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Bachelor thesis

Towards the generation of a safe lentiviral vector based vaccine

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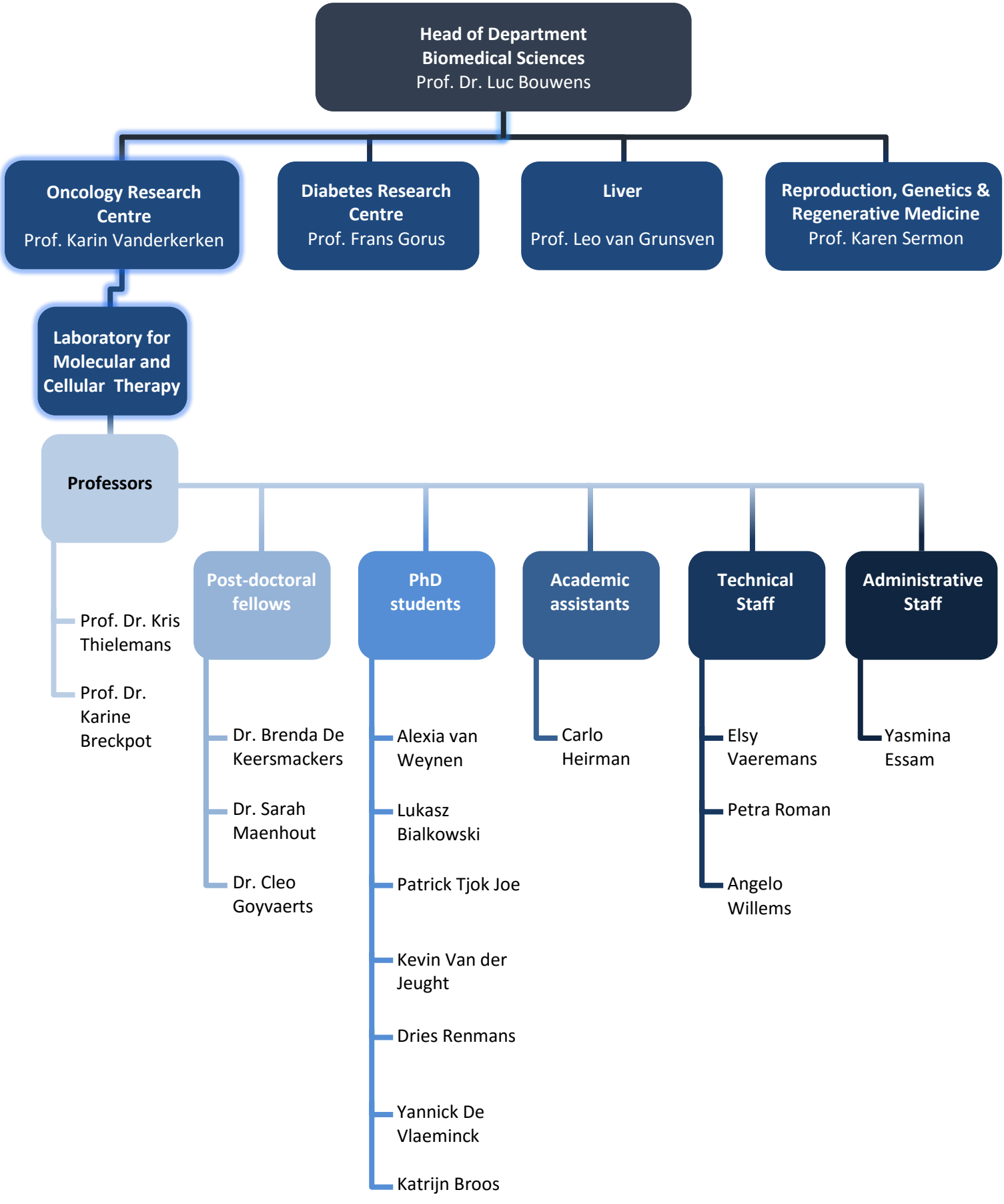
II Organization Chart



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IV List of abbreviations

ABTS	2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
AIDS	Acquired immunodeficiency syndrome
Anti-DIG-POD	Anti-digoxigenin-peroxidase
APC	Allophycocyanin
ATCC	American type culture collection
BIV	Bovine immunodeficiency virus
BMDC	Bone marrow derived dendritic cells
Bp	Basepairs
C57BL/6	C57 black 6 mouse
CAEV	Caprine arthritis encephalitis virus
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CMV	Cytomegalovirus
cPPT	Central polypurine tract
DC	Dendritic cells
DMEM	Dulbecco's modified eagle medium
DMEM+	Dulbecco's modified eagle medium +: 87,5 $^{v}/_{v}$ % Dulbecco's Modified Eagle Medium / 10$^{v}/_{v}$ % Fetal Bovine Serum/ 2,5$^{v}/_{v}$ % Penicillin-Streptomycin with L-glutamine
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's phosphate buffered saline
ds DNA	Double stranded deoxyribonucleic acid
ds RNA	Double stranded ribonucleic acid
E.coli	*Escherichia coli*
*Eco*RI	First discovered restriction enzyme of the RY 13 strain of *Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
eGFP	enhanced green fluorescent protein
EIA	Equine infectious anemia
Env	Envelope gene of the lentiviral genome

FACS	Fluorescence-activated cell sorting
FCS or FBS	Fetal calf serum or fetal bovine serum
FIV	Feline immunodeficiency virus
FSH	Forward scatter
Gag	Group specific antigen gene of the lentiviral genome
Gen	Generation
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOI	Gene of interest
gp100	Glycoprotein 100 or melanocyte protein PMEL
HEK293T	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HIV-I	Human immunodeficiency virus type I
IDLV	Integrase deficient lentiviral vector
IRES	Internal ribosomal entry site
iv	Intravenous
Kana res	Kanamycin resistance gene
Kb	Kilo basepairs = 10^3 basepairs
LB	Lysogeny broth
LMCT	Laboratory for molecular and cellular therapy
LMP	Low melting point
LTR	Long terminal repeat
LV	Lentiviral vector
MA	Matrix
MCS	Multiple cloning Site
MHC II	Major histocompatibility complex two
Milli-Q® H_2O	Ultrapure water: distilled and passed through an ion exchange filter
MOI	Multiplicity of infection
MP	Microplate
Nef	Negative regulatory factor

ORF	Open reading frame
ORI	Origin of replication
OVA	Ovalbumin
PBS/BSA/azide	Phosphate buffered saline /bovine serum albumin/azide
PE	R-phycoerythrin
PEI	Polyethylenimine
PLV	Puma lentivirus
pol	Polymerase gene of the lentiviral genome
pro	Protease gene of the lentiviral genome
PS-Lglu	Penicillin, streptomycin and L-glutamine
QC	Quality Control
RBS	1% RBS AM 35 MD detergents
RCL	Replication competent lentiviral lectors
RNA	Ribonucleic acid
rpm	Rates per minute
RPMI	Roswell Park Memorial Institute
RRE	Rev response element
RT	Reverse transcriptase
S/MAR	Scaffold matrix attachments regions
SAP	Shrimp alkaline phosphatase
SIINFEKL	Immunodominant epitope of OVA, presented by the class I MHC molecule H-2Kb
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
SMART-DCs	Self-differentiated myeloid derived antigen presenting cells reactive against tumors
*Spe*I	First discovered restriction enzyme of *Sphaerotilus natans*
ss DNA	Single stranded deoxyribonucleic acid
ss RNA	Single stranded ribonucleic acid
SSC	Sideward scatter
SU	Surface glycoprotein

SV 40	Simian virus 40
TADC	Tumor-associated dendritic cell
TAM	Tumor-associated macrophage
TAR	Trans activated region
TBE	Tris-borate-EDTA
TM	Transmembranary glycoprotein
TME	Tumor microenvironment
TMV	Tobacco mosaic virus
tNGFR	truncated nerve growth factor receptor
TU	Transducing units
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein unique
*Xba*I	First discovered restriction enzyme of *Xanthomonas badrii*
*Xho*I	First discovered restriction enzyme of *Xanthomonas holcicola*
XL1 blue	Strain of *Escherichia coli* used for cloning experiments
λ	Lambda
Ψ	Psi

V Samenvatting – Abstract

Achtergrond

Zo'n twintig jaar geleden werden lentivirale vectoren (LVn) ontwikkeld om genen efficiënt en permanent over te dragen naar cellen. De overdracht gebeurde door het LV genoom te insereren in het gastheercel DNA. Vandaag lijkt hun potentieel groter dan aanvankelijk voorgenomen sinds ze worden getest voor zowel het fundamenteel alsook klinisch kader van gen therapie en immunotherapie onderzoek. Desalniettemin zijn LVn afgeleid van lentivirussen zoals het humane immunodeficiëntie virus type I (HIV-I) waardoor hun toepassingen gepaard gaan met twee belangrijke bioveiligheidsrisico's. Enerzijds bestaat het risico dat replicatie competente lentivirussen (RCLs) gegenereerd worden tijdens het LV productie proces en anderzijds bestaat het risico op insertionele mutagenese. Daarom werden in het verleden reeds verschillende veiligheidsmaatregelen getroffen zoals de ontwikkeling van verschillende 'generaties' LVn om de kans op RCLs te verlagen. Daarnaast werd ook reeds een integratie deficiënte LV (IDLV) ontwikkeld om de kans op insertionele mutagenese tot een minimum te beperken.

Doel

In het verleden werd op het LMCT steeds gewerkt met integratie competente tweede generatie (gen) LVn. Om tegemoet te komen aan de verhoogde veiligheidsnormen, is het echter noodzakelijk om de tweede gen LVn af te lossen met de veiligere IDLVn en derde gen LVn te werken. Het doel van deze thesis is dan ook om het productie en transductie proces te optimaliseren en het vergelijken van de transductie efficiëntie en immunogeniciteit van tweede gen IDLVn en derde gen LVn met onze "standaard" tweede gen LVn.

Methoden

Om LVn te produceren, werden 293T cellen getransfecteerd met een mix aan plasmiden namelijk een envelop plasmide, één of twee packaging plasmide(n) en het transfer plasmide. Om derde gen LVn te kunnen genereren, kloneerden we eerst drie verschillende derde gen transfer plasmiden. Vervolgens werd de productie van enhanced green fluorescent protein coderende IDLVn en derde gen LVn geoptimaliseerd door hun respectievelijke plasmiden in verschillende ratio's te transfecteren in 293T cellen. Daaropvolgend werden de geoptimaliseerde IDLV en derde gen LV productie protocols gebruikt om deze LVn aan te maken en hun titer te vergelijken met die van de tweede gen LVn. Hiervoor werd een titratie alsook een reverse transcriptase (RT) assay uitgevoerd. Verder werd hun transductie efficiënte en de tijdsduur van transgene expressie nagegaan op beenmerg afgeleide dendritische cellen (BMDCs) en 293T cellen respectievelijk. Tot slot werd ook het potentieel van de

verschillende LVn coderend voor ovalbumine getest om een antigen specifieke immuun respons op te wekken *in vivo* met behulp van een cytotoxische T cel assay.

Resultaten

Na optimalisatie bleek het voor de IDLVn en de derde gen LVn best om 45 µg of tweemaal 30 µg van hun respectief packaging plasmide te gebruiken. Vervolgens werden deze geoptimaliseerde LV productie protocols aangewend om LVn te genereren en hun titers te vergelijken met 'onze standaard' tweede gen LVn. Hieruit bleek dat we met deze laatste een vier tot acht keer hogere titer (transducing units/ml) konden bekomen, in vergelijking met IDLVn en derde gen LVn respectievelijk. Opmerkelijk was wel dat de titer uitgedrukt in ng reverse transcriptase/µl tot tweemaal hoger was in de IDLVn t.o.v. de tweede en derde gen LVn. Wanneer we vervolgens hun transductie efficiëntie nagingen in BMDCs, resulteerde de tweede gen LVn wederom tot het hoogste percentage getransduceerde cellen. Daaropvolgend, verloopt de transgene expressie in getransduceerde 293T cellen door middel van tweede en derde gen LVn stabiel over de tijd terwijl deze in het geval van de IDLVn volledig afneemt tussen dag drie en zeven na de IDLV transductie. Tot slot toonden we aan dat na subcutane injectie van muizen met 40 ng RT van elk van de LVn coderende voor ovalbumine, de tweede en derde gen LVn een evenwaardige immuun respons konden induceren terwijl deze erg verlaagd was na injectie met IDLVn.

Conclusie

Hoewel de meeste resultaten in deze thesis niet statistisch significant zijn, geven ze toch aan dat het productie en transductie protocol van de IDLVn en derde gen LVn nog verder geoptimaliseerd dient te worden alvorens het niveau van onze 'gouden standaard' tweede gen LVn bereikt zal worden. Belangrijk is wel dat indien we een vergelijkbare hoeveelheid tweede of derde gen LVn toedienen *in vitro* of *in vivo*, hun transgene expressie verloop alsook immunogeniciteit vergelijkbaar is. Dit laatste moedigt de verdere optimalisatie van het derde gen LV systeem aan om alsnog de tweede gen LVn hierdoor op termijn volledig te vervangen.

Introduction

About 20 years ago, lentiviral vectors (LVs) were developed to efficiently and permanently transfer genes to cells. The latter is achieved by inserting their own genome into the host cell DNA. Today their potential appears even wider than originally anticipated as they are being tested for both fundamental and clinical gene therapeutic and immunotherapeutic research. Nevertheless, LVs are derived from lentiviruses such as the Human Immunodeficiency Virus Type I (HIV-I) and therefore their applications still hold two important biosafety risks. On the one hand, the risk of generating replication competent lentiviruses (RCL) during LV production and on the other hand the risk of insertional mutagenesis. Hence various safety measures were introduced such as the development of disparate LV generations in order to reduce the possibility of RCLs. Furthermore the integrase deficient LV (IDLV) was engineered to restrict the chance of insertional mutagenesis.

Issue

The host laboratory performed a lot of research with 2nd generation (gen) integrase competent LVs in the past. In order to meet the requested safety measures however, it is paramount to exchange the 2nd gen LVs for their safer integrase deficient (IDLV) or 3rd gen counterparts. The purpose of this thesis was to optimize the production of and compare the transduction efficiency and immunogenicity for 2nd gen IDLVs and 3rd gen LVs with our “standard” 2nd gen LVs.

Methods

In order to produce LVs, 293T cells were transfected with a mixture of plasmids namely an envelope plasmid, one or two packaging plasmid(s) and a transfer plasmid. In order to generate 3rd gen LVs, we first cloned three different 3rd gen LV transfer plasmids. Subsequently, the production of IDLVs and 3rd gen LVs encoding enhanced green fluorescent protein or ovalbumin, was optimized by altering the ratio of their respective plasmids. Next, we utilized the optimized IDLV and 3rd gen LV production protocols to produce these LVs and compare their titer with 2nd gen LVs. Therefore a titration and reverse transcriptase (RT) assay were performed. Next, transduction efficiencies and extent of transgene expression was monitored on bone marrow derived dendritic cells (BMDCs) and 293T cells respectively. Finally, the potential of the different LVs encoding ovalbumin was tested in order to elicit an immune response *in vivo* by means of a cytotoxic T cell assay.

Results

After optimization, it appeared that the IDLVs and 3rd gen LVs are preferably produced with 45 µg or two times 30 µg of their respective packaging plasmids. These optimized LV production protocols were applied to generate the respective LVs and compare their titers to our "standard" 2nd gen LVs. This revealed that the latter attained four to eight fold higher titers (transducing units/ml), compared to the IDLVs and 3rd gen LVs respectively. Of note, the titer expressed in ng reverse transcriptase/µl was up to two times higher in the case of IDLV when compared to the 2nd and 3rd gen LVs. When we subsequently checked their transduction efficiency in BMDCs, the 2nd gen LVs resulted in the highest percentage of transduced cells. Further, the transgene expression in transduced 293T cells with 2nd or 3rd gen LVs was stable over time whilst this completely diminished between days three and seven after IDLV transduction. Finally we demonstrated that after subcutaneous injection of mice with 40 ng RT of each of the LVs encoding ovalbumin, the 2nd and 3rd gen LVs induced a similar immune response whilst this response was almost undetectable upon IDLV injection.

Conclusion

Although the most results in this thesis aren't statistically significant, they do indicate that the production and transduction protocols of the IDLVs and 3rd gen LVs require further optimization before they can level up to our "golden standard" 2nd gen LVs. It is important to note that when a comparable amount of 2nd and 3rd gen LVs is administered *in vitro* or *in vivo*, their transgene expression course as well as immunogenicity is comparable. The latter encourages the further optimization of the 3rd gen LV system to ultimately replace the 2nd gen LVs.

1 Introduction & Issue

The main aim of gene therapy is to deliver one or more genes to cells or tissues of interest and as such transiently or permanently provide them with a new gene encoded molecule. As viruses are intracellular obligate parasites that completely rely on the host's replication, transcription and translation machinery, it's not surprising that viruses have been evaluated for their suitability to be transformed into gene therapeutic vectors. Virus derived vectors are called viral vectors and they have been fine tuned to become exquisite gene transfer vectors for mammalian cells for both *in vitro* and *in vivo* applications (Goyvaerts, Bricogne, Escors, & Breckpot, 2012).

Of these, lentiviral vectors (LVs) confer several advantages in comparison to other viral and nonviral vectors. A first one is their large genetic capacity which they can integrate in the host cell genome. As such they enable the stable transfer of one or more genes of interest (GOI). Secondly, they can be pseudotyped or in other words their envelope glycoproteins can be changed (Cronin, Zhang, & Reiser, 2005). As such their tropism or ability to target a specific cell type can be altered. Another benefit of LVs is that the lentiviruses from which they are derived, have no pre-existing immunogenicity in contrast to the adenoviral- and adeno-associated viral vectors (Addgene, 2015). Hence LV transduced cells won't be cleared immediately by the immune system of the patient. Finally, LVs are, in contrast to their retroviral counterparts, able to infect both dividing and non-dividing cells.

All these advantages led to the wide use of LVs as tools for stable gene transfer in the fields of fundamental biological research, functional genomics, vaccination and translational gene therapy (Bukrinsky et al., 1993; Lewis & Emerman, 1994; Mátrai, Chuah, & VandenDriessche, 2010).

However, as LVs are derived from lentiviruses such as Human Immunodeficiency Virus type I (HIV-I), safety concerns have to be taken into account. First of all there is a chance that replication competent LVs (RCLs) are generated during the LV production process. LVs are produced by transient transfection of Human Embryonic Kidney (HEK293T) cells with a number of plasmids that encode the viral components. Subsequently, recombination between these plasmids can lead to RCL formation. As for the production of first generation (gen) LVs only two plasmids are used, the possibility of RCL formation is quite high since only one recombination event needs to occur. In contrast, to make second gen LVs, three plasmids are used which reduces the chance for RCLs. Yet third gen LVs have the virion encoding sequences scattered over four plasmids in order to further reduce the risk of RCLs.

Furthermore, while their integrative capacity bestows the LVs with the ability to induce stable gene transfer, this also holds the possibility that insertional mutagenesis is induced (Pauwels et al., 2009). Therefore integrase deficient LVs (IDLVs) have been developed as well, which do not integrate their genome in the host's genome hence significantly reduce the risk for insertional mutagenesis.

The intention of this thesis is to compare the production and transduction efficiency between a second gen IDLV, second gen LV and a third gen LV. As such we hope to optimize the generation of a safer LV tool and determine their suitability for different gene therapeutic applications to enhance the safety of gene therapy for future experiments.

2 Literature Review

2.1 From virus to gene vector

Viruses are a relative new subject of biology as their discovery took place in 1892 by Dimitri Ivanovsky, hence all obtained information concerning viruses has been made over the past 124 years. Dimitri Ivanovsky was a Russian scientist who conducted research on the tobacco mosaic disease and presented a paper in which he concluded that the juice of infected leaves retains its infectious properties after filtration through bacteria-proof Chamberland filter candles (Ivanovsky, 1892; Lustig & Levine, 1992). The last study Dimitri Ivanovsky undertook with tobacco mosaic disease was published in 1903 in which he described the presence of abnormal intracellular inclusion bodies in the host cells of virus diseased plants (Ivanovsky, 1903). Independently from Dimitri Ivanovsky's published work about mosaic tobacco disease, Martinus Beijerinck conducted a likewise experiment with the same conclusion namely that the causal agent of the disease was filterable. Furthermore he determined that the infectious agent was able to multiply and therefore described it as "contagium vivum fluidum" (Beijerinck, 1898). This incited a 25-year long debate about viruses, liquids and particles which was laid to rest thanks to d'Herelle's plaque assay (D'Herelle, 1917) and the first electron micrographs of the Tobacco Mosaic Virus (TMV).

The origin of viruses remains a mystery up until this day, but their lineage can be traced back through Paleovirology. The human genome consists of five to eight percent of endogenous retroviral sequences which are remnants of ancient viral sequences that integrated into the genome and up to 90% of non-coding mobile sequences of the human genome are retroviral remnants (Zwolińska, 2006). Thanks to these endogenous retroviral sequences we are able to trace back when and which virus integrated into a genomic sequence based on the phylogeny of species. In the case of HIV and other lentiviruses, integration into the human genome was made very recently through cross-species transmission (Compton, Malik, & Emerman, 2013). The two most popular theories of the rise of viruses are that viruses are degenerated cells which lost or discarded many cellular functions until the essence remained allowing viruses to ensure their survival. The second theory comprises the transfer of nucleic sequences from one cell to another cell from a different species and instead of being degraded the nucleic sequence survived and replicated.

Today it is generally known that all viruses rely on the host's replication, transcription and translation machinery. Viruses are infectious, obligate intracellular parasites with a diameter size ranging from 17 nm (Mankertz et al., 2000) to 0,5 µm and a length of 1,0 µm (Philippe et al., 2013).

Furthermore their genome consists either of single or double stranded DNA or RNA. The differences in their genomic type and replication cycle allow us to classify viruses. This classification is known as the Baltimore classification and is represented in Figure 1.

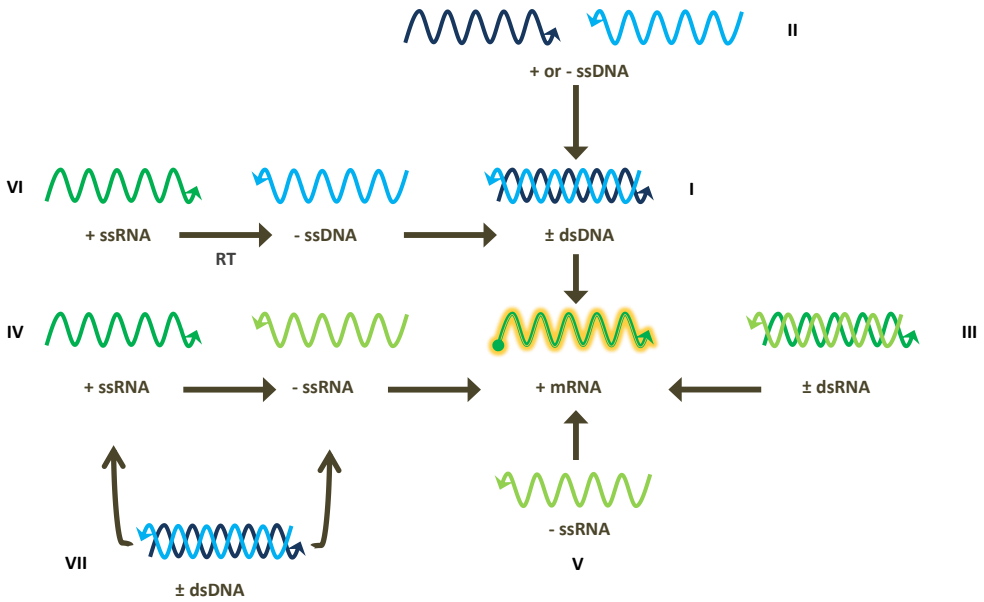

Figure 1: Representation of the Baltimore classification system for viruses.
The seven different classes of viruses are based on the genome type and its transcription pathway. The first class comprises viruses with a double stranded (ds) DNA genome. The second class comprises viruses with a positive (+) or negative (-) single stranded (ss) DNA genome while the third class carries a ds RNA genome. The fourth class comprises viruses with a + ssRNA genome that has to be transcribed to – ssRNA in order to function as a template for the production of a + mRNA strand. The fifth class comprises virions that consist of – ssRNA and the sixth class of viruses have a genome made up of + ssRNA which is reverse transcribed into - ssDNA in order to function as a template for transcription of + mRNA. Finally the seventh class of viruses carries a ds DNA genome which is transcribed into – ssRNA or + ssRNA. Whenever 'RT' is placed under an arrow, this indicates that reverse transcriptase (RT) was involved in the process.

The viral genome is enclosed by a capsid, which consists of capsomeres that form a protective measure for the viral genome against the hostile environment (Figure 2). These can be physical threats i.e. temperature and natural radiation next to chemical threats i.e. pH extremes, proteolytic and nucleolytic enzymes. Some viruses such as lentiviruses, possess a second protective measure which is derived from the host's cellular membrane into which viral glycoproteins are embedded. This second protective measure is referred to as the envelope and surrounds the capsid (Figure 2). Non enveloped viruses such as the lambda (λ) phage virus, TMV and adenovirus, target cells through the incorporation of cell specific viral receptors. While the former utilizes a head-tail structure in which the head contains the viral genome and the tail functions as the cell specific receptor (Figure 2A), the latter two incorporate specific viral receptors into their capsid (Figure 2B). In the case of enveloped viruses, the cell specific targeting function of the capsid is replaced by the envelope in which cell specific glycoproteins are embedded. Since the latter contain binding sites for host cell specific surface receptors, they can mediate fusion of the virion with the cellular membrane of the target cells.

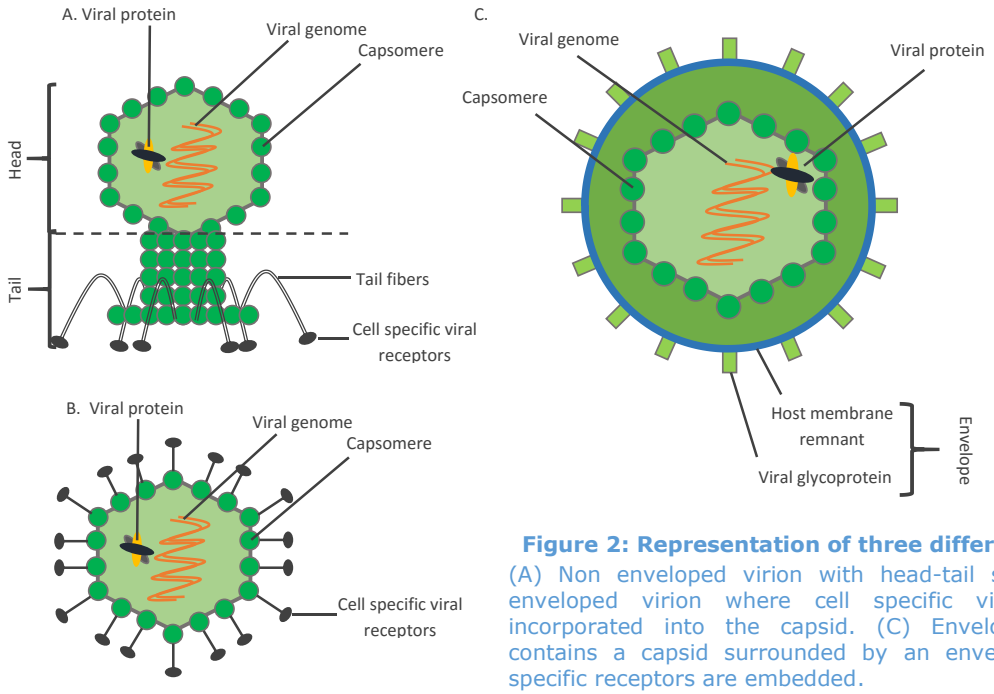

Figure 2: Representation of three different virion types.
(A) Non enveloped virion with head-tail structure. (B) Non enveloped virion where cell specific viral receptors are incorporated into the capsid. (C) Enveloped virion which contains a capsid surrounded by an envelope in which cell specific receptors are embedded.

After target cell specific attachment, viral cell entry (infection) is induced upon which the viral genome is made available through uncoating (Figure 3). Of note, some viruses combine the penetration and uncoating process into one and some integrate their genome after uncoating into the host's genome. After the viral genome has been made available in one-way or another, the biosynthesis process is initiated which involves the production of viral proteins and newly replicated viral genomes. After the production of viral proteins and newly replicated viral genomes, new viral particle assembly commences. Finally, these viral particles are released and mature to infect new cells hence the vicious cycle of viral replication can repeat itself.

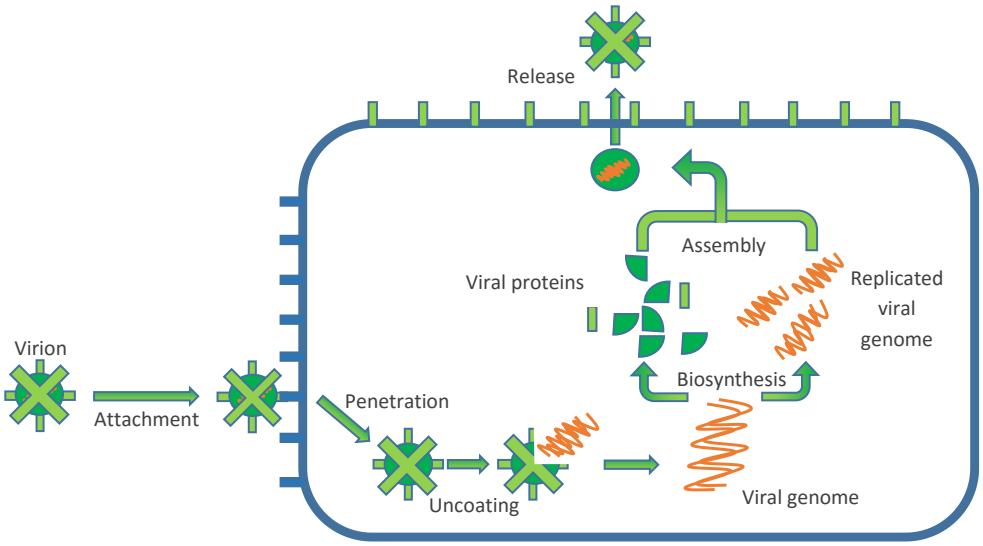

Figure 3: Schematic representation of the general virus multiplication cycle.
The viral multiplication cycle starts with the attachment of the virion to specific host cell receptors. After attachment, the virion penetrates the host cell and uncoats its viral genome. Subsequently the viral genome is made available to the host's replication and translation machinery to induce the biosynthesis of new viral proteins and viral genomes. Next the assembly of new viral particles takes place which in turn results in the release and maturation of virulent viral particles (virions). These virions are able to infect other cells, allowing the virus multiplication cycle to repeat itself (Adapted from Dimmock, Easton, & Leppard, 2007).

Every organism known to man is infected by species-specific viruses due to the fact that viruses have naturally evolved to transfer their genes into host cells and ensure gene expression. Due to this property viruses are a subject of interest for gene therapeutic applications (Walther & Stein, 2000). Hence viruses are excellent blueprints for gene transfer vehicles, provided that the viral genome is modified in such a way that it can no longer induce viral disease. Since the first human gene therapy experiment in 1970, a lot of viral candidates have been evaluated for their use in gene therapeutic applications, such as adenovirus, adeno-associated virus, retrovirus, herpes virus, lentivirus etc. (Friedmann & Roblin, 1972; Goyvaerts et al., 2012). In 1983 the first viral vectors were developed by combining a transfer plasmid and packaging cell line providing the viral genome and proteins respectively (Mann, Mulligan, & Baltimore, 1983; Watanabe & Temin, 1983). In general, the transfer plasmid contains a partial viral genome in *cis* comprising a GOI next to viral sequences necessary for the packaging proteins to recognize the transcribed transfer plasmid as viral genome (Figure 4). The packaging proteins themselves are provided via the packaging plasmid or cell line in *trans*.

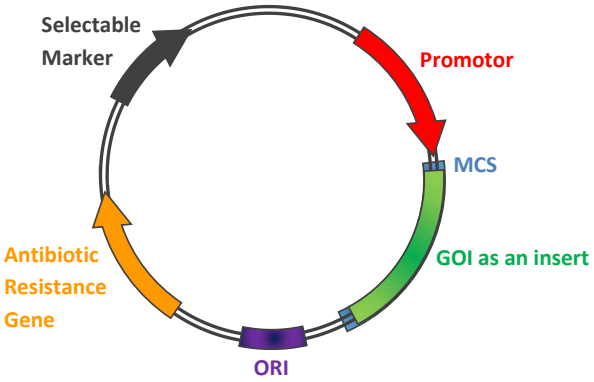

Figure 4: Basic representation of a plasmid.
An antibiotic resistance gene allows a transformed bacterium to survive in a antibiotic selective environnement. The promotor ensures the expression of the GOI cloned near the plasmid's MCS. The plasmid can be copied due to its Origin of Replication (ORI) which has to correspond with the ORI of the cell in which it resides. The selective marker allows a second selection of transfected (eukaryotic) cells to survive in an antibiotic selective environnement.

2.2 Development of lentiviral vectors

Lentiviruses are members of the *Retroviridae* and their genome consists of a diploid 10 kb + ssRNA strand with positive polarity. As such they are members of the 'sixth class' according to the Baltimore classification system (Dufait et al., 2012; Vogt, 1997). The 'lenti' prefix means slow in *Latin* and refers to the extent of time between initial infection and onset of disease, which can take up several months to years. The property of retroviruses to reverse transcribe their ssRNA genome into a ds proviral DNA strand is unique. Moreover this feature influences their mutation rate as the error rate of RNA synthesis is 10^5 times higher than the error rate of DNA synthesis (Berg, Tymoczko, & Stryer, 2002; Springgate, Battula, & Loeb, 1973). In addition the possibility of recombination, their replication rate, viral population size and selective forces accumulate their genetic variation (Svarovskaia, Cheslock, Zhang, Hu, & Pathak, 2003). This allows retroviruses to mutate rapidly and adapt to the host's immunity, develop resistance to therapy and cause variations in viral clades. The best-known lentivirus is HIV-I, which causes the acquired immunodeficiency syndrome (AIDS) and has claimed around 34 million lives up until 2014. Of note also other primates as well as non-primates can get infected by lentiviruses such as simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), PLV, equine infectious anemia virus (EIA), bovine immunodeficiency virus (BIV), caprine arthritis encephalitis virus (CAEV) and Visna virus.

A lentiviral virion is enveloped, spherical and measures 80 to 120 nm in diameter (Figure 5) Furthermore the envelope embeds viral glycoproteins that are also known as spikes and consist of two components. The first component is the surface glycoprotein (SU), which interacts with the host's cell membrane receptors and as such determines the tropism or host cell specificity of the virion.

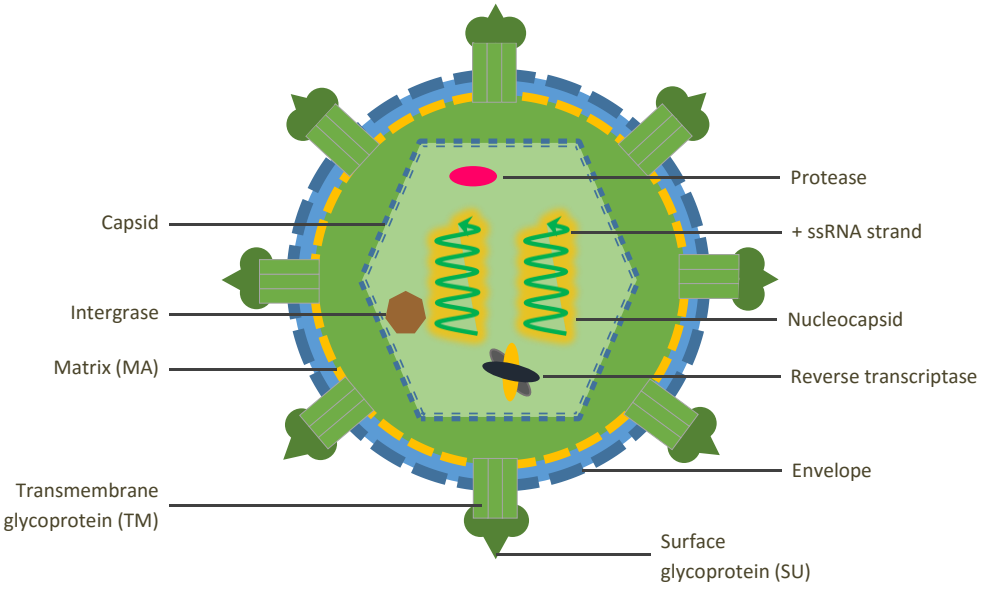

Figure 5: Visual representation of a lentivirus.
The viral genome is protected by the nucleocapsid, which is together with viral proteins such as reverse transcriptase, protease and integrase surrounded by the capsid. The capsid consists of capsid proteins and is surrounded by the matrix and the envelope. The latter is on his turn encircled by an envelope which is derived from the host's cell membrane and embeds target cell binding glycoproteins.

The second component is the transmembranary glycoprotein (TM), which is attached to the non-glycosylated structural matrix (MA) proteins and *via* a disulphide bridge to the SU. Both components are encoded by the *Env* Open Reading Frame (ORF, Figure 6). Within the envelope the capsid contains RT, integrase and protease enzymes. The former two are transcribed and translated from the *Pol* gene while the latter is encoded by the *Pro* gene. The capsid proteins, matrix proteins and the genome enclosing nucleocapsid proteins are encoded by the *Gag* gene. Lentiviruses are also called complex retroviruses because besides their *Pol, Gag* and *Env* gene, they also encode two regulatory (*Tat* and *Rev)* and four accessory proteins *(Vif*, *Vpr*, *Vpu* and *Nef)* (Figure 6).

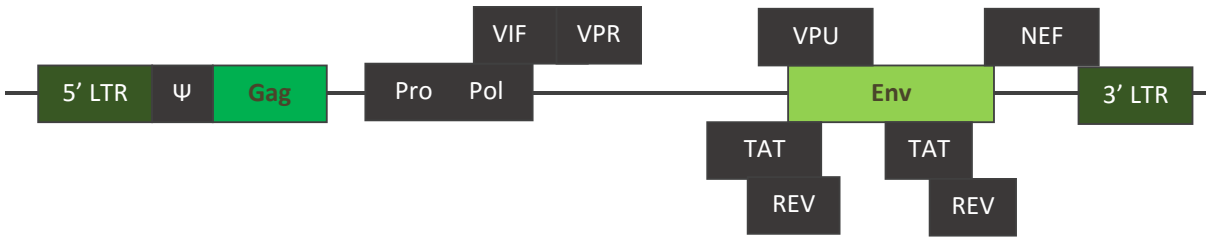

Figure 6: Representation of the integrated lentiviral genome.
The reverse transcribed lentiviral genome (ds DNA) is flanked by two identical Long Terminal Repeats (LTR's) which activate the viral genomic RNA transcription. Ψ represents the psi (Ψ)-packaging recognition site. *Pro, pol, env, and gag* are genes which are necessary for the assembly of viral particles. V*if*, *vpr*, *vpu* and *nef* are four accesory genes and (*tat* and *rev*) are two regulatory genes (Adapted from Addgene, 2015).

Lentivirus derived vectors are called lentiviral vectors (LVs) and confer numerous advantages compared to both viral and non-viral gene transfer vectors. A first benefit is their large genetic capacity, which they integrate in the host cell genome. As such they enable stable transfer of the GOI. Secondly, they can be pseudotyped or in other words their envelope glycoproteins can be changed (Cronin et al., 2005). As such their tropism can be altered. Another benefit of LVs is that the lentiviruses from which they are derived, have no pre-existing immunogenicity in contrast to the adenoviral- and adeno-associated viral vectors (Addgene, 2015). Hence LV transduced cells won't be cleared immediately by the immune system of the patient. Finally, LVs are, in contrast to their retroviral counterparts, able to infect both dividing and non-dividing cells. Thus lentiviral vectors are suitable candidates for gene therapeutic applications.

In order to generate a replication deficient and safe lentivirus derived vector, their pathogenic features needed to be deleted. Therefore a lot of effort has been put in the generation of recombinant and safe LVs. In order to prevent replication of a complete viral genome, the latter has been scattered across different plasmids. While the genes required for viral assembly and infectivity are expressed in *trans*, only the genes that need to be encoded by the LV itself act in *cis*.

In general LVs are produced by transient transfection of HEK293T cells with these *cis*- and *trans*-acting plasmids. Since the HEK293T cells are derived from human cells the possibility of RCL formation increases during LV production. This RCL formation can occur through homologous recombination of the different transfected plasmids with the retroviral mobile sequences (Pauwels et al., 2009). Based on risk for RCL formation during LV production as well as insertional mutagenesis during LV infection, several safety measures have been undertaken that resulted in the development of several LV generations. A visual representation of the production and transduction of IDLVs, 2nd gen and 3rd gen LVs is represented on Figure 7.

For the production of first gen LVs, transfection of a *trans*-acting helper cell line with only one *cis*-acting plasmid or one *cis*-acting plasmid and one *trans*-acting plasmid is enough to generate LVs (Watanabe & Temin, 1983). The *trans*-acting helper cell line encodes the *Env*, *Gag* and *Pol* genes to complement the replication deficient lentiviral genome on the *cis*-acting plasmid whilst producing replication deficient virions. However the possibility of recombination into RCLs remains and as such holds a major safety risk.

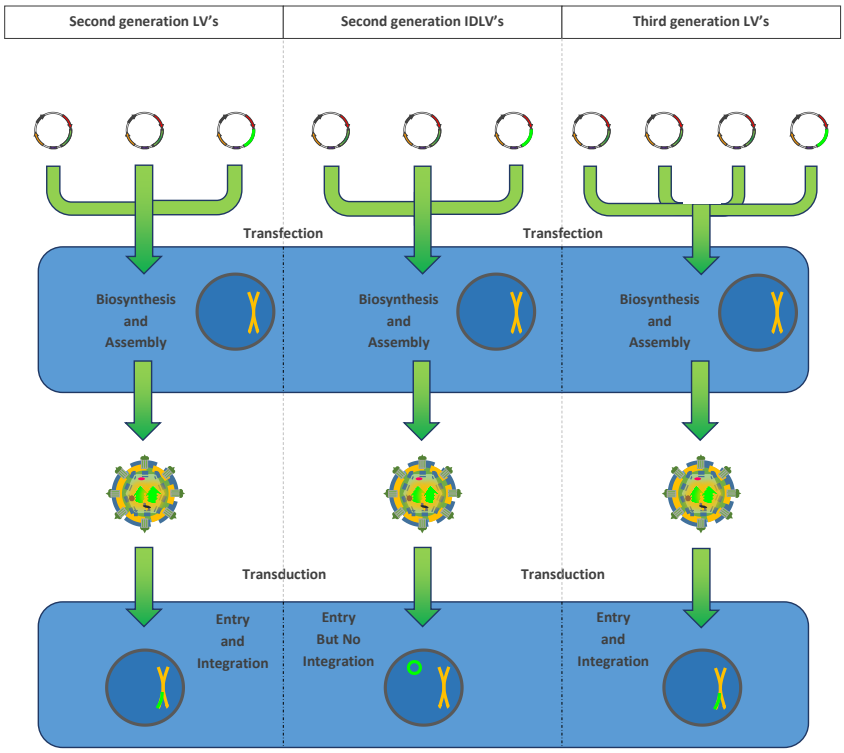

Figure 7: Overview of the production and transduction of IDLVs, 2nd and 3rd generation LVs.
HEK293T cells are transfected with the required plasmids in order to produce LVs. Subsequently these LVs can transduce their respective target cells. Subsequently, their genome (encoded by the transfer plasmid) is reverse transcribed into a provirus and shuttled into the target cell's nucleus. Depending on the type of LV, the proviral sequence integrates randomly or not at all (for IDLVs) into the target cell's genome.

Therefore all components necessary for the production of second gen LVs, are scattered over three plasmids (Figure 8). Furthermore the virulence genes *vif*, *vpr*, *vpu* and *nef* are removed from the vector system as they are dispensable for LV production (Seelamgari et al., 2004). Furthermore the *trans*-acting envelope and packaging plasmids carry the *Env* and *Gag*, *Pol*, *Rev* and *Tat* genes respectively. The *Rev* gene produces a protein that binds to the RRE on the transfer plasmid and as such facilitates nuclear export of the GOI after integration into the host's genome. The Tat protein triggers the Trans Activator Region (TAR) within the LTRs of the proviral genome to activate expression.

The *cis*-acting transfer plasmid contains the GOI, central polypurine tract (cPPT) and the RRE. All these elements are flanked by long terminal repeats (LTRs) in order for the plasmid to be integrated into the host's genome. To further improve safety, the 3' LTR has been altered via a deletion in the sequence which renders the transfer vector replication incompetent and results in self-inactivation (SIN) after integration into the host's genome (Zufferey et al., 1998). On the one hand, GOI expression is still driven by the 5' LTR promotor via the presence of the *TAR* region. On the other hand an internal constitutive or tissue-specific promotor can be present to modulate the expression pattern of the GOI.

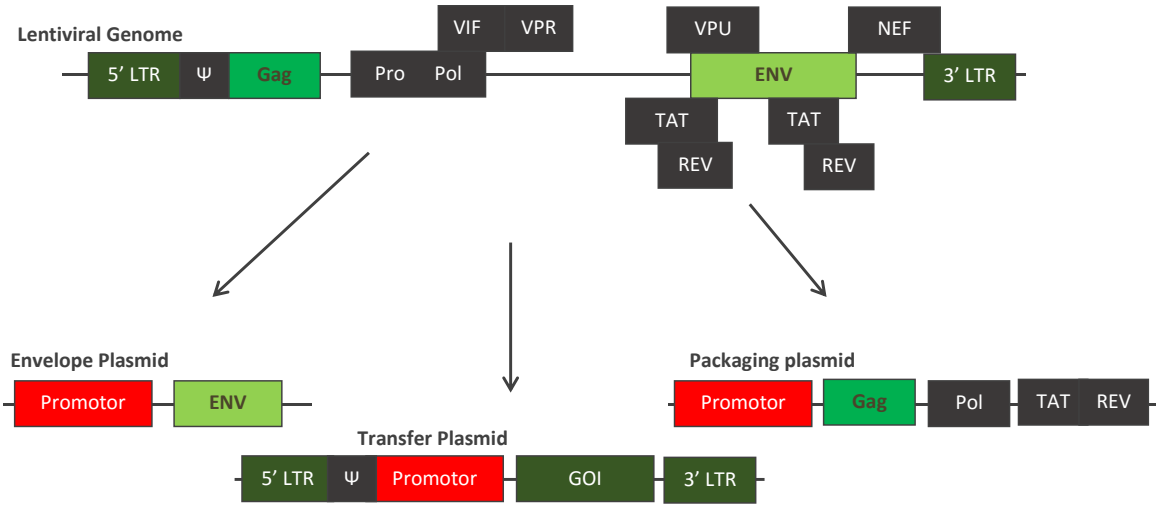

Figure 8: Second generation LV encoding plasmids

The envelope plasmid encodes all genes for the production of the envelope embedded glycoproteins. Further the packaging plasmid contains all the genes for the production of all other structural and enzymatic proteins while the transfer plasmid contains two truncated LTR precursors and encodes the Gene of Interest (GOI).

On the other hand an internal promotor can be present to modulate the expression pattern of the GOI. The Ψ gene is the RNA target site for packaging by the nucleocapsid, which ensures that a virion contains the transfer plasmid encoded LV genome. Unique for LVs is the cPPT which functions as a recognition site for proviral DNA synthesis and subsequent nuclear import of retro-transcribed proviral DNA in non-dividing cells (Durand & Cimarelli, 2011).

Although the second gen LVs had been customized to become much safer LVs with a reduced RCL formation risk, they had no reduced chance of inducing insertional mutagenesis. Therefore, second gen IDLVs have been developed as well. They differ from the normal second gen LVs in the *Pol* gene encoding part of the packaging plasmid in the sense that the integrase encoding part is truncated or mutated in such a way that it can no longer mediate integration of the retro-transcribed provirus after infection. Instead, it forms a circular episomal DNA, that doesn't replicate along with the host cell. However, support episomal replication sites such as the scaffold matrix attachment regions (S/MAR) or crafted artificial chromosomes can allow its replication and as such enable the prolonged expression of the GOI in dividing cells (Argyros, Wong, & Harbottle, 2011). If not incorporated, IDLVs are useful tools for transient transduction of dividing cells and stable transduction of non-dividing cells.

To further reduce the risk of RCL formation, third gen LVs scattered the viral genome over four plasmids with reduced homology as depicted in Figure 9. Concrete, the viral packaging system is separated over two plasmids encoding the *Rev* gene or the *Gag* and *Pol* genes respectively. When third gen LVs are produced, the *Gag/Pol* and transfer plasmid require Rev for nuclear export of their RNA transcripts. Within the vector genome (encoded by the transfer plasmid), the RRE is located between splice donor and acceptor sites to ensure that only unspliced (full-length) transcripts are exported into the cytoplasm and packaged into vector particles. The *Gag/Pol* transcript also requires the RRE for its nuclear export, which would otherwise be inhibited due to RNA secondary structures. Once in the cytosol, G*ag/Pol* is translated, yielding the vector structural and enzymatic proteins (Tareen et al., 2013). Furthermore, the *Tat* gene is removed and expression is regulated by a constitutive promotor such as the promotor derived from the cytomegalovirus (CMV). The transfer plasmid further contains, in comparison to the second gen LVs, an altered 5' LTR which is fused with a heterologous promotor resulting in *Tat* independent expression of the GOI.

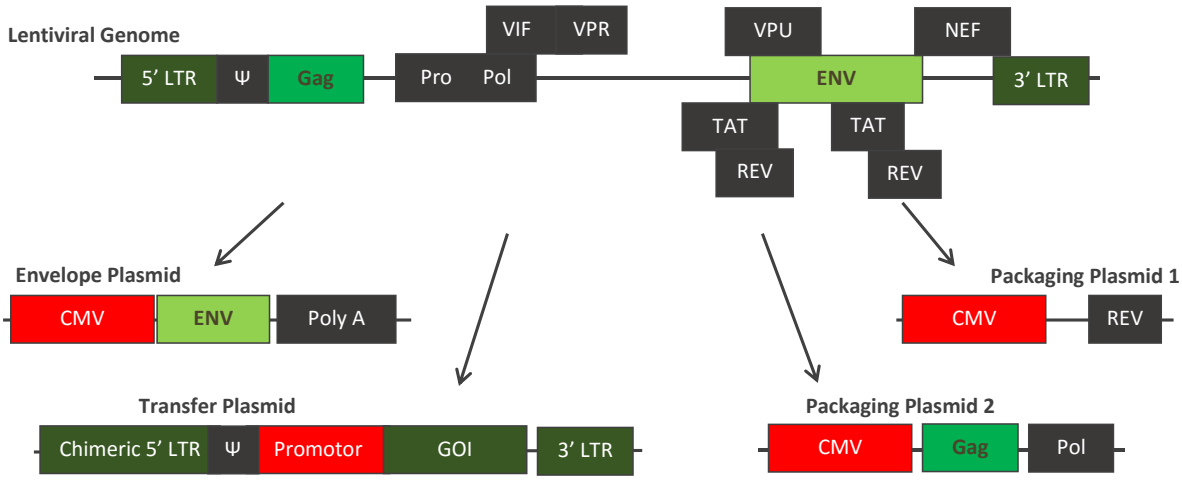

Figure 9: Third generation LV encoding plasmids
The envelope plasmid encodes all genes for the production of the envelope embedded glycoproteins. Further the packaging plasmid is squatterd over two different plasmid encoding Rev or all genes for the production of all structural and enzymatic proteins within the envelope respectively. Finally, the transfer plasmid contains truncated and chimeric LTRs precursors and encodes one ore more GOIs.

2.3 Applications

Today, most applications with LVs are conducted in the field of fundamental genetic research. Nonetheless, until now 114 clinical trials with LVs have been initiated, comprising five percent of all vectors used in gene therapy trials (Figure 10, B). Up to 46 % of all LV trials concern cancer diseases, thus LVs are mostly used in the struggle against cancer. The second most represented disease category involving LVs is represented by monogenic diseases. These diseases are the result from modifications in a single gene occurring in all cells of the body causing pathological conditions. Typical examples hereof are the inherited blood disorders such as beta thalassemia, sickle cell anemia and hemophilia next to severe combined immunodeficiency syndrome and cystic fibrosis. The third most represented disease category are the infectious diseases such as HIV-I (Figure 10, A).

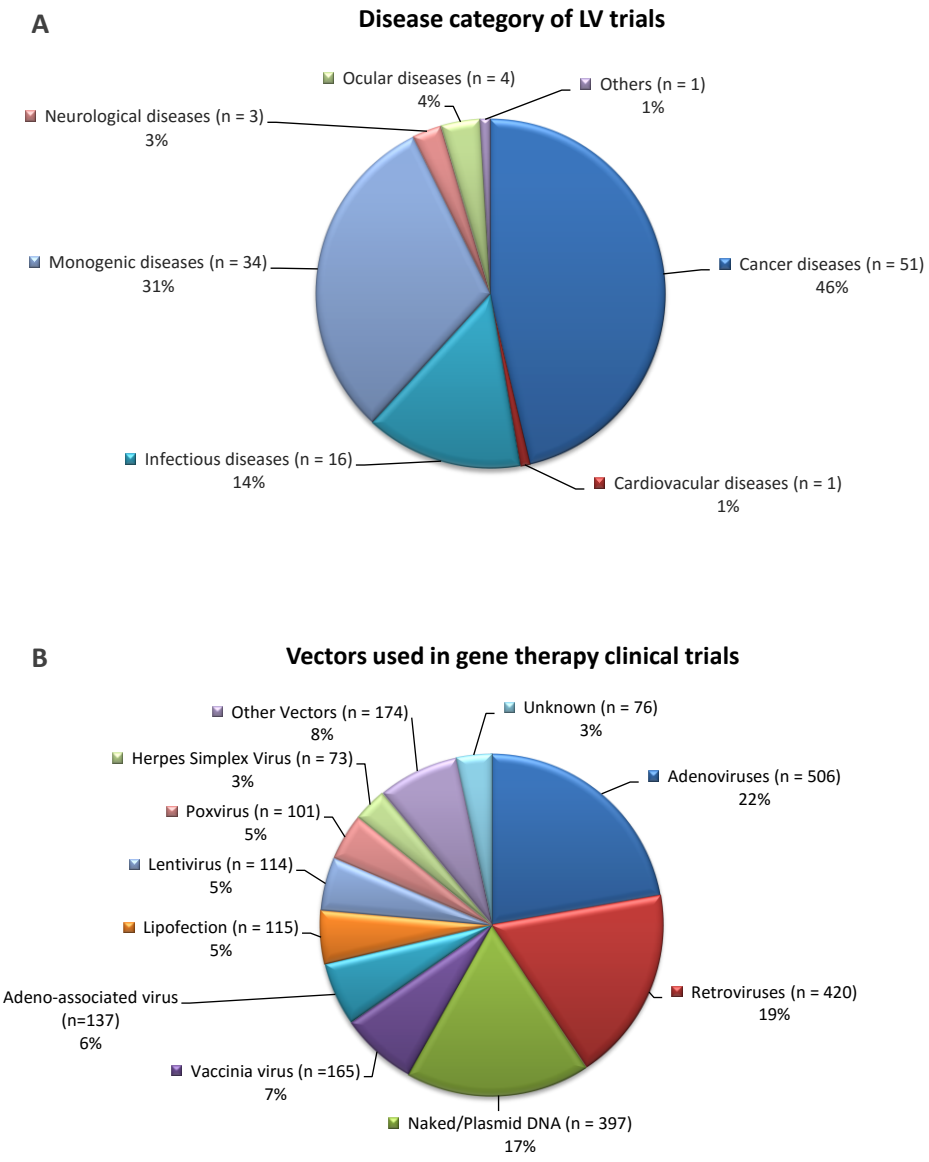

Figure 10:(A) Disease categories of LV trials and (B) Vectors used in gene therapy clinical trials. All data concerning the graphics are obtained from "Wiley Online Library," 2015, the trials used in B are all open, ongoing trials which involve the use of LVs.

Lentiviral vectors have a multifaceted potential in the field of oncology. First, LVs allow targeting and destruction of tumor tissue directly by transducing tumor cells with LVs encoding a pro-apoptotic molecule or suicide gene which is able to convert a harmless prodrug into a cytotoxic agent (Emeagi et al., 2012; Lumniczky & Sáfrány, 2006). Secondly, the tumor microenvironment (TME) could also be targeted. This TME consists of a variety of tumor growth promoting cells such as tumor-associated dendritic cells (TADCs) and tumor-associated macrophages (TAMs) which help the tumor to thrive through suppression of the immune system and stimulation of angiogenesis (Movahedi et al., 2012). Subsequently, also these cells could be targeted by LVs, an experimental set up which is currently tested at the LMCT. Thirdly, LVs have been extensively tested for their potential to stimulate antitumor immunity whereby the patient's own immune system is prompted to attack the tumor (Adachi & Tamada, 2015). Therefore two main strategies have been developed namely an 'active' and a 'passive' strategy. For the active one, dendritic cells (DCs) are stimulated in such a way that they actively induce a cytotoxic T cell response *in vivo* towards the tumor cells. Previously, DCs were isolated from the patient, loaded with tumor associated antigens with extra DC-stimulating factors and subsequently reinfused into the patients' body (Bonehill et al., 2009). An example of such stimulated DCs with LVs are "self-differentiated myeloid derived antigen presenting cells reactive against tumors" or "SMART-DCs" (Pincha et al., 2012). Concrete these DCs are transduced overnight *ex vivo* with tricistronic LVs co-expressing two cytokines (granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin IL-4) as well as a melanoma associated antigen. Recently, it became clear however that isolation, manipulation and reinfusion of patient specific DCs is a very labor-intensive, costly and therefore cumbersome procedure. Therefore research shifted towards the *in vivo* modification of DCs, a field in which the LVs have also contributed to a large extent (Goyvaerts et al., 2014; Odegard et al., 2015).

The main aim of passive antitumor immunotherapy is to enhance an existing anti-tumor response. The strategies that are used to accomplish this include the use of monoclonal antibodies, immune-boosting cytokines and adoptively transferred tumor specific T lymphocytes. The latter are made tumor specific by genetic modification of autologous (patient-derived) T lymphocytes with a gene encoding a T cell receptor specific for a tumor associated antigen (Figure 12). For the latter, LVs have been used comprehensively to transduce and modulate patient derived T cells into tumor cell specific cytotoxic T cells (pathway β, Figure 12).

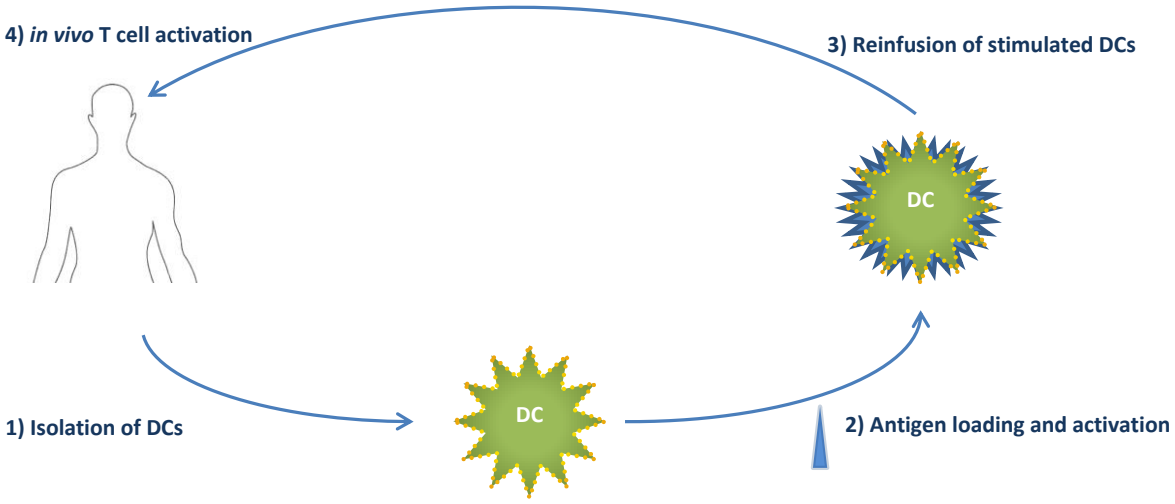

Figure 11: Diagrammatic overview of active tumor vaccination.
At first, DCs (green twelve point star) are isolated from the subject who's about to undergo tumor vaccination. Next the isolated DCs are stimulated and loaded with tumor associated antigens (blue triangle). Subsequently, the stimulated DCs are reinfused into the patient where they will finally activate cytotoxic T cells *in vivo* in order to elicit an immune response against the tumor. Adapted from (Eggermont et al., 2014)

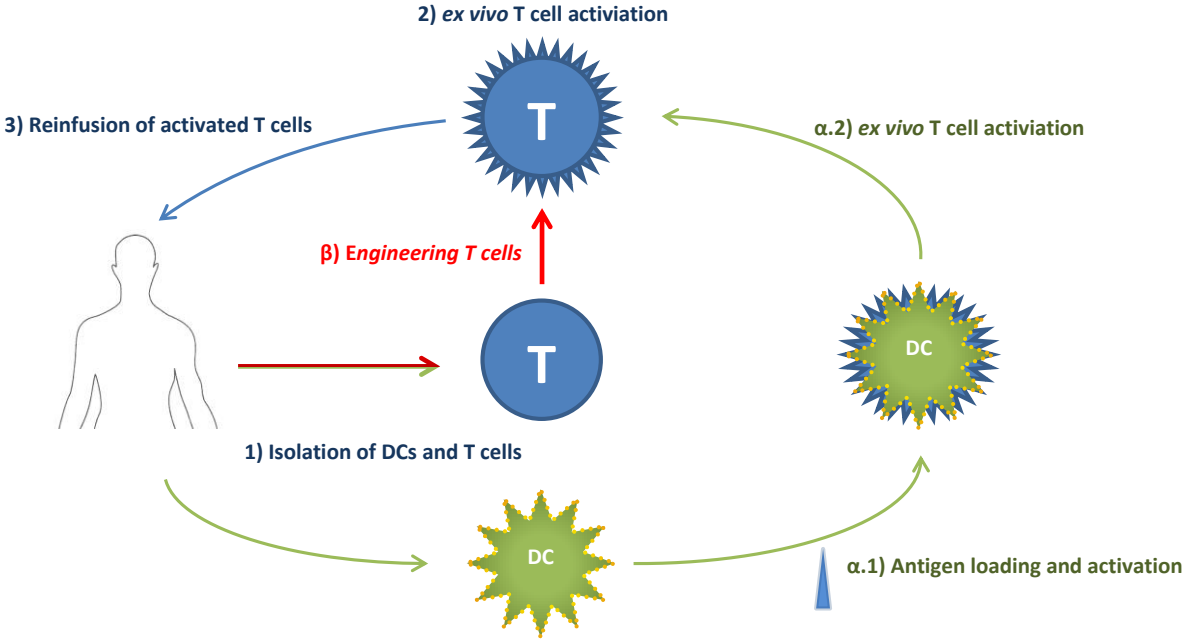

Figure 12: Diagrammatic overview of passive tumor vaccination.
At first, DCs (green twelve point star) and T cells (blue sphere) are isolated from the subject who is about to undergo adoptive T cell transfer. Next the isolated T cells could be activated in two different ways. The first one is through *ex vivo* activation of cytotoxic T cells by stimulated and peptide loaded DCs according to the α pathway (green arrows). The second one is through *ex vivo* activation of cytotoxic T cells by engineering T cells through transgenic delivery of T cell receptors according to the β pathway (red arrows). Subsequently, the activated T cells are reinfused into the subject where they will finally elicit an immune response against the tumor. Adapted from (Eggermont, Paulis, Tel, & Figdor, 2014)

To conclude LVs are currently extensively used in different fields of fundamental as well as clinical research. Due to their putative safety risks however, there use does not correlate with their application potential yet. Therefore, it is important that research keeps on searching for safer and more effective ways to generate LVs and as such pave the way towards a more extensive use of LVs for the treatment of all pathologies for which they could make the difference.

3 Experiments

3.1 Cultivation of HEK293T cells

3.1.1 Context of HEK293T cells

Human Embryonic Kidney or HEK293T cells are an immortalized cell line that is frequently used for retroviral generation, gene expression and protein production. The 293T cell line is a highly transfectable derivative of the 293 cell line into which the SV40 T-antigen was inserted, hence the T in 293T. The expression of the SV 40 large T antigen allows the replication of plasmids which bear an SV40 ORI within mammalian cells (Mahon, 2011). The HEK293T cells have an epithelial morphology and are adherent thus well attached to a substrate, which results in very few cells floating in suspension. In this thesis we used the HEK293T cell line to produce LVs, determine the amount of transducing units in each concentrated LV stock and monitor their transgene expression course over time upon LV transduction. The cultivation of this cell line was performed as described in the American Type Culture Collection (ATCC) protocol, which asserts the cells should be split every other day in order to sustain a logarithmic growth curve and expand their number (American Type Culture Collection (ATCC), 2014).

3.1.2 Thawing

Frozen HEK293T cells originating from the ATCC were stored in liquid nitrogen (-196°C) at a concentration of $5x10^6$ $cells/_{ml}$ freeze medium (fetal bovine serum or FBS, Merck Millipore with 10% dimethylsulfoxide or DMSO, Acros Organics). On a monthly basis one vial was thawed by placing it at 37°C for one minute. Next the thawed cells were transferred to a 15 ml falcon with 9 ml OptiMem (Gibco) and centrifuged at 1500 rates per minute (rpm) for 5 minutes. Next supernatant was removed, the pellet resuspended in 20 ml prewarmed DMEM+ (87,5% DMEM, Sigma with 10% FBS and 2,5% penicillin, streptomycin and L-glutamine or PS-L-Glu) and transferred to a T175 culture flask (Falcon) stored in an incubator at 37°C and 5%CO_2.

3.1.3 Sustainment

Attached HEK293T cells were split every other day in order to sustain a logarithmic growth curve and expand their number. First the cell containing T175 flasks were washed by removing the DMEM+, adding 5 ml Dulbecco's Phosphate Buffered Saline (DPBS, Sigma) and removing the DPBS again. Next 3 ml of trypsin-EDTA (Sigma) was added to the flasks. After five minutes of incubation, the detached cells could be transferred to a DMEM+ containing 15 or 50 ml falcon. After centrifugation (5 min, 1500 rpm), the pellet was resuspended in DMEM+, the cells were counted and according to the purpose, plated at a certain number into new T 175 culture flasks and maintained in the incubator.

3.1.4 Cryopreservation

In order to replenish the HEK293T cell stock in the liquid nitrogen tank, HEK293T cells were resuspended at $5x10^6$ $cells/_{ml}$ of freeze medium. Next, this suspension was aliquoted over cryovials of 1 ml which were then transferred into a Mr. Frosty® Cryo 1°C Freezing Container (Nalgene®) that was transferred on his turn to the -80°C freezer. After two days the vials from the freezing container were moved to the liquid nitrogen storage at -196°C.

3.2 Cloning of third generation transfer plasmids

3.2.1 Context of cloning

The cloning process comprises two main parts (Figure 13). The first part comprises the digestion of two different plasmids to obtain an empty vector and a GOI sequence respectively. The digestion is conducted with restriction enzymes that create sticky or blunt ends at particular restriction sites. The digestion to obtain the empty transfer plasmid (vector) is performed with the same restriction enzymes as those used to obtain the GOI sequence (insert). As such the vector plasmid obtains sticky ends that overlap with those of the digested GOI. The second part of the cloning process includes the ligation of the digested GOI into the vector. This ligation process requires a ligase protein in order to attach the sticky ends of the GOI to those of the accessible vector. This ultimately leads to the creation of a new transfer plasmid encoding the preferred GOI.

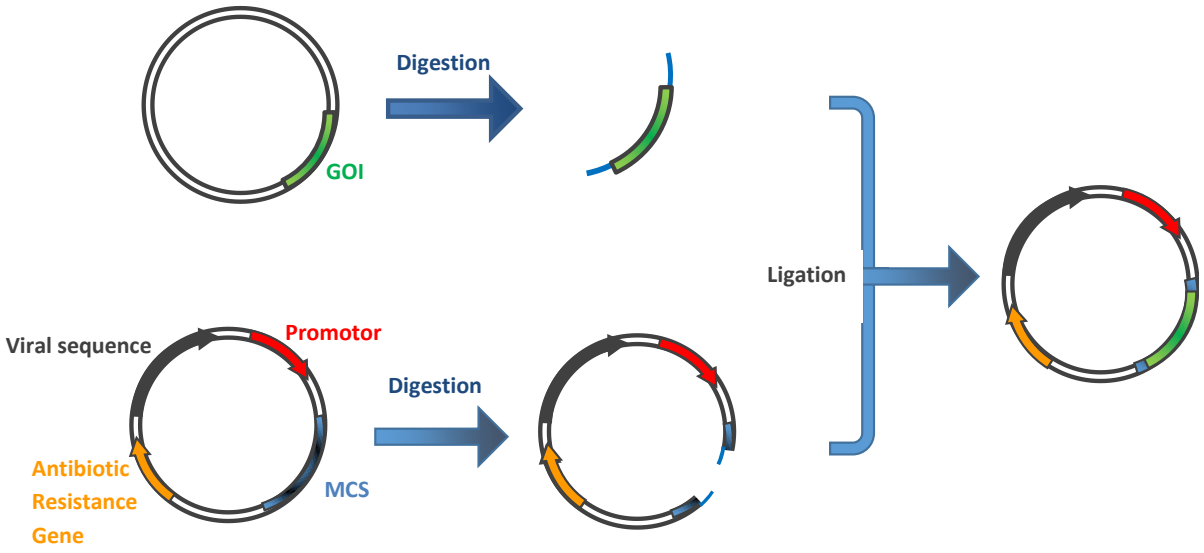

Figure 13: Representation of the entire cloning process.
The entire cloning process comprises the digestion of start plasmids and the ligation of the resulting digested products into a new plasmid.

3.2.2 Digestion

In order to obtain third gen LV transfer plasmids encoding one or more GOIs, we used an empty pLenti-puro vector (Plasmid Map 4, Addgene plasmid # 39481, Guan, Wang, & Shih, 2011) and three different GOIs as inserts namely: (1) enhanced green fluorescent protein (eGFP), (2) glycoprotein 100 (gp100) and (3) ovalbumin (OVA) and truncated nerve growth factor receptor (tNGFR) linked with an internal ribosomal entry site (IRES). While the former two were obtained by digesting the pST1-eGFP (Plasmid Map 3) and pST1-gp100-DCL.OPT (Plasmid Map 1) plasmids respectively with the restriction

enzymes *Xho*I and *Spe*I (Fermentas), the latter was derived from pHR'-huli80-tOVA-IRES-tNGFR (Plasmid Map 2) via a *Xba*I restriction (Fermentas). The digestions were performed with restriction enzymes in such a way that the sticky ends of the digested GOIs overlapped with the sticky ends of the digested empty transfer vector. Therefore pLenti-puro was also digested with *Xho*I/*Spe*I or *Xba*I to allow the respective ligation of eGFP, gp100 or OVA-IRES-tNGFR. The samples were prepared according to Table 1 and Table 2 with buffers G and Tango respectively (Fermentas). After assembly of the digestion mixes the samples were incubated at 37°C for two hours. To prevent self-ligation of the pLenti-puro plasmid digested with *Xba*I, 1 µl of Shrimp Alkaline Phosphatase (SAP, Thermo Fisher Scientific) was added to the sample and incubated at 37°C for another 15 minutes.

3.2.3 Separation of digested fragments through electrophoresis

After melting of a 1,2% Low Melting Point (LMP, Lonza) gel and the addition of 1 µl of nucleic acid staining solution Midori Green, the liquefied gel was casted with a wedge and put at 4°C to speed up congelation. Once the gel was hardened, it was transferred to the electrophoresis appliance and submerged with 1 X Tris-borate-EDTA (TBE) buffer. Next, 2 µl of loading dye was added to 20 µl of each digestion mix after which they were loaded onto the gel. Furthermore one well was filled with 5 µl of a 1 kb DNA ladder (Genescript, Thermo Fisher Scientific). The gel electrophoresis appliance (Labnet) was set to run for 40 minutes at 100 V. Finally the gel was analyzed under ultraviolet light.

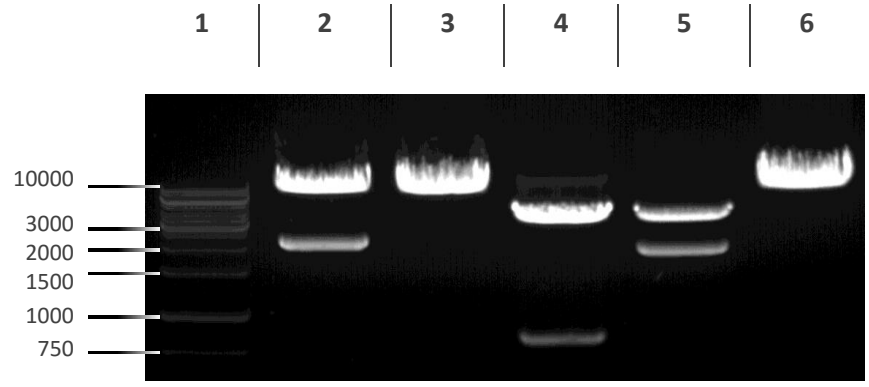

Figure 14: Digestion of 3rd and 2nd generation LV transfer plasmid.
One kb DNA ladder (1). pHR'-huli 80-tOVA digested by *Xba*I (2). pLenti-puro digested by *Xba*I (3). pST1-eGFP digested by *Xho*I and *Spe*I (4). pST1-gp100-DCL-OPT digested by *Xho*I and *Spe*I (5). pLenti-puro digested by *Xho*I and *Spe*I (6) on a 1,2% LMP gel

Figure 14 shows that the digestions of pHR'-huli80-tOVA-IRES-tNGFR by *Xba*I, or pST1-eGFP and pST1-gp100-DCL.OPT by *Xho*I and *Spe*I, all resulted in two DNA fragments of which the 'smallest' DNA fragment represents the fragment or GOI. For the fragments encoding tOVA-I-tNGFR (lane 2), eGFP (lane 4) and gp100-DCL (lane 5) we could furthermore demonstrate that the digestion resulted in fragments of the expected sizes namely 2500 bp, 750 bp or 2100 bp respectively (Figure 14). The digestion of pLenti-puro by *Xba*I or *Xho*I and *Spe*I resulted in the linearization of the plasmid and as such in only one fragment of about 7067 or 7013 bp respectively (Figure 14, lanes 3 and 6).

3.2.4 DNA purification

To purify the desired DNA fragments we applied the GeneJET Gel Extraction Kit (Thermo Fischer Scientific®, Catalog number: K0691). Therefore each gel slice containing the digested linearized vector or GOI was excised with a scalpel and transferred to a micro centrifuge tube with 200 µl binding buffer. These mixtures were incubated for ten minutes on a heat block set at 55°C until the gel slices completely dissolved. Next the solutions were transferred to GeneJET purification columns and centrifuged at 14000 rpm for 1 minute. The flow through was discarded, 700 µl of wash buffer was added after which the purification columns were centrifuged again at 14000 rpm for one minute and an additional minute in order to ensure the complete removal of any residual wash buffer. Finally the GeneJET purification columns were transferred to new micro centrifuge tubes and centrifuged for 2 min at 8000 rpm with 50 µl of elution buffer. After centrifugation, the GeneJET purification columns were discarded and the purified DNA fragments stored at -20°C.

3.2.5 Ligation and transformation

Ligations were performed by assembling three µl 'ligation mix with vector' (one µl T4 ligation buffer, one µl T4 ligase and one µl of the corresponding vector) with insert and Milli-Q at different ratios as depicted in Table 3. After assembly, the ligation mixes were incubated for 1 hour at room temperature to achieve ligation as depicted in Figure 13. Subsequently the TransformAid Bacterial Transformation Kit (Thermo Fischer Scientific®, Catalog number: K2710) was used to generate competent XL1 blue bacteria and transform the ligated 3rd gen transfer plasmids in these bacteria. The XL1 blue bacteria were cultivated in a shaker at 37°C overnight in five ml C-medium the day before transformation. On the day of transformation, the overnight culture was diluted tenfold with C-medium and incubated for 30 minutes at 37°C in a shaker.

After incubation, the culture was divided over micro centrifuge tubes, centrifuged at 1100 rpm for one minute and subsequently the pellets were resuspended in 300 µl assembled T buffer which consisted of a 50:50 ratio of T buffer A and T buffer B. The tubes were centrifuged again at 1100 rpm for one minute, the pellet was resuspended in 120 µl of assembled T buffer and the culture was then kept on ice for five minutes. Next, cultures were aliquoted over micro centrifuge tubes with 50 µl of culture in each tube. To each tube, five µl of ligation mix was added. After five minutes incubation on ice, the cultures were complemented with 200 µl LB-medium and incubated at 37°C in a shaker for 30 minutes. Finally, all cultures were plated on pre-warmed ampicillin agar plates which were incubated overnight at 37°C. The next day colonies were selected, cultivated in five ml LB with ampicillin (50 $^{\mu g}/_{ml}$) at 37°C in a shaker overnight and the GeneJET Plasmid Miniprep Kit® (Thermo Fischer Scientific®, Catalog number: K0503) was used to isolate their DNA.

3.2.6 Test digestion

In order to evaluate that the ligation of the GOI in the corresponding transfer vector occurred correctly, we performed test digestions on eight selected colonies/ligation as depicted in Tables 4 and 5. The assembled digestion samples were incubated for two hours in a hot water bath at 37°C. After incubation, the digested fragments were visualized with a 1,2% (for pLenti-eGFP & pLenti-gp100-DCL-OPT) or a 1% (pLenti-OVA-I-tNGFR) agarose gel (Invitrogen) with ethidium bromide (Apex) and a one kb DNA ladder (Genescript, Thermo Fisher Scientific). The gel was run at 100 V for 20 minutes and analyzed under ultraviolet light. The eight minipreps of the pLenti-gp100-DCL.OPT (Plasmid Map 6) and of the pLenti-eGFP (Plasmid Map 7) transfer plasmids were digested by *Spe*I and *Xba*I. If ligated correctly, this resulted in a fragment of 1900 bp and 717 bp respectively. As depicted in Figure 15, A, this was the case for the minipreps of the pLentigp100-DCL.OPT colonies 1-4, 6 and 7 loaded in lanes 2-5, 7 and 8 respectively. Also for colonies 1-7 of the pLenti-eGFP ligation a fragment

of 717 bp was demonstrated as depicted in lanes 2-8 (Figure 15, C). Finally, the eight minipreps containing pLenti-OVA-I-tNGFR (Plasmid Map 5) were digested by *Eco*RI which should have resulted in a fragment of 750 bp. As shown in Figure 15, B, this was the case for colonies number 1, 3, 5 and 6 loaded in lanes 2, 3, 5 and 6.

Based on these test digestions, one bacterial clone was selected and cultivated for each transfer plasmid in 500 ml LB with ampicillin (50 $^{\mu g}/_{ml}$). The multiplied plasmid DNA was isolated through the use of the NucleoBond® Xtra Midi EF kit (Macherey-Nagel®).

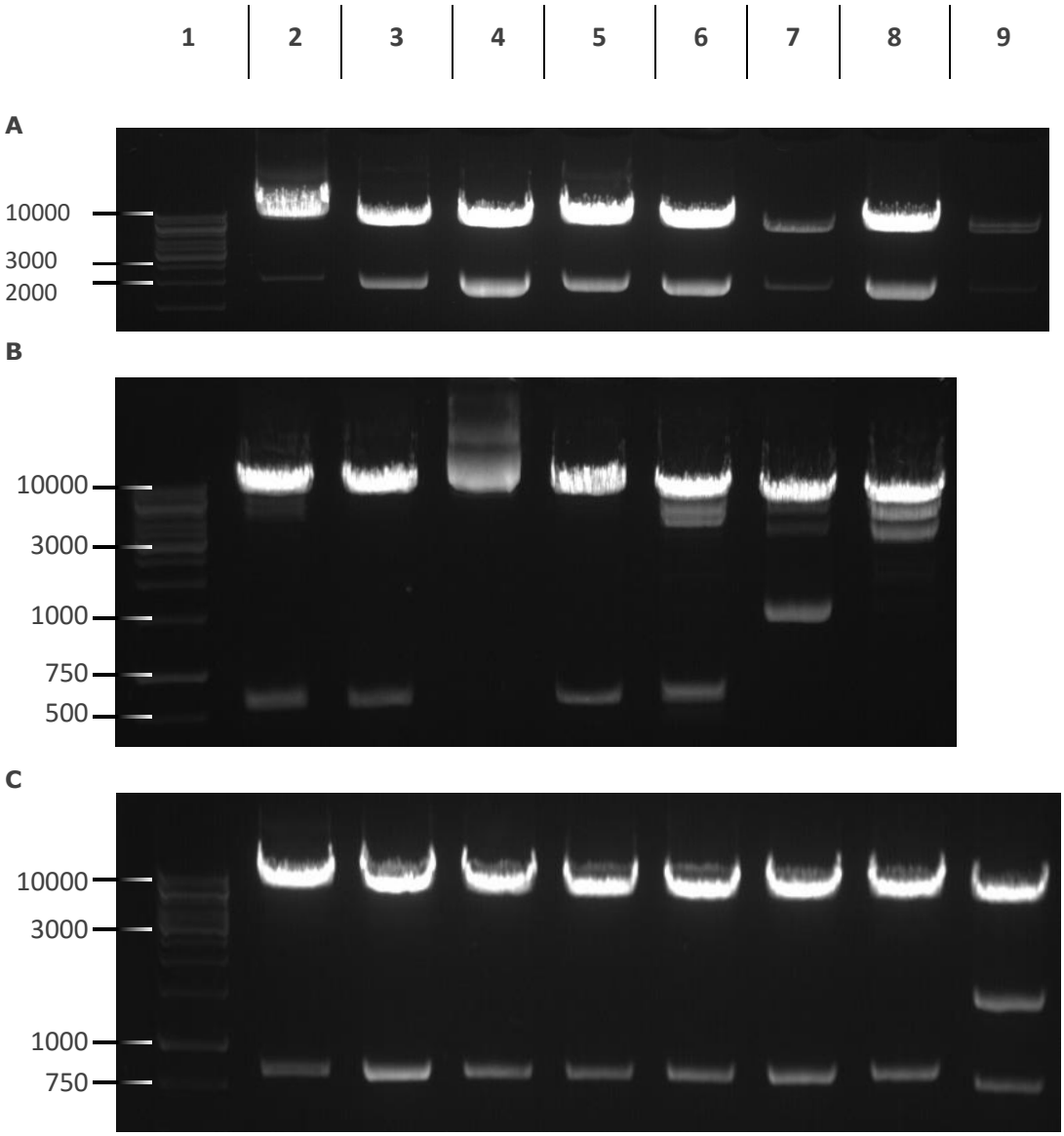

Figure 15: Overview of all test digestions.
(A) pLenti gp 100 DCL-OPT (2 → 9) digested by *Spe*I and *Xba*I on a 1,2 % agarose gel with a one kb DNA ladder (1). (B) pLenti OVA-I-tNGFR (2 ➔ 8) digested by *Eco*RI on a 1,0 % agarose gel with a one kb DNA ladder (1). (C) pLenti-eGFP (2 ➔ 8) digested by *Spe*I and *Xba*I on a 1,2 % agarose gel with a one kbDNA ladder (1)

3.3 Optimization of lentiviral-production

3.3.1 Context

In order to compare the generation of 2nd gen LVs, 2nd gen IDLVs and 3rd gen LVs, we started with the optimization of their production. While the production protocol for 2nd gen LVs was already optimized in the host laboratory in the past, we started by testing different ratios of the envelope, packaging and transfer plasmid for the generation of 2nd gen IDLVs and 3rd gen LVs as described below.

3.3.2 Transfection

HEK293T cells were plated the day before at $12x10^6$ cells per T175 flask. Further, the necessary amount of each plasmid was determined, precipitated in ethanol and resuspended in milli-Q to a concentration of 1 $^{\mu g}/_{\mu l}$. On the day of transfection, each transfection mix was made by adding one volume of OptiMem with polyethyleneimine (PEI, 1 $^{mg}/_{ml}$, Polysciences Inc.) to one volume of OptiMem with the required plasmids as described in Table 6, Table 7 & Table 8. After 30 minutes of incubation at room temperature, 10 ml transfection mix was transferred to the corresponding T175 flask from which the medium was removed. After a four hour incubation period at 37°C and 5%CO_2, the transfection mixes were replaced by 15 ml DMEM+. Two and three days later, the LV containing medium was harvested and centrifuged at 3000 rpm for three minutes after which the LV containing supernatant was stored at -80°C.

3.3.3 Determination of transfection efficiency

On day three, +/- 10^6 LV producer cells were collected, transferred to a FACS-tube with 1 ml of PBS/BSA/azide solution and centrifuged for three minutes at 2800 rpm. Next, the pellet was resuspended in 500 µl PBS/BSA/azide and the transfection efficiency was analyzed via flow cytometry (LSR FORTESSA, Becton Dickinson). For the cells that produced eGFP encoding LVs, eGFP expression was evaluated as depicted in Figure 16D to F. For the samples encoding OVA-I-tNGFR, cells were prestained with an anti-tNGFR (CD271) antibody linked to allophycocyanin (APC, Biolegend) protected from light at 4°C for 20 minutes. After incubation and an additional wash step, tNGFR expression was analyzed in flow cytometry (Figure 16G to I). As depicted in Figure 16D to I and Figure 17A, the transfection efficiency was similar for IDLVs and 3rd gen LVs produced with different ratios of the envelope, packaging and transfer plasmid. Also when we compared this with the transfection efficiency of 2nd gen LVs, we could demonstrate similar transgene expression three days after transfection (Figure 19A).

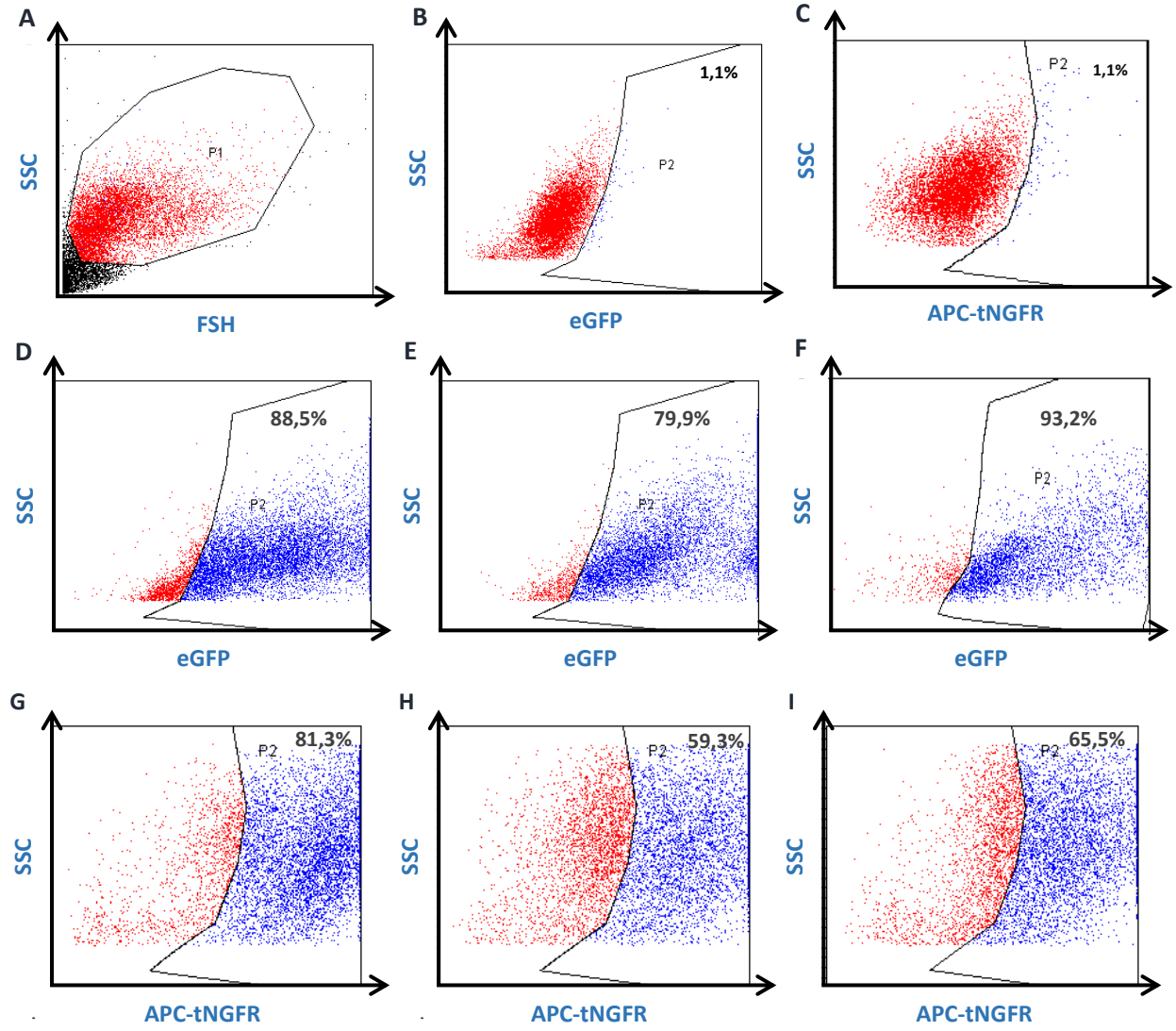

Figure 16: Transfection efficiency of eGFP or OVA-IRES-tNGFR encoding LV production cells three days after transfection.

(A-C) Untransfected cells and more specifically; (A) gating of living HEK293T cells (P1), (B) background eGFP signal within P1 set at 1,1%, (C) background signal for APC signal within P1 set at 1,1%. Next the transfection efficiency is displayed for HEK293T cells producing 2nd gen LV encoding eGFP (D), 2nd gen IDLV encoding eGFP (E) or 3rd gen LV encoding eGFP (F) 2nd gen LV encoding OVA & tNGFR (G), 2nd gen IDLV encoding OVA & tNGFR (H) and 3rd gen LV encoding OVA & tNGFR (I). (A) The x-axis represents the forward scatter (FSH) and the y-axis represents the side scatter (SSC). (B-I) The x-axis represents the intensity of the detected fluorochrome signal per cel in a logaritmic scale and the y-axis represents the SSC.

3.3.4 Ultracentrifugation

In order to concentrate the harvested LV containing supernatants, they were thawed and filtered through a 0,22 µm filter (Corning). Next the filtrate was transferred to ultracentrifuge tubes together with 2 ml 25% sucrose gradient solution, added to the bottom of the centrifuge tube. After balancing the centrifuge tubes, the tubes were centrifuged at 22000 rpm for one hour and a half at 4°C. After centrifugation, the viral pellets were resuspended in a protamine sulphate (PS, LeoPharma) - PBS solution (10 $^{\mu g}/_{ml}$) in order to concentrate the LV containing harvest a 1000 fold. Finally the concentrated LV stocks were aliquoted over cryovials and stored at -80°C.

3.3.5 Titration of the concentrated virus stock

To determine the concentration of each concentrated LV stock, we performed a titration series on HEK293T cells. Therefore these cells were plated at 10^5 $^{cells}/_{ml}$ per well of a 6 well plate in the morning. In the afternoon of the same day we diluted the LV stock in DMEM+ with PS (10 $^{mg}/_{ml}$) at a 1/250, 1/2500, 1/5000 and 1/25000 dilution. After a 30-minute incubation period, 1 ml of each dilution was transferred to 1 ml of DMEM+ with 10^5 $^{cells}/_{well}$, resulting in a LV dilution serie of 1/500, 1/5000, 1/10000 and 1/50000. Seventy-two hours later, the cells were analyzed for transduction efficiency and as such transgene expression via flow cytometry as depicted in Figure 17 and Table 10. The respective titers were calculated by the following formula:

$$\frac{\%\ transgene\ positive\ cells \times dilution}{2\ ml\ of\ volume \times 100} = \#\ {}^{TU}/_{ml}.$$

Figure 18B shows that the titer of the 2nd gen IDLVs produced with 45µg of pCHELP packaging plasmid is about one and a half times higher than for IDLVs produced with 30µg of pCHELP packaging plasmid. Therefore we decided to use 45µg of pCHELP to produce all following 2nd gen IDLVs. In the case of the 3rd gen LVs, the difference was less clear. As the titer of the LVs produced with a 30/30 ratio of pRSV/pMDL was slightly higher than the 15/15 and 45/45 ratio, we decided to use the 30/30 ratio for all following 3rd gen LV productions.

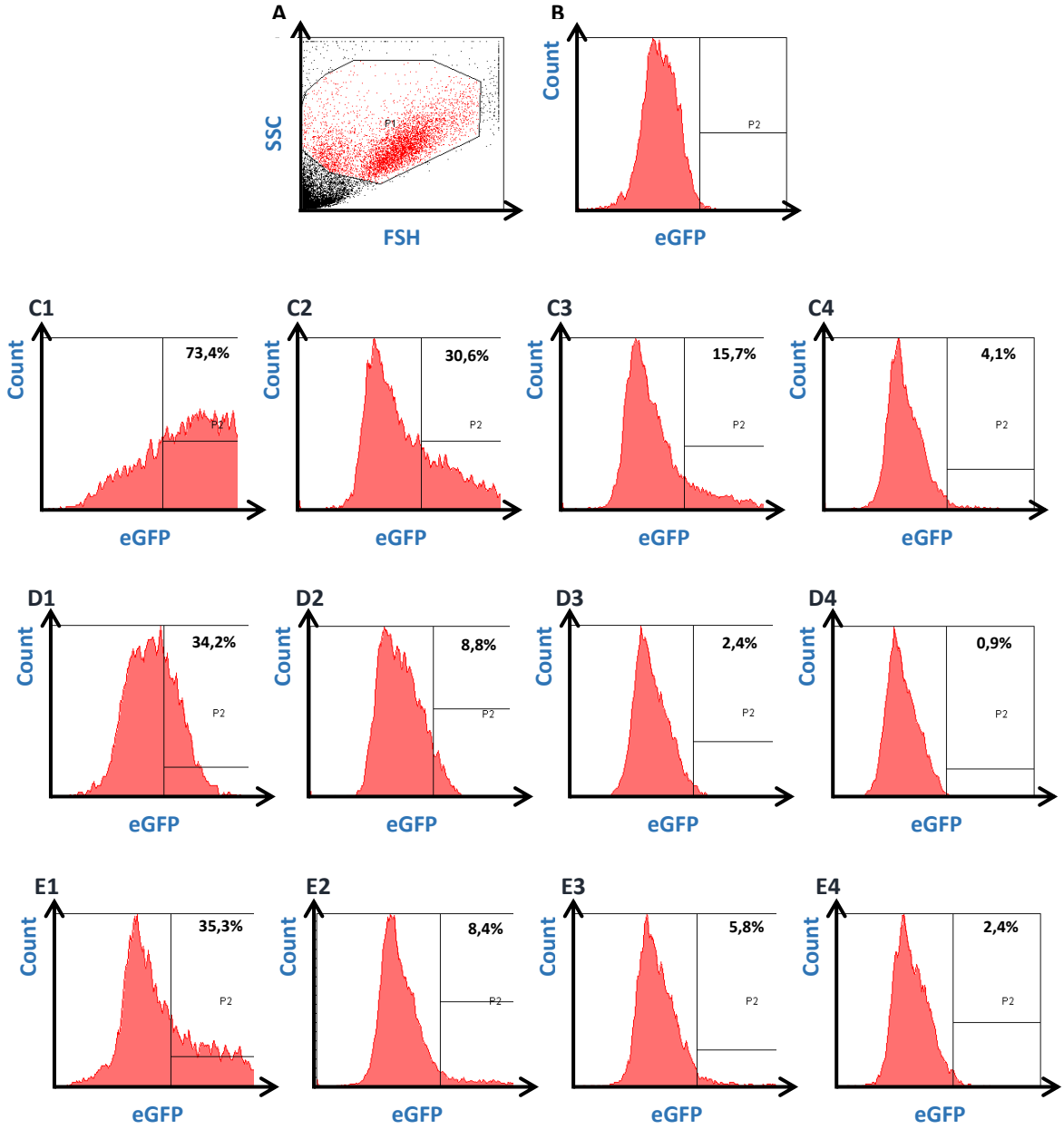

Figure 17: Overview of the titration of a single eGFP encoding virion production.
(A) Gating of living cells (P1) within an untransduced HEK293T cell suspension. (B) Background eGFP signal within P1 set at 1,1%, (C1 → 4, D1 → 4 and E1 → 4) Transduction efficiency of HEK293T cells with 2nd gen LV, IDLV or 3rd gen LV encoding eGFP respectively at a 500, 5000, 10000 and 50000 fold dilution of the respective virus stocks. (A) The x-axis represents the FSH and the y-axis represents the SSC. (B, C1 → 4, D1 → 4 and E1 → 4) The x-axis represents the intensity of the detected fluorochrome signal per cel in a logaritmic scale and the y-axis represents the amount of detected events.

When we subsequently compared the titer of the different LVs produced with their 'optimized' production protocol, we could clearly deduce that the amount of $^{TU}/_{ml}$ was the highest in the 2nd gen LV productions, compared to the IDLV productions which showed a fourfold decrease of $^{TU}/_{ml}$. Finally the 3rd gen LV productions generated the lowest amount of effective virions of all three LV production processes with an eight fold decrease of $^{TU}/_{ml}$ in comparison to the 2nd gen LV productions (Figure 18B).

3.3.6 Reverse transcriptase assay

An alternative way to determine the concentration of each LV stock, is by measuring the amount of functional RT. Therefore we applied the Reverse Transcriptase Assay® kit (Roche). Briefly, the LV stocks were 50 and 100 fold diluted with lysis buffer in a 96 well plate and incubated for 30 minutes. During incubation, the RT standard array was assembled according to Table 9. Next, 2 µl of each LV dilution was transferred to a new well which contained 38 µl of lysis buffer. In addition 40 µl of each standard dilution was transferred to new wells. Next 20 µl of reaction mixture was added to the samples, standard array and a blanc with 40 µl lysis buffer only, and the 96 well plate was incubated for three hours at 37°C and 5%CO_2. After the incubation, all reaction mixtures were transferred to microplate (MP) strips, and incubated for one hour at 37°C and 5%CO_2. After incubation, the solution was removed, the MP strips were washed five times with 200 µl washing buffer and 100 µl of anti-digoxigenin-peroxidase (anti-DIG-POD) was added to each well. After one hour of incubation at 37°C, the solution was removed, the MP strips were washed five times with 200 µl washing buffer and 200 µl ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate buffer was added to each well. After at least 15 minutes of incubation in the dark, the colorimetric absorbance of each well was measured at 405 nm with a microplate reader (Thermo Max). As depicted in Figure 20C, the amount of ng RT was higher for the 2nd gen IDLVs than for the 3rd gen LVs with no significant differences between the different ratios within each LV generation. Furthermore, also when the ng RT values were compared between the 2nd gen LVs, IDLVs and 3rd gen LVs, we could demonstrate the highest among of RT in the 2nd gen IDLV stocks.

3.3.7 Statistical Analysis

The data concerning the improvement of virion production represents one performed experiment. The data concerning the analysis overview of all lentiviral productions represents two performed experiments. Via a one-way ANOVA followed by a Bonferroni's multiple comparison test, statistical test was performed. Sample sizes and number of times experiments were repeated are indicated in the figure legends. All statistical analyses were performed with the software GraphPad Prism, and the error bars indicate the standard error of the mean.

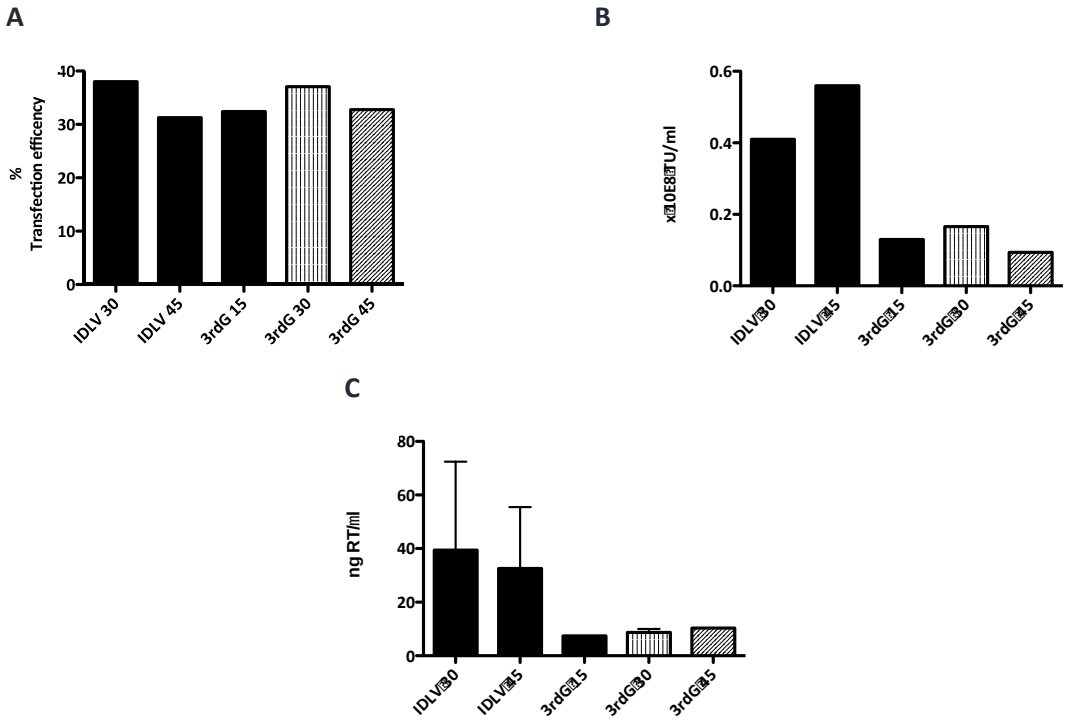

Figure 18: Optimization of the virion production protocol for 2nd gen IDLVs and 3rd gen LVs.

All figures represent data from 2nd gen IDLVs produced with 30 or 45 µg of the packaging plasmid pCHELP or from 3rd gen LVs produced with a 15/15, 30/30 or 45/45 µg ratio of the packaging plasmids pRSV/pMDL. (A) The transfection efficiency was determined via flow cytometry on the third day after transfection of HEK293T cells with the plasmids required for the respective LV productions (n = 1). (B) The titer in $^{TU}/_{ml}$ was determined via flow cytometry of 293T cells transduced three days earliers with a dilution serie of the respective LV stocks (n = 1). (C) The titer in $^{ng\ RT}/_{\mu l}$ after performance of an RT assay (n = 1).

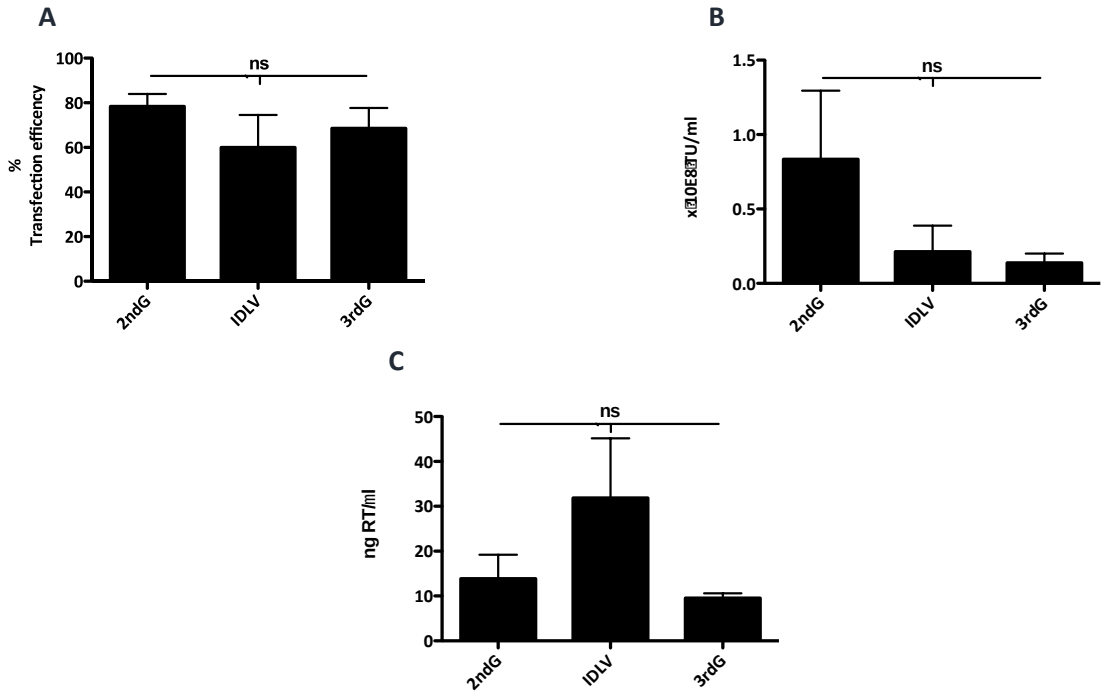

Figure 19: Comparison of 2nd generation LV, IDLV and 3rd generation LV production.

The results of the evaluated (A) transfection efficiencies, (B) titer in $^{TU}/_{ml}$ and (C) in $^{ng\ RT}/_{\mu l}$ are depicted for the respective 293T cell production cells or concentrated LV stocks (n = 3, six T175 flasks per production). The abbreviation "ns" above the columns stands for not significant.

3.4 Transduction efficiency & kinetic profile *in vitro*

After production of the different LV generations encoding eGFP or OVA-IRES-tNGFR, we evaluated their transduction efficiency as well as the duration of transgene expression (kinetics). Therefore we applied two cell types. On the one hand we tested the transduction efficiency in primarily, bone marrow derived DCs (BMDCs). On the other hand we tested the kinetic profile of transgene expression after transduction of HEK293T cells, as they are, in contrast to BMDCS, easy to keep in culture for several weeks.

3.4.1 Transduction of bone marrow derived dendritic cells

Primary BMDCs were kindly provided by Yannick De Vlaeminck and Kevin Van der Jeught. Briefly BMDCs were obtained by isolating bone marrow from C57BL/6 mice and cultivating this in DMEM+ medium with 10% FBS, supplements (5 %, Sigma) and β-mercaptoethanol (50 µM, Sigma) together with 20 $^{ng}/_{ml}$ GM-CSF. Six days later, the BMDCs were transduced by plating them in a 96 well plate (Sarstedt) at 10^5 cells per well and adding 10^6 TU of the different LV stocks. This corresponded to a multiplicity of infection (MOI) of 10 in DMEM+ with 10 $^{\mu g}/_{ml}$ PS. Three days later, the transduced BMDCs were transferred to FACS tubes and washed with PBS/BSA/azide. To evaluate the transduction efficiency, the BMDCs transduced with LVs encoding OVA-IRES-tNGFR, were stained with anti-tNGFR-APC for 20 minutes at 4°C. The BMDCs transduced with LVs encoding eGFP could be evaluated as such as eGFP is a fluorescent molecule detectable in the flow cytometer. Furthermore, all BMDCs were stained with anti-MHCII-R-phycoerythrin (PE), anti-CD11c-PerCPCy™ 5.5, anti-CD86-eFluro450 and anti-CD80-biotin + streptavidin-PECy7. This was done on the one hand to ensure all BMDCs expressed the DC characteristic markers CD11c and major histocompatibility complex class II (MHCII). The CD11c marker is a peptide, which ensures adhesion, cell migration, survival and proliferation, and MHCII is a peptide present on antigen presenting cells. On the other hand the expression of CD80 and CD86, two costimulatory molecules, was evaluated to assess the degree of maturation after transduction.

When we evaluated the transgene expression of BMDCs at day three and five or six after transduction with LVs encoding eGFP or OVA-IRES-tNGFR respectively, we could demonstrate more transgene positivity after transduction with the 2nd gen LVs than with the 2nd gen IDLVs or 3rd gen LVs (Figure 20). Furthermore, the transduction process had no significant effect on the CD11c and MHCII expression levels Figure 21. Finally, the expression level of the CD80 marker was only elevated in the case of the 2nd gen and 3rd gen LVs, while the CD86 marker was elevated in all transduced conditions.

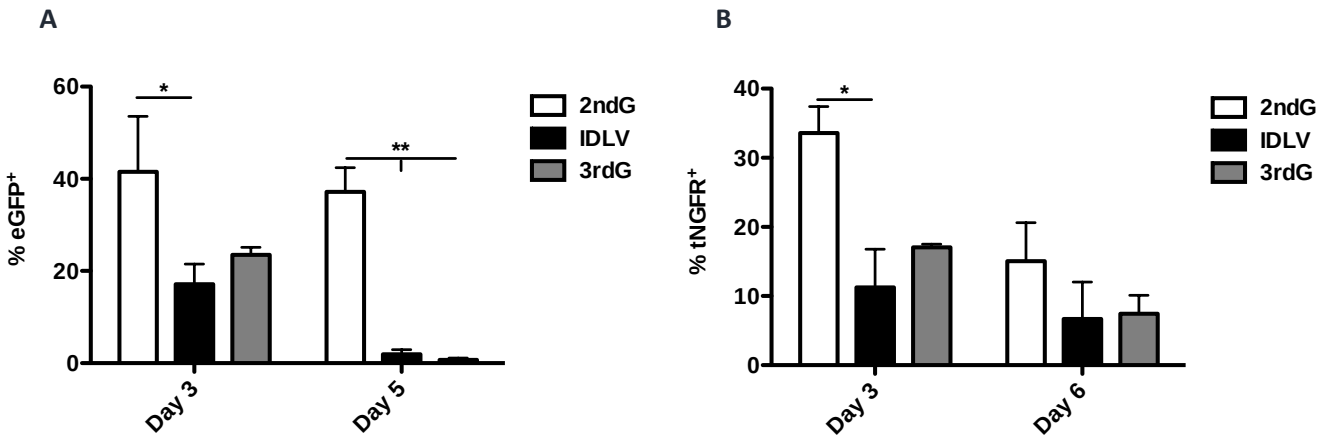

Figure 20: Overview of the transduction efficiency of BMDCs with 2nd gen LVs, IDLVs and 3rd gen LVs.

(A)Transduction efficiency of BMDCs with eGFP encoding virions on day three and day five after transduction (n = 2) (B) Transduction efficiency of BMDCs with OVA and tNGFR encoding virions on day three and day six after transduction (n = 2). Number of asterisks in the figures indicates the level of statistical significance as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

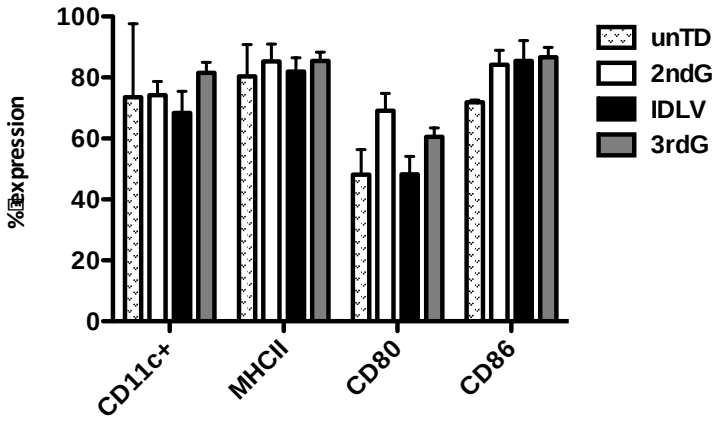

Figure 21: Phenotyping of untransduced and transduced BMDCs.

Three days after transduction with nothing (unTD), 2nd gen LVs (2ndG), 2nd gen IDLVs (IDLV) or 3rd gen LVs (3rdG), BMDCs were analyzed for their expression of CD11c, MHCII, CD80 and CD86 using flow cytometry (n = 4).

3.4.2 Kinetics of transgene expression

To evaluate the duration of transgene expression after transduction of HEK293T cells with 2nd gen LVs, IDLVs and 3rd gen LVs, we transduced HEK293T cells with the respective LVs encoding eGFP at an MOI of ten. From day three after transduction till day 12, eGFP expression was followed up via flow cytometry (Figure 24). As expected, the kinetic experiment clearly shows that eGFP expression was durable after transduction with 2nd or 3rd gen LVs. In contrast, transgene expression started to decrease from day three till it reached an undetectable level on day seven, when cells were transduced with the IDLVs.

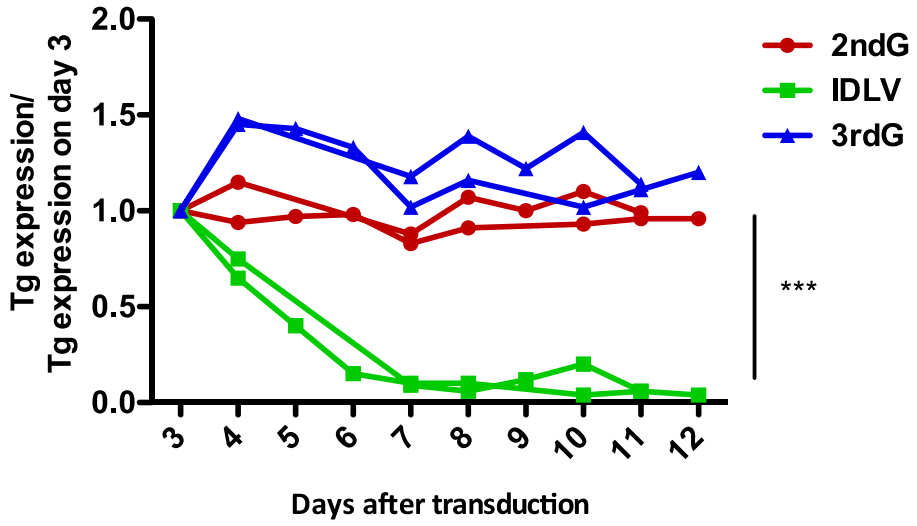

Figure 22: Kinetic experiment in transduced 293T cells with different eGFP encoding LVs.
Transduction of HEK293T with eGFP encoding IDLVs, 2nd and 3rd gen LVs (n = 2). Number of asterisks in the figures indicates the level of statistical significance as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3.4.3 Statistical analysis

All statistical analyses were performed with the software GraphPad Prism, and the error bars indicate the standard errors of the mean. A one-way ANOVA followed by a Bonferroni's multiple comparison test was performed.

3.5 Immunogenicity *in vivo*

In the past the host laboratory regularly applied 2nd gen LVs encoding a tumor associated antigen to test their potency as antitumor vaccine via both *ex vivo* and *in vivo* modulation of DCs (Breckpot et al., 2003; Goyvaerts et al., 2014, 2015). Since we want to shift the use of 2nd gen LVs to the use of safer 2nd gen IDLVs and/or 3rd gen LVs, we compared their potency to induce an antigen specific immune response with the 2nd gen LVs. More specifically, we evaluated the capacity of the different LV types encoding OVA (model antigen) to induce OVA specific cytotoxic $CD8^+$ T cells (CTLs) using an *in vivo* CTL assay as described below.

3.5.1 Cytotoxic T lymphocyte assay

On day zero, 15 C57BL/6 mice were immunized subcutaneously at the tail base with 40 ng RT of OVA encoding LVs resuspended in 50 µl PBS with 1 $\mu g/_{ml}$ PS. Three mice were injected with 50 µl PBS/PS, four with 2nd gen LV-OVA, four with IDLV-OVA and four with 3rd gen LV-OVA. On day five, spleens of three C57BL/6 mice were isolated and pooled in 5 ml red blood cell lysis buffer. Next spleens were reduced to a single cell suspension, filtered through a 40 µm nylon filter (Falcon) and centrifuged for five minutes at 1500 rpm. Next, the cell pellet was resuspended in 10 ml DMEM+ with β-mercaptoethanol (50 µM) and divided over two fractions containing 150 x 10^6 spleen cells each. Only to the first fraction 5 µM SIINFEKL peptide was added. This peptide represents the MHC class I immunodominant epitope of OVA in C57BL/6 mice. Next, both fractions were incubated for one hour and a half at 37°C and 5%CO_2. After incubation, both tubes were centrifuged (7 min at 1500 rpm) and pellets were resuspended in 5 ml PBS with 0,1% BSA and 5 or 0,5 µM carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) to the peptide and non-peptide loaded tube respectively. Next, the tubes were incubated for ten minutes at 37°C, centrifuged and washed with 5 ml cold RPMI with 10% FCI (Harlan). Finally the tubes were centrifuged again and after supernatant removal, cell pellets were resuspended in 3,5 ml DPBS. Before injection, the CFSE ratio was checked via flow cytometry. Next, 200 µl of the cell suspension, accounting for ± 20x10^6 spleen cells, was injected intravenously into the 15 immunized C57BL/6 mice. On day six, two lymph nodes from each immunized mouse were isolated, transferred to a 24 well plate and incubated for 30 minutes in 200 µl liberase solution at 37°C and 5 %CO_2. After incubation, the lymph nodes were smashed, cell suspensions filtered and subsequently the CFSE ratios were analyzed in flow cytometry (Figure 23).

The percentage specific lysis was calculated by the following formula:

$$\left(1 - \frac{\%\ CFSE_{high} / \%\ CFSE_{low}\ \ of\ immunized\ mouse}{\%\ CFSE_{high} / \%\ CFSE_{low}\ \ of\ unimmunized\ mouse}\right) \times 100$$

We could clearly demonstrate that both the 2nd gen and 3rd gen LVs encoding OVA are able to induce a functional CTL reaction *in vivo*. Although not significant, we furthermore show that the OVA encoding IDLVs are much less potent to induce antigen specific lysis upon their subcutaneous injection.

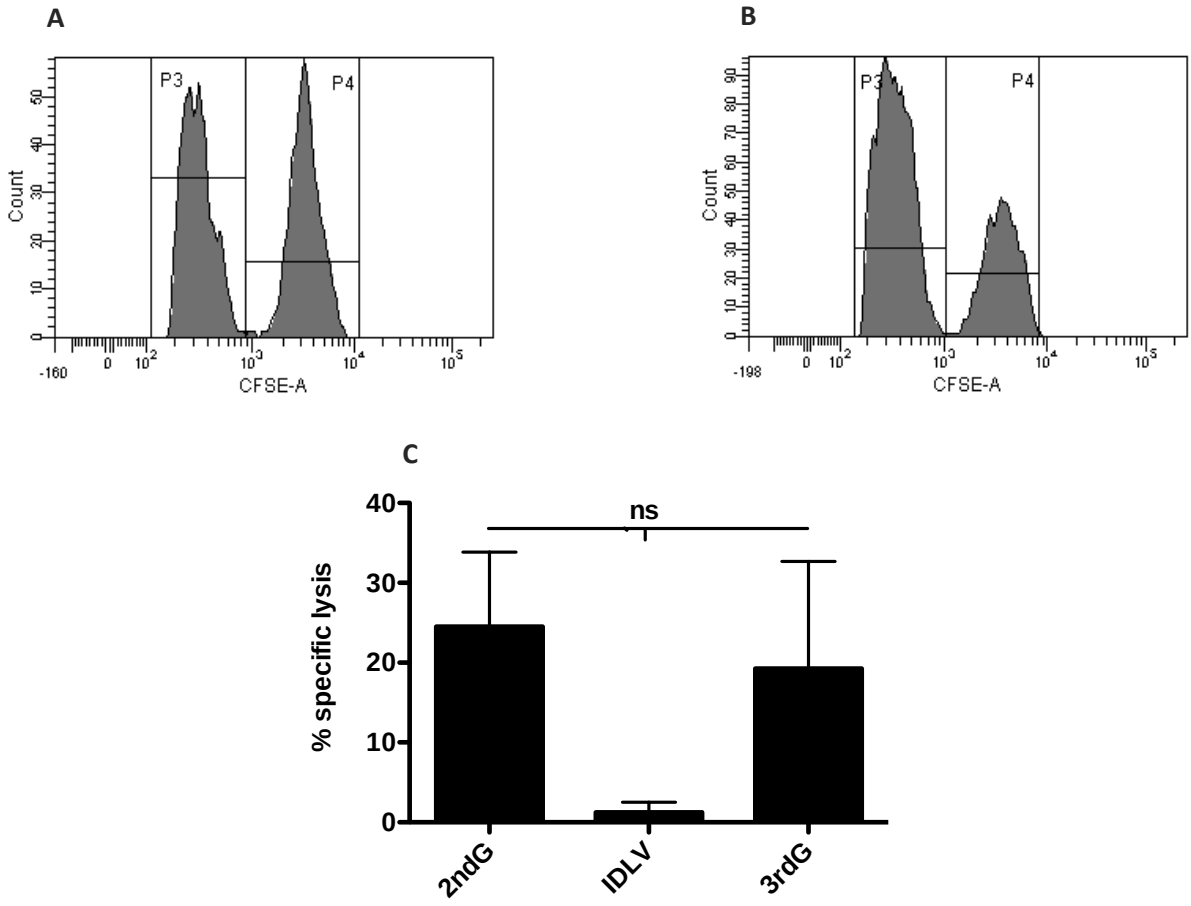

Figure 23: Cytotoxic T-lymphocyte assay

Example of two CFSE low (left) and high (right) labeled splenocyte populations as detected within the lymph nodes on the day of OVA specific lysis detection in a non-immunized (A) or immunized (B) mouse. (C) Representaion of the percentage specific lysis after subcutaneous immunization with 40 ng RT of OVA encoding 2nd gen LV (2ndG), IDLV or 3rd gen LV (3rdG) Therefore the immunized mice were euthanized on day 6 and two lymph nodes from each mouse were analysed via flow cytometry (n = 1, 4 mice per experiment). The abbreviation "ns" above the columns stands for not significant.

3.5.2 Statistical analysis

The data represents one experiment using cells or extracts from fifteen separate isolations, of which four mice were injected per LV stock. All of the statistical analyses were performed with the software GraphPad Prism and the error bars indicate the standard errors of the mean. A one-way ANOVA followed by a Bonferroni's multiple comparison test was performed.

4 General discussion

Lentiviral vectors represent powerful gene transfer vehicles and as such also vaccine moieties for antitumor immunotherapy. Therefore the host laboratory already invested a lot of effort in the application potential of 2nd gen LVs for immunotherapy (Breckpot et al., 2007, 2010). Furthermore the laboratory has been comparing broad tropism 2nd gen LVs with mRNA (Dullaers et al., 2004) or with DC subtype specific targeted LVs for their immunogenic potential (Goyvaerts et al., 2014, 2015). Since the putative biosafety risks currently hamper their translation from bench-to-bedside, this thesis project was set out to generate safer LV platforms such as the 2nd gen IDLV and 3rd gen LV. Therefore my thesis project embraced the following goals: (1) generation of transfer plasmids for the production of 3rd gen LVs encoding eGFP, gp100 or OVA-IRES-tNGFR, (2) optimize the production protocol of 2nd gen IDLVs and 3rd gen LVs, (3) compare their titer in $^{TU}/_{ml}$ and $ng\ ^{RT}/_{\mu l}$ with 2nd gen LVs, (4) compare their *in vitro* transduction efficiency and kinetic profile on BMDCs and HEK293T cells respectively and (5) compare their potency to stimulate CTLs *in vivo*.

After accomplishment of goal (1), we started with the optimization of the 2nd gen IDLV and 3rd gen LV production process. First we defined the optimal plasmid ratio for their production (goal 2). Next we compared their production efficiency with 2nd gen LVs, which served as 'the golden standard'. Here we demonstrated that while the transfection efficiencies of the 2nd and 3rd gen (ID)LV production cells didn't show big differences, this similarity was not reflected in their titers. Especially, when comparing the transfection efficiencies with the transduction efficiencies and with the results from the RT assay, two remarkable discrepancies are observed (respectively Figure 19, A ; Figure 19, B and Figure 19, C). The first discrepancy is seen when the transfection efficiency of the 3rd gen LV productions is compared with their respective titer expressed in $^{TU}/_{ml}$. The observed difference is remarkable given the fact that the transfection efficiency of the 3rd gen LV productions is comparable to the 2nd gen LV productions. However the titer of the 3rd gen LV stocks shows an eight-fold decrease in comparison to the 2nd gen LVs. This could be explained by the fact that the transfection efficiency is the result of the sum of two different processes. On the one hand the transgene will be expressed from the transfected transfer plasmid. On the other hand, the transgene will also be expressed upon transduction of the LV production cells with their own generated LV virions. In the case of the 2nd gen LV production system, only three plasmids are necessary to produce effective virions in contrast to the 3rd gen LVs, which need four plasmids in order to produce effective virions. Hereby the chance that all four plasmids are present in one HEK293T cell at the same time is much lower than the chance of three plasmids being present in one HEK293T cells at the same time. This can subsequently result in a similar amount of transgene positive production cells but a different LV

titer as observed for the LV production cells during 3rd gen LV production in comparison to 2nd gen LV productions. So in the end, the transfection efficiency doesn't necessarily signify a high transduction efficiency although it could be used as an indication. The second discrepancy is observed when the titers of the different LV generations expressed in $^{TU}/_{ml}$ are compared with their respective titers in $^{ng\ RT}/_{\mu l}$ (respectively Figure 19, B with Figure 19, C). In the case of the IDLV stocks it is remarkable that the titer expressed in $^{TU}/_{ml}$ is four fold lower compared to the 2nd gen LV productions, while their amount of RT is four fold higher than the amount of RT present in the 2nd gen LV stocks. This could be explained by an inefficient IDLV production process resulting in a lot of uninfectious virions with a lot of packaged RT. The latter discrepancy further shows that the amount of RT in a LV stock and especially an IDLV stock is only an approximation of the amount of completely matured, infectious virions. Of note, a one-way ANOVA followed by a Bonferroni's multiple comparison test indicated that the differences observed among the different LV generations weren't statistically significant. This is explained by the limited number of experimental repeats due to lack of time. In order to obtain reliable statistical results, the experiments require to be repeated.

Evaluating goal 4 resulted in the observation that the transgene expression of BMDCs three days after transduction with 2nd gen LVs was the highest compared to a twofold lower expression level after transduction by IDLVs and 3rd gen LVs and this irrespective of the evaluated transgene (Figure 20). Furthermore, this difference was even more pronounced at day five when the LVs encoded eGFP. In the case of OVA-IRES-tNGFR encoding LVs, the difference in transgene expression became much smaller on day 6. These results indicate that within the 2nd gen LV stocks, more infectious $^{TU}/_{ml}$ are present than in the 2nd gen IDLV and 3rd gen LV stocks. Furthermore, we evaluated the phenotype of the transduced BMDCs. An overview of all results obtained from BMDCs transduced three days before with LVs encoding eGFP or OVA-I-tNGFR allow us to evaluate their maturation state as reflected in their CD80 and CD86 positivity (Figure 21). Since CD80 was only elevated after transduction with 2nd gen LVs and 3rd gen LVs, while the CD86 marker also elevated upon IDLV transduction, this could indicate that CD80 upregulation represents an effort to stimulate the immune system in response to repel the 2nd and 3rd gen LVs while the elevation of CD86 could indicate an effort to stimulate the immune system in response to both integrative and non-integrative LVs.

After analyzation of the kinetic profile, it was clear but not unexpected that the transgene expression of the 2nd and 3rd gen transduced HEK293T cells remained more or less constant over time whereas this completely dropped between day three and seven for the IDLV transduced cells. For the latter this corresponded with the bisection of transgene expression as previously described (Wanisch & Yáñez-Muñoz, 2009).

Finally, the results of the CTL assay (goal 5), show that 2nd and 3rd gen LVs encoding OVA generated a comparable $CD8^+$ T cell mediated immune response (Figure 23, C). In contrast, immunization with OVA encoding 2nd gen IDLVs generated a much lower immune response, which could be explained by several factors. First, as we injected 40 ng RT per stock in each mouse, this resulted in a much lower amount of $^{TU}/_{ml}$ injected per mouse for both the 3rd gen LVs but especially the IDLVs where the discrepancy between its titer in $^{TU}/_{ml}$ and $^{ng\ RT}/_{\mu l}$ was the highest. Subsequently a much lower amount of $^{TU}/_{ml}$ were injected in the case of the IDLVs compared to the 2nd gen LVs. Furthermore it has been shown in the past that the transgene expression level and duration is of paramount importance for the induction of a potent immune response (Garmory, Brown, & Titball, 2003). This is explained by the fact that it takes three up to five days for transduced DCs *in vivo* to migrate to the draining lymph node, select and activate the proper $CD8^+$ T cells (Figure 10.8, from Charles A Janeway, Travers, Walport, & Shlomchik, 2001). As we showed in goal 4, that the transgene expression of HEK293T cells transduced with IDLVs starts to decline from day three, this could result in less potent stimulation of the T cells by the transduced DCs *in vivo* after IDLV injection. In addition to the transient transgene expression of IDLVs, the transduction of murine BMDCs was also lower compared to the 3rd and especially 2nd gen LV BMDCs transduction. In the case of IDLV, this lower transduction efficiency could also contribute to the lack of a detectable immune response *in vivo* (Figure 20). Of note, a one-way ANOVA followed by a Bonferroni's multiple comparison test indicated that differences in induction of specific lysis amongst the LV generations aren't statistically significant. This is explained by the fact that the experiment was only performed once and that it should be repeated at least two more times to obtain reliable statistical results.

5 Conclusion and future perspectives

In this thesis we premised to compare the production protocol, transduction efficiency and induction of an immune response between a 2nd gen LV, IDLV and 3rd gen LV.

First, we can conclude that the 2nd gen LV system is able to generate LV stocks with the most infectious virions at the highest titer (in $^{TU}/_{ml}$). In contrast, the IDLV and 3rd gen LV system do not perform as well and result in a four- and eight-fold decrease in titer (in $^{TU}/_{ml}$) respectively. Secondly, the RT-assay to evaluate the titer expressed in $^{ng\ RT}/_{\mu l}$ does not correspond with the titer expressed in $^{TU}/_{ml}$ for the IDLVs and 3rd gen LVs. Thirdly, the 2nd gen LVs also outperformed the other two LV systems in terms of murine BMDC transduction. Fourthly, the kinetic experiment demonstrated stable transgene expression after transduction of HEK293T cells with the integration efficient 2nd gen and 3rd gen LV systems while for the IDLV system a gradual decline in transgene expression was observed. Finally, both the 2nd and 3rd gen LVs were capable of inducing equal antigen specific immunity, whilst the IDLV system did not induce a detectable immune response.

In prospect of the low viral titers of the 2nd gen IDLV and 3rd gen LV, their low BMDC transduction efficiencies, the discrepancy between their titers expressed in $^{TU}/_{ml}$ and $^{ng\ RT}/_{\mu l}$ and lack of statistical significance, a few measures will be undertaken in the near future. On the one hand we will further optimize the packaging plasmid ratio's during the transfection protocol. Furthermore we will test the addition of caffeine to the HEK293T production cells in order to boost their LV production. Finally we will repeat all conducted and planned experiments at least three times in order to attain more reliable statistical results.

6 References

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7 Appendix

7.1 Tables

Table 1: Components and volumes for assembling the digestion mixes of p-HR'-huli80-tOVA-IRES-tNGFR and pLenti-puro digested by *XbaI*

Component \ Samples		p-HR'-huli80-tOVA-IRES-tNGFR	pLenti-puro
Restriction enzyme: *Xba*I	(µl)	1	1
Template DNA	(µl)	0,8	1,9
$c_{Template\ DNA}$	$(\mu g/ml)$	2469	1050
Buffer G	(µl)	2	2
Milli-Q® H_2O	(µl)	16,2	15,1
Total	(µl)	20	20

Table 2: Components and volumes for assembling the digestion mixes of pST1-eGFP@3, pST1-gp100-DCL.OPT & pLenti-puro 3 digested by *XhoI* and *SpeI*

Component \ Samples		pST1-eGFP@3	pST1-gp100-DCL.OPT	pLenti-puro 3
Restriction enzyme: *Spe*I	(µl)	1	1	1
Restriction enzyme: *Xho*I	(µl)	1	1	1
Template DNA	(µl)	0,97	2	1,9
$c_{Template\ DNA}$	$(\mu g/ml)$	2070	1000	1050
Buffer Tango	(µl)	2	2	2
Milli-Q® H_2O	(µl)	15,03	14,3	14,1
Total	(µl)	20	20	20

Table 3: Components and volumes for assembling ligation mixes

Component		Ligation Mix 1	Ligation Mix 2	Negative control 1
Ligation pool	(µl)	3	3	3
Insert	(µl)	7	1	/
Milli-Q H_2O	(µl)	0	6	7
Total	(µl)	10	10	10

Table 4: Components and volumes for assembling a sample digested by one restriction enzyme (*Eco*RI)

Component		Samples	pLenti-OVA-I-tNGFR
Restriction enzyme: *Eco*RI	(µl)		1
Template DNA	(µl)		17
Buffer Tango	(µl)		2
Total	(µl)		20

Table 5: Components and volumes for assembling a sample digested by two restriction enzymes (*Spe*I & *Xba*I)

Component		Samples	pLenti-eGFP	pLenti-gp100-DCL-OPT
Restriction enzyme: *Spe*I	(µl)		1	1
Restriction enzyme: *Xba*I	(µl)		1	1
Template DNA	(µl)		16	16
Buffer G	(µl)		2	2
Total	(µl)		20	20

Table 6: Components and volumes of 2nd gen LV transfection mix per T175 flask with HEK293T cells

Component / Samples		2nd gen LV Transfection mix	PEI-solution
pMDG	(µl)	15	/
pCMV DR8.9	(µl)	30	/
2nd gen transfer plasmid	(µl)	45	/
OptiMem	(µl)	$5x10^3$	$5x10^3$
PEI ($1\ ^{mg}/_{ml}$)	(µl)	/	180
Total (roughly)	(µl)	$5x10^3$	$5x10^3$

Table 7: Components and volumes of IDLV transfection nmix per T175 flask with HEK293T cells

Component / Samples		Integrase Deficient LV Transfection mix	PEI-solution
pMDG	(µl)	15	/
pCHELP-IN	(µl)	30 or 45	/
2nd gen transfer plasmid	(µl)	45	/
OptiMem	(µl)	$5x10^3$	$5x10^3$
PEI ($1\ ^{mg}/_{ml}$)	(µl)	/	180 or 270
Total (roughly)	(µl)	$5x10^3$	$5x10^3$

Table 8: Components and volumes of 3rd gen LV transfection mix per T175 flask with HEK293T cells

Component / Samples		3rd gen LV Transfection mix	PEI-solution
pMDG	(µl)	15	/
pRSV.Rev	(µl)	15 or 30 or 45	/
pMDL g/RRE	(µl)	15 or 30 or 45	/
3rd gen transfer plasmid	(µl)	45	/
OptiMem	(µl)	$5x10^3$	$5x10^3$
PEI ($1\ ^{mg}/_{ml}$)	(µl)	/	120 or 240 or 360
Total (roughly)	(µl)	$5x10^3$	$5x10^3$

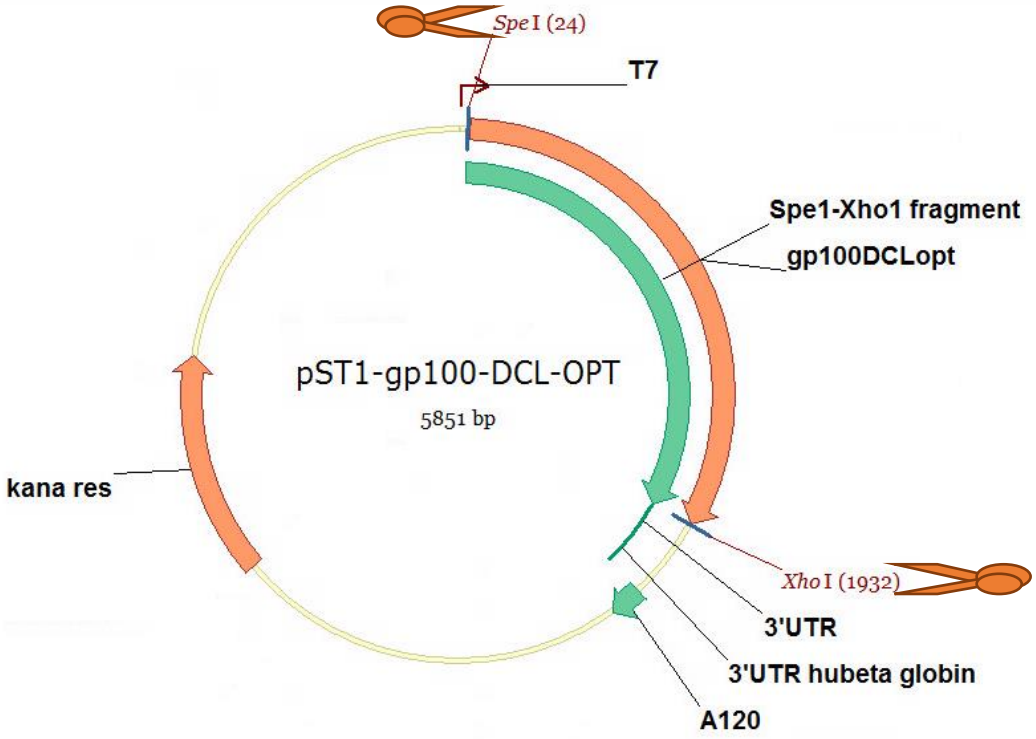
Table 9: Assembly of the RT-array for the RT-assay

Well	RT concentration	Lysis buffer (µl)	RT-solution
Well 1	blanc	75	0
Well 2	$2\ ^{ng}/_{well}$	195	5 µl of RT stock
Well 3	$1\ ^{ng}/_{well}$	75	75 µl of well 2
Well 4	$0{,}5\ ^{ng}/_{well}$	75	75 µl of well 3
Well 5	$0{,}25\ ^{ng}/_{well}$	75	75 µl of well 4
Well 6	$0{,}125\ ^{ng}/_{well}$	75	75 µl of well 5
Well 7	$0{,}0625\ ^{ng}/_{well}$	75	75 µl of well 6
Well 8	$0{,}03125\ ^{ng}/_{well}$	75	75 µl of well 7

Table 10: Calculation of the amount of transducing units present in a virusstock on a titration of 100.000 HEK293T cells at the start of the titration together with a template

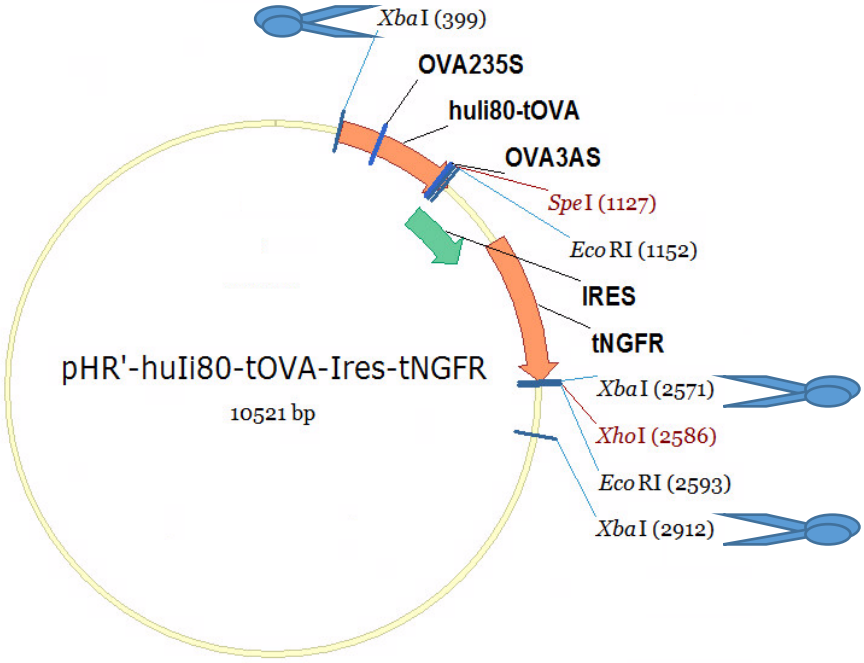
LV Dilution	2nd gen	IDLV	3rd gen	Template
500 fold dilution	73,4%	34,4%	35,5%	A % ➔ $^{TU}/_{ml} = \frac{100.000 * \text{A \%} * 500}{2\ ml * 100}$
5000 fold dilution	30,6%	8,8%	8,4%	B % ➔ $^{TU}/_{ml} = \frac{100.000 * B\ \% * 5000}{2\ ml * 100}$
10000 fold dilution	15,7%	2,4%	5,8%	C % ➔ $^{TU}/_{ml} = \frac{100.000 * C\ \% * 10000}{2\ ml * 100}$
50000 fold dilution	4,1%	0,9%	2,4%	D % ➔ $^{TU}/_{ml} = \frac{100.000 * D\ \% * 50000}{2\ ml * 100}$
$^{TU}/_{ml}$	6,89 x 10^7	1,63 x 10^7	2,97 x 10^7	= mean $^{TU}/_{ml}$
$^{TU}/_{\mu l}$	6,89 x 10^4	1,63 x 10^4	2,97 x 10^4	= $^{TU * 10^{-3}}/_{ml * 10^{-3}}$

7.2 Plasmid maps



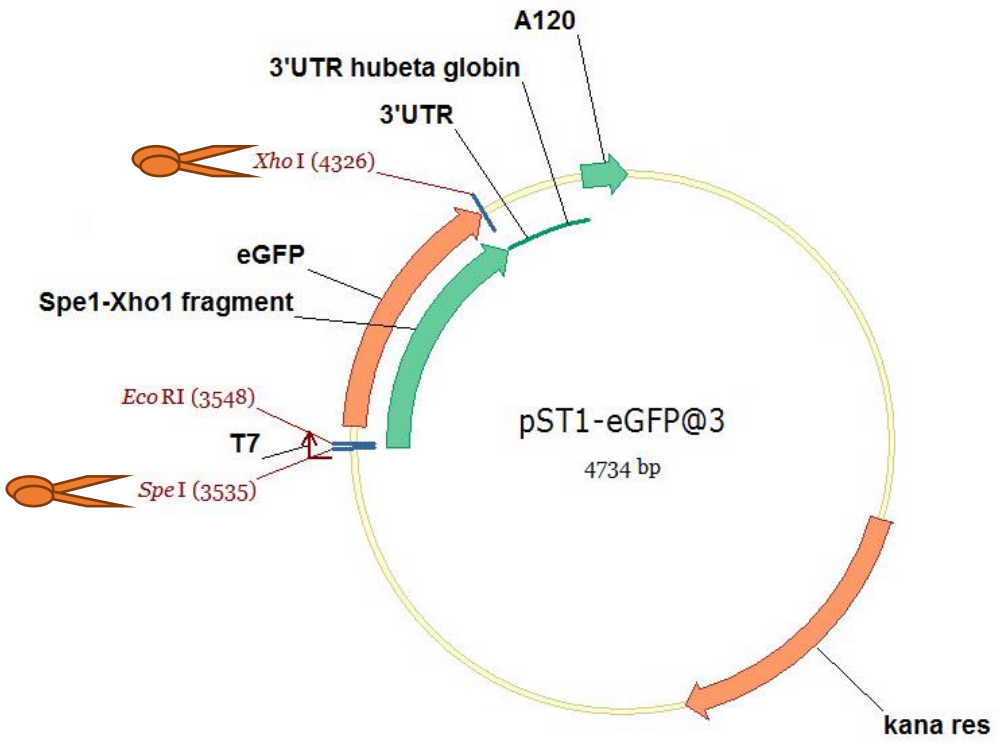
Plasmid Map 1: Plasmid map of pST1-gp100-DCL.OPT

The restriction sites are marked with an orange scissor next to the restriction enzyme. The orange scissors indicate the *Spe*I or *Xho*I restriction site.
The plasmid map shows sequences such as the kanamycin resistance gene (kana res), Three prime untranslated region (3´ UTR) and 3´UTR hubeta globin



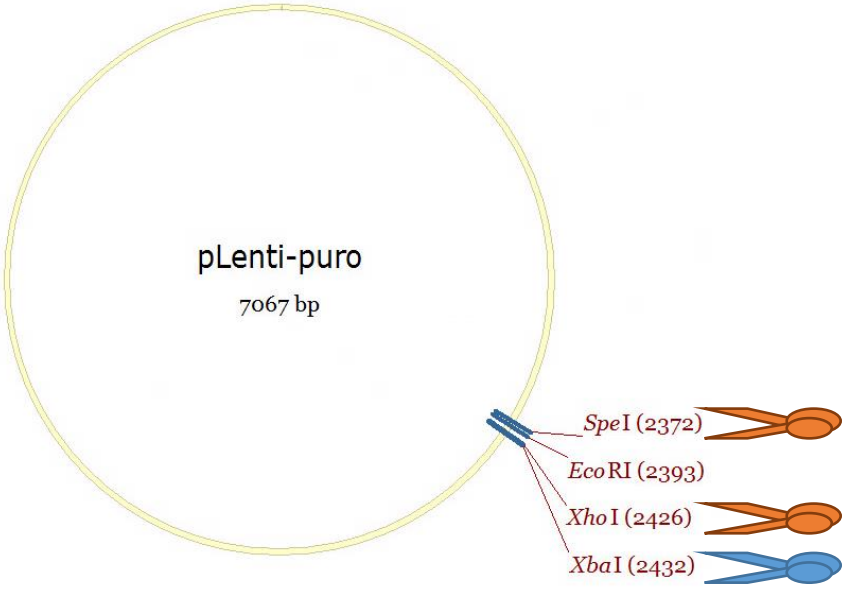
Plasmid Map 2: Plasmid map of pHR'-huli80-tOVA-Ires-tNGFR

The restriction sites are marked with a blue scissor next to the restriction enzyme. The blue scissors indicate the *Xba*I restristion site.



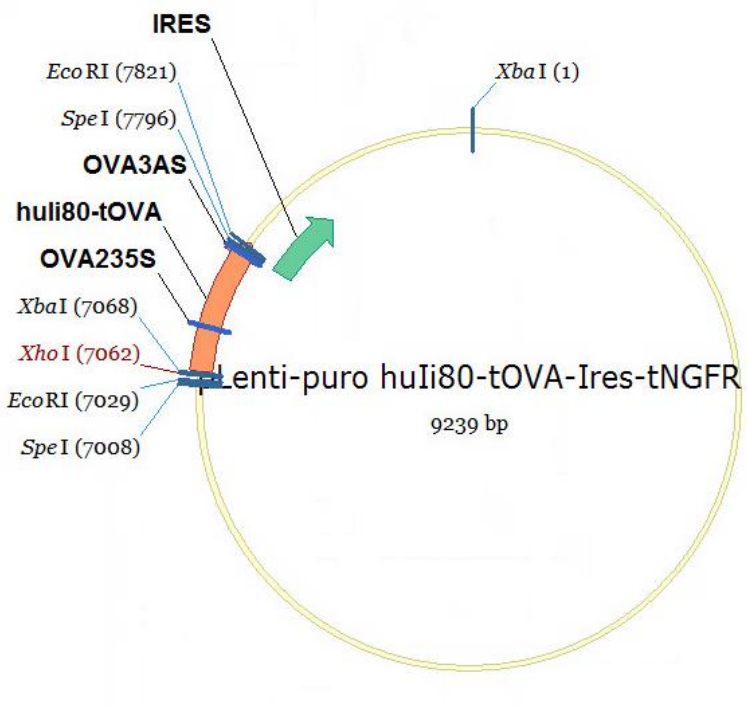
Plasmid Map 3: Plasmid map of pST1-eGFP@3.

The restriction sites are marked with an orange scissor next to the restriction enzyme. The orange scissors indicate the *Spe*I or *Xho*I restriction site.
The plasmid map shows sequences such as the kanamycin resistance gene (kana res), Three prime untranslated region (3´ UTR) and 3´UTR hubeta globin



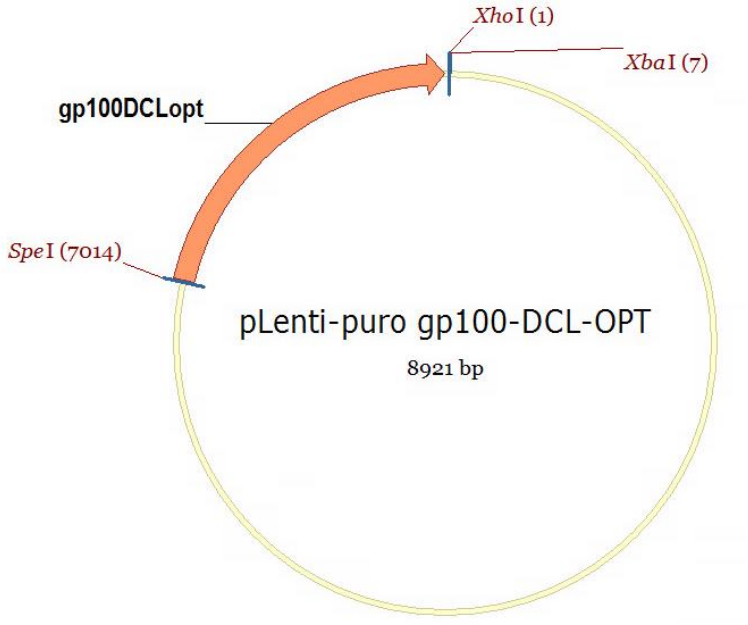
Plasmid Map 4: Plasmid map of pLenti-puro

The restriction sites are marked with an orange and a blue scissor next to the restriction enzyme. The orange scissors indicate the *Spe*I or *Xho*I restriction site and the blue scissor indicates the *Xba*I restristion site.



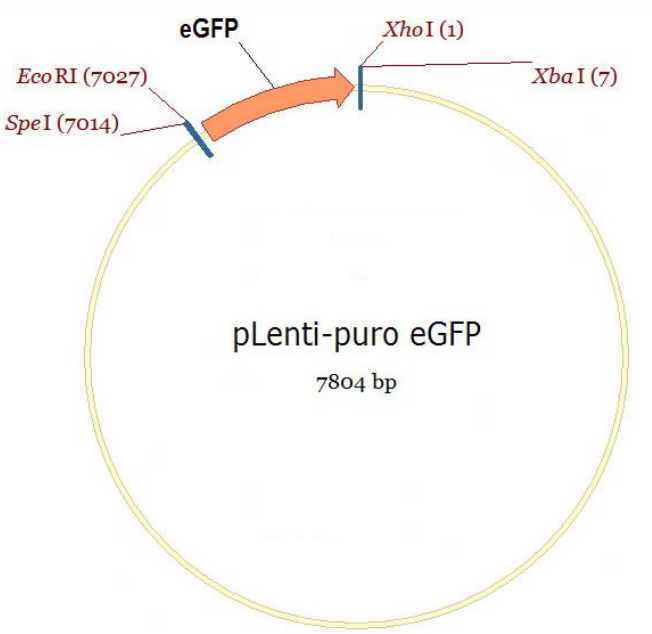
Plasmid Map 5: Plasmid map of pLenti-puro huli80-tOVA-IRES-tNGFR

In the thesis, we refer to the plasmid as pLenti OVA-I-tNGFR.



Plasmid Map 6: Plasmid map of pLenti-puro gp100-DCL-OPT

In the thesis, we refer to this plasmid as pLenti gp100-DCL-OPT



Plasmid Map 7: Plasmid map of pLenti-puro eGFP

In the thesis, we refer to this plasmid as pLenti eGFP