universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Antiviral strategies by interferon-γ to control intestinal adenovirus infections“

verfasst von / submitted by

Armin Fejzic

angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna, 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
Betreut von / Supervisor:	Prof. Thomas Lion, MD, PhD, MSc

This page intentionally left blank

Acknowledgments

First and foremost, all praises and thanks belong to God, the Almighty for being able to complete my master studies.

I would like to express my deep and sincere gratitude to my research supervisor, Dr. Karin Kosulin, for her continuous support during my whole work in the laboratory, but also during the writing of this thesis. I would like to thank her for everything she taught me in the best and nicest possible way, for being patient with me and for the guidance through all time. Also, I would like to thank the professor and our group leader Dr. Thomas Lion, for giving me the opportunity to do my master's thesis in his team and providing invaluable advice. Karin and Thomas taught me the methodology to carry out the research and to present the research works in the best possible way. It was a great privilege and honor to work and study under their guidance.

I am extremely grateful to my parents for their love, prayers, caring and sacrifices for educating and preparing me for my future. Also, I am thankful to my brother for his support and care. The last three years of my life I spend with my beloved wife. I would like to thank her for love, understanding, prayers and continuing support to complete my master's degree. I also express my thanks to my father- and mother-in-law, the closest family and friends for their support.

Armin Fejzic

Vienna, October 2020

This page intentionally left blank

Summary

Background: Human adenoviruses (HAdVs) mainly cause infections of the gastrointestinal (GI) and the respiratory tracts in early childhood and were shown to persist in infiltrating lymphocytes in adenoids, tonsils and the GI tract. The latter is a potential source of virus reactivation, especially in the pediatric immunosuppressed patient setting. The mechanism of HAdV persistence is not entirely clear, but an interferon (IFN)-dependent repression of the adenoviral early gene E1A transcription was demonstrated in bronchial epithelial cells and fibroblasts. The principal idea for the present study was to investigate the antiviral potential of interferon-γ (IFN-γ) in colon epithelial cells and its relevance for HAdV reactivation upon treatment with immunosuppressive agents.

Methods: The effect of IFN-γ on HAdV replication and mRNA expression of E1A and IFN-γ stimulation upon infection has been investigated by RQ-PCR in human intestinal colon epithelial cells (1CT), colon epithelial carcinoma cell lines (HCT116 and CaCo2) and a lung carcinoma cell line (A549). The inhibition of virus progeny production by IFN-γ in 1CT cells has been assessed by tissue culture infectious dose (TCID50) analyses. Additionally, the promoting effect of immunosuppressive agents on HAdV replication and expression of IFN-γ mRNA in peripheral blood mononuclear cells (PBMCs) has been examined.

Results: The findings revealed that 1CT and carcinoma cell lines do not secrete IFN-γ, but exogenous addition of IFN-γ to 1CT cells resulted in significant inhibition of HAdV at the levels of DNA, RNA and infectious virus particle production. By contrast, IFN-γ failed to inhibit HAdV replication in the colon cancer cell lines. Moreover, the data indicate that immunosuppressive agents apparently promote HAdV replication in PBMCs by inhibiting IFN-γ mRNA expression.

Conclusion: The present study provided novel data assessing the putative role of IFN-γ in HAdV reactivation during immunosuppression and the antiviral capacity of IFN-γ in colon epithelial cells.

This page intentionally left blank

Zusammenfassung

Hintergrund: Humane Adenoviren (HAdVs) verursachen bereits in der frühen Kindheit hauptsächlich Infektionen des Gastro-Intestinal-Trakts (GI) und der Atemwege und wurden in Adenoiden bzw. Tonsillen sowie im GI-Trakt auch persistierend in infiltrierenden Lymphozyten gefunden. Diese Viruspersistenz stellt insbesondere für immunsupprimierte pädiatrische Patienten ein Risiko dar, weil eine Reaktivierung im GI-Trakt schwere systemische Infektionen hervorrufen kann. Der genaue Mechanismus der adenoviralen Persistenz ist nicht geklärt, jedoch konnte eine interferon-abhängige Hemmung der frühen adenoviralen E1A-Transkription in Bronchialepithelzellen und Fibroblasten gezeigt werden. Die grundlegende Idee dieser Arbeit war, die antivirale Wirkung von Interferon-γ (IFN-γ) in Darmepithelzellen zu untersuchen sowie seine Bedeutung für die Reaktivierung von HAdV nach Immunsuppression zu analysieren.

Methoden: Die Wirkung von IFN-γ auf die HAdV-Replikation, die mRNA-Expression von E1A sowie die zelleigene IFN-γ-Stimulation durch HAdV-Infektion wurden mittels RQ-PCR in menschlichen Darmepithelzellen (1CT), Darmepithelkarzinomzelllinien (HCT116 und CaCo2) und einer Lungenkarzinomzelllinie (A549) untersucht. Die Inhibierung von neu produzierten infektiösen Viren durch IFN-γ in 1CT-Zellen wurde mit *tissue culture infectious dose* (TCID50) Analysen gezeigt. Zusätzlich wurde die Wirkung von Immunsuppressiva auf die HAdV-Replikation und Expression von IFN-γ-mRNA in mononukleären Zellen des peripheren Blutes (PBMCs) untersucht.

Ergebnisse: Die Ergebnisse zeigen, dass 1CT- und Karzinomzelllinien kein IFN-γ sezernieren, aber die exogene Zugabe von IFN-γ zu einer signifikanten Hemmung der HAdV-Replikation, der viralen mRNA-Expression und der infektiösen Viruspartikelproduktion in 1CT-Zellen führt. Im Gegensatz dazu war die inhibierende Wirkung von IFN-γ bei Karzinomzelllinien nicht zu beobachten. Darüber hinaus zeigen die Daten, dass Immunsuppressiva offensichtlich die HAdV-Replikation in PBMCs fördern, bei gleichzeitiger Hemmung der IFN-γ-mRNA-Expression.

Conclusio: Die vorliegende Studie liefert neue Daten zur Bewertung der mutmaßlichen Rolle von IFN-γ bei der HAdV-Reaktivierung unter Immunsuppression und seiner antiviralen Kapazität in Darmepithelzellen.

This page intentionally left blank

Table of Contents

List of figures

*Ich habe mich bemüht, sämtliche Inhaber*innen der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.*

List of tables

1. Introduction

1.1. Human adenovirus (HAdV) – an overview

Adenoviruses are non-enveloped viruses with icosahedral nucleocapsid (Gr. *eîkosi*, twenty; *hédra*, seat) with 20 triangular faces, 30 edges, and 12 vertices.[1] The genome of adenoviruses is constituted of a linear, double-stranded DNA (dsDNA). Adenoviruses were first isolated from adenoids and described in 1953. They are named *adenoviruses* based on the tissue in which they were discovered.[2,3] In immunocompetent individuals human adenoviruses cause mild, self-limiting infections which can result in acute respiratory morbidity. In children they are responsible for about 5-10% of respiratory illness, but can also be responsible for epidemic outbreaks.[3,4] In individuals with an intact immune system, HAdV infections are usually cleared by cytotoxic T cells.[5,6] On the contrary in immunocompromised individuals, HAdV infections are associated with life-threatening clinical manifestations and disseminated disease correlating with high mortality.[7–10]

1.1.1. Classification

Adenoviruses belong to the family of viruses called *adenoviridae*. They have been isolated only from vertebrates, from fish to mammals.[3] Four major genera have been determined based on the genome sequences: *Mastadenovirus*, isolated from mammals, including all human adenoviruses; *Aviadenovirus*, isolated from birds; *Atadenovirus*, isolated from reptiles, birds, a marsupial and mammals; and *Siadenovirus*, isolated from a reptile and birds.[3,11]

Table 1. 1. Species and types of HAdVs[12]

Specie	Type[I]
A	12, 18, 31, **61**
B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55, **66, 68, 76–79**
C	1, 2, 5, 6, **57, 89**
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, **53, 54, 56, 58–60, 63–65, 67, 69–75, 80–88, 90–103**
E	4
F	40, 41
G	**52**

Genera of HAdVs currently comprising 103 types (July 2019) classified into seven species (A-G) listed in Table 1.1.[3,12,13] In the past, classification of HAdVs has been done based on

[I] The bolded types were identified by genomic and not by serological analyses.[13]

serology, hemagglutination, oncogenicity in rodents, transformation of cultured primary cells. However, nowadays genome sequencing is used, and due to improved sequencing technologies a number of new HAdV types has been explored in the last decade (see Table 1).[3,12] The majority of newly identified HAdV types belong to species HAdV-D (over 70 types) followed by species HAdV-B (currently 16 types).[12,14] The emergence of these new HAdV types is mainly a result of homologous recombination between capsid genes (hexon, penton, and fiber).[15]

1.1.2. Virion structure

The icosahedral particle (virion) of adenoviruses can vary in size from 70 to 100 nm in diameter, along with the fibers extruding from the vertices of an icosahedron.[1,3] The virion consists of a protein shell (capsid) that surrounds the DNA and it has a mass of ~$150x10^6$ D, from which 13% comprises DNA and 87% protein.[3]

The capsid is composed of hexon, as main capsid protein and penton subunits (capsomers; Gr. *capsa*, box; méos, poion). The hexon protein is composed of three tightly associated molecules of hexon polypeptides. This trimeric protein is referred to as a hexon capsomere. The penton is composed of five polypeptide copies that form the penton base protein. One capsid is made of 252 capsomers, from which 240 are hexon (12 per triangular facet of the icosahedron) and 12 penton capsomers at each vertex of the icosahedron. From each penton base, one fiber protein extends.[16] A comparison of electrophoretic results with genomic open reading frames (ORFs) suggests there are 12 virion proteins numbered by convention II-IX, IIIa, IVa, μ, terminal protein, and the p23 viral protease. The function of minor capsid proteins IIIa, VI, VIII, and IX is to ensure the stability of the shell and play roles after entry of the virus into the cell, such as endosome penetration, transcriptional activation and nuclear organization, for which protein IX is liable. The core of the virion is made of seven proteins and DNA, from which proteins V, VII, and X (μ) interact with viral DNA (Figure 1.1).[3,17]

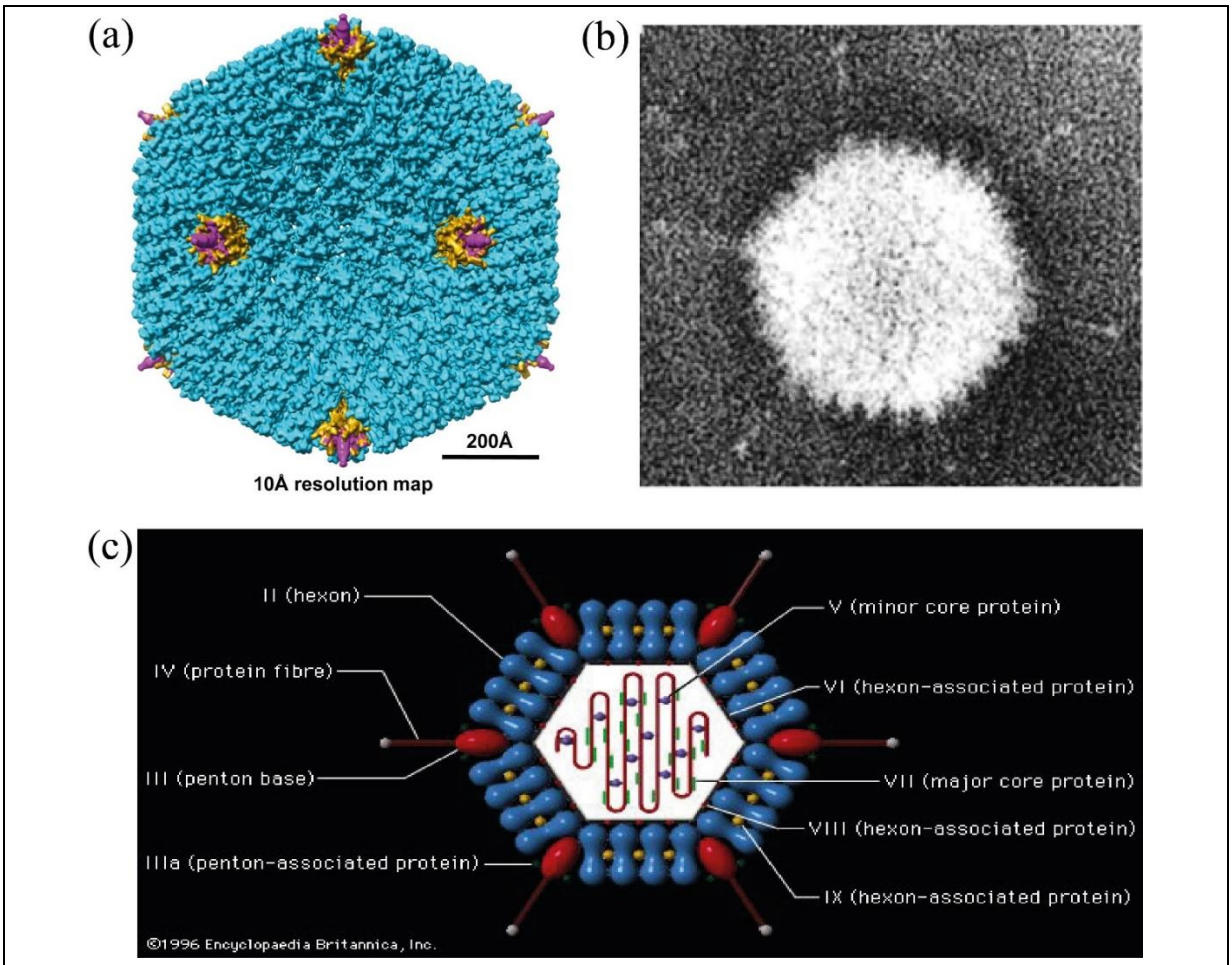

Figure 1. 1. The overall structure of HAdV-C5 and the penton base protein at 10 Å, centered on a 2-fold axis of the icosahedron, the fiber (magenta) associating with the penton base (yellow) located at the vertices of an icosahedron. The remaining capsid proteins are colored blue.[18] (a) Electron microscopic image of HAdV with projecting fibers and terminal knobs.[19] (b) Structural components and polypeptides of adenovirus.[20] (c)

The majority of HAdVs encode one single type fiber protein, however types 40, 41, 52 encode two fiber proteins. These fiber proteins are necessary for interaction with different cellular receptor proteins.[3] The fiber protein is made of three structural domains: the tail, the shaft, and the knob. The tail domain is the binding site for the penton base. The shaft domain can vary in length between HAdV types, resulting in different flexibilities of the fiber and differences in the interaction with the host cell integrins. At a distal, C-terminal end of the protein, the fiber knob domain is located which operates as a major attachment tool for the virus to the cellular receptors (Figure 1.2). The penton base interacts with the integrins of the host to enable entry of the virus.[1,1,20]

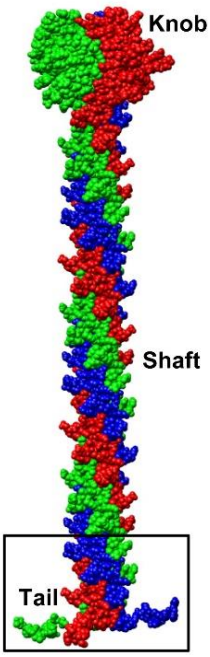

Figure 1. 2. Full space-filling model of the HAdV-C5 fiber consisting of a global knob, a long shaft, and three short N-terminal tails. The three subunits are colored red, green, and blue.[18]

1.1.3. Attachment and entry of HAdVs

Multiple receptors are involved in the recognition and binding of the virus to the host cell. But most of the HAdV species (A, C-F) enter the host cell through the coxsackie-adenovirus receptor (CAR), with exception of HAdV species D type 37 (HAdV-D37) and the species B types using CD46 as a receptor for the entry.[23,24]

CAR is a transmembrane protein, member of the immunoglobulin (Ig) superfamily receptors and consists of two immunoglobulin-like domains.[17] It is expressed in the heart, pancreas, the central and peripheral nervous system, prostate, testis, lung, liver, and intestine, but on hematopoietic and adult muscle cells it is very little or is not expressed.[25] CAR is located on the basolateral surface of polarized epithelial cells, bellow the tight junctions between the neighboring cells, whereas many tumor cells have CAR absent.[17,26,27] The subject of discussion is how do adenoviruses gain access to the receptor since it is not expressed on the apical side of epithelial cells.[28] It has been suggested that adenoviruses may use a transient break in the cell monolayer which may allow initial virus binding to CAR or it uses lactoferrin as a bridge for attachment to host cells.[29,30] Lactoferrin is a multifunctional protein of the transferrin family, one of its functions is to play a role in the first line of defense against microbial infections. Adenovirus hijacks lactoferrin and uses it as a bridge for attachment to host cells.[30,31]

In contrast to CAR, which is utilized by most of the HAdV species, HAdV-D37 and B species types, except types 3 and 7, use CD46 as a receptor.[17,24,32] CD46 is a member of the family of complement regulatory proteins, present on the plasma membrane of most cell types including hematopoietic cells. Compared to CAR, CD46 is much more accessible for the adenoviruses, but also for some other viruses and bacteria. This is because it contains four short consensus repeat domains (SCR 1-4), which serve as a binding site for mentioned organisms.[17,33]

It has been also reported that ocular pathogens HAdV-D (HAdV-8, -19a and -37) use sialic acid as a receptor rather than CAR.[34] HAdV-37 is capable of binding CAR and CD46, but cannot use them efficiently. This suggests that host cell factors may be involved in virus tropism.[17]

Following the successful binding of the virus to a receptor, integrins are involved in the uptake of the virus particle into the host cell. Integrins are heterodimeric proteins composed of two non-covalently associated α and β subunits. These two subunits recognize RGD (Arg-Gly-Asp) peptide which is located within the penton base of almost all adenoviruses, except HAdV-40 and -41. Proteins known to facilitate adenovirus entry include the vitronectin-binding integrins αvβ3, αvβ5, and αvβ1.[17]

Heparan sulfate proteoglycans (HSPG), blood coagulation factor (e.g. X, C3, C4Bp), lactoferrin, CD80, CD86, integrins αMβ2 and αLβ2 and other protein and non-protein receptors (e.g. class I major histocompatibility complex, vascular cell adhesion molecule) can also serve for binding and internalization of adenoviruses.[17,21]

1.1.4. HAdV tissue tropism and diseases

HAdVs show a broad tissue tropism and despite their diversity, only one-third of them are associated with human diseases.[35] Due to a lack of humoral immunity, 80% of these infections take place in children under five years old and rarely in adults.[36] HAdVs can be frequently found to infect and replicate in different sites of the respiratory tract, in the eyes and gastrointestinal tract (GI). Rarely they can be found in the urinary bladder and liver. In some cases, adenoviruses can trigger diseases in the pancreas, myocardium, or central nervous system. Infections of the respiratory tract are mainly caused by species B, C and E types, occasionally also by D types. The gastrointestinal tract is mainly infected by species F and G, intriguingly only species F types cause intestinal diseases in children. Infections of the eyes are

mainly caused by species B, C, D and E types. Species A types can be found in the respiratory and the GI tract.[13,35]

HAdV may be responsible for causing epidemic outbreaks of febrile respiratory illnesses, pharyngoconjunctival fever, keratoconjunctivitis or gastroenteritis and diarrheal illness.[35–38] Infections of the upper and lower respiratory tract by HAdV can be caused in at least 5-10% of pediatric and up to 7% of adult patients. The most common symptoms include fever, pharyngitis, tonsillitis, cough, and sore throat. Generally, these infections do not lead to fatalities but cases of small airway dysfunction and bronchiectasis have been documented. HAdVs are also associated with ocular infections (keratoconjunctivitis), gastroenteritis and diarrhea. In pediatric individuals with an immunocompromised immune system severe diseases have been documented like hepatitis, meningoencephalitis, myocarditis, pneumonia and disseminated diseases. Gastroenteritis is also known for immunosuppressed patients, in whom it can also lead to more severe manifestations.[9,14,36,39–41] In cases of non-inflammatory HAdV-mediated conditions, a putative association of HAdV-D36 with obesity has been reported.[42,43]

1.1.5. Genome organization

The dsDNA linear genome of adenoviruses can vary in size from 26 to 45 kb. All adenovirus types share some homologous genes which encode proteins required for viral replication (terminal protein, viral DNA polymerase and viral single-stranded DNA (ssDNA) binding protein) and major structural components of the virion. On the phosphates at 5’ ends of both strands, the terminal protein is covalently bound, which acts as a primer for replication. Genes for major structural components of the virion are highly conserved, with the exception that genes encoding the core protein V are only found in the *mastadenoviruses* isolated from mammals. In the central portion of the genome commonly shared genes are located, whereas genes specific for some adenoviruses are mainly found at the terminal part of the genome. The genomes of all adenoviruses contain inverted terminal repeat sequences which can vary in size (36-200 bp) and serve as a DNA replication origin at each end of the genome.[11]

The HAdV genome is divided into three different sections at which viral transcription units are expressed. The early phase starts as soon as the virus enters a host cell and the five early transcription units (E1A, E1B, E2, E3, and E4) are first expressed. Genes expressed from these transcription units interact with different cellular and viral proteins. Early and late transcription units are separated by the expression of intermediate transcription units (IX, IVa2, L4

intermediate, and E2 late). The late transcription unit is further processed and spliced into five families of late mRNAs (L1-L5) from which capsid proteins are encoded (Figure 1.3). All of these transcription units are transcribed by RNA polymerase II.[3]

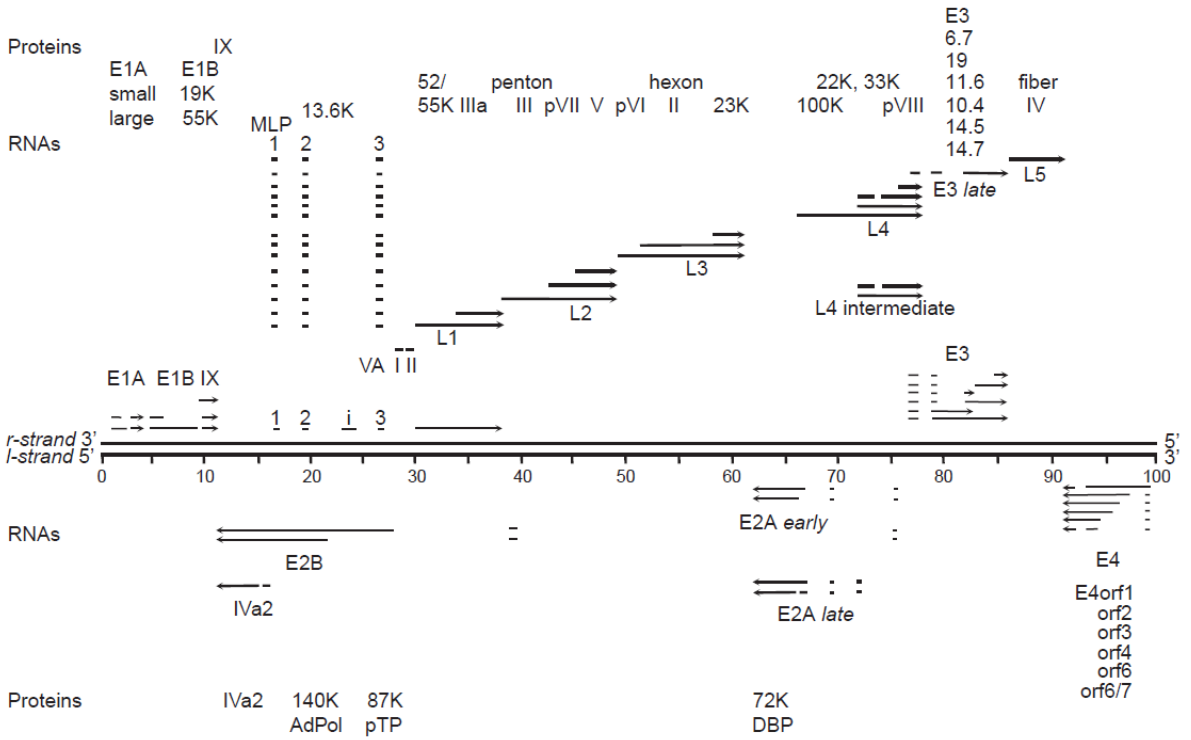

Figure 1. 3. The HAdV-C2 genome consisting of 35 937 bp divided into a map of 100 units.[3; II]

As soon as adenovirus enters a host cell, it has three goals related to the early gene expression. In order to provide the optimal conditions for viral replication, it brings the cell to enter the S phase of the cell cycle. In this step products of E1A and E4 genes play the main role. Afterwards, E1A, E1B, E3 and VA RNA establish a viral system that will protect the infected cell from being detected by the immune system of the host. In the end, the virus has to transcribe the proteins necessary for viral DNA replication. Without successful transcriptional activation of the viral genome and promoting the host cell to enter the S phase of the cell cycle, these three goals cannot be achieved.[3]

[II] Early mRNAs are marked with thin lines represent, late mRNAs with heavy thick lines and intermediate mRNAs with intermediate thick lines

1.1.5.1. Early transcribed genes - E1A region

E1A is the first transcribed adenoviral gene. It has a very strong enhancer located ~500 bp upstream of its promoter site. E1A mRNA is alternatively spliced into two mRNAs with different sedimentation coefficients (13S and 12S) encoding proteins of 289 and 243 amino acids respectively.[3] These two E1A proteins, are heavily phosphorylated by cyclin-dependent kinase (CDK1,2 and 4) a mitogen-activated protein kinase (MAPK) and casein kinase II.[44] In the later phases of infection three additional E1A mRNAs are detected but with still unknown functions. The 13S E1A protein has an additional 46 amino acids segment, the conserved region 3 (CR3), which encodes a transcriptional activation domain.[3]

The E1A proteins are of huge importance for successful infection since they are capable to activate other transcription units from the adenoviral and cell genome and also to modulate the cell cycle. They do not bind DNA sequences directly but alter the function of host proteins and transcription factors (TFs). The five conserved regions (CR1-CR4 and N-terminal) in the E1A proteins are rather different among the HAdV species and show several binding sites for important proteins of the host cell (Figure 1.4).[3,44]

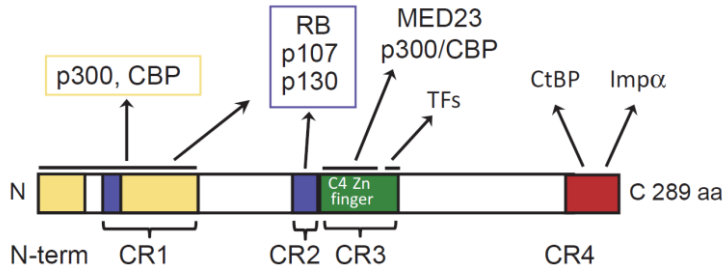

Figure 1. 4. The structure of E1A protein with indicated proteins that bind specific regions [3]

HAdVs generally infect resting or terminally differentiated cells. Each of two E1A proteins can induce infected cells, without any of the mitogenic signals, to enter from G0- or G1-phase into the S-phase of the cell cycle. For this process, it is necessary that E1A proteins interact with a variety of cellular proteins like nuclear lysine acetyltransferase p300, CREB-binding proteins (CBP) and retinoblastoma (Rb) proteins (RB1, RBL1 (p107) and RBL2 (p130)), supported by E2F TFs family that controls the expression of genes required for entry of cells into S-phase.[3,44]

The 13S E1A protein is capable to activate transcription of E1A, but also of other early transcribed HAdV genes (E1B, E2 early, E3 and E4). For this activation, it is necessary to have contact between the conserved region 3 (CR3) and the MED23 subunit of the human mediator of transcription complex.[3] On the other hand, the small E1A protein is capable to activate

transcription specifically from the E2 early promoter, to which the host's E2F TFs family binds. E2Fs are heterodimeric TFs that regulate the expression of genes required for entry of the host cells into the S-phase of the cell cycle. E2F TFs are regulated by the Rb proteins family which binds E2F directly. Rb proteins inhibit E2Fs by binding histone deacetylase complexes and polycomb histone methyltransferase. E1A proteins displace bound proteins from E2F TFs, together with associated transcriptional repression complexes. This displacement gives rise to constitutive activation of E2 promoter and other cellular genes required for the cell to enter into S-phase (e.g., dihydrofolate reductase, DNA polymerase α, CDK2, cyclin A and E, and c-MYK). E1A also blocks the action of CDK inhibitors which target Rb proteins family and regulate their activities. The hyperphosphorylated Rb-family proteins do not inhibit E2F activation, chromatin structure is reconfigured, allowing the TFs to stimulate entry into S-phase.[44]

1.2. Viral persistence

When a virus is not completely cleared by the host by the immune system, but remains in specific cells, a persistent infection can be established. Viral particles, proteins and genomes continue to be produced or persist for longer periods and sometimes also life-long. There are three types of interaction between the host and virus which are defined as latent, chronic and slow infection. A latent or hidden infection is characterized by the lack of symptoms until it becomes active again. By contrast, chronic infection is defined by continuously presence following primary infection and may lead to chronic or recurrent disease. A slow infection has a prolonged period of incubation followed by progressive disease.[45]

Many viruses such as human immunodeficiency virus (HIV), several herpes viruses, e.g. Epstein-Barr virus (EBV), human cytomegalovirus (CMV), lymphocytic choriomeningitis virus develop persistent infection.[45] It has been shown that HIV persists in resting CD4+ T cells, whereas latent EBV can be found in resting memory B cells and is capable of reactivation. CMV has been found in epithelial cells of salivary glands and kidneys, but also in macrophages and HAdVs have been described to persist in mucosal T lymphocytes of tonsillar, adenoidal and intestinal tissuet.[8,13,35,46–49] Multiple cellular mechanisms were described and suggested to support HAdV persistence. It seems to be mainly dependent on the defense mechanisms and regulation of apoptosis in the infected cell.[13,35,50]

1.2.1. HAdV persistence in immunocompetent individuals

HAdVs are known to establish long-term infection and persistence in a latent state. They were first found to persist in tonsillar and adenoidal tissue, where their DNA was detected. By more detailed analyses, HAdVs were found to persist in the mucosal T lymphocytes derived from children.[35,51,52] Besides, there is strong evidence that HAdVs persist in the gut. Shedding of the virus into the stool in asymptomatic individuals, several months post infection, has been reported since the 1980s.[53] A more recent study showed convincing evidence for intestinal HAdV persistence. In one-third of immunocompetent pediatric patients investigated, the viral DNA was detected in biopsies derived from the gut, with the highest prevalence in the terminal ileum.[49] Also, it has been shown that HAdVs persist in the T lymphocytes of intestinal lamina propria. In almost half of the samples of intestinal tissue and almost 90% of lamina propria lymphocyte samples, adenoviral DNA was detected.[52] These findings provided strong evidence for HAdV persistence in the gut.

Virus infections are usually controlled by the innate immune system, and this also applies to adenoviral infections, which may be regulated by cytokines. The entry of the virus into the cell triggers increased secretion of cytokines, such as interferon-gamma (IFN-γ), tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-2 and macrophage inflammatory protein.[6,54,55] IFNs are the essential key players in the innate and adaptive immunity and they have broad antiviral properties by activating the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway promoting the expression of IFN stimulated genes (ISGs).[56]

1.2.2. Possible mechanism of human adenovirus persistence

Very little is known about the mechanism of HAdV persistence but it has been found to persist mainly in T lymphocytes derived from patient samples. It is still not clear how the virus is kept in a persistent state and prevented to progress to an active infection, but several hints are available that the inhibition of early viral gene expression might play a role.[50] A study with the T cell lines keeping the virus in a persistent state for a long period has been reported showing no expression of early genes and a reduced CAR expression.[57] Compared with an *in-vivo* model for HAdV persistence in humanized mice. The repression of early genes has been observed, except for the E1A expression in the bone marrow. Moreover, in mice with persistent infection, HAdV-specific CD8+ cells were generated and the production of IFN-γ was significantly increased compared to the mock-infected mice. This provides additional hints about the

influence of the immune system and specific cytokines on persistent HAdV infection.[58] The cytotoxic T-cells usually eliminate cells infected with the virus. Since HAdVs are capable to block apoptosis through the early expressed proteins, this could be an underlying mechanism why the infected cells are not destroyed and the virus is not cleared.[3,55]

It has been shown that in general IFNs have an inhibitory effect on HAdVs. Chahal et al. 2012 have reported that in the human fibroblasts and primary human lung epithelial cells universal IFN type I inhibited HAdV-C5. The inhibitory effect was even stronger when the gene for the expression of the E1B protein was knocked out. This might be because one of the functions of the E1B protein is to inhibit the expression of interferon-stimulated genes (ISGs).[59] Furthermore, it has been documented that IFN type I (IFN-β) and type III (IFN-λ) have an inhibitory effect on HAdV replication in the human intestinal organoids.[60] Zheng et al. showed how type I (IFN-α) and type II (IFN-γ) interferons inhibit HAdV replication and progeny production in primary human bronchial epithelial cells and fibroblasts. Inhibition of early gene expression has been observed, especially of the E1A. The enhancer region of E1A consists of several binding sites: two for the cellular TF GABP and binding sites for E2F and Rb proteins. The initial binding of GABP promotes the transcription of the important early transcribed viral gene E1A. However, in cells treated with IFN-α or IFN-γ, binding of GABP to the E1A enhancer region was blocked, whereas the inhibitory effect of IFN-γ was stronger than IFN-α. Additionally, it has been shown that IFN treatment also induces the binding of E2F/Rb protein complex to the E1A enhancer region and represses viral immediate-early transcription (Figure 1.5).[54]

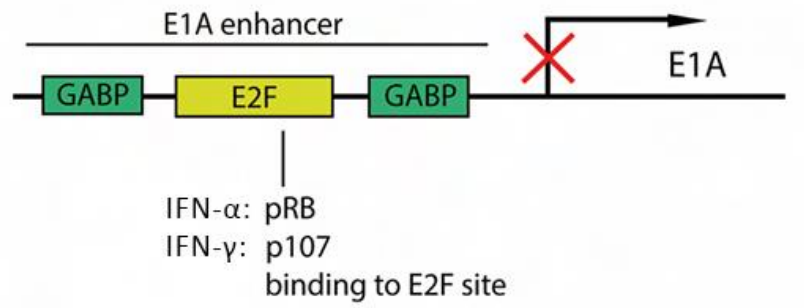

Figure 1. 5. Inhibition of E1A gene expression through IFN-α or IFN-γ

In a persistent infection model with fibroblasts, the infected cells were cultivated for more than 100 days, regularly supplied with IFN-γ. When IFN-γ was withdrawn, the virus load escalated. On the other side, the cells which were supplied with IFN-α died after 45 days and the reason for that was unknown. These findings suggest that IFN-γ may play a major role in keeping

HAdV in a persistent state and preventing it from reactivation.[54] These studies indicate that different IFNs might play a role for the repression of the virus.

1.3. Human adenovirus infections in immunosuppressed individuals

1.3.1. Opportunistic virus infections and risk for severe diseases

Immunosuppressed individuals like HSCT or organ recipients have a major risk for the reactivation of persistent virus infections which can be life-threatening.[9,13,14,49] Due to administered immunosuppressive agents, to reduce rejection of the donor material or graft-versus-host disease (GvHD), the viruses take the opportunity to reactivate. This so-called opportunistic infection can be caused by CMV, EBV, BKV, HIV but also by HAdVs.[9,13,46–49,61,61]

In immunocompetent individuals HAdVs usually cause mild and self-limiting infections, nevertheless severe and even fatal courses have been reported, but generally they are cleared by the innate immune system. Reactivation of persistent HAdV infections can be life-threatening for immunocompromised individuals.[9] Diseases caused by HAdVs are more severe in immunocompromised then in immunosuppressed individuals like hepatitis, encephalitis, or pneumonia (see Table 1.2).[9,39,40] Specific cohort are pediatric HSCT-recipients in which HAdV opportunistic infections seem to be a specific problem. These infections have been described in adult and pediatric HSCT-recipients, nevertheless the occurrence of severe invasive infections is noticeably higher in pediatric patients.[14] It has been shown that individuals whose HAdV DNA copies exceeds $1x10^6$ copies per gram stool represent a 70% higher risk for developing adenoviremia, than those with lower HAdV DNA copies per cell.[10] Furthermore when increased virus shedding takes place before transplantation, this implies that there is a huge possibility that HAdV-related complications might occur in these patients. Currently, there are data available that show that HAdVs persistence and reactivation under immunosuppression occurs in the intestine of pediatric patients. This indicates that GI may not be the main place of adenoviral persistence and reactivation, nevertheless for pediatric HSCT-recipients, this represents the main issue of morbidity and mortality.[13,14,49,62]

Table 1. 2. Adenovirus infections associated with disease in immunocompetent and immunocompromised individuals [9,39,40]

Immunocompetent individuals	Immunocompromised individuals
Acute febrile pharyngitis	Hepatitis
Gastroenteritis	Gastroenteritis
Keratoconjunctivitis	Encephalitis
	Pneumonia
	Disseminated disease

1.3.2. Immunosuppressive agents

Immunosuppressive agents are a class of drugs that are used to inhibit the functions of the immune system. These drugs are used in the treatment of autoimmune and immune-mediated diseases and transplantation. The immunosuppressive drugs can have different targets like glucocorticoid receptors, DNA, calcineurin, c-Jun N-terminal kinases (JNK), p38 kinase and many different proteins. In clinics, transplant recipients are treated with immunosuppressive agents and corticosteroids, which causes depletion of T cells and NK cells, that are known interferon-γ producing cells.[63,64]

Immunosuppressive agents for which is known that they inhibit the production of IFN-γ are calcineurin inhibitors (CNIs). Additionally, they inhibit the production of other cytokines like IL-2, IL-4, tumor necrosis factor α (TNF-α). CNIs inhibit the action of calcineurin, which is an enzyme that activates T-cells of the immune system. Currently known and used CNIs are Cyclosporine (Cyclosporine A; CsA) and Tacrolimus. CsA is an antifungal agent that was first isolated from two strains of fungi (*Cylindrocarpon lucidum* Booth and *Trichoderma polysporum* Rifai) and has been introduced for clinical use in the 1980s. Tacrolimus was isolated in 1984 from the fermentation broth of *Streptomyces* tsukubaensis, and it is introduced into clinics in the 1990s.[65,66]

The activity of calcineurin is dependent on the level of cytosolic Ca2+. Increased level of Ca2+ leads to the activation of calcineurin, which in turn dephosphorylates the cytoplasmic NFAT transcription factor. CsA and Tacrolimus have similar mechanisms of action.[66] CsA binds cyclophilins and Tacrolimus binds FK-506 binding protein (FKBP12). This binding has constituted a complex that inhibits the activity of calcineurin. This prevents the dephosphorylation and following translocation of NFAT into the nucleus prevented. Inhibition of calcineurin activity leads to reduced transcription of cytokines.[67] Activation of NFAT transcription factors is prevented and with this also cytokine production. In addition to the

calcineurin-NFAT pathway, calcineurin inhibitors also prevent IκB degradation which has a consequence that NF-κB. Also, they block the activation of JNK and p38 (MAPK14) signaling pathways (Figure 1.6).[66,67]

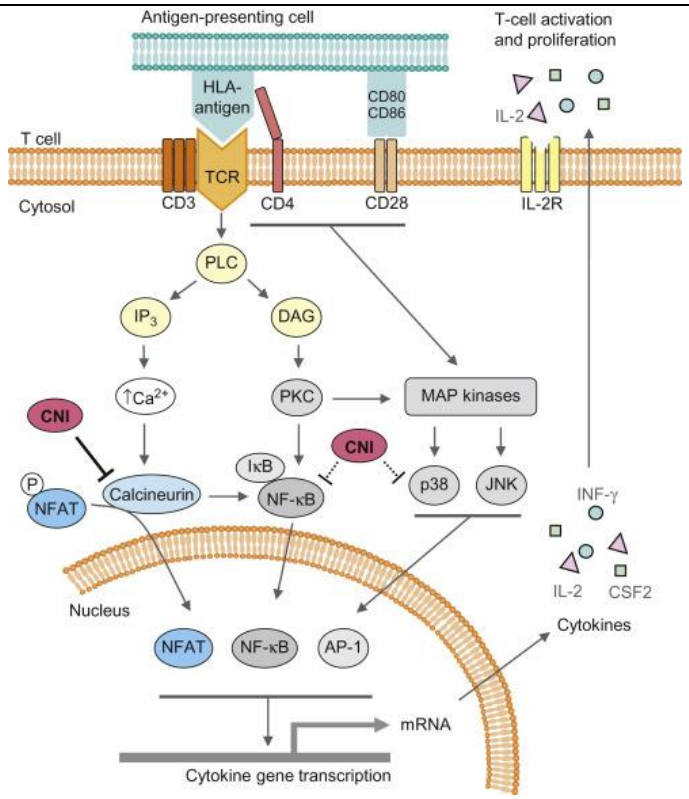

Figure 1.6. Mechanism of action of CNIs.[66]

Corticosteroids are anti-inflammatory immunosuppressive drugs used in clinics. They interact with NF-κB and AP-1 families of transcription factors, but also with the Jak-STAT pathway, and in this way inhibit secretion of various cytokines and chemokines like IL-2, IL-6, IFN-γ, and IL-8. An immunosuppressive drug which is used in clinics is Hydrocortisone (HCor) which is known for its downregulation and inhibitory function in the NF-κB signaling pathway.[68–70]

1.3.3. Therapy against HAdV

Depending on the situation of the individual patient, the optimal time for antiviral treatment is determined.[14] Based on the data published in the last 15 years, a group of experts recapped a set of recommendations for the treatment of HSCT-recipients based on their situation.[62] Currently there is no reliable effective therapy against HAdVs available, compared for example to herpes viruses.[13,14,71] There are few antivirals against DNA viruses used in the setting of HSCT-recipients like cidofovir and ribavirin, but also brincidofovir (a derivate of cidofovir).[13,14]

Cidofovir is an acyclic nucleoside monophosphate and cytosine analog nucleotide that is phosphorylated when it reaches the cell competes with cytosine to be incorporated into a viral DNA leading chain. Successful incorporation of cidofovir into a DNA chain leads to termination and inhibition of viral DNA synthesis.[35,72] Cidofovir is a standard antiviral drug used for treatment of adenoviremia. Nevertheless, it has been shown that the cellular uptake of cidofovir is poor and higher concentrations of it can lead to toxicity. Still it is used since there is no other more effective antiviral drug.[13,14] Brincidofovir, a lipid-conjugated derivative of cidofovir showed better antiviral activity in controlling adenoviremia and cellular uptake in the HSCT-recipients, whereas in some patients diarrhea occurred.[13,73]

Ribavirin is a synthetic guanosine used against several RNA and DNA viruses. It resembles RNA nucleotide guanosine when it is metabolized and can interfere with RNA metabolism required for viral replication. More than five direct and indirect mechanisms have been proposed for its mechanism of action.[74] For the treatment of HAdV infections, ribavirin showed low or no efficiency and toxicity.[13,14]

The recent approach which showed promising results is the transfer of donor-derived HAdV specific cytotoxic T cells (CTLs). With this immunotherapy donor-derived CTLs are enriched *ex-vivo* and injected into the blood of the HSCT-recipients. The use of CTLs as immunotherapy resulted in a helpful inhibition of HAdV infection in some patients, but there are still some limitations.[14,75–77]

Optimization of current and emergence of the new therapeutic strategies is needed considering that ones currently utilized are not efficient. For new therapeutic strategies a better understanding of the mechanisms of HAdV persistence and reactivation. This would be beneficial to specifically target the reactivation of HAdV in HSCT-recipients.[13,14]

1.4. Aims

In the setting of pediatric HSCT-recipients, the shedding of HAdV into stool and intestinal infections correlate with severe manifestations such as viremia leading to disseminated disease and high mortality.[13,14] Moreover, HAdV persistence has been shown in pediatric intestinal tissue and a previous study revealed the repression of E1A expression by IFN-γ in bronchial epithelial cells and fibroblasts, revealing a possible mechanism for HAdV persistence.[54]

The administration of immunosuppressive agents, inhibiting cytokine expression, in HSCT-recipients might promote the reactivation of HAdVs in the gut. To investigate more about the mechanism of HAdV persistence it was planned to characterize adenoviral infections in intestinal epithelial cells and the antiviral effect of IFN-γ in these cells.[13,14] Additionally, the impact of immunosuppressive agents on HAdV production in PBMCs was examined.

Following investigations were performed:

1. To characterize HAdV infection in intestinal epithelial cells (primary and cancer cell lines) RQ-PCR analysis detecting the HAdV hexon gene and the E1A mRNA expression were employed. Moreover, the specific receptor (CAR) and IFN-γ mRNA expression has been assessed in these cells.
2. Investigation of the inhibitory effect of IFN-γ on HAdV propagation in intestinal epithelial cells (primary and cancer cell lines). The effect of INF-γ on adenoviral replication and on the formation of infectious HAdV particles was studied by RQ-PCR and the TCID50 assay, respectively.
3. The assessment of HAdV replication in peripheral blood mononuclear cells (PBMCs) and IFN-γ mRNA expression upon treatment with immunosuppressive agents (Cyclosporine A and Hydrocortisone) has been investigated by RQ-PCR.

2. Materials and Methods

2.1. Cell culture

The human cells were handled in the laminar flow biosafety cabinet and grown in sterile cell culture plates or bottles at 37°C, in humidified air containing 5 % CO_2. All cell media were pre-warmed to 37°C before application to the cells and changed every second or third day. In addition, all plastic ware, buffers or media that came in contact with HAdV were discarded in a separate waste container and the laminar airflow was decontaminated with UV light for 45 minutes.

2.1.1. General equipment

Laminar flow biosafety cabinet LP2-4S1 (Labculture®)
Serological pipettes (VWR®)
Beads Bath (Lab Armor™)
Freezer -80°C Forma 88000 Series (Thermo Scientific™)
Freezer -20°C Premium no–frost (Liebherr)
Microscope (Motic AE21)
Tissue culture Flask 75 cm^2, treated, w/vent cap, sterilized (VWR®)
Cell culture dish 100mm, surface-modified polystyrene (Corning® Primaria™)
Falcon tubes 15 ml and 50 ml, canonical bottom, sterile (Corning® Falcon®)
Centrifuge 40 (Heraeus™ Megafuge™)
Incubator 150i CO2 with stainless-steel chambers (ThermoFisher Heracell™)
Centrifuge 5424 (Eppendorf®)
Safe-lock tubes 1.5 and 2 ml (Eppendorf®)

2.1.2. General reagents

Dulbecco's Modified Eagle Medium (DMEM) high glucose, GlutaMAX™ (Thermo Fisher Scientific™ Gibco™)
Dulbecco's phosphate-buffered saline (DPBS), no calcium, no magnesium (Thermo Fisher Scientific™ Gibco™)
Trypsin 2.5% 1X no phenol red (Thermo Fisher Scientific™ Gibco™)
Accutase - Enzyme Cell Detachment Medium (Thermo Fisher Scientific™ Invitrogen™)
Fetal Bovine Serum (FBS), qualified, heat inactivated; origin Brazil (Thermo Fisher Scientific™ Gibco™)
Pen-Strep – Penicillin Streptomycin - 10.000 Units/ml Penicillin; 10.000 µg/ml Streptomycin (Thermo Fisher Scientific™ Gibco™ by Life Technologies)
Medium 199 (10X) (Thermo Fisher Scientific™ Gibco™)
Cosmic Calf Serum (CCS), US origin; heat inactivated SH30087.03HI (THP Medical Products)

Gentamicin Solution 50 mg/ml G1397-10ML (Sigma-Aldrich)
Epidermal Growth Factor (EGF), human recombinant, 100µg (Corning®)
Hydrocortisone - Water-Soluble H0396-100MG (Sigma-Aldrich;)
Insulin-Transferrin-Selenium (ITS-G) 100X (Thermo Fisher Scientific™ Gibco™)
HEPES 1M (ThermoFisher Scientific™ Gibco™)
Sterile Water (Aqua bidest)
AF liquid (Mikrozid®)
Cell culture media (Table 2.1.)
Cell lines and virus (Table 2.2.)

Table 2. 1. Cell culture media

Media	Material	Stock Conc.	Final Conc.
DMEM 10 % FBS			
Used for HCT116 and A549 cell lines	**DMEM high glucose**		
	FBS		**10 v/v % ml medium**
	Pen-Strep	**10.000 Units/ml Penicillin; 10.000 µg/ml Streptomycin**	**1 v/v % ml medium** **100 Units/ml Penicillin; 100 µg/ml Streptomycin**
DMEM 20 % FBS	**DMEM high glucose**		
Used for CaCo2 cell line	**FBS**		**20 v/v % per ml medium**
	Pen-Strep	**10.000 Units/ml Penicillin; 10.000 µg/ml Streptomycin**	**1 v/v % ml medium** **100 Units/ml Penicillin; 100 µg/ml Streptomycin**
1CT cell medium			
	DMEM high glucose		
	Medium 199 10x	**10X**	**10 ml/500 ml medium**
	Sterile water		**90 ml/500 ml medium***
	CCS		**2 v/v % per ml medium**
	Gentamicin	**50 mg/ml**	**60 µg/ml medium**
	EGF	**100 µg/ml**	**0,02 µg/ml**
	Hydrocortisone	**5 mg/ml**	**1 µg/ml**
	ITS-G	**100X**	**1X per ml medium**
	HEPES 1M	**1 M**	**20 mM per ml medium**

** 90 ml of water was added to dilute Medium 199 10x.*

Table 2. 2. Cell lines and virus

Name	Origin	Reference
HCT116	Human colon carcinoma	ATCC® CCL-247™;
CaCo2	Human colon carcinoma	ATCC® HTB-37™;
A549	Human lung carcinoma	DSMZ® ACC 107
HCEC-1CT	Human colon epithelium	Roig et al. 2010;
Human adenovirus 2	Clinical patient	(ATCC® VR-1786™)

2.1.3. Thawing and freezing of cells

Reagents

DMSO (SERVA; Dimethyl sulfoxide research grade 20385.01)

Equipment

Biobanking and Cell Culture Cryogenic Tubes1.8 ml (Thermo Scientific™ Nunc™)
LX Cell Freezing Container 12 x 1 mL or 2 mL cryogenic vial (Sigma-Aldrich Corning® CoolCell™)

Procedure – Thawing

- A cryogenic tube with frozen cells was taken out from the freezer (-80°C).
- 14 ml of the appropriate cell culture medium were pipetted into a falcon tube.
- 13 ml of the appropriate cell culture medium were pipetted into a culture flask/plate.
- Cells were allowed to thaw for 5 minutes on room temperature (RT).
- Immediately after cells started to thaw, 500 µl of cell culture medium was pipetted into the tube and mixed by pipetting. This procedure was done quickly to avoid cell lysis by DMSO. The cells suspension was transferred to the prepared tube with a 13 ml cell culture medium.
- Cells were centrifuged for 5 minutes at 1600 rpm.
- The supernatant was poured out and the pellet was resuspended in 1 ml cell culture medium.
- The whole-cell suspension was transferred into a cell culture flask/plate (1CT cells were cultivated in Primaria™ cell culture dishes, whereas all other cell lines were cultured in culture flasks).
- Cells were gently shaken to spread evenly in the flask/plate and incubated overnight in the incubator.

Procedure- Freezing

- Confluent cells were washed with 5 ml of PBS.
- 3 ml of Trypsin or Accutase[III] were added.
- Cells were incubated for 5 minutes in the incubator and then collected with 12 ml of cell culture medium into a 50 ml falcon tube.
- Centrifuged (5 min/1600 rpm).
- The supernatant was poured out the cell pellet was resuspended in 1 ml of freezing medium- The freezing medium consists of 50% FBS/CCS[IV] + 40% cell culture medium + 10% DMSO.
- The cell suspension was transferred into a 1.8 ml cryogenic tube.
- The tube was put in a cell freezing container (all empty wells were fulfilled with empty tubes) and stored in the freezer at -80°C. In the next days, frozen cells were transferred to the liquid nitrogen tank for longer storage.

2.1.4. Cells splitting

Principle

Confluent cells cover the complete surface of a flask or plate. For further cultivation they have to be split in different ratios (1:10, 1:5 or 1:3), depending on their growth kinetics.

Reagents

Trypan blue dye 0,4% (Bio-Rad)

Procedure

- The cell culture medium was taken out from the culture flask/plate.
- Cells were washed with 5 ml of PBS.
- 3 ml of Trypsin or Accutase were added.
- Cells were incubated for 5 minutes in the incubator at 37°C, 5% CO_2 and subsequently collected with 12 ml of cell culture medium in a 50 ml falcon tube.
- Cells were centrifuged (5 min/1600 rpm).

[III] To detach cells from the culture dish, Trypsin was used for all cells except for 1CT cells Accutase was applied.
[IV] For 1CT cells.

- Into a new culture flask/plate 12 ml of cell culture medium were added and part of the resuspended cell pellet was given to this new flask/plate.
- The cells were evenly distributed in the flask/plate and incubated overnight in the incubator.

2.1.5. Human Adenovirus titration with endpoint dilution assay

Principle

Tissue culture infection dose 50 ($TCID_{50}$) assay was performed to measure the virus titer of infectious virus particles. $TCID_{50}$ quantifies the amount of virus required to achieve a cytopathic effect in 50% of cells.[78] A549 cells, as known producer cell line for adenovirus were infected with HAdV. Virus titer was calculated according to the Spearman-Käber method.[79]

Equipment

96-well Tissue Culture Plates, Surface treated, Sterile (VWR®)
Biobanking and Cell Culture Cryogenic Tubes1.8 ml (Thermo Scientific™ Nunc™ 375418)
Cell Scraper 25 cm Sterilized by Gamma Irradiation (VWR®)
8-Channel, variable pipette 10 – 100 µL, yellow (Eppendorf Research®)
Plastic Reagent Reservoir 50 ml, pre-sterile (VWR®)

Reagents

DMEM 2% FCS cell culture medium

Procedure – Virus propagation

- Subconfluent A549 cells in a 75 cm2 culture flask were infected with 200 µl of HAdV stock solution (containing about $1x10^6$ virus particles/µl).
- After a few days when cells started to lyse and swim in the medium, the rest of the attached cells were harvested using a cell scraper and collected into a 50 ml falcon tube.
- Freezing and thawing was done three times to provide completely lysed cells and have all virus particles released in the supernatant.
- Centrifuged for 10 minutes at 400g.
- 600 µl of the supernatant was aliquoted in cryogenic tubes and stored at -80°C.

Procedure – $TCID_{50}$ assay

- The cell culture medium was taken out from the cell culture flask with confluent A549 cells.
- Cells were washed with 5 ml of PBS.
- 3 ml of Trypsin were added, and cells were incubated for 3 minutes in the incubator.
- Cells were collected with 12 ml of cell culture medium into a 50 ml falcon tube and centrifuged (5 min/1600 rpm).
- The supernatant was poured out and the cell pellet was resuspended in 1 ml of DMEM 2% FBS[V] cell culture medium, afterwards 39 ml of medium were added additionally.
    - One culture flask with confluent A549 cells is necessary for seeding cells in four 96-well plates.
- 40 ml of cell suspension were poured out into a 50 ml plastic reagent reservoir. Using a multichannel pipette, 90 µl of cell suspension/well was pipetted in 96-well plates.
- Cells were incubated for 24 hours in the incubator.
- 10 µl of HAdV was added in each well of the first column and mixed by pipetting.
    - If it is needed, the virus should be diluted in DMEM (without supplements).
- From the wells of the first column 10 µl was transferred in the wells of the second column. The same was done until the 11th column. The 12th was left uninfected (mock).
- Cells were infected for seven days. On the seventh day, cells were examined under the microscope to check how many wells show a cytopathic effect. The results were documented for calculation.
- Virus titer was calculated according to the Spearman-Käber method (Spearman, 1908; Käber, 1931)[79].

2.1.6. In-vitro infection of mammalian cells with HAdV

Principle

Mammalian cells were infected with different multiplicity of infection (MOI) of HAdV. MOI represents the ratio between the number of viruses and the number of cells to be infected.[80] Prior infection, cells need to be in a subconfluent state in order to have a better infection.

[V]To slow down cell growth 2% FBS medium was used.

Equipment

6-well cell clear flat bottom surface-modified multi-well culture (Corning® Primaria™)
6-well tissue culture plates, surface treated, sterile (VWR®)
12-well tissue culture plates, surface treated, sterile (VWR®)
Automated cell counter TC20™ (Bio-Rad)
Counting slides, dual chamber for cell counter (Bio-Rad)

Procedure – Seeding and infection

- The cell culture medium was removed, and cells were washed with 5 ml of PBS.
- 3 ml of Trypsin or Accutase were added and cells were incubated for 5 minutes in the incubator at 37°C, 5% CO_2.
- Cells were collected with 12 ml of cell culture medium into a 50 ml falcon tube and cells were centrifuged (5 min/1600 rpm).
- A new culture flask/plate was prepared with 12 ml of cell culture medium.
- After centrifugation, the supernatant was poured out and the pellet was resuspended in 1 ml of cell culture medium for cell counting.
- 10 µl of the cell suspension was mixed with 10 µl of Tryptan blue dye into a 1.5 ml tube.
- 10 µl of the mixture was loaded on the counting slide and inserted into the cell counter.
- The number of cells seeded per well:

Table 2. 3. Number of cells seeded in plates

Cell line	Number of cells/well	Plate
1CT	$5x10^5$ (2 ml cell culture medium/well)	6 well plate (Primaria™)
HCT 116, CaCo2	$2x10^5$ (2 ml cell culture medium/well)	6-well plate (VWR®)
	$5x10^5$ (1 ml cell culture medium/well)[VI]	12-well plate (VWR®)
A549	$2x10^5$ (2 ml cell culture medium/well)	6-well plate (VWR®)

- To calculate the volume of cell suspension we needed for a specific number of cells, this formula was used:

$$x = \frac{\text{number of cells needed} * \mathbf{1\ ml}}{\text{number of cells}/\mathbf{1ml}}$$

- The calculated volume (x) of cell suspension was transferred into a falcon tube with cell culture medium and mixed using a serological pipette.

[VI] To have cells in confluent state, more cells were seeded in a smaller wells.

- 2 ml/well (6-well plate) or 1 ml/well (12-well plate) were/was applied.
- 1CT and A549 cells were incubated in the incubator at 37°C, 5% CO2 for 24 h prior to infection, whereas HCT116 and CaCo2 cells needed 48 h to be completely attached and ready for further infection.
- The infection of mammalian cells was done with different MOIs ranging from 10 to 500. The individual volume of HAdV added per well was calculated using these equations.

$$number\ of\ cells\ per\ well * \ MOI = needed\ number\ of\ viruses\ per\ well$$

$$x(\mu l\ virus/well) = \frac{needed\ number\ of\ viruses\ per\ well * 1\ \mu l}{virus\ titer\ (stock\ solution)}$$

- The calculated volume of the virus was mixed with 1 ml (6-well plate) or 0.5 ml (12-well plate) of DMEM (without supplements) in a falcon tube.
- Cell culture medium was taken out from the wells which should be infected and 1 ml or 0.5 ml per well of the prepared and diluted HAdV was pipetted to the cells.
- Cells were incubated for 2 hours in the incubator.
- After 2 hours of incubation, medium with the virus was taken out and 2 ml or 1 ml of culture medium was pipetted to the cells.
- Cells were returned in the incubator.

Procedure – Harvesting

- The cell medium was taken out from the well and cells were washed with 500 µl of PBS.
- 500 µl or 250 µl of Trypsin were added and cells were incubated for 5 minutes in the incubator.
- 1 ml of cell culture medium was added and cells were collected into a 1.5 ml tube.
    - When necessary, the cell suspension was divided into two tubes, one for DNA and another one for RNA extraction.
- Cells were centrifuged (5 min/1600 rpm).
- The supernatant was removed and the pellet was resuspended:
    - For DNA isolation cells were resuspended in 200 µl of PBS and stored at -20°C.
    - For RNA isolation cells were resuspended in 350 µl of RLT+DTT buffer and stored at -80°C.

2.1.7. Incubation of intestinal epithelial cells with IFN-γ prior to HAdV infection

Principle

We wanted to investigate whether IFN-γ inhibits HAdV in intestinal epithelial cells. Cells were incubated with IFN-γ 24 hours before infection, based on previously reported studies.[54,59]

Equipment

6-well Cell Clear Flat Bottom Surface-Modified Multiwell Culture (Corning® Primaria™)
6-well Tissue Culture Plates, Surface treated, Sterile (VWR®)
12-well Tissue Culture Plates, Surface treated, Sterile (VWR®)
Automated Cell Counter TC20™ (Bio-Rad)
Counting Slides, Dual Chamber for Cell Counter (Bio-Rad)

Reagents

IFN-γ Recombinant Human Protein 100 µg $2.0x10^6$ to 3.3x105 units/mg (Thermo Fisher Scientific™ Gibco™)

Procedure

- Cells were seeded, as written in the chapter 2.1.6.
- 24 hours before infection and approximately 5 hours after seeding the cells, 2 µl (6-well plates) or 1 µl (12-well plates) of IFN-γ (1000 U/ml medium) were/was pipetted per well.
- Gently mixed by shaking and the plate was returned in the incubator.
- The cells were incubated for 0, 3- and 6-days post-infection. To all cells incubated for 6 days, additional 2 µl of IFN-γ were added on the third day after infection.

2.1.8. Model for persistent HAdV infections in human intestinal epithelial cells

Principle

Our aim was to establish a model of a persistent HAdV infection in 1CT cells. For this purpose, the infected cells were cultivated for nine weeks under treatment with IFN-γ. In addition, reactivation upon withdrawal of IFN-γ after five weeks infection was examined by analyzing the HAdV DNA copies.

Procedure

- 1CT cells were seeded, incubated with IFN-γ and infected like it is explained in chapters 2.1.6. and 2.1.7.
- Seeded cells in the 6-well plates for the persistence model were splitted with Accutase[VII] as described in chapter 2.1.6.After the centrifugation step, 1 ml of cell culture medium was added to the pellet and cells were resuspended.
- 1/3 µl of the cell suspension was transferred into a 1.5 ml tube and centrifuged for 5 minutes at 1600 rpm. The pellet was resuspended in 200 µl PBS and stored at -20°C for later DNA isolation.
- Another 1/3 µl of the cell suspension was also transferred into a 1.5 ml tube, centrifuged for 5 minutes at 1600 rpm and the cells were resuspended in 100 µl of cell culture medium and seeded in a new well of the 6-well plate. The rest of the cell suspension was discarded.
- 24 h after splitting the cells, they were further incubated with fresh 2 µl IFN-γ (1000U/ml). At 96 h after splitting 2 µl IFN-γ (1000U/ml) were added again. Once a week the cells of this well were splitted and IFN-γ was added like described above. This procedure was repeated until day 62 and aliquots of cells in PBS have been stored every week for further DNA extraction.
- At day 42 post-infection the part of cells, which was discarded the weeks before was also seeded in another well in the 6-well plate. These cells were cultivated without IFN-γ and harvested aliquots were stored at day 43, 48 and 53 in 200 µl at -20°C for further DNA extraction.

2.1.9 Assessment of infectious HAdV particles in 1CT cells upon IFN-γ incubation

Principle

Exploiting the $TCID_{50}$ assay, the inhibition of infectious HAdV particle production upon IFN-γ incubation in 1CT cells has been examined. For the $TCID_{50}$ assay, A549 cells were seeded and then infected with the lysate of harvested 1CT cells.

[VII] Because these cells were further cultivated, instead of Trypsin, Accutase was used. Hence, Accutase is not harsh for the cells like Trypsin.

Equipment

6-well Cell Clear Flat Bottom Surface-Modified Multiwell Culture (Corning® Primaria™)
96-well Tissue Culture Plates, Surface treated, Sterile (VWR®)
Cell scraper 25 cm sterilized by gamma irradiation (VWR®)

Reagents

IFN-γ recombinant human protein 100 µg, $2.0x10^6$ to $3.3x10^5$ units/mg (Thermo Fisher Scientific™ Gibco™)

Procedure

- 1CT cells were seeded into a 6-well plate, incubated with IFN-γ and infected with HAdV at an MOI of 100. This was performed as described in the chapters 2.1.6 and 2.1.7.
- Subsequently, after infection for 2 h, the cells were washed with PBS two times to remove any residual virus particles from outside of the cells.
- Cells were harvested using a cell scraper at day 0 (2 h post-infection), 3 and 6 post-infection and gently scraped from the surface of the well.
- The cells and the supernatant were collected together into a 15 ml falcon tube and stored at -20°C.
- Cells were three times frozen and thawed in order to destroy the cell walls and to release all virus particles into the supernatant.
- The cell lysate was centrifuged for 10 minutes at 400 g.
- 1 ml of the supernatant was pipetted into a 1.5 ml tube and stored in the fridge at 6°C.
- TCID50 assay was performed on A549 cells, following the procedure described in chapter 2.1.5. ($TCID_{50}$ assay) and the virus titer was calculated.

2.1.10. Isolation of peripheral blood mononuclear cells (PBMCs)

Principle

Isolation of PBMCs was done by Ficoll gradient centrifugation. Ficoll aggregates the erythrocytes, thereby increasing their sedimentation rate. The sedimentation of leukocytes is only slightly affected and nucleated cells, particularly mononuclear cells, can be collected from the upper part of the tube when the erythrocytes have settled.

Equipment

Heparin coated tubes 9 ml (Greiner Bio-one)
Sysmex cell counter (KX-21N, Sn. A2842)

Reagents

Blood from healthy donors
Ficoll-Paque™ PLUS density 1.077 g/mL (GE Healthcare)
RPMI 1640 medium, no phenol red (Thermo Fisher Scientific™ Gibco™)

Procedure

- 15-20 ml of blood were transferred into a 50 ml falcon tube and were diluted with PBS (1:1).
- 20 ml of Ficoll were pipetted in two 50 ml falcon tubes.
- Ficoll reagent was overlaid with 15-20 ml of diluted blood in each falcon tube. For this purpose, the tube was held at an angle of 45°, the pipette was leaned on the wall of the falcon tube and the blood was pipetted very slowly on the top of the Ficoll reagent. This was done in order to prevent the mixing of blood and Ficoll.
- Cells were centrifuged for 20 minutes at 2200 rpm without brake (acceleration and deceleration at 1) to prevent the mixing of separated layers.
- 10 ml of PBS were prepared in a fresh falcon to collect and wash all PBMCs.
- After centrifugation, the cells were collected using a 1 ml pipette and transferred in the already prepared falcon tube with 10 ml of PBS. This step was done carefully in order to collect only the cells without Ficoll reagent.
- Cells were centrifuged (5 min/1600 rpm) to wash the cells.
- The supernatant was carefully poured out and cells were resuspended in 5 ml of RPMI medium.
- 50 µl of the cell suspension was taken into a 1.5 ml tube and cells were counted using the Sysmex cell counter.

2.1.10.1. HAdV infection of PBMCs treated with immunosuppressive agents

Principle

To investigate the promoting effect of immunosuppressive agents on HAdV propagation in PBMCs, the cells were infected with the virus and subsequently treated with Cyclosporine A

(CsA) and Hydrocortisone (HCor). CsA and HCor are supposed to repress cytokine expression, thereby these drugs might also support the HAdV replication.

Equipment

24-well tissue culture plates, surface treated, sterile (VWR®)

Reagents

RPMI 1640 Medium, no phenol red (Thermo Fisher Scientific™ Gibco™)
+ 10 v/v % ml medium FBS
+ 1 v/v % ml medium Pen-Strep
Cyclosporine A 1 mg/ml (Sigma Adrich)
Hydrocortisone 50 µM (Sigma Adrich)

Procedure – Seeding, infection and adding of immunosuppressive agents

- Depending on the number of isolated cells, 1 or 2 million cells per well were seeded in a 24-well plate in 1ml RPMI Medium + 10% FCS and 1% Pen/Strep.
- To calculate the cell density in each well and the virus copies needed for MOI 100 the same formulas were used as described in the chapter 2.1.6.
- The calculated volume of the virus was added directly into the medium. The medium was not changed.
- After 24 hours of infection, immunosuppressive agents were added and on the third day after the first addition using the following concentrations described in the Table 2.4.

Table 2. 4. Immunosuppressive agents

Immunosuppressive agent	Stock concentration	Final concentration
Cyclosporine A	1 mg/ml diluted[VIII] to 100 µg/ml	0,1 µM, 1 µM/ml medium
Hydrocortisone	50 µM	50 nM, 250 nM/ml medium

Procedure – Harvesting

- After six days of infection, the cells were harvested by pipetting to detach adhered monocytes from the surface of the well.
- The cell suspension was divided into two 1.5 ml tubes. One for DNA and another for RNA extraction.

[VIII] Diluted in DMSO.

- Cells were centrifuged for 5 minutes at 2200 rpm.
- The supernatant was taken out and the cells were resuspended.
    - For the DNA extraction cells were resuspended in 200 µl PBS and stored at -20°C.
    - For the RNA extraction cells were resuspended in 350 µl RLT+DTT buffer and stored at -80°C.

2.2. Analysis of adenoviral DNA

Principle

The number of DNA copies from the genes of interest was calculated in Microsoft Excel using the obtained Ct-values, and previously investigated standard curves of the gene (including slope and Y-intercept). Y-intercept represents the theoretical detection limit of the reaction and slope represents the amplification efficiencies. This is the point at which the standard curve intersects with the ordinate. The value of the Y-intercept for the hexon genes of HAdV-C is 39,33 and -3,36 of the slope. For the human housekeeping gene beta2-microglobulin (β2MG) the value of Y-intercept is 39,5 and -3,57 of the slope.[81]

The number of detected DNA molecules of HAdV was normalized to the number of B2MG. The following formula was used:

$$\frac{HAdV\ hexon\ gene\ copies * \ 2E+6}{B2MG\ gene\ copies} = HAdV\ DNA\ copies\ in\ 1E+6\ cells$$

2.2.1 DNA extraction

Principle

For the isolation of DNA from infected cells the QIAamp® DNA Mini Kit was used. Cell wall and cellular proteins were lysed by Proteinase K. To ensure the binding of the DNA, Ethanol 96% was added and the DNA was adsorbed on a silica membrane. After washing away possible contaminants, the bounded DNA was eluted from the column by enriching the salt concentration.[82]

Equipment

QIAamp DNA Mini Kit (Qiagen)
Thermomixer Compact T1317 with 1.5 mL block, AC/DC input 115 V AC (Sigma Eppendorf®)

Reagents

QIAamp® DNA Mini Kit (Qiagen, Germany)
- Proteinase K
- Buffer AL
- Buffer AW1
- Buffer AW2
- Buffer AE

Ethanol 96% (Merck, Emprove®)

Procedure

- 20 µl of Proteinase K and 200 µl of AL Buffer were added in the tube with cells resuspended in 200 µl of PBS. Mixed by pulse-vortexing for 5-10 seconds.
- Samples were incubated in the thermomixer at 56°C for 10 minutes.
- Samples were shortly centrifuged (5 seconds) to remove drops from the inside of the lid.
- 200 µl of 96% Ethanol 96% were added and mixed by pulse-vortexing for 10 seconds. After mixing, briefly centrifuged to remove drops from the inside of the lid.
- The mixture from the step above was applied to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Centrifuged at 8000 rpm for a 1 minute.
- A collection tube with the filtrate was discarded and the QIAamp Mini spin column was placed into a clean 2 ml collection tube.
- 500 µl of Buffer AW1 was added in QIAamp Mini spin column and centrifuged at 8000 rpm for a 1 minute. The filtrate was discarded, the QIAamp Mini spin column was placed into a clean 2 ml collection tube.
- 500 µl Buffer AW2 was added in QIAamp Mini spin column and centrifuged on 14000 rpm for 3 minutes.
- A collection tube containing the filtrate was discarded and the QIAamp Mini spin column was placed into a clean 1.5 ml tube.
- 200 µl Buffer AE was added in the QIAamp Mini spin column.
- Samples were incubated at room temperature for 1 minute and then centrifuged at 8000 rpm for 1 minute.

- Samples were either stored in the fridge at 6°C, if they are going to be used soon, or in the freezer at -20°C for long-term storage.

2.2.2. RQ-PCR

Principle

Detection of adenoviral and mammalian DNA by RQ-PCR was done using a TaqMan hydrolysis probe to increase the specificity for the target sequence.[83] This principle is based on the 5'-3' exonuclease activity of a *Taq* polymerase to cleave the dual-labeled probe which is bound to the complementary target sequence.[84] The sequence-specific primer and TaqMan probe bind to the target sequence. To the sequence-specific primer a *Taq* polymerase binds and reaction starts. The TaqMan probe has a fluorophore which is covalently attached to the 5'-end and a quencher at the 3'-end. When the reporter molecule is stimulated by an appropriate light source to emit fluorescence, the energy is transferred to the quencher, thereby suppressing the emission of fluorescence. This is a physical principle known as fluorescence resonance energy transfer (FRET) (Figure 2.1).[85]

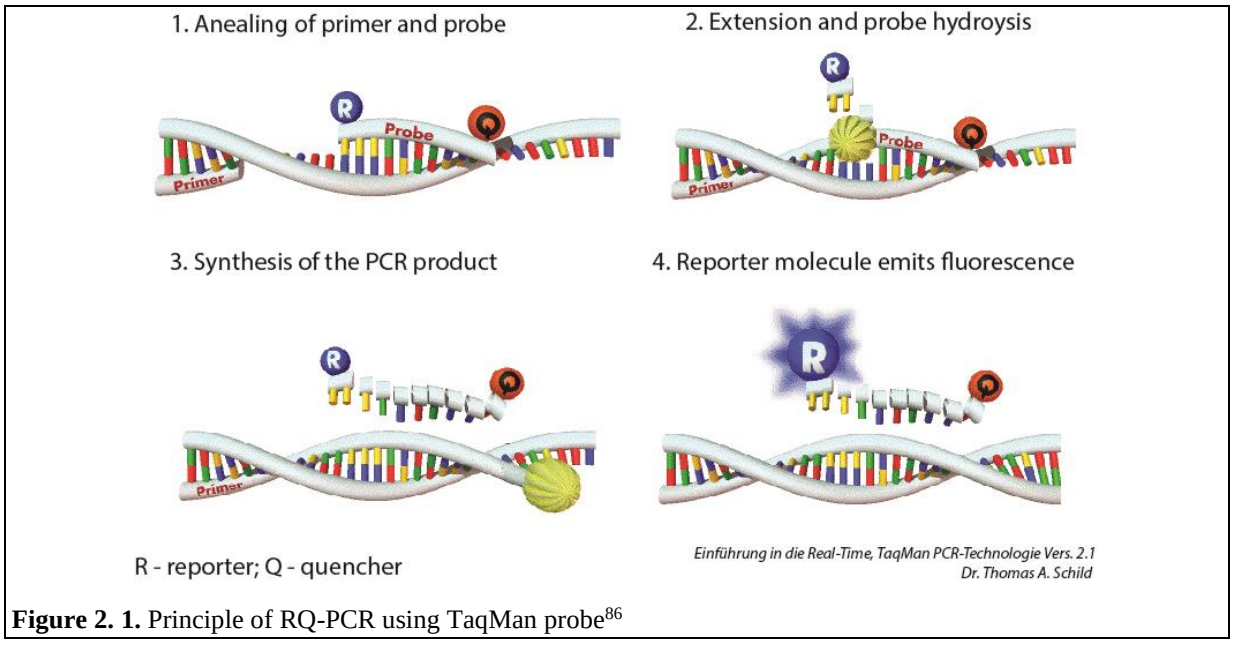

Figure 2. 1. Principle of RQ-PCR using TaqMan probe[86]

During the extension the AmpliTaq Gold® DNA polymerase cleaves the probes, thereby destroying the FRET effect by bringing the fluorophore and the quencher in a greater distance. This results in an emitted fluorescence signal by the reporter. The dNTPs with dUTP are used to prevent carryover from previous amplifications. To normalize signal fluorescence fluctuations during data analysis, ROX™ passive reference dye was used as an internal reference.[87]

Equipment

Fast optical 96-well reaction plate with barcode, 0.1 ml (Applied Biosystems™ MicroAmp™)
Optical adhesive film (Applied Biosystems™ MicroAmp™)
7500 Fast Real-Time PCR System (Applied Biosystems™)
7500 Fast System with 21 CFR Part 11 Software, Version 1.4.0.27 (Applied Biosystems™)
Microsoft Excel
Primers and probes for RQ-PCR (Table 2.5)

Table 2. 5. Primers and probes for RQ-PCR

Primer name	5‘ – 3‘	5’ labeling 3’labeling
Beta-2 MG forward	TGA GTA TGC CTG CCG TGT GA[81]	
Beta-2 MG reverse	ACT CAT ACA CAA CTT TCA GCA GCT TAC[81]	
Beta-2 MG probe	CCA TGT GAC TTT GTC ACA GCC CAA GAT AGT T[81]	FAM-TAMRA
HAdV-C forward	ACC TGG GCC AAA ACC TTC TC[81]	
HAdV-C reverse	CGT CCA TGG GAT CCA CCT C[81]	
HAdV-C probe	AAC TCC GCC CAC GCG CTA GA[81]	FAM-TAMRA

Reagents

Universal PCR Master Mix (TaqMan™)
- AmpliTaq Gold® DNA polymerase, ultra-pure
- Uracil-N glycosylase (UNG)
- dNTPs with deoxyuridiner tiphosphate (dUTP)
- ROX™ passive reference dye
- Optimized buffer components

Primers and probes (Metabion AG, Germany)

Procedure

- All primers were ordered in a dried state and were diluted in water to stock solutions of 100 pmol/µl. Primers and probes were stored in the freezer at -20°C.
- After thawing, primers and probes were briefly vortexed and centrifuged.
- Master mixes were prepared as follows (Table 2.6):

Table 2. 6. RQ-PCR master-mixes

Beta 2-MG	Volume for 60 reactions	Stock concentration	Final concentration
Universal Master-Mix	750 µl	2x	1x
Beta2-MG forward	4,5 µl	100 pmol/µl	7,5 pmol
Beta2-MG reverse	4,5 µl	100 pmol/µl	7,5 pmol
Beta2-MG probe	3 µl	100 pmol/µl	5 pmol
H_2O	378 µl		
Total:	**1.500 µl**		
HAdV-C			
MM	750 µl	2x	1x
HAdV-C forward	4,5 µl	100 pmol/µl	7,5 pmol
HAdV reverse	13,5 µl	100 pmol/µl	22,5 pmol
HAdV probe	3	100 pmol/µl	5 pmol
H2O	369 µl		
Total:	**1.500 µl**		

- After adding all ingredients, the mixture was briefly mixed and centrifuged.
- 19 µl of the appropriate master mix and 6µl DNA was pipetted in one well of the 96-well reaction plate. Each sample was done in duplicates.
- Controls: 6 µl water served as a negative control for HAdV and beta-2MG master-mix; 6µl of HAdV-C DNA (isolated from cell culture supernatants) as a positive control.
- The plate was covered with an adhesive film and briefly centrifuged to remove drops from the wall.
- The RQ-PCR was carried out at the TaqMan 7500 Fast System.

PQ-PCR setup

Table 2. 7. Temperature and time profile of the RQ-PCR program

Stage	Step	Time	Temperature	Function
Stage 1		2 minutes	50°C	UNG incubation
Stage 2		10 minutes	95°C	Taq DNA polymerase activation, DNA and UNG denaturation.
Stage 3 (50 repeats)	Step 1	15 seconds	95°C	DNA denaturation
	Step 2	1 minute	60°C	Annealing and Taq DNA polymerase activity

- The Ct-values were analyzed with the 21 CFR Part 11 Software and the evaluated Ct-values were exported in the ‘xls’ files.

- The number of DNA copies from the genes of interest was calculated in Microsoft Excel using the obtained Ct-values, and previously investigated standard curves of the gene (including slope and Y-intercept).

2.3. Analysis of adenoviral RNA

2.3.1. RNA extraction

Principle

For the isolation of RNA from mammalian cells the RNeasy® Plus Mini Kit has been used. To ensure isolation of intact RNA, cells were lysed and homogenized in a highly denaturing guanidine-isothiocyanate–containing buffer. Genomic DNA has been removed and ethanol 70% was added to provide appropriate binding conditions for RNA to the silica membrane. After washing away possible contaminants, the bounded RNA was eluted from the column by RNase free water.[88]

Equipment

RNeasy® Plus Mini Kit (Qiagen, Germany)
- gDNA Eliminator spin column
- 2 ml collection tube
- RNeasy spin column

Reagents

RLT-buffer and dithiothreitol (DTT) 40 mM (Qiagen RNeasy Plus Mini Kit)
RNeasy® Plus Mini Kit (Qiagen, Germany)
- Buffer RW1
- Buffer RPE
- RNase-free water

Ethanol 70% (Merck, Emprove®)

Procedure

- Cells resuspended in 350 µl RLT-buffer+DTT 40nM were lysed and homogenized.
- Cells were pipetted through a gDNA Eliminator spin column and placed in a 2 ml collection tube.
- Centrifuged at 10000 rpm for 30 seconds. The column was discarded, and the flow-through was saved.

- 350 µl of ethanol 70% was added to the flow-through and mixed well by pipetting.
- The mixture was transferred into an RNeasy spin column, placed in a 2 ml collection tube. The lid was closed and centrifuged at 10000 rpm for 30 seconds. The flow-through was discarded.
- 700 µl of Buffer RW1 was added to the RNeasy spin column and centrifuged at 10000 rpm for 30 seconds, to wash the spin column membrane. The flow-through was discarded.
- 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged at 10000 rpm for 30 seconds. The flow-through was discarded.
- 500 µl of Buffer RPE was added to the RNeasy spin column and centrifuged at 10000 rpm for 2 minutes. The flow-through was discarded.
  - After centrifugation, the RNeasy spin column was carefully removed from the collection tube so that the column does not contact the flow-through.
- The RNeasy spin column was placed in a new 1.5 ml collection tube. 50 µl of RNase-free water was added directly into the spin column membrane and centrifuged at 10000 rpm for 1 minute to elute the RNA.
  - RNA samples were frozen at -80°C or it has proceeded with reverse transcription.

2.3.2. DNA digestion

Principle

Before the reverse transcription reaction, DNA digestion was performed to remove residual genomic DNA. This procedure was done only with samples in which viral mRNA expression was assessed. Since adenoviral DNA has no introns and primers cannot be designed over different introns in order to include the exon of interest. Because of this, the RQ-PCR can not distinguish between genomic DNA and cDNA and DNA digestion has to be performed.

Equipment

Thermomixer Compact T1317 with 1.5 mL block, AC/DC input 115 V AC (Sigma Eppendorf®)
Ice

Reagents

RQ1 RNase-Free DNase 10X Reaction Buffer (Promega)
RQ1 RNase-Free DNAse (Promega)
RQ1 DNase Stop Solution (Promega)

Procedure

- For 8 µl of RNA, 1 µl RQ1 RNase-Free DNase 10X Reaction Buffer and 1 µl RQ1 RNase-Free DNAse were added and mixed by pipetting.
- The digestion reaction was incubated at 37 °C for 30 minutes.
- To terminate the reaction 1 µl of RQ1 DNase Stop Solution was added and incubated at 65 °C for 10 minutes to inactivate the DNase.
- Samples were centrifuged for 5 seconds to remove drops from the inside of the lid and proceeded with reverse transcription.

2.3.3. Reverse transcription

Principle

In order to analyze the isolated RNA by RQ-PCR, it must be transcribed into cDNA by reverse transcriptase. The use of a random primer ensures a reliable transcription of the complete sequence into a complementary DNA.

Equipment

Thermomixer Compact T1317 with 1.5 mL block, AC/DC input 115 V AC (Sigma Eppendorf®)
Ice

Reagents

- M-MLV Reverse Transcriptase (Promega)
- 5X RT Buffer (Promega)
- dNTP (Invitrogen)
- Recombinant RNasin® Ribonuclease Inhibitor (Promega)
- Random primers (Promega)

Procedure

- 15 µl of the RNA was pipetted into a 1.5 ml tube and incubated for 5 minutes at 70^{o}C to denature the RNA.
- Samples were shortly centrifuged for 5 seconds and then put on ice for 5 minutes.
- 15 µl of the reverse transcription mix (Table 2.8.) was mixed with the RNA and incubated for 60 minutes in thermomixer at 37^{o}C.

- cDNA samples were either stored in the fridge at 6°C, if they are going to be used soon or frozen at -20°C for long-term storage.

Table 2. 8. Reverse transcription mix

Component	Volume (15µl)	For 20 reactions	Final concentration
5X RT Buffer	6	120	**1X**
dNTP	1,2	24	**200 µM**
pd(N_6) Random Hexamers	0,75	15	**100 ng**
Recombinant RNasin® Ribonuclease	0,75	15	**25 Units**
M-MLV Reverse Transcriptase	0,75	15	**200 Units**
RNase free water	5,55	111	**-**
Total	**15µl**	**300**	

2.3.5. RQ-PCR

Principle

Detection of adenoviral and mammalian cDNA by RQ-PCR was done by using a TaqMan hydrolysis probe, as described in chapter 2.2.2.

Equipment

Fast optical 96-well reaction plate with barcode, 0.1 ml (Applied Biosystems™ MicroAmp™)
Optical adhesive film (Applied Biosystems™ MicroAmp™)
7500 Fast Real-Time PCR System (Applied Biosystems™)
7500 Fast System with 21 CFR Part 11 Software, Version 1.4.0.27 (Applied Biosystems™)
Microsoft Excel
Primers and probes (Metabion AG)

Table 2. 9. Primers and probes for RQ-PCR

Primer name	5‘ – 3‘	5’ labeling 3’labeling
CAR Ex7 forward	TGCCAGAAGCTACATCGGCAGTAA[89]	
CAR Ex7 reverse	ATAGACCCATCCTTGCTCTGTGCT[89]	
CAR Ex7 probe	AAGTCGAATGGGTGCGATTCCTGTGA[89]	FAM-TAMRA
E1A forward	GAC GGC CCC CGA AGA TC*	
E1A reverse	CGA GGA GGC GGT TTC GCA GA*	
E1A probe	TCC TGC ACC GCC AAC ATT*	FAM-TAMRA
IFN-γ forward	AAACGAGATGACTTCGAAAAGC*	
IFN-γ reverse	TGCTGGCGACAGTTCAGCC*	
IFN-γ probe	GACTTGAATGTCCAACGCAAAGCA*	FAM-TAMRA
	** one part of the table is on the next page	

Primer name	5'-3'	5'labeling 3'labeling
Beta-2 MG forward	TGA GTA TGC CTG CCG TGT GA[81]	
Beta-2 MG reverse	TGA TGC TGC TTA CAT GTC TCG AT[81]	
Beta-2 MG probe	CCA TGT GAC TTT GTC ACA GCC CAA GAT AGT T[81]	FAM-TAMRA

* *new designed oligonucleotides*

Reagents

Universal PCR Master Mix (TaqMan™)

Procedure

- Needed primers were ordered in a dry state and were diluted in water to have stock concentrations of 100 pmol/µl. Primers and probes were stored in the freezer at – 20°C.
- After thawing, primers and probes were briefly vortexed and centrifuged.
- Master mixes were prepared in the following way (Table 2.10):

Table 2. 10. Preparation of cDNA master-mixes

CAR	Volume µl for 60 react.	Stock conc.	Final conc.
Universal Master-Mix	750 µl	2x	1x
CAR for	6 µl	100 pmol/µl	10 pmol
CAR rev	6 µl	100 pmol/µl	10 pmol
CAR probe	3 µl	100 pmol/µl	5 pmol
H_2O	375 µl		
Total:	**1.500 µl**		
E1A			
Universal Master-Mix	750 µl	2x	1x
E1A for	6 µl	100 pmol/µl	10 pmol
E1A rev	6 µl	100 pmol/µl	10 pmol
E1A probe	3 µl	100 pmol/µl	5 pmol
H_2O	375 µl		
Total:	**1.500 µl**		
IFN-γ			
Universal Master-Mix	750 µl	2x	1x
IFN-γ for	4,5 µl	100 pmol/µl	7,5 pmol
IFN-γ rev	4,5 µl	100 pmol/µl	7,5 pmol
IFN-γ probe	3 µl	100 pmol/µl	5 pmol
H_2O	378 µl		
Total:	**1.500 µl**		
Beta2-MG			
Universal Master-Mix	750 µl	2x	1x
Beta2-MG for	6 µl	100 pmol/µl	10 pmol
Beta2-MG rev	6 µl	100 pmol/µl	10 pmol
Beta2-MG probe	3 µl	100 pmol/µl	5 pmol
H_2O	378 µl		
Total:	**1.500 µl**		

- The loading and running procedure was the same as described in chapter 2.2.2.

- For the genes CAR, E1A, IFN-γ and B2-MG we did not have a standard curve, thus mRNA fold expression values of genes were calculated using the ‘’Delta-Delta Ct Method’’ by Kenneth Livak and Thomas Schmittgen.

3. Results

3.1. HAdV infection of human intestinal epithelial cells

3.1.1. The HAdV life cycle in primary human intestinal epithelial cells (1CT cells)

Previous studies showed an inhibitory effect of immune regulators only in primary cells.[54,59,60] Thus, the 1CT cells, immortalized primary colon epithelial cells have been used for further experiments in this project. A MOI of 100 was chosen for the infection and different cell densities have been tested. HAdV DNA copies have been analyzed in 1CT cells at days 3 and 6 post-infection by exploiting a RQ-PCR targeting the adenoviral hexon gene. These analyses showed a more successful infection when seeding a higher cell number ($5x10^5$ instead of $1x10^5$ cells per well in a 6-well plate) (Figure 3.1.a). The number of detected HAdV-C2 DNA molecules was normalized to the number of DNA molecules of the human house-keeping beta-2 macroglobulin gene referring to two copies per cell. The standard curves, including defined Y-intercept and slopes for these respective RQ-PCR assays (HAdV species C and beta-2 MG) have been described before and were considered for the calculation of the results herein.[81] All quantifications of HAdV-C2 DNA copies per cell in this study have been performed in the same manner.

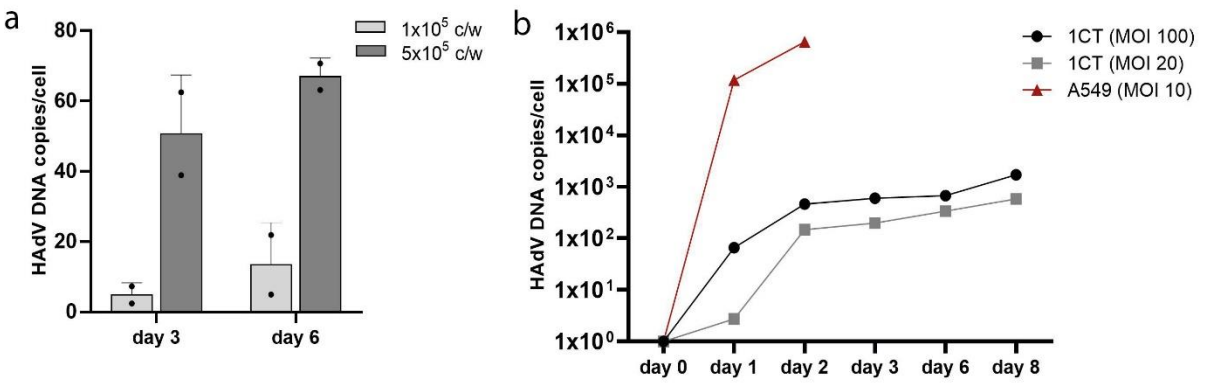

Figure 3. 1. (a) **HAdV infection of 1CT cells at different cell density.** RQ-PCR has been exploited to quantify the number of HAdV DNA copies/cell in 1CT cells. The standard curves of hexon- and beta2-MG- gene have been considered for the calculation. Cells were infected with MOI 100 and harvested at days 3 and 6 post-infection. (b) **HAdV replication in 1CT cells over the time.** RQ-PCR has been also exploited to quantify the number of HAdV DNA copies/cell in 1CT and A549 cells. The standard curves of hexon- and beta2-MG- gene have been considered for the calculation. A549 cells were infected with HAdV-C2 MOI 10 for 48 h. 1CT cells were infected with MOI 100 and MOI 20 for 8 days.

The fast replication of HAdV in A549 cells, as adenoviral producer cell line, is known.[90] On the Figure 3.1.b can be seen that in A549 cells two days post-infection the number of HAdV DNA copies per cell reached $1x10^6$. Afterwards, cells started to enter a lytic infectious cycle.

Whereas 1CT cells have been infected with a double and even ten times higher MOI but show a decreased virus replication in comparison with A549 cells. In infected 1CT cells an increase of adenoviral replication can be seen from day 0 to day 2, thereafter a plateau has been reached from day 2 until day 8. Cell lysis was not documented even after 8 days of infection (Figure 3.1.b). Further, the mRNA expression of one of the most important and first expressed adenoviral E1A gene has been investigated.[35] An increase from day 0 to day 1 was observed. Afterwards, the level of E1A mRNA expression was without big deviations until day 8 (Figure 3.2.a). The mRNA fold change of the E1A mRNA was calculated by the delta-delta Ct method considering the Ct-values for the E1A expression in relation to the Ct-value for the human housekeeping gene beta-2 microglobulin. All quantifications of E1A mRNA fold expression in this study have been performed as described here.

Furthermore, to the investigated increases of adenoviral DNA and RNA in 1CT cells, it was of interest to assess also the possibility of virus progeny production in these cells. HAdV-C2 infected 1CT cells have been harvested at specified days and A549 cells have been reinfected with the collected virus suspension from the 1CT cells. The titter has been calculated according to the Spearman-Käber method. $TCID_{50}$ resulted in a four-log increase of virus particles from day 0 to day 6, with a vast virus production already within the first three days (Figure 3.2.b). In this study all $TCID_{50}$ assays were performed in this manner.

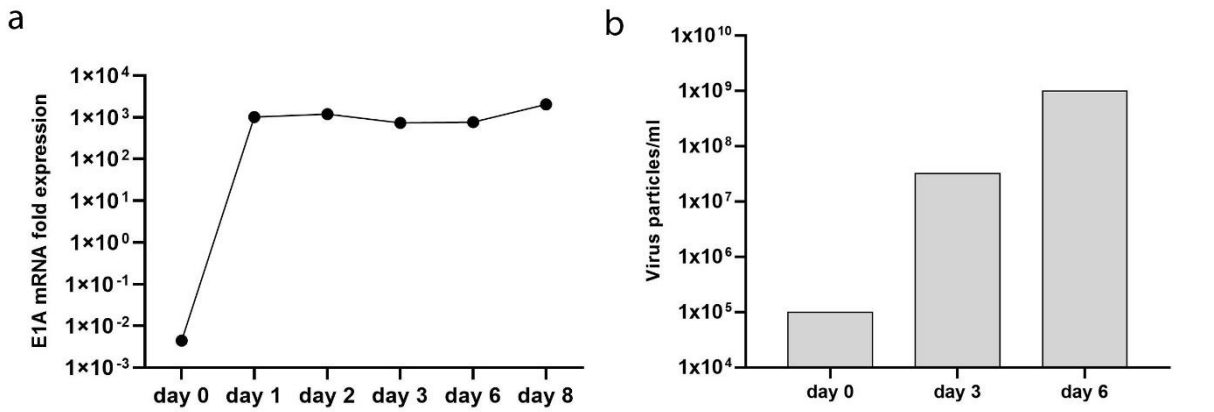

Figure 3. 2. (a) **E1A mRNA expression in the 1CT cells.** RQ-PCR has been exploited to quantify the E1A expression. E1A mRNA fold expression was calculated by the delta-delta Ct method related to the housekeeping gene beta2-MG. Cells have been infected with HAdV-C2 with MOI 100 for 8 days. (b) **HAdV particle production in 1CT cells.** TCID50 assay was exploited to quantify HAdV-C2 infectious particle production in 1CT cells. The titter has been calculated according to the Spearman-Käber method. 1CT cells were infected with MOI 100 and harvested at days 3 and 6 post-infection.

3.1.2. HAdV infection in colon cancer cell lines

In addition to the characterization of HAdV-C2 infection in the primary cells, infection of colon cancer cell lines has been investigated. The colon cancer cell lines show a much faster proliferation rate than 1CT cells and seem to have characteristics more similar to A549 cells. Thus, a ten times lower MOI has been used for *in-vitro* infection. RQ-PCR analysis showed an increase up to one-log from day 1 to day 2 in the number of HAdV DNA copies in CaCo2 and HCT116 cells (Figure 3.3.a). Considering that the infection success was similar in both cell lines, the expression of E1A was only investigated in the CaCo2 cells. Expectedly, an increase of the E1A mRNA expression has been seen from 16 to 48 hours post-infection (Figure 3.3.b), and this increase is much higher compared with the one in the 1CT cells (Figure 3.2a).

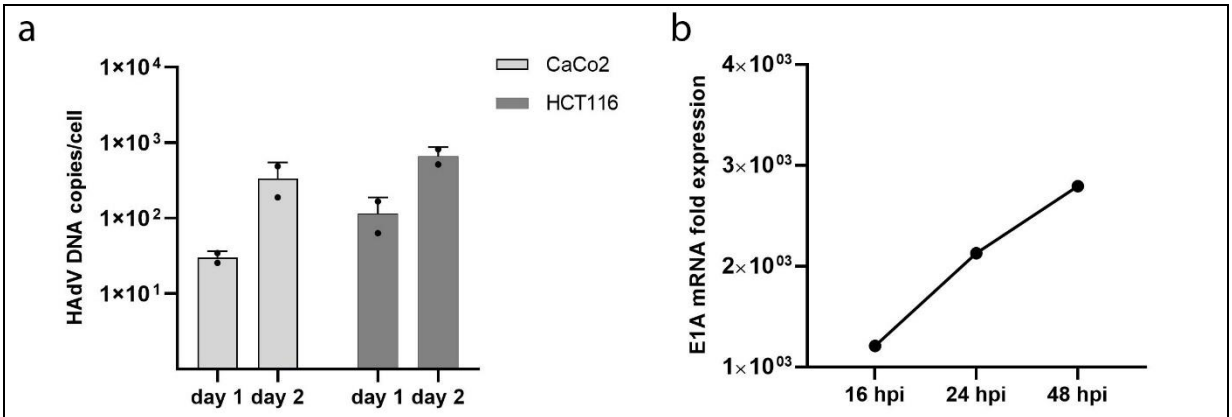

Figure 3. 3. (a) **HAdV-C2 infection of CaCo2 and HCT116 cells**. RQ-PCR has been exploited to quantify the number of HAdV DNA copies/cell in the CaCo2 and HCT116 cells. The standard curves of hexon- and beta2-MG- gene have been considered for the calculation. Cells have been infected with HAdV-C2 MOI 10 for 2 days. (b) **E1A expression in CaCo2 cells**. E1A mRNA fold expression was calculated by the delta-delta Ct method related to the housekeeping gene beta2-MG. Cells have been infected with HAdV-C2 MOI 10 for 2 days.

3.1.3. CAR mRNA expression in intestinal cells

Since very limited data are available about the *in-vitro* infection of human intestinal epithelial cells by HAdV,[13] characterizing the conditions of HAdV infections in these cells has been performed. The mRNA expression of the most commonly used receptor for the species C types,[3] CAR, has been analyzed for different colon cancer cell lines (CaCo2 and HCT116), lung cancer cells and in the non-tumor, but immortalized colon epithelial cells 1CT. RQ-PCR analysis showed the highest CAR expression in CaCo2 cells, lower receptor expression in HCT116 cells and the lack of the receptor in 1CT cells (Figure 3.4). A549 has been included as a control. The relative fold change of the CAR mRNA was calculated by the delta Ct method considering the Ct-values for the CAR expression in relation to the Ct-value of the human housekeeping gene beta-2MG. Moreover, to examine a possible difference in CAR mRNA between infected cells

and non-infected cells, the cells have been infected with HAdV-C2, but there are no significant changes observed (Figure 3.4).

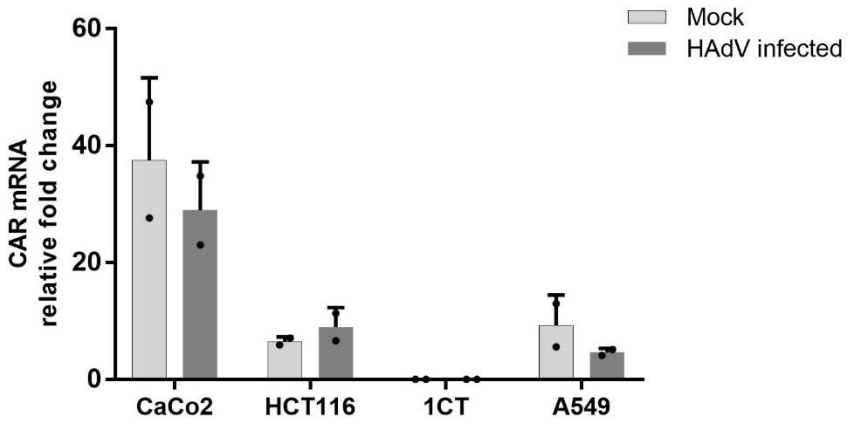

Figure 3. 4. CAR mRNA expression in human intestinal epithelial cells. RQ-PCR has been exploited to quantify the CAR expression. The relative fold change was calculated by the delta Ct method. All cells were infected with HAdV-C2 for 48h. The tumor cell lines were infected with HAdV-C2 with MOI 10 and 1CT cells with MOI 100 for 48 h.

3.2. Antiviral properties of IFN-γ on HAdV infection in human intestinal epithelial cells

3.2.1. IFN-γ expression in different colon epithelial cell lines and PBMCs

Previous to experiments with external administration of IFN-γ and assessing the antiviral properties of this cytokine on intestinal epithelial cells, the physiological expression of IFN-γ has been investigated in different cell types. Moreover, a potential stimulation of the cytokine expression upon the infection with HAdV-C2 has been investigated. RQ-PCR analysis showed that there was no IFN-γ mRNA expression in all examined cells, except in the PBMCs. In the PBMCs, which are known IFN-γ producing cells,[55] upon the infection an increased expression of IFN-γ mRNA has been documented as a defensive mechanism against HAdV-C2 (Figure 3.5). This can be also seen in Table 3.1 with the obtained Ct-values. Even though the virus barely stimulated IFN-γ expression in the above-mentioned cells, this is negligible in comparison with the PBMCs, which also have a basal IFN-γ expression regardless of the presence of pathogens (Table 3.1).

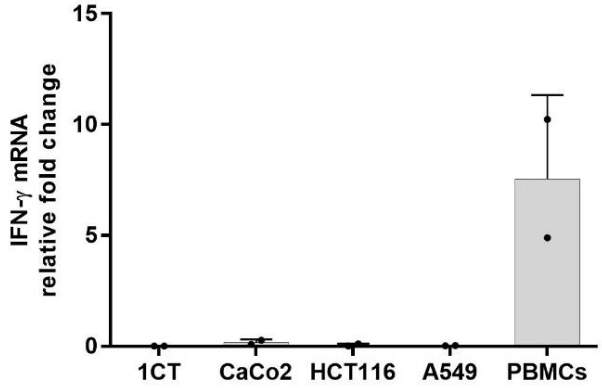

Figure 3. 5. IFN-γ mRNA expression in the intestinal epithelial cells and PBMCs. RQ-PCR has been exploited to quantify the IFN-γ expression. The relative fold change was calculated by the delta Ct method. All intestinal epithelial cells have been infected with HAdV-C2 for 48h. The tumor cell lines were infected with MOI 10 and 1CT cells with MOI 100. PBMCs were infected for 6 days with HAdV-C2 MOI 100.

Table 3. 1. Ct-values of IFN-γ expression in different cell types

Cell type	Ct-value (I)	Ct-value (II)
CaCo2 mock	39,2	39,45
CaCo2 HAdV-C2 48h	38,5	37,82
HCT116 mock	50,0	44,88
HCT116 HAdV-C2 48h	36,6	38,37
A549 mock	50,0	45,13
A549 HAdV-C2 48h	39,5	39,23
1CT mock	50	45,43
1CT HAdV-C2 48h	50	45,13
PBMCs mock	35,30	36,18
PBMCs HAdV-C2 48h	30,34	32,35

3.2.2. IFN-γ inhibits HAdV only in primary colon epithelial cells

Hence there was no IFN-γ production in the intestinal epithelial cells (Figure 3.5), IFN-γ was administered to the cells 24 hours before infection. Through RQ-PCR analysis, highly significant inhibition of HAdV-C2 by the addition of IFN-γ was observed on the DNA level after 3 and 6 days (Figure 3.6.a). Besides, 1CT cells were also infected with different MOIs. Even though significant inhibition was observed by the cytokine when cells were infected with MOI of 500, but less effective compared with MOI 20 and 100 (Figure 3.6.b). For this and all other experiments which have been done in three or more repeats, statistical analysis has been performed in the GrapPad Prism 8.0 software, where average values, standard deviation, t-test (two-stage step-up method of Benjamini, Krieger and Yekutieli) and significance (p-values) values were calculated.

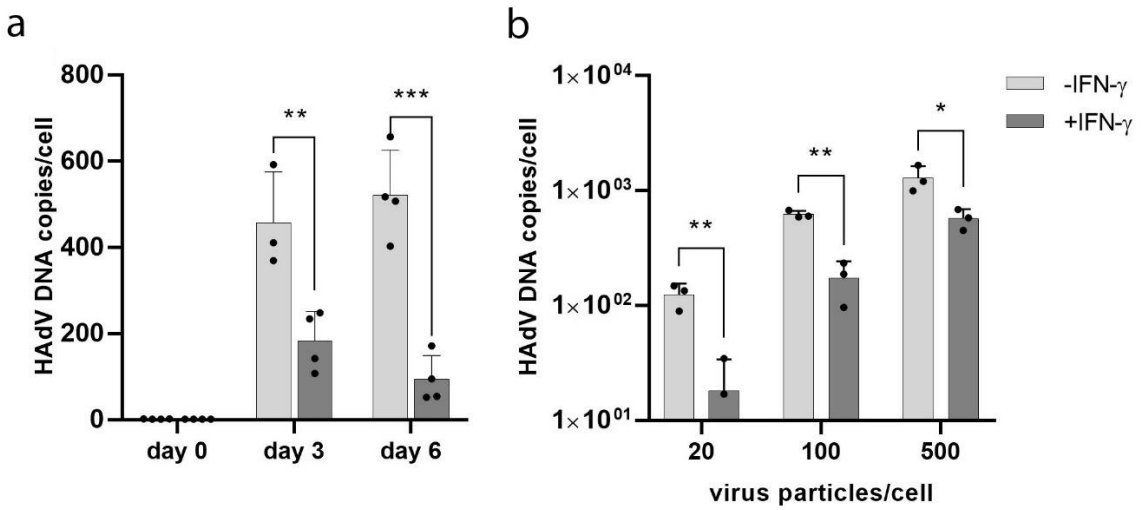

Figure 3. 6. IFN-γ inhibitory effect on HAdV-C2 replication. RQ-PCR has been exploited to quantify the number of HAdV DNA copies/cell in 1CT cells. The standard curves of hexon- and beta2-MG- gene have been considered for the calculation. P-values: * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. (a) Cells were infected with MOI 100 and harvested at days 3 and 6 post-infection (n=3). (b) Cells were infected with MOI 20, 100 and 500 and harvested at day 3 post-infection.

Besides the inhibitory effect of IFN-γ on the HAdV-C2 replication, significant inhibition of adenoviral E1A mRNA by IFN-γ up to one log has been observed too (Figure 3.7.a). Since the formation of infectious particles in the primary epithelial cells has been shown (Figure 3.2.b) and the significant inhibition has been achieved on the DNA and mRNA level in the 1CT cells (Figure 3.6.a, 3.6.b and 3.7.a), the next step was to examine the inhibitory effect of IFN-γ on the formation of infectious adenoviral particles. Likewise, using the $TCID_{50}$ assay after 3 days of infection, IFN-γ induced a significant inhibition of two logs for the formation of virus particles (Figure 3.7.b).

Following the investigation of the antiviral effect by IFN-γ in the primary cells, this effect was also inspected in the colon cancer cell lines. However, IFN-γ failed to inhibit HAdV replication in the CaCo2 and HCT116 cells. There was a neglectable difference in the number of HAdV DNA copies between the treated and non-treated cells (Figure 3.8).

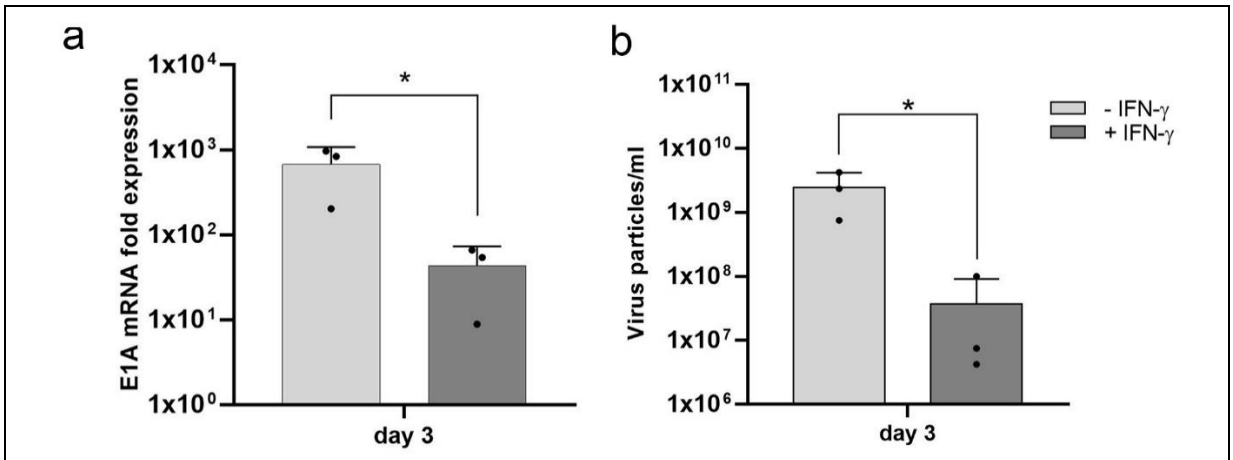

Figure 3. 7. (a) **Inhibitory effect of IFN-γ on E1A mRNA in expression in 1CT cells.** RQ-PCR has been exploited to quantify the E1A expression. E1A mRNA fold expression was calculated by the delta-delta Ct method. Cells have been infected for 3 days with HAdV-C2 with MOI 100. P-value * - p<0.05. (b) **IFN-γ prevents HAdV production in 1CT cells**. TCID50 assay was exploited to quantify HAdV-C2 infectious particle production in 1CT cells. The virus titer was calculated according to the Spearman-Käber method. 1CT cell were infected with MOI 100 for 3 days. P-value * - p<0.05.

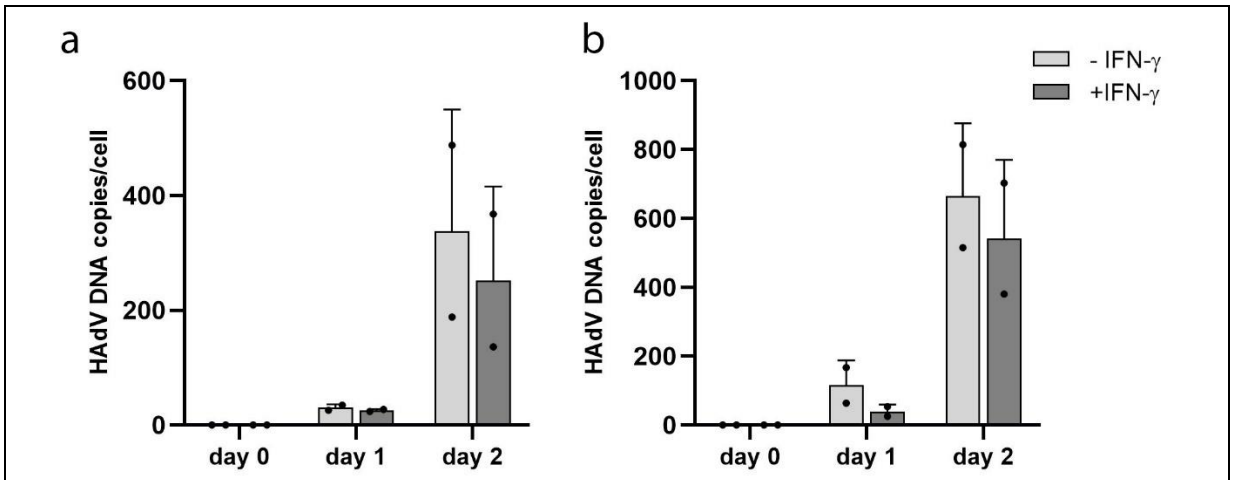

Figure 3. 8. Effect of IFN-γ on HAdV replication in the cancer cell lines. RQ-PCR has been exploited to quantify the number of HAdV DNA copies/cell in the CaCo2 and HCT116 cells. The standard curves of hexon- and beta2-MG- gene have been considered for the calculation. (a) CaCo2 cells were infected for 48 hours with HAdV-C2 MOI 10. (b) HCT116 cells were infected for 48 hours with HAdV-C2 MOI 10.

3.3. IFN-γ promotes HAdV persistence in human intestinal epithelial cells

It has been shown that IFN-γ significantly inhibited adenoviral replication after 3- and 6-days post-infection in the primary cells (Figure 3.6). Additionally, significant inhibition by IFN-γ of virus production has been also documented (Figure 3.7.b). However, long term incubation of infected epithelial cells with IFN-γ might mimic the *in-vivo* situation with a permanent cytokine release. To investigate if IFN-γ supports the persistence of HAdV over an extended period of time, infected 1CT cells were weekly splitted and regularly supplied with IFN-γ for five weeks. After five weeks, IFN-γ was withdrawn from one well, whereas the HAdV infected cells from

another well were continuously supplied with IFN-γ until week 8 (Figure 3.9a). In the cells which were cultivated with IFN-γ, after eight weeks no further increase of HAdV DNA copies per cell has been documented (Figure 3.9.b). In comparison, the cells from which INF-γ was withdrawn, from week six to week eight an increase of 1,5 log of HAdV DNA copy numbers in relation to the treated cells has been documented (Figure 3.9.c) showing that IFN-γ supports HAdV persistence in the primary colon epithelial cells.

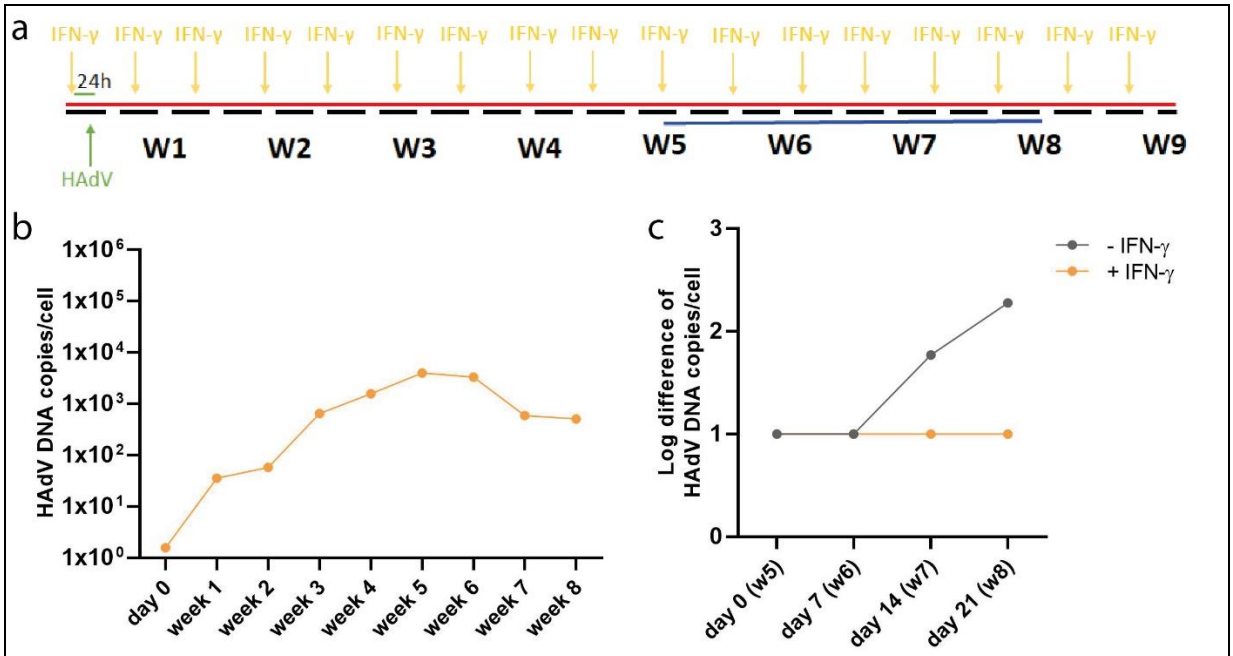

Figure 3. 9. Persistent HAdV infection in 1CT cells. (a) Cultivation timeline from week 0 to week 8. Dotted black line – a period of cells cultivation. Gold arrows represent time points when IFN-γ has been administered. Red line represents the cells that were continuously cultivated with IFN-γ. Blue line represents the cells that were not cultivated with IFN-γ. (b) RQ-PCR has been exploited to quantify the number of HAdV DNA copies/cell in the 1CT cells. The standard curves of hexon- and beta2-MG- gene have been considered for the calculation. Cells were supplied twice weekly with IFN-γ and splitted every week. (c) Log difference between the cells supplied with INF-γ until week 8 and those cultivated without IFN-γ between week 5 and week 8.

3.4. Immunosuppression supports HAdV replication in peripheral blood mononuclear cells (PBMCs)

To investigate the effect of immunosuppression on HAdV persistence an *in-vitro* model with immune cells was necessary. PBMCs infected with HAdV-C2 were treated separately, 24 hours post-infection, with immunosuppressive agents Cyclosporine A (CsA) and Hydrocortisone (HCor) at different concentrations. The infected and treated cells have been cultivated for 6 days with additional administration of CsA and HCor at day 3 post-infection. PBMCs treated with these immunosuppressive drugs resulted in a clear downregulation of basal IFN-γ mRNA expression in our *in-vitro* setting. Moreover, infection of PBMCs with HAdV-C2 showed

stimulation of the cytokine expression, which is strongly reduced by CsA and less pronounced but also reduced under the treatment with HCor (Figure 3.10.a).

Showing that the immunosuppressive agents inhibit the IFN-γ mRNA expression in PBMCs (Figure 3.10.a), their effect on HAdV replication has been further investigated. Infected PBMCs were treated with already mentioned immunosuppressive agents. Compared with the untreated and infected PBMCs, 1 µM CsA induces an increase of HAdV DNA copies per cell up to one log. However, in PBMCs treated with 0,1 µM CsA (data not shown here) or with 50 nM HCor this promotion of HAdV replication has not been observed (Figure 3.10.b). Calculations have been conducted in Microsoft Office® Excel and statistical analysis in the GraphPad Prism 8.0.

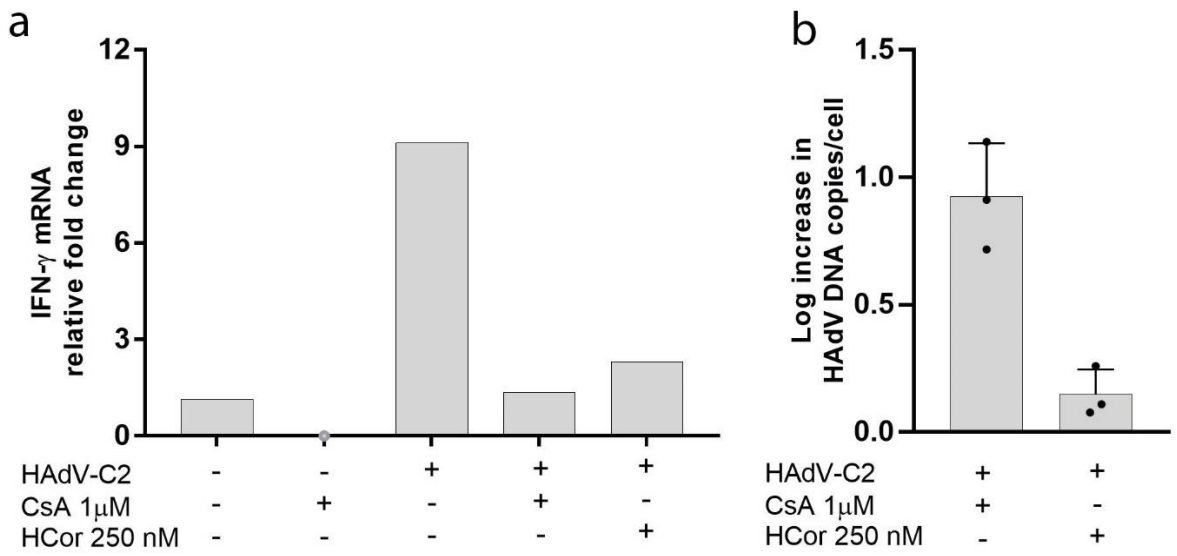

Figure 3. 10. (a) **IFN-γ mRNA expression in PBMCs treated with immunosuppressive agents.** RQ-PCR has been exploited to quantify the IFN-γ mRNA expression. The relative fold change was calculated by the delta Ct method related to the housekeeping gene beta2-MG. PBMCs were infected with HAdV-C2 MOI 100 and harvested at day 6 post-infection. (b) **HAdV replication in the PBMCs.** RQ-PCR has been exploited to quantify the number of HAdV DNA copies/cell in the PBMCs. The results were represented in log difference between the infected and untreated PBMCs and those which were treated with immunosuppressive agents. The cells were infected with MOI 100 and harvested at day 6 post-infection.

4. Discussion

Reactivation of persisting HAdVs in the gut of pediatric HSCT-recipients is a risk factor for invasive infections, which are associated with high morbidity and mortality rates.[9,13,14,49,62] Since there are rare data available characterizing adenoviral infections in the intestinal epithelial cells,[13] the experiments in this study tried to explore more about the virus entry and regulation of HAdV infections by IFN-γ. Due to the occurrence of invasive and severe HAdV infections, and the current lack of effective antivirals for HSCT-recipients, it is of importance to understand the regulation of HAdV persistence and reactivation in the intestine.[13,14] A study by Zheng et al. showed an inhibitory effect of IFN-γ on the adenoviral gene expression in fibroblasts over a span of several months.[54] Thus, the potential role of IFN-γ for the inhibition of HAdV infections in intestinal epithelial cells was addressed in the present study.

Previous reports about *in*-vitro adenovirus infections in intestinal epithelial cells were mainly based on the exploitation of CaCo2 cells.[91,92] *In-vitro* infection of immortalized non-tumor colon epithelial cells (1CT) with HAdV-C2 showed replication, transcription of E1A mRNA and formation of infectious virus particles over a time of 3 and 6 days post-infection. Thus, 1CT cells are suitable for the investigation of adenoviral infections but compared with colon cancer cell lines (CaCo2 and HCT116), they show reduced replication in terms of copy number kinetics. However, the infection of CaCo2 and HCT116 cell lines seemed to be similar to A549 cells. For the infection of cancer cell lines, 10-times lower MOI has been used in comparison to primary cells. A reason for reduced infectivity could be the different growth kinetics of the primary cells, but also the absent CAR expression, in comparison to CaCo2 and HCT116 or A549 cells. Additionally, Roig et al. showed that 1CT cells have characteristics of intestinal stem cells and express the specific marker LGR5. Maybe only differentiated epithelial cells express CAR,[93] but this needs to be clarified in further experiments. Other experiments, not part of this master's thesis, exploiting a GFP-tagged virus already showed the infection of 1CT cells with HAdV by a small amount of GFP positive cells. These immunofluorescence-based analyses provide evidence that the virus managed the entry, and the expression of viral genes did not seem to be blocked. Moreover, the lower HAdV DNA copies might be due to a minority of infected 1CT cells, compared with A549, CaCo2 or HCT116 cells. Based on these observations all *in-vitro* infections in 1CT cells have been performed with a MOI of 100, whereas for the tumor cell lines a MOI of 10 was sufficient to result in the production of high virus titers.

The 1CT cells seem to be a suitable cell culture system for analyses addressing HAdV inhibition and persistence in the intestinal epithelium. Especially in patients lacking a functional immune system because of the treatment with immunosuppressive drugs, a massive HAdV production in the intestinal epithelium has been documented.[49] Since no effective therapy against adenoviruses is currently available,[13,14,50] the idea was to investigate the effect of IFN-γ on HAdV propagation in intestinal epithelial cells. Chahal et al. 2012 and Zheng et al. 2016 reported about the inhibitory effect of IFN-α and IFN-γ on HAdV in human fibroblasts and primary human lung epithelial cells.[54,59] The inhibitory effect was even stronger by knocking-out the E1B gene, due to its known inhibitory function on ISGs.[59] Nevertheless, type II IFN seems to have a stronger effect on HAdV repression than type I IFN.[54] Most experiments characterizing HAdV infections are done in fibroblasts and some in lung epithelial cells. Taking into account that HAdVs are frequently observed in stool samples and the reactivation of persisting virus might occur in the gut,[14,49] investigating a potential inhibitory effect of IFN-γ on intestinal HAdV infection might be of clinical importance. Similar to already described findings in fibroblasts we were able to inhibit HAdV replication, E1A mRNA expression and HAdV progeny production by IFN-γ in the intestinal epithelial cells. Furthermore, our cooperation partners from the Helmholtz Center in Munich (Sabrina Schreiner) showed the inhibition of the adenoviral polymerase (E2A) and the hexon protein by IFN-γ in 1CT cells (these results are not included in this master's thesis).

Others also reported an inhibitory effect of IFN-β and IFN-λ on HAdV replication in human intestinal organoids.[60] This shows that different IFNs might play a role for repression of the virus. However, for IFN-γ and IFN-α, the inhibitory effect on the expression of E1A has been elucidated,[54] whereas for other IFNs, especially for IFN-λ, different underlying mechanisms can be assumed.

Zheng et al. demonstrated how IFN-γ is capable to repress HAdV replication in fibroblasts over a long time and that withdrawing IFN-γ leads to recovery and reactivation of persistent HAdV.[54] We were also able to establish a HAdV persistence model with primary intestinal cells. Virus replication has been repressed by IFN-γ for 6 weeks but after withdrawing the cytokine, HAdV replication was restored and increased by more than one log in comparison to cells still treated with IFN-γ. The *in-vivo* data suggested that persisting HAdVs are usually found in lymphocytes, which are known IFN-γ producing cells.[52,55,57] This indicates that IFN-γ might keep HAdV in a persistent state in lymphocytes, and we were able to mimic this inhibitory effect in primary intestinal cells. Along with 1CT cells, for additional investigation, the

cultivation of large bowel organoids has been established in our laboratory. HAdV infection of the organoids and treatment with IFN-γ also resulted in the inhibition of virus replication like in 1CT cells. In regard to the inhibitory effect of IFN on HAdV replication, 1CT cells show more comparable characteristics to these tissue-like structures and fewer similarities with CaCo2 and HCT116 cells. These experiments cumulatively provide evidence for a significant inhibitory effect of IFN-γ on HAdV in primary intestinal cells. The failure of IFNs to inhibit HAdV in cancer cell lines has been shown before with A549 cells, a lung epithelial cancer cell line.[54,60] The mechanism underlying this observation might be linked to the fact that genes encoding proteins involved in the E2F/Rb pathway are mutated in nearly all human cancer cells. Considering that the binding of pRb and p107 is essential for the inhibitory effect by IFNs, this might be one of the reasons for the failure of virus suppression by IFN-γ in cancer cell lines.[94]

Adenoviral infections are a major challenge for HSCT-recipients due to the immunosuppressive treatment during conditioning and after transplantation to prevent a reaction of donor lymphocytes against patient tissue.[13,14,49,63,64] Thus, the immunosuppressive agents usually attack lymphocytes and it has been shown that HAdV can establish persistent infections in this cell subset *in-vivo* and *in-vitro*.[52,57,58] A valid question emanating from these studies is whether the immunosuppression in patients results in inhibition of IFN secretion, which, in turn, promotes HAdV reactivation and dissemination. [8,52,54,57,58,65,95] IFN-γ is a key player for the innate and adaptive immune system and its secretion is triggered by the presence of pathogens.[55,96,97] CsA and HCor are immunosuppressive drugs used in the clinics and are known to repress several cytokines, including IFN-γ.[65–68,70] Herein we tested if this inhibitory effect of CsA and HCor on IFN-γ mRNA expression has an impact on HAdV replication in PBMCs. An increased IFN-γ expression in PBMCs after HAdV infection has been observed together with the inhibition of IFN-γ mRNA upon treatment with immunosuppressive agents. Interestingly, we found evidence for increased HAdV replication in PBMCs upon treatment with CsA, whereas no effect with HCor was observed. This might be related to a weaker inhibitory effect of HCor on IFN-γ expression (shown in Figure 3.10.a) and needs additional investigations. These data further support an important role of IFN-γ for HAdV persistence and reactivation. For future experiments, co-cultivation of primary intestinal cells and PBMCs are planned. In addition, examination of the specific hematopoietic cell type responsible for reactivation of HAdV under immunosuppression needs to be identified.

Our data show that IFN-γ has a strong inhibitory effect on HAdV in primary epithelial cells of the intestine, but not in corresponding cancer cell lines. Based on the obtained results, IFN-γ

might be of relevance for antiviral therapy against HAdV, but more data are still needed. IFN-γ is already an FDA approved drug and its use in clinical trials started since in 1986.[98] Today a recombinant form of IFN-γ-1b is used for treatment of chronic granulomatous disease and osteopetrosis. Many studies are examining the effect of IFN-γ for treatment of various diseases e.g. systemic sclerosis, but there are also adverse effects. The most common adverse effects which occur due to treatment with IFN-γ are mild fever and flu-like symptoms, headache, and moderate reactions at the injection site. More severe manifestations include cardiac pain, atrioventricular block, reversible loss of hearing, and impotence.[99] In summary, this study provides data about on the role IFNs can play for HAdV reactivation upon immunosuppression and on the specific antiviral capacity of IFN- γ.

5. References

1. Chroboczek, J., Ruigrok, R. W. H. & Cusack, S. Adenovirus Fiber. in *The Molecular Repertoire of Adenoviruses I: Virion Structure and Infection* (eds. Doerfler, W. & Böhm, P.) 163–200 (Springer, 1995). doi:10.1007/978-3-642-79496-4_10.

2. Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H. & Ward, T. G. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Exp. Biol. Med.* **84**, 570–573 (1953).

3. Knipe, D. M., Howley, P. M. & Howley, P. M. *Fields' Virology; Chapter 55 p.1704-1731*. vol. 2 (Wolters Kluwer Health/Lippincott Williams & Wilkins, 2013).

4. Kunz, A. N. & Ottolini, M. The Role of Adenovirus in Respiratory Tract Infections. *Curr Infect Dis Rep* **12**, 81–87 (2010).

5. Chirmule, N. *et al.* Immune responses to adenovirus and adeno-associated virus in humans. *Gene Therapy* **6**, 1574–1583 (1999).

6. Nazir, S. A. & Metcalf, J. P. Innate immune response to adenovirus. *J. Investig. Med.* **53**, 292–304 (2005).

7. Lion, T. *et al.* Molecular monitoring of adenovirus in peripheral blood after allogeneic bone marrow transplantation permits early diagnosis of disseminated disease. *Blood* **102**, 1114–1120 (2003).

8. Kosulin, K., Hoffmann, F., Clauditz, T. S., Wilczak, W. & Dobner, T. Presence of Adenovirus Species C in Infiltrating Lymphocytes of Human Sarcoma. *PLOS ONE* **8**, e63646 (2013).

9. Lion, T. Adenovirus Infections in Immunocompetent and Immunocompromised Patients. *Clin Microbiol Rev* **27**, 441–462 (2014).

10. Lion, T. *et al.* Monitoring of adenovirus load in stool by real-time PCR permits early detection of impending invasive infection in patients after allogeneic stem cell transplantation. *Leukemia* **24**, 706–714 (2010).

11. Davison, A. J., Benkő, M. & Harrach, B. Genetic content and evolution of adenoviruses. *Journal of General Virology*, **84**, 2895–2908 (2003).

12. HAdV Working Group. http://hadvwg.gmu.edu/.

13. Kosulin, K. Intestinal HAdV Infection: Tissue Specificity, Persistence, and Implications for Antiviral Therapy. *Viruses* **11**, (2019).

14. Lion, T. Adenovirus persistence, reactivation, and clinical management. *FEBS Letters* **593**, 3571–3582 (2019).

15. Dhingra, A. *et al.* Molecular Evolution of Human Adenovirus (HAdV) Species C. *Scientific Reports* **9**, 1039 (2019).

16. Rux, J. J., Kuser, P. R. & Burnett, R. M. Structural and Phylogenetic Analysis of Adenovirus Hexons by Use of High-Resolution X-Ray Crystallographic, Molecular Modeling, and Sequence-Based Methods. *Journal of Virology* **77**, 9553–9566 (2003).

17. Smith, J. G., Wiethoff, C. M., Stewart, P. L. & Nemerow, G. R. Cell Entry by Non-Enveloped Viruses: Adenovirus. in *Cell Entry by Non-Enveloped Viruses* (ed. Johnson, J. E.) 195–224 (Springer, 2010). doi:10.1007/82_2010_16.

18. Liu, H., Wu, L. & Zhou, Z. H. Model of the Trimeric Fiber and Its Interactions with the Pentameric Penton Base of Human Adenovirus by Cryo-electron Microscopy. *Journal of Molecular Biology* **406**, 764–774 (2011).

19. Williams, R. C. & Wyckoff, R. W. G. Electron Shadow-Micrography of Virus Particles. *Proceedings of the Society for Experimental Biology and Medicine* **58**, 265–270 (1945).

20. Adenovirus | Encyclopedia Britannica. *Encyclopedia Britannica* https://www.britannica.com/science/adenovirus.

21. Zhang, Y. & Bergelson, J. M. Adenovirus Receptors. *Journal of Virology* **79**, 12125–12131 (2005).

22. Nemerow, G. R., Pache, L., Reddy, V. & Stewart, P. L. Insights into adenovirus host cell interactions from structural studies. *Virology* **384**, 380–388 (2009).

23. Roelvink, P. W. *et al.* The Coxsackievirus-Adenovirus Receptor Protein Can Function as a Cellular Attachment Protein for Adenovirus Serotypes from Subgroups A, C, D, E, and F. *Journal of Virology* **72**, 7909–7915 (1998).

24. Gaggar, A., Shayakhmetov, D. M. & Lieber, A. CD46 is a cellular receptor for group B adenoviruses. *Nature Medicine* **9**, 1408–1412 (2003).

25. Meier, O. & Greber, U. F. Adenovirus endocytosis. *The Journal of Gene Medicine* **6**, S152–S163 (2004).

26. Ma, Y.-Y. *et al.* Loss of coxsackie and adenovirus receptor expression in human colorectal cancer: A potential impact on the efficacy of adenovirus-mediated gene therapy in Chinese Han population. *Molecular Medicine Reports* **14**, 2541–2547 (2016).

27. Walters, R. W. *et al.* Basolateral Localization of Fiber Receptors Limits Adenovirus Infection from the Apical Surface of Airway Epithelia. *J. Biol. Chem.* **274**, 10219–10226 (1999).

28. Stonebraker, J. R. *et al.* Glycocalyx Restricts Adenoviral Vector Access to Apical Receptors Expressed on Respiratory Epithelium In Vitro and In Vivo: Role for Tethered Mucins as Barriers to Lumenal Infection. *J Virol* **78**, 13755–13768 (2004).

29. Walters, R. W. *et al.* Adenovirus Fiber Disrupts CAR-Mediated Intercellular Adhesion Allowing Virus Escape. *Cell* **110**, 789–799 (2002).

30. Johansson, C. *et al.* Adenoviruses Use Lactoferrin as a Bridge for CAR-Independent Binding to and Infection of Epithelial Cells. *Journal of Virology* **81**, 954–963 (2007).

31. Sánchez, L., Calvo, M. & Brock, J. H. Biological role of lactoferrin. *Arch Dis Child* **67**, 657–661 (1992).

32. Marttila, M. *et al.* CD46 Is a Cellular Receptor for All Species B Adenoviruses except Types 3 and 7. *Journal of Virology* **79**, 14429–14436 (2005).

33. Cattaneo, R. Four Viruses, Two Bacteria, and One Receptor: Membrane Cofactor Protein (CD46) as Pathogens' Magnet. *Journal of Virology* **78**, 4385–4388 (2004).

34. Arnberg, N., Pring-Åkerblom, P. & Wadell, G. Adenovirus Type 37 Uses Sialic Acid as a Cellular Receptor on Chang C Cells. *J Virol* **76**, 8834–8841 (2002).

35. Knipe, D. M. & Howley, P. M. *Fields' Virology; Chapter 56 p.1732-1767*. vol. 2 (Wolters Kluwer Health/Lippincott Williams & Wilkins, 2013).

36. Iii, J. P. L. & Kajon, A. E. Adenovirus: Epidemiology, Global Spread of Novel Serotypes, and Advances in Treatment and Prevention. *Semin Respir Crit Care Med* **37**, 586–602 (2016).

37. Ishiko, H. *et al.* Novel Human Adenovirus Causing Nosocomial Epidemic Keratoconjunctivitis. *Journal of Clinical Microbiology* **46**, 2002–2008 (2008).

38. Gonçalves, G. *et al.* Outbreak of acute gastroenteritis caused by adenovirus type 41 in a kindergarten. *Epidemiology & Infection* **139**, 1672–1675 (2011).

39. Marshall S. Horwitz. *Fields' Virology - Ch. 68 p.2309*. vol. 17 (2002).

40. Ison, M. G. Adenovirus infections in transplant recipients. *Clin. Infect. Dis.* **43**, 331–339 (2006).

41. Echavarría, M. Adenoviruses in Immunocompromised Hosts. *Clin Microbiol Rev* **21**, 704–715 (2008).

42. Esposito, S., Preti, V., Consolo, S., Nazzari, E. & Principi, N. Adenovirus 36 infection and obesity. *Journal of Clinical Virology* **55**, 95–100 (2012).

43. Shang, Q. *et al.* Serological data analyses show that adenovirus 36 infection is associated with obesity: A meta-analysis involving 5739 subjects. *Obesity* **22**, 895–900 (2014).

44. Knipe, D. M. & Howley, P. M. *Fields' Virology; Chapter 63 p. 2355-22394*. (Lippincott Williams & Wilkins, 2007).

45. Boldogh, I., Albrecht, T. & Porter, D. D. Persistent Viral Infections. in *Medical Microbiology* (ed. Baron, S.) (University of Texas Medical Branch at Galveston, 1996).

46. S. Mocarski, Jr, E., Shenk, T., D. Griffiths, P. & F. Pass, R. *Fields' Virology; Chapter 62 p.1960-2014*. vol. 2 (Wolters Kluwer Health/Lippincott Williams & Wilkins, 2013).

47. Gosselin, A. *et al.* HIV persists in CCR6+CD4+ T cells from colon and blood during antiretroviral therapy. *AIDS* **31**, 35–48 (2017).

48. Thorley-Lawson, D. A. EBV Persistence—Introducing the Virus. *Curr Top Microbiol Immunol* **390**, 151–209 (2015).

49. Kosulin, K. *et al.* Persistence and reactivation of human adenoviruses in the gastrointestinal tract. *Clin. Microbiol. Infect.* **22**, 381.e1-381.e8 (2016).

50. Radke, J. R. & Cook, J. L. Human adenovirus infections: update and consideration of mechanisms of viral persistence. *Curr. Opin. Infect. Dis.* **31**, 251–256 (2018).

51. Garnett, C. T. *et al.* Latent species C adenoviruses in human tonsil tissues. *J. Virol.* **83**, 2417–2428 (2009).

52. Roy, S. *et al.* Adenoviruses in Lymphocytes of the Human Gastro-Intestinal Tract. *PLOS ONE* **6**, e24859 (2011).

53. Adrian, T., Schäfer, G., Cooney, M. K., Fox, J. P. & Wigand, R. Persistent enteral infections with adenovirus types 1 and 2 in infants: no evidence of reinfection. *Epidemiol. Infect.* **101**, 503–509 (1988).

54. Zheng, Y., Stamminger, T. & Hearing, P. E2F/Rb Family Proteins Mediate Interferon Induced Repression of Adenovirus Immediate Early Transcription to Promote Persistent Viral Infection. *PLOS Pathogens* **12**, e1005415 (2016).

55. Abbas, A. K., Lichtman, A. H. & Pillai, S. *Cellular and Molecular Immunology; Ch.4 p. 55-86*. (Elsevier Health Sciences, 2014).

56. Nan, Y., Wu, C. & Zhang, Y.-J. Interplay between Janus Kinase/Signal Transducer and Activator of Transcription Signaling Activated by Type I Interferons and Viral Antagonism. *Front Immunol* **8**, 1758 (2017).

57. Markel, D. *et al.* Type dependent patterns of human adenovirus persistence in human T-lymphocyte cell lines. *Journal of Medical Virology* **86**, 785–794 (2014).

58. Rodríguez, E. *et al.* Humanized Mice Reproduce Acute and Persistent Human Adenovirus Infection. *J Infect Dis* **215**, 70–79 (2017).

59. Chahal, J. S., Qi, J. & Flint, S. J. The Human Adenovirus Type 5 E1B 55 kDa Protein Obstructs Inhibition of Viral Replication by Type I Interferon in Normal Human Cells. *PLOS Pathogens* **8**, e1002853 (2012).

60. Holly, M. K. & Smith, J. G. Adenovirus Infection of Human Enteroids Reveals Interferon Sensitivity and Preferential Infection of Goblet Cells. *Journal of Virology* **92**, (2018).

61. Leen, A. M. & Rooney, C. M. Adenovirus as an emerging pathogen in immunocompromised patients. *British Journal of Haematology* **128**, 135–144 (2005).

62. Hiwarkar, P. *et al.* Management of adenovirus infection in patients after haematopoietic stem cell transplantation: State-of-the-art and real-life current approach: A position statement on behalf of the Infectious Diseases Working Party of the European Society of Blood and Marrow Transplantation. *Rev. Med. Virol.* **28**, e1980 (2018).

63. Wiseman, A. C. Immunosuppressive Medications. *Clin J Am Soc Nephrol* **11**, 332–343 (2016).

64. Allison, A. C. Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacology* **47**, 63–83 (2000).

65. Mejia, J. C., Basu, A. & Shapiro, R. Chapter 17 - Calcineurin Inhibitors. in *Kidney Transplantation–Principles and Practice (Seventh Edition)* (eds. Morris, P. J. & Knechtle, S. J.) 231–249 (Content Repository Only!, 2014). doi:10.1016/B978-1-4557-4096-3.00017-9.

66. Bremer, S., Vethe, N. T. & Bergan, S. Chapter 11 - Monitoring calcineurin inhibitors response based on NFAT-regulated gene expression. in *Personalized Immunosuppression in Transplantation* (eds. Oellerich, M. & Dasgupta, A.) 259–290 (Elsevier, 2016). doi:10.1016/B978-0-12-800885-0.00011-4.

67. Mechanisms of action of cyclosporine. - Abstract - Europe PMC. http://europepmc.org/article/med/10878286.

68. Olnes, M. J. *et al.* Effects of Systemically Administered Hydrocortisone on the Human Immunome. *Sci Rep* **6**, 23002 (2016).

69. Hu, X., Li, W.-P., Meng, C. & Ivashkiv, L. B. Inhibition of IFN-γ Signaling by Glucocorticoids. *The Journal of Immunology* **170**, 4833–4839 (2003).

70. Cesario, T. C. *et al.* The Effect of Hydrocortisone on the Production of γ-Interferon and Other Lymphokines by Human Peripheral Blood Mononuclear Cells. *Journal of Interferon Research* **6**, 337–347 (1986).

71. Kimberlin, D. W. & Whitley, R. J. Antiviral therapy of HSV-1 and -2. in *Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis* (eds. Arvin, A. et al.) (Cambridge University Press, 2007).

72. Cidofovir. in *LiverTox: Clinical and Research Information on Drug-Induced Liver Injury* (National Institute of Diabetes and Digestive and Kidney Diseases, 2012).

73. Hiwarkar, P. *et al.* Brincidofovir is highly efficacious in controlling adenoviremia in pediatric recipients of hematopoietic cell transplant. *Blood* **129**, 2033–2037 (2017).

74. Graci, J. D. & Cameron, C. E. Mechanisms of action of ribavirin against distinct viruses. *Rev Med Virol* **16**, 37–48 (2006).

75. Leen, A. M. *et al.* Cytotoxic T lymphocyte therapy with donor T cells prevents and treats adenovirus and Epstein-Barr virus infections after haploidentical and matched unrelated stem cell transplantation. *Blood* **114**, 4283–4292 (2009).

76. Leen, A. M. *et al.* Multicenter study of banked third-party virus-specific T cells to treat severe viral infections after hematopoietic stem cell transplantation. *Blood* **121**, 5113–5123 (2013).

77. Keib, A., Mei, Y.-F., Cicin-Sain, L., Busch, D. H. & Dennehy, K. M. Measuring Antiviral Capacity of T Cell Responses to Adenovirus. *The Journal of Immunology* **202**, 618–624 (2019).

78. *Principles of Virology, 3rd Edition, Volume I: Molecular Biology*. (ASM Press).

79. Kangro, H. O. & Mahy, B. W. J. *Virology Methods Manual*. (Elsevier, 1996).

80. Encyclopedia of Genetics | ScienceDirect. https://www.sciencedirect.com/referencework/9780122270802/encyclopedia-of-genetics.

81. Watzinger, F. *et al.* Real-time quantitative PCR assays for detection and monitoring of pathogenic human viruses in immunosuppressed pediatric patients. *J. Clin. Microbiol.* **42**, 5189–5198 (2004).

82. *QIAamp DNA Mini and Blood Mini Handbook*. (QIAGEN, 2016).

83. Holland, P. M., Abramson, R. D., Watson, R. & Gelfand, D. H. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A* **88**, 7276–7280 (1991).

84. TaqMan. https://www.ncbi.nlm.nih.gov/probe/docs/projtaqman/.

85. Bustin, S. A. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *Journal of Molecular Endocrinology* **25**, 169–193 (2000).

86. Schild, D. T. A. Einführung in die Real-Time TaqMan PCR-Technologie. 118.

87. *TaqMan® Universal PCR Master Mix User Guide Publication Number 4304449*.

88. *Qiagen RNeasy® Plus Mini Handbook*. (2014).

89. Excoffon, K. J. D. A. *et al.* Isoform-Specific Regulation and Localization of the Coxsackie and Adenovirus Receptor in Human Airway Epithelia. *PLOS ONE* **5**, e9909 (2010).

90. Kovesdi, I. & Hedley, S. J. Adenoviral Producer Cells. *Viruses* **2**, 1681–1703 (2010).

91. Lee, J. Y. *et al.* Bacterial RecA Protein Promotes Adenoviral Recombination during In Vitro Infection. *mSphere* **3**, (2018).

92. Sherwood, V., King, E., Tötemeyer, S., Connerton, I. & Mellits, K. H. Interferon treatment suppresses enteric adenovirus infection in a model gastrointestinal cell-culture system. *Journal of General Virology,* **93**, 618–623 (2012).

93. Roig, A. I. *et al.* Immortalized Epithelial Cells Derived From Human Colon Biopsies Express Stem Cell Markers and Differentiate In Vitro. *Gastroenterology* **138**, 1012-1021.e5 (2010).

94. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).

95. Rhen, T. & Cidlowski, J. A. Antiinflammatory Action of Glucocorticoids — New Mechanisms for Old Drugs. *New England Journal of Medicine* **353**, 1711–1723 (2005).

96. Kang, S., Brown, H. M. & Hwang, S. Direct Antiviral Mechanisms of Interferon-Gamma. *Immune Netw* **18**, (2018).

97. Schoenborn, J. R. & Wilson, C. B. Regulation of Interferon-γ During Innate and Adaptive Immune Responses. in *Advances in Immunology* vol. 96 41–101 (Academic Press, 2007).

98. Miller, C. H. T., Maher, S. G. & Young, H. A. Clinical Use of Interferon-γ. *Ann N Y Acad Sci* **1182**, 69–79 (2009).

99. Interferon gamma. in *Meyler's Side Effects of Drugs (Sixteenth Edition)* (ed. Aronson, J. K.) 220–222 (Elsevier, 2016). doi:10.1016/B978-0-444-53717-1.00902-1.