TRANSDUCTION OF BOVINE PERIPHERAL BLOOD CELLS WITH RECOMBINANT BOVINE ADENOVIRUS-3 EXPRESSING GREEN FLUORESCENT PROTEIN

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School of Public Health

University of Saskatchewan

Saskatoon, Canada

By

Sugandhika Khosa

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ABSTRACT

Bovine adenovirus type 3 (BAdV-3), a non-enveloped icosahedral particle with a double-stranded DNA genome of 34,446 base pair, has been developed as a vaccine vector (Zakhartchouk et al., 1999). It belongs to *Mastadenovirus* genus in *Adenoviridae* family. Like other *Mastadenovirus* members, BAdV-3 genome is composed of early, intermediate and late regions (Reddy et al., 1998). A number of characteristics including lack of virulence and ability to grow to high titers have made BAdV-3 a vector of choice for further development as a vaccine delivery vehicle for cattle. One of the ways to improve the efficacy of immune response is by targeting recombinant BAdV-3 to immune cells. However little is known about the interaction of BAdV-3 with different immune cells. Determining the tropism of BAdV-3 for specific leukocyte subpopulations may help in devising ways to target BAdV-3 to appropriate leukocyte population for the induction of robust and efficient immune responses in calves immunized with recombinant BAdV-3 expressing vaccine antigens.

The specific aim of this work is to determine the interaction of recombinant BAV304a (GFP expression cassette inserted in E3 deleted region of BAdV-3 (Du and Tikoo, 2010)) with different leukocyte populations in the blood. Different leukocyte populations present in bovine blood (consisting of monocytes, B-cells, T-cells, NK cells and dendritic cells) and PMNs (neutrophils) were transduced with BAV304a. The transduction of bovine peripheral blood mononuclear cells (PBMCs) with BAV304a revealed a GFP expression of 12-15%. Transduction efficiencies of bovine PBMC were shown to be highest with an MOI of 2. Further analysis of bovine PBMC subpopulations transduced with BAV304a at MOI 2 was completed by monoclonal antibody labeling of

lineage specific proteins. The following subpopulations were analyzed: CD14+ & CD11c+ (monocytes); CD3 (T-cells); CD21 (B-cells); CD335 (NK cells) and CD209+ & CD14- (dendritic cells). I observed 100% transduction of monocytes, while 2%, 1% and 4% transduction was observed in B-cells, T-cells and NK-cells, respectively. Similar results were obtained following transduction of monocytes purified from PBMC cells using high-speed cell sorter. However, transduction of purified dendritic cells (CD209+CD14-) and purified PMNs revealed GFP expression of 1-2% and 9-18%, respectively. This result indicates that BAV304a does not transduce myeloid dendritic cells efficiently but both monocytes and PMNs can be transduced by BAV304a.

Though monocytes and neutrophils showed 100% and 9-18% transduction by BAV304a, respectively, Western blot analysis detected a block in the expression of BAV304a specific proteins. While, expression of most of the late proteins could not be detected in transduced monocytes, no expression of any BAV304a specific protein (early or late) could be detected in transduced PMNs. Moreover, no progeny virions could be detected in BAV304a infected monocytes or PMNs confirming a block in the later stages of virus replication. The $TCID_{50}$ assay showed no detectable viral CPE and GFP expression with infected lysates of monocytes and PMNs. These results demonstrate that BAV304a transduces some subpopulations of PBMCs primarily monocytes and PMNs without viral replication. In contrast, dendritic cells, the primary cells involved in the induction of T cell responses, were refractory to transduction by BAV304a.

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ABBREVIATIONS USED IN THIS THESIS

A	Adenine
Ag	Antigen
ADP	Adenovirus death protein
APC	Antigen presenting cell
ARD	Acute respiratory disease
BAdV	Bovine Adenovirus
BAV304a	Bovine Adenovirus 304a
BCR	B cell receptor
BHV	Bovine herpes virus
BoMtch1	Bovine Mitochondrial carrier homolog 1
BoPSAP	Bovine Presenilin-1-Associated Protein
bp	Base pair
C	Cytosine
CAdV	Canine Adenovirus
CAR	Coxsackie and adenovirus receptor
CMV	Cytomegalovirus
CD	Cluster of Differentiation
CPE	Cytopathic Effect
CpG	Guanosine-cytosine hexamer
CsCl	Cesium Chloride
CTL	Cytotoxic T Lymphocytes
dCMP	Deoxycytidine monophosphate
DBP	DNA binding protein
DC	Dendritic cell
cDC	Conventional dendritc cell
pDC	Plasmacytoid dendritic cell
DDX	DEAD box protein
DNA	Deoxyribonucleic acid
DYNLL	Dynein light chain
E	Early
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FcR	Fragment crystallization receptor

FrAdV	Frog Adenovirus
FITC	Fluorescein isothiocyanate
G	Guanine
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colony stimulating factor
Gp	Glycoprotein
HAdV	Human adenovirus
HMGB1	High mobility group protein B1
INF	Interferon
IL	Interleukin
Ig	Immunoglobulin
ITR	Inverted Terminal Repeat
kb	Kilo base
kDa	Kilodalton
L	Late
MAb	Monoclonal antibody
MAMP	Microbial associated molecular patterns
MALT	Mucosal associated lymphoid tissues
MDBK	Madin-Darby Bovine Kidney
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MLP	Major late promoter
MMP	Mitochondrial membrane potential
MP	Membrane protein
MOI	Multiplicity of infection
MFI	Mean fluorescence intensity
mDC	Myeloid dendritic cell
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular traps
NF	Nuclear factor
NFBP	NFkB-binding protein
NLS	Nuclear localization signal
NK	Natural Killer
NPC	Nuclear pore complex
ORF	Open reading frame
PAdV	Porcine Adenovirus
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline

PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-OH kinase
PMN	Peripheral morphonuclear cells
Pol	Polymerase
PRR	Pattern recognition receptors
pTP	Precursor terminal protein
RAdV	Raptor Adenovirus
RBC	Red blood cells
Rb	Retinoblastoma
RGD	Arginine-glycine-aspartic acid
RI-67	Respiratory infectious agent 67
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SAdV	Simian Adenovirus
SDS	Sodium dodecyl sulfate
SV40	Simian Virus 40
T	Thymine
Th	T helper cell
$TCID_{50}$	Tissue culture infectious dose50
TCR	T cell receptor
TLR	Toll-like receptors
TNF	Tumor necrosis factor
TP	Terminal protein
TPL	Tripartite leader
VA RNA	Virus-associated RNA
WSAdV	White sturgeon adenovirus

1. LITERATURE REVIEW

1.1 Adenoviruses

Adenoviruses previously known as ‘adenoid degeneration agent’ or ‘respiratory infectious agent RI-67’ were first isolated in 1953 by two scientists attempting to identify the causative agents of an acute respiratory epidemic in 1952-1953 (Rowe et al., 1953; Hilleman et al., 1954). They were named adenoviruses as they were initially isolated from human adenoids (Enders et al., 1956), and then linked with acute respiratory disease (ARD) subsequently. Presently, over 120 serotypes have been identified infecting diverse species of mammals, birds, reptiles and fish (Ayalew et al., 2015).

All adenoviruses have a non-enveloped, double-stranded DNA genome, with size ranging from 24 to 45 kb (Davison et al., 2003), which is contained in an icosahedral capsid containing 252 capsomers (Berk, 2007). The icosahedral capsid (Stewart et al., 1993) comprises of three major proteins, fibre (IV), penton base (III) and hexon (II) and minor proteins, IIIa, IVa2, VI, VIII, and IX. The genome of adenovirus virus is double-stranded DNA which have inverted terminal repeats (ITRs) with a terminal protein (TP) attached covalently to the 5’ termini (Rekosh et al., 1977) and is closely linked with a small peptide named mu and extremely basic protein VII (Anderson et al., 1989). Another protein V provides structural linkage to the capsid through protein VI (Matthews et al., 1995) and is packaged with this DNA–protein complex. In addition, adenovirus also contains a virus-encoded protease (Weber, 1976; Webster et al., 1989), which is essential for generation of mature infectious virion by cleavage and processing of some of the structural and scaffolding proteins.

Adenovirus replicate in the nucleus of vertebrate cells such as epithelial cells lining the gut and respiratory tract, eye, urinary bladder and the liver using the host's replication machinery, but different serotypes display varied tissue tropism. Even though adenoviruses infect in a wide array of species, their replication is restricted to their host species. Nevertheless, occurrence of neutralizing antibodies against more than one adenovirus serotype indicates the likelihood of infections that are asymptomatic in species that are not the natural host (Shenk, 2001).

As they are one of the largest and most complex non-enveloped viruses, they offer several advantages as a vaccine vector by meeting the most important criteria of an ideal vaccine vector in terms of efficacy, safety, and stability (Tastis et al., 2004). Because of the capability of these vectors to infect various target cells effectively and to express the transgene, recombinant adenoviral vectors have been used extensively for *in-vivo* gene transfer (Crystal, 1995).

1.1.1 Adenovirus taxonomy

Adenoviruses belong to the family *Adenoviridae*. Many serotypes infecting a broad range of species, including humans, birds, reptiles, fish and livestock have been identified till date (Berk, 2007) and are clustered into five phylogentically distinct genera: Aviadenovirus (isolated from birds), *Mastadenovirus*, (isolated from mammals), *Atadenovirus* (isolated from reptiles, birds, ruminants and have high Adenine + Thymine genome content), *Siadenovirus* (isolated from birds and amphibians and contains sialidase homologue gene) and *Ichtadenovirus* (contains a single species virus) (International Committee on Taxonomy of Viruses and King, 2012). All of these virus groups possess a unique and characteristic genome structure.

The members of genus *Mastadenovirus* have been characterized most extensively. They are limited to mammalian species including 7 species of human adenovirus (HAdV-A to G) (Chroboczek et al., 1992), 1 species of canine adenovirus (CAdV)-2 (Szelechowski et al., 2009), 1 species of Simian adenovirus (SAdV) (Kovács et al., 2005), 3 species of bovine adenovirus (BAdV-A, B, C) (Reddy et al., 1998), 3 species of porcine adenovirus (PAdV-A, B, C) (Nagy et al., 2001; Reddy et al., 1998), 3 species of murine adenovirus (MAdV-A, B, C), 2 species of ovine adenovirus (OAdV-A and B), 2 species of bat adenovirus and 1 species of three shrew adenovirus. The genomes of adenoviruses consists of sixteen genus-common genes that includes genes encoding for DNA replication such as DNA polymerase, terminal protein (TP), DNA binding protein (DBP), proteins for DNA encapsidation such as 52K, IVa2, and for formation of virion capsid such as pIIIa, pV, pVI, pVII, pVIII, pX, 100K, 33K, hexon, fibre and protease (Davison et al., 2003). There are four genus-specific genes, which are located at the ends of the genome. The protein pV and many proteins present in the E1, E3, and E4 regions of the genome are unique to the *Mastadenovirus* genus (Davison et al., 2003). The *Mastadenovirus* genome contains five transcriptional units namely E1, E2, E3 and E4 region along with two delayed early transcriptional units, IVa2 and pIX.The early regions E1 and E4 are located at either end of the genome whereas E2 and E3 are located inside in the genome. The late region comprises of the core of the adenovirus. As these proteins are involved in viral gene expression and virus cell interaction, variations in any of the protein expression may result in different pathogenicity and host range of different *Mastadenoviruses* members.

Aviadenovirus genus comprises of species of adenoviruses infecting several avian host species (International Committee on Taxonomy of Viruses and King, 2012), including chicken (Chiocca et al., 1996), falcon (Schrenzel et al., 2005), goose (Zsak et al., 1984) and turkey (Kajan et al., 2010). *Aviadenoviruses* code for 47 to 54 ORFs on the basis of their genome sequence (Kajan et al., 2012). Although their genomes are fairly large ranging from 43,804 bp (Chiocca et al., 1996) to 45,667 bp (Griffin and Nagy, 2011), their ITRs are rather short and found in the same positions as *Mastadenoviruses* (Griffin and Nagy, 2011). There is a significant difference in the G+C % content amongst the members of this genus; highest being that of turkey adenovirus 1 with 66.9 % and lowest being that of goose adenovirus 4 with 44.7 % (Kajan et al., 2012). The E1, E3, and E4 regions are absent in *Aviadenoviruses* genomes but both ends of the genome are rich in open reading frames (Chiocca et al., 1996), which are predicted to encode proteins that help perform the functions of E1, E3 and E4 regions (Chiocca et al., 1996).

The homologues of pIX, pV, E2 and E3 regions of this genus are absent as compared to other genera of adenoviruses (Davison et al., 2003). Each vertex of *Aviadenovirus* capsid contains two fiber proteins; regardless whether the virus encodes two fiber genes, like Fowl Adenovirus 1 (Griffin et al., 2011), or only one gene, like the Duck Adenovirus 2 (International Committee on Taxonomy of Viruses and King, 2012; Marek et al., 2014)

Atadenovirus genus includes five species of adenoviruses (Davison et al., 2003; International Committee on Taxonomy of Viruses and King, 2012). A very high A+T content in their genomes gives this genus its name (Fields et al., 2007) and include mammals (bovine adenovirus D, ovine adenovirus D, possum adenovirus A), a bird (duck

adenovirus A) and a reptile (snake adenovirus A) (Benko et al., 2002). *Atadenovirus* genomes range between 29,576 bp (Vrati et al., 1995) and 33,213 bp (Hess et al., 1997) in length with high AT content and relatively small ITRs between 46 (Vrati et al., 1995) and 118 (Benko et al., 2002). Similar to *Aviadenoviruses*, the members of *Atadenovirus* genus does not encode proteins pV and pIX (Benko et al., 2002; Hess et al., 1997; Vrati et al., 1995). Two genus specific genes; p32K and RH belong to *Atadenoviruses* (Benko et al., 2002; Davison et al., 2003; Hess et al., 1997; Vrati et al., 1995).

Members of *Siadenovirus* genus infect reptiles (Kovacs et al., 2009), frogs (Davison et al., 2000) and turkey (Pitcovski et al., 1998). So far, only five adenoviruses namely raptor Adenovirus A (RAdV-A) (Kovacs et al., 2009), Frog Adenovirus 1 (FrAdV-1) (Davison et al., 2000) and turkey Adenovirus A (TAdV-A) (Pitcovski et al., 1998), Great tit adenovirus A and Skua adenovirus A are included in this genus. These *siadenoviruses* comprise of shortest ITRs, ranging from 29 to 39 bp and smallest genomes varying from 26,163 bp to 26,282 bp (Davison et al., 2000; Kovacs and Benko, 2009; Pitcovski et al., 1998), which makes them the smallest known adenoviral genomes (Kovacs et al., 2010; Kovacs et al., 2011). They are called *Siadenoviruses* because they contain sialidase gene homologue in the E1 region of their genomes (Davison et al., 2003). Unlike *Mastadenoviruses*, the early regions E1, E3 and E4 and genes encoding for pV, pIX are absent from their genomes (Davison et al., 2000; Kovacs et al., 2009; Pitcovski et al., 1998).

Ichtadenovirus belongs to the fifth genus and has been added recently to the *Adenoviridae* family (International Committee on Taxonomy of Viruses and King, 2012). Only one specie White Sturgeon Adenovirus 1 (WSAdV-1) is included in *Ichtadenovirus*

genus. It has the longest genome of 48,396 bp in the members of all known adenovirus genuses. Two nucleotides between the hexon gene and protease gene are present in WSAdV-1; and two stop codons at the end of hexon genes as compared to only one in all other four recognized adenoviral genera is characteristic of this genera (Kovacs et al., 2003). DNA sequencing and phylogenetic analysis of WSAdV-1 places it as a separate and new genus (Davison et al., 2003).

1.1.2 Human adenovirus

Human adenoviruses (HAdVs) belong to *Adenoviridae* family and the genus *Mastadenovirus*, which contains seven known HAdV species HAdV-A to G (Huang et al., 2013), consisting of 67 different serotypes reported till date (http://hadvwg.gmu.edu/; Ghebremedhin, 2014). In 1953, adenoviruses were first isolated by Rowe and colleagues from human adenoid tissues, which they identified as a cytopathogenic agent undergoing spontaneous degeneration in tissue culture (Huebner et al., 1953). Adenoviruses can cause a range of clinical manifestations in humans like conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia (Maranhão et al., 2009; Sambursky et al., 2007; Chang et al., 2008; Lewis et al., 2009). The adenovirus infects primarily respiratory, enteric, ocular, renal or hepatic organs in humans (Arnberg, 2012). Most of these appear in children below the age of 5 years and are usually self-limiting infections. It does not cause clinical diseases in healthy adults but adenovirus induced diseases can cause substantial illness in immunocompromised patients and some vulnerable populations (La Rosa et al., 2001; Kojaoghlanian et al., 2003; Leen and Rooney, 2005). Presently, no therapeutic anti-adenoviral drug is available for the treatment of adenoviral infections (Kinchington, 2005; San Martin, 2012).

The human adenovirus genome comprises of the early genes (E1 through E4), intermediate genes or delayed-early (pIX, IVa2) and late genes, which encode structural proteins required for the viral capsid formation (IIIa, V, VI, VII, VIII, IX, μ, hexon, penton and fiber) and non-structural proteins (22K, 33K, 52K, 100K and the viral protease), the expression of which is controlled by major late promoter (MLP) (Russell, 2009). The DNA sequence of variable region of fiber, hexon, penton and other genes have been used for the classification of human adenovirus isolates (Robinson et al., 2011; Walsh et al., 2011; Matsushima et al., 2012).

1.1.3 Bovine adenovirus

Bovine adenovirus (BAdV) was initialy isolated in 1959 from the respiratory secretions of a cow suffering from pneumoenteritis (Klein et al., 1959). Most prototype strains of many serotypes of bovine adenovirus were first isolated from healthy calves (Klein et al., 1959; Darbyshire et al., 1965; Rondhuis, 1968; Burkis et al., 1978). Currently, fourteen serotypes of bovine adenovirus have been identified in cattle (International Committee on Taxonomy of Viruses, 2012) (Lehmkuhl et al., 2008; Sibley et al., 2011). These viruses were isolated from the sick cattle (Horner et al., 1989; Graham et al., 2005), healthy cattle (Mattson et al., 1988), or from environment samples (Sibley et al., 2011).

Table 1. Major structural and non structural adenoviral proteins

Role of major and minor adenoviral structural proteins in the viral life cycle	
Viral proteins	**Role in viral life cycle**
Hexon	Major capsid protein, which forms trimers and arranged as 12 molecules on each of 20 facets of the icosahedron (Athappilly, 1994).
Penton base	Associates with four hexon trimers to form the icosahedral asymmetric unit of capsid (Komoriya et al., 1991). Endocytosis of the virus particle and internalization (Cupelli and Stehle, 2011).
Fiber	The fiber tail interacts with the penton and the fiber knob interacts with the CAR receptor and these interactions are essential for the attachment of the virus with the cellular surface (Zhang et al., 2005).
Polypeptide IIIa	Consists of group of six-glue domain, hexon-binding domain, pVIII binding domain and core-proximal domain (Liu et al., 2010).
pVI	Located on the inner side of the capsid essential for endosomal escape of virus (Wiethoff et al., 2005; Liu et al., 2010).
pVIII	Each molecule interacts with the four molecules of hexon in the capsid and hence also known as the hexon associated protein (Liu et al., 2010).
pIX	Present as trimer on facet at four positions, which act as cementing protein for the group of nine hexons (Reddy et al., 2010)
pV	Located inside the capsid beneath the major capsid proteins in mature virion.
pVII	Associated with the viral DNA and get imported to the nucleus of infected cells (Xue et al., 2005).
Mu	Execute conformational change to adenoviral DNA and help in the packaging of the genome in to the capsid (Russell, 2009).
Terminal protein	Forms a heterodimer and is essential for viral DNA replication (Pronk and van der Vliet, 1993).
IVa2	Bind with 22K and 52K proteins and assist in viral DNA packaging in to the capsid (Ostapchuk and Hearing, 2005; Ewing et al., 2007).
Protease	Cleaves six viral proteins (pIIIa, pVI, pVIII, pVII, pTP and μ) and help in maturation of virion (San Martin, 2012).

Role of important non-structural proteins in the adenovirus life cycle	
Viral proteins	**Role in viral life cycle**
E1A region 289R 243R 217R 171R 55R	Produced before viral DNA replication and act as the hub and essential for the productive viral infection (Jones and Shenk, 1979; Stephens and Harlow, 1987; Ulfendahl et al., 1987; Pelka et al., 2008).

E1B region 55K 19K 156R 93R 84R	E1B-55K and 19K proteins are involved in the viral replication and transformation of cells in culture (Takayesu et al., 1994; Sieber and Dobner, 2007). 55K stimulate the export of viral late mRNA (Blanchette et al., 2008). Prevents apoptosis by interacting with proapoptotic mitochondrial BCL-2 antagonist (Lomonosova et al., 2005).
E2 region DNA polymerase DBP pTP	Pol is essential for viral DNA replication by interacting with the pTP for initiation (Liu et al., 2000). DBP enhances the ability of the viral DNA polymerase to commence the replication of viral DNA (van Breukelen et al., 2003). pTP forms a heterodimer and is essential for viral DNA replication (Pronk and van der Vliet, 1993).
E3 region 11.6K/ADP gp19K 10.4K 14.5K 6.7K 14.7K 12.5K	The E3-10.4K and 14.5K helps in immune evasion (Hilgendorf et al., 2003; Sharma and Andersson, 2009). E3-gp19K in HAdV-2 infected cell curbs the expression of major histocompatibility complex (MHC) class I molecules (Fu et al., 2011). Responsible for the cell death and release of the progeny viruses (Tollefson et al., 1996). Protein 14.7K inhibit tumor necrosis factor (TNF) mediated cell lysis in the adenovirus-infected cells (Krajcsi et al., 1996). Protein 6.7K with 10.4K and 14.5K prevent the apoptosis by replacing the TNF-related apoptosis-inducing ligand receptor-2 from the adenovirus infected cell surface (Lichtenstein et al., 2004).
E4 region E4orf1 E4orf2 E4orf3 E4orf4 E4orf3/4 E4orf6 E4orf6/7	E4 region codes for seven proteins (Bridge and Ketner, 1989). E4orf1 protein was found to interact with the PI3K pathway leading to mTOR activation (O'Shea et al., 2005). E4orf4 responsible for induction of apoptosis transfected cells (Brestovitsky et al., 2011). E4orf6 is 294 amino acid protein with a role in degradation of p53 (Nevels et al., 2000).
33K protein 22K	L4-33K is involved in control of late gene expression and viral DNA packaging in infected cells (Wu et al., 2013). L4-22K activates late gene expression and suppression of early gene expression. It is required for Ad genome packaging and contributes to adenovirus-induced cell death regulated by ADP (Wu et al., 2012)
100K protein	Involved in translation of tripartite leader (TPL) containing late viral mRNA and in the nuclear localization, translation, folding and trimerization of hexon protein in the cytoplasm (Hong et al., 2005; Xi et al., 2004; Xi et al., 2005; Koyuncu et al., 2012).
52K protein	Serotype specific viral DNA packaging, gets cleaved by proteases for generation of mature virus (Wohl and Hearing, 2008).

1.1.3.1 Classification

Bovine adenoviruses are earlier classified into ten serotypes (International Committee on Taxonomy of Viruses, 2012). The serotypes BAdV-1, -2, -3, -9, -10 belong to *Mastadenovirus* genus, and the serotypes BAdV-4, -5, -6, -7, -8 belong to *Atadenovirus* genus (http://www.ictvdb.org). Now, all bovine serotypes are divided into 4 species and two subgroups based on their biological and serological distinctiveness (Bartha, 1969; Horner et al., 1989). Species A, B, C belong to *Mastadenovirus* genus. Specie D belongs to *Atadenovirus* genus. The subgroup 1 bovine adenoviruses serotypes (BAdV-1, -2, -3, and -9) propagate in established bovine cell lines and contain common complement-fixing antigens, which cross-react with other *Mastadenoviruses* members in the complement fixation tests (Zhu et al., 2011). However, the subgroup 2 bovine adenoviruses serotypes (BAdV-4, -5, -6, -7, -8, and -10) can be propagated solely in low-passage cultures of calf testicular or thyroid cells and do not cross-react with any other members of mammalian adenovirus in the complement fixation test (Reddy et al., 1998, Tikoo et al., 2002; Zhu et al., 2011).

1.1.3.2 Bovine adenovirus type 3

Bovine adenovirus-3 (WBR I strain), a respiratory tract pathogen of cattle, particularly of newborn calves is a member of subgroup 1 of bovine adenovirus (Mattson et al., 1988). Although it was first isolated in 1965 in Britain from the eye swab of a healthy cow (Darbyshire et al., 1965a), BAdV-3 causes clinical disease in colostrum-deprived calves following intranasal immunization (Darbyshire et al., 1965). As recombinant human adenoviruses are established as excellent mucosal vaccine vectors

(Imler, 1995), recombinant BAdV-3 has been evaluated as a vector for vaccine delivery in cattle (Zakhartchouk et al., 1999).

1.1.3.2.1 Genome structure and organization

Bovine adenovirus-3 is a non-enveloped, icosahedral virus with a double-stranded DNA genome of 34,446 nucleotides with 54% G+C content (Reddy et al., 1998). Viral DNA coupled with the core proteins are enclosed in an icosahedral capsid, with 20 triangular faces comprising mainly of hexon; the major capsid protein (Rux et al., 2004). All of the 12 capsid vertices contain a protrusive elongated fiber. The fiber is bound to a pentameric structure at its proximal end and forms a globular "knob" domain, while the penton base at its distal end. Generally, the primary attachment site for cellular receptors is the fiber knob, while the secondary interactions of virus with its cellular receptor is mediated through penton base which is required for entry of virus into the cell (Zhang et al., 2005). It has been shown that sialic acid acts as primary receptor for the attachment and entry of BAdV-3 into the cell (Li et al., 2009).

The genome of BAdV-3 is similar to that of HAdVs and is organized into early (E1 [E1A and E1B], E2 [E2A and E2B], E3 and E4), intermediate (IVa2, pIX), and late (L1 to L7) regions (Reddy et al., 1998). The transcription map of BAdV-3 is shown in (Fig1.1). The ITR of BAdV-3 is 195 bp that is fairly long as compared to the ITR of HAdV-2 (102 bp) and HAdV-5 (103 bp) (Reddy et al., 1998; Davison et al., 2003). The distinct features of BAdV-3 genome include arrangement of late region in seven families and absence of RGD motif in and the absence of VA RNA genes (Reddy et al., 1998).

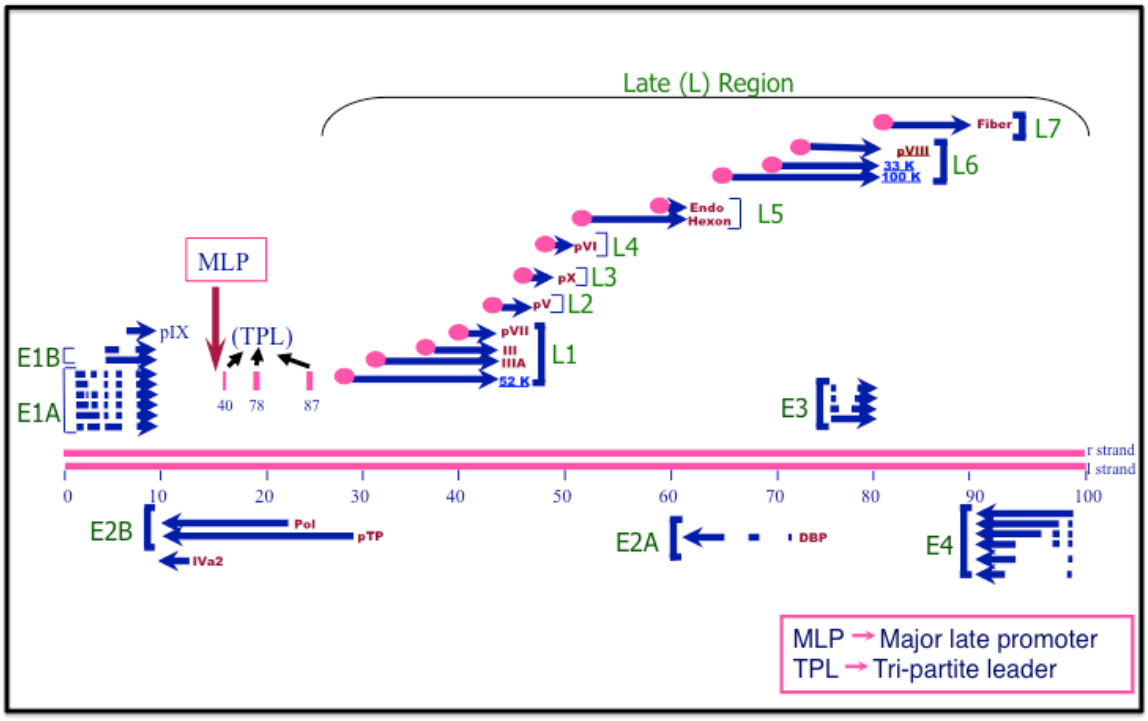

Fig. 1.1. Transcription map of BAdV-3. Transcript locations and directions are indicated by arrows. Adapted from (Ayalew et al., 2015), with permission to use.

1.1.3.2.2 Viral proteins

Adenoviral proteins are classified into structural proteins and non-structural proteins. The structural proteins are further classified into major capsid proteins, minor capsid protein and core proteins (Fig.1.1.2). Major capsid proteins comprise of Hexon, Penton base and Fibre. Minor capsid proteins comprise of IIIa, VI, VIII and IX. The core proteins comprise of V, VII, mu, TP, IVa2 and protease. The non-structural proteins comprise of 100K, 33K/22K and 52K/55K proteins that are not the central part of the mature virions (Davison et al., 2000). These proteins are involved in the cleavage and processing of precursor virion proteins alongwith the transport and assembly of virion proteins required for the maturation of viruses.

Major capsid proteins

The BAdV-3 encodes three major capsid proteins (hexon, fiber and penton base). The hexon (910 amino acid) is encoded by the L5 region of BAdV-3 genome and makes up the majority of the viral capsid and forms the facets of icosahedron (Reddy et al., 1998). It does not have a recognised NLS and remains in the cytoplasm when expressed in the absence of other viral proteins (Wodrich et al., 2003). Hexon has a high sequence identity of about 66-71% with the hexon proteins of other *Mastadenovirus* genus members. However, three regions from the external loops on the surface of the virion in the protein sequence of BAdV-3 hexon display significant differences from that of HAdV-2 (Hu et al., 1984; Crawford-Miksza and Schnurr, 1996). In BAdV-3,

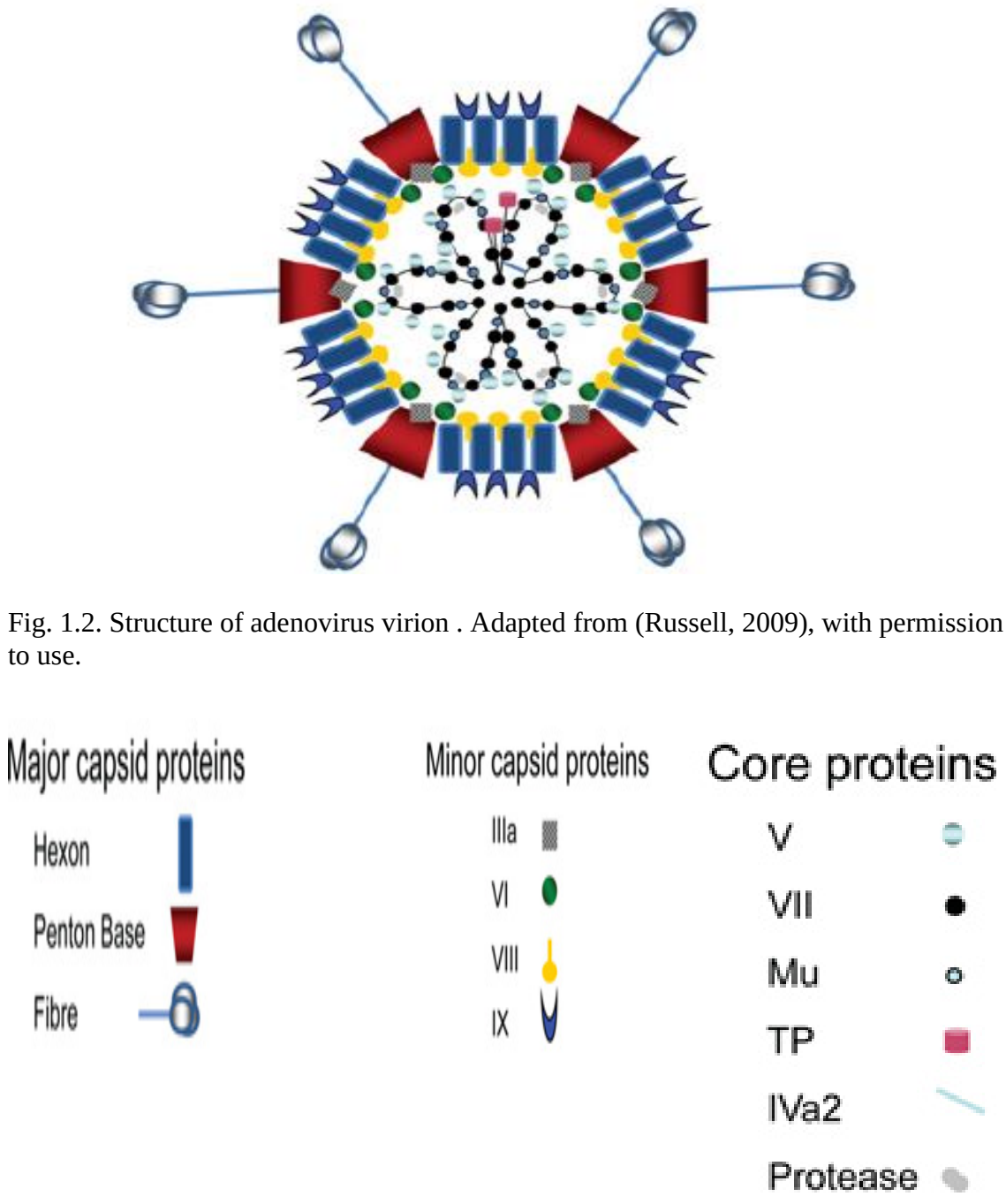

Fig. 1.2. Structure of adenovirus virion . Adapted from (Russell, 2009), with permission to use.

the L1 loop is shorter than that of HAdVs (Athappilly et al., 1994). The amount of differences between adenovirus serotypes is due to two of these loops in the hypervariable regions. The hexon protein of BAdV- 3 is thus anticipated to have the same general structure as that of HAdV (Reddy et al., 1998).

The penton base (482 amino acids) is encoded by L1 region of BAdV-3 genome and is present on the capsid vertices, which unlike HAdVs (Komoriya et al., 1991; Wickham et al., 1993) does not contain an RGD motif (Reddy et al., 1998). The RGD motif of penton interacts with cellular integrins and facilitates entry of HAdV-5 in cells (Shayakhmetov et al., 2005 and Nemerow, 2000). Absence of the RGD motif in the penton of BAdV-3 suggests that the virus does not use integrins as receptor for virus attachment and entry (Reddy et al., 1998). Interestingly, BAdV-3 penton base protein contains a MDA motif rather than RGD motif found in HAdV penton, which is shown to interact with α4β1 integrin (Komoriya et al., 1991; Reddy et al., 1998) to trigger the internalization for virus through receptor mediated endocytosis via formation of clathrin coated pits (Wickham et al., 1993).

The fibre protein (976 amino acids) is encoded by L7 region of BAdV-3 genome and projects from the penton base mediating the initial attachment of virus to the to host cells (Bangari et al., 2005). BAdV-3 fiber protein, like other *Mastadenoviruses* can be divided into knob, shaft and tail regions (Reddy et al., 1998). The knob region of BAdV-3 primarily interacts with the cellular receptors initially (Bangari et al., 2005) followed by the shaft region, which is long and bent (Ruigrok et al., 1994). The tail region of BAdV-3 includes the conserved FNLVYPYKA motif and is proposed to play a significant role in the interaction of fiber with the penton protein (Caillet-Boudin. 1989; Reddy et al., 1998).

The fiber protein of BAdV-3 is detected as 102 kDa in virus infected cells and contains a nuclear localization signal (NLS), which is required for the translocation of the protein to the nucleus of the infected cells (Wu et al., 2004). Moreover, NLS plays an important role in the replication of virus in MDBK cells (Wu et al., 2004).

Minor capsid proteins

The pIX (125 amino acids) encoded by intermediate region makes the external faces of the capsid and is required for the stabilization of virion. The pIX protein is expressed as 14 kDa protein in purified virions and in virus infected cells (Zakhartchouk et al., 2004). Based on the kinetics of the gene expression, the gene coding for BAdV-3 pIX protein is also classified as intermediate region gene (Reddy et al., 1999a). The detection of pIX in CsCl gradient purified BAdV-3 suggested that pIX is a component of the viral capsid (Reddy et al., 1999a). Analysis of mutant BAdV-3 suggests that conserved N –terminal and and putative lucine zipper element is essential for the replication of BAdV-3 (Zhang et al., 2016). The C-terminus of pIX is exposed on the surface of the virion, which is utilized to incorporate heterologous peptides like RGD to enhance the transduction in integrin positive cells (Zakhartchouk et al., 2004). An RGD motif fused to the C-terminus of pIX enhances the ability of BAdV-3 to infect integrin-expressing cells (Zakhartchouk et al., 2004).

The pVIII (216 amino acids) encoded by L6 region is a minor capsid protein connecting the core with the inner surface of the capsid. It is detected as 24kDa (precursor) and 8kDa (cleaved) protein in BAdV-3 empty capsid and mature virion, respectively, due to the cleavage of this precursor protein by protease to produce mature progeny virion. The pVIII protein localizes in the nucleus of BAdV-3 infected cells and

utilizes the classical importin α /β dependent nuclear import pathway (Ayalew, et al., 2014). It interacts with cellular protein DDX3 and inhibits the cap-dependent cellular mRNA translation at late times post-infection (Ayalew et al. 2016). Recently, our laboratory has demonstrated that pVIII (amino acids 147-174) appear to interact with N-terminus of eIF6 (amino acids 44-97), which helps to impair the formation of 80 ribosomes (Gaba, 2016). In addition, partial cleavage of pVIII appears to produces thermolabile virions suggesting that cleavage at both potential sites of BAdV-3 pVIII is required for production of infectious progeny virions (Gaba, 2016).

Not much is known about IIIa and pVI. The IIIa (568 amino acids) encoded by L1 region is present underside of the penton base and is required to stabilise the vertices. The pVI (263 amino acids) encoded by L4 region is present in the inner hexon cavity. Like HAdV-2 (Honkavuori et al., 2004) the C-terminus of BAdV-3 VI protein may act as the cofactor for the adenoviral protease (Reddy et al., 1998). It is also required for the virus assembly, endosome disruption and nuclear import of hexon.

Core proteins

The pV (423 amino acid) encoded by L2 region is a core protein that functions as a bridge between the viral capsid and the DNA genome. It is detected as 55 kDa protein in BAdV-3 infected cells and is localised in the nucleus and in the nucleolus in both infected and transfected cells (Zhao, 2016). Deletion mutant analysis identified amino acid 80-120, 190-210 and 380-423 acting. As nuclear localization signals and use importin α3 of importin α/β pathway. Similar analysis identified N-terminal amino acids 21-50 and C-terminal amino acid 380-389 as nucleolar localization signals (Zhao, 2016). The pV appears essential for BAdV-3 replication and maintaining the integrity and

stability of mature virions (Zhao and Tikoo, 2016). In addition, BAdV03 pV interacts with cellular protein nucleolin, which may play a role in ribosomal biogenesis (Zhao, 2016).

pVII (171 amino acids) encoded by L1 region is mainly used to target viral genome to the nucleus. The BAdV-3 VII protein contains a mitochondrial localization signal and localizes the protein into the mitochondria. The retention of Ca2+ in the mitochondria is aided by the localization of BAdV-3 pVII in mitochondria which in turn increases the ATP concentration and maintain the mitochondrial membrane potential (MMP) in transfected and virus infected cells (Anand et al., 2014).

The protease (204 amino acids) encoded by L5 region recognizes two consensus cleavage motifs (M/I/L)XGX-G and (M/I/L)XGG-X (where X is any amino acid) and cleaves selected precursor proteins (Webster et al., 1989) before maturation of infectious progeny virions. Analysis of the protease cleavage of pVIII encoded by members of *Mastadenoviruses* including BAdV-3 suggest that the potential recognition sites and mechanism of cleavage appear conserved in members of *Mastadenoviruses* including BAdV-3. In addition to cleaving structural proteins, interestingly BAdV-3 protease also cleaves non-structural protein 1000 (Makadiya et al., 2015), which is required for the nuclear localization of 100K protein.

The X protein encoded by L3 region is 80 amino acids long (Anderson et al., 1989; Reddy et al., 1998) and contains two potential protease cleavage sites (Reddy et al., 1998). TP (649 amino acid) encoded by E2B region is present at the 5’ end of the genome and is shown to prime the DNA replication process. IVa2 (376 amino acids) encoded by E2B region is a core protein involved in DNA packaging.

Non-structural proteins

The E1 region comprises of E1A and E1B proteins. The E1 region of BAdV-3 is divided into E1A and E1B. In contrast to HAdV, same transcriptional unit are used for the expression of E1A and E1B regions and contain the same polyadenylation site (Reddy et al., 1999a). Though the regions between the left ITR and upstream of the E1A start codon does not contain TATA or CAAT boxes, the promoter situated within the ITR drives the expression of the E1A open reading frame (Xing and Tikoo, 2006). Six transcripts are produced in the E1A region by alternate splicing that encode for three proteins; 211R, 115R and 100R (Reddy et al., 1999a). The E1A proteins are required for the activation of the other early viral genes transcription and for virus replication (Reddy et al., 1999b; Zhou et al., 2001). However, two overlapping mRNAs are encoded by E1B; E1Bsmall and E1Blarge, which are homologues of HAdV-5 E1B 19K and HAdV-5 E1B 55K proteins (Zheng et al., 1994; Reddy et al., 1998). The E1Bsmall (157 amino acids) and E1B large (420 amino acids) are expressed as 19kDa and 48kDa proteins, respectively in BAdV-3 infected cells (Reddy et al., 1999a.) The E1Blarge protein seems crucial for virus replication in MDBK cells (Zakhartchouk et al., 2001). Although E1B small appears essential in fibroblast cells but not in MDBK cells (Zhou et al., 2001).

The E2 region of BAdV-3, codes for proteins that are necessary for viral DNA replication and are transcribed from the complementary strand, (Reddy et al., 1998). Two ORFs E2A and E2B are encoded by E2 region where E2A transcript codes for the protein DBP (432 amino acids) that shares 38 to 47% sequence identity with other *Mastadenoviruses* DBP (Reddy et al., 1998). The DBP is expressed during the early and late phase of the life cycle of BAdV-3 in high amounts and plays a key role in DNA

binding, initiation of DNA replication and for the determination of titer in BAdV-3 infected cells by immunohistochemistry (Linne and Philipson, 1980, Zhou et al., 2001a). The E2B transcripts encode for the proteins DNA Pol and pTP (Reddy et al., 1998) and share 56 to 62% and 58 to 60% homology, respectively with other *Mastadenovirus* DNA Pol and pTP proteins. Both DNA Pol and pTP proteins are important for viral DNA replication (Baxi et al., 1998).

The E3 region of BAdV-3 is 1.4 kb, and produces transcripts encoding four proteins of 284R, 121R, 86R and 82R amino acids (Idamakanti et al., 1999). The 284R is a unique glycoprotein in BAdV-3. The 121R displays reduced homology to HAdV-5 E3-14.7 kDa protein (Mittal et al., 1993). It is detected as 14.5 kDa protein in BAdV-3 infected cells (Idamakanti et al., 1999) and is involved in the inhibition of TNF-α mediated apoptosis (Zakhartchouk et el., 2001). The E3 proteins are not crucial for the BAdV-3 replication *in-vitro* (Zakhartchouk et al., 1998) as well as *in-vivo* (Zakhartchouk et al., 1999). Thus, E3 deleted replication-competent vectors are being evaluated for vaccine antigen delivery in cattle (Ayalew et al., 2014).

The E4 region produced seven mRNA transcripts that code for five unique proteins containing 69, 143, 143, 219 268 long amino acids (Baxi et al., 1999). None of these unique proteins are essential for BAdV-3 replication as suggested by mutational analysis of E4 region (Baxi et al., 2001).

The 52K (370 amino acids) is a non-structural protein encoded by L2 region is expressed as 40kDa protein in BAdV-3 infected cells. 52K localizes in the nucleus of the BAdV-3 transfected or infected cells (Paterson et al., 2012) with the help of nuclear import receptor importin α3 and has a bipartite nuclear localization signal. The 52K

protein interacts with BAdV-3 encoded pVII and cellular protein NFkB-binding protein and aids the reallocation of NFBP from nucleolus to nucleus (Paterson, 2010). Moreover, many mitochondria localization signals (MLS) have been indicated in the sequence analysis of BAdV-3 52K protein. The expression of 52K in transfected cells has shown to considerably increase ROS/SO production without substantial change in ATP production, mitochondrial Ca2+ or MMP demonstrating that 52K alone can cause an oxidative stress and apoptosis in infected cells (Anand et al., 2014)

The 33K (279 amino acids), 22K (274 amino acids) and 100K (850 amino acids) are non-structural proteins encoded by the L6 region of BAdV-3 (Reddy et al., 1998; Kulshreshtha et al., 2009). The 33K (spliced) and 22K (unspliced) proteins share N-terminal 138 amino acids. The 33K protein is expressed as two major proteins of 42 kDa and 22 kDa and five minor proteins of 39 kDa, 35 kDa, 29 kDa 25 kDa and 19 kDa in BAdV-3 infected cells (Kulshreshtha et al., 2014), and is involved in the capsid assembly especially in the viral DNA encapsidation (Kulshreshtha et al., 2004). Moreover, 33K activate the transcription from the major late promoter, which is mediated by leucines residues (217, 224, 232 and 240) of lucine zipper present in the conserved region (amino acid 201-240) of BAdV-3 33K (Kulshrestha et al., 2015). Moreover, analysis of deletion mutant showed that BAdV-3 33K amino acids 201 to 240 containing RS (arginine/serine) repeats (Kulshreshtha et al., 2014) are necessary for the nuclear localization of 33K, which is mediated by both transportin and importin α/β dependent nuclear import pathways (Kulshreshtha et al., 2014). The 33K protein of BAdV-3 interacts with the 100K and pV proteins of BAdV-3 (Kulshreshtha and Tikoo, 2008), and cellular bovine

presenilin-1-associated protein / mitochondrial carrier homolog 1 (BoPSAP / BoMtch1) (Kulshreshtha, 2009).

The 22K protein is expressed as 42 kDa and 39 kDa proteins in infected cells which seems important for BAdV-3 replication (Kulshreshtha, 2015), capsid assembly and/or viral DNA encapsidation (Kulshreshtha et al., 2004). Unlike HAdV-5 (Ali et al., 2007), BAdV-3 22K does not appear to activate the transcription from major late promoter (Kulshreatha et al., 2015)

The 100K (850 amino acid) protein encoded by L6 region is the largest non-structural protein encoded by *Mastadenoviruses* members (Reddy et al., 1998). BAdV-3 100K protein is shown to have 27 to 52% sequence identity with other members *Mastadenoviruses* encoding 100K protein (Reddy et al., 1998; Kulshreshtha et al., 2004) and is expressed as 130 kDa, 100 kDa, 95 kDa and 15 kDa proteins in BAdV-3 infected cells (Makadiya et al., 2015). Like other adenoviruses, the C-terminus of BAdV-3 100K also coincides with 33K/22K proteins of BAdV-3 (Kulshresthat et al., 2004). Interestingly, 100K protein is cleaved by BAdV-3 protease at two adenovirus specific protease cleavage sites; amino acids 740-745 and 781-786. The C-terminal of cleaved 100K fragment contains a bipartite nuclear localization signal (amino acid 624 to 637), which help to localize the protein to the nucleus using importin α3 receptor of classical importin-α/β transport pathway for nuclear transport (Makadiya et al., 2015). Although BAdV-3 protease cleavage of 100K promotes the localization of 100K to the nucleus of the infected cells, it does not appear to be essential for efficient replication of BAdV-3 (Makadiya et al., 2015). The BAdV-3 100K protein has shown to interact with dynein light chain (DYNLT)-1 cellular protein and 33K protein of BAdV-3 (Makadiya, 2013).

1.1.3.2.3 Replication cycle

The adenovirus replication cycle can be divided into five stages: adsorption and entry, activation of early genes, DNA replication, activation of late genes, assembly and release.

The entry of BAdV-3 in the cell has been reported to be independent of CAR and integrin-dependent mechanisms (Bangari et al., 2004; Mittal et al., 2005). BAdV-3 uses sialic acid as a primary receptor for attachment and entry to the cell (Li et al., 2009). The infection of MDBK cells by BAdV-3 is inhibited by removing sialic acid residues with neuraminidase treatment or blocking it with a lectin. Many different viruses including some HAdV serotypes have been reported to use sialic acid as a receptor for cellular entry (Lehmann et al., 2006 and Arnberg et al., 2000). There is no RGD motif in penton of BAdV-3. Instead it contains a MDV motif (Reddy et al., 1998), which may interact with α4β1 integrin and help in virus entry into the cells (Komoriya et al., 1991; Reddy et al., 1998). Like HAdV-5 (Greber et al., 1993; Wiethoff et al., 2005), after internalization of virus into the cell, BAdV-3 is presumed to stimulate the cell by acidification of the endosome to respond to the infection, which helps adenovirus disassembly. All the capsid proteins except pVII and few hexons are detached from the capsid and viral DNA alongwith core protein complex escapes the endosome by cleavage of pVI with viral proteases (Wiethoff et al., 2005). The hexons bind to microtubule motor protein dynein and enters the nucleus by minus-end directed movement (Bremner et al., 2009). Finally, the viral genome is injected into the nucleus via nuclear pore complex (NPC) for initiating the transcription and DNA replication.

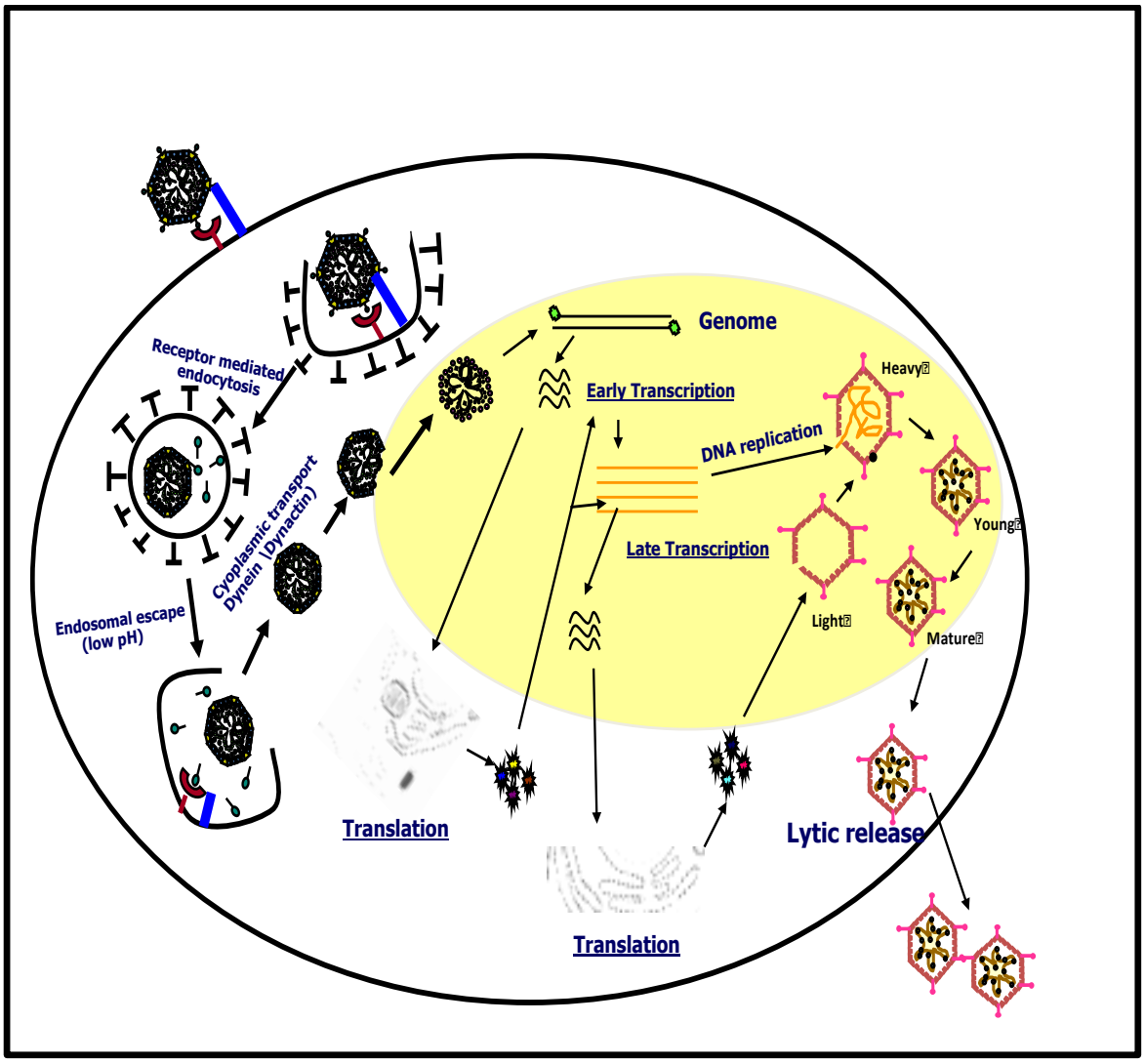

Fig. 1.3. Adenovirus Replication Cycle. The stages include adsoption and entry of adenovirus in host cells, early genes expression, DNA replication, late genes expression, virus assembly and release (Tikoo, SK) used with permission.

The BAdV-3 replication cycle of in MDBK cells appears to be slower and occurring later than HAdV (Zhou et al., 2001b). The early proteins such as E1A are detected after 12 hours post-infection (Reddy et al., 1999a). In HAdV, the early gene expression takes about 5 to 6 hours before the beginning of DNA replication and late gene expression (Berk, 2007).

Not much is known about the DNA replication in BAdV-3 infected cells. In general, three viral proteins encoded by the E2 region: Pol, pTP, and DBP are essential for adenoviral DNA replication. Adenovirus DNA replication initiation requires a protein complex composed of several viral and cellular proteins (Hoeben and Uil, 2013). The ITRs in the viral DNA contains site for the origin of replication. Pol and pTP interact together and form a heterodimer and bind to this origin of replication site (Temperley and Hay, 1992). Two transcription factors namely nuclear factor NFI and NFIII interact with Pol and pTP, respectively, and stabilises the protein-DNA interaction. This complex is recruited to their binding site on the viral DNA thereby initiating DNA replication (Chen et al., 1990; Mul et al., 1990; Mul and van der Vliet, 1992; Armentero et al., 1994; Coenjaerts et al., 1994). Adenoviral replication begins with the formation of the ester linkage between the deoxycytidine monophosphate (dCMP) α-phosphoryl group and the β-OH of serine residues in pTP (Lichy et al., 1981). After pTP-dCMP complex formation, 3'-OH group starts to serve to the new strand synthesis by DNA pol. Two E2-encoded proteins, DNA pol and DBP, and a cellular factor NFII are required for DNA elongation. DBP binds to single stranded DNA tightly and serves as DNA helicase to unwind the double stranded template in DNA replication (Lindenbaum et al., 1986). NFII

functions as a topoisomerase I, and is used in the regulation of DNA supercoiling and decreasing torsional stress created by by the replication forks (Schaack et al., 1990).

Much of the BAdV-3 assembly is derived from studies with HAdVs (Ahi and Mittal, 2016). Two viral structural proteins, IVa2 and pIX, are known as delayed-early or intermediate proteins since they are expressed at the same time as the viral DNA replication starts but their transcripts appear before than the other late proteins transcripts (Binger and Flint, 1984). The late genes code for structural proteins (hexon, penton, fiber, IIIa, V, VI, VII, VIII, IX and μ) that form the viral capsid and non-structural proteins (22K, 33K, 52K, 100K and the viral protease). The late genes expression is regulated by the major late promoter (MLP) using host machinery. Several transcripts of the late viral genes are created by the splicing of a single mRNA transcript produced by MLP transcription (Goldberg et al., 1978; Imperiale et al., 1995), which are then spliced and polyadenylated into mature mRNAs. These mRNAs primarily encode viral structural proteins and scaffolding proteins facilitating virion assembly (Iwamoto et al., 1986). Hexon trimers are formed in the cytoplasm shortly after translation, even though virion assembly takes place in the nucleus (Cepko and Sharp, 1983). After the nuclear translocation, the hexon trimers alongwith the penton base and other minor proteins start the formation of the capsid (Wodrich et al., 2003). After the formation of the empty capsid, the viral DNA genome is transported to the capsid to complete genome encapsidation (Edvardsson et al., 1976). Before virion assembly is complete, several structural proteins are cleaved by virus-coded protease to produce mature virus particles (Mangel et al., 2003). After 30 hrs post-infection, the infectious progeny viruses are released from the lysed cell.

BAdV-3 replication is obstructed in human cells (Wu and Tikoo, 2004). Several human cell lines are poorly transduced by BAdV-3, but the modification of the BAdV-3 fiber with the knob of HAdV-5 has shown to increase the transduction efficiency in human cells. BAdV-3 efficiently expresses early and late proteins without the production of infectious progeny virus, suggesting that there is a species-specific block of replication, probably at a late stage of viral infection. BAdV-3 is able to efficiently transduce 293 cells, but capsid assembly does not occur (Patel and Tikoo, 2006). Other human cells like HeLa, Hep-2, and A549 cells when transduced with BAdV-3 show the expression of late proteins but 293 cells do not express any late proteins although they express the E1 proteins of HAdV-5 suggesting that BAdV-3 DNA replication is inhibited in 293 cells. This can be counteracted by expression of the simian virus 40 (SV40) T antigen in 293T cells which allows the virus to replicate, but virus titres are significantly lower with much slower replication than the MDBK cells (Patel and Tikoo, 2006). As these recombinant BAdV-3 vectors have shown to increase the transduction and efficient replication in several human and bovine cells, they can be used as vaccine delivery vehicle in cattle. One way to further improve the efficacy of immune response may be to target recombinant BAdV-3 to immune cells such as dendritic cells, which are involved in the induction of primary T cell responses.

1.2 The Immune system

The immune system is a host defense system that comprises of complex network of cells, tissues and organs that function collectively to guard the body from harmful and toxic substances and organisms and protect against disease. The immune system can be categorized into two major responses, such as innate immunity and adaptive immunity

(humoral immunity and cell-mediated immunity) (Fig. 1.2). The innate and the adaptive immune system use many soluble molecules present in blood and other body fluids like enzymes, antibodies and short amino acid chains. Both arms of the immune system, the innate and the adaptive, use cellular and humoral defense mechanism. The mammalian immune systems of mice and humans are the most extensively studied immune systems.

Leukocytes also known as white blood cells are the cells of immune system that protect our tissues from infection and other forms of damage. The study of leukocytes found in humans and mice (CD2, 3, 4, 8) and its differentiation has shown that their structure and function is extremely conserved throughout the mammalian species (Davis and Hamilton, 1998). Leukocytes are classified as cells of the innate immune system (macrophages, neutrophils and dendritic cells) or of the adaptive immune system (for B cells and T cells).

1.2.1 Innate Immunity

All the organisms can protect themselves from infection by preventing the exposure from the harmful and infected materials (Medzhitov et al., 2012). Mucous and cilia on epithelia, the tight junctions between epithelial cells and the enzymes in tears and saliva, safeguard the organism from infection (Roth and Perino, 1998; Keele and Estes, 2011). For the virus to infect the cells, correct receptors are required. Many non-human animal or plant viruses are incapable of infecting humans as they do not posses the required receptors for virus entry (Mayer, 2011).

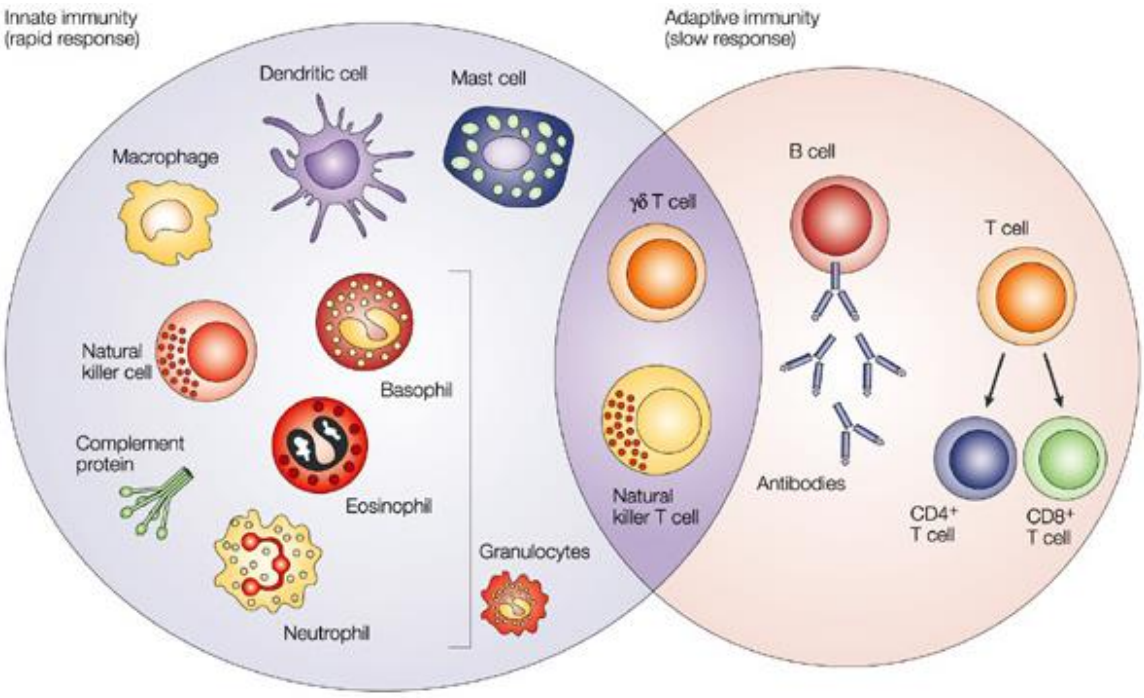

Fig 1.4 The cells of Immune system. Adapted from Nature reviews. Cancer, with permission to use

Once infected or colonized, the host may tolerate the foreign organism (Medzhitov et al., 2012). The first response to viral infection involves the innate immune system, which recognize and attack or kill foreign organisms. If the infection persists, the innate response primes the more powerful adaptive immune response (Iwasaki and Medzhitov, 2010; Shetnten and Medzhitov, 2011), which in turn utilises many of the tools available in the innate system.

1.2.2 Adaptive Immunity

For the development of efficient adaptive immune response the antigen-specific lymphocytes need to go through activation, expansion and differentiation. This course of development is based on the interaction of the T-cell with the antigen presenting cells (APC), where the processed antigen peptides in association with the APC's major histocompatibility complex (MHC) molecules are presented to the T-cell antigen receptor (TCR) (Janeway et al., 2001; Levings, 2012). The adaptive immune response is characterized by two main criteria's, firstly by the specificity of T and B lymphocyte receptors as a result of gene segment rearrangement and clonal selection; and secondly by the memory of the immune response (Bonilla and Oettgen, 2010). The surface epitopes on proteins are recognised B cells through the immunoglobulin B-cell receptor (BCR). The T cells is equipped with special receptors called TCR which recognize peptides that are broken down by other cells and displayed on that cell's surface in a association with an MHC molecule (Murphy et al., 2008). The adaptive response is generally composed of two arms, cell-mediated and humoral, enabled by T-helper (Th1 and Th2) responses, respectively, so the adaptive immune response generated by T cells in both the arms is

said to be MHC-restricted unlike innate immune responses that are non specific (Levings, 2012).

A fully functional and effective APC, satisfies the following criteria's successfully, that include: a) the ability to take up and cleave antigens b) generation of antigen epitopes c) formation of stable antigenic peptide complexes with MHC class I or class II molecules and d) constitutively express co-stimulatory molecules. APCs also secrete cytokines for T cells to differentiate into a specific subset, forming an environment favourable for T-cell differentiation (Janeway, 2001; Banchereau et al., 1998; Heath et al., 2004; Pardoll, 2002; Stoll et al., 2002). Although, any cell expressing MHC molecules is a potential APC, the APCs differ in their capacity to stimulate T cells. Dendritic cells (DCs) and macrophages are professional APCs that are well equipped than other types of immune cell to boost T-cell stimulation (Kim et al., 2004). However, for efficient proliferation, generation of effector cells, and producing memory cells by lymphocytes, a 'second signal' after antigen recognition by BCR or TCR is needed, like interaction with a co-receptor and cytokine stimulation (Levings, 2012). A third signal involving the innate response by toll like receptors (TLRs) is also proposed to be needed for efficient activation of naïve T cells and B cells (Levings, 2012; Ruprecht et al., 2006).

1.3 Bovine Immune System

The bovine immune system is made up of two arms: an innate (native) response, that occurs instantly, and the adaptive (acquired) response that is slower and develops within 10-14 days post exposure to the invading pathogen. The innate immune system comprises of a combination of natural defensive barriers - mainly mucosal surfaces like epithelial cells, connective tissues, skin, phagocytes and neutrophils, natural killer cells,

dendritic cells, cytokines, complement and anti-microbial peptides. The innate immune system is constantly active, responds rapidly to injuries or the manifestation of foreign pathogens non-specifically and does not improve with repeated exposures. The macrophages and dendritic cells contain unique receptors known as cellular pattern recognition receptors (PRRs), which recognize pathogen-associated molecular patterns (PAMPs). They get activated upon exposure to pathogen and secrete cytokines - interleukin-1 (IL-1) and interleukin IL-6, tumour necrosis factor alpha (TNF-α) and high-mobility group protein B1 (HMGB1).

The Toll-like receptors (TLRs) bind microbial associated molecular patterns (MAMPs) and trigger innate immune responses leading to secretion of cytokines such as IL-1β, IL-6, interferon gamma (IFN-γ) and TNF-α, and chemokines such as IL-8 (Akira and Takeda 2004). The adaptive immune systems include humoral and cell-mediated immune responses. Antibodies produced by B cells facilitate humoral immunity, and T cells facilitate cell-mediated immunity. However, unlike the innate system, the adaptive system is antigen specific and generates a long-term immunity against specific pathogens. In adaptive immunity, the cell-mediated immune responses involve T lymphocytes like CD4 Helper T cells which induce Th1 and Th2 immune responses, CD8 cytotoxic T cells and a unique subset of T cells known as gamma delta (γδ) T cells whereas humoral immune responses involve antibody producing B cells. The adaptive immune system can be enhanced with vaccination to become stronger, better and quicker in response to a specific pathogen.

1.3.1 Polymorphonuclear cells

Neutrophils, the most abundant type of phagocyte, normally represent 20 to 30% of the total circulating leukocytes in cattle, and are typically the first cells to arrive at the site of an infection (Grewal et al., 1980, Rouse et al., 1980 and Bertram, 1985). They are also known as granulocytes due to the presence of granules in their cytoplasm, or as polymorphonuclear cells (PMNs) due to their distinctive segmented nucleus. In addition to recruiting and stimulating other cells of the immune system, neutrophils wield their anti-microbial activity by three mechanisms that usually leads to the killing of the pathogen: phagocytosis (ingestion), release of soluble anti- microbial molecules (including granule protein- Azurophil) (Peyron et al., 2000), and generation of neutrophil extracellular traps (NETs). Like macrophages, neutrophils capture and kill pathogens by activating a respiratory burst. The bovine PMN respiratory burst includes the activation of the enzyme NADPH oxidase that produces large quantities of strong oxidizing agents including hydrogen peroxide, free oxygen radicals and hypochlorite which can kill the pathogens that are phagocytosed by the neutrophil (Paape et al., 2003).

Neutrophils are guided towards a site of infection or inflammation by chemotaxis. The neutrophils are able to identify biochemical molecules such as interferon gamma (IFN-gamma), interleukin-8 (IL-8), C5a, and Leukotriene B4 through their cell surface receptors (Kunkel et al., 1995), which are used to direct the path of their migration. Neutrophils have many different types of receptors, such as complement receptors, cytokine receptors for interleukins and interferon gamma (IFN-gamma) (Sengelov et al., 1995), receptors for chemokines, receptors to detect and adhere to endothelium, receptors for leptins and proteins, and Fc receptors for opsonin. Being extremely motile,

neutrophils quickly assemble at a site of infection, attracted by cytokines released by activated endothelium, mast cells, and macrophages. The cytokines released by neutrophils in turn augment inflammatory reactions by several other cells (Ear and McDonald, 2008). Earlier study demonstrated that bovine PMNs interact with T cells at sites of inflammation with secretion of pro-inflammatory cytokines, interferon (IFN)- γ and granulocyte macrophage colony stimulating factor (GM-CSF), and bacterial lipopolysaccharide (LPS) (Whale et al., 2005). Evidence for the formation and release of membrane proteins from peripheral blood mononuclear cells (PBMCs) and the attachment of these membrane proteins (MPs) to bovine PMNs has also been reported showing that MPs were one of the mechanisms by which bovine PMNs could passively acquire membrane lipids and integral membrane proteins (Whale et al., 2006). A significant increase in green fluorescent protein (GFP) transgene expression observed following PMN infection using the GFP expressing bovine adenovirus vector BAV304 (BAdV-3 expressing GFP cassette in E3 deleted region) shows the passive acquisition of a functional viral receptor protein from an adenovirus permissive cell line (Whale et al., 2006).

1.3.2 Natural Killer cells

Natural killer (NK) cells are among the first cells of the innate immune system to respond during infection or inflammation through secretion of toxic substances and immunoregulatory cytokines by these cells. In humans, NK cells are identified as $CD56^{+}/CD3^{-}$. In cattles NK cells can be characterized by their expression of NKp46 (CD335) and fights against viral infections and cancer. IL-15 is a crucial cytokine in NK cell proliferation, development and survival. $CD2^{+}$ subset circulating in peripheral blood

and $CD2^{-/low}$ subset circulating in lymph nodes are the two bovine NK cells subsets (Boysen et al. 2006). Both subpopulations are cytotoxic and produce IFN-γ upon infection with virus (Boysen et al. 2006). In cattle, CD16 has been reported to be expressed on natural killer (NK) cells (Storset et al., 2004) and sheep (Elhmouzi-Younes et al., 2010). In mice and humans, CD1d present Ag to NKT cells, a specialized population of T cells that possesses both properties of T cells and NK cells. It is a T cell lineage that is characterized by a unique TCR and NK cell markers and is capable of rapidly secreting large amounts of IFN-gamma and IL-4. In cattle, two CD1D pseudogenes are present but no intact CD1D genes are present which suggests the absence of NKT cells in cattle (Rhijn et al., 2006). By contrast, it has been reported that the bovine CD1D gene has an unusual gene structure and CD1d protein is expressed in bovine but cannot present α-galactosylceramide variants with shorter fatty acids (Nguyen et al., 2012).

1.3.3 Dendritic cells

Dendritic cells (DCs) are a heterogeneous population making up less than 1% of the leukocyte cell population in blood (Steinman and Cohn 1973; Palucka and Banchereau, 1999; Banchereau et al., 2000; Banchereau et al., 2003; Ju et al., 2010). The main role of DCs is to present antigen to T and B cells. They have the ability to prime naïve T cells by taking up and processing the antigen and presenting it to naïve T cells and initiate a primary T cell-mediated response and induce a Th1 or Th2 response. All the DCs subtypes have some dissimilarity in the morphology, phenotypes and functions between them. About 0.1–0.7% of bovine PBMCs have been recognised as DCs (Renjifo et al., 1997). Various DC subpopulations have been identified in human blood but their

functional characterization has been restricted due to their insufficient amounts in blood (Dzionek et al., 2000). Cattle have been used as an ideal model for the phenotype and functional studies as sufficient DCs can be isolated from their blood. Like human DCs (Dzionek et al., 2000; Strobl et al., 1998), bovine DCs do not contain the lineage markers for B cells (CD21), T cells (CD3), monocytes (CD14) or NK cells (CD335) but show the expression of a multiple adhesion molecules and co-express CD205 at either a high (CD205Hi) or low (CD205Lo) level (Patricia González-Cano et al., 2014).

DCs are primarily categorized into four major cell types, i.e., Conventional DCs (cDCs), Plasmacytoid DCs (pDCs), Langerhans cells and monocyte-derived DCs (Belz and Nutt, 2012). They are differentiated on the basis of their different origin, distribution in tissues and expression of surface receptors. The cDCs, also named as myeloid DCs, contain migratory cells and lymphoid-resident cells (Freer and Matteucci, 2009). Bovine monocyte derived DCs (MoDCs) display myeloid markers such as CD11a, CD11b, CD14, and CD172a (SIRPa) like human and mouse cDCs. Upon activation, the expression levels of CD40, CD80, and CD86 are up- regulated (Sei et al. 2014; Miyazawa et al. 2006). Peripheral blood DCs express MHC II, CD11c, and CD172a; DCs in the thymic medulla express CD1 and CD172a and DCs within the Peyer's patches express CD11b and CD172a (Brimczok et al. 2005). The cDCs have specialized mechanisms for Ag capturing and processing; they migrate through afferent lymph nodes to effector sites in lymphoid tissues and initiate immune response; and mature rapidly in response to a range of microbes (e.g., cytokines produced by innate immune cells) (Steinman and Hemmi, 2006; Levings, 2012). Upon activation, the cDCs markedly increase IFN-γ secretion by T-cells and NK cells by producing IL-12 and IL-15, and

promote differentiation of CD4+ and CD8+ T cells (Lambotin et al., 2010; Levings, 2012). In short, they act as a major link between innate and adaptive immune response. The cDCs have a very short half-life (approx. 3-5 days) and are constantly produced in bone marrow and situated in the skin, mucosal surfaces and the blood, so they tend to rapidly encounter pathogen and get activated by invading pathogens (Murphy et al., 2008). The cDCs can be infected by viruses themselves, can phagocytose infected cells, or can micropinocytose antigen. Migrating cDCs take up and process the antigen and deliver the antigen to DCs residing in the lymph node (Murphy et al., 2008; Singh and Cresswell, 2010). Unlike human and mouse pDCs, bovine pDCs require better characterization but some studies show that they express MHC II, CD4, CD172a, CD32, and CD45RB and are major source of type 1 interferons in response to viral infections (Reid et al. 2011). Bovine pDCs do not express either CD11c or CD205 but produce interferon-α in response to CpG ODN stimulation (Gibson et al., 2012; Patricia González-Cano et al., 2014).

1.3.4 Monocytes

Monocytes are the largest type of leukocytes that originate in the bone marrow and are released into the peripheral blood, where they circulate for one to three days and enter into the tissues where they mature and differentiate into tissue macrophage or dendritic cell populations acting as the first line of defense against pathogens (Auffray C et al., 2009; Hussen et al, 2013). These tissue resident macrophage and dendritic-cell perform three key functions in the immune system, which are phagocytosis, antigen presentation, and cytokine production. Macrophages express PRRs such as TLRs, which recognize microbial PAMPs (Rue-Albrecht et al. 2004). Monocytes are well equipped

with many receptors that identify several pathogens and mediate phagocytosis (Hussen et al, 2013). Large quantities of chemokines and cytokines like chemokine (C-X-C motif) ligand 8 (CXCL8), CXCL1, interleukin (IL)-1β, reactive oxygen species (ROS), and tumor necrosis factor (TNF)-α) that are involved in the defense against pathogens are generated by the activated monocytes (Auffray et al., 2007; Ziegler-Heitbrock, 2000; Hussen et al, 2013). The monocytes/macrophages in cattle express CD14 constitutively (Sopp et al., 1996; Berthon et al., 1996; Corripio et al., 2015). Monocytes are divided into three subsets on the basis of surface marker CD14 and CD16 expression namely a) classical monocytes cM (CD14++ CD16−), b) intermediate monocytes intM (CD14++ CD16+) and c) non-classical monocytes ncM (CD14+ CD16++) (Ziegler-Heitbrock et al., 2010; Hussen et al, 2013; Corripio et al., 2015). Some studies suggest that monocytes depart the bone marrow as classical cells, which after directly attacking the inflamed tissues may either differentiate into macrophages or dendritic cells, or else they may differentiate into (CD14++CD16+) intermediate monocytes in the circulation (Ziegler-Heitbrock et al., 2010; Heine et al., 2012; Hussen et al, 2013). A recent report has suggested that, a bovine non-classical CD14+CD16++ population exists in peripheral blood monocytes and is non-inflammatory and compared to cM and intM showed a considerably low phagocytic capacity, a considerably reduced production of reactive oxygen species, and reduced mRNA expression of CXCL8 and CXCL1 stimulation (Hussen et al, 2013; Corripio et al., 2015).

1.3.5 T cells

T cells are a type of lymphocyte, which are produced in bone marrow and play an essential role in cell-mediated immunity. The presence of a T-cell receptor (TCR) on

their cell surface makes them distinguishable from other lymphocytes, like B cells and natural killer cells (NK cells). T cells are named so as they mature in the thymus. T lymphocytes are primarily divided into four types; T helper cells (TH cells), T effector cells, cytotoxic T cells (Tc cells or CTLs) and memory T cells. Once activated by the suitable antigen, helper T cells secrete chemical signals called cytokines, which stimulate the differentiation these T cells into B cells that further differentiate into plasma cells (antibody-producing cells). Tregs also known as regulatory T cells help in the regulation of immune responses. Cytotoxic T cells, which are activated by various cytokines, bind to and kill infected cells and cancer cells. Similar to humans and mice (Magombedze et al., 2014), cattle also induce both Th1/Th2 responses to antigens. During the initial stage, the infected animal evokes a strong cell-mediated CD4 T cell response with the production of interferon gamma (IFN-γ) (Th1 response). Interleukin-4 (IL-4) and IL-10 secreted by CD4+ T cells causes the loss of Th1 response and gets replaced with a Th2 response (antibody production) over a period of time.

IL-17 is a pro-inflammatory cytokine, which plays a protective role against infection and promotes inflammation in autoimmunity. Two discrete populations of T cells in cattle (CD4+ and WC1+γδT-cell) secrete IL-17 efficiently under suitable cytokine stimulation (TGF-β1, IL-6 and/or IL-1β). Th17 cells are also known to be negatively regulated by IFN-γ and do not produce IL-17 and IFN-γ simultaneously (Peckham et al. 2014).

Another small yet specialized subset of T cells called the Gamma delta T cells (γδ T cells) is present in cattle, which possess a distinct T cell receptor (TCR) on their surface and can effectively present antigens to other T cells (Vantourout et al., 2013).

Cattle have high concentrations of γδ TCR+ T cells in blood (15-60%) and they are considered to be the chief regulatory T cell in bovine (Hoek et al. 2009).

The TCRs are heterodimers that comprise either an alpha/ beta polypeptide chain, and appear in about 95% of the TCR population, or a gamma/delta polypeptide chain (Pitcher and van Oers 2003; Malissen, 2008). The cytoplasmic tail of the TCR lacks the ability to signal, requiring intracellular signalling to be initiated by the CD3 protein complex. The CD3 protein complex is an important T cell marker that is localized in the cytoplasm. As the T cell maturation occurs, the cytoplasmic CD3 expression on T cells is lost and the CD3 antigen is expressed on the cell surface. The specificity of the CD3 antigen is the defining feature for T cells and its presence at all phases of T cell development makes it an ideal and effective T cell marker for the detection of normal T cells and T cell neoplasms. It is also used as an immunohistochemical marker for T cells in tissue sections (Salvadori et al. 1994, Vernau and Moore 1999).

1.3.6 B cells

B-lymphocytes are a type of white blood cell that makes antibodies each of which recognize a unique antigen, and may have the capacity to neutralize specific pathogens. They develop from stem cells in the bone marrow. They are classified into three types (Murphy and Kenneth 2012); a) Plasma B cells - which are large B cells that have been exposed to the antigen and produce\ secrete large amounts of antibodies by binding to them and making them easier targets for phagocytes and activation of the complement system, b) Memory B cells - which are formed after B cell activation and are specific to the antigen it encounters during the primary immune response and c) Regulatory B cells – which are involved in immune regulation via various mechanism including secretion of

IL-10 and TGF-β. Each B cell produces a specific antibody, each with a unique antigen-binding site. The bound antigen molecules are engulfed into the B cell by B-cell receptors (BCR). When a naïve or memory B cell is stimulated by antigen (with the help of a helper T cell), it proliferates and differentiates into an effector cell that secrets antibody (Alberts, 2002). Such cells have the unique antigen-binding site similar to the cell-surface antibody that functioned initially as the antigen receptor. The effector B cells begin to secrete antibody at a very early stage when they are small lymphocytes, but after maturation they become a large plasma cell, which secretes antibodies continuously at the astounding rate of about 2000 molecules per second (Alberts, 2002). Although many plasma cells die after several days, some plasma cells survive in the bone marrow for months or years where they continue producing antibodies into the blood. In cattle, expression of the CD21 molecule is restricted to B-lymphocytes (Naessens et al. 1990). In B-cell development, CD21 emerges after the pre-B-stage, is sustained during peripheral B-cell development and is lost when differentiated terminally into plasma cells. CD21 interacts with CD23 and associates with CD19, CD81 and Leu13 activate B cell by forming a large signal-transduction complex (Cherukuri et al., 2001).

1.3.7 Mucosal Immune System

The immune system can be divided into different functional anatomical compartments. The two most important compartments of adaptive immune response are the peripheral lymphoid system and the mucosal lymphoid system (Janeway et al., 2001). The mucosal immune system is an adaptive immune system that is located near the surfaces from where most pathogens can gain entry. Organized lymphoid tissues in this system are referred to as mucosal associated lymphoid tissues (MALT) (Fig. 1.3). The

mucosal surfaces of the body act as thin and permeable barriers to the internal body as per their physiological roles in food absorption (the gut), sensory activities (eyes, nose, mouth, and throat), gas exchange (the lungs) and reproduction (uterus and vagina). This makes these surfaces more vulnerable to infections as well (Janeway et al., 2001).

Although gut functions as a portal of entry to a wide range of foreign antigens in the form of food, these antigens do not necessarily induce an adaptive immune response. Soluble antigens administered by mouth may elicit antigen-specific tolerance or antigen-specific suppression. However, pathogenic microorganisms stimulate strong protective Th1 responses (Janeway et al., 2001). In cattle, intranasal immunization of calves with a replication-competent BAdV-3 expressing full-length and truncated forms of BHV-1 gD has shown to induces both gD-specific mucosal and systemic immune responses (Zakhartchouk et al., 1999). Also, different routes of mucosal and systemic immunization with recombinant adenovirus stimulate different levels of antigen-specific IgA antibodies in mice (Papp et al., 1998).

The mucosal associated lymphoid tissues differ from the peripheral lymphoid system in the types and distribution of T cells, with significantly greater numbers of γδ T cells in the gut mucosa as compared to peripheral lymph nodes and blood. The dominant antibody type in mucosal immunity secreted by the epithelial cells lining mucosal surfaces is secretory polymeric IgA that is found as a monomer in blood as opposed to a dimer in mucosal secretions. Adenoviruses have been reported as excellent vaccine vectors for the inducing mucosal immunity (Imler, 1995; Rosenthal et al., 1996). Intranasal immunization of calves with BAdV-3 expressing gD, induced a gD-specific IgA response in the nasal secretions and the presence of gD- specific IgA-secreting cells

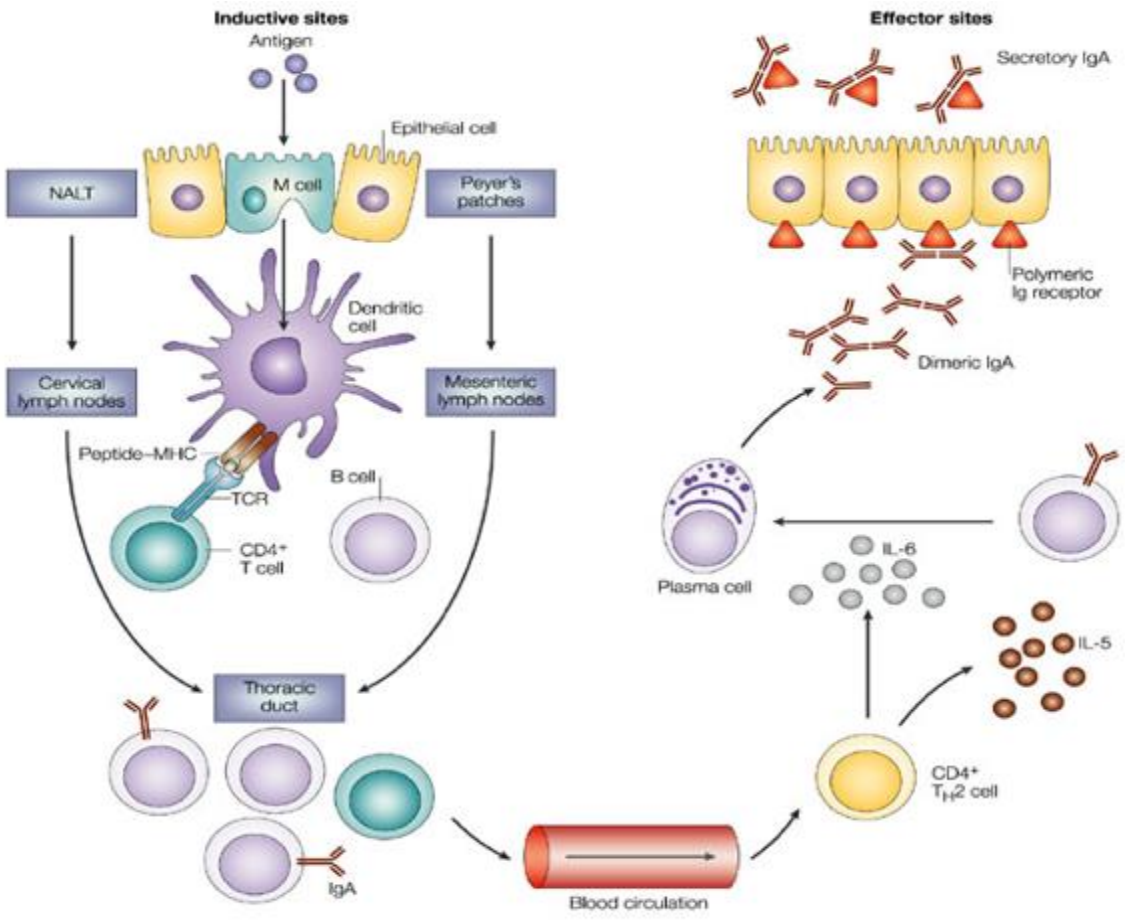

Fig. 1.5 Mucosal Immune System. Adapted from Nature Reviews Immunology (Hiroshi, 2004), used with permission

in the nasopharyngeal tonsil but not in the lymph node or PBMC suggesting that antibody was locally produced in the nasal passage (Zakhartchouk et al., 1999).

Thus, mucosal vaccines activate immune cells mainly dendritic cells, macrophages and T cells in mucosal inductive sites and consequently induce immunity in both the mucosal and systemic compartments (Boyaka et al., 1999). Intranasal immunization of mice with adenovirus serotype 5 vector expressing FL (growth factor that binds to flt-3 kinase receptor), were found to elicit Th1- and Th2-type responses, thereby providing both Ag-specific S-IgA Ab and cell-mediated immune responses (Sekine et al., 2008).

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1.3.8 Systemic Immune System

The primary lymphoid organs generate lymphocytes from immature progenitor cells. These lymphocytes are generated in thymus and the bone marrow that constitute the primary lymphoid organs (Boundless, 2016). The bone marrow is the site for the formation of T cells and for the production and maturation of B cells. In cattle the Ileal Peyer's patches are the primary site for naïve B cell production (Yasuda et al., 2006). From the bone marrow, B cells immediately migrate to the circulatory system and travel to secondary lymphoid organs in search of pathogens. Secondary or peripheral lymphoid organs, which include lymph nodes and the spleen, maintain mature lymphocytes and initiate an adaptive immune response. These peripheral lymphoid organs serve as the sites of lymphocyte activation by antigens (Janeway et al., 2001). The two major types of peripheral lymphoid tissue are the spleen, which collects antigens from the blood and the lymph nodes, which collect antigen from sites of infection in the tissues and from the epithelial surfaces of the body. Spleen is a lymphoid tissue that is known to produce immune response against blood-borne antigens and is involved in removal of aged red blood cells (Bronte et al., 2013). It synthesizes antibodies and, removes antibody-bound bacteria and antibody-bound blood cells by way of blood and lymph node circulation. One of the studies suggested that the mouse spleen contains half of the body's monocytes that upon recruiting to injured tissue (such as the heart) differentiate into dendritic cells and macrophages and help in tissue healing (Swirski et al, 2009). Other lymphoid tissue

that is associated with immune functions in protecting the body against infections is connective tissue formed of reticular fibers, with various types of leukocytes, mostly lymphocytes enmeshed in it. Adaptive immune responses are originated in these peripheral lymphoid tissues. The T cells that are presented with the antigen proliferate and differentiate into antigen-specific effector cells, whereas B cells upon maturation proliferate and differentiate into antibody-secreting cells (Janeway et al., 2001). In cattle, replication defective and replication competent BAdV-3 virus expressing gD of BHV-1 induced gD-specific IgG and IgA antibodies in the serum and the nasal secretions respectively, showing a significant induction of systemic immune reponse (Reddy et al., 2000).

1.3.9 BAdV-3 as vaccine delivery vehicle

Adenoviruses are the intensively studied as vectors for gene transfer into mammalian cells, vaccine delivery, oncolysis and other therapeutic uses (San Martin, 2012) due to several reasons such as their ability to transduce and infect both dividing and non-dividing cells, they can package large foreign genes, can easily be produced as high titer recombinants in cell culture (Russell, 2000), elicit strong antigen specific T cell responses and are avirulent (Yang, 2003; Millar, 2007). Many studies are also continuing to improve their efficiency as vectors for *in vivo* gene transfer studies as well (Crystal, 1995). The use of recombinant HAdVs to deliver vaccine antigens to domestic animals and birds has been shown in many studies but its use may be limited due to regulatory concerns regarding safety in domestic animals (Ayalew et al., 2015). Moreover, species specificity limiting host range, restricted replication in non-host species and stability of non-human adenoviruses has led to the evaluation of animal specific adenoviruses

(Hammond et al., 2001; Tordo et al., 2008; Zakhartchouk et al., 1999) as vaccine delivery vectors. Since BAdV-3 is a naturally avirulent virus with restricted host-range, the capacity to grow to high titers in cell cultures and can be delivered intranasally, BAdV-3 is being evaluated as vaccine delivery vector in animals including cattle (Baxi et al., 2000; Brownlie et al., 2014; Kumar et al., 2014; Reddy et al., 2000; Zakhartchouk et al., 1999).

2. HYPOTHESIS AND OBJECTIVES

Although human adenovirus can interact with leukocytes in the bloodstream, the study of freshly isolated leukocytes show they do not express conventional adenovirus receptors (Mentel. et al., 1997) and are not permissive for adenovirus infection during immediate *ex-vivo* culture (Mentel et al., 1997; Huang et al., 1995). Nevertheless, an interaction between adenovirus and freshly isolated human peripheral blood mononuclear cells (PBMC) maintained in fetal calf serum (FCS; 10%) has led to the production of cytokines (Higginbotham et al., 2002).

A number of characteristics including lack of virulence and the ability to grow to high titers, have made BAdV-3 a vector of choice for further development as a vaccine delivery vehicle for cattle. In fact, intranasal immunization of calves containing BAdV-3 specific antibodies induces effective protective immune responses against virus challenge (Zakhartchouk et al., 1999). One way to further improve the efficacy of immune response may be to target recombinant BAdV-3 to immune cells. However little is known about the interaction of BAdV-3 with different types of immune cells. Determining the tropism of BAdV-3 for specific leukocyte subpopulations may help devise better strategies to

target BAdV-3 to appropriate leukocyte population for the induction of robust and efficient immune responses in calves immunized with recombinant BAdV-3 expressing vaccine antigens.

Based on these observations, I hypothesized that recombinant BAdV-3 vector expressing green florescent protein(GFP) (GFP expression cassette inserted in E3 deleted region of BAdV-3 (Du and Tikoo, 2010) can efficiently infect and transduce specific population of leukocyte such as dendritic cells (DCs), which are required for the induction of an efficient protective immune response in vaccinated animals.

The specific aim of this work was to investigate and quantify the interaction of recombinant BAV304a (GFP expression cassette inserted in E3 deleted region of BAdV-3 (Du and Tikoo, 2010) with different leukocyte subpopulations in the blood.

3. INTRODUCTION

Adenoviruses are double-stranded DNA viruses, which offer several advantages as vaccine delivery vectors by meeting the most important criteria of an ideal vaccine vector in terms of efficacy, safety, and stability (Tatsis et al., 2004). Because of the ability of these vectors to enter many different target cells and to efficiently express the transgene, recombinant adenoviral vectors are widely used for in-vivo gene transfer (Crystal, 1995). Although adenoviruses of have been used for delivering vaccine antigens to cattle (Tatsis et al., 2004), we have focused on developing species specific adenoviruses as vaccine delivery vehicles (Ayalew et al., 2015). Earlier, molecular characterization of bovine adenovirus (BAdV) -3 (Lehmkuhl et al., 1975) has led to the development of BAdV-3 as vaccine vector for immunization of calves. Although intranasal immunization of calves with recombinant BAdV-3 expressing bovine herpesvirus gDt induces protection against

BHV-1 challenge, the parental immunization has not induced efficient protective immune responses in calves (Zakhartchouk et al., 1998). The efficient induction of protective immune response in intranasal immunized calves could be in part due to efficient mode of cell entry in respiratory mucosa.

Despite importance of blood cells including lymphocytes, macrophages, B-lymphocytes and dendritic cells (DC) in the induction of protective immune responses, little is known about interaction of BAdV-3 with bovine blood cells. In particular, DCs are the most potent stimulators of antigen-specific T-lymphocyte responses and play an important role in inducing protective immunity against viral infection (Kim et al., 2004). Since DCs can serve as potent antigen-presenting cells (APC), enhancing the interaction of bovine DCs (myeloid DCs) with BAdV-3 vector may not only help in inducing robust protective immune responses, but may also help to produce cost effective BAdV-3 vector based vaccine.

In this report, we analyzed the interaction of BAdV-3 with different bovine leukocyte subpopulations. Our results suggest that BAdV-3 efficiently transduces monocytes, and neutrophils without any detectable viral replication.

3.1 Materials and Methods

3.1.1 Cell lines and virus

Madin-Darby bovine kidney (MDBK) cells were grown in the minimum essential medium (MEM) (Sigma Aldrich) with 10 % fetal bovine serum (FBS) (Sera care life sciences), 10 mM HEPES buffer (Life Technologies), 0.1 mM non-essential amino acids (NEAA) (Life Technologies), and 50µg/ml gentamicin (HyClone® Laboratories, Inc.).

Recombinant BAV304a virus (BAdV-3expressing green fluorescent protein inserted in E3 deleted region) (Du and Tikoo, 2010) was propagated in MDBK cells in MEM supplemented with 2% FBS, purified by cesium chloride density-gradient centrifugation (Tollefson et al., 2007). The virus titer was determined by $TCID_{50}$ assay (Kulshreshtha et al., 2004).

3.1.2 Culture media and reagents

All peripheral bovine blood cell cultures were maintained in AIM-V Serum Free Lymphocyte Medium (Gibco BRL, Burlington. ON) containing 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco BRL) and unless otherwise indicated, cultures were incubated at 37^0C in a humidified atmosphere with 5% CO_2.

3.1.3 Monoclonal antibodies

Primary monoclonal antibodies (mAbs) specific for bovine leukocyte antigen and fluorochrome-conjugated secondary antibodies are listed in Table 2.

3.1.4 Purification of BAV304a

The recombinant BAV304a was purified as described earlier (Tollefson et al., 2007).

3.1.5 BAdV-3 specific antibodies

Production and characterization of BAdV-3 E1B small 19K (Zhou et al., 2001b), DNA binding protein (Zhou et al., 2001a), 52K protein (Paterson et al., 2010) and 100K protein (Makadiya et al., 2013) specific sera has been described. Anti-hexon sera recognizes a protein of 110 kDa in BAdV-3 infected cells (Ayalew et al., 2014).

3.1.6 Purification of bovine blood cells

All animal experimental procedures were evaluated and approved by University of Saskatchewan – University Committee on Animal Care and Supply. All procedures were executed following guidelines approved and permitted by the Canadian Council on Animal Care.

3.1.6.1. Peripheral blood mononuclear cells (PBMCs)

Venous blood was collected from castrated male Holstein calves between 9 and 12 months of age, housed at the VIDO-InterVac facility (Saskatoon, SK, Canada), using 7.5% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich). The Percoll density gradient centrifugation procedure (Arsenault et al., 2009) was used for isolation of bovine PBMCs. To separate buffy coat layers, the whole blood was centrifuged at 1400 g for 20 min without brake. The buffy coat of mononuclear cell population was layered over a 60% Percoll (GE Healthcare Life Science, Quebec, CA) and centrifuged at 2000 g for 15 min without brake. The cells were aspirated and washed twice with autoclaved phosphate buffer saline (PBS; pH 7.4) solution. Total number of cells was counted using a haemocytometer and viability of the cells was determined by staining with trypan blue.

3.1.6.2. PMNs

The PMNs were purified as described earlier (Whale et al., 2006). Briefly, the venous blood was collected from castrated male Holstein calves between 9 and 12 months of age using 7.5% ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich). Whole blood was centrifuged at 1400 g, without brake for 20 min to separate the buffy coat layer, which was removed at the interface between the red blood cell pellet and the plasma. After removal of plasma and buffy coat, the top half of the remaining red blood

Table 2. Monoclonal antibodies and fluorochrome conjugated secondary antibodies used to detect bovine leukocyte antigens

Bovine leukocyte antigen[1]	Isotype	Clone	Supplier
CD3	IgG1	MM1A	WSU[a]
CD11c	IgM	BAQ153A	WSU
CD14	IgG1	MM61a	WSU
CD21	IgG1	MCA1424G	VMRD[b]
CD209	IgG2b	DCN46	WSU
CD335	IgG1	AKS1	AbD serotec[c]
Secondary ab	IgG2b/PE		Invitrogen[d]
Secondary ab	IgG1/APC		Invitrogen
Secondary ab	IgM/APC		Invitrogen

a Washington State University (Pullman, WA).
b Veterinary Medical Research & Development (Pullman, WA).
c AbD Serotec (Raleigh, NC).
d Invitrogen (Burlington, ON).

cell/PMN (RBC/PMN) fraction was also discarded. The RBCs in the remaining red blood cell/PMN (RBC/PMN) fraction were lysed by adding 5-10mL of the RBC/PMN fraction to 40mL of a lysis solution (distilled water with 0.17M NH4Cl, 1mM KHCO3, and 0.11mM EDTA; pH 7.3) followed by three washes with PBSA.

3.1.6.3. Bovine monocytes

For sorting monocytes ($CD14^{+}CD11c^{+}$) cells, 3 x 10^8 bovine PBMC, at a final concentration of 1 x 10^8 cells/mL PBS, were labeled with anti-bovine CD11c and anti-bovine CD14 antibody (5ul/ml) for 20 min at 4 °C, with gentle mixing every 10 min. The cells were then pelleted by centrifuging at 311 g for 8 min at 4 °C and washed twice with ice-cold PBS. The cell pellet was re-suspended in 3 mL PBS and incubated with goat anti-mouse IgG1-PE (5ul/ml) and goat anti- mouse IgM-APC (4ul/ml) in the dark at 4 °C. After 20 min of incubation, the labeled cells were washed twice with ice-cold PBS. Labeled PBMCs were adjusted to a final concentration of 1 x 10^8 cells/mL in ice-cold PBS, and to remove cell clumps, the purified PBMCs were filtered through 35 um cell strainer capped 12 x 75 mm polystyrene round bottom tubes (BD Falcon ON, CA). The labeled PBMCs were subjected to high-speed cell sorting with a MoFlo XDP (Beckman Coulter Inc. CA, USA). PE and APC fluorescence was gathered through 575/25 and 670/30 band-pass filters, respectively. The first sort region (R1) was defined by dot scatter plots exhibiting forward light scatter (FSC) and side scatter (SSC) and dead cells and debris were excluded from it. The second sort region (R2) was defined by dot plots exhibiting FSC-Height and FSC-Width to exclude doublets or bigger cell clumps.

To sort monocytes, R3 and R4 regions were established on the basis of co-expression of CD14 and CD11c. The conditions for the sort were: 60psi sheath pressure,

70 um nozzle, differential pressure of 0.3psi, and a sort rate of 18–22,000 events/s. Sterile 12 x 75 mm polypropylene round bottom tubes (VWR international, Mississauga, ON, CA) were used to collect the sorted cells which were kept on ice throughout the sort and cells were suspended in ice-cold AIM-V medium (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON) after every 30 minutes.

3.1.6.4. Dendritic cells

For dendritic cell sorting, $1x10^8$ PBMCs were labeled with anti-bovine CD14 and anti-bovine CD209 mAb (4ul/ml) (Davis et al., 2015) for 20 min at 4 °C with gentle mixing very 10 min. The cells were then pelleted by centrifuging at 311 g for 8 min at 4 °C and washed twice with ice-cold PBS. The cell pellet was re-suspended in 3 mL PBS and incubated with goat anti-mouse IgG1-APC (5ul/ml) and goat anti- mouse IgG2a-PE (3ul/ml) antibody in the dark at 4 °C. After 20 min of incubation, the labelled cells were washed twice with ice-cold PBS. Labeled PBMCs were adjusted to a final concentration of 1×10^8 cells/mL in ice-cold PBS. To remove cell clumps, the purified and labeled PBMCs were filtered through 35 um cell strainer capped 12 x 75 mm polystyrene round bottom tubes (BD Falcon ON, CA). The labeled PBMCs were subjected to high-speed cell sorting with a MoFlo XDP (Beckman Coulter Inc. CA, USA). PE and APC fluorescence was collected through 575/25 and 670/30 band-pass filters, respectively. The first sort region (R1) was defined by dot scatter plots exhibiting forward light scatter (FSC) and side scatter (SSC) and dead cells and debris were excluded from it. The second sort region (R2) was defined by dot plots exhibiting FSC-Height and FSC-Width to exclude doublets or bigger cell clumps.

To sort DCs, a sort region was set to include CD209+ (R3) and exclude CD14- cells (R4). Events in R4 were viewed with FSC and FL1 to visualize CD209+ populations. R1 and R2 regions were also used when sorting monocytes based on co-expression of CD14 and CD209. The conditions for the sort were: 60psi sheath pressure, 70 um nozzle, differential pressure of 0.3psi, and a sort rate of 18–22,000 events/s. Sterile 12 x 75 mm polypropylene round bottom tubes (VWR international, Mississauga, ON, CA) were used to collect the sorted cells which were kept on ice throughout the sort and cells were suspended in ice-cold AIM-V medium (Invitrogen, Burlington, ON) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Burlington, ON) after every 30 minutes.

3.1.7 *In-Vitro* transduction of bovine peripheral blood mononuclear cells (PBMCs)

About $0.5x10^6$ cells (PBMCs) / well were transduced with BAV304a at a multiplicity of infection (MOI) of 1, 2, 5 or 10. After incubation for 18 hrs at 37 °C, the infected PBMCs were harvested using 0.5% trypsin, resuspended in phosphate buffered saline (PBS; pH 7.4) and fixed with 2% paraformaldehyde. Finally, the fixed cells were stored at 4°C in the dark until analyzed for GFP expression with a FacsCalibur (Becton–Dickinson, Franklin Lakes, NJ) using Cell Quest acquisition and analysis software (version 3.3). Each sample was analyzed by capturing a minimum of 10,000 events during analysis.

3.1.8 PBMC subpopulations transduced by BAV304a

To identify specific PBMC subpopulation transduced by BAV304a, 20×10^6 bovine PBMC were transduced with BAV304a at MOI 2. About 1×10^6 transduced PBMCs were plated in separate wells of a 96-well plate followed by labeling with anti-

bovine CD3 (T cell marker), anti-bovine CD14 and anti-bovine CD11c (monocyte marker), anti-bovine CD21 (B cell marker), anti-bovine CD335 (NK cell marker) antibody individually to each well. Labeling with fluorochrome-conjugated secondary antibodies was performed by adding goat anti- mouse IgG1-PE and goat anti-mouse IgM-APC.

3.1.9 *In-vitro* transduction of monocytes and DCs

Bovine PBMCs were isolated from bovine blood of three animals using Percoll density gradient centrifugation. CD11c+CD14+ subpopulation (monocytes) and CD209+CD14- subpopulation (myeloid DCs) were purified by fluorescence activated cell sorting (FACS) (Gonzales-Cano et al., 2014). About 0.5 x 10^6 cells/well (monocytes) and 50,000 cells/well (mDCs) were transduced with BAV304a at MOI of 2. After 18 hours post transduction, the transduced monocytes and DCs were harvested using 0.5% trypsin and analyzed by flow cytometry for GFP expression.

3.1.10 *In-vitro* transduction of PMNs

About 0.5 x 10^6 cells/well (PMNs) were transduced with BAV304a at MOI of 2. After 18 hrs post transduction, the transduced PMNs were harvested using 0.5% trypsin and analyzed by flow cytometry for GFP expression.

3.1.11 Western blot analysis

Western blot analysis was performed as described before (Kulshreshtha et al., 2004). Briefly, sorted monocytes and PMNs were seeded in 6-well plates, and were either mock-infected or infected with BAV304a. After 48 h post infection, the cells were collected, washed twice in 1X PBS; pH7.4 and centrifuged at 1500 rpm before lysing the cell pellets with radio-immunoprecipitation assay (RIPA) buffer (distilled water with 50

mM Tris pH 8, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% Triton X-100) containing protease inhibitors (Roche). Finally, the lysed cell samples were added to the sample loading buffer (1 M Tris-HCl, pH 6.8, 0.8% SDS. 0.4% glycerol, 0.15% β-mercaptoethanol, 0.004% bromophenol blue), heated at 95°C for 5 min, separated by 12% SDS polyacrylamide gel electrophoresis (PAGE), and then blotted onto nitrocellulose membrane (Bio-Rad). The membrane was probed with BAdV-3 protein-specific rabbit polyclonal antisera followed by alkaline phosphatase-conjugated secondary antibodies. The BCIP®/NBT solution (Sigma) were used as a substrate to visualize target protein bands.

3.1.12 Virus titer assay

The titer of the BAV304a virus collected from transduced monocytes or PMNs cells was determined using the 50% tissue infectious dose ($TCID_{50}$) assay (Kulshreshtha et al., 2004). The purified monocytes and PMNs cells were transduced with BAV304a at MOI 2. After 48 h p.i, the transduced cells were harvested, subjected to 5 rounds of freeze-thaw cycles and supernatant containing virus was separated by centrifuging the lysates at 2500 rpm at 4^{o} C for 5 min. Five-fold serial dilutions of the virus was prepared in MEM medium supplemented with 2% FBS, which was used to infect the MDBK cells cultured in 96 well plates (100 µl\well) in triplicates. After adding medium (100 µl\well), the cells were incubated at 37 °C, and observed daily for cytopathic effects (CPE). The titer of the virus was determined by counting the number of fluorescent foci using a fluorescence microscope, and expressed as $TCID_{50}$\ml (Kulshreshtha et al., 2004).

3.2 Data Analysis

All of the results presented are non-parametric in their distribution. All data were analysed with the aid of the GraphPad Prism program (GraphPad software Inc. California USA).

3.3 Results

3.3.1 Purification of BAdV304a

The caesium chloride (CsCl) density gradient combined with ultracentrifugation procedure was used for BAV304a (Fig. 3.1A) purification. MDBK cells were grown in 25 150 cm^2 flask, infected with BAV304a at an MOI of 5 and incubated at 37 °C (Fig. 3.1B). After 2 days post infection, the cell pellets from 25 of 150 cm^2 flasks were combined in a small volume (10 mls) of medium, and subjected to five cycles of freezing and thawing, before centrifuging the suspension at 1500 rpm for 10 min at 4 °C to remove the cell debris. The supernatant was subjected to CsCl density gradient centrifugation at 35000 rpm for 1hr at 4°C. Two bands were visible after centrifugation, upper band containing empty capsids, and lower band containing mature virions (Fig. 3.1C). The band containing mature virions was collected and used for a second round of CsCl density gradient centrifugation at 35000 rpm for 16 hrs at 4°C (Fig.3.1C). The virus was collected, dialyzed using Slide-A-Lyzer Dialysis Cassette (Thermo Scientific) against three changes of dialysis buffer to remove traces of cesium chloride and stored in small aliquots at - 80°C. $TCID_{50}$ assay was performed with 10 fold serial dilution of the purified virus following infection in MDBK cells. A very high titre of 10^{10} $TCID_{50}$ \ mL was observed in the CsCl purified BAV304a virus.

3.3.2 Bovine peripheral blood mononuclear cells

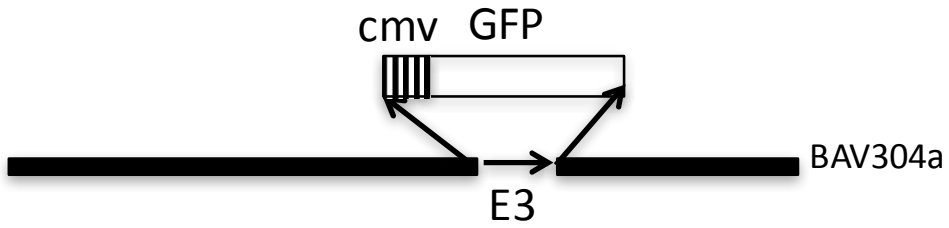
The bovine PBMCs were purified by Percoll density gradient centrifugation procedure as described earlier (Arsenault et al., 2009). About 400 x 10^6 PBMC were obtained from 200 ml of EDTA treated bovine blood. The cells prepared by this method were consistently >98% viable and contained less than 1% red blood cells.

To determine the concentration of BAV304a (BAdV-3 containing GFP inserted in E3 deleted region) required for optimal transduction of PBMCs, purified bovine PBMCs from four different animals were either mock-infected or infected with BAV304a at a MOI of 1, 2, 5 or 10. After incubation for 18 hrs at 370C, the infected cells were trypsinized, fixed in 2% paraformaldehyde in PBS and analyzed by flow cytometry.

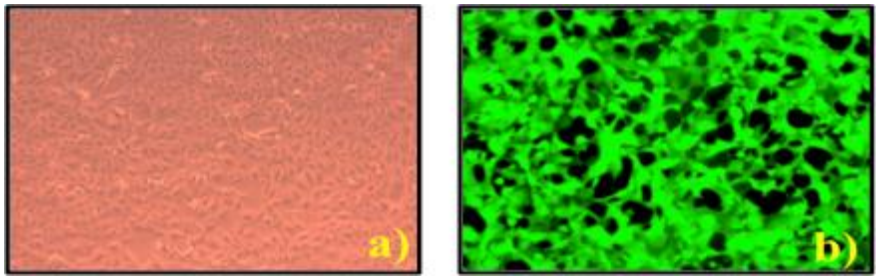
The percentages of transduced cells based on GFP expression and Mean fluorescence intensity (MFI) were analyzed by flow cytometer. As seen in Fig. 3.2A, flow cytometric analysis detected about 10-20% cells expressing GFP irrespective of MOI used. Animal #34 showed about 20% PBMC transduction with BAV304a at MOI 1 and 2. Moreover, MOI 2 showed a better transduction efficiency than MOI 1 and also there was not much decrease in the percentage of GFP expression at MOI 2 as compared to the higher MOIs suggesting that MOI 2 is optimal for future experiments.

The mean fluorescence intensity data (intensity of GFP expression displayed by every transduced cell) also shows a high amount of transduction in PBMC at all MOIs (Fig 3.2B), with MOI 10 being the highest for all animals, which reflects more virus particles in each cell (forced transduction). The MFI of transduced PBMCs at MOI 2 was better than MFI at MOI 1 and equivalent to MFI at MOI 5 (Fig 3.2B), suggesting MOI 2 as an optimum working MOI for future experiments.

(A)



(B)



(C)

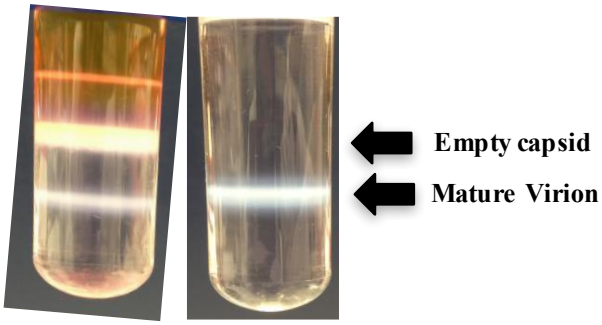


Fig. 3.1. Purification of BAV304a. (A) Schematic diagram of BAV304a genome. Human cytomegalovirus immediate early gene promoter (cmv)' green fluorescent protein gene (GFP), Early (E) -3. **(B).** Direct fluorescent image of mock infected (a) or BAV304a infected (b) MDBK cells at 18 hrs post infection. **(C)** CsCl density gradient purification. The lysates of BAV304a infected cells were separated by CsCl gradient centrifugation and centrifuge tubes are photographed

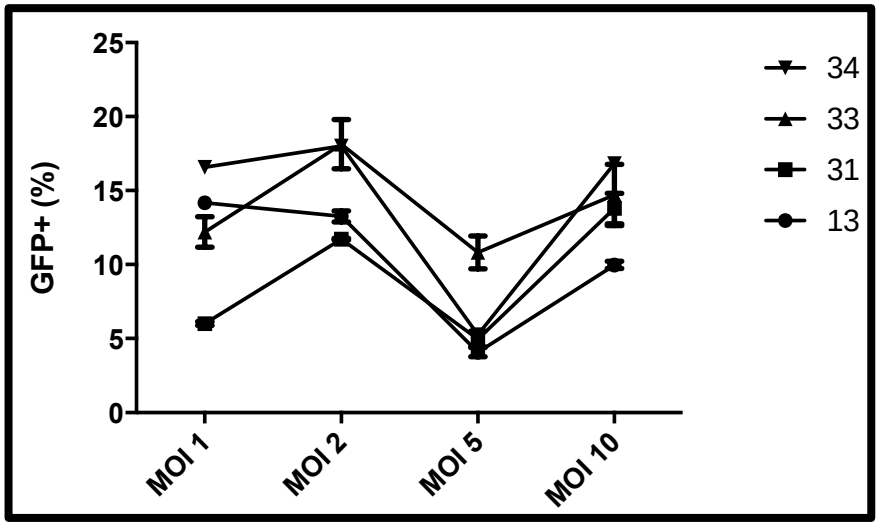
The mean fluorescence intensity data (intensity of GFP expression displayed by every transduced cell) also shows a high amount of transduction in PBMC at all MOIs (Fig 3.2B), with MOI 10 being the highest for all animals, which reflects more virus particles in each cell (forced transduction). The MFI of transduced PBMCs at MOI 2 was better than MFI at MOI 1 and equivalent to MFI at MOI 5 (Fig 3.2B), suggesting MOI 2 as an optimum working MOI for future experiments.

3.3.3. Bovine peripheral blood polymorphonuclear cells (PMNs)

The PMNs were purified as described earlier (Whale et al., 2006). The cells were consistently >98% viable as determined by trypan blue dye exclusion and contained less than 3-4% mononuclear cells (eiosinophils and basophils). A total of 100 x 10^6 PMNs could be isolated from 50 ml of EDTA treated bovine venous blood.

Bovine PMNs from four different animals were either mock-infected or infected with BAV304a at a MOI of 1, 2, 5 or 10. After incubation for18 hrs at 37 ^{0}C, the infected cells were trypsinized, fixed in 2% paraformaldehyde in PBS and analyzed by flow cytometry. As seen in (Fig 3.3A), 9-18% PMNs were transduced by BAV304a at all MOIs with MOI 2 showing a high GFP expression as compared to higher MOIs. Moreover, the mean fluorescence intensity (Fig 3.2B) of transduced PMNs showed to be a little enhanced at MOI 2 than MOI 1, suggesting that MOI 2 is optimal for future experiments.

A)



B)

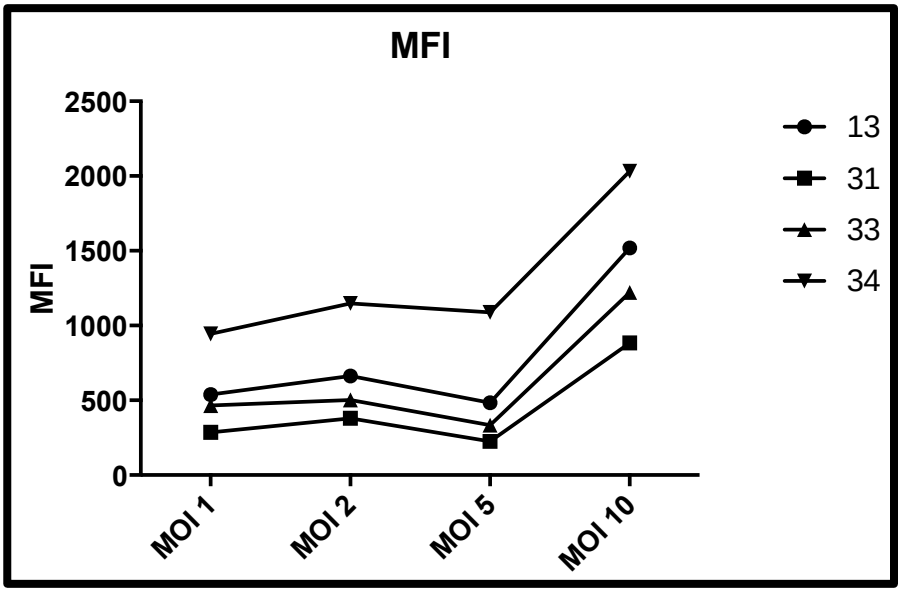


Fig. 3.2. Transduction of bovine PBMCs by BAV304a. Purified bovine PBMCs were infected with indicated MOI of BAV304a. At 18 hrs post infection, the cells were collected and analyzed by flow cytometry for GFP expression (A) and mean florescent intensity of GFP (B). Animal numbers are shown on the right of the panel.

3.3.4 PBMC subpopulations transduced with BAV304a in bovine blood

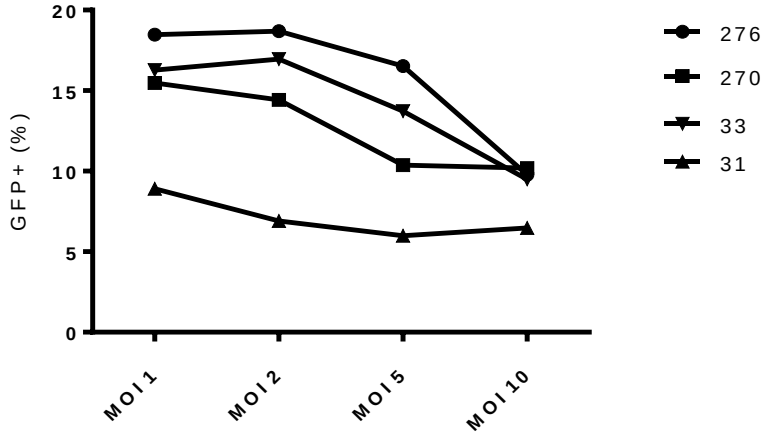
To test the transduction efficiency of immune cells (monocytes, T-cells, B-cells, NK-cells and dendritic cells) present in bovine blood with BAV304a, initially, the freshly isolated bovine PBMC were infected with BAV304a at MOI 2. At 18 h.p.i, the transduced PBMCs were labeled with mAbs recognizing different leucocyte antigens in PBMC cells, namely, CD14+ & CD11c+ (marker for monocytes), CD3 (T-cells), CD21 (B-cells), CD335 (NK-cells) followed by labeling with fluorochrome-conjugated secondary antibodies - goat anti- mouse IgG1-PE and goat anti-mouse IgM-APC.

These labeled cells were analysed on flow cytometer for GFP expression. Our results demonstrate that expression of GFP was detected in monocytes with very few B-cells, T-cells and NK-cells showing expression of GFP. The percentage of GFP-positive cells was 100% in the purified monocytes, while 2%, 1% and 4% transduced B-cells (CD21), T-cells (CD3) and NK-cells (CD335) showed GFP expression (Fig. 3.4).

3.3.5 *In-vitro* transduction of monocytes, PMNs and DCs

To confirm these results, purified monocytes, PMNs and dendritic cells were infected with BAV304a at a MOI of 2. At 18 h.p.i, the infected cells were tested for the expression of GFP using flow cytometric analysis. As seen in Fig 3.5, (A) the monocytes and (B) PMNs (neutrophils) showed GFP expression with 100% and 9% of GFP positive cells respectively, while (C) dendritic cells did not show GFP expression on transduction with BAV304a at MOI 2.

A)



B)

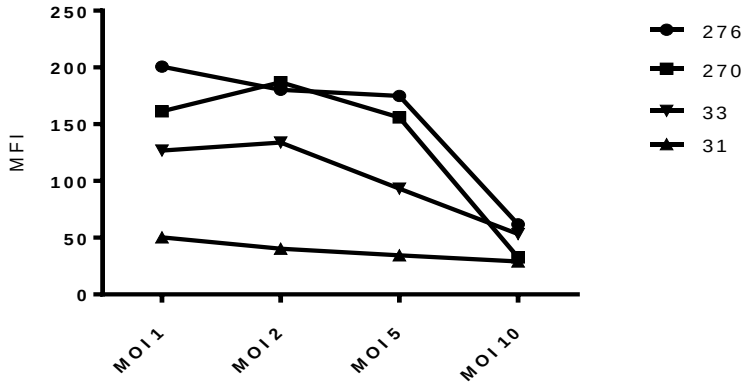


Fig. 3.3. Transduction of bovine PMN by BAV304a. Purified bovine PMNs were infected with indicated MOI of BAV304a. At 18 hrs post infection, the cells were collected and analyzed by flow cytometry for GFP expression (A) and mean florescent intensity of GFP (B). Animal numbers are shown on the right of the panel

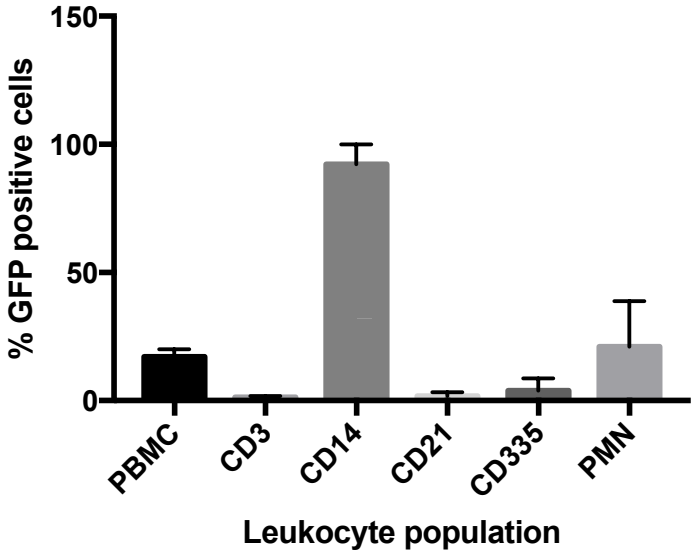
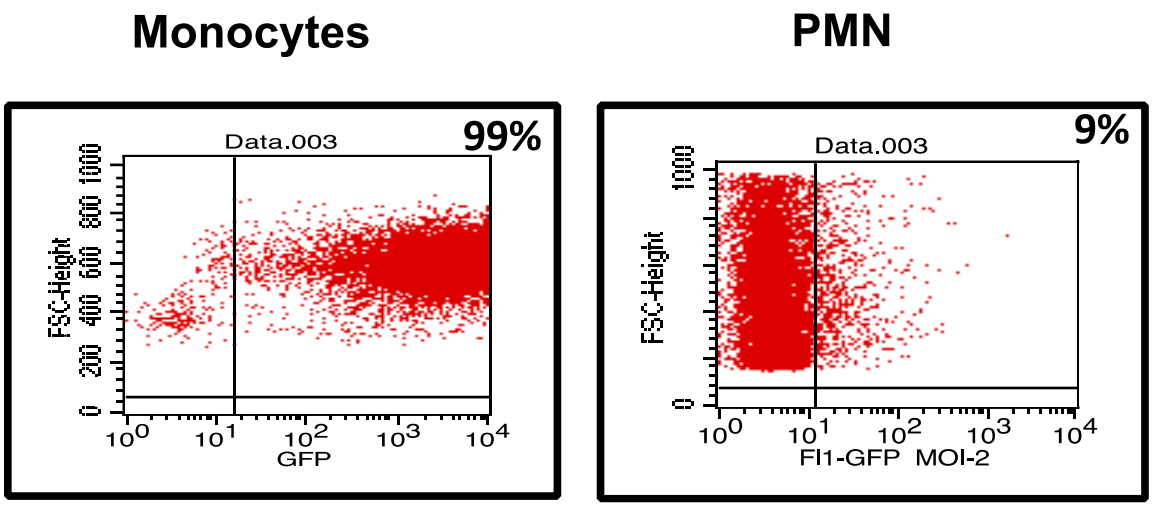


Fig 3.4. Leukocyte subpopulations transduced by BAV304a at MOI 2. Transduced PBMCs labeled with individual leukocyte antigen expressing GFP analysed by flow cytometer. Each bar represents the number of cells in individual leukocyte subpopulation expressing GFP. This experiment was repeated with five different animals.

(A) **(B)**



(C)

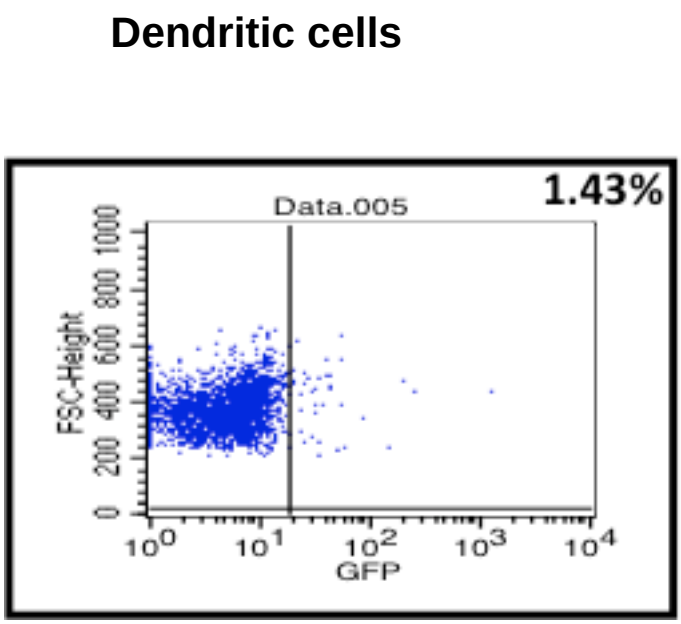


Fig. 3.5. Transduction of purified leukocyte subpopulations with BAV304a at MOI 2. Different leukocyte subpopulations were sorted from PBMC using FACS and transduced with BAV30a at MOI 2. Flow cytometric analysis showing the percentage GFP positive cells in (A) Monocytes (B) PMNs (neutrophils) and (C) Dendritic cells post transduction. This experiment was repeated on 3 different animals as biological replicates.

3.3.6 Production of progeny virus in transduced leukocyte subpopulations

The above results demonstrate that some of the monocytes are efficiently transduced by BAV-304a. To determine if BAV304a produces progeny virions in monocytes and PMNs, the purified monocytes and PMNs were transduced with BAV304a at MOI of 2 as described above. At 48 h.p.i, the infected cells were harvested, freeze-thawed and analysed for the production of progeny virions by $TCID_{50}$ assay. As seen in (Fig 3.6A), no detectable expression of GFP or viral CPE was observed in the monocyte after 14 days post infection. These results suggest that BAV304a infection of monocytes does not produce progeny virus.

To determine if BAV304a produces progeny virions in PMNs, the purified PMNs were transduced with BAV304a at MOI of 2 as described above. At 48 h.p.i, the infected cells were harvested, freeze-thawed and analysed for the production of progeny virions by $TCID_{50}$ assay. As seen in (Fig 3.6B), no detectable expression of GFP or viral CPE was observed in the PMNs after 14 days post infection. These results suggest that BAV304a infection of PMNs does not produce progeny virus.

To determine if BAdV-3 specific proteins are expressed, about 2 x 10^6 purified monocytes were infected with BAV304a at MOI of 2. At 48 h.p.i, the proteins from the lysates of infected cells were separated by SDS-PAGE, transferred to nitrocellulose and probed in Western blot using BAV-3 protein specific antibodies followed by alkaline phosphatase-conjugated secondary antibodies. As seen in (Fig. 3.7), expression of early (E) E1 19K (panel a) and E2 DNA binding protein (panel b) and late (L) L1 52K (panel c) could be detected in BAV304a infected monocytes. However, no expression of L5 hexon (panel d) or L6 100K (panel e) could be detected in BAV304a infected monocytes.

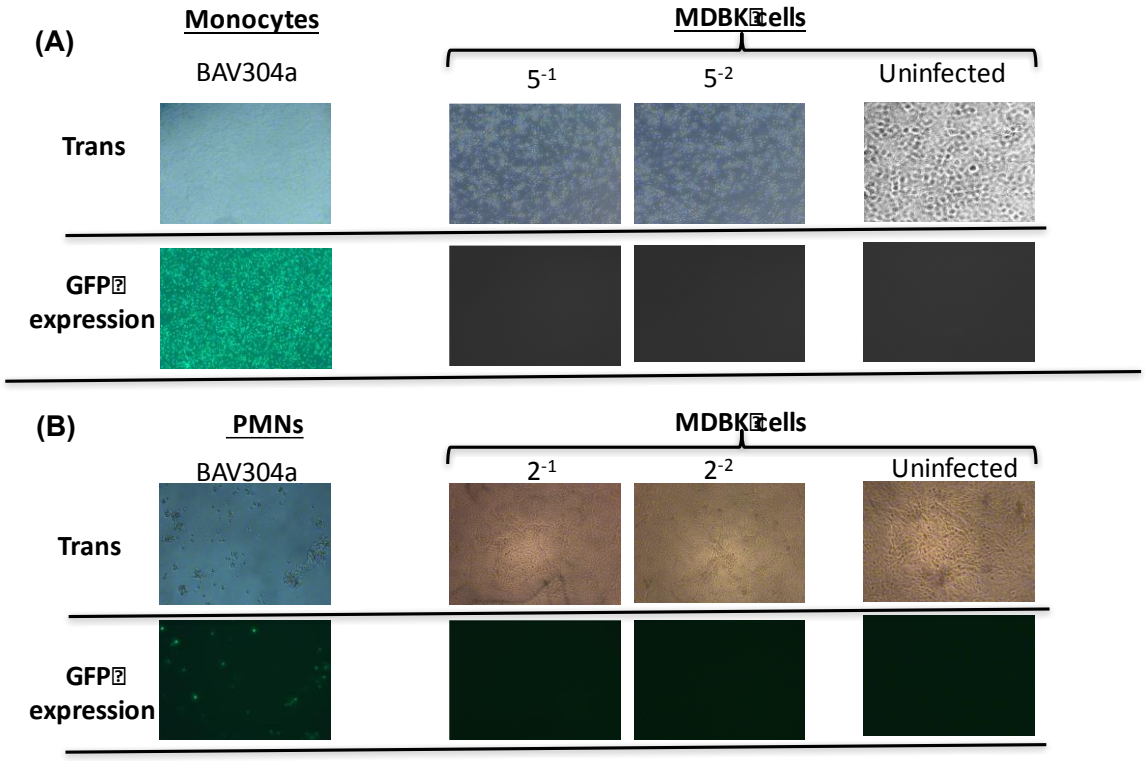


Fig. 3.6 Replication of BAV304a in monocytes and PMNs. Purified monocytes (panel A) or PMNs (panel B) were infected with BAV304a at a MOI of 2**.** At 48 hrs post infection, the cells were collected, freeze-thawed and virus was titrated on MDBK cells as described in the text. The cells were visualized for GFP expression and development of cytopathic effects using fluorescent microscope. The results were repeated two independent times, with three replicates each time. Five fold (monocytes) and 2 fold (PMNs) dilutions were used for titration.

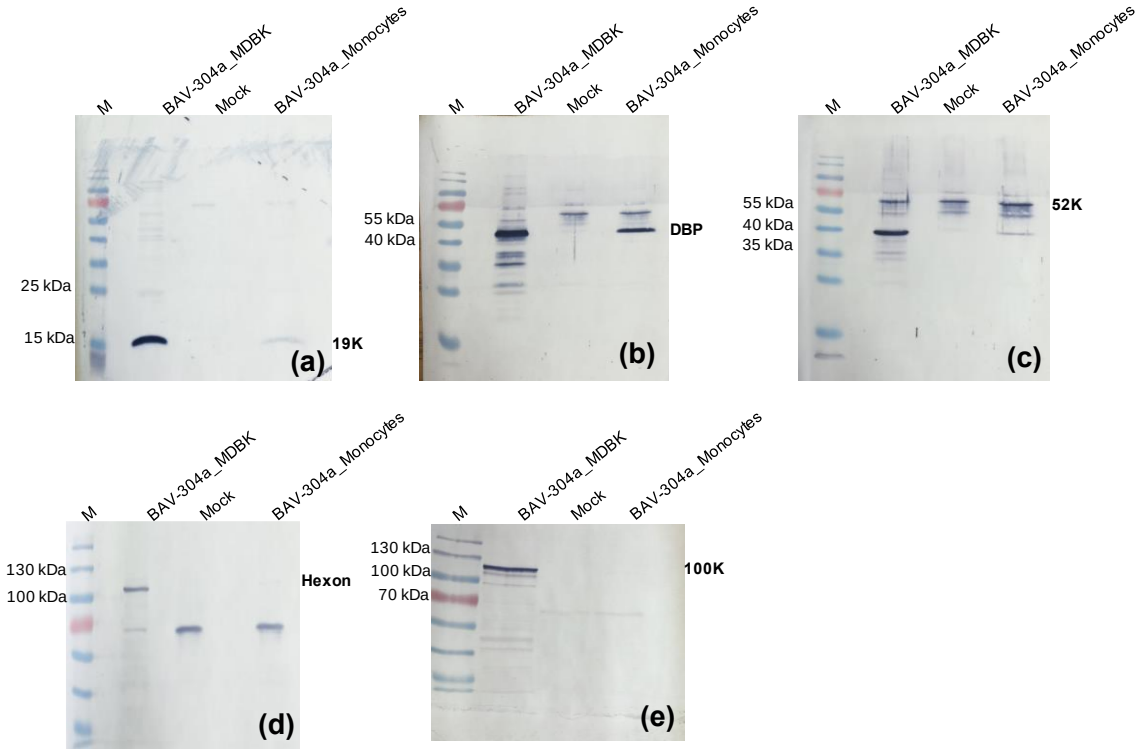


Fig. 3.7. Protein expression in BAV304a infected monocytes. Proteins from the lysates of mock or BAV304a infected MDBK cells (panels a-e), BAV304a infected bovine monocytes were separated by 12% SDS-PAGE, transferred to nitrocellulose and probed in Western blot with BAdV-3 anti-19K serum (panel a), BAdV-3 anti DBP serum (panel b), BAdV-3 anti-52Kserum (panel c), BAdV-3 anti-hexon serum (panel d) and BAdV-3 anti-100K serum (panel e).

These results suggest that BAV304a specific gene expression is blocked in the later stages of virus infection cycle.

To determine if BAdV-3 specific proteins are expressed, about 2 x 10^6 purified PMNs were infected with BAV304a at MOI of 2. At 48 h.p.i, the proteins from the lysates of infected cells were separated by SDS-PAGE, transferred to nitrocellulose and probed in Western blot using BAV-3 protein specific antibodies followed by alkaline phosphatase-conjugated secondary antibodies. As seen in Fig.3.8, no BAV304a specific proteins could be detected in infected PMNs suggesting the BAV304a gene expression is blocked at the early stage of infectious cycle.

4. DISCUSSION

Although recombinant human adenoviruses have been shown to deliver vaccine antigens to animals effectively (Callebaut et al., 1996; Prevec et al., 1989; Torres et al., 1996), their role as a vaccine-delivery system is limited partially due to safety concerns in domestic animals. As non-human adenoviruses are species specific, the development of animal-specific adenovirus based vectors may be a better option for delivering vaccine antigens to cattle (Baxi et al., 2000; Brownlie et al., 2015; Reddy et al., 1999b; Zakhartchouk et al., 1998).

Although two shot (prime and booster) BAdV-3 vectored vaccine induces protection in calves (Zakhartchouk et al., 1999), an ideal BAdV-3 vectored vaccine should induce protection in single shot. (Brownlie et al., 2015). A number of approaches including directing the BAdV-3 vector to most potent antigen presenting cells known as

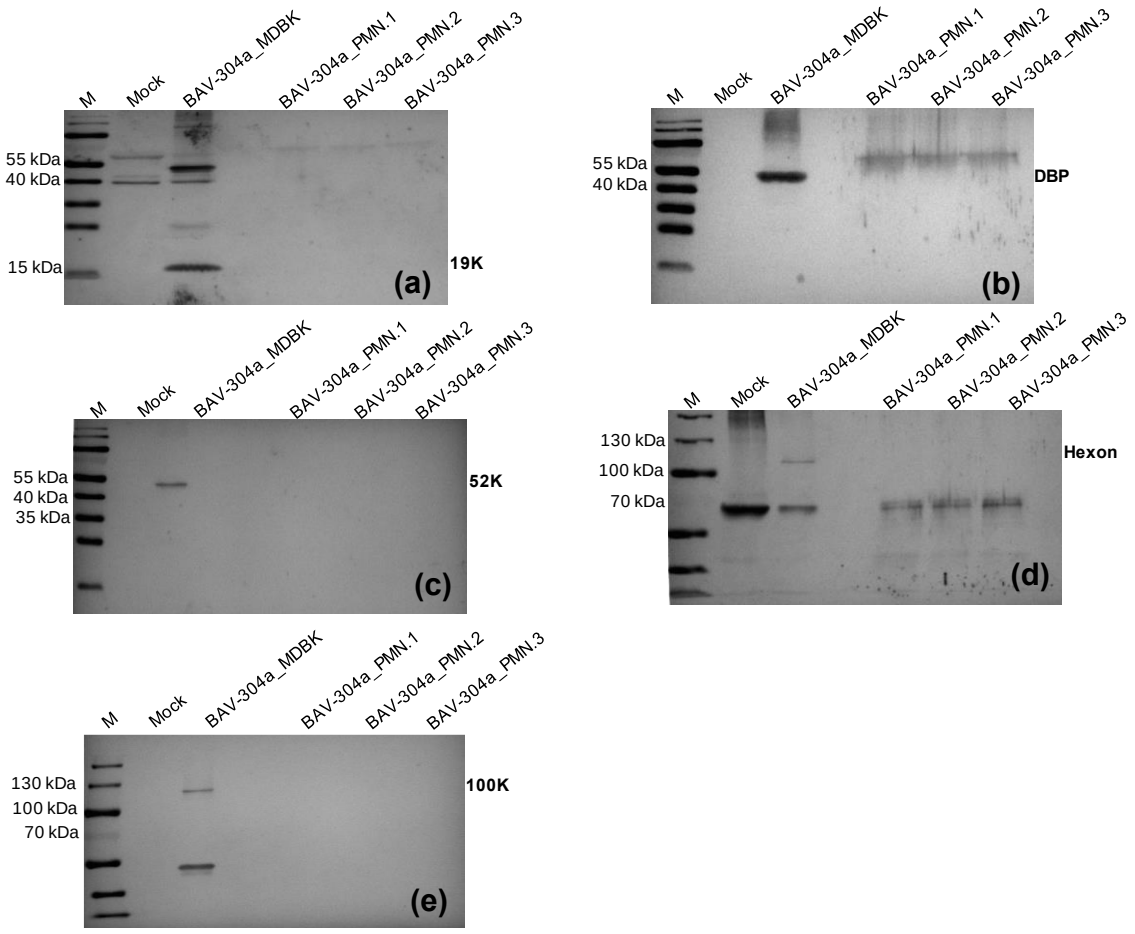


Fig. 3.8. Protein expression in BAV304a infected PMNs. Proteins from the lysates of mock or BAV304a infected MDBK cells (panels a-e), BAV304a infected bovine PMNs isolated from three animals (PMN1, PMN2, PMN3) were separated by 12% SDS-PAGE, transferred to nitrocellulose and probed in Western blot with BAdV-3 anti-19K serum (panel a), BAdV-3 anti DBP serum (panel b), BAdV-3 anti-52Kserum (panel c), BAdV-3 anti-hexon serum (panel d) and BAdV-3 anti-100K serum (panel e).

dendritic cells can help in developing and improving the efficacy of BAdV-3 delivered vaccines by increasing antigen expression, antigen presentation and development of potent adaptive immune responses. However, altering the tropism of BAdV-3 for a specific population of PBMCs require the knowledge of normal interaction of wild-type BAdV-3 with different subpopulations of PBMCs. Here, we determined the interaction of recombinant BAV304a with different subpopulations of bovine PBMC and PMN.

About 12-15% PBMC were transduced with BAV304a irrespective of the MOI used. The transduced cells expressed a high GFP content as well as a high mean fluorescence intensity suggesting a great transduction capacity of the virus in these cells. Very low transduction was observed in T-cells, B-cells, dendritic cells and NK cells. Although little is known about the cell receptor(s) recognized by BAdV-3 (Wu and Tikoo, 2004) to enter permissive cells, it is possible that low transduction of these cells by BAV304a could be due to absence of cell receptors leading to inefficient virion binding and internalisation of the virus in these cells. Similar results have been reported for transduction of lymphocytes (T and B cells) with of selected human adenovirus serotypes HAdV-11p and HAdV-35 (Segerman et al., 2006).

Interestingly, BAV304a efficiently transduces monocytes as is evident from expression of GFP and, expression of BAdV-3 19K (E1) and 52K (L1) proteins in transduced bovine monocytes. However, absence of expression of hexon (L5) and 100K (L6) proteins in BAV304a transduced monocytes and absence of expression of GFP in MDBK cells infected with lysates of monocytes (infected with BAV304a) suggests that BAV304a does not produce progeny virions in transduced bovine monocytes. Earlier, results suggest that human adenovirus also transduces human monocytes and leads to the

expression of E1A (E1) and hexon (L3) without production of progeny virions (Adam et al., 2009; Kessler et al., 2010).

Human neutrophils are internalized but not transduced by HAdV-5 vectors expressing luciferase under CMV promoter (Cotter et al., 2005). Moreover, the uptake of HAdV-5 vector is independent of receptor binding to neutrophils, which leads to efficient transduction of the virus in neutrophils. However, the interaction of AdLuc with neutrophils has been suggested to reduce the HAdV-5 vector transduction of neutrophils due to complement and antibodies in the human sera (Cotter et al., 2005). Earlier study has demonstrated the transduction of PMNs by BAV304 (Whale, 2005; Reddy et al., 1999b). Our study confirms the earlier results suggesting that neutrophils are transduced by BAV304a. However, absence of BAdV-3 specific gene expression and absence of expression of GFP in MDBK cells infected with lysates of PMNs infected with BAV304a suggests that BAV304a does not produce progeny virions in transduced bovine neutrophils. Unlike other species, endogenous MHC class II expression has not been detected in bovine neutrophils, thus questioning its role in the induction of adaptive immune responses is not clear (Whale, 2005). However, passive acquisition of MHC II protein(s) by bovine PMNs has been proposed to help them in acting as antigen presenting cells (Whale, 2005).

Dendritic cells initiate and regulate adaptive immune responses (Guzman et al., 2016) and are capable of initiating antigen-specific T cell response by naïve T cells (Senesac et al., 2014). The transduction of human dendritic cells varies depending upon the use of an adenovirus serotype (Guzman et al., 2016). HAdV-2 enters and transduces human dendritic cells by CAR independent receptor (Adams et al., 2009), and express

HAdV specific 19K (E1) and hexon (L3) without production of progeny virus (Kessler et al., 2010). HAdV-5 transduces circulating DCs and monocytes even though they are CAR deficient cells (Adams et al., 2009). Several CAR-deficient cells are susceptible to HAdV-35 infection and have shown to stimulate polyfunctional memory T cell responses (Lore et al., 2007). In contrast, bovine dendritic cells appear refractory to BAV304a infection as neither GFP expression (regulated by cmv promoter) nor BAdV-3 specific protein expression could be observed in transduced bovine dendritic cells.

These results suggest that BAV304a does not transduce bovine dendritic cells.

In summary, we have demonstrated that though bovine monocytes are transduced efficiently by BAV304a, the bovine dendritic cells are transduced poorly by BAV304a. Since DCs are the most potent and primary antigen presenting cells (APC), targeting of BAdV-3 based vectors to bovine DCs may increase the efficacy of BAdV-3 vectored vaccines.

5. GENERAL DISCUSSION AND CONCLUSION

The inherent property of viruses to efficiently deliver genetic material to eukaryotic cells has led to the development of viruses as gene delivery vectors (Choi and Chang, 2013; Draper and Heeney, 2010). Based on the successful development of human adenoviruses as a vector for humans (Draper and Heeney, 2010; Wold and Toth, 2013), species-specific adenoviruses are being evaluated as gene delivery vehicles for animals (Hammond et al., 2001; Mittal et al., 1995; Singh et al., 2008; Wright et al., 2013). Our laboratory is evaluating bovine adenovirus-3 as a vaccine delivery vehicle for vaccination of cattle (Baxi et al., 2000; Brownlie et al., 2015; Reddy et al., 1999b; Zakhartchouk et al., 1998) and have demonstrated the potential of using replication- competent bovine

adenovirus-3 as vaccine delivery vector in cattle (Baxi et al., 2000; Brownlie et al., 2015; Reddy et al., 1999b;). The target antigen expressed by the adenovirus vectors can induce protective immunity in cattle when administered twice four weeks apart by mucosal route (Ayalew et al., 2015; Zakhartchouk et al., 1998). In order to develop more efficient BAdV-3 based vectors, which are economical to use, and can induce efficient immune responses with single immunization, it is important to determine the interaction of BAdV-3 with immune cells particularly dendritic cells. Knowledge of such interactions will help to devise strategies for improving the interaction of BAdV-3 with antigen presenting cells particularly bovine dendritic cells leading to the induction of more potent protective immune responses in cattle (natural host).

In the present study, we have determined the interaction of recombinant BAV304a with different leukocyte populations in bovine blood. Our study demonstrates thatBAV304a transduces some bovine blood cells efficiently and some bovine blood cells poorly.

Interestingly, monocytes showed 100% transduction efficiency with BAV304a. Monocytes are recognized as key immune effector cells that can mediate protection against a number of different pathogens. At the same time, monocytes and macrophages are also associated with immune suppression (Gabrilovich et al., 2009). Peripheral blood derived monocyte serves as reservoirs for many viruses like HIV, HCV, Influenza A virus and adenovirus (Kumar et al., 2014; Coquillard et al., 2009; Hoeve et al., 2012; Huang et al., 1996). They have a short life span in bloodstream but tend to live and persist longer upon viral infection. In some cases, monocytes internalize the virus and rapidly differentiate in macrophages or dendritic cells while in other cases; they remain in

latency while harbouring the virus in them. The studies with human adenovirus show that HAdV-infected monocytes cannot be differentiated into immature DC (Kessler et al., 2010) as the virus hijacks the normal biosynthetic capacity of the monocytes and does not let it differentiate into macrophages or DCs and serve as more stable hideouts for the virus. This results in prevention of antigen presentation by these DCs and generation of adaptive T cell mediated immune response. However, adenoviruses have shown to induce innate immune responses in transduced PBMCs (Higginbotham et al., 2002). HAd-2 infection has shown to induce increase in the levels for TNF-alpha and IL-1 beta in both monocytes and in macrophages. We speculate that high transduction of BAV304a in bovine monocytes may play an important part in the induction of innate immune responses but not adaptive immunity. Moreover, induction of innate immunity may result in tissue damage and rapid clearance of the vector. Thus, it can be a challenge for the clinical use of the bovine adenovirus vectors.

In contrast to monocytes, neutrophils showed a comparatively low (9-18%) transduction with BAV304a with no viral gene expression or production of progeny virus. Previous studies have demonstrated that BAV304 transduces bovine neutrophils and interact with T cells at sites of inflammation following secretion of cytokines (Whale et al., 2005). Neutrophils have shown passive acquisition of a functional viral receptor protein from adenovirus permissive cell line upon transduction with BAV304 and MHCII proteins, thus can act as antigen presenting cells (Whale et al., 2006).

In order to generate strong adaptive immune response, targeting of adenovirus to dendritic cells is critical. Dendritic cells (DCs) are professional APCs, which play an essential role in bridging the innate and adaptive immune system and, in promoting the

Ag specific activation and expansion of CD8+ and CD4+ T cells (Banchereau et al., 1998). They can also be found in an immature state in the blood. Once activated, the mature DCs migrate to the lymph nodes where they stimulate T cells and B cells to initiate adaptive immune response. HAdV-5 transduces circulating DCs and monocytes even though they are CAR deficient cells (Adams et al., 2009). Several CAR-deficient cells are susceptible to HAdV-35 infection and have shown to stimulate polyfunctional memory T cell responses (Lore et al., 2007). However, we could not detect any transduction of bovine dendritic cells with BAV304a, suggesting these cells do not possess receptors for the binding and internalization of BAdV-3 vector. Therefore, they cannot efficiently prime the naïve T cell and generate primary immune response to infection.

Taken together, we can suggest from our results that further analysis is required to support the development of more robust recombinant BAdV-3 vectors, which can transduce bovine immune cells especially dendritic cells and efficiently deliver vaccine antigen genes to animals to protect against diseases of zoonotic importance.

6. FUTURE DIRECTIONS

Low transduction of dendritic cells could be due to the absence of cell receptors leading to inefficient virion binding and internalization of BAV304a in these cells. So, the development of more robust recombinant BAdV-3 vectors is required for the efficient targeting of the vaccine vector to dendritic cells. One way is to alter tropism of BAdV-3 is by genetic manipulation of capsid proteins. Incorporation of the RGD motif into the pIX has shown a significant augmentation of BAdV-3 fiber knob-independent infection

of the integrin-positive cells, suggesting that RGD motifs are displayed on the surface of virion capsids and are accessible for binding to integrins (Zakhartchouk et al., 2004).

Since, DCs are integrin positive cells, transduction of BAdV-3 expressing RGD motif with dendritic cells may result in enhanced transduction in dendritic cells. Therefore, the strategies for genetic retargeting of adenovirus by insertion of targeting ligands in fiber protein (Bouri et al., 1999) or C-terminus of pIX protein (Vellinga et al., 2004) may improve the transduction efficiency of adenoviruses in dendritic cells.

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