Strategies for Improved Cancer Virotherapy: *In vivo* migration and expansion of dendritic cells enhance cross-presentation of tumor antigens in virotherapy

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This work is dedicated to
the goddess of study
and my family members

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Contents

1 Abstract

Triggering of dendritic cell (DC) migration into tumor tissue and subsequent expansion of dendritic cells may be useful to generate anti-tumor immune response by adenoviral gene transfer employing cytokines and growth factors for dendritic cell migration and expansion to improve cross-presentation of tumor antigens during oncolytic virotherapy.

Appropriate syngenic mouse models were established for lung carcinoma cells CMT64 and KLN205. *In vitro* replication assay showed that CMT64 and KLN205 are permissive for replication of the oncolytic telomerase-dependent adenovirus hTERT-Ad. Further *in vivo* replication was studied using replication defective adenovirus expressing Luciferase gene in combination of telomerase dependent adenovirus.

We used adenoviral expression of Macrophage Inflammatory Protein 1α (MIP-1α) to recruit DCs into hTERT-Ad infected tumors and Ad-Flt3L to expand DCs within the tumor nodules during virotherapy.

Compared with virotherapy alone or virotherapy with expression of a single CC chemokine, expression of both MIP1α and Flt3L during virotherapy significantly increases infiltration of DCs and T cells, as shown by histochemistry. Interferon-γ, and CTL assay showed that the combination of cytokines improve cross-presentation of tumor antigens and induces an anti-tumor immune response during virotherapy. Combination of viroimmunotherapy and DC vaccination showed nearly complete regression of the subcutaneous tumors and very strong inhibition of established lung metastasis.

In conclusion mobilizing of DCs to tumors during virotherapy improves cross-presentation of tumor antigens and induced anti-tumor immune response, which facilitates an effective viroimmunotherapy of established solid tumors *in vivo*. The results show high efficacy of the viroimmunotherapy, which may be considered as safe even, when translated to human cancer therapy.

Key words: virotherapy, immune answer, dendritic cell migration

2 Zusammenfassung

Die Stimulation der Wanderung und die nachfolgende Expansion von dendritischen Zellen in Tumore, die durch tumor-spezifisch replizierende Viren infiziert sind, könnte ein attraktiver Ansatz sein, um die Tumorimmunantwort durch Kreuzpräsentation von Tumorantigenen im Rahmen einer Virotherapie zu verstärken.
Es wurden geeignete syngene Mausmodelle für diese immunologischen Untersuchungen mit den Tumorzelllinien CMT64 und KLN205 etabliert. In vitro Replikations-Assays zeigten, dass sowohl in KLN 205 als auch in CMT64 das humane telomerase-abhängige Adenovirus hTert-Ad repliziert. Weitere in vivo-Untersuchungen durch die Generierung und Verwendung eines Lufizerase-exprimierenden Adenovirus bestätigten die virale Replikation von hTert-Ad in den entsprechenden subkutanen Tumoren.
Um die Migration und Expansion der dendritischen Zellen in den Tumoren im Rahmen der Virotherapie zu induzieren, wurden zwei nicht replikative Adenoviren kloniert, die das Macophage Inflammatory Protein 1α (MIP-1α) und Flt3L exprimieren.
Im Vergleich zur Virotherapie alleine, oder zur Virotherapie mit einem einzelnen CC Chemokine, resultierte die gemeinsame Expression von MIP-1α und Flt3L in einer signifikant gesteigerten Infiltration der Tumoren mit dendritischen Zellen und T-Zellen. IFN-γ-ELISpots und CTL-Assays zeigten, dass die Kombination von MIP-1α und Flt3L die Kreuzpräsentation von tumorspezifischen Antigenen stark stimuliert und eine starke antitumorale Immunantwort auslöst. Durch die weitere Kombination der MIP-1α /Flt3L-Virotherapie mit einer dendritischen Zell-Impfung, konnten nahezu komplette Remissionen der subkutanen Tumore und eine sehr starke Hemmung von Lungenmetastasen erzielt werden, sodass über 50% der Tiere geheilt werden konnten.
Zusammenfassend zeigt diese Arbeit, dass die Wanderung und Expansion von dendritischen Zellen in Tumoren, die durch tumorspezifische Viren infiziert wurden, die Kreuzpräsentation von tumorspezifischen Antigenen verstärkt und hierdurch eine starke tumorspezifische Immunantwort auslöst. Dies ermöglicht eine effektive Viroimmuntherapie von etablierten soliden Tumoren und ihren Metastasen. Die

Ergebnisse dieser Arbeit zeigen die hohe Effektivität der Viroimmuntherapie, die hoffentlich als sichere Tumortherapie Eingang in die klinische Praxis finden wird.
Key words: Virotherapie, immune antwort, dendritische zell migration

3 Introduction

3.1 Cancer

Cancer is a group of over 100 diseases characterized by uncontrolled growth of transformed cells. Cancer is classified according to the cell types and organs from which they originate. Carcinoma is the most common form of cancer that arises in the epithelium, the layers of cells covering body surfaces or lining internal organs and various glands. In this thesis, two lung cancers are discussed, namely small lung carcinoma cell line (CMT 64) and squamous cell carcinoma (KNL205).

Cancer is a world wide health problem which was responsible for the death of millions of people every year. Even though our knowledge of molecular mechanisms, diagnostic methods and traditional treatments of cancer have all improved during the last decades, most cancer types still have a poor prognosis and evoke a high mortality, this being especially the case with metastatic types. Thus, more efficient approaches and improved therapeutic strategies are needed for the treatment of cancer.
The first clinical gene therapy trial was carried out in the early 1990s. From that beginning, gene therapy has become a widely studied concept for treatment of various diseases. Even though gene therapy was initially thought to be more suitable for the treatment of inherited monogenic diseases, gene therapy has been increasingly utilized in the treatment of acquired and complex diseases such as cancer. In fact, by the year 2004, the majority of clinical gene therapy trials (66%) have focused on cancer diseases.

Even though several approaches have been successfully developed to improve the gene transfer efficiency, one major obstacle in cancer gene therapy is still insufficient transduction of the gene and consequently a poor therapeutic effect. There are several possibilities that can be utilized to circumvent this problem. First, new alternative viral vectors can be explored to find optimal gene transfer vehicle for each purpose. Secondly, viral vectors can be re-targeted to cancer cells, which simultaneously

enhance gene transfer rates in tumors and diminish undesired side effects in healthy tissue. In addition, it is possible to exploit viral replication per se to destroy cancer cells by transcription regulation. To avoid side effects and to increase the safety of these oncolytic agents, replication can be restricted to tumor cells by partially deleting the viral genome or by using tissue specific promoters to drive the viral genes responsible for replication.

3.2 Cancer Associated Genes

3.2.1 Overview

Three major gene types have been shown to be closely related to tumorigenesis: proto-oncogenes, tumor-suppressor genes and DNA mismatch repair genes. Since cancer is a group of neoplastic diseases involving multiple steps and genetic events, tumor development usually requires alterations in a number of cancer-associated genes. A transformed phenotype when combined with a metastatic capability typically involves both oncogene activation and tumor suppressor gene loss or inactivation (Bast et al., 2000). Furthermore, it is noteworthy that the same genes causing genetic predisposition to cancer are most often associated and involved in sporadic forms of carcinogenesis (Knudson et al, 1991).

3.2.2 Proto-oncogenes

The proto-oncogenes encode proteins that have a crucial role in cell signaling pathways. The proto-oncogenic products (including various growth factors and their receptors, signal transducers and transcription factors) control the growth and differentiation of normal cells (Aaronson, 1991; Bast et al., 2000). These proto-oncogenes can be activated to carcinogenic oncogenes through mutation, gene amplification or chromosomal rearrangement resulting in an altered proto-oncogene structure or an increased proto-oncogene expression (Bast et al., 2000). Thus activation of oncogenes offers a growth advantages to the cell and eventually normal cells become transformed.

3.2.3 Tumor suppressor genes

Tumor suppressor genes can prevent cell growth by several pathways either by blocking the cell cycle or by promoting cell cycle arrest and apoptosis (McCormick et al, 2001). In cancer cells, these genes are inactivated and block the control of normal cell cycle and thus increase the probability of tumor formation (Knudson et al, 2001).

3.2.4 DNA mismatch repair (MMR) genes

The DNA mismatch repair (MMR) system is one of the three cellular mechanisms involved in DNA repair (Charames and Bapat, 2003). MMR proteins recognize and eliminate potential misincorporated nucleotides on the newly synthesized DNA strand during DNA replication (Fedier and Fink, 2004). Hence, MMR machinery is a crucial mechanism for maintaining genome integrity during cell proliferation. Mutations in genes encoding MMR proteins attenuate or inactivate the DNA repair machinery and thus interfere with genetic stability and increase the incidence of further mutations during the DNA synthesis (Fedier and Fink, 2004). This increased mutation frequency thus augments the susceptibility to undergo cellular transformations and tumor formation (Eshleman and Markowitz, 1996). Mutated MMR proteins have been identified and associated with various cancer types including hereditary nonpolyposis colon cancer (Bronner et al., 1994; Fishel et al., 1993), breast (Balogh et al., 2003) and bladder cancer as well as gliomas. In addition, tumor cells with a defective MMR system have been shown to display reduced sensitivity to the cytotoxic, DNA damaging chemotherapeutic drugs such as cisplatin (Brown et al., 1997) and doxorubicin (adriamycin).

3.3 The Immune System

The immune system is an organization of cells and molecules that have evolved in order to protect an organism from foreign pathogens and possibly against altered self-cells that may lead to cancer. The immune system can be divided into two parts, the innate (natural) and adaptive (acquired) immune systems. In reality, a cross talk

between the innate and adaptive immune response is needed in order to eliminate many pathogens.

3.3.1 Innate immunity

The very first lines of defense against foreign pathogens are physical barriers to the outer world such as mucosal epithelial surfaces that contain lysozymes and other secretary factors that prevent pathogen growth. Other molecular components include the complement system, cytokines and acute phase proteins. Cells of the innate immune system include phagocytic cells (neutrophils, monocytes, macrophages and DCs), cells that release inflammatory mediators (basophils, eosinophils and mast cells), and natural killer (NK) cells, all with pre-existing receptors for pathogens. In mammalian cells, TLRs are important PRRs responsible for a large proportion of the innate immune recognition of pathogens (Akira S, 2004).There is strong evidence that these TLRs are responsible for sensing the PAMPs or providing the "danger signal" for activation of the adaptive immune system (Guermonprez P, 2002). The innate system protects us but may only delay and not eliminate certain pathogens until the adaptive immune system becomes activated.

3.3.2 Adaptive immunity

Adaptive immunity is unique for vertebrates. The adaptive immune system mounts its response during an infection and is therefore slower, but in contrast to innate immunity it leads to immunologic memory. DCs are the key activators that link innate and adaptive immunity. The adaptive immune response consists of a humoral and a cellular response. Humoral immunity is mediated by B lymphocytes and their secreted products, namely antibodies. Antibodies are produced in response to antigens which often derive from extracellular pathogens such as bacteria and virus. Cell-mediated immunity involves macrophage and NK cell activation, the production of antigen-specific T cells and the release of various cytokines in response to antigens. Cell-mediated immunity is primarily a response to intracellular pathogens such as viruses.

3.3.3 Antigen Processing and Presentation

Antigens are macromolecules that elicit immune response in the body. Majority of antigens for T cells are proteins, and these must be fragmented and recognized in association with MHC products expressed on the surface of nucleated cells, not in soluble form. Antigen processing and presentation are processes that occur within a cell that result in fragmentation (proteolysis) of proteins, association of the fragments with MHC molecules, and expression of the peptide-MHC molecules at the cell surface where they can be recognized by the T cell receptor on a T cell. However, the path leading to the association of protein fragments with MHC molecules differs for class I and class II MHC. MHC class I molecules present degradation products derived from intracellular (endogenous) proteins in the cytosol. MHC class II molecules present fragments derived from extracellular (exogenous) proteins that are located in an intracellular compartment.

3.4 Major Histocompatibility Complex (MHC)

3.4.1 Self MHC Restriction

In order for a T cell to recognize and respond to a foreign protein antigen, it must recognize the MHC on the presenting cell as self MHC. This is termed self MHC restriction. Helper T cells recognize antigen in context of class II self MHC. Cytotoxic T cells recognize antigen in context of class I self MHC. The process whereby T cells become restricted to recognizing self MHC molecules occurs in the thymus.

3.4.2 Presentation by MHC I

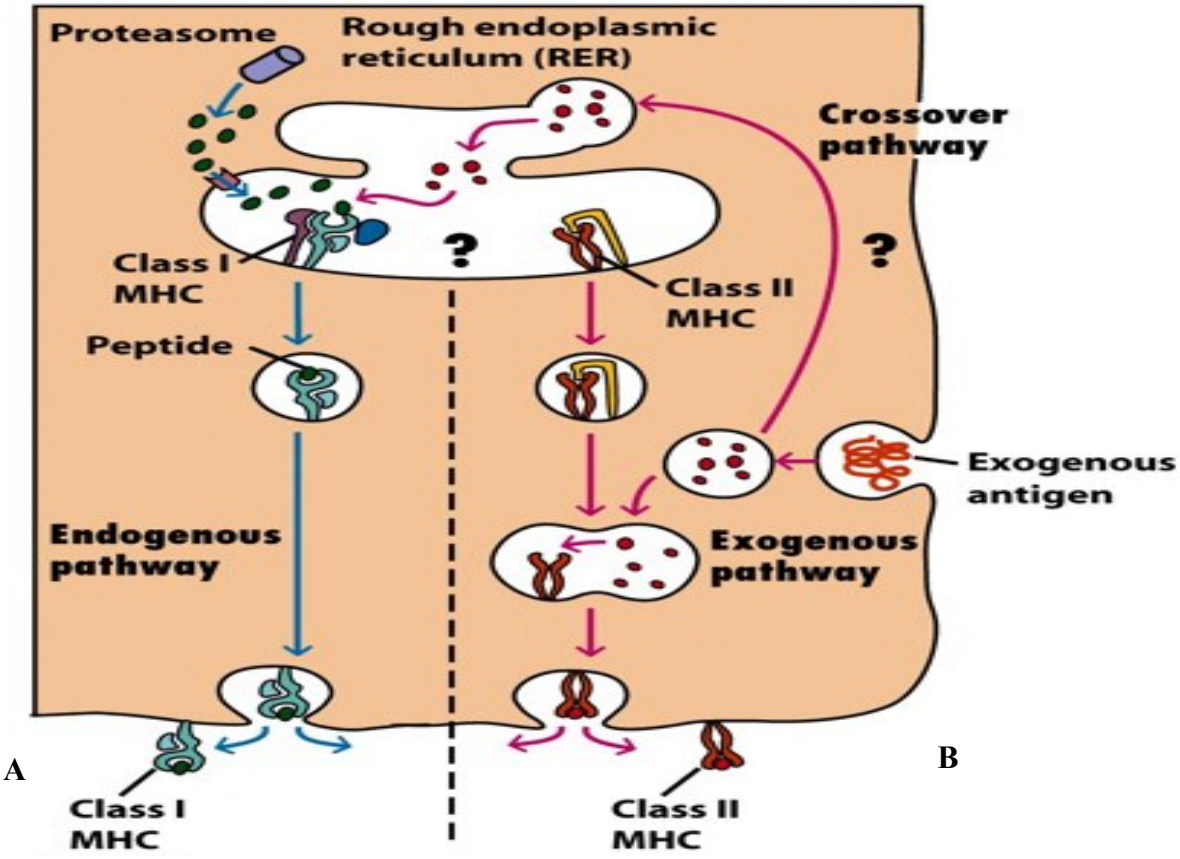
Professional antigen presenting cells process intracellular antigens, such viruses or virus infected cells are degraded by the proteasome and the resulting peptides are shuttled into endoplasmic reticulum (ER) by TAP proteins. These peptides are loaded onto MHC class I molecules and the complex is delivered to the cell surface, where it stimulates naïve CD8 T cells.

3.4.3 Presentation by MHC II

In contrast to MHC class I, extracellular antigens are engulfed by phagosomes or endocytosis. Inside phagosome, the antigen derived peptides are loaded directly onto MHC class II molecules, which activate helper T cells.

3.4.4 Cross-Presentation

Conventional presentation of exogenous antigen in the context of MHC-I pathway refers as cross presentation **(Figure 3.1)**. It is the fundamental mechanism of induction of anti tumor immune responses.



This figure obtained from Janis Kuby, Immunology, 6th edition

Figure 3.1 Antigen Processing and Presentation.

A. Exogenous antigen ingested via endocytosis/phagocytosis. Once in the phagosome these antigens degraded by several processing enzymes and the antigen is degraded into small peptides, which complexes with MHC class II molecules and transported to the membrane of antigen presenting cells.

B. Endogenous antigen produced with in the cells, or viral infected cells is degraded with in the cytoplasm by proteasome into peptides, these peptide move ER via transporter associated for antigen presentation where they bind to MHC class I molecules. These MHC-peptide complexes move to Golgi complex then to the cell surface

3.4.5 T cell priming by DCs

Immature DCs are distributed throughout the body. They have receptors for chemokines such as macrophage inflammatory protein (MIP)-3α, MIP-1α and RANTES. They accumulate rapidly, within an hour, at the sites of antigen deposition. Immature DCs are very efficient in capturing antigens in the forms of apoptotic and necrotic cell fragments (Banchereau J, 2000). Upon antigen uptake, DCs mature, downregulate their antigen internalization capacity and upregulate the surface expression of the co-stimulatory molecules CD80 and CD86 (Lanzavecchia A et al, 2000). They also upregulate expression of chemokine receptors CXCR4 and CCR7. The upregulation of CCR7 promotes responsiveness to the chemokines MIP-3β and 6Ckine that initiate DC migration. 6Ckine helps to co-localize mature DCs and naive T cells in the T cell zone of secondary lymphoid organs, i.e. the spleen and the lymph nodes, and contributes to T cell activation. It has long been thought that apoptotic cell death is poorly immunogenic (tolerogenic), whereas necrotic cell death is immunogenic. This difference was thought to result from an intrinsic capacity of cells dying from non-apoptotic cell death to stimulate the immune response: for example, by stimulating local inflammatory responses and/or by triggering the maturation of DCs. It seems, however, that the classification of necrosis as immunogenic and apoptosis as tolerogenic is an oversimplification. It has recently been shown that the capacity of apoptotic cells to trigger an immune response depends on the inducer of apoptosis (Casares N et al, 2005), indicating that qualitative differences in the biochemical mechanisms of cellular apoptosis could lead to different immune response outcomes.

T cell receptors (TCRs) present on T cells recognize antigens in the form of peptide fragments bound to major histocompatibility complex (MHC) molecules on the surface of DCs. There are two types of MHC, class I and class II, which stimulate

CD8+ and CD4+ T cells, respectively (Banchereau J, 2000). Endogenously expressed antigens are processed in the cytosol of the DC and peptides are presented in the context of MHC class I molecules to naïve CD8+ T cells. Exogenously expressed antigens taken up by DCs through endocytosis are processed and generally presented as peptides by MHC class II molecules to CD4+ Th cells (Banchereau J et al, 2000). However, DCs have evolved a unique cross-priming mechanism to transport extracellular antigens to the cytosol where they are degraded and loaded onto MHC class I molecules for presentation to CD8+ T cells. This is called cross-presentation. Moreover, one DC can prime up to ten T cells at the same time. Engagement of TCRs by MHC-peptide complexes on the surface of APCs provides the first activation signal to naïve T cells to become CTLs. Activated CTLs migrate to the site of inflammation and kill target cells via Fas ligand and/or perforin / granzyme mediated killing (Igney FH et al, 2002).

3.5 Cancer Immunotherapy

Cancer immunotherapy is the use of the immune system to reject cancer. The main principle is stimulating the host immune system to attack the malignant tumor cells that are responsible for the disease. This can be either through immunization of the host, in which case the hosts own immune system is trained to recognize tumor cells as targets to be destroyed, or through the administration of therapeutic drugs, in which case the hosts immune system is recruited to destroy tumor cells by the therapeutic drugs. An efficient way to activate an individual's immune system is through infiltration of immune cells into inflammatory tissue in case of cancer, tumor tissue where the interaction among tumor cells, T and DCs would activate immune system specifically to tumor antigens.

3.5.1 Adenoviral vectors

Cancer immunotherapy based on the delivery of genes encoding TAAs to DCs or immunomodulatory molecules to tumor cells requires an efficient gene transfer method. The genes being transferred are included in vectors that are either of viral or non-viral origin to improve transduction efficiency of therapeutic gene delivery; the

resulting therapeutic gene expression can transduced efficiently in to the local damaged tissue and compensate low transduction efficiency, to enhance therapeutic applications and low toxicity.

3.5.2 Adenoviral vectors for Transgene delivery

In the recent years, adenoviruses have received considerable attention for use in gene therapy because of their large cloning capacity and ease of genetic manipulation and growth. In addition their ability to transduce a wide range of tissue type containing both dividing and non-dividing cells. The most commonly used recombinant adenoviral vectors are generated from human adenoviruses of serotypes 2 and 5 (Volpers C et al, 2004). Adenovirus genome is a double-stranded linear DNA molecule that codes for early and late proteins (Goncalves MA et al, 2006). Early genes encode proteins involved in host cell cycle regulation, anti-apoptosis, immune system deviation and virus replication. Late genes encode structural viral proteins and proteins involved in virus assembly. First generation Ad vectors, replication defective by deletion of E1A have proven an inadequate for long term, stable transgene expression necessary for correction of most genetic diseases. This could be because of foreignness versus self transgenes, strong innate immunity and generation of anti-Ad cytotoxic T lymphocytes and anti-adenoviral antibodies. It is likely that poor performance of clinical studies is of several reasons.

The first interaction of an adenoviral vector with the host cell occurs through binding of the virus fiber knob to the coxsackie adenovirus receptor (CAR).

Thereafter, the RGD (Arg-Gly-Asp) motif on the adenoviral penton base interacts with αV integrins, which act as a co-receptor for viral endocytosis. The virus is internalized into the cell and escapes from the endosome, a step in which the fibers and penton bases detach from the virus capsid. The partly uncoated adenoviral capsid is translocated along microtubules toward the nucleus. The adenoviral capsid is uncoated and the adenovirus genome enters the nucleus through nucleopores. The adenoviral vector stays episomal and the gene of interest carried by the adenoviral vector is expressed.

Adenoviruses are airborne viruses and therefore the primary targets for infection are the respiratory, gastrointestinal, and urinary tracts, as well as the eye (Kojaoghlanian T et al, 2003) However, adenoviruses can infect all cell types expressing CAR and integrins. Therefore, adenoviral vectors can provide highly efficient delivery of therapeutic genes in most cell cultures. It is easy to produce high viral titers for *in vitro* and *in vivo* purpose. The major disadvantage of adenoviral vectors is immunogenic and most of us have already antibodies against adenoviruses. Virus neutralizing antibodies induced by adenoviral infections or upon adenoviral vector delivery are primarily directed against the adenoviral surface hexon protein (Kojaoghlanian T et al, 2003). Moreover, adenoviruses activate the innate immune system to produce proinflammatory cytokines that can initiate differentiation of immature DCs (Muruve DA et al, 2004). Systemic administration of high doses of adenoviral vectors into mice (Zhang Y, 2001) was shown to trigger the rapid release of IL-6, IL-12, and TNF-α and show accumulation of immune cells in lymphatic tissue. However, in oncolytic virotherapy of cancers, specifically when using conditionally replicating adenoviruses in cancer therapy, combination of immunotherapy setting would be an additional induction of the immune system by the adenoviral vectors expressing cytokine like co-stimulatory molecules or cytokine which enhance immune cell infiltration into local tissue could lead to a desired immunological effect against tumor specific antigen delivered by adenoviral replication.

Theoretically, metastatic disease may only be treated by the systemic delivery of targeted gene vectors. However, for locally advanced prostate cancer (T3 and T4) there are several ways to deliver the vectors. Steiner MS et al compared three routes of administering adenoviral vectors in a canine model: intravenous, intra-arterial and intraprostatic injections (Steiner MS et al, 1999). This study provides direct support for the use of intratumoral prostatic injections of gene therapy vectors. Generally, when the vector is administered directly to the target tissue or organ, toxicity is dose-dependent and confined to the injection site. Another way to overcome side effects is to use a tumor or tissue-specific promoter to control transgene expression, which is also known as transcriptional targeting. Here again we used tumor specific promoter **(Figure 3.2)**, human telomerase reverse transcriptase, the catalytic subunit of telomerase which drives E1A protein of adenovirus replication (Wirth T et al, 2003).

3.6 Merging virotherapy and immunotherapy

Several gene therapy approaches have been demonstrated to cancer cells, clinical trails that the early generation viral vectors have rather limited ability to completely eradicate advanced tumor masses. In order to improve the efficacy of virotherapy, combination of immunotherapy approaches might be useful to eradicate established cancer specifically when using tumor specific viral replication to enhance tumor antigen specific immune response. In the present study we used adenoviral vectors for cytokine gene (Replication deficient Ad vectors) delivery into tumor tissue, which are E1A and E3 genes deleted (except hTERT-Ad) and are replication-deficient (MIP-1α, Flt3L, LacZ, GFP and Luciferase Ad-viruses) viruses. The deletions create space for introducing foreign promoters and genes of interest (Robbins PD et al, 1998).

3.6.1 MIP-1α (Macrophage Inflammatory Protein 1α)

Macrophage inflammatory protein 1α (MIP-1α) is a member of the C-C subfamily of chemokines, a large super family of low-molecular weight, inducible proteins that exhibit a variety of proinflammatory activities *in vitro* including leukocyte chemotaxis. MIP-1α (CCL3) is of a particularly interesting chemokine, because in addition to its proinflammatory activities, it inhibits the proliferation of hematopoietic stem cells in vitro and *in vivo*. The MIP-1α null mice have no overt abnormalities of peripheral blood or bone marrow cells, indicating that MIP-1α is not necessary for normal hematopoiesis. However, the MIP-1α null mice have reduced inflammatory response to influenza virus and are resistant to coxsackievirus-induced myocarditis. These data demonstrate that MIP-1α is required for a normal inflammatory response to these viruses.

MIP-1α is structurally and functionally related CC chemokine. MIP-1α participate in the host response to invading bacterial, viral, parasite and fungal pathogens by regulating the trafficking and activation state of selected subgroups of inflammatory cells e.g. macrophages, lymphocytes and NK cells. MIP-1α exerts effect on lymphocyte differentiation and in selectively attracting CD8+ lymphocytes. Additionally, MIP-1α has also been shown to be potent chemo attractant for T cells and dendritic cells (New JY et al, 2002). Mostly immature DCs infiltrated by CC and CXC chemokines such as MIP-1a, MIP-5, and RANTES **(**MC Dieu-Nosjean et al,

1999).

3.6.2 Flt3 ligand

The fms-like tyrosine kinase 3 (Flt3), also known as stem cell tyrosine kinase-1 or fetal liver tyrosine kinase-2 (Lyman SD et al, 1993; Rosnet O et al, 1991). Flt3 (CD135) is expressed on multipotent HSC and progenitor cells, suggesting a critical role in stem cell development and differentiation (Rosnet O et al, 1996). Analysis of Flt3 knockout mice has shown that mainly primitive B cell progenitors are reduced. Yet, normal numbers of functional B- cells are present in periphery. Flt3- Ligand knockout mice on the other hand show an overt reduction of leukocyte counts in bone marrow, spleen, thymus as well as peripheral blood. In addition these animals also show reduced numbers of myeloid and lymphoid dendritic cells and are deficient in NK cells. Similar to the receptor knockout mice these animal show slightly reduced numbers of myeloid progenitors (McKenna HJ et al, 2000). Many animal studies shown that adenoviral expressing Flt3L enhances number of dendritic cells *in vivo* and enhances immune response to specific antigens (Wang H et al, 2005) Another study showed that immunogenicity of DNA vaccine can be enhanced with dendritic cell migration (Sumida SM et al, 2004).

3.6.3 Telomerase-dependent conditionally replicating adenovirus (hTERT-Ad)

Telomeres are essential elements at chromosome termini that preserve chromosomal integrity by preventing DNA degradation, end to end fusions, rearrangements and chromosome loss (Blasco MA et al, 1997; Lee HW et al, 1998; Rudolph KL et al, 1999). Each cell replication is associated with the loss of 30-150 bps of telomeric DNA that can compensated by telomerase, an RNA-dependent DNA polymerase (Greider CW et al, 1996). Ectopic expression of hTERT, the catalytic subunit of telomerase, is capable to reconstitute telomerase activity in telomerase negative cells, indicating that hTERT is the major determinant of telomerase activity in mammalian cells (Weinrich SL et al, 1997; Vaziri H et al, 1998).

Most of human somatic cells neither exhibit hTERT expression not telomerase activity, hence limited number of cell divisions because of the reduction in telomeres

to a critical length. In contrast to quiescent somatic cell, in highly proliferative cells, such as germ-line, HSC or transformed cancer cells, diverse molecular mechanisms are necessary to maintain telomere length. Most of human cancer cells acquire immortality by expression of the hTERT. It has been shown that hTERT expression is regulated at the transcriptional level (Poole JC et al, 2001). Previous studies demonstrated that the hTERT promoter is inactive in normal cells but is activated during carcinogenesis, thereby providing a promising tool for tumor specific gene expression (Abdul Ghani R et al, 2000; Koga S et al, 2000).

In the present study, an adenoviral vector (hTERT-Ad) was constructed by replicating the internal adenoviral E1a promoter by a 255bp hTERT promoter fragment (Wirth et al, 2003). This altered transcriptional control of E1A expression was restricted to telomerase positive tumor cells.

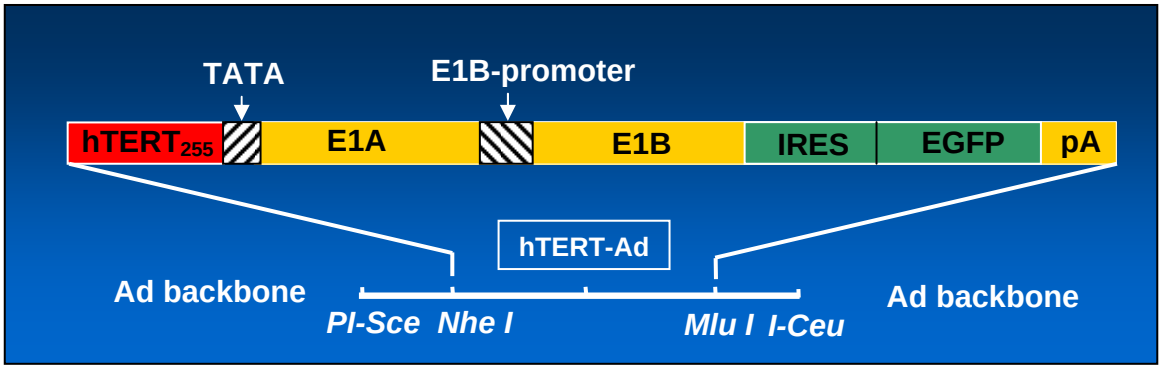


Figure 3.2 Schematic diagram of construction of hTERT-Ad (Wirth T et al, Cancer Research, 2003)

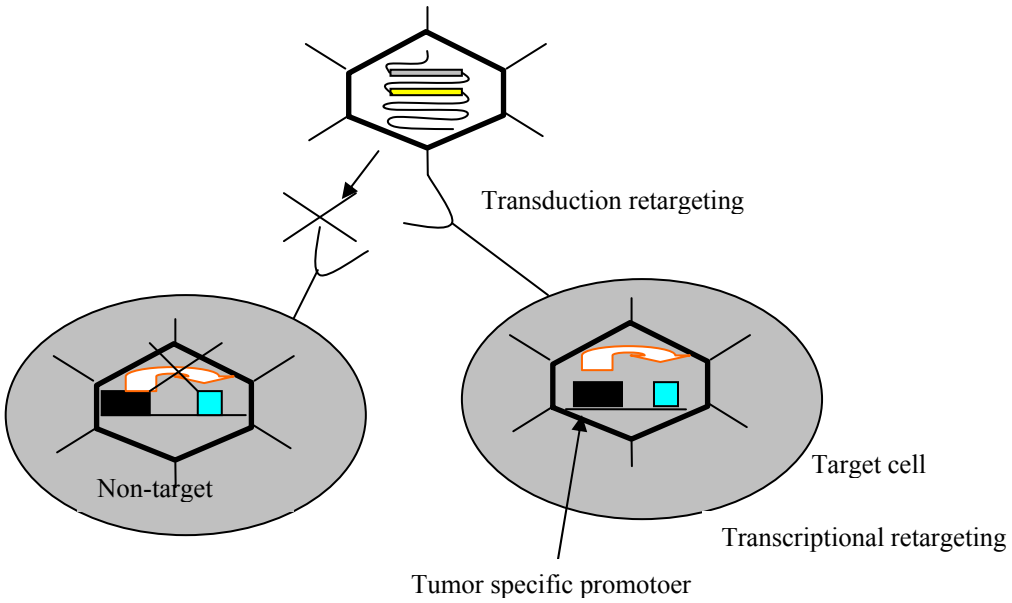


Figure 3.3 To achieve transgene expression specifically in the tissue of interest it is possible to either change the homing of the virus (transductional retargeting) or to alter the expression profile of the transgene (transcriptional retargeting).

In this study, we examined several potential approaches to improve oncolytic virotherapy. First, we explored the feasibility of replication of murine tumor cells using conditionally replicating adenoviral vectors (hTERT-Ad) in cancer gene therapy (Wirth et al, 2003) **(Figure 3.2 and 3.3)**. In addition, we used cytokines expressing in adenoviral vectors to attract immune cells into inflamed tissue. Finally, we established an efficient method for generating immune responses against tumor specific antigens using conditionally replicative adenovirus in combination of immunotherapy and evaluated its oncolytic potency and immune response for cancer gene therapy in murine model.

4 Aims of the study

The major goal of this work was to define a protocol for the cancer therapy using oncolytic virotherapy and immunotherapy in small lung carcinoma and squamous carcinoma tumors in murine model.

1. Evaluation of replication of murine tumor cells with conditionally replicating human adenovirus serotype 5 *in vitro and in vivo*

2. To develop new strategies to enhance virotherapy efficacy by immunotherapeutic approaches in cells with defective in transporters associated with antigen processing lung carcinoma cells by recruiting immune cells into tumors

3. To determine whether modulation of immune responses with viroimmunotherapy and antigen pulsed dendritic cell vaccination can modulate immune responses to virus and tumor

4. To investigate whether viroimmunotherapy and dendritic cell vaccination has any effect on distant lung metastasis for further translation of viroimmunotherapy to humans

5 Material and methods

5.1 Animals

Female C57Bl/6 (B6, $H2^b$) and DBA/2 mice (6-12 weeks old) were purchased from Charles River, Germany. The animals were housed in sterilized individually ventilated cages at the animal facility of the Medical School of Hannover (MHH) and received sterilized food and water. All manipulations were performed under aseptic conditions in a laminar-flow hood.
Transgenic OT-I mice (OVA323-339–SIINFEKL specific H2b-restricted TCR-transgenic mice on a C57/BL6 background) (1) were generously provided by Dr. Martin Sauer, MHH. All experiments were conducted under approved animal protocols of the state government of Lower Saxony, Germany.

5.2 Cell lines

CMT64, a C57/BL6 (H-2b) derived lung carcinoma cell line exhibits low expression of tumor-associated antigen presentation in the context of MHC class I antigens because of defects in the antigen presentation pathways of transporters associated with antigen processing (TAP). KLN 205 squamous carcinoma cells derived from DBA/2 mice. The murine cell line EL4 (H-2b) is a lymphoma cell line (kind gift from Prof. Christopher Klein, MHH) and was grown in 10% DMEM. HEK293 cells are human embryonic kidney cells from the ATCC, (Rockville, MD, USA).

5.3 Creation of stable cell line

Plasmid MMP-OVA-IRES-GFP was a kind gift from Dr. Martin Sauer (MHH). Cells were seeded one day before transfection. Co-transfection of MMP-OVA-IRES-GFP (4 μg) and retrovirus pseudotyped with vesicular stomatitis virus (VSV G) (2 μg) and gag-pol (2 μg)) plasmids into producer cell line Phoenix-Amphoteric packaging cell

line ATCC (Rockville, MD) was carried out using the lipofectamine or calcium phosphate method by transient transfection. After 48 hrs, the cellular supernatant was collected over a period of a further 48 hrs. For retroviral transduction of the CMT64 cell line, supernatant containing viral particles was centrifuged at 2000 rpm for 10 minutes and taken 1.5 ml of virus-containing supernatant and 1.5 ml DMEM, plus 10% fetal calf serum (FCS), were added together in the presence of 8 μg/ml polybrene. The cells were incubated in an incubator at 37°C for 3 hrs. After 4 days, green fluorescent protein (GFP)-expressing cells were sorted and cultured. The new cell line CMT64-OVA-IRES-GFP is referred to as CMT64-OVA.

5.4 Transfection procedure

5.4.1 Lipofectamine-2000

One day before transfection, approximately $2*10^6$ cells in 5 ml of growth medium without antibiotics were seeded in 25 mm flasks so that they would be 90-95% confluent at the time of transfection.

Dilute DNA (adenoviral transfection: 10 μg DNA; normal DNA transfections: 1 μg DNA) was added to 500 μl of opti-MEM reduced serum medium and mix gently.

Lipofectamine-2000 was gently mixed before use, and then 18 μl of the lipofectamine-2000 was diluted in 482 μl of Opti-MEM. The solution was mixed gently once more and incubated for 5 minutes at room temperature (RT). The diluted DNA was then immediately combined with the diluted lipofectamine-2000, mixed gently and incubated for 20 minutes at RT to allow DNA-lipofectamine-2000 complexes to from.

The DNA-lipofectamine-2000 complexes (1 ml) were added to the flasks containing cells and medium. The flasks were gently agitated back and forth.

The cells were incubated for 4-6 hrs at 37°C in an incubator and washed with phosphate-buffered saline (PBS), following which new medium with 10% FCS was added to the cells. After 24-48 hrs, the cells were ready to assay for transgene expression. In the case of adenoviral DNA transfection, the cells were incubated for 7-10 days until a cytopathic effect was visible.

5.4.2 Calcium Phosphate Transfection

2X HBS buffer (Transfection buffer)

280 mM of NaCl (1,637 gm/100ml)

50 mM BES (1,066 gm/100ml)

1.5 mM Na_2HPO_4 ($2H_2O$)

PH - 6.95 to 7.2 to test for optimum transfection efficiency. This solution must be filtered to sterilize it and should be kept at 4^oC. It should be brought to RT before use.

$CaCl_2$ preparation

2.5 M $CaCl_2$ (27.75 gm anhydrous $CaCl_2$ or 36.75 gm $CaCl_2$, ($2H_2O$))

Both reagents were brought to RT before transfection. DNA was added to the $CaCl_2$ (300 µl) solution and kept for 10 minutes, following which an equal volume (300 µl) of 2X HBSS solution was added, and incubation was continued for 10 further minutes. This solution was added to the cells drop-wise. After overnight incubation, the cells were washed, and new medium was added. Transgene expression was measured 48 hrs later.

5.5 Establishment of subcutaneous tumors

A total of $5X10^6$ KLN205 or CMT64 (OVA) cells were injected into the right flank of mice. After 7-10 days, tumors had grown to approximately 0.5-1cm. Mice were anesthetized with isofluorane, and viruses were injected intratumoral in a volume of 100-150 µl.

5.6 Lung metastasis

100,000 CMT64 or CMT64-OVA cells were injected intravenously into C57Bl6 mouse, two days before viroimmunotherapy. After 10-15 days, the lungs were full of metastases. In the treatment group, mice were sacrificed only when the experiment ended.

5.7 Bone-marrow-derived dendritic cell isolation and antigen pulsing

Mice were sacrificed by cervical dislocation. Bone marrow was flushed out from the femur and tibia of mice as previously described (Weigel BJ et al, 2002). Briefly, red blood cells were lysed by ammonium chloride incubation, and single cell suspensions were then incubated at 2 x 10^6 cells/ml RPMI medium with penicillin and streptomycin, sodium pyruvate, non-essential amino acids, and 1 M 2-mercaptoethanol at 37°C and 5% CO_2 in 6-well plates with 150 U/ml granulocyte/macrophage colony-stimulating factor (GM-CSF; Sigma, Munich, Germany) and

75 U/ml interleukin-4 (IL-4; Sigma, Munich, Germany) for 7 days or GM-CSF supernatant generated from EL4 stable cells. On day 8, dendritic cells (DC) were pulsed with tumor lysate or ovalbumin antigen (OVA). This was followed by maturation of DC by using anti-CD40 as a maturing agent on day 7.

5.8 Spleen cell isolation

Mice were sacrificed by cervical dislocation, and their spleens were taken. To make single cell suspension, spleens were crushed gently and transferred through 70-µM mesh into PBS. Splenocytes were centrifuged and resuspended in 1ml of PBS and 5 ml of red blood cell (RBC) lysis buffer and centrifuged again. The pellet was resuspend in PBS and kept on ice until used.

5.8.1 Erythrocyte lysis buffer

4.14 gm of (NH_4) Cl

0.5 gm of $KHCO_3$

0.1 mM EDTA or 100 µl of 500 mM EDTA in 500 ml of distilled water. This solution must be sterilized for cell culture use.

5.9 Flow cytometry

1*10^6 cells were washed and incubated with Fc blcok (CD16/CD32) (2.4G2) (BD PharMingen, Heidelberg, Germany) at 4°C for 10 minutes to block nonspecific

binding of fluorochromes. The following directly conjugated antibodies were incubated with splenocytes at 4°C for 30 minutes with anti-CD11c, anti-CD8a, anti-CD4, anti-CD25, anti-FoxP3, and labeled with Ova Pentamer-R-PE (only 10-minute incubation with pentamers, according to the protocol).

OVA-reactive CTLs were tracked by using R-PE-labeled Pro5 MHC Pentamer H2-Kb/SIINFEKL (ProImmune, Oxford, UK). After the cells had been stained, they were washed twice, centrifuged for 5 minutes at 1000 rpm, suspended in FACS buffer, and counted by FACS Caliber or FACSConto II. The data were analyzed by cell quest or FlowJo.

5.9.1 FACS buffer

0.1% bovine serum albumin (BSA), 0.1%NaN3, 10mM HEPES and 2mM EDTA in 500 ml of PBS.

5.9.2 MACS buffer

One liter of MACS buffer was prepared with 0.5% BSA (5 gm), penicillin and streptomycin (10 ml), 1 mM EDTA, pH 8 (4 ml of 0.5 M stock), 4 ml of 1 M stock HEPES, 1 mM sodium pyruvate (10 ml of 100mM stock) and sterile-filtered for cell culture use.

5.10 ELISpot

ELISpot membrane was activated with 100 µl of 50% methanol/well for a few seconds and washed three times with PBS. Immediately, 100 µl/well of the capture antibody (0.25 µg of interferon-γ; IFN-γ) was added. The plate was covered with a lid and incubated overnight at 4^{o}C. The primary antibody was washes out with PBS, and the membrane was blocked with RPMI plus 10% FCS for 1-2hrs. Spleenocytes were plated $2*10^{5}$ cells/well and stimulated with different parameters and incubated in the ELISpot plate in a 37^{o}C incubator without disturbance for 24-36 hrs. Plates were washed three times with PBS, followed by the addition of biotin-conjugated secondary antibody with 1% BSA in PBS and incubated at RT for 2 hrs. After a further three washes and incubation with streptavidin-HRP conjugate for 1hr, the plates were washed again extensively and then developed with DAB 1X substrate

with peroxidase 1X solution (both from Pierce, Germany) substrate until spots were developed. Development was stopped carefully with PBS.

5.11 ELISA

5.11.1 Measurement of total anti-adenovirus antibodies in serum

ELISA plates (from Nunc Maxi Sorb) were coated with 10^8 particles/100µl/well and incubated overnight at 4°C. They were washed three times with PBS containing 0.1% Tween 20, blocked with 5% milk powder for 1 hr at RT, washed three times with PBS, and incubated with serum samples (serial dilutions in 100 µl/well) in duplicate for 2 hrs at RT. The plates were washed three times with PBS, incubated with peroxidase-conjugated goat-anti-mouse IgG (H+L) (1:3000, 100 µl/well) for 2 hrs, washed three times, and finally incubated with TMB substrate (100 µl/well) until color developed. The reaction was stopped with 1M H_3PO_4 (50 µl / well). The optical density of the reaction products was measured at 450 nm.

5.11.2 Cytokine ELISA

The capture antibody was diluted to the working concentration in PBS without carrier protein. A 96-well microplate was immediately coated with 100 µl per well of the diluted capture antibody, sealed, and incubated overnight at RT. Each well was aspirated and washed with wash buffer, three times. The plates were blocked by adding 300 µl of reagent diluent to each well and incubated at RT for a minimum of 1 hr, followed by three times with wash buffer. Sample or standards in reagent diluent (100 µl/well) were added to the plates, which were covered with an adhesive strip and incubated for 2 hrs at RT. The plates were washed three times with washing buffer. Detection antibody diluted in reagent diluent (100 µl) was added to each well, and the plate was covered with a new adhesive strip and incubated for 2 hrs at RT. Subsequently, the plate was washed three times with washing buffer. An aliquot of 100 µl of the working dilution of streptavidin-HRP was added to each well, and the plate was once more covered and incubated for 20 minutes at RT in the dark, followed by three washes with wash buffer. Substrate (TMB substrate, BD OptE1A,) solution

(100 µl) was added to each well and incubated for 20 minutes at RT or until standards reached maximum color (in the dark).
Following the addition of 50 µl of stop solution to each well, the plates were gently tapped to ensure thorough mixing and the OD of each well was immediately determined by using a microplate reader (Lambda Scan 200e; data analyzed by KC4 software) set to 450 nm.

5.12 Construction of adenoviral vectors

The adenoviral plasmids were constructed by using shuttle-CMV vectors and pAd-Easy-1 recombinant bacterial cells (BJ5183-AD-1 from Stratagene, Europe). The AdEasy-1 adenoviral plasmid contained all adenovirus-5 (Ad5) sequences, except for the E1 genes and E3 genes. pShuttle vector was used for the expression of murine MIP-1α and Flt3 ligand (these plasmids were a kind gift from Prof. Dan H Barouch, Harvard Medical School, Boston, USA). The pShuttle vector contains a multiple cloning site for the insertion of exogenous transgenes. This site is surrounded by adenoviral sequences (arms) that allow homologous recombination in Ad-Easy-1 (Tong Chun He et al, 1998). The left arm contains Ad5 nucleotides 34,931-35,935 (which mediate homologous recombination with pAdEasy vectors in *E. coli*), the inverted terminal repeat, and packing signal sequences (nucleotides 1-480 of Ad5) required for viral production in mammalian cells. The right arm contains Ad5 nucleotides 3534-5790, which mediate homologous recombination with AdEasy vectors. An artificially created *Pac* I site surrounds both arms.

BJ5183-AD-1 electroporation-competent cells are recombination-proficient bacterial cells carrying the pAdEasy-1 plasmid that encodes the Ad5 genome (E1/E3 deleted). These cells supply the components necessary to execute a recombination event between the pAdEasy-1 vector and pShuttle-CMV vector containing the murine MIP-1α and Flt3 ligand, thus generating a recombinant adenovirus that contains these genes.

pShuttle-CMV-MIP-1α and pShuttle-CMV-Flt3 ligand plasmids were taken (around 2-5 µg DNA), linearized with restriction endonuclease *Pme* I, and then purified by

phenol-chloroform extraction and ethanol precipitation. The precipitated DNA was suspended in 5-10 µl distilled water depending on the precipitate, and 1 µl of DNA was transformed into electrocompetent BJ5183-AD-1 bacterial cells by using electroporation (Bio-Rad gene pulser electroporator). The cells were immediately placed in 900 µl of L-Broth (LB) and grown at 37°C for 60 minutes. Aliquots of 100 µl of the cell suspension were inoculated onto agar plates containing kanamycin. After 16-18 hrs of growth at 37°C, 25-50 small colonies were picked and grown in 3 ml of LB medium containing 50 µg of kanamycin and grown for 8 hrs at 37°C. The positive clones were screened and selected by restriction endonuclease digestions with *Bam*H I and *Hind* III. Depending on the special restriction pattern of *Hind* III digestion, further positive clones **(Figure 5.1)** were selected and sequenced.

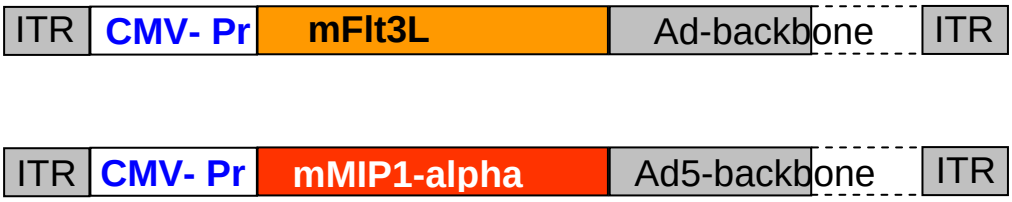


Figure 5.1 Schematic Diagram representing the construction of MIP-1α and Flt3L expressing in adenoviral vector

Ad-LacZ (contain β-Gal protein) was a kind gift from Dr. D. Brenner and Dr. K. Streetz. hTERT-Ad virus was constructed by Dr. Thomas Wirth from our lab (Wirth T et al, 2003). hTERT is the catalytic component of human telomerase reverse transcriptase and is not expressed in primary somatic cells, whereas the majority of cancer cells have reactive telomerase following the transcriptional up-regulation of hTERT. The hTERT-promoter drives the production of the E1A protein of the adenovirus.

5.12.1 Large-scale production, purification, and titration of recombinant adenoviruses

Approximately 1.5 million cells (HEK 293) were plated in 25cm^2 flasks without penicillin and streptomycin and allowed to reach 90% confluency, 24 hrs before transfection with Lipofectamine 2000. Cells were washed with optiMEM medium and

3 ml of optiMEM medium and kept in an incubator at 37°C. A sample of 10 µg of *Pac*1-digested (overnight digestion) adenoviral DNA containing the gene of interest and purified by the phenol-chloroform method was transfected and further followed according to the protocol provided with kit. After 7-10 days, when cytopathic effects were visible, cells were harvested, freeze/thawed three times and reinfected HEK 293 cells.

The plaque-purified adenoviral recombinant was used to infect HEK293 cells grown in 250 ml culture flasks when cells were 80% confluent. After 48 hours, when the cytopathic effect was visible, the cells were collected, and the pellet was resuspended in PBS. The cell-sludge was freeze-thawed three times. The debris was then separated by low-speed centrifugation, and the supernatant containing the virus was layered onto two discontinuous CsCl gradients and centrifuged at 27000 rpm for 4 hours in a Beckman LE80K ultracentrifuge. The virus band was then collected and stored in storage buffer. The OD of the harvested fractions (500 µl) was measured by spectrophotometry (at 260 nm) and titrated by Rapid Titer assay carried out by infecting 293 cells with a serial dilution of virus ranging from 10^{-2}-10^{-7}, followed by analysis with the kit provided.

The virus stock was used immediately or stored at –20^{o}C in storage buffer.

5.12.2 Reagents

CsCl gradient preparation

1.2 gm/ml CsCl and 1.4 gm/ml CsCl were prepared as follows:

1.2 gm/ml	13.25 gm CsCl
1.4 gm/ml	26.75 gm CsCl

were weighed separately and to each was added 0.5 ml of 1 M Tris, pH 7.5. Each solution was made up to 50 ml with distilled water and sterile-filtered.

The gradient was prepared by layering 3.5 ml of 1.4 gm/ml CsCl on the bottom of tube and 3.5 ml of 1.2 gm/ml as the upper layer, followed by the virus-containing lysate, and centrifugation for 4 hrs in a Beckman ultracentrifuge. After 4 hrs, two distinct layers were observed; the bottom layer held the transgene-containing virus and was collected gently by making a small hole with a 26-gauge needle in the centrifuge tube, whereas the upper layer contained contaminating wild-type virus and

was discarded. After collection of virus, 2X storage buffer was added, depending on the volume of the virus-containing layer, and kept at –20^{o}C.

2X storage buffer for 10 ml (virus)

1 M Tris PH-8.0	100 µl
5 M NaCl	200µl
1 M $MgCl_2$	10 µl
Glycerol	5 ml
H_2O	4.59 ml
10% BSA (sterile-filtered)	100 µl

Dialysis buffer for 2 liters (virus)

20 ml of 1M Tris, pH 8.0
2 ml of 1 M $MgCl_2$
56 ml of 5 M NaCl or 16.36 g NaCl made up to 2 liters with distilled H_20.

5.12.3 Rapid Titer Assay (for titrating adenoviruses)

One day before experiments, 1ml of healthy HEK293 cells ($2*10^5$) were seeded in each well of a 24-well plate in DMEM, 10%FCS. With medium as the diluent, 10-fold dilutions of the viral sample were prepared from 10^{-2} to 10^{-7} ml. Aliquots of 100 µl of the viral dilution were added drop-wise to each well in duplicate. The cells were incubated at 37^{o}C in 5% CO_2 for 48hrs. The medium was aspirated, and the cells were fixed gently by adding 1 ml ice-cold 100% methanol to each well at –20^{o}C for 10 minutes, followed by a rinse 1 ml of PBS +1%BSA. Anti-hexon antibody (1:2000 in PBS + 1 %BSA) was added to each well and incubated at 37^{o}C in a shaker at 100 rpm. The primary antibody was aspirated, and the wells were washed gently three times with PBS + 1% BSA. Rat anti-mouse antibody (HRP conjugate; 1:500 in PBS+1%BSA) was added to each well and incubated at 37^{o}C in a shaker at 100 rpm. Prior to removal of the secondary antibody, the DAB working solution was prepared by diluting 10X DAB substrate (stored cold) 1:10 with 1X stable peroxidase buffer and allowing the DAB working solution to come to room temperature. The secondary

antibody was aspirated, and each well was gently rinsed three times, followed by incubation in 250 µl of DAB working solution at RT for 10 minutes. The DAB solution was removed and replaced by 1ml PBS for storage at 4^{o}C. Ten fields of brown and black positive cells were counted by using microscope with a 20X objective and the mean number of positive cells in each well was calculated.
The infectious units (ifu/ml) for each well were calculated as follows:

$$\frac{\text{(Infected cell/field) x (fields/well)}}{\text{Volume of virus (ml) x (dilution factor)}}$$

5.13 Replication of murine tumor cells

5.13.1 Quantitation of infectious particles in tumor cells

A total of $2*10^5$ KLN and CMT64 cells were infected with various multiplicities of infections (MOIs) ranging from 0.1 to 10 MOI. After 48hrs, cells were harvested and freeze-thawed to release infectious particles. The supernatant of the freeze/thaw lysate was used to infect 293 cells, which were incubated for 48 hrs when cytopathic effects were visible. The preparation was further stained by using the Rapid Titer kit for infectious particles.

5.13.2 *In vitro* Ad-Luciferase expression

A total of 600,000 KLN and CMT64 cells were seeded in 60 mm plates and co-infected with 1 MOI of hTERT-Ad or control virus in combination with 1 MOI of Ad-Luciferase (replication defective). After 24, 48, and 72 hrs infection, cells were harvested and analyzed by luminometry (Lumat LB 5901, Berthold) for luciferase expression (Luciferin from Applichem, Germany).

5.13.3 Luciferin preparation for *in vitro* replication assay

A stock solution was prepared by dissolving 10 mg of luciferin in 1.325 ml of distilled water. For the working solution, a 1:1000 (25 mM) dilution of the stock was used and

always kept at –20°C: This was brought to RT before using, since luciferase expression is temperature sensitive.

5.13.4 *In vivo* imaging of Ad-Luciferase expression

Mice were subcutaneously injected with $5*10^6$ KLN205 or CMT64 tumor cells in the right flank. When tumors reached approximately 1 cm in diameter, mice were injected intratumoral (i.t.) with hTERT-Ad virus or control virus in combination with Ad-Luciferase virus, each injection containing $1*10^9$ and $1*10^9$ infectious viral particles and in some cases $1*10^9$ and $1*10^8$ viral particles.

After i.t. injections, mice were imaged for Luciferase expression every day until 8 days. Then, 4.5 mg of D-Luciferin (Applichem, Germany) in 100 µl of RPMI medium was co-injected with ketamine and Rampum intraperitoneal. After 10 minutes, images were captured with the IVIS imaging system series 200 by using living imagev2.5 software (Xenogen, USA), and photon emission values were calculated as recommended by the manufacturer.

5.14 *In vivo* CTL killing assay

Cytotoxic activity and specificity of CTL were determined by using a carboxy fluorescein succinimidyl ester (CFSE)-based *in vivo* killing assay as previously described (14). Untreated mice were used as controls. Target cells were prepared by using splenocytes from Black 6 mice. RBC-depleted splenocytes were pulsed with SIINFEKL peptide (1 µg/spleen) and incubated for 60 minutes at 37°C. Coated splenocytes were intensely labeled with high levels of CFSE (Molecular probes, Germany) at a final concentration 10 µl of 5 µM. As a control, non-coated splenocytes were weakly labeled at a lower concentration of CFSE 1 µl of 5 µM. The coated and non-coated fractions were mixed 1:1 and then injected intravenously at 2 x 10^7 (together) cells per treated or untreated mouse. Specific *in vivo* cytotoxicity was determined by flow cytometric analysis of CFSE-positive cells within spleens that were harvested 18 hrs later. The ratio *r* between $CFSE^{hi}$ and $CFSE^{lo}$ cells was calculated for determining the cytotoxicity of the effector cells.

Cytotoxicity was calculated by the formula $[1-(r_{unprimed} / r_{primed})] \times 100$.

5.15 Immunohistochemistry

Immunofluorescence analysis allows the investigation and characterization of tumor and lung tissues in terms of the proteins expressed on the surface of cells that have infiltrated into the tumor bed. The method is especially valuable in detecting infiltrating cells into the tumor and lungs. Immunofluorescence reveals even a small number of antigen-positive cells against a largely negative background. Two methods of fixation were used (1) methanol:acetone, and (2) paraformaldehyde (suitable for intracellular proteins).

Paraformaldehyde fixation solution:

A mixture of 0.4 g paraformaldehyde and 10 ml PBS was heated at 60°C and stirred until the solution became clear. It was then cooled to RT (prepared shortly before use).

Preparation of sodium citrate buffer:

(10 mM sodium citrate, 0.05% Tween 20, pH 6.0):

Tri-sodium citrate (dihydrate)	2.94 g
Distilled water	1,000 ml

2.94 g of Tri-sodium citrate was completely dissolved in 1000 ml distilled water. The pH was adjusted to 6.0 with 1 N HCl, and then 0.5 ml Tween 20 was added and mixed well. Sodium citrate buffer was stored at RT for 3 months or at 4°C for longer storage.

DAPI (4,6-diamidino-2-phenylindole) solution (Sigma):

1:500 dilutions were prepared in PBS and stored in the dark at 4°C.

Fluorescence microscope:

The model Leica DM 3000 was used for capturing fluorescence and hematoxylin and eosin (H & E) stained images of tissue sections.

Fixation and staining of tissue:

Cryosections (5 µm thick) of tumors were cut on a cryostat, attached to Super Frost Plus slides, and fixed in methanol:acetone for 10 min at –20°C or in paraformaldehyde for 20 minute at RT. The sections were washed with PBS, three times, permeabilized with Triton-X 100 (1%) for 5 min (10 ml PBS+100 µl Triton-X 100), and then rinsed (2x3 min) with PBS. The cell membranes were polarized with 0.1% glycine for 5 min (100 mg glycine in 100 ml PBS). The sections were then washed (2x3 min) with PBS. To prevent unspecific binding, the slides were incubated with FCS in a humidified chamber at RT for 60 min. Aliquots of 60-100 µl of the primary antibody were prepared by dilution in PBS (the optimal dilution of each antibody was determined separately). The sections were incubated with the primary antibody in a humidified chamber at 4°C overnight, followed by gentle washing (three times) with PBS. Aliquots of 100 µl of the secondary fluorescence-conjugated antibody were prepared by dilution in PBS (the optimal dilution for each was determined separately). The sections were incubated with 100 µl secondary antibody at 37°C in a humidified chamber for 60 min, rinsed three times with PBS at RT, incubated with 200 µl DAPI solution for counter-staining of the nuclei in a humidified chamber at 37°C for 10 min, rinsed three times with PBS at RT, mounted in mounting medium (MOVIOL), and analyzed by using a fluorescence or confocal microscope.

Staining of paraffin sections:

Paraffin-embedded tissue sections were deparaffinized in two changes of xylene (4 min each), hydrated in two changes of 100% ethanol for 4 min each, followed by changes in 95% and 80% ethanol for 2 min each and a rinse in distilled water for 2 min. In a water bath, a staining dish containing sodium citrate buffer was pre-heated until the temperature reached 98°C. Slides were immersed in the staining dish and incubated for 60 min. The staining dish was removed from the water bath, and the slides were allowed to cool for 20 min. The tissue sections were rinsed in washing buffer (2x2 min) and blocked with normal FCS for 60 min at RT. FCS was removed from sections carefully, taking care not to dry the section. Primary antibody was added at the appropriate dilution and incubated overnight at 4°C. Sections were rinsed in washing buffer (3x5 min). Secondary antibody was added and incubated for 1 hr at RT and then rinsed with washing buffer (3x5 min). Sections were counter-stained with DAPI for 10 min, rinsed again with washing buffer three times, and mounted with mounting medium.

Hematoxylin-Eosin (H & E) staining

Cryosections of lung (7µm) were mounted on Super Frost Plus Gold slides or polylysine-coated slides (Menzler Glaeser, Braunschweig, Germany), air-dried for 20 minutes, and immediately fixed in 10% neutral buffered formaldehyde (Carl Roth, Karlsruhe, Germany) for 20 minutes. After being washed with running tap water for 3 minutes, the sections were stained with Mayer´s hemalaun (Merck, Darmstadt, Germany) for 10 minutes. After differentiation in freshly prepared 3.75% HCl (in 70% ethanol), the sections were washed with running tap water for another 5 minutes, dehydrated in 70% and 90% ethanol for 2 minutes, respectively, stained with alcoholic eosin (containing 0.1% phloxine in 90% ethanol; Sigma-Aldrich, Steinheim, Germany) for 5 minutes, dehydrated in 100% ethanol and xylene, and finally mounted with Entellan New (Merck, Darmstadt, Germany) mounting medium.

Images were acquired with a Leica DM 3000 Microscope for the "stitching" of images from lung sections; different parts of the organ were taken and manually stitched with Adobe Photoshop.

5.16 Tumor infiltrating dendritic cell isolation (TID)

DBA/2 mice were subcutaneously injected in their right flank with $5*10^6$ KLN205 tumor cells. Tumors were treated with various viruses when they reached approximately 0.5-1 cm. After 7 days, tumors were removed, and tissues were cut into small fragments and incubated in 1 mg/ml collagenase (PAN biotech, Germany) at 37^oC for 45-60 minutes. Single cell suspensions were prepared by depressing the digested tumor tissue, added to RPMI medium with 10% FCS, and incubated for 4 hrs in 37^oC. Non-adherent TID cells were then purified anti-CD11c microbeads (Miltenyi Biotec, Germany) according to the manufactures instructions and adherent cells were removed (tumor cells). These purified TID cells were further stained with anti-CD11c, anti-CD40, anti-86, anti-CD83, and anti-MHC-II anti-sera.

5.16.1 Tumor lysate pulsing

Briefly, CMT64 tumor lysates were prepared as follows. CMT64 cells were resuspended in the same medium in which they were grown (approximately 1 ml),

freeze/thawed three times, and centrifuged for 5 minutes at 1000 rpm to remove cellular debris. The supernatant was taken as the tumor lysate for pulsing T cells and dendritic cells (DCs). For antigen-specific stimulation of DCs, either peptides or anti-CD40 antibody (or some times both) were used.

5.17 Reagents and antibodies used

Alexa 488	FITC	Molecular Probes
H-2Kb SIINFEKL pentamers	R-PE	Proimmune
anti-Mouse CD3e	PE	145-2C11
anti-Mouse CD4	FITC	L3T4
anti-Mouse CD8a	FITC	53-6.7
anti-Mouse CD11b	FITC	M1/70
anti-Mouse CD11c (Integrin a_x)	APC	N418
anti-Mouse CD11c (Integrin a_x)	Biotin	N418
anti-Mouse monoclonal CD16/CD32 (FcγIII/II Receptor) (Fc Block™)BD		
anti-Mouse CD19	Biotin	MB19-1
anti-Mouse CD25 (IL-2 Rezeptor α)	PE	PC61.5
anti-Mouse CD40	FITC	HM40-3
anti-Mouse CD40	unconjugated	1C10
anti-Mouse CD80 (B7-1)	PE	16-10A1
anti-Mouse CD83	PE	Michel-17
anti-Mouse CD86 (B7-2)	FITC	GL1
anti-Mouse/Rat Foxp3	APC	FJK-16s
Goldener syrian Hamster Isotype control	PE	-/-
Rat IgG2a Isotype control	FITC	eBioscience
Rat IgG2a Isotype control	PE	eBioscience
Rat IgG1 Isotype control	FITC	eBR2a
Rat IgG Isotype control	APC	eBioscience
anti-Mouse polyvalent IgG	Peroxidase	Zymed
anti-Mouse Interferon-gamma	unconjugated	AN-18,
eBioscience		

anti-Mouse Interferon-gamma	Biotin	R4-6A2, eBioscience
anti-Mouse MHC class II (I-A/I-E)	APC	M5/114.15.2, eBioscience
anti-Mouse MHC class I, H-2K^{d}	FITC	SF1-1.1, BD PharMingen
anti-Mouse MHC class I, H-2K^{b}	FITC	AF6-88.5, BD PharMingen
anti-Mouse MHC class I, H-2D^{b}	Biotin conjugated	KH95, BD PharMingen
Rat IgG2a κ isotype control		BD PharMingen
Rat IgG2b κ isotype control		BD PharMingen
Hamster IgG1, κ isotype control		BD PharMingen
Anti-Mouse Rae pan specific	APC	R&D Systems
Hamster anti-Mouse CD-3ε	unconjugated	BD PharMingen
Flt3 Ligand ELISA Kit		R&D Systems
MIP1α ELISA Kit		R&D Systems
Biotinylated mouse anti-hamster cocktail		BD PharMingen
Hamster anti-Mouse CD11c	Clone HL3 (IHC)	BD PharMingen
CellTrace CFSE cell proliferate kit		Molecular Probes
CD11c (N418) MicroBeads		Miltenyi Biotec
Streptavidin-FITC		
Streptavidin-PE		
Streptavidin-PE-Cy7		
Streptavidin-PerCP		BD PharMingen
Streptavidin-APC		
Streptavidin-APC-Cy7		
TMB Substrate		BDOptE1A
DAB Substrate		Pierce
Peroxidase 1X		Pierce
BD Cytofix/cytoperm		BD
Collagenase		PAN Biotech
OCT Compound		Sakura

D-Luciferin, Sodium salt	Applichem,Germany

6 Results

6.1 Replication kinetics of conditionally replicating adenovirus in murine tumor cells measured by using coinfection of Ad-luciferase-expression

To assess replication kinetics in vitro, KLN205 and CMT64 cells were infected with conditionally replicating virus (hTERT-Ad) and control virus Ad-LacZ in combinations of replication-deficient luciferase-expressing Ad5 virus with each MOI of 1 and harvested at 24, 48 and 72 hrs. Harvested cell lysate was analyzed for luciferase expression and revealed that KLN205 cells had higher luciferase expression than CMT64, thereby revealing that hTERT was replicated to a greater extent in KLN than in CMT64. Luciferase expression was high at 48hrs and decreased at 72 hrs because of the cytopathic effect of the virus (**Figure 6.1 and 6.2**); the control virus exhibited no replication.

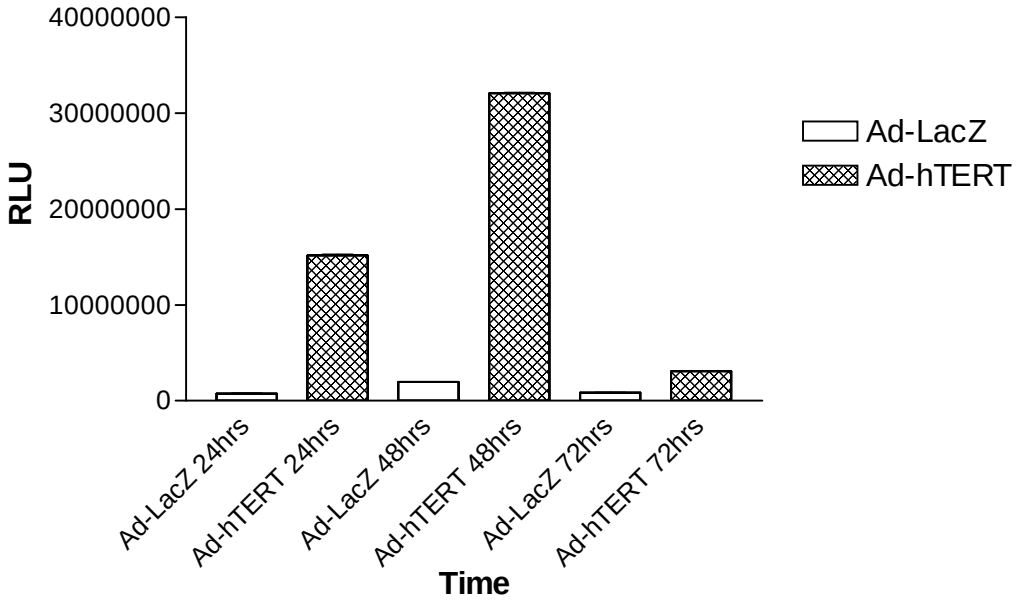


Figure 6.1 KLN205 squamous carcinoma cells were infected with the indicated viruses in combinations of control virus, hTERT-Ad and Ad-Luciferase. After 24, 48, and 72 hrs cells were analyzed for luciferase expression

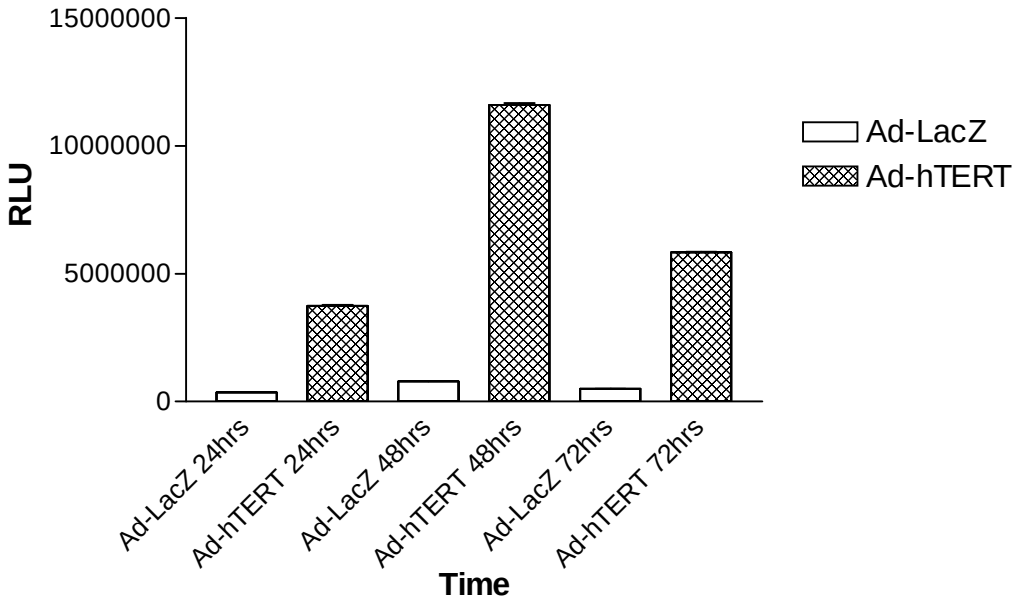


Figure 6.2 CMT64 small lung carcinoma cells were infected with the indicated viruses in combinations of control virus, hTERT-Ad and Ad-Luciferase. After 24, 48, and 72 hrs cells were analyzed for luciferase expression

To assess the replication of conditionally replicating virus in murine tumor cells, KLN205 and CMT64 cells were plated at $2*10^5$ cells /well/ml in 24 well plate. Next day, the cells were infected with different MOIs from 0.1 to 10. After 48 hrs, cells from each well were harvested separately in the same medium, freeze/thawed three times to release viral particles into the medium, and centrifuged for 5 minutes at 1000 rpm at 4^oC. Aliquots of 100µl supernatant was taken to infect HEK 293 cells in order to assess infectious particles by using Rapid Titer assay kit according to the protocol described above. The results showed that KLN205 and CMT64 tumor cells were permissive to replication and lysis by producing viral particles upon infection with low MOIs. KLN205 produced more viral particles than CMT64, which further confirmed that murine tumor cells were able to replicate human viruses and to produce virions **(Figure 6.3)**. These results correlated with *in vitro* luciferase expression in cells with combinations of hTERT-Ad and control virus.

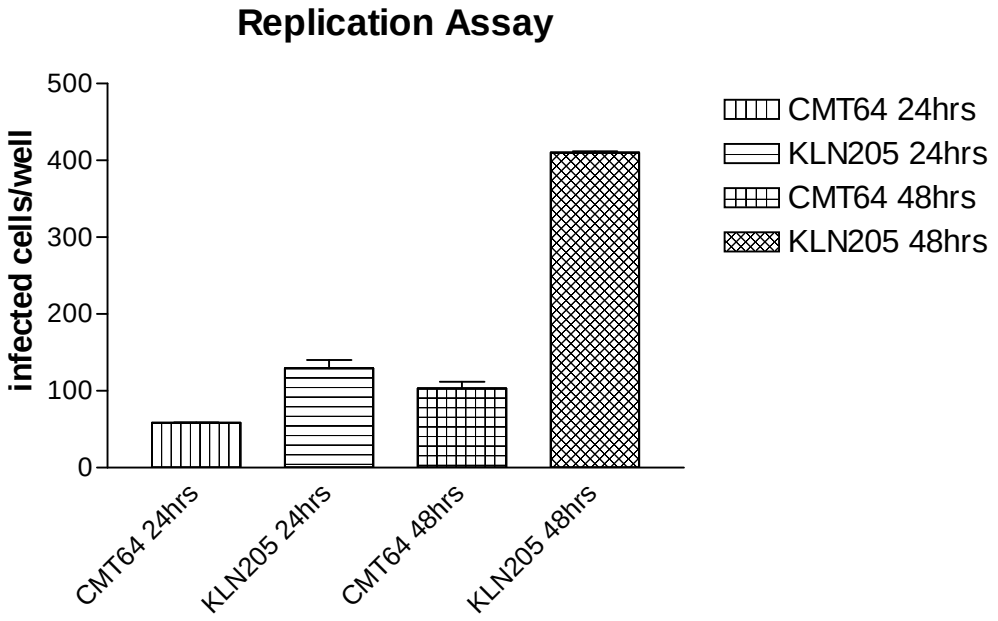


Figure 6.3 Monolayers of KLN205 and CMT64 cells were infected with an MOI of 0.1-5.0. Virus production was assessed by TCID assay after 24 and 48hrs infection, showing hTERT-Ad virus replication in murine tumor cells.

6.2 Bioluminescence imaging of co-infected subcutaneous tumors

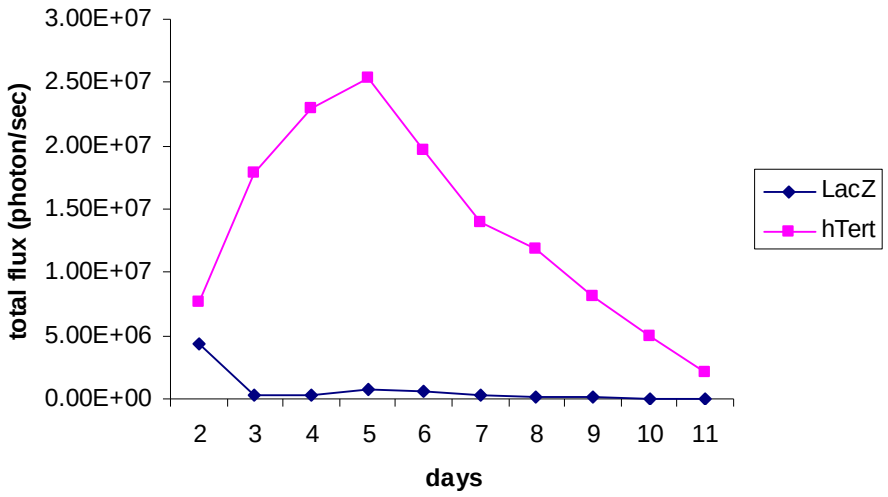
To analyze the *in vivo* replication efficiency of hTERT-Ad virus in murine tumor cells, KLN205 and CMT64 cells were injected subcutaneously (s.c.) in the right flank of DBA/2 and C57Bl6 mice respectively. These cells grow rapidly in mice and are difficult to treat, despite the use of a variety of treatments. Conditionally replicating oncolytic virus featuring the hTERT promoter, the catalytic subunit of telomerase, which is expressed in most tumor cells, and which drives the E1A protein of adenovirus (hTERT-Ad, $1*10^9$ PFU), and a control LacZ-Ad or Ad-GFP ($1*10^9$ PFU) virus, which is deficient in replication, were coinjected with replication-deficient Ad-luciferase ($5*10^8$ PFU) into subcutaneous tumors (intratumoral injections), when tumors had grown to about 1 cm in diameter. After 24hrs of virus injection, treated tumors showed photon emission, suggesting the efficient transduction and replication

of virus in tumor cells and the subsequent *in vivo* conversion of D-luciferin by luciferase.

Imaging of tumors on subsequent days showed an increase in the conversion of D-luciferin by luciferase, suggesting that viral particles increased with conditionally replicating hTERT-Ad virus compared with replication-deficient LacZ-Ad control virus in DBA/2 mice model **(Figure 6.4a, 6.4c)** and in CMT64 **(Figure 6.4b)**.

These results indicate that the presence of a replicating virus with an E1-deleted (Ad-Luciferase) virus allows the replication of both viruses, whereas replication-deficient LacZ-Ad control virus values are low throughout the experiment because of replication deficiency. The increase in the replication-deficient control virus could be attributable to the transduction efficiency of the virus but not to the replication of the virus when compared with the *in vitro* replication assay and luciferase expression assay described above.

6.4a



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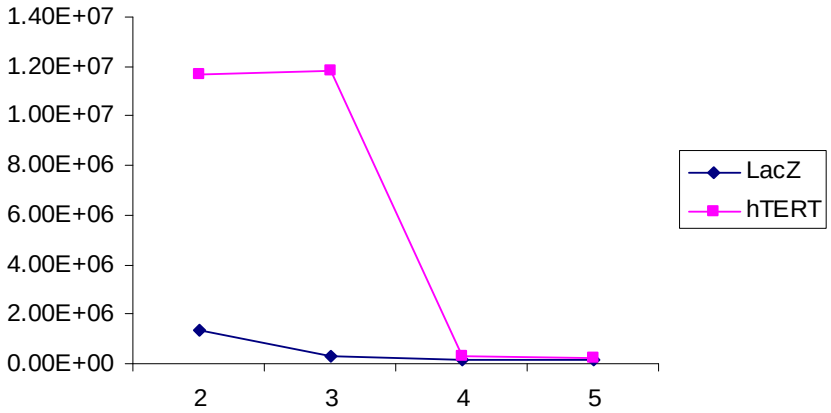
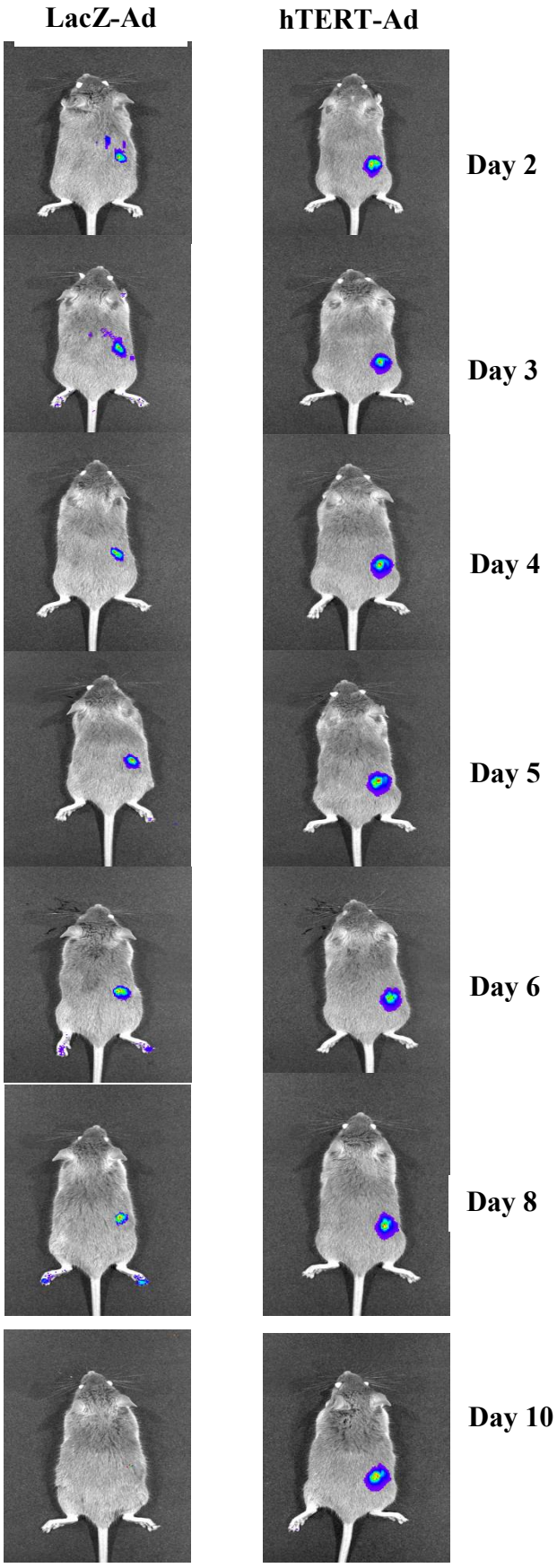


Figure 6.4 Mice were s.c injected with KLN205 and CMT64 cells. Tumors were treated with Ad-Luciferase and hTERT-Ad. (a) Photon emission in KLN205, (b) Photon emission in CMT64 and (c) *In vivo* bioluminescence of subcutaneous tumors

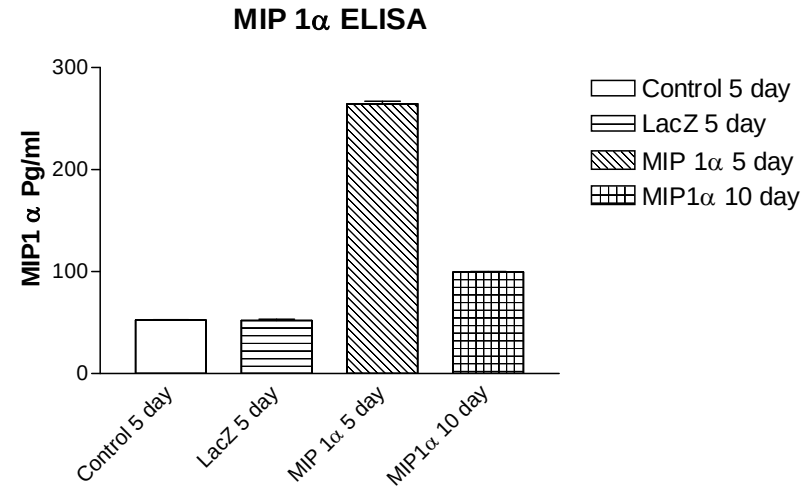
6.4c



6.3 Construction and expression of Ad-MIP-1α and Ad-Flt3L proteins *in vivo*

First we have constructed murine MIP-1α and Flt3L in adenoviral vectors. To determine the expression of adenoviruses expressing MIP-1α and Flt3 ligand, dialyzed viral particles were injected at $1*10^9$ pfu intravenously (i.v.) into DBA/2 mice. Serum samples were collected at days 5 and 10 and tested for secretion, stability and survival of viral particles. MIP-1α secretion was decreased by day 10, whereas Flt3L secretion was constant on day 10 **(Figure 6.5a, 6.5b)**.

6.5 a



6.5 b

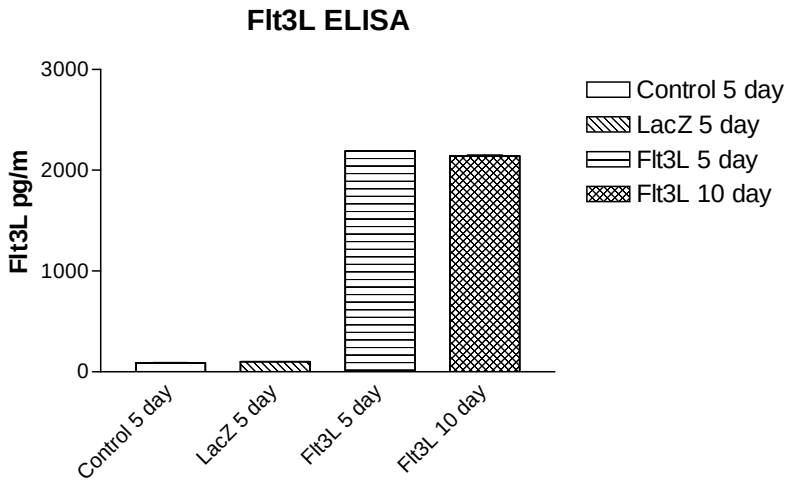


Figure 6.5a Mice were injected with 1x 10^9 pfu of Ad- MIP-1α. Serum samples were collected at days 5 and 10. ELISA was performed to test secretion of MIP-1α.

Figure 6.5b Mice were injected with 1x 10^9 pfu of Ad-Flt3L. Serum samples were collected at days 5 and 10. ELISA was performed to test secretion of Flt3L.

6.4 Infiltration of dendritic cells into tumor tissue allows efficient antigen presentation in oncolytic therapy

Dendritic cells (DCs) are critical for priming adaptive immune responses to foreign antigens, but harnessing these cells *in vivo* to enhance immunogenicity against cancer cells has not been studied in oncolytic virotherapy. Here, we used replication-defective adenovirus expressing macrophage inflammatory protein 1α (MIP-1α), a chemotactic factor that mobilizes DCs, and fms-like tyrosine kinase 3 ligand (Flt3L) to expand DCs *in vivo* to enhance the immunogenicity of antigens of tumors experiencing ongoing replication with conditionally replicating hTERT-Ad virus.

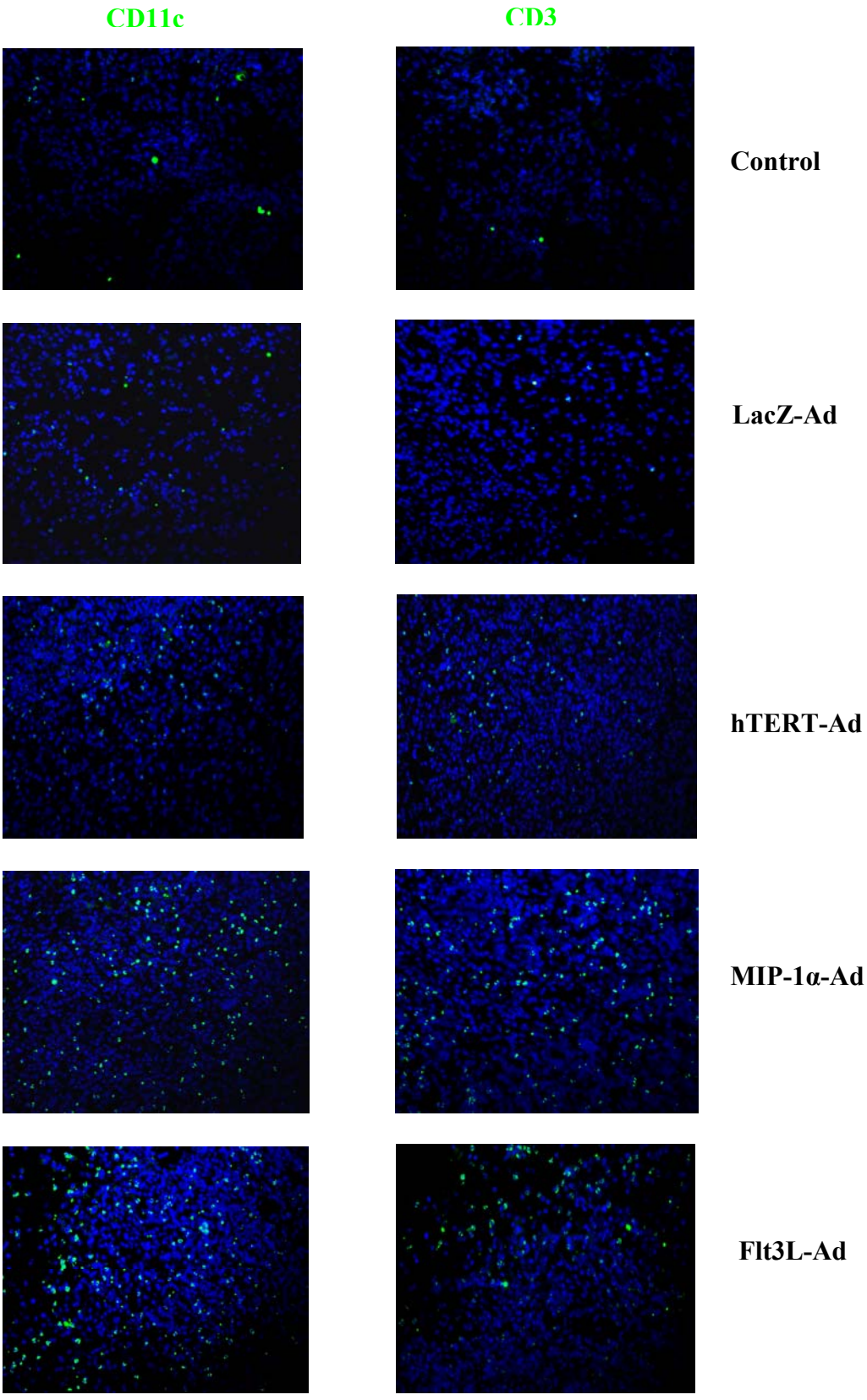
MIP-1α is able to recruit immune cells, such as T cells and DCs, to the site of injection. Most of these cells are immature in status. Flt3L is a potent growth factor for DCs and is known to expand DCs *in vivo* in humans and mice and to expand mature DCs.

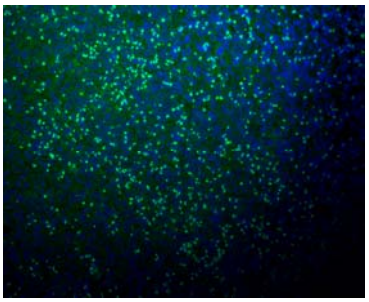
In this study, we hypothesized that conditionally replicating virus in tumor tissue lyse the tumor tissue. Expression of MHC-I antigens on the surface of virus-infected cells down-regulated by adenovirus might cause further inhibition of antigen presentation and immune response against tumor antigens. Hence, our interest was to recruit immature DCs in ongoing virotherapy with adenovirus expressing MIP-1α and to expand them by using Flt3L to enhance antigen-specific immune responses in virotherapy.

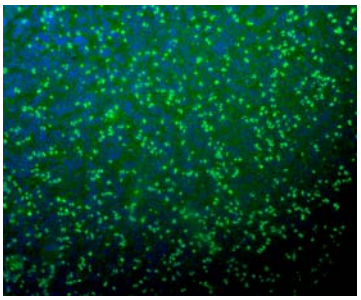
We determined whether a combination of these chemotactic and growth factors expressed in adenovirus would lead to the recruitment and expansion of infected tumor bed with DCs.

We used KLN205 (DBA/2 mice) and CMT64 (C57Bl6 mice) cells with 25% infected and 75% uninfected cells injected s.c. After 6 days, tumors were harvested in OCT compound and kept at –80^{o}C. Tumors were cut by cryostat into 5 µm serial sections and fixed on Super Frost slides. Immunohistochemistry of tumor sections revealed the presence of a few local cellular infiltrates with hTERT-Ad, Ad-MIP-1α, and Ad-Flt3L alone. Co-injection of Ad-MIP-1α and Ad-Flt3L resulted in large clusters of tumor-infiltrating immune cells. Further, we stained these tissues with the CD3e and CD11c antibodies and found that the recruited cells were T lymphocytes and DCs. The combination of chemotactic factor MIP-1α and DC growth factor Flt3L resulted in

greater number of T lymphocytes and DCs compared with virotherapy alone or treatment with a single chemokine. **(Figure 6.6)**







MIP-1α-Ad & Flt3L-Ad

Figure 6.6 Infiltration of immune cells by MIP-1α-Ad & Flt3L-Ad.

KLN205 cells were infected with an MOI of 25 and injected s.c. into DBA/2 mice. Tumors were harvested at day 6 and stained for dendritic cell (anti-CD11c) and T cell (CD3) markers. Magnification 20X.

6.5 Migration and expansion of DCs enhance immune response to tumor antigens in virotherapy

Oncolytic therapy with conditionally replicating virus causes cell death in tumors depending on the replication efficiency of the tumor cells. hTERT-Ad virus has been shown to replicate efficiently in human tumor cells.

In our experimental model, we used murine tumor cells KLN205 and CMT64-OVA. Tumor cells were injected s.c. into either DBA/2 or C57Bl6 mice. When tumors had grown to around 0.5-1 cm in size, tumors were treated intratumoral with hTERT-Ad, Ad-MIP1α, Ad-Flt3L, or control virus Ad-LacZ alone or with various combinations of hTERT/Ad-MIP1α, hTERT/Ad-Flt3L, hTERT/Ad-MIP1α/Flt3L, and control virus Ad-LacZ. All virus combinations were always normalized to $2*10^9$ with control virus. We used hTERT-Ad at $1*10^9$ and Ad-Flt3L and Ad-MIP1α each at $5*10^8$ viral particles in 150 µl. We always supplemented with control virus to make up to $2*10^9$ viral particles in cases of single viruses used in our experimental setup. To evaluate immune response to tumor antigen, mice were sacrificed on day 14, and splenocytes were stimulated with either tumor lysate or ovalbumin peptide at 1 µg/ml. The cells were incubated for 48hrs.

The results of the experimental approach with different combinations of chemokine and growth factors showed that single chemokine Ad-MIP1α or Ad-Flt3L or hTERT-Ad virus exhibited an immune response to tumor antigen, but that a combination of Ad-MIP1α and Ad-Flt3L had synergistic effects by secreting high IFN-γ **(Figure 6.7).**

These results indicate that the combination of virotherapy with immunotherapy not only enhances virotherapy efficacy, but also generates tumor antigen-specific immune responses in ongoing virotherapy.

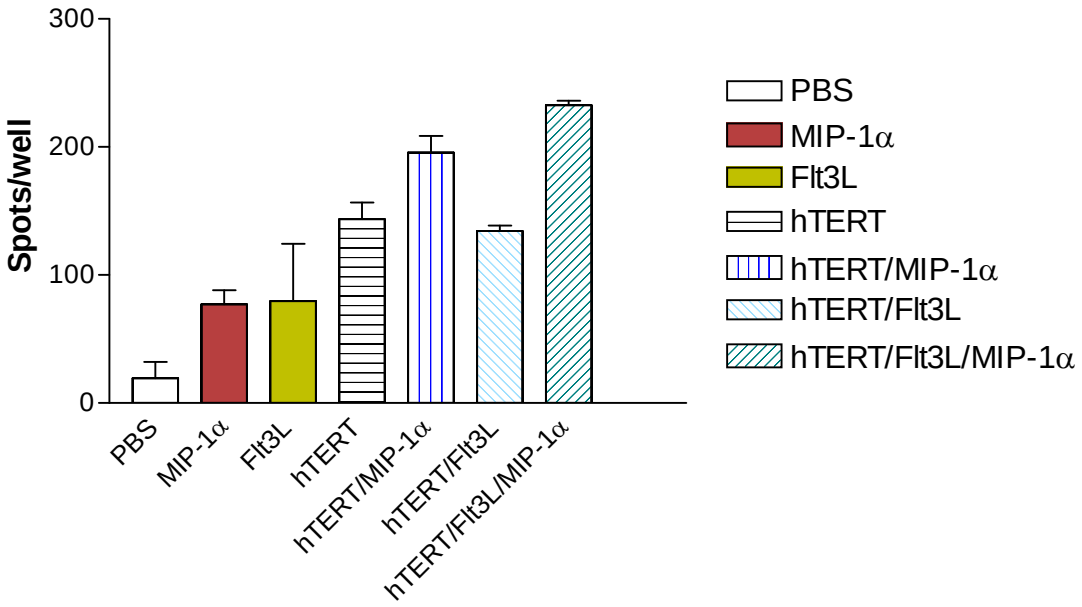


Figure 6.7 Immune response against tumor lysate augmented by MIP-1α and Flt3L in oncolytic therapy. Intra-tumoral injection with hTERT-Ad, Ad-MIP-1α, or Ad-Flt3L or a combination of both cytokine viruses with oncolytic virus. The immune response was measured by IFN-γ ELISpot assay.

6.6 Migration and expansion of DCs enhances immune response against virus

In this experimental approach, hTERT-Ad virus replication in KLN205 and CMT64 cells was efficient and produced viral particles as shown in Fig 1 above. DC migration into tumor tissue after treatment with different combinations of MIP1α and Flt3L with hTERT not only showed captured tumor-associated antigen and produced antigen-specific immune responses, but also revealed captured viral particles and produced a virus-specific CD8 T cell immune response, as shown by IFN-γ **(Figure 6.8)** secretion by mouse splenocytes upon stimulation with virus with an MOI of 100.

From these results, DC migration can be seen not only to enhance tumor antigen-specific immune responses, but also viral antigen-specific immune responses as shown by IFN-γ secretion (ELISpot). Further modulation of the immune response to tumor antigen is however necessary.

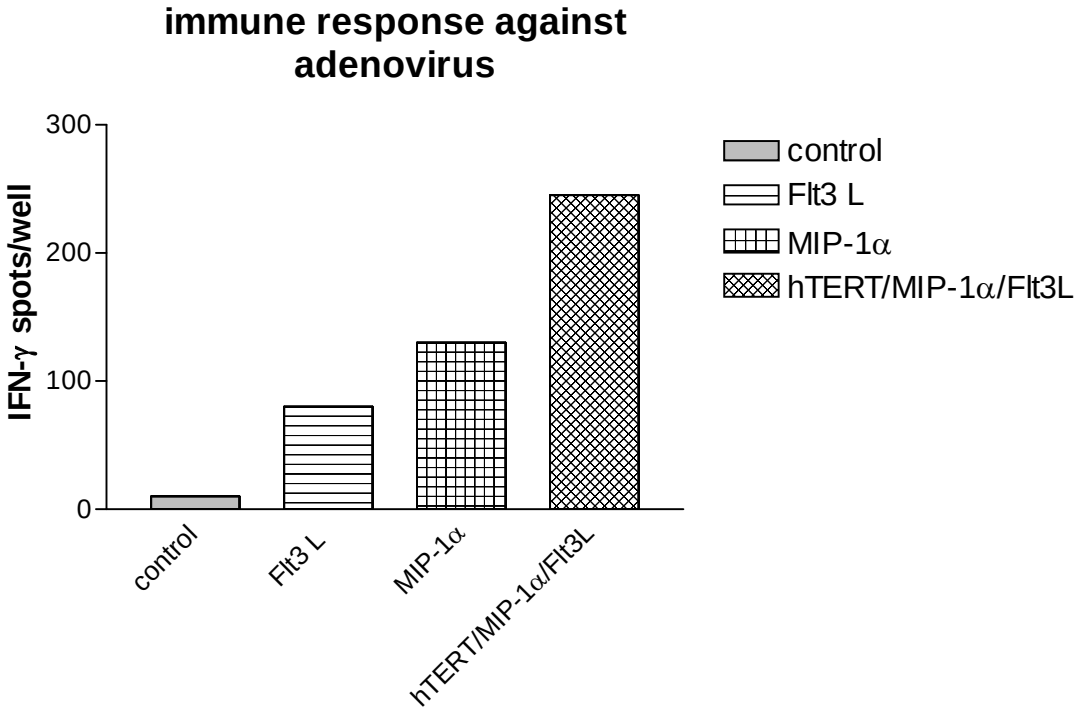


Figure 6.8 Immune response against virus. Tumors were injected with various virus combinations and IFN-γ secretion was analyzed by ELISpot against virus.

6.7 Modulation of immune responses by DC vaccination enhances tumor antigen-specific immune responses and reduces humoral immune response to virus

DC vaccination is one of the most promising approaches for cancer therapy. To investigate whether DC vaccination could modulate the immune response toward tumor antigens in viroimmunotherapy, we investigated various sequence-specific treatments of viroimmunotherapy and subsequent DC vaccination, or vise versa.

Mice were injected with s.c. CMT64-OVA tumors. When tumors had grown to approximately 0.5-1 cm, tumors were treated with viroimmunotherapy (injection of MIP1α/Flt3L/hTERT-Ad; 2X) for three days continuously, during a one-week time interval and, in another set up, one viroimmunotherapy and a subsequent vaccination

of DC loaded with peptide at $5*10^5$ cells per mouse. Control groups were subjected only to DC vaccination and only to virotherapy (hTERT-Ad virus).

The results for IFN-γ secretion show that viroimmunotherapy (1X) and a subsequent DC vaccination not only enhances tumor antigen-specific CD8 T cell responses, but also reduces humoral response to virus, which is highly important in a clinical setting to improve viral persistence in ongoing therapy. Treatment with viroimmunotherapy only showed no significant IFN-γ secretion compared with viroimmunotherapy plus subsequent DC vaccination. DC vaccination alone also resulted in no significant IFN-γ secretion. These results suggest that viroimmunotherapy provides an inflammatory environment, which provides precondition for effective DC vaccination. **(Figure 6.9)**

The major hurdle of virotherapy is the inactivation of viral vectors or the virus itself. Our aim has been to enhance the antigen-specific immune response to tumor antigens and to reduce the immune response to viral antigens in oncolytic virotherapy.

Our experimental set up tested whether viroimmunotherapy and subsequent ovalbumin tumor antigen loaded DC vaccination could enhance the antigen-specific immune response and reduce antibody titers to adenovirus. Our results with viroimmunotherapy alone showed no significant antigen-specific immune response but high humoral immune response to adenovirus, which was measured by total IgG. The humoral immune response might inactivate viral persistence in mice, further reducing tumor antigen-specific immune responses.

Interestingly, viroimmunotherapy and subsequent ovalbumin tumor antigen loaded DC vaccination enhanced IFN-γ secretion to ovalbumin-specific peptide and reduced viral antibody titers. However, ovalbumin peptide-pulsed DC vaccination and subsequently viroimmunotherapy (DC-viroimmunotherapy) also gave no significant immune responses to ovalbumin peptide and no significant reduction in viral antibody titers.

In conclusion, viroimmunotherapy alone was not very effective, but viroimmunotherapy plus subsequent tumor antigen loaded DC vaccination was effective in eradicating established tumors and reducing anti-adenoviral antibody titers **(Figure 6.10).**

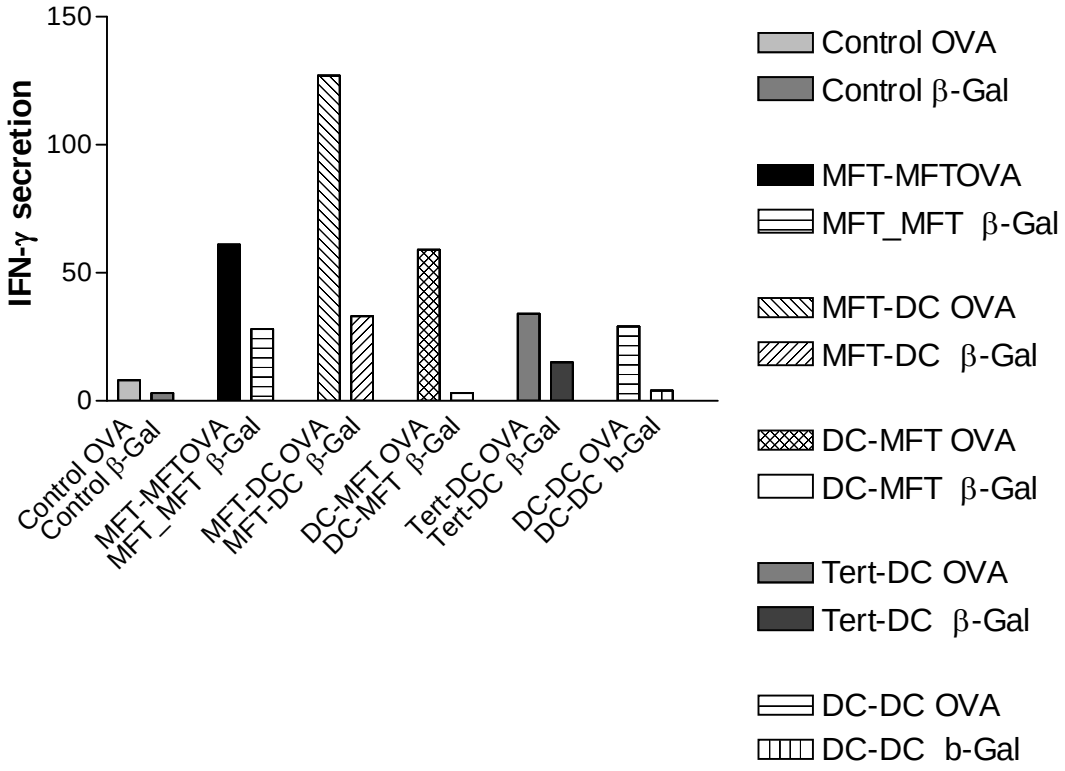


Figure 6.9 Viroimmunotherapy and subsequent DCs vaccination enhances tumor antigen-specific immune response. Combination of cytokines with virotherapy and subsequent antigen-specific pulsed DC vaccination (MFT-DC) enhances antigen-specific immune responses compared with viroimmunotherapy (MFT-MFT), virotherapy (hTert-DC), peptide-pulsed DC vaccination plus subsequent viroimmunotherapy (DC-MFT), and DC vaccination alone (DC-DC).

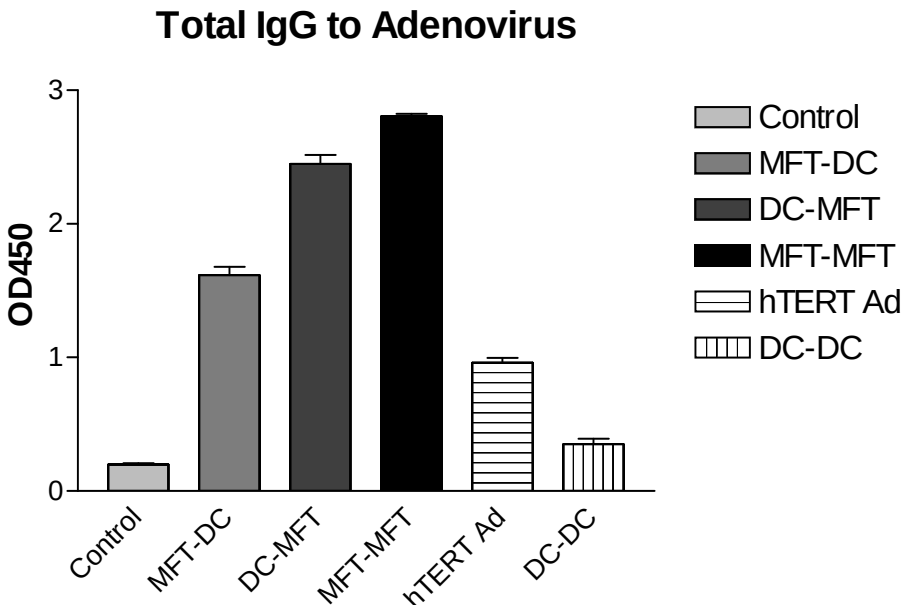


Figure 6.10 Anti-adenoviral antibody ELISA. Mice were injected with various combination of viruses as mentioned and DC vaccination (before or after viroimmunotherapy). After two weeks, serum was collected, and total IgG was measured by ELISA.

6.8 Viroimmunotherapy expands cytotoxic T cells *in vivo* and kills antigen-specific target cells

A cytotoxicity assay was performed to determine the presence of cytotoxic T cells (CTLs) *in vivo*. CMT64-OVA cells were injected s.c. and, when tumors were palpable, the tumors were injected with control virus, hTERT-Ad, and viroimmunotherapy. After two weeks, mice were injected with ovalbumin-pulsed splenocytes together with high CFSE, whereas control peptide (β-gal peptide) containing splenocytes were injected i.v. with low CSFE, with each population at $1*10^7$ (total of $2*10^7$ cells). After 18 hrs, mice were sacrificed, and isolated splenocytes were examined, by fluorescence-activated cell sorting (FACS), for CFSE-labeled cells and CD8 T cell populations for specific killing by CTLs.

Mice from untreated control and hTERT-Ad-treated mice shown no killing of ovalbumin-pulsed target cells compared with viroimmunotherapy-treated mice. These results showed that MIP-1α-Ad and Flt3L-Ad viruses infiltrated and caused the

expansion of DCs into the tumor bed and then mature DCs migrated to lymph nodes where they activated naïve T cells to become CTLs, but the hTERT-Ad did not generate CTLs or only in extremely low numbers **(Figure 6.11a, 6.11b)**. Thus, virotherapy alone is not effective for generating high enough numbers of CTLs, whereas a combination of immunotherapy is successful in generating effective antigen-specific lysis of target cells **(Figure 6.11c, 6.11d)**.

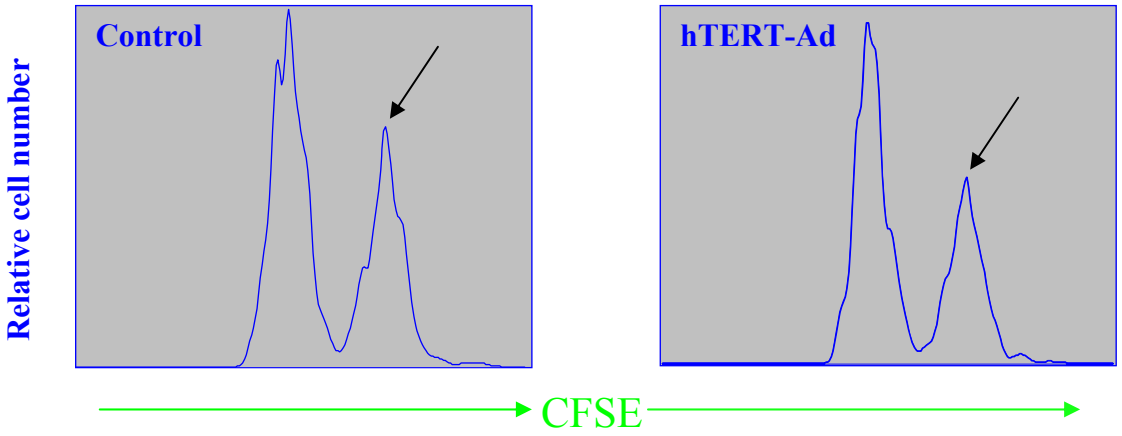


Figure 6.11a *In vivo* cytotoxicity of target cells in virotherapy in cross-presentation of tumor antigen. Mice were bearing s.c tumors with ovalbumin expressing CMT64 cells. Splenocytes were labeled with CFSE ^{hi} (target cells) and CFSE ^{low} (control) and injected i.v. into treated mice. After 18 hrs, splenocytes were analyzed for CFSE. hTERT-Ad alone treated mice shown killing of target cells by cross-presenting the ova peptide (SIINFEKL) expressing tumor antigen.

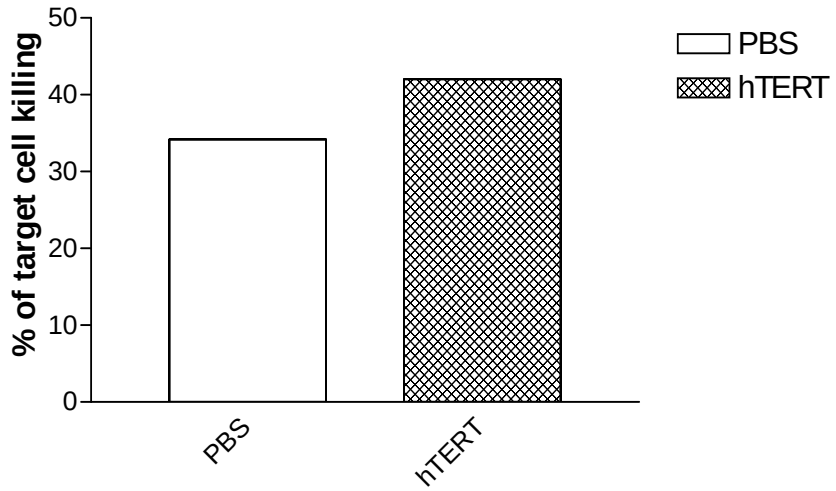


Figure 6.11b *In vivo* cytotoxicity of target cells in virotherapy in cross-presentation of tumor antigen.

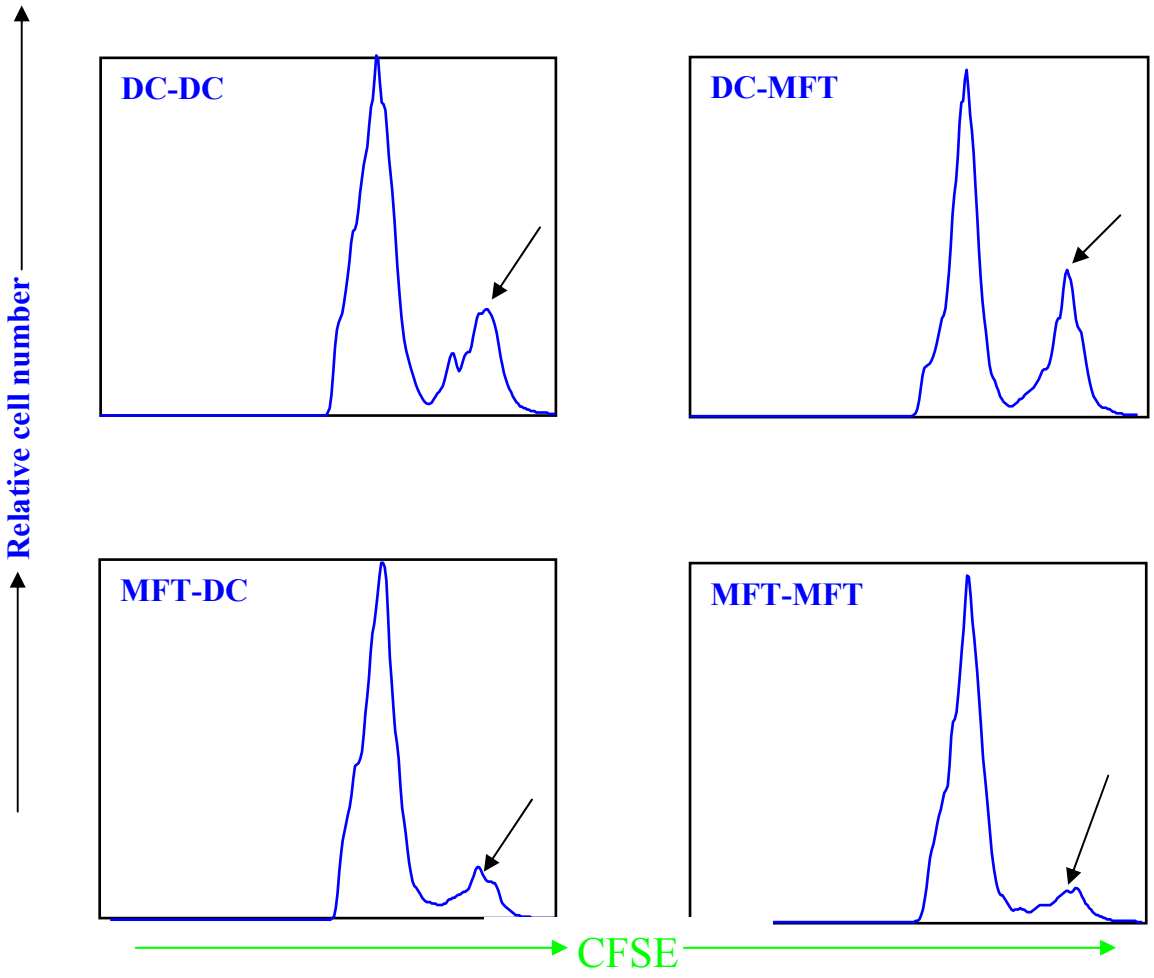


Figure 6.11c *In vivo* killing of target cells in viroimmunotherapy and DC vaccination. Mice were treated with different vaccinations and treated mice were injected i.v. with CFSE ^{hi} labeled target cells (ova peptide SIINFEKL) and CFSE ^{low} (control peptide). After 18 hrs, splenocytes were analyzed for CFSE.

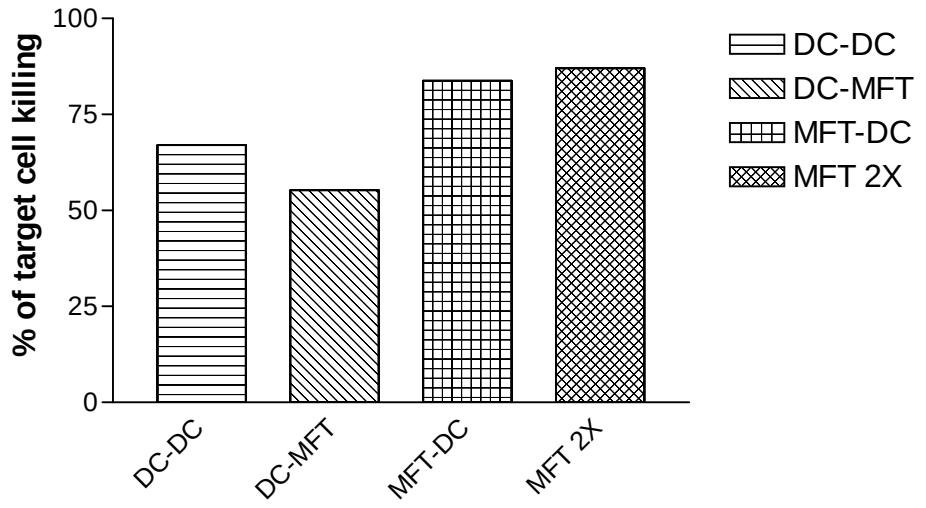
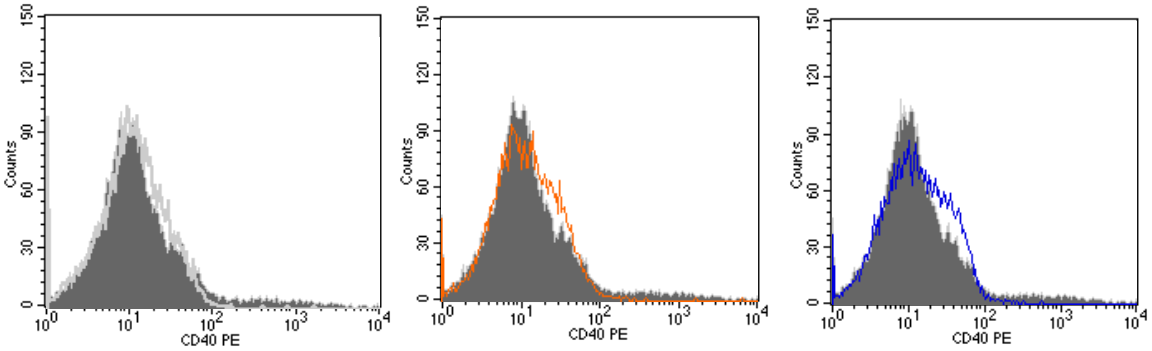


Figure 6.11d ***In vivo*** **cytotoxicity of target cells in viroimmunotherapy subsequent DC vaccination in cross-presentation of tumor antigen.**

6.9 *In vivo* maturation of DCs leads to migration of DCs to lymph nodes to activate naïve T cells

To test that tumor-infiltrating immature DCs were matured by viral infection or the take-up of apoptotic tumor cells and migrated to lymph nodes to activate naïve T cells in order to generate an immune response, TIDs (tumor infiltrating dendritic cells) were isolated and purified by CD11c beads and analyzed by FACS for CD40 markers. The presence of MHC II, CD80, and CD83 was demonstrated by immunohistochemistry.

An increase in the expression of maturation markers CD40, CD80, CD83 and MHC-II **(Figure 6.12a, 12b, 12c)** upon infection with MIP1α and Flt3L adenoviruses shown that TIDs had matured and migrated to lymph nodes to activate naïve T cells



Control **MIP-α1+Flt3L** **MIP-1 α +Flt3L+hTERT**

Figure 6.12a Expression of CD40 on TIDs after treatment with viroimmunotherapy

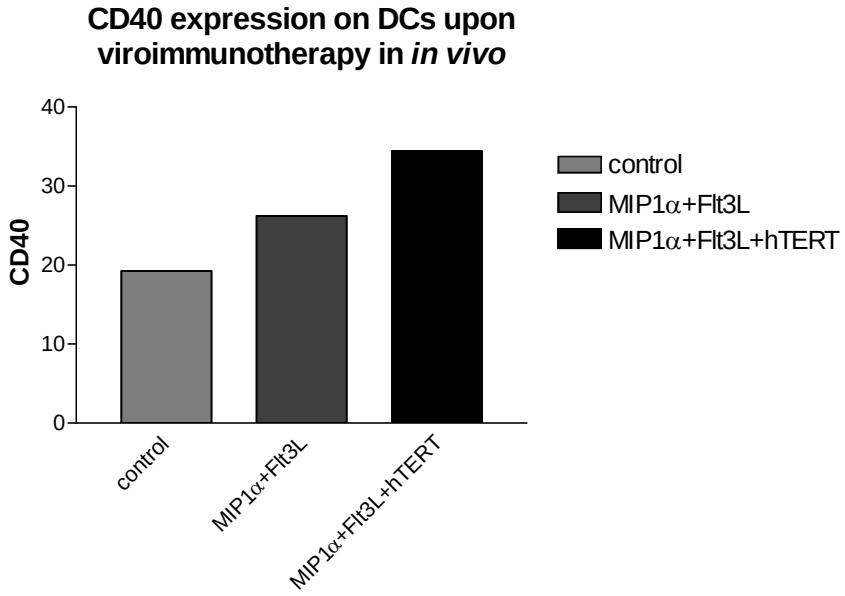


Figure 6.12b Representative expression of CD40 on DCs after treatment with viroimmunotherapy

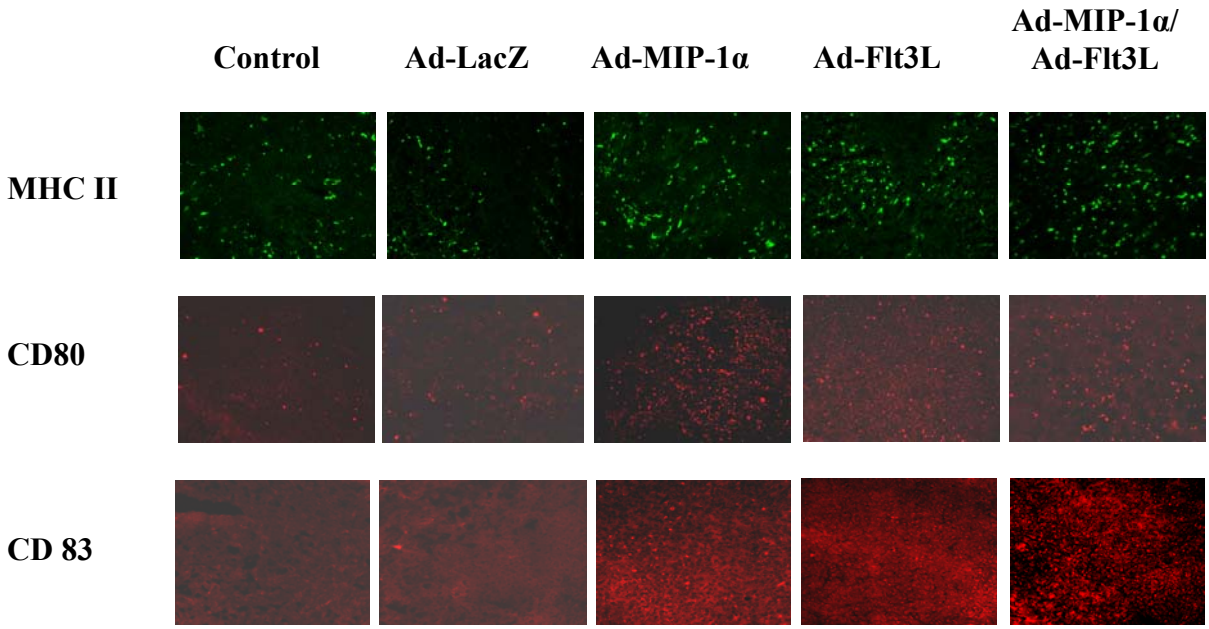


Figure 6.12c KLN205 s.c tumor sections were stained for maturation markers on TIDs

6.10 Viroimmunotherapy and subsequent tumor antigen loaded DC vaccination inhibit established lung metastasis

To investigate whether viroimmunotherapy and DC vaccination has any clinical relevance, we injected i.v. CMT64-ova tumor cells, two days before treatment. Mice were grouped into six groups as follows

1. Untreated control, n = 6
2. Only virotherapy (hTERT-Ad only), n = 6
3. Viroimmunotherapy, n= 6
4. Viroimmunotherapy and subsequent DC vaccination, n = 6
5. DC vaccination and subsequent viroimmunotherapy, n = 6
6. Only DC vaccination, n = 6

Mice were treated intratumoral with viroimmunotherapy and vaccination with DCs pulsed with ovalbumin peptide (1 μg/ml) and stimulated with anti-CD40 overnight. CD11c-purified DCs were injected s.c. After 2 weeks, lungs were harvested, and sections prepared for H&E staining.

Interestingly, untreated controls and mice subjected only to virotherapy exhibited no inhibition in lung metastasis, whereas viroimmunotherapy and viroimmunotherapy-DC vaccination inhibited lung metastasis. The group treated with only viroimmunotherapy and subsequent DC vaccination showed an inhibition of more than 50% in lung metastasis **(Figure 6.13a, 6.13b)** and rule out that adenoviral protein infiltrated to lungs which further inhibited metastasis **(Figure 6.14).**

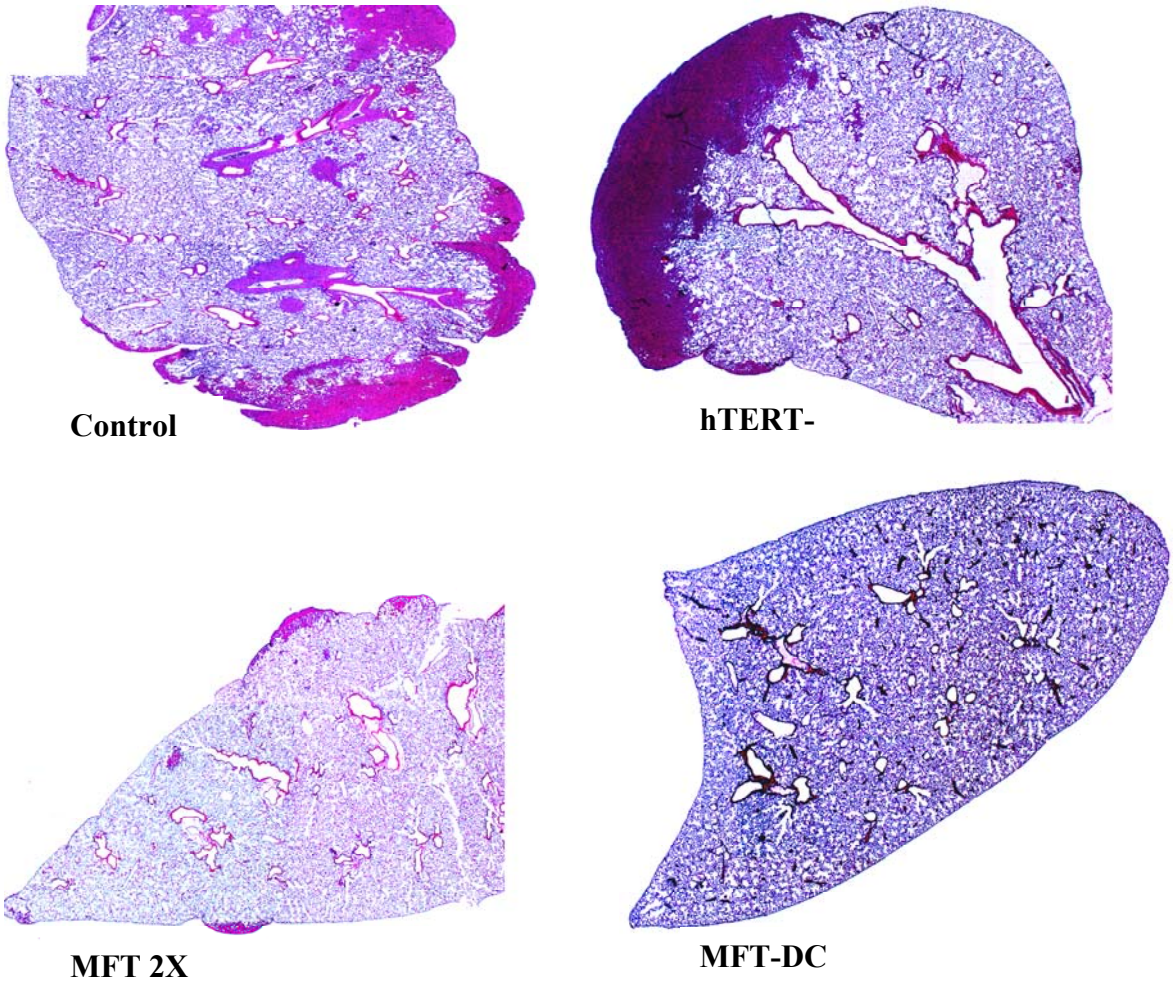


Figure 6.13a Inhibition of established lung metastasis. Mice were injected i.v CMT64 cells and treated viroimmunotherapy and DC vaccination. After last injection, mice were killed on day 14th and lung sections were stained with H & E.

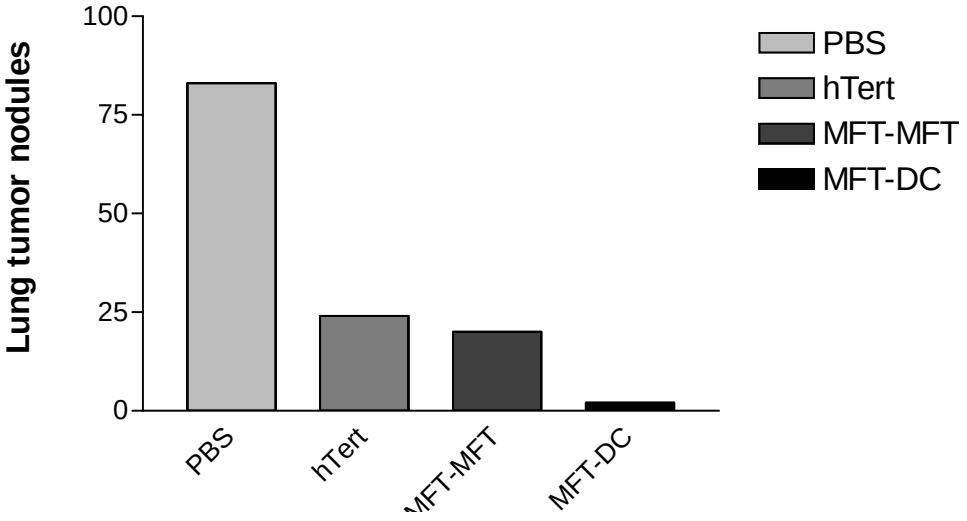
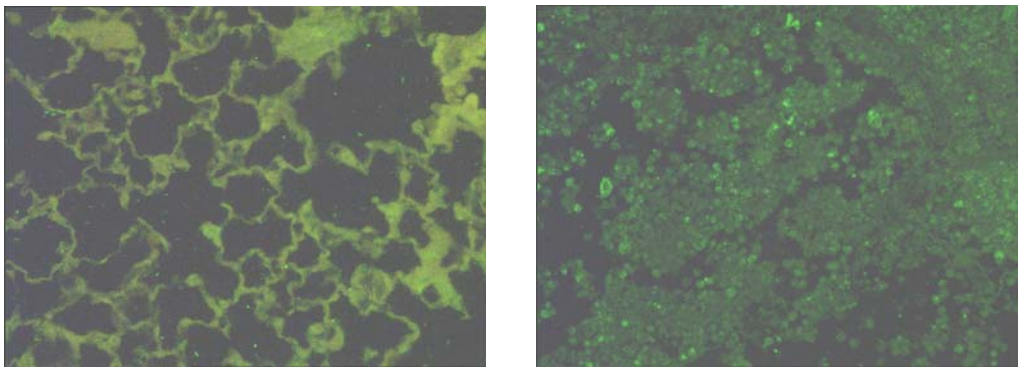


Figure 6.13b Representative value of tumor nodules in lung after vaccination



Lung **Tumor**

Figure 6.14 Adenoviral hexon protein staining of lung and tumor

6.11 Combination of oncolytic virotherapy and immunotherapy increases spleen size and eradicates established subcutaneous tumors

Oncolytic therapy has shown that replicates in KLN 205 cells produce viral particles by various methods. We have studied whether this has any effect on tumor regression in the treatment groups. Control groups, PBS, and LacZ-Ad injected tumors grew continuously. Virotherapy and immunotherapy alone retarded growth. However, a combination of viroimmunotherapy resulted in almost total tumor regression **(Figure 6.15)** and spleen size increased to twice that of the untreated group **(Figure 6.16).**

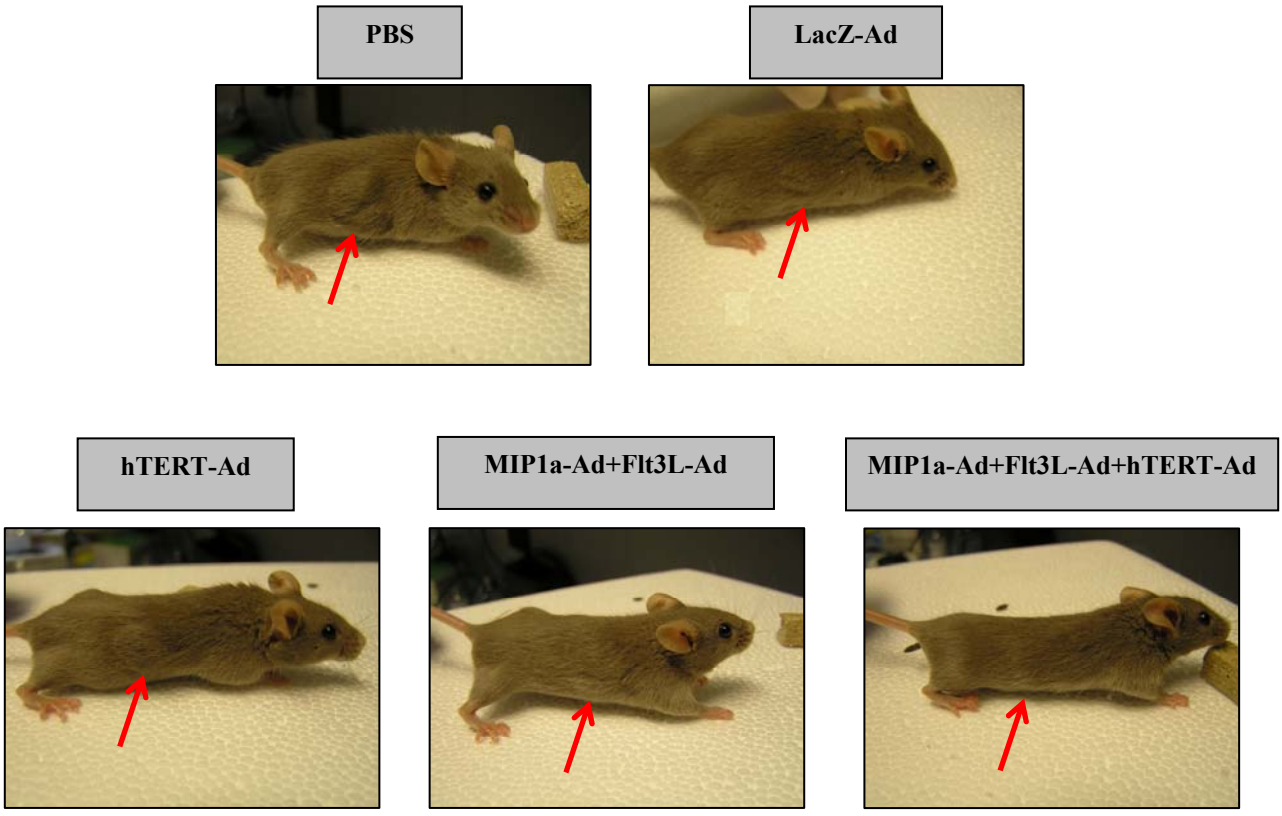


Figure 6.15 Eradication of tumors after viroimmunotherapy. After treatment with the above combinations, mice were photographed at day 21.

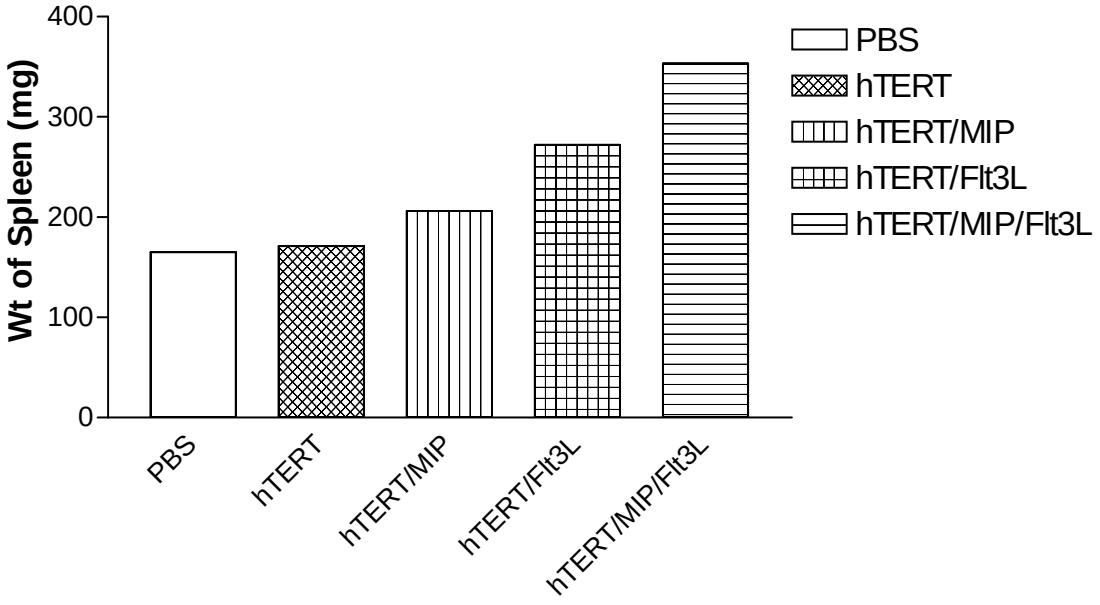


Figure 6.16 Spleens were weighed at day 21 after treatment with the different combinations of viruses.

7 Discussion

Several strategies have been used to eradicate solid tumors. Conventionally, cancer is treated with chemotherapy, radiotherapy, and the surgical removal of solid tumors. The genetic mutations responsible for carcinogenesis may increase the resistance of malignant cells to conventional treatments, thereby allowing the further survival of cancer cells and tumor growth (Fojo T, 2003). Increased knowledge of the molecular basis of cancer has also provided the possibility to exploit gene therapy as a novel strategy to treat cancer. The principle of gene therapy is to introduce, into a target cell, a therapeutic gene whose expression either stops or hinders the progression of the disease.

The major obstacle to adenoviral therapy is the transduction of viral vectors into the total tumor mass. Tumor specific, conditionally replicating viruses are promising tools to overcome low tumor cell transduction (Kirn D et al, 2001, Wirth T et al, 2003).

7.1 Efficient replication of conditionally replicating human adenovirus (hTERT-Ad) in murine tumor cells *in vitro* and *in vivo*

Oncolytic viruses kill tumor cells by tumor-specific replication. Clinical data have shown the safety of the approach but also the need for improvements in potency. Here, we have investigated the effects of a conditionally replicating adenovirus that is under the control of the hTERT promoter, which drives the E1A protein of adenovirus and causes the replication of adenovirus specific to the tumor cells. Most of previous studies have examined conditionally replicating adenoviruses in xenograft models and the regression of tumor growth, but not the immune response to tumor antigen in immunocompetent mice.

In this study, we have shown the kinetics of conditionally replicating adenovirus (hTERT-Ad) in immunocompetent murine models. We have chosen two cell lines: KLN205, a squamous carcinoma cell line, and CMT64, a lung cell carcinoma, both of them cause's lung metastasis.

To assess the replication kinetics in vitro, KLN205 and CMT64 cells were plated with hTERT-Ad and replication-defective LacZ-Ad virus or in combination with replication-defective Luc-Ad viruses. Each virus dose was MOI of 1 (together always MOI 2). In this study, we hypothesized that, when replication-defective Luc-Ad occurred in combination with hTERT-Ad, both viruses would replicate when present in the same cell. Cells were infected with various viral combinations and analyzed at 24, 48, and 72 hrs for luciferase expression.

Replication-defective control LacZ adenovirus in combination with Luc-Ad virus showed no replication in KLN205 and CMT64 cells for all analyzed parameters in terms of luciferase expression. Interestingly, the combination of hTERT-Ad with Luc-Ad showed a gradual increase in luciferase expression from 24hrs to 48hrs, but this then decreased by 72hrs (Figure 6.1, 6.2). The decrease of luciferase expression at 72hrs was attributable to all cells having undergone lysis and the further non-availability of new tumor cells to produce new viral particles (VPs).

Furthermore, in order to reconfirm that whether these tumor cells replicated hTERT-Ad virus and produce new virions, we performed a Rapid Titer Assay. KLN205 and CMT64 cells were infected with low MOI. After 48hrs, the cells were harvested and freeze/thawed three times to release viral particle into medium. The supernatant was used to infect HEK293 cells. After 48hrs, infected cell were stained with a Rapid Titer kit. These data showed that replication-defective adenovirus LacZ-Ad produced no new virions (Figure 6.3), whereas hTERT-Ad produced a high number of VPs at 48hrs.

An additional interest of mine has been to study the *in vivo* replication of the hTERT-Ad virus in tumors. To this end, we injected KLN205 cells and CMT64 cells s.c. into DBA/2 and C57Bl6 mice respectively. When the tumors were palpable, $1*10^9$ VPs of hTERT-Ad in combination of $5*10^8$ Luc-Ad VPs were injected i.t., or control

replication-defective virus and Luc-Ad were injected i.t. with same number of VPs as for hTERT and Luc-Ad, respectively. After 24hrs, we measured luciferase expression by using the XENOGEN, IVIS System. The tumors showed photon emission at this time point, suggesting the successful transduction of tumor cells and the subsequent conversion of D-Luciferin by luciferase. Interestingly, in replication-defective viruses, photon emission was always constant as a small increase, whereas replication competent hTERT-Ad showed gradual increases in photon emission every day until day 5 and decreased slowly thereafter (Figure 6.4a, 6.4b and 6.4c).

These data thus reveal that human telomerase-dependent conditionally replicating adenovirus replicates efficiently in murine tumor cells *in vitro* and *in vivo* and produces new VPs.

7.2 Expression of Ad-MIP-1α and Ad-Flt3L

To investigate the stability and expression of viruses expressing Ad-MIP-1α and Ad-Flt3L, viruses were injected at $1*10^9$ VPs i.v. into DBA/2 mice. Serum samples were collected at days 5 and 10. ELISA was performed for each cytokine. Ad-MIP-1α exhibited high expression at day 5, but this decreased by day 10, in comparison with irrelevant virus (Ad-LacZ). The increase at day 5 was high because of the high transduction of virus in the liver. The decrease by day 10 showed the high immunogenicity caused by Ad-MIP-1α and the antiviral immunity stimulated by humoral response (Figure 6.5a). On the other hand, Ad-Flt3L expression increased by day 5 and was consistent until day 10 (Figure 6.5b), showing tolerance to adenovirus and that this tolerance persisted for long periods, thereby helping further longer periods of transgene expression.

7.3 Recruitment and expansion of DCs by expressing Ad-MIP-1α and Ad-Flt3 ligand in the tumor

DCs are professional antigen-presenting cell that play a central role in priming the immune response to foreign antigens. However, the limited availability of DCs in various vaccine strategies impairs these therapeutic approaches. We have hypothesized that a combination of virotherapy with immunotherapy not only will improve virotherapy efficacy, but also will generate an anti-tumor immune response and cross-presentation of the tumor antigen. To assess this, we used replication-defective Ad-MIP-1α to mobilize DCs into the tumor bed and replication defective Ad-Flt3L to expand the mobilized DCs (Sumida et al, 2004). Other studies have shown that tumor vaccines generated by the transfection of immune-regulating molecules *in vitro* and *in vivo* are also effective as a therapeutic vaccine in generating the host immune response against tumors in various models (Shilin Yang et al, 2004). Another investigation examining Ad-Flt3L expression alone has demonstrated that, in hepatocellular carcinoma cells, gene therapy by local injection of Ad-Flt3L is an alternative to genetically modified tumor cells for stimulating host immune responses (Wang H et al, 2005).

As shown above, replication-defective virus in combination with hTERT-Ad virus can replicate and produce more virions **(**Figure 6.1 and 6.2). In our experimental set up, we injected tumor cells KLN205 and CMT64 s.c. into DBA/2 and CMT64 mice respectively. When tumors had grown to 1 cm in diameter, tumors were injected i.t. with $1*10^9$ hTERT-Ad or $5*10^8$Ad-MIP-1α or $5*10^8$ Ad-Flt3L or a combination of Ad-MIP-1α /Ad-Flt3L. In some cases 25% infected and 75% uninfected with these combinations and injected s.c in DBA/2 and C57bl6 mice. Ad-LacZ was used as a control virus in order to keep the total dose of virus constant in all animals. On day 6 after the viruses had been injected into tumors, the tumors were harvested to determine the migration of immune cells into tumor bed. Tumor sections were analyzed by immunohistochemistry with specific antibodies for DC cells and T cells.

As shown in Figure 6.6, control Ad-LacZ-virus-infected cells showed no infiltration or a low number of immune cells. Ad-hTERT-infected cells showed a small

infiltration of DCs and T cells, whereas after treatment with Ad-MIP-1α or Ad-Flt3L alone, large number of DCs and T cells were observed. The combination of Ad-MIP-1α and Ad-Flt3L resulted in even larger number of DCs and T cells infiltrating the tumors. Indeed, the coadministration of both cytokine viruses resulted in a greater than 10-fold increase in inflammatory cell number as compared with hTERT-Ad alone. Furthermore, the apoptotic cells produced by hTERT-Ad were accessible to these recruited DCs and T cells.

These results demonstrate that Ad-MIP-1α or Ad-Flt3L alone recruit small number of DCs and T cells to the injected site. When these viruses are administered together, a massive number of DCs and T cells infiltrate the tumor. Probably Ad-MIP-1α recruit DCs and T cells, and Ad-Flt3L expanded these cells resulting in large number of DCs and T cells at the site of injection.

7.4 Infiltration of DCs and T cells augments immune response to tumor antigens and viral antigens in virotherapy

Our aim was to infiltrate and expand immature dendritic cells into the tumor bed where replication-competent virus could produce apoptotic tumor cells. Infiltrated immature DCs directly take up tumor antigens and mature by inflammation caused by viruses. These mature DCs migrate to draining lymph nodes (DLN) where they activate naïve T cells to generate antigen-specific immune responses.
Here, we have used the antigen presentation pathway-deficient cell line CMT64 together with transporter associated with antigen processing 1 gene deficient (TAP1). Expression of TAP1 in lung carcinoma cells increases tumor-specific immune responses and survival (Lou Y, 2005, Jefferies WA, 1993); others have shown TAP-dependent MHC I presentation of exogenous antigen by bone marrow DCs (BMDCs) (Norbury CC et al, 1997).

On the basis of this, we have hypothesized that the infiltration of DCs into tumor cells, in which ongoing replication of adenovirus (virotherapy) is taking place, would enhance tumor-specific immune responses by cross-presenting the tumor antigens

released by virotherapy. CMT64 tumor cell were injected s.c. into C57Bl 6 mice, which are defective in TAP expression. When tumors had grown to approximately 1 cm in diameter, various combinations of viruses (control, Ad-hTERT, Ad-MIP-1α, and Ad-Flt3L and a combination of Ad-hTERT and cytokines) were injected i.t. (Figure 6.7). After 2 weeks, mice were killed, and their splenocytes were isolated. Viroimmunotherapy-elicited $CD^{+}8$ T lymphocyte responses specific for the tumor lysate were assessed. As demonstrated in Figure 6.7, a single injection of virotherapy alone showed secretion of IFN-γ. Control mice treated with PBS exhibited no specific immune response, but virotherapy alone resulted in a high secretion of IFN-γ compared with cytokines alone. This could be that hTERT-Ad virus contains E1A gene under the control of hTERT promoter, which drives E1A expression specifically in tumor cells (Wirth T et al, 2003) and produces more E1A protein molecules and transduction of virus, further enhancing the immune response through various pathways. Recent studies have shown that adenovirus serotype 5 E1A expression sensitizes tumor cells to NKG2D-mediated NK cell lysis and tumor rejection in a murine model (Routes JM, 2005).

Interestingly, the combination of a single cytokine with hTERT-Ad virus showed a synergistic effect, with high levels of IFN-γ being secreted. Infiltration of DCs and T cells into tumor bed and secretion of inflammatory cytokines by infiltrated cells would enhance the MHC expression on CMT64 cells, which mediates antigen specific immune response and target cell killing. Further, a combination of both cytokine viruses with hTERT-Ad showed an extremely high secretion of IFN-γ specific to tumor-associated antigens. The secretion of IFN-γ presented in Figure 4.7 is the mean value of 8 wells of each parameter.

One drawback of virotherapy is the anti-viral immune response to viral vectors or to the virus. Since the migration and expansion of DCs enhances the anti-tumor immune response, we expected an anti-viral immune response too. As shown in Figure 6.8, treatment with a single cytokine, Ad-Flt3L, leads to a lower immune response to virus than treatment with Ad-MIP-1α alone. Moreover, in Figure 6.5a, MIP-1α can be seen to have more immunogenicity than Flt3L, i.e., Flt3L is more tolerance to virus. Nevertheless, the combination of both cytokines with hTERT-Ad results in a high anti-viral T cell responses (Figure 6.8). This reveals another of the major obstacle

concerning the use of viral vaccine in tumor therapy, namely, that the strong viral antigens will dominate, thereby inhibiting responses to the subdominant tumor antigens. However, quenching of the immunodominant antigens is rarely seen with recombinant viral vaccines. Hence, a balancing/modifying the immune responses to tumor and virus are important in virotherapy (Carroll MW et al, 1997 and Paoletti E et al 1995).

7.5 Modulation of immune responses by DC vaccination in a sequence specific manner enhances tumor-specific T cell responses and decreases the humoral response to viruses

One chief barrier to cancer virotherapy is the anti-viral immune response, as discussed above. To break or modulate anti-tumor and anti-viral responses, we performed an elegant experiment, in which we injected s.c. the ovalbumin (SIINFEKL-H2K^b restricted) model antigen-expressing stable cell line CMT64-OVA and treated the s.c. tumors as follows.

1. Control
2. Viroimmunotherapy (hTERT/MIP-1α/Flt3L); in subsequent weeks, (MFT-MFT)
3. Viroimmunotherapy; in subsequent week, tumor antigen pulsed DC vaccination (MFT-DC)
4. Tumor antigen pulsed DC vaccination; in subsequent week, viroimmunotherapy (DC-MFT)
5. Virotherapy alone (hTERT-Ad-DC)
6. Only tumor antigen pulsed DC vaccination

M- MIP1α-Ad

F-Flt3 Ligand-Ad

T- hTERT-Ad

Following treatment, splenocytes were isolated on 28th day and assessed for the cellular immune responses by IFN-γ ELISPOT. As shown in Figure 6.9, control and DC vaccination alone resulted in no detectable immune response, whereas viroimmunotherapy, viroimmunotherapy with subsequent DC vaccination, and DC

vaccination with subsequent viroimmunotherapy showed detectable ovalbumin antigen-specific immune responses. Interestingly, only viroimmunotherapy with subsequent ovalbumin antigen pulsed DC vaccination showed a three to four-fold increase compared with viroimmunotherapy and with DC-vaccination plus subsequent viroimmunotherapy. Viroimmunotherapy thus seems to provide a conditioned environment in the tumor and subsequent antigen pulsed DC vaccination leads to direct migration of DCs to DLN and activation of naïve T cells to become effector T cells. As a control peptide, β-Gal ($H2K^b$ restricted) was used.

To measure the immune response to the virus, serum samples were collected from all treated animals on 28^{th} day following treatment. Further serum samples were measured for total anti-adenoviral antibody titers in the serum sample by ELISA. Interestingly, viroimmunotherapy with subsequent ovalbumin pulsed DC vaccination showed lower anti-adenoviral antibody titers (Figure 6.10) compared with MFT-MFT and DC-MFT treatment.

These results indicate that viroimmunotherapy with subsequent ovalbumin pulsed DC vaccination is effective in generating an antigen-specific immune response and in reducing anti-adenoviral antibody titers. Tumor growth inhibition is also seen in the DBA/2 mice model.

7.6 Generation of cytotoxic T cells by viroimmunotherapy

We also considered that it is important to determine whether viroimmunotherapy had generated enough cytotoxic T cells (CTLs) to kill target cells. We performed an *in vivo* cytotoxicity assay after the following treatments. Mice were injected s.c. with CMT64-OVA cells. Tumors were treated with virotherapy or with viroimmunotherapy with or with out ovalbumin peptide pulsed DC vaccination, or first with ovalbumin peptide pulsed DC vaccination and subsequent viroimmunotherapy, or only ovalbumin pulsed DC vaccination. At day 28, mice were transferred i.v. with ova-peptide pulsed target cell with $CFSE^{hi}$ and control peptide with $CFSE^{low}$ labeled splenocytes.

As we expected, mice receiving viroimmunotherapy or viroimmunotherapy plus subsequent DC were able to kill OVA peptide pulsed CFSE labeled target cells more efficiently compared with controls or with those treated with virotherapy (hTERT) alone or DC vaccination alone (Figure 6.11a, b, c and d).

Although DC vaccination alone and DC vaccination plus subsequent viroimmunotherapy caused cytotoxicity, the percentage of target cell killing is high after viroimmunotherapy with or without DC vaccination. Indeed, the cytotoxicity of viroimmunotherapy plus subsequent DC vaccination was lower than that after viroimmunotherapy alone; however, the cytotoxicity of MFT-MFT and MFT-DC treatments were more or less similar, but the anti-adenoviral antibody titers were lower in MFT-DC vaccination mice. This again shows the importance of viroimmunotherapy with subsequent antigen pulsed DC vaccination of poorly immunogenic carcinomas.

7.7 Maturation and migration of DCs to lymph node play an important role in the activation of naïve T cells

Localization in tissues and migration to lymphoid organs are essential steps in the immunobiology of DCs. Chemokines play an important role in guiding DC migration and regulating their maturation (Lin CL, 1998). In the present study, MIP-1α and Flt3L have been used as chemokines to attract DCs into the tumor bed.

Among the antigen-presenting cells, DCs often acquire foreign antigens, and the maturation stimulated by inflammatory agonists causes rapid mobilization of DCs. Upon interaction of DCs with apoptotic tumor cells, CCR7 expression is induced, which further enhances the migration of mature DCs to DLNs (Hirao M, 2000). These mature DCs migrate to lymph nodes through lymphatic vessels and activate naïve T cells in DLN to become effector T cells (Randolph GJ, 2005). In our study, we have performed immunohistochemistry for MHC-II, CD80, and CD83 co-stimulatory molecules and FACS analysis of CD40 expression from tumor-infiltrating dendritic cells after treatment with MIP1α-Ad or Flt3L-Ad or a combination of them. The data indicates that these DCs exhibit high expression of CD40, MHC class-II, CD83, and

CD80 co-stimulatory molecules upon treatment (Figure 6.12a, 6.12b and 6.12c). Hence, these DCs are functionally active and efficiently migrate to the T cell zone of DLN. From the previous results concerning the immune response and cytotoxicity of target cells, this again confirms that these infiltrated DCs have matured, and migrated to DLN and activated naïve T cells to generate antigen-specific effector T cells.

7.8 Viroimmunotherapy with subsequent tumor antigen loaded DC vaccination inhibits established lung metastasis

Metastasis is the most lethal attribute of cancer cells, and clinical decisions regarding treatment are based largely upon the likelihood of the development of metastases (Danny R. Welch, 2006). When cancer survivor Clifton Leaf lamented the state of cancer research in early 2004, he concluded that progress in the "War on Cancer" was limited because research into the prevention and treatment of metastasis had been inadequate (Leaf C, 2004). He asserted that failure to control the establishment of secondary colonies was the major contributor to failure, and that winning against cancer would require increasing resources devoted to the study of metastasis.

Some tumors are highly aggressive, forming secondary lesions with high frequency, such as small lung carcinoma and melanoma, whereas others are rarely metastatic, despite being locally invasive, for instance, basal cell carcinoma. Therefore, metastasis is not an inherent property of all invasive or neoplastic cells. Most secondary tumors arise from tumor cells migrating via the lymphatics (lymph node metastasis) and across body cavities.
Hence, a study of the clinical relevance of viroimmunotherapy is important. In the present investigation, we used CMT64, a lung carcinoma cell line. This cell line causes aggressive tumors with an ability to cause distant metastasis.CMT64 cells were injected i.v. to establish lung metastasis followed by various regimes as follows:

Control, virotherapy, viroimmunotherapy, and viroimmunotherapy with subsequent antigen pulsed DC vaccination. After two weeks of treatment, lungs were perfused with 3.7% formalin, fixed, embedded in paraffin, and prepared for light microscopy.

Deparaffinized sections were stained with hematoxylin-eosin according to a standard protocol.

After viroimmunotherapy, lungs were identified with metastatic lesions in controls, and in animals treated with virotherapy alone, whereas viroimmunotherapy with or with out DC vaccination showed the inhibition of lung metastasis. Interestingly, viroimmunotherapy with subsequent DC vaccination showed very strong inhibition of lung metastasis and more than 50% of the cases of the animals cured lung metastasis (Figure 6.13a and 6.13b). Whether the adenovirus proteins migrated to the lungs and inhibited metastasis was not clear. We therefore performed immunohistochemistry of adenovirus hexon protein and ruled out the absence of the spreading of viral protein in lungs, except only in locally infected tumors (Figure 6.14).

These results indicate the promising clinical relevance of viroimmunotherapy with subsequent DC vaccination, at least with regard to the inhibition of lung metastasis.

7.9 Increased size of spleen and inhibition of tumor growth followed by viroimmunotherapy

In addition to the inhibition of lung metastasis, we also observed that a significant delay occurred in the tumor growth of viroimmunotherapy treated mice compared with those in mice treated with control virus or virotherapy alone. Despite the delay of tumor growth after treatment with a combination of Ad-MIP-1α and Ad-Flt3L, a further combination of hTERT-Ad eradicated s.c. tumors (Figure 6.15)**.** This can be explained by the replication of adenovirus in KLN205 tumor cells.

Further, we observed that the treatment with viroimmunotherapy not only inhibited the tumor growth, but also increased size and number of splenocytes (Figure 6.16).
These findings suggest that a combination of oncolytic virotherapy with immunotherapy is effective in curing tumor growth in more than 50% of animals and increasing spleen size.

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9 Abbreviations

Ad	Adenovirus
APC	Antigen presenting cells
BMDC	Bone marrow dendritic cells
CFSE	Carboxy Fluoroscein Succinimidyl Ester
CTL	Cytotoxic T cells
DC	Dendritic cells
DMEM	Dulbecco´s Minimal Essential Medium
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
GrB	Granzyme B
hTERT	Human telomerase reverse transcriptase promoter
IFN	Interferon
IL	Interleukin
i.v.	Intravenous
i.p.	Intraperitoneal
i.t	intratumoral
lacZ	β-galactosidase gene
Luc	Luciferase
MHC	Major histocompatibility antigen
MOI	Multiplicity of infection
MFT	MIP-1α/Flt3L/hTERT-Ad
NK	Natural killer cells
NKT	Natural killer T cells

PAMP	Pathogen-associated molecular patter
PRR	Pattern recognition receptor
Pfu	Plaque forming unit
s.c	Subcutaneous
TAA	Tumor-associated antigen
TLR	Toll-like receptor
TCR	T cell receptor
TID	Tumor infiltrating dendritic cells
TNFα	Tumor necrosis factor alpha
VP	Viral particles

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Erklärung

Hiermit erkläre ich, dass die Dissertation "**Strategies for Improved Cancer Virotherapy: *In vivo* migration and expansion of dendritic cells enhance cross-presentation of tumor antigens in virotherapy"** selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

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