounter prolonged exposure to enzymes and uptake by other none DC cells. Another important application of the strategy is data showing the delivery of multiple pathogen antigens for combination vaccines including anthrax protective antigen. The first emergency responders and the defense personnel could use such low dose multiple vaccines in fewer injections.

Naked DNA antigens were also administered at low doses using the DC targeting strategy. Conventional DNA vaccine or therapy strategy often requires high concentration, multiple immunizations, and can not efficiently induce immune responses. More importantly the widely used experimental viral vector based gene targeting strategies may cause severe adverse side-effects. Thus, with the above advantages, the bfFp targeting of DC may be a potential candidate for development of a universal vector for vaccine or therapy in cancer and infectious diseases.

In the last strategy, a universal ovarian cancer cell targeting vehicle that can deliver biotinylated therapeutic formulations was developed. A single chain antibody variable domain (scFv) that recognizes the CA125 antigen of ovarian cancer cells was fused with a core-streptavidin domain (core-streptavidin-VL-VH and VL-VH–core-streptavidin orientations) using recombinant DNA technology and then expressed in *E. coli* using the

T7 expression system. The bifunctional fusion protein was expressed and affinity purified for ovarian cancer cell targeting. The two distinct activities (biotin binding and anti-CA125) of the bfFp were demonstrated using ELISA, Western blot and confocal laser-scanning microscopy methods. The ELISA method utilized human OVCAR-3 cells and biotinylated bovine serum albumin or biotinylated liposomes, whereas, the Western blot involved probing with B-BSA. The CLSM study has shown specificity in binding to the OVCAR-3 cell-line. ELISA and Western blot studies have confirmed the bifunctional activity and specificity. In the presence of bfFp, there was enhanced binding of biotinylated antigen and liposome to OVCAR-3 cells. In contrast, the control EMT6 cells, which do not express the CA125 antigen, showed minimal binding of the bfFp.

Ovarian cancer patients usually undergo surgery and/or chemotherapy as part of their primary treatment; however, metastatic disease and/or drug resistant variants usually occur. Newer strategies such as targeting the CA125 antigen with mouse monoclonal antibody have shown early promise. It has been recently reported that antibody targeted treatment is well tolerated without serious adverse events, CA125 targeting with monoclonal antibody has limitations. Antibody targeting strategies could not consistently activate immune responses and overcome immune escape mechanisms, especially in patients with a significant disease burden. Consequently, utilizing multiple therapeutic strategies could add value as an effective treatment. However, multiple therapies are costly and therapeutic complications are likely; therefore, the development of a universal ovarian cancer cell targeting vehicle that could deliver multiple therapies might be desirable.

Pretargeting radioimmunotherapy of a single-chain antibody-streptavidin fusion protein has been applied in clinical studies and is very promising as a therapy. Radioimmunotherapy of solid tumors has been largely ineffective due to suboptimal tumor uptakes, slow serum elimination, and bone marrow toxicity. Unlike the direct radiolabeled antibodies, in pretargeted therapy, a non-radioactive tumor-reactive fusion antibody is first administered. After maximal accumulation of the fusion protein in the tumor, the radiolabeled biotin is administered for targeting and irradiation of the tumor. The pretargeted therapy enables the delivery of high doses of radioactivity to tumors resulting in a significantly increased therapeutic index as compared to the traditional

radioimmunotherapy approaches. The disadvantage is that streptavidin is known to be taken up by the kidney and several studies have described renal radiotoxicity using the pretargeting approach. As a result, development of a smaller single-chain antibody-core-streptavidin fusion protein would be desirable. The smaller fusion protein could potentially be applicable for the pretargeted radioimmunotherapy of ovarian cancer. The bfFp that have been developed could be tested *in vivo* to deliver any biotinylated therapeutic-biomaterials to ovarian cancer cells including biotinylated drugs, toxins, therapeutic proteins, biotin liposomal encapsulated vesicles or biotinylated nanoparticles with drugs or radioisotopes. Furthermore, the bfFp could be applied in pretargeting radioimmunotherapy of ovarian cancer. The bfFp targeting of biotinylated therapeutic materials or clinical relevant pretargeting therapy to ovarian cancer could be an alternative and convenient method to deliver effective therapy in an adjuvant setting for ovarian cancer patients.

The above three studies have demonstrated the versatility of bispecific targeting systems which may present as the potential strategies for future vaccine or therapeutics.

6.2. Future Work

A future modification could incorporate a human DEC-205 scFv fused with CS for human vaccines. Such constructs could also eliminate the need for traditional adjuvants. Further characterization of the bfFp constructs targeting DC or ovarian cancers includes biodistribution, kidney toxicity, immune responses and immunogenicity study. The ovarian bfFp could be tested in *vivo* to deliver other biotinylated therapeutic-biomaterials to ovarian cancer cells including biotinylated drugs, toxins, therapeutic proteins, biotin liposomal encapsulated vesicles or biotinylated nanoparticles with drugs or radioisotopes. Furthermore, the same bfFp could be applied in pretargeting radioimmunotherapy of ovarian cancer. The bfFp targeting of biotinylated therapeutic materials or clinically relevant pretargeting therapy to ovarian cancer could be an alternative and convenient method to deliver effective therapy to ovarian cancer. The bfFp targeting methodology can also be adopted for treatment of other cancer such as breast cancer by appropriate specific antibodies.

BfFp targeting of biotinylated antigens to DC could be a convenient method to deliver single or multiple antigens to DC. Such formulations can induce immune responses towards peptide, protein, glycolipids and DNA (DNA vaccine). The targeting strategy of DC may present as the potential candidate for development of vaccine or therapy for cancer (GM3, GM2, MUC-1) and infectious diseases (WEEV, EBOV, SARS-CoV, Anthrax). Protection and challenge study of cancer and infectious diseases need to be investigated in future studies. The bfFp pharmacokinetic profile, kidney toxicity profile and vaccine optimization (antigen dose response and bfFp concentration required to generate sufficient immune response) should also be studied. Different routes of immunization can be considered, such as respiratory (nasal) and gastrointestinal routes since DC DEC-205 receptor is found in the nasal mucosa and gut epithelial cells. Potential formulation of bfFp delivery of the biotinylated nanoparticle (encapsulated with antigen of interest and co-stimulatory molecule) can be applied in aerosol formulation. Gastrointestinal targeting *via* oral rectal routes can also be explored: oral feeding of the capsule and rectal suppository delivery of bfFp with biotinylated antigen complex (including co-stimulatory molecule). Targeting of DEC-205 receptor without co-stimulatory molecule would generate tolerance (anergy) towards the targeted antigens. Lastly, the bfFp delivery vector may also be applied to prevent autoimmune diseases, transplantation rejection and allergy by inducing tolerance in the absence of the co-stimulation.