TRANSDUCTION OF BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS WITH RECOMBINANT BOVINE ADENOVIRUS-3 EXPRESSING CHIMERIC PIX

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Saskatoon

By

Maria Bravo Araya

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Canada

ABSTRACT

BAdV-3, like many other members of the family *Adenoviridae*, has been developed and evaluated as a vaccine delivery vehicle in cattle (Ayalew *et al*., 2014). However, protective immune responses using recombinant BAdV-3 based vaccines are achieved after two immunizations (Kumar *et al*., 2014; Zakhartchouk *et al*., 1999). One way to increase the efficiency of BAdV-3 based vectors is by increasing the transduction to antigen presenting cells (APCs). Since C-terminus of BAdV-3 minor capsid protein pIX is exposed to the exterior of virion capsid, efforts have been made to use it to add targeting ligands, so that recombinant BAdV-3 expressing chimeric pIX can be targeted to a particular cell (Zakhartchouk *et al*., 2004).

In the present study, we constructed and characterized a recombinant BAdV-3, designated as BAV888, expressing chimeric minor capsid protein pIX fused to RGD. The RGD motif, which is not present in the BAdV-3 virion, is an important pathway for the internalization of the virus into integrin-positive cells, such as APCs.

I*n vitro* studies have demonstrated that at MOI 1, both BAV888 and BAV304a (BAdV-3 expressing GFP inserted in E3 region of the genome) can transduce PBMCs without a significant difference in the percentage of cells transduced. However, BAV888 transduced cells exhibited a mean fluorescence intensity significantly higher than BAV304a transduced cells.

Further, analysis in different PBMC subpopulations (T-cells, B-cells, monocytes, NK-cells and dendritic cells) showed that BAV888 and BAV304a have tropism mainly for monocytes and NK-cells. Furthermore, the mean fluorescence intensity of GFP+ monocytes is up to four times higher in BAV888-transduced cells, suggesting that our recombinant virus is more efficiently internalized by monocytes. To prove this, we compared viral copy number of transduced monocytes and transcriptional analysis of viral proteins using qPCR. The copy number of BAV888 is significantly higher than BAV304a in transduced

monocytes at the same MOI. Moreover, the expression of GFP and early and late viral proteins is higher in BAV888 transduced monocytes.

To determine the expression of costimulatory molecules in BAV888 and BAV304a transduced monocytes, flow cytometry, and transcriptional analyses were performed. Although there is no significant difference in the expression of MHC class I and MHC class II between viruses using flow cytometry, analysis of the mRNA expression of showed a decrease in CD40 and CD86 expression in BAV888-transduced monocytes compared to BAV304a and mock transduced cells.

In terms of cytokine production, BAV888-transduced monocytes show an increased production of TNFα and IL-12, compared to BAV304a transduced monocytes. In mRNA analysis, there was a significant increase in expression of IFNβ in BAV888-transduced monocytes relative to BAV304a-transduced and mock cells, which could suggest an anti-viral response against the virus.

Our results demonstrate that, although there is no change in tropism of the virus, the addition of an RGD motif in the C-terminus of pIX of BAdV-3 can increased its uptake in PMBCs and, in particular, monocytes.

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DEDICATION

To my parents, for giving me wings to reach “the unreachable star”. *Infinitas gracias.*

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED IN THIS THESIS

A	Adenine
ADP	Adenovirus death protein
AIM2	Absent in melanoma 2
APC	Antigen Presenting Cell
ARD	Acute Respiratory Disease
BAdV	Bovine Adenovirus
BCR	B-cell receptor
BHV-1	Bovine herpesvirus 1
BMDC	Bone marrow-derived dendritic cells
Bp	Base pairs
BSA	Bovine Serum Albumin
BVDV	Bovine viral diarrhea
CAdV	Canine Adenovirus
CAR	Coxsackie adenovirus receptor
CD	Cluster of differentiation
CLRs	C-type lectin receptors
cM	Classical monocytes
CPE	Cytopathic effect
CsCl	Cesium Chloride
CSR	Class switch recombination
CTL	Cytolytic T-lymphocyte
DAI	DNA-dependent activator of IFN-regulatory factors
DAMPs	Damage-associated molecular patterns
DBS	DNA-binding protein
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrins
DNA	Deoxyribonucleic acid
DNApol	DNA polymerase

dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
Foxp3	Forkhead Box P3
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Germinal center
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HAdV	Human Adenovirus
HSPG	Heparan sulfate proteoglycans
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
intM	Intermediate monocytes
ITR	Inverted Terminal Repeat
kb	Kilo base
LPS	Lipopolysaccharide
LSECs	Liver sinusoidal endothelial cells
MDBK	Madin Darby Bovine Kidney
mDCs	Myeloid dendritic cells
MEM	Minimum Essential Medium
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MLP	Major late promoter
MoDC	Monocyte-derived dendritic cells
MOI	Multiplicity of Infection
MR	Mannose receptor
ncM	Non-classical monocytes
NLRs	Nucleotide oligomerization domain-like receptors

NF-κB	Nuclear factor kappa B
NK	Natural killer cells
ORF	Open Reading Frame
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-Buffered Saline
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pDCs	Plasmacytoid dendritic cells
PIC	Pre-initiation complex
pMHC class II	Peptide-major histocompatibility complex class II
PRRs	Pattern Recognition Receptors
pTP	Terminal protein precursor
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I like receptors
RGD	Arginine – Glycine - Aspartic acid
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SHM	Somatic Hypermutation
T	Thymine
TCR	T-cell receptor
T_{FH}	Follicular T-helper cells
T_H	T-helper cells
TI	T-independent
TLRs	Toll-like receptors
TNF	Tumor Necrosis Factor
TP	Terminal Protein
Treg	Regulatory T-cell
VCAM1	Vascular cell adhesion molecule

1. LITERATURE REVIEW

1.1 Adenoviruses

In 1953, Wallace Rowe noticed changes in epithelial cells morphology after incubating adenoids from infants, followed by the destruction of the tissue days later (Rowe *et al*., 1953). Hilleman and Werner had similar findings while investigating an outbreak of an acute respiratory illness, claiming that they found a novel viral agent, which they called "RI-67" (Hilleman and Werner, 1954). Later on, with isolates from RI-67 in conjunction with sera from patients with a syndrome called "acute respiratory disease (ARD)", Ginsberg found evidence that ARD syndrome might be caused by a complex mixture of pathogens, including RI-67, which they proposed to call "ARD virus" (Ginsberg *et al*., 1955).

Huebner named a new group of respiratory viruses as "adenoidal-pharyngeal-conjunctival agents" based on the symptomatology and anatomical areas from where these viruses could be isolated. Although they describe six types based on results from immunology tests, all cause the same cytopathogenic effects in the epithelium (Huebner *et al*., 1954). Later, Rowe *et al*. found eight new serologically distinct types from the ones previously described, for a total of 14 serotypes for the "adenoidal-pharyngeal-conjunctival" group of viruses. All these viruses possess similar characteristics including sharing a complement-fixing antigen and producing similar cytopathogenic effects (Rowe *et al*., 1956). Cells infected with adenovirus present similar characteristics, such as cellular aggregation and rounding, and nuclear inclusions (Harrach, 2008).

The term "Adenovirus" was proposed for the group of viruses isolated primarily from adenoids, which share common characteristics such as producing infection of ocular and respiratory epithelium, a distinctive cytopathogenic effect in cell culture, sharing a common antigen in the complement-fixation test all without causing visible disease in laboratory animals (Enders *et al*., 1956).

1.1.1 Adenovirus taxonomy and biology

The host range of adenovirus is narrow, restricted to one or a few usually closely related species. Most human adenoviruses (HAdV) infect humans, without producing any clinical sysmptoms but some HAdV types are associated with respiratory, ocular or venereal disease. HAdV is one of the primary causes of diarrhea in young children and can be transmitted through ocular and throat secretions, feces and urine. In mammals, infections by adenoviruses are common, but usually subclinical, unless the animals are under stress or immunocompromised. The only exception is Canine adenovirus 1(CAdV-1) that can cause fatal disease in young and wild animals; and CAdV-2, which causes very contagious respiratory infections in domestic dogs (King *et al*., 2011).

1.1.1.1 Adenovirus classification

The family *Adenoviridae* is composed of five genera: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus*, and *Ichtadenovirus*. Recently, a sixth genus, *Testadenovirus*, is proposed to include adenoviruses from turtles (Order *Testudin*) (Doszpoly *et al*., 2013). These genera can be differentiated using the complement fixation assay, where every genus reacts to a specific antigen, and there is an evident separation of each cluster in phylogenetic analyses (Fig.1.1) (King *et al*., 2012).

The genus *Mastadenovirus* is composed of more than 20 species that infect mammals only. The size of the genomes of *Mastadenovirus* members ranges between 30.2 to 38kb, with longer and more complex ITRs than other genera in the *Adenoviridae* family (Harrach, 2008). Several polypeptides encoded by early regions E1A, E1B, E3 and E4, minor capsid protein IX and core protein V are unique to members of this genus. Except proteins encoded by E3 and E4 regions, most of its genome is conserved in all mastadenoviruses. *Human adenovirus C* is the most studied species in the genus (King *et al*., 2011; Vrati *et al*., 1995).

Members of Aviadenoviruses infect only birds. Morphologically, they differ from other genera because they possess two fibers on each penton base (Chiocca *et al*., 1996; Marek *et al*., 2014). Although, they are known for causing diverse symptoms in bird species, mainly in the respiratory system (pulmonary congestion and bronchitis), they can also induce abnormalities in liver, heart and egg production (Benkő, 2008; Hess *et al*., 1997). Their genome size ranges - between 43.8 and 46.6kb. These viruses contain unique transcription units at one of the extremes of their genome, but the gene content in this region has not been well studied (King *et al*., 2011).

The genus *Atadenovirus* received its name because of a unique characteristic: its DNA has a high A+T content, up to 66% in some species. Compared to other genera, members of this genus can infect a broader range of species including ruminants, marsupials, birds, and reptiles (Harrach, 2008). Most members of *Atadenovirus* do not cause severe disease, even though an outbreak of Odocoileus adenovirus killed a large population of deer in North America. Virions are less sensitive to heat compared to other adenoviruses and have unique essential structiural proteins like p32k and LH3 (ÉlŐ *et al*., 2003; Gorman *et al*., 2005).

Members of *Siadenovirus* genus include frog adenovirus 1, turkey adenovirus 3 and raptor adenovirus 1 and contain the shortest genomes of all adenoviruses, between 26.1 and 26.2kb (Kovács and Benkő, 2009; Pitcovski *et al*., 1998). Member of *Ichtadenovirus* genus include white sturgeon adenovirus 1, a non-pathogenic virus, which infect fish. It contains the longest genome (48.3 kb) identified in members of adenoviridae family (Kovács *et al*., 2003).

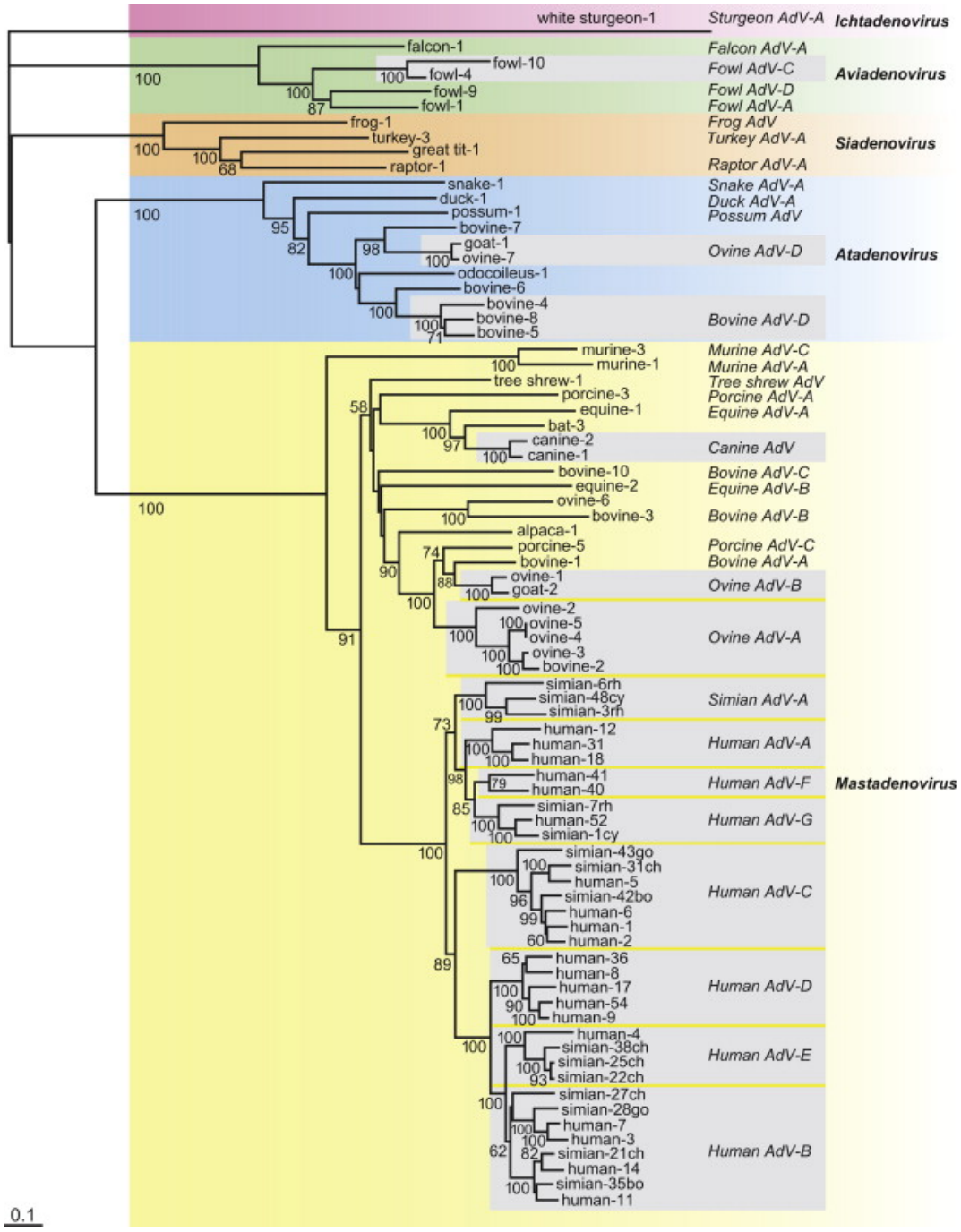

Figure 1.1 Phylogenetic analysis of family *Adenoviridae.* Reproduced with permission from Fig. 5 in (King *et al*., 2012) *Family – Adenoviridae*. Place: Elsevier.

1.1.1.2 Virion structure

Adenovirus virions are non-enveloped, possess icosahedral symmetry, and their capsid is formed by 240 hexons and 12 penton bases and fibers (Harrach, 2008). One-third of the almost 50 different polypeptides encoded by the DNA of the virion correspond to capsid proteins (Acheson, 2011), which are usually conserved in all genera, except for protein V and IX, that can be only found in members of the *Mastadenovirus* genus (King *et al*., 2011).

Hexons (trimers of polypeptide II) are considered major capsid proteins; they are firmly arranged together, forming a protective shell for inner proteins and nucleic acids (Leppard, 2008). Hexons contain conserved regions and hypervariable regions (HVRs) in their sequence; interesingly, the latter ones do not seem to interact with any other viral protein (Crawford-Miksza and Schnurr, 1996). However, HVRs have been found to contain serotype-specific epitopes (Rux and Burnett, 2000).

The penton base is a bucket-shaped structure, with a fiber shaft emerging from its center (Ruigrok *et al*., 1990). Its sequence is highly conserved between adenoviruses, and in most cases they contain an RGD motif (Zubieta *et al*., 2005).

There is one fiber per penton base, constituted by several polypeptides IV and III at each vertex (Leppard, 2008; Steward *et al*., 1993; van Oostrum and Burnett, 1985). Despite their structural functions, fibers are essential for attachment to the cell, which constitutes the first step in viral infection (Chroboczek *et al*., 1995).

The group of minor capsid proteins is constituted by IIIa, VIII, IX, and VI that maintain the hexon proteins in the capsid together (King *et al*., 2011; Acheson, 2011). Protein IX is the smallest of them and it is only present in the genus *Mastadenovirus* (Everitt and Philipson, 1974). The N-terminus of the protein is inside the virion, between the hexons, while the C-terminus faces the exterior of the capsid (Akalu *et al*., 1999). Each facet of the virion contains twelve pIX molecules which are not essential for the virus but provide stability to the capsid at high temperatures (Boulanger *et al*., 1979; Colby and Shenk, 1981; Furcinitti *et al*., 1989; Rosa-Calatrava *et al*., 2001). Crystallography studies of adenovirus virions confirm that pIX interacts primarily with the hexons, and form triskelion structures (Natchiar *et*

al., 2018). The N-, C- terminus and a central alanine-rich portion are conserved between serotypes, but the N-terminus is only responsible for capsid insertion (Rosa-Calatrava *et al*., 2001; Vellinga *et al*., 2005). Protein IX can also affect the capacity of the virus to package its DNA (Ghosh-Choundry *et al*., 1987) and has the ability to stimulate several promoters (Lutz *et al*., 1997).

Protein VIII structure is generally well-conserved among adenoviruses (Yu *et al*., 2017). It contains motifs recognized by adenoviral protease (AVP) (Liu *et al*., 2010). The protease cleavage appears essential for maturation of progeny virus (Gaba et al., 2017). To hold the hexons together, pVIII interacts with pIIIa forming cementing interactions (Natchiar *et al*., 2018).

The capsid protein pIIIa (Benevento et al., 2014) is a cement protein, located beneath the vertex region in the interior of the viral capsid (Natchiar *et al*., 2018). To hold the hexons together, pVIII interacts with pIIIa forming cementing interactions (Natchiar *et al*., 2018). Protein IIIa is also involved in determining the serotype specificity of adenovirus DNA packaging (Ma and Hearing, 2011). Protein pIIIa appear to be cleaved by adenovirus protease, which is potentially essential for the maturation of progeny virus (Gaba *et al*., 2017).

The capsid protein pVI is involved in nuclear localization and assembly of hexons in nucleus through interaction of N -terminus of pIV with hexons (Moyer *et al*., 2015; Natchiar *et al*., 2018). Protein pIV is cleaved by adenovirus protease, which appears essential for virus entry and capsid assembly (Moyer *et al*., 2015). In addition, while C-terminal factor acts as a co-factor for adenovirus protease cleavage of selected adenovirus proteins, the lytic region of N-terminal fragment appears to be involved in endosome lysis during intial stages of virus infection (Mangel *et al*., 1993 Wiethoff *et al*., 2005; Luisoni *et al*., 2015).

Mastadenovirus genus specific polypeptide V is also part of the core proteins, and it interacts with protein VI, facing the interior of the capsid, and the dsDNA (Flint, 2016). pV also associates with the host cell nucleoli (Matthews and Russel, 1998). It contains multiple nuclear and nucleolar localization signals, and it can redistribute major nucleolar proteins to the cytoplasm (Matthews, 2001).

Protein VII, the most abundant core protein in the virion, is tightly associated with the viral DNA (Benevento *et al*., 2014). In the early phases of viral infection, pVII remains associated with the adenoviral DNA (Walkiewicz *et al*., 2009), but it is released when the transcription of early genes is initiated (Chen *et al*., 2007). A recent report suggests that pVII is not required for DNA condensation,, DNA packaging or virion assembly, but appears vital for the viral escape from the endosome in early stages of infection (Ostapchuk *et al*., 2017).

Mu is a 9 kDa protein and is present between 100 and 300 copies per virion (Anderson *et al*., 1989). Like protein VII, during maturation mu is cleaved by the viral protease (Blainey *et al*., 2013). Proteins V, VII and mu interact with each other in the viral core (Chatterjee *et al*., 1985) and with the viral dsDNA, neutralizing its negative charge to allow its condensation and tight packaging within the icosahedral capsid (Pérez-Berná *et al*., 2015).

The Terminal protein (TP) is essential for the virus since it primes its genome replication (Liu *et al*., 2003) . It is cleaved by adenovirus protease before maturation of progeny virus (Webster *et al*., 1997).

The genome of adenoviruses is linear, double-stranded DNA (dsDNA), with a TP at the 5' end of each strand (Acheson, 2011), and contains inverted terminal repeat (ITR) sequences with variable sizes, between 36 to more than 200 bp (Davison *et al.*, 2003). The middle region is conserved among the different genera in the family, while the 5' and 3' ends vary in composition and dimension. The genomes of members of adenoviridae family range in size from 26,163 to 48,395 bp (King *et al*., 2011). Many adenoviral genes are spliced in both conserved and non-conserved regions (Harrach, 2008).

Virions are stable at freezing temperatures, and their heat sensitivity is variable, depending on the genera (Harrach, 2008). They are resistant to mild acid and cannot be inactivated using lipid solvents, because of their lack of envelope (Leppard, 2008).

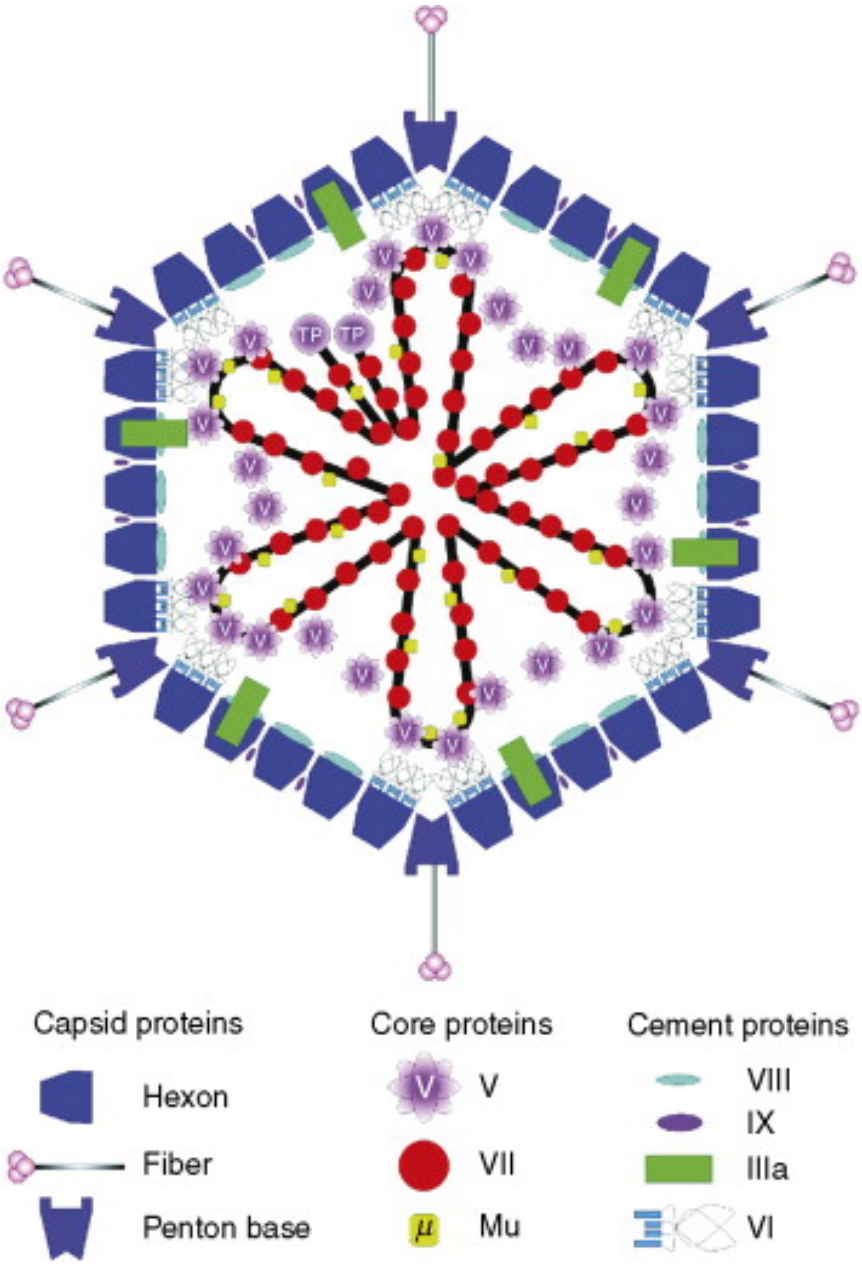

Figure 1.2 Adenoviral structure. Reproduced with permission from Fig. 5 in (Leppard, 2008). *Adenoviruses: Molecular Biology*. Place: Elsevier.

1.1.1.3 Virus life cycle

Adenoviruses interact primarily with the cell-surface protein (e.g coxsackie-adenovirus receptor [CAR] for HAdV-5) through adenovirus fiber knob (Bergelson, 1999; Nemerow *et al*., 2009). However, there are other receptors that a may be involved in the adenovirus primary attachment to the cell, such as CD46, CD80/CD86, MHC I α-2, heparan sulfate proteoglycans (HSPG), and vascular cell adhesion molecule 1 (VCAM-1) (Defer *et al*., 1990; Hong *et al*., 1997; Dechecchi *et al*., 2000; Chu *et al*., 2001; Short *et al*., 2004). Once the primary attachment has occurred, integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$, present in the cell surface, interact with the Arg-Gly-Asp (RGD) motif located in the viral penton base, allowing the virus internalization via receptor-mediated endocytosis (Wickham *et al*., 1993).

The virus enters the cell through endocytosis (Meier and Greber, 2004; Varga *et al.*, 1991), and the consequent acidification of the vesicle leads to loss of some proteins of the virion, like fiber and pVI (disassembly), which is vital to complete the viral cycle (Moyer *et al*., 2016). This process is responsible for the lysis of the membrane of the endosome and the release of the virion in the cytoplasm of the cell (Wiethoff *et al*., 2005). Recent report suggest that pVI binds to the endosome membrane, resulting in a positive curvature that leads to fragmentation of the endosome and release of the viral particle to the cytoplasm (Maier *et al*., 2010)

Once in the cytoplasm, the viral genome is transported to the nucleus with the aid of microtubules and dynein (Leopold *et al*., 2000; Bremner *et al*., 2009; Kelkar *et al*., 2006; Zhou *et al*., 2018). Finally, the adenovirus protein hexon associates with nucleoporin Nup214 outside the nuclear pore complex (Cassany *et al*., 2015), which helps to finally disrupt the capsid and transport the adenovirus genome to the nucleus.

Transcription of the entire viral genome occurs in the nucleus is mostly facilitated by the host enzyme RNA polymerase II. The gene expression is temporally regulated by viral and host proteins that activate specific regions of the adenovirus genome (Leppard, 2008). Early regions, E1 to E4, encode proteins: a) responsible for modulating the transcription of viral DNA using the host machinery, b) help to

avoid the detection of virus infection by host immune response immune, and c) required for DNA replication. The intermediate and late regions encode proteins involved in virion assembly and production of infectious progeny virions (Harrach, 2008).

At the beginning of the infection, the E1A region is the first one to be transcribed. It encodes two proteins, 286R and 243R, that are known for regulating the host cell-cycle to allow viral replication, and acting as transcription activators for the early gene promoters (Avvakumov *et al*., 2002; Crisostomo *et al*., 2017). The early region E1B encodes two proteins, 19K and 55K, which suppress apoptosis (Debbas and White, 1993).

The E2 region encodes proteins required for DNA replication including DNA polymerase (DNA pol), DNA-binding protein (DBP), and terminal protein precursor (pTP) (de Jong *et al*., 2003).

Early region E3 encodes proteins that are not essential for viral replication, but mainly regulate the host immune responses (Orwitz, 2004). Proteins encoded by the E4 region of the viral genome have a variety of functions in different cellular and viral processes such as apoptosis, transcription, control of the cell cycle and regulation of the host innate antiviral response (Täuber and Dobner, 2001; Ullman and Hearing, 2008).

The accumulation of DBP, DNA pol, and pTP is a signal to start viral DNA replication. Replication of the genome does not require the host DNA helicase, and elongation is reliant on the viral DBP (Dekker *et al*., 1997). The origin of replication of this virus is situated at the extremes of the genome, within the ITR regions. DBP has an important role again because it can modify the conformation of the origin of replication, which enhances the ability of the DNApol to bind to it (Lindebaum *et al*., 1986; van Breukelen *et al*., 2003). After the assembly of a pre-initiation complex (PIC), which includes the viral proteins pTP DNApol and DBP, and cellular proteins such as NFI and NFIII, pTP serves as a primer to start the replication, where the DNApol has both polymerase (5’ to 3’) and exonuclease activity (3’ to 5’) (Bosher *et al.*, 1990; Challberg *et al*., 1980; Mul *et al.*, 1990; Nagata *et al*., 1983).

The DNA replication marks the transition between the expression of early and late genes of the adenovirus genome. Proteins IVa2 and pIX are considered intermediate genes, and they get activated when DNA replication is initiated. Protein IVa2 has very important roles in the viral life cycle since it participates in the activation of the major late promoter (MLP) and packaging of the virion (Lutz and Kedinger, 1996; Pardo-Mateos and Young, 2004; Tribouley *et al*., 1994; Zhang and Imperiale, 2003). On the other hand, protein IX is a small polypeptide that holds hexon proteins together in the viral capsid, increasing its stability and has also been shown to stimulate the MLP activity (Boulanger *et al*., 1979; Lutz *et al*., 1997). After DNA replication, the late region of adenovirus genome is transcribed as a single transcript using the major late promoter, which produces overlapping transcripts based on use of different poly A signals, additional sites, and alternate splicing (Backström *et al*., 2010; Ziff and Evans, 1978). These overlapping transcripts encode both structural and non-structural proteins. Although viral capsid proteins are synthesized in the cytoplasm of the infected cell, the assembly of the virus takes place in the nucleus, where progeny genomes are produced (Velicer and Ginsberg, 1970). The non-strutural 100K viral protein is responsible for assembly of the hexons trimers (Cepko and Sharp, 1982; Shah *et al*., 2017). Next step is the encapsidation of the DNA in the capsids, which requires a cis acting signal(s) located at the left end of its genome (Hammarskjöld and Winberg, 1980; Hearing *et al*., 1987). Several viral (IIIa, 52k, 22k, IVa2) and cellular proteins are involved in interacting with genome packaging DNA sequences to encapsidate adenovirus genome in empty capsids. (Ma and Hearing, 2011; Ostapchuk *et al*., 2011; Guimet and Hearing, 2013; Wu *et al*., 2013). Recent reports suggest that capsid formation and DNA encapsidation may occur simultaneously (Condezo and San Martín, 2017).

Once the genome is packaged inside the capsid, next step is the removal of non-structural proteins (52 K, 22 K, 33 K, 100 K). Recent studies have shown that the mechanism of cleavage of one of these proteins, NS 52 K, is DNA dependent and it causes the disruption of the interaction with this protein and other capsid and core proteins, which results in the absence of 52K in mature virions (Pérez-Berná *et al*., 2014). Finally, maturation of progeny virions is associated with cleavage of capsid and core precursor proteins (pIIIa, pVII, pVIII, pX 52K, and pTP) by adenovirus protease (Mangel and San Martin, 2014).

Recent reports have suggested that cleavage of pVIII (Gaba *et al*., 2017) and pVI (Moyer *et al*., 2017) appear essential for production of infectious progeny virions.

Finally, mature virions are released by cell lysis, with the aid of the Adenovirus death protein (ADP) (Tollefson *et al*., 1996).

1.1.2 Bovine adenovirus

In 1959, viral isolates from apparently healthy cattle were found to share antigenic properties with human adenoviruses (Klein *et al*., 1959). Recent reports suggest that bovine adenoviruses are present in high percentages in bovine population and can be detected in cattle feces, urine and, even in soil/water in feedlots and residential areas (Sibley, 2011).

1.1.2.1 Classification of Bovine Adenoviruses

Bovine adenoviruses belong to two different genera: *Mastadenovirus* (BAdV-1, -2, -3, -9 and -10) and are *Atadenovirus (*BAdV-4, -5, -6, -7 and -8). BAdV-1, -2, -3 and -9 are known for growing well *in vitro* and for cross-reacting in complement fixation tests, while members of subgroup 2 (BAdV-4, -5, -6, -7, -8 and -10) are harder to propagate in cell cultures and do not have any cross-reactivity with other mammalian adenoviruses antibodies (Bartha, 1969; King *et al.*, 2011).

1.1.2.2 Genome organization of BAdV-3

The complete genome of BAdV-3 is 34,446 bp in length, and possesses a G+C content of 54%; similar to other members of *Mastadenovirus,* BAdV-3 genome can be divided into early, intermediate and late regions (Fig. 1.3) (Reddy *et al*., 1998).

The E1 region is located at the left end of the genome which is divided into E1A and E1B (Reddy *et al*., 1999) sharing a common poly(A) site along with pIX (Zheng *et al*., 1994; Zheng *et al*., 1999). The E1A region is the first to be transcribed and produces three small non-structural proteins, which have a role in BAdV-3 replication and activation of viral genes (Reddy *et al*., 1999; Zheng *et al*., 1999; Zhou *et*

al., 2001b). In contrast, the E1B region encodes two overlapping mRNAs: $E1B^{small}$ and $E1B^{large}$ (Reddy *et al*., 1999) The $E1B^{small}$ and $E1B^{large}$ expresses a 19 kDa and 48 kDa protein, respectively in BAdV-3 infected cells (Reddy *et al*., 1999). While $E1B^{large}$ 48 kDa protein appears essential for virus replication, $E1B^{small}$ 19 kDa protein appear essential for virus replication only in certain cells (Zhou *et al*., 2001b).

Early (E) region 2 is composed of E2A and E2B regions and contain genes required for DNA replication (Reddy *et al*. 1998). DBP (DNA binding protein) encoded by E2A region is expressed as a 50 kDa protein from 6 hrs to 48 hrs post infection (Reddy et al., 1998; Zhou *et al*., 2001a). The detection of DBP in BAdV-3 infected cells using immunohistochemical methods can be used for titration of the virus (Zhou *et al*., 2001a). On the other hand, early region 2B encodes a terminal protein precursor (pTP) and the viral DNA polymerase (Baxi *et al*., 1998).

Region E3 appears to encode four proteins: 284R, 121R, 86R, and 82R that are not essential for virus replication (Idamakanti *et al*., 1999; Zakhartchouk *et al*., 1999). Protein 284R is a glycoprotein which does not show homology to proteins encoded by E3 region of members of other Mastadenoviruses (Idamakanti *et al*., 1999). In addition, protein 121R has been shown to inhibit apoptosis mediated by TNFα (Zakhartchouk *et al*., 2001).

E4 region has the potential to encode five ORFs. Of these, ORF1, ORF2, and ORF4 appear unique to BAdV-3 (Lee *et al*., 1998). Mutational analysis of this region showed that none of these ORFs are required for viral replication (Baxi *et al*., 2001).

Intermediate region encode two proteins named IVa2 and IX (Reddy *et al*., 1998). Protein IVa2 is a structural and shares up to 69% of identity with its counterparts encoded by other members of *Matadenoviruses* (Reddy *et al*., 1998). On the other hand, protein IX is minor structural protein which shares a low percentage of identity (16-28%) with homologs encoded by other members of *Mastadenoviruses* (Reddy *et al*., 1998; Zheng *et al*., 1999). Interestingly, the C-terminus of pIX, facing the exterior of the viral capsid, has been successfully modified to alter the tropism of the virus (Zakhartchouk *et al*., 2004), which has helped to enhance the transduction efficiency of the virus to a wider range of cells (Zakhartchouk *et al*., 2004).

The late (L) region, which encodes structural and non-structural proteins is transcribed as a single primary transcript using major late promoter. Subsequently, alternate splicing and usage of polyA addition signal produces overlapping transcripts which can be grouped into seven regions L1-L7 (Reddy *et al*., 1998). L1 region encodes proteins 52K, IIIa, III, and pVII.

Protein 52K, a non-structural protein expressed as 40 kDa protein, which localizes to nucleus using nuclear localization signal and importin α-3 (Paterson *et al*., 2012). The 52K protein interacts with protein pVII and redistributes the cellular NFκB-binding protein inside the nucleus (Paterson, 2010; Paterson *et al*., 2012). It has been suggested that the interaction between NFBP and 52K could possibly inhibit the cellular processing of rRNA and cause changes in the activity of NFκB in infected cells (Paterson, 2010). Moreover, 52K protein localizes to mitochondria using mitochondria localization signals (MLS), which coincide with an increase in ROS/SO production in transfected cells without major changes in mitochondrial Ca2+ or ATP production, indicating that 52K could be responsible for oxidative stress and apoptosis in BAdV-3 infected cells (Anand *et al*., 2014)

Protein IIIa is a structural protein (Reddy *et al*., 1998), and appears to be involved in stabilizing the capsid (Vellinga *et al*., 2005). In addition, protein III, also recognized as penton base protein, contains an "RGD" motif which is shown to be involved in adenovirus internalization and viral entry into the cell (Wickham *et al*., 1994). However, BAdV-3 penton base protein contains "MDV" motif instead of "RGD" motif, which suggests that BADV-3 may use an alternate mode of virus entry (Reddy *et al*., 1998).

Protein VII is localized in the core of the virus, and its sequence contains a protease cleavage site (Reddy *et al*., 1998) suggesting at pVII may be cleaved by adenovirus protease. Recent study has shown that pVII localizes to the mitochondria using a mitochondrial localization signal localized at the N-terminus of pVII (Anand *et al*., 2014). pVII appears to modulate the mitochondrial activity of the infected cell and inhibits apoptosis (Anand *et al*., 2014).

Protein V is a structural protein (Reddy *et al*., 1998) which appears essential for maintaining the integrity of BAdV-3 virion capsids and production of infectious virions (Zhao and Tikoo, 2016). Protein

pV localizes to nucleus using three nuclear localization signals and importin α3 (Zhao 2016). pV also contains two nucleolar localization signals, which appear essential for production of progeny virions (Zhao, 2016).

L3 and L4 regions encode just one protein each: protein X and VI, respectively (Reddy *et al*., 1998). The protein X of BAdV-3 is rich in basic amino acids, and it contains one nuclear localization signal and two protein cleavage sites, which suggests that it is cleaved by the viral protease (Reddy *et al*., 1998). pVI is a structural protein, and it contains two consensus sequence motifs for cleavage by endoproteases (Reddy *et al*., 1998). Although its function is not well understood in BAdV-3, the precursor of this protein has been related to transport of hexon capsomers to the nucleus in HAdV (Kauffman and Ginsberg, 1976).

The L5 region of BAdV-3 encodes the hexon protein and a protease (Reddy *et al*., 1998). Hexon is a major component of the capsid, and it is a potent adjuvant for activation of cell-mediated immunity in HAdV (Molinier-Frenkel *et al*., 2002). The protease is essential for the attainment of infectious virions since many viral protein precursors need to be cleaved by this protein for maturation (Cai *et al*., 1990; Reddy *et al*., 1998).

Non-structural proteins 100K, 33K, and 22K, together with the structural protein pVIII are encoded in L6 region of the BAdV-3 genome (Reddy *et al*., 1998). The 100K protein is localized in the cytoplasm and nucleus of infected cells (Makadiya *et al*., 2015) and it interacts with protein 33K (Kulshreshtha and Tikoo, 2008). Although cleavage by viral protease is required for nuclear localization of 100K in BAdV-3 infected cells, this is not essential for replication (Makadiya *et al*., 2015). 33K and 22K are produced by alternative splicing of a single transcript and share the same N-terminal (Kulshreshtha *et al*., 2015). 33K plays a major role in the activation of the major late promoter (Kulshreshtha *et al*., 2015), while protein 22K appears to be necessary for viral replication (Kulshreshtha *et al*., 2004).

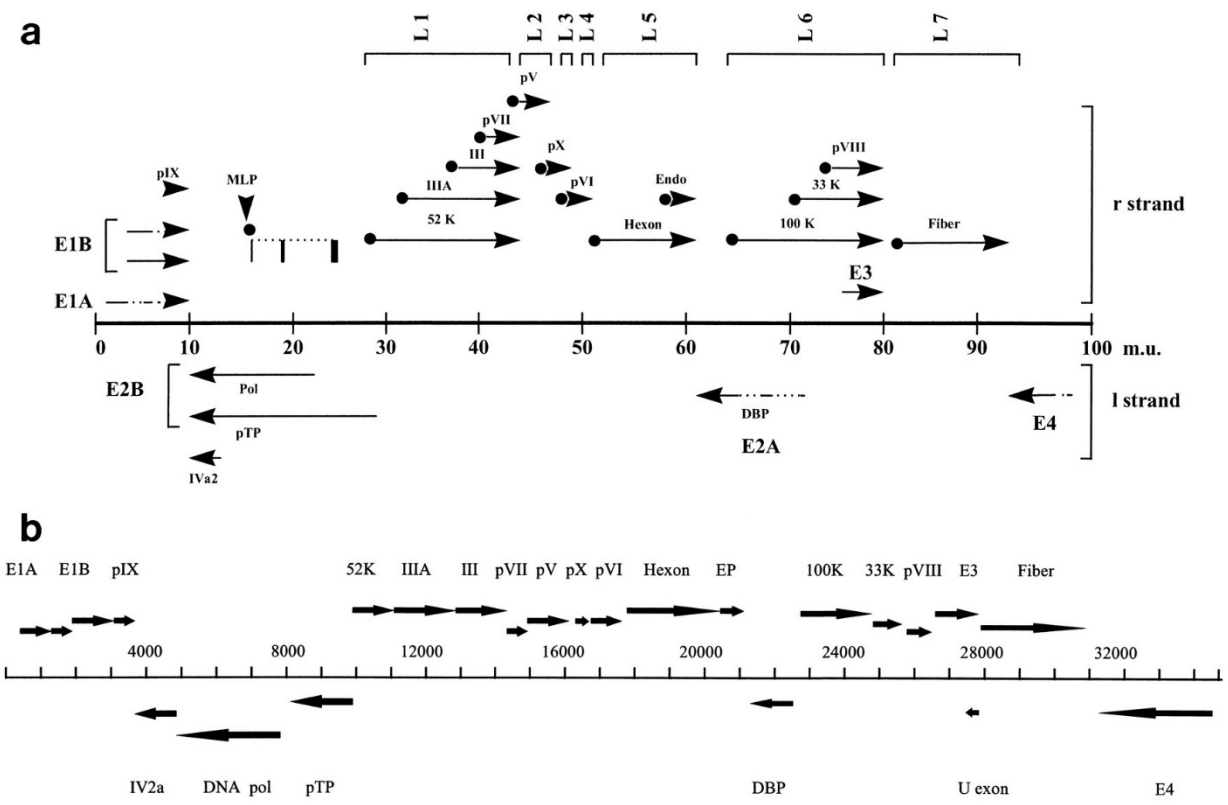

Figure 1.3 Schematic representation of Bovine Adenovirus-3 genome. Reproduced with permission from Fig. 1 in (Reddy *et al*., 1998). *Nucleotide Sequence, Genome Organization, and Transcription Map of Bovine Adenovirus Type 3.* Place: American Society for Microbiology.

Minor structural protein VIII is localized in the nucleus of infected cells and interacts with importin α3 (Ayalew *et al*., 2014), and its two protease cleavage sites are vital for replication (Gaba *et al*., 2017).

Finally, the L7 region encodes the fiber protein, which is long and bent in BAdV-3 virions (Ruigrok *et al*., 1994)., The knob region of fiber is involved in the initial attachment to host cell receptor, and interacts initially with cellular receptor (sialic acid) (Bangari *et al*., 2005). The fiber protein is expressed as 102- kDa protein in infected cells (Wu and Tikoo, 2004). The fiber protein is localized to the nucleus of infected cells using nuclear localization signal (amino acids 14-20) and fiber protein seems essential for the successful replication of the virus (Wu and Tikoo, 2004).

1.1.2.3 Viral attachment and internalization

The primary interaction between the viral particle and the cell is through the attachment of virion using knob region of fiber to a cell surface receptor. For instance, in HAdV-5, the fiber knob interacts with Coxsackievirus-adenovirus receptor (CAR) in the cell (Bergelson *et al*., 1997), followed by a secondary interaction between the penton base of the virus and integrins in the surface of the cell (Sharma *et al*., 2009). In contrast, BAdV-3 does not interact with CAR for attachment to the cell, but it uses sialic acid as a primary receptor instead (Bangari *et al*., 2005; Li *et al*., 2009). Moreover, due to the lack of an RGD motif in its capsid, it does not utilize the integrin pathway for internalization (Reddy *et al*., 1998). Interestingly, BAdV-3 penton protein contain "MDV" motifs instead (Reddy et al., 1998). Although little has not been reported regarding different steps of BAdV-3 replication, it appears that once internalized, BAdV-3 replication follows steps similar to HAdV-5 (Flint *et al*., 2009).

Level of relative transduction	Cell line	Species	Origin	CAR expression
Higher than HAdV-5 (≥101%)	Jurkat	Human	T cell leukemia	Below detection level
	NIH 3T3	Murine	Mouse fibroblast	Below detection level
	FBRT HE1	Bovine	Fetal retina transformed with HAdV-5 E1	High
	MDBK	Bovine	Kidney	High
	FBK-34	Bovine	Kidney transformed with BAdV-3 E1	High
	BT-5705	Bovine	Turbinate	Below detection level
	EBL	Bovine	Embryonic lung	Below detection level
	PK-15	Porcine	Kidney	High
	PT-K75	Porcine	Turbinate	Below detection level
	SBC-1765	Porcine	Derived from buffy coat cells	Below detection level
	EPL	Porcine	Embryonic lung	Below detection level
High (50-100%)	293	Human	Embryonic Kidney	High
	MTIA2	Murine	Mammary tumor transformed with SV40 T antigen	High
	WEHI	Murine	Fibrosarcoma	Below detection level
	EL4	Murine	T cell	High
	FPRT HE1-5	Porcine	Fetal retina transformed with HAdV-5 E1	High
	PK-M12	Porcine	Kidney cell transformed with SV40 T antigen	Moderate
Moderate (25-49%)	MCF-10A	Human	Mammary epithelium	Moderate to low
	AML12	Murine	Liver	Below detection level
	MS-K68	Murine	Spleen	Moderate
Low (15-24%)	MDA-MB-231	Human	Malignant breast cancer cell	Moderate to low
	PC-3	Human	Prostate cancer metastases site	Below detection level
	LNCaP	Human	Prostate cancer metastases site	High
	DU145	Human	Prostate cancer metastases site	High
Very low (≤14%)	HepG2	Human	Hepatocarcinoma	High
	T24	Human	Transitional cell carcinoma	Below detection level
	TCCSUP	Human	Transitional cell carcinoma	Below detection level

Table 1.1 Relative transduction of BAdV-3 compared to HAdV-5 in cell lines from a variety of origins and CAR expression levels (Bangari *et al*., 2005).

1.1.2.4 Tropism

The tropism of HAdV-5, the most studied adenovirus, is thought to be mostly dependent on the expression of the coxsackie-adenovirus receptor (CAR) in the cell surface; therefore, cells lines expressing a high quantity of CAR will be transduced more efficiently by HAdV-5 (Bergelson *et al*., 1997).

BAdV-3 tropism is independent of CAR, which explains its ability to efficiently transduce even CAR-deficient cells. Although BAdV-3 transduces bovine and porcine cells, human cells are poorly transduced (Table 1.1) (Bangari *et al*., 2005; Rasmussen *et al*., 1999; Wu and Tikoo, 2004).

1.2 Bovine immune system

The immune system in cattle is composed of different tissues and organs: the thymus, spleen and lymph nodes (Suleymanov, 2005). The thymus undergoes morphological and functional changes during the lifetime of the animal (Gazizova, 2001) and is a critical organ of the immune system (Galaktionov, 2005). Furthermore, it has endocrine functions, producing hormones involved in the maturation of pre T-cells into functional T-lymphocytes (Gazizova and Murzabekova, 2007; Gazizova and Atkenova, 2015). The central organ of the embryonic immune system, the thymus, is where lymphoid cells are formed and mature, and where the processes that occur in the peripheral lymphoid organs, spleen, and lymph nodes, are regulated in the early stages of life in calves (Drannik, 2003). As the host ages, lymphopoiesis decreases gradually in these organs, although some functional tissue in the thymus still remains active in animals of old age (Bodrova, 2010).

In addition, Peyer's patches constitute one of the primary sources of systemic B-cells in ruminants, and they serve as a major site for B-cell proliferation and antigen-independent somatic hypermutation of immunoglobulins (Griebel and Hein, 1996).

Besides the thymus and Peyer´s patches, lymphocytes are also produced in the bone marrow and then travel in the bloodstream until reaching peripheral lymphoid organs (Gridnev and Mironov, 2003). Antigenic exposure in these organs causes complex interactions between immune cells that trigger lymphocyte differentiation, proliferation and transformation of B and T cells into effector cells (Kondrakhin, 2004).

The spleen participates in the production of IgM, synthetizes tuftsin tetrapeptide that stimulates NK cells, promotes phagocytosis and enhances the activity of macrophages (Kapitonov *et al*., 2005; Kierszenbaum and Tres, 2015; Lustig *et al*., 2007).

The system in the bovine can be further divided into two components: the innate and the adaptive immunity that work together with different mechanisms to recognize and eliminate pathogens that threaten the animal's health (Carroll and Forsberg, 2007).

1.2.1 Innate immunity

Contrary to the adaptive immune system which is present only in highly complex organisms such as vertebrates, the innate immune system is found in all multicellular organisms (Pancer and Cooper, 2006). There is cellular, but also a humoral component in innate immunity, where proteins such as complement, C-reactive protein, LPS binding protein, and other antimicrobial peptides facilitate the clearance of the potential infection (Turvey and Broide, 2010).

The cells involved in the innate immune system originate from from hematopoietic origins (e.g. dendritic cells, mast cells, macrophages, neutrophils, basophils, eosinophils and NK cells) and non-hematopoietic origin (e. g. epithelial cells composing different organs, like the skin and gastrointestinal and respiratory tracts) to prevent pathogens from infecting the host (Turvey and Broide, 2010).

The innate immune system identifies potentially harmful elements through soluble proteins or cell surface receptors (Stahl, 1992). This system relies on three main strategies to recognize pathogens. The first example is the molecules recognized by the pattern recognition receptors, which are known as pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). The second kind of molecules

recognized by the innate immune system is the damage-associated molecular patterns (DAMPs), which are molecules that are upregulated in tissue damage and cell lysis when inflammation and infection occur (Bianchi, 2007). Finally, there are certain molecules that are not expressed in infected cells or in pathogens, but are present in healthy cells; the innate immune system can recognize the lack of these molecules: for example, NK cells can detect the downregulation of MHC class I molecules in the cells due to an infection and attack them (Joncker and Raulet, 2008).

1.2.1.1 Pattern Recognition Receptors

The detection of pathogens through innate immunity is achieved using a variety of pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are usually found in the plasma membrane and endosome (Kaisho and Akira, 2006). These receptors recruit adaptor proteins that finally activate nuclear factor kappa b (NF-κB), interferon regulatory factors, and other molecules that will culminate with the expression of a variety of cytokines, chemokines, and interferons, and interfere with the cell metabolism (Honda *et al*., 2005).

The second class of innate sensors, known as RIG-I like receptors (RLRs), which are particularly important as anti-viral defenses, recognize foreign cytosolic RNA from viral origins (Yoneyama *et al*., 2004) and induce the expression of type I interferons and antiviral cytokines (Kawai *et al*., 2005; Meylan *et al*., 2005).

The nucleotide oligomerization domain-like receptors (NLRs) detect PAMPs and DAMPs (Martinon *et al*., 2009) and many of them are responsible for inflammatory responses after sensing pathogens like bacteria or viruses, activating caspase-1, that will finally lead to the activation of pro-inflammatory cytokines IL-1β and IL-18 (Martinon *et al*., 2002).

Another group of innate sensors is the C-type lectin receptors (CLRs). This group is composed of three members: mannose receptor (MR), Dectin-1, and dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN), which are all expressed in the plasma membrane of immune cells and recognize mainly fungal pathogens (Areschoug and Gordon, 2008).

In addition to TLR, RLR, NLR, and CLR receptors, there are cytosolic DNA sensors such as DAI (DNA-dependent activator of IFN-regulatory factors) and IFI16 that lead to the production of type I interferons (Takaoka *et al*., 2007; Tsuchida *et al*., 2010), and AIM2 (absent in melanoma 2) that can initiate the inflammasome formation, leading to the production of inflammatory cytokines such as IL-1β and IL-18 (Jones *et al*., 2010).

1.2.1.2 Antigen presentation

Although theoretically, any cell subtype expressing MHC molecules could act as an antigen presenting cell, only professional APCs, such as DCs, B-cells, and macrophages can successfully stimulate T-cells. Professional APCs have certain characteristics that make them effective to initiate adaptive immune responses, including the capacity to process antigens and generate antigenic determinants, form complexes between MHC molecules and antigens, express co-stimulatory molecules and secrete cytokines and adhesion molecules that will stimulate T-cells (Kim *et al*., 2004).

To initiate an adaptive immune response, the first step involves the recognition of a peptide-major histocompatibility complex class II (pMHC class II) on the surface of an APC by T-cells located mostly in secondary lymphoid organs, since they rarely have access to body compartments other than blood, lymph nodes, mucosal lymphoid tissues and spleen (Banchereau and Steinman, 1998; von Andrian and Mackay, 2000; Reinhardt *et al*., 2001). The site of entrance of the antigen to the body can determine where the T-cell response will begin; antigens entering the skin will be transported to the peripheral lymph nodes, while pathogens internalizing through mucosal surfaces or blood, will be presented to T-cells in mucosal lymphoid organs or spleen, respectively (Jenkins *et al*., 2001).

Three main cell lineages expressing MHC class II have been identified in murine lymph nodes: macrophages, B-cells and dendritic cells (DCs) (Witmer and Steinman, 1984). However, macrophages are unlikely to act as initial APCs, because they usually locate in non-lymphoid tissues and areas where naïve T-cells are not present (Witmer-Pack *et al*., 1993). Moreover, studies in macrophage-deficient mice have

shown that these animals can still present T-cell dependent immune responses, which suggests that macrophages are not essential for the initiation of T-cell responses (Chang *et al*., 1995).

On the other hand, even though B-cells are able to express MHC class II on their surface, they are not efficient at taking up antigens and activating T-cells (Lanzavecchia, 1985; Itano *et al*., 2003). Studies in B cell-deficient have also shown that antigen-specific T-cell priming after immunization *in vivo* is not affected by the lack of these cells (Epstein *et al*., 1995; Topham *et al*., 1996).

Since macrophages and B-cells are proven not essential for initial activation of naïve T-cells, it can be inferred that DCs are the only subpopulation of cells in the lymph nodes expressing MHC class II capable of priming naïve T-cells *in vivo* (Itano and Jenkins, 2003). This is supported by the evidence of studies showing that DCs are capable of efficiently taking up antigens like ovalbumin, ovalbumin-specific T-cells can be found around them in 12-24 hrs, indicating that there is a direct interaction between them for antigen presentation (Ingulli *et al*., 1997; Byersdorfer and Chaplin, 2001).

In the absence of systemic illness or inflammation, DCs patrol the organism through the blood, are able to process antigens from a variety of origins and load them onto MHC class I and II molecules, for antigen presentation (Banchereau *et al*., 2000). However, immature DCs are not efficient APCs, and they need signals from pathogens and inflammatory molecules (e.g. cytokines) to mature and be able to successfully activate naïve T-cells (Banchereau and Steinman, 1998; Banchereau *et al*., 2000; Bell *et al*., 1999). Maturation of DCs not only involves modifications such as increased half-life and expression of MHC and costimulatory molecules but also their migration to secondary lymphoid organs, where T-cells are located (Gallucci and Matzinger, 2001).

On the other hand, monocytes usually are not considered an APC, but just a precursor of certain populations of macrophages and DCs (Randolph *et al*., 2008). However, it has been demonstrated that monocytes can take up antigens, retain them and present them on MHC II molecules after maturation, similarly to DCs (Tacke *et al*., 2006; Delamarre *et al*., 2005). If monocytes are able to capture antigens while they are in the bone marrow or in the blood, they could possibly act as vehicles, and present a wide variety of antigens in different tissues after they differentiate into DCs (Randolph *et al*., 2008).

1.2.2 Adaptive immunity

The adaptive immune system originated and evolved in order to be able to protect complex organisms from mutating pathogens and to be able to recognize an extensive variety of antigenic structures (Cooper and Alder, 2006). Contrary to the innate immune system, where all the recognition receptors are encoded in the germline genome, the adaptive immunity relies on highly specific receptors generated from somatic recombination of a wide variety of gene segments (Murphy and Weaver, 2016). These receptors remain for the lifetime of the host and will be able to elicit an expeditious response in re-infection, due to the immunologic memory (Janeway *et al*., 2005).

The primordial cells of the adaptive immune system are T- and B-lymphocytes, which develop in the primary lymphoid organs, bone marrow and thymus, . These cells will migrate to the spleen and lymph nodes (secondary lymphoid organs) where they will orchestrate adaptive immune responses influenced by innate immunity signals from pathogens or activated APCs (Bonilla and Oettgen, 2010). Once activated, B- and T-cells travel through the organism to different tissues guided by different chemokines and adhesion molecules (Capra *et al*., 1999).

1.2.2.1 T-cell immune response

The process of antigen recognition by T-cells is started by an immunological synapse between the T-cell receptors (TCR) and costimulatory molecules expressed on APCs. These events will trigger a signaling cascade that could recruit naïve T-cells to the site, depending on the level of stimulation (Dustin and Cooper, 2000). The synapse efficiency depends on three main factors: a) the number of complexes between MHC and antigen in APCs, b) the level of costimulatory molecules expressed, and c) the duration of the signaling process, determined by the stability of the synapse (Lanzavecchia and Sallusto, 2001).

The T-cell response is not homogeneous, and it varies depending on the developmental stage and the kind of APC priming the cells. Activated effector and memory T-cells are very efficient in responding

to low doses of antigens, and they do not necessarily require co-stimulation. On the other hand, naïve T-cells are only activated in the presence of costimulatory molecules, or non-physiological high doses of antigen. To exemplify this, naïve T-cells could require more than 30 hours of TCR stimulation for activation, while effector or memory T-cells could be activated in 0.5 to 2 hours (Lanzavecchia and Sallusto, 2001). Activated T-cells produce IL-2 in response to antigenic stimulation, which will enhance their proliferation.

Although all T-cells share the same mechanisms of activation, they differentiate into a variety of effector and intermediate cells, depending on the duration of TCR stimulation and the cytokines produced (Lanzavecchia and Sallusto, 2000). Effector cells play different roles in the immune response, killing infected cells in order to eliminate pathogens, while helper and regulatory T-cells enhance B- and T-cell immune responses and limit the extension of the inflammatory response, respectively (Bonilla and Oettgen, 2010).

Helper T-cells (T_H), members of the CD4+ αβ TCR population can be divided into T_H1 and T_H2 depending on the cytokines they produce after being activated, which are mostly mutually exclusive. While T_H1 cells produce IFNγ and IL-2 that lead to cell-mediated immunity with the activation of phagocytes, NK cells and cytolytic T-cells that destroy infected cells, T_H2 cells produce IL-4, IL-5, IL-10 and IL-13, that enhances the antibody production and regulate immunity against parasites and hypersensitivity (Mossman *et al*., 1986). When T_H1 and T_H2 are differentiated, they leave the lymph nodes and are able to migrate to different tissues to act as effector cells (Lanzavecchia and Sallusto, 2001).

Previously, all T-cell immune responses were thought to be driven by either T_H1 or T_H2 cells. However, newly discovered subsets such as T_H9 and T_H17 (Steinman, 2007) have been identified. T_H9 cells, resulting from exposure of T_H2 cells to IL-4 and TGFβ, produce IL-9, which is involved in anti-parasitic immunity and acts as a mast-cell growth factor (Veldhoen *et al*., 2008). In addition, follicular T-helper cells (T_{FH}) are memory CD4+ cells that drive B-cell activation in germinal follicles (Bonilla and

Oettgen, 2010). T_H17 cells produce mostly pro-inflammatory cytokines from the IL-17 group (IL-17A-IL-17F) and are important in the pathogenesis of autoimmunity and asthma (Wang *et al*., 2008).

Another group of cells involved in T-cell immunity are regulatory T-cells (Treg) (part of the CD4+ αβ TCR population), which have a critical role in a very complex system that regulates T-cell responses (Chatila, 2005).

One of the largest groups of circulating T-cells are CD8+, which recognize cells containing intracellular pathogens. Cytolytic T-lymphocytes (CTLs) recognize cytosolic antigens through its TCR and produce immunologic synapse with the MHC class I molecules of the infected cell, which leads to apoptosis in the target cell mediated by CTL granules (Bonilla and Oettgen, 2010).

There is a very small subset of T-cells that recognize antigens using non-classical MHC molecules. These cells express γδ TCRs, and seem to be particularly important in the response against mycobacterial infections (D'Souza *et al*., 1997).

Finally, NK cells, another T-cell subset, recognize antigens presented by non-classical MHC molecules, similarly to γδ-cells. When activated, these cells produce a high amount of cytokines, which links them to various inflammatory processes including allergies and asthma (Meyer *et al*., 2008).

1.2.2.2 B-cell immune response

The adaptive B-cell immune response or humoral immunity is mediated by B-cells that differentiate into plasma cells and produce antibodies as a response to antigens with the help of Th2 type cells (Bonilla and Oettgen, 2010). For B-cell development, the rearrangement of V, D, and J gene segments in the H chain, and V and J genes in the L chain loci of Ig are indispensable (Alt *et al*., 1986).

Antibody production can be initiated in mature B-cells in different ways, depending on the involvement of T-cells in the B-cell activation (Bonilla and Oettgen, 2010). T-independent (TI) antigen responses are meant to provide a rapid response, even when T-cell recruitment has not been initiated. It can be divided into two groups, TI type 1 and type 2. While TI type 1 antigens by themselves can induce

cell proliferation and antibody production in mature B cells (Richards *et al*., 2008), TI type 2 antigens need to be recognized by B-cell receptors in conjunction with dendritic cells or cytokine signals (Vos *et al*., 2000). T-cell dependent antibody responses are much more common and involve antigens known as "T-dependent." In this case, B-cells receive a first signal from the antigen, which will activate intracellular signaling pathways allowing them to interact with T-cells in secondary lymphoid tissues and receive a secondary signal for activation. Like APCs, B-cells can express processed antigens in their surface, in a complex between the peptide and MHC class II molecules, and they can interact with the mature B-cells, and activate them for further differentiation into memory or plasma cells (Batista and Harwood, 2009). The interaction between T- and B-cells is similar to the interaction that occurs between T-cells and dendritic cells. Moreover, B-cells can express the same costimulatory molecules as dendritic cells, such as CD40, CD80, and CD86 (Hartmann and Krieg, 2000).

Activated B cells can follow one of 2 pathways: to become plasma cells and produce low-affinity antibodies for a reduced period of time or to establish a germinal center after entering follicles in secondary lymphoid tissue (Batista and Harwood, 2009). In the germinal center, B-cells can go through a process called "class-switching," where they can change the production of antibodies from IgM or IgD to IgA, IgG or IgE, depending in part to the type of cytokines they are exposed to (Stavnezer *et al*., 2008; Oettgen, 2000). Along with class switching, there is another process occurring at the same time known as SHM (somatic hypermutation), where nucleotide substitutions in the sequences of the heavy and light chains of immunoglobulins lead to point mutations, causing affinity maturation that will finally result in antibodies with higher affinity for the antigen (Steele, 2009; Peled *et al*., 2008).

In the primary immune response, occurring after the first exposure to an antigen, IgM low-affinity antibodies are produced initially while the production of isotypes with higher affinity for the antigen, such as IgA, IgE and IgG can take 15 days or longer. In this first exposure, memory T- and B-cells are generated. If the immune system is challenged with the same pathogen for the second time (secondary response), the immune response will be faster, and the production of different high-affinity immunoglobulin isotypes is established in the first few days after infection (Bonilla and Oettgen, 2010).

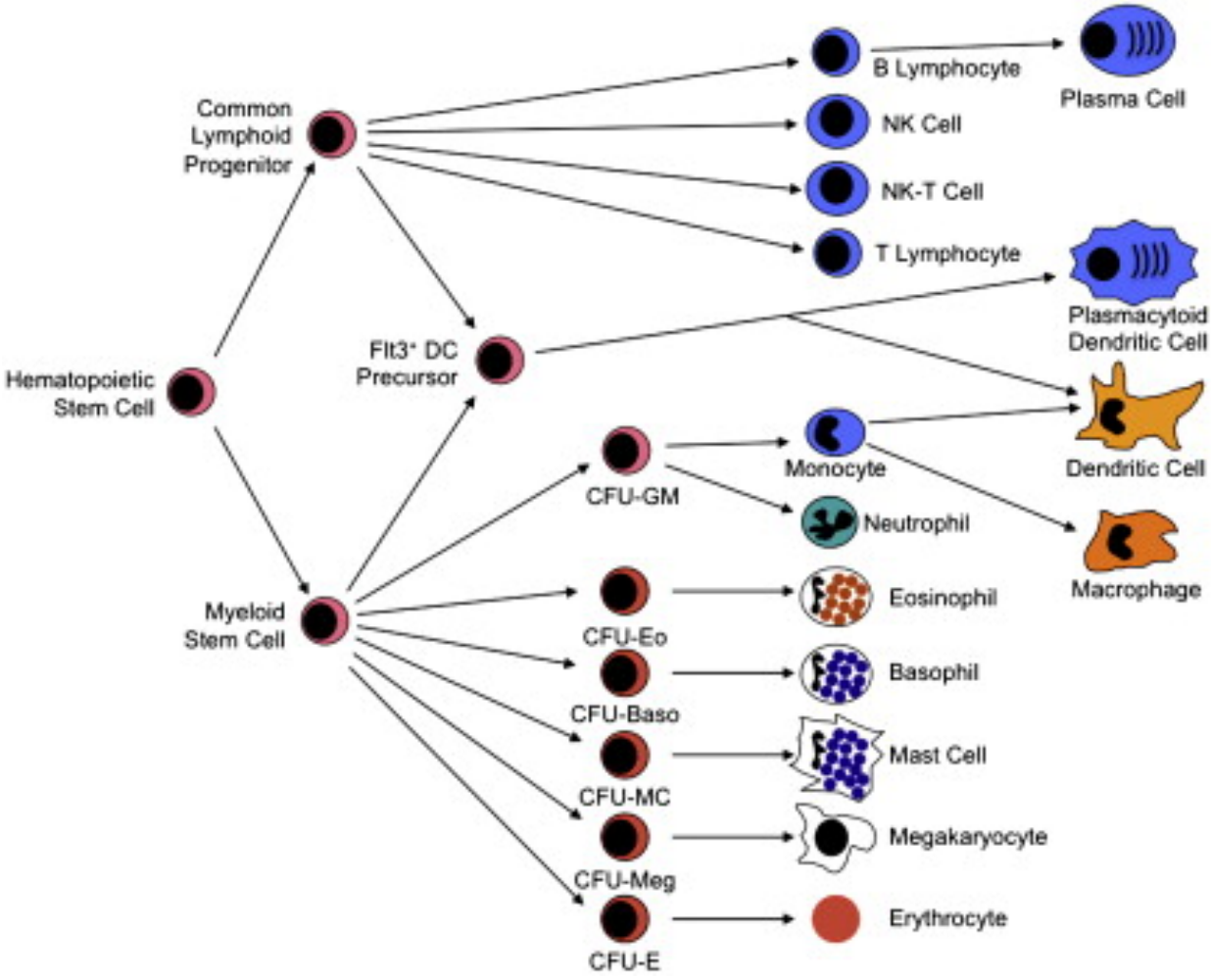

Figure 1.4 Schematic representation of cell lineages derived from a common hematopoietic stem cell predecessor. Reproduced with permission from Fig. 1 in (Chaplin, 2010). *Overview of the immune response*. Place: Elsevier.

1.2.3 Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) are the cellular components of blood that contain a round nucleus, such as lymphocytes, monocytes, dendritic cells, and macrophages. Other cellular components like erythrocytes and platelets are excluded from this group, since they have no nuclei, and granulocytes (eosinophils, basophils, neutrophils) that have a multi-lobed nucleus (Delves *et al*., 2001).

1.2.3.1 Lymphoid cells

The common lymphoid progenitor, developed from hematopoietic stem cells, can differentiate into a variety of populations of lymphocytes: T-cells, B-cells, NK cells and NK-T cells (Figure 1.4), each with a distinctive surface phenotype. B-cells express membrane-anchored immunoglobulins that act as receptors for the antigens, although there are several subtypes of these cells, depending on the types of antibody they can produce and the antigens to which they react. T-cells can be recognized because they express TCR on their surface to interact with APCs, while NK cells lack either TCR or immunoglobulins on their surface, but they are distinctively large and granular. Finally, NK-T cells are distinguished from NK and T-cells, because they share characteristics of both of those populations (Brignier and Gewirtz, 2010; Balato *et al*., 2009).

1.2.3.1.1 B cells

In 1948, plasma cells were suggested to be responsible for antibody production, after the first identification of serum gammaglobulins (Tiselius and Kabat, 1938) that led to the discovery of B-cells (Fagraeus, 1948). B-cells are produced in the bone marrow and go through a series of developmental stages that allow them to acquire antigen specificity, even though these cells lack contact with the exogenous antigen through their development in a process called "antigen-independent B-cell development" (LeBien and Tedder, 2008). Immature B-cells, characterized mostly in mice, are also known as transitional cells (T1, T2) acquire cell surface markers CD21 and CD22, as well surface IgD when they exit the bone marrow (Chung *et al*., 2003). These cells can get activated in the presence of T

cell-independent antigens, such as lipopolysaccharides (Coutinho and Möller, 1975). However, once B-cells encounter antigen, they go through an "antigen-dependent phase" of development, get activated and become either a memory cell or a plasma cell depending on the stimuli they receive (Desiderio, 1992). A combination of cytokines and transcription factors will command the B-cell development; for example in mice, IL-7 promotes the rearrangement of V, D and J genes, and sends signals needed for proliferation and survival of B-cells (Milne and Paige, 2006). At least 10 transcription factors are involved in B-cell development, although Pax5, E3A, and EBF are the best known for being involved in B-lineage commitment and B-cell differentiation (Nutt and Kee, 2007).

After maturation, B-cells reside mostly in gut-associated lymphoid tissue (GALT) or in lymphoid follicles in lymph nodes and spleen, where they can encounter T cell-dependent antigens, proliferate and differentiate into plasma cells or start GC reactions. B-cells are activated and differentiate in secondary lymphoid tissues in a series of events named germinal center (GC) reaction, where class switch recombination (CSR), somatic hypermutation (SHM) and affinity maturation of BCR for specific antigens occur (LeBien and Tedder, 2008).

Long lasting antigen-specific antibody responses require long-lived plasma cells. Plasma cells are generated in the spleen and eventually migrate to the bone marrow, where they can live for as long as the lifetime of the individual. They can be produced by primary and secondary immune responses (Radbruch *et al*., 2006). There are plasma cell pools in the spleen and bone marrow. When depleted, they are replenished from the pool of memory B-cells, that can be differentiated into long-lived plasma cells and produce antibodies if they sense certain cytokines or Toll-like receptor signals (DiLillo *et al*., 2008).

Even though B-cells are known for their role in humoral immunity, they also have other functions related to immune homeostasis. B-cells can initiate T-cell immune responses by processing and presenting antigens to T-cells through MHC, although this activation can produce different immune responses than the one started by DCs (LeBien and Tedder, 2008). Furthermore, B-cells can produce cytokines that will influence T-cell differentiation (Harris *et al*., 2000). B-cells can also secrete immunomodulatory cytokines that regulate a variety of functions like tumor development and immunity, tissue rejection, and

wound healing among others (LeBien and Tedder, 2008). In addition, there is a specific subset of regulatory B-cells that produce IL-10 and regulate inflammatory responses mediated by T-cells (Yanaba *et al*., 2008).

1.2.3.1.2 T cells

T-cells are able to recognize antigens when their TCR interacts with the MHC-antigen complex displayed by APCs (Germain, 1994; Davis *et al*., 1998). This interaction initiates T-cell proliferation and further differentiation into a variety of cells that can determine the type of immune response that will follow. CD4+ T-cells can differentiate into T_H1 or T_H2 that can protect against intracellular or extracellular microorganisms and will produce different cytokines: IFNγ or IL-4, IL-5, and IL-13, respectively (Abbas *et al*., 1996; Romagnani, 1994). When CD8+ T-cells differentiate into cytotoxic T-cells, they gain the capability of detecting and destroying virus-infected cells (Zinkernagel, 1996). There are also some subsets of regulatory T-cells, such as T_H cells that visit B-cell areas to trigger T cell-dependent antibody responses or T-cells that can down-regulate immune responses producing inhibitory cytokines (Faria and Weiner, 1999).

The primary response against an antigen can generate T-cells that can survive as memory cells (for years), which can get activated and, provide fast and effective immune responses in case of re-infection with the same pathogen (Ahmed and Gray, 1997; Dutton *et al*., 1998).

In bovine, γδ T-cells are one of the most important subsets of circulating lymphocytes (15-60%) (Davis *et al*., 1996). These cells can be activated without the presence of MHC-peptide complexes, and perform varied functions, such as antigen presentation, cytokine production and immune regulation (Pang *et al*., 2012; Kabelitz *et al*., 2013). γδ T-cells seem to have a role in immune suppression in ruminants (Rhodes *et al*., 2001; Brown *et al*., 1994). Bovine γδ T-cells have an important regulatory role, secreting IL-10 spontaneously that will inhibit proliferation of CD4 and CD8 T-cells *in vitro*, in cells activated in either antigen-specific or non-specific pathways (Guzman *et al*., 2014). In contrast, regulatory T-cells

(Tregs) are vital for the immune system balance, and they usually express CD4, CD25, and Foxp3 (Hori *et al*., 2003). However, $CD4^{+}CD25^{high}$ $Foxp3^{+}$ T-cells in the bovine do not seem to have a regulatory function *in vitro* (Hoek *et al*., 2009).

1.2.3.1.3 NK cells

Natural killer (NK) cells were first identified because of their large size, granularity, and due to their ability to provoke lysis in tumor cell lines (Trinchieri, 1989). Natural killer cells are known for protecting the organism against a great variety of pathogens and cancers. Their role in the innate immune responses is to fight primarily against viruses, in an NK cell-mediated toxicity induced by type I IFN (Biron *et al*., 2002).

NK-cells can be divided into two groups: classical NK-cells and NKT-cells. Classical NK-cells do not express TCR, are developed in the bone marrow and, are mostly present in the bloodstream and spleen (Biron *et al*., 1999). On the other hand, NKT-cells are found within T-cell populations, in small numbers in the thymus and spleen, but in a significant number in the liver. NKT-cells express TCR although they have a restricted repertoire (Eberl *et al*., 1999).

Classical NKs play an important role in anti-viral immunity, not just through NK cell-mediated cytotoxicity but also by producing IL-12 that will stimulate IFNγ production, which is known for having direct anti-viral functions and an activator of pathways that will promote protective immune responses (Biron *et al*., 2002). NK-cells can also produce TNF and trigger chemokine secretions that promote inflammatory processes and attract other cell populations (Bluman *et al*., 1996).

1.2.3.2 Myeloid cells

Myeloid stem cells or common myeloid progenitors are the precursor cells of erythrocytes, platelets, megakaryocytes, and the cells of the granulocyte lineage, such as neutrophils, eosinophils, basophils, monocytes, macrophages and mast cells (Figure 1.4). These cells are very important in the innate immune system because they can trigger the adaptive immune system acting as APCs. They have

phenotypic and functional differences based on their location and the variety of tissues they have been seen previously (Serbina *et al.*, 2008).

1.2.3.2.1 Monocytes

Monocytes are not just precursors for dendritic cells and macrophages, but they also have an active role in the innate immune system, especially in infection and inflammation (Lavau *et al*., 2014). They have been characterized based on their phagocytic, pathogen-sensing, ability to produce cytokines/chemokines and ability to present antigens to T-cells (Geissmann *et al*., 2010).

Monocytes in humans and mice are divided into three subpopulations depending on their expression of CD14 and CD16 that share similar phenotypical and functional characteristics between the two species (Ziegler-Heitbrock, 2007). The largest monocyte subpopulation is usually designated as "classical" (cM), and the smallest group is referred to as "non-classical" (ncM), while the level of markers in the intermediate population (intM) is not well defined (Ziegler-Heitbrock, 1996).

Studies in mice have shown that the classical monocyte subpopulations have an active role in inflammation and in containing infections as they can be found at the site of infection attracted by cytokine and chemokine signals, to later be differentiated into dendritic cells and macrophages (Wong *et al*., 2011). In humans, classical monocytes respond in a similar way to TLR ligands, leading to upregulation of pro-inflammatory cytokines and IL-10, although intermediate monocytes could be responsible for IL-10 production (Wong *et al*., 2012). Alternatively, non-classical monocytes are mostly located in the blood vessels, patrolling the endothelium, and express mostly tumor necrosis factor alpha (TNFα) (Frankenberger *et al*., 1996; Belge *et al*., 2002).

In bovine, monocytes express CD172a (signal-regulatory protein alpha) among all subpopulations, although they are commonly subdivided into three subpopulations depending on the expression of CD14 and CD16, similarly to humans: cM are $CD14^{++}$ $CD16^{-}$, intM are $CD14^{++}$ $CD16^{+}$, and ncM are $CD14^{-}$ $CD16^{++}$, being cM the most abundant of bovine monocytes (89%) (Hussen *et al*., 2013).

The functions of bovine monocytes also appear to be similar to the ones found in humans; bovine cM, like their human counterparts, have the highest capacity for bacteria phagocytosis (Zawada *et al*., 2011; Cros *et al*., 2010). Bovine intM produce high levels of reactive oxygen species (ROS) and inflammatory cytokines, such as IL-1β and TNFα, while ncM evidence a very low ability to produce ROS and pro-inflammatory cytokines and to phagocyte (Hussen *et al*., 2013).

Although human and murine ncM are known for their ability for neutrophil recruitment to inflamed tissues, this characteristic has not been found in bovine ncM (Auffray *et al*., 2007; Hussen *et al*., 2013).

Bovine intM seem to have clinical significance in inflammatory processes, especially in the acute phase, when they are recruited in large numbers in the draining lymph nodes and elicit a high gene expression of a large variety of inflammatory mediators and cytokines (Düvel *et al*., 2014; Lund *et al*., 2016).

1.2.3.2.2 Macrophages

Macrophages are derived from monocytes localized in the bloodstream and bone marrow. These mononuclear phagocytes acquire certain abilities when they relocate to different tissues. While Kupffer cells in the liver have a phagocytic function to remove foreign toxins and materials in that organ, alveolar macrophages in the lung can produce and secrete cytotoxic oxidants to fight air-borne pathogens (Laskin *et al*., 2011).

Macrophages are considered cellular components of the innate immune response because they eliminate a diverse variety of pathogens, tumor cells, apoptotic cells, and debris. In addition, they can trigger inflammatory responses when they encounter danger signals in case of infection or injury (Zhang and Mosser, 2008). Moreover, these cells can act like professional APCs and induce activation of T-cells that will initiate a specific adaptive immune response (Mosser and Edwards, 2011).

There are two well-characterized major subpopulations of macrophages: M1, or classically activated macrophages, and M2 or alternatively activated macrophages. M1 macrophages are activated by

PAMPs, type I cytokines (such as TNFα and IFNγ) and endogenous danger signals, eliciting very strong activity against microbes and tumors by releasing IL-12 and IL-23 that promote Th1 immune responses (Laskin, 2009). On the other hand, M2 macrophages are responsible for downregulating inflammation through the production of anti-inflammatory cytokines (IL-4, IL-10, IL-13) and mediators involved in tissue repair (Martinez *et al*., 2008).

1.2.3.2.3 Dendritic cells

Dendritic cells are the most efficient APCs and the only APC capable of priming naïve T-cells (Palucka and Banchereau, 1999; Sh ortman and Liu, 2002). Although mature DCs play a main role in antigen presentation and activation of T-cells, recent studies suggest that they also play a role in induction of immunological tolerance (Akbari *et al*., 2001; Joffre *et al*., 2009). To process the antigens, DCs can internalize them through phagocytosis, receptor-mediated interactions and micropinocytosis (Inaba *et al*., 1993; Moll *et al*., 1993; Sallusto *et al*., 1995). They also play a role in interactions between T- and B-cells leading to antibody production, due to their ability to produce large amounts of IL-12 (Cella *et al*., 1996; Koch *et al*., 1996; e Sousa *et al*., 1999).

DC maturation occurs while they migrate through the blood or lymph, to the spleen or lymph nodes, respectively, which involves changes in MHC class II expression, costimulatory molecules and their capacity to process antigens (De Smedt *et al*., 1996; Kudo *et al*., 1997; Inaba *et al*., 1994; Schuler and Steinman, 1985). The stage of development of DCs play a role in their ability act as APCs. Although immature DCs are very efficient in processing the antigen, they lack the ability to activate naïve T-cells, whereas mature DCs cannot take up proteins efficiently, but have a high capacity of antigen presentation (Winzler *et al*., 1997; Cella *et al*., 1997).

There are many subpopulations of DCs which possess unique functional and phenotypical characteristics, different tissue distributions and trigger different T-cell responses (Steinman and Cohn, 1973; Fries *et al*., 2011; de Jong *et al*., 2005). The four major subsets of DCs are conventional DCs, Langerhans cells, monocyte-derived DCs (MoDCs) and plasmacytoid DCs (pDCs). Conventional DCs are

specialized in antigen presentation and they can circulate (migratory DCs) or reside in tissues. Langerhans cells are located in the skin and migrate to the lymph nodes when they find an antigen to present to T-cells. MoDCs are professional APCs and can be rapidly recruited to the site of inflammation or when a danger signal is present (Belz and Nutt, 2012). In contrast, pDCs have very poor antigen-presentation abilities, and their role in the immune response is unclear (Reizis *et al*., 2011).

Since the DC population in blood is very low (less than 1% of PBMCs), different techniques have been developed in order to acquire a higher number of cells. The most common methods involve the stimulation of $CD14^{+}$ monocytes and bone marrow precursors with IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF), to generate MoDCs and BMDCs (Pinchuk *et al*., 2003). However, the T-cell and humoral immune responses induced by BMDCs and MoDCs are different than the ones elicited by blood DCs in humans and bovine (Osugi *et al*., 2002; Pinchuk *et al*., 2003).

In bovine, the lineage markers in DC are not compatible with B-cells (CD21), monocytes (CD14), T-cells (CD3) or NK cells (CD335) (Renjifo *et al*., 1997). Bovine DCs express adhesion molecules and CD205 at high or low levels which have helped to identify two defined myeloid DC (mDCs) populations: $CD205^{Hi}$ and $CD205^{Lo}$. Although both are very efficient in processing antigens, they have different abilities to activate T-cells. When compared to $CD205^{Hi}$, $CD205^{Lo}$ has a higher capacity to upregulate costimulatory molecules CD40, CD80 and CD86, and TGFβ in T cells (González-Cano *et al*., 2014).

1.3 Adenovirus as a vaccine delivery vehicle

Recombinant adenoviruses were first used as a delivery vehicle for gene therapy (Wilson, 1996), until their potential as vaccine vectors to carry pathogen or tumor genes was discovered (Tatsis and Ertl, 2004; Lasaro and Ertl, 2004). One of their advantages is that they are able to trigger strong and long-lasting immune responses against specific antigens after administration via systemic and mucosal routes (Fitzgerald *et al*., 2003; Xiang *et al*., 2003). Furthermore, adenovirus-based vectors can accommodate large segments of foreign DNA in their genomes, they can be grown to high titers in cell lines under

laboratory conditions, and there are well-established protocols for their production and purification, which makes them a convenient option for clinical use (Tatsis *et al*., 2006).

The most widely studied adenovirus as a vaccine vector is human adenovirus serotype 5 (HAdV-5), which causes mild respiratory symptoms in naturally-infected patients. Replication-competent and defective recombinant HAdV-5 vectors have been developed and used to deliver vaccine antigens in dogs, mice, and even non-human primates (Xiang *et al*., 1996; Fischer *et al*., 2002; Shiver *et al*., 2002).

In humans, adenoviral vectors have been studied as vaccine candidates against hepatitis C, influenza A, HIV, tuberculosis, malaria, SARS, and Ebola, with the vaccines specific forHIV and malaria the most widely tested (Appaiahgari and Vrati, 2015). Some adenovirus-based vaccines have been evaluated in animals against diseases such as rabies and foot and mouth disease (Li *et al*., 2006a).

Although pre-clinical and clinical testing of adenoviral vectors for vaccine delivery have been promising, there are concerns about the safety of these vectors, especially after a patient that received adenovirus-based gene therapy died due to a systemic inflammatory response attributed to the virus (Raper *et al*., 2003). Adenovirus vectors come in contact with several blood factors after administration, including platelets, which can produce adenovirus-induced thrombocytopenia (Othman *et al*., 2007).

When adenovirus vectors are inoculated systemically (intravenous route), there is an activation of the innate immune response that occurs between 30 minutes to 6 hours post-inoculation, after the interaction between the virus and TLRs (Toll-like receptors) (Appledorn *et al*., 2008; Rhee *et al*., 2011), blood clotting factors (Shayakhmetov *et al*., 2005; Greig *et al*., 2009), and mediators of common, classical, and alternative complement factors (Tian *et al*., 2009). Later on, in a process that can occur from hours to a few days after inoculation, adenovirus-associated molecular patterns are recognized by TLR2 and TLR9, which activate pathways responsible for the induction of inflammatory cytokines and chemokines such as IL-6, IL-8, IL-12, TNFα, and interferons γ and λ, among others (Appledorn *et al*., 2008; Cerullo *et al*., 2007). In this process, thrombocytopenia and liver damage can occur (Seiler *et al*., 2007). Finally, adaptive immune responses against the adenovirus vector can develop in response to viral

gene expression and subsequent antigen presentation, which results in the generation of adenovirus-specific CD8+ T-cells and humoral immune response (Segura, 2008).

Pre-existing antibodies against HAdV-5 have been found in 30-100% of the human population, depending on the geographical location (Mast *et al*., 2010; Barouch *et al*., 2011), which results in early clearance of the cells transduced with the vector (Buchbinder *et al*., 2008; Gray *et al*., 2010). Moreover, in individuals previously exposed to the virus, the adaptive immune response after administration is strong, and the inflammatory responses can be fatal (Zaiss *et al*., 2009). Another challenge is that more than 85% of the vector accumulates in the liver shortly after administration, where Kupffer cells and LSECs (liver sinusoidal endothelial cells) limit the transduction of target cells, although some virus can enter the liver parenchyma and transduce hepatocytes, which results in high-level expression of transgene (Khare *et al*., 2011).

Recent *in vivo* studies in mice have shown that specific antibody titers against adenovirus vectors decrease between 6 and 10 months post-inoculation, while specific humoral and cell-mediated immunity against the vaccine antigen increases (Mittal *et al*., 2018). These results suggest that, even with pre-existing immunity against the vector, repeated vaccination using the same vector can be performed if there is a window period between inoculations.

1.3.1 Altered tropism

A number of characteristics have led to the development and evaluation of adenovirus vectors for vaccination and gene therapy (Kotterman *et al*., 2015). These include wide tropism, easy construction of recombinant adenovirus, relatively large insertion capacity for foreign genes and high virus titers can be produced in laboratory conditions (Nicklin and Baker, 2002). However, the efficiency of adenovirus vectors is decreased if the target cells lack expression of the primary receptors for adenoviral attachment, or because the primary receptor is expressed in a great variety of cells in the host (Mizuguchi and Hayakawa, 2004).

To overcome these limitations, researchers have developed and used several approaches for developing adenovirus vectors with altered tropism (Table 1.2).

1.4 BAdV-3 as a vaccine vector

BAdV-3 has been studied as a vaccine delivery vehicle in cattle because it has a restricted host range, is non-pathogenic, can be grown to high titers in laboratory and can be delivered intranasally, which is advantageous in food-producing animals immunization by this route does not affect meat quality (Ayalew *et al*., 2015).

Biodistribution studies in mice have demonstrated that BAdV-3 vectors efficiently transduce mostly liver and spleen cells, but also heart, kidney, and lung cells, and have a longer persistence in those organs than HAdV-5 or PAdV-3 vectors (Mittal *et al*., 2009).

Initial efforts focused on developing recombinant replication-competent BAdV-3 vaccine vectors containing the deletion of E3 region (a non-essential region for viral replication) for insertion of foreign genes (Zakhartchouk *et al*., 1998; Reddy *et al*., 2000; Baxi *et al*., 2000). Vaccine antigens from both DNA and RNA viruses were successfully expressed by replication-competent BAdV-3 (Zakhartchouk *et al*., 1998; Baxi *et al*., 2000; Reddy *et al*., 2000b; Brownlie *et al*., 2015). Vaccines using recombinant replication competent BAdV-3 expressing antigenic proteins of BHV-1 and BVDV, administered intranasally to cotton rats have shown antigen-specific immune responses after two doses (Zakhartchouk *et al*., 1998; Baxi *et al*., 2000). However, there was a concern about the use of BAdV-3 as a vector in cattle because of the level of pre-existing antibodies in the field (Ghirotti *et al*., 1991; Lehmukhul *et al*., 1979; Roshtkhari *et al*., 2012). Because of this issue, calves with significant levels of pre-existing neutralizing antibodies against BAdV-3 were immunized with the recombinant vector expressing a BHV-1 protein intranasally in two doses, and challenged with BHV-1 two weeks later. Immunization of calves induced BHV-1 specific humoral immune responses and absence of development of clinical disease, which shows that intranasal immunization with BAdV-3 in calves can overcome the effect of BAdV-3 specific pre-existing neutralizing antibodies in calves (Zakhartchouk *et al*., 1999). Interestingly, calves

Approach	Modification	Examples
Modification of capsid proteins	Fiber	Addition of peptides in C-terminus or HI loop (Wickham *et al*., 1997; Dmitriev *et al*., 1998; Mizuguchi *et al*., 2001; Koizumi *et al*., 2003b; Sharma *et al*., 2017) Substitution with fibers from different Adenoviruses (Stevenson *et al*., 1997; Shayakhmetov *et al*., 2000; Mizguchi and Hayakawa, 2002; Chondronasiou *et al*., 2018). Removal of knob domain from fiber protein (Hong *et al*., 2003; Gaden *et al.*, 2004).
	pIX	Insertion of ligands at C-terminus of pIX (Dmitriev *et al*., 2002; Le *et al*., 2004; Vellinga *et al*., 2004; Salisch *et al*., 2017).
	Penton base	Deletion of RGD motif to prevent binding to α_v integrins (Mizguchi *et al*., 2002; Einfeld *et al*., 2001; Koizumi *et al*., 2003a).
Use of adaptor molecules	Anti-fiber monoclonal antibody (mAb)	Anti-fiber mAb conjugated with different ligands (folate, fibroblast growth factor 2, epidermal growth factor receptor, anti-CD40) and complexed with adenovirus vector (Douglas *et al*., 1999; Sosnowski *et al*., 1999; Miller *et al*., 1998; Tillman *et al*., 1999).
	Modified capsid proteins	Combination of genetically-modified capsids and adaptor molecules (Henning *et al*., 2002; Volpers *et al*., 2003) Metabollically biotinylated adenovirus vectors (Parrott *et al*., 2003; Campos *et al*., 2004)
Chemical modification	Polyethylene glycol	Modified adenovirus with PEG interacting with lysine residues in capsid proteins (Alemany *et al*., 2000; Croyle *et al*., 2002; Ogawara *et al*., 2004). Addition of functional molecules on the tip of PEG in adenovirus vectors (Lanciotti *et al*., 2003; Ogawara *et al*., 2004; Eto *et al*., 2005; Kasala and Yun, 2016).
	Others	Use of multivalent hydrophilip polymers (Fisher *et al*., 2001; Green *et al*., 2004)

Table 1.2. Approaches for elaboration of adenovirus vectors with altered tropism.

immunized with replication-defective BAdV-3 expressing BHV-1 vaccine antigen subcutaneously did not show induction of protective immune response, and protection against BHV-1 challenge (Reddy *et al*., 2000).

Subsequently, replication-defective BAdV-3 vectors containing deletion of E1A and E3 region were developed (Reddy *et al*., 1999). However, immunization of calves with replication defective recombinant BAdV-3 expressing BHV-1 vaccine antigen did not show either an induction of an immune response or protection against BHV-1 challenge (Reddy *et al*., 2000b).

Studies in mice comparing BAdV-3and HAdV expressing HA of influenza virus demonstrated that the BAdV-3 based vector stimulated more potent cellular immune responses than HAdV and offered complete protection against challenge when delivered using intramuscular route. Moreover, intranasal inoculation with BAdV-3-HA vector enhanced cellular and humoral immune responses in mice and provided complete protection in mice, even at doses 30-fold lower than intramuscular (Mittal *et al*., 2018).

1.4.1 Altering tropism of Bovine Adenovirus-3

Although BAdV-3 has been demonstrated to be a suitable vaccine vector for the delivery of antigens to respiratory mucosal surfaces in cattle, two vaccinations are required to induce protective immunity (Zakhartchouk *et al*., 1999). Targeting BAdV-3 to different cells could improve the vector efficiency, and allow vaccine administration by different routes, even orally (Ayalew *et al*., 2015), which could prove more economical.

Efforts have been made to change the tropism of BAdV-3 through genetic manipulation of capsid proteins fiber and pIX. BAdV-3 expressing chimeric fiber (knob region of BAdV-3 fiber replaced with knob region of HAdV-5 fiber) significantly increased the transduction efficiency of recombinant BAdV-3 (Wu and Tikoo, 2004) in non-bovine cells. The recombinant virus transduction efficiency was between 3-67 fold higher than wild-type BAdV-3 with an unmodified fiber. In addition, early and late viral

proteins could be detected in transduced human cells, which suggests that BAdV-3 tropism can be modified for non-bovine cells transduction (Wu and Tikoo, 2004). On the other hand, pIX can be modified inserting large polypeptides in its C-terminus region, and it has been shown that the insertion of targeting ligands in this protein can enhance the tropism in a fiber-independent manner (Zakhartchouk *et al*., 2004). Targeting BAdV-3 to different cells could improve the vector efficiency, and allow vaccine administration in different routes, even orally (Ayalew *et al*., 2015).

2. HYPOTHESIS AND OBJECTIVES

Like other adenoviruses, BAdV-3 has been developed and evaluated as a vaccine delivery vehicle in cattle (Ayalew *et al*., 2015). However, development of protective immune responses using recombinant BAdV-3 based vaccines require two immunizations (Kumar *et al*., 2014; Zakhartchouk *et al*., 1999). One way to develop a single shot BAdV-3 vectored vaccine is to target BAdV-3 to antigen presenting cells. Since the C-terminus of BAdV-3 minor capsid protein pIX is exposed to the exterior of the virion capsid, efforts have been made to use it to add targeting ligands, so that recombinant BAdV-3 expressing chimeric pIX can be targeted to a particular cell (Zakhartchouk *et al*., 2004).

We hypothesize that a recombinant BAdV-3 expressing an RGD motif fused to the C-terminus of the pIX protein can enhance the transduction efficiency of bovine peripheral bovine mononuclear cells (PBMCs), especially the population of antigen presenting cells (APCs), compared to the wild-type BAdV-3 virus.

The objectives of this work are:

a) Construct and characterize a recombinant BAdV-3 expressing GFP and a chimeric pIX (RGD fused to C-terminus of pIX).

2) Determine the transduction efficiency of recombinant BAdV-3 expressing RGD in different populations of peripheral blood mononuclear cells (PBMCs).

3) Evaluate the expression of activation markers and cytokine expression in bovine monocytes after transduction with recombinant BAdV-3 expressing RGD.

3. TRANSDUCTION OF BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS WITH RECOMBINANT BOVINE ADENOVIRUS-3 EXPRESSING CHIMERIC PIX

3.1 INTRODUCTION

Due to the natural ability of viruses to enter cells and efficiently deliver genetic material, different viruses have been evaluated for delivery of foreign DNA into cells (Liu, 2010). Through the use of molecular virology, the development of highly attenuated recombinant viruses has led to production of efficient and safe vaccine vectors for vaccination of animals and humans (Rollier *et al*., 2011). A number of viruses including pox virus (Townsend *et al*., 2017), herpesvirus (Palya *et al*., 2014), adeno-associated virus (Büning and Schmidt, 2015) retrovirus (Wang *et al*., 2015) and adenovirus (Tatsis and Ertl, 2004) have been developed and evaluated as vaccine delivery vectors.

Adenoviruses have been studied as vaccine vectors because they constitute an efficient method of gene delivery in a variety of human and animal cells (Maheshri *et al*., 2006). They are not only able to trigger strong and long-lasting immune responses against specific antigens when administered through different routes (Fitzgerald *et al*., 2003; Xiang *et al*., 2003), but are also easily grown and purified in laboratory conditions (Kamen and Henry, 2004). Moreover, the adenovirus genome can integrate large inserts of foreign DNA (Tatsis *et al*., 2006).

Bovine adenovirus-3 (BAdV-3) is being developed and evaluated as a vaccine delivery vehicle for cattle (Ayalew *et al*., 2015). It is a non-enveloped DNA virus with an icosahedral capsid. The genome of BAdV-3 is 34,446bp and is organized into early, intermediate, and late regions (Reddy *et al*., 1998). BAdV-3 has been studied as a vaccine vector candidate because is non-pathogenic, has restricted host-range, grows to high titers and can be delivered intranasally (Ayalew *et al*., 2015; Zakhartchouk *et al*., 2004). However, *in -vivo* studies using recombinant BAdV-3 expressing bovine herpesvirus-1 gDt show that induction of protective immunity against BHV-1 challenge in calves requires two immunizations

three weeks apart (Kumar *et al*., 2014; Zakhartchouk *et al*., 1999). Since economy plays an important role in developing and marketing veterinary vaccines, the prospect of developing single shot vaccines may help in increasing economic benefits.

In order to achieve a more efficient immune response, and a more cost-effective vaccine vector candidate, one of the approaches is to improve the transduction efficiency of the BAdV-3 in antigen presenting cells (APCs) that would initiate strong adaptive immune responses. Since BAdV-3 utilizes primarily sialic acid receptors to enter the cells, this may hinder the efficient transduction of BAdV-3 based vectors in integrin-positive cells, such as leukocytes, including APCs (Li *et al*., 2009; Khosa, 2016).

To overcome this, selected viral capsid protein(s) could be modified so that recombinant adenovirus can enter non-permissive cells (Belousova *et al*., 2002; Dmitriev *et al*., 2002). Earlier studies demonstrated that modification of major capsid protein fiber (Wu and Tikoo, 2004) or minor capsid protein pIX (Zakhartchouk *et al*., 2004) alters the tropism of BAdV-3. Here, we describe the construction and evaluation of a recombinant BAdV-3 that incorporates an RGD motif in the C-terminus of the minor capsid protein pIX of the virus. The results indicate that recombinant BAdV-3 expressing chimeric pIX (c-terminus of pIX fused to RGD) increases the viral transduction of PBMCs, especially monocytes.

3.2 MATERIALS AND METHODS

3.2.1 Cells and viruses

Madin Darby Bovine Kidney (MDBK) (Madin and Darby, 1958; ATCC CCL-22) and VIDO DT1 (Du and Tikoo, 2010) cells were grown in minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) enriched with 2% sterile filtered fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 0.1 mM non-essential amino acids (NEAA, Thermo Fisher Scientific, Waltham, MA, USA), 10 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA) and 50 μg/ml gentamicin (Thermo Fisher Scientific, Waltham, MA, USA). Recombinant BAV304a (BAdV-3 expressing GFP inserted in E3 deleted region) (Du and Tikoo, 2010), recombinant BAV951 (BAdV-3 with C-terminus region of pIX fused to EYFP) (Zakhartchouk *et al*., 2004), and recombinant BAV888 (BAV304a expressing chimeric pIX [C-terminus of pIX fused to a 10GS spacer, cathepsin cleavage site and RGD motif]) were propagated, purified by CsCl and titrated as previously described (Kulshreshtha *et al*., 2004).

3.2.2 Antibodies

Production and characterization of anti-BAdV-3 pIX sera has been described (Zakhartchouk *et al*., 2004). Antibodies used to perform flow cytometry experiments are listed in tables 3.1 and 3.2.

3.2.3 Plasmid construction

To isolate recombinant BAdV-3, full length BAdV-3 plasmid containing chimeric pIX was constructed following standard DNA manipulation techniques, as described earlier (Zakhartchouk *et al*., 2004).

a) Plasmid pBAVNdA-10GS-CATRGD. Plasmid pBAVNdA plasmid (Zakhartchouk *et al*., 2004) (containing E1A and E1B regions and protein pIX DNA sequence of the BAdV-3 genome followed by a 10GS linker sequence) was digested with *HpaI -XhoI* (nt 4181) restriction enzymes. Oligonucleotides

containing a cathepsin cleavage site (de Vrij *et al*., 2012) and Arg-Gly-Asp (RGD) sequence were annealed creating overhangs for *HpaI* and *XhoI* sites and ligated to the *HpaI-XhoI* digested plasmid pBAVNdA DNA creating plasmid pBAVNdA-10GS-CATRGD.

b) Plasmid pUC304a-CATRGD. Homologous recombination in *E. coli* BJ5183 (Chartier *et al*., 1996) was performed between the digested plasmid pUC304a DNA (containing the genome of BAdV-3 with a GFP cassette in the E3 deleted region; Du & Tikoo, 2010), and an Ase1-Not1 11kb DNA fragment of plasmid pBAVNdA-10GS-CATRGD, creating plasmid pUC304a-CATRGD.

3.2.4 Isolation of recombinant BAdV-3

VIDO DT1 (Du and Tikoo, 2010) cells in a 6-well plate ($1x10^6$ cells/well) were transfected with 4-6µg/well of plasmid *pUC304a-CATRGD* DNA using Lipofectamine 2000 (Invitrogen, CA, USA). At 6 hrs post-transfection, the medium was replaced with fresh MEM containing 2% FBS (MEM; Sigma-Aldrich, St. Louis, MO, USA; FBS; Sigma-Aldrich, St. Louis, MO, USA) and the cells were observed daily under the microscope for any signs of cytopathic effect (CPE). After 15 to 20 days post transfection, the cells showing CPE were collected, freeze-thawed three times and used for viral purification (Tollefson *et al*., 2007).

3.2.5 Virus growth

MDBK cells were grown in MEM with 10% FBS (MEM; Sigma-Aldrich, St. Louis, MO, USA; FBS; Sigma-Aldrich, St. Louis, MO, USA) in T150 flasks (Corning Inc., NY, USA) until they reached a confluency of ~70-80%. Before infection, the media was removed from the flask and cells were washed once with MEM without FBS. Next, the cells were infected with individual virus at MOI 1. The infected flasks were incubated at 37°C on a rocking platform for 90 min. After incubation, the medium in the flasks was discarded and replaced with MEM with 2% FBS. Cells were examined daily, and were

collected between 36 and 48 hrs post-infection, when at least 80% of the cells were showing signs of cytopathic effect (CPE) or fluorescence, and stored at -80°C in 5ml of medium.

3.2.6. CsCl gradient centrifugation

MDBK cells infected with each individual virus were harvested at 48 hrs post infection. After five rounds of freezing and thawing, 1.5% Nadeoxycholate was added to the lysate and the mixture was incubated at room temperature for 30 min. The cell lysate was then combined with 150 µl 2 M $MgCl_2$ and 75 µl DNase I solution and incubated for additional 30 min at 37°C before centrifuging for 15 min at 5000 *rpm*. The supernatant was collected and subjected to CsCl density gradient centrifugation at 35000*rpm* at 10°C for 1 hour. Two bands were observed after centrifugation. The lower band corresponding to mature virus was collected and subjected to a second round of CsCl gradient centrifugation at 4°C for 20 hrs. The band containing the virus was collected and dialyzed using dialysis cassettes (Thermo Fisher Scientific, Waltham, MA, USA) submerged in 0.1M Tris-HCl overnight to remove any traces of CsCl. Afterward repeating the process three times, the content of each cassette was recovered, pooled. The titer of the virus was determined as described earkier (Kulshrestha et al., 2004). Finally, the virus was stored at -80 °C, in 100 µl aliquots.

3.2.7 Western Blotting

The proteins were analyzed by Western blot as described earlier (Kulshrestha et al., 2004). Briefly, MDBK cells grown in 6-well plates were mock-infected or infected with individual virus at a MOI of 1. After 48 hrs, the cells were collected, lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) and proteins were separated by 15% SDS-PAGE gel. Finally, the proteins were transferred to nitrocellulose membrane (Bio-Rad, CA, USA) and probed using protein specific antisera produced in rabbit followed by alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody

(Invitrogen, CA, USA). The target protein bands were visualized using BCIP solution (Sigma-Aldrich, St. Louis, MO, USA).

3.2.8 Purification of bovine blood cells

All animal experimental procedures were evaluated and approved by University of Saskatchewan – University Committee on Animal Care and Supply (Phlebotomy Protocol #20180015). All procedures were executed following guidelines approved and permitted by the Canadian Council on Animal Care.

3.2.9 Peripheral blood mononuclear cells (PBMCs)

The PBMCs were purified as described previously (Arsenault *et al*., 2009). Briefly, venous blood from male calves (9 and 12 months old) was collected in syringes containing 7.5% EDTA (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 1400 x g at room temperature for 20 min. The buffy coat layer was collected, mixed with PBSA + 0.1% EDTA to complete 20 ml before cautiously layering over a 60% Percoll (GE Healthcare, Chicago, IL, USA) solution in PBSA. After centrifugation at 2000 x g for 20 min, the cell layer containing PBMCs was collected and washed first with PBSA + EDTA 0.1% followed with PBSA. The cells were diluted in PBSA, counted using a hemocytometer, and tested for viability using Trypan blue exclusion test.

3.2.10 Monocytes

The monocytes (CD14+) were isolated from isolated PBMCs as described previously (Arsenault *et al*., 2009). Briefly, PBMCs isolated from calf venous blood using Percoll gradients (described above) were washed with magnetic activated cell sorting (MACS) running buffer (50ml of 10% BSA, 4 ml of 0.5 M EDTA and 946 ml of PBSA) and resuspended to a density of 1.25 x 10^8 cells/ ml. About 2.4 ml of the cell suspension was mixed with 600µl of anti-CD14 magnetic microbeads (Miltenyi Biotec Inc., San Diego, CA, USA). After incubation at 4°C for 15 min, the cells were washed with MACS running buffer. The cell suspension was added to an LS Midi Column (Miltenyi Biotec Inc., San Diego, CA, USA),

containing magnetic spheres, attached to a magnet that will allow the positive selection of the cells. Once all the cell suspension had passed though the magnetic column, the column was washed three times with MACS buffer 5ml\wash). Next, the columns were removed from the magnets and placed into a sterile 15 ml conical centrifuge tube (Corning Inc., NY, USA). To obtain the purified cells that remain in the column, 5 ml of MACS running buffer was added, and the contents were pushed through the column using the plunger provided. The adherent fraction was counted using a hemocytometer, and cell viability was assessed using the Trypan blue exclusion test. The purified CD14+ population was coincident in size and complexity (FSC and SSC) with bovine monocytes in flow cytometry analysis (data not shown).

3.2.11 *In vitro* transduction of PBMCs and monocytes

About 1 x 10^6 cells per well, plated in 12-well plates (Corning Costar, Corning Inc., NY, USA) in 500µl AIM-V medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) without FBS were transduced with indicated amount of BAV304a (Du and Tikoo, 2010) or BAV888 (this study). After incubation at 37 °C for 90 mins, 500 µl of AIM-V medium supplemented with 20% FBS was added to each well. After incubation for 16 hrs at 37°C, the supernatant from each well was collected and stored at -20°C for ELISA assays. After washing with PBSA + 0.1% EDTA, the cells were collected using StemPro Accutase Cell Dissociation Reagent (Gibco, Thermo Fisher Scientific, Waltham, MA, USA).

3.2.12 Flow cytometry

Purified PBMCs and monocytes (CD14+) transduced with BAV304a or BAV888 were resuspended in Facola (0.1 M PBS, 0.20/0 gelatin, 0.030/0 NaH3) and plated in a 96-well plate (Corning Costar, Corning Inc., NY, USA), with a density of approximately 1 x 10^6 cells per well. To identify the different PBMC populations and activation markers, the cells were incubated with protein specific primary antibody (Table 3.1) on ice for 20 min. After washing 3 times with Facola (PBSA supplemented with 1% fetal calf serum, 0.09% sodium azide, pH 7.5), the cells were incubated with fluorochrome-conjugated secondary antibodies (Table 3.2) for 20 min on ice. After 3 washes, the cells were fixed with

2% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBSA and stored at 4°C in the dark. Flow cytometry was performed using a FACS Calibur instrument (BD Biosciences, Franklin Lakes, NJ, USA), with 10000 events captured per sample. Analysis of flow cytometry data was performed using Cell Quest acquisition and analysis software (BD Biosciences, Franklin Lakes, NJ, USA).

3.2.13 Quantitative RT-PCR

About 5 x 10^6 purified bovine monocytes (CD14+) were transduced with BAV304a or BAV888 at a MOI of 1. At 16 hrs post-infection, the supernatant from each well was collected and centrifuged at 300 x g for 8 min to collect the floating cells. Meanwhile, TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added directly to each well containing attached cells. After incubation for 10 min at room temperature, the TRIzol disrupted cells were collected from each well and added to the floating cell pellet obtained above. After adding 0.2 mL of chloroform, the contents were mixed vigorously before centrifuging at 14000 x g at 4°C for 15 min. The aqueous phase was collected, transferred to a new tube and mixed with an equal volume of 70% ethanol. Finally, RNA was extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. The RNA samples were analyzed for quality and quantity using the Agilent 2100 Bioanalyzer (G2938B, Agilent Technologies, Santa Clara, CA, USA). Samples with a RNA Integrity Number (RIN) above 7 were used to synthesize cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) as per manufacturer's instructions.

The samples were analyzed with the iCycler iQ PCR detection system (BioRad, CA, USA). Each reaction contained 10 ng of cDNA, 0.5 µl of each primer pair at a concentration of 10 µM (Table 3.3), 0.5 µl of nuclease-free water (Sigma-Aldrich, St. Louis, MO, USA) and 9 µl of PerfeCTa SYBR Green SuperMix for iQ (Quanta BioSciences Inc., MD, USA) and the program followed consisted in: 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 30 secs; 45 cycles of 95°C for 15 sec, 60° for 30 secs and 72°C for 30 sec. β-actin was used as reference gene, and all reactions were performed in duplicate.

Table 3.1: List of primary antibodies used for flow cytometry experiments

Cell surface marker	Isotype	Catalog number	Supplier
CD14	IgG1	BOV2109	Washington State University
CD3	IgG1	BOV2009	Washington State University
CD21	IgG1	BOV2031	Washington State University
CD335	IgG1	BOV2147	Washington State University
CD209	IgG2a	BOV2133	Washington State University
MHC I	IgG2a	BOV2120	Washington State University
MHC II	IgG2a	BOV2115	Washington State University

Table 3.2: List of secondary antibodies used for flow cytometry experiments

Reactivity	Fluorescent conjugate	Target Ig class	Catalog number	Supplier
Rat anti-mouse	APC	IgG1	17-4015-80	ThermoFisher Scientific
Goat anti-mouse	APC	IgG2a	17-4010-82	ThermoFisher Scientific
Rat anti-mouse	APC	IgM	550676	BD Biosciences

3.2.14 qPCR assay for viral quantification

About $2x10^6$ purified bovine monocytes (CD14+) were transduced with BAV304a or BAV888 at a MOI of 1. At 16 hrs post-infection, the cells were collected by centrifugation at 300 g for 7 mins and resuspended in PBS. The DNA was extracted from the cells using the DNA Blood and Tissue kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Finally, the extracted DNA was subjected to quantitative PCR using the iCycler iQ PCR detection system (BioRad, CA, USA), using BAdV-3 pVIII specific primers (Table 3.3). Viral copy numbers were calculated using a standard curve for wild-type BAdV-3 as described earlier (Gaba *et al.*, 2018).

3.2.15 Cytokine detection – ELISA assays

Supernatants from mock infected or virus infected $5x10^6$ monocytes (CD14+) were collected after 16 hrs of incubation and stored at -20 °C. The plates were primarily coated with mouse anti-recombinant bovine IL-12 (BioRad, CA, USA) or mouse anti-rBoTNFα monoclonal antibody (VIDO-InterVac, SK, Canada) in coating buffer (BioRad, CA, USA) and left at 4°C overnight. The next day, plates were washed four times with TBST, and standards were added to the plate in two-fold dilutions, followed by 100ul of the samples, and incubated for 2 hrs at room temperature. After washing again four times with TBST, rabbit anti –BoTNFα (VIDO-InterVac, SK, Canada) antibody was added to the TNFα plate. After incubation, each plate was washed with TBST and the conjugates were added according to each protocol (mouse anti -bovine IL-12 biotin [BioRad, CA, USA] and Goat anti Rabbit IgG (H+L) biotin [Thermo Fisher Scientific, Waltham, MA, USA] and incubated for one hour at room temperature. After washing with TBST, samples were incubated with streptavidin conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories, PA, USA) for one hr at room temperature. After washing plates (as described above), substrate PNPP (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. After incubating for 30 mins to one hr at room temperature, the reaction was stopped by adding 0.3M EDTA to the plate. Finally, the plates were read using the SpectraMax Plus 384 Microplate Reader (Molecular

Devices Corporation, CA, USA) and values for each cytokine were determined from the standard curve calculated by the Softmax Pro software (Molecular Devices Corporation, CA, USA).

3.2.16 Statistical analysis

GraphPad Prism 7 software (La Jolla, CA, USA) was used for statistical analysis of all the data. Differences among viruses were assessed with Student t-tests for multiple comparisons. Two-way ANOVA was used to determine differences between different MOI and when more than three groups where compared in q-RT-PCR and flow cytometry experiments. Differences were considered significant with a p-value of less than 0.05.

Table 3.3. List of primers

Target gene	Direction	Sequence (5' to 3')	Annealing Temperature (°C)
GFP	Forward	AAGCTGACCCTGAAGTTCATCTGC	62.4
	Reverse	CTTGTAGTTGCCGTCGTCCTTGAA	
pVIII BAdV-3	Forward	CAGGTGCCAGTCAAGATTAC	52.8
	Reverse	ATGGCCGACTGAGTCATAAG	
β-actin	Forward	GATCTGGCACCACACCTTCTAC	57.9
	Reverse	AGGCATACAGGGACAGCACA	
Hexon BAdV-3	Forward	TGCTTCTTGCAAACACGACG	61
	Reverse	CCAATCTGAACCCCCGACAA	
19K BAdV-3	Forward	ATCGCACTGGAGTGTGGAAG	64.8
	Reverse	GGCACCACAAACACGTCAAA	
52K BAdV-3	Forward	ACCCTGGGTTTGATGCACTT	59.3
	Reverse	AGCTTCCCCAAAAATGCCCT	
CD40	Forward	CTGCCCATATGCCAAGAGTT	56
	Reverse	GGCATCTTCTCTTTCCCCAT	
CD80	Forward	ACCACCCAAGCGCCCATG	62.6
	Reverse	AGGCAGGATGGCCAGCAC	
CD86	Forward	TGTGCCCTGCAACTTGAGCCA	64.8
	Reverse	AGAGGGGCCAGGCTGCTTCT	
IFNβ	Forward	RTCTGSAGCCAATCCARAAG	56.9
	Reverse	CAGGCACACCTGTYGTACTC	

3.3 RESULTS

3.3.1 Isolation of recombinant BAV888

To determine if addition of an"RGD" motif altered the tropism of BAV304a (E3 deleted BAdV-3 containing CMV-GFP cassette inserted in E3 region) (Du and Tikoo, 2010), we constructed a plasmid pUC304a-CATRGD (Fig. 3.1[a]) containing full length BAV304a genomic clone expressing chimeric pIX (C-terminus of pIX fused in frame to a cathepsin cleavage site (ccs) and a RGD motif). Transfection of VIDO DT1 cells (cotton rat lung [CRL] expressing I-SceI protein) (Du and Tikoo, 2010) with plasmid pUC304a-CATRGD DNA produced cytopathic effects in 15-20 days indicating successful infection. The cells showing cytopathic effects (CPEs) and GFP expression were harvested, freeze-thawed three times, propagated in MDBK cells and purified by CsCl banding method. As shown in Fig. 3.2 (panel b) BAV951 which does not contain cathepsin cleavage sequence between pIX and EYFP (Fig 3.2[a]) (Zakhartchouk *et al*., 2004), the virus particle appeared to be inefficiently released from the endosomes. In contrast, BAV888, which contains cathepsin cleavage sequence between pIX and "RGD" motif was released efficiently.

The identity of recombinant virus named BAV888 was confirmed by sequencing of viral DNA and expression of recombinant protein IX was analysed by Western blot using anti- pIX serum (Zakhartchouk *et al*., 2004). As seen in Fig.3.1 (c), anti-pIX detected a 14 kDa protein in BAV304a infected cells (lane 2) which is the anticipated size. In contrast, the anticipated 20 kDa protein was detected in BAV888 infected cells (lane 3). No such proteins were detected in mock-infected cells (lane 1).

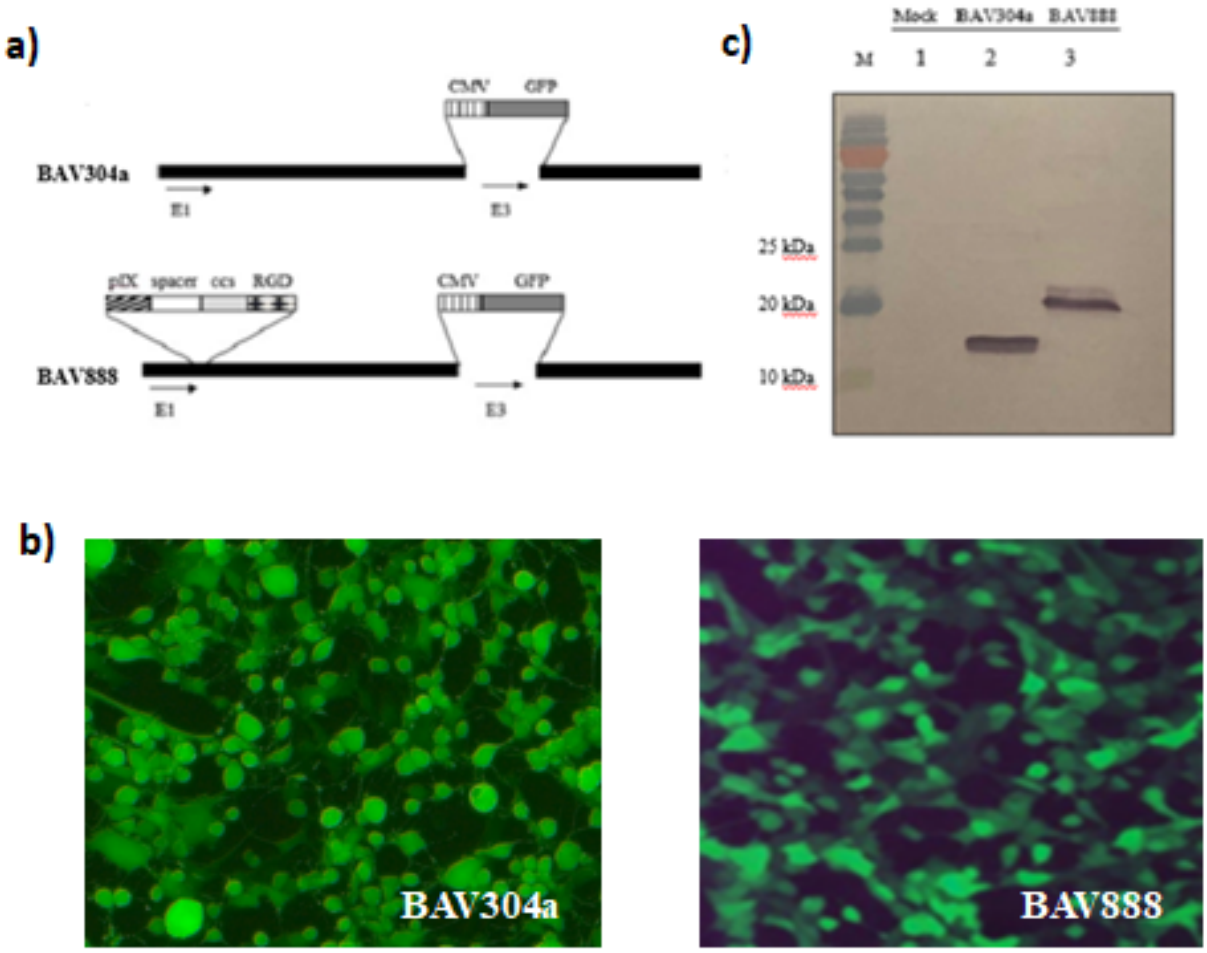

Figure 3.1 Isolation and characterization of BAV304a and BAV888. (a) Schematic diagram of genome of BAV304a and BAV888; cathepsin cleavage site (ccs); 10 repeats of glycine-serine (spacer). The black box represents the BAdV-3 genome sequence, while the blank space in early (E)-3 region represents the deletion of this region. Human cytomegalovirus immediate early gene promoter (CMV); Green fluorescent protein (GFP) gene. The direction of the transcription is indicated with arrows. (b) Fluorescent microscopy of BAV304a and BAV888 infected cells (c) Western Blot. Proteins from the lysates of cell mock-infected (lane 1), BAV304a infected (lane 2) or BAV888 infected (lane 3) were separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane and probed in Western blot using anti-pIX serum (Zakhartchouk *et al*., 2004).

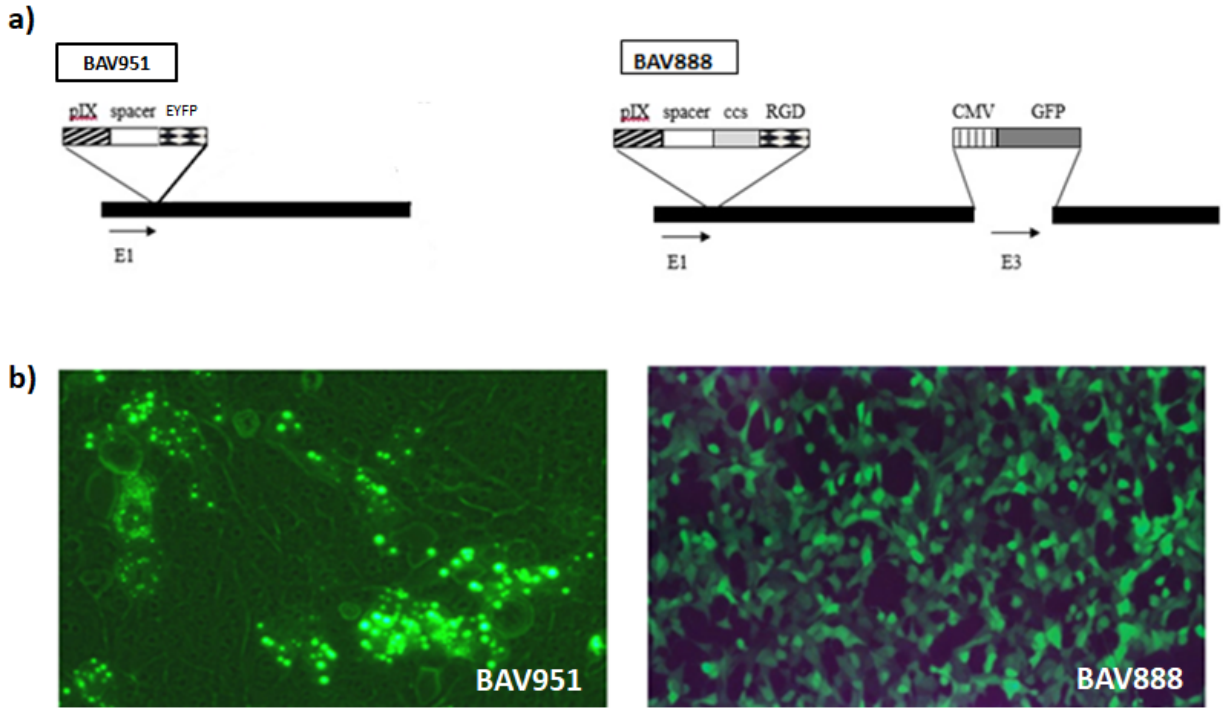

Figure 3.2 Propagation of BAV888 and BAV951 in MDBK cells. (a) Schematic representation of BAV888 and BAV951 genomes; cathepsin cleavage site (ccs); 10 repeats of glycine-serine (spacer). The BAdV-3 genome sequence is represented with a black box, while the blank space in early (E)-3 region represents the deletion of this region. Human cytomegalovirus immediate early gene promoter (CMV); Green fluorescent protein (GFP) gene; Enhanced yellow fluorescent protein (EYFP). The direction of the transcription is indicated with arrows. (b) Fluorescent microscopy of BAV888 and BAV951 infected MDBK cells.

3.3.2 Transduction of peripheral blood mononuclear cells (PBMCs)

To establish the optimal concentration of BAV888 required for transduction of bovine PBMCs, purified PBMCs from each of the five animals were mock-infected or infected with either BAV304a or BAV888 at a MOI 0.2, 0.5, 1 and 2. After a 16-hr post infection, the cells were collected and analyzed by flow cytometry. Transduction was assessed as percentage of cells expressing GFP and mean fluorescence intensity (MFI) of GFP displayed by each transduced cell.

As seen in Fig. 3.3 (a), there is a significant difference in transduction of PBMCs between MOI of 0.2 and 2, irrespective of BAV304a or BAV888. Although more PBMCs appear to be transduced (percentage of cells expressing GFP) by BAV888 compared to BAV304a at respective MOIs, there is no significant difference in the transduction of PBMCs by indicated viruses at respective MOI (Fig. 3.3[a]). Interestingly, there are significant differences between the MFI of PBMCs (intensity of fluorescence exhibited by each cell) transduced with BAV888 compared to BAV304a at MOI 0.5, 1 and 2 (Fig. 3.3[b]). The MFI is three times higher in PBMCs transduced with BAV888 compared to BAV304a at MOI 1 and 2. Therefore, the most efficient concentration to use for the next experiment is MOI 1.

3.3.3 Transduction of subpopulations of PBMCs

To determine if there is any difference in tropism of BAV304a and BAV888 for sub populations of PBMCs, we isolated bovine PBMCs from five different animals and transduced them *in vitro* with either BAV304a or BAV888 at MOI 1. After incubating the cells for 16 hrs, the transduced cells were incubated with cocktail of antibodies specific for CD3 (T cells), CD14 (monocytes), CD21 (B cells), CD335 (NK cells) and CD209 (dendritic cells) surface markers, followed by allophycocyanin (APC) conjugated secondary antibody. The labelled cells were analysed by flow cytometry for GFP expression, cell marker protein expression, and mean GFP fluorescence intensity. BAV304a transduced about 60% of monocytes (CD14+), which correspond to about 6% of the total PBMCs, as seen in Fig. 3.4 (a) and (b). In contrast, BAV304a transduced about 35% of dendritic cells (CD209+) (0.21% of PBMCs). However, when considering the total number of PBMCs, 2% of the total number of PBMCs correspond to

BAV304a-transduced T-cells (CD3+), followed by about 2% NK cells (CD335+) and 1% are transduced B-cells (CD21+). Interestingly, there was no significant difference in the transduction of monocytes (CD14+), NK (CD335+) cells, dendritic cells (209+), T cells (CD3+) or B cells (CD21+) by BAV304a or BAV388. In contrast to the number of GFP positive cells, the mean fluorescence intensity of GFP is significantly higher in BAV888 transduced T cells (CD3+) and monocytes (CD14+ cells) compared to BAV304a transduced T cells (CD3+) and monocytes (CD14+) (Fig. 3.4 [c]).

3.3.4 Genome copy number in transduced monocytes (CD14+)

To determine the viral genome copy number, the monocytes (CD14+) cells were purified from purified PBMCs using magnetic-activated cell sorting (MACS). The purified monocytes were transduced with either BAV304a or BAV888 at MOI 1 and incubated for 16 hrs. The viral DNA present in transduced bovine monocytes was analysed by quantitative real time PCR. As seen in Figure 3.5 (a), virus genome copy number is significantly higher in monocytes transduced with BAV888 compared to monocytes transduced with transduced BAV304a.

3.3.5 Viral gene transcription in transduced monocytes (CD14+)

To determine the transcription of viral genes, total RNA isolated from MACS purified monocytes infected with either BAV304a or BAV888 was analysed by q-RT-PCR using gene specific primers (Table 3.3). As seen in Fig. 3.5 (panel b), there was significant difference in the level of GFP specific transcripts in monocytes transduced with BAV888 compared to monocytes transduced with BAV304a. Similarly, there was a significant difference in the level of BAdV-3 19K or hexon-specific transcripts in monocytes transduced with BAV888 compared to monocytes transduced with BAV304a. However, there is no significant difference in the level of BAdV-3 52K-specific transcripts in monocytes transduced with BAV888 compared to monocytes transduced with BAV304a.

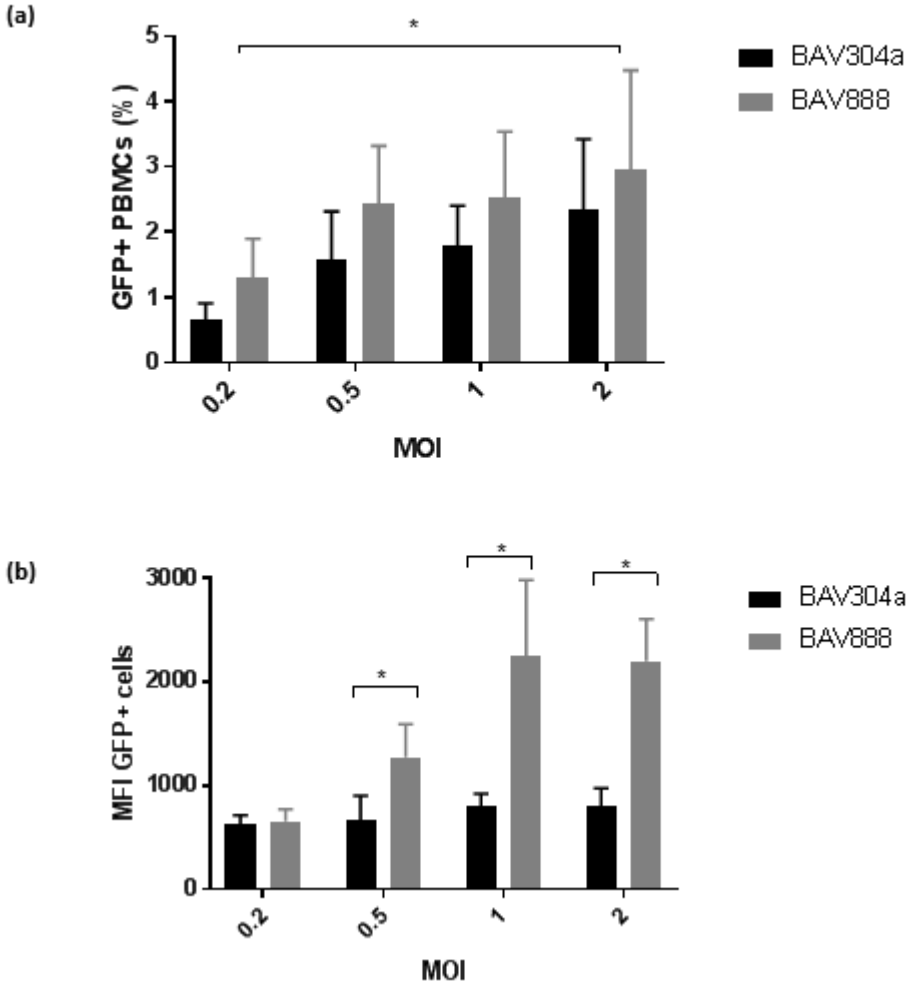

Figure 3.3 Transduction of bovine PBMCs by BAdV-3. PBMCs were isolated from bovine blood and transduced with BAV304a or BAV888 *in vitro* at MOI 0.2, 0.5, 1 and 2. After 16 hrs post transduction, the cells were collected and analyzed by flow cytometry. a) Percentage of GFP positive cells. b) Mean fluorescence intensity (MFI) of GFP of each cell analyzed. Data is representative of five independent repeats, and it is expressed as mean ±SD. For statistical analysis, the differences between BAV304a and BAV888 were analyzed using multiple t-tests, and Two-way ANOVA for differences between MOI, using GraphPad Prism 7 (GraphPad Software).

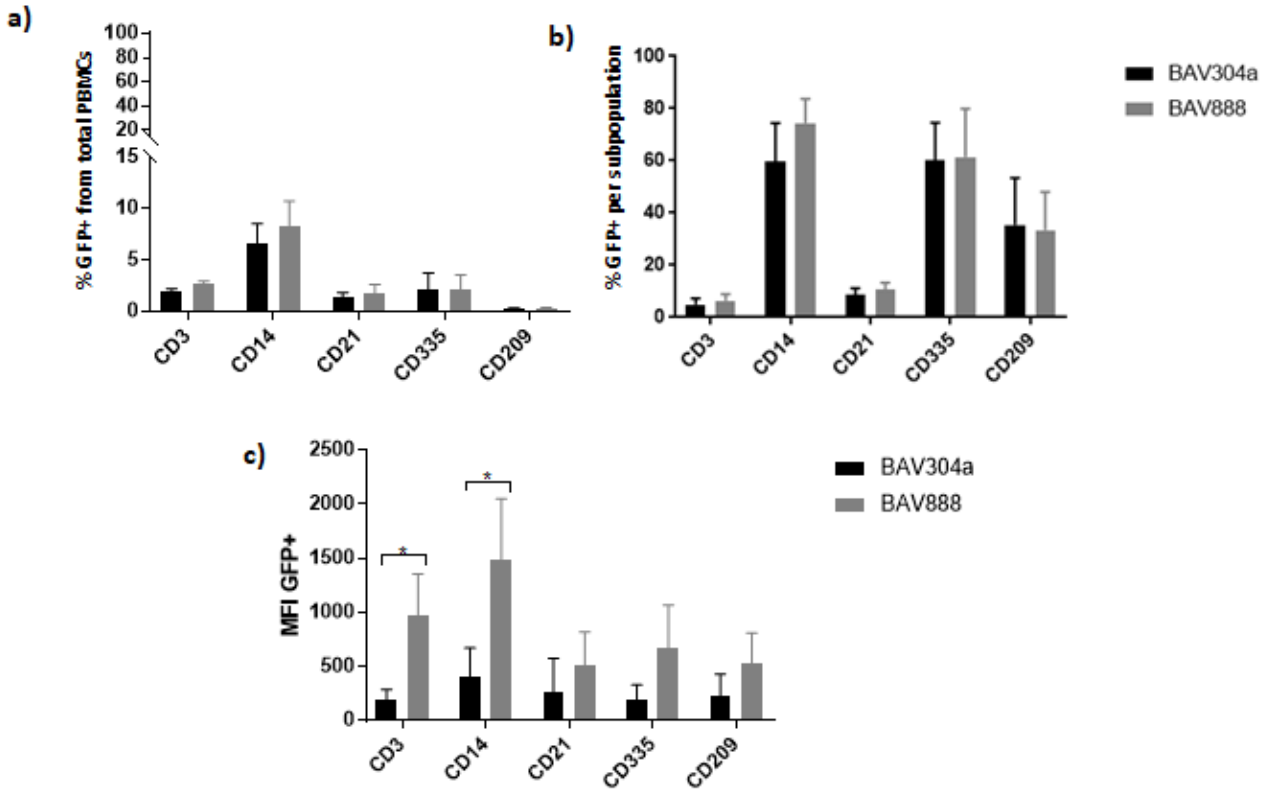

Figure 3.4 Transduction of subpopulations of PBMCs by BAdV-3. Bovine PBMCs from five animals were isolated from blood and transduced with BAV304a or BAV888 *in vitro* at MOI 1. After 16 hrs post transduction, the cells were collected and stained with antibodies recognizing CD3 (T cells), CD14 (monocytes), CD21 (B cells), CD335 (NK cells) or CD209 (dendritic cells) surface markers, followed by APC conjugated secondary antibodies. The samples were analyzed by flow cytometry, analyzing GFP and surface molecules expression in the total PBMC population (a) and percentage of GFP+ cells per subpopulation (b), in addition to mean fluorescence intensity (MFI) of GFP (c) of each cell. Data is representative of five independent repeats, and it is expressed as mean ±SD. For statistical analysis. The differences between BAV304a and BAV888 were analyzed using multiple t-tests, with GraphPad Prism 7 (GraphPad Software).

There are significant differences in the level of GFP specific transcripts compared to the level of BAdV-3 (19k, 52K, hexon) specific transcripts in monocytes transduced with either BAV888 (Fig. 3.5, panel c) or BAV304a (Fig. 3.5[d]). Interestingly, unlike monocytes transduced with BAV888, there is no significant difference in the BAdV-3 specific transcripts in monocytes transduced with BAV304a (Fig. 3.5 panel c and d).

3.3.6 Expression of costimulatory molecules and MHC molecules

To determine if the transduction of monocytes with either BAV304a or BAV888 can increase the expression of MHC molecules, MACS purified monocytes (CD14+) were transduced with either virus at a MOI of 1. After 16 hrs post transduction, the cells were stained with anti-MHC I or anti-MHC II antibodies, and analysed by flow cytometry. As seen in Figure 3.6 (a), there was no significant difference in the expression of MHC class I or class II proteins on the surface of monocytes transduced with either BAV304a or BAV388. Similarly, there was no significant difference in the mean GFP fluorescent intensity of MHC-II (panel b) or MHC- I (panel c) in monocytes transduced with either BAV304a or BAV888.

To determine if transduction of monocytes with either BAV304a or BAV888 alter the expression of co-stimulatory molecules (CD40, CD80 and CD86), MACS purified monocytes (CD14+) were transduced with either virus at a MOI of 1. After 16 hrs post transduction, RNA was isolated and analysed by qRT-PCR using gene specific primers. As seen in 3.6 (panel d), there were significant decreases in the transcription of CD40 and CD86 mRNAs in monocytes transduced with BAV888 compared to untransduced and BAV304a-transduced monocytes. However, there is no difference in the transcription of CD80 mRNA in monocytes transduced with either BAV304a or BAV888 or untransduced.

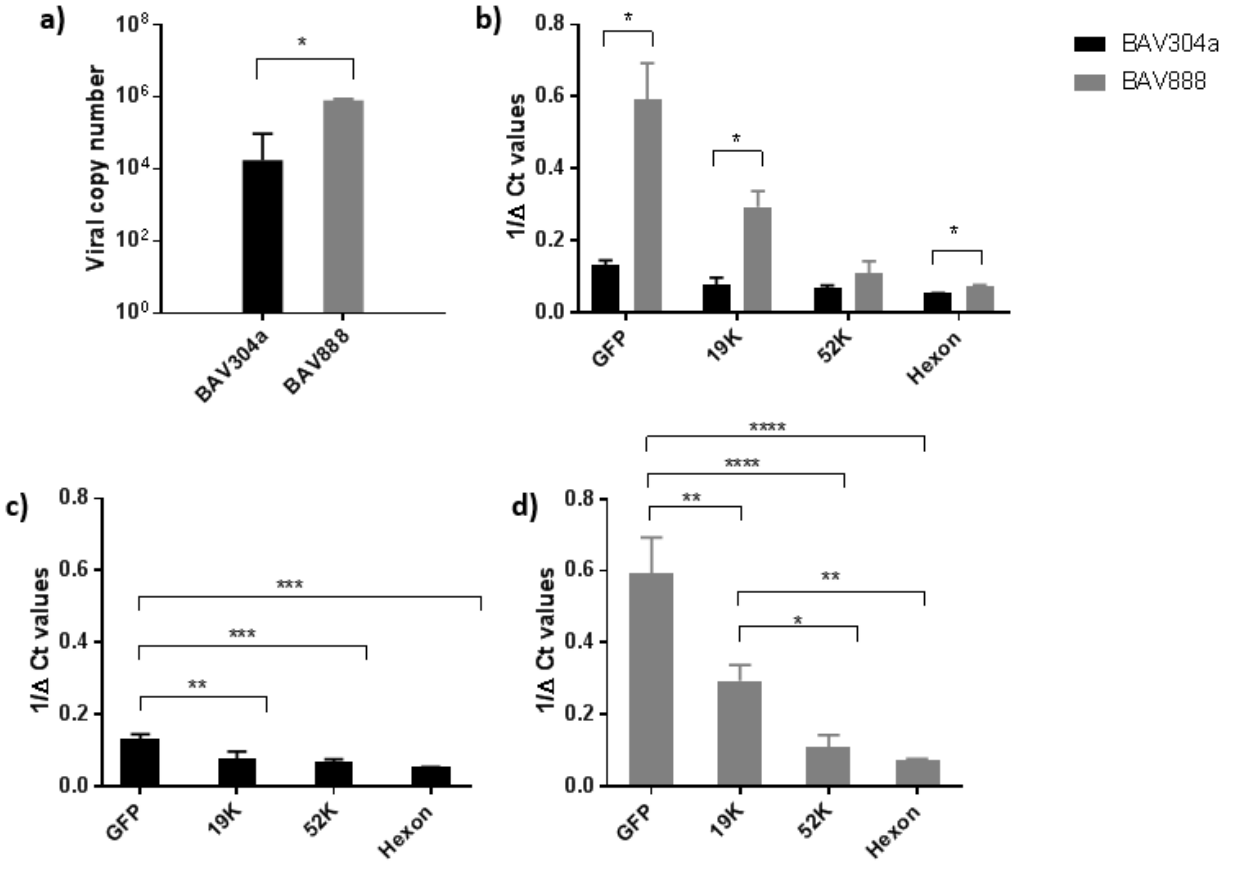

Figure 3.5 Viral uptake and gene expression are higher in BAV888-transduced monocytes. Bovine CD14+ cells were isolated from bovine blood using MACS and transduced with BAV304a and BAV888 *in vitro* at MOI 1. After 16 hours of incubation, cells were collected, and DNA and RNA extraction were performed. DNA samples were analyzed with qPCR to obtain the viral copy number, using a standard curve for wild-type BAdV-3 (a); the differences between BAV304a and BAV888 were analyzed using multiple t-tests. RNA samples were used for transcriptional analysis of GFP and early and late viral genes, using RT-qPCR. Ct values of BAV304a and BAV888 genes were normalized with β-actin (ΔCt) and represented as 1/ΔCt (b). Gene expression value for BAV304a (c) and BAV888 (d) genes was standardized by β-actin gene values (ΔCt) and expressed as 1/ΔCt. Data is representative of three independent repeats, and it is expressed as mean ±SD.

3.3.7 Production of cytokines in transduced monocytes (CD14+)

To determine cytokine production in monocytes (CD14+), MACS-purified monocytes were transduced with BAV304a or BAV888 at a MOI of 1. After 16 hrs post transduction, the supernatants were collected and analyzed for expression of cytokines by capture ELISA (IL-12 and TNF-α) or detection of cytokine mRNA by qRT-PCR (IFN-β). As seen in Fig. 3.7, there was a significant increase in the level of TNF-α produced by monocytes transduced with BAV888 compared to monocytes transduced with BAV304a (panel a). Similarly, the levels of IL-12 were significantly higher in supernatants from monocytes transduced with BAV888 compared to monocytes transduced with BAV304a (panel b). Furthermore, the level of IFN-β mRNA was significantly higher in monocytes transduced with BAV888 compared to monocytes transduced with BAV304a (panel c).

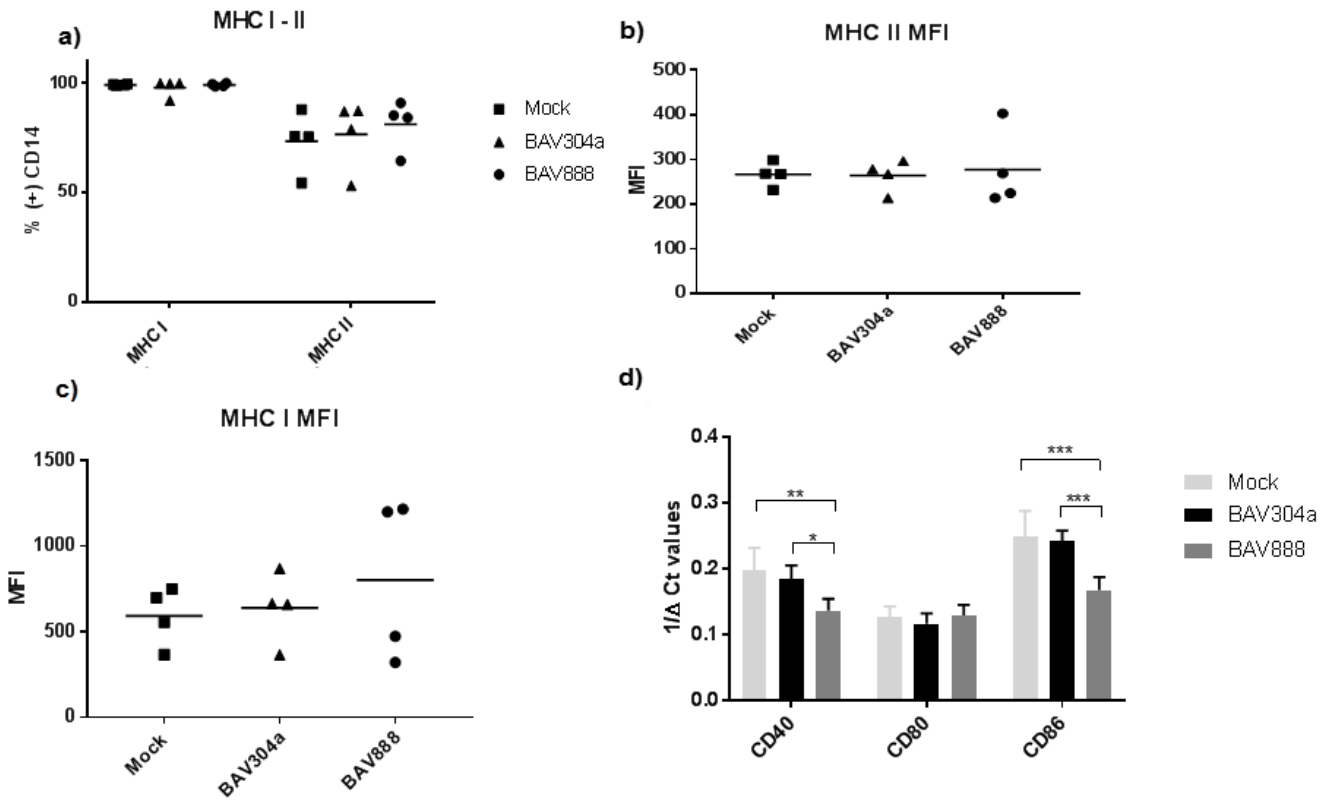

Figure 3.6 Activation of co-stimulatory molecules in transduced bovine monocytes. Bovine CD14+ cells were transduced with BAV304a and BAV888 *in vitro* at MOI 1. After 16 hours of incubation, cells were collected and stained for flow cytometry, and RNA extraction was also performed. Samples were stained with MHC I and MHC II primary antibodies to evaluate the percentage of cells expressing these proteins in transduced cells (a) and the differences in the level of expression through MFI of each cell (b and c). RNA samples were used for transcriptional analysis of costimulatory molecules CD40, CD80 and CD86 using RT-qPCR (d). The Ct values for each gene were normalized by β-actin (ΔCt) and expressed as 1/ΔCt. The differences between mock, BAV304a and BAV888 transduced monocytes were analyzed using Two-way ANOVA. Data is representative of four independent repeats, and it is expressed as mean ±SD.

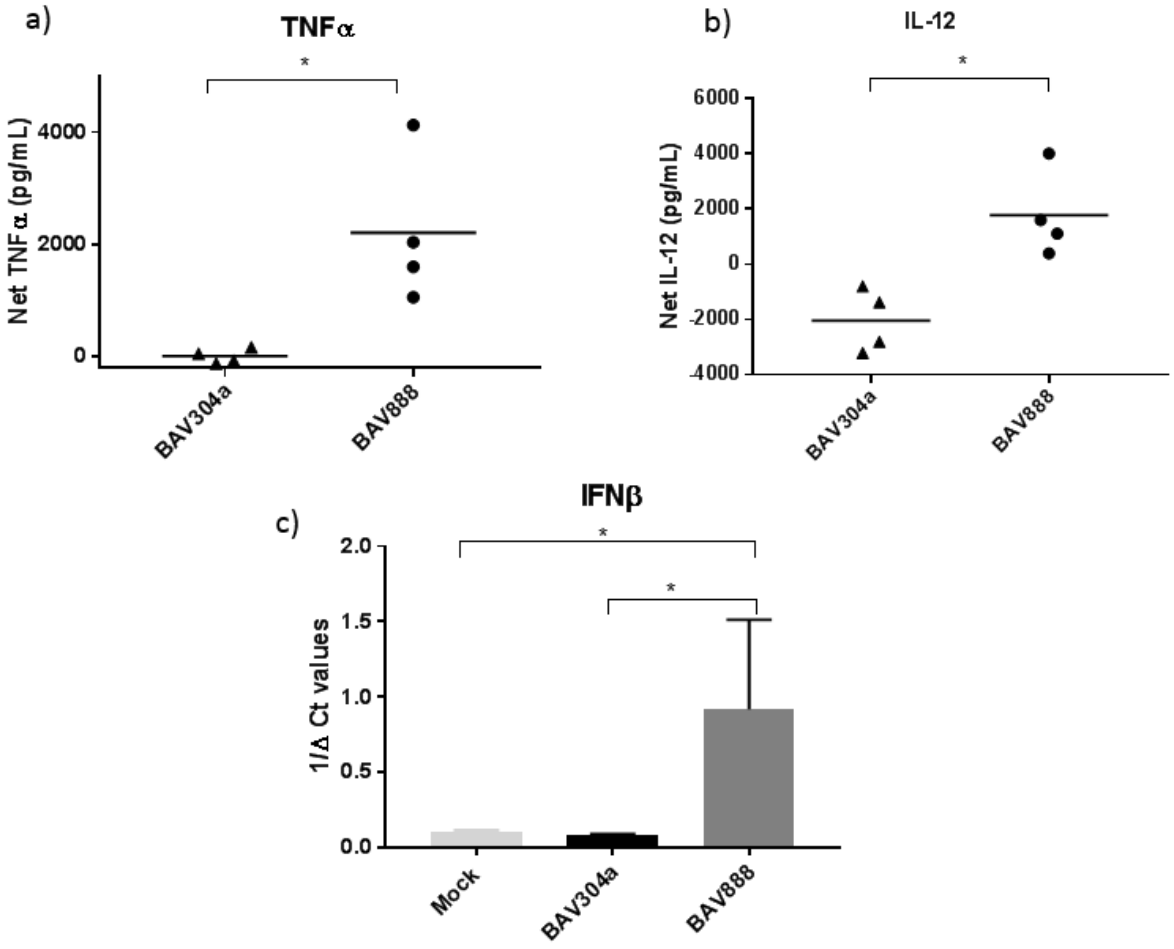

Figure 3.7 Cytokine production in transduced monocytes. Bovine monocytes were isolated and transduced with BAV304a and BAV888 *in vitro* at MOI 1. After 16 h incubation, the supernatant was collected to perform ELISA assays, and RNA was isolated for RT-qPCR. We performed capture ELISA against bovine TNFα (a) and IL-12 (b). The data shows to the concentration of cytokines for each virus-transduced cells – cytokines produced by mock-infected cells (net concentration). RNA samples were obtained to analyze the expression of IFNβ (c). The IFNβ gene expression in BAV304a and BAV888 transduced cells was normalized by β-actin (ΔCt) and expressed as 1/ΔCt. The differences between BAV304a and BAV888 were analyzed using multiple t-tests. The differences between mock, BAV304a and BAV888 transduced monocytes were analyzed using Two-way ANOVA. Data is representative of four independent repeats, and it is expressed as mean ±SD.

3.4 DISCUSSION

Many viruses including retroviruses, herpes viruses, baculoviruses, and adenoviruses, have become very important tools for gene delivery (Maheshri *et al*., 2006). A number of properties of adenovirus (Wilson, 1996), including efficient transfer of genes *in vivo*, even to slowly-proliferating or non-dividing cells (Graham, 1990; Lemarchand *et al*., 1992; Rosenfeld *et al*., 1992; Stratford-Perricaudet *et al*., 1990) has made it attractive as gene delivery vehicle. Although replication-defective recombinant human adenovirus-5 has been used in delivering vaccine antigens to mucosal surfaces of cattle, the amount of recombinant HAdv-5 required does not appear to result in producing an economical vaccine delivery vehicle in cattle (Gogev *et al*., 2004).

One way is to increase the transduction of antigen presenting cells by capsid modified BAdV-3 vector leading to enhanced availability of vaccine antigen, which can result in the initiation of stronger vaccine antigen specific adaptive immune responses. A very good candidate for use of this approach is the addition of targeting ligand(s) to C-terminus of minor capsid protein pIX of BAdV-3 which tolerates the addition of peptides that could potentially alter the transduction efficiency of BAdV-3 (Zakhartchouk *et al*., 2004).

Leukocyte surfaces including antigen presenting cells are rich in integrins that allow them to interact with different cell types and structures (Harris et al., 2000; Stewart *et al*., 1995). In contrast to HAdV-5 (Meier and Greber, 2003), BAdV-3 penton protein does not contain "RGD" motif (Reddy *et al.*, 1999) suggesting that BAdV-3 internalization may be independent of integrin receptors (Bangari *et al*., 2005). Thus, addition of an "RGD "motif at the C-terminus of BAdV-3 minor capsid protein pIX could enhance the ability to the virus to enter the integrin positive APCs (Wu *et al*., 2002). Here, we present the construction and evaluation of recombinant BAdV-3 expressing chimeric pIX (C-terminus of pIX fused to RGD motif). Moreover, we demonstrate that recombinant BAV888 (Recombinant BAV304a expressing chimeric pIX-RGD) is more efficient than BAV304a (BAdV-3 with GFP cassette in E3 deleted region) in transducing PBMCs, especially monocytes.

The addition of a cathepsin cleavage site in pIX protein of BAV888 allowed an efficient release of the virions from the endosomes, resulting in effective propagation to the adjacent cells. This is also supported by comparing BAV888 to BAV951 (BAdV-3 fused with EYFP at C-terminus of minor capsid protein pIX) (Zakhartchouk *et al*., 2004), where the escape of recombinant BAV951 from the endosome to the cytoplasm appeared inefficient (Zakhartchouk *et al*., 2004). Several reports have suggested that transduction efficiency of recombinant HAdV-5 displaying a ligand on the surface of virus using C-terminus of pIX appears inefficient (de Vrij *et al*., 2008; Campos and Barry, 2006; Corjon *et al*., 2008). It has been proposed that inefficient release of recombinant HAdV-5 particle from the endosome to cytoplasm may be due to high -affinity binding of pIX fused ligand and its receptor (Campos and Barry, 2006). Since cathepsin protease resides in lysozymes, inclusion of cathepsin cleavage sequence between pIX and targeting ligand may help in efficient release of virus from endosomes. Therefore, the insertion of cathepsin cleavage site between pIX and "RGD" motif resulted in increased transduction by BAV888. Similar results have been reported when cathepsin cleavage site has been inserted between pIX and targeting ligand in recombinant HAdV-5 (de Vrij *et al*., 2012).

Though HAdV-5 entry into the cell involves a primary interaction (fiber with the coxsackievirus-adenovirus receptor [CAR]) (Bergelson *et al*., 1997), and a secondary interaction (penton base "RGD" with $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins in the cell surface) (Wickham *et al*., 1993), little is known about requirement of both primary and secondary interactions for BAdV-3 entry in the cells. While primary interaction of BAdV-3 fiber with sialic acid has been reported (Li *et al*., 2009), the nature of the existence of secondary interaction is unclear (Bangari et al., 2005).

Although a slight increase in percent of GFP positive cells could be observed in BAV888 transduced PBMCs compared to BAV304a transduced PBMCs, the difference does not appear significant, suggesting that there is no apparent alteration in the primary interaction of BAdV-3 fiber with its receptor. However, there was significant difference in the mean GFP fluorescence intensity in PBMCs transduced with recombinant BAV888 compared to PBMCs transduced with BAV304a. This could be

due to increased attachment of recombinant BAV888 to PBMCs. Alternatively, it is possible that addition of "RGD" motif to pIX facilitated additional interaction with integrin receptors resulting in increased entry of recombinant BAV888 in bovine PBMCs.

Compared to other PBMC subpopulations, high percentage of GFP positive monocytes (CD14+) are detected either in BAV304a or BAV888 infected cells with no significant difference in the number of GFP positive cells. Moreover, the mean GFP florescence intensity is significantly higher in monocytes transduced with BAV888 compared to monocytes transduced with BAV304a. Similar results are observed in T-lymphocytes ($CD3^{+}$). These results suggest that additional interaction of "RGD" motif could result in secondary interaction between "RGD" and cellular integrins, thus modulating the uptake of BAV888. A number of observations support this speculation. First, viral genome copies are significantly higher in monocytes (CD14+) transduced with BAV888 compared to BAV304a transduced monocytes. Secondly, there is significant difference in the detection of GFP and BAdV-3 19K ($E1B^{small}$ region) specific transcripts in monocytes (CD14+) transduced with BAV888 compared to monocytes transduced with BAV304a. Thirdly, the level of 19K specific transcripts is significantly different than other BAdV-3 specific transcripts in monocytes transduced with BAV888 compared to monocytes transduced with BAV304a.

These findings are relevant for the development of vaccines, since even though monocytes are not considered professional APCs, they can differentiate into macrophages or dendritic cells once they reach tissues, and the latter ones can effectively initiate an adaptive immune responses with naïve T cells (Steinman and Hemmi, 2006; Trombetta and Mellman, 2005). Moreover, earlier studies suggest that immature monocytes themselves can act as APCs, processing antigens, retaining them and presenting them to T-cells in the lymph nodes after differentiation to monocyte-derived dendritic cells (Tacke *et al*., 2006).

Analysis of recombinant virus transduced monocyte (CD14+) showed no significant increase in the expression of MHC I or MHC II or mean fluorescence intensity of MHC I and II in monocytes

transduced with either with BAV304a or BAV888. MHC class I and II molecules play an important role in antigen presentation; while MHC I presents intracellular peptides to CD8+ T-cells, MHC II presents processed exogenous antigens to CD4+ T-cells (Kurts *et al*., 2010). Interestingly, some adenoviruses have shown the ability to downregulate MHC class I molecules in infected cells, to avoid recognition by CD8+ T-cells (Proffitt *et al*., 1994; Sester *et al*., 2010).

Transduction of bovine monocytes with either BAV304a or BAV888 led to significant downregulation of CD40 or CD86 specific transcripts compared to un-transduced bovine monocytes. However, there was no significant difference in the CD80-specific transcripts in monocytes untransduced or transduced with BAV304a or BAV888. Activation of monocytes leads to alterations in the expression of activation markers (e.g. CD40, CD80, CD86 etc.). CD40 is expressed in a greater variety of cells, including B cells, activated monocytes, and endothelial cells, among others (Grewal & Flavell, 1996). The main role of CD40 is in APC activation and in the efficient presentation of the antigen to T-cells (Schoenberger *et al*., 1998). In antigen-presenting cells, the absence of co-stimulatory signals could lead to low stimulation of T-cells, leading to decreased proliferative T response or an anergic state (Boussiotis *et al*, 1996; Pinelli *et al*., 1999).

Our study show that the level of TNF-α is significantly higher in bovine monocytes transduced with BAV888 compared to bovine monocytes transduced with BAV304a. It is possible that higher amount of TNF-α detected in BAV888 transduced monocytes could be due to higher number of viral genome copies which can trigger an anti-viral immune response. TNF-α appears to be important in differentiation of monocyte-to-dendritic cell phenotype (Lyakh *et al*., 2002). Adenoviruses induce a strong innate immune response (Liu & Muruve, 2003) resulting in the induction of inflammatory cytokines such as IL-12 and TNFα (Zhang *et al*., 2001).This early inflammatory response seems to be dose-dependent, but it reaches a point of saturation with high viral titers, resulting in no further increases in the inflammatory response (Hidaka *et al*., 1999; Higginbotham *et al*., 2002).

The level of IL-12 protein detected in bovine monocytes transduced with BAV888 was significantly higher than bovine monocytes transduced with BAV304a. The presence of IL-12 indicates that monocytes in culture were activated, and the higher level of IL-12 expression in BAV888 transduced monocytes may indicate a higher level of activation than mock- or BAV304a-transduced monocytes. IL-12 is be produced by many different cell types including activated monocytes (Hamza *et al*., 2010), and it induces the production of IFN-γ and regulates the induction of type of adaptive immune responses (Mosmann *et al*., 1989). Studies have shown that even the attachment of monocytes to plastic culture plates results in their activation (Kelley, 1987).

The level of IFN-β transcripts were significant higher in bovine monocytes transduced with BAV888 compared to bovine monocytes transduced with BAV304a.There is no difference between mock and BAV304a induction of IFN-β. This lack of cytokine expression could be due to low viral genome uptake. Alternatively, BAV304a could have managed to counteract the anti-viral response by blocking induction of an IFN response. Interferon (IFN) type I responses involving IFNα and IFNβ can be induced directly in the presence of viruses as primary protection in recently-infected cells (Randall & Goodbourn, 2008). Adenoviruses have been shown to induce type I interferon *in vitro* and *in- vivo* (Huarte et al., 2006; Zhu et al., 2007). However, recent studies suggest that BAdV-3 protein V can prevent the IFN-β promoter activation (Hao, 2017).

Bovine dendritic cells (CD209+) appear to be permissive to transduction by BAV304a and BAV888. Around 40% of the dendritic cells were transduced with both viruses at MOI 1. However, there was no significant difference the transduction efficiency or mean GFP fluorescence intensity in bovine dendritic cells transduced with either BAV304a or BAV888. Dendritic cells are key components of the immune system because they are the only antigen presenting cells capable of priming naïve T-cells (Allenspach *et al*., 2008; Inaba *et al*., 1990; Trombetta & Mellman, 2005). Because of this characteristic, dendritic cells are an optimal target for vaccination, especially when a cellular immune response is desirable (Cohn and Delamarre, 2014). It has been demonstrated that HAdV-5 is able to infect human

peripheral blood monocyte-derived dendritic cells using lactoferrin and CD209 as primary receptors (Adams *et al*., 2009). Moreover, recent studies have shown that HAdV-5 based vectors can internalize bovine skin dendritic cells via actin-dependent endocytosis, which is an alternative pathway to CAR, integrins, and other known receptors (Guzman *et al*., 2016). This integrin-independent mechanism could explain the lack of difference in transduction with BAV304a and BAV888.

3.5 CONCLUSIONS

In conclusion, here we describe the transduction efficiency of two recombinant BAdV-3: BAV304a (with GFP insertion in E3-deleted region) and BAV888 (with RGD motif in C-terminus of pIX and GFP cassette in E3-deleted region) in bovine PBMCs. We have demonstrated that BAV888 is more efficient in transducing some bovine PBMCs subtypes than BAV304a. Both viruses seem to have similar tropism for the distinct subpopulations, and share a higher percentage of transduction of monocytes. However, data of mean fluorescence intensity of GFP and viral copy number show that the viral uptake of BAV888 is higher in monocytes compared to BAV304a. This difference in uptake suggests that the addition of an RGD motif in the BAdV-3 capsid may enhance its ability to internalize the cells, without interfering with the viral tropism.

Although the transduction of monocytes with either BAV304a or BAV888 do not increase the level of costimulatory molecules expressed in the cells, the cytokines pattern suggest that there is an induction of innate immune response in monocytes transduced with BAV888, especially because of the significantly higher level of IFN-β expressed in those cells. Since we demonstrated that BAV888 is present in a higher copy number in monocytes than BAV304a, this immune response may be dose-dependent, which may pose a challenge for clinical use.

Future studies should further explore if the dose of BAV888 has to be adjusted to avoid an anti-viral response against the vector. Since dendritic cells are the most important professional antigen presenting cells, the interaction between our recombinant BAV888 and DCs should be further analyzed. In addition, the higher MFI in T-cells transduced with BAV888 virus could indicate that there is a higher virus uptake in these cells, therefore, the repercussions of this for vaccination or gene therapy could be explored in future studies. Moreover, the insertion of an antigen in BAV888 and immunization *in vivo* could help to analyze the type of immune response generated and if it can deliver vaccine antigens efficiently as a recombinant vector.

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