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Adenovirus Death Protein: The Switch Between Lytic and Persistent Infections in Lymphocytes?

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ADENOVIRUS DEATH PROTEIN: THE SWITCH BETWEEN LYTIC AND PERSISTENT INFECTIONS IN LYMPHOCYTES?

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ABSTRACT

Adenovirus Death Protein (ADP) expression during late stages of a lytic infection releases mature virions to promote viral spread, thus leading to death of the host cell. We sought to investigate ADP expression patterns in persistently infected human lymphocytes cells. We hypothesized that low expression of ADP allows the virus to persist while high expression would promote lytic infection in lymphocytes. Accordingly, we found ADP expressed in low amount in BJAB and KE37 cells, while lytically infected Jurkat cells demonstrated higher ADP expression in both protein and transcript levels. ADP overexpression in persistently infected lymphocytes did not alter the viability of these cells, or their level of ADP expression. In contrast, Jurkat cells infected with an ADP-deleted virus had increased survival and maintained viral DNA for greater than 1-month, suggesting conversion to a persistent infection. Also manipulating ADP expression had minimal impact on the total virus yield from infected lymphocytes.

INDEX WORDS: Adenovirus, ADP, Persistent infection, Lymphocytes

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DEDICATION

To my late friend Venkatramanan, who succumbed to bone cancer on September 11, 2005 at a tender age of 18. You have always been my source of inspiration and will continue to motivate me until my last breath. Wherever you are in this Universe I wanted to let you know I miss you so much, wish you were here with me.

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CHAPTER 1: INTRODUCTION

1.1 Adenovirus Species C

Human species C Adenoviruses (Ad 1, 2, 5 and 6) are commonly associated with mild upper respiratory tract and gastrointestinal infections (acute infection) in children under 5 yrs of age and immunocompromised individuals (Avila et al., 1989). Most studies characterizing the virus life cycle have been evaluated and described in epithelial cells. Infection of epithelial cells results in a lytic infection with release of newly synthesized virions, and death of infected cells within 72h (Hall et al., 1998).

Following primary infection, Species C adenoviruses are known to enter a persistent stage during which live virus can be intermittently detected in stool after virus detection in the nasopharyngeal cavity is lost (Fox et al 1969 and Fox et all 1977).

Adenoviruses were first isolated and characterized by scientists exploring the etiological agent for acute respiratory infections (Rowe et al., 1953). In these studies investigators discovered a spontaneous degeneration of adenoid tissues, which they speculated was caused by viral replication in the adenoid tissues. A year later, Hilleman and his group isolated a similar agent amongst military recruits (Hilleman and Werner, 1954). Since, these agents were isolated from adenoid tissues, they were referred to as adenoid degeneration factors or acute respiratory disease agents only to be rechristened as Adenovirus (Ad) later (Enders et al., 1956). Human Ad is now divided into 6 species, A-F. Presently, there are more than 100 members in the adenovirus family, which infect a wide range of vertebrate hosts (Norrby et al., 1976). Each member, or serotype, is defined based on the neutralization capability with specific antisera. The different serotypes can cause a wide variety of common and sporadic infections.

Adenoviruses are non-enveloped, icosahedral shaped with a diameter of 70-90 nm (**Figure 1A**)(Athappilly et al., 1994; Bewley et al., 1999; Durmort et al., 2001; Rux and Burnett, 2000, 2004) Their make-up is comprised of DNA (13%) and protein (87%). The outer core of the virus is made of capsid proteins. The capsids are built of 252 smaller subunits called capsomeres (Ginsberg et al., 1966). The capsomeres are comprised of 240 hexons and 12 pentons, forming the vertex of the icosahedron. As the name suggests, 6 and 5 neighbors surround the hexons and pentons respectively **(Figure 1A, 1D, 1E).** Ad also encodes a fiber protein, which interacts with cellular receptors to gain entry.

The Ad subgroup C has four distinct serotypes- Ad1, Ad2, Ad5 and Ad6, and the vast majority of studies on adenoviruses have been carried out using Ad2 and Ad5. Species C causes upper respiratory infections predominantly in infants and young children (Krilov, 2005). An acute infection might take 6-7 days to resolve, however, Ad is also known to be persistent with recurrent and sporadic shedding of the virus from feces observed for years following resolution of the acute infection (Fox et al., 1969).

1.2 Gene Organization and Expression Patterns

All known adenovirus genomes have the same genetic organization, i.e., all viral genes encoding specific functions are positioned at the same location on the viral genome (Larsson et al., 1986). The 35kbp (kilo-base pair) adenovirus genome is a linear, double stranded DNA molecule with inverted repeat sequences that play a crucial role in the viral DNA replication (Shenk, 1996). The adenoviral genome can be divided into the immediate early genes (E1A), early genes (E1B, E2, E3 and E4) and the late genes (L). Each region is transcribed to yield multiple mRNAs through differential splicing and polyadenylation (Shenk 2001). An unique feature of the Ad genome is the orientation of the genes. Certain genes such as the E2A DNA binding protein (DBP), and E4 run in the reverse direction when compared to the trans-

activating genes E1A and E1B **(Figure 2).** These genes work together in a coordinated fashion to setup a productive lytic infection. In addition to a variety of specialized genes Ads also possess cis-packaging elements within a few hundred base pairs from the end of the chromosome, which facilitate the interaction of viral DNA with some encapsidating proteins (Grable and Hearing, 1992; Hammarskjold and Winberg, 1980; Hearing and Shenk, 1983). The expression patterns of early genes (E1A, E2A, E1B55k and E4) along with the E3 11.6kDaAdenovirus Death Protein (ADP) are of particular interest to our study.

The first viral gene to be transcribed is E1A (34-48kDa) that is processed by differential splicing (Nevins et al., 1979). This leads to 5 distinct messages 13S, 12S, 11S, 10S and 9S (Stephens and Harlow, 1987; Ulfendahl et al., 1987). 13S and 12S variants are found in abundance during the early stage of infection, whereas 9S is observed at a later stage (Stephens and Harlow, 1987; Ulfendahl et al., 1987). E1A genes also encode products that play a major role in the transcriptional regulation of several viral and cellular genes(Nevins, 1995; Shenk and Flint, 1991). The multifunctioning E1A protein plays a role in transcriptional activation of other early genes, transcriptional repression of cellular proteins, and initiation of DNA synthesis. E1A has the ability to immortalize primary cells in collaboration with E1B (Bayley and Mymryk, 1994). In the nucleus, the immediate early genes are expressed first (E1A) and they induce the delayed transcription of other early genes such as E2A, E1B, E3, &E4 (Thimmappaya et al., 1982).

The products of the E1B region of the Ad genome are involved in a variety of functions such as viral DNA replication, host cell transformation, viral and cellular protein interactions and also inhibit apoptosis induced death of infected cells by inactivating the p53 tumor suppressor gene(Boyer and Ketner, 2000; Cathomen and Weitzman, 2000; Querido et al., 1997). The E1B products are also know to counter the cellular responses such as blocking transcriptional

activation of p53 in the absence of E4orf6 and induction of apoptosis by Cytotoxic T lymphocytes and macrophages (Teodoro and Branton, 1997b). The 19kDa product of E1B is a functional homolog of anti-apoptotic cellular protein family Bcl-2 that inhibits apoptosis and premature death of infected cells that would hamper virus production (Cuconati and White, 2002). This product counter balances the effect of E1A, which induces apoptosis in infected cells. E1B55kDa cooperates with E1A and E4orf6/7 in the process of cellular transformation; the E1B55kDa-E4orf6 complex also degrades cellular proteins associated with DNA damage repair complex and hampers the DNA repair machinery (Teodoro and Branton, 1997b; White et al., 1992; Yew et al., 1994).

The E2 gene has two distinct regions E2A and E2B (de Jong et al., 2003). The E2 gene runs in the reverse orientation and the various E2 products are made as a result of differential splicing. The E2 proteins play a role in initiation of viral DNA replication and ensure the transcription of several late proteins by the activation of the late E2 promoter (Baker and Ziff, 1981; Bhat et al., 1987). The E2A gene codes for a 72kDa DNA binding protein (DBP). This protein is critical for viral reproduction in a host cell, since it aids the virus in taking the first step in successfully propagating itself in host cells by replicating the viral genome. The E2B region encodes a 140kDa polymerase needed for DNA replication. The E2 region also encodes a precursor terminal protein, which serves as the primer from which the polymerase enzyme extends (de Jong et al., 2003).

Ad2 and Ad5 encode 6 different proteins from the E3 transcription unit. Most products of the E3 region of adenovirus are specific for immune evasion (Horwitz, 2001). They are categorized based on their size as 6.7kDa (Wilson-Rawls et al., 1990), gp19kDa (Persson et al., 1980), 11.6kDa (Wold et al., 1984), 10.4kDa (Teodoro and Branton, 1997a), 14.5kDa (Tollefson et al., 1990) and 14.7kDa (Tollefson and Wold, 1988). The function of the 6.7kDa protein is not known however the other E3 products affect the proper functioning of cell surface receptors,

and a variety of proinflammatory signaling cascades. For example, E310.4kDa and E314.5kDa complex, referred to as receptor internalization and degradation complex (RID), protects the virus-infected cells from death by apoptosis by down regulating death receptors such as Fas/CD95, and tumor necrosis receptor apoptosis inducing ligand(TRAIL)(Tollefson et al., 1991). The E3 gp19K membrane proteins target systematic down regulation of MHC molecules from the cell surface thereby countering recognition by immune surveillance system. The E3/10.4-14.5K product however, down-regulate apoptosis receptors by rerouting them into lysosomes. Interestingly, the 11.6kDa product of E3 transcription unit is synthesized in small quantities during the early stage of infection, but expressed in high amounts at a later stage from a major late promoter (MLP)(Zou et al., 2004). In contrast to the immune evading activities of the other E3 genes, Tollefson et al., investigated the release mechanism of newly synthesized virions after a productive adenovirus infection (Tollefson et al., 1996) and proposed that the 11.6K protein encoded by the E3 region (Wold et al., 1984) aids in the release of newly synthesized virions (Doronin et al., 2003; Tollefson et al., 1996). Expression of this glycoprotein increased the rate of cell death and is now referred to as Adenovirus Death Protein (ADP-E3 11.6kDa). ADP is an integral membrane glycoprotein with complex O and N-linked oligosaccharides (Scaria et al., 1992). During the final stages of infection, ADP abrogates the activity of apoptosis inhibiting proteins, such as Bcl2 and E1B19k (Boyd et al., 1994). The exact mechanism involved in the induction of cell death of infected cells is not clearly understood. A study by Zou et al., suggests both caspase dependent and independent mechanisms of cell killing when ADP is overexpressed(Zou et al., 2004). Several viruses that are known to persistently infect lymphocytes transcribe products that help in efficiently releasing newly synthesized virions during late stage of infection. Such proteins also act as a switch that turns on or off latency depending on cellular conditions. Epstein - Barr virus, which, like Adenovirus, establishes persistent infection of B-lymphocytes, encodes a gene BZLF1 (Wen et al., 2007).

This protein is expressed as an immediate early gene and functions to similar to ADP by aiding cell-to-cell spread of the virus. Similarly, Kaposi sarcoma associated Herpes virus encodes orf50, which acts as a switch to turn on or off viral latency and aid in efficient cell-to-cell spread of the virus. Expression of Rta/Orf50 is required and sufficient to reactivate the Kaposi Sarcoma associated herpes viruses from latency (Damania et al., 2004). ADP expression by adenovirus differs from the above-mentioned genes in the timing of gene expression. Even though ADP is predominantly an early gene, it is not expressed in abundance until final stages of infection. As it is widely known to aid the release of newly synthesized virions from an infected cell in a manner similar to the proteins that have been characterized to facilitate the latency shift in other persistent viruses, we were interested in determining if ADP has a similar biological role in adenoviral persistence. .

The E4 region encodes multiple proteins through different open reading frames (orf) with varying functions. The E4 orf3 (11kDa) and orf6 (34kDa) are critical for DNA replication (Bridge and Ketner, 1989; Huang and Hearing, 1989). These products play a role in the efficient initiation of viral DNA replication, late Ad mRNA splicing and inhibition of viral DNA concatemer formation. The E4 product also associates with E1B55kDa protein to shut down cellular protein synthesis and down regulation of p53 tumor suppressor genes(Dobbelstein et al., 1997; Hobom and Dobbelstein, 2004). The highly conserved orf3 region and orf6 of E4 inhibits the double stranded break repair (DSBR) mechanisms by binding and inhibiting the activity of DNA protein kinase (Boyer et al., 1999). This mechanism of DSBR inhibition is independent of the E1B55k protein. The E4 region is also known to compensate for the loss of E1A functionality by promoting the expression of key proteins such as the E2A-DBP(O'Connor and Hearing, 2000). E4orf6/7 induces the binding of cellular transcription factor on the E2A promoter region driving

the E2A transcription.E4 products also govern the enhanced expression of late genes (Bridge and Ketner, 1989).

After the DNA replication process has been initiated following early gene expression, the late phase of Ad infection begins. The proteins synthesized during the late stage are as a result of translation of late mRNAs. In order to complete the lytic cycle, the L4 100kDa non-structural protein expressed during this phase oversees to the inhibition of cellular protein synthesis and selective translation from tripartite leader (TL)-containing viral late mRNAs like Adenovirus Death Protein. In addition, L4-100K has been implicated in the efficient production of virions. During this phase the expression of early genes is greatly reduced. Protein synthesis during the late stage of infection is dominated by a very high expression of structural capsid proteins including hexon and fiber. As a result, Hexon expression is also an indicator of a productive lytic infection. Several non-structural proteins are also synthesized during this phase like the delayed early region E3-ADP, which is expressed in high amounts late in infection. Late proteins such as L52/55kDa are essential for the assembly of new virions and packaging of DNA into them(Wohl and Hearing, 2008). These proteins are localized in the nucleus distinct from viral DNA replication centers. (Wohl and Hearing, 2008)

1.3 Lytic infection of Epithelial Cells

Ad efficiently infects lung epithelial cells, such as A549 cells, and these cells serve as an ideal model to study the lytic infection cycle. A549 cells thoroughly support lytic infection and die 24-48hr-post infection (hpi). Within 24h post-infection, they express several key viral proteins. High expression of hexon by 18h post infection is an indicator of successful infection.

1.4 Adenovirus interaction with Human Lymphocytes

Adenoviruses were first isolated from adenoid and tonsil tissues in a non-infectious form that appeared to be a latent type of infection (van der Veen and Lambriex, 1973) and persistent viral infections have been observed up to 24 months after primary infection. In 1973, Van der Ween and his group were the first to suggested that lymphocytes in tonsils and adenoids might be naturally infected with Ad (van der Veen and Lambriex, 1973). Ad was isolated in 62% of the tonsil and adenoid specimens after periods of cultivation that was not detected at the initiation of the cultures. Interestingly, the serotypes that were isolated were Species C Ad1, Ad2, Ad5 and Ad6. The possibility of lymphocytes harboring the virus during latency was further investigated after traces of Ad DNA were detected in peripheral lymphocytes (Flomenberg et al., 1996). Ad DNA was detected even in the absence of significant viral production (Garnett et al., 2002; van der Veen and Lambriex, 1973). The existence of latent adenovirus in tonsillar tissues was not experimentally confirmed until 2002 when a significant amount of adenoviral DNA was reported in tonsillar T-lymphocytes in 79% of patient sample tested (Garnett et al., 2002). However, no infectious virus was observed, even after several passages of the cells suggesting true viral latency (Garnett et al., 2009). Furthermore viral transcription was also absent unless the lymphocytes were stimulated with lymphocyte activation agents (Garnett 2009).

Since these initial reports of latent adenovirus in lymphocytes, investigation into the dynamics of virally infected lymphocytes have been further evaluated in experimental models of infection (Zhang et al., 2010). These studies have demonstrated that while the infection of some lymphocytes (Jurkat) is lytic, Ad DNA persists in other lymphocyte cell lines (BJAB, KE37 and Ramos) for as long as 365 days after infection **(Figure 3A-C)**(Zhang et al., 2010). The Ad DNA was shown to remain episomal inside the lymphocytes and not integrate with the host cell

genome. Furthermore, infection of persistent cells does not result in the cell death observed in infected epithelial cells or Jurkat cells **(Figure 4)**.

In sharp contrast to the Ad gene expression patterns in A549 cells, the time course of protein expression is delayed in the lymphocyte cell lines. McNees et al., (2004) studied the kinetics of adenovirus protein expression in A549 cells and observed DBP and E1A expression as early as 6hpi and the late proteins detected at 12hpi. 90% of infected A549 cells expressed viral proteins 24hpi (McNees et al., 2004) (**Figure 5**). Adenovirus has also been shown to lytically infect Jurkat cell line derived from an individual with T cell leukemia (McNees et al., 2004; Zhang et al., 2010). The gene expression in Jurkat cells is very similar to that seen in A549 cells, except that the gene expression is delayed taking one day for early gene expression and two days for late gene expression to be observed in this lymphocyte cell line (**Figure 5B**).

McNees et al., 2004, comparing the protein expression patterns in A549 and T-lymphocyte cell lines and observed peak protein expression 72hpi with 80-90% cells showing positive results for viral protein expression. Of the several viral proteins observed, the E1A and DBP proteins are expressed as early as 6 hrs post infection. However during the late stage of infection (36hrs p.i.) in A549 cells the DBP appears to be expressed in abundance in comparison with the transactivating E1A **(Figure 5)**. In contrast the lymphocyte cells express the viral proteins several days later into infection highlighting a clear differential gene regulation pattern between the different cell types.**(Figure 5)**(McNees et al., 2004).

1.5 Importance of the study

Adenovirus is popularly used in gene therapies to treat other diseases and even some cancers (Choi et al., 2012; Yang et al., 2012). Now, that it has been shown that adenoviruses

can infect human lymphocytes and remain latent within them, there is a need to revisit and further understand this virus's interaction with the host, and in particular to fully characterize its latency in lymphocytes. Furthermore, a viral agent has long been suspected to play a role in the initiation of some types of cancer such as leukemia. However, no viral genes or gene products were detected from the leukemia cancer cells to support the hypothesis. Given adenoviruses unique interaction with lymphocytes, the ability of the E1A proteins to transform cells and the fact that the virus encodes proteins such as E4orf 6 that inhibit cellular DNA repair pathway perhaps it is time to revisit the old question of whether adenovirus are responsible for some leukemia's. Hence, it is becoming increasingly more important to fully understand the dynamics of gene expression of adenovirus in lymphocytes.

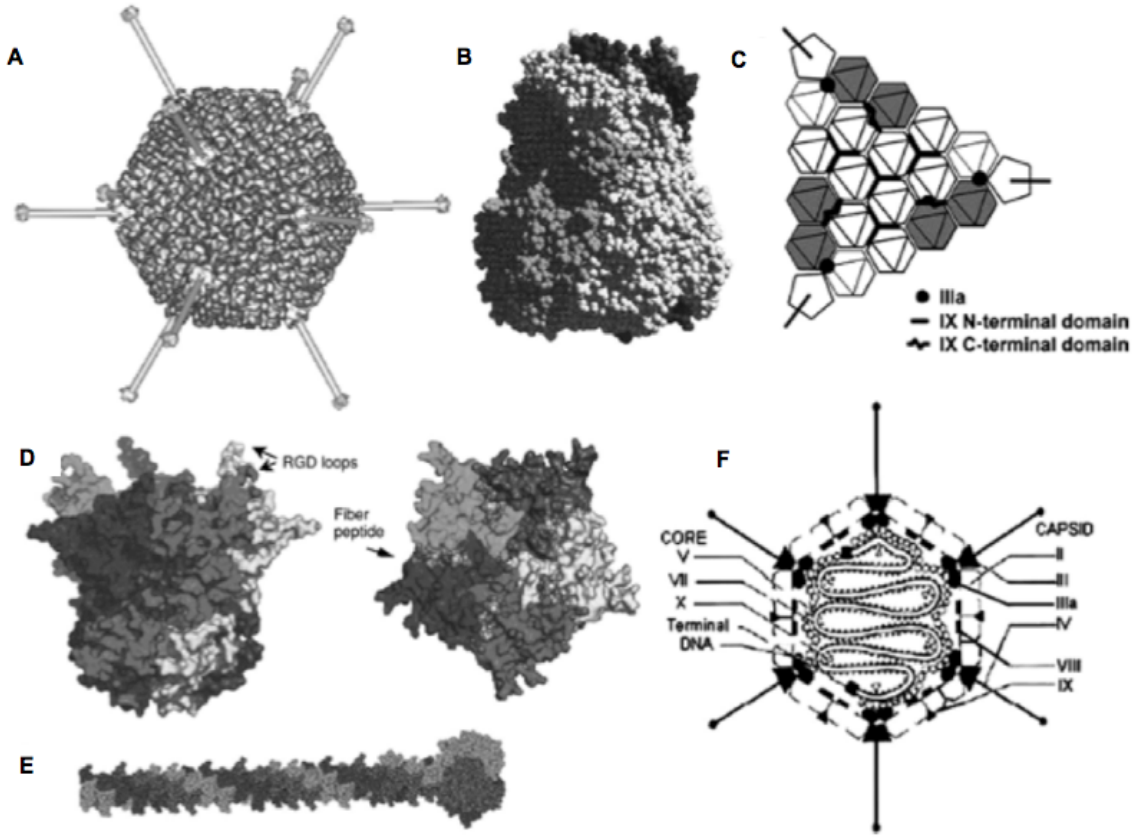

Figure 1 Structure of the adenovirus virion

A. Adenovirus model virion from computer reconstructed cry0-electron microscopy images. **B-E.** Space filling model of Ad5 hexon trimer, penton base & Ad2 fiber shaft. **F.** Current model of adenovirus virion. Picture courtesy: Fields Virology, 5th Edition. Copyright 2007. Lippincott Williams and Wilkins.

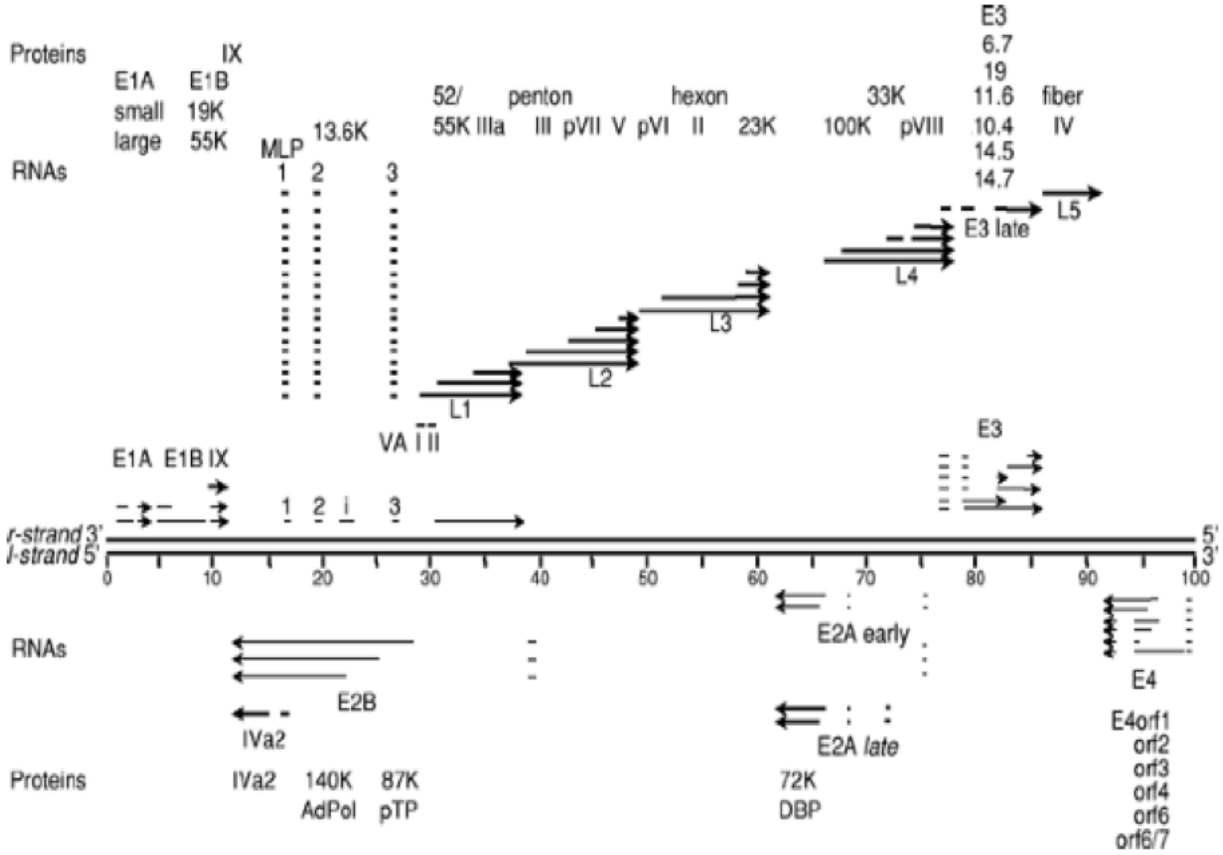

Figure 2 The gene organization in species C Adenovirus.

The genes are arranged in the order of immediate early genes, early genes and late genes. The orientation of the Ad5 genes is bidirectional. Picture Courtesy: Fields Virology 5th Edition. Copyright 2007. Lippincott Williams and Wilkins.

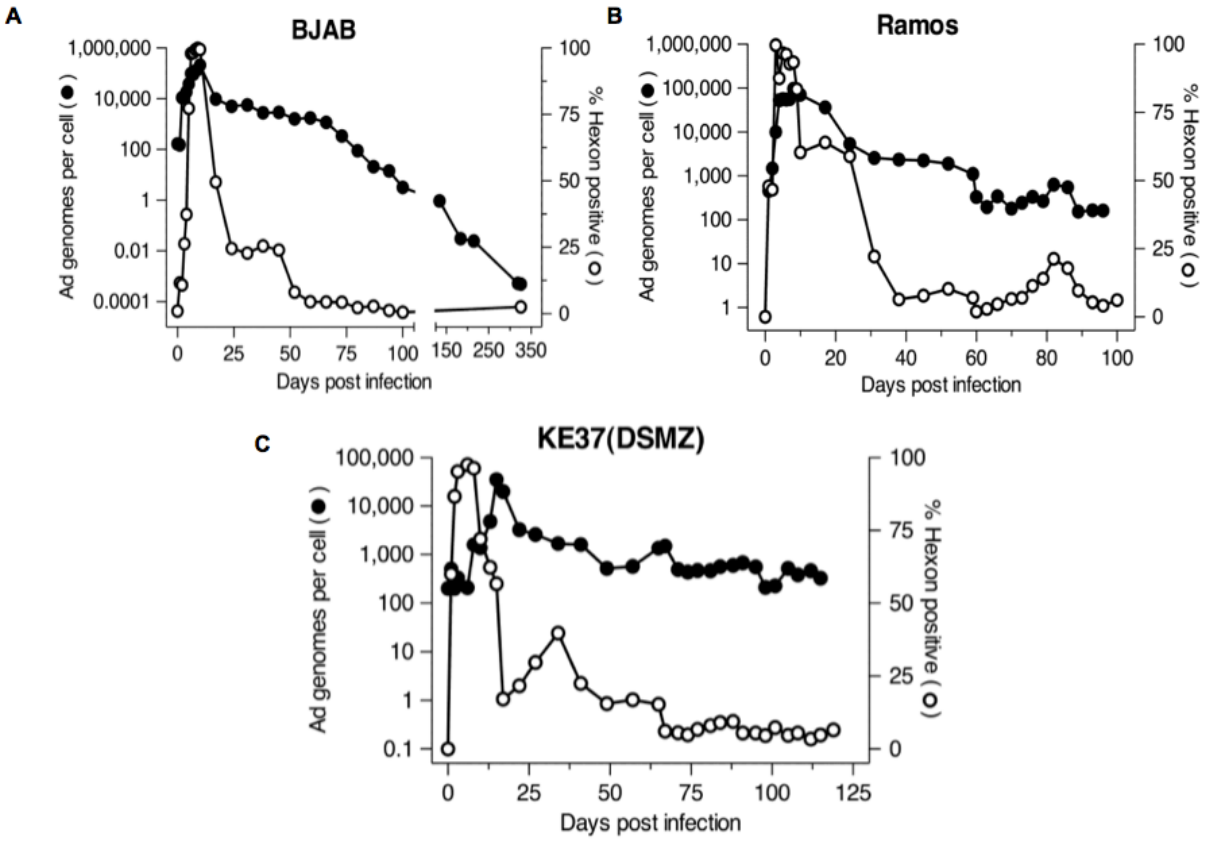

Figure 3 The long-term maintenance of Ad genome in lymphocytes.

A & B. B lymphocytes (BJAB and Ramos) support a productive infection at early stages of infection as indicated by high levels of hexon expression as early as day 7. **C**. T-cell line KE37 has a robust infection during early stages of infection before the virus goes latent. A large amount of Ad genome is maintained throughout the course of infection. The genome levels were detected by QPCR for hexon DNA and hexon protein levels determined by Flow cytometry (Zhang et al., 2010).

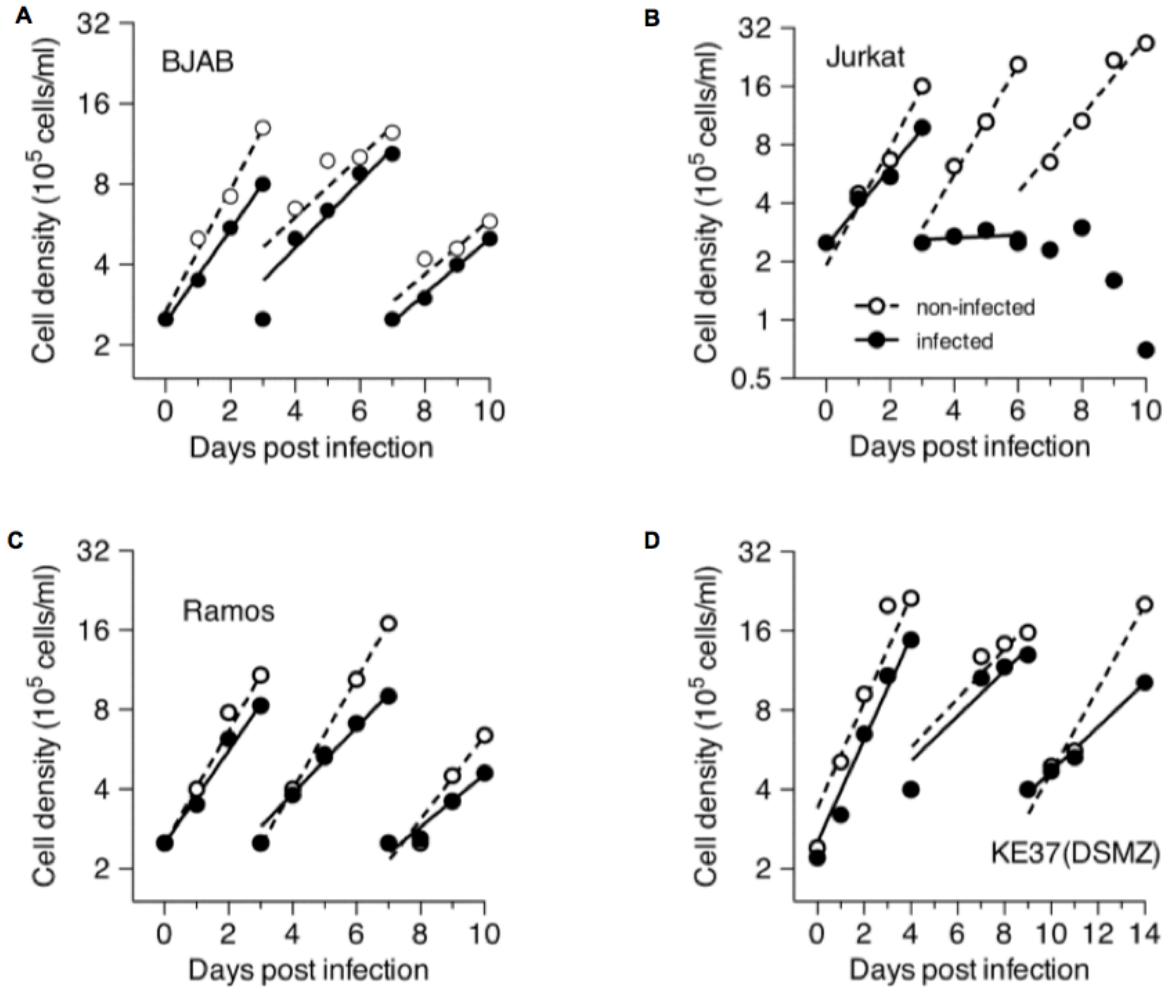

Figure 4 Viability of Lymphocytes infected with wild type Ad5 dl309 virus at different time points post infection.

A, C & D: Viability of persistently infected lymphocytes BJAB, Ramos and KE37 was determined by Trypan Blue Exclusion Assay and compared with mock-infected cells during the early stages of infection. Dark circles correspond to cells infected with dl309 and open circles correspond to uninfected cells. **B.** Comparing the viability of T-cell Jurkat infected with dl309 and those that are uninfected.

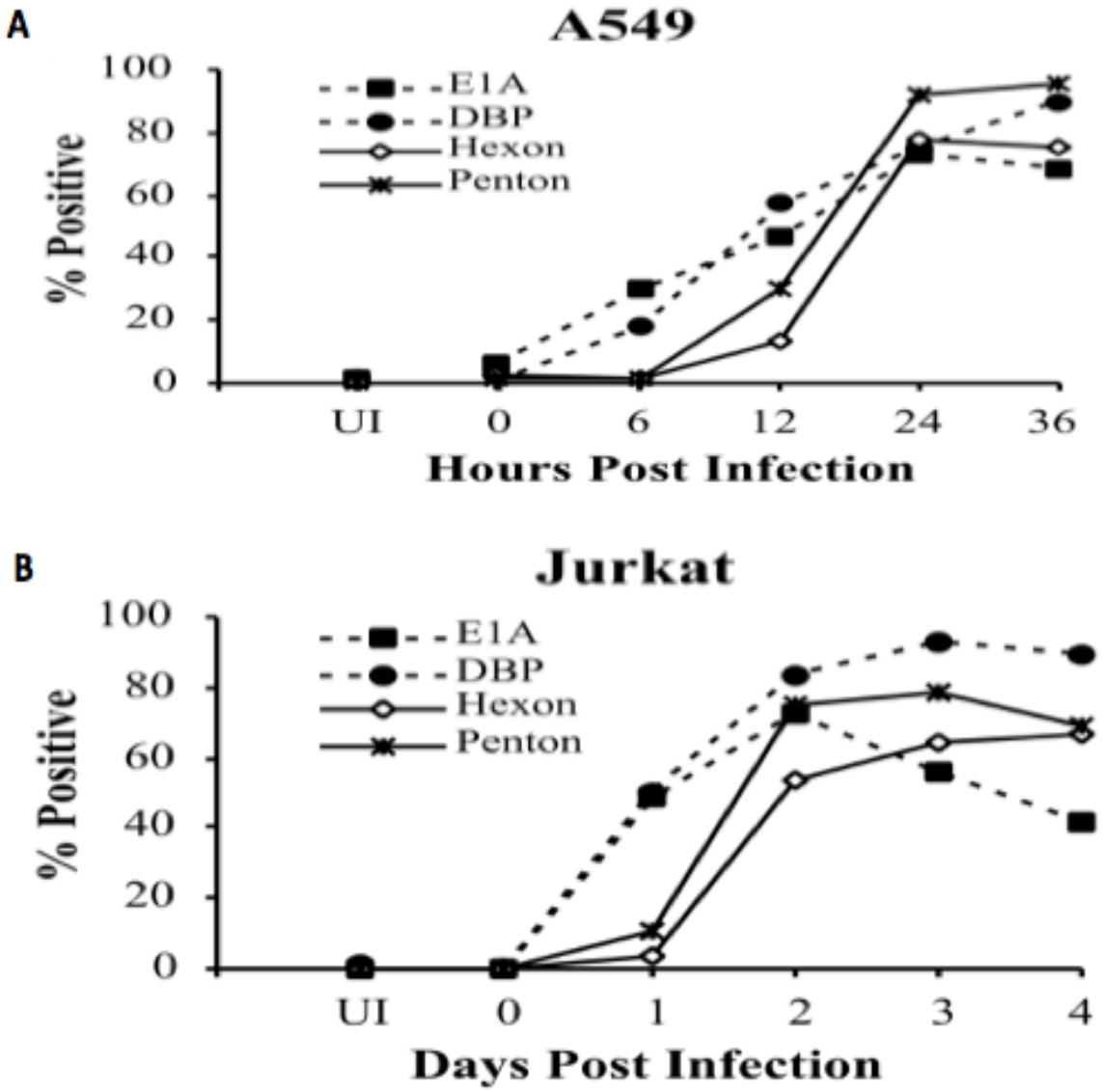

Figure 5 Gene expression in lytically infected cell types

A. Early gene expression in lytically infected lung epithelial cells (A549). Almost all of the critical genes are expressed within 24hrs into infection. B. Early gene expression in a lytically infected T lymphocyte (Jurkat cells). The expression patter is similar to A549 but it is delayed. The data was acquired by flow cytometry (McNees et al., 2004).

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines

Cell culture media, supplements and fetal bovine sera were obtained from Mediatech, Inc. (Manassas, VA). The lung carcinoma epithelial cells, A549 (ATCC CCL-185; American Type Culture Collection, Manassas, VA) were maintained as confluent monolayer cultures in Dulbecco-modified Eagle's minimal essential medium (DMEM) supplemented with 10% Fetal Bovine Serum and 10mM Glutamine and incubated at 37C in a humidified atmosphere containing 8% CO_2. The Burkitts Lymphoma B-cell line (BJAB), and Jurkat T-cell lines were also obtained from ATCC and maintained as suspension cultures in Roswell Park Memorial Institute 1640 medium (RPMI) supplemented with 10% Fetal Bovine Serum and 10mM Glutamine. The KE37 cell line (immature T-cell ALL) was purchased from the German Collection of Microorganisms and Cell Cultures. These cells were maintained by incubating at 37 degrees C in a humidified atmosphere containing 5% CO_2.

2.2 Viruses

The wild-type virus used in this study is the adenovirus type 5 strain wt300, which has all of its genes intact, was kindly provided by Ann E. Tollefson (St. Louis University). dl309 (kindly provided by David A. Ornelles, Wake Forest University), a phenotypically wild-type virus, lacks a portion of E3 region (10.4K, 14.5K and 14.7K) that has been shown to be dispensable for growth in tissue culture (Jones and Shenk, 1979). The ADP mutant virus pm534 was constructed by introducing two nonsense mutation on methionine codons and expresses no ADP protein (Doronin et al., 2003). The VRX-021 virus which over-expresses ADP, was constructed by using a shuttle plasmid encoding a version of the E3 region that is a hybrid between VRX-006 and VRX-007 as previously described by (Tollefson et al., 2007). Ann E.

Tollefson, St. Louis University, kindly provided these viruses.. Virus infectivity was determined for each of the cell lines (A549, BJAB, Jurkat and KE37) used in this study prior to infection. Epithelial cells were plated 24h before infection and then infected with 10 PFU per cell in serum-free media. After 2 hrs, complete media was added to the cells. For adenovirus infections of lymphocytes, cells were seeded at least 24 hrs before infection at a density of $1x10^6$ cells/ml. Cells were infected with 100 plaque-forming units (pfu) per cell and incubated for 2h at 37 degrees C. The non-absorbed virus was then washed from the infected cells twice with complete medium. The cells were incubated at 37 C for two to three hrs.

2.3 RT-PCR analysis of ADP expression

RNA was isolated and purified from the infected cells using RNA miniprep kits by Qiagen (Cat No.74104). 500ng of the isolated mRNAs were reverse transcribed using DyNAmo cDNA synthesis kit (Thermofisher) according to manufacturer's instructions using Bio-Rad Thermocycler. PCR of the cDNA was then performed to amplify the ADP transcript. For the qPCR, duplicate 25uL reaction mix was assembled containing the cDNA; 2X master mix (Thermofisher), 10mM each of forward and reverse primers and RNase free water. The primers designed for Ad5 ADP are placed on the 2nd and 3rd exon of the tripartite leader sequence and the 5' non-translated exon ADP 5' (TPL 2) GTACTCTTGGATCGGAAACCC (Ad5 genome 7147-7167), ADP3' (ADP non-translated exon) ACTCCGTCTGGGTTGAAAC (Ad 5 genome 28316-28298), ADP probe (TPL 3) TCGAGAAAGGCGTCTAACCAGTCAC (Ad 5 genome 9700-9724**).** CD44 or E1F1 mRNA was amplified to serve as an endogenous control for RNA input. The primers and probe used for CD44 are 5'-TTCAAATCTTCCACCAAACCT-3', 5'-TCTTCAACCCAATCTCACACC-3'and5'-/56-FAM/ACGCTTCAG/ZEN/CCTACTGCAAATCCA/3IABkFQ/-3'. The primers and probe used for E1F1 are 5'-/56-FAM/CTCCACTCT/ZEN/TTCGACCCCTTTGCT/3IABkFQ/-3', 5'-

GTATCGTATGTCCGCTATCCAG-3', 5'-GATATAATCCTCAGTGCCAGCA-3'. The threshold cycle (C_T) values were calculated for each reaction using the ΔΔCT method for comparing relative values of gene expression(Livak and Schmittgen, 2001).

2.4 Intracellular Staining and Flow Cytometry

Infected cells were harvested at different times post infections and washed with ice cold Phosphate Buffered Saline (PBS). The cells were then fixed in the dark with 1% Para formaldehyde obtained from Affymetrix Inc. (Cat. No. 19943 1 LT) and incubated at room temperature (RT) for 30 mins. The fixed cells were then resuspended in 0.2% Tween20 (Permeabilize buffer) at a density of 10^6 cells/ml for 15 mins at RT. This was followed by centrifuging at 1500 rpm for 5 mins at 20 degrees C. The cells were then incubated at RT for 30 mins with Blocking Buffer (PBS/2%BSA/2000ug/ml Rabbit IgG). The cells were centrifuged and resuspended in cell staining buffer obtained from BioLegend (Cat.No.420201). The antibodies specific to adenovirus hexon (Chemicon mAb 8051) was used at 1:100 dilution in cell staining buffer, monoclonal mAb to ADP was obtained from Ann E. Tollefson (St. Louis School of Medicine, Missouri) was used at 1:50 and an Isotype control (Purified Mouse IgG) was used at 1:25 (BD Pharmigen, Cat. No.557273) dilution in cell staining buffer. After incubation with the primary antibody, cells were staining with a fluorescently labeled secondary antibody. An APC labeled secondary antibody (rabbit anti mouse) was used at a dilution of 1:50. Cells were washed and resuspended in *600μL* of cell staining buffer and acquired by flow cytometry on a BD Fortessa using FACSdiva software.

2.5 Trypan Blue Exclusion Assay

Trypan Blue Exclusion Assay was used to measure cell death of A549, BJAB, Jurkat and KE37 cells as described in (Tollefson et al., 1996). 10uL of cells were diluted 1:1 with 0.4%

Trypan blue dye and cell death was determined. These experiments were conducted three times, with similar results. The data shown is representative of one such experiment.

2.6 Western Blotting and Antibodies

At each time point, protein lysates were collected using Radioimmunoprecipitation assay (RIPA) buffer in the presence of protease and phosphatase inhibitors (PPI). The collected lysates were then suspended in 2x Sample buffer (20% SDS, 0.5M Tris, P.H 6.8, glycerol, H2O, bromophenol blue, β-Mercaptoethanol). The adherent cells and suspension cells were washed with ice cold PBS and the former was treated with 0.1% Trypsin (incubated at RT for 2 mins) in order to disrupt the adherent monolayer. The cells were then washed with complete cell culture medium and PBS-PPI and resuspended in one-tenth volume of PBS and inhibitors. The cell concentration was adjusted to 100ul/1x10^6 cells. Protein lysates to measure the expression levels of E1A, E2A-DBP and ADP were boiled at 95C for 5 mins, whereas protein lysates to measure E4orf6/7 levels were heated at 65C for 20 mins. The E1A and E2A-DBP antibodies were used at 1:50 dilution; ADP at 1:400 with polyclonal anti-rabbit Ab kindly provided by Ann E. Tollefson, St. Louis University and E4orf6/7 was used at 1:20 in Tris Buffer Saline supplemented with 0.1%Tween.

Proteins from identical number of cells was resolved by SDS-PAGE and then transferred to 0.2microM nitrocellulose membrane. The transferred membrane was blocked with TBS-Tween supplemented with 5% non-fat dry milk obtained from Bio-Rad (Cat.No.170-6404). β-Actin was used as loading control used at a dilution of 1:5000. The horseradish peroxidase- conjugated secondary antibodies were used at 1:5000-1:10000. The band signals were detected following minimal incubation with SuperSignal chemiluminiscent substrate from Pierce.

2.7 Virus Yield

For assay of viral growth in the lymphocyte cell types ($3x10^6$ cells) were infected at 100PFU per cell in 500µl of serum-free RPMI 1640 1X. After 2 hrs of incubation at 37C, the cells were washed with RPMI 1640 (10%FBS), then the infected lymphocyte cells were cultured in 10ml of RPMI 1640 (10% FBS) at 37C. At a time indicated in the figures, 500µl of sterile PBS and 37.5µl of Tris-HCl (pH 7.8) was added to 1ml of cells and freeze thawed three times. The supernatant and pellets were assayed by plaque assay to determine the virus yield.

CHAPTER 3: RESULTS

Adenovirus is known to persistently infect lymphocytes with very different kinetics than the well-studied lytic infections. Our study aims to investigate the adenoviral gene expression patterns in persistently infected human lymphocytes. We analyzed the role of Adenoviral Death Protein (ADP) in maintaining persistent infections in lymphocytes. Furthermore, we also analyzed the difference in the expression of several well studied viral genes including transactivating E1A, DNA binding protein E2A and DNA repair inhibiting E4orf6/7 between lytically infected lung epithelial cells and persistently infected human lymphocyte cell lines.

3.1 ADP is down regulated in persistently infected lymphocytes

Human species C adenoviruses are known to establish a lytic infection in human lung epithelial cells that results in virion production and death of the cells 48-72h-post infection (Hall et al., 1998). In contrast, persistent infections with the same viruses have been demonstrated in several human lymphocyte cell lines, including BJAB, Ramos and KE37 (Zhang 2010). During such persistent infections, the lymphocytes exhibit continued cell proliferation and no subsequent overt cell death (Zhang 2010). Interestingly, substantial loss of cell viability occurred in Jurkat cells by 3 days post infection (Zhang 2010) **(Figure 4)**. Thus, infection of Jurkat cells appears to result in a lytic infection that is similar to infection of epithelial cells resulting in cell death. As ADP has been reported to facilitate the release of virus from infected cells resulting in cell lysis and death, we first compared the expression of ADP in persistently infected human lymphocytes (BJAB and KE37 cells), with expression of ADP in lytically infected cells (Jurkat and A549 cells). All cells were infected with wild-type Ad5 (dl309) and subsequently evaluated for expression of ADP by intracellular staining **(Figure 6; black bars)**. A higher fraction of lytically infected A549 cells expressed ADP when compared with the

persistently infected lymphocyte cell lines BJAB and KE37. While 82% of A549 cells infected with Ad5 dl309 expressed ADP 24hr post infection, only a small fraction of infected BJAB cells expressed ADP (<20%). Similarly, less than 15% of infected KE37 cells expressed ADP. Surprisingly, while ADP levels were low in both BJAB and KE37 cells, expression of the structural protein hexon was much higher **(Figure 6; gray bars).** Maximum expression of hexon protein observed in BJAB cells was 79% and 77% in KE37 cells. As expected, greater than 80% of infected A549 cells expressed hexon protein and this high level of expression was detected as early as 1 day after infection. Evaluation of Jurkat cells, which are lytically infected with the virus, revealed much higher levels of ADP when compared to KE37 or BJAB cells **(Figure 6).** Three days after infection, ADP protein could be detected in approximately 60% of Jurkat cells. High hexon protein expression was consistently detected in most of the Jurkat cells 3, 5 and 7 days post-infection, while ADP expression declined after day 3. However, Jurkat cells also begin to die as early as 3 days post infection **(Figure 7).** By day 3 post-infection 40% of cells are dead and 60% are dead on day 5. As a result, very few cells remain in culture for evaluation of protein expression at day 7 and beyond. Thus, in our studies, low expression of ADP appears to correlate with lymphocytic cells reported to establish long-term persistent infections with Adenovirus.

Given the difference in expression of ADP among the different lymphocyte cell lines we next wanted to investigate the levels of ADP mRNA transcripts in these cells. mRNA was isolated from persistently infected cell types BJAB and KE37on day 1, 3, 5 and 7-post infection with wild-type virus and the relative amounts of ADP transcripts were quantified from cDNA. mRNA for ADP were detected in persistently infected cell types **(Figure 8).** One-day post-infection BJAB cells contained the least amount of ADP mRNA. We normalized this level of expression, compared to endogenous housekeeping gene (E1F1) expression, to one and

compared relative changes in expression across the infected cell types. BJAB increased ADP transcript levels 130-fold-fold 3-days p.i., however there was no further increase in expression on day five or day seven. KE37 cells expressed higher relative levels of ADP mRNA one-day p.i. however level of expression decreased five and seven days p.i. In contrast, Jurkat cells expressed 5-logs more ADP mRNA than BJAB cells one-day p.i. This high level of expression was maintained the second day after infection **(Figure 8).** Jurkat cells were not evaluated beyond three-days post infection due to considerable amounts of cell death in the cultures. Overall, Jurkat cells, which expressed the highest amount of ADP protein among the lymphocyte cell lines, also expressed the highest amounts of ADP mRNA compared to the other two lymphocytic cells. These high levels of expression in Jurkat cells were detected as early as one-day post infection. Interestingly, though protein expression of ADP was low in both KE37 and BJAB cells, ADP mRNA was not completely absent from KE37 and BJAB cells.

3.2 Characterization of ADP mutant viruses

Our previous results suggested a potential link between ADP non-expression and long-term cell survival and probable viral persistence. We hypothesized that over expressing ADP in persistently infected lymphocytes could reverse the latent infection cycle and allow the virus to productively infect them. To test our hypothesis we utilized ADP mutant viruses, which either did not express ADP (pm534- deletion mutant virus) or overexpressed ADP (VRX021- ADP over expression virus) and compared these cells with cells infected with wild type dl309. To characterize the expression of ADP, we infected lung epithelial cells with 20PFU of wt, pm534 or VRX021 and determined the viability and ADP expression at 48hrs p.i. by acquiring them by flow cytometry **(Figure 9).** At 48h p.i. similar percent of A549 cells expressed hexon protein (94%). As expected cells no ADP protein (4.6 %) could be detected in the cells infected with the

ADP deletion mutant virus pm534 **(Figure 9)**. In contrast ADP expression was detected when epithelial cells were infected with wt ADP expressing virus (dl309) or over-expressing ADP virus (VRX021). Though hexon levels are comparable between these two infections (93% vs. 87%), there is more ADP expression in the cells infected with VRX021 (MFI of 230) when compared to ADP expression in cells infected with dl309 (MFI of 116). These data demonstrate that pm534 does not express ADP in infected cells, and that the VRX021 virus results in higher levels of ADP expression than that observed in cells infected with wt virus. Levels of ADP peak earlier than late hexon protein levels in VRX021 infected cells. ADP levels remain absent 48h p.i. in pm534 infected cells.

As ADP has been reported to be responsible for cell death of infected cells, we next evaluated viability of epithelial cells infected with the different ADP-expressing viruses. Pm534 infected cells had minimal cell death and contained similar percent of viable cells as mock infected cells 48h p.i. In contrast, less than 10% of the cells infected with VRX021 overexpressing virus were alive 48h p.i. As expected, 48h p.i. less than 30% of the epithelial cells infected with the wt virus remained viable **(Figure 7**).

3.3 ADP overexpression has no impact on the cell survival in persistently infected lymphocytes.

We next evaluated the effect of over expressing ADP in persistently infected cell types. BJAB, KE37 were infected with wt and vrx021 viruses and the viability of cells determined by Trypan Blue exclusion assay. Cell viability was determined on day 1, 3, 7 and 10 post infection. Both BJAB cells and KE37 cells exhibited minimal differences in the amount of cell death between cells infected with wt ADP expressing viruses and cells infected with ADP over-expressing viruses. Some limited amount of cell death was observed in KE37 cells infected with

both wt and overexpressing viruses at day 7 and 10 p.i. In KE37 cells infected with wt dl309 and vrx021 the percentage of viable cells was considerably higher (90% and92% respectively) on day 3-post infection before the fraction of viable cells fell considerably to 55%and 45% respectively on day 5 before recovering on day 7 and at later time points (data not shown). There was minimal effect on the cell viability of BJAB cells even when infected with ADP overexpression virus with high fraction of viable cells (90-95%) **(Figure 10 A)**. Interestingly infection with the ADP-deletion virus did not increase the percent viable fraction in either BJAB or KE37 cells.

3.4 ADP is differentially regulated in persistently infected cells

We saw no difference in cell death induced following infection of persistently infected lymphocytes with the over-expression virus so we next wanted to confirm if this was happening in spite of high expression of ADP in our cells. We were surprised to see ADP expression in BJAB and KE37 cells infected with a wt virus similar to ADP expression levels observed when infected with VRX021 virus (ADP over expression) **(Figure 10 B).** Again, the percentage of cells expressing ADP is much lower than the percentage of cells expressing hexon protein in both cells infected with wt or the ADP overexpressor. We also looked the ADP transcripts in lymphocytes infected with ADP overexpressing virus (VRX021) **(Figure 11)** to compare with transcript levels detected in cells infected with wt dl309 **(Figure 8).** Again, both BJAB and KE37 cells contained ADP mRNA; however, the levels remained logs below those observed in Jurkat cells infected with the same virus. For example, one-day p.i. Jurkat cells contained two and five logs more ADP mRNA than KE37 cells and BJAB cells, respectively.

3.5 Loss of ADP can convert lytically infected lymphocytes to a persistent infection

Jurkat cells were infected with the 3 viruses and cell viability was monitored 1, 3, 7 and 10 days p.i. As expected all Jurkat cells infected with wt or ADP overexpression viruses were dead by 10-day p.i. and both infections demonstrated higher expression of ADP protein than observed in either BJAB or KE37 cells **(Figure 12A&B).** In sharp contrast, the Jurkat cells infected with ADP deletion mutant (pm534) remained viable. The T cell line infected with pm534 (ADP deletion mutant) had a remarkably high percentage of viable cells (70% on day 10) and continued to live well past day ten (data not shown). Moreover, thirty-three days p.i. high levels of viral DNA were still detected in these cells (4.2×10^8 Ad genomes/ 10^7 cells) suggesting that the virus did indeed persist in these cells. Our findings suggest that in order for the virus to persistently infect host cells, ADP expression needs to be down regulated. Loss of ADP seems to have allowed the lytically infected cells to survive much longer than they are known to, and could potentially lead to viral persistence in them. This is interesting because Jurkat infected with Ad5 usually dies 6 to 7 days post infection due to abundant ADP expression in them.

3.6 ADP expression has minimal impact on the total virus yield

As the loss of ADP expression seems to correlate with persistent infections and lytically infected cells have higher expression of ADP we wanted to see if ADP expression had an impact on virus yield. To study the impact of ADP expression on the amount of virus produced, the three-lymphocyte cell types BJAB, KE37 and Jurkat cells were infected with 100PFU per cell of dl309, pm534 and VRX021. Cells and supernatant were harvested on day 3, 8 and 11 post infection and assayed for virus yield by plaque assays. Jurkat cells expectedly produced a higher titer of the viruses than the persistently infected KE37 cells at all times p.i., and higher than infected BJAB cells at 3 and 8 days p.i. Interestingly, BJAB cells infected with pm534 produced more viruses than BJAB cells infected with WT or VRX021 **(Figure 13).** We are

currently continuing this experiment by evaluating cell associated virus production versus virus in the supernatant.

3.7 Differential early gene expression patterns in persistently infected lymphocytes

In order to evaluate the difference in gene expression dynamics in lytic and latent infections, we evaluated the early gene expression patterns in BJABs. BJAB cells were infected with 100PFU per cell of dl309 and protein lysates were collected at different time post infection. We used western Blotting to detect the Ad5 early protein (E1A and E2A) levels during the early phase of lymphocyte (BJAB) infection and was compared with viral protein expression in lung epithelial cells (A549). The uninfected BJAB cells were used as the negative control and wt dl309 infected A549 cells were used as the positive control in this study. We examined the protein expression of E1A and E2A from one to seven days p.i. Our preliminary data shows that E1A is expressed beginning on day one post infection, but in very low amounts. The expression of E1A is however detected in higher levels as the infection proceeds into day seven **(Figure 14).**

From previous studies and based on our preliminary data, it appears as if the 13S isoform (48kDa) is expressed first and the 12S isoform begins to be detected from day seven post infection. It is also noted that the E1A is expressed in abundance from 7 to 14 days post infection (data not shown). In contrast to E1A expression, E2A-DBP expression is less robust than E1A, with increasing expression moving onto day seven **(Figure 14).** Similar to the trend observed in trans-activating E1A, the DNA binding protein E2A also shows increased expression pattern as the infection moves into its second week (data not shown). In comparison to E1A and E2A expression in the lymphocytes, these genes are expressed in high levels within 20hrs post infection in permissive lung epithelial cells **(Figure 5)** (McNees et al., 2004) indicating a delayed expression of Ad genes in a lymphocyte population.

3.8Transactivating E1A may not be required for the expression of DNA binding protein

In order to study the role of E1A in the expression of other early Ad genes, we evaluated the expression pattern of DNA binding E2A in the absence of E1A using western blotting analysis. In order to validate the effect of the mutant virus we performed western blotting analysis on mutant's infected A549 cells. Protein lysates were collected at 24 hrs and 48 hrs post infection. We then blotted for E1A and E2A. As expected there were no bands in lanes containing viral proteins, whereas those lanes containing lysates prepared from cells infected with wild type dl309 did contain bands at appropriate molecular weight. Surprisingly, we noticed E2A expression in the mutant lanes despite the absence of E1A **(Figure 15).** This prompted us to evaluate E1A less E2A expression in persistent cell types. The uninfected A549s were used as the negative control and the Ad5 infected A549s were used as the positive control. We examined the expression of E2A in the absence of E1A (by replacing E1A with early promoter of CMV virus) on day one to seven-p.i. As shown in **Figure 15**, despite the lack of E1A region, E2A is detected at day one and three p.i., however, the proteins levels drastically decreased on day five post infection and only traces of E2A were observed on day seven p.i. E2A expression in the absence of E1A raises the possibility of an alternate gene expression pattern in the adenovirus **(Figure 16).**

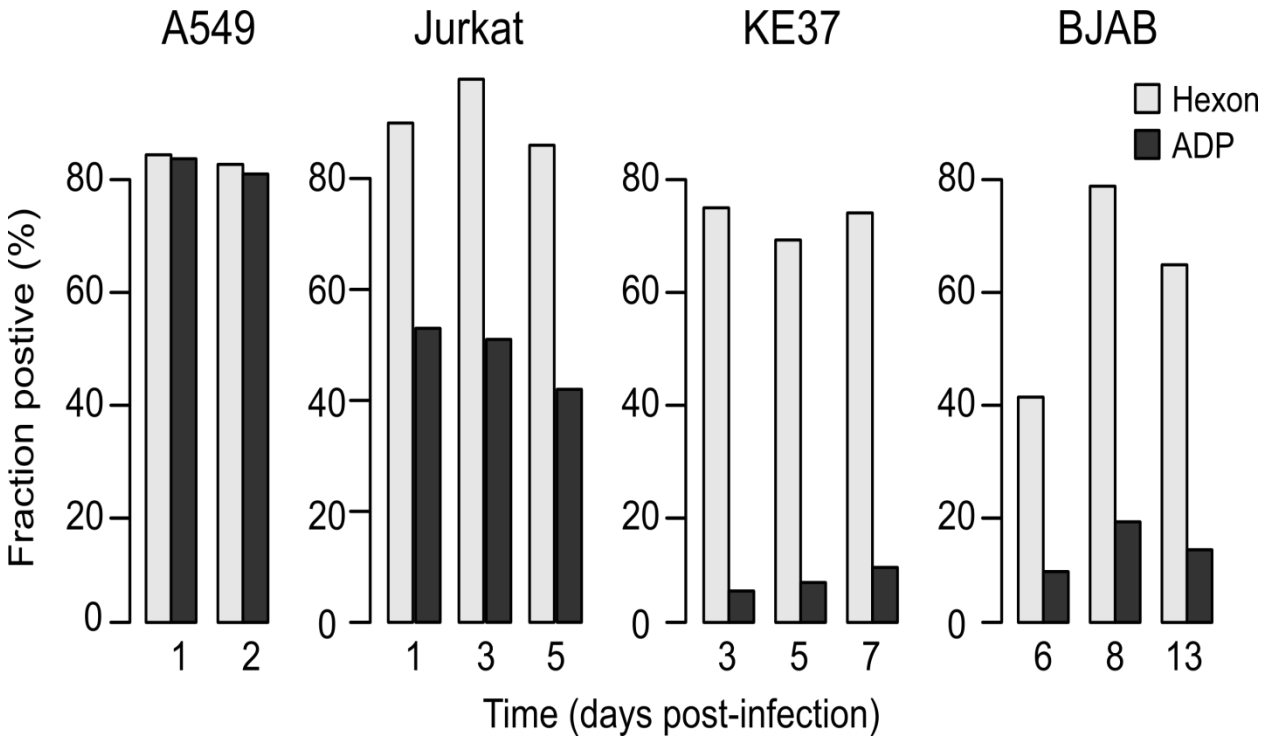

Figure 6 Expression of ADP and the hexon gene differs among persistently and lytically infected cell.

Flow Analysis of ADP vs. Hexon expression in different cell types. Monoclonal mAb specific for ADP was used at a dilution of 1:50 and intracellularly stained before acquiring the data in Flow Cytometry. Hexon is an indicator of a productive infection. Ab specific for hexon was used at a dilution of 1:100. FITC labeled secondary Ab was used at a dilution of 1:100. The data is representative of three identical repeat experiments.

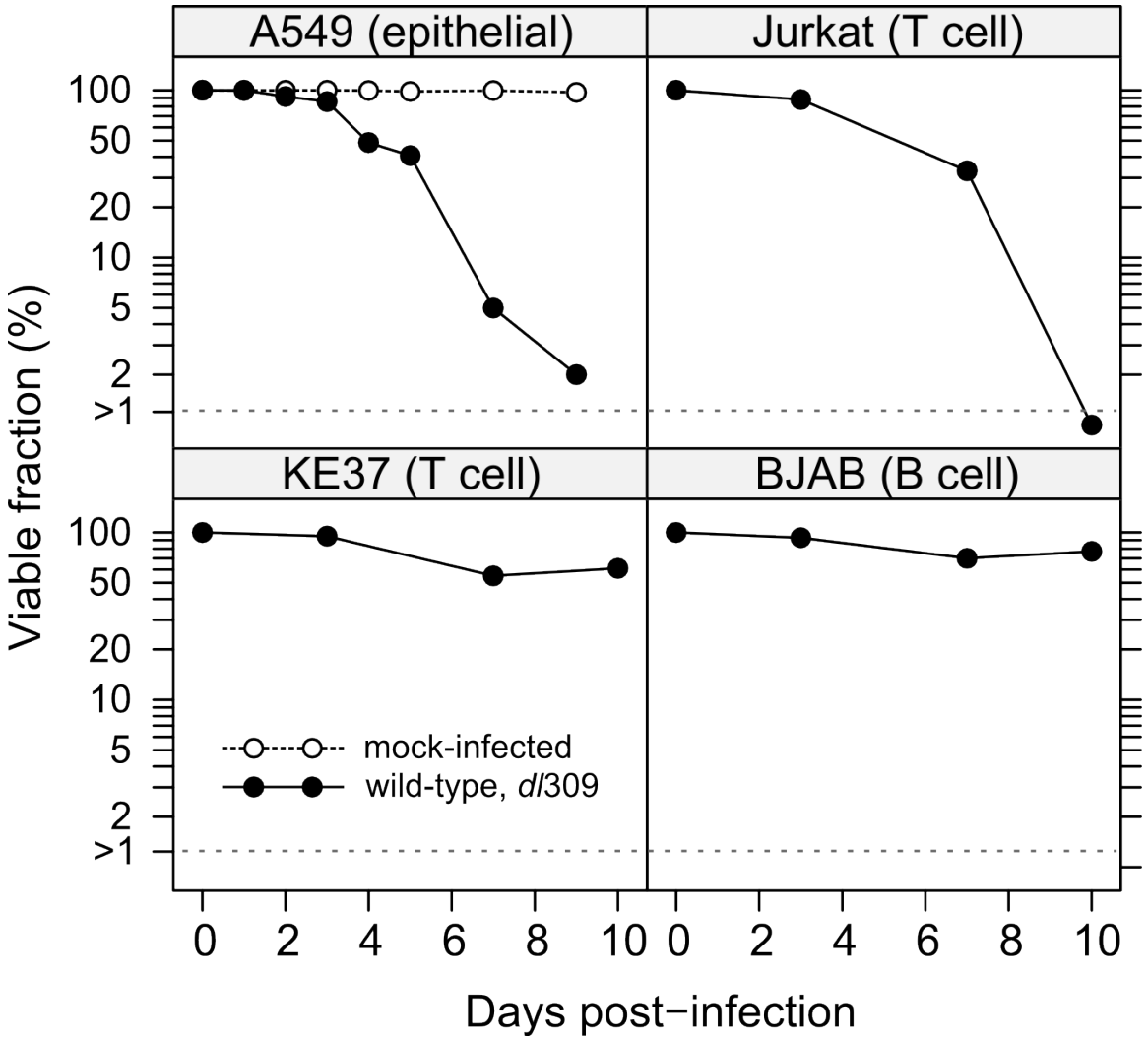

Figure 7 Viability of Lymphocytes infected with wild type Ad5 dl309 virus at different time points post infection compared with viability of lytically infected A549 cells.

Trypan Blue Exclusion Assay determined viability of persistently infected lymphocytes BJAB and KE37 in comparison to lytically infected T lymphocyte cell type Jurkat. Lung epithelial A549 cells were used as a control. Cell viability was conducted by diluting the cells with 0.2% Trypan Blue at a ratio of 1:1 and viability obtained by automated cell counter.

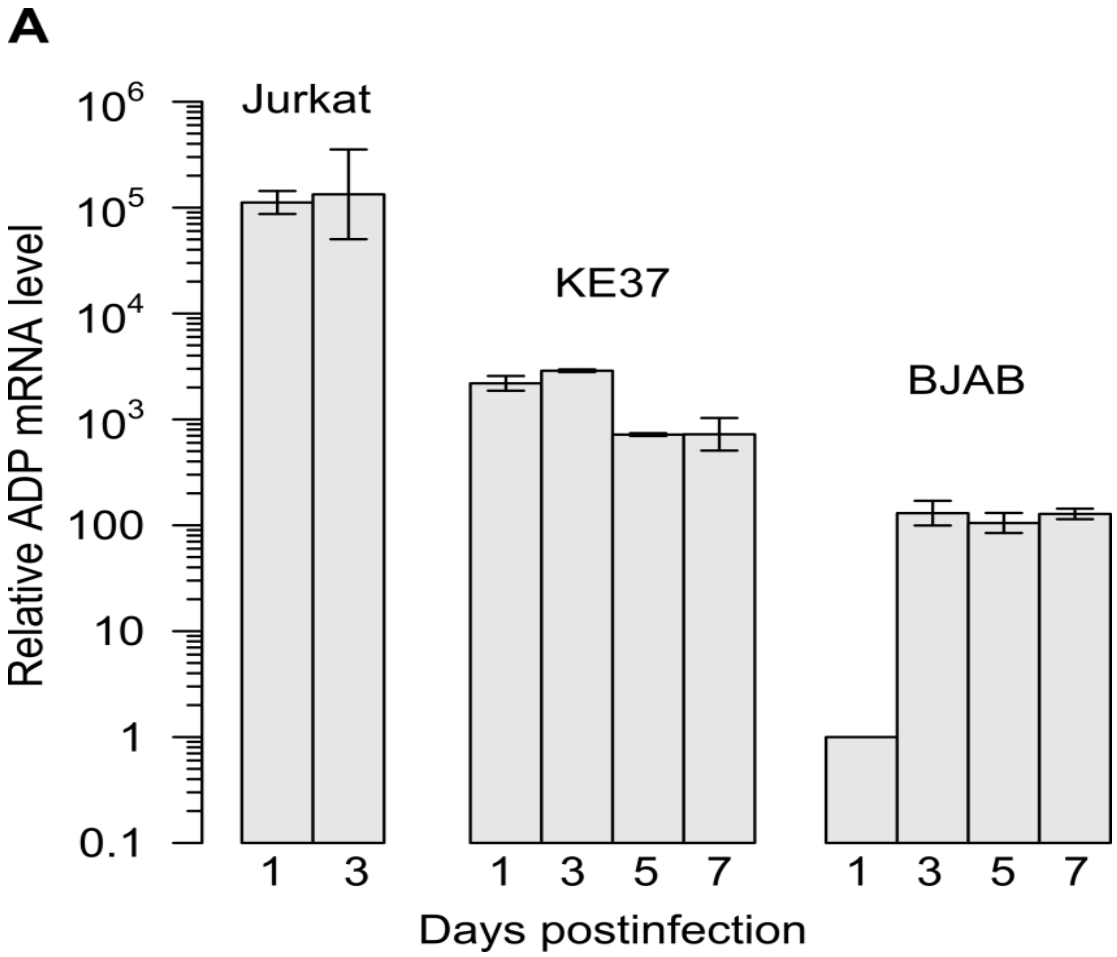

Figure 8 ADP mRNA transcript levels in different cell types.

The mRNA levels were quantified by RT-PCR with primers designed from Integrated DNA Technology software. The different cell types were infected with 100pfu/cell of wild type dl309. The cells were harvested for mRNA isolation at different time post infection. mRNA obtained were converted to cDNA by RT-RXN and then quantified using Real Time Q-PCR. The data was normalized to E1F1 and against BJAB day 1 post infection.

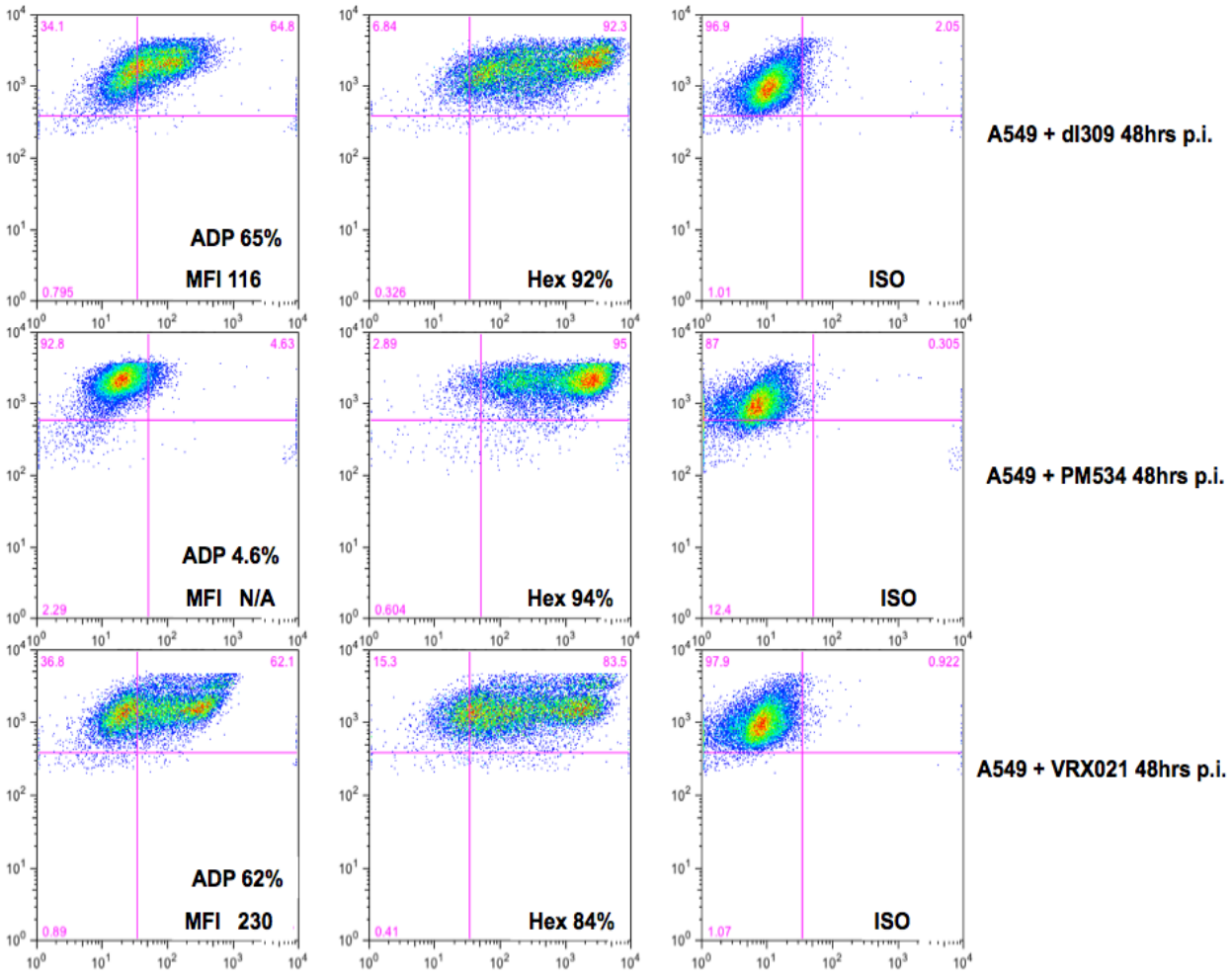

Figure 9 Validation of ADP mutant viruses.

Epithelial cells infected with Ad5 mutant viruses (pm534; ADP deleted, vrx021; ADP over-expressor and wt dl309; wt ADP) at 24h and 48hpost infection were fixed in dark with 1% HCHO and then subjected to permeabilization. Monoclonal mAb specific to ADP were used at a dilution of 1:25 followed by APC labeled secondary Ab at a dilution of 1:50. Hexon was used as an indicator of productive infection used at 1:100 dilution. The cells were then acquired by flow cytometry and analyzed using FlowJo software. The Isotype IgG was used as a control.

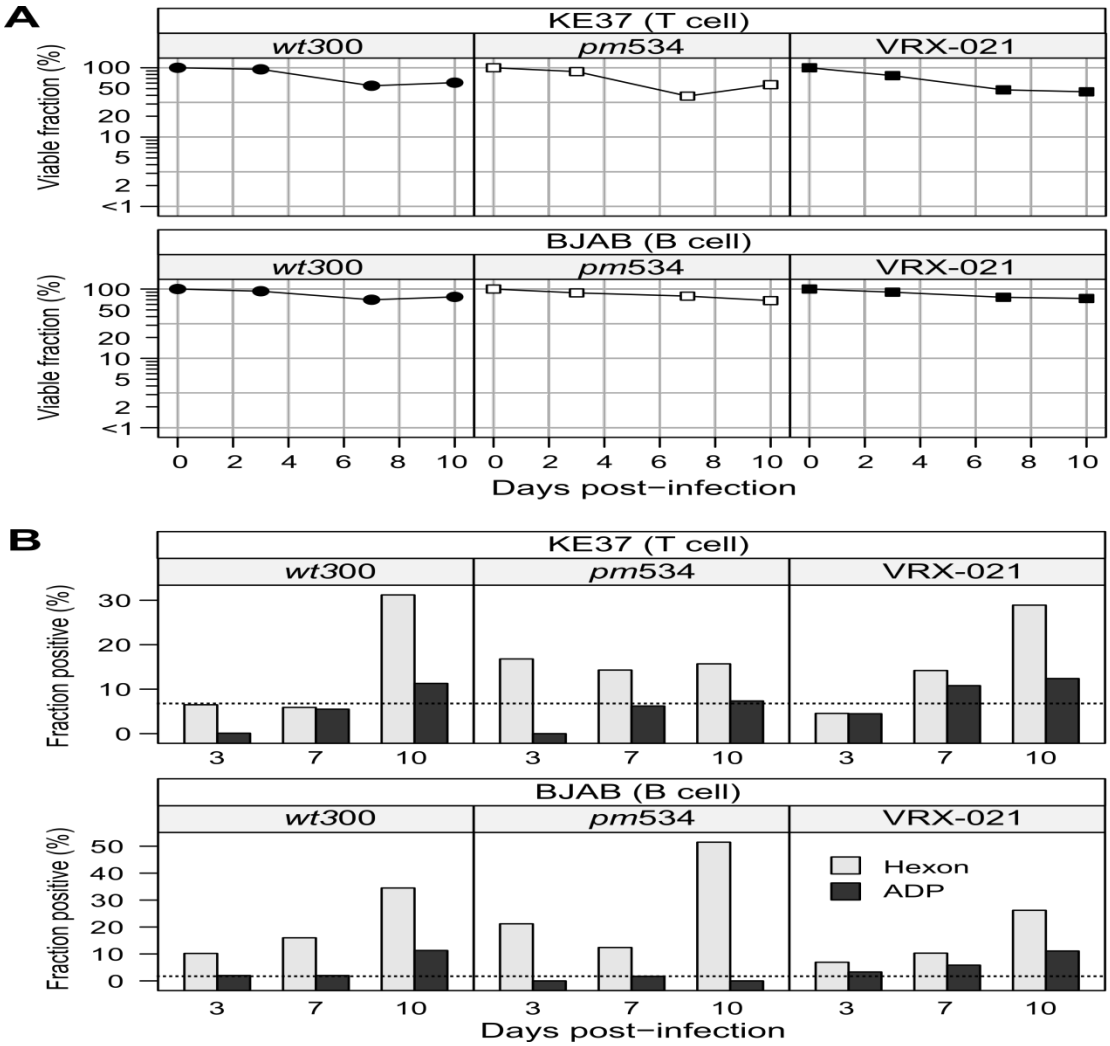

Figure 10 A. Infection of BJAB and KE37 cells with ADP over-expressing virus (VRX021) have no overt impact on cell survival. 10 B. Differential regulation of ADP in persistent cell lines

A.Trypan Blue Exclusion Assay on BJAB, KE37 cells infected with wt300, pm534 and VRX021. 10 uL of infected sample was diluted in 10 uL of Trypan blue and the cells were counted using Countess-Automated Cell Counter. **B.** The suspension cells on day 10 post infection were fixed in dark with 1% HCHO and then subjected to permeabilization. Monoclonal mAb specific to ADP were used at a dilution of 1:25 followed by APC labeled secondary Ab at a dilution of 1:50. Hexon was used as an indicator of productive infection used at 1:100 dilutions. The cells were then acquired by flow cytometry and analyzed using FlowJo software. The Isotype IgG was used as a control.

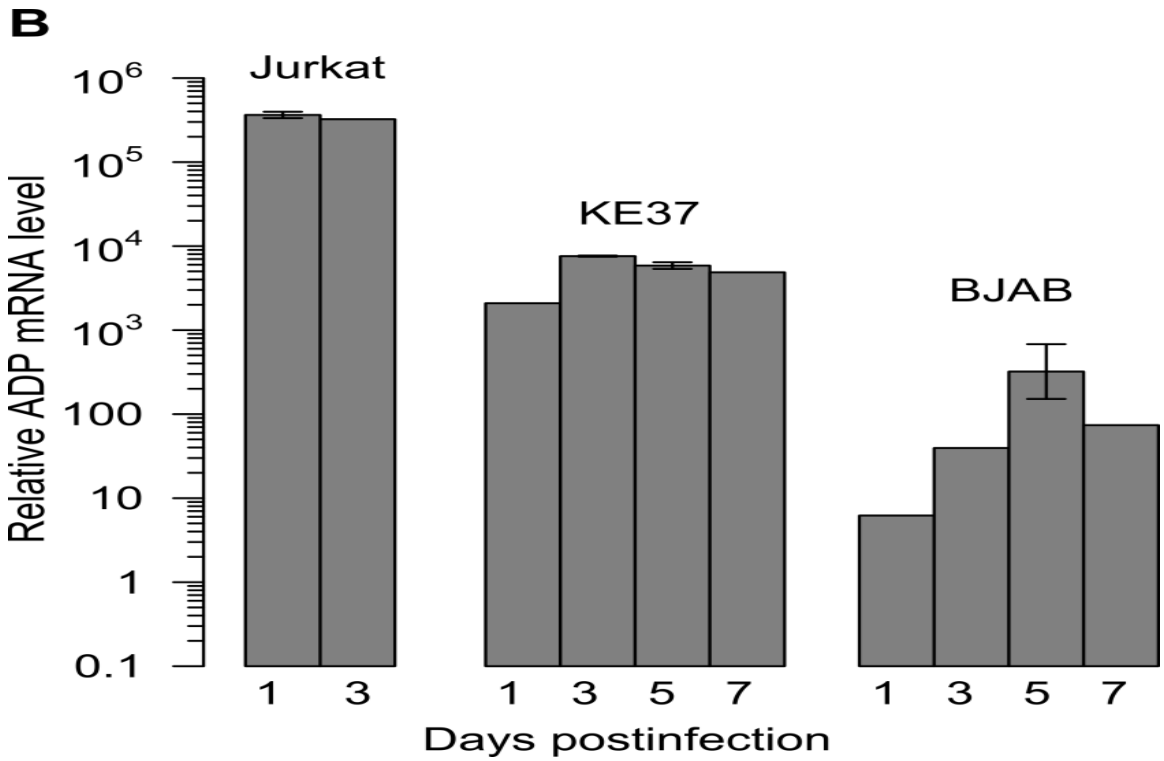

Figure 11 ADP mRNA transcript levels in different cell types.

The mRNA levels were quantified by RT-PCR with primers designed from Integrated DNA Technology software. The different cell types were infected with 100pfu/cell of ADP overexpressor VRX021. The cells were harvested for mRNA isolation at different time post infection. mRNA obtained was converted to cDNA by RT-RXN and then quantified using Real Time Q-PCR. The data was normalized to E1F1 and against BJAB day 1 post infection.

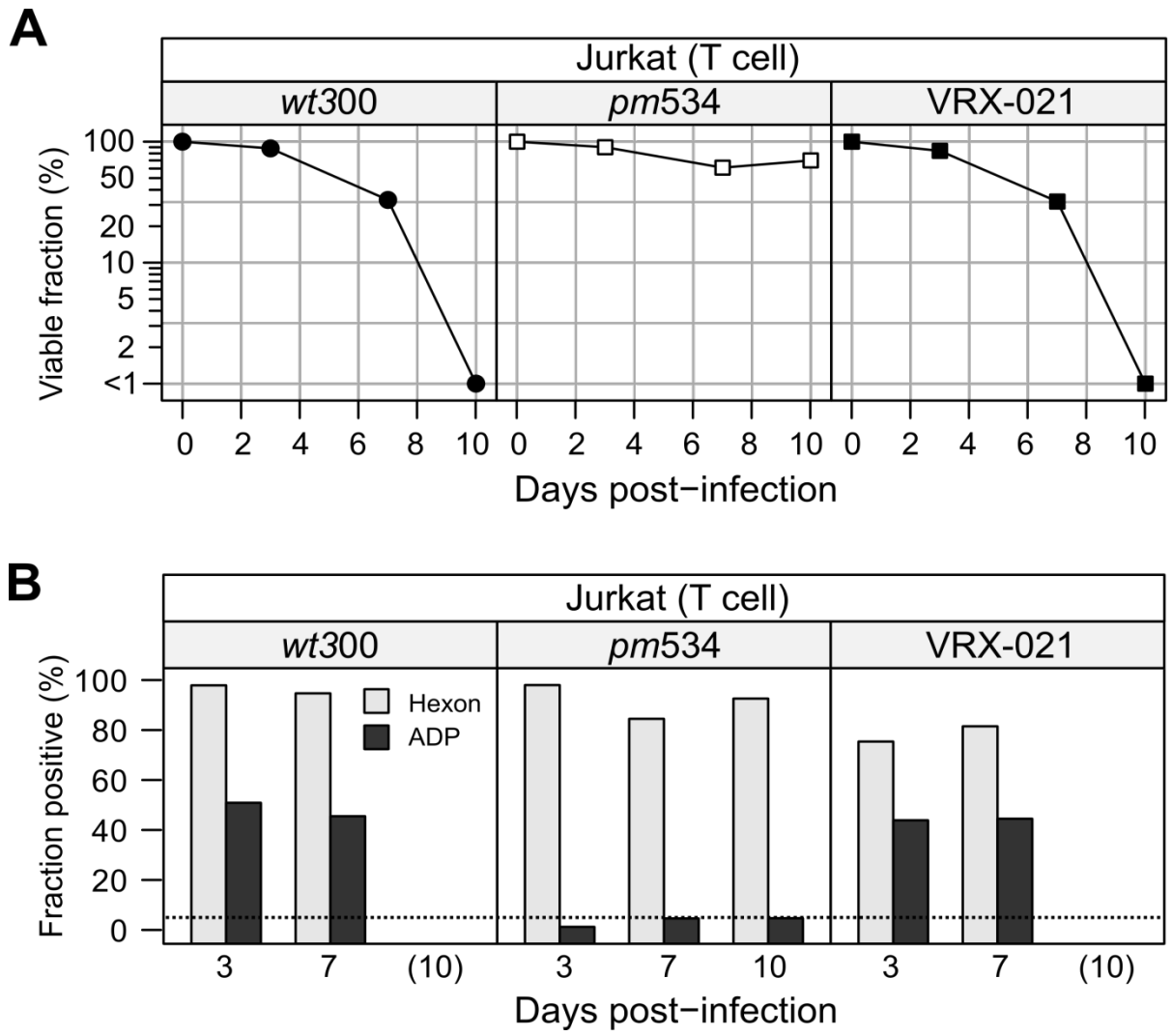

Figure 12 A. Cell viability of Jurkat cells infected with Ad5 mutants. B. Viral gene expression in Jurkat cells infected with ADP mutant and wt viruses

A. Trypan Blue Exclusion Assay on BJAB, KE37 and Jurkat cells infected with wt300, pm534 and VRX021. 10 uL of infected sample was diluted in 10 uL of Trypan blue and the cells were counted using Countess-Automated Cell Counter. **B.** The suspension cells on day 10 post infection were fixed in dark with 1% HCHO and then subjected to permeabilization. Monoclonal mAb specific to ADP were used at a dilution of 1:25 followed by APC labeled secondary Ab at a dilution of 1:50. Hexon was used as an indicator of productive infection used at 1:100 dilutions. The cells were then acquired by flow cytometry and analyzed using FlowJo software. The Isotype IgG was used as a control.

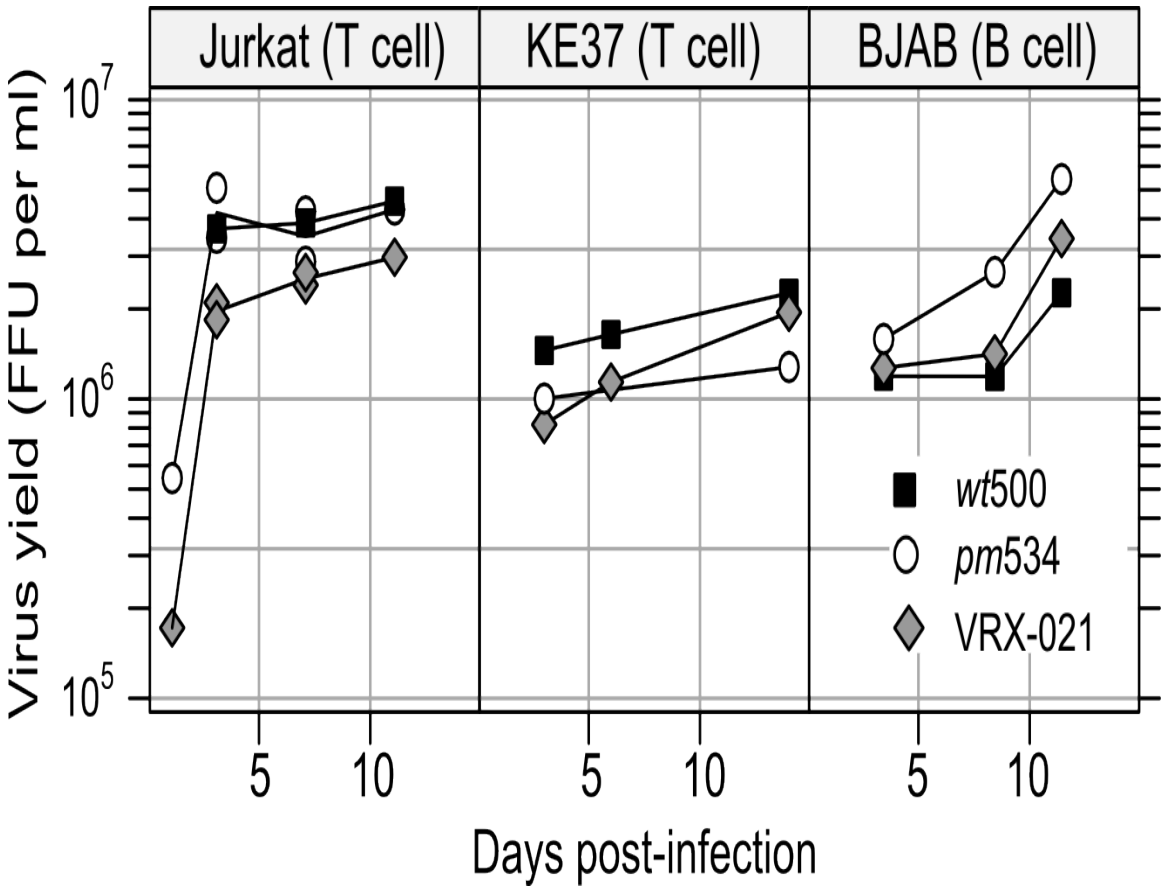

Figure 13 Infection of lymphocytes with ADP-overexpressing virus does not greatly impact virus titer.

Virus titer was determined by plaque assay at a time when the respective infections is at its peak measured by the peak hexon expression. The above figure shows the virus titer determined at day 3, 5 and 10-post infection.

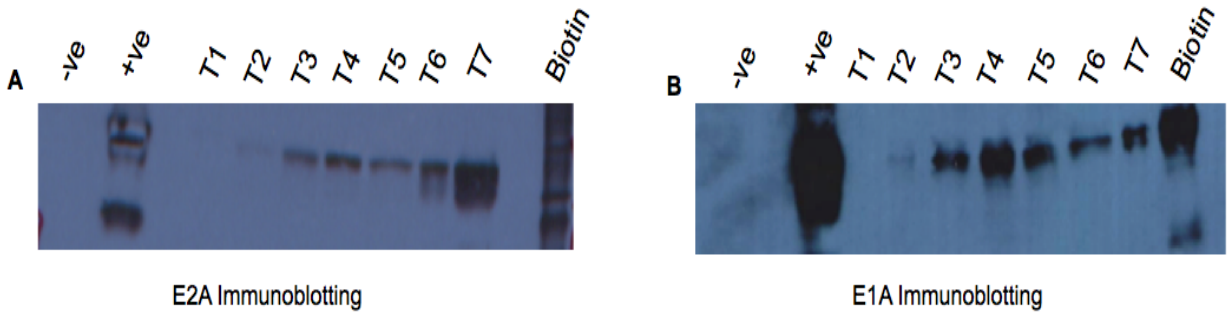

Figure 14 Changes in the expression of viral proteins in E1A less mutant infected BJAB cells.

BJAB cells were infected with 100PFU/cell of dl309. Protein lysates were made at different time post infection by using RIPA buffer and prepared a lysate cocktail by diluting it in 2X SDS buffer. The samples were run on 12% Mini Protean Gel obtained from Bio-Rad at 100 V and transferred at 100 V for 1 hour. The transferred membrane was then blocked overnight with milk and primary antibodies used at a dilution of 1:20. Horseradish peroxidase labeled secondary antibody was then used at 1:5000 in addition to 1:1000 of anti-biotin. Images were developed at 5 seconds, 30 seconds and 5 mins exposure to X-ray sensitive film in a dark room.

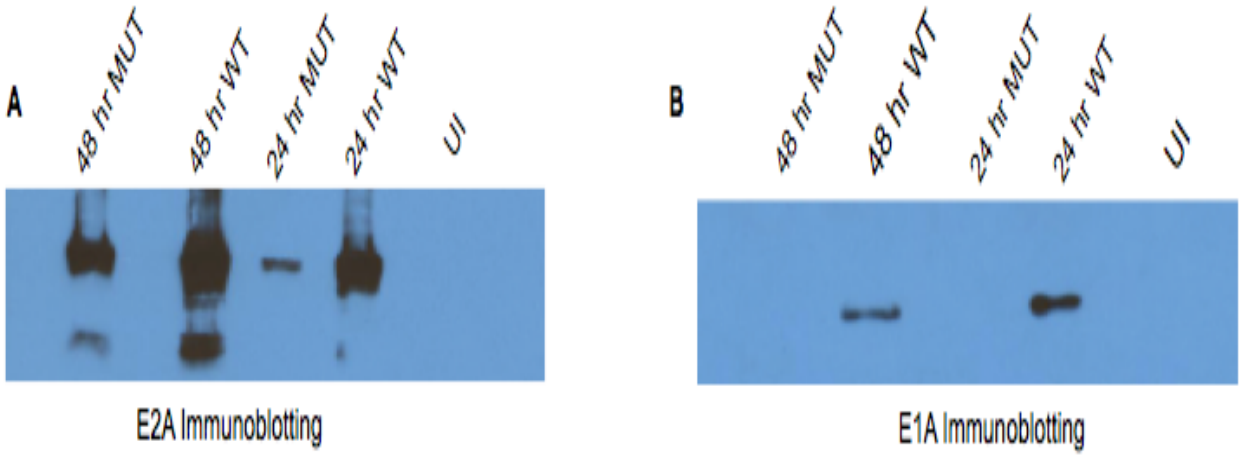

Figure 15 Changes in the expression of viral proteins in E1A less mutant infected A549 cells.

A549 cells were infected with 10PFU/cell of Ad-CMV-GFP, which replaced the E1A gene with a CMV promoter. Protein lysates were made at different time post infection by using RIPA buffer and prepared a lysate cocktail by diluting it in 2X SDS buffer. The samples were run on 12% Mini Protean Gel obtained from Bio-Rad at 100 V and transferred at 100 V for 1 hour. The transferred membrane was then blocked overnight with milk and primary antibodies used at a dilution of 1:20. Horseradish peroxidase labeled secondary antibody was then used at 1:5000 in addition to 1:1000 of anti-biotin. Images were developed at 5 seconds, 30 seconds and 5 mins exposure to X-ray sensitive film in a dark room.

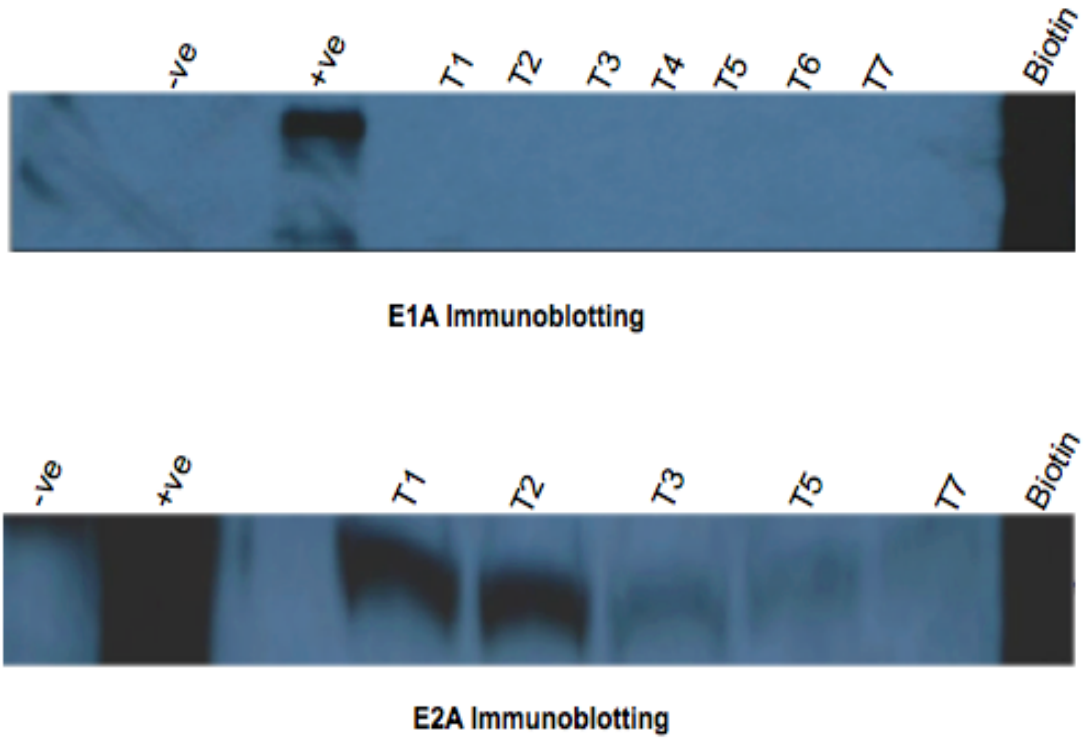

Figure 16 Western Blotting for DBP in the absence of E1A in BJAB infected with dl309.

The BJAB cells were infected with 100PFU/cell of Ad-CMV-GFP, which replaced the E1A gene with a CMV promoter. Protein lysates were made at different time post infection by using RIPA buffer and prepared a lysate cocktail by diluting it in 2X SDS buffer. The samples were run on 12% Mini Protean Gel obtained from Bio-Rad at 100 V and transferred at 100 V for 1 hour. The transferred membrane was then blocked overnight with milk and primary antibodies used at a dilution of 1:20. Horseradish peroxidase labeled secondary antibody was then used at 1:5000 in addition to 1:1000 of anti-biotin. Images were developed at 5 seconds, 30 seconds and 5 mins exposure to X-ray sensitive film in a dark room.

CHAPTER 4: DISCUSSION

Adenovirus Death Protein is encoded presumably to aid the effective cell-to-cell spread of adenovirus during the final stages of infection. ADP is believed to achieve this by forcing the cell to lose its membrane integrity and lyse. The exact mechanism of ADP action in releasing the virions however is unclear. One of the differences between a lytic life cycle and persistent infections is the ability of the virus to encode gene products leading to death of its host cell and ability to make progeny virions. In order to understand the viral gene expression in persistently infected lymphocytes in comparison with well-studied lytic infection in epithelial cells, this study investigated the role of ADP in viral persistence by evaluating cell death associated with its expression, and the total virus yield. This study has also potentially discovered one of the most important functions of ADP in determining the fate of viral life cycle in cell types that is previously not known.

We began evaluating ADP expression in lymphocytes (BJAB, KE37 & Jurkat cells) and compared the expression in lytically infected A549 cells. Expectedly, we observed low ADP expression in cell types harboring persistent infections (BJAB & KE37 cells) and in abundance in Jurkat cells **(Figure 6).** Interestingly, there exists a clear difference in the levels of ADP expression in A549 and the different lymphocytes. While Jurkat cells expressed ADP levels much higher than those seen in BJAB and KE37, the levels did not match those observed in A549 cells. Given the role ADP is known to play in infected cells, low expression in persistent cell types is understandable, as any ADP in these cell types would result in cell death and jeopardize long-term survival of the viral genome inside the host. These findings suggest that though the virus lytically infects the Jurkat cells, there exists a tighter regulation of this gene in lymphocyte cells than in epithelial cell types.

We also evaluated the viability of lymphocytes infected with wild type Adenovirus. While BJAB and KE37 cells had a higher fraction of viable cells throughout the time period under study, the percentage of viable Jurkat cells decreased drastically by day seven-post infection and eventually died. A549 cells typically die 48-72 hrs post infection (**Figure 7**). These findings supported our previous results, which relate the ADP expression and cell death.

Since ADP expression is associated with death of the host cell, we further investigated to determine if overexpressing ADP in persistent cell types would alter the viral behavior in lymphocytes by reversing viral persistence to adopt a lytic cycle consequently killing the cell. We evaluated the ADP expression in all three lymphocytic cells infected with ADP over expression virus VRX021. Disappointingly, our attempt to overexpress ADP was unsuccessful as the ADP levels in BJAB and KE37 continued to be dismal, while Jurkat cells did not show much increase in ADP expression in comparison with the wild type levels. Since ADP were not expressed at expected levels the viability of persistent cells were high though Jurkat cells had higher percentage of dead cells at time points measured **(Figure 10).**

Differential gene regulation is further illustrated when we evaluated the levels of ADP transcripts in these cell types. Jurkat cells with high ADP expression also expressed the highest levels of ADP mRNA at all-time points evaluated when compared to the other two lymphocytic cells. Since BJAB and KE37 cells did not express adequate ADP, we expected to see lower levels of ADP transcripts in them. To our surprise, we detected at least 130 fold increase in ADP transcripts in BJAB 3 days post infection and KE37 cells also expressed relatively high levels of ADP transcripts on day one before the expression is down regulated **(Figure 8 & 11)**. Production of ADP transcripts without materializing into a protein product indicates a potential regulation of ADP at the level of mRNA transcripts. This is interesting given that other gene products in the E3 region are expressed while only ADP is consistently and intensely regulated in these cell types. The exact mechanism underlying this differential regulation is not clearly

understood. According to our study so far ADP is tightly regulated in certain lymphocytic cells that attempts to over express ADP in those cell types were unsuccessful. Future studies to determine if ADP does act as switch between lytic and persistent infections, ADP can be overexpressed artificially by introducing retrovirus based vectors carrying ADP gene with a promoter that is constitutively active.

We also analyzed the effect of ADP knockout on lymphocytic cell types that support lytic infection like Jurkat cells. Our results confirmed our initial hypothesis that ADP knockout would allow the virus to persist in cell types that typically harbor lytic infection. Jurkat cells, which usually die 7-8 days post infection-survived up to indicative of viral persistence. This clearly demonstrates that ADP could potentially act as a switch between lytic and persistent infections **(Figure 12).** The fact that ADP belongs to early E3 region of adenoviral genome and that it is expressed in abundance during late stages of infection provides us a cue that the E3 11.6kDa product indeed has a specialized function.

It is well known that ADP is expressed during late stages to facilitate release of new virions. However, it is unclear if ADP itself has any impact on the total amount of virus produced in a cell type. We explored this possibility by determining the virus yield in these cell types. It appears as though ADP has no impact on the total virus yield as Jurkat cells, which are robustly infected typically produced higher titers than persistently infected BJAB and KE37**(Figure 13).** Function of ADP seems limited to causing cell death by lysis, which could be utilized by the virus to turn on and off latency in a cell type.

It is evident that ADP expression appears to be closely related to the corresponding late gene expression hexon in lytically infected cell types. In sharp contrast, persistently infected lymphocytic cells also yield a productive infection (measured by the levels of late gene hexon), but express ADP in low levels. We also showed that BJAB and KE37 cells yield far less titer of virus than the Jurkat cells. There is a possibility where even though late structural genes are

expressed in BJAB and KE37 cells, products necessary for the completion of virus assembly is not made. Those products may impact the overall ADP expression. Another possible stimulus that may affect ADP expression could be the amount of virus made in a specific cell type. The stimulatory signals could be due to excessive crowding of the cell following the assembly of new virions.

Also in our study, we evaluated the early viral gene expression in persistently infected BJAB to determine if other viral genes are also differentially regulated in these cell types. Our preliminary results did support our hypothesis, where transactivating E1A and DNA binding protein E2A had a delayed expression in comparison with lytically infected A549 cells **(Figure 14).** It was necessary to evaluate the expression of E1A and E2A-DBP as they drive the viral gene replication helping maintain viral genome over a period of time. We detected higher levels of E2A than E1A from day 7-14 (data not shown). Since, it was previously shown that E1A loss can be compensated by E4orf6/7, we sought to investigate if E2A-DBP expression is independent of E1A expression. Our preliminary results suggest that E1A may not be required to drive the expression of E2A-DBP **(Figure 15 & 16).** This unique behavior might help maintain the Ad5 genome without full viral replication in latently infected cells. Future studies need to confirm our findings and characterize their gene expression pattern over longer period of time and analyze the change in gene expression pattern, when latently infected cells are reactivated.

All studies characterizing adenovirus have been carried in lytically infected epithelial cells. Adenoviruses are also known to infect some lymphocytes and go latent in them. Not much is known about the mechanism of viral persistence. Given that adenovirus is used in gene therapy to treat several diseases, it is important to fully understand how adenovirus infection of lymphocytes could change its biology, and in particular to fully characterize its latency. A viral agent has long been suspected to play a role in the initiation of cancer.

However, no viral genes or gene products were detected from the cancer cells to support the hypothesis. The natural history of leukemia in children evolves around the pre-natal initiation of pre-leukemic clones (more so by chromosomal translocation) followed by postnatal promotion and series of secondary mutation. The adenovirus possesses certain specialized oncogenes that can initiate the cellular transformation by a hit and run mechanism. The adenovirus can mediate cellular transformation through an initial hit and then maintain the transformed state even in the absence of the viral molecules (Nevels et al., 2001). The Ad5 protein E4orf6/7 and E4orf3 are known to inhibit double stranded DNA break repair mechanisms. While this may not be an issue during acute lytic infections, inhibition of this process in cells persistently infected with adenovirus could have a potentially devastating impact on the host cell. This includes possibly promoting DNA translocations, which could initiate the first step in leukogenesis. Honkaniemi E. et al. reported the increased detection of adenoviral DNA in Guthrie cards of children who develop Acute Lymphoblastic Leukemia (ALL) when compared to non-leukemic controls (Gustafsson et al., 2007). Given adenoviruses unique interaction with lymphocytes, there is a growing belief that adenovirus could initiate leukogenesis in neonates and develop into ALL in children under 5 years of age. Hence, it is important to fully understand the dynamics of gene expression of adenovirus in lymphocytes.

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