PROTEINS pV AND pVIII ENCODED BY BOVINE ADENOVIRUS-3 ARE POTENTIAL VIRAL SUPPRESSORS OF THE INTERFERON RESPONSE

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ABSTRACT

Bovine adenovirus 3 (BAdV-3), which belongs to the *Mastadenovirus* genus, is a non-enveloped virus with an icosahedral capsid of 75 nm diameter, which wraps a double-stranded linear DNA genome with size of 34,446 base pairs (Reddy et al., 1999). It has been studied for potential application as gene delivery vector (Zakhartchouk et al., 1999). But one of the major limitations of adenovirus vector application is the induction of innate immune response. It has been reported that multiple adenoviral components could be detected by several host sensors leading to multi-layered innate immune response, which limit the viral infection (Hendrickx et al., 2014a, Thaci et al., 2011). Like many other viruses, it has also been shown that adenoviruses have developed strategies to modulate the innate antiviral by expression of adenoviral early proteins E1A, E1B, E3, E4 as well as structural proteins pVI and pVII (Avgousti et al., 2016, Burgert et al., 2002, Schreiner et al., 2012).

Here, the objective of this study is to identify BAdV-3 viral proteins that are involved in regulating the primary signaling cascade of innate immune response. This could provide insight into methods of engineering BAdVs in future with attenuated innate response and enhanced vaccine delivery features. The initial luciferase reporter assay results of monitoring type I interferon (IFN) response suggest that several BAdV-3 viral proteins may act as potential suppressors of the interferon response. Since both pV (a core protein connecting the viral capsid and DNA genome) and pVIII (a structural protein connecting the core with the viral capsid) significantly reduced the expression of luciferase due to reduced activation of IFN-β promoter in both cell lines tested, we choose pV and pVIII for further analysis (Ayalew et al., 2016, Gaba, 2016).

Overexpression of pattern recognition receptors (RIG-1, MDA-5, TLR-3) did not increase the IFN-β activity in cells expressing pV, suggesting that pV acts at a common step(s) of IFN-β signaling pathways used by different PRRs. Overexpression of TBK1 but not IKKε significantly down-regulated the IFN-β promoter activity in pV expressing cells suggesting that pV may suppress the activation of IFN-β promoter by targeting the activity down-stream of TBK1 activation including activation of interferon regulatory factor(s) (IRF) or at the level of IKKε kinase activation. Using co-immunoprecipitation assays, we demonstrated that pV interacts with IRF3. Further analysis using Western blotting and immunofluorescence microscopy suggested that expression of pV leads to significant reduction in the phosphorylation and translocation of transcription factor IRF3 to the nucleus. These results suggest that BAdV-3 pV modulate the activation of IFN-β promoter by affecting the required activation of IRF3 for its transcription activation function.

Although pV significantly reduced the transcription factor NF-kB or IRF7 responsive promoter activity, there was no effect on the nuclear translocation of NF-kB and IRF7 in the presence of pV, suggesting that pV may not act to modulate IFN-β promoter activity by targeting NF-kB and IRF7 transcriptional factors.

Similar experiments using overexpression of different PRRs (RIG-1, MDA-5, TLR-3) or kinases (TBK1 and IKKε) in pVIII expressing cells suggested that pVIII may act down-steam of TBK1 and IKKε in the signaling pathway of IFN-β promoter activation. Moreover, although pVIII significantly reduced the transcription factor NF-kB responsive promoter activity, there was no effect on the nuclear translocation of NF-kB in the presence of pVIII, suggesting that pVIII may not act to modulate IFN-β promoter activity by targeting NF-kB and IRF7 transcriptional factors. Further experiments are needed to explore the possibility that pVIII interferes with the DNA

binding activity of IRF3 by assessing the function of CBP/p300 transcriptional co-activating proteins.

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ABBREVIATIONS USED IN THIS THESIS

ALRs	AIM2-like receptors
BAdV	Bovine Adenovirus
cGAMP	Cyclic guanine-adenine dinucleotide
cGAS	Cyclic GMP-AMP synthase
CBP	CREB-binding protein
CPE	Cytopathic Effect
Co-IP	Co-immunoprecipitation
CLRs	C-type lectin receptors
CsCl	Cesium Chloride
DAPI	4'-6-Diamidino-2-phenylindole
DDX	DEAD Box Protein
FACS	Fluorescence activated cell sorting
poly I:C	Polyinosinic-polycytidylic acid
IKKε	Inhibitor of nuclear factor kappa-B kinase subunit epsilon
IRES	Internal Ribosome Entry Site
IRF	Interferon regulatory transcription factor
ISGs	Interferon stimulated genes
Kb	Kilo base
KDa	Kilodalton
mAb	Monoclonal antibody
MDA5	Melanoma Differentiation-Associated protein 5

MOI	Multiplicity of infection
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	Nucleotide oligomerization and binding domain (NOD)-like receptors
PRRs	Pattern recognition receptors
RLRs	Retinoic acid inducible geneI-like receptors
RIG-I	Retinoic acid-inducible gene I
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gels
$TCID_{50}$	Tissue culture infectious dose50
TLRs	Toll-Like Receptors
TBK1	TANK-binding kinase 1
VA RNA	Virus-associated RNA

1 LITERCATURE REVIEW

1.1 Adenovirus

Adenoviruses are non-envelope virions, which were first isolated from adenoid tissue (Hilleman and Werner, 1954, Rowe et al., 1953). Adenoviruses contain an icosahedral capsid which encloses a double stranded linear DNA genome of variable size (26 to 45 kilobases) (Kovács et al., 2003). So far, more than 100 serotypes of adenoviruses have been identified affecting various vertebrate hosts (Babiuk and Tikoo, 2000), mostly associated with only mild clinical infections (Chiocca et al., 1996). Adenoviruses have been intensively studied for its molecular biology, which has contributed to the research of the splicing mechanism (Berget et al., 1977, Broker et al., 1977). Recently, intense studies have also focused on using adenoviruses as potential vectors for gene delivery (Babiuk and Tikoo, 2000, Nash and Parks, 2016, Toth et al., 2010).

1.1.1 Adenovirus classification

Adenoviruses belong to the family Adenoviridae and are classified into five genera including *Mastadenovirus*, *Aviadenovirus*, *Ataadenovirus*, *Siadenovirus* and *Ichtadenovirus* according to their host origins, phylogenetic relationships (Davison et al., 2003).

Although members of *Mastadenovirus* and *Aviadenovirus* genuses only originate from mammals or birds respectively, members of *Atadenovirus* and *Siadenovirus genuses* have a wide variety of hosts. So far genus *Ichtadenovirus* has been reported to contain a single species confirmed in fish (Davison et al., 2003).

Mastadenovirus is the largest genus, which is comprised of 25 adenovirus species infecting mammals (Knowles et al., 2012). They have been shown to infect human, bovine, monkeys, tree shrews, equine, murine, ovine, porcine, bats, canines. Human adenovirus (HAdV) is the first

member isolated in early 1950s from human adenoids (Rowe et al., 1953). So far 67 human adenovirus serotypes have been identified (Ghebremedhin, 2014, Rosen, 1960). The adenovirus genome is flanked by inverted terminal repeats (ITR) which also act as replication origin sites. The longest ITR is identified in bovine adenovirus (BAdV)-10 (Dán et al., 2001). Sixteen adenoviral genes shared by members *of Mastadenovirus* genus including DNA polymerase (pol), terminal protein (pTP), DNA binding protein(DBP), 52K, IVa2, pIIIa, III, pVII, pX, pVI, hexon, protease, 100K, 33K, pVIII, fiber have been presumed to be inherited from a common ancestral. The proteins encoded by these genes function in DNA replication (pol, pTP, DBP), DNA encapsidation (52K and IVa2) and virion capsid formation (pIIIa, III, pVII, pX, pVI, hexon, protease, 100K, 33K, pVIII, fiber) (Davison et al., 2003). However, capsid protein pIX, core protein pV and some of the protein products from E1, E3 and E4 regions are specific for members of *Mastadenovirus* genus (Davison et al., 2003).

Members of *Aviadenovirus* genus only infect avian host, including chicken, falcon, goose, and turkey (Knowles et al., 2012) and contain genomes with the size of 43 to 45 kilobases encoding 47 to 54 open reading frames (Chiocca et al., 1996, Griffin and Nagy, 2011, Kaján et al., 2012). Unlike *Mastadenoviruses,* the genes encoding pIX, pV, E2 region proteins, E3 region proteins are absent in *Aviadenoviruses* genomes (Davison et al., 2003, Grgić et al., 2011). Interestingly, two fiber proteins per vertex are present in *Aviadenovirus* virion, irrespective of the presence of one or two fiber genes in the virus genome (Griffin and Nagy, 2011, Marek et al., 2014). Compared to members of *Mastadenovirus* genus, the ITRs in the genomes of members of *Aviadenovirus* genus are at the same locations but relatively shorter. Some *Aviadenoviruses* including fowl adenovirus-4, turkey adenovirus-3 and duck adenovirus-1 are associated with causing economically important diseases like hydropericardium syndrome (Brash et al., 2009, Pitcovski et al., 1998).

Atadenovirus genus is named because members of this genus contain high AT content in their genomes. Members of *Atadenovirus* genus can infect various hosts such as birds, reptiles, ruminant and marsupial (Benkö and Harrach, 1998, Benko et al., 2002, Hess et al., 1997). Their genomes have relatively smaller ITRs (46 to 118 bps) and don't contain the genes encoding pV and pIX proteins (Benko et al., 2002, Vrati et al., 1995). However, members encode *Atadenovirus* genus-specific genes in E1 and E4 regions encoding proteins namely p32K and LH3 (ÉlŐ et al., 2003, Gorman et al., 2005).

Siadenovirus genus is named because members of the genus contain viral gene encoding sialidase. Members of *Siadenovirus* can infect reptiles, frogs and turkeys (Davison et al., 2003, Kovács and Benkő, 2009, Pitcovski et al., 1998). They have relatively short genomes with the size of about 26 kilobases containing short ITRs (Davison et al., 2003, Pitcovski et al., 1998). Similarly, the E1, E2, E3 and E4 early regions and the genes encoding proteins V and IX are absent in their genomes (Davison et al., 2003, Kovács and Benkő, 2009, Pitcovski et al., 1998).

Ichtadenovirus is the latest genus that has been reported in the *Adenoviridae* family (Davison et al., 2003). So far it has only one species, White Sturgeon Adenovirus 1 (WSAdV-1), which was isolated from white sturgeon (Davison et al., 2003). The genome of WSAdV-1 is 48,395 bps, which is the longest genome identified in all known adenoviruses (Kovács et al., 2003).

1.1.1.1 Virion structure of Adenoviruses family

Adenoviruses are non-enveloped viruses with a double stranded linear DNA genome, which is surrounded by an icosahedral capsid (Gallaher and Berk, 2013, Kovács et al., 2003). The adenovirus capsid contains 13 proteins including major structural proteins (hexon, penton, fiber), minor structural proteins (IIIa, pVI, pVIII, pIX) and core proteins (pV, pVII, Mu, terminal protein, IVa2, adenovirus protease) (Fig 1.1). It is needed to mention that, the information below are based

on human adenovirus type 5(HAdV-5) since most of the current research on adenovirus are based on HAdV-5.

For the major capsid proteins (hexon, penton, fiber), 720 copies of hexon are present at capsid facets as trimers per virion, 5 copies of penton are present at each of the 12 vertices, which makes a complex with a trimer of fiber (Russell, 2009). For the four minor capsid proteins (IIIa, pVI, pVIII, pIX), 240 copies of protein IX are present with C-terminus exposed outside of the capsid (Vellinga et al., 2005). 60 copies of protein IIIa are present at the inner capsid surface per virion. 360 copies of the protein VI are also present at the inner surface of capsid, which interact with protein IIIa, hexon, V (Matthews and Russell, 1998, Reddy and Nemerow, 2014, San Martín, 2012, Vellinga et al., 2005). 120 copies of the protein VIII are present at the inner surface of capsid as well, provide a bridge between the major capsid protein hexon and the capsid (San Martín, 2012). The core proteins (pV, pVII, Mu, terminal protein, IVa2, adenovirus protease) connect the capsid and the viral DNA genome to form the adenovirus core (Corden et al., 1976). Protein VII is associated with adenovirus genome (van Oostrum and Burnett, 1985). Protein Mu and V also function in connecting the capsid and core through their association with viral DNA genome.(Chatterjee et al., 1986, Hosokawa and Sung, 1976) . The terminal protein is found binding to the 5′ ends of viral DNA (Rekosh et al., 1977). Protein IVa2 present at only one vertex of the virion, involved in viral DNA encapsidation (Christensen et al., 2008, Ewing et al., 2007). Adenovirus protease has been reported to be involved in the cleavage of some virus-encoded precursor proteins for the virion maturation (Greber, 1998).

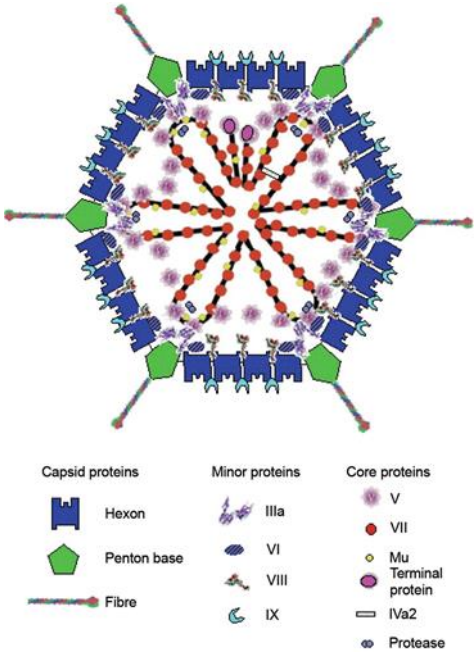

Figure 1.1 Virion structure of Adenoviruses (Russell, 2009).

1.1.1.2 Genome organization

Adenovirus genome is divided into four early transcription regions (E1-E4), two intermediate genes (pIX and IVa2) and one major late unit which is alternatively spliced into five groups (L1-5) after transcription. DNA replication initiates from two DNA replication origin sites, namely inverted terminal repeats (ITRs). The viral genes are transcribed by RNA polymerase II (Mishoe et al., 1984) but the viral associated RNAs (VA RNAs) are reported to be transcribed by RNA polymerase III (Kidd et al., 1995).

1.1.1.3 Adenovirius life cycle

The adenovirus life cycle can be divided into six steps: virus entry, expression of early genes, DNA replication, expression of intermediate genes, expression of late genes, virus assembly and release (Fig.1.2).

The initial non-specific and electrostatic interactions between host cells and adenovirus have been observed, which help with the association between viral fiber protein and diverse cellular membrane receptors (Arnberg, 2009). After the initial attachment, virus internalization is triggered by the interaction between cellular surface integrins and arginine-glycine-aspartic acid (RGD) motif of viral penton protein (Shayakhmetov et al., 2005). The interaction activates phosphotidylinosito-3-OH kinase, which is involved in the rearrangement of actin cytoskeleton required for endocytosis (Varga et al., 1991). Moreover, the clathrin-mediated virus endocytosis also requires dynamin, GTPase, and the adaptor protein 2 for its regulation (Meier and Greber, 2004).

After virus internalization into the endosome, virus stimulates the endosome acidification and the partial uncoating of viral capsid proteins including penton, fiber, IIIa, hexon, VI, VIII, pIX (Gastaldelli et al., 2008, Greber et al., 1993), the lytic portion of pVI gets exposed after

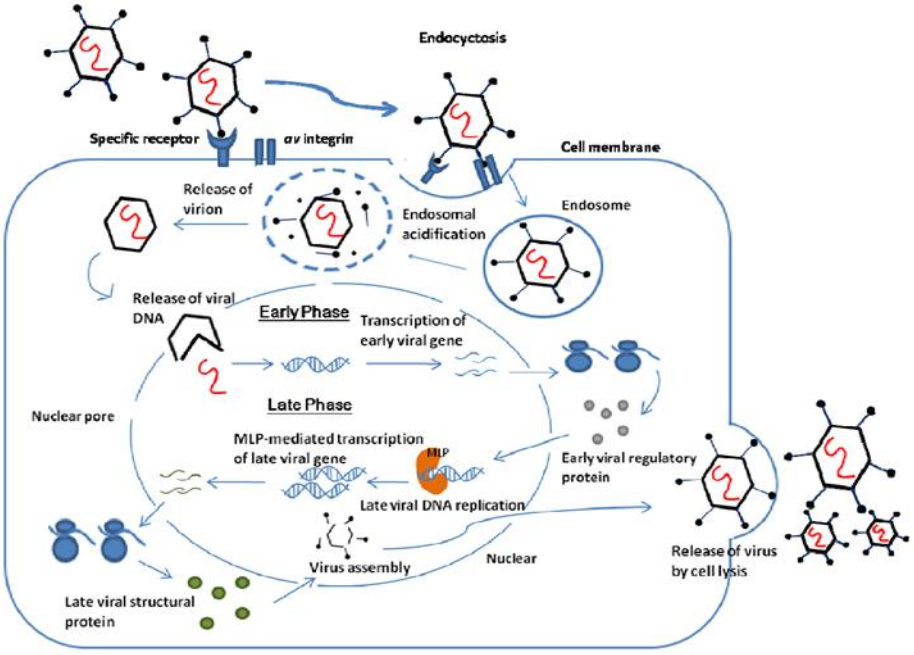

Figure 1.2 Adenovirus life cycle (Waye and Sing, 2010)

cleavage by adenovirus protease, leading to the disruption of endosomal membrane and transportation of virus into the microtubule organization center (MTOC) located near the nucleus by using the microtubule motor protein dynein in cytoplasm (Bremner et al., 2009, Kelkar et al., 2006, Wiethoff et al., 2005). The virus attaches to the nuclear pore complex (NPC) through the association between virus hexon protein with cytoplasmic nucleoporins (Nups) Nup214 and Nup358 which are located in fibrils on NPC (Cassany et al., 2015, Leopold et al., 2000). In addition, molecular motor kinensin 1 has been reported to be required for further virus uncoating at the NPC. Finally, the cellular transportation factors such as transportin, importin and histone H1 are required for the transportation of viral DNA genome complex into the nucleus through NPC (Hindley et al., 2007, Strunze et al., 2011, Wodrich et al., 2006). The expression of the early genes first starts with the transcription of E1A region, which produces two main E1A proteins namely 289R and 243R with various functions including the induction of S phase in the infected cells, control of cell cycle and the transcription of other early genes (Avvakumov et al., 2002).

The E1B region encodes two proteins, namely E1B-55K and E1B-19K, both have functions in apoptosis prevention. The E1B-19K has been shown to inhibit the programmed cell death by suppressing the activity of the pro-apoptotic proteins BAK and BAX (Sundararajan et al., 2001). The E1B-55K together with viral protein E4orf6 and some cellular proteins has been reported to block tumor suppressor p53 protein-dependent apoptosis by causing the p53 degradation (Sarnow et al., 1984). The E2 region encodes three proteins: DNA binding protein (DBP), DNA polymerase (Pol) and pre-terminal protein (pTP), involved in viral DNA replication (de Jong et al., 2003). DNA binding protein has been shown to be involved in the binding of viral DNA polymerase to the DNA genome, the unwinding of viral double-stranded DNA and the removal of the secondary structures during the elongation of DNA replication (Dekker et al., 1997,

van Breukelen et al., 2003). The transcription of E3 region is regulated by promoter responsive to E1A and produces four proteins, namely E3-19K, 10.4K, 14.5K and 14.7K, which are involved in the evasion of host immune defense (Horwitz, 2004) (described in details in section 1.2.4). The E4 region encodes six proteins, namely orf1, orf2, orf3, orf4, orf6 and orf7, which are involved in the control of cell cycle, regulation of the viral DNA replication, the expression of late viral proteins and counteraction of the antiviral innate immune response (Greer et al., 2011, O'Shea et al., 2005, Ullman and Hearing, 2008, Weitzman and Ornelles, 2005). For example, E4orf3 and E4orf6 have been shown to increase the expression of late viral genes by contributing to the cytoplasmic accumulation and stability of viral mRNA (Imperiale et al., 1995).

Adenovirus DNA replication starts after accumulation of the E2 gene products (DBP, DNA Pol and pTP). The inverted terminal repeats (ITRs) which locate at both ends of viral DNA genome are two origin sites of DNA replication (Hay, 1985). The pre-terminal protein (pTP) binding to the 5′ end of DNA works as a protein primer to initiate the viral DNA replication (de Jong et al., 2003). Moreover, the pre-initiation complex (PIC) comprised of all E2 gene products (pTP, Pol and DBP) and two cellular proteins, namely nuclear factor I (NFI) and NFIII is also required for the replication initiation (Bosher et al., 1990, Hoeben and Uil, 2013, Mul et al., 1990). Virus-encoded DNA polymerase has both 5’ to 3’ polymerase activity and 3’ to 5’ exonuclease activity. The nuclear factor NFII and E2 gene products (Pol and DBP) have been shown to be required for the replication elongation (Challberg et al., 1980, Lichy et al., 1981, Nagata et al., 1983). During the initiation of viral DNA replication, two intermediate proteins IVa2 and pIX are also produced. Protein IVa2 has been reported to be involved in the regulation of major late promoter (MLP) activity, viral capsid formation and DNA encapsidation (Lutz and Kedinger, 1996, Tribouley et al., 1994, Zhang and Imperiale, 2003). Protein IX is a minor structural protein with various

functions including the stabilization of viral capsid and activation of major late promoter (MLP) (Boulanger et al., 1979, Lutz et al., 1997). Moreover, it has been recently studied as a target for the alteration of adenovirus tropism by addition of other ligands (Dmitriev et al., 2002).

Late (L) genes are expressed after the initiation of DNA replication. All late genes are encoded by a major late transcription unit (MLTU) to produce a single pre-mRNA, which is subsequently spliced and polyadenylated with differential poly A sites into approximate 20 mature late mRNAs (Fraser et al., 1979, Nevins and Darnell, 1978). In HAdV-5, the mature late mRNAs are divided into five groups (L1-L5) according to the use of poly A sites (Nevins and Darnell, 1978, Ziff and Evans, 1978). They are expressed under the regulation by major late promoter (MLP), which has very low activity during the early infection but gets stimulated by viral proteins IVa2 and L4-22k during the late phase of infection (Backström et al., 2010, Pardo-Mateos and Young, 2004, Tribouley et al., 1994).

Virus assembly starts in the cytoplasm where protein hexon forms the trimer with the assistance of L4-100K protein (Horwitz et al., 1969, Velicer and Ginsberg, 1970). Transport of hexon trimmers along with other proteins including penton to nucleus leads to the formation of the empty capsids in the nucleus of infected cells (Cepko and Sharp, 1982). Then, adenovirus DNA genome is packaged into the empty capsids starting with the left end of genome which contains seven AT-rich repeat elements (A1-A7) necessary for adenovirus DNA encapsidation (Edvardsson et al., 1976, Hammarskjöld and Winberg, 1980, Hearing et al., 1987, Ostapchuk and Hearing, 2003). Moreover, protein IVa2 has also been shown to be involved through the interaction with DNA packaging sequence. Finally, some structural proteins are cleavaged by viral protease for the maturation of infectious virus progeny (Mangel et al., 2003, Weber, 1976). Although virus particles have been found to be released from the infected cells about 30 hrs post-infection, the

exact mechanism for adenovirus release is unknown. Viral E3-11.6kDa protein has been suggested to be involved in the lysis of infected cells (Tollefson et al., 1996).

1.1.2 Bovine adenovirus

The isolation of Bovine adenovirus (BAdV) was first reported in 1959 from cattle respiratory secretion (Klein et al., 1959). To date, fourteen different serotypes of bovine adenoviruses mostly associated with only mild-clinical infections have been identified from both cattle samples and environment (Lehmkuhl et al., 1975, Lehmkuhl and Hobbs, 2008, Sibley et al., 2011).

1.1.2.1 Classification of Bovine adenoviruses

Based on the phylogenetic proximit, the currently known fourteen serotypes of bovine adenovirus can be divided into six groups (A-F). Based on various criteria including nucleotide content, genus specific antigens and restriction enzyme analysis pattern, bovine adenovirus serotypes are classified in *Mastadenovirus* and *Atadenovirus* genuses.

1.1.2.2 Characteristics of BAdV-3

Bovine adenovirus 3 (BAdV-3) is a non-enveloped virus with an icosahedral capsid of 75 nm diameter, which wraps a double-stranded linear DNA genome with size of 34,446 base pairs (Reddy et al., 1999). The isolation of BAdV-3 was first reported in 1965 from the conjunctiva of a healthy cattle (Darbyshire et al., 1965). Recently, isolation of BAdV-3 from sick calves was also reported in China (Zhu et al., 2011).

1.1.2.3 Genome organization of BAdV-3

BAdV-3 has a linear double-stranded DNA genome of 34,446 base pairs, with a G+C content of 54%, which is close to G+C content reported for genomes of HAdV-2, HAdV-5 and HAdV-12 (Chroboczek et al., 1992, Reddy et al., 1999, Sprengel et al., 1994). Like other

Mastadenoviruses, the genome of BAdV-3 is also divided into early regions, intermediate regions and late regions (Fig. 1.3) (Reddy et al., 1998). Unlike other *Mastadenoviruses,* the DNA packaging sequence of BAdV-3, which is present at the left end of genome also contains a part of E1A region, in addition, the E1A promoter of BAdV-3 has been reported to present in the left ITR region (Xing and Tikoo, 2006). Unlike HAdV-5 late genes, the late genes of BAdV-3 genome are divided into 7 families (Chroboczek et al., 1992, Xing and Tikoo, 2006).

1.1.2.3.1 Early genes

The E1 region of BAdV-3 is divided into two parts, namely E1A and E1B (Berk and Sharp, 1978, Reddy et al., 1998, van Olphen and Mittal, 2002). Unlike HAdV-5, the inverted terminal repeats (ITRs) contain E1A sequence promoter as BAdV-3 (containing deletion of the sequence between the left ITR and the ATG of the E1A protein) retains replication-competence (Xing and Tikoo, 2006).The E1A region is the first to be transcribed at 12-36 hrs post infection in infected cells, producing three relatively small proteins (211, 115 and 100 amino acids) by alternatively spliced mRNAs with the same N-terminus (Reddy et al., 1998). Like HAdV-5, the E1A proteins of BAdV-3 are non-structural proteins, which are crucial for BAdV-3 replication and transactivation of other viral genes (Reddy et al., 1998, Zheng et al., 1994, Zhou and Tikoo, 2001). The E1B region produces two proteins (157 and 420 amino acids) at 6-48 hours post infection by two overlapping mRNAs with the same N-terminus, which have been shown important for the BAdV-3 replication (Zhou and Tikoo, 2001). The BAdV-3 E2 region is divided into E2A and E2B, which produces proteins required for virus replication(Reddy et al., 1998).

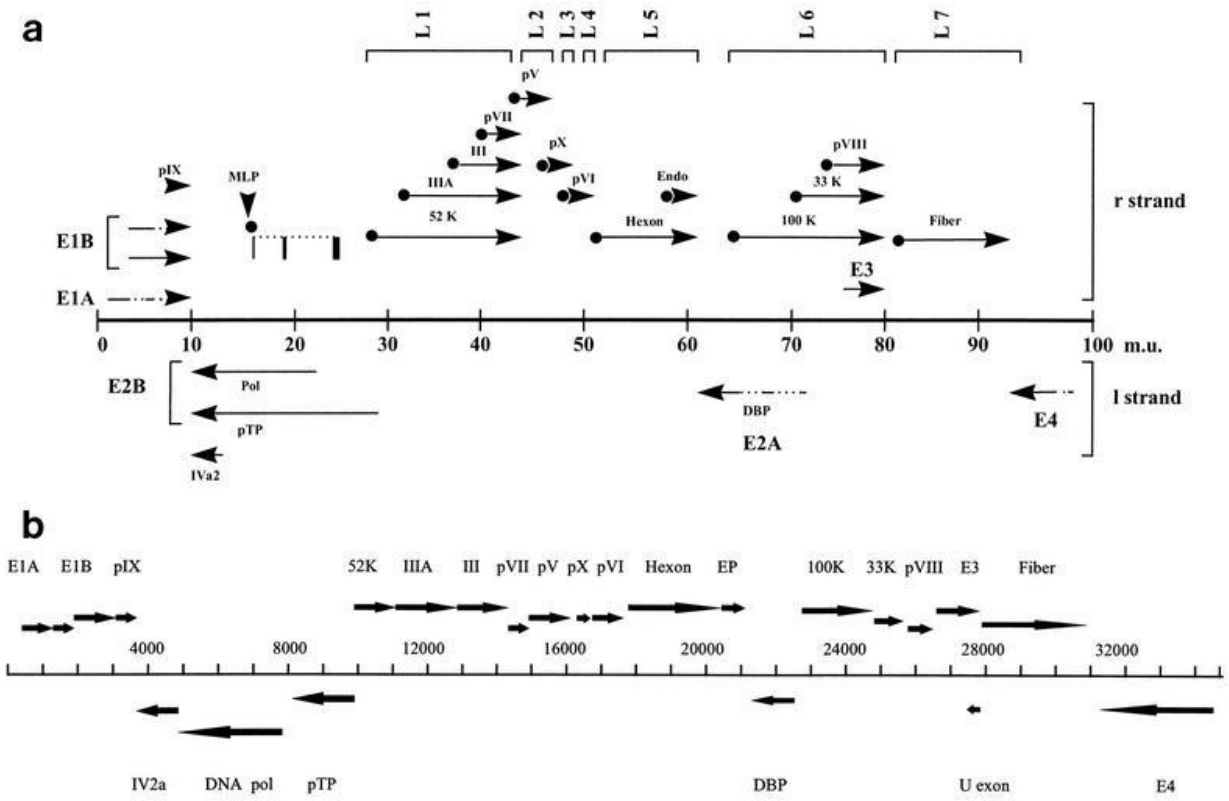

Figure 1.3 Schematic representation of Bovine adenovirus 3 genome (Reddy et al., 1998).

The E2A region encodes DNA binding protein (DBP), which shows 18 to 47% amino acid sequence identities with other *Mastadenovirus* DBPs (Chase and Williams, 1986, Reddy et al., 1998). The DBP of BAdV-3 contains a variable N-terminus, which has been shown to determine the host range, and a highly conserved carboxy-terminal region which has been shown to be involved in DNA binding, activation of the major late promoter and virus replication (Anderson and Klessig, 1984, Kitchingman, 1985, Tucker et al., 1994). In addition, motifs with basic amino acid in BAdV-3 DNA binding protein, for example $_{29}$PRKK$_{32}$, $_{35}$RKRR$_{38}$, and $_{53}$KRAK$_{56}$, have the potential to act as nuclear localization signals (NLS) for the transportation of DBP into the nucleus (Reddy et al., 1998). The E2B region produces two proteins, namely DNA polymerase and precursor terminal protein (pTP) with 59-60% and 58-60% amino acid identities with similar proteins encoded by other members *Mastadenovirus* genus. (Baxi et al., 1998, Reddy et al., 1998). Moreover, YSRLVYR motif in BAdV-3 precursor terminal protein has been shown to be essential to function as primer for the initiation of viral DNA replication (Baxi et al., 1998). However, unlike HAdV-2 pTP, two potential protease cleavage sites have been identified in BAdV-3 precursor terminal protein (Baxi et al., 1998, Webster et al., 1994).

The BAdV-3 E3 encodes four proteins (284, 121, 86 and 82 amino acids) by spliced mRNAs. Although E3 is not essential for viral replication (Zakhartchouk et al., 1998), it has been speculated to influence the adenovirus pathogenesis (Idamakanti et al., 1999, Morin et al., 1987, Reddy et al., 1998). The 284R protein is a glycoprotein, which has been found to be exclusive to BAdV-3 without any sequence identities to E3 proteins of HAdV-2 (Idamakanti et al., 1999). Like HAdV-5 14.7K, the 121R protein has been found to inhibit the tumor necrosis factor (TNF)-α mediated apoptosis (Gooding et al., 1988, Mittal et al., 1992, Zakhartchouk et al., 2001).

The E4 region, located at the right end of BAdV-3 genome (Bridge and Ketner, 1989, Reddy et al., 1998) encodes five potential proteins (ORF 1-5) encoded by alternatively spliced mRNAs (Baxi et al., 2001, Reddy et al., 1998). Although BAdV-3 E4ORF3 and E4ORF5 show limited homology with HAdV-5 E4orf6 protein, BAdV-3 E4ORF 1, E4ORF2 and E4ORF4 show no amino acid sequence identities with corresponding Orfs encoded by HAdV-5 E4 mRNAs (Baxi et al., 2001, Bridge and Ketner, 1989).

Two copies of non-coding RNAs, namely viral associated (VA) RNAs have been reported in HAdV-2, they are transcribed by genes mapped between the 52/55K and pTP encoding genes and RNA polymerase III (Ma and Mathews, 1996, Reich et al., 1966). Various functions including antagonism of the host defense response has been identified for VA RNAs (Kitajewski et al., 1986, O'Malley et al., 1986, Sano et al., 2006). However, no VA RNAs have been reported in BAdV-3 so far.

1.1.2.3.2 Intermediate genes

Like HAdV-5, two intermediate proteins IVa2 and IX are produced by BAdV-3(van Oostrum and Burnett, 1985) .The protein IVa2 is a structural protein (376 amino acids), which shares 29 to 69% sequence identity with other IVa2 proteins encoded by adenoviruses (Reddy et al., 1998). The protein IX is a minor structural protein (125 amino acids), which shares 16-28% sequence identity with pIX encoded by other members of *Mastadenovirus* (Reddy et al., 1998, Zheng et al., 1999). Like HAdV-5, the C-terminal portion of BAdV-3 protein IX has been found to be exposed outside of the viral capsid (Ayalew et al., 2015, Zakhartchouk et al., 2004). Protein pIX has been exploited as a potential target to alter viral tropism by insertion of other targeting ligands. The fiber-independent tropism of BAdV-3 has been enhanced by the integration of RGD motif at C-terminus of protein IX. This has the potential to improve the delivery efficiency of

BAdV-3 vectored vaccine and the host range of targets (Ayalew et al., 2015, Zakhartchouk et al., 2004).

1.1.2.3.3 Late genes

Unlike HAdVs that have five families (L1-L5) of late mRNAs, the major late transcription unit (MLTU) of BAdV-3 is divided into seven late regions (L1-L7) according to the use of polyadenylation sites (Fraser et al., 1982, Reddy et al., 1998).

The BAdV-3 L1 region encodes for four proteins namely 52K, IIIA, III and VII, which share the same polyadenylation site in the mRNAs (Kreivi et al., 1991, Reddy et al., 1998). BAdV-3 protein 52K is a non-structural protein (331 amino acids), which shares 21.4 to 61.6% identity with 52K proteins encoded by other adenoviruses (Reddy et al., 1998). BAdV-3 protein 52K is expressed as a 40 kDa protein both in transfected and infected cells, and predominantly localizes in the nucleus (Paterson et al., 2012). The three residues $_{105}$RKR$_{107}$ of the identified nuclear localization signal (amino acids 102 to 110) have been shown to be crucial for BAdV-3 52K nuclear transportation (Paterson et al., 2012). The cellular nuclear import receptor importin α3 of the classical importin α/β-dependent pathway is utilized by 52K protein for nuclear transport (Paterson et al., 2012). Protein 52K has been shown to interact and redistribute the cellular protein NFκB-binding protein (involved in the ribosomal RNA processing) from the nucleolus to the other parts of nucleus (Paterson et al., 2012, Paterson, 2010). Moreover, BAdV-3 52K has also been shown to be interact with BAdV-3 protein pVII (Paterson et al., 2012).

BAdV-3 protein IIIa is a structural protein (568 amino acids) shows25 to 75% identity with IIIa proteins encoded by other adenoviruses (Reddy et al., 1998). BAdV-3 IIIa contains a potential viral protease cleavage site at 19 amino acids from the C-terminus. The adenovirus IIIa protein has

been shown to be involved in the capsid stabilization by connecting the hexon trimmers (Vellinga et al., 2005).

BAdV-3 protein III shows 44% to 64% identity with penton proteins encoded by other adenoviruses (Reddy et al., 1998). Like HAdVs (Caillet-Boudin, 1989; Zubieta et al., 2005), BAdV-3 protein III is also predicted to be involved in virus internalization through interaction with fiber protein. However, unlike HAdV-5, BAdV-3 protein III doesn't contain the integrin binding motif - tripeptide Arg-Gly-Asp (RGD) motif, which is required for the viral endocytosis during virus internalization (Neumann et al., 1988, Reddy et al., 1998).

BAdV-3 protein VII is a core protein with 29.7 to 53.2% identity with protein VII encoded by other adenoviruses. A potential viral protease cleavage site has been suggested at location adjacent to the N-terminus (Reddy et al., 1998). Moreover, a mitochondria (MLS) localization signal has been identified in BAdV-3 protein VII (amino acid 1-54) which directs the mitochondria localization of protein VII leading to the modulation of mitochondrial Ca^{2+} and ATP production, and inhibition of apoptosis (Anand et al., 2014).

The BAdV-3 L2 region encodes only protein V (423 amino acids), which shows 28 to 40% identity with protein V encoded by other members of *Mastadenovirus* genus (Reddy et al., 1998). Protein V is detected as a 55kDa protein within transfected or BAdV-3 infected cells (Zhao and Tikoo, 2016) and appears essential for efficient viral replication (Zhao and Tikoo, 2016). Protein V uses three nuclear localization signal (amino acids 80-120, 190-210 and 380-389) and two nucleolar localization signals (amino acids 21-50 and 380-389) to locate in the nucleus and nucleolus, respectively of BAdV-3 infected cells, and the nucleolar signals appear essential for replication of BAdV-3 (Zhao and Tikoo, 2016). In addition, protein V interacts with 33K protein (Kulshreshtha and Tikoo, 2008).

The L3 region of BAdV-3 encodes protein X with38 to 64% identity with the pX encoded by other adenoviruses (Reddy et al., 1998). Amino acid sequence analysis have identified a potential bipartite nuclear localization signal and two potential protease cleavage sites (27LRGG↓S31) and (47LRGG↓F51) in BAdV-3 protein X (Reddy et al., 1998, Russell and Kemp, 1995, Weber, 1995).

The L4 region of BAdV-3 encodes protein VI (263 amino acids), a structural protein, which shares 15 to 38% identity with other adenoviruses (Reddy et al., 1998). Analysis of amino acid sequence of BAdV-3 protein VI identified two potential viral protease cleavage sites at both the N-and C-terminus (Reddy et al., 1998, Russell and Kemp, 1995). Moreover, protein VI has been postulated to be a cofactor for the function of adenoviral protease and mediate the endosome membrane disruption (Honkavuori et al., 2004, Kauffman and Ginsberg, 1976, Reddy et al., 1998).

The BAdV-3 L5 region encodes two proteins hexon and protease (Reddy et al., 1998). BAdV-3 protein hexon is a major capsid protein (910 amino acids), which shows 45 to 70% amino acid sequence identity with hexon proteins encoded by other adenoviruses (Reddy et al., 1998). It is expressed as 98kDa protein in BAdV-3 infected cells (Kulshreshtha et al., 2004). BAdV-3 protease shows 30 to 65% amino acid identity with proteases encoded by other adenoviruses (Reddy et al., 1998). During BAdV-3 infection, the precursors of structural proteins pIIIa, pVI, pVII, pVIII, pX, and pTP supposed to be cleaved by viral protease, which is essential for the production of infectious virion (Reddy et al., 1998).

The BAdV-3 L6 region encodes three non-structural proteins 100K, 33K and 22K, and one structural protein pVIII (Reddy et al., 1998). The BAdV-3 100K protein shows 27 to 52% amino acid sequence identity with 100K protein encoded by other adenoviruses (Reddy et al., 1998). BAdV-3 100K protein is expressed as 130 kDa protein in infected cells and localizes to the

cytoplasm and the nucleus (Makadiya et al., 2015). It has been reported that cleavage of 100K by adenoviral protease is required for nuclear localization in infected cells but not for BAdV-3 replication (Makadiya et al., 2015). Interestingly, although C-terminus of full length 100K contains bi-partite nuclear localization signal, only cleaved C-terminal 100K containing bipartite NLS localizes to nucleus by interacting with importin α3 (Makadiya et al., 2015). In addition, BAdV-3 100K interacts with another non-structural protein 33K (Kulshreshtha and Tikoo, 2008). BAdV-3 33K and 22K proteins, produced from a single transcript by alternative splicing, contain the same N-terminal portion of 138 amino acids but different C-terminal portions (Kulshreshtha et al., 2015). BAdV-3 33K is expressed as five proteins of 42 kDa, 39 kDa, 37 kDa, 21 kDa and 19 kDa in infected cells, which localize to nucleus by interacting with importin-α5 import receptor of classical α/β nuclear transport pathway and transportin-3 import receptor of transportin pathway (Kulshreshtha et al., 2014). The C-terminus conserved residues (201-240 amino acids) of BAdV-3 33K are required for the activation of major late promoter (Kulshrestha et al., 2015). Moreover, leucine residues at 217, 224, 232 and 240 appear required for the binding of 33K protein to major late promoter of BAdV-3 (Kulshrestha et al., 2015). BAdV-3 33K bind to 100K protein and protein V in infected cells (Kulshreshtha and Tikoo, 2008)

BAdV-3 22K is expressed as 42kDa and 37 kDa proteins in infected cells and 42kDa protein in transfected cells, it appears to be essential for virus replication (Kulshreshtha et al., 2004). Unlike 33K, 22K does not appear to stimulate the transcription from major late promoter (Kulshrestha et al., 2015).

BAdV-3 protein VIII is a structural protein (216 amino acids), which shows 19 to 56% amino acid identity with protein VIII encoded by other adenoviruses (Reddy et al., 1998, Sussenbach, 1984). While protein VIII is expressed as 8 kDa and 24 kDa proteins in infected cells,

only 8 kDa protein could be detected in purified mature virion (Ayalew et al., 2014). Protein VIII localizes to the nucleus in infected cells by interacting with importin α3 (Ayalew et al., 2014). Interaction between BAdV-3 pVIII and cellular protein DEAD box (DDX) 3 abrogates the translation of capped mRNA at late times post infection (Ayalew et al., 2014). Moreover, two potential viral protease cleavage sites (108IAGG↓G112, 143LGGG↓S147) have been reported in pVIII, which appear necessary for the BAdV-3 replication (Gaba et al., 2017).

The BAdV-3 L7 region encodes protein named fiber with 17% to 26% amino acid sequence identity with fiber proteins encoded by other adenoviruses (Reddy et al., 1998). The knob region of BAdV-3 fiber has been suggested to be involved in the initial interaction with cellular receptor during BAdV-3 infection (Bangari et al., 2005). A conserved FNLVYPYKA motif has been found in the tail region, which is suggested to interact with viral penton protein (Caillet-Boudin, 1989, Reddy et al., 1998). In addition, a nuclear localization signal at the amino acids 14 to 20 has been identified in BAdV-3 fiber protein, which appears essential for efficient virus replication (Wu and Tikoo, 2004).

1.2 Innate immune system and virus evasion strategies

The innate immune system provides host cells the first line of protection from pathogen infections. Among all the sensing strategies utilized by host innate immune system of viral components known as pathogen-associated molecular patterns, the fundamental mechanisms based on pattern recognition receptors (PRRs) are well characterized.

1.2.1 Pattern recognition receptors and Pathogen-associated Molecular Patterns

Based on the protein domain homology, the pattern recognition receptors (PRRs) can be divided into several classes including Toll-Like Receptors (TLRs) and the C-type lectin receptors (CLRs) that are transmembrane proteins expressed at the cell surface or in endocytic

compartments; retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs), the AIM2-like receptors (ALRs) that are intracellular receptors expressed in cytosol or within endosomes (Jang et al., 2015, Kumar et al., 2011).

Toll-Like Receptors (TLRs), first identified in Drosophila are the best-characterized PRRs (Lemaitre et al., 1996). While, eleven functional TLRs have been reported in mice, namely TLR 1-7, TLR 9 and TLR 11-13, ten TLRs have been found in human, known as TLR 1-10 (Akira and Takeda, 2004, Takeda et al., 2003). Most TLRs are expressed by immune cells such as dendritic cells and macrophages (Kawai and Akira, 2009). The TLRs (3, 7, 8 and 9) that recognize viruses located in endosomal membrane and get to sense viral RNA or DNA nucleic acids after virus endocytosis. For example, TLR3 has been found to be activated by dsRNA, TLR7 by ssRNA and TLR 9 by unmethylated CpG-DNA motifs (Shayakhmetov et al., 2010). In addition, TLR3 is expressed in most cell types while TLR7 and TLR9 are mainly expressed in plasmacytoid dentritic cells (Colonna et al., 2004). After synthesis of these TLRs in endoplasmic reticulum (ER), the ER membrane protein UNC-93B transport TLRs into the endosome (Brinkmann et al., 2007, Tabeta et al., 2006). After the transportation into the endosomal compartment, these TLRs require proteolytic processing by endosomal proteases to be competent to recruit adaptors and bind to viral ligands. For example, studies have suggested that the ectodomains of TLR9, TLR3 and 7 are cleaved by cathepsins (Ewald et al., 2008, Park et al., 2008). Moreover, the proteolytic processing of these TLRs in endolysosome has also been shown to avoid the aberrant activation of TLRs such as upon the recognition of self DNA (Diebold and Brencicova, 2013, Kawai and Akira, 2010). All TLRs are type I transmembrane proteins that consist of ectodomains with leucine-rich repeats which mediate PAMP recognition, transmembrane domains which help in receptor localization on

cell surface or in endosomes primarily and, cytoplasmic Toll-interleukin-1 receptor homology (TIR) domains, which bind to adaptor proteins and transfer downstream signaling (Akira and Takeda, 2004). The TLRs engage various adaptors located at the cell surface or in the endosomal compartments for downstream signaling cascade, including the TIR-containing adaptor protein (TIRAP), the TRIF-related adaptor molecule (TRAM), the TIR domain–containing adaptor-inducing IFN-β (TRIF), and the protein myeloid differentiation primary response 88 (MyD88). Most TLRs utilize MyD88 adapter except for TLR3, which only uses TRIF adapter. Moreover, TLR4 is unique in that it utilizes both MyD88 and TRIF adaptors sequentially (Feng and Chao, 2011, Takeda and Akira, 2005, Yamamoto et al., 2002). Stimulation of TLRs after the recognition of virus infection activates complex signaling cascades that a) lead to the synthesis of pro-inflammatory cytokines, antiviral cytokines and type I IFNs (Thompson and Iwasaki, 2008), b) result in the activation of the mitogen-activated protein kinases (MAPKs), which can induce the expression of various genes that regulate the inflammatory response (Arthur and Ley, 2013) and may c) lead to the induction of adaptive response after the recognition by dendritic cells, which are the most potent antigen-presenting cells (Iwasaki and Medzhitov, 2004).

In addition to TLRs, C-type lectin receptors (CLRs) are also transmembrane receptors, which are expressed on the plasma membrane (Areschoug and Gordon, 2008). Langerin has been reported to be competent to clear HIV in Birbeck granules through endocytosis and degradation (de Witte et al., 2007). Dectin-1 and DC-SIGN are involved in anti-fungal defense (Den Dunnen et al., 2009).

The RIG-I-like receptors (RLR) are expressed by most cell types and constitute a family of cytoplasmic RNA helicases including retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and Laboratory of Genetics and Physiology 2(LGP2,

also known as DHX58) (Yoneyama et al., 2004). Both RIG-I and MDA5 have been found to recognize viral RNA through the utilization of a common adaptor localized at the mitochondrial membrane named mitochondria antiviral signaling protein (MAVS), which activates two protein kinase complexes: complex 1 consists of TANK (TRAF family member associated NF-κB activator), TBK1 (TANK-binding kinase 1), TRAF3 (TNF receptor associated factor 3) and IKKε (inhibitor of κB kinase ε); complex 2 consists of RIP1 (receptor interacting protein), TRAF-6, TAK1 (TGF-β activated kinase 1), IKKα and IKKβ and NEMO (NF-κB essential modulator) (Seth et al., 2005), which results in the induction of antiviral cytokines and type I interferons expression (Kawai et al., 2005, Meylan et al., 2005, Seth et al., 2005). Two caspase activation and recruitment domains (CARDs) have been reported in N-terminus of both RIG-I and MDA5, which initiate downstream signaling and facilitate a structure realignment after getting exposed. Moreover, RIG-I has a C-terminal regulatory domain (RD), which confers RIG-I an auto-inhibited state (Saito et al., 2007, Yoneyama and Fujita, 2008). Despite the similar structural domains shared by RLRs, they have significant differences in activation mechanisms and preferences for recognizing viral ligands (Gack et al., 2007).

RIG-I recognizes single-stranded (ssRNA) with 5' triphosphate end, and also double-stranded (dsRNA) without terminal triphosphates with a preference for short dsRNA. RIG-I detects blunt, short, double-stranded 5'-triphosphate RNA discriminatingly, which is common in the panhandle region of ssRNA viral genomes (Hornung et al., 2006, Kato et al., 2008). Longer dsRNA (over 1kb) are preferably recognized by MDA5 compared to those recognized by RIG-I (Kato et al., 2008). Based on different preferences on viral ligands recognition, RIG-I and MDA5 function as critical PRR for different viruses. While RIG-I has been found engaged by Sendai virus (SeV), vesicular stomatitis virus (VSV), influenza, hepatitis C virus, Japanese encephalitis virus

and small RNA encoded by the DNA virus Epstein-Barr virus (Kato et al., 2006, Saito et al., 2007, Yoneyama and Fujita, 2008), MDA5 has been found to induce innate immunity in picorna viruses (Gitlin et al., 2006).

Laboratory of genetics and physiology 2 (LGP2) protein, which has similar helicase domain as RIG-I and MDA5 has been suggested to act as both positive and negative regulator of RIG-I and MDA5 depending on the type of virus infection (Yoneyama et al., 2005). For example, studies have shown that while lgp2 knockout in mice can augment type I IFN induction following poly I:C transfection or VSV infection, LGP2 is required for the induction of antiviral response during EMCV infection (Komuro and Horvath, 2006, Venkataraman et al., 2007). However, the underlying mechanisms of these LPG2 conflicting functions are unclear.

NOD like receptors (NLRs) consist of a large family of 22 members identified in human that recognize various PAMPs and stress signals (Martnon et al., 2009)**.** They comprise multiple domains including a C-terminal leucine-rich repeat (LRR) domain responsible for detecting PAMPs and danger associated molecular patterns (DAMPs) leading to the activation of NLRs, a central nucleotide oligomerization and binding domain (NOD) domain and a N-terminal effector domain. Depending on the N-terminal domains, the NLRs can be divided into three groups: pyrin domain (PYD)-containing subfamily (NLRPs) and CARD-containing subfamily (NOD1, NOD2, NLRCs, CIITA) which have been shown involved in the regulation of the antiviral innate immunity, and baculovirus inhibitor repeat (BIR) domain-containing subgroups (Ting et al., 2008). Moreover, some NLRPs such as NLRP1, NLRP2, NLRP3 and NLRP6 show an important role in the inflammatory response by forming an inflammasome complex and leading to the activation of caspase-1, which leads to the proteolytic activation of the pre-formed pro-inflammatory cytokines IL-1ß and IL-18 (Elinav et al., 2011, Keller et al., 2008, Martinon et al., 2002). For example,

NLRP3, also known as NALP3, forms an ASC (Apoptosis–associated speck-like protein containing a caspase recruitment domain)–dependent inflammasome complex after sensing stimuli including bacterial or viral infections, cellular stress such as membrane disruption and ATP or uric acid released from damaged/dying cells (Martnon et al., 2009). Studies have shown that Sendai virus, influenza virus and adenovirus can activate the NLRP3-ASC-caspase-1 inflammasome (Kanneganti et al., 2006, Muruve et al., 2008). However, poly I:C transfection has been found not able to induce inflammasome activation, which suggests that viral RNAs alone are insufficient for inflammasome activation (Jacobs and Damania, 2012). In addition, some NLRPs have shown negative effect on immune response. For example, NLRP4 has been shown to downregulate type I IFN response through the degradation of TBK1 (Cui et al., 2012).

In addition, more cytosolic DNA sensors have been elucidated recently. For example, DNA-dependent activator of IFN-regulatory factors (DAI) has been found to bind to cytosolic dsDNA and lead to the production of type I IFNs through the TBK1-IRF3 signaling (Takaoka et al., 2007). The DNA sensor IFI16, which belongs to a protein family named the pyrin and HIN domain (PYHIN) family has been found to recruit an adaptor named STING (The endoplasmic reticulum (ER)-resident transmembrane protein stimulator of IFN genes) to activate IFNβ induction by TBK1-IRF3 signaling (Burdette et al., 2011, Tsuchida et al., 2010). The helicase DDX41 has also been identified to be involved in DNA sensing through the engagement of STING (Zhang et al., 2011). AIM2 (absent in melanoma 2) is another member of the PYHIN family, which can trigger the formation of inflammasome upon activation and result in the production of mature IL-1β and IL-18 (Jones et al., 2010). p202 is another member of the PYHIN family which has shown to repress caspase-1 activation in contrast to AIM2 (Roberts et al., 2009).

1.2.2 Type I Interferon response induced antiviral state

The detection of the pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) will activate the transcriptional innate immune responses by different signaling pathways particularly the production of type I interferons (IFNs). These IFNs in turn induce a battery of genes that directly suppress viral replication (Brubaker et al., 2015). The PRR-mediated recognition has also been shown to activate non-transcriptional responses such as phagocytosis, autophagy and cell death (Deretic et al., 2013, Lamkanfi and Dixit, 2014). The type I Interferon response can also direct the activation of the adaptive immune response, which is more antigen-specific and long-lasting (Palm and Medzhitov, 2009).

Interferons, discovered in 1957 in chicken, were the first cytokines to be described. They are named because of the ability to interfere with viral replication (Isaacs and Lindenmann, 1957). Interferons are divided into three groups namely type I, II, III IFNs (Randall and Goodbourn, 2008). Type I IFNs identified in humans include twelve IFN-α subtypes, one IFN-β and multiple less-known ones such as IFN-ω, IFN-δ, IFN-ε, IFN-τ and IFN-κ (Hardy et al., 2004). IFN-α subtypes are mostly induced by plasmacytoid dendritic cells (pDCs), but IFN-β can be produced by most cell types (Hardy et al., 2004). Type II IFN also named IFN-γ, was first identified in human leukocytes has also been detected in activated immune cells including T cells, NK cells, B cells and NKT cells (Wheelock, 1965; Schroder et al., 2004). Type III IFNs comprising of four subtypes (IFN-λ (IFN-λ1, IFN-λ2, IFN-λ3, IFN-λ4). have been detected primarily in plasmacytoid dendritic cells and epithelial cells (Kotenko, 2002, Kotenko et al., 2003, Sheppard et al., 2003, Sommereyns et al., 2008).

Type I IFNs have been shown to be significant for the induction of innate immunity and regulation of the adaptive immune response (Stetson and Medzhitov, 2006). Recognition of viral

components by PRRs triggers the activation and induction of IFNs (described in Section 1.2.1). The activated PRRs initiate various signaling, which result in the activation of transcription factors named IRF-3, IRF-7, AP-1 and NFκB by cellular kinases. The activated transcription factors induce the transcription of the IFN genes after the translocation into nucleus (Kawai and Akira, 2010). In addition, the type I IFNs can also be induced by host factors such as tumor necrosis factor (TNF), which signals using IRF1 (Yarilina et al., 2008). After their induction, the interferons exert the antiviral effect in neighbouring and infected cells by utilization of different receptors including IFN-αR1/R2 for type I IFNs, IFN-γR1/R2 for type II IFN and IFN-λR1 for type III IFN (Kotenko et al., 2003, Sheppard et al., 2003). For example, binding of type I IFN to IFNAR activates the transcription factors named STAT1 and STAT2 (signal transducer and activator of transcription 1 and 2) through the activated receptor-associated protein tyrosine kinases Janus kinase I (JAK1) and Tyrosine kinase 2 (TYK2) (Balachandran et al., 2000). Phosphorylated STAT1 and STAT2 translocate into the nucleus, binds to DNA sequences known as IFN-stimulated response element (ISREs) together with IRF9 and result in the transcription of over 300 interferon stimulated genes (ISGs) (Schoggins et al., 2011). The ISGs interfere with various steps of virus infection including virus entry, virus replication and viral protein translation (Fig. 1.4) (Bowie and Unterholzner, 2008). For example, 2'-5' oligoadenylate

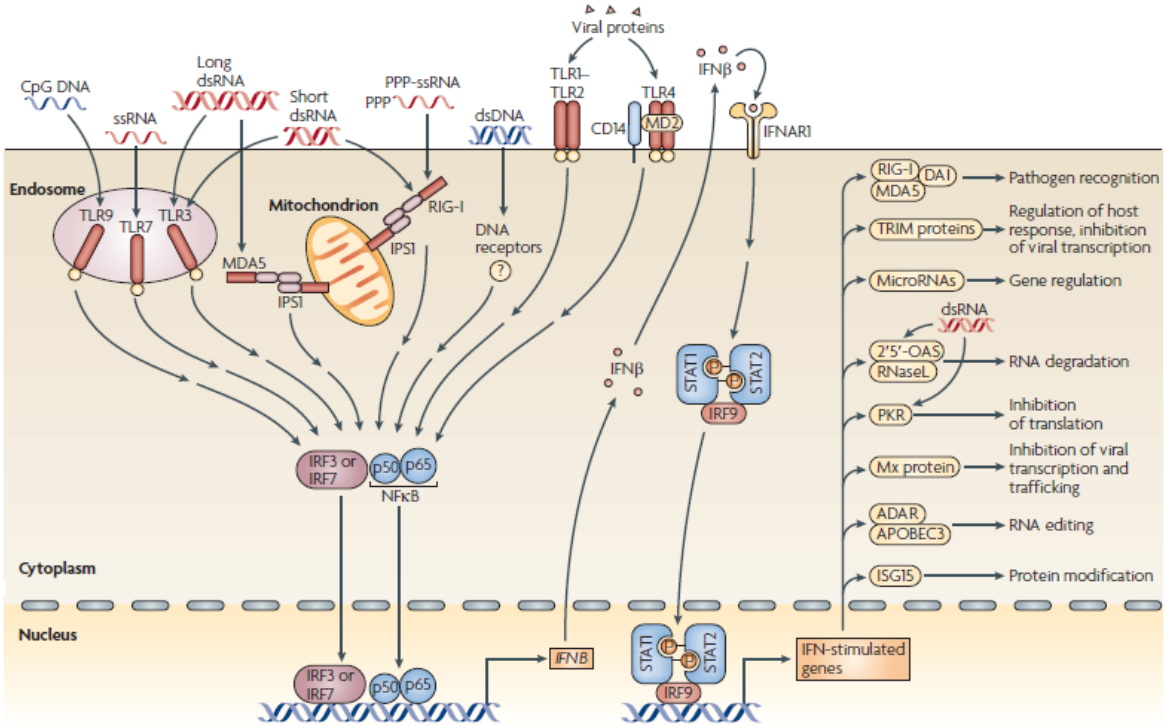

Figure 1.4 Activation of the interferon response and IFN-induced antiviral mechanisms (Bowie and Unterholzner, 2008).

synthetase (OAS) is an IFN stimulated gene which leads to the degradation of viral RNA (Rutherford et al., 1988). OAS produces 2'-5' oligoadenylates which activate the RNaseL, leading to the cleavage of the viral RNA in cytosol and inhibition of viral protein synthesis (Silverman, 2007). In addition, the cleaved products of RNase L have been found to activate the RLRs by functioning as ligands for RIG-I and MDA5 (Malathi et al., 2007). Moreover, RNase L has been found to be primarily responsible for IFN antiviral activity during influenza virus infection, but the roles of OAS and RNase L in restriction of DNA virus infection remain unclear (Malathi et al., 2007). Protein kinase R (PKR) is a serine/threonine kinase, which also has been found to be involved in the inhibition of viral protein translation through phosphorylation of the eukaryotic translation initiation factor 2 α (eIF2α), it can also mediate apoptosis, cell-growth arrest, and autophagy to control viral replication and spread (Sadler and Williams, 2007).

Orthomyxovirus Resistance Gene (Mx) proteins named MxA and MxB have been identified in human, and related Mx proteins namely Mx1 and Mx2 have been reported in mice (Haller et al., 2007). They show antiviral activities against several viruses including orthomyxomaviruses, paramyxomaviruses, rhabdoviruses, togaviruses and bunyaviruses, probably by blocking the early steps of viral replication through mediating the vesicle trafficking to trap essential viral components (Haller et al., 2015). For example, overexpression of human MxA protects IFN-αβR -deficient mice from fatal infection with Thogoto virus, influenza virus, VSV, LaCrosse virus, Semliki and Forest virus (Hefti et al., 1999, Pavlovic et al., 1990, Pavlovic et al., 1995). The ISG15 is an ubiquitin-like protein that mediates ISGylation of targeted viral proteins (Der et al., 1998). For example, the ISGylation of human papillomavirus L1 capsid protein plays an essential role in the inhibition of HPV16 pseudoviruses infectivity (Durfee et al., 2010).

In addition, studies have shown that ISG15 deficient mice have increased susceptibility to virus infection including herpes virus, influenza and sindbis viruses (Lenschow et al., 2007).

Tetherin and Viperin are important ISGs that inhibit virus infection by the restricting virus release. Tetherin is a GPI-anchored protein that has been shown to inhibit the release of some retrovirus, filovirus, arenavirus members (Gnirss et al., 2015, Neil et al., 2008, Venkatesh and Bieniasz, 2013). Viperin is known to prevent the replication of various viruses including HCMV, influenza virus, hepatitis C virus, dengue virus, alphaviruses, and HIV-1 (Fitzgerald, 2011)**.**

In addition, type I IFN-independent antiviral mechanisms including RNA interference (RNAi) and autophagy have been reported. RNAi antiviral mechanism was identified in plants, fungi, nematodes and insects, but not in infected mammalian cells (Li and Ding, 2006). However, RNAi suppressor proteins have been reported to be produced by various plants and invertebrate viruses (Agrawal et al., 2003). Dicer endonuclease members recognize and process viral dsRNA into siRNA, which mediate the cleavage and degradation of viral RNA (Agrawal et al., 2003). Autophagy has been found to be involved in the lysosomal degradation of cytosolic organelles, which are important for the cellular homeostasis (Deretic and Levine, 2009). Studies have illustrated that signalling through PRRs can lead to the induction of autophagy, resulting in the engulfing of intracellular virion or viral products into autophagosome and their degradation in the lysosome, which is referred to "xenophagy" (Levine and Deretic, 2007). This antiviral mechanism has been shown to be crucial to protect Drosophila from infection by VSV (Shelly et al., 2009).

1.2.3 Virus-mediated modulation of innate immunity

Most viruses possess two major mechanisms to counteract the innate immune responses at multiple stages. First is the general inhibition of cellular protein expression. In this mechanism, some RNA viruses such as picornaviruses, influenza viruses, and rhabdoviruses have been shown

to block general expression of cellular genes with antiviral functions through diverse viral proteins (Lyles, 2000). For example, the VSV M protein has been shown to interfere with multiple steps in host gene expression including transcription, host RNA processing and transportation (Her et al., 1997).

Second is the interference with molecules involved in the IFN signaling through sequestration or disruption (Versteeg and García-Sastre, 2010). In this mechanism, multiple components in the IFN signalling can be targets for various viruses such as the initial PRR sensors and Interferon regulatory factors (IRFs). For example, the poliovirus $3C^{pro}$ protease and the EBOV (Ebola virus) VP35 protein can lead to the degradation of RIG-I senor or blocking of viral RNA sensing through RIG-I respectively (Chan and Gack, 2016). The Rabies virus protein P and Hepatitis E virus RF3 can prevent the phosphorylation of IRF3 and IRF7 respectively (Chan and Gack, 2016, Pfaller and Conzelmann, 2008).

1.2.4 Adenovirus-induced host innate immune responses

Adenoviruses have been shown to induce intensive innate and adaptive immune responses, which hamper both the efficiency and safety of the use of adenovirus as gene delivery vectors (Shayakhmetov et al., 2010). Thus, it is important to identify the viral and cellular components which are involved in the antiviral responses. Currently, many mechanisms have been reported to be involved in the adenovirus-induced innate immune responses, including Toll-

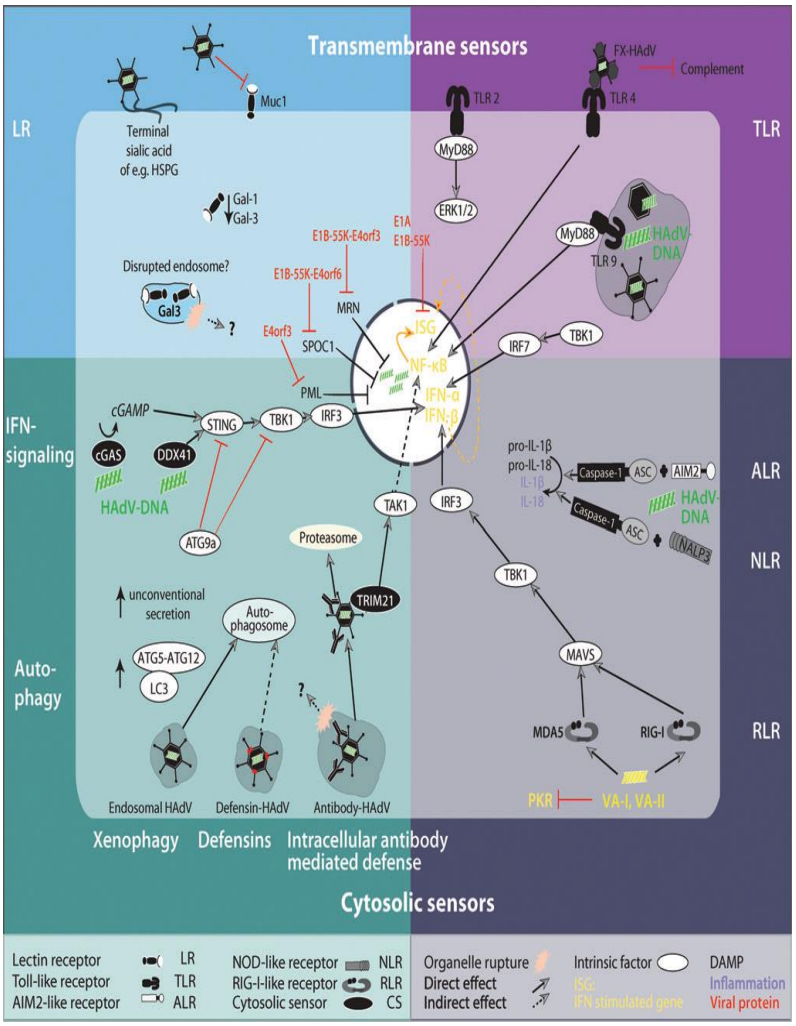

Figure 1.5 Adenovirus-induced host innate responses (Hendrickx et al., 2014)

Like Receptor dependent mechanisms, and some other Toll- Like receptor independent mechanisms (Fig. 1.5).

It has been reported that innate immune responses can be detected right after the application of adenovirus into human and nonhuman mammals (Raper et al., 2003, Schnell et al., 2001). Moreover, it has been suggested that adenovirus-induced innate immune responses are dose-dependent and without the requirement of viral gene expression (Schnell et al., 2001, Stilwell et al., 2003).Mechanisms of adenovirus sensing have been suggested to engage many different sensors, which are often cell-specific. In plasmacytoid dendritic cells (pDCs) which are the principal producer of type I IFNs, TLR9 and MyD88 were suggested to be involved in the sense of HAdV-B by recognition of viral DNA (Zhu et al., 2007). In addition, TLR2-knockout mice showed diminished NF-kB activation and humoral responses to human adenoviruses (Appledorn et al., 2008b) suggesting involvement of TLR2.

The innate immunity induced by HAdV-5 infection has been found to be mediated by TLR4 (Doronin et al., 2012). Since studies showed that CD46-utilizing adenovirus (Ad5.F16) was preferential to stimulate TLR9-mediated innate responses than CAR-utilizing adenovirus (Ad5.F5), a connection between the activation of TLR signalling and cell entry pathways mediated by specific receptors has also been suggested to exist (Iacobelli-Martinez and Nemerow, 2007). However, the underlying mechanisms by which cell entry pathways affect innate immune responses against adenoviruses remain unclear. Furthermore, studies have shown that while transcription factor interferon response factor 3 (IRF-3) is critically involved in sensing adenovirus genomic DNA in murine bone marrow-derived macrophages (Nociari et al., 2007), IRF7 is also critical to mediate adenovirus-induced type I-IFN response *in-vivo* by using adenovirus infected mice deficient for IRF3 or IRF7 (Fejer et al., 2008, Nociari et al., 2007). Other pathways

independent of TLR signalling are suggested to be involved as mice deficient in TLR-adaptors TRIF and MyD88 also induced the immune responses against HAdV-3 (Fejer et al., 2008). Two inflammasome-associated DNA sensors AIM2 (absent in melanoma 2) and NALP3 have been found to be involved in HAdV-infected myeloid cells through the recognition of HAdV DNA (Stein and Falck-Pedersen, 2012). AIM2 appears to be involved in the adenovirus-induced innate immune response (Stein and Falck-Pedersen, 2012). The activation of NLRP3 inflammasome was also shown in human adenovirus type 5 (Ad5) infected THP1 cells (Barlan et al., 2011) or transfection of Ad5 DNA into human keratinocytes (Schulte et al., 2013).

In addition, various cytosolic DNA sensors have been reported in anti-adenoviral response (Fig. 1.5). The knockdown of DDX41 or STING in mouse DCs significantly decreased the type I IFN response against HAdV infection (Zhang et al., 2011). In addition, the knockdown of DDX41 in the HAdV-5 infected RAW 264.7 murine macrophage-like cells resulted in dramatic reduction in the phosphorylation of transcription factor interferon response factor 3 (IRF3), which is a primary antiviral response marker (Stein and Falck-Pedersen, 2012). All these evidence suggest that DDX41 is involved in the recognition of HAdV DNA in certain cells. Recently, knockdown of cGAS, TBK1 or STING reduced the phosphorylation of interferon response factor 3 (IRF3) after infection by HAdV-5 (Ad5CiG) suggested that cGAS (Cyclic GMP-AMP synthase), a DNA sensor that produces a cyclic guanine-adenine dinucleotide (cGAMP), TBK1 kinase and adapter molecule STING are also involved in sensing HAdV DNA in murine RAW 264.7 macrophage-like cells (Lam et al., 2014). These studies suggested that cGAS is an additional cytosolic PRR involved in adenovirus infection, which is responsible for detection of internalized adenovirus DNA to induce the type I interferon response. Moreover, RIG-I has also been found to mediate the activation of innate immune responses against adenovirus infection. Studies have shown that the

adenoviral genome encodes two RNA by host RNA polymerase III, namely virus associated RNA I (VA-I) and VA-II with the size of 157 and 158 nucleotides respectively, VA-I and VA-II were found to bind to RIG-I upon transfection (Minamitani et al., 2011, Weber et al., 2013). Moreover, unknown cytosolic DNA sensors have also been suggested to sense adenovirus DNA in conventional DCs (Zhu et al., 2007).

1.2.5 Modulation of Innate immune response by adenovirus

So far, proteins encoded by early (E) regions E1, E3 and E4 regions, the VA-RNAs and viral structural proteins VI and VII have been demonstrated to act as innate antiviral response antagonists (Schreiner et al., 2012, Weitzman and Ornelles, 2005).

Multiple HAdV genes have been found to counteract host immune responses. E1A produced proteins have been shown involved in the apoptosis, immune evasion, and other viral gene expression (Berk, 1986, Burgert et al., 1987). Specifically, E1A can inhibit the IFN-α-activated antiviral response since E1A has been found to interfere with the formation of the crucial ISGF3 transcriptional complex which has been found to activate the transcription of downstream antiviral genes through binding to ISREs (Darnell Jr et al., 1994). This has also been confirmed by studies showing that E1A can rescue other viruses from the effects of IFN response by inhibiting the formation of the ISGF3 complex (Kalvakolanu et al., 1991). Moreover, E1A inhibits transcription of JAK1, which is essential for signaling for some type I and type II cytokine in epithelial cells (Shi et al., 2007). In addition, E1A has been reported to be involved in the suppression of ISG activation through interfering with the ISG transcription (Fonseca et al., 2012). E1A can also reduce antigen presentation in infected cells through interference with the peptides presentation to the immunoproteasome. Studies showed that two E1B proteins E1B-19K and E1B-55K proteins can block the tumor suppressor protein p53-mediated apoptosis and help the virus to

persist in an infected host (Sabbatiniet al., 1995; Teodoro and Branton, 1997, Chahal et al., 2013). In addition, E1B-55K interferes with the induction of ISG genes as HAdVs lacking 55K protein are notably sensitive to type I IFN (Chahal et al., 2012). Furthermore, E1B-55K together with E4orf6 protein forms a complex to block the DNA damage signaling through the immobilization of damage sensor during virus infection and degradation of the antiviral factor SPOC1 involved in DNA damage response by the proteasome thereby leading to the increase of virus yield in infected cells (Carson et al., 2009, Schreiner et al., 2013).

E3 proteins are known for the immunomodulatory functions. E3-14.7K and E3-6.7K interfere with the cytolytic and pro-inflammatory activities by inhibiting the NF-κB induced transcription of inflammatory mediator (Carmody et al., 2006). E3-49K has been found to inhibit peptide presentation by MHC class I through retargeting of MHC I to the endoplasmic reticulum (Jackson et al., 1993), it also has immunomodulatory functions as it reduces the expression of activation markers on NK cells, and inhibits the activation of T cell receptor (Windheim et al., 2013).

Some E4 proteins are involved in the regulation of apoptosis and antiviral responses. For example, E4orf6 together with E1B-55K can block the DNA damage signaling as a complex (Carson et al., 2009, Schreiner et al., 2013). In addition, E4orf3 can inhibit IFN production through disrupting the PML-ND (promyelocyticleukemia protein bodies) function, which is important for virus detection in antiviral innate immunity (Bernardi and Pandolfi, 2007). Moreover, E4orf3 is suggested to antagonize species-specific innate immune responses against adenoviruses (Hendrickx et al., 2014b).

Recently, viral protein VII has been found to have an impact on antiviral responses in human cells through the formation of complexes with nucleosomes and limitation of DNA

accessibility (Avgousti et al., 2016). Both *in-vitro* and in-vivo studies showed that protein VII sequesters HMGB1 protein, which is inflammation-induced and functions as antiviral immune responses activation signal via direct protein interaction (Lotze and Tracey, 2005). In addition to these antagonist proteins, HAdV VA-RNAs (non-coding virus-associated RNA) also have been found to modulate host IFN responses by blocking dsRNA-activated protein kinase R (PKR) activity (Mahr and Gooding, 1999, Wold et al., 1999).

1.2.6 Attempts to modify AdV vectors to diminish immune responses

Currently, various viral vectors derived from adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, herpes simplex viruses have been studied in clinical trials over the decades (Appaiahgari and Vrati, 2015, Giacca and Zacchigna, 2012) (Table 1.).

Adenoviruses are one of the most intensively studied vectors in decades for gene therapy and vaccination as they have been shown to have several advantages, for example they can infect a broad range of dividing and non-dividing cells, can be grown to high titers easily and their molecular biology has been well understood. Moreover, they can be manipulated to express foreign genes with the size up to 37 kb without host genome integration especially HAdV-2 and -5, which are commonly used as vectors (Bett et al., 1993, Harui et al., 1999, SM Wold and Toth, 2013). In addition, recently it has been reported that desiccated adenovirus vectors are thermostable at 45°C for up to 6 months and can be stored at 4°C for up to 15 months with minimal losses in titer or immunogenicity (Alcock et al., 2010). However, further studies and widespread utilization of adenoviruses have been hampered because of some disadvantages. The studies have indicated that activation of innate immunity by adenoviral vectors both *in-vitro* and *in-vivo* is associated with significant reduction in the efficacy of gene transfer, as well as notable damage in transduced tissue and host (Schnell et al., 2001, Worgall et al., 1997). Often, increase

Table 1.1 Major properties with different viral vectors (Thomas et al., 2003).

Vector	Genetic Material	Packaging Capacity	Tropism	Vector genome forms	Main Limitations	Main advantages
Retrovirus	RNA	8kb	Dividing cells only	Integrated	Only transduces dividing cells; integration might induce oncogenesis in some applications	Persistent gene transfer in dividing cells
Lentivirus	RNA	8kb	Broad	Integrated	Integration might induce oncogenesis in some applications	Persistent gene transfer in most tissues
HSV-1	dsDNA	40kb-150kb	Strong for neurons	Episomal	Inflammatory; transient transgene expression in cells other than neurons	Large packaging capacity; strong tropism for neurons
AAV	ssDNA	<5kb	Broad, with possible exception of haematopoietic cells	Episomal and Integrated	Small packaging capacity	Non-inflammatory; Non-pathogenic
Adenovirus	dsDNA	8kb-30kb	Broad	Episomal	Capsid mediates a potent inflammatory response	Extremely efficient transduction of most tissues

of the vector capacity for foreign gene insertion involves the deletion of other viral genes. Those deleted genes are often required for counteraction of host innate immunity and thus may make adenovirus vectors more prone to induce strong innate antiviral responses.

To deliver foreign genes effectively and safely, adenovirus-based vectors are required to be engineered with attenuated innate response induction. Although the "gutless" adenoviral vectors lacking all viral structural proteins show reduced adaptive immunity and improved gene delivery, the innate immunity still remains intact (Brunetti-Pierri et al., 2004, Muruve et al., 2004). As such attempts are being made to overcome this limitation by pre-emptive immune modulation of the host and further modification of the virus vector. For pre-emptive immune modulation of the host, immunosuppressive compounds are always transiently utilized to block general immune system or the immune pathways, which are specifically involved in adenovirus infection. For example, pre-treatment with anti-inflammatory glucocorticoid such as dexamethasone (DEX) before systemic adenovirus administration block adenovirus-triggered innate immune responses including pro-inflammatory gene induction in liver / spleen tissues, cytokine induction, and reduced adenovirus-specific humoral responses (Seregin et al., 2009). In addition, it has shown that pre-treatment with liposome-encapsulated clodronate results in transient depletion of macrophages, reduced induction of cytokines and chemokines (IL-6 and RANTES) leading to enhanced adenovirus-based hepatic gene delivery (Kuzmin et al., 1997, Zhang et al., 2001). TLR9 specific-antagonist oligonucleotide ODN-2088 can significantly suppress the induction of cytokines such as IL-6 and IL-12 in mice (Cerullo et al., 2007). Furthermore, utilization of the neutralizing antibodies to type I IFNs can effectively block the innate and adaptive immune responses to adenovirus leading to prolonged foreign gene expression *in -vivo* (Zhu et al., 2007).

Efforts have been made to further modify the adenovirus vector. For example, E3 region, which is well known for the immunomodulatory functions is retained in adenovirus vector together with appropriate E1-independent promoter since the activity of E3 gene promoter is dependent mostly upon trans-activation of E1 gene products. Use of such modified adenovirus in rats showed prolonged transgene expression with reduced humoral antiviral responses (Ilan et al., 1997). In addition, the modification of adenovirus capsid, has been shown to block the detection of viral capsid epitopes such as hexon and fiber (O'Riordan et al., 1999, Roy et al., 1998). For example, attaching the polymer polyethylene glycol to the adenovirus capsid (PEGylated HAdVs) have shown to reduce adenovirus-triggered innate antiviral responses significantly such as the induction of pro-inflammatory cytokines and chemokines (Geest et al., 2005, Wonganan and Croyle, 2010). Moreover, studies with PEGylated adenovirus vector injection showed prolonged transgene expression in murine livers as well as in the lungs (Croyle et al., 2002). Studies also have shown that the use of receptor or tissue specific ligand can reduce the immune response induction in the off-target cells or tissues (De Geest et al., 2003, Ding et al., 2002).

Since conventional adenoviruses induce robust complement activation *in-vivo*, leading to the amplification of innate anti-adenovirus responses and significant activation of humoral immune responses, researchers have developed HAdV based vectors with capsid that displays peptides with ability to inhibit complement system (Appledorn et al., 2008a, Kiang et al., 2006, Tian et al., 2009). For example, a natural complement inhibitor in host cells named DAF (decay accelerating factor) has been utilized to modified adenovirus. Intravenous injection of such adenovirus (capsid displaying DAF) in mice showed significant reduction in antiviral responses including thrombocytopenia and induction of pro-inflammatory cytokines \ chemokines (Seregin et al., 2010).

Taken together, understanding of the strategies to counteract the antiviral immune responses may improve both the efficacy and safety of adenovirus-mediated gene delivery.

2 HYPOTHESIS AND OBJECTIVES

Viruses have developed strategies to evade the innate immunity to establish infection. Like many other viruses, it has also been shown that adenoviruses modulate the innate immunity through expression of adenoviral early proteins E1A, E1B, E3, E4 as well as structural proteins pVI and pVII (Avgousti et al., 2016, Burgert et al., 2002, Schreiner et al., 2012).Thus in this project, we hypothesized that bovine adenovirus serotype 3 (BAdV-3) also encodes viral proteins that are involved in regulating the induction of innate immune response.

Since innate immune response induction is crucial in early infection of BAdV-3, we hypothesized that bovine adenovirus-3 (BAdV-3) structural proteins are involved in the suppression of innate immune response. The objective of this project is to identify the potential BAdV-3 structural proteins involved in regulating innate immune response, and determine the regualtion mechanisms of BAdV-3 induced innate immune response, and also determine the pathways involved in BAdV-3 innate immune response regulations.

3 BOVINE ADENOVIRUS-3 PROTEINS pV AND pVIII ARE POTENTIAL VIRAL SUPPREESORS OF THE INTERFERON RESPONSE

3.1 INTRODUCTION

Virus host interaction leads to the induction of cytokines including type I interferons, which prevent viral replication by initiating the establishment of an antiviral state in the infected host cells (Brubaker et al., 2015). Recognition of pathogen associated molecular patterns (PAMPs) of viral nucleic acid (RNA or DNA) by host pattern recognition receptors leads to the recruitment of adapter molecules. This signaling activates certain kinases, which phosphorylate and translocate specific transcription factors to the nucleus where they activate the transcription of antiviral genes by binding to promoters containing specific transcription factor binding sequences (Kawa and Akira, 2009).

The signaling pathways activated by recognition of RNA by pattern recognition receptors including RIG-1 induced gene 1 (RIG-I), melanoma differentiation associated gene 5 (MDA5) or DNA by pattern recognition receptors including DNA-dependent activator of IFN-regulatory factors (DAI), RNA polymerase III, interferon-inducible protein16 (IFI16), Dead box helicase 41 (DDX41) , absent in melanoma 2 (AIM 2) and cGAS\STING (Holm et al., 2013; Lam et al., 2014) have been reported to activate family of transcription factors including IRF3 (interferon regulatory transcription factor), which lead to the induction of IFN-β. Although those different PRRs use different adapter molecules, all lead to the activation of IKK (IκB kinase) related kinases including TBK1 (TANK binding kinase 1) and IKKε (IκB kinase epsilon), which usually result in the activation of IRF3 (Clark et al., 2011; Gu et al., 2013) leading to transcriptional activation of promoters containing IRF3 binding domains including IFN-β promoter (Honda et al., 2006).

Phosphorylation at C-terminal serine \ threonine of cytoplasmic IRF3 leads to the dimerization and translocation of IRF3 to the nucleus (Clement et al. 2006; Servant et al. 2003).

Although PRRs including DDX41, AIM2, DAI and cGAS have been reported to act as cytosolic adenovirus DNA sensors for the induction of type I IFNs (Hendrickx et al., 2014), cGAS appears to act as the dominant cytosolic DNA sensor involved in the detection of adenovirus DNA in antigen presenting cells (Lam et al.,2014). Moreover, involvement of RIG-1 like receptor (MDA5) in the adenovirus induced innate immune response particularly in the induction of type 1 inferferons has also been reported (Schulte et al., 2013).

Bovine adenovirus (BAdV)-3 is a non-enveloped icosahedral particle containing 34446 bp linear double-stranded DNA genome (Reddy et al., 1998). The icosahedral capsid of adenovirus including BAdV-3 is made of major and minor structural proteins (Reddy et al., 1998; Russell, 2009). In addition, it contains core proteins including pV, which binds to adenovirus genome and is involved in connecting the core with capsid proteins (Perez-Vargas et al., 2014, Reddy and Nemerow, 2014). Bovine adenovirus-3 pV, expressed as a 55kDa protein localizes predominantly in nucleolus and interacts with 33K protein in virus infected cells (Kulshreshtha and Tikoo, 2008; Zhao, 2016). Moreover, BAdV-3 pV appears essential for the production of infectious progeny virions (Zhao and Tikoo, 2016). Our results suggest an additional role of viral protein V as potential suppressor of the interferon response.

3.2 MATERIALS AND METHODS

3.2.1 Cells and viruses

Madin Darby bovine kidney (MDBK) cells, cotton rat lung (CRL) cells, and CRL.pV (CRL cells expressing bovine adenovirus [BAdV]-3 protein V) cells (Zhao and Tikoo, 2016) were grown in minimal essential medium (MEM) (Sigma Aldrich) supplied with 10% heat-inactivated fetal

bovine serum (FBS, Invitrogen). Bovine monocytes were maintained in AIM-V Serum Free Lymphocyte Medium (Invitrogen) containing 10% heat inactivated fetal bovine serum (FBS). Mouse embryonic fibroblast (MEF) cells which was kindly provided by Dr. Joyce Wilson, University of Saskatchewan, Hela cells, and HEK293T cells (ATCC® CRL-3216™) were all cultivated in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS. All cell cultures were incubated at 37°C with 5% CO_2. BAdV304a (E3 region replaced by GFP gene) and BAdV-dV which has the deletion of protein V (Du and Tikoo, 2010, Zhao and Tikoo, 2016) were propagated in MDBK or CRL cells with MEM supplemented with 2% FBS and purified by cesium chloride density-gradient centrifugation. The virus titer was determined by $TCID_{50}$ assay.

3.2.2 Construction of plasmids

Plasmid expressing hepatitis C virus -NS3/4A (Yu et al., 2010), a well-known inhibitor of IFN-β induction as positive control was kindly provided by Dr. Qiang Liu from VIDO-Intervac. University of Saskatchewan. Plasmids expressing BAdV-3 viral proteins V (pcV), VIII (pc.pVIII) , 33K (pC.33K), IVa2 (pC.IVa2) and IX (pc.IX) have been described (Zhao and Tikoo, 2016; Ayalew et al., 2016; Kulshreshtha et al., 2014; Kulshreshtha et al., 2015). FLAG tagged RIG-I, MDA5, TRIF plasmids were kindly provided by Dr. Takashi Fujita, Japan. pEGFP-p65, luciferase reporter plasmids (pNF-kB-FireFly Luc or pIRF7-FireFly Luc) encoding luciferase under the NF-kB and IRF7 promoter element were kindly provided by Dr. Fengyi Wan, Johns Hopkins University. Plasmids pGFP-IRF3, pGFP-IRF7, pEGFPC1-TBK1, pcDNA3-Flag-IKKε and luciferase reporter plasmids encoding luciferase under IFN-β promoter (pIFN-β-luc) were kindly provided by Dr. Rongtuan Lin from Mcgill University. Plasmids pEY.p1-111(amino acid 1-111), pEY.p112-216 (112-216), pEY.p111-148 (111-148), pEY.p142-216 (142-216) cloned into pcDNA-EYFP backbone have been described earlier (Gaba, 2016).

Plasmid pcDNA.pVII: A 522bp fragment, which contains gene encoding protein VII was amplified by PCR by using primers pVII-R-BamHI and pVII-F-EcoRI (Table 3.1) and plasmid PUC304A+ DNA as a template (Anand et al., 2014). The EcoRI-BamHI digested PCR product was ligated to EcoRI-BamHI digested plasmid pCDNA3 DNA creating the plasmid pcDNA.pVII

Plasmid pDsRed.pV: A1280bp fragment which contains gene encoding protein V was amplified by PCR by using primers F-XhoI-pV, R-EcoRI-pV (Table 3.1) and plasmid pcV (Zhao and Tikoo, 2016) as a template. The EcoRI- XhoI digested PCR product was ligated to EcoRI-XhoI digested plasmid pDsRed-Monomer-C1 creating the plasmid pDsRedpV.

Plasmid pDsRed.pVIII: A 659bp fragment, which contains gene encoding protein VIII was amplified by PCR by using primers F-XhoI-pVIII and R-EcoRI-pVIII (Table 3.1) and plasmid pc.pVIII as template. The EcoRI-XhoI digested PCR product was ligated to EcoRI-BamHI digested plasmid pDsRed-Monomer-C1 creating the plasmid pDsRed.pVII.

Plasmid pCDNA3-HA-IKKε: A 2158bp fragment, which contains gene encoding protein IKKε was amplified by PCR by using primers IKKε-F-EcoRI, IKKε-R-NotI (Table 3.1) and plasmid pcDNA3-Flag-IKKε DNA as a template. The EcoRI-BamHI digested PCR product was ligated to EcoRI-BamHI digested plasmid pcDNA3-HA creating the plasmid pCDNA3-HA-IKKε.

Table 3.1 List of primers

ATGCGGATCCATGATGCATCCCGCTTTACGG	52K-BamHI-F
ATGCGAATTCTCAGAATCGCCAGTGGTTAG	52k-EcoRI-R
AGGCGGATCCATGGCCATTCTAATCTCTCCTAG	pVII-F-BamHI
ACGCGAATTCTCAAACGGTGTTGCTGACCGTAG	pVII-R-EcoRI
GGCCCGAATTCATGCAGAGCACAGCCAATTAC	IKKε-F-EcoRI
AATTGCGGCCGCTCAGACATCAGGAGGTGCTGGGACTC	IKKε-R-NotI
CTCGAGCTATGGCCTCCTCTCGGTTGATTAAAG	F -XhoI-pV
GAATTCTTAACGGCGGAGCCGGGTTACTG	R-EcoRI-pV
CTCGAGCTATGAGCAAAGAAATTCCCACACCTTATG	F-XhoI-pVIII
GAATTCTCAGCTATAACCGCTCACAGAGTTG	R-EcoRI-pVIII
ATGACAGCAGAAATGAGTCTTCC	IFNAD-1-R1
ACACACACCTGGTTCAACAC	IFNAD-1-F1
RTCTGSAGCCAATCCARAAG	IFNβ-F
CAGGCACACCTGTYGTACTC	IFNβ-R
AGGCCTCGAGATGAGCAAAGAAATTCCCACACCTTATG	F-XhoI-pVIII-1-111
ATTAGAATTCTCCGCCCGCGATTTGAGCGC	R-EcoRI-pVIII-1-111
ATTACTCGAGGGCGCTGCGGGCGATTACTT	F-XhoI-pVIII-112-216
GCCGGAATTCTCAGCTATAACCGCTCACAG	R-EcoRI-pVIII-112-216
ATTACTCGAGGGCGCTGCGGGCGATTACTT	F-XhoI-pVIII-112-146
ATCCGAATTCCGAGCCTCCTCCTAGTTGAA	R-EcoRI-pVIII-112-146
ATTCCTCGAGGCGTTCATCTTTCAACCCCC	F-XhoI-pVIII-147-216
GCCGGAATTCTCAGCTATAACCGCTCACAG	R-EcoRI-pVIII-147-216

3.2.3 Transfection

HeLa cells, HEK293T cells and MEF cells were transfected with various plasmids using Lipofactamine 2000 or 3000 reagents (Invitrogen) as described by the manufacturer.

For analysis of the IFN-β promoter activity, HEK293T cells or MEF cells grown in 24-well tissue culture plate at a density of 2×10^5 cells per well were co-transfected with 120 ng reporter plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc DNA and with either 300 ng plasmid DNA expressing individual viral protein or 300 ng of control plasmid. At 20 hrs post-transfection, the cells were left untreated or further treated with 300 ng poly I:C (Polyinosinic-polycytidylic acid, InvivoGen) or poly dA:dT (a synthetic analog of B-DNA, InvivoGen). After 20 hrs of incubation, the samples were analyzed by dual luciferase reporter assay.

For the analysis of involved pathways, HEK293T cells grown in 24-well plate at a density of 2×10^5 cells per well were co-transfected with 300ng plasmid DNA pCDNA3 or pcV or pc.pVIII individually, 120 ng of reporter plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc and 500 ng of plasmid DNA expressing RIG-I (pEF-flag-RIG-I) or MDA-5 (pEF-flag-MDA5) or TRIF (pEF-Bos-flag-TRIF) or TBK1 (pEGFPC1-TBK1) or IKKε (pCDNA3-HA-IKKε) individually without poly I:C or poly dA:dT stimulation, After 48 hrs of incubation, the cells were harvested for analysis by dual luciferase reporter assay.

For analysis of the NF-kB and IRF7 promoter activity, HEK293T cells grown in 24-well plate at a density of 2×10^5 cells were co-transfected with 5 ng pCMV-Rennila Luc, and 300ng plasmid pCDNA3 or pcV or pc.pVIII individually, and with either 300 ng NF-kB-FireFly Luc or IRF7-FireFly Luc. At twenty hrs post-transfection, the cells were treated with 300ng poly I:C. After 20 hrs of incubation, the cells were analyzed by dual luciferase reporter assays.

3.2.4 Luciferase reporter assay

For dual luciferase reporter assay, the transfected cells were washed with ice-cold phosphate-buffered saline (PBS) three times and lysed in 0.1 ml Passive Lysis Buffer (Promega). Luciferase reporter gene activities were measured by Dual luciferase assay kit following the manufacturer's instructions (Promega). Relative firefly luciferase activity was analyzed by the normalization to Renilla luciferase control. Results were expressed as fold changes compared to the empty vector controls. In all experiments, data was a representative of three independent experiments. Each experiment was performed in triplicate.

3.2.5 CsCl gradient centrifugation and Endotoxin quantitation

Monolayers of MDBK or CRL cells cultured in T-150 flasks were infected with BAdV304a or BAdV-dV, respectively at a multiplicity of infection (MOI) of 5. At 40 or 48 hrs post-infection, the infected cells were collected in 15ml medium and freeze-thawed for three times. Then, 1.5 ml 5% Na deoxycholate was added into the cell lysates. After incubating for 30 mins at room temperature, 150 µl 2M $MgCl_2$and 75 µl DNase I solution were added. After another 60 mins incubation at 37°C, the lysate was subjected to centrifugation at 5000rpm at 4°C for 15 mins and the supernatant was subjected to cesium chloride density gradient centrifugation at 35000 rpm at 10°C for 1 hr. After the first centrifugation, the band that contained virus was collected and then subjected to the second centrifugation at 35000 rpm at 4°C for 20 hrs. After the second centrifugation, the virus band was collected again and dialyzed for three times with 0.1M Tris-HCl overnight in Dialysis Cassettes (Thermo Scientific) to remove the cesium chloride and stored in small aliquots at -80°C. Viral titer was calculated by $TCID_{50}$ assay. LAL Chromogenic Endotoxin Quantitation Kit was used to measure the amount of endotoxin in virus stock following the manufacturer's instructions (Thermo Scientific).

3.2.6 Immunofluorescence microscopy

Monolayer of Hela cells cultured in 4-well chamber slides were transfected with 2 µg of plasmid DNA expressing DsRed-pV (pDsRed.pV) or DsRed-pVIII (pDsRed.pVIII) respectively. At 20 hrs post transfection, the cells were left untreated or treated with poly I:C (1mg/ml). After incubating for 12 hrs the cells were washed twice with phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde for 30 min and permeabilized with 0.1 M PBS solution containing 0.5% Triton X-100 for 10 min. Then, the cells were incubated with 10% goat serum in PBS for blocking for 1 hr at room temperature, and finally incubated with anti-IRF3 mAb (Cell Signalling) and Alexa Fluor 488 conjugated goat anti-rabbit IgG antibody (Invitrogen) for 2 hrs, respectively. After final three washes with PBS, the cells were mounted in mounting media (Vector Laboratories Inc.) and analyzed with a Leica fluorescence microscope TCS SP5. To determine the localization of IRF7 and NF-kB, the Hela cells were transfected with plasmids DNAs expressing GFP-IRF7 (pGFP-IRF7) and plasmid expressing either BAdV-3 pV (pDsRed.pV) or BAdV-3 pDsRed.pVIII individually; or GFP-p65 (pEGFP-p65) and plasmid expressing either BAdV-3 pV (pDsRed.pV) or BAdV-3 pDsRed.pVIII individually; and analysed with a Leica fluorescence microscope TCS SP5.

3.2.7 Flow cytometry

Bovine peripheral blood mononuclear cells (PBMC) were isolated from bovine venous blood using Percoll density gradient centrifugation as described earlier (Khosa, 2017). Purified bovine PBMCs resuspended at a density of 1×10^8 cells/ml were labeled with monoclonal antibodies specific for bovine CD11c (BAQ153A), CD14 (Clone MM61A) from VMRD (Pullman, WA) for 20 min at 4°C and remixing every 10 minutes gently. The cells were washed twice with ice-cold PBSA solution before incubating with fluorochrome-conjugated secondary

antibodies (Allophycocyanin (APC)-conjugated rat anti-mouse secondary antibody (BD Bioscience) and phycoerythrin (PE)-conjugated goat anti-mouse secondary antibody (Thermofisher scientific) for 20 min at 4°C. The cells were washed twice with ice-cold PBSA again and re-suspended with PBSA to make a final density of 1×10^8 cells/ml.

For monocyte sorting, labelled PBMC cells were filtered through 35 µm cell strainer capped 12×75 mm polystyrene round bottom tubes (BD Falcon) to remove cell clumps and isolated by a FACScan flow cytometer using the Cell Quest program (Becton Dickinson, Mountain View, CA). Sorted monocyte cells were centrifuged and resuspended in AIMV medium supplemented with 10% fetal bovine serum.

3.2.8 Western blotting

The whole-cell extracts were obtained by two times washing with ice-cold phosphate-buffered saline (PBS) and lysis with radio-immunoprecipitation assay buffer (1 mM EDTA, 10 mM Tris-Cl (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) supplemented with protease inhibitors (Roche) and/or phosphatase inhibitor cocktail (Sigma). The cell lysates were centrifuged at 13000×g at 4°C for 15 min, and amount of proteins in the supernatant was calculated using NanoDrop 2000/2000c Spectrophotometer (Thermofisher scientific). Known amount of proteins were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane (Bio-Rad) and probed by protein specific primary antibodies and Alexa Fluor 680 conjugated goat anti-rabbit secondary antibody (Invitrogen) or IRDye800 conjugated goat anti-mouse secondary antibody (Rockland) respectively. Finally, the membranes were analyzed by Odyssey® CLx Imaging System (LI-COR).

3.2.9 Total RNA extraction and cDNA synthesis

The isolated monocytes were infected with virus at a MOI of 2. At indicated hrs post infection, the infected cells were lysed by adding TRIzol (Life Technologies) following) directly to cell culture plates. The RNA was extracted by adding chloroform to the cell lysate in TRIzol, vortexing and centrifuging at 14000×g at 4°C for 15 minutes. The aqueous phase was transferred to the Eppendorf tube containing 100% isopropanol with glycogen and incubated at -80°C for 5 min. The RNA pellet was collected by centrifuging at 14000×g at 4°C for 15 min and resuspended in 70% ethanol. This step was repeated once with 70% ethanol and then with 100% ethanol. The pellet was finally air-dried and resuspended in RNAse-DNAse free water (Invitrogen). Finally, RNA quality and quantity was determined using the Agilent 2100 Bioanalyzer (G2938B, Agilent technologies).

The cDNAs were synthesized by reverse transcription using QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's instructions: About 300 ng of total RNA in 12 µl RNAse-DNAse free water (Invitrogen) and. 2 µl of gDNA wipe-out buffer (to eliminate genome) were incubated at 42°C for 2 min with GeneAmp 9700 PCR System (Applied Biosystems) and placed on ice immediately. The cDNA was generated by adding the prepared mix to the wiped RNA (4 µl of Quantiscript RT buffer, 1 µl of reverse transcriptase and 1 µl the primer mix). The reaction mix was incubated at 42°C for 30 min and then at 95°C for 3 min with GeneAmp 9700 PCR System (Applied Biosystems).

3.2.10 Quantitative RT-PCR

Primers used to amplify gene transcripts have been previously described (Rahwa Osman, Philip Griebel, unpublished results). For quantitative reverse transcription-PCR (RT-PCR), we used PerfeCTa® SYBR® Green SuperMix (Quanta BioScience) with iCycler iQ PCR detection

system (Bio-Rad) following the manufacturer's instructions. Briefly, the reaction was carried out in a total volume of 15 µl by using a mix of 15ng cDNA product, 3 µl of the indicated primer set at a concentration of 3.3 uM (Table 3.1) and 9 µl of the perfecta SYBR green master mix (2×) following the below program: 1 cycle at 50°C for 2 min; 1 cycle at 95°C for 30 s; 45 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. All reactions were performed in triplicate. Amplification data of IFNα and IFNβ genes was expressed as threshold cycle (Ct) and relative gene expression values were standardized by β-actin reference gene control to obtain ΔCt (ΔCt = Ct gene-Ct βactin).

3.2.11 Co-immunoprecipitation analyses

HEK-293T cells were co-transfected with different plasmid mix. At 40 hrs post-transfection, the transfected cells were washed three times with ice-cold phosphate-buffered saline (PBS) and then lysed in 200 µl of lysis buffer for 30 min at 4°C (250mM Tris-HCl (pH 7.4), 15mM $MgCl_2$, 150mM NaCl, 0.1% NP-40, and 5mM EDTA, 1mM dithiothreitol) supplemented with protease inhibitor (Roche). The cell lysates were centrifugated at 1000×g for 10 min at 4°C, and the proteins in the supernatants were immunoprecipitated overnight at 4°C with protein specific anti-serum and protein G or A sepharose beads (GE Healthcare). The immunoprecipitates were washed with 1 ml of lysis buffer for three times and analyzed with immunoblotting procedures using indicated specific primary antibodies.

3.2.12 Statistical analysis

All experiments were carried out three times independently with triplicate samples each time. Data are expressed as means ± SEM and statistical analyses were performed by One way ANOVA with Tukey's Multiple Comparison Test using GraphPad program (GraphPad Software). Differences were considered significant at * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.3 Results

3.3.1 BAdV-3 pV and pVIII are involved in the down-regulation of type I IFN response

To determine whether any BAdV-3 encoded structural proteins have antagonistic effect on innate antiviral response, we screened six BAdV-3 proteins by assessing their potential effect on the IFN-β promoter activation. HEK293T cells were co-transfected with plasmid pIFNβ-Rennilaluc-IRES-Firefly luc (luciferase reporter gene under IFNβ promoter) together with plasmid expressing individual viral protein or empty control vector. At 20 hrs post transfection, the cells were stimulated by transfection with poly I:C (a double-strand RNA analog which is ligand for PRRs such as RIG-I,MDA5 and TLR3). After additional incubation for 20 hrs (to activate the IFN-β promoter activity), the cells were harvested and analyzed for luciferase activity. As shown in Fig 3.1(panel a), poly I:C stimulation enhanced the IFN-β promoter-driven luciferase activity up to 30-fold compared with the mock treated control. Interestingly, expression of hepatitis C virus NS3/4A, BAdV-3 pV or pVIII significantly inhibited the IFNβ promoter activation. No such inhibition could be observed in the cells expressing BAdV-3 pVII, IVa2, pIX or 33K proteins.

Similarly, stimulation of the MEF cells by transfection of poly dA:dT (act via intracellular DNA sensors) enhanced the IFN-β promoter-driven luciferase activity up to 60-fold compared with the mock treated control (Fig 3.1, panel b). Moreover, expression of BAdV-3 proteins including pV and pVIII significantly inhibited the IFN-β promoter activation (Fig 3.1b). No such inhibition could be observed in the cells expressing BAdV-3 pIX or 33K proteins (Fig 3.1b).

Since cleaved forms of pVIII are present in the infectious BAdV-3 (Gaba, 2016), we determined if different cleavage forms of pVIII are required for inhibiting the activation of IFN-β promoter. As seen in Fig 3.2 (panels a, b), unlike full length pVIII, none of the cleaved pVIII forms

expressed in cells transfected with indicated plasmid DNAs down regulated the activation of IFN-β promoter.

3.3.2 Protein V antagonizes the IFNs response by disrupting IRF3 signaling

Since many viruses have been shown to attenuate the type I IFN response by interference with the central kinases namely TANK-binding kinase 1 (TBK1) or IκB kinase ε (IKKε) involved in the activation of IFN transcription factors, we determined if BAdV3 protein V and VIII also have similar antagonistic mechanism to suppress the interferon response. To accomplish this, HEK293T cells were co-transfected with indicated amount of luciferase reporter plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc DNA, plasmid pEGFPC1-TBK1 expressing TANK-binding kinase 1 (TBK1; activating IFN-β promoter) or plasmid pCDNA3-HA-IKKε expressing IκB kinase ε (IKKε; activating IFN-β promoter) together with plasmid expressing either BAdV-3 pV (pcV) or pVIII (pcpVIII) protein. After 40 hrs of transfection, the cell were collected and the cell lysates were analysed for luciferase activity. As shown in Fig 3.3 (panels a, b), overexpression of kinases TBK1 or Ikkε significantly induced the activation of IFN-β promoter in plasmid pcDNA3 DNA (control) transfected cells. The co-expression of pV significantly inhibited the activation of IFN-β promoter in cells overexpressing TBK1 kinase but showed no effect in the cells overexpressing IKKε kinase. In contrast, co-expression of pVIII significantly inhibited the activation of IFN-β promoter in the cells overexpressing TBK1 or IKKε kinases.

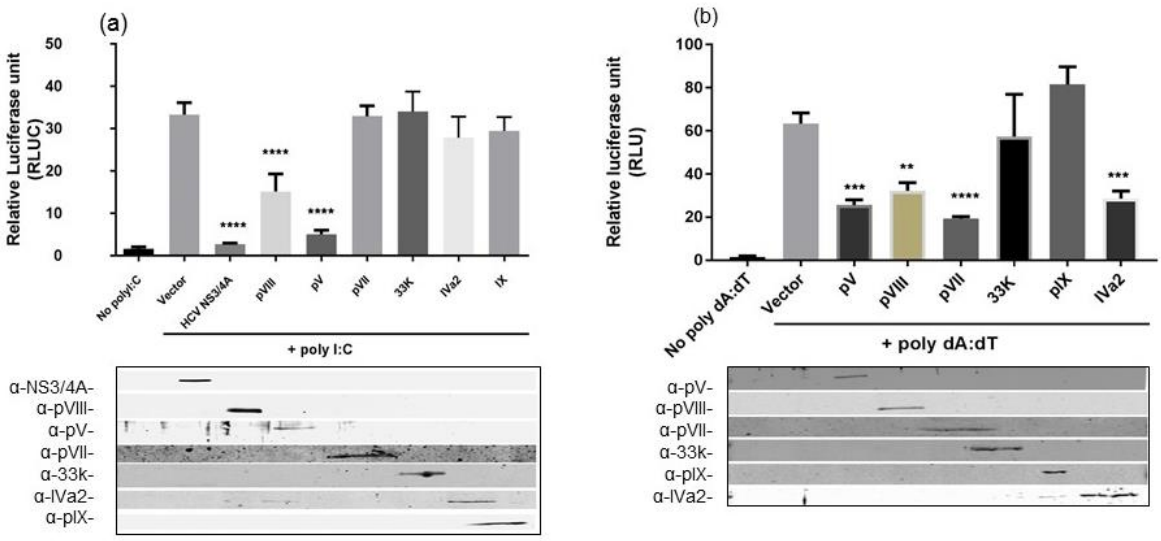

Figure 3.1 Multiple viral proteins block IFN-β promoter activation triggered by poly I:C or poly dA:dT stimulation. HEK293T cells **(a)** or MEF cells **(b)** in 24 well plate were cotransfected with indicated amount of reporter plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc and plasmid expressing individual viral protein or empty vector for 20 hours. Then the cells were stimulated by the transfection with 300 ng poly I:C **(a)** or poly dA:dT **(b)** for another twenty hours, then the cells were harvested for dual luciferase reporter assay. Data is a representative of three independent repeats and expressed as means±SEM. Statistical analysis was performed by One way ANOVA with Tukey's Multiple Comparison Test using GraphPad program (GraphPad Software). Immunoblot analysis was used to determine the expression of various constructions**(c)**, samples are representatives of three triplicates from three independent repeats in both **(a)** and **(b)**.

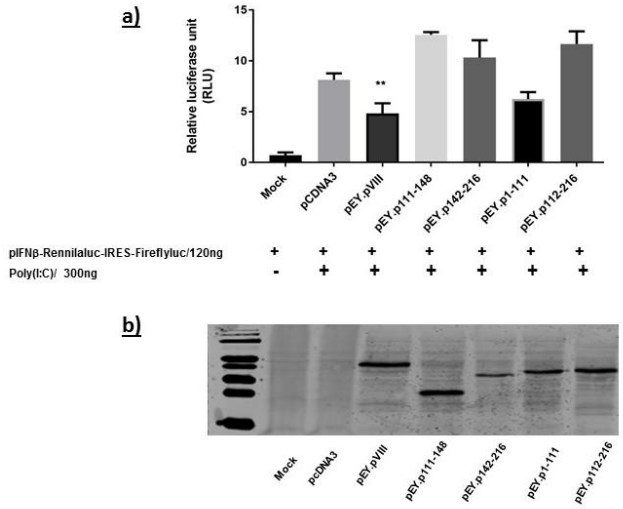

Figure 3.2 Full length protein VIII is required for the down-regulation of IFN-β promoter activity induced by poly I:C stimulation HEK293T cells in 24 well plate were co-transfected with indicated amount of reporter plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc and plasmid expressing different fragement of protein VIII or empty vector for 20 hours. Then the cells were stimulated by the transfection with 300 ng poly I:C for another twenty hours, and the cells were harvested for dual luciferase reporter assay **(a)**. Data is a representative of three independent repeats and expressed as means±SEM. Statistical analysis was also performed by One way ANOVA with Tukey's Multiple Comparison Test using GraphPad program (GraphPad Software). Immunoblot analysis was used to determine the expression of various constructions **(b)**.

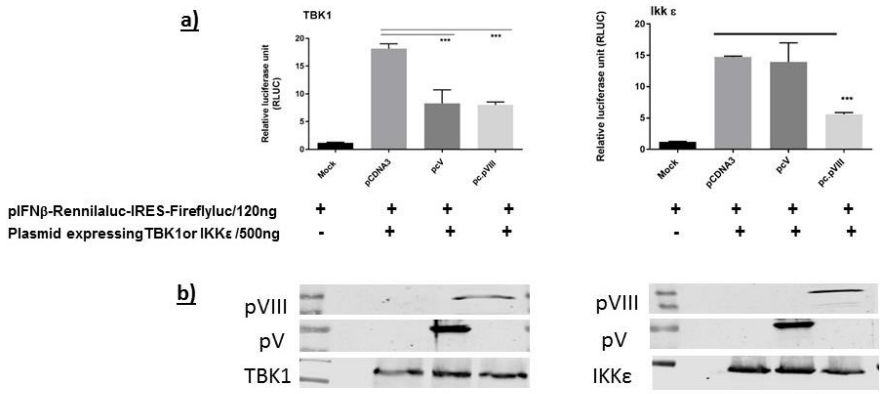

Figure 3.3 Protein V and VIII significantly downregulate the IFN-β promoter activity induced by overexpression of TBK1 or IKKε. HEK293T cells were co-transfected with indicated amount of luciferase reporter plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc, plasmid expressing TANK-binding kinase 1 (TBK1) or IκB kinase ε (IKKε) together with plasmid expressing viral protein V or VIII for forty hours. Then the cell lysates were harvested for dual luciferase reporter assay. Data is a representative of three independent repeats and expressed as means±SEM **(a).** Statistical analysis was performed by One way ANOVA with Tukey's Multiple Comparison Test using GraphPad program (GraphPad Software). Immunoblot analysis was used to determine the expression of various constructions **(b).**

3.3.3 Protein V interferes with the phosphorylated activation and nuclear translocation of IRF-3.

To examine the effect of BAdV-3 pV or pVIII on the phosphorylation dependent transactivation activity of IRF3 (Fig 3.4, panel a), Hela cells were transfected with plasmid pCDNA3 (lane 3), pcV (expressing protein V) (lane 4) or plasmid pcpVIII (expressing pVIII) (lane 5) DNAs. After 30 hrs of transfection, the transfected cells were transfected with poly I:C (1 mg/ml). After additional incubation for 12 hrs, the cells were collected and analyzed by Western blot for expression of phosphorylated IRF3 using antibodies detecting phosphorylated IRF3 (Ser396) (Fig 3.4, panel a). No phosphorylated IRF3 could be detected in the cells not stimulated with poly I:C (lane 1). However, phosphorylated IRF3 could be detected in the cells stimulated with poly I:C (lane 2) only. Moreover, no detectable difference could be observed in the amount of phosphorylated IRF3 detected after poly I:C stimulation of cells transfected with plasmid pcDNA3.1 (control) (lane 3) or plasmid pcpVIII (expressing pVIII, lane 5) DNAs. In contrast, significant difference could be observed in the amount of phosphorylated IRF3 detected after poly I:C stimulation of the cells transfected with plasmid pcV (expressing pV) DNA (lane 4) compared to the cells transfected with plasmid pcDNA3.1 (lane 3) or pcpVIII (expressing pVIII) (lane 5) DNAs.

To examine the effect of BAdV-3 pV or pVIII on the nuclear translocation of IRF3, Hela cells in 4 well chamber slide were transfected with indicated plasmid pDsRed.pV (expressing DsRed-pV) or plasmid pDsRed.pVIII (expressing DsRed-pVIII) DNA (Fig 3.4, panel b). At 20 hrs post transfection, the cells were treated with poly I:C (1 mg/ml). After 12 hrs of incubation, the cells were examined by direct fluorescence (panels a1, b1, c1, d1) or indirect fluorescent (panels a3, b3, c3, d3) using anti-IRF3 serum. As seen in Fig 3.4 (panel b),

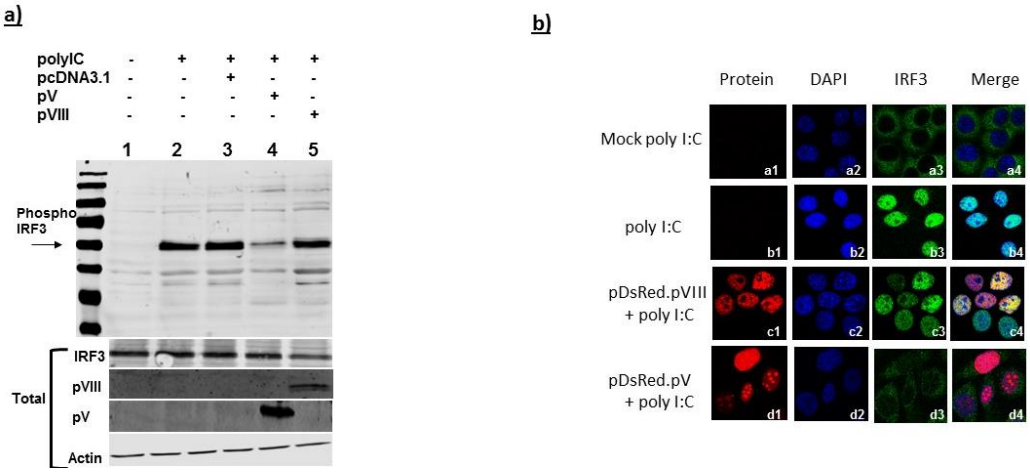

Figure 3.4 protein V interferes with the phosphorylated activation and nuclear translocation of IRF-3 induced by poly I:C stimulation **(a)** Hela cells in 6 well plate were transfected with empty vector or indicated plasmid DNA expressing protein V or VIII (Lanes 3-5) for thirty hrs, then the transfected cells were treated with poly I:C (1 mg/ml) for another twelve hrs. The cells without poly I:C treatment or only with poly I:C treatment without any plasmid transfection were shown as controls (Lane 1-2). Immunoblot analysis with antibodies directed against phosphorylated IRF3 (Ser396) and total IRF3 was used to determine the phosphorylation level of IRF3. Expression of protein V and VIII was also shown by immunoblot analysis. **(b)** Hela cells in 4-well chamber slide were transfected with plasmid DNA expressing DsRed-pV or DsRed-pVIII for twenty hours, then the cells were left untreated or treated with poly I:C (1 mg/ml) for another 12 hrs. The localization of IRF-3 was analyzed with IRF3 antibody and DNA was stained with mounting media.

while the IRF3 is localized in the cytoplasm of unstimulated cells (panels a3, a4), the IRF3 translocates to nucleus in poly I:C stimulated cells (panels b3, b4). Expression of DsRed-pVIII (panel c1, c4) does not affect the nuclear localization of IRF3 (panel c3, c4) after poly I:C stimulation. In contrast, expression of DsRed-pV (panel d1, d4) significantly inhibits the nuclear localization of IRF3 (panel d3, d4) after poly I:C stimulation.

3.3.4 pV and pVIII suppress the NF-kB or IRF7 promoter activity, but do not prevent nuclear translocation of NF-kB or IRF7

To determine if pV or pVIII affects NF-kB or IRF7 promoter activity, we performed luciferase assay using the luciferase reporter plasmids namely pNF-kB-FireFlyLuc or pIRF7-FireFly Luc that confer NF-kB-dependent or IRF7-dependent inducibility to firefly-luciferase expression, respectively. As seen in Fig 3.5 (panels a, c), the expression of pV or pVIII significantly reduces the expression of luciferase activity in plasmid pNF-kB-FireFlyLuc DNA transfected cells stimulated with poly I:C compared to control (plasmid pcDNA3.1 DNA transfected cells). In contrast, expression of pV but not pVIII significantly reduces the expression of luciferase in plasmid pIRF7-FireFlyLuc DNA transfected cells stimulated with poly I:C compared to control (plasmid pcDNA3.1 DNA transfected cells).

To determine the effect of pV or pVIII on the nuclear translocation of NF-kB in poly I:C stimulated cells, we employed immunofluorescence assay (Fig 3.5b). Hela cells were co-transfected with plasmid pEGFP-p65 (expressing EGFP-65) (Fig 5b-GFP65, panela3, b3, c3, d3) DNAs and either plasmid pDsRed.pV DNA expressing DsRed-pV (panel c1, c4) or plasmid pDsRed.pVIII expressing DsRed-pVIII (d1, d4). After 20 hrs post transfection, the cells were stimulated by poly I:C for 12 hrs before analyzing the cells by direct immunofluorescence.

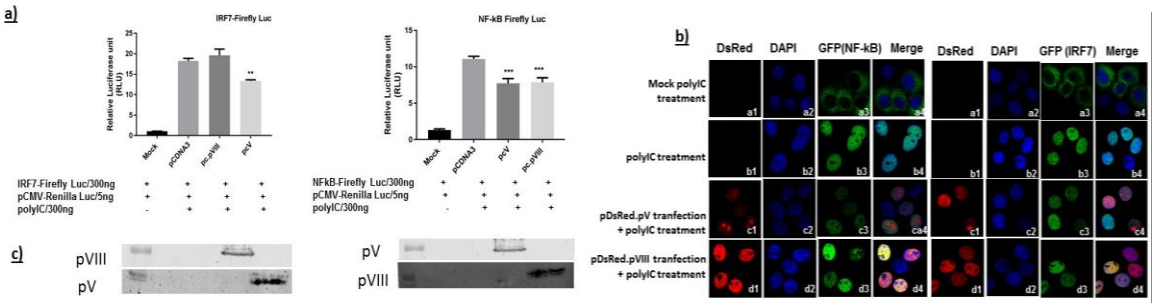

Figure 3.5 Protein V and VIII suppress the NF-kB and IRF7 promoter activity but do not affect the nuclear translocation of NF-kB and IRF7 **(a)** HEK293T cells in 24 well plate were cotransfected with indicated amount of plasmid expressing viral protein V or VIII, reporter plasmid NF-kB-FireFly Luc or IRF7-FireFly Luc together with pCMV-Renilla as normalization control. At twenty hrs post-transfection, the cells were stimulated by the transfection with 300 ng poly I:C for another twenty hours before harvested for dual luciferase reporter assay. Data is a representative of three independent repeats and expressed as means±SEM. Statistical analysis was also performed by One way ANOVA with Tukey's Multiple Comparison Test using GraphPad program (GraphPad Software). **(b)** Hela cells in 4-well chamber slide were transfected with plasmid DNA expressing DsRed-pV or DsRed-pVIII together with plamid expressing GFP-IRF7 or GFP-p65 for twenty hours, then the cells were left untreated or treated with poly I:C (1 mg/ml) for another 12 hrs. DNA was stained with mounting media and the localization of indicated protein was analyzed by Leica fluorescence microscope TCS SP5. **(c)**Immunoblot analysis was used to determine the expression of various constructions.

As seen in Fig 3.5 (panel b-GFP65), the expression of pV (panel c1, c3, c4) or pVIII (panel d1, d3, d4) does not alter the nuclear translocation of NF-kB subunit p65 fused to EGFP in poly I:C stimulated cells.

Similar experiment was conducted to determine the effect of pV or pVIII on translocation of IRF7 in poly I:C stimulated cells. As seen in Fig 3.5 (panel b-GFP-IRF7), expression of pV (panels c1, c3, c4) or pVIII (d1, d3, d4) does not alter the nuclear translocation of IRF7 fused to GFP in poly I:C stimulated cells.

3.3.5 Protein V does not interact with TBK1 or IKKε, but interacts with IRF3

To explore the possible mechanisms underlying the interference with IRF3 signaling by pV, we determined if BAdV-3 pV interacts with kinases TBK1 or IKKε which are primarily responsible for the IRF3 activation. HEK293T cells were co-transfected with plasmid pDsRed.pV (expressing DsRed-pV) or either plasmid pcDsRed (control) DNAs, and plasmid pEGFPC1-TBK1 (expressing GFP-TBK1) or plasmid pcDNA3-Flag-IKKε (expressing HA-IKKε) DNAs and analyzed by co-immunoprecipitation coupled with Western blotting. As seen in Fig 3.6 (panel a), anti-GFP serum detects GFP-TBK1 specific protein in cells expressing DsRed-pV and GFP-TBK1 (lane 1) or DsRed and GFP-TBK1 (lane 4). No such protein could be observed in the lysates of DsRed-pV and GFP-TBK1 expressing cells immunoprecipitated with anti-pV serum (lane 3) or rabbit IgG and probed in Western blot with anti-GFP serum.

As seen in Fig 3.6 (panel b), anti-HA mAb detects HA-IKKε specific protein in the cells expressing HA-IKKε and DsRed-pV (lane 1) or HA-IKKε and DsRed (lane 4). No such protein could be observed in the lysates of DsRed-pV and HA-IKKε expressing cells immune-precipitated with anti-pV serum (lane 3) or rabbit IgG (lane 2) and probed in Western blot with anti-HA mAb. To next determine if pV interacts with IRF3, HEK293T cells were co-transfected

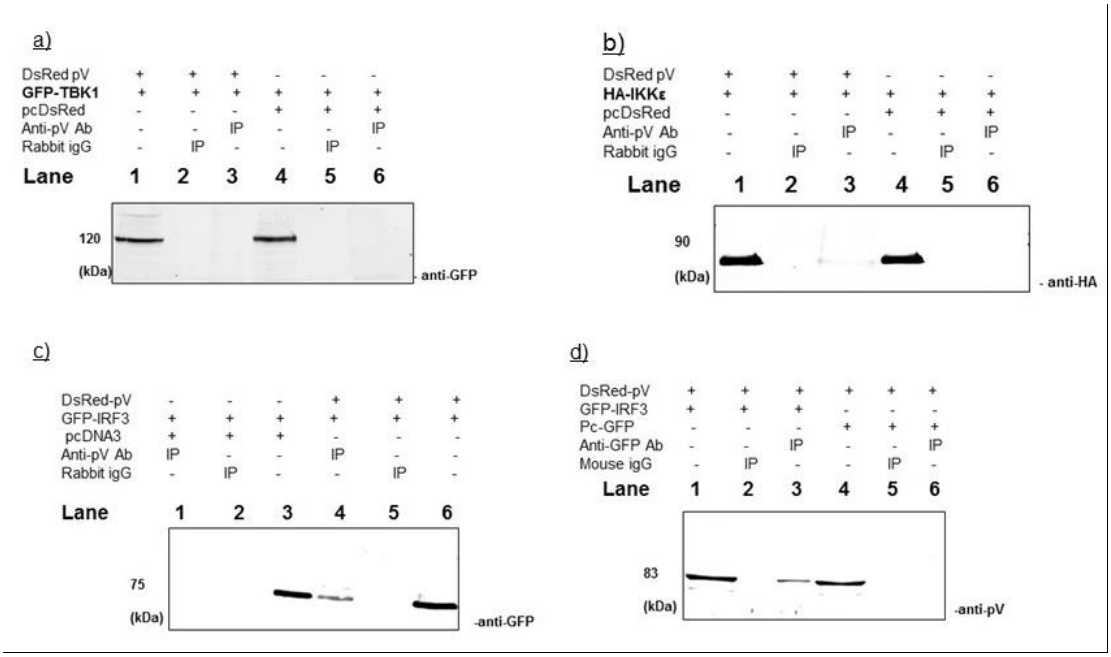

Figure 3.6 Protein V does not interact with TBK1 or IKKε directly, but interact with IRF3

HEK-293T cells were co-transfected with plasmid expressing DsRed-pV(Lanes 1-3) or empty vector(Lanes 4-6) together with plamid expressing GFP-TBK1**(a)** or HA-IKKε**(b)**. At forty hrs post-transfection, the transfected cells were harvested for protein immunoprecipitation by indicated antibody against protein V (Lanes 3 and 6) or control rabbit serum (Lanes 2 and 5) , and immunoblotting analysis was performed with antibodies against GFP**(a)** or HA**(b)**. Whole cell extract was also shown as control by immunoblotting analysis (Lanes 1 and 4). **(c)**HEK-293T cells were co-transfected with plasmid expressing GFP-IRF3 together with plasmid expressing DsRed-pV (Lanes 4-6) or empty vector (Lanes 1-3). At forty hrs post-transfection, the transfected cells were harvested for protein immunoprecipitation by indicated antibody against protein V (Lanes 1 and 4) or control rabbit serum(Lanes 2 and 5) , and immunoblotting analysis was performed with antibodies against GFP. Whole cell extract was shown as control by immunoblotting analysis (Lanes 3 and 6). **(d)**HEK-293T cells were co-transfected with plasmid expressing DsRed-pV together with plasmid expressing GFP-IRF3 (Lanes 1-3) or empty vector (Lanes 4-6). At forty hrs post-transfection, the transfected cells were harvested for protein immunoprecipitation by indicated antibody against GFP (Lanes 3 and 6) or control mouse serum(Lanes 2 and 5) , and immunoblotting analysis was performed with antibodies against protein V. Whole cell extract was shown as control by immunoblotting analysis (Lanes 1 and 4).

pDsRed.pV (expressing DsRed-pV) DNA or either plasmid pcDysRed (control) with plasmid pGFP-IRF3 DNA (expressing GFP-IRF3), analyzed by co-immunoprecipitation coupled with Western blotting. As seen in Fig 3.6 (panel c), anti-GFP serum detects GFP-IRF3 specific protein in cells expressing GFP-IRF3 and DsRed (lane 3) or DsRed-pV (lane 6). Similar protein could be observed in the lysates of DsRed-pV and GFP-IRF3 expressing cells immunoprecipitated with anti-pV serum (lane 3) and probed in Western blot with anti-GFP serum. As seen in Fig 3.6 (panel d), anti-pV serum detects pV specific protein in the cells expressing DsRed-pV and GFP-IRF3 (lane 1) or pcGFP (lane 4). Similar protein could be observed in the lysates of DsRed-pV and GFP-IRF3 expressing cells immunoprecipitated with anti-GFP serum (lane 3) and probed in Western blot with with anti-pV serum.

3.3.6 pV and pVIII inhibit IFNβ promoter activity mediated by by RIG-I / MDA-5/ TLR3

To examine the potential of specific pathways that can be affected by protein V and VIII, we assessed the ability of pV and pVIII to block the IFN-β promoter induced by overexpression of pathway-specific molecules RIG-I or MDA5 or TRIF (an adaptor molecule downstream of TLR3). As seen in Figure 3.7 (panel a), the cells co-transfected with plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc (luciferase under IFN-β promoter) DNA, plasmid pcDNA3.1 (control) DNA and either plasmid pEF-flag-RIG-I DNA expressing RIG-I (lane 1), plasmid pEF-flag-MDA5 DNA expressing MDA5 (lane 6) or plasmid pEF-Bos-flag-TRIF DNA expressing TRIF (lane 10) resulted in up to 70, 50, 200 fold induction of the IFN-β activity, respectively. In contrast, expression of pV (lanes 3,7,11) or pVIII (lanes 4,8,12) led to significant suppression of IFN-β promoter activation, in cells co-transfected with plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc and either plasmid pEF-flag-RIG-I DNA expressing RIG-I (lane 3,4), plasmid pEF-flag-MDA5 DNA

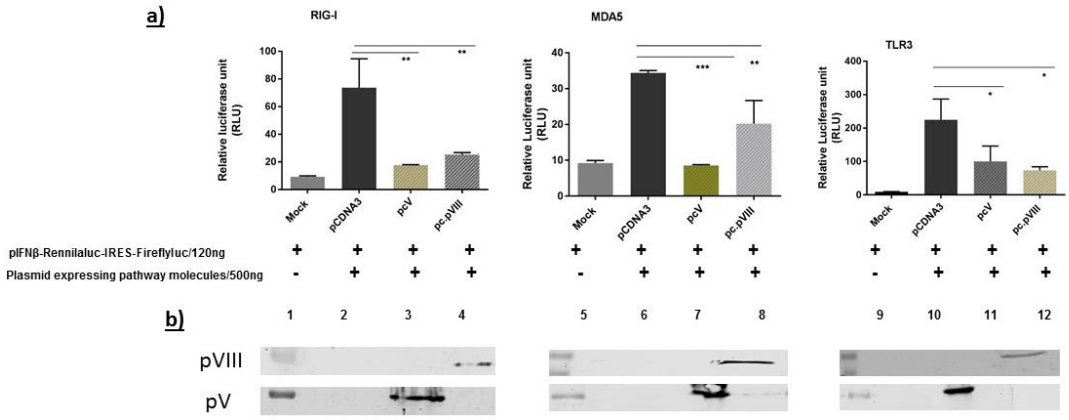

Figure 3.7 Protein V and pVIII inhibit IFNb promoter activity mediated by by RIG-I / MDA5/ TLR3. HEK293T cells were co-transfected with indicated amount of luciferase reporter plasmid pIFNβ-Rennilaluc-IRES-Fireflyluc, plasmid expressing viral protein V or VIII, plasmid expressing RIG-I or MDA5 or TRIF (an adaptor molecule downstreams of TLR3) individually for forty hours **(a).** Then the cell lysates were harvested for dual luciferase reporter assay. Data is a representative of three independent repeats and expressed as means±SEM. Statistical analysis was performed by One way ANOVA with Tukey's Multiple Comparison Test using GraphPad program (GraphPad Software). Immunoblot analysis was used to determine the expression of various constructions **(b).**

expressing MDA5 (lanes 7, 8) or plasmid pEF-Bos-flag-TRIFDNA expressing TRIF (lanes 11,12).

3.3.7 IFNα and IFNβ transcripts in BAV.dV infected cells

To determine the impact of protein V on the type I IFN response in virus infected cells, we next analyzed the involvement of pV in suppression of IFNβ and IFNα induction in BAV.dV (Zhao and Tikoo, 2016) infected cells. MDBK cells in 12 well plate were either mock-infected or infected with BAV304a or BAV.dV (pV deleted BAdV-3) at a multiplicity of infection (MOI) of 2. The virus stock appeared to be endotoxin free (<0.1 endotoxin unit/mL). At indicated times post infection, the total RNA was extracted and used to analyze the transcription levels of IFNα and IFNβ by real-time RT-PCR. As seen in Fig 3.8 (panel a), deletion of protein V significantly enhanced the level of both IFN-α and IFN-β transcripts at 12 and 24 hrs post of BAdV-dV infection of MDBK cells. Same analysis was performed in FACS-isolated bovine monocytes which is the most potent cell type to induce type I IFNs during bovine adenovirus infection, and similar result was shown in Fig 3.8 (panel b), the deletion of protein V significantly enhanced the level of both IFN-α and IFN-β transcripts at 6, 12 and 24 hrs post of BAV.dV infection of bovine monocytes cells confirming the Figure 3.1 result by dual luciferase reporter assay that protein V significantly suppresses the IFN-β promoter activation, confirming that the type I IFN response could be restrained by protein V.

3.4 DISCUSSION AND CONCLUTION

Adenovirus infection of host cells induces strong innate immune response including type I IFN (Muruve, 2004). To avoid elimination, adenovirus encodes proteins which can help to evade the host innate immune response. So far, structural proteins pVI (Schreiner et al., 2012) and pVII (Avgousti et al., 2016) have been shown to aid modulating the innate antiviral response

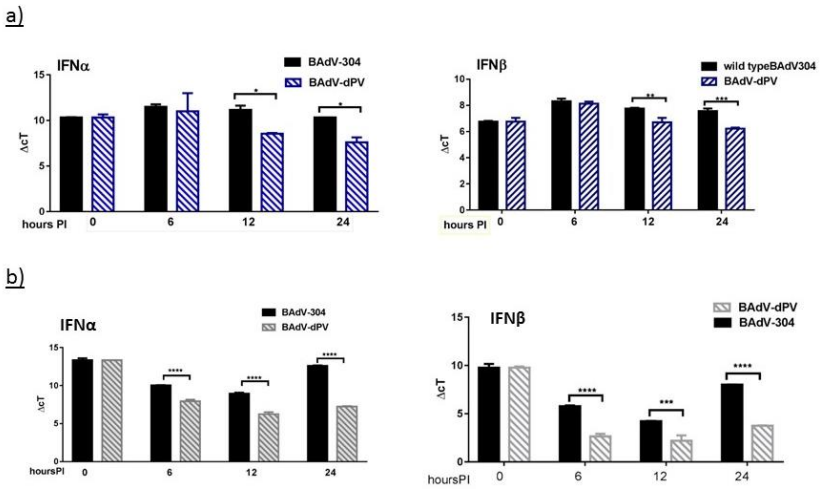

Figure 3.8 Deletion of protein V significantly enhances the transcript level of IFNα and IFNβ under BAdV-dV infection. MDBK cells **(a)** and bovine monocytes **(b)** in 12 well plate were either mock-infected or infected with BAdV-304a or BAdV-dV virus lacking protein V at a multiplicity of infection (MOI) of 2 respectively. Total RNA were extracted from the mock-infected cells and infected cells at the indicated time points 6, 12, 24 hours post-infection, the transcription levels of IFNα and IFNβ were analyzed by real-time RT-PCR. The data is expressed as relative gene expression value standardized by β-actin reference gene control: ΔCt (ΔCt = Ct interest gene-Ct βactin). Data is a representative of three independent repeats and expressed as means±SEM.

by eliminating the DAXX mediated repression of E1A promoter and elimination of HMGB1 release from chromatin, respectively. Several early proteins including E1A have been demonstrated to inhibit type I IFN inducible gene expression (Ackrill et al.; 1991; Hendrickx et al., 2014). Moreover, it has been shown that E1A can also suppress IRF3 dependent induction of IFN-α\β (Juang et al., 1998). Here, we demonstrate that BAdV-3 structural proteins pV and pVIII modulate the activation of IFN-β promoter. Though sensing of adenovirus DNA by cytosolic DNA sensor cGAS appears to be dominant mechanism used to induce type I IFN (Lam et al., 2014) in virus infected cells, earlier reports indicate that PAMPs of adenovirus nucleic acid are recognized by PRRs sensing both cytosolic DNA (e.g cGAS) and cytoplasmic RNA (e,g. RIG-1 like receptors) leading to the induction of type I IFN (Muruve, 2004). Nevertheless, those different cGAS and RIG-I/MDA5 signaling pathways involve common kinases (TBK1 and IKKε), which activate and phosphorylate IRF3 and IRF7 (Wu and Chen, 2014) leading to the activation IFN-α or β promoter.

The IFN-β promoter activity mediated by over expression of RIG-I, MDA5, TLR3 or molecules was inhibited by BAdV-3 pV or pVIII. Moreover, BAdV-3 pV or pVIII also significantly reduced the activation of IFN-β promoter in the cells stimulated with either poly I:C or poly dA:dT. These results suggest that both proteins appear to act at a step common to signal pathways used by different PRRs. However, BAdV-3 pV and pVIII appear to act at different components of the common step used by different PRR signalling pathways.

Overexpression of TBK1 but not IKKε did not help in increasing the activation of IFN-β promoter in cells expressing BAdV-3 pV, suggesting that pV may suppress the activation of IFN-β promoter by targeting the activity at IKKε kinase activation or level down stream of TBK1 activation including activation of interferon regulatory factor(s) (IRF). Although both TBK1 and IKKε exibit different substrate specificity and expression pattern, they are indistinguishable in

inducing IFN expression (Fitzgerald et al., 2003, Yu et al., 2012) as both TBK1 and IKKε appear to possess a partially redundant function in the activation of IFN-β promoter (Hemmi et al., 2004).

IRF3, a constitutively expressed cytoplasmic protein is a transcription factor, which plays an important role in IFN-β signaling pathway leading to the expression of IFN- β (Tsuchida et al., 2009). Phosphorylation of IRF3 by activated kinaseTBK1 leads to its homo-dimerization and translocation into the nucleus, where it binds to IFN-β promoter DNA (Kawai and Akira, 2009). Viruses have used a number of mechanisms including binding of viral protein to DDX3 complexed to TBK1\IKKε (Schroder et al., 2008), TBK1 scaffolding protein complex (Unterholzner et al., 2011), directly to TBK1\IKKε (Otsuka et al., 2005) or directly to IRF3 (Zhang et al, 2016) to inhibit the activation of IRF3 by TBK1 kinase.

The significant reduction in the phosphorylation and translocation of IRF3 to the nucleus suggest that pV affected the activation of IRF3. It could be due to observed interaction of pV with IRF3, which could prevent the interaction between TBK1 and IRF3 required for the activation of IRF3. Similar mechanism has been reported for inhibition of IRF3 activation by VP24 of herpes simplex virus-1 (Zhang et al, 2016).

Type I IFN induction is also regulated by transcription factors IRF7 and NF-kB (Levy et al., 2011). Although BAdV-3 pV significantly reduced the promoter activity of NF-kB or IRF7, there was no effect on the nuclear translocation of NF-kB and IRF7, suggesting that the activation of NF-kB and IRF7 transcription factors may not be targeted by the antagonism functions of protein V.

Over expression of TBK1 or IKKε, did not help in increasing the activation of IFN-β promoter in cells expressing BAdV-3 pVIII suggesting that pVIII may also suppress the activation of IFN-β promoter by targeting the activity down stream of TBK1 and IKKε activation including

activation of IRFs. DDX3 binds to IKKε, which leads to TBK1\IKKε mediated IRF activation and IFN-β promoter activation (Schroder et al., 2008). Interaction of vaccinia virus K7 protein with DDX3 has been reported to abolish such IFN-β promoter activation (Schroder et al., 2008) as K7 protein binds to N-terminus of DDX3 required for IFN-β promoter activation. Recent report demonstrating interaction of BAdV-3 pVIII with DDX3 (Ayalew et al., 2016) led to our speculation that such interaction should also inhibit IFN-β promoter activation by suppressing the activation of IRFs mediated by TBK1\IKKε. However, pVIII did not lead to the suppression of activation of IFN-β transcriptional factors including NF-kB, IRF7 or IRF3. It is possible that the motif of DDX3 involved in binding to BAdV-3 pVIII is required for the activation of IFN-β promoter activation (Ayalew et al., 2016). Alternatively, it is possible that pVIII may block the IFN gene induction at a subsequent step, downstream of IFN transcription factor activation. For example, it may interfere with the DNA binding activity of IRF3 (Lin et al., 2001). Future studies should explore the possibility if pV and pVIII can interfere with the DNA binding activity of IRF3 by assessing the function of CBP/p300 transcriptional co-activating proteins, as well as the possibility of involvement of DNA sensors such as DDX41 and cGAS in the regulation of IFN response by pV and pVIII.

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