HOST RESPONSES TO RECOMBINANT BACULOVIRAL VECTORS IN ANIMAL MODELS

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Publications

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3. Yang J, **Balasundaram G*,** Lo SL, Guang EC, Xue JM, Song J, Wan AC, Ying JY, Wang S. Microfibers fabricated by non-covalent assembly of Peptide and DNA for viral vector encapsulation and cancer therapy. Adv Mater. 2012 Jun 26;24(24):3280-4. Epub 2012 May 25.(*-co-first author)

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Summary

Baculoviruses (BVs) are rod shaped, double stranded DNA viruses infecting insects. They have been engineered to deliver genes into mammalian cells for more than two decades now. Because of their large cloning capacity, non-pathogenic nature, low cytotoxicity and ease of production, they are emerging as a novel and promising gene therapy vector for a number of *ex vivo* and *in vivo* applications, especially for the treatment of brain tumors and are gearing up to enter clinical trials. However, there have been reports that BV elicits immune responses in mammalian hosts and undergoes inactivation by serum complement system, thus questioning their suitability for clinical trials. Mammalian host responses to baculoviral transduction or inoculation is not yet completely known, the understanding of which is necessary to overcome the obstacles put forth by the immune system. Hence, this study aims to assess the host responses to baculoviral inoculation, particularly in the brain and develop methods to attenuate them.

Foreseeing clinical applications for BV, to substantially reduce unwanted immune responses due to insect cell culture derived impurities such as host cell DNA, proteins and endotoxins in BV formulations, membrane chromatography based baculovirus purification method was improvised. Purified BV formulation was checked for host responses in mice brains by cDNA microarray gene expression profiling. This purification method yielded highly pure *in vivo* grade BV formulation that induced lesser immune responses in mice brains compared to a commonly used laboratory method of high-speed centrifugation to purify BV.

To investigate the host responses to BV purified by membrane chromatography, non-human primates - cynomolgous macaques that share a high genomic similarity with humans were used. This is the first study to explore the host

response to BV in non-human primates. Analysis of global gene expression profiles using cDNA microarray technology upon intracranial administration revealed that rBV inoculation caused no major abnormality to the animals but induced anti-viral, primarily, innate immune response and complement protein activation as major reactions. The humongous data generated from the microarray studies also revealed the major pathways of IFN induction in macaque brains thus offering better understanding of the primates' responses to BV and clues to overcome them.

To overcome the major hurdle of *in vivo* inactivation of BV by serum complement proteins, a novel method of coating rBV with cationic lipids – Lipofectamine 2000 and Cellfectin II was developed and tested *in vitro*. It was established that cationic lipid coated BV greatly improved the transduction efficiency of BV in the presence of serum though there was not much improvement in the absence of serum. These findings together will facilitate the optimization of BV vector design and serve as a guide to rational therapeutic applications of BV vectors.

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List of Abbreviations

AAV	Adeno-associated virus
AcMNPV	*Autographa californica* multiple nucleopolyhedrosis virus
Ad	Adenovirus
BEVS	Baculovirus expression vector system
BMDC	Bone marrow - derived DCs
BmNPV	*Bombyx mori* nucleopolyhedrosis virus
BMSC	Bone marrow-derived mesenchymal stem cell
BP	Biological process
BV	Baculovirus
cDNA	Complementary DNA
CMV	Cytomegalovirus
CNS	Central nervous system
DAF	Decay accelerating factor
DAI	DNA-dependent activator of IRFs
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cells
DNA	Deoxyribo nucleic acid
ds	Double-stranded
EGFP	Enhanced green fluorescent protein
FT	Flow through
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCV	Ganciclovir
GFAP	Glial fibrillary acidic protein
GO	Gene Ontology
gp64	Glycoprotein 64
GV	Granulovirus
hESC	Human embryonic stem cell
HIV	Human immunodeficiency virus
HS	High speed centrifugation
HSV	Herpes simplex virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory gene
kb	Kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
luc	Luciferase
LV	Lentivirus
MC	Membrane chromatography
MES	2-(N-morpholino)ethanesulfonic acid
MHC	Major histocompatibility
miRNA	Micro RNA
mRNA	messenger RNA

MOI	Multiplicity of infection
MSC	Mesenchymal stem cell
MyD	Myosin D
NFkB	Nuclear factor kappa B
NHP	Non-human primate
NK cell	Natural killer cell
NPV	Nucleopolyhedrosis virus
NSC	Neural stem cell
ODV	Occlusion derived virus
OV	Occluded virus
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PEC	Peritoneal macrophage
PEG	Polyethylene glycol
PEI	Polyethyleneimine
pfu	Plaque forming unit
PRR	Pathogen recognition receptor
RLR	Rig1-like receptor
RNA	Ribonucleic acid
RT	Room temperature
RV	Retrovirus
Sf	*Spodoptera frugiperda*
SIV	Simian immunodeficiency virus
STING	Stimulator of IFN genes
TAA	tumor associated antigen
TEM	Transmission electron microscopy
tk	Thymidine kinase
TLR	Toll-like receptor
TNF	Tumor necrosis factor
VSV	Vesicular stomatitis virus

1. Introduction

1.1. Baculoviruses – an overview

Baculoviruses (BVs) constituting the family Baculoviridae are a very diverse group of viruses infecting a wide range of invertebrates of which well documented are insects from the orders of Lepidoptera, Dipteria and Hymenoptera. They possess double stranded, super-coiled, circular genomes with sizes varying from 80 kb to over 180 kb packed into rod shaped nucleocapsids of dimensions 230-385 nm x 40-60 nm (Rohrmann, 2011b). Discovered as early as 5000 years ago when the silk industry originated, BVs initially received interests because of the threat they posed to the silk industry. Later, in 1980s, when it was first demonstrated that BVs can be genetically modifed to carry genes of interest, they moved in to the main stream of biotechnology for three main reasons. The inherent insecticidal activity of baculovirus together with recombinant technology have been exploited in making insecticides with enhanced host specificity and killing speed marking a new era in insect selective peptide toxin (Inceoglu *et al.*, 2006). Together with insect cells that support post translational modifications similar to that in mammalian cells, BV has been utilised to make recombinant proteins that serve as vaccines and therapeutics (Drugmand *et al.*, in press). Non-replicative nature of BV in mammalian cells together with the wise use of promoters has been taken advantage of to develop them as gene delivery vectors to a number of mammalian cells and animal models (Hu, 2006). Of these varied applications, this section shall focus on the dimension of BV as a gene delivery vector gearing up to enter clinical trials.

More than 20 unique BV genomes have been sequenced so far. The best-characterized BV, *Autographa californica* multicapsid nucleo-polyhedrosis virus

(AcMNPV) has a genome of 134kb and is estimated to contain 154 genes. BV genes are expressed in a transcriptional cascade where each phase is dependent on the expression of genes in immediate previous phase. Generally, they can be divided into two categories, early genes, which are transcribed by the host RNA polymerase and late genes which are transcribed by a virus-specific RNA polymerase. Expression of late genes is completely dependent on the expression of early genes and is indispensible for the expression of two very late genes, namely polyhedrin gene, which encodes the major occlusion body protein, and p10, which encodes a small poorly conserved protein that may be involved in occlusion body formation or cell lysis. These two proteins are highly expressed due to very strong promoters but are not really necessary for infectious virus formation. Replacing these genes with foreign genes appended to suitable mammalian cell promoters convert these viruses into expression vectors for recombinant protein production and therapeutic gene delivery (summarized from Ahrens C H, 1996, Okano *et al.*, 2006, Vlak, 2008).

1.2. Fate of baculovirus (BV)

Baculoviruses being insect viruses, their fate in insect cells is different from that in mammalian cells. It is these differences that are used to the advantage of the researchers to develop them as promising gene delivery vectors that awaits entry into clinical trials.

1.2.1. In insect cells

In insect cells, BVs follow a complex replication cycle involving two types of virions. One is occluded virus (OV) adapted for stability outside the host and for infection of the insect midgut cells. Other is the budded virus adapted for cell to cell and systemic infection within the host. Based on the morphology of their occlusion bodies, BVs can be divided into two major genera – nucleopolyhedrosis viruses

(NPVs) characterised by single or multiple virions occluded in polyhedrins and granuloviruses (GVs) characterized by single virion occluded in granulin matrix. When both genera are widely used as insectides, NPVs are majorly researched as gene delivery vectors. Especially *Autographa californica* multiple nucleopolyhedrosis virus (AcMNPV) is revered as widely used gene delivery vector. Upon consumption by insects, once inside the midgut, OVs are sensitive to the alkaline insect gut fluid containing enzymes that break down the crystalline protein matrix and release the occluded virions called occlusion-derived virions (ODVs). The released ODVs fuse with the midgut cells releasing nucleocapsids and initiating viral replication. During the early stage of infection, nucleocapsids bud through the plasma membrane to form budded viruses. These budded viruses help spread the viral infection through out the insect. During the later stage of infection, i.e. about 5-7 days after ingestion, OVs are formed. After the insect dies, the OVs are ready to infect other larva (summarized from Inceoglu *et al.*, 2006, Rohrmann, 2011a). This is summarized in Figure 1.1. This inherent lytic capacity of BVs is exploited to develop them as full-fledged insecticide.

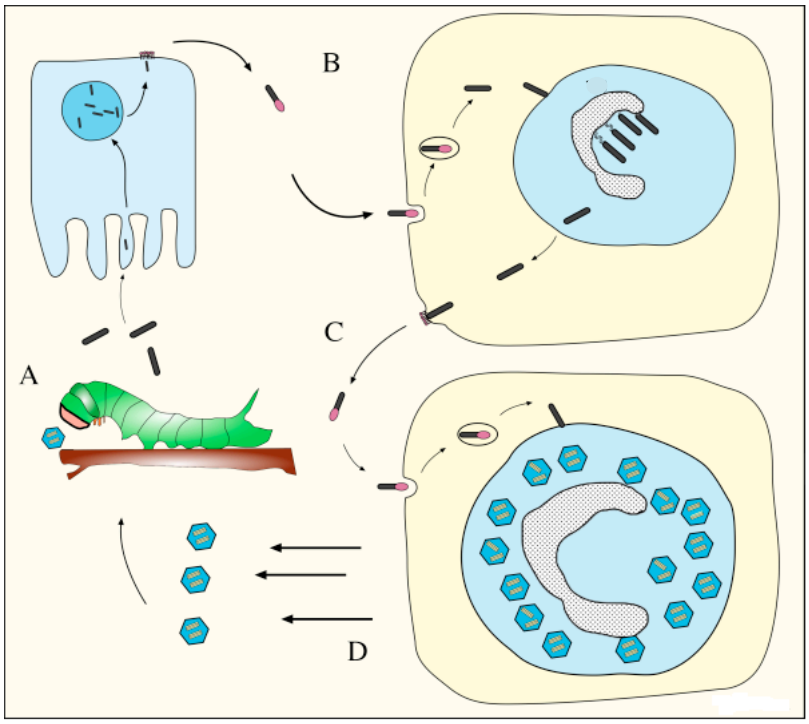

Figure 1.1. Fate of BV in insect cells. (A) Occlusion bodies ingested by an insect dissolve in the alkaline environment of midgut to release ODVs which then infect midgut cells (B) Virions bud out of the cell to initiate infection (C) Early stage infection where more budded viruses are produced to spread the infection through out the insect (D) Late stage infection where virions are occluded and the cell dies releasing them. Image adapted from Rohrmann, 2011a.

1.2.2. In mammalian cells

In mammalian cells, BVs cannot replicate because of the transcriptional silencing of its major regulatory genes. Unlike in insect cells, occluded viruses have no role in mammalian cells. It is the budded baculoviruses accomodating transgenes harvested from insect cells that are used as gene delivery vectors for mammalian cells. Baculovirus entry into mammalian cells is not yet clearly understood. There are evidences for several of the factors and several routes to be involved in its entry. For instance, while Duisit et al showed that the entry depended on electrostatic interactions and heparan sulfate (Duisit *et al.*, 1999), Tani et al demonstrated it to depend on phospholipids (Tani *et al.*, 2001). Clathrin dependent (Matilainen *et al.*, 2005, Long *et al.*, 2006) and independent endocytosis (Laakkonen *et al.*, 2008),

macropinocytosis (Matilainen *et al.*, 2005) and phagocytosis (Laakkonen *et al.*, 2008) have been demonstrated to be the routes of entry. These diverse data suggest that BV might follow different entry routes depending on the cell type. Nevertheless, there is a unified notion that the envelope glycoprotein gp64 is essential for its entry as blocking it abrogated the transduction efficiency of BV (Abe *et al.*, 2005, Niu *et al.*, 2008) and also its capacity to activate dendritic cells (Schutz *et al.*, 2006).

Once inside the cell's endosome, triggered by the pH change, gp64 fuses with the endosomal wall delivering the contents of BV into cytoplasm which is then transported to the nucleus where the transgene is transcribed. With the right kind of promoters that support replication in mammalian cells, baculoviruses are known to express a wide variety of transgenes (Hu, 2006, Chen *et al.*, 2011). This is illustrated elaborately in Figure 1.2. Out of the 155 genes that BV codes for, 43 transcripts have been detected in BV transduced Hela cells using BV DNA chips (Fujita *et al.*, 2006, Liu *et al.*, 2007) and overexpression of immediate early genes *ie1* and *ie2* increased the number of activated transcripts to 59 in mammalian Vero E6 cells (Liu *et al.*, 2007). Also, three immediate early genes from another baculovirus *Bombyx Mori* NPV, BmNPV, were activated in transduced HEK 293 cells (Kenoutis *et al.*, 2006). However, there have been no reports of baculovirus replicating in mammalian cells (Kost *et al.*, 2005).

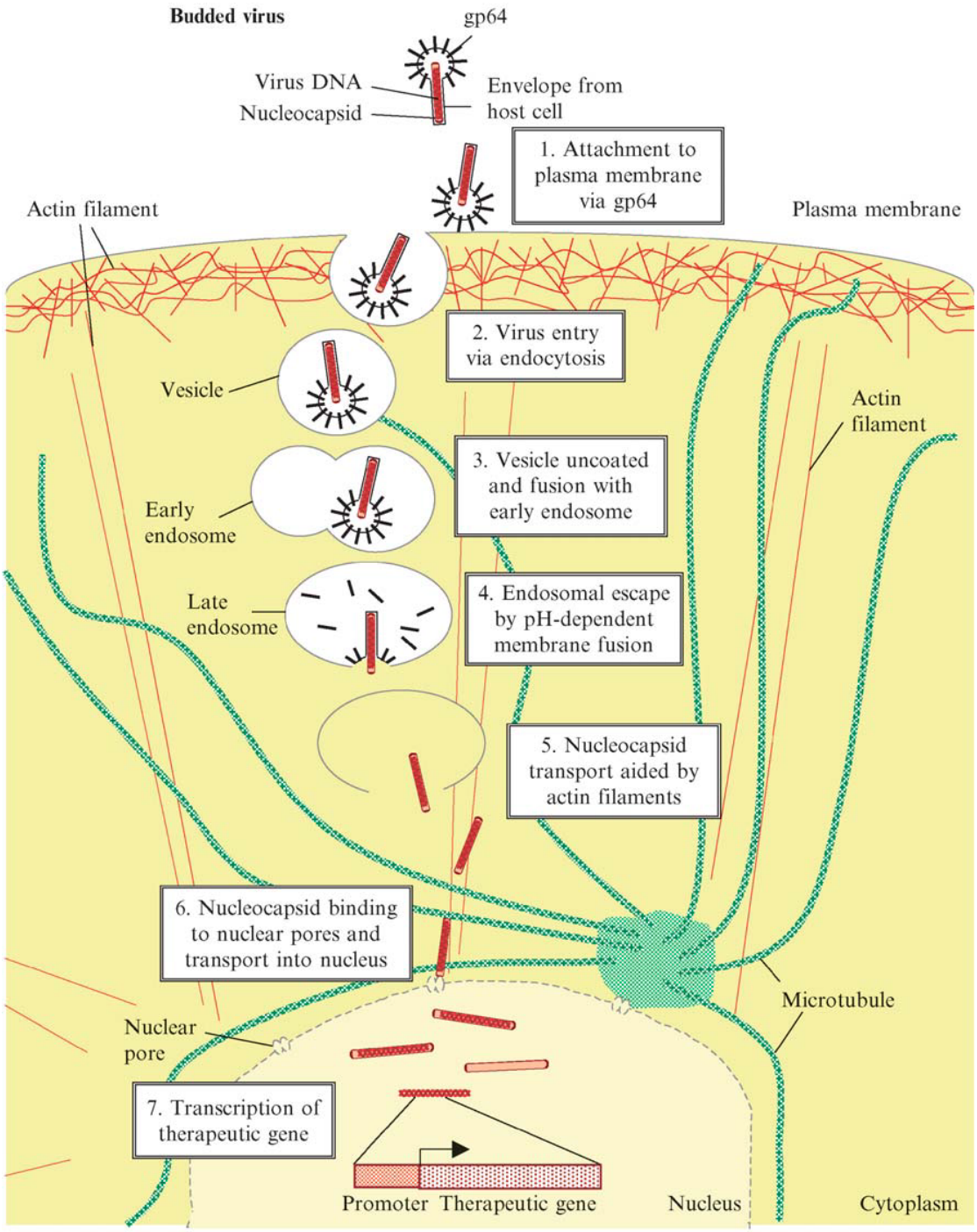

Figure 1.2. Fate of BV in mammalian cells. Illustrated is the proposed entry and intracellular trafficking of a baculovirus vector for expression of a therapeutic gene in a mammalian cell. Image adapted from Hu, 2006.

1.3. Advantages of BV in comparison with other gene delivery vectors

Gene delivery vectors can be broadly classified into non-viral and viral vectors. Non-viral vectors include nucleotide or amino acid sequences either naked or coated with artificially synthesized cationic lipids or polymers. Though they have low immunogenicity, they are often restricted by the poor transduction efficiency (Boeckle *et al.*, 2006). Viral vectors possess the capacity of high transduction efficiency and the flexibility to be genetically modified to attain target specificity. Some of the commonly used viral vectors include retroviruses (RV), lentiviruses (LV), adenoviruses (Ad) and adeno-associated viruses (AAV). Though several of these vectors are already in clinical trials (Edelstein *et al.*, 2007), they are not without limitations. For instance, the life cycle of retroviruses include an integrated state in the host genome that allows for long-term, stable expression of therapeutic genes. The preferential integration of these vectors into transcriptionally active regions of host genomes, however, occassionally leads to insertional mutagenesis, oncogene activation and cellular transformation, the most severe case being the development of leukemia in several children in France and the UK following RV gene therapy for SCID-X1 (Hacein-Bey-Abina *et al.*, 2003). Lentiviruses derived from human or simian immunodeficiency viruses (HIV or SIV) are emerging as capable gene delivery vectors for dividing and non-dividing cells. However, the pathogenic nature of HIV or SIV does not eliminate the safety concerns associated with their usage. Ad and AAV derived viral vectors have a much lower risk of insertional mutagenesis. However, as infectious human viruses, both can activate the human immune system (Jooss *et al.*, 2003, Bessis *et al.*, 2004, Huang *et al.*, 2009). This is of serious concern in view of the possible undesired rejection responses. Pre-existing immunity against adenovirus has been detected in the majority of the human population (Jooss *et al.*, 2003, Nayak

et al., 2010) and in a worse case, this has brought about death following fever and multiple organ failure in an individual who was treated for ornithine transcarbamylase partial deficiency with a high dose of adenoviral vector (Bostanci, 2002, Thomas *et al.*, 2003). Pre-existing immune responses to AAV vectors are no less than that to Ad vectors (Thomas *et al.*, 2003). AAV vectors are efficient in activating B cells (Bessis *et al.*, 2004) and specific antibodies against AAV2 are detected in 35 to 80% of individuals depending on age group and geographic location (Jooss *et al.*, 2003). The unfortunate death events have brought intense scrutiny to the potential risk associated with the viral vectors. Even if the pre-existing antiviral immunity does not trigger severe pathological changes, it can still inactivate the viral vectors, therefore affecting their transduction efficiency. The demerits of various gene transfer vectors in dealing with safety and efficacy underscore the importance of the development of new vectors.

In comparison with these vectors, BVs have numerous advantages. The most commonly used BV vectors are derived from *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) and for this reason, all through out this thesis, AcMNPV are commonly refered to as BV. As mentioned earlier, BV has the ability to enter mammalian cells and express the transgene but, it neither replicates inside the transduced cell nor causes obvious toxicity to the cell (Kost *et al.*, 2005). The viral DNA degrades over time thus eliminating the possibility of causing side effects to the host cells (Ho *et al.*, 2005, Wang *et al.*, 2005b). The vector has a high cloning capacity (at least 38 kb) allowing accommodation and delivery of a large functional gene or multiple genes (Cheshenko *et al.*, 2001). Unlike many other gene therapy viral vectors, BVs can be produced in serum-free cell culture medium, which eliminates the potential hazard of serum contamination with viral and prion agents

from the donating animal. Most importantly, BVs do not have pre-existing antiviral immunity in humans since it is not infectious to them. Strauss *et al.* have reported that none (n=20) of the serum samples tested positive for BV neutralizing antibodies while 65% of the samples tested positive for Ad type 5 (Strauss *et al*., 2007). In the same study, pre-existing Ad-specific T cells were detectable but there were no pre-existing BV-specific T cells in humans. Thus, BV has relatively low immunogenicity as indicated by the induction of lesser number of virus-specific T-cells (Strauss *et al.*, 2007). All these advantages and safety associated with BV have been motivating the increasing efforts to employ it for a wide range of applications.

Vectors	Packaging capacity	Host range	Features	Clinical trials
Retrovirus (RV)	Medium 8 kb	Restricted, dividing and non-dividing cells	Genome integration, long-term expression	Yes
Lentivirus (LV)	Medium 8 kb	Broad, dividing and non-dividing cells	Genome integration, long-term expression, safety concerns low titres, production inefficient	No
Adenovirus (Ad)	Medium < 7.5 kb	Broad, Low transduction of neurons	Transient expression, strong immunogenicity	Yes
Adeno-associated virus (AAV)	Low <4 kb	Broad, infects both non-dividing and dividing cells	Slow expression onset, genome integration, long-term expression, inefficient large scale virus production	Yes
Baculovirus (BV)	High ≈ 130 kb	Broad, dividing and non-dividing cells	Easy production and large cloning capacity, safety (lack of pre-existing immunity)	No

Table 1.1. Viral vectors for gene therapy. Adapted from Kim et al., 2009a.

1.4. Applications of BV as a gene delivery vector

Since the first finding in 1995 that BV can transduce mammalian cells, a number of studies have demonstrated their capacity to deliver genes *in vivo*. For instance, BV could successfully deliver genes into carotid artery of rabbits (Airenne *et al.*, 2000), liver (Huser *et al.*, 2001) and brain (Sarkis *et al.*, 2000, Lehtolainen *et al.*, 2002, Wang *et al.*, 2005a) of rats, as well as brain (Sarkis *et al.*, 2000), skeletal muscle (Pieroni *et al.*, 2001), cerebral cortex, testis (Tani *et al.*, 2003), liver (Hoare *et al.*, 2005) and brains of mice (Balani *et al.*, 2009, Wu *et al.*, 2009). *In vivo* gene delivery testing phase using reporter genes was soon replaced by therapeutic gene delivery. The following paragraphs will briefly discuss the therapeutic applications of BV gene delivery in *in vivo* studies.

1.4.1. *In vivo* studies involving direct injection

Cancer therapy is one of the major areas in which BV has been widely evaluated for its gene delivery and therapeutic efficacy. The suitability of BV for cancer gene therapy was first demonstrated in 2006 when recombinant baculovirus (rBV) expressing bacterial diphtheria toxin A (DT-A) gene impeded the growth of cultured mouse and human glioma cells and glioma xenograft in immune-competent rat brains (Wang *et al.*, 2006a). Following that, rBV expressing HSVtk governed by a truncated high mobility group box2 (HMGB2) promoter suppressed the growth of human glioblastoma xenograft in the presence of the prodrug ganciclovir (GCV) and extended the survival in mice models. HMGB2 restricted the expression of the suicide gene to the glioma cells thus minimizing the damage to neighboring cells (Balani *et al.*, 2009). Alternatively, GFAP promoter active in normal and glioma cells was used to govern the HSVtk gene and the target specificity of BV was improved by

exploiting the idea of transgene regulation by endogenous micro RNAs (miRNA) (Brown *et al.*, 2006, Brown *et al.*, 2007). Target sequences of three miRNA (has-miR31, has-miR127 and has-miR143) that were down-regulated in gliomas but not in normal glial cells and neurons were appended to the 3' end of HSVtk gene to restrict the expression of *tk* to glioma cells. In the presence of GCV, this rBV effectively inhibited human glioma xenograft and imparted negligible toxicity to normal astrocytes (Wu *et al.*, 2009).

rBV could successfully deliver a number of tumor suppressor and apoptotic genes to arrest the solid-tumor growth and induce apoptosis. For instance, rBV has been used with tumor suppressor genes, p53 to inhibit the growth of U251 glioma cell induced subcutaneous tumors, normal epithelial cell specific gene-1 (NES1), to treat gastric cancer (Huang *et al.*, 2008b) and programmed cell death 4, Pdcd4 to suppress the growth of oral squamous carcinoma xenografts (Kim *et al.*, 2010) and apoptotic protein, apoptin to suppress the growth of hepatocellular carcinoma in mice models (Pan *et al.*, 2010). Recently, BVs have been used to inhibit angiogenesis, the process of formation of new blood vessel that is indispensible for the growth and spread of tumors. Hu and colleagues constructed rBVs accommodating anti-angiogenic fusion protein hEA (human endostatin and angiostatin) with/without inverted terminal repeat (ITR) sequences from adeno-associated virus (AAV). *In vivo,* rBV-hEA with AAV-ITR resulted in stronger angiogenic effects, potent tumor growth inhibition and prolonged the survival of mice with pancreatic xenograft compared to control treated mice and rBV-hEA with no ITR treated mice (Luo *et al.*, 2011b). Alternatively, rBV constructed with a hybrid sleeping beauty (SB) transposon not only hindered the growth of prostate tumor allografts and human ovarian tumor xenografts and extended the survival of animals by inhibiting angiogenesis *in vivo* but also

prolonged the expression up to 77 days without any antibiotic selection (Luo *et al.*, 2011a).

rBV has also been used to deliver immunogenic molecules thus marking a niche for itself as vaccine for a number of animal and human diseases (Madhan *et al.*, 2010) including cancer (Wang *et al.*, 2010a). The concept of baculovirus as a vaccine vector was first described by Aoki et al (Aoki *et al.*, 1999) when intramuscular immunization of recombinant baculovirus expressing rabies viral glycoprotein B induced antibody response specific to that protein. Following that, in 2007, Kim et al demonstrated the potential of a pseudotyped baculovirus expressing mTERT, a potential tumor associated antigen (TAA) driven by CMV promoter as an anti-cancer gene delivery vaccine (Kim *et al.*, 2007) for GL26 glioma cell challenge in mice. Baculoviruses engineered to express antigens on their surface are much more potent in eliciting antigen-specific immune responses than those that express immunogenic molecules upon transduction. The capsid protein VP39 and viral envelope glycoprotein gp64 are the most commonly exploited proteins for this purpose. Recently, BV engineered to express a fragment of ovalbumin (OVA) on the capsid protein VP39 was found to have adjuvant activity and efficiently deliver OVA antigens to potentiate anti-tumor immune response in melanoma mice models (Molinari *et al.*, 2011). Several different antigens have been expressed as a fusion protein with gp64 and have proved successful as vaccines against infections by foot and mouth disease virus, Plasmodium berghei, malaria, H5N1 and bovine herpesvirus-1 (BHV-1) (Madhan *et al.*, 2010). However, the efficiency of this site for TAA display remains unexplored. Also, the natural immunogenicity of BV that elicits non-specific immune responses have certified them as adjuncts for antiviral and anti-tumor therapies (Kitajima *et al.*, 2008).

1.4.2. *Ex vivo* transduction of cells by BV for cancer therapy and regenerative medicine

Besides being directly used as gene delivery vectors, rBVs have been used to transduce cells *ex vivo* and used in cell-based cancer therapies and tissue and bone regeneration. Bone marrow-derived DCs (BMDCs) transduced with wild type baculovirus *ex vivo* and administered intravenously suppressed the growth of lung cancer and melanoma in mouse models and improved their cell survival. The anti-tumor activity could be contributed partly to the induction of CD8+ T cell- and NK cell-dependent, CD4+ T cell-independent antitumor immunity. Interestingly, the intravenous injection did not bring about significant damage to the liver and kidney as revealed by the minimal disturbance to the serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and creatine levels (Suzuki *et al.*, 2010). Other cells used in cancer therapy and at the same time possessing tumor tropism are stem cells like mesenchymal stem cells (MSCs) and neural stem and precursor cells (NSCs/NPCs) that respond to inflammatory mediators secreted from tumor cells and migrate to tumor tissues. These stem cells engineered to carry therapeutic genes have been exploited for delivering gene products to main tumor mass as well as metastatic tumors. BV vectors have not only been demonstrated to successfully transduce stem cells, including human embryonic stem cells (hESCs) (Zeng, 2007, Du *et al.*, 2010), hESC derived stem cells (Zhao *et al.*, 2011), and human bone marrow-derived MSCs (BMSCs) (Ho *et al.*, 2005, Ho *et al.*, 2006) without significant changes in their stemness and characteristics but exhibit cancer therapeutic efficacy. Tail vein injection of BMSCs transduced with BV expressing HSVtk significantly repressed human glioma xenograft in the presence of GCV and prolonged the survival of animals (Bak *et al.*, 2010). BV-tk transduced MSC like cells (Bak *et al.*, 2011) and NSC like cells (Zhao *et al.*, 2011) derived from hESCs also exhibited

similar therapeutic effect on glioma bearing mice models in the presence of GCV and extended the survival of tumor bearing mouse. BVs have also been used to direct BMSCs differentiation *ex vivo* into specific lineage by delivering appropriate growth factors. For example, BMSCs transduced with BV accommodating bone morphogenic protein-2 (BMP-2) differentiated into osteoblasts *in vitro* (Chuang *et al.*, 2007). Administration of these cells into mice (Chuang *et al.*, 2007) and rats (Chuang *et al.*, 2010) resulted in progressive mineralization and ectopic bone formation. BVs have also been used to transduce adipose-derived stem cells (ASCs), another promising stem cell source for regenerative medicine (Lo *et al.*, 2009). ASCs requiring sustained expression of growth factors promoting osteogenesis and angiogenesis for successful differentiation into osteoblasts were transduced with dual baculoviral vector system containing flippase recombination enzyme in one and bmp-2 or vegf in another flanked by the flippase recognition target sequences to acquire persistent gene expression for more than 28 days. BV transduced ASCs accelerated the healing, improved the bone quality and angiogenesis for repairing large segmental bone defects (Lo *et al.*, 2009).

1.4.3. Other applications

Apart from applications as vaccines and cancer gene delivery vectors, BV, because of its efficient gene delivery have found use as RNA interference mediator. BV mediated delivery of shRNA has been shown to effectively inhibit the replication of porcine reproductive and respiratory syndrome virus (PRRSV) (Lu *et al.*, 2006), peste des petits ruminants virus (PPRV) (Nizamani *et al.*, 2011) and Hepatitis B virus (HBV) (Starkey *et al.*, 2009) *in vitro*. BV mediated miRNA delivery has also been demonstrated to knock down the target gene *in vitro* thus implicating their potential for antiviral therapy (Chen *et al.*, 2011). Also, several recombinant proteins produced

in baculovirus expression vector system (BEVS) are being tested in clinical trials as vaccines with Cervarix® (a product of GSK) managing to reach the market first as an effective prophylactic vaccine for cervical cancer and other HPV infections. Cervarix is a formulation of L1 proteins from human papilloma viruses (HPV) 16 and 18 produced in BEVS and ASO4, an adjuvant containing 3-O-desacyl-4'-monophosphoryl lipid A (MPL) adsorbed on aluminium hydroxide and hydrated (Al(OH)3). Cervarix gives rise to better immunogenicity by inducing higher serum neutralizing antibody titers and has a safe profile on patients compared to the GARDASIL (a product of Merck), another vaccine containing L1 proteins produced in yeast for HPV infections and cervical cancer (Einstein *et al.*, 2009). Another product with proteins raised in BEVS is the vaccine Provenge for advanced, metastatic, asymptomatic hormone refractory prostate cancer (HRPC) that is recently approved by the US FDA. Provenge is a product of Dendreon Corporation consisting of autologous dentritic cells (DCs) loaded *ex-vivo* with a recombinant fusion protein consisting of prostatic acid phosphatase (PAP) linked to granulocyte-macrophage colony-stimulating factor produced in BEVS. Shortly after FDA approval, Provenge was added to compendium of cancer treatment published by National Comprehensive Cancer Network as the highest recommended drug for HRPC.

1.5. Host responses to BV

Despite all these successes as a gene delivery vector, BV has certain limitations that delay its advancement into clinical trials. Among them, the most crucial are the host immune responses to BV, susceptibility of BV to the host complement system and the purification and concentration methods of active BV particles.

1.5.1. Immune responses to BV

BV is capable of eliciting immune responses in mammalian cells and conferring antiviral and anti-tumor protection in animal models. This has been exploited in making vaccines. However, only a few definitive findings have been reported on such use. *In vitro,* in mammalian cells, they have been demonstrated to induce the expression or promote the release of inflammatory cytokines including interferons (IFNs), tumor necrosis factor – alpha (TNF-α), interleukin (IL)-1α, IL-1β, IL-6, IL-8 and IL-12 (Abe *et al.*, 2010). *In vivo*, they could elicit significant innate immune responses which were exploited to protect animals from lethal encephalomyocarditis virus (Gronowski *et al.*, 1999) and influenza virus (Abe *et al.*, 2003). They can also stimulate natural-killer (NK) cell mediated increase in serum levels of IFN-γ that is involved in antiviral and antitumor effects and demonstrate antitumor immunity (Kitajima *et al.*, 2008). Apart from cytokine production, they can activate mouse dendritic cells (DCs) through their interaction with Toll-like receptor 9 (Abe *et al.*, 2005) and also human DCs (Schutz *et al.*, 2006) thus leading to the induction of adaptive immunity. They were also demonstrated to potentiate adaptive immune responses by inducing IFN-α and IFN-β (Hervas-Stubbs *et al.*, 2007). Previous studies from our lab demonstrated that BV could mount immune responses in “immune privileged” brains in rats by activating the TLR signaling pathway and IFN α/β signaling pathway (Boulaire *et al.*, 2009). However, the precise mechanism of elicitation of immune responses by BV remains unclear. A clear understanding of this is essential to make maximal use of the advantageous and promising baculovirus as gene delivery vectors.

1.5.1.1. Components of BV responsible for eliciting immune responses

Given the production of proinflamatory cytokines and type I IFNs upon BV administration, they are believed to possess immuno-stimulatory capacity. However, the exact component responsible for immune stimulation is not yet known. Grownowski et al demonstrated that only live BV could elicit immune responses but not the inactivated BV or dsRNA, DNA or lipopolysaccharides derived from BV preparation (Gronowski *et al.*, 1999). Particularly, gp67, probably identical to gp64 was shown to be responsible for the IFN production in cultured cells and protection from a lethal encephalomyocarditis virus infection in mice. Addition of monoclonal antibodies raised against gp67 bound to them tightly and neutralized their IFN inducing capacity (Gronowski *et al.*, 1999). As mentioned previously, the envelope glycoprotein gp64 is believed to play a major role in the entry of BV into mammalian cells. Since BV is amplified in insect cells and proteins derived from insect cells do not pass through N-linked oligosaccharides to form complexes containing outer-chain galactose and sialic acid residues, gp64 contains mannose, fucose and N-acetyl glucosamine but no detectable galactose or terminal sialic acid residues like those in mammalian cell derived proteins (Jarvis *et al.*, 1995). Mannose receptors are primarily expressed on the immune competent cells like macrophages and dendritic cells and play a key role in host defense and induce an innate immune response. It is suggested that the mannose residues on gp64 may be recognised by the mannose receptor (MR) and contribute to the immune responses. However, Abe et al demonstrated that recombinant gp64 produced in insect cells produced no pro-inflammatory cytokines and type-I IFNs in mouse macrophage cell lines (Abe *et al.*, 2005) suggesting that viral component other than gp64 may participate in the immune activation by BV. The same study also demonstrated that the genomic DNA

of BV with amounts of bioactive CpG sequences similar to that in E.coli or herpes simplex virus (HSV) was recognized by the pathogen recognition receptors (PRRs), toll-like receptors (TLRs) or TLR independent cytoplasmic recognition (Abe 2005 and 2009). Another study by Jordan et al demonstrated that BV infected insect cells expressing MHC peptide complexes have been recognised, processed and presented by host antigen presenting DCs which further generate functional antigen specific CD8+ T cell responses (Jordan *et al.*, 2008). This emphasizes the contribution of unwanted insect cells and proteins to the elicitation of immune responses by the host. Thus, several components of BV formulation are known to elicit immune responses.

1.5.2.1. Involvement of various PRRs in IFN production upon BV recognition

TLRs

Once inside the immune competent cell, BVs are recognized by pathogen recognition receptors (PRRs) like membrane bound toll-like receptors (TLRs) and cytoplasmic RIG-1 like receptors (RLRs). TLRs are the most well studied PRRs with respect to viral infections (Kawai *et al.*, 2008). Upon infection, TLRs follow MyD88 dependent pathway to activate NFkB and c-Jun, the transcriptional regulators of a number of chemokines and cytokines responsible for cellular immune response (Wang *et al.*, 2001). BVs are known to induce type I interferons (IFNs) in immune cells like peritoneal macrophages (PECs), splenic CD11c+ DCs and murine macrophage line RAW264.7 through TLR-9/MyD88-dependent pathway (Abe *et al.*, 2005). However, IFN production was not completely restricted upon BV inoculation in cells derived form MyD88 or TLR-9 deficient mice or in PECs treated with endosomal inhibitors, suggesting the existence of TLR-9 and endocytosis independent signaling

pathways (Abe *et al.*, 2005, Abe *et al.*, 2009). Recently, Chen and colleagues reported that BVs can induce type I IFN production in mesenchymal stem cells through TLR-3 dependent pathway (Chen *et al.*, 2009a). Our laboratory has demonstrated through microarray studies that neurons, which do not possess TLR-9, respond to BV transduction by inducing IFN signaling pathway involving STAT1 and STAT2 through TLR-3 dependent pathway. In rat brains, TLR2 is involved in IFN production (Boulaire *et al.*, 2009).

RLRs

RLRs are a second class of PRRs including RNA helicases like RIG-1, MDA5 and LGP2 that reside in the cytoplasm of immune and non-immune cells and recognize viral dsRNA species produced in the cytoplasm. RLRs are known to induce type I IFN production through an adaptor molecule IPS-1 that localizes in the mitochondria (Kawai *et al.*, 2005). LGP2 serves as a negative or positive regulator of RIG-1 and MDA-5 depending on the type of RNA viruses (Venkataraman *et al.*, 2007). Though not many studies have demonstrated the involvement of RLRs in BV transduction, a recent study has noted that RIG-1 and MDA5 mRNA levels were elevated in BV-transduced cells (Wang *et al.*, 2010b). Involvement of RLR signaling pathway in BV transduction is a relatively new field ready for exploration.

Cytosolic DNA sensors

Apart from TLR and RLR signaling pathways, there are evidences for other pathways involving cytosolic DNA sensors to participate in IFN production. Recently, Abe et al, upon examining the molecular mechanism of type I IFN induction by BV in a number of immune and non-immune cells found that type I IFN production in PECs

was TLR independent but did not involve RLRs. At the same time, pretreatment with endocytosis inhibitors did not affect the IFN production significantly suggesting the involvement of cytoplasmic recognition of BV by TLR-independent immune sensors (Abe *et al.*, 2009). Recently, several receptors for cytoplasmic DNA sensors such as DNA-dependent activator of IRFs (DAI) and stimulator of IFN genes (STING) have been identified to play roles in IRF3 activation and thus IFN production in response to dsNDA derived from viruses, bacteria and synthetic analogues (Takaoka *et al.*, 2007, Ishikawa *et al.*, 2008, Zhong *et al.*, 2008) Though interrupting the function of DAI produced sufficient amounts of type I IFN in response to bacterial DNA and synthetic B-form DNA (Charrel-Dennis *et al.*, 2008, Ishii *et al.*, 2008), MEFs deficient in STING that positively regulates RLR mediated type I IFN production suffered severe impairment in IFN production (Ishikawa *et al.*, 2009). These studies suggest a role for the cytoplasmic DNA sensors DAI and STING in type I IFN induction.

Though these immune responses are used to the advantage of researchers in making vaccines against a number of human and animal diseases (Madhan *et al.*, 2010), there are evidences that these immune responses interfere with the transgene expression. For example, intramuscular injection of BV in mice stimulated the production of neutralizing antibodies and this is suspected to be the reason for clearance of transduced muscle fibers (Pieroni *et al.*, 2001). Also there are chances for these immune responses to further prevent efficient transduction upon repeated injections of BV. The effect of immune responses on transduction upon repeated BV administration is yet to be studied.

1.5.2. BV susceptibility to complement-mediated lysis

A major hurdle to *in vivo* BV transduction is its inactivation as a consequence

of virus recognition by complement proteins, a major component of innate immune system. BV activates both classical (Hofmann *et al.*, 1998) and alternate (Hoare *et al.*, 2005) pathways of the complement system and naturally occurring IgM antibodies with affinity for BV may be partially responsible for complement activation (Hoare *et al.*, 2005). Activation of the complement system leads to the inactivation of viruses or the lysis of infected cells. In the case of virus-based gene transfer, activation of the complement system can dramatically reduce gene transfer efficiency and occassionally eliminate transgene expression. Sometimes, excessive complement activation can result in serious tissue damage and systemic inflammatory responses (Kiang *et al.*, 2006). To overcome the barrier imposed by the complement system to BV-mediated *in vivo* gene transfer, immune privileged organs that possess intrinsic ability to prevent the activation of innate and adaptive immune responses were chosen. These include brain, eye and testis (Simpson, 2006). BV administration by stereotaxic injection into the brain (Sarkis *et al.*, 2000, Lehtolainen *et al.*, 2002, Tani *et al.*, 2003, Li *et al.*, 2004, Li *et al.*, 2005, Wang *et al.*, 2005a, Liu *et al.*, 2006, Wang *et al.*, 2006b), intrathecal injection into the spinal cord (Wang *et al.*, 2005c) subretinal injection (Haeseleer *et al.*, 2001) and intravitreal injection (Haeseleer *et al.*, 2001, Li *et al.*, 2004, Luz-Madrigal *et al.*, 2007) into the eye and injection via the efferent ductules that resulted in efficient transgene expression in basal and Sertoli cells of the testis (Tani *et al.*, 2003)serve as examples for this strategy.

BV was also genetically manipulated to accommodate the complement-regulatory protein human decay-accelerating factor (DAF) incorporated into its envelope, which improved the efficiency of BV transduction in complement-sufficient neonatal rats after direct injection of the viruses into the liver parenchyma (Huser *et*

al., 2001). However, the presence of DAF protein in the envelope interfered with the BV replication cycle and the systemic use of the modified virus was not reported (Tani *et al.*, 2003, Hoare *et al.*, 2005). BVs possessing vesicular stomatitis virus envelope glycoproteins (VSV-G) were also produced for this purpose. Although these viruses exhibited greater resistance to inactivation by animal sera than unmodified BV *in vitro*, there was no detectable transgene expression following injection of VSV-G pesudotyped BV into mice by the intravenous, intraperitoneal, or intrahepatic route (Tani *et al.*, 2003). Another approach is to inhibit the complement proteins at the site of BV injection. Co-administration of the soluble complement inhibitor 1 (sCR1) with intraportal injection of BV vectors led to a small amount of transgene expression in the liver parenchyma, but no detectable transgene expression after tail vein injection (Hofmann *et al.*, 1999, Hoare *et al.*, 2005). Other inhibition strategies that were able to increase the survival of BV include treatment of human serum with cobra venom factor that inhibits the complement component C3 and a functional antibody blocking complement component 5 (C5) that are involved in both classical and alternative pathways (Hofmann *et al.*, 1998).

Chemical modification offered another promising approach to overcome the problem of complement inactivation. Previous studies have reported enhancement of transgene expression when synthetic polymers were used to modify adenoviral vectors, retroviral vectors and AAV vectors. As demonstrated in adenovirus, modification with synthetic polymers reduces innate immune responses, evades pre-existing anti-Ad antibodies, and allows for repeated vector delivery, thus serving as an effective strategy in overcoming barriers to *in vivo* delivery of viral vectors (Kreppel *et al.*, 2008). It is worth investigating in future whether polymer modification of BV can reduce immune recognition, thus allowing a significant level of transgene

expression upon repeated administration of BV vectors.

1.5.3. Purification and concentration of active viral particles

In vivo gene therapy demands highly pure, heavy doses of viral vectors to achieve therapeutic effects, necessitating the development of efficient scalable purification and concentration methods. Current purification and concentration methods for BV involve ultracentrifugation of virus supernatants harvested from the insect cell culture. Budded BV obtains its envelope and glycoprotein gp64, a protein enabling infection and transduction of BV, from the host insect cell membrane and these lipid and protein components are vulnerable to mechanical forces. The high speed associated with ultracentrifugation causes a significant loss of virus infectivity due to the damage of viral envelope by hydrodynamic shear stress and often results in badly aggregated viral particles. Another limitation with ultracentrifugation procedure is the small volume capacity of ultra-high speed rotors, making the scale up process difficult.

Chromatography-based purification schemes are viewed as the most versatile methods for virus purification and concentration, with a great potential for large-scale manufacturing of high-purity virus stocks for clinical applications (Burova *et al.*, 2005, Segura *et al.*, 2006). Several chromatographic methods have been reported for concentrating and purifying BV vectors. However, the peculiar rod shape of BV, unlike the usual spherical or hexagonal shape of other viral vectors makes its recovery from the column very difficult. A cation exchange column based chromatographic method used to concentrate baculovirus earlier in 1999 reported a recovery rate close to 79% of the starting viruses (Barsoum, 1999). In a method using immobilized metal affinity chromatography, BV was modified to display

hexahistidine (His6) tags on the envelope so that the tagged viruses can be purified by the affinity chromatography (Hu *et al.*, 2003). The purity was up to 87% but the recovery by this method was only 2-3%. When a size exclusion chromatography method was used, a final virus recovery of 25% was achieved (Transfiguracion *et al.*, 2007). However, the purity of the BV preparation obtained by this method was significantly lower than sucrose gradient purified viruses. More recently, Con A chromatography has been used for BV purification (Chen *et al.*, 2009b). As baculovirus is strongly bound to the column, >99% of protein impurities can be washed away, achieving 16% of recovery after elution. Another new process comprises three steps for BV purification - depth filtration, ultra/diafiltration and membrane sorption achieving the recovery yield of 40% and the purity over 98% (Vicente *et al.*, 2009). We have developed a scalable cation exchange membrane chromatographic method to purify BV (Wu *et al.*, 2007). The procedure allowed for a final recovery of 78% of infective viral particles from the original supernatant with satisfactory purity. Membrane chromatography characterized by faster flow rates and easy scalability promises further investigation for scale-up of our method.

1.6. Aims and Objectives

Having discussed the major limitations of BV that pose problems for them entering clinical trials, the need for more studies to address these issues can be realized. Therefore, the aim of this thesis is to understand the host responses to BV inoculation in brains of animal models, especially non-human primates that better reflect the human responses and develop methods to reduce them or mask BV from the host immune system. Three lines of investigation were pursued in this study.

1. In chapter 3, membrane chromatography based method to purify BV was

improvised to obtain *in vivo* grade BV formulation. The method was validated for reducing immune responses in mice brains compared to a high speed centrifugation method by high throughput cDNA microarray gene expression profiling.

2. In chapter 4, the host responses to BV purified by membrane chromatography method was evaluated by high throughput cDNA microarray gene expression profiling in non-human primates' brains. Toxicology of BV to the host brains was also evaluated by various analyses.

3. In chapter 5, a novel method involving coating BV with cationic lipids – Lipofectamine 2000 and Cellfectin II to reduce its inactivation by the complement system, a major host response, was developed and tested *in vitro.*

Findings from this investigation, for the first time, revealed the molecular level responses and the pathways activated in non-human primate brains upon BV inoculation and highlighted the differences in the response of mice and macaque to BV inoculation thus serving as a guide to rational therapeutic application of baculoviral vectors in the central nervous systems. Furthermore, the findings demonstrated the usefulness of a purification method in terms of immune responses and the suitability of cationic lipids in offering serum protective effect to BV.

2. Materials and Methods

2.1. Cell lines

Spodoptera frugiperda (Sf9) insect cells preadapted to Sf-900 II SFM (serum-free medium) were purchased from Invitrogen (Carlsbad, CA) and grown in T175 flasks at 27.5º C. Sf9 cells were passaged when ≈ 80-90% confluent and used for experiments. U87MG glioma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml), and streptomycin (100 µg/ml). U87MG cells were passaged when ≈ 80% confluent and used for experiments within 20 passages from the time of initiation of the culture.

2.2. Recombinant plasmids/baculoviral (BV) vector production

pFastBac1 plasmid vectors containing the reporter genes encoding luciferase (luc) enzyme and enhanced green fluorescent protein (EGFP) under the control of cytomegalovirus (CMV) enhancer/promoter were constructed as described previously (Wang and Wang 2006). These vectors were used to produce recombinant baculovirus (rBV) accommodating *luc* and *EGFP* genes respectively in accordance with the Bac-to Bac baculovirus expression system (Invitrogen). P3 baculovirus containing herpes simplex virus thymidine kinase (HSV*tk*) therapeutic gene under the control of CMV enhancer-glial fibrillary acidic protein promoter (CMV E/GFAP), an engineered cell type specific promoter were constructed as described previously (Wang *et al.*, 2006b, Wu *et al.*, 2009). Sf9 cells were infected with various P3 rBV at an MOI of 0.05 at 50% confluence in 175-cm^2 tissue culture flasks. Supernatants

containing rBVs were collected 72 hr post infection.

2.3. Determination of infectious titer and total viral particles

Infectious viral titres were determined by plaque assay. Sf9 cells were seeded in 6-well plates at 50% confluence. One ml of different dilutions (10^{-3} to 10^{-8}) of baculovirus supernatants in Sf-900 II SFM was added to each well. After 1 hour of incubation at room temperature, medium containing virus was aspirated and 1% nutrient agarose was overlaid on to Sf9 cells. Plaques were counted after 7-10 days. Total viral particles were determined by quantitative real-time PCR (qRT-PCR) assay. Viral genomic DNA was isolated from 200 µl of budded virus using High Pure Viral Nucleic Acid kit (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. qRT-PCR reactions to quantify baculovirus copy number was carried out in a final volume of 25 µl. Each reaction contained 12.5 µl of iQ supermix (Biorad, Hercules, CA), 100 nM of forward primer (5'-AAAGCAACCTCATAACCACCATG), 100 nM of reverse primer (5'-CCAATTCGCCTTCAGCCATG) and 100 nM of Taqman probe (6-FAM-5'-CAGACTGGTGCCGACGCCGCC-BHQ1). DNA amplification was carried out using iCycler iQ™ Real Time PCR Detection system (Bio-rad) in triplicates with the following cycling conditions: 1) 95 °C for 3 minutes 2) 40X of 95 °C for 15 seconds and 60 °C for 1 minute. For each run, triplicates of five 10 fold dilutuions (10^3 to 10^8 copies of gp64) of the viral standard DNA (pFastBac-CMV-gp64), viral DNA samples and non-template controls were subjected for analysis.

2.4. Virus purification

Budded viruses harvested from Sf9 cells were clarified by centrifugation at 1000 g for 5 minutes and filtered through 0.45 µm filter to remove cell debris. rBVs

were then purified and concentrated by two methods- one involving a single step of high-speed centrifugation (HS) and the other comprising two steps – membrane chromatography for purification and high speed centrifugation for concentration (MC+HS).

2.4.1. HS - High-speed centrifugation method

Baculoviral supernatant was centrifuged at a high speed of 28000 g for 1 hour at 4º C in Avanti™ J-25 centrifuge (Beckman Coulter, Leeds, UK). The supernatant was aspirated out and the pellet was resuspended in appropriate volumes of 1X PBS with a salt concentration of 150 mM NaCl and pH 7.4. The concentrated virus formulation was put on Stuart orbital shaker (Bibly Scienctific, Staffordshire, UK) for at least 1 hour for even dispersion.

2.4.2. MC+HS - Membrane chromatography + high speed centrifugation method

Acrodisc chromatography unit with a Mustang S cation exchange membrane (Pall Corporation, Port Washington, NY) was preconditioned with 5 ml of 0.2 N NaOH and equilibrated with 10 ml of 25 mM MES buffer (pH 6.0). Ten ml of viral supernatant either at room temperature or 4º C was passed through the membrane chromatography unit at a flow rate of 3 ml/min. The membrane was washed with 5 ml of equilibration buffer. Baculovirus was eluted first with 5 ml of PBS-150 mM NaCl and later with PBS-500 mM NaCl at pH 7.4. The combined eluate was subjected to high-speed centrifugation at conditions mentioned previously and the pellet was dissolved in appropriate volume of 1X PBS with a salt concentration of 150 mM NaCl at pH 7.4. The concentrated virus formulation was evenly dispersed by placing it on orbital shaker for at least 1 hour.

2.5. Protein gel electrophoresis, western blot and silver staining

Western Blot for gp64, the major envelope glycoprotein of baculovirus, was performed to check for the presence of baculovirus in the purified formulation. Mock infected Sf9 cell cultured medium, baculovirus containing supernatant, flow-through, wash and collected elution fractions were mixed with 4X LDS sample buffer (Invitrogen) and 10X sample reducing agent (Invitrogen) and heated at 70º C for 10 minutes for denaturing the proteins. Samples (40 µl) and SeeBlue® Pre-Stained Standard (Invitrogen) were loaded on to precast 4-12% NuPAGE Novex Bis-Tris polyacrylamide gels (Invitrogen) and electrophoresed in MES running buffer for 2 hours at 80 V. Following electrophoresis, separated proteins on the gel were transferred onto a nitrocellulose membrane using the iBlot transfer stack on an iBlot dry blotting system (Invitrogen) following the manufacturer's instructions. Complete transfer of protein was assessed by the visible transfer of ladder proteins. Membranes were blocked in blocking solution [5% (w/v) non fat dry milk, 0.1% (v/v) TritonX-100 in PBS] for 1 hr at room temperature. After blocking, membranes were incubated with baculoviral gp64 antibody (sc-65499, Santacruz Biotechnology, Santa Cruz, CA) raised in mouse (dilution - 1:1000) in blocking solution, overnight at 4 °C on an orbital shaker. After incubation with primary antibody, membranes were washed 3 times for 10 mins each on an orbital shaker in PBST [0.1% (v/v) TritonX-100 in PBS] at room temperature. Membranes were then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody, diluted in PBST, for 1 hr at room temperature. After incubation, membranes were washed 3 times for 10 mins each in PBST with shaking. Visualization of bound antibody was done using ECL Plus western blotting detection reagent (GE Healthcare, Amersham Place, Little Chalfont, Buckinghamshire). Resulting chemiluminescent signal was

detected with CL-XPosure Film (Thermo Scientific, Rockford, IL). Exposed films were imaged on FluorChem HD Gel Doc System using white light transillumination (Alpha Innotech, Santa Clara, CA).

Silver staining was used to analyse the purity of the virus formulation. SDS-PAGE for various virus samples was carried out as described earlier and the gels were stained with silver iodide solution in accordance with the Silver Quest Silver staining kit protocol (Invitrogen). Stained gels were imaged on FluorChem HD Gel Doc System using white light transillumination (Alpha Innotech).

2.6. Determination of transduction efficiency

The transduction efficacy was determined on the basis of baculoviral transduction of human U87MG cells with BV-luc and BV-EGFP. Cells were seeded at the count of 10000/well in 96-well plates and after attachment, equal number of BV-luc viruses corresponding to MOI 50 from various samples were used in transduction in serum free medium. After incubation at 37 ºC for 4 hours, the medium containing viruses was replaced with 10% FBS containing fresh growth medium. After 36-48 hours, when luciferase gene expression stablised, the cells were lysed by freeze-thaw in Reporter Cell Lysis Buffer (Promega, Madison, WI) and luciferase activities were measured with a Berthold Lumat LB detecting 9507 luminometer (Berthold Detection Systems, Pforzheim, Germany). Protein concentrations were quantified using DC protein assay kit (Bio-Rad). For quantification, 5 µl of protein lysates were mixed with 25 µl working Reagent A and 200 µl Reagent B in a 96-well plate and incubated for 15 mins at room temperature. Absorbance was measured at 750 nm in a microplate reader (Bio-Rad). Concentration of protein lysates was obtained from a standard curve produced with dilutions of bovine serum albumin solution (BSA)

prepared in Reporter Cell Lysis Buffer (Promega), ranging from 0.125 - 2 mg/ml protein concentration.

For measuring transduction efficiency of BV-EGFP, cells were seeded at a density of $2x10^6$ per well in 6-well plates and imaged after 36-48 hours using fluorescence microscopy (Olympus). Percentage of EGFP positive cells was determined by flow cytometry using FACSCalibur (BD Biosciences). Cells from each well were trypsinized, washed with PBS twice and resuspended in 1 ml of PBS containing 5% FBS and transferred to appropriate tubes (5 ml polystyrene tubes from BD Falcon) for flow cytometry analysis. Fluoresecent measurements were taken on the FL-2 channel for at least 10, 000 events gated for EGFP positive cells.

2.7. *In vivo* studies

2.7.1. Virus inoculation into mouse brains

For *in vivo* viral vector inoculation into mouse brains, three adult male immunocompetent BalB/c mice were used for each group of Mock-, MC+HS- and HS- purified BV injections. 10 µl of BV-HSVtk (10^8 viral particles) was injected stereotaxically into each side of the striatum of the mouse brain (anteroposterior: 0.0 mm, mediolateral: +2.0 mm, and dorsoventral: –3.0 mm from bregma and dura) using a 10 µl Hamilton syringe connected with a 30G needle. Brain samples around the injection sites were collected 2 days (≈ 48 hours) after virus inoculation and stored in RNA Later (Ambion, Austin, TX) at 4 ºC until used for RNA extraction. Studies on mice were performed following the Guidelines on the Care and Use of Animals for Scientific Purposes issued by National Advisory Committee for Laboratory Animal Research, Singapore. The experimental protocols of the current study were approved by the Institutional Animal Care and Use Committee (IACUC),

National University of Singapore (NUS) and Biological Research Centre (BRC), the Agency for Science, Technology and Research (A*STAR), Singapore.

2.7.2. Virus inoculation into macaque brains

For studying the host response and toxicology to recombinant baculovirus in non-human primates, 3 adult male cynomolgous macaques (Macaca *fascicularis)* weighing approximately 3-4 kg were used. BV-HSVtk (10^8 viral particles in 10 µl per injection site) purified by MC+HS was administered as 4 separate intrastriatal microinjections into the left hemisphere (anteroposterior: 0.0. mm and +3.0. mm to the bi-lateral commissural line targeting the centre of caudate and putamen) (Figure 2.1). The right hemisphere was injected with mock virus preparation. Mock virus preparation refers to Sf9 culture medium containing no baculovirus subjected to purification by MC+HS method. Brain samples were collected approximately after 2 days (≈ 48 hours) of virus inoculation and stored in RNA Later at 4º C unti further use in RNA extraction. Real time PCR for gp64 was used to verify the successful injection of BV-HSVtk into the animal brains. Samples for real-time PCR were collected in dry tubes and stored at -80 ºC until further use in genomic DNA extraction. Studies on macaques was performed following the IACUC protocol of Maccine Pte Ltd and to the standards of GLP though not formally monitored by Maccine Quality Assurance.

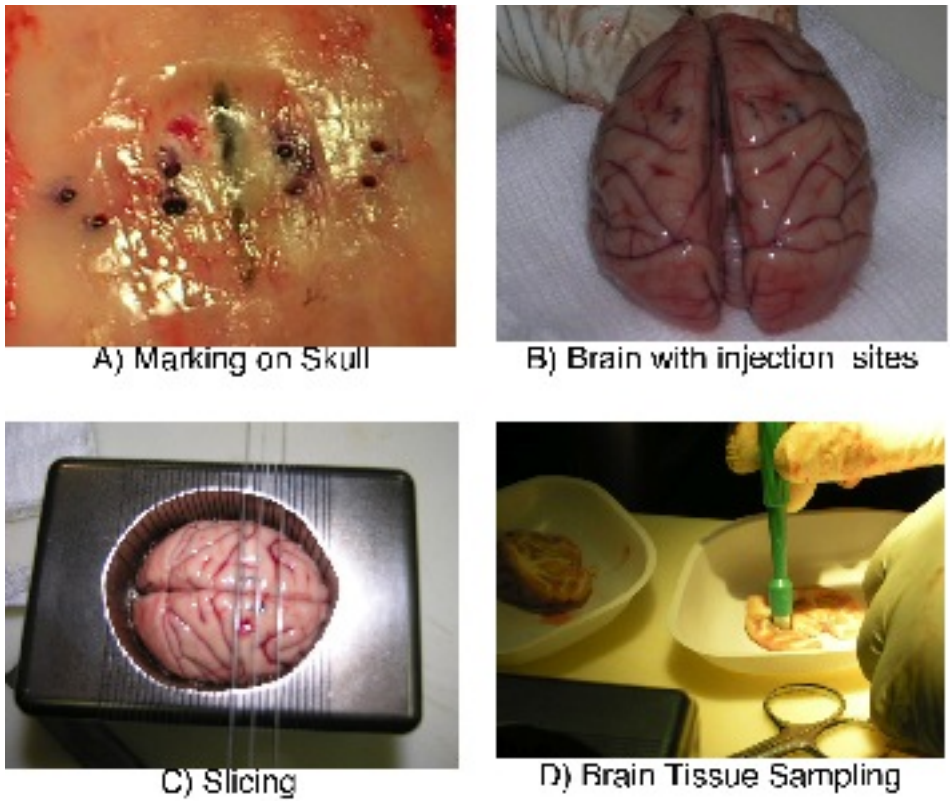

Figure 2.1. Virus inoculation into macaque brains. (A) BV-tk purified by MC+HS was administered as 4 separate intrastriatal microinjections into the left hemisphere and mock into the right at sites marked on the skull (B) shows the injection sites after collecting brain 48 hours from the time of injection (C) shows the slicing procedure and (D) shows the sampling of injected sites from sliced brain samples.

2.8. Sample preparation for cDNA microarray analysis

2.8.1. Total RNA isolation

Total RNA comprising all RNAs including mRNAs, rRNAs and small RNAs was isolated using Trizol (Invitrogen) reagent according to the manufacturer's instructions. Precisely, tissues up to 100 mg were lysed and homogenized in 1 ml of Trizol reagent using Vibra-cell sonicator (Sonics & Materials, Newtown, CT). Following lysis, all samples were processed for phase separation after adding the recommended amounts of chloroform. RNA in the aqueous phase was carefully removed and precipitated using appropriate amounts of 100% ethanol. The gel-like RNA pellet was washed with 75% ethanol, dried thoroughly for ≈ 10 minutes to

remove as much ethanol as possible and resuspended in 50 µl RNAse free water. The amount and purity of isolated RNA samples were determined by measuring absorbance at 230 nm (A230), 260 nm (A260), 280 nm (A280) with a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA). An A260/A280 ratio of 1.8-2.0 and A230/A260 ratio of 1.9-2.2 indicated RNA free of contaminants and was used for subsequent procedures. RNA was stored at -80 °C until use and freeze-thawing was limited to less than 5 times.

2.8.2. RNA clean up

Total RNA was cleaned up to remove RNAs < 200 nucleotides (usually 5.8S rRNA, 5S rRNA, and tRNAs) and enriched for mRNAs using RNeasy mini kit (Qiagen, Hilden, Germany). Precisely, total RNA isolated by Trizol method was made up to 100 µl and mixed with 350 µl of buffer RLT and 250 µl of 100% ethanol to constitute 35% v/v ethanol in the mixture. The mixture was loaded on to the RNeasy spin column. At this concentration of ethanol, the RNeasy spin column binds only RNA > 200 nucleotides (otherwise only mRNAs). The bound mRNAs were washed twice with Buffer RPE and eluted using 30-50 µl RNase free water. Cleaned up RNA was again quantified using a NanoDrop ND-1000 spectrophotometer.

2.8.3. RNA amplification and labeling

RNA amplification and labeling was done using Kreatech's RNA ampULSe amplification and labeling kit (Kreatech, Amsterdam, The Netherlands). Starting with one µg of good quality RNA (i.e. A260/A280 > 2.0 and A230/A260 > 2.1) in 10 µl, first strand cDNA was synthesized using T7 oligo dT primer following the instruction manual. The reaction mixture for first strand cDNA synthesis additionally contained diluted eukaryotic poly(A) RNA controls (Affymetrix) as recommended by Affymetrix

protocol to serve as controls for all steps of sample preparation and array hybridization. The end product of first strand cDNA synthesis was entirely used for second strand cDNA synthesis using DNA polymerase and purified according to the instruction manual. Double stranded cDNA obtained at the end of this process was used as a template in a 4 hour *in vitro* transcription process performed at 37 ºC for synthesizing amplified RNA (aRNA). aRNA was purified using columns provided in the kit. The yield and purity of aRNA was determined using NanoDrop. Thirty µg of aRNA was labeled with 5 µl biotin-coupled ULS for 30 mins at 85 °C. Following this, unincorporated biotin-ULS was removed using KREApure bead columns as recommended. Purified labeled RNA (20 µg) was then fragmented using RNA fragmentation reagent (Ambion, Foster city, CA) following the manufacturer's instructions.

2.8.4. Hybridisation, washing and scanning

A hybridisation mix of total volume 300 µl was prepared as follows:

Component	Volume (µl)	Source
Fragmented and Labeled aRNA	30	Product of amplification and labeling (Kreatech)
Control Oligonucleotide B2 (3nM)	5	Affymetrix
20X Eukaryotic Hybridization Controls	15	Affymetrix
2X Hybridization mix	150	Affymetrix
Krea Bloc	75	Kreatech
Nuclease free water	25	Promega

For mouse brain samples, GeneChip Mouse Genome 430A 2.0 Array and for macaque brain samples, GeneChip Rhesus Macaque Genome Array were used. The arrays were pre-hybridized with 200 µl Prehybridization mix (Affymetrix) for 10 mins

at 45 °C with rotation. Meanwhile, the hybridization mix was heated to 99°C and centrifuged to pellet insoluble materials, as recommended by Affymetrix hybridization protocol. Finally, 200 µl of each hybridization mix was hybridized to a GeneChip array for 16 hours at 45 ºC. Array washing and staining was done on the GeneChip Fluidics Station 450 (Affymetrix) following the recommended protocol. For washing and staining, the fluidics protocol FS450_0004 was used. Scanning of arrays was done on the GeneChip Scanner 3000. Array quality was determined from the signal intensities of internal control and the percentage of absent and present calls made by the GeneChip Operating Software for each array.

2.9. Data analysis

2.9.1. By Genespring GX 11

Data from the chips were acquired using the GCOS software and all data files were exported using the Data Transfer Tool (DTT). Data files in .CHP format were transferred to GeneSpring GX 11 (Agilent) software. The files were normalized using the default setting of the software i.e to the median of percentile target (75.0) of all samples and were applied a baseline transformation to median of all samples. Replicates (at least 3 per group) were grouped together based on treatment (virus injected/mock injected) and the probes in the normalized data were subjected to quality control based on expression and flags. Probes with either present or marginal flags in at least 2/3 of the samples in both control and experimental samples were then filtered such that there is a fold change of at least 2 in experimental samples pooled together against the control samples pooled together and then subjected to statistical analysis by t-test with a p-value of 0.05.

2.9.2. Genelists analysis by web-based services

To understand the biological meaning of the list of genes significantly modified by baculovirus injection against the mock injection in brain samples, Metacore™ (GeneGo) and Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 Bioinformatics Resources (Huang *et al.*, 2008a) (http://david.abcc.ncifcrf.gov/) were used. The list of both upregulated and downregulated genes were analysed together in Metacore or DAVID using their default settings for obtaining the Gene Ontology (GO) processes and pathways affected.

2.10. Quantitative real time-PCR of mRNAs

A portion of the total RNA isolated was used for the validation of microarray data using quantitative Real Time-PCR.

2.10.1. DNAse treatment of RNA samples

Total RNA was DNAse-treated to remove traces of genomic DNA contamination that would contribute to subsequent PCR amplification product. Typically, two µg of total RNA was treated with 1 µl (2 units) of Turbo DNAse (Ambion) in 20 µl volume for 30 min at 37 °C, followed by addition of another 1 µl of Turbo DNAse to the sample for further 30 minutes incubation at 37 °C. At the end of the incubation, 4 µl (0.2 volumes of 20 µl) of DNAse inactivation reagent was added to the sample and incubated at 25 °C for 5 minutes, with repeated dispersal of the inactivation reagent every minute by flicking the tube. For removal of DNAse inactivation reagent, tubes were centrifuged at 10,000 x g for 1.5 mins and ≈ 15 µl of DNAse treated sample was carefully removed without disturbing the inactivation

reagent pellet, and transferred to a fresh tube.

First strand cDNA was synthesized from 1 µg of DNAse-treated total RNA using Superscript III reverse transcriptase (Invitrogen). cDNA was diluted 10-fold before amplification in a 25 µl reaction with iQ5 SYBR-Green PCR Mix (Biorad). Each reaction consisted of 2 µl diluted cDNA, 1 µl (2.5 µM) of the RT^2 qPCR Primers corresponding to each gene (SABiosciences, Fredrick, MD), 9.5 µl nuclease-free water and 12.5 µl PCR mix. The cycling conditions included an initial denaturation step of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95 °C and 30 seconds at 60 °C. All reactions were done in triplicates on Bio-Rad iCycler. Expression levels were normalized to mouse and rhesus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for mouse and macaque mRNA samples respectively. For each primer pair, negative control PCR reaction with nuclease-free water was performed in duplicates. Relative gene expression level was obtained by triplicate experiments from each of the mouse brain samples and at least two of the monkey brain samples. The fold changes between the experimental and control samples were calculated according to instructions in the manual.

2.11. Preparation of cationic lipids coated BV

rBV coding for luciferase enzyme (BV-luc) were coated with either of the two cationic lipids - Lipofectamine 2000 and Cellfectin II (both from Invitrogen). Lipofectamine 2000 is an advanced version of Lipofectamine, a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy -N- [2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water majorly used in the transfection of DNA into cultured eukaryotic cells. Cellfectin II reagent is the

improved Cellfectin, a 1:1.5 (M/M) liposome formulation of the cationic lipid N, NI, NII, NIII-Tetramethyl- N, NI, NII, NIII-tetrapalmityl-spermine (TM-TPS), and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water used in the transfection of DNA into insect and mammalian cells especially Sf9 insect cells. Both cationic lipids are available in stock solutions of 1mg/ml. For coating, the cationic lipids were diluted in PBS to the required concentrations (10^4 - 10^7 molecules/particle BV), mixed with BV-luc concentrated by HS method and incubated at room temperature for 20-30 minutes.

2.12. Size and zeta potential characterization of uncoated and cationic-lipid coated BV

Uncoated and cationic lipid coated BV-luc in distilled water were characterized for their size by dynamic light scattering (DLS) and zeta potential by electrophoresis using Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK). Precisely, 750 µl of the uncoated or coated BV-luc were loaded into folded capillary cell using 1 ml syringe. Care was taken to see that no air bubbles were introduced while loading. A minimum of $5x10^9$ particles/per sample was used for size and zeta potential measurements. Data were exported in .csv format to excel and graphs were plotted using Graphpad Prism 5.

2.13. Cytotoxicity assay

Cytotoxicity of cationic lipid coated BV was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay System (MTS) (Promega) on U87MG glioma cells as a representative mammalian cell line. U87 cells were seeded in 96 well plates at a confluency of 50% and allowed to attach overnight. Uncoated and coated BV (10^4 - 10^7 molecules/BV particle) at an MOI (multiplicity of infection) of 10,

50, 100 and 500 were added to the cells in serum free and antibiotic free medium. After 4 hours of incubation, medium containing uncoated and coated BV were removed and cells were replenished with serum containing medium. After 48 hours of incubation, 40 µl of the MTS reagent (20 µl MTS solution/100 µl of medium) was added directly to the medium, incubated at 37º C for 2-3 hours and absorbance was measured at 490 nm in a microplate reader (Bio-rad).

2.14. Transmission electron microscopy (TEM)

Changes in the morphology of uncoated/ coated baculovirus when treated with serum complement were observed using transmission electron microscopy (TEM). Baculovirus (uncoated/coated and serum treated/noserum treated) samples were prepared in deionised water. 4 µl of the various samples were placed on Formvar/Carbon coated Copper 200 mesh (Polysciences, Warrington, PA) for a minute and the remaining solution was wiped off using a filter paper. The grid was washed with 4 µl deionized water once for 1 minute and the sample was stained with 4 µl of 2% phosphotungstic acid (PTA). Excess stain was washed away using deionized water. The grid was air dried for a few minutes and imaged under a 200kV TEM (FEI, Hillsboro, OR).

2.15. Complement assays and *in vitro* transduction studies

The protective effect of cationic lipid coating on BV-luc against serum complement system was tested *in vitro* using mouse, rat and human serum complement. Balb/C mouse and Wistar rat serum complement were obtained from Innovative Research (Sarasota, FL). Lyophilized powders of human serum (S-1764) were obtained from Sigma Aldrich (St. Louis, MO) and reconstituted in 1 ml of sterile deionised water. For inactivation treatment, mouse, rat or human serum complement

were mixed with coated/uncoated baculoviruses in the ratio of 1:1 (volume not exceeding 5% of the medium in a 96 well plate) for 30 minutes at 37 ºC. Transduction efficacy of serum treated, uncoated and coated BV-luc were tested on U87 glioma cells as described earlier and compared against no serum treated uncoated/coated BV as positive controls.

3. Purification of BV by Membrane Chromatography Method Reduces Immune Responses in Mouse Brains

3.1. Introduction and Aims

With growing applications of baculovirus (BV) in various fields including pesticide development, recombinant protein production and gene therapy vectors, several circumstances require the use of concentrated and purified BV. For instance, large scale protein production in insect cells require highly concentrated and pure BV inoculum. *In vivo* gene therapy applications require a high titre of BV in a small volume of physiological buffer. BVs being insect viruses and are propagated in insect cells, the chances of contamination of recombinant BV (rBV) formulation with insect cell DNA and proteins are high. Insect cells infected with rBV expressing MHC peptide complexes have been shown to be recognised, processed and presented by host antigen presenting dendritic cells (DCs) which further generate functional antigen specific CD8+ T cell responses (Jordan *et al.*, 2008). This highlights the contribution of insect cells and proteins to the immune responses of the host and underscores the necessity to remove the unwanted insect cell contaminants when rBV are used as gene therapy vectors for *in vivo* studies.

Several different methods have been developed so far for purifying rBV. Each method has its own pros and cons. For example, immobilized metal affinity chromatography improved the purity of BV up to 87% but compromised the final recovery yield which was only 2-3% (Hu *et al.*, 2003). Size exclusion chromatography resulted in recovery of 25% of total virus particles but the purity was compromised as judged by the SDS-PAGE results (Transfiguracion *et al.*, 2007). While concanavalin A affinity chromatography resulted in 29.3% overall recovery (Chen *et al.*, 2009b), a multistep downstream processing involving anion exchange

chromatography resulted in an overall recovery of 38% (Vicente *et al.*, 2009). Making use of the easy scalability and faster processing times associated with membrane chromatography (Zuo *et al.*, 1999), previous studies in our laboratory demonstrated cation exchange membrane chromatography to provide good purity and 78.1% recovery of rBV (Wu *et al.*, 2007). However, none of these studies demonstrated the biosafety associated with the material purified from these methods *in vivo,* particularly, in terms of immune responses in the host upon delivery of rBV prepared by various methods. With DNA microarrays becoming increasingly popular to monitor host responses to virus and virus-derived gene therapy vectors (Piersanti *et al.*, 2004), the task of evaluating molecular impact by different formulations on the host is much easier. Therefore, this chapter aims to improvise the existing cation exchange membrane chromatography purification protocol (Wu *et al.*, 2007) in order to substantially improve the purity of BV to generate *in vivo* grade formulation and demonstrate the biosafety associated with this method *in vivo* in mouse brains using global gene expression profiling.

3.2. Recovery of rBV from cation exchange membrane chromatography unit

The cation exchange membrane chromatography unit preconditioned with 0.2 N NaOH was equilibrated with 25 mM MES buffer, the working pH range of which accommodates rBV-supernatant of pH ~6. Virus supernatant at room temperature was passed through the membrane chromatography unit and the flow through (FT) was collected. The chromatographic unit was washed with MES buffer of same pH to remove unbound proteins and viruses. PBS of pH 7.4 with NaCl of concentrations - 150 mM and 500 mM were used to elute the virus. Real-time PCR assay for gp64 was performed on rBV supernatant, FT, wash and eluates to calculate the

percentage recovery. Most of the viruses bound to the membrane as evidenced from lesser percentage of viral particles in FT and wash (Table 3.1), thus indicating a strong interaction between viruses and the membrane. The first two eluates (E1-150 and E2-150) in PBS-150 mM NaCl contained 25% and the first two eluates (E1-500 and E2-500) in PBS-500 mM NaCl contained 47% of the viral particles. Totally, 72% of the viral particles were recovered in 2 ml of the eluate (Table 3.1). Recovery could be further improved to ≈ 90% by applying more elution buffer. A representative data set presenting recovery by real-time PCR and plaque assay is provided in Table 3.1.

However, the recovery was not always as high as 90% and drastically varied with the temperature of the rBV-supernatant passed through the membrane chromatography unit. When cold (≈ 4 ºC) rBV-supernatant was passed through the acrodisc unit, most of the viral particles bound to the unit as evidenced by the lesser percentage of rBV particles in FT (Figure 3.1). But, only ≈ 40% of virus particles were recovered in the eluate suggesting too strong an interaction between viruses and the membrane making it difficult for the elution buffer to break it. These data underscored the importance of temperature of rBV-supernatant in obtaining high recovery.

Sample	Volume (ml)	Real Time PCR			Plaque assay		
		VP/ml	Total VP[1]	% recovery[2]	PFU/ml	Total PFU[1]	% recovery[2]
BV Load	10	1.60E+09	1.60E+10		5.20E+07	5.20E+08	
Flow through (FT)	10	1.19E+08	1.19E+09	7.43	-	-	
Wash	5	2.23E+07	1.11E+08	0.70	-	-	
E1-150	0.5	5.58E+09	2.79E+09	17.40	-	-	
E2-150	0.5	2.30E+09	1.15E+09	7.17	-	-	
E1-500	0.5	1.09E+10	5.47E+09	34.16	-	-	
E2-500	0.5	4.21E+09	2.10E+09	13.12	-	-	
E1-150+E2-150 +E1-500+E2-500	2		1.15E+10	71.86	-	-	
Total Eluate	10	1.44E+09	1.44E+10	90.12	4.49E+07	4.49E+08	86.35

Table 3.1. Recovery of recombinant baculoviruses after purification by membrane chromatography.VP - viral particles; PFU – plaque forming units; 1-Total viral particles (TVP) and Total PFU was calculated by multiplying viral particles and PFU respectively with volume; 2 - Recoveries were calculated by normalizing the data against the loaded sample; E1, E2 – First and second elute; 150, 500 – Concentration of NaCl (mM) in PBS. Total Eluate (combined) – Virus eluted in PBS-150 mM +Virus eluted in PBS-500 mM.

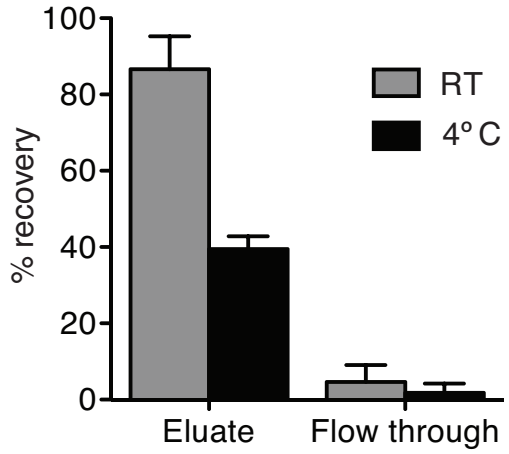

Figure 3.1. Effect of temperature of rBV-supernatant on recovery from membrane chromatography method. rBV-supernatant at 4 ºC and room temperature (RT) were passed through the membrane chromatography unit and eluted using PBS-150 mM and 500 mM NaCl. Using real-time PCR for gp64, the major BV envelope glycoprotein, number of rBV particles in eluate and flow through were measured and expressed as % recovery by normalizing against those in loaded samples.

3.3. Transduction efficiency of recovered rBV

To test the transducing ability of rBV purified by membrane chromatography method, rBV encoding reporter genes – luciferase and enhanced green fluorescent protein (EGFP) were used. rBV eluates were collected in fractions and 5 µl of each fraction was used for analysing the transduction efficiency on U87 cells in a 96 well plate. Reporter gene expression was analyzed after 48 hours of transduction. BV-luc eluted with PBS-150 mM NaCl, (E1-150 and E2-150) showed higher luciferase expression (Figure 3.2A) than non-concentrated load sample. This could be attributed to the increased number of viral particles in the eluates. Further fractions collected using PBS-150 mM NaCl did not show better luciferase expression than that used to load the chromatographic unit (data not shown) which might be because of the lesser number of rBV particles present in them.

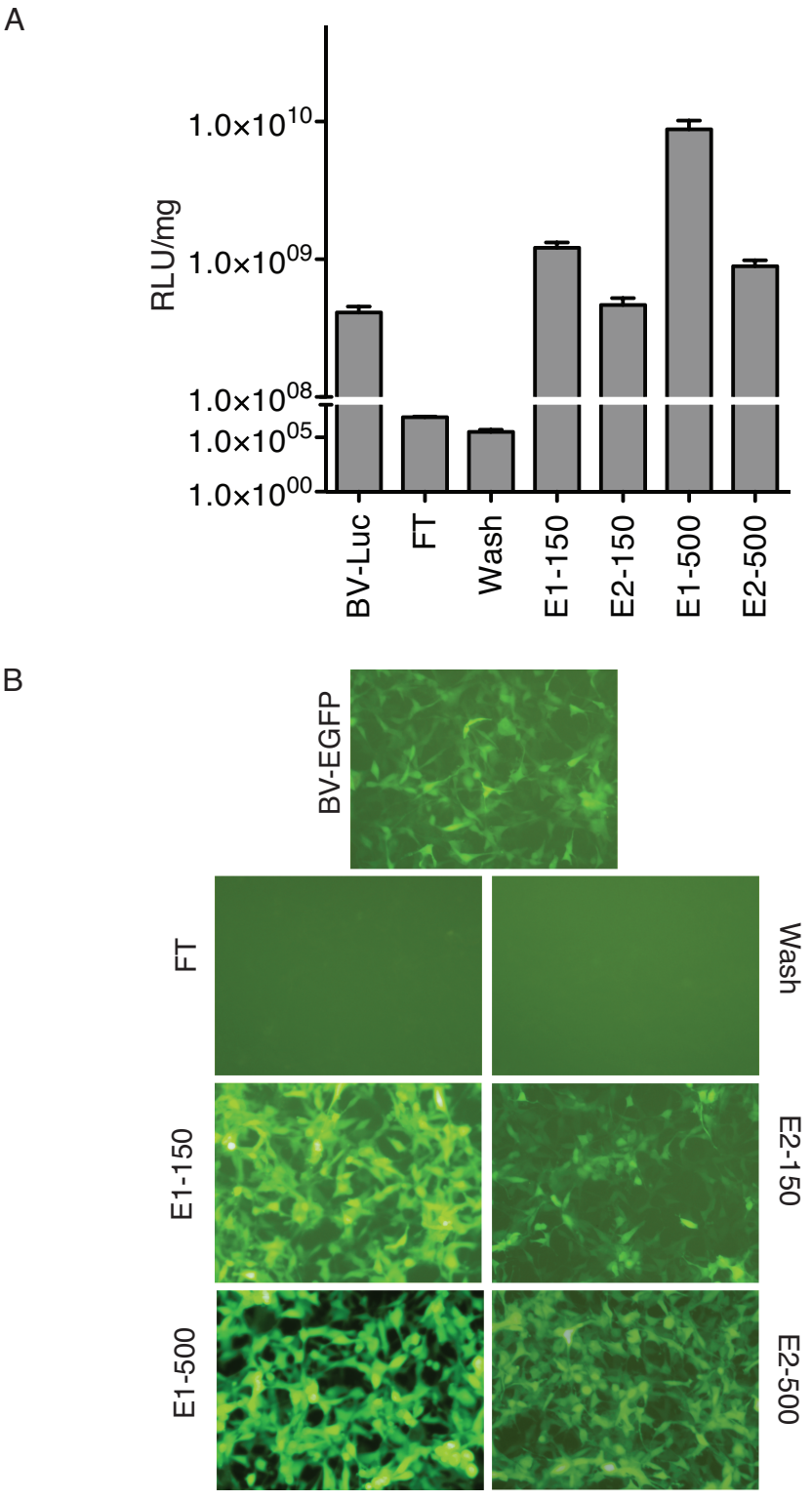

Figure 3.2. Transduction efficiency of rBV expressing reporter genes purifed by cation exchange membrane chromatography. Luciferase (A) and EGFP (B) gene expression for various virus fractions were measured after 48 hours on U87 cells transduced with BV-luc and BV-EGFP respectively. FT-Flow through; E1, E2 – first and second elute; 150, 500 – Concentration of NaCl (mM) in PBS..

Similar trend was observed with fractions eluted using PBS-500 mM NaCl. Precisely, first two eluates (E1-500 and E2-500) displayed higher luciferase expression than load sample whereas the eluates obtained later showed lesser luciferase expression. Consistent with the number of viral particles recovered, U87 cells transduced with E1-500 and E2-500 expressed more luciferase than E1-150 and E2-150.

The same set of experiments were performed with rBV encoding enhanced green fluorescent protein (EGFP) encoding gene and similar expression patterns were observed under fluorescence microscope (Figure 3.2B).

3.4. Analysis of purity for recovered rBV

To examine the purity of the virus samples eluted from membrane chromatography unit, eluted virus fractions together with mock supernatant (Sf9 cultured supernatant not infected with BV), rBV load, FT and wash were denatured by boiling in 1% SDS sample buffer and proteins were resolved on SDS-PAGE and viewed using silver iodide staining (Figure 3.3A). As expected, virus supernatant contained an enormous amount of contaminating proteins. Comparison with the proteins in the lane of mock supernatant indicated that most of the proteins in BV-load were of insect cell origin thus, emphasizing the need to remove them for minimizing the immune responses to rBV as a gene therapy vector. Minimal amounts of gp64 and large amounts of other contaminating proteins could be spotted on FT lane. This indicated the strong interaction between rBV and the cation exchange membrane at pH 6. Relatively lesser contaminating proteins and trace amounts of gp64 were present in wash thus proving helpful in washing away some of the contaminating proteins. Far lesser contaminating proteins and enrichment for gp64 characterized the lanes containing eluates. This indicated the successful elution of

rBV at pH 7.4. Abundant proteins studded on the lane – proteins on the membrane indicated the retainment of humongous amounts of proteins on the membrane even after elution of ≈ 90% of the rBV particles thus indicating the selectivity of pH to elute only rBV from the membrane. A western blot analysis for gp64 performed on various samples reconfirmed the presence of rBV. The results were in agreement with the findings from real-time PCR and SDS-PAGE (Figure 3.3B). Thus, these results provide evidences for the usefulness of membrane chromatography method in purifying BV without considerable loss in recovery and transduction efficiency.

3.5. Concentration of rBV

Though purification by membrane chromatography method offered better purity, in an attempt to maximize the elution to ~90% by increasing the volume of elution buffer, the concentration was compromised. To concentrate BV, a second step involving centrifugation of the eluate fractions at a high speed of 28,000 g at 4 ºC for one hour was included. The pellet was dissolved in appropriate amounts of PBS (pH 7.4) containing 150 mM NaCl. To examine if the two step purification process affected virus quality, the prepared formulation was tested for recovery, transduction efficiency and purity and compared against virus formulation purified by the commonly used laboratory method involving only high speed centrifugation (HS) at 28,000 g for 1 hour at 4 ºC. This will be discussed in the following sections.

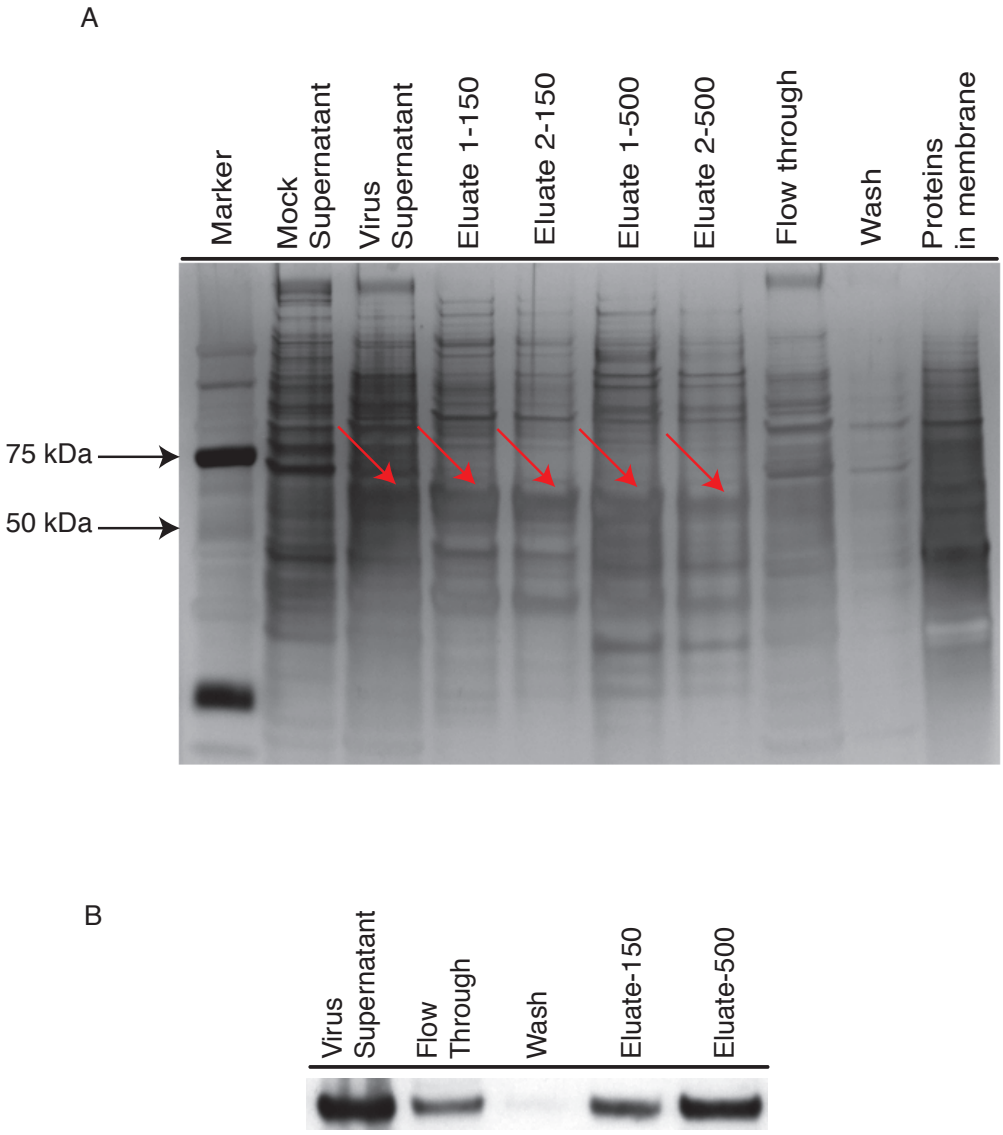

Figure 3.3. Analysis of rBV formulation before and after purification by membrane chromatography. Various virus fractions were electrophoresed on sodium dodecyl sulphate-polyacrylamide gels and total proteins were viewed using silver iodide staining (A) and gp64 viewed by western blot (B). ➔denotes gp64. Note the lesser amount of total proteins in eluates indicated by fewer and lighter protein bands compared to BV-load and abundant amounts of total proteins in the last lane indicating the removal of much of the proteins from the BV load.

3.6. Comparison of rBV purification methods for recovery, transduction efficiency and purity

Purification by high-speed centrifugation (HS) method yielded a final recovery of ~55% by real time PCR assay for gp64 and the two-step purification process (MC+HS) yielded a final recovery of 29% ± 5 (represented as Mean ± SD of three independent sets of purification). Since the high speed centrifugation method of purifying baculovirus is always associated with a loss of ~50%, it was not surprising that the recovery by MC+HS dropped to ~30%. A representative run of BV purification by MC+HS and HS method is given in Table 3.2.

Method		Sample	Voulme (ml)	VP/ml	Total VP[1]	% recovery[2]
		BV-luc	10	6.08E+09	6.08E+10	
HS		HS	0.3	1.12E+11	3.35E+10	55
MC+HS	Step1	E-150	5	8.08E+09	4.04E+10	66
		E-500	5	2.71E+09	1.35E+10	22
	Step 2	MC+HS	0.5	3.91E+10	1.95E+10	32

Table 3.2. Summary of total BV yield and final recovery after purification by High Speed Centrifugation and Membrane Chromatography+high speed centrifugation. VP- viral particles; 1-Total viral particles (TVP) was calculated by multiplying viral particles with volume; 2 - Recoveries were calculated by normalizing the data against the loaded BV-luc sample E-150- BV eluted with PBS-150mM NaCl at pH 7.4; E-500- BV eluted with PBS-500mM NaCl at pH 7.4.

The reverse of this process, that is, purification of baculovirus by centrifugation at high speed followed by membrane chromatography was also tried (data not shown). However, when membrane chromatography was used as the second step, virus could not be efficiently eluted using a smaller volume of elution buffer and the concentration was compromised when trying to maximize the recovery yield. To check the effect of purification procedures on the transduction efficiency,

equal number (MOI 50) of purified BV-luc from both methods and BV-luc supernatant were tested for their transduction activity. Transduction by MC+HS-purified BV-luc resulted in transgene expression comparable to that resulted from transduction by HS-purified BV. Though there were slight differences in luciferase gene expression between transduction by MC+HS purified and HS-purified BV, the variations were not statistically significant. (Figure 3.4A). The purity of the samples were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining. As shown in Figure 3.4B, BV-luc supernatant collected from Sf9 cell cultures, clarified by centrifugation at 1000 g for 5 minutes and filtered through 0.45 µm pore sized filter contained abundant proteins. MC+HS purified virus formulation displayed a single major band of molecular mass ≈ 64 kDa correlating to gp64 whereas HS purified virus formulation displayed a major band of gp64 together with other protein bands indicating the presence of contaminating proteins. The presence of BV in various formulations were confirmed by western blot for gp64 (Figure 3.4C). These results suggested that MC+HS purification method offered better purity virus formulation with little or no loss in transduction efficiency.

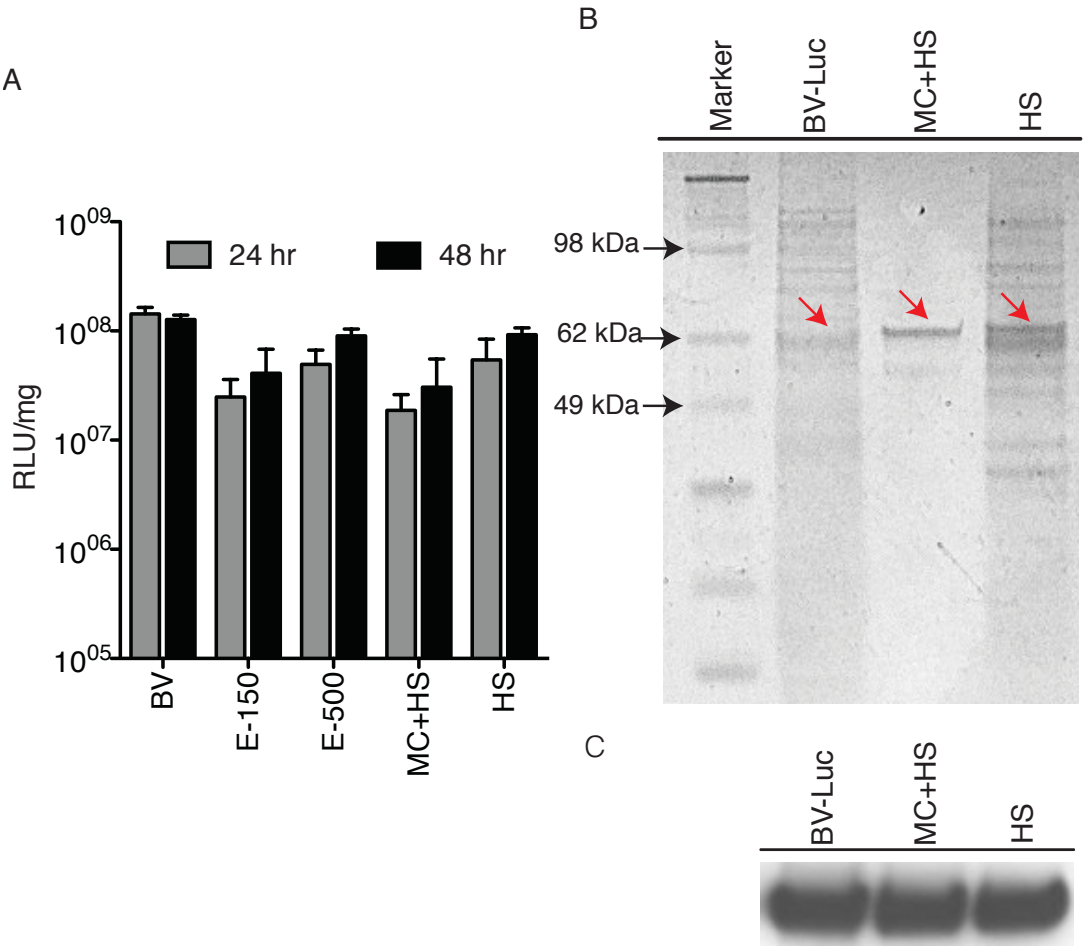

Figure 3.4. Transduction efficiency and purity of BV-luc purified by membrane chromatography + high speed centrifugation (MC+HS) method and high speed centrifugation (HS) method alone. (A) Comparison of transduction efficiency of BV-luc before and after purification by the two methods. Luciferase activities were measured after 24 and 48 hours. Each group consisted of at least three repeats and values are expressed as mean + SD. Note that there is no significant differences in luciferase expression (B) Purity of virus preparations before and after MC+HS and HS purification by silver staining. Note that MC+HS lane has a single band of proteins corresponding to molecular weight of ≈ 64 kDa as against multiple protein bands in other lanes. ➔denotes gp64. (C) Confirmation of the presence of BV in MC+HS- and HS-purified formulations by western blot for gp64.

3.7. Comparison of rBV purification methods for immune responses in mice brains by cDNA microarray analysis

3.7.1. rBV formulations prepared by MC+HS and HS methods considerably alters gene expression in mice brains

To verify the usefulness of MC+HS method in adding to the biosafety profile of BV for treating CNS disorders, microarray analysis on mice brain striatum inoculated with MC+HS-purified rBV was performed and compared with those inoculated with HS-purified rBV using Affymetrix GeneChip Mouse Genome 430 2.0 Array. rBV accommodating HSVtk gene under the control of GFAP promoter and appended to three repeats of three micro RNA sequences (hsa-miR-31, hsa-miR-127 and hsa-miR-143) shortly referred to as BV-tk that were used to treat gliomas in mice models (Wu *et al.*, 2009) were used for this purpose. The chip offers a comprehensive analysis of genome wide expression on a single array comprising of 45,000 probe sets for over 39,000 well characterized mouse genes. At least three mice brain samples from separate animals, each injected with mock, MC+HS-purified and HS-purified viruses were collected for microarray analysis. We observed approximately 61.3%, 59.37% and 60.1% of gene probes on the chip being flagged as present in MC+HS-purified BV-, HS purified BV- and mock - injected mouse brain samples respectively. GenespringGX 11.5 from Agilent Technologies was used for microarray data analysis. Using a two fold difference in normalized expression level and a t-test p-value of ≤ 0.05 as an arbitrary cut off value against the mock injected brain samples, we observed 379 probes (0.84%) in MC+HS purified BV injected brain samples and 431 probes (0.94%) in HS purified BV injected brain samples to be altered. The set of 379 gene probes from MC+HS purified BV injected brain samples represented 351 up regulated and 28 down regulated probes with a total of

378 IDs available in Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources 6.7 and the set of 431 gene probes from HS purified BV injected brain samples represented 406 up regulated and 25 down regulated probes with a total of 429 IDs available in DAVID. This is summarized in Table 3.3**.** These data indicate that rBV formulations prepared by both purification methods bring about alterations in considerable number of genes.

	HS	MC+HS
Microarray Chip Used	Affymetrix GeneChip Mouse Genome 430 2.0 Array	Affymetrix GeneChip Mouse Genome 430 2.0 Array
Total No. of Transcripts present	45101	45101
Total No. of transcripts examined (present/marginal)	27438	27004
Probes modified (2 fold change+p<0.05)	431	379
Probes Up regulated	406	351
Probes Down regulated	25	28
% of modified genes	0.94	0.84

Table 3.3. Summary of microarray data analysis on mice brains inoculated with HS and MC+HS purified BV by Genespring GX11.

3.7.2. Validation of microarray results by real-time PCR assay

To validate the fold changes in expression from microarray analysis, 8 significantly modified (fold changes >= 2 and t-test p-value <= 0.05) genes participating in important KEGG pathways were analyzed by real-time PCR assay. The various KEGG pathways and the genes tested are as follows:

RIG-1 like receptor signaling pathway - MDA5, Isg15 and Irf7

Antigen presentation and processing - Tap1 and Tapbp

Chemokine signaling pathway - RANTES, CXCL11 and CXCL12

Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal normalization control. All of the selected genes displayed similar trend in up or down regulation consistent with those from the microarray analysis even though the actual fold changes were not identical (Figure 3.5). Due to the intrinsic differences in the techniques used and normalization methodology, absolute values could be different between the results obtained with microarray and real-time PCR assay. For example, it is likely that pixel saturation occurs when the pixel intensity exceeds a threshold in a microarray, thus compressing the true induction magnitude of transcription (Dodd *et al.*, 2004).

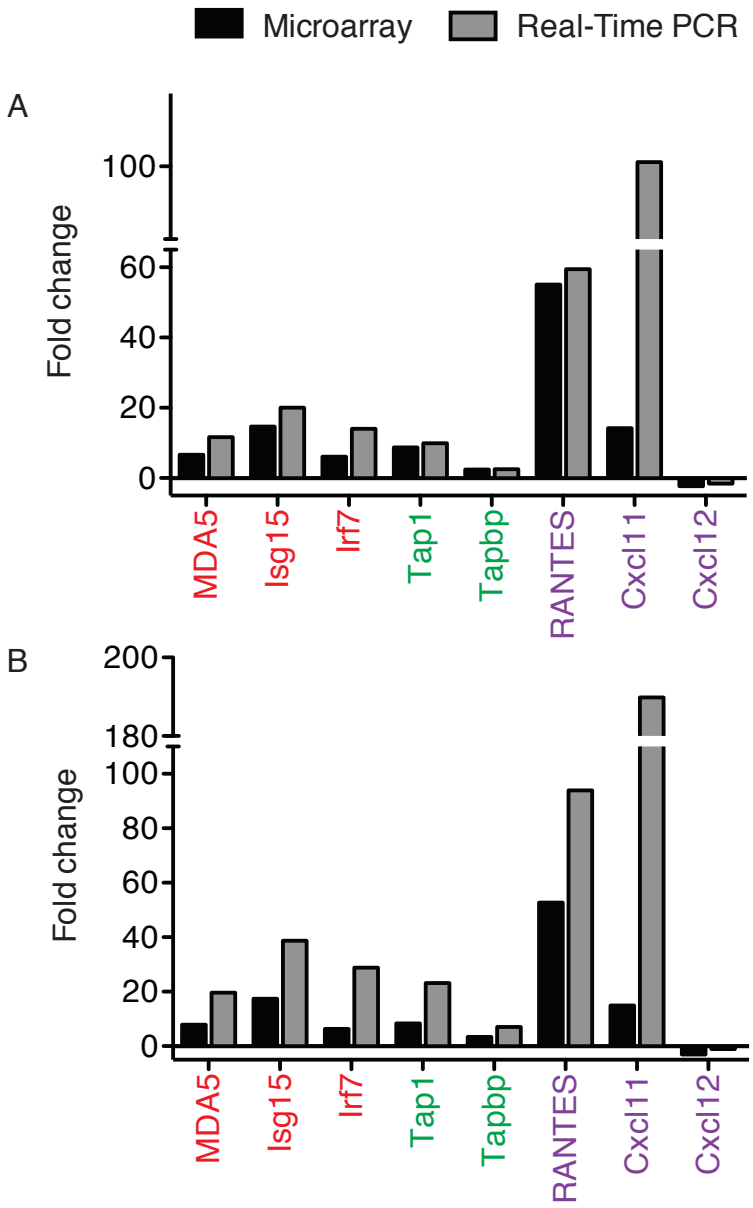

Figure 3.5. Validation of fold changes in expression by microarray results using quantitative real time PCR assay for mouse genes. Eight (seven upregulated – MDA5, Isg15, Irf7, Tap1, Tapbp, RANTES, Cxcl11 and one downregulated - Cxcl12) of the significantly modified (fold changes >= 2 and t-test p-value <= 0.05) genes from MC+HS (A) and HS (B) purified BV-tk injected mouse brain samples were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal normalization control.

3.7.3. Molecular responses in mice brains to MC+HS- and HS-purified BV

Molecular responses to rBV in mouse brains were analyzed using three different softwares – Genespring GX11.5, Metacore™ and DAVID Bioinformatics to gain insight into different issues. To obtain the list of genes differentially expressed between MC+HS and HS lists, Genespring GX 11.5 was used. For convenience, the probe lists containing significantly modified genes (fold change >= 2 and t-test P-value <=0.05) in MC+HS- and HS-purified BV injected mouse brains will hereafter be mentioned as MC+HS and HS probe lists. Of the significantly modified probes in MC+HS and HS lists, a large number (353) were common to both as expected since BV is the major component of both formulations. Of the 353 probe IDs, one way Anova test with unequal variance followed by Tukey HSD post hoc test revealed 19 probes (corresponding to 18 genes) to be differentially regulated (Table 3.4). The fold changes of these differentially regulated genes in HS probe list were remarkably higher compared to that in MC+HS probe list. Using DAVID, Gene Ontology – Biological Processes (GO-BP) that these genes participate in were obtained. Upon using Amigo Visualize Tool (http://amigo.geneontology.org/cgi-bin/amigo/amigo?mode=visualize) on these GO-BPs, antiviral immune responses were found to be the major responses (Figure 3.6).

Other than the commonly affected probes, there were 35 probes in MC+HS list and 77 probes in HS list. To gather information about the difference that the changes in these probes make to the host, Metacore from GeneGO was used. Metacore is an integrated software suite based on a manually curated database for functional and comparative analysis of microarray data across gene lists. Both lists were subjected to comparative enrichment analysis for various ontologies in

Metacore. Comparative enrichment analysis option in Metacore allows for the analysis of contribution of common and unique genes across the two gene lists to various ontologies. We restricted our focus to Gene Ontology (GO) processes and GeneGO pathways. Top 10 significantly affected GO processes and GeneGO pathways in MC+HS and HS are listed along with the contribution of common and unique genes to various ontologies in terms of negative log (P-value) in Figure 3.7A. The more the negative log (P-values), the stronger and more reliable are the chances for the ontology to be affected. The over represented GO processes with the most significant P-values included immune response, immune system response, defense response, cellular response to cytokine stimulus, response to cytokine stimulus, response to other organism, innate immune response, response to biotic stimulus, cytokine-mediated signaling pathway and multi-organism process. The fact that they were listed among the top 10 GO processes indicated that these were the most important biological changes that define the host responses to BV transduction in mouse brains. This is similar to our previously published results defining the responses of BV inoculation in the rat brain, human astrocytes and neurons (Boulaire *et al.*, 2009).

Comparative enrichment analysis for affected GeneGO pathways also revealed the top 10 significant pathways to be related to immune responses. Complement activation pathways topped the list across the two gene lists. Complement system consists of nearly 30 small proteins that can be activated through three pathways namely classical, alternate and lectin-binding complement pathway (Taylor *et al.*, 1998). All three pathways were activated across the two lists. Other pathways significantly affected were involved in eliciting innate immune response to viral infection and resulting in interferon signaling and production

followed by antigen presentation by MHC class I molecules. The list of genes affected across various significant pathways and their fold changes are given in Table 3.5**.** Most of the genes from MC+HS were modified by lesser fold changes compared to those from HS.

A close scrutiny at the graphs revealed that the contributions of the large number of genes common to both MC+HS and HS list towards the P-values of both GO processes and GeneGO pathways were highly significant. Apart from them, those genes unique to HS list contributed significantly to all top 10 GO processes. However, genes unique to MC+HS either did not contribute or contributed to a smaller extent to the P-values of GO processes compared to those genes unique to HS. Of the 10 significant GeneGO pathways, genes unique to HS contributed to the complement activation pathways, IFN alpha/beta signaling pathways and antigen presentation by MHC class I whereas genes unique to MC+HS did not participate in these pathways. These results suggested that genes from MC+HS list give rise to same immune responses elicited by the genes common to both MC+HS and HS list and the genes unique to MC+HS did not contribute to immune responses unlike the unique genes from HS list thus confirming the safety associated with this method.

Gene Symbol	Fold Change	
	HS vs Mock	MC+HS vs Mock
9530028C05	15.64	10.4
BC013712	3.02	2.51
C2	2.76	2.38
Ctsc	3.79	3.04
Gbp2	12.5	10.29
Ifit2	18.78	13.02
Igtp	16.41	12.11
Tor3a	3.03	2.4
Parp12	8.05	5.76
Parp14	6.06	4.65
Parp9	7.54	5.73
Plac8	15.02	12.08
Rsad2	18.53	12.92
Slfn8	9.96	7.56
Stat1	10.6	7.77
Trim25	4.63	3.72
Ugt1a	3.86	3.09
Zc3hav1	5.66	3.95

Table 3.4. Differentially expressed genes across HS and MC+HS gene lists and their fold changes. Probes common to HS and MC+HS list were analyzed by Genespring GX 11.5 for differentially expressed genes by one way Anova test with unequal variance followed by Tukey HSD post hoc. Fold changes of the various differentially expressed genes are represented.

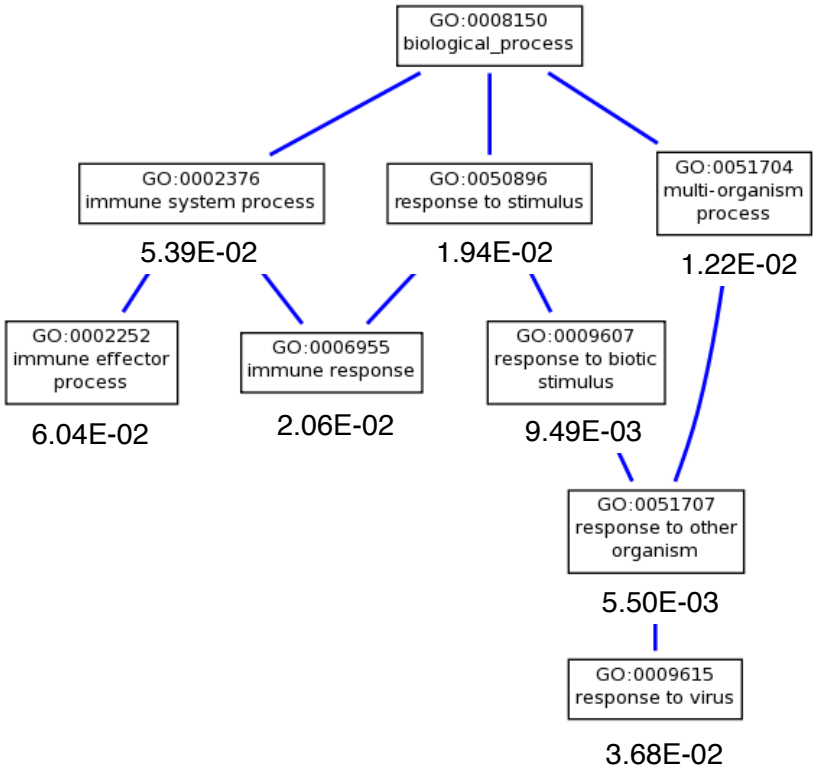

Figure 3.6. Treeview of Gene Ontology Biological Processes (GO-BP) that the differentially expressed genes in HS and MC+HS involved in Significantly affected GO-BP from DAVID enrichment analysis were fed into Amigo Visualization Tool for obtaining the GO tree view. The P-values of the affected terms are given below each term.

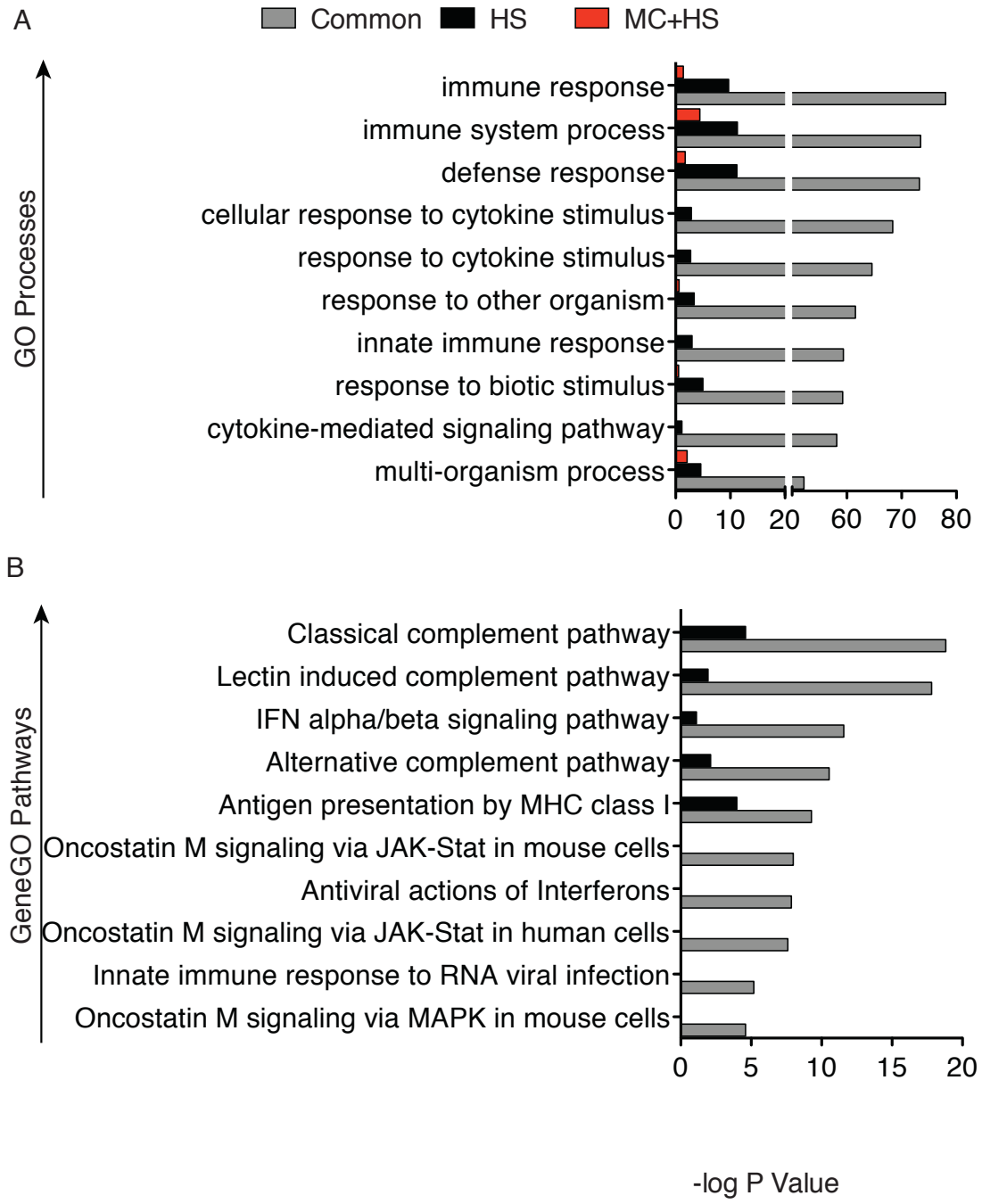

Figure 3.7. Comparative enrichment analysis for GO processes and GeneGO pathways for significantly modified mouse genes. Significantly affected GO processes (A) and GeneGO pathways (B) indicated major host responses to baculovirus purified by the two methods. Significantly modified genes are those with fold changes ≥ 2 and t-test p-value ≤ 0.05.

GeneGO Pathways	Fold Changes	
	MC+HS	HS
Complement Pathways		
C3a/C3b/C3dg/C5 convertase/iC3b	13.88	18.81
alpha-M/beta-2 integrin	-	2.04
alpha-X/beta-2 integrin	-	2.04
C5AR	2.90	2.72
Factor B	8.71	7.62
C1 inhibitor	5.61	6.15
C1	-	2.07
C1q	-	2.07
C1qRp	3.04	3.34
C3 convertase (C2aC4b)	3.47	4.08
C4b	2.72	2.97
IgM	-2.15	-2.20
IFN a/b signaling pathway- P-value		
SOCS1	-	2.38
SHP-1	2.09	2.45
STAT2	2.58	2.62
PML	2.18	2.75
IRF9	3.65	4.26
ISGF3	3.85	5.24
STAT1/STAT2	3.85	5.24
STAT1	3.85	5.24
ISG15	10.22	11.25
USP18	10.39	11.87
ISG54	8.88	13.02
Antigen presentation by MHC class I		
Immunoproteasome (11S regulator)	2.14	2.48
Tapasin	2.62	3.52
Beta-2-microglobulin	2.63	2.65
TAP2 (PSF2)	7.25	8.17
Immunoproteasome (20S core)	7.66	8.80
TAP1 (PSF1)	8.69	8.31
MHC class I	9.22	9.82
PSME1	-	2.19
PSME2	2.14	2.48
PSMB8(LMP7)	7.66	8.80
Antiviral actions of Interferons		
STAT2	2.58	3.25
IRF9	3.65	4.26
PKR	4.92	5.18
STAT1	6.30	7.58
ISGF3	6.30	7.58
2'-5'-oligoadenylate synthetase	7.80	8.53
OAS1	5.16	5.76
OAS2	7.80	8.53
HLA-A	9.22	9.82
MHC class I	9.22	9.82

Oncostatin M signaling via JAK-Stat & MAPK in mouse and human cells		
OSM receptor	2.74	3.05
SOCS3	4.86	5.38
TIMP1	5.82	6.04
STAT1	6.30	7.58
SERPINA3 (ACT)	5.03	5.15
CCL2	9.53	12.68
Innate immune response to RNA viral infection		
I-kB	2.46	3.11
RIG-I	4.18	6.42
IRF7	6.06	6.26
MDA-5	6.62	9.25
LGP2	8.15	11.23
TLR3	3.46	4.12

Table 3.5. Significantly affected GeneGO pathways in MC+HS and HS purified BV innoculated mouse brains. Significantly modified genes (fold changes >= 2 and t-test p-value <= 0.05) from MC+HS and HS were subjected to comparative enrichment analysis for GeneGO pathways. Note the lesser number of genes and lesser fold changes of the affected genes in MC+HS compared to HS.

3.8. Discussion

The overriding objective of this chapter was to develop an efficient purification method for BV which can minimize insect cell contaminants thus eliminating much of the unwanted immune responses. The main finding of our study is that immune responses to BV decreased as the purity of the formulation increased. The method we developed met our objective as the purity of BV formulation in MC+HS method as shown by SDS-PAGE was much better than that obtained by HS method. It also yielded a decent recovery of more than 30% of total viral particles after the two-step purification process. Recovery after purification by membrane chromatography was up to 94% in one of our experiments. Such high recoveries are possible only with membrane chromatography because it uses absorptive membranes with highly porous structures and active chemical groups attached to the surface of the membrane. The greater pore size offers more active surface area for binding of particles as large as viruses unlike column chromatography with resins or beads with

active surface areas contained within the small pores of the matrix. Also, the membranes are associated with shorter diffusion times compared to the resins or beads which are usually closely packed (Charcosset, 2006). Also, membrane chromatography allows for easy scale up. The mustang S cation exchange membrane chromatography unit used in this study has an open pleat of 16 layers of polyethersulfone (PES) membrane with pendant sulfonic functional groups and pore size of 0.8 μm which is larger than the size of BV. The binding of BV to the cationic membrane was mainly through gp64 protein (Wu *et al.*, 2007). At slightly acidic pH of the endosomes, BV envelope is known to fuse with the host cell triggered by the conformational changes in gp64 (Blissard *et al.*, 1992, Zhou *et al.*, 2006). The same principle might apply here. At pH ≈ 6 (the pH of the Sf9 supernatant containing BV), gp64 protein is in its native conformation. When passed through the membrane, virus bound to it through gp64 proteins in its native conformation. Upon passing PBS of pH 7.4, change in pH caused conformational changes in gp64 thus allowing the elution of BV. Since this method utilizes the conformational changes of BV gp64 for purification, besides BV purification, it can also be applied to purify other viruses psedotyped with gp64.

However, the recovery varied largely depending upon the temperature of the BV supernatant passed through the chromatography unit. Maximum recovery could be obtained when the supernatant was at room temperature and the recovery decreased with the decrease in temperature suggesting that binding of virus to the membrane chromatography unit was stronger at lower temperatures thus decreasing the elution whereas the binding at room temperature was weak enough for the elution buffer to recover most of the viruses. Similar trend in baculoviral supernatant temperature playing a role in virus binding and elution has been reported before in

concanavalin A chromatography for baculovirus purification (Chen *et al.*, 2009b). The second step of high speed centrifugation not only concentrated the virus but also purified them by removing more proteins (Figure 3.4). Also, the lesser speeds used for centrifugation (28000 g) as against the ultra high speed of 80,000 g in sucrose gradient ultracentrifugation method resulted in little loss in infectivity. This method could be used for virus volumes up to 40 ml without much changes in recovery and scaling up to larger volumes awaits further investigation.

Although other purification methods like size exclusion chromatography and multistep downstream processing involving the use of anion exchange membranes yield an overall recovery of ~67% (Transfiguracion *et al.*, 2007) and ≈ 38% (Vicente *et al.*, 2009) respectively, our study is the first to establish the biosafety associated with a purification method *in vivo* using global gene expression profiling. MC+HS method brought significant changes only to 379 probes as against the 431 probes in HS method. Further analysis of these gene lists, in general, resulted in highly enriched immune response related GO processes and GeneGO pathway maps (as denoted by lower P-values) for genes in HS list than in MC+HS list. The lower the p-value, the higher is the confidence that the effect is true and not by chance. In particular, BV purified by MC+HS method resulted in the elicitation of lesser number of genes with lesser fold changes in the complement pathways, interferon signaling pathway and antigen presentation by MHC class I (Table 3.5) suggesting that removal of contaminating proteins could be the reason for this. Thus, this chapter demonstrated the usefulness of MC+HS purification method in reducing the immune responses *in vivo*.

4. Host Responses to rBV inoculation into brains of non-human primates

4.1. Introduction and Aims

With the successful demonstration of therapeutic effect of rBV expressing diptheria toxin A gene (Pieroni *et al.*, 2001, Wang *et al.*, 2006a) or HSV-tk gene under the control of cell-specific promoters (Balani *et al.*, 2009, Wu *et al.*, 2009) on rodent glioma xenograft models, hopes on rBV as gene delivery vectors for the treatment of gliomas or other central nervous system (CNS) disorders continue to be increasing. However, reports that baculovirus elicits immune responses in hosts (Abe *et al.*, 2010) that sometimes nullify the transgene expression (Pieroni *et al.*, 2001) question their suitability as gene delivery vectors for clinical trials. A complete understanding of the baculovirus-host interactions has not yet been established. The fact that limited information is available so far on the effects of baculoviral vectors on transduced mammalian cells at the molecular level necessitates the usage of high throughput analysis to clarify virus-host interactions. DNA microarray based gene expression profiling is one such technology that has been suggested to characterize host responses to viral-vector mediated transduction (Piersanti *et al.*, 2004). The humungous data generated from microarray studies allow for the recognition of major pathways of virally induced responses and thus risk assessment of the vectors and identification of new target genes for specific viruses. Our laboratory has been applying DNA microarray technology that allows global gene expression profiling as a systems level approach in exploring host-BV interactions in human cell lines and rat models (Boulaire *et al.*, 2009). Though rodents have been the experimental model for majority of the research groups to provide an insight into various research problems, especially the immune system, because of the differences in the time of evolution,

size and lifespan, they are not the same as humans. Variations in the structure, general characteristics, innate and adaptive immunity between mice and man have been reviewed in detail to caution the researchers about the possible differences in outcome (Mestas *et al.*, 2004). This underscores the necessity of valid animal models to accurately evaluate the safety and efficacy of vectors designed for treatment of patients.

Figure 4.1. Cynomolgous monkey (*Macaca fascicularis*).This macaque sharing high genome similarity with humans has become an important model for preclinical evaluation of viral-based gene therapy vectors.

Non-human primates (NHPs), with their greater immunological and biological similarities to humans are in great demand as models for preclinical evaluation of highly specific biological therapeutics (t'Hart *et al.*, 2003, Fiandaca *et al.*, 2010). The most commonly used NHPs in research are Old World monkey species especially, rhesus and cynomolgus macaques (*Macaca mulatta* and *M. fascicularis* (Figure 4.1)), the common ancestor of which and man dates back to 25 million years ago (Gibbs *et al.*, 2007). This evolutionary proximity has reflected a high degree of similarity between man and macaques at the level of polymorphic gene families, such as those encoding major histocompatibility complex or T-cell receptor molecules or

immunoglobulins, and also leukocyte surface molecules and immune regulatory molecules, such as cytokines and chemokines (Bontrop *et al.*, 1995, Bontrop *et al.*, 1999). Also, the draft genome sequence of rhesus macaque published in 2007 (Gibbs *et al.*, 2007) transforms the animal from a physiological model in to a "whole-organism system". Together with these, the fact that both humans and NHPs can be infected with similar types of viruses and that infections often follow a similar course makes NHPs important models to study virus-based constructs for gene therapy. Thus, envisaging the advancement of baculoviral vectors into clinical trials as gene delivery vectors to treat CNS disorders, this chapter aims to understand baculovirus-host interactions at the molecular level in non-human primates' brains and evaluate the toxicology of BV upon acute central administration.

4.2. BV-tk inoculation into macaque brains considerably alters gene expression

Three cynomolgous monkeys (*Macaca fascicularis*) – 5A5C, 557A and 5D0D were used for this study. Having verified the improved safety associated with MC+HS purification method and the therapeutic efficacy of recombinant baculovirus accommodating HSVtk gene under the control of GFAP promoter and appended to three repeats of three micro RNA sequences (hsa-miR-31, hsa-miR-127 and hsa-miR-143) shortly referred to as BV-tk to treat gliomas in mice models (Wu *et al.*, 2009), BV-tk purified by MC+HS method was used to assess the host responses upon acute central administration into macaque brains. Four injections each containing 10^8 pfu of test virus was injected into right hemisphere against the mock supernatant purified by MC+HS method into the left hemisphere. After 48 hours, the samples were collected and successful injection of virus into macaque brains was verified by real time PCR for gp64 on one of the four test virus injected brain samples

against its corresponding control in each animal (Figure 4.2). All three virus injected brain samples tested positive for gp64 but with varying copy numbers. Differences in the copy number may be attributed to the differences in the sample collection (a bit further away from the injection site) or processing of the tissues for measuring gp64 copy number.

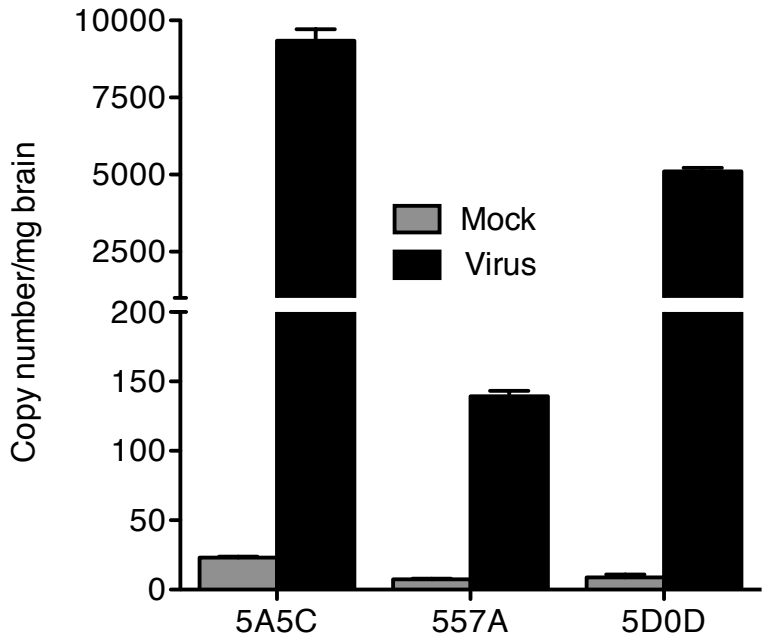

Figure 4.2. Confirmation of successful injection of rBV-tk into macaque brains by real time-PCR analysis for gp64. In all three animals, one of the four BV-tk injected brain samples and their corresponding control brain samples were analyzed for the presence of virus.

Gene expression profiling in response to BV-tk inoculation in to macaque brains was examined using Affymetrix GeneChip Rhesus Macaque Genome Array that provides a comprehensive coverage of the transcribed rhesus genome, comprising 52,024 probe sets for approximately 47,000 Macaca *mulatta* transcripts. Two pairs of samples, BV-tk-injected and mock-injected brain tissues from each animal with a total of 6 samples each in BV-tk-injected and mock-injected category were collected for microarray analysis. Approximately 53.5% and 50.43% of the gene

probes on the chips were called present in the virus-injected and mock-injected brain samples. After performing quality control based on expression and flags, 205 probes (0.39%) were observed to show two or more folds difference in expression and to be statistically significant (t-test, p-value ≤ 0.05) between the BV-tk-injected and mock-injected samples. This set of genes represented 184 up regulated and 21 down regulated probes with 171 IDs recognized by DAVID Bioinformatics 6.7. Microarray data analysis by GenespringGX 11.5 from Agilent Technologies is summarized in Table 4.1.

Microarray Chip Used	Affymetrix GeneChip Rhesus Macaque Genome Array
Total No. of Transcripts present	52,865
Total No. of transcripts examined (present/marginal)	25491
Probes modified (2 fold change+p<0.05)	205
Probes Upregulated	184
Probes Downregulated	21
% of modified genes	0.39

Table 4.1. Summary of Microarray Data Analysis on macaque brains inoculated with MC+HS purified BV by Genespring GX11.5

4.3. Validation of microarray results by real time PCR assay

To validate the findings from microarray analysis, real time PCR assays were carried out on 9 genes – MDA5, ISG-15, IL-15, TAP-1, MAMU-A, MAMU -E, MAMU-F, C1S and FCGR3. The selection was made in such a way that genes from important KEGG pathways were validated. The various KEGG pathways involving these genes are as follows:

RIG-1 like receptor signaling pathway – MDA5, ISG-15 and IL-15

Antigen presentation and processing - TAP1, MAMU-A, MAMU -E and MAMU -F

Complement and coagulation cascades – C1S

Fc gamma R-mediated phagocytosis – FCGR3

Rhesus GAPDH was used as an internal normalization control. All of the selected genes displayed similar trend in up or down regulation (Figure 4.3) consistent with those from the microarray analysis even though the actual fold changes were not identical.

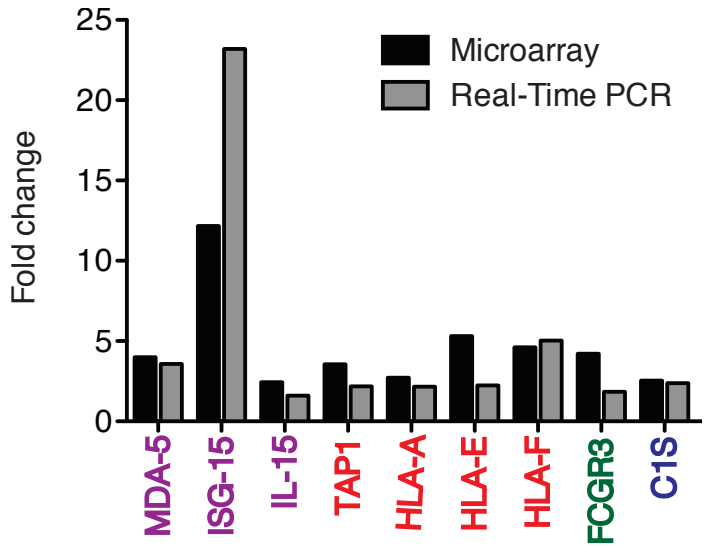

Figure 4.3. Validation of fold changes in expression by microarray using quantitative real time PCR assay for macaque genes. Nine of the significantly modified (fold changes >= 2 and t-test p-value <= 0.05) genes from macaque brains participating in different KEGG pathways were analyzed. Genes in purple - RIG-1 like receptor signaling, genes in red - Antigen Presentation and Processing, genes in green - Complement and Coagulation Cascades, genes in blue - Fc gamma R-mediated phagocytosis. Rhesus glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal normalization controls.

4.4. rBV inoculation into macaque brains elicits antiviral immune responses

To obtain the biological meaning of significantly modified genes in MC+HS purified BV-tk injected macaque brains, the web-based services, DAVID Bioinformatics Resources v6.7 and Metacore from GeneGO were used. The resultant probe list containing 205 IDs was fed into Metacore to gather insights into the GO processes and GeneGO pathways responsive to BV-tk inoculation in macaque brains. The top 10 significantly affected GO processes included immune response, defense response, immune system process, innate immune response, type I interferon-mediated signaling pathway, cellular response to type I interferon, response to type I interferon, interferon-gamma-mediated signaling pathway, cytokine-mediated signaling pathway and cellular response to interferon-gamma. Upon visualizing them on Amigo Visualize tool (http://amigo.geneontology.org/cgi-bin/amigo/amigo?mode=visualize), all 10 GO processes were eventually either a response to stimuli or a part of immune system process (Figure 4.4). Also, in an attempt to map the significantly affected genes to various GeneGO pathways, seven out of the top 10 significantly affected pathways were observed to be involved in immune response elicitation (Figure 4.5). The pathways included Lectin induced complement pathway, classical complement pathway, antiviral actions of IFNs, IFN alpha/beta signaling pathway, innate immune response to RNA viral infection, antigen presentation by MHC class I and IL-5 signaling. These results indicated that antiviral immune responses were the major responses to BV inoculation in the brains of non-human primates.

Upon using DAVID to map significantly genes to KEGG pathways, three immune response related pathways namely antigen presentation and processing (P-

value= 4.30E-05), RIG-1 like receptor signaling pathway (P-value= 2.81E-03) and cytokine-cytokine interactions (P-value= 6.50E-02) were found to be majorly elicited. In agreement with the notion that astrocytes and microglial cells of the brain present antigens and stimulate T-cells (Aloisi *et al.*, 2000), 13 genes from antigen processing and presentation pathway were found to be upregulated (Table 4.3). RIG-1 like receptors (RLRs) are a class of pathogen recognition receptors (PRRs) including RNA helicases like RIG-1, MDA5 and LGP2 that reside in the cytoplasm of immune and non-immune cells and recognize viral RNA species produced in the cytoplasm. RLRs are known to induce type I interferon (IFN) production through an adaptor molecule IPS-1 that localizes in the mitochondria (Kawai *et al.*, 2005). LGP2 serves as a negative or positive regulator of RIG-1 and MDA-5 depending on the type of RNA viruses (Venkataraman *et al.*, 2007). In macaque brains, five of the RLR pathway genes were up regulated and one down regulated (Table 4.2). Apart from IFN production, activation of PRRs in antigen presenting cells also leads to the production of immunologic messenger molecules - cytokines and chemokines, that interact with their respective receptors to bring about more innate and adaptive immune responses. Four cytokine genes were found to be upregulated in macaque brains (Table 4.3).

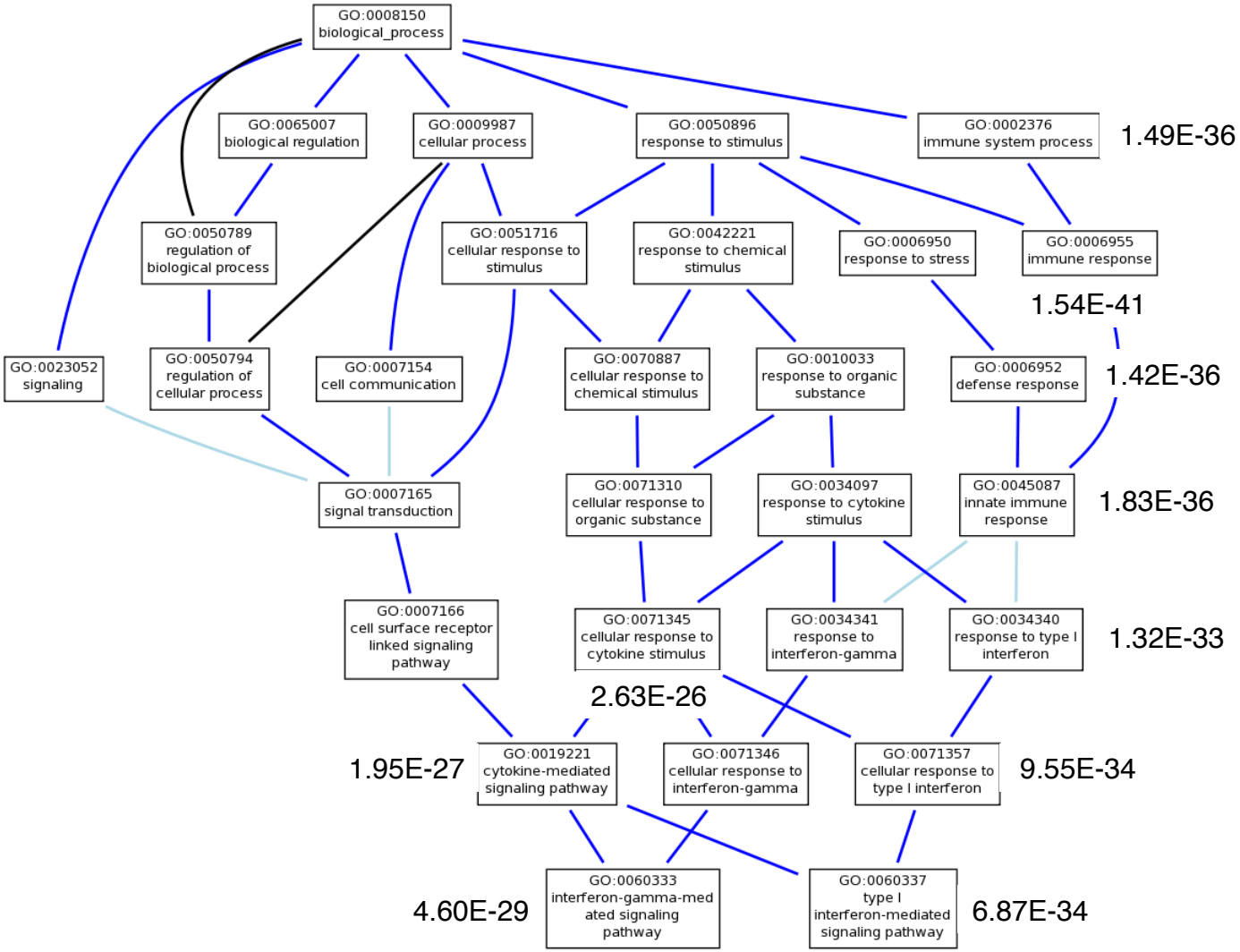

Figure 4.4. Tree view of significantly affected Gene Ontology - Biological Processes (GO-BP) in MC+HS purified BV-tk injected macaque brains samples. Top 10 significantly affected GO-BP from GeneGO enrichment analysis were fed into Amigo Visualization Tool for obtaining the GO tree view. The P-values of the affected terms are given below each term.

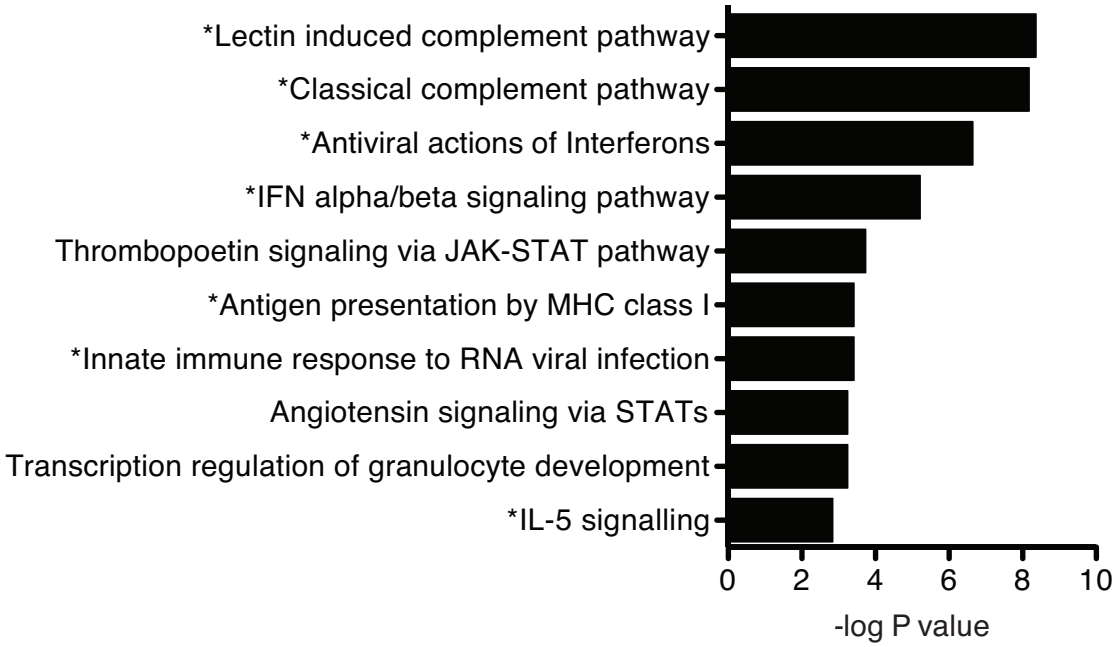

Figure 4.5. Significantly affected GeneGO pathways in BV-tk injected macaque brain samples. List of significantly affected genes in macaque brain upon BV inoculation was fed into Metacore for enrichment analysis using the default threshold for P-values. Top 10 significantly affected GeneGO pathways are presented here along with their negative log P-values.* indicates immune response related pathways. Note that immune response related pathways are dominant in the top ten pathways.

4.5. Differential responses to rBV inoculation in macaque and mouse brains

Though BV-tk elicited immune responses in macaque and mouse brains, remarkable differences were observed in the type of immune responses and interferon production pathways, which are discussed below

4.5.1. Interferon production pathway

In both macaque and mouse, interferon (IFN) production pathways were activated as an innate immune response to BV-tk inoculation. Induction of interferon production can occur as a result of activation of different PRRs. A close scrutiny of genes involved in the GeneGO pathway – Innate Immune responses to RNA viral

infection across macaque and mouse MC+HS gene list revealed that none of the TLR genes were significantly modified in eliciting innate immune responses in macaque brains (Table 4.3). This may suggest that TLR independent pathway may be involved in the induction of IFN production. To gain insight into the particular pathways that result in IFN induction, mouse and macaque gene lists were mapped to various KEGG pathways using DAVID bioinformatics. Significantly modified genes (fold change ≥2 and P-value ≤0.05) from mouse mapped to three interferon production inducing pathways namely Toll-like receptor (TLR) signaling, RIG-1 like receptor (RLR) signaling and cytosolic DNA sensing pathway whereas, gene list from macaque mapped only to RLR pathway (Figure 4.6) suggesting that RLR signaling is the major pathway of interferon production upon rBV inoculation in macaque brains.

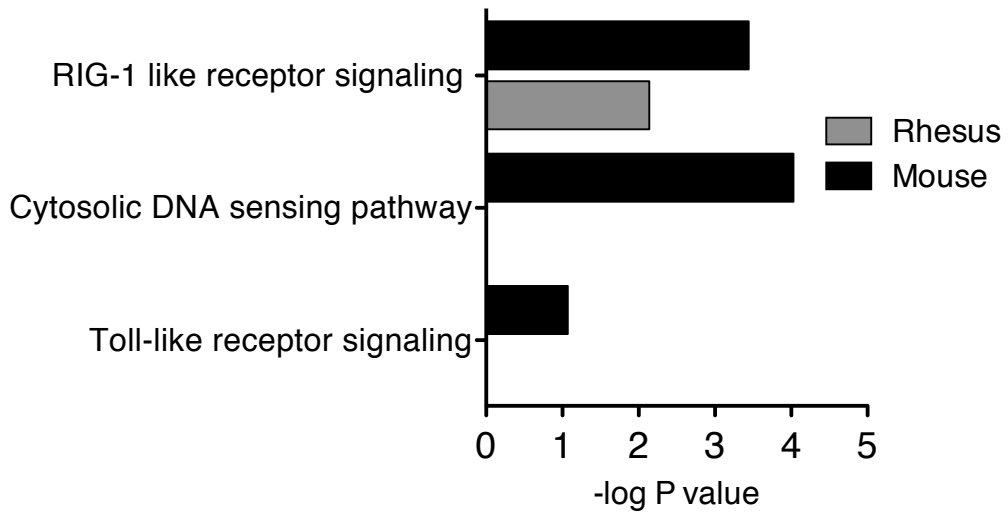

Figure 4.6. Interferon production pathways affected in mouse and macaque brains upon BV-tk innoculation. Significantly modified genes from mouse and macaque brains were subjected to DAVID analysis for enriched KEGG pathways. Note that only RIG-1 like receptor signaling pathway was affected in macaque brains whereas three pathways were affected in mouse brains upon BV-tk injection.

Gene Symbol	Mouse	Macaque
RIG-1 like receptor signaling pathway *p-value*	3.62E-04	2.81E-03
CXCL10	15.46	-
IL-8	-	2.21
IRF7	6.06	-
ISG15	10.22	12.19
LGP2	8.15	-
MDA5	6.62	3.99
POLR3C	-	-2.13
RIG1	3.77	3.85
STING	2.02	-
TRIM25	3.42	2.69
Cytosolic DNA-sensing pathway *p-value*	9.38E-05	
RIG1	4.18	
IFI202B	19.29	
STING	2.02	
IRF7	6.06	Pathway
PYCARD	2.23	not affected
CCL5 (RANTES)	55.13	
DAI	12.99	
CXCL10	15.46	
Toll-like receptor signaling pathway *p-value*	3.36E-03	
MYD88	2.01	
IRF7	6.06	
TLR2	2.44	
TLR3	3.46	Pathway
TLR4	2.12	not affected
CCL5	55.13	
STAT1	4.93	
CXCL10	15.46	

Table 4.2. Interferon production pathways affected in mouse and macaque brains upon BV-tk innoculation. Significantly modified genes (expression level ≥ two-fold and t-test p-value of ≤ 0.05) from mouse and macaque brains were subjected to DAVID analysis for enriched KEGG pathways. Fold changes of the various genes affected in the mentioned KEGG pathways are represented.

4.5.2. Major histocompatibility molecules

BV-tk induced antigen processing and presentation pathway in both macaque and mice brains. In macaque brains, antigen processing and presentation pathway involved both class I and II molecules of major histocompatibility molecules (MHC)

whereas in mouse brains, it involved only MHC class I molecules. Analysis by DAVID Bioinformatics for enriched KEGG pathways revealed this. The list of significantly modified genes involved in these pathways together with their fold changes are listed in Table 4.3.

4.5.3. Cytokines and chemokines

Another remarkable difference between mice and macaque responses to BV-tk inoculation was in the type and number of cytokines and chemokines. Chemokines are a class of small cytokines secreted by immune cells with immunomodulatory and chemo-attractive properties to recruit immune cells to the site of viral infection. In mice, 13 chemokine and 7 chemokine receptor genes were significantly up regulated suggesting the effective amplification of chemokine genes to recruit immune cells to the site of infection. However, in macaque brains, only four cytokines were significantly up regulated (Table 4.3).

These results suggested that macaque brains respond to BV by inducing interferon production through a TLR-independent RLR signaling pathway and that the injected dose of baculovirus per injection site did not elicit stronger immune responses and produced only fewer cytokines than in mouse.

Gene Symbol	Mouse	Macaque
RIG-1 like receptor signaling pathway (GeneGO pathway)		
I-kB	2.46	-
IL-8	-	2.21
IRF7	6.06	-
LGP2	8.15	-
MDA-5	6.62	4
RIG-I	4.18	3.35
TLR3	3.46	-
Antigen Processing and Presentation (KEGG pathway)		
MHC class 1		
(H2-Q6/H2-Q7)/HLA-B	13.4	2.52
(H2-T10/H2-T22)/-	6.09	-
B2m/B2M	2.52	-
Cd74/CD74	-	3.42
H2-D1/HLA-C	6.41	2.52
H2-Ea/HLA-DRA	-	2.86
H2-K1/HLA-C	6.87	2.52
H2-M3/HLA-G	2.91	5.21
H2-M5/MAMU-F	-	2.21
H2-Q8/-	6.87	-
H2-Q10/MAMU-B18	-	3.21
H2-Q10/MAMU-I	-	3.36
H2-T23/MAMU-E	7.5	3.18
H2-T24/-	3.62	-
H2-T9/-	13.4	-
H2-Dma/LOC717870	-	2.12
Psme2/-	2.14	-
Tap1/TAP1	8.69	3.29
Tapbp/-	2.41	-
Tap2/TAP2	7.25	-
MHC class II		
H2-Dma/LOC717870	-	2.12
H2-Ea/HLA-DRA	-	2.86
Ifi30/LOC719379	-	2.4
Cytokines-Cytokine receptor interaction		
CCL12 (MCP-5)	9.53	-
CCL2 (MCP-1)	12.5	-
CCL5 (RANTES)	55.13	-
CCL6 (MRP-1)	2.61	-
CCL9 (MRP-2)	3.24	-
CCR6 (CD196)	-2.04	-
CLCF1 (BSF-3)	2.71	-

CSF1 (MCSF)	2.17	-
CSF2RB2 (IL-3R)	2.19	-
CXCL10 (IP-10)	15.46	-
CXCL12	-2.32	-
CXCL13 (BCA-1)	3.99	-
CXCL16	2.47	-
CXCL2 (MIP-2)	3.24	-
IL2RG	4.94	-
IL4RA	2.72	-
OSMR	2.74	-
TNFRSF12A	2.21	-
TNFRSF1A	2.03	-
TNSF10	2.07	
IL15	-	2.44
CXCL11	-	5.29
TNFSF13B	-	4.53
IL8	-	2.21

Table 4.3. Differential Responses of mouse and Macaque brains to baculoviral transduction. Fold changes are shown for genes with expression level >= two-fold difference between baculovirus injected mouse and Macaque brains samples and corresponding control samples and a t-test p-value <= 0.05.

4.6. Toxicology and behavioral assessment of rBV vectors in non-human primates

To evaluate the toxicology and behavioral effects of rBV-tk following acute central administration in macaques, the animals were observed for various tests listed in Table 4.4 before and after virus injection. During the observation period of 48 hours after test virus injection, there was no death, abnormal clinical signs or behavioral changes in any animal (Table 4.5). There were no major changes in body weights (Table 4.6). Gross pathological findings also showed no detectable abnormality (Table 4.7). Urinalyses performed pre and post injection just before necropsy showed no abnormality (Table 4.8). Blood, liver and kidney samples tested negative for baculovirus by real time PCR assay for gp64 (data not shown). Also, histopathology report revealed no rBV-associated lesions in any of the tissues examined including brain (Figure 4.7). These data suggest that baculovirus has a good safety profile with minimal to no toxicity in macaque.

Observations	Results – 3 Macaque
Mortality	None
Clinical Observations	No abnormality
Body weight Measurement data	No major change
Haematology and Coagulation	Normal
Clinical Chemistry	Normal
Urinalysis	Normal
Gross pathology	No abnormality
Histopathology	No test article associated histopathologic lesions

Table 4.4. Summary of toxicology assessment of BV-tk vectors in macaque brains.

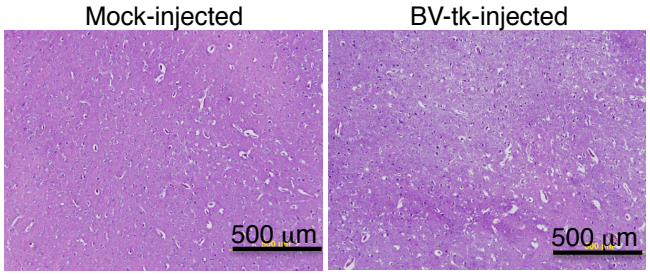

Figure 4.7. Representative images of Haematoxylin and Eosin stained brain sections around the mock and BV-tk injected sites.

Animal No.	Observation	
	Day 1	Day 2
5A5C	NA	NA
557A	NA	NA
5D0D	NA	NA

NA - No Abnormality Noted

Table 4.5. Clinical Observations of Individual Animals

Animal No.	Body Weights (kg)	
	Pre-Dose	Day 2
5A5C	2.98	2.86
557A	2.99	2.87
5D0D	2.67	2.73

Table 4.6. Body Weight Measurements

Animal Number	Findings
5A5C	NAD
557A	NAD
5D0D	NAD

No Abnormality Detected

Table 4.7. Gross Pathological Findings

Pre-Dose

Animal #	Urinalysis										
	Appearance	VOL (ml)	SG	pH	PROT	GLU	KET	BIL	ERY	UBG	LEU (Leu/ul)
5A5C	Light yellow / clear	5.5	1.015	9	neg	norm	neg	neg	neg	norm	25
557A	Straw / sl turbid	5.5	1.006	8	neg	norm	neg	neg	neg	norm	neg
5D0D	Light yellow / sl turbid	5	1.01	6	neg	norm	neg	neg	neg	norm	neg

Day 2- Post Dose

Animal #	Urinalysis										
	Appearance	VOL (ml)	SG	pH	PROT	GLU	KET	BIL	ERY	UBG	LEU (Leu/ul)
5A5C	Light yellow / clear	11	1.019	8	neg	norm	neg	neg	25	norm	neg
557A	Straw / sl turbid	5.5	1.013	9	neg	norm	neg	neg	neg	norm	neg
5D0D	Light yellow / sl turbid	5.5	1.012	9	neg	norm	neg	neg	neg	norm	neg

Table 4.8. Urinanalysis pre and post rBV dose.
VOL – Volume; SG - Specific Gravity; PROT – Protein; GLU – Glucose; KET – Ketone; BIL – Bilirubin; ERY - Blood Pigments; UBG – Urobilinogen; LEU - White Blood Cells; norm – normal & neg- negative

4.7. Discussion

The primary objective of this study was to understand the host responses and evaluate the toxicity in macaque brains to BV innoculation. Once inside the immune competent cell, BVs are recognized by pathogen recognition receptors (PRRs) like membrane bound TLRs and cytoplasmic RLRs. TLRs are the most well studied PRRs with respect to viral infections (Kawai *et al.*, 2008). Upon infection, TLRs follow MyD88 dependent pathway to activate NFκB and c-Jun, the transcriptional regulators of a number of chemokines and cytokines responsible for cellular immune response (Wang *et al.*, 2001). Endosomal TLRs (TLR-3, 7, 8 and 9) are traditionally associated with viral recognition (Brennan *et al.*, 2010) while cell membrane bound receptors TLR-2 and TLR-4 are involved in interacting with the viral envelope glycoproteins (Barton, 2007). Baculoviruses are known to induce type I IFNs in immune cells like peritoneal macrophages (PECs), splenic CD11c+ DCs and murine macrophage line RAW264.7 through TLR-9/MyD88-dependent pathway (Abe *et al.*, 2005). However, treatment of PECs with endosomal inhibitors failed to stop type I IFN production suggesting the existence of TLR-9 independent pathways (Abe *et al.*, 2009). Recently, Chen and colleagues reported that baculoviruses can induce type I IFN production in mesenchymal stem cells through TLR-3 dependent pathway (Chen *et al.*, 2009a). We have demonstrated through microarray studies that neurons, which do not possess TLR-9, respond to BV inoculation by producing IFNs through TLR-3 dependent pathway. In rat brains, TLR2 is involved in IFN production (Boulaire *et al.*, 2009). In this study, TLR-2, 3 and 4 were up regulated in the mouse brain (Table 4.2) suggesting the recognition of envelope glycoproteins by TLR-2 and TLR-4 and genomic DNA or transcribed RNA products by TLR-3. IFN-β secreted as a result of TLR induction acts on neighboring cells and activates Jak-STAT pathway via IFN receptor to induce the expression of IFN

regulatory factor 7 (IRF7) which may result in the amplification of type I IFN through a positive feedback mechanism (reviewed in Kawai *et al.*, 2008). STAT1 and IRF7 were also up regulated in this study (Table 4.2). However, none of the TLRs were significantly modified in virus injected macaque brain samples (Table 4.2 & Table 4.3) suggesting the involvement of a TLR independent pathway in the induction of IFN production in the macaque brains.

RLRs are a second class of PRRs including RNA helicases like RIG-1, MDA5 and LGP2 that reside in the cytoplasm of immune and non-immune cells and recognize viral RNA species produced in the cytoplasm. RLRs are known to induce type I IFN production through an adaptor molecule IPS-1 that localizes in the mitochondria (Kawai *et al.*, 2005). LGP2 serves as a negative or positive regulator of RIG-1 and MDA-5 depending on the type of RNA viruses (Venkataraman *et al.*, 2007). In this study, RIG-1 (DDX58), LGP2 (DHX58) and several other genes in RLR pathway were found to be up regulated (Table 4.3) suggesting its involvement in IFN production upon baculoviral transduction in mouse brains. In macaque brains, STAT1 (fold change = 2.17) and RLR pathway genes - RIG-1, ISG15, MDA5, IL-8, POLR3C and TRIM25 were up regulated. This may suggest that RLR pathway is the major IFN production pathway in macaque brains. Though not many studies have demonstrated the involvement of RLRs in baculoviral transduction, a recent study has noted that RIG-1 and MDA5 mRNA levels were elevated in baculovirus-transduced cells (Wang *et al.*, 2010b). Involvement of RLR signaling pathway in BV transduction is a relatively new field ready for exploration.

TLR-3 and RLR signaling have so far been associated with the recognition of double and single stranded RNA. A common query that would strike any researcher is how baculovirus accommodating double stranded DNA could be recognized by PRRs recognizing RNA molecules. One possible explanation given by Chen and colleagues was

that the immediate early gene products expressed by baculovirus upon entry into mammalian cells could be recognized by RNA recognizing PRRs (Chen *et al.*, 2009a). Recently, RLR pathway was demonstrated to contribute to IFN responses activated by cytosolic DNA as well (Kim *et al.*, 2009a). The two RLRs - RIG-1 and MDA-5 act as non-redundant cytosolic DNA receptors resulting in the direct activation of IRF3 leading to the induction of IFN. This suggests that RLRs can recognize BV DNA as such excluding the need for the transcribed immediate early gene to elicit immune responses. Furthermore in mice, other cytosolic DNA sensors like DNA-dependent activators of IRFs (DAI) and stimulator of IFN genes(STING) which have been identified to play roles in IRF activation and IFN production (Choi *et al.*, 2009) were upregulated.

PRR triggering leads to the production of different immunologic messenger molecules like IFNs, cytokines and chemokines. Chemokines are secreted by immune cells and possess immunomodulatory and chemo-attractive properties to recruit immune cells to the site of viral infection. In the current study, as a result of BV inoculation, 13 cytokines and 7 cytokine receptors have been up regulated in mouse brains and 4 cytokines in macaque brains, suggesting that active recruitment of immune cells to the site of infection is low for the given dose of 10^8 pfu BV per injection site and over the given observation period of 48 hours. Upregulation of IL-2Rγ in mouse brains and IL-15 in macaque brains may indicate the induction of host adaptive immune responses to BV inoculation. IL-2Rγ is shared by cytokines IL-2 and IL-15 both of which are T-cell growth factors. IL-2 plays an important role in adaptive immunity by generating and maintaining Treg cells whereas IL-15 is involved in maintaining the survival of CD8+ memory T cells (Commins *et al.*, 2010). BV transduction of bone marrow derived DCs has been shown to up regulate MHC class I and II molecules and co-stimulatory molecules CD40, CD80 and CD86 (Suzuki *et al.*, 2010). Signals sent out by these molecules can eventually induce

the simultaneous secretion of IL-2 and the expression of high-affinity IL-2R on the effector T-cells (Thornton *et al.*, 2004) thus inducing adaptive immune responses.

In spite of all these immune responses, baculoviruses are generally viewed as gene delivery vectors with a good safety profile compared to other vectors and experimental evidences for the same have been mounting for years now. Even though baculoviruses co-exist with humans in close proximity, so far, there are no evidences for them to infect hosts other than insects. No baculovirus neutralizing antibodies were detected in human serum samples whereas adenovirus neutralizing antibodies were detected in 65% of the humans tested. Also, the immunogenicity of baculovirus is relatively lower as indicated by the induction of lower number of T-cells compared to adenovirus (Strauss *et al.*, 2007). Feeding or intraperitoneal injection of live nuclear polyhedrosis viruses (NPVs) and granulosis viruses (GV) to mice and hamsters did not cause chromosomal aberrations or sister chromatid exchanges in rapidly dividing bone marrow cells unlike other mammalian viral vectors (Reimann *et al.*, 1982). In another study, guinea pigs and mice tested with high doses of polyhedral or free baculovirus rods for intradermal allergenicity, intranasal and intravenous administration confirmed the safety associated with these viruses (Heimpel, 1966). NPVs tested for propagation, immune reactions and acute infections in pigs of commercial and agricultural importance demonstrated a good safety profile (Doller *et al.*, 1983). A recent study evaluated the safety of Bombyx mori (another widely known baculovirus)-based vaccines in rhesus macaques and reported its safety even at high doses (Jin *et al.*, 2008). However, toxicology studies of replication defective adenoviral vectors was associated with lethargy and death within 1 hour post-high dose upon intravenous injection in rats (Morrissey *et al.*, 2002b) and acute cardiovascular and hemodynamic effects upon intrahepatic injection in pigs (Morrissey *et al.*, 2002a). Moreover, our study to assess the toxicology of AcMNPV in

macaque brains has also guaranteed its biosafety profile in spite of the immune responses mounted against baculovirus at the molecular level. Significant modifications in 0.39% of the total probes did not bring about any behavioral changes thus highlighting the safety associated with them. Also, considerably less immune response were elicited in macaque brains for the same dose of 10^8 pfu/injection site compared to mouse brains suggesting that BV may be safer to higher animal models than rodent models.

In this study, however, we have observed the response in macaque brains only upon single injection of BV for a time span of 48 hours. This pilot study may not be sufficient to provide a comprehensive understanding of the host brain response to BV. For instance, single higher dosing or multiple dosing of viral vector to achieve the therapeutic effect may activate the immune system more, eventually compromising the therapeutic effect. The degradation time of BV in macaque brains can be figured out only by observing the animals for a longer time period. Also, the toxicity may vary upon repeated injections. To answer these unknowns and to further the understanding of host brain response to BV, in future, a dose-dependent ($4x10^8$ pfu/ml and $4x10^9$ pfu/ml) study for extended time point (14 days) and multiple dosing (3 BV inoculations at an interval of 2 weeks) study will be carried out.

5. Cationic Lipid Coating Improves Serum-Resistance in Recombinant Baculovirus

5.1. Introduction

Despite the efficient transducing ability in a wide variety of quiescent and non-quiescent mammalian cells including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS), *in vivo* gene delivery by recombinant baculovirus (rBV) is known to be hampered by its susceptibility to complement-mediated inactivation (Sandig *et al.*, 1996, Hofmann *et al.*, 1998, Hofmann *et al.*, 1999, Kaikkonen *et al.*, 2011). Complement system consists of nearly 30 small proteins contributing to specific or non-specific immune reactions. Upon encountering foreign bodies like viruses, the complement system initiates a series of enzymatic reactions eventually resulting in their inactivation and lysis of infected cells. In the case of BV based gene delivery, complement-mediated immune reactions either reduced or shut down the transgene expression (Hofmann *et al.*, 1995, Sandig *et al.*, 1996, Pieroni *et al.*, 2001). Sometimes, the immune reactions may be too strong resulting in serious tissue damage and systemic immune response. Therefore, to achieve efficient transduction and minimize the immune reactions, it is necessary to improve the serum resistance of rBV or camouflage it from the complement system.

Several strategies have been developed to overcome this hurdle and improve the serum-resistant capacity of rBV vectors. This is discussed in (Kaikkonen *et al.*, 2011). One of the commonly used strategies to prepare rBV capable of withstanding the serum complement system is the genetic modification of BV. Genetically modifed BV with the complement regulatory protein - human decay accelerating factor (DAF) incorporated into its envelope had improved transduction efficiency in complement sufficient neonatal rats

upon direct injection into the liver parenchyma (Huser *et al.*, 2001). However, the injection did not bring about successful transduction in adult rats and BV-DAF could not be produced in sufficient titres as the DAF protein in the envelope interfered with its normal replication cycle (Huser *et al.*, 2001, Hoare *et al.*, 2005). Also, genetically modified BV possessing vesicular stomatitis virus envelope glycoprotein (VSVG) demonstrated increased resistance to inactivation by animal sera *in vitro* but failed in *in vivo* studies upon intravenous, intraperitoneal or intrahepatic routes (Tani *et al.*, 2003). Second commonly used strategy is the inhibition of complement proteins at the site of virus delivery. Though the use of soluble complement inhibitor (sCR1) increased the survival of BV in human serum (Hoare *et al.*, 2005), suppressing the immune system might lead to other complications.

Recently, the use of hybrid vectors formed by merging viral and non-viral vectors has been suggested as the best possible solution to overcome the viral-vector associated hurdles (Boeckle *et al.*, 2006). Hybrid vectors are an attempt to merge the complementary strengths of viral vectors – the high transduction efficiency and that of non-viral vectors - low immunogenicity and high systemic potential. Formation of hybrid vectors can otherwise be called as chemical modification of viruses as it involves the use of non-viral vectors like polymers or lipids that are chemicals. Hybrid vectors have proved successful in producing synergistic effects (Boeckle *et al.*, 2006). Especially, when the non-viral component of the hybrid vector is conjugated to a ligand having receptors on target cells, enhanced targeting is taken care of while the viral component looks after the enhanced transduction efficiency (Wagner *et al.*, 1992, Kim *et al.*, 2009b). rBV have been used with polymers like polyethylene glycol (PEG) and polyethyleneimine (PEI) *in vitro* and *in vivo* to achieve enhanced transduction efficiency and resistance to serum complement system. Evidences that cationic lipids enhance adenoviral infection efficiency of several cell lines

including primitive human hematopoietic cells (Byk *et al.*, 1998) and augment gene transfer to cerebral arteries *in vivo* (Toyoda *et al.*, 2001) turned our attention towards them as the non-viral counterparts to merge with rBV to form hybrid vectors that can evade the complement mediated attack. With the improvised versions of cationic lipids - Lipofectamine 2000 and Cellfectin II that can deliver genes into mammalian cells in the presence of serum, we reasoned that coating rBV with these lipids could help rBV escape the complement mediated inactivation and achieve efficient transduction. Therefore, this chapter aims to test this hypothesis.

5.2. Formation of cationic lipid coated BV

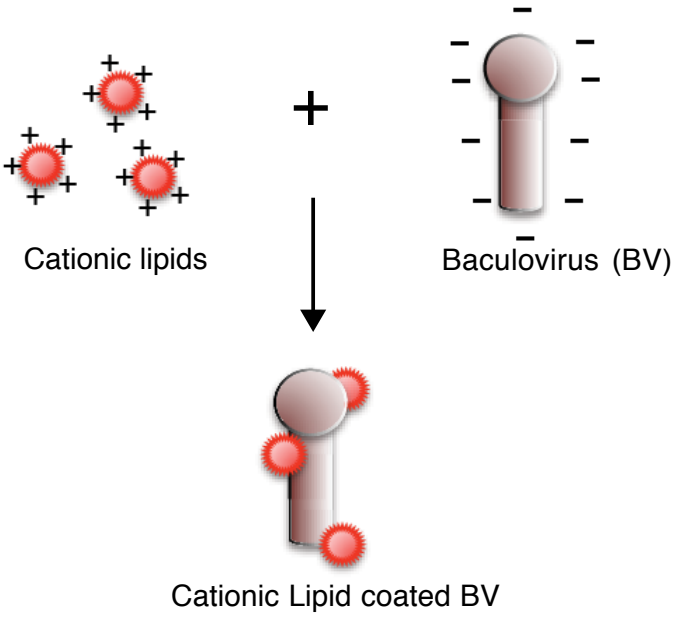

Figure 5.1. Schematic representation of cationic lipid coating on recombinant baculovirus. The electrostatic interaction between the negatively charged viruses and positively charged cationic lipids results in the hybrid vector formation.

Hybrid vectors combining rBV and cationic lipids were formed by mixing rBV and appropriate amounts of cationic lipids and incubating for 20-30 minutes at room temperature. The electrostatic interaction between the negatively charged BV and the

positively charged cationic lipids aided in the hybrid vector formation. Schematic representation of the interaction between BV and cationic lipids is represented in Figure 5.1.

5.3. Zeta potential and size characterization of uncoated/cationic lipids coated BV

rBV encoding luciferase gene (BV-luc) was used all through out the experiments of this chapter and the terms rBV and BV-luc are used interchangeably. To understand the interaction between rBV and cationic lipids - Cellfectin II and Lipofectamine 2000 and to optimize the amount of lipids to coat rBV, uncoated/cationic lipids coated BVs were characterized for their zeta potential using laser Doppler electrophoresis and size using dynamic light scattering (DLS) on a Zetasizer. At neutral pH, BV in water was negatively charged with a zeta potential of ~-20 mV. As the number of molecules of cationic lipids used to coat BV increased, the zeta potential of the samples moved towards zero and increased further to ~+15 mV and ~+22 mV at 10^7 molecules of Cellfectin II and Lipofectamine 2000 respectively per rBV particle (Table 5.1). This drastic change in zeta potential upon addition of cationic lipids indicated the strong electrostatic interaction between them and the virus particles.

Uncoated rBV measured a hydrodynamic diameter of ~407 nm. This was more than the dimension of BV in the literature which is ~300 nm. This difference could be explained by the fact that hydrodynamic diameter of the virus particle is influenced by hydration and solvation effects unlike size measurements using transmission electron microscopy (TEM), where the virus sample is a dehydrated hard sphere. Upon addition of varying amount of cationic lipids, the size of the complex increased gradually indicating the coating of lipids on to BV. Z.average (intensity weighted harmonic mean size) peaked at ~5557 d.nm for Cellfectin II coated BV at a ratio of 7.5×10^6 molecules/virus particle and

~8262 d.nm for Lipofectamine 2000 coated BV at a ratio of $2.5x10^6$ molecules/virus particle. This sharp increase in size was an indication of particle aggregation. Particles with zeta potential in the range of -20 mV to +20 mV tend to aggregate and the degree of aggregation depends on the proximity of zeta potential value to neutral zeta potential value. When the amount of cationic lipids was increased further, the size decreased, following the increased zeta potential that increased the electrostatic repulsion to form well - dispersed cationic lipid coated BV complexes.

# molecules/ BV particle	Hydrodynamic diameter (Z.ave d.nm)	Polydispersity Index (PDI)	Zeta Potential (mV) in Water
BV-luc			
0	407.07 ± 5.5	0.42 ± 0.03	-19.7 ± 0.5
BV-luc-Cellfectin II			
1.00E+05	430.63 ± 30.66	0.4 ± 0.03	-19.93 ± 0.31
1.00E+06	1159.67 ± 42.36	0.44 ± 0.03	-13.93 ± 0.23
2.50E+06	2757.67 ± 83.39	0.45 ± 0.01	-9.31 ± 0.25
5.00E+06	3988.33 ± 207.37	0.45 ± 0.04	-4.98 ± 0.31
7.50E+06	5557.33 ± 344.92	0.53 ± 0.01	1.24 ± 0.37
1.00E+07	637.43 ± 71.21	0.6 ± 0.02	15.63 ± 0.32
BV-luc-Lipofectamine 2000			
1.00E+05	544.77 ± 24.58	0.46 ± 0.01	-16.73 ± 0.31
1.00E+06	7007.33 ± 273.43	0.53 ± 0.02	-6.67 ± 0.19
2.50E+06	8262 ± 868.88	0.65 ± 0.04	7.14 ± 0.41
5.00E+06	2573 ± 87.5	0.87 ± 0.01	16.83 ± 0.42
7.50E+06	644.47 ± 25.41	0.7 ± 0.13	20.87 ± 0.5
1.00E+07	376.6 ± 37.35	0.65 ± 0.17	22.17 ± 0.42

Table 5.1. Characterization of uncoated/cationic lipid coated BV-luc for size and zeta potential. Cationic lipids – Cellfectin II and Lipofectamine 2000 complexed with BV-luc (1e4 – 1e7 molecules/BV particle) for 30 minutes and uncoated BV-luc particles were measured for size and zeta potential. Note the increase in the charge and size of rBV upon addition of increasing amounts of cationic lipids. Also note the fall in size of the complexes with increasing amounts of cationic lipids due to the electrostatic repulsion between the complexes. The values are expressed as the Mean ± SD for at least 3 readings.

5.4. Cytotoxicity of cationic lipid coated BV

Cellfectin II, as a transfection reagent, is known to have low cytotoxicity compared to most other cationic lipid based transfection reagents whereas Lipofectamine 2000 is relatively more cytotoxic to mammalian cells. Therefore, it was important to test the cytoxicity of these cationic lipids coated BV-luc on mammalian cells. Consistent with our previous studies, BV transduction did not result in significant cytotoxicity on cultured U87 cells at MOI up to 100. Even at MOI 500, the cell viability decreased only to ~62% (Figure 5.2). BV coated with varying amounts (10^5-10^7 molecules/per virus particle) of Cellfectin II and Lipofectamine 2000 showed cell viability not less than 57% even at at MOIs 500.

5.5. Effect of cationic lipids on BV transduction

Lipofectamine 2000 and Cellfectin II have been reported to improve adenoviral infection efficiency of several cell lines including primitive human hematopoietic cells (Byk *et al.*, 1998). To test if the cationic lipid coating has a similar effect on BV transduction of U87 cells, BV-luc coated with varying amounts of cationic lipids (10^4 – 10^7 particles/BV) for 30 minutes were added to U87 cells. Based on the cytotoxicity assay performed, BV-luc of MOI 50 was used for this study. Luciferase activities were measured after 48 hours. No significant alterations were noted in luciferase expression when Cellfectin II of concentration up to 10^6 molecules/BV particle and Lipofectamine 2000 of concentration up to $2.5x10^6$ molecules/BV were used. However, beyond these concentrations, coating with Cellfectin II and Lipofectamine 2000 significantly reduced luciferase expression (Figure 5.3). Thus, these results show that cationic lipids do not improve but interfere with the transduction efficiency of BV vectors at higher concentrations.

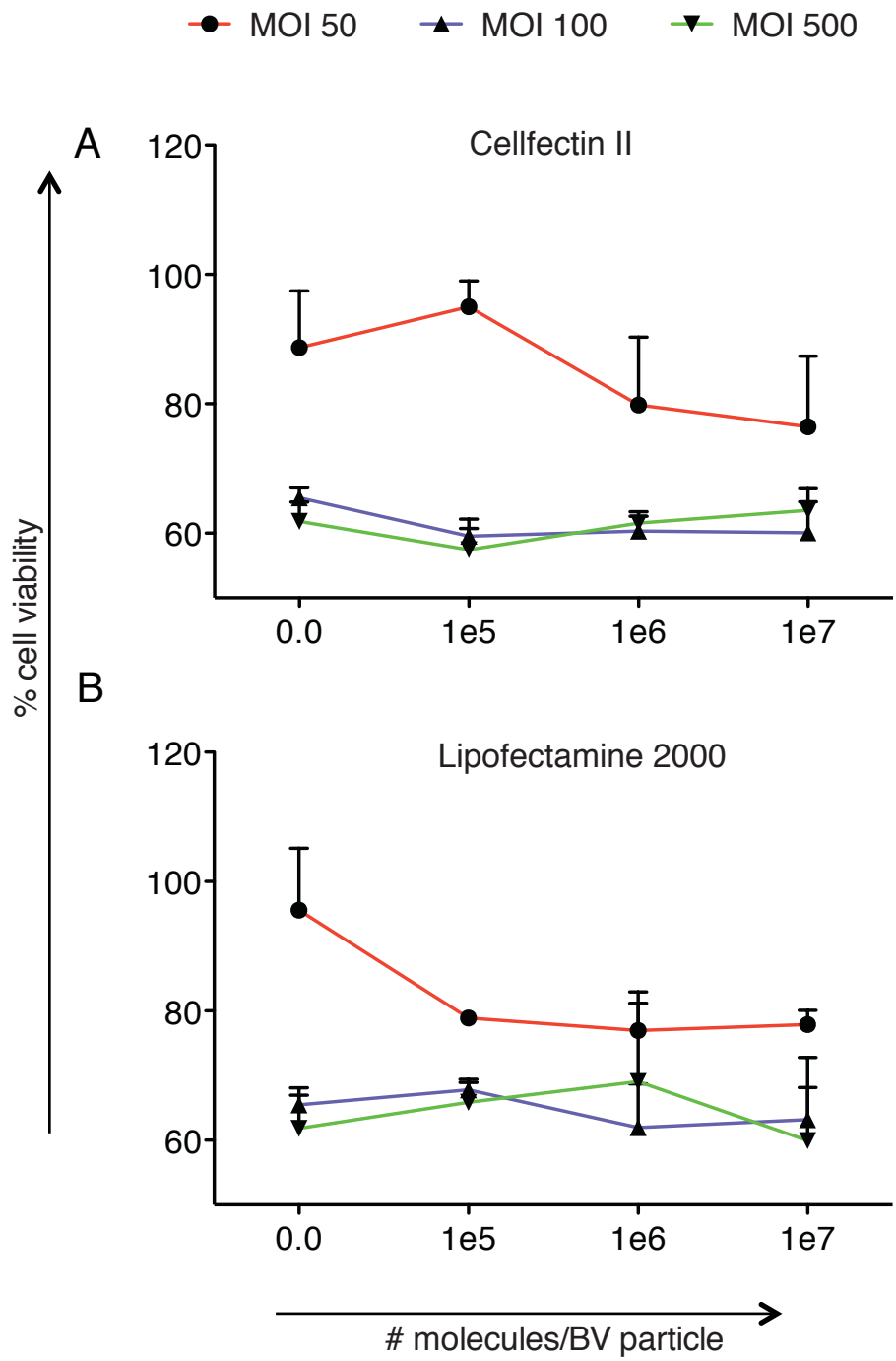

Figure 5.2. Cytotoxicty of Cellfectin II (A) and Lipofectamine 2000 (B) coated BV-luc on U87 glioma cells. Cellfectin II and Lipofectamine 2000 (1e4 – 1e7 molecules/BV particle) molecules complexed with BV-luc of MOI 50, 100 and 500 for 30 minutes were added to U87 cells and the % viability was measured after 48 hours. Values are shown as mean ± SD of at least 3 wells.

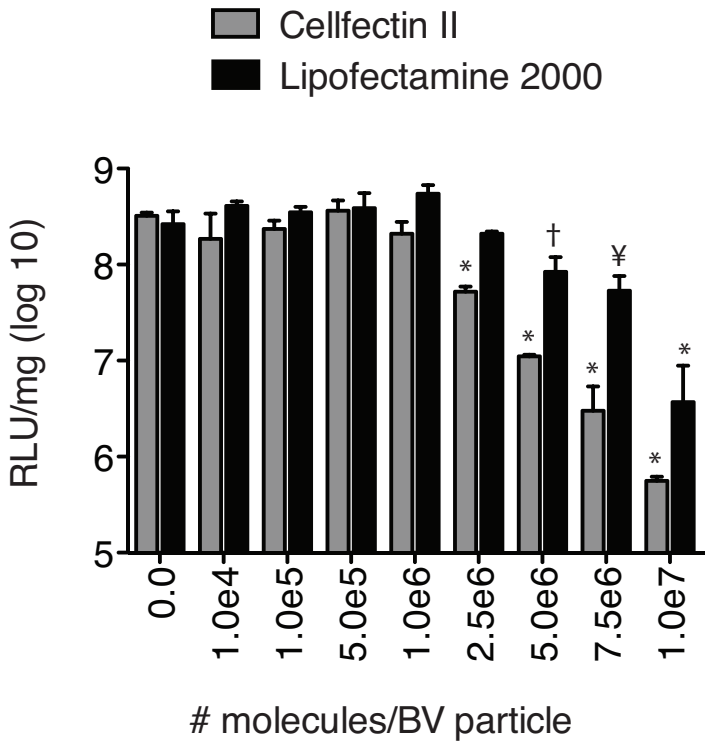

Figure 5.3. Effect of cationic lipids coating on BV-luc transduction of U87 cells. BV-luc complexed with varying amounts of Cellfectin II and Lipofectamine 2000 for 30 minutes were added to U87 cells and luciferase gene expression was measured after 48 hours. The values are represented as mean ± SD of at least 3 readings. Statistical analysis was done by one way ANOVA followed by Tukey's multiple comparison tests. † - $p < 0.05$; ¥ - $p < 0.01$; * - $p < 0.001$.

5.6. Serum protective effect of cationic lipids on BV

Several reports have been published to demonstrate that BV is inactivated by the serum complement system (Sandig *et al.*, 1996, Hofmann *et al.*, 1998, Hofmann *et al.*, 1999, Kaikkonen *et al.*, 2011). Significant reduction in BV transduction efficiencies were especially observed with sera from humans, rats and guinea pigs (Tani *et al.*, 2003). These results were confirmed when the luciferase expression by U87 cells upon transduction with BV-luc significantly decreased in the presence of human (by ≈ 1000 fold) and rat serum (by ~300-500 fold) but not by mouse serum (Figure 5.4). Therefore,

the serum protective effect of cationic lipids on BV was tested against rat and human serum in subsequent experiments.

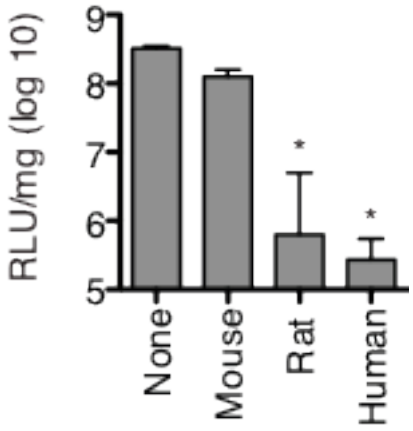

Figure 5.4. Effect of serum on BV transuction in U87 cells. BV-luc treated with sera from mouse, rat and human for 30 minutes at 37 ºC were added to U87 cells. Transgene expression was measured after 48 hours. Values are presented as Mean ± SD. Note the considerable decrease in transgene expression upon treatment of BV-luc with rat and human serum. Statistical analysis were carried out by one-way ANOVA followed by Tukey's multiple comparison test. * - P value < 0.001 against no serum treated sample.

To examine whether cationic lipids – Cellfectin II and Lipofectamine 2000 coated BV-luc offered better transduction in the presence of serum complement system, BV coated with varying amounts of these lipids (10^4-10^7 molecules/per virus particle) preincubated with 50% human/rat serum were assayed for luciferase expression in U87 cells after 48 hours. Based on the cytotoxicity assay performed, MOI 50 was chosen to study the serum protective effect. Luciferase expression that reduced by ≈ 1000 fold in the presence of human serum and ≈ 500 fold in the presence of rat serum gradually increased with the increase in the amount of cationic lipid used for coating BV. Cellfectin II coating increased the transduction efficiency significantly between concentrations $2.5x10^6$ and $7.5x10^6$/BV particle reaching the maximum increase of ≈ 24 fold at $5x10^6$/BV particle in the presence of human serum. In the presence of rat serum, the transduction efficiency significantly increased between $1x10^6$ and $2.5x10^6$/BV particle reaching the maximum

increase of ≈ 180 fold at $2.5x10^6$/BV particle (Figure 5.5). Lipofectamine 2000 coating increased the transduction efficiency significantly between concentrations $1x10^6$ and $5x10^6$/BV particle by ≈ 10 fold in the presence of human serum whereas in the presence of rat serum, the transduction efficiency significantly increased at $1x10^6$ by ≈ 13 fold (Figure 5.5). Thus, Cellfectin II coated on rBV at a concentration of $2.5x10^6$/BV particle and Lipofectamine 2000 coated on rBV at a concentration of $1x10^6$/BV particle offered the maximum serum protective effect in the presence of human and rat sera and Cellfectin II was observed to offer better protection to rBV than Lipofectamine 2000 from the serum complement system *in vitro.*

To gain insights in to the morphological changes that occurred upon coating rBV with cationic lipids and how the coating offers serum protective effect, transmission electron microscopy (TEM) was used (Figure 5.6). Coating ratio of $2.5x10^6$ molecules of the cationic lipids/BV particle that offered maximum serum protective effect on BV *in vitro* and 50% human serum were chosen for this investigation against uncoated BV. Electron micrographs of the uncoated, no-serum treated samples displayed rod shaped particles of length 200-250 nm and width ≈ 50 nm complying with BV dimensions in the literature. Human serum treated uncoated BV samples were observed to have slightly swollen, distorted and disrupted envelope structure providing evidence for the action of complement proteins on BV. When BV coated with cationic lipids did not differ much in morphology from the uncoated BV, serum-treated, Cellfectin II and Lipofectamine 2000 coated BV seemed to have an extra uneven layer around the normal rod shaped morphology and an increase in size. This may be due to the coating of cationic lipids on to rBV thus, suggesting that cationic lipid coating provided protection against complement-mediated rBV inactivation.

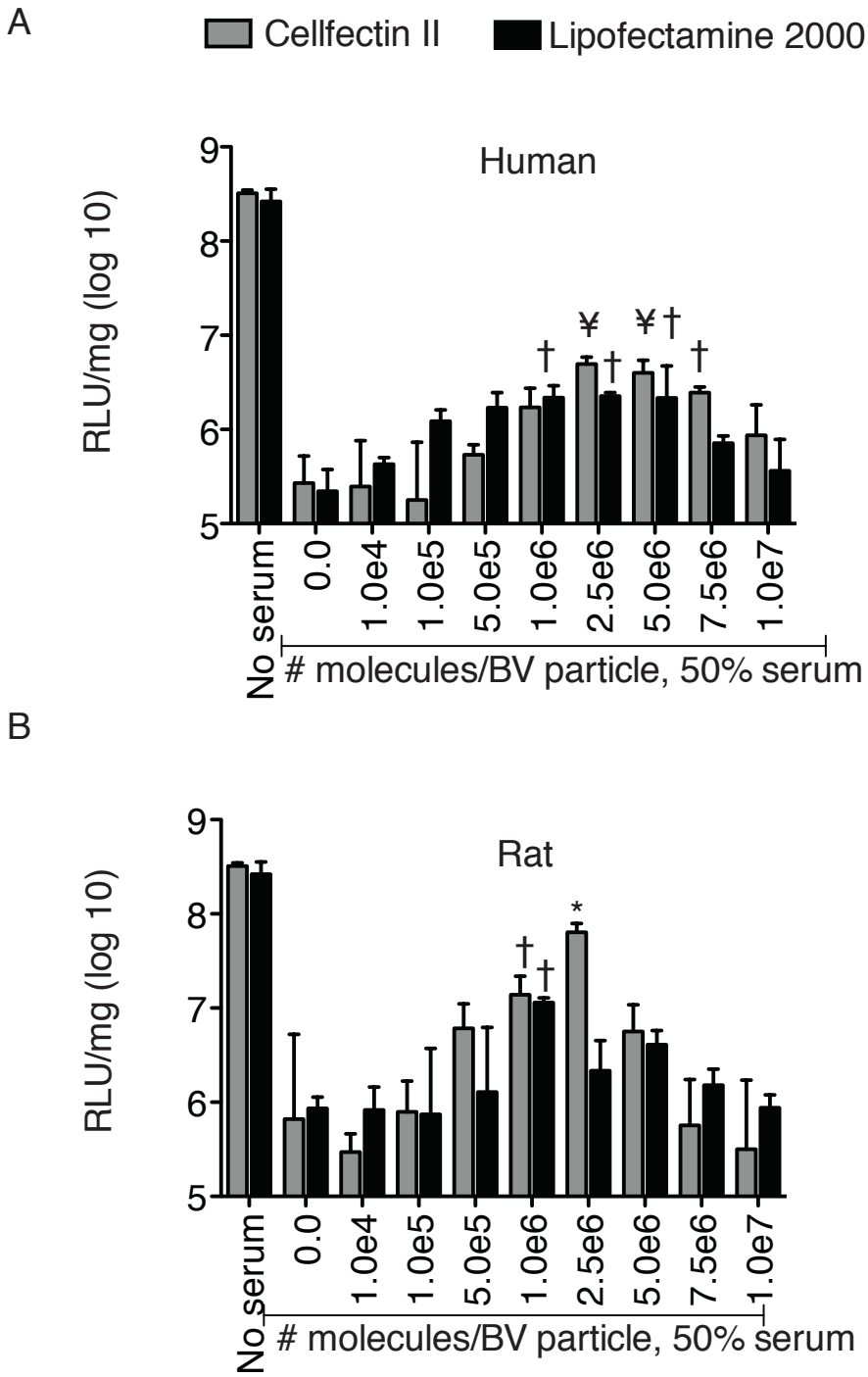

Figure 5.5. Serum Protective effect of cationic lipids on BV-luc. Uncoated and cationic lipid coated BV-luc were pre-treated with (A) human and (B) rat serum and tested for transduction efficiency against no-serum treated sample. Luciferase activities were measured after 48 hours. Note the increase in the luciferase expression peaking at $2.5x10^6$ molecules of Cellfectin II/BV particle and at $1x10^6$ molecules of Lipofectamine 2000/BV particle in the presence of human and rat sera. The values are represented as Mean ± SD of atleast three values. Statistical analysis was done by one way ANOVA followed by Tukey test. † - $p < 0.05$; ¥ - $p < 0.01$; * - $p < 0.001$.

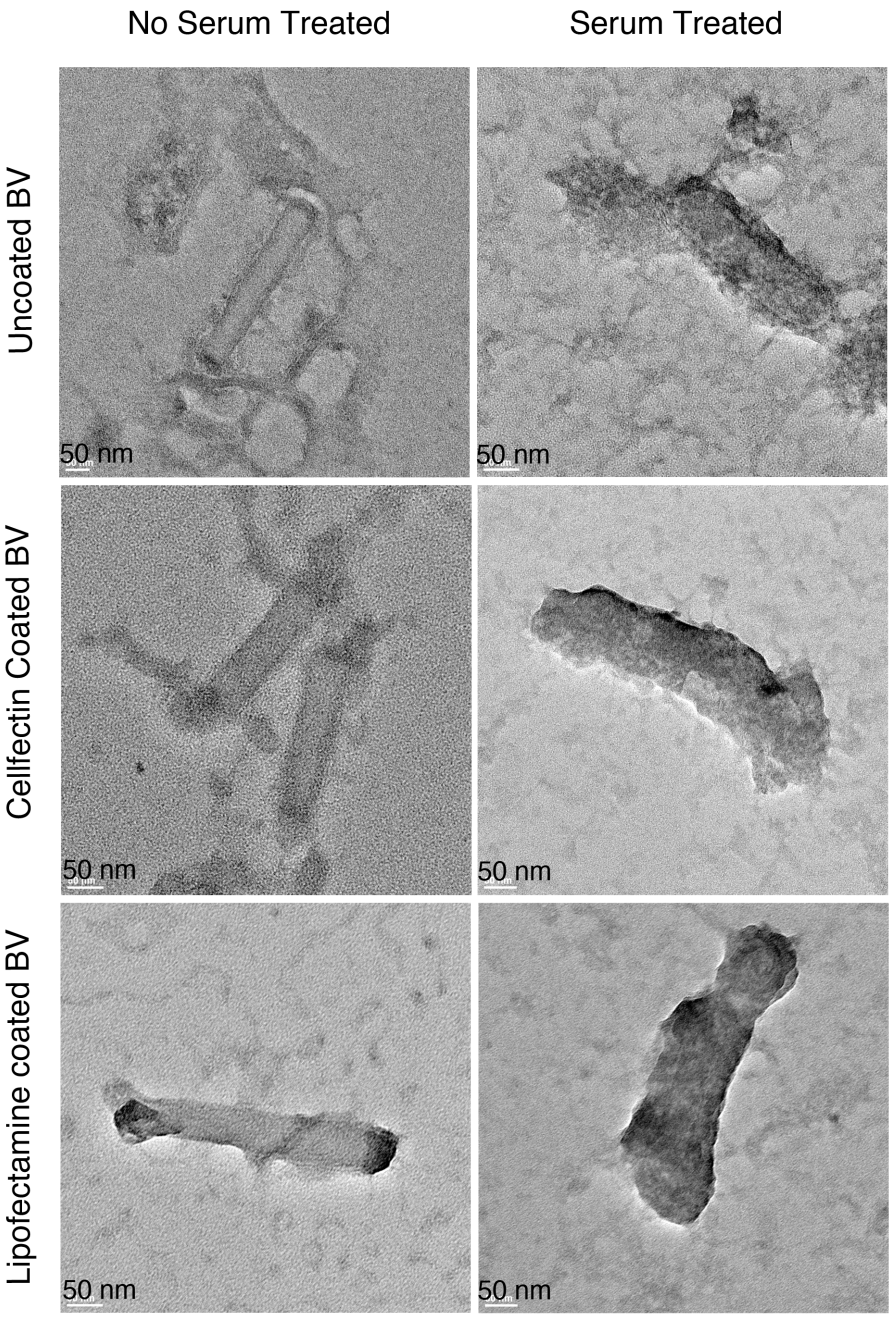

Figure 5.6. Transmission electron microscopic analysis of uncoated/coated rBV without or with serum treatment. Uncoated no serum treated rBV showed clear rod-shaped particles with dimensions, 200 – 250 nm in length and 50 nm in width whereas uncoated serum treated BV showed rod shaped BV particles with disrupted envelope (upper panel). Cationic lipid coated BV showed similar morphology like uncoated rBV whereas serum treated cationic lipid coated rBV showed increase in size and an extra layer around the rod shape indicating the efficient coating and protection from serum complement proteins (middle and bottom panels).

5.7. Discussion

One of the major obstacles that hinder the progress of BV into clinical trials is its inactivation mediated by the complement system in the blood (Hofmann *et al.*, 1998) and organs (Hofmann *et al.*, 1995, Sandig *et al.*, 1996). To prevent this, rBV has been used to deliver genes to immune privileged sites like eye, brain and testis or used in *ex vivo* transduction of cells and tissues. However, to fully exploit the advantages associated with BV, strategies to improve their serum resistance of BV need to be devised and this chapter addressed this issue. The major findings of this study include the following: firstly there is a strong electrostatic interaction between recombinant BV vectors and the cationic lipids – Lipofectamine 2000 and Cellfectin II which can be tapped to optimize the size of the virus-cationic lipid complex. Secondly, in the absence of serum complement, the coating of these cationic lipids on rBV does not improve the transgene expression but at higher concentrations, negatively affect it. Thirdly, in the presence of 50% serum complement *in vitro*, cationic lipid coating augment the transgene expression thus, demonstrating the suitability of using cationic lipids with baculoviruses for enhanced gene delivery in the presence of serum complement *in vitro* and opening up the possibility to test them *in vivo*.

Cationic lipids and polymers have widely been used for *in vitro* and *in vivo* non-viral gene delivery. Several gene delivery protocols using cationic lipids have entered clinical trials (summarized in Zhang *et al.*, 2012). Among them, lipofectamine has been reported as vectors for gene transfer *in vivo* in blood vessels (Muller *et al.*, 1994) and cerebral arteries (Toyoda *et al.*, 2001) and is involved in almost 7% of the world wide gene therapy trials (Zhang *et al.*, 2012). Also, Lipofectamine 2000 and Cellfectin II have been improvised to deliver genes *in vitro* in the presence of serum. Therefore, we reasoned that merging BVs and cationic lipids will result in hybrid vectors possessing

advantages of both. Because this strategy involved no genetic modifications but exploited the electrostatic interaction between the cationic lipids and BV, the fear of reduced transduction efficiency was almost eliminated.

Of the two cationic lipids used, Cellfectin II was observed to form complexes of smaller diameter compared to Lipofectamine 2000. These could be attributed to the smaller size of the hydrophobic region of Cellfectin II compared to Lipofectamine 2000 and higher charge of +4 at the cationic end that aids in its strong and tight binding to BV. Both the cationic lipids neither seemed to affect nor improve the transduction efficiency much at lower concentrations but affected the transduction efficiency negatively at higher concentrations (> $5x10^6$ molecules/BV particle). This could be because the extremely high concentration of cationic lipids may be toxic to the cells (Figure 5.2) or may result in aggregation of virus particles (Table 5.1) making it difficult for them to enter the cells. Similar problems have been reported with cationic lipid coated adenovirus (Toyoda *et al.*, 2001). Hence, it was important to characterize the cationic lipid coated BV for optimal transduction efficiency.

In the presence of serum complement, Cellfectin II seemed to offer better transgene expression than Lipofectamine 2000. One of the reasons for this could be differences in the route of entry of cationic lipid coated BV. Cellfectin II as a gene delivery vector enters the cells via a direct route through the cell cytosol (Wagner *et al.*, 1993) as opposed to endocytosis by lipofectamine. Transmission electron micrographs to gain insight into the morphological changes that occur upon serum complement treatment revealed similar morphology (increased size and presence of cationic lipid coating) for Lipofectamine 2000 and Cellfectin II coated BV. However, it does not provide any information about whether cationic lipid coating brought about any changes in the pathway of uptake of BV. Further *in vitro* experiments blocking the usual routes of BV

intake and cyro TEM will help gain better insight into the mechanism behind serum protective effect of cationic lipids. Furthermore, since BV is capable of eliciting innate and adaptive immune responses (Abe *et al.*, 2010) that have been tapped to produce BV-based vaccines (Madhan *et al.*, 2010), it is worth investigating whether modification of rBV with these cationic lipids can suppress cellular and humoral immune responses and add to the biosafety profile of BV like how the polyethylene glycol (Kreppel *et al.*, 2008) coating modified responses to intravenously delivered adenoviruses.

In summary, rBV coated with cationic lipids can effectively escape complement-mediated inactivation *in vitro* to qualify as an efficient gene transfer vector in an environment containing complement components. With further evidences of its success *in vivo,* this simple technique of coating with biomaterial will aid in overcoming other hurdles to BV gene delivery such as elicitation of immune responses thus reinforcing the applications of BV in clinical gene therapy.

6. Conclusion

The prime objective of this thesis is to understand the host responses to baculovirus in the brains of mice and macaques and develop methods to mask them from the host immune system. The main findings of this study include the following - (1) immune responses reduce with the reduction in the impurities of BV formulation (2) BV can mount antiviral immune responses in the brains which are generally considered to be immune privileged organs and (3) cationic lipid coating can effectively camouflage BV from the host complement system *in vitro*.

In chapter 3, it was demonstrated that membrane chromatography purification method (MC+HS) offered *in vivo* grade BV formulation. In comparison with the widely used laboratory method of purifying baculovirus - high speed centrifugation, though the yield was lesser by MC+HS (≈ 30% against ≈ 55% from HS), purity was highly improved and there was little or no compromise in the transducing ability of BV. Scaling up of this method to purify large volumes of BV will be tested in the near future. The cDNA microarray gene expression profiling to investigate the immune responses elicited by BV prepared by the two methods – MC+HS and HS provided evidence in support of MC+HS method for providing *in vivo* grade BV formulation. The humongous data generated from the microarray studies also offered insights into the various means of IFN induction and production in mouse brains.

In chapter 4, the first evidence for antiviral immune responses in macaque brains to BV was demonstrated by high throughput cDNA gene expression profiling. BV inoculation brought about significant changes in the expression of fewer number of probes (205 or 0.39% of all probes on the microarray chip) compared to mice brains (379 or 0.84 of all probes on the microarray chip) suggesting that BV inoculation might not elicit

stronger immune responses in non-human primates than in rodents. Further experiments to test the host responses by varying the dose and time of observation can further our knowledge about the responses in non-human primates' brain to BV inoculation. Significant fold changes in the genes belonging to RIG-1 like receptor signaling pathway but not other PRR signaling pathways in BV inoculated macaque brains compared to the control injected macaque brains suggests RIG-1 like receptor signaling to be the major pathway of IFN induction. The effects of higher doses or repeated doses of BV on eliciting other IFN induction pathways are yet to be studied. Findings from the toxicology studies demonstrate the safety profile of BV for use as gene delivery vectors in higher animal models.

In chapter 5, methods to avoid the most common problem of complement mediated BV inactivation were developed. rBV coated with cationic lipids – lipofectamine 2000 and cellfectin II were demonstrated to effectively escape complement-mediated inactivation *in vitro* to qualify as an efficient gene transfer vector in an environment containing complement components. With further evidences of its success *in vivo,* this simple technique of coating with biomaterial will aid in overcoming other hurdles to baculovirus gene delivery such as elicitation of immune responses thus reinforcing the applications of baculovirus in clinical gene therapy.

To conclude, the findings from this thesis help in gaining better understanding of BV responses in rodent and non-human primate brains that would allow for the facilitation of optimization of baculoviral vector design to escape the hurdles and successfully deliver genes into the CNS and also serve as a guide to rational therapeutic applications of baculoviral vectors for other organs.

7. References

Abe T, Hemmi H, Miyamoto H*, et al.* (2005) Involvement of the toll-like receptor 9 signaling pathway in the induction of innate immunity by baculovirus. J Virol 79:2847-2858.

Abe T, Kaname Y, Wen X*, et al.* (2009) Baculovirus induces type i interferon production through toll-like receptor-dependent and -independent pathways in a cell-type-specific manner. J Virol 83:7629-7640.

Abe Tand Matsuura Y (2010) Host innate immune responses induced by baculovirus in mammals. Curr Gene Ther 10:226-231.

Abe T, Takahashi H, Hamazaki H*, et al.* (2003) Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. J Immunol 171:1133-1139.

Ahrens C H LDJ, Rohrmann G F (1996) Baculovirus DNA replication. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Airenne KJ, Hiltunen MO, Turunen MP*, et al.* (2000) Baculovirus-mediated periadventitial gene transfer to rabbit carotid artery. Gene therapy 7:1499-1504.

Aloisi F, Ria Fand Adorini L (2000) Regulation of t-cell responses by cns antigen-presenting cells: Different roles for microglia and astrocytes. Immunol Today 21:141-147.

Aoki H, Sakoda Y, Jukuroki K*, et al.* (1999) Induction of antibodies in mice by a recombinant baculovirus expressing pseudorabies virus glycoprotein b in mammalian cells. Vet Microbiol 68:197-207.

Bak XY, Lam DH, Yang J*, et al.* (2011) Human embryonic stem cell-derived mesenchymal stem cells as cellular delivery vehicles for prodrug gene therapy of glioblastoma. Hum Gene Ther 22:1365-1377.

Bak XY, Yang Jand Wang S (2010) Baculovirus-transduced bone marrow mesenchymal stem cells for systemic cancer therapy. Cancer Gene Ther 17:721-729.

Balani P, Boulaire J, Zhao Y*, et al.* (2009) High mobility group box2 promoter-controlled suicide gene expression enables targeted glioblastoma treatment. Mol Ther 17:1003-1011.

Barsoum J (1999) Concentration of recombinant baculovirus by cation-exchange chromatography. Biotechniques 26:834-836, 838, 840.

Barton GM (2007) Viral recognition by toll-like receptors. Semin Immunol 19:33-40.

Bessis N, GarciaCozar FJand Boissier MC (2004) Immune responses to gene therapy

vectors: Influence on vector function and effector mechanisms. Gene therapy 11 Suppl 1:S10-17.

Blissard GWand Wenz JR (1992) Baculovirus gp64 envelope glycoprotein is sufficient to mediate ph-dependent membrane fusion. J Virol 66:6829-6835.

Boeckle Sand Wagner E (2006) Optimizing targeted gene delivery: Chemical modification of viral vectors and synthesis of artificial virus vector systems. Aaps J 8:E731-742.

Bontrop RE, Otting N, de Groot NG*, et al.* (1999) Major histocompatibility complex class ii polymorphisms in primates. Immunol Rev 167:339-350.

Bontrop RE, Otting N, Slierendregt BL*, et al.* (1995) Evolution of major histocompatibility complex polymorphisms and t-cell receptor diversity in primates. Immunol Rev 143:33-62.

Bostanci A (2002) Gene therapy. Blood test flags agent in death of penn subject. Science 295:604-605.

Boulaire J, Zhao Yand Wang S (2009) Gene expression profiling to define host response to baculoviral transduction in the brain. J Neurochem 109:1203-1214.

Brennan Kand Bowie AG (2010) Activation of host pattern recognition receptors by viruses. Curr Opin Microbiol 13:503-507.

Brown BD, Gentner B, Cantore A*, et al.* (2007) Endogenous microrna can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nature biotechnology 25:1457-1467.

Brown BD, Venneri MA, Zingale A*, et al.* (2006) Endogenous microrna regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. Nat Med 12:585-591.

Burova Eand Ioffe E (2005) Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: Methods and implications. Gene therapy 12 Suppl 1:S5-17.

Byk T, Haddada H, Vainchenker W*, et al.* (1998) Lipofectamine and related cationic lipids strongly improve adenoviral infection efficiency of primitive human hematopoietic cells. Hum Gene Ther 9:2493-2502.

Charcosset C (2006) Membrane processes in biotechnology: An overview. Biotechnology Advances 24:482-492.

Charrel-Dennis M, Latz E, Halmen KA*, et al.* (2008) Tlr-independent type i interferon induction in response to an extracellular bacterial pathogen via intracellular recognition of its DNA. Cell Host Microbe 4:543-554.

Chen CY, Lin CY, Chen GY*, et al.* (2011) Baculovirus as a gene delivery vector: Recent understandings of molecular alterations in transduced cells and latest applications.

Biotechnology Advances 29:618-631.

Chen G-Y, Shiah H-C, Su H-J*, et al.* (2009a) Baculovirus transduction of mesenchymal stem cells triggers the toll-like receptor 3 pathway. J Virol 83:10548-10556.

Chen GY, Chen CY, Chang MD*, et al.* (2009b) Concanavalin a affinity chromatography for efficient baculovirus purification. Biotechnol Prog 25:1669-1677.

Cheshenko N, Krougliak N, Eisensmith RC*, et al.* (2001) A novel system for the production of fully deleted adenovirus vectors that does not require helper adenovirus. Gene therapy 8:846-854.

Choi MK, Wang Z, Ban T*, et al.* (2009) A selective contribution of the rig-i-like receptor pathway to type i interferon responses activated by cytosolic DNA. Proc Natl Acad Sci U S A 106:17870-17875.

Chuang CK, Lin KJ, Lin CY*, et al.* (2010) Xenotransplantation of human mesenchymal stem cells into immunocompetent rats for calvarial bone repair. Tissue Eng Part A 16:479-488.

Chuang CK, Sung LY, Hwang SM*, et al.* (2007) Baculovirus as a new gene delivery vector for stem cell engineering and bone tissue engineering. Gene therapy 14:1417-1424.

Commins SP, Borish Land Steinke JW (2010) Immunologic messenger molecules: Cytokines, interferons, and chemokines. The Journal of allergy and clinical immunology 125:S53-72.

Dodd LE, Korn EL, McShane LM*, et al.* (2004) Correcting log ratios for signal saturation in cdna microarrays. Bioinformatics 20:2685-2693.

Doller G, Groner Aand Straub OC (1983) Safety evaluation of nuclear polyhedrosis virus replication in pigs. Appl Environ Microbiol 45:1229-1233.

Drugmand J, Schneider Yand Agathos S (in press) Insect cells as factories for biomanufacturing. Biotechnology Advances.

Du J, Zeng J, Zhao Y*, et al.* (2010) The combined use of viral transcriptional and post-transcriptional regulatory elements to improve baculovirus-mediated transient gene expression in human embryonic stem cells. J Biosci Bioeng 109:1-8.

Duisit G, Saleun S, Douthe S*, et al.* (1999) Baculovirus vector requires electrostatic interactions including heparan sulfate for efficient gene transfer in mammalian cells. J Gene Med 1:93-102.

Edelstein ML, Abedi MRand Wixon J (2007) Gene therapy clinical trials worldwide to 2007—an update. J Gene Med 9:833-842.

Einstein MH, Baron M, Levin MJ*, et al.* (2009) Comparison of the immunogenicity and safety of cervarix and gardasil human papillomavirus (hpv) cervical cancer

vaccines in healthy women aged 18-45 years. Hum Vaccin 5:705-719.

Fiandaca MSand Bankiewicz KS (2010) Gene therapy for parkinson's disease: From non-human primates to humans. Curr Opin Mol Ther 12:519-529.

Fujita R, Matsuyama T, Yamagishi J*, et al.* (2006) Expression of autographa californica multiple nucleopolyhedrovirus genes in mammalian cells and upregulation of the host beta-actin gene. J Virol 80:2390-2395.

Gibbs RA, Rogers J, Katze MG*, et al.* (2007) Evolutionary and biomedical insights from the rhesus macaque genome. Science 316:222-234.

Gronowski AM, Hilbert DM, Sheehan KC*, et al.* (1999) Baculovirus stimulates antiviral effects in mammalian cells. J Virol 73:9944-9951.

Hacein-Bey-Abina S, Von Kalle C, Schmidt M*, et al.* (2003) Lmo2-associated clonal t cell proliferation in two patients after gene therapy for scid-x1. Science 302:415-419.

Haeseleer F, Imanishi Y, Saperstein DA*, et al.* (2001) Gene transfer mediated by recombinant baculovirus into mouse eye. Invest Ophthalmol Vis Sci 42:3294-3300.

Heimpel AM (1966) Exposure of white mice and guinea pigs to the nuclear-polyhedrosis virus of the cabbage looper, trichoplusia ni. J Invertebr Pathol 8:98-102.

Hervas-Stubbs S, Rueda P, Lopez L*, et al.* (2007) Insect baculoviruses strongly potentiate adaptive immune responses by inducing type i ifn. J Immunol 178:2361-2369.

Ho YC, Chung YC, Hwang SM*, et al.* (2005) Transgene expression and differentiation of baculovirus-transduced human mesenchymal stem cells. J Gene Med 7:860-868.

Ho YC, Lee HP, Hwang SM*, et al.* (2006) Baculovirus transduction of human mesenchymal stem cell-derived progenitor cells: Variation of transgene expression with cellular differentiation states. Gene therapy 13:1471-1479.

Hoare J, Waddington S, Thomas HC*, et al.* (2005) Complement inhibition rescued mice allowing observation of transgene expression following intraportal delivery of baculovirus in mice. J Gene Med 7:325-333.

Hofmann C, Huser A, Lehnert W*, et al.* (1999) Protection of baculovirus-vectors against complement-mediated inactivation by recombinant soluble complement receptor type 1. Biol Chem 380:393-395.

Hofmann C, Sandig V, Jennings G*, et al.* (1995) Efficient gene transfer into human hepatocytes by baculovirus vectors. Proceedings of the National Academy of Sciences 92:10099-10103.

Hofmann Cand Strauss M (1998) Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene therapy 5:531-536.

Hu Y-C, Tsai C-T, Chung Y-C*, et al.* (2003) Generation of chimeric baculovirus with histidine-tags displayed on the envelope and its purification using immobilized metal affinity chromatography. Enzyme and Microbial Technology 33:445-452.

Hu YC (2006) Baculovirus vectors for gene therapy. Advances in Virus Research 68:287-320.

Huang DW, Sherman BTand Lempicki RA (2008a) Systematic and integrative analysis of large gene lists using david bioinformatics resources. Nat Protocols 4:44-57.

Huang W, Tian XL, Wu YL*, et al.* (2008b) Suppression of gastric cancer growth by baculovirus vector-mediated transfer of normal epithelial cell specific-1 gene. World journal of gastroenterology : WJG 14:5810-5815.

Huang Xand Yang Y (2009) Innate immune recognition of viruses and viral vectors. Hum Gene Ther 20:293-301.

Huser A, Rudolph Mand Hofmann C (2001) Incorporation of decay-accelerating factor into the baculovirus envelope generates complement-resistant gene transfer vectors. Nat Biotechnol 19:451-455.

Inceoglu A, Kamita Sand Hammock B (2006) Genetically modified baculoviruses: A historical overview and future outlook. Advances in Virus Research 68:323-360.

Ishii KJ, Kawagoe T, Koyama S*, et al.* (2008) Tank-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature 451:725-729.

Ishikawa Hand Barber GN (2008) Sting is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 455:674-678.

Ishikawa H, Ma Zand Barber GN (2009) Sting regulates intracellular DNA-mediated, type i interferon-dependent innate immunity. Nature 461:788-792.

Jarvis DLand Finn EE (1995) Biochemical analysis of the n-glycosylation pathway in baculovirus-infected lepidopteran insect cells. Virology 212:500-511.

Jin R, Lv Z, Chen Q*, et al.* (2008) Safety and immunogenicity of h5n1 influenza vaccine based on baculovirus surface display system of <italic>bombyx mori</italic>. PLoS ONE 3:e3933.

Jooss Kand Chirmule N (2003) Immunity to adenovirus and adeno-associated viral vectors: Implications for gene therapy. Gene therapy 10:955-963.

Jordan KR, McMahan RH, Oh JZ*, et al.* (2008) Baculovirus-infected insect cells expressing peptide-mhc complexes elicit protective antitumor immunity. J Immunol 180:188-197.

Kaikkonen MU, Yla-Herttuala Sand Airenne KJ (2011) How to avoid complement attack in baculovirus-mediated gene delivery. J Invertebr Pathol 107 Suppl:S71-79.

Kawai Tand Akira S (2008) Toll-like receptor and rig-i-like receptor signaling. Ann N Y Acad Sci 1143:1-20.

Kawai T, Takahashi K, Sato S*, et al.* (2005) Ips-1, an adaptor triggering rig-i- and mda5-mediated type i interferon induction. Nat Immunol 6:981-988.

Kenoutis C, Efrose RC, Swevers L*, et al.* (2006) Baculovirus-mediated gene delivery into mammalian cells does not alter their transcriptional and differentiating potential but is accompanied by early viral gene expression. J Virol 80:4135-4146.

Kiang A, Hartman ZC, Everett RS*, et al.* (2006) Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. Mol Ther 14:588-598.

Kim CH, Yoon JS, Sohn HJ*, et al.* (2007) Direct vaccination with pseudotype baculovirus expressing murine telomerase induces anti-tumor immunity comparable with rna-electroporated dendritic cells in a murine glioma model. Cancer Lett 250:276-283.

Kim Y-K, Choi JY, Jiang H-L*, et al.* (2009a) Application of baculovirus for gene therapy. Viruses.

Kim YK, Choi JY, Jiang HL*, et al.* (2009b) Hybrid of baculovirus and galactosylated pei for efficient gene carrier. Virology 387:89-97.

Kim YK, Kwon JT, Choi JY*, et al.* (2010) Suppression of tumor growth in xenograft model mice by programmed cell death 4 gene delivery using folate-peg-baculovirus. Cancer Gene Ther 17:751-760.

Kitajima M, Abe T, Miyano-Kurosaki N*, et al.* (2008) Induction of natural killer cell-dependent antitumor immunity by the autographa californica multiple nuclear polyhedrosis virus. Mol Ther 16:261-268.

Kost TA, Condreay JPand Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nature biotechnology 23:567-575.

Kreppel Fand Kochanek S (2008) Modification of adenovirus gene transfer vectors with synthetic polymers: A scientific review and technical guide. Mol Ther 16:16-29.

Laakkonen JP, Kaikkonen MU, Ronkainen PH*, et al.* (2008) Baculovirus-mediated immediate-early gene expression and nuclear reorganization in human cells. Cell Microbiol 10:667-681.

Lehtolainen P, Tyynela K, Kannasto J*, et al.* (2002) Baculoviruses exhibit restricted cell type specificity in rat brain: A comparison of baculovirus- and adenovirus-mediated intracerebral gene transfer in vivo. Gene therapy 9:1693-1699.

Li Y, Wang X, Guo H*, et al.* (2004) Axonal transport of recombinant baculovirus vectors. Mol Ther 10:1121-1129.

Li Y, Yang Yand Wang S (2005) Neuronal gene transfer by baculovirus-derived vectors

accommodating a neurone-specific promoter. Exp Physiol 90:39-44.

Liu BH, Yang Y, Paton JF*, et al.* (2006) Gal4-nf-kappab fusion protein augments transgene expression from neuronal promoters in the rat brain. Mol Ther 14:872-882.

Liu CY, Wang CH, Wang JC*, et al.* (2007) Stimulation of baculovirus transcriptome expression in mammalian cells by baculoviral transcriptional activators. J Gen Virol 88:2176-2184.

Lo WH, Hwang SM, Chuang CK*, et al.* (2009) Development of a hybrid baculoviral vector for sustained transgene expression. Mol Ther 17:658-666.

Long G, Pan X, Kormelink R*, et al.* (2006) Functional entry of baculovirus into insect and mammalian cells is dependent on clathrin-mediated endocytosis. J Virol 80:8830-8833.

Lu L, Ho Yand Kwang J (2006) Suppression of porcine arterivirus replication by baculovirus-delivered shrna targeting nucleoprotein. Biochem Biophys Res Commun 340:1178-1183.

Luo WY, Shih YS, Hung CL*, et al.* (2011a) Development of the hybrid sleeping beauty-baculovirus vector for sustained gene expression and cancer therapy. Gene Ther.

Luo WY, Shih YS, Lo WH*, et al.* (2011b) Baculovirus vectors for antiangiogenesis-based cancer gene therapy. Cancer Gene Ther 18:637-645.

Luz-Madrigal A, Clapp C, Aranda J*, et al.* (2007) In vivo transcriptional targeting into the retinal vasculature using recombinant baculovirus carrying the human flt-1 promoter. Virol J 4:88.

Madhan S, Prabakaran Mand Kwang J (2010) Baculovirus as vaccine vectors. Curr Gene Ther 10:201-213.

Matilainen H, Rinne J, Gilbert L*, et al.* (2005) Baculovirus entry into human hepatoma cells. J Virol 79:15452-15459.

Mestas Jand Hughes CCW (2004) Of mice and not men: Differences between mouse and human immunology. The Journal of Immunology 172:2731-2738.

Molinari P, Crespo MI, Gravisaco MJ*, et al.* (2011) Baculovirus capsid display potentiates ova cytotoxic and innate immune responses. PLoS One 6:e24108.

Morrissey RE, Horvath C, Snyder EA*, et al.* (2002a) Porcine toxicology studies of sch 58500, an adenoviral vector for the p53 gene. Toxicological Sciences 65:256-265.

Morrissey RE, Horvath C, Snyder EA*, et al.* (2002b) Rodent nonclinical safety evaluation studies of sch 58500, an adenoviral vector for the p53 gene. Toxicological Sciences 65:266-275.

Muller DW, Gordon D, San H*, et al.* (1994) Catheter-mediated pulmonary vascular gene transfer and expression. Circ Res 75:1039-1049.

Nayak Sand Herzog RW (2010) Progress and prospects: Immune responses to viral vectors. Gene therapy 17:295-304.

Niu M, Han Yand Li W (2008) Baculovirus up-regulates antiviral systems and induces protection against infectious bronchitis virus challenge in neonatal chicken. Int Immunopharmacol 8:1609-1615.

Nizamani ZA, Keil GM, Albina E*, et al.* (2011) Potential of adenovirus and baculovirus vectors for the delivery of shrna against morbilliviruses. Antiviral Res 90:98-101.

Okano K, Vanarsdall AL, Mikhailov VS*, et al.* (2006) Conserved molecular systems of the baculoviridae. Virology 344:77-87.

Pan Y, Fang L, Fan H*, et al.* (2010) Antitumor effects of a recombinant pseudotype baculovirus expressing apoptin in vitro and in vivo. Int J Cancer 126:2741-2751.

Pieroni L, Maione Dand La Monica N (2001) In vivo gene transfer in mouse skeletal muscle mediated by baculovirus vectors. Hum Gene Ther 12:871-881.

Piersanti S, Martina Y, Cherubini G*, et al.* (2004) Use of DNA microarrays to monitor host response to virus and virus-derived gene therapy vectors. Am J Pharmacogenomics 4:345-356.

Reimann Rand Miltenburger H (1982) Cytogenetic studies in mammalian cells after treatment with insect pathogenic viruses [<i>baculoviridae</i>]. I. <i>in vivo</i> studies with rodents. BioControl 27:267-276.

Rohrmann GF (2011a) The baculovirus replication cycle: Effects on cells and insects. (Rohrmann, G. F., ed) Bethesda (MD): National Center for Biotechnology Information (US).

Rohrmann GF (2011b) Introduction to the baculoviruses, their taxonomy, and evolution. (Rohrmann, G. F., ed) Bethesda (MD): National Center for Biotechnology Information (US).

Sandig V, Hofmann C, Steinert S*, et al.* (1996) Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum Gene Ther 7:1937-1945.

Sarkis C, Serguera C, Petres S*, et al.* (2000) Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. Proc Natl Acad Sci U S A 97:14638-14643.

Schutz A, Scheller N, Breinig T*, et al.* (2006) The autographa californica nuclear polyhedrosis virus acnpv induces functional maturation of human monocyte-derived dendritic cells. Vaccine 24:7190-7196.

Segura MM, Kamen Aand Garnier A (2006) Downstream processing of oncoretroviral and

lentiviral gene therapy vectors. Biotechnology Advances 24:321-337.

Simpson E (2006) A historical perspective on immunological privilege. Immunol Rev 213:12-22.

Starkey JL, Chiari EFand Isom HC (2009) Hepatitis b virus (hbv)-specific short hairpin rna is capable of reducing the formation of hbv covalently closed circular (ccc) DNA but has no effect on established ccc DNA in vitro. J Gen Virol 90:115-126.

Strauss R, Huser A, Ni S*, et al.* (2007) Baculovirus-based vaccination vectors allow for efficient induction of immune responses against plasmodium falciparum circumsporozoite protein. Mol Ther 15:193-202.

Suzuki T, Chang MO, Kitajima M*, et al.* (2010) Baculovirus activates murine dendritic cells and induces non-specific nk cell and t cell immune responses. Cell Immunol 262:35-43.

t'Hart BA, Vervoordeldonk M, Heeney JL*, et al.* (2003) Gene therapy in nonhuman primate models of human autoimmune disease. Gene Therapy 10:890-901.

Takaoka A, Wang Z, Choi MK*, et al.* (2007) Dai (dlm-1/zbp1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 448:501-505.

Tani H, Limn CK, Yap CC*, et al.* (2003) In vitro and in vivo gene delivery by recombinant baculoviruses. J Virol 77:9799-9808.

Tani H, Nishijima M, Ushijima H*, et al.* (2001) Characterization of cell-surface determinants important for baculovirus infection. Virology 279:343-353.

Taylor P, Botto Mand Walport M (1998) The complement system. Current Biology 8:R259-R261.

Thomas CE, Ehrhardt Aand Kay MA (2003) Progress and problems with the use of viral vectors for gene therapy. Nature reviews Genetics 4:346-358.

Thornton AM, Donovan EE, Piccirillo CA*, et al.* (2004) Cutting edge: Il-2 is critically required for the in vitro activation of cd4+cd25+ t cell suppressor function. J Immunol 172:6519-6523.

Toyoda K, Nakane Hand Heistad DD (2001) Cationic polymer and lipids augment adenovirus-mediated gene transfer to cerebral arteries in vivo. J Cereb Blood Flow Metab 21:1125-1131.

Transfiguracion J, Jorio H, Meghrous J*, et al.* (2007) High yield purification of functional baculovirus vectors by size exclusion chromatography. J Virol Methods 142:21-28.

Venkataraman T, Valdes M, Elsby R*, et al.* (2007) Loss of dexd/h box rna helicase lgp2 manifests disparate antiviral responses. J Immunol 178:6444-6455.

Vicente T, Peixoto C, Carrondo MJ*, et al.* (2009) Purification of recombinant baculoviruses

for gene therapy using membrane processes. Gene therapy 16:766-775.

Vlak MMvOM (2008) Baculovirus insect cell expression system. Chichester: John Wiley & Sons, Ltd.

Wagner E, Zatloukal K, Cotten M*, et al.* (1992) Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes. Proc Natl Acad Sci U S A 89:6099-6103.

Wagner R, Matteucci M, Lewis J*, et al.* (1993) Antisense gene inhibition by oligonucleotides containing c-5 propyne pyrimidines. Science 260:1510-1513.

Wang CY, Li F, Yang Y*, et al.* (2006a) Recombinant baculovirus containing the diphtheria toxin a gene for malignant glioma therapy. Cancer Res 66:5798-5806.

Wang CYand Wang S (2005a) Adeno-associated virus inverted terminal repeats improve neuronal transgene expression mediated by baculoviral vectors in rat brain. Hum Gene Ther 16:1219-1226.

Wang CYand Wang S (2006b) Astrocytic expression of transgene in the rat brain mediated by baculovirus vectors containing an astrocyte-specific promoter. Gene therapy 13:1447-1456.

Wang KC, Wu JC, Chung YC*, et al.* (2005b) Baculovirus as a highly efficient gene delivery vector for the expression of hepatitis delta virus antigens in mammalian cells. Biotechnol Bioeng 89:464-473.

Wang Q, Dziarski R, Kirschning CJ*, et al.* (2001) Micrococci and peptidoglycan activate tlr2-->myd88-->irak-->traf-->nik-->ikk-->nf-kappab signal transduction pathway that induces transcription of interleukin-8. Infect Immun 69:2270-2276.

Wang Sand Balasundaram G (2010a) Potential cancer gene therapy by baculoviral transduction. Curr Gene Ther 10:214-225.

Wang X, Wang C, Zeng J*, et al.* (2005c) Gene transfer to dorsal root ganglia by intrathecal injection: Effects on regeneration of peripheral nerves. Mol Ther 12:314-320.

Wang X, Yin J, Huang X*, et al.* (2010b) DNA methyltransferase inhibitors increase baculovirus-mediated gene expression in mammalian cells when applied before infection. Analytical Biochemistry 396:322-324.

Wu C, Lin J, Hong M*, et al.* (2009) Combinatorial control of suicide gene expression by tissue-specific promoter and microrna regulation for cancer therapy. Mol Ther 17:2058-2066.

Wu C, Soh KYand Wang S (2007) Ion-exchange membrane chromatography method for rapid and efficient purification of recombinant baculovirus and baculovirus gp64 protein. Hum Gene Ther 18:665-672.

Zeng X (2007) Human embryonic stem cells: Mechanisms to escape replicative senescence? Stem Cell Rev 3:270-279.

Zhang XX, McIntosh TJand Grinstaff MW (2012) Functional lipids and lipoplexes for improved gene delivery. Biochimie 94:42-58.

Zhao Y, Lam DH, Yang J*, et al.* (2011) Targeted suicide gene therapy for glioma using human embryonic stem cell-derived neural stem cells genetically modified by baculoviral vectors. Gene therapy.

Zhong B, Yang Y, Li S*, et al.* (2008) The adaptor protein mita links virus-sensing receptors to irf3 transcription factor activation. Immunity 29:538-550.

Zhou Jand Blissard GW (2006) Mapping the conformational epitope of a neutralizing antibody (acv1) directed against the acmnpv gp64 protein. Virology 352:427-437.

Zuo L, Zhou Jand Yao T (1999) Purification of outer membrane proteins in pseudomonas aeruginosa by high performance ion-exchange liquid chromatography. Chinese journal of chromatography / Zhongguo hua xue hui 17:46-48.