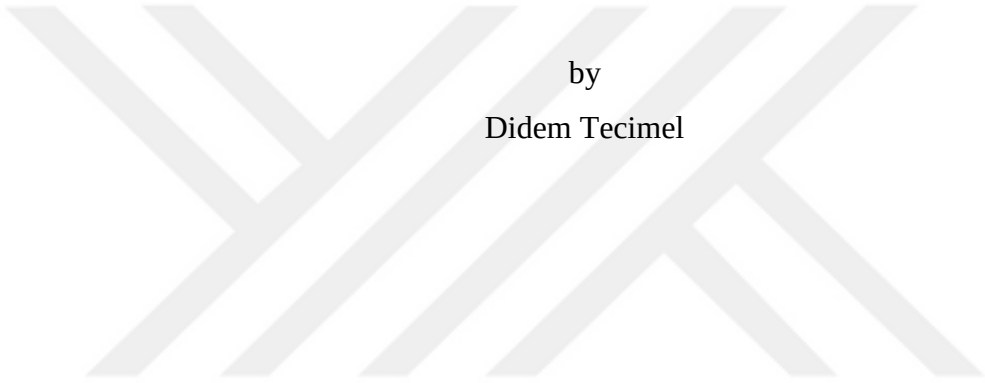


PD-L1/PDGFR- α TARGETED CHIMERIC ANTIGEN RECEPTOR-NATURAL
KILLER CELL THERAPY FOR CHORDOMA



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ABSTRACT

PD-L1/PDGFR- α TARGETED CHIMERIC ANTIGEN RECEPTOR-NATURAL KILLER CELL THERAPY FOR CHORDOMA

Chordoma, a rare neoplasm, is a type of bone tumor that mostly appear in the axial skeleton. The Food and Drug Administration (FDA)-approved chemotherapeutics for chordoma treatment is unavailable. Conventional treatment options include en bloc resection or radiation therapy. Additionally, receptor tyrosine kinase (RTK) inhibitors are widely recommended. Therefore, a targeting strategy against RTKs by chimeric antigen receptors is promising. Growing interest also evaluates checkpoint inhibitors in chordoma and several studies yielded encouraging outcomes both in the pre-clinical and clinical stages. Chimeric antigen receptor (CAR) fused cytotoxic cells fulfilling components of immunotherapeutic applications. However, both in the bench side and clinical utilization, benefits are restricted by the challenges in design and transfer to T and natural killer cells. Target, promoter or ,co-stimulatory domain selections lead the fate of chimeric antigen receptor-mediated cytotoxic function. Still, a comprehensive investigation of basic CAR structure and designing the structure accordingly to research outcomes does not guarantee the functionality. Therefore, each individual CAR structure requires its own optimization, and pre-clinical evaluation. In this thesis, a PD-L1 and PDGFR- α targeting combinatorial CAR aimed to be designed for chordoma treatment. The PD-L1/PDGFR- α CAR structure cloned into lentiviral or sleeping beauty transposon backbones. The expressibility of the designed CAR structure demonstrated with “easy-to-transfect/transduct” cell lines (Human immortalized astrocytes and HEK293T cells) to avoid labor intensive evaluation. Later, transfection and transduction optimizations were performed in natural killer cell lines for the transfer of CAR vectors. Following CAR vector transfer expression analysis within the cell and cell surface was evaluated. Our findings demonstrate that vector size, transfection/transduction methods, choice of cell line and the design itself has profound effects on CAR-NK cell generation.

ÖZET

KORDOMALARDA PD-L1/PDGFR- α HEDEFLİ KİMERİK ANTİJEN RESEPTÖRÜ TAŞIYAN DOĞAL KATİL HÜCRE TERAPİSİ

Nadir görülen bir neoplazma olan kordoma, çoğunlukla aksiyel iskelette görülen bir kemik tümörü türüdür. Güncel olarak kordoma tedavisi için Gıda ve İlaç İdaresi (FDA) onaylı kemoterapötikler mevcut değildir. Geleneksel tedavi seçenekleri arasında en blok rezeksiyon veya radyasyon tedavisi bulunur. Ek olarak, reseptör tirozin kinaz (RTK) inhibitörleri yaygın olarak tavsiye edilir. Bu nedenle, kimerik antijen reseptörleri tarafından RTK'lara karşı bir hedefleme stratejisi umut vericidir. Artan ilgi, kordomadaki kontrol noktası inhibitörlerini de değerlendirir ve birçok çalışma hem klinik öncesi hem de klinik aşamada cesaret verici sonuçlar vermiştir. Kimerik antijen reseptörü (CAR) ile kaynaşmış sitotoksik hücreler, immünoterapötik uygulamaların tamamlayıcı bileşenleridir. Bununla birlikte, hem tezgah tarafında hem de klinik kullanımda, faydalar, tasarım ve T ve doğal öldürücü hücrelere transferdeki zorluklarla sınırlıdır. Hedef, promotör veya yardımcı uyarıcı alan seçimleri, kimerik antijen reseptörü aracılı sitotoksik fonksiyonun kaderini belirler. Yine de, temel CAR yapısının kapsamlı bir şekilde araştırılması ve değerlendirmelere göre tasarlanması, işlevselliği garanti etmez. Bu nedenle, her bir CAR yapısı kendi optimizasyonunu ve klinik öncesi değerlendirmeyi gerektirir. Bu tezde, kordoma tedavisi için kombinatoriyal CAR'ı hedefleyen bir PD-L1 ve PDGFR- α tasarlamak amaçlanmıştır. Lentiviral veya uyuyan güzel transpozon omurgalarına klonlanan PD-L1/PDGFR- α CAR yapısının ifade edilebilirliği, "transfekte edilmesi/aktarılması kolay" hücre hatları (İnsan ölümsüzleştirilmiş astrositler ve HEK293T hücreleri) ile gösterildi. Daha sonra, CAR vektörlerinin transferi için doğal katil hücre hatlarında transfeksiyon ve transdüksiyon optimizasyonları yapıldı. Hücre ve hücre yüzeyi içindeki CAR vektör transfer ekspresyon analizinin ardından değerlendirildi. Bulgularımız, vektör boyutunun, transfeksiyon/transdüksiyon yöntemlerinin, hücre hattı seçiminin ve tasarımın kendisinin CAR-NK hücre üretimi üzerinde derin etkileri olduğunu göstermektedir.

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LIST OF SYMBOLS/ABBREVIATIONS

ζ	Zeta
α	Alpha
γ	Gamma
WHO	World health organization
LOH	Loss of heterozygosity
RTK	Receptor tyrosine kinase
SMARCB1	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1
IFN γ	Interferon gamma
TNF α	Tumor necrosis factor alpha
FDA	The food and drug administration
ILCs	Innate lymphoid cells
DCs	Dendritic cells
NKs	Natural killers
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
ITAM	Immunoreceptor tyrosine-based activating motifs
PD1	Programed cell death protein 1
PD-L1	Programed cell death protein ligand 1
PDGFR- α	Platelet derived growth factor receptor alpha
EGFR	Epidermanl growth factor receptor
c-MET	Hepatocyte growth factor receptor

CLPs	Common lymphoid progenitors
NKP	Natural killer progenitors
iNK	Immature natural killer
Dap10	NKG2D/DNAX-activating protein of 10 kDa
MHC	Major histocompatibility complex
HLA	Human leukocyte antigen
GVHD	Graft versus host disease
TCR	T-cell receptor
NCR	Natural killer cell receptor
KIR	Killer-cell immunoglobulin-like receptor
NCR	Natural cytotoxicity receptors
TIL	Tumor infiltrating lymphocytes
CAR	Chimeric antigen receptor
TIM-3	T cell immunoglobulin and mucin-domain containing-3
ICI	Immune checkpoint inhibitor
ACT	Adoptive cell therapy
LAMP-1	Lysosome-associated membrane protein-1
NKG2D	Natural killer group 2D

1. INTRODUCTION

1.1. CHORDOMA

Chordoma, a rare neoplasm, is a type of bone tumor that mostly appear in the axial skeleton. Chordoma throughout the axial skeleton mainly occurs in the sacrum (50%), 35% arise at the skull base, whereas only 15% appear in the mobile spine (1). The mobile spine consists of the thoracic, lumbar, and cervical regions. The location distribution of mobile spine chordoma is as follows, 15-52% cervical, 33-35% lumbar, and 14-17% thoracic (2). Even though chordomas are referred to as low-grade and slow-growing tumors, they are locally destructive (1). Three types of chordoma are recognized by World Health Organization (WHO); conventional, dedifferentiated, and poorly differentiated chordoma (3). Dedifferentiated chordoma encounters the worst prognosis and highest recurrence rate, though all other subtypes exert malignant properties with high recurrence rates and metastatic potential (4).

The rarity of chordoma restricts epidemiologic accuracy. However, the current statistical information demonstrates that chordoma represents 3% of all bone tumors while encountering 20% of primary spinal tumors. As rare cancer, the incidence of chordoma is 0.8 cases in 1.000.000 per year (5,6). Sex, age, tumor size, and histological type are prognostic factors (7). Depending on the sex, male predominance is observed over the female population (2:1), except for the skull base chordoma, which presents no sex-dependent association (8). Chordoma may be diagnosed at any age, but most cases are detected in 40-60 years old. Patients diagnosed below 20 years comprise approximately 5% of chordoma cases (9).

It is widely accepted that chordoma arises from the remnants of notochordal cells (10). Those residual cells are proposed to be derived from the embryonic notochord, a temporary structure of embryonic cells crucial in developmental processes (11,12). Notochord cells expressing type X collagen are differentiated during developmental stages and contribute to bone formation. However, notochord cells that do not have type X collagen expression participate in forming the nucleus pulposus between the vertebrae. Notochord cells are characterized by SOX9, collagen, and aggrecan expressions similar to chordoma cells (13). Chordoma cells are typically physaliferous, represented by mucin and glycogen-rich

extracellular matrix. Those physaliferous cells can be seen as clusters separated into lobules by a fibrous septum (14). As well as the gene profile, this distinguishing morphological characteristic of chordoma embodies similarities with notochordal cells (15).

The benign lesions that emerge from notochordal remnants located through the axial skeleton, from the clivus to the sacrococcygeal region, are called ecchordosis physaliphora (16). The incidence of ecchordosis physaliphora is reported to be 0.5-2%, depending on the radiological and autopsic evaluations (17). The incidence of notochordal remnants is much higher than the occurrence of chordoma. Therefore, the idea is proposed that residual notochordal cells remain quiescent and are transformed into chordoma cells by mutations or environmental factors (12).

The similarities in gene expression profile, morphology, and location of notochord and chordoma support the notochordal origin hypothesis of chordoma.

1.1.1. Differential Diagnosis and Clinical Symptoms

Clinical symptoms differ between clival and sacral chordoma cases. Skull base chordomas often cause neurological symptoms such as headaches and vision problems. Depending on the tumor size, clival tumors may also cause speech or facial expression problems. On the other hand, sacral chordomas usually are not diagnosed until a physical lump is observed. They may cause symptoms like weakness in the arms and legs and dizziness (18).

The WHO classification of chordoma is dependent on the histological properties (3). The most commonly diagnosed chordoma type is conventional, represented by a “bulky” appearance. Histological preparations demonstrate a lobular fraction that compromises tumor cells, trabeculae, and nests within a myxoid matrix. The chondroid chordoma, a subtype of conventional chordoma, has a hyaline cartilage appearance in some regions within the sections (19). Also, conventional chordoma can be distinguished by brachyury protein expression. Brachyury is a transcription factor functioning in embryonic development, notochord development specifically. Unlike many neoplasms, e.g., chondrosarcoma, renal, colorectal, and hepatocellular carcinomas, chordoma cells have nuclear localization and expression of brachyury. Brachyury expression implies a distinctive and sensitive immunohistochemical marker for differential diagnosis of chordoma (20).

Dedifferentiated chordoma contains both conventional chordoma and high-grade sarcoma properties. These two counterparts are either abruptly transitive or intermixed (3). Poorly differentiated chordoma is a rare type affecting females more than men in children or young adults. It generally occurs in the clivus, but a few cases of sacral origin is presented. Unlike conventional chordoma, poorly differentiated types do not contain physaliphorous cells, and myxoid stroma is nonexistent or only exists locally. The primary diagnostic marker is the loss of SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily B member 1 (SMARCB1) expression. The loss of SMARCB1 expression exerts its effect on histological characteristics such as focally rhabdoid morphology. Also, nuclei structure differs from round to ovoid. The cytoplasm is defined as eosinophilic, and periodic acid Schiff staining reveals positivity (19,21–24).

The differential diagnosis of chordoma also depends on the triple positivity of immunohistochemical stainings of cytokeratins (CK; CK8, CK18, and CK19), embryonic membrane antigen (EMA), and S100 protein (25,26).

1.1.2. Molecular Biology of Chordoma

Karyotyping involves the pairing and ordering of chromosomes exhibiting the chromosomal complement of an organism (27). Cytogenetic studies began with the discovery of Tjio and Levan, demonstrating that human cells have 46 chromosomes (28). In 1962, with the discovery of the Philadelphia chromosome, it was proposed that cancer results from a chromosomal abnormality (29). Later in 1973, the formation of the Philadelphia chromosome was revealed as a result of a translocation between the long arms of chromosomes 9 and 22 (30). Those discoveries led to a new era of cancer diagnosis, and genetic evaluation is considered a valuable tool for diagnosing, classifying, progressing, and emerging specialized therapeutical approaches (31). In 2008, the cytogenetic-based categorization of acute myeloid leukemia was introduced by WHO to clinical applications (32). Therefore cytogenetic evaluation has become a golden standard in clinical utilization for cancer diagnosis.

Chromosomal abnormalities are observed as late-onset events in chordoma progression (33). Though, not all chordoma cases exhibit abnormal chromosomal profiles. Chordoma karyotyping revealed the loss of chromosomes 1, 3, 4, 9, 10, 13, and 18, while a

chromosomal gain in 7 and 20,2–4,9,31–33,36 chromosomes is observed. However, none of those chromosomal loss or gain cases found chordoma-specific breakpoints (34). In 2011, chordomas were defined as having notably unstable genomes. In all chordoma samples used in the study, partial or complete loss of chromosome 1p is determined. Other detected chromosomal losses (3, 4, 9p, 9q, 10, 13, 14, 18, and 22) and gains (7 and 19) were similar to those previously presented (35).

Loss of heterozygosity (LOH) is a frequently observed genetic event during tumorigenesis. The loss of heterozygosity results in a differential genetic landscape between tumor and healthy cells, which may be used to determine novel therapeutic targets (36). During tumorigenesis, the loss of specific chromosomal regions results in the inactivation of tumor suppressor genes.

The first introduced LOH in chordoma belongs to intron 17 of the retinoblastoma gene. Only 2 of 7 skull base chordoma samples showed the LOH in the retinoblastoma gene. Still, those were aggressive tumors implying an association between the aggressiveness of skull base chordoma and related LOH (37). Most recently, it has been demonstrated that 70% of conventional chordoma (sacral origin) has LOH of chromosome 3q, which is associated with prolonged relapse-free survival (38). Microsatellite instability studies also revealed LOH patterns of chordomas (39,40).

Due to the rarity of chordoma, genome-wide studies are limited, or the sample size is small. The largest sample size contained 104 sacral chordoma samples, but genome-wide sequencing only applied to 11. The results of this study revealed that 27% of the cases exhibit brachyury duplications. Also, significant mutations of the phosphoinositide-3 kinase (PI3K) signaling pathway-related genes were detected in 16% of cases. A frequent inactivating mutation of the lysosomal trafficking regulator (LYST) gene was found in 10% of cases proposing a novel chordoma marker (41). In 2021, the genome sequencing of 80 skull-base chordoma samples identified mutated polybromo 1 (PBRM1) as a driver gene. The most frequently detected event was the PBRM1 alterations and deletions in the cyclin-dependent kinase 2A/2B (CDKN2A/2B) locus. Chromosome 22q deletion involves the loss of the SMARCB1 gene. With PBRM1 mutation and 22q deletion, a strong relationship with poor survival has been observed (42). The latest research mapping the genetic dependencies of chordoma used a loss-of-function approach introduced by the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)- Cas9 system (43). Additional to

previously known dependencies, this study provided a range of new gene lists, including Protein Tyrosine Phosphatase Non-Receptor Type 1 (PTPN1), RNA-specific adenosine deaminase 1 (ADAR), Protein Activator Of Interferon Induced Protein Kinase EIF2AK2 In (PRKRA), Putative RNA-binding protein Luc7-like 2 (LUC7L2), Solute Carrier Family 2 Member 1 (SLCA2A1), Solute Carrier Family 7 Member 5 (SLC7A5), FA Complementation Group M (FANCM) and THAP Domain Containing 1 (THAP1) (43). Alongside this list, Sharifnia et al. represent a correlation between ADAR dependence and interferon-stimulated gene expression, which is up-regulated in chordoma. Also, confirmative results were obtained regarding the therapeutic potential of chordoma's genetic dependencies (43). In another recent study, a 60-year-old female skull base chordoma patient was identified with a frame-fusion between exon 5 of Rabphilin 3A Like Domains (RPH3AL) and exon 2 of Telomerase reverse transcriptase (TERT). TERT gene rearrangements were detected in only 3.6% of a chordoma cohort constituting 55 patients. TERT rearrangement-positive chordoma cases exhibited low tumor mutational burden (TMB) with no specific driver mutation (44).

Receptor tyrosine kinases (RTKs) are plasma membrane receptors with enzymatic activity. They catalyze the transfer of phosphate groups to another protein, which subsequently mediates cellular processes such as proliferation, motility, and differentiation. All RTKs consist of a ligand binding domain, a transmembrane region, an intracellular region, a tyrosine kinase domain, and a carboxyl-terminal tail (45). Dysregulation of RTK signaling is a prominent characteristic of cancer cells. Mutations, up-regulation of gene expressions, chromosomal abnormalities, and autocrine activation are among the factors that regulate excessive RTK activation (45).

Several studies were conducted on the expression of RTKs and their downstream molecules in chordoma. Mainly, epidermal growth factor receptor (EGFR), hepatocyte growth factor receptor (c-MET), and platelet-derived growth factor receptor (PDGFR) were prominent RTKs. In a study comprising 160 patients, EGFR expression was positive in 69% of all cases. 38% of cases were observed to have high-level copy number gain. Further, phospho-receptor tyrosine kinase arrays were performed in the chordoma cell line U-CH1 and three chordoma patients, revealing aberrant activation of EGFR signaling in chordoma (46). Also, high levels of EGFR were associated with poor prognosis in chordoma patients (47). One of the most common chromosomal abnormalities in chordoma involves chromosome 7, in

which the c-MET gene resides. In a study containing skull-base chordoma samples, c-MET was positive in 70% of primary cases, whereas positivity increased to 80% in recurrent cases (48). Younger patient age and high c-MET expression are correlated with more prolonged survival. However, the c-MET expression was also associated with matrix metalloproteinase expression, which may contribute to the invasive ability of chordoma cells (48). In a study containing 49 chordoma patients, none of the recurrent chordoma cases were positive in c-MET staining, supporting the strong correlation between c-MET expression and recurrence (49). Up-regulation of PDGFR- β expression and functional activation is common in chordoma. Several attempts to treat chordoma patients with PDGFR- β suppressors yield promising results leading to clinical trials (50). Evaluation of 31 chordoma patients exposed that PDGFR- α is expressed to a lesser extent than PDGFR- β but phosphorylated. Thus, PDGFR- α is also considered activated in chordoma (51). Similar to c-MET, younger patient age was correlated with higher PDGFR- α expression. The study comprising 52 primary and 104 recurrent chordoma cases showed that 75% exhibit PDGFR- α positivity. The higher PDGFR- α expression is associated with poor prognosis (47).

Immune checkpoints regulate immune response initiation, maintenance, termination, and self-tolerance. The duration and amplitude of the immune response are crucial to prevent immune disorders (52). Immune checkpoints exert inhibitory functions via signaling through mono-tyrosine motifs like immunoreceptor tyrosine-based inhibitory motifs (ITIM). Many inhibitory receptors have also been identified in cancer cells and studied regarding their role and therapeutic potential. Examples of immune checkpoints include but are not limited to programmed cell death protein 1 (PD-1), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and lymphocyte-activation gene-3 (LAG-3) (53). PD-1/ PD-L1 axis regulates the cytotoxic T-cell response, intervening the anti-tumor responses (54).

In 2015, the PD-1/PD-L1 axis was studied in chordoma for the first time. The study revealed that PD-1/PD-L1 is not constitutively expressed in chordoma cell lines. However, their expression is induced upon interferon-gamma (IFN γ) exposure. PD-L1 expression is also found to be up-regulated upon long-term tumor necrosis factor-alpha (TNF- α) exposure (55). Regardless of the non-significant expression of PD-L1 in chordoma cells, tumor-infiltrating macrophages and lymphocytes were detected as positive for PD-L1 expression. On the other hand, tumor-infiltrating lymphocytes were positive for PD-1 expression in 3 of 6 tissue samples (56). The same research group performed tissue microarray for nine chordoma

samples and detected positive PD-L1 expression in 94.4% of all cases (57). Correlation studies demonstrated that PD-L1 expression is significantly associated with metastatic status and the presence of tumor-infiltrating lymphocytes (TILs) (57).

1.1.3. Diagnosis and Treatment of Chordoma

Radiological techniques are used as a typical method for the diagnosis of chordoma. Osteolytic lesions and soft-tissue masses are observed on radiographs of chordoma tumors. Rarely intratumoral calcification may be present. Magnetic resonance imaging (MRI) is the best option for chordoma diagnosis. Characterization is performed using T1/T2 weighted and post-contrast settings. However, the best identification of chordoma is obtained in the T2 setting because the vacuolated structures containing high fluid components yield a hyperintense signal (58). As T2 is much more specific, T1 signals may be variable among the cases (59). In addition to MRI, computed tomography (CT) is recommended since the intratumoral calcifications could be detected at better resolution. CT scan also is used to detect the extent of the tumor (60). Positron emission tomography (PET) is usually a helpful method in cancer diagnosis. The principle of PET scans relies on cancer cells' rapid glucose consumption yielding a radiance (61). However, PET scan is not commonly used for chordoma detection since the chordoma cells slow grown cells and have a slower metabolism than any other neoplasm (62). Imaging techniques are only sufficient to present the chordoma suspicion, but a differential diagnosis is required following the biopsy (62).

The Food and Drug Administration (FDA)-approved chemotherapeutics for chordoma treatment is unavailable (63). Conventional treatment options include surgery or radiation therapy (59). Also, both chordoma-specific and chordoma-included clinical trials are ongoing. Chordoma-specific clinical trials are at phase 2 and embrace locally advanced, unresectable, and metastatic chordoma cases. Currently, Anlotinib Hydrochloride for tyrosine kinase inhibition (VEGFR, PDGFR, and EGFR targeting) (NCT04042597), Cetuximab for EGFR inhibition (NCT05041127), Nivolumab and relatlimab combination for checkpoint inhibition (NCT03623854), and Palbociclib for CDK4/6 inhibition (NCT03242382) are under investigation. Chordoma-included clinical trials compromise EGFR and transforming growth factor-beta (TGF- β) inhibitor combination with PD-1 inhibitor Pembrolizumab (NCT04429542), CDK9 inhibitor KB-0742 (NCT04718675),

Brachyury vaccine (NCT04246671), and combination of an oncolytic virus, Talimogene laherparepvec, a checkpoint inhibitor, nivolumab, and a chemotherapy drug, trabectedin (NCT03886311) (64).

1.2. NATURAL KILLER CELL BIOLOGY

The immune system fights pathogens and disease-causing changes in the body and recognizes and neutralizes hazardous materials from the environment (e.g., allergens) (65). The immune system is complex and mainly classified into innate and adaptive immune systems. The fundamental principles of the eukaryotic immune response are evolutionary conserved, but distinct differences exist between organisms (66). One important conserved property is immunological memory (67). The rudimentary form of memory, exerted by innate immunity, is a consequential outcome of chromatin structure rearrangement and induces an enhanced but non-specific response upon reinfection. Adaptive system memory, on the other hand, is a much more complex mechanism originating from epigenetic changes and genetic recombination, resulting in both enhanced amplitude and specificity of the immune response (67).

The innate system is evolutionarily ancient and responds to an infection or a disease as the first line (68). The defense mechanism of the innate system is divided into physical barriers (skin and mucosa), cellular components (e.g., effector cells, receptors, soluble factors), and humoral components (complement system, contact system, naturally occurring antibodies (NAb), and pentraxins) (69–71). Effector cells encompass granulocytes, monocytes/macrophages, dendritic cells (DCs), innate lymphoid cells (ILCs), epithelial and endothelial cells, and platelets (70). Effector cells mediate the immune response for maintaining cellular homeostasis. Pathogen recognition, phagocytosis of pathogens and cellular debris (by granulocytes), cytokine, protein and enzyme production and secretion, and cell lysis are the mechanisms that effector cells use (70).

ILCs, localized at epithelial surfaces and lacking acquired antigen receptors, exert a similar function with T-cell subsets acting like their innate counterparts (72–74). Unlike T-cells, clonal selection and expansion upon stimulation are not characteristics of ILCs. They respond to signals from infected/injured/transformed tissues producing cytokines and

mediating a prompt immune response (75). Therefore, it can be emphasized that ILCs represent the early responder and highly reactive effector components of the innate immune system by the rapid activation through stress signals, microbial peptides, or cytokine induction (75). Cytokine and transcription factor profile is used for the classification of ILCs and mainly subtyped into cytotoxic (Natural killer (NK) cells) and helper (ILC-1/ 2/ 3)-ILCs (76–78). ILCs may also be classified into three leading groups 1) ILC-1 and NK-cells, 2) ILC-2, and 3) ILC-3 and lymphoid tissue inducer (LTi) (79). Indeed, the NK cells are the best characterized and known subtype of the ILCs (76).

1.2.1. Natural Killer Cell Production, Development, and Maturation

In the early 1970s, lymphoid cells subtyped into two as B and T-cells. However, later in 1973 and the following years, a new group of effector cells was discovered with a spontaneous cytotoxic effect requiring no prior stimulation. Rolf Keissling et al. identified that this novel subtype of killer cells was genetically controlled and capable of eliminating tumorigenic cells. After all, Keissling referred to those cytotoxic cells as "Natural" killer (NK) cells (80,81).

The bone marrow is the well-established primary site of NK cell development (82). However, evidence demonstrates that different populations of NK cells might arise in the thymus, liver, or lymph node however, whether those are distinct NK cell populations or just poorly matured bone marrow-originated cells (83–85).

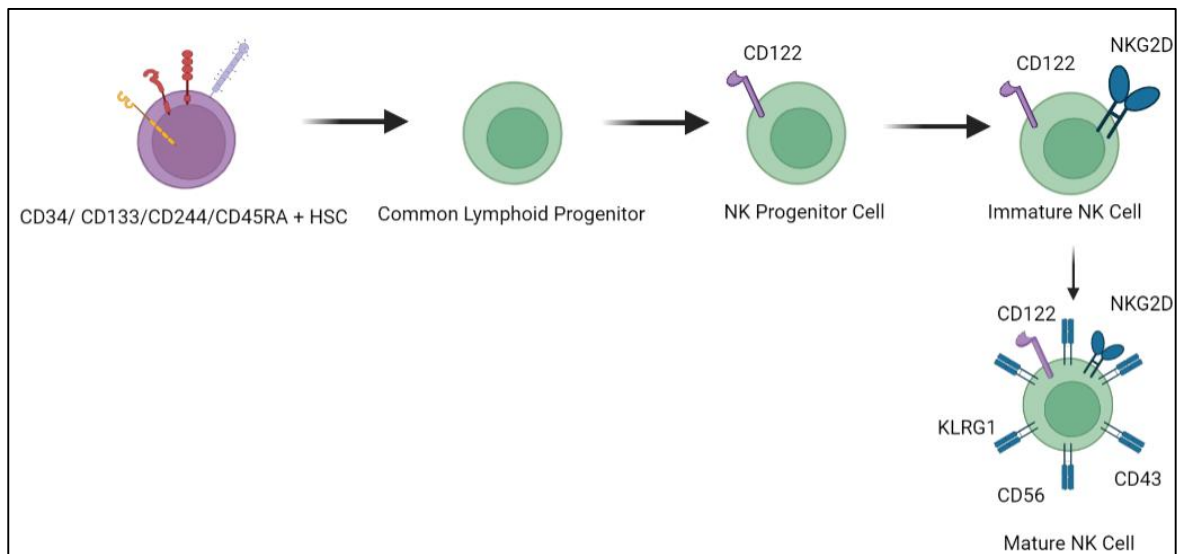


Figure 1.1 Developmental stages of natural killer cells (illustrated by BioRender) (99).

The acquired expression of CD45RA by CD34/CD133/CD244 positive hematopoietic stem cells (HSCs) leads to a fate towards differentiation into common lymphoid progenitor (CLPs) cells (86). CLPs further differentiate into pre-T cells, NK cell progenitors, pro-B cells, and other ILCs (87). NK cell progenitors are classified depending on the sequential maturation stages as follows stage 1 (NK cell progenitors), stage 2 (pre-NK cells), and stage 3 (immature NK cells) (88). Continuous CD34 expression sustains the differentiation potential into other lymphoid cells (e.g., T-cells). However, the downregulation of CD34 and acquisition of IL-2 receptor β chain expression (CD122) direct the irreversible differentiation of CLPs into NK cell progenitors (NKP) (88,89). NKP progression towards immature NK cells (iNK) proceeded by transcription factor activation (e.g., an inhibitor of DNA binding 2 (Id2), E4-binding protein 4 and NKG2D/DNAX-activating protein of 10 kDa (DAP10), an activation receptor, expression. The expression of NK cell receptors (e.g., NKG2A, DNAM-1) and cell adhesion molecules such as Leukosialin (CD43) defines the iNK stage (90–96). Mature NK cells are characterized by the expression of CD244, CD122, Nkp46, DX5, CD11b, and Ly49, besides the lack of expression of CD3, CD27, and TNF-related apoptosis-inducing ligand (TRAIL) (97). CD56, neural cell adhesion molecule, expression is not a unique feature of NK cells, yet an important one (98). Mature NK cells are further subtyped depending on the CD56 expression levels, such as CD56^{dim} and CD56^{bright}. CD56 negative NK cell population also exists and is associated with dysfunction since their presence in healthy individuals is very rare. Increased levels of CD56 negative population were observed in pathologies, such as human immunodeficiency virus (HIV)

infections, chronic hepatitis C, or autoimmune diseases (99–101). Why immune cells express CD56 needs to be better understood, but its role in immune activation is undeniable (98). Previously, the CD56^{dim} NK cells were believed to produce pro-inflammatory cytokines, whereas the CD56^{bright} NK cell subset claimed to be the cytotoxic counterpart. Instead, CD56^{bright} and CD56^{dim} NK cell populations differ in responsiveness against the target. For instance, CD56^{bright} NK cells respond better to soluble factors, while CD56^{dim} NK cells respond better against receptors (102). CD56 expression levels might be adjusted upon activation or immunosuppression. The downregulation impairs the cytotoxic function (103–105). During NK cell maturation, most iNK cells differentiate into CD56^{dim} population and are also the prominent NK cells found in the peripheral blood. The final stage of CD56^{dim} NK cell maturation is the Leu-7 (CD57) expression (106). Conversely, only a minority (approximately 5%) of iNKs converted into CD56^{bright} population (107–109). The interplay between bright and dim populations is a two-edged sword in anti-tumor activity. Even though the CD56^{bright} cells exert the primary cytotoxic function, the induction of an inflammatory environment by cytokine production exacerbates tumor progression (107,109).

1.2.2. Natural Killer Cell Education

Self-major histocompatibility complex class 1 (MHC-1) and NK cell inhibitory receptor interactions provide the self-tolerance. Self-tolerance and functional integrity is obtained by a fine-tuning process called NK cell education (110,111). Different education mechanisms (classical MHC-1 dependent (licensing), non-classical MHC-1 dependent, and MHC-1 independent) elucidate the reaction variability and inhibition sensitivity observed between the individuals (112,113). Diversity of NK cell responsiveness directed by the germ-line encoded ligands and their associated killer cell immunoglobulin-like (KIR) receptors (110). Education mechanisms embodied by inhibitory (arming) /activating (disarming), rheostat and receptor distribution-cell adhesion (confining) models (112). Uneducated NK cells either do not express inhibitory receptors or have low binding affinity to self- MHC-1 molecules. Therefore, the uneducated NK cells are non-autoreactive but hypo-responsive to the MHC-1 deficient targets.

The downstream signaling cascade of licensing and arming model remains to be discovered (113). Yet, it's clear that a higher effector function is achieved when the expression of self-MHC molecules and associated receptors are concurrent. In brief, licensing and arming models postulate the functional enhancement of responsiveness by inhibitory receptors (110,113). Disarming model, on the other hand, proposes the retention of NK cell reactivity by inhibitory signaling. Chronic stimulation triggers NK cells to enter an anergic state and become hypo-responsive (113,114). Disarming models utilize this information to hypothesize that all NK cells are notably responsive but lose the effector function due to an anergy state. Hypo-responsive NK cells are rescued by inhibitory signaling caused by the interaction of self-MHC molecules and associated receptors (115). The rheostat model of education proposes the adjustability of the effector potential. Accordingly, NK education is enhanced as the inhibitory receptor or self-MHC ligand availability increases. It can be emphasized that tuning the educated state depends on the quantitative interactions of inhibitory receptors and self-MHC ligands (113,116–121).

Non-classical MHC molecules (e.g., Human Leukocyte Antigen (HLA)-E/G/F, MHC class I chain-related A/B) and inhibitory receptor (except KIRs) interactions are another mechanisms of education. The prominent receptor in non-classical MHC-dependent education is the C-type lectin-like receptor CD94/NKG2A heterodimer (122–124). In fetal studies, KIR-expressing NK cells could not educate the NK cells. In contrast, NKG2A-expressing NK cells get educated not only circulating adult NK cells but also fetal NK cells, proofing NKG2A proficiency in education (125). However, distinct mechanisms between KIR and NKG2A-dependent education remain unknown (112).

Signaling lymphocytic activation molecule (SLAM) family member 6 (SLAMF6), the C-type lectin-like receptor NKRP-1B, and T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (ITIM) domain (TIGIT) receptors are distinguished inhibitory receptors interacting with non-MHC molecules. The absence of either the receptors or related ligands impairs NK cell target recognition and cytotoxic effects (112,126–130). Therefore, NK self-tolerance and self-recognition are regulated by educating the cells through MHC-1 independent mechanisms.

Upon viral infections, infected cells suffer from the lack or diminished levels of HLA molecules on the surface (131,132). Similarly, tumorigenic cells have reduced HLA-1 expression concurrent with the increased expression of NK-activating receptor ligands (133). In this case, infected and tumorigenic cells are recognized as “missing self” by the educated NK cells. NK cells also play a crucial role in allogeneic organ transplant applications. If the donor HLA-1 and recipient KIRs are mismatched, foreign cell recognition may result in graft rejection and destruction (134).

1.2.3. Natural Killer Cell Activation and Cytotoxicity

NK cells recognize danger signals and self-missing cells and exert cytotoxic effects toward foreign or infected cells. First, though, cellular activation requires a threshold of activation signals. If the activating signals exceed the inhibitory signaling, then an activating immune synapse formation occurs at the NK and target cell contact. The requirement of such connection to initiate the effector functions provides specific targeting without affecting the surrounding healthy cells (135,136). The cytotoxic response may be directed by lytic granule secretion or induction of death receptor-mediated apoptosis (137).

NK cell receptors (NCRs) encompass transmembrane inhibitory and activating receptors such as KIRs, CLRs, and natural cytotoxicity receptors (NCRs). NCRs, belonging to the immunoglobulin superfamily, help to distinguish NK cells from T cells in function since responding to targets requires no prior immunization (138). Currently, NCR expression is also identified in T cell subsets. However, the function is assumed independent of the T-cell receptor signaling. It has been postulated that T-cell subsets utilize NCR signaling for rapid response, surpassing the antigen-restricted responses (139). NCR expression on the T-cell surface has also been considered an indication of chronic activation and subsequent immune desensitization (139). In the NK cell aspect, they are important in mediating cytotoxicity and immunosurveillance. NCRs include NKp46, NKp30 (found in resting/activated NK cells), and NKp44 (induced upon activation) (140,141). Those receptors have both activating and inhibiting immunoligands. For example, NKp44 has an ITIM domain that is inactive until the binding of proliferating cell nuclear antigen (PCNA) (142). The most well-known ligand is natural killer cell cytotoxicity receptor three ligand one interacting with NKp30 (B7-H6; NCR3LG1). B7-H6 is widely expressed in tumor cells (e.g., gastric cancer, leukemia) and

induced upon the inflammatory state (143). Other ligands (e.g., Bcl-2 associated athanogene 6) were identified for NKp30, but B7-H6 is the only one expressed on the cell surface (143,144). Once the adaptor proteins (e.g., DNAX activating protein; DAP12) mediate receptor phosphorylation and subsequent recruitment of sarcoma homology 2 (SH2) domains of spleen tyrosine kinase (Syc)/zeta-chain-associated protein kinase 70 (ZAP70), NCRs initiate the cell activating signalization through immunoreceptor tyrosine-based activation motives (ITAMs) (145). NKp46 and NKp44 cross-linking may result in proinflammatory (IFN γ and TNF) cytokine production supporting dendritic cell maturation. In this aspect, NK cells are considered effectors and immune regulatory cells (139).

Natural killer group 2D (NKG2D), a calcium-dependent lectin-like receptor, is not unique to NK cells, but unlike other cell subsets, it directly mediates cytotoxicity (146). NKG2D has two isoforms, NKG2D-short (interact with DAP10/12) and NKG2D-long (interact with DAP10). Humans only have the NKG2D-long isoform, and by phosphorylation (aided by DAP10), phosphatidylinositol 3-kinase (PI3K) and growth factor receptor bound protein 2 (GRB2) are recruited initiating the cytotoxicity and cell survival-related signaling cascades (147,148).

Activation of PI3K and GRB2 signaling cascade consequently results in actin cytoskeleton recombination and transcription factor activation. Rearranged actin cytoskeleton enables immunologic synapse formation, whereas activated transcriptional factors are crucial in proinflammatory cytokine secretion, which induces cell death pathways (149).

Adhesion molecules (e.g., Lymphocyte function-associated antigen 1; LFA-1, and Macrophage-1 antigen; Mac-1/CD11b) gather into the outer space of the immunologic synapse forming the peripheral supramolecular activation cluster (pSMAC). Adhesion molecule gathering contribute to actin polymerization and not only provide the tight conjugation of the cells but the subsequent signaling is critical in the phosphorylation of signaling molecules such as ZAP70 (150,135,151,152). The pSMAC formation leads to activating receptor accumulation at the center of the immunological synapse, which is referred to as the central supramolecular activation cluster (cSMAC) (135). Activating receptors synergise in signaling and trigger actin polymerization at the cell-cell connection site. The rearrangement of actin cytoskeleton by both activating receptor signaling and adhesion molecules leads to microtubule organizing center (MTOC) translocation along with lytic granules to the immunological synapse (152). Cytoskeleton rearrangement is pivotal to

inducing the cytotoxic function. Impairment in this reorganization inhibits NK cell adhesion and lytic granule translocation and secretion, circumventing the NK cell cytotoxicity (153).

Lytic granules comprise a bilayer membrane structure, lysosomal enzymes, anti-microbial peptides, and proteins. Proteins include; perforin, granzymes (A/B/M/H/K), Fas ligand (FasL), and TRAIL. Lytic granules within NK cells are not monotypes but rather exert heterogeneity with type I, type II and intermediate granules subtypes. Type I granules (50–700 nm) contain a dense core and encircled with thin layer of vesicles. Type II (200–1000 nm) and intermediate (<50 nm) granules both contain multivesicular bodies but differ in abundancy (more abundant in type II). They considered as lytic granule forms observed in developmental stage while type I proposed as mature (154). The dense core include chondroitin-sulfate proteoglycan serglycin, perforin and granzymes whereas multivesicular bodies encompass cathepsin and lysosome-associated hydrolases (155). Lysosomal lumen acidification, protein import, membrane fusion, and translocation of degraded products to cytoplasm require lysosomal membrane proteins (156). However, the function of those protein varieties remains elusive (152). Lysosome-associated membrane protein (LAMP)-1 (CD107a) is one of the most abundant ones found in the granule membrane. Although LAMP-1 aggregates at the synapse, indicating a potential utilization in membrane internalization, the exact function remains unknown. Additionally, LAMP-1 used as a granulation marker for NK cells (151,157).

Upon activation, NK cells do not secrete the whole lytic granule repertoire instead a subset within the pool is secreted (158). Although the polarized lytic granules are not fated to secretion and require additional molecular events to be transefer and fuse to membrane, it demonstrated that in human NK cells, the translocation of lytic granules along with polarized MTOC are sufficient to deliver them to the secretory area (159,160). The plasma membrane fusion is aided by soluble N-ethylmaleimide-sensitive factor adaptor proteins receptors (SNARE) family members. SNARE proteins and lytic granules constitutes a complex and subsequently a fusion pore is formed. Those pores might be fully-open, transient or incomplete. Incomplete pores retain the most of the content within the cell while completely open pore associated with maximal and rapid secretion (161).

Once the lytic granules are secreted, calcium and neutral pH inside the cell mediate the self-recruitment of perforin to the membrane and create pores. Pore formation enables the release of vesicular content of lytic granules such as granzyme. In this aspect, perforin function is

indispensable since even a slight decrease impairs the cytotoxic function (162,163). Apoptosis is either induced via caspase-dependent or caspase-independent pathways. Irrelevant from the apoptotic pathway, reactive oxygen species (ROS) increment, mitochondrial damage and consequent cytochrome C release, and DNA fragmentation is the induction mechanisms (164). All types of granzymes involve in apoptotic induction. Granzyme B can cleave a range of caspases and pro-apoptotic protein Bid (Granzyme K cleaves Bid as well), resulting in both caspase-dependent and caspase-independent apoptotic pathway induction. Granzyme A/B/K cleaves DNA repair enzymes such as Poly (ADP-ribose) polymerase (PARP). Granzyme M also induces caspase-independent death. However, unlike the granzymes A and K, the mitochondrial damage pathway is not involved in Granzyme M-induced apoptosis since ROS production, and cytochrome C release is not observed (152,165).

In recent years, the importance of NK cell-mediated cytotoxicity has gained prominence due to its potential in clinical utilization, especially for cancer therapies. Therefore, increasing knowledge of NK cell regulating mechanisms is essential to enhance clinicians' potential to generate individualized and targeted NK cell-mediated approaches.

1.3. IMMUNE SURVEILLANCE OF CANCER

Immunosurveillance is a crucial host-defense mechanism that involves a harmonized cooperation of innate and adaptive immune systems. The surveillance directs rapid eradication of invading pathogens and transformed cells. However, inhibition of cancerous growth is compelling due to evolving evasion mechanisms by cancer cells (166,167).

In 1909, Paul Ehrlich first postulated that the host-defense mechanism might prevent cancerous growth. However, the inadequacy of knowledge and the insufficiency of experimental procedures and tools precluded the proof of this hypothesis (168). In the 1950s, the concept of immune system recognition and subsequent elimination of cancerous cells was proposed by Burnet and Thomas. (169). Burnet considered the existence of neoantigens, specific to cancer cells, might direct the immunological recognition and consequent response leading to eradication of the cancerous growth (170,171). On the other hand, Thomas's postulation was based on evolutionary development as complex organisms acquire protection mechanisms against neoplastic disease. He thought those mechanisms might be

similar to homograft rejection responses (172). In the later years, studies demonstrated that chemically and virally induced tumor formation in mice considerably correlated with Burnet's and Thomas's postulations and served as the proof-of-concept (173,174). It was observed that athymic nude mice had not shown an increased incidence of tumor formation compared to wild-type mice. Because of this outcome, it was initially thought that the immune response was not against cancer but against transforming viruses (169). Currently, it is known that athymic nude mice's immune system compromise NK cells but fewer T cells than wild-type mice. The presence of immune cells contributes to immunosurveillance. Though chemically induced tumor formation varies, enhanced tumor formation was detected in both athymic nude mice and immunodeficient (SCID) mice from wild-type mice (175–177). This variation also suggests the importance of the presence and quantity of immune cells in surveillance. The comprehensive research over the years revealed that surveillance is just a part of the immunological control over cancer cells due to the paradigm of tumor formation despite the immunosurveillance. Immunodeficient and immunocompetent mice-derived tumors exert differential immunogenicity proposing that the immune system is a selective pressure for cancer cells but an induction, at the same time, to modulate the surrounding immune system. This modulation process referred as immunoediting which is the currently accepted hypothesis (178–180). Interplay between host immune system and tumor cells are divided into three phases; elimination, equilibrium and escape (181).

The elimination phase is a successful consequence of immunosurveillance. Elimination step processing is investigated into four phases. As the tumor size increases, cells require vascularization and stromal remodeling for nutrient supply which induces pro-inflammatory signaling. Phase 1 of elimination compromises the innate immune cells' recognition and cytotoxicity by the recruitment to the tumor site due to the tumor-induced pro-inflammatory signaling (182,183). IFN- γ is secreted from innate cells, which exerts anti-proliferative and anti-angiogenic effects while inducing apoptosis (184–186). Phase two involves the maturation and recruitment of dendritic cells (DCs). Immature DCs ingest the necrotic cancer cells, matured under a pro-inflammatory environment, and migrate to tumor-draining lymph nodes for antigen-presentation to the T-cells (181). In phase three- tumor-infiltrating macrophages and NK cells continue to exert cytotoxic function. Meanwhile, in tumor-draining lymph nodes, naïve CD4 positive T-cells differentiated into effector CD4

positive T-cells by the antigen presentation by DCs. Thus, clonal expansion of CD8-positive T-cells specific to the presented antigen is triggered (187). Final stage (phase four) comprehend the homing of tumor antigen specific CD4- positive and CD8 positive T-cells (181,188). Elimination process through a cascade induced by pro-inflammatory signaling. If this signaling manipulation does not occur, how the immune system gets activated is unclear. The danger theory hypothesis postulated that tumor cells do not provide adequate pro-inflammatory signals to activate the immune cells. In this case, tolerance or responsiveness may happen (183). Still, danger signals (e.g., uric acid) may be sufficient to trigger pro-inflammatory signaling and activate innate immune cells against pathogens. Danger signals directly stimulate DC maturation and following T-cell priming (189,181).

The equilibrium phase of the immunoediting hypothesis is the most putative stage, as more evidence is urgent to prove its existence. It is postulated that the equilibrium stage is an interphase between the end of the elimination and the beginning of the evasion. The cells in equilibrium are proposed to be in a latent stage similar to tumor dormancy (178). Even the immune system applies selective pressure and eliminates a part of cancer cells, increasing immune resistance-related mutations acquired in the arising variants. The acquired mutations provide a survival advantage in the immunocompetent host (178). Finally, the equilibrium phase is pushed into the escape phase since the emerged cells are reprogrammed by the surrounding immune cells and become familiar, developing resistance to elimination (178).

The immune response-related elimination of cancerous cells surpasses, and the growth continues in the escape phase. Immune system evasion constitutes complex molecular mechanisms include, but not limited to, decreased antigen representation by cancer cells, increased expression of immune cell inhibiting receptors (e.g., PD-1), protein production (e.g., Transforming Growth Factor- β (TGF- β)) that suppress the immune system or increase the angiogenesis.

Current research indicate that the immune and chordoma cell interaction might be directing the locally aggressive characteristics of chordoma, leading to immune evasion. Especially, PD-1/PD-L1 axis and correlations with overall and local recurrence free survival is extensively studied in chordoma. Accordingly, PD-L1 expression in tumor tissue is found to be associated with advanced stages while TIL expression of PD-L1 correlated with better survival outcomes. Contrary, PD-1 expression in TILs correlated with worse survival of the

patients (190,191). T cell immunoglobulin and mucin-domain containing-3 (Tim-3), a checkpoint receptor, is expressed in IFN- γ secreting T-cells and co-expression with PD-1 mediates the T-cell exhaustion and immunotherapy response (192,193). Galectin-9 interacts with TIM3 receptor and induces T-cell death. Chordoma is positive for Galectin-9 expression and TIM3 expressing in TILs which is predictive for invasiveness and prognosis (193). TILs and chordoma tissue also found to be expressing high levels of Cytotoxic T-lymphocyte Antigen-4 (CTLA-4), a checkpoint receptor, that is correlated with a worse disease-free survival rate (194).

Immunoediting is an emerging hallmark of cancer (195). Understanding of immunoediting mechanisms should be extended to evaluate more accurately the relationship between host immune cells and cancerous cells and to design more effective immune system-centered therapeutic strategies. The benefits and potential of immunotherapeutic approaches in the treatment of cancer are undeniable and need further investigation.

1.4. CANCER IMMUNOTHERAPY

Immunotherapeutics trigger or slow down the immune system to control immunologically related disorders such as immunodeficiencies, autoimmune diseases, and malignancies. Immunotherapeutics include drugs (e.g., immunosuppressors), biologicals (e.g., monoclonal antibodies), vitamins and minerals (e.g., vitamin C), adoptive cell transfer (e.g., T-cell transfer), transplantation (e.g., bone marrow) and immunizations (e.g., vaccines) to mediate the immune system reactions (196).

The focus on cancer treatment was resection of the tumor, chemotherapeutics, and radiation therapy for decades. In 2013, immunotherapy was the breakthrough of the year, and in 2018 Nobel prize winners were nominated for the discovery of inhibiting the negative immune regulation in cancer therapy (198,199). Developments in immunotherapy in oncology expedite. Currently, FDA-approved monoclonal antibodies (e.g., Imatinib Mesylate (Gleevec)), checkpoint inhibitors (e.g., pembrolizumab (Keytruda)), chimeric antigen receptor-T-cells (e.g., CD19-CAR-T (Kymriah)) and cytokines (e.g., IFN- γ and interleukin-2 (IL-2)) are in the use of cancer treatment (200–203).

Eradication of cancerous cells by immunotherapies relies on boosting the immune system and conceptualizing the effectiveness extended over the 19th century. For example, William Coley observed that his patient with a type of skin infection (erysipelas) had regressed osteosarcoma. Osteosarcoma regression in the presence of infection made him hypothesize if inducing erysipelas in patients might be a treatment. Later, the bacteria responsible for erysipelas was identified, *Streptococcus pyogenes*, and Coley started injecting his patients with the bacteria. Over many formulations Coley finalized his toxin as a combination of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* (204–206). In 1921, Albert Calmette and Camille Guérin developed a tuberculosis vaccine (BCG) constituting *Mycobacterium bovis*. Coley's toxins were under investigation for clinical utilization simultaneously encouraging the investigation of BCG for cancer treatment. During the 1970s, BCG was approved for use in bladder cancer patients. Still, the BCG vaccine is a standard therapy for treating high-risk nonmuscle-invasive bladder cancer patients. Until the discovery of the early implications of immune surveillance and experimental mice models (immunocompetent and immunodeficient models), the working mechanism for Coley's toxin was unknown. Extending understanding of immune system regulation and control over cancer cells increased the attention towards immunotherapeutics. Today, the essential aim of cancer immunotherapy is to circumvent immune evasion to mediate anti-tumor immunity (206).

1.4.1. Immunomodulators

Immunomodulators are widely used to stimulate or suppress the immune system. Tumors possess intrinsic resistance mechanisms to surpass the immune system elimination by creating an immunosuppressive environment through cytokines or inhibitory molecules. In cancer treatment, the fundamental goal of immunomodulatory agents is to overwhelm immunosuppressive mechanisms and boost the immune system.

1.4.1.1. Cytokine Therapy

Cytokines are soluble and small proteins with a molecular weight lower than 30 kDa. They may exist as polypeptides or glycoproteins acting in cell-to-cell communication. Cellular communication may be in an autocrine, paracrine, or endocrine manner inducing cellular signaling, which in turn induces growth and differentiation. Cytokine secretion occurs upon

stimuli, and the half-life is limited. Functionally, they are classified as pro- or anti-inflammatory cytokines. In cancer immunity, pro-inflammatory cytokines support the recruitment of immune cells to the tumor site, enhance antigen priming and improve the activation of cytolytic cells. However, pro-inflammatory cytokine balance in the tumor microenvironment is crucial since the increased inflammation exacerbates cancer progression (207–209).

Currently, only IL-2 (for metastatic renal cell carcinoma, metastatic melanoma) and IFN- γ (for hairy cell leukaemia, follicular non-Hodgkin lymphoma, melanoma and AIDS-related Kaposi's sarcoma) are FDA-approved regardless of the low efficiency (210–217). TNF- α (for high grade soft tissue sarcoma) is approved for cancer therapy in Europe (218,219). In addition, IL-12, IL-15, IL-21, and Granulocyte-macrophage colony-stimulating factor (GM-CSF) are under clinical trials for cancer treatment. Cytokines can be used both as monotherapy or as an adjuvant (220). For example, clinical trials are ongoing for using IL-15 as an adjuvant in adoptive T and NK cell transfer (NCT01875601, NCT02465957, and NCT01385423) (64,208). Due to the short half-life, a major drawback in cytokine treatment is the requirement of high doses in clinical usage. However, high doses employ adverse effects. Adverse effects are caused by the downstream cytokine cascade (involving IL-1, IL-6, and TNF- α), which is initiated upon systemic administration (208,221). Mild side effects include flu-like symptoms. However, the downstream cytokines may stimulate the hypothalamus and/or autoimmune conditions leading to depression or thyroid dysfunction (221). Though, high-risk and mostly seen adverse effect involves the cytokine storm. Cytokine storm is an immune dysregulation condition encompassing systemic inflammation and multiorgan dysfunction. Severe and uncontrolled cytokine storm reactions may lead to multiorgan failure (222).

1.4.1.2. Immune Checkpoint Inhibitors

Immune suppression may be directed in cell-dependent manner mediated by enhanced expression of inhibitory receptors both in cancer and immune cells (223). Inhibitory regulators of immune cells are fundamental for sustaining self-tolerance and maintaining the balance between the immune response's length, amplitude, and termination. For example, immune checkpoints are such regulatory mechanisms. However, in cancer cells, immune checkpoints are generally overexpressed, constraining the anti-tumor activity of the immune system. Such immune evasion mechanisms are partially surpassed by the revolutionary

development of immune checkpoint inhibitors (ICIs). The most prominent ICIs' mechanism of function involves the inhibition of PD-1/PD-L1 and CTLA-4. Many monoclonal antibodies inhibiting ICIs are FDA-approved, and clinical trials are also enduring (206,224,225).

To date, chordoma has no approved treatment. However, imatinib (targeting BCR-ABL, c-KIT, and PDGFRA) and broad spectrum tyrosine kinase inhibitor dasatinib is recommended for targeted therapy (226,227). First report on clinical response of chordoma patients towards immunotherapy was published in 2017. Three recurrent chordoma patient that failed to respond in standard therapeutics either treated with tumor-based vaccine or anti-PD1 antibodies (Pembrolizumab and Nivolumab). All patients had favorable clinical and radiological responses including tumor shrinkage (228). In 2020, second clinical report is published demonstrating the pembrolizumab response in a metastatic chordoma patient. Patient had a progression free survival 9.3 months and after 4 months from initial administration of pembrolizumab CT evaluation exhibited partial response (229). A retrospective study evaluated the clinical activity of ICI therapy in recurrent chordoma patients (230). As the results were promising exerting a favorable durability, benefits of ICI incorporation into first-line therapies of chordoma is supported (230).

1.4.2. Cancer Vaccines

Cancer vaccines are one of the most promising types of cancer immunotherapy. For many years, vaccines have been used to prevent and treat infectious diseases. However, to date, only a few cancer vaccines have been approved. Cancer vaccines are predicted to become more prompt within years, considering the approval processes of commonly used immunotherapeutics. For example, until the FDA-approval of rituximab and nivolumab, monoclonal antibody trials and PD-1 targeted therapy efficiencies were clinically inconsistent. Though, they eventually led to considerable success (231–233).

Cancer vaccine design principle is to induce an immune response to promote tumor regression, produce an anti-tumor memory while avoiding off-target and side effects (234). Cancer vaccines deliver tumor antigens combined with adjuvants. The delivered tumor antigens are aimed to provoke DC activation and subsequent T-cell response and memory. Thus, antigen selection, quality of the selected antigen, DC activation, T-cell response and

maintenance of the response are the fundamentals of cancer vaccines (234,235). The common strategies in antigen selection are tumor-associated (TAA) or neo-antigens. However, neoantigens has reduced efficacy in tumor mutational burden-low patients while TAAs need improvements to overcome tolerance-related responses (236).

In 2013, a yeast-based Brachyury-targeted vaccine is developed. GI603 was clinically tested in locally advanced and unresectable chordoma concurrently with the radiotherapy. However, phase II randomized study was discontinued early due to the inadequency of statistical power (237). Though, this trial failed to exert a synergistic effect of GI603 and radiotherapy, modified formulations are being tested. For example, modified vaccinia Ankara-Bavarian Nordic-Brachyury vaccine was reported to be well-tolerated and induce immune response against Brachyury and downstream cascade antigens in solid tumors. In chordoma patients it was well-tolerated as well and a partial efficiency was reported in phase I trials (238).

1.4.3. Adoptive Cell Transfer

Adoptive cell therapy (ACT) in cancer treatment involves autologous or allogeneic lymphocyte transfer to cancer patients. Clinical trials were initiated after discovering lymphocyte cell culture maintenance aided with IL-2 and *in vitro* cytotoxicity of cultured lymphocytes against cancer cells. 34% of metastatic melanoma patients reported to met the RACIST criteria, though only 5/29 response was complete. The addition of lymphodepletion prior to ACT increased the response rate to ACT up to 72%, while the complete response reached 20/93. ACT in melanoma studies focused on utilizing TILs which remains challenging to maintain and isolate from other cancers (239–241). In the ACT, the repetitious stimulation of allogeneic or autologous T-cells to cancer antigens is another approach (242). ACT has revolutionized by introducing genetic engineering of T cells expressing either T-cell receptor (TCR) or chimeric antigen receptor (CAR). The first example of CAR-T cells dates to 1989. Eshhar and colleagues fused the variable domain of an antibody to the CD3 ζ , a TCR constant domain, to redirect patients' T cells independently from MHC interactions (243). TCR engineering, on the other hand, dates to 2006 report of Rosenberg. He and his colleagues established an melanocyte-differentiating antigen recognizing TCR towards metastatic melanoma and demonstrated a promising effect on tumor regression (244). CAR

technologies are the most advanced ACT approach with six FDA-approved CAR-T therapy by 2022. Though, every improvement lead to new challenges and questions.

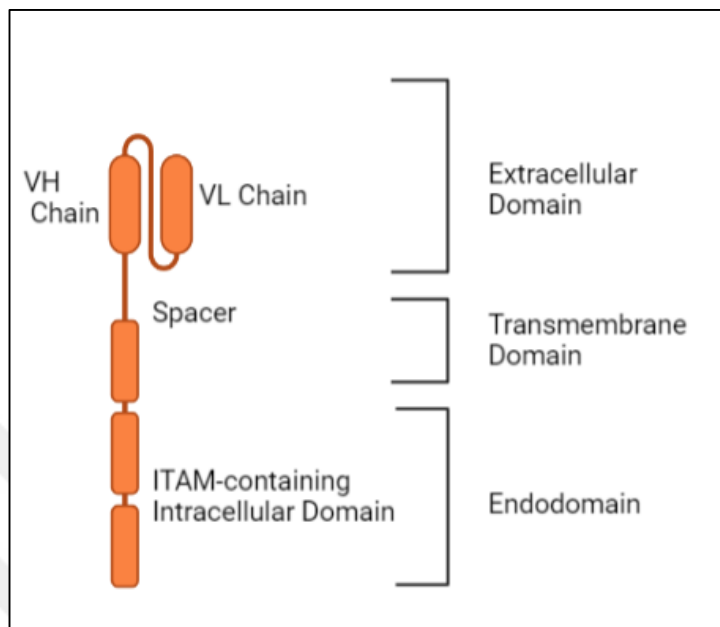


Figure 1.2 Developmental stages of natural killer cells (illustrated by BioRender) (99).

1.4.3.1. Chimeric Antigen Receptor Based Cell Therapies

The fundamental design of a CAR structure includes an extracellular domain linked to intracellular signaling domains that mediate and enhance T-cell activation. The extracellular domain consists of a single chain variable fragment (scFv) derived from variable heavy (VH) and variable light (VL) chains of an antibody. scFV region is conjoint to a spacer/hinge, which is joined to intracellular domains. The intracellular domain consists of an ITAM motif carrying protein, mostly CD3 ζ (245,246). Initial clinical results was dissappointing due to the poor persistence and expansion *in vivo*. Therefore, modifications in CAR structure was inevitable. The most potent regulation in CAR structure is the modulations of endodomain compositions. Currently, five generations of CAR structure are present; first, second, third, fourth (T cells redirected for universal cytokine-mediated killing, TRUCKs), and fifth(next generation).

First generation CARs consist of a single intracellular domain, CD3 ζ . However, clinical results yielded low cytotoxicity and proliferation due to the lack of co-stimulatory signaling along with cytokine signaling. Therefore, second and third generations focused on addition of co-stimulatory domains such as CD28, and 4-1BB. CAR structure refered as second generation if only one co-sitimulatory domain is added or third generation when two co-

stimulatory domain is joined to CD3 ζ . TRUCKs were designed as second generation CARs but with an addition of a cytokine (e.g., IL-12) whose expression either constitute or inducible. Fifth generation is the most advanced form of CAR structures that designed with the intention of providing the all required signals that physiologically drive T-cell activation, and proliferation. Similar to fourth generation, base design is the second generation CAR structure. Distinctly, fifth generation CARs comprehend a truncated cytoplasmic IL-2 receptor β -chain domain providing a binding site for STAT3 transcription factor. So that, antigen binding stimulates CD3 ζ and its signaling is enhanced by the co-stimulatory and cytokine signaling (247–249). CARs therapeutic improvements aim to avoid tumor escape and enhance cytotoxicity. Therefore, multitargeted (dual, tandem, and combinatorial CARs), syn-Notch co-receptor or different switch strategies (ON-switch, and universal CARs) were developed. Regardless of the design strategies, CAR-T cells' drawbacks (e.g., Graft vs. host disease (GVHD), cytokine storm, and neurocytotoxicity) are still a problem. Therefore, CAR-ACT introduced NK-cells because of their unique property of eliminating the HLA restriction and subsequent adverse effects (250).

2. METHODS AND MATERIALS

2.1. CELL CULTURE CONDITIONS

The human chordoma cell lines were obtained from Chordoma Foundation.

Table 2.1 Chordoma Cell Lines and Properties

Cell Line	Location	Status
U-CH1	Sacral	Recurrent
U-CH2	Sacral	Recurrent
MUG-Chor1	Sacral	Recurrent
JHC7	Sacral	Primary
CH22	Sacral	Recurrent
UM-Chor1	Clival	Primary

Chordoma cells were grown on 0.1% gelatin coated flask containing 4:1 ratio of IMDM:RPMI (GIBCO, UK) combination. Culture medium supplemented with 10% Fetal Bovine Serum (FBS) (Sigma F9665, Germany), 1% Penicillin-Streptomycin (Gibco, UK). Exceptionally, JHC7 were grown in DMEM-F12 (Gibco, UK) supplemented with 10% Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin.

HEK293T, and HEK293FT cells were purchased from ATCC and grown in DMEM (Gibco,UK) supplemented with 10% Fetal Bovine Serum (FBS). Only in HEK293FT cell culture 1X Gentamicin (Gibco, UK) is supplemented.

NK-92 cells were purchased from ATCC and grown in α -MEM (Gibco, UK) supplemented with 0.2mM inositol (Sigma, Germany), 0.1 mM β -mercaptoethanol (Gibco, UK), 0.02 mM folic acid (Sigma, Germany), 500 U/mL IL-2 (Gibco, UK), 12.5% horse serum (Gibco, UK) and 12.5% FBS, unless otherwise is indicated.

KHYG-1 cells were purchased from DSMZ and grown in RPMI supplemented with 150 U/mL IL-2, and 10% FBS, unless otherwise is indicated. Jurkat cells were grown in RPMI supplemented with 10% FBS.

Immortalized human astrocytes were purchased from abmgood and grown on collagen coated flasks. Cells were maintained in Prigrow IV (abmgood, Canada) supplemented with

1X glutamax (Gibco, UK), 10% FBS, 1% Penicillin-Streptomycin and 0.01% epidermal growth factor.

All the cells were maintained in humidified air conditions at 37°C constituting 5% CO₂. Cells were passaged when the confluency reached to 70-80%. Adherent cells were trypsinized with 0.25% trypsin-EDTA (Gibco, UK). Suspension cells were splitted every 2-3 days when the confluency is 70-80%. Otherwise, medium change or medium addition was made.

2.2. CHIMERIC ANTIGEN RECEPTOR VECTOR DESIGN

The chimeric antigen receptor structure is designed following the current advances presented in the literature. In principle, benefits of CAR technology are fulfilling for clinical utilization. However, in practice, the benefits restricted by the challenges in design and transfer to immune cells. During a CAR design many factors need to be evaluated such as a appropriate target selection, and promoter selection. Yet, the comprehensive evaluations during the design does not guarantee the functionality of the CAR structure. For example, accurate and precise sequence of ScFv domains is essential for the proper target binding. The different antibody sequences for the same target might differ in response when used in ScFv generation. Therefore, each designed CAR vector requires its own optimization studies in design, delivery methods and functional assays.

In this thesis, it was aimed to design a dualCAR vector targeting the PD-L1 and PDGFR- α . Target selection was based on the chordoma literature and the data obtained in our lab. In principle, our design provides the individual expression of PD-L1 and PDGFR- α targeting receptors on the cell surface provided by the ribosomal skipping sequences. This is the most critical point of our design since the main goal is the initiate signaling in NK cells via PD-L1 binding and enhance the effect only if the secondary binding of PDGFR- α happens. General design exhibits a 3rd generation CAR properties constituting CD3 ζ , CD28, and 4-1BB signaling domains. Plasmids contains two marker gene (eGFP/mCherry & puromycin/gentamicin resistance gene) providing detection and selection advantage of the CAR-positive cell population.

The size of dualCAR vector is 11.846 bp long and a 3rd generation lentiviral backbone is used.

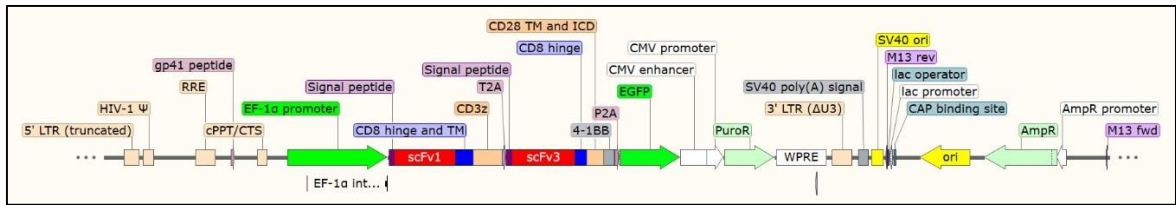


Figure 2.1 dualCAR construct design

A second design constructed utilizing a 3rd generation lentiviral backbone. However, this time PD-L1 and PDGFR- α CAR structures are divided into different plasmids yielding a relatively smaller size, (PD-L1-CAR, 10.569 bp; PDGFR- α CAR, 10.665 bp). The smaller size plasmids were designed to evaluate if the viral packaging capacity differs between dualCAR and divided CAR vectors. Another question asked is if the full length and functional dualCAR could be sufficiently achieved by the consecutive transduction of NK cells by divided CAR vectors. The designed plasmids were synthesized by Creative Biolabs.

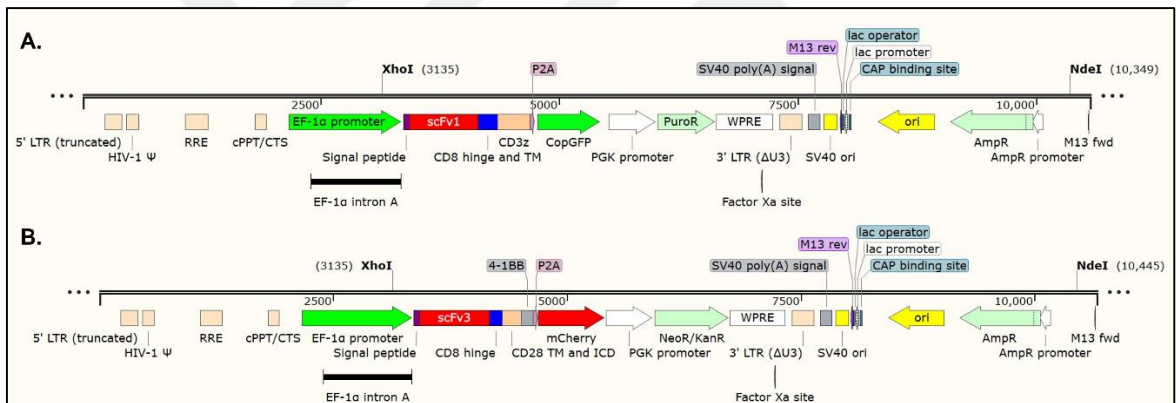


Figure 2.2 Divided CAR construct design A. PD-L1-CAR B. PDGFR- α CAR

Additionally, PDGFR- α CAR was cloned into a 2nd generation lentiviral plasmid (pLVX-EF1alpha-IRES-mCherry), yielding a 10.177 bp long construct. PDGFR- α CAR was cloned from the original dualCAR vector. Briefly, pLVX-EF1alpha-IRES-mCherry plasmid has the XbaI ve BamHI single-cut regions within its sequence. However, the insert must be restricted by the same restriction enzymes for cloning, but the original vector does not constitute XbaI ve BamHI single-cut regions. Therefore, an initial PCR step was added before restriction to add restriction sites to the original vector. Primer sequenced used for PCR is given below.

Table 2.2 PDGFR- α sequence specific primer sequences adding XbaI and BamHI restriction sites to the dualCAR vector

Primers	Sequence	Added Restriction Site
PDGFR α Forward	5'— atataTCTAGAATGGCCTTACCAGTGACC —3'	TCTAGA (XbaI)
PDGFR α Reverse	5'— atataGGATCCGAATTCCAGTTCGCAGCC —3'	GGATCC (BamHI)

Both the insert and backbone prepared by restriction with XbaI ve BamHI enzymes. Briefly, a reaction mix was prepared constituting nuclease-free water (Invitrogen, USA), 1X fast digest green buffer, and DNA (0.2 μ g-1 μ g) in a total volume of 20 μ l. Mixtures were incubated at 37°C in thermal cycler for 25 minutes. Then, samples were loaded into 1.2% agarose gel. Gel was prepared by dissolving the agarose (Thernofisher, USA) in 1X Tris Acetate Edta (TAE) buffer (Sigma, Germany). Samples were run onto gel at 120 V for 1 hour, and visualized under UV lamb. The desired products were isolated from agarose gel using gel extraction kit (Qiagen, USA). Then, the products were ligated. For ligation 1:1, 1:2, 1:3, 1:5, and 1:7 ratios of insert:backbone were used. Backbone vector is used as 100 ng and related insert amount is calculated by NEBBioCalculator (nebiocalculator.neb.com/ligation). Ligation performed by T4 ligase (Promega, USA) and T4 ligase buffer (Promega, USA). Before T4 ligase addition to ligation mix, samples were incubated at 45°C for 3 minutes. Then, T4 ligase were added and samples incubated in thermalcycler at 16°C for 18 hours, and 65°C for 10 minutes. Plasmid referred as self-ligated PDGFR- α CAR.

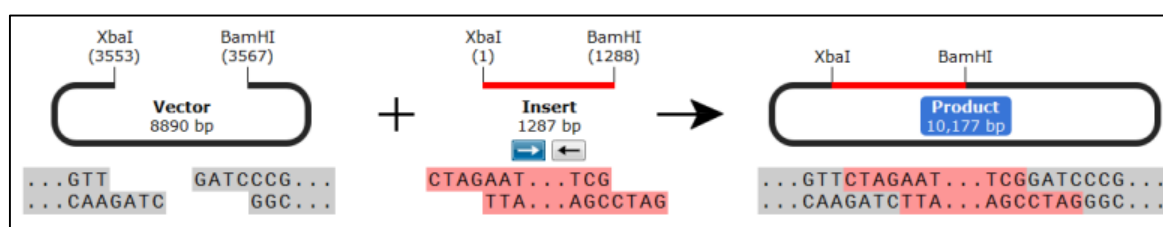


Figure 2.3 Cloning strategy for the PDGFR- α -CAR construction

The third strategy used in dualCAR design is using a sleeping beauty (SB) transposon system. The same design implicated in a dualCAR vector is integrated into an SB transposon backbone. SB system works through a “cut and paste” principle for genomic integration by using a transposase. In this study, transposase is supplied by SB100X plasmid. Both plasmids were obtained from Creative Biolabs. The vector referred as SBCAR.

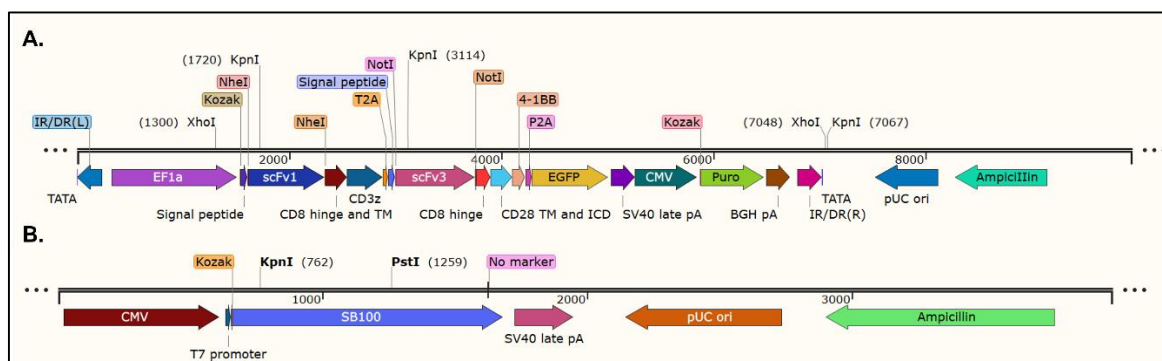


Figure 2.4 Sleeping beauty transposon system based design of dualCAR construct
A. SBCAR design B. SB100X plasmid design

2.3. BACTERIAL TRANSFORMATION

The bacterial transformation studies conducted with *E.coli* strains DH5 α and Stab13 (ThermoFisher, USA). Stab13 strain commercially available as chemically competent.

DH5 α *E.coli* cells were made competent by calcium chloride method. Briefly, *E.coli* from frozen stock is expanded in 5 mL of antibiotic free LB broth (Sigma, Germany) at 37°C overnight. Bacteria is inoculated onto antibiotic free LB agar (Sigma, Germany) plates. Single colony from agar plate inoculated in 5 mL of antibiotic free LB broth at 37°C overnight. Next day, 500 μ l from 5 mL stock is inoculated in 50 mL antibiotic free LB broth at 37°C for 3 hours. Later, cells were incubated on ice for 20 minutes and centrifuged at 3000x g for 10 minutes at 4°C. Supernatant is discarded and pellet is resuspended in cold 0.1M CaCl₂ (Sigma, Germany) solution. Subsequently, transferred into cold falcon tubes and incubated on ice for 30 minutes. Cells centrifuged at 3000x g for 10 minutes at 4°C. Supernatant is discarded and pellet is resuspended in cold 0.1M CaCl₂ solution containing 15% glycerol (Sigma, Germany). Competent cells were aliquoted and stocks were kept at -80°C.

Table 2.3 List of plasmids used in the study

Plasmids	Supplier
pCMV-dR8.2 dvpr	Addgene, 8455
pCMV-VSV-G	Addgene, 8454
psPAX2	Addgene, 12260
pMD2.G	Addgene, 12259
pMDLg/pRRE	Addgene, 12251
pRSV-Rev	Addgene, 12253
pLVX-EF1α-IRES-mCherry	Kindly provided from Prof. Dr. Fikretin Şahin
pLenti CMV eGFP Puro	Addgene, 17448
pLVX-EF1α-IRES-ZsGreen1	Clontech, 631982
dualCAR Vector	Creative Biolabs, USA
PD-L1-CAR Vector	Creative Biolabs, USA
PDGFR-α CAR Vector	Creative Biolabs, USA
SBCAR Vector	Creative Biolabs, USA
SB100X	Creative Biolabs, USA

For the transformation, all equipment were used cold. Competent bacterial cells were thawed on ice. Then, 100-300 ng plasmid were added onto 100 μ l of competent cells. Cells were incubated on ice for 30 minutes. After incubation, cells were heat shocked by 42°C for 90 seconds and put onto ice for another 90 seconds. For recovery, 800 μ l of super optimal broth (S.O.C) medium was added onto cells and incubated at 37°C in water bath for 45 minutes. At the end of the incubation, cells were spreaded onto agar plates containing ampicillin (Sigma, Germany) or not (control plates for bacterial growth). Agar plates incubated overnight at 37°C incubator. Plates were kept at 4°C.

Single colony from agar plate containing the transformed bacteria was inoculated in 5 mL of LB broth ampicillin or not (control plates for bacterial growth) at 37°C for 1-2 hours. Then, LB broth was completed to 50 mL and incubated overnight at 37°C. Next day, cells were centrifuged at 2300x g for 20 minutes. Supernatant was discarded and plasmid DNA was isolated by GeneJET Plasmid Miniprep Kit (Thermofisher, USA).

2.4. RESTRICTION DIGESTION

Before proceeding with further experiments, plasmids obtained by single-colony expansion are prone to restriction/digestion reaction to check the vector identity. Fast digest enzymes (Thermofisher, USA), and fast digest green buffer (Thermofisher, USA) were used.

Table 2.4 Plasmids and the restriction enzymes used for the vector identity confirmation

Plasmids	Enzymes
pCMV-dR8.2 dvpr	KpnI
pCMV-VSV-G	BamHI
psPAX2	PvuII
pMD2.G	NdeI
pMDLg/pRRE	PvuII
pRSV-Rev	PvuII
pLVX-EF1alpha-IRES-mCherry	KpnI
pLenti CMV eGFP Puro	PvuII
pLVX-EF1 α -IRES-ZsGreen1	Ssp1 & Nhe1
dualCAR Vector	KpnI
PD-L1-CAR Vector	XhoI & SspI
PDGFR- α CAR Vector	XhoI & SspI
SBCAR Vector	XhoI
SB100X	PstI & KpnI
Self-ligated PDGFR- α CAR Vector	SmaI

Briefly, a reaction mix was prepared constituting nuclease-free water (Invitrogen, USA), 1X fast digest green buffer, and DNA (0.2 μ g-1 μ g) in a total volume of 20 μ l. Mixtures were incubated at 37°C in thermal cycler for 25 minutes. Then, samples were loaded into 1.2% agarose gel. Gel was prepared by dissolving the agarose (Thermofisher, USA) in 1X Tris Acetate Edta (TAE) buffer (Sigma, Germany). Samples were run onto gel at 120 V for 1 hour, and visualized under UV lamp.

2.5. SANGER SEQUENCING

Designed dualCAR was synthesized by Creative Biolabs. ScFv regions of plasmids were prone to sanger sequencing in order to compare designed and synthesized sequences.

Additionally, self-cloned PDGFR- α CAR was sequenced. Sequencing comprehends the nucleotides before and after between XbaI restriction site 3553-3558. bases and BamHI restriction site 4040-4845. Therefore, the compatibility of restriction and ligation processes would be confirmed if the results were as designed. Sequencing results were aligned using an open-source bioinformatics tool, Unipro UGENE v. 38. Multiple sequence alignment resulted in a consensus sequence. Consensus sequences were compared with the sequencing results provided by the manufacturer. This comparison is performed by nucleotide-BLAST (BLASTn) established by NCBI (National Center for Biotechnology Information). Using the megaBLAST algorithm, only high-similarity results were detected. Details in sanger sequencing protocol is given below.

Table 2.5 Primer designs used in sanger sequencing

Primers	Sequence
Sanger Primer Reverse	CTGCTGCTGTTGATGCTG
Sanger Primer Forward	AGCGACTACATGAACATGAC
Primer 1	CTGTGCTGCTGAAGGTGAAG
Primer 2	TCAATCTTGGTCGGAGAGA
Primer 3	CTGCTGCTGTTGATGCTG
Primer 4	CAGCAGAGAAGCAACTGG
Primer 5	ACCTATGACGCTCTTCACATG
Primer 6	CTTGGTTCATTCTCAAGCCTC

The working temperature of designed primers was concluded by gradient PCR. Temperatures between 55- 65°C were tested. For each temperature that was tested, a different mixture was prepared. After the PCR reaction, samples were loaded to 1.2% agarose gel and run onto gel at 120 V for 1 hour, then visualized under UV lamb. Details of PCR mixture and reaction conditions were given in table 2.5.

Table 2.6 Gradient PCR reaction mixture compotion and conditions

PCR Mixture	PCR Conditions
2μl DNA	95°C 4 min- 1x
25 μl DreamTaq	95°C 30 sec
2 μl GC Buffer	65-55°C 30 sec
1 μl Forward Primer	72°C 1 min
1 μl Reverse Primer	72°C 10 min
19 μl dH₂O	4°C Hold

The sequences that will be analyzed were amplified by PCR reaction. Touch-down PCR method was used. First, sample preparation was made, as explained in table 2.6. The PCR conditions were the same as gradient PCR. However, instead of preparing different samples for each temperature, the same sample was exposed to a temperature range between 65-55°C, and the temperature change interval was 30 seconds. PCR products were confirmed by agarose gel electrophoresis. Later, ExoSAP-IT (ThermoFisher, USA) treatment was used for PCR product cleanup. The reagent directly added onto PCR product and incubated at 37°C for 15 minutes, at 80°C for 15 minutes in thermalcycler. Using BigDye kit (ThermoFisher, USA) sequence PCR was established. For the purification step PCR products were incubated with 125 mM EDTA, 3M sodium acetate and 100% ethanol containing mixture for 15 minutes and centrifuged for 45 minutes. Then, plate containing the samples were reversed and centrifuged shortly (~30 seconds). Each well supplemented with injection buffer and samples were denaturated at 95°C for 5 minutes followed by an incubation on ice for 2 minutes. Then, samples were loaded into sanger sequencing device and results were analyzed as described above.

2.6. VIRAL PACKAGING OF PLASMIDS

In this study, both the 2nd and 3rd generation lentiviral plasmids were used. Lentiviral plasmids were packaged by using HEK293T and HEK293FT as host cells. 2nd and 3rd generation packaging plasmids were used.

Table 2.7 Lentiviral packaging plasmids

Plasmids	Generation
pCMV-dR8.2 dvpr	2 nd
pCMV-VSV-G	2 nd
psPAX2	2 nd
pMD2.G	2 nd
pMDLg/pRRE	3 rd
pRSV-Rev	3 rd

Briefly, HEK293T and HEK293FT cells were seeded in 6-well plates at 4×10^5 cells/well density. Before transfection, cells were starved with Opti-MEM (Gibco, UK) for 2 hours.

Then, the medium changed, and cells were transfected either using FuGENE HD (Promega, USA), Lipofectamine 3000 (Thermo Fisher Scientific, USA), Viafect (Promega, USA), or Calcium chloride precipitation method. Unless otherwise mentioned, FuGENE HD is used as the standard transfection reagent in experiments. In all transfection studies, the DNA amount is 2 µg/well. For the commercially available reagents, manufacturers' protocols were used. For calcium chloride precipitation, DNA and 2.5 mM CaCl₂ were mixed and added into HEPES-buffered saline (HBS, pH=7.0) while gently swirling. The mixture was incubated at room temperature for 30 minutes and then added onto HEK293FT cells dropwise. HBS buffer formulation given in table 2.6. Ratio of packaging plasmids and plasmid of interest as follows pCMV-dR8.2 dvpr: pCMV-VSV-G: plasmid of interest; 1:0.1:1 while psPAX2: pMD2.G: plasmid of interest; 4:2:1 and pMDLg/pRRE: pRSV-Rev: VSV-G: Plasmid of interest; 4:1:1:2.

Table 2.8 2X HBS Buffer Composition

Chemical	Concentration
HEPES	50 mM
NaCl	280 mM

After 16-18 hours of transfection, the medium was changed. The medium was collected from the cells at 48 and 72 hours. The collected medium was filtered through 0.45 µm membrane filters, and viral supernatant was kept at -80°C until use. Viral titer was measured by Lenti-X Gostix Plus (Takara, Japan).

2.7. VIRAL TRANSDUCTION

Viral transduction procedure for HEK293T, HEK293FT, U87-MG, HGC-27 and Immortalized human astrocytes 4x10⁴ cells/well seeded into 6 well plates. Before transduction cells were starved for 2 hours with opti-MEM. 8 µg/mL polybrene (Sigma, Germany), 6 µg/mL for astrocytes, was added into viral supernatants. Opti-MEM was removed from the wells and 1 mL of viral supernatant was added and plates were spinoculated at 1200 RPM for 2 hours. Subsequently, viral supernatant is removed and fresh supernatant added which diluted in 1:2 ratio with normal cell culture media. Cells were

incubated overnight at 37°C. Then, the medium was changed and fluorescence positivity was checked by flow cytometry (BD,FACSCalibur, BD Biosciences, San Jose, CA).

For the viral transduction of NK-92 cells, different medium compositions and transduction reagents were used. Details about medium compositions and transduction-aiding reagents were given in table 2.8 and table 2.9, respectively. All transduction protocols applied by spinoculation method explained for HEK293T, HEK293FT, U87-MG, and HGC-27 cells. Before transduction protocols, NK-92 cells were either exposed to 20 ng/mL IL-12 or not for 4-6 hours.

Table 2.9 Medium compositions tested in NK-92 cell transduction

Medium A	Medium B	Medium C
2% Serum No IL-2 No β -mercaptoethanol	25% Serum 1000 UI/mL IL-2 No β -mercaptoethanol	4% Serum 1000 UI/mL IL-2 No β -mercaptoethanol

Table 2.10 Transduction aiding reagents and working concentrations

Reagent	Working Concentration
Polybrene (Sigma, Germany)	8 μ g/mL
Protamine Sulfate (Sigma, Germany)	8 μ g/mL
Dextran (Sigma, Germany)	8 μ g/mL
RetroNectin (TaKara, Japan)	100 μ g/mL

For the RetroNectin-dependent transduction procedure, non-culture treated and round bottom 96-well plates were coated with 100 μ g/mL RetroNectin and overnight incubation at 4°C. Coated wells were blocked by 2% BSA solution (Gibco, UK) for 30 minutes at room temperature. Then, wells were washed with HBSS buffer (Thermofisher, USA). Viral supernatants were added into wells and centrifuged at 2000 x g, 32°C for 2 hours. Next, wells were washed with 1X PBS (Gibco, UK), and 8×10^4 cells/well were seeded. Cells were incubated overnight on retronectin-coated plates. The next day, cells were either transferred into normal culture conditions or a second transduction was performed.

KHYG-1 cells were transduced by dual transfection with RetroNectin and prior exposure to IL-12 for 4-6 hours. Jurkat cells were transduced with a combination of 8 µg/mL polybrene and RetroNectin.

Transduction efficiency was checked by flow cytometry (BD, FACSCalibur, BD Biosciences, San Jose, CA).

2.8. TRANSFECTION OF SBCAR VECTOR

SBCAR vectors were transfected by chemical (FuGENE HD) and physical (Nucleofection) methods to NK cell lines. HEK293T was transfected using the chemical method only.

Literature examples of nucleofection in NK cells have varying buffers and related program usage. Therefore, optimization to determine the most efficient condition is required. KHYG-1 cells were transfected with CD90 KO-CRISPR plasmid, previously used in our lab. However, NK-92 cells do not express CD90. Therefore, NK-92 transfection optimization was performed by GFP plasmid provided by the nucleofactor kits (Lonza, USA).

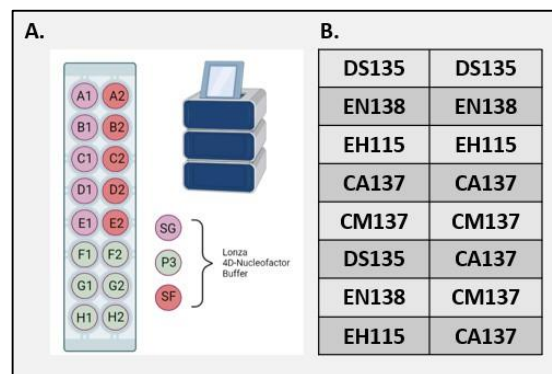


Figure 2.5 Nucleofection buffers (A) and protocols (B) used in NK cell transfection

2.9. CONVENTIONAL PCR

Genomic integration of lentiviral plasmids was detected by conventional PCR with the primers designed specifically for the puromycin selection region of the plasmids. The working temperature of primers were identified by gradient PCR (see section 2.5). Primer sequences were given in table 2.10.

Table 2.11 Puromycin sequence specific primer sequences

Primers	Sequence
Puromisin Forward	5'— CAGCAACAGATGGAAGGCC —3'
Puromisin Reverse	5'— TCGTAGAAGGGGAGGTTGCG —3'

DNA isolation was performed using DTAB (dodecyl trimethyl ammonium bromide) /CTAB (cetyltrimethylammonium bromide) method. Briefly, the cell pellet was resuspended in DTAB and incubated at 65°C. Chloroform was added to the samples. After centrifugation, phase separation is observed. The upper phase was transferred to a new tube, mixed with CTAB and nuclease-free water, and incubated at -20°C. Samples were centrifuged, and the pellet was resuspended in 1.2% NaCl and 100% Ethanol. Samples were centrifuged and washed with 70% ethanol. After centrifugation, the supernatant was discarded, and the remaining ethanol was evaporated at 56°C for 3 minutes. Finally, pellets were resuspended in TE buffer and quantified by Nanodrop measurement.

Table 2.12 DTAB/CTAB buffer formulations

DTAB	CTAB
8% DTAB (Sigma, Germany)	
0.1 M Tris (Sigma, Germany)	5% CTAB (Sigma, Germany)
1.2 M NaCl (Sigma, Germany)	400 mM NaCl (Sigma, Germany)
0.05 EDTA (Sigma, Germany)	

PCR reaction is applied as shown in Table 2.5 with the determined working temperature of primers. PCR products were loaded to 1.2% agarose gel and run onto gel at 120 V for 1 hour, then visualized under UV lamp.

2.10. WESTERN BLOT

For the confirmation of CAR expression western blotting is used. Protein isolations were made using 1X RIPA (Cell Signaling Technology, USA) buffer. Briefly, cell pellets were resuspended in 1X RIPA buffer and mixed well. After incubation on ice for 15 minutes, samples were centrifuged at 16.000 x g for 15 minutes. Supernatant was aliquoted and kept at -80°C until use. Protein concentrations were determined using Pierce BCA Protein Assay (Thermofisher, USA). Proteins were separated with 15% SDS-PAGE (Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis). Composition of SDS-PAGE gel is given in table 2.11.

Table 2.13 SDS-Polyacrylamide gel composition

15% Separating Gel (2 gels)	Stacking Gel (2 gels)
2.4 ml H ₂ O	2.7 ml H ₂ O
5 ml acrylamide (30 %)	0.8 ml acrylamide (30 %)
2.5 ml 1.5 M tris (pH 8.8)	0.5 ml 1 M tris (pH 6.8)
50 µl SDS (20%)	20 µl SDS (20%)
100 µl APS (ammonium persulfate) (10%)	40 µl APS (10%)
10 µl TEMED	4 µl TEMED

Proteins were transferred onto 0.45 µm PVDF (polyvinylidene difluoride)(Thermofisher, USA) membranes. Membrane were blocked by 5% BSA solution and incubated overnight with CD3ζ (Invitrogen, USA) and β-actin (CST, USA) specific antibody at 4°C. Next day, membranes were incubated with HRP (Horseradish peroxidase)-conjugated secondary antibody and chemiluminescence was detected by using Clarity Western ECL Substrate kit (BIO-RAD).

2.11. PROTEIN L STAINING

For detection of CAR expression on the cell surface Protein L staining was used. Briefly, 1x10⁶ cells were washed with ice-cold wash buffer (0.04% BSA/PBS (1X)) for 3 times. Cells were resuspended in 200 µl wash buffer and 1 µg Protein L (Sigma, Germany) was added. Cells were incubated on ice for 45 minutes. Then, the wash step is repeated for 3 times. Cells

were resuspended in 200 μ l wash buffer and biotin-conjugated Streptavidin-PE (ThermoFisher, USA) was added at 5 μ g/mL working concentration. Cells were incubated on ice for 45 minutes. After incubation, cells were washed 3 times and resuspended in DAPI/PBS. Cells were analyzed by flow cytometry. As a unstained control, only secondary antibody treated cells were used to avoid false positive fluorescence. Analysis was made by FlowJo v10 Software (BD, CA).

2.12. DETECTION OF PROTEIN LEVELS BY FLOW CYTOMETRY

The basal surface expression of PD-L1 and PDGFR- α on chordoma cells lines were detected by flow cytometric analysis. Cells were trypsinized and washed with 1X DPBS (Gibco, UK). Cells were treated with FcR blocker (BD, CA) for 5 minutes. Then, incubated with fluorophore conjugated antibodies (BD, CA) for PD-L1 and PDGFR- α for 30 minutes on ice. At the end of incubation cells were washed and resuspended in DAPI/PBS. Cells were analyzed by flow cytometry. As a positive control for PD-L1 and PDGFR- α expression H1975 cells were used. Analysis was made by FlowJo v10 Software (BD, CA).

2.13. STATISTICAL ANALYSIS

Statistical analysis was made by Graphpad Prism 5 software. Biological replicate dependent calculation of standard deviation (SD) presented as means $\pm$ SD. Statistical calculations were made by t-test and two-way ANOVA; *** $P \leq 0,001$, ** $P \leq 0,01$, * $p \leq 0,05$, ns $p > 0,05$.

3. RESULTS

3.1. CAR VECTOR DESIGNS

The dual targeting strategy aims to provide an increased specificity toward chordoma cells. In design, receptors are synthesized separately from each other, and only the PD-L1 compromising part includes the CD3 ζ for signal initiation. PDGFR- α containing part of the dual receptor system includes signal enhancer domains, CD28 and 4-1BB. This design provides a logic-gated model of CAR technology. “And logic gate” of the CAR vector is designed for achieving efficient lytic activity only if both antigens bind to their targets. In this case, the PD-L1 part containing the CD3 ζ chain will provide the initial signaling when bound to its target. However, the killing efficiency will be low and can only be enhanced by the signalization provided by the CD28 and 4-1BB upon target binding.

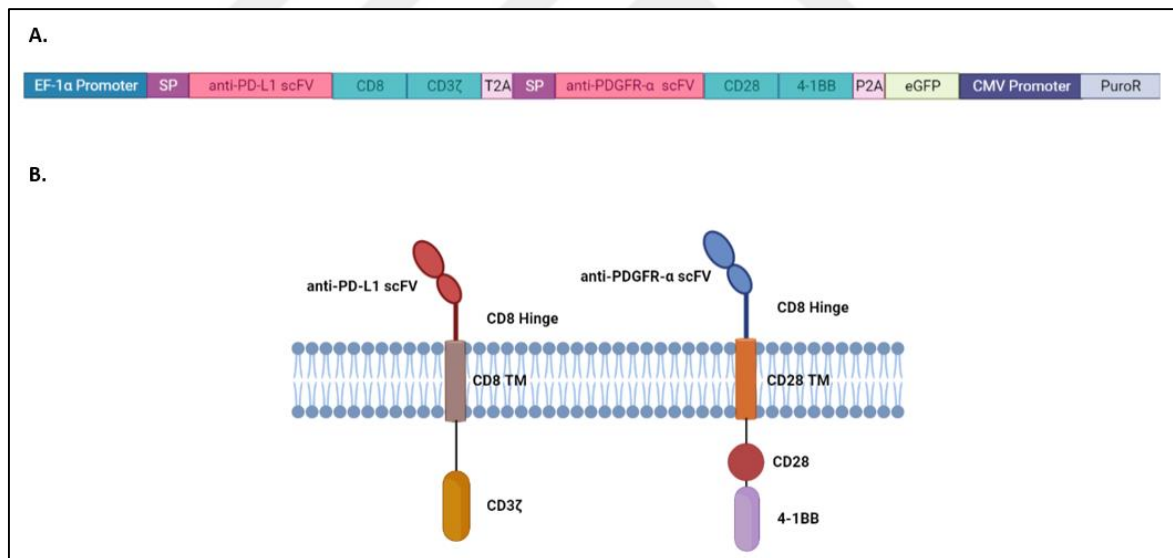


Figure 3.1 Logic-gated (and-logic) chimeric antigen receptor design. A. Schematic of dualCAR vector. B. Predicted cell surface expression of chimeric antigen receptor system on natural killer cells. Images were illustrated with BioRender (251).

Currently, there is no approved treatment option for chordoma. However, receptor tyrosine kinase (RTK) inhibitors are widely recommended. Therefore, a targeting strategy against RTKs by chimeric antigen receptors is promising. RTK expression in chordoma cells was evaluated by literature research. PDGFR- α , PDGFR- β , and c-Met expressions were the most evaluated among chordoma patient tissues and cell lines (47). PD-L1 inhibitors are FDA-approved drugs used in cancer therapy. Promising results of PD-L1 targeting strategies are

growing in immuno-oncology. Patient-based studies revealed positive expression of PD-L1 in chordoma cells (56,190). Therefore, PD-L1 and PDGFR- α were selected as promising targets for chordoma treatment. Then, previously established transcriptomic profiling of four chordoma patients and one primary chordoma cell line (Yu-Chor) was checked for the PD-L1 and PDGFR- α expressions. Expression positivity detected to varying degrees.

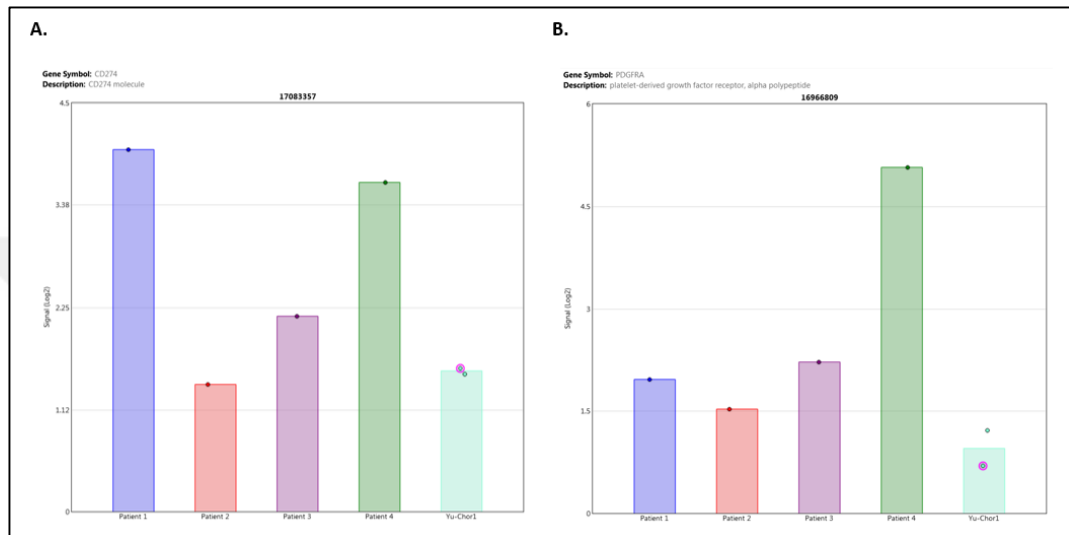


Figure 3.2 PD-L1 and PDGFR- α signal(log2) levels established by transcriptomic profiling of four chordoma patient and one primary chordoma cell line (Yu-Chor) A. PD-L1(CD274) signal(log2) levels established by transcriptomic profiling of four chordoma patients. B. PDGFR- α signal(log2) levels established by transcriptomic profiling of four chordoma patient

Cell surface expressions of PD-L1 and PDGFR- α were also evaluated in chordoma cell lines.

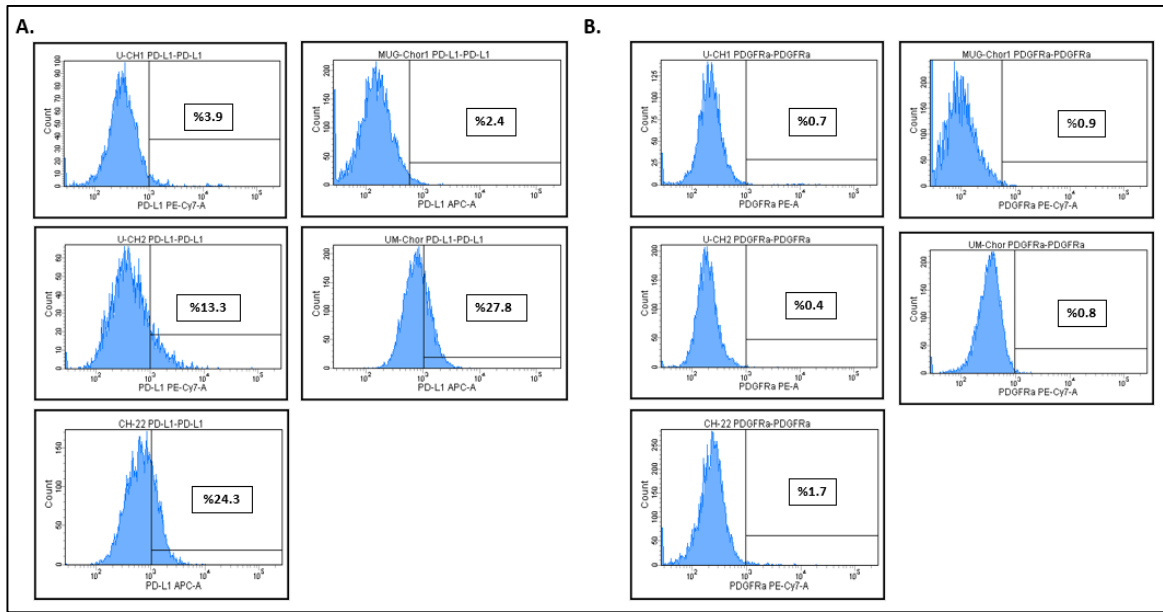


Figure 3.3 Basal expression levels of PD-L1 and PDGFR- α in chordoma cell lines. A. Basal expression of PD-L1 in chordoma cell lines. B. Basal expression of PDGFR- α in chordoma cell lines.

Finally, PD-L1 and PDGFR- α levels were compared between chordoma tumorspheres and parental cells. Data is retrieved from transcriptomic profiling analysis performed by Transcriptome Analysis Console (TAC) software. Calculated fold change values were given in figure 3.4. This comparison were made since there are many examples of NK cells selectively kill cancer stem-like cells (252). Therefore, whether selected targets could be directed to a tumor-initiating population was questioned. Results showed that PDGFR- α levels, except MUG-Chor1 tumorspheres, significantly upregulated. On the contrary, PD-L1 expression was not differential between tumorspheres and parental cells, except MUG-Chor1. As a result, the selected target combination might be promising in increasing recurrence-free survival in chordoma patients.

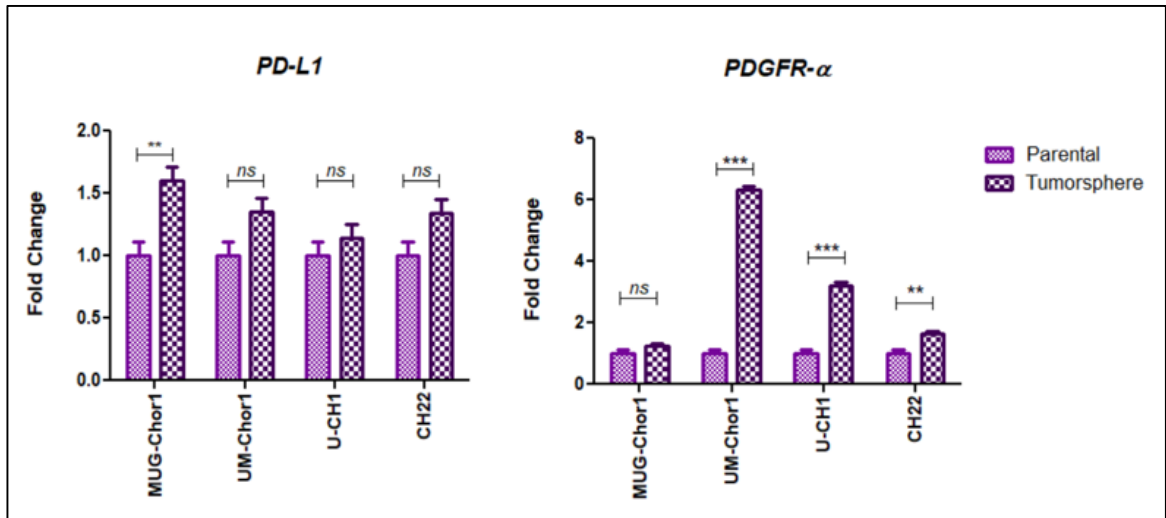


Figure 3.4 Fold changes of PD-L1 and PDGFR- α between tumorspheres and parental cell of chordoma cell lines.

Synthesis of dualCAR, divided CAR, and SBCAR was established by Creative Biolabs. The maps belonging to synthesized vector are given in figure 3.5, 3.6 and 3.7.

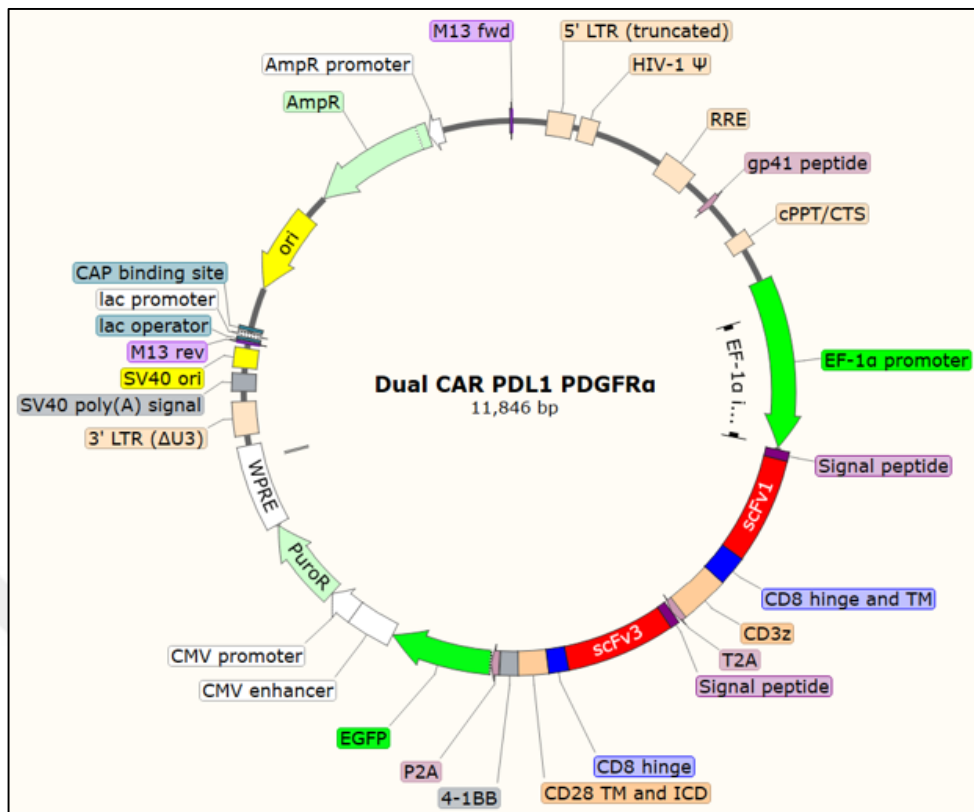


Figure 3.5 dualCAR vector map.

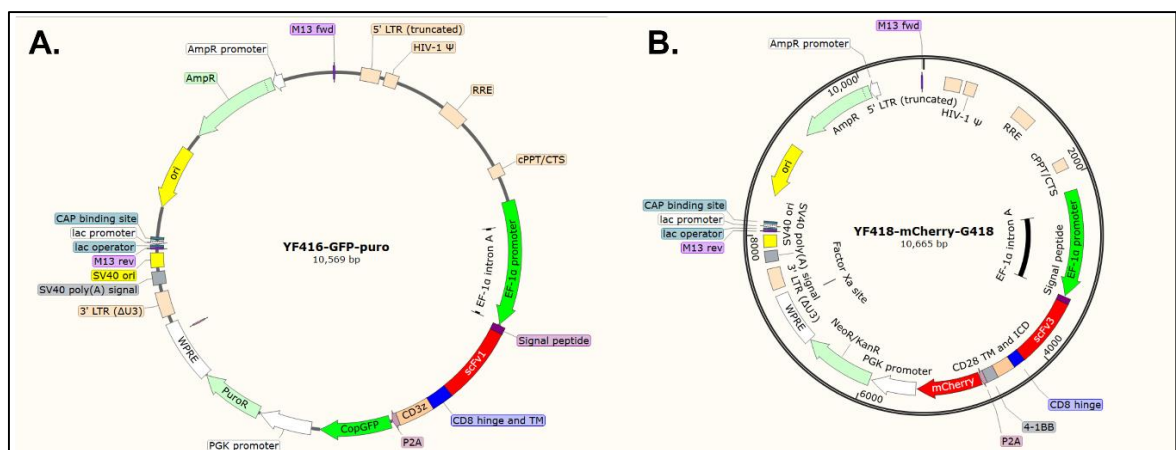


Figure 3.6 Divided CAR vector maps A. PD-L1-CAR vector map B. PDGFR-α-CAR vector map.

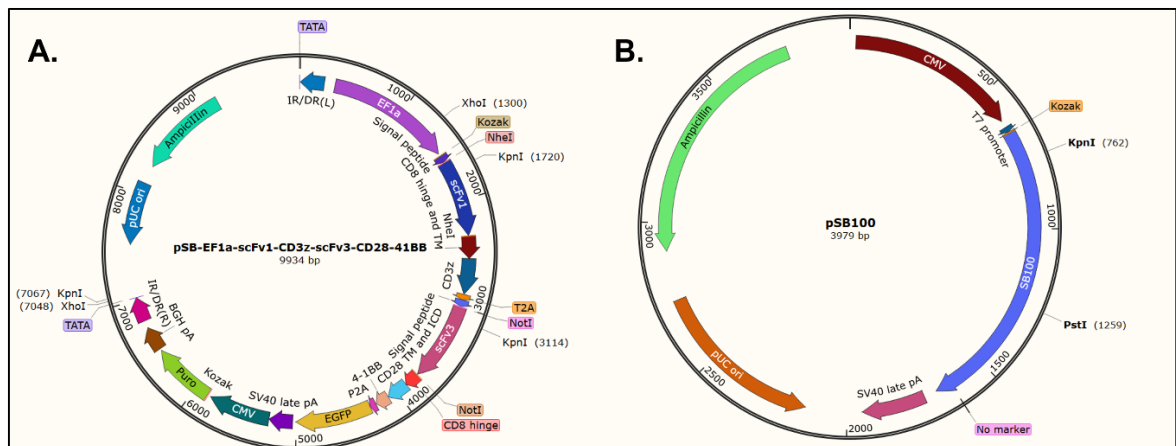


Figure 3.7 SB system vector maps A. SBCAR vector map B. SB100X vector map.

For the production of self-ligated PDGFR- α CAR, a dualCAR vector is used as a DNA template, and the PDGFR- α region is amplified with the addition of restriction sites. Figure 3.5 presents the agarose gel image of the PCR product. A high amount of PCR product is loaded into the gel to obtain a good quantity of DNA from gel isolation, causing the smear appearance. The primer used in the PCR reaction has non-specific binding because of the repeated regions in the DNA template. Therefore, separating the bands and cutting the band clearly that belongs to the PDGFR- α PCR product was crucial.

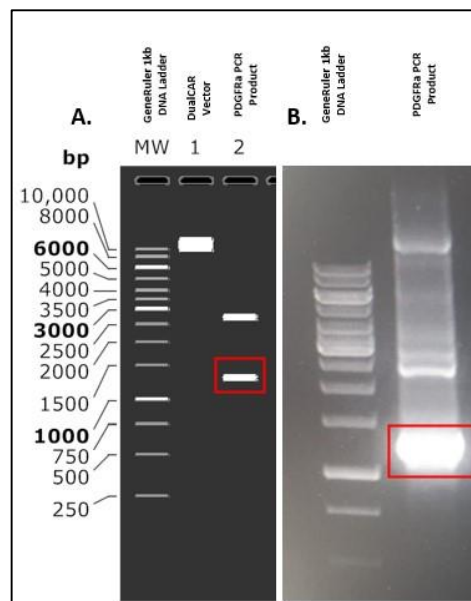


Figure 3.8 PDGFR- α amplicon A. The expected band appearance of the PDGFR- α amplicon in 1.2% agarose gel. PDGFR- α amplicon (1287 bp) is indicated in the red box B. The band appearance of the PDGFR- α amplicon in 1.2% agarose gel. Band of PDGFR- α amplicon (1287 bp).

PCR products excised from the gel were attached to XbaI and BamHI restriction sites provided by the primers. After the restriction/digestion reaction, a 14 bp difference in size was expected. However, distinguishing such a small-sized band and subsequent prediction if the restriction occurred is not applicable. Yet, the restriction product was loaded onto agarose gel and the band was excised. DNA was isolated from the gel to eliminate the salts in the reaction mixture to prevent their inhibition of ligation.

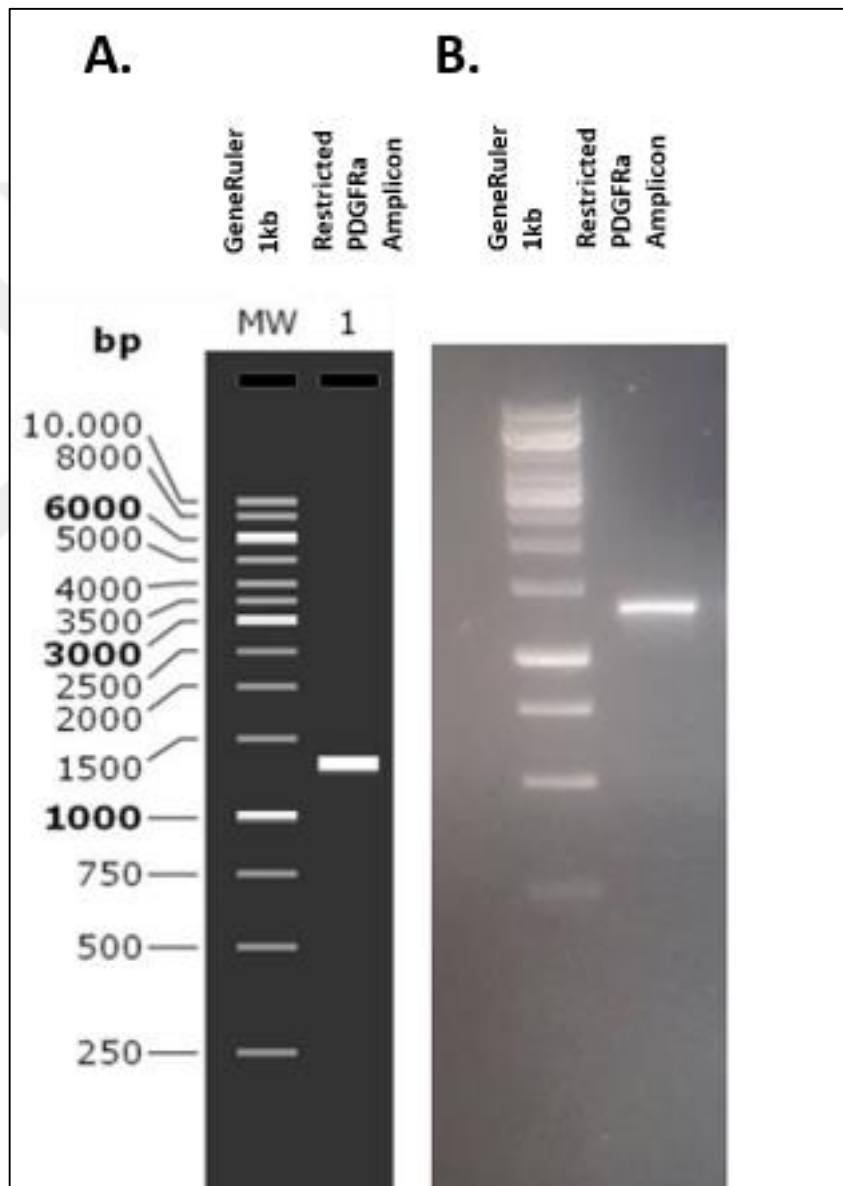


Figure 3.9 Restricted PDGFR- α amplicon with XbaI and BamHI restriction enzymes. A. The expected band appearance of the restricted PDGFR- α amplicon in 1.2% agarose gel. B. The band appearance of the restricted PDGFR- α amplicon in 1.2% agarose gel.

The cloning backbone (pLVX-EF1alpha-IRES-mCherry) was linearized by restriction/digestion reaction with XbaI and BamHI enzymes. After restriction, two band observations of sizes 8890 bp and 14 bp were expected. Again, the 14 bp band was not distinguishable. 8890 bp band represents the backbone used for cloning (shown in red box in figure 3.7). This band was visualized under a UV lamp and extracted for DNA isolation.

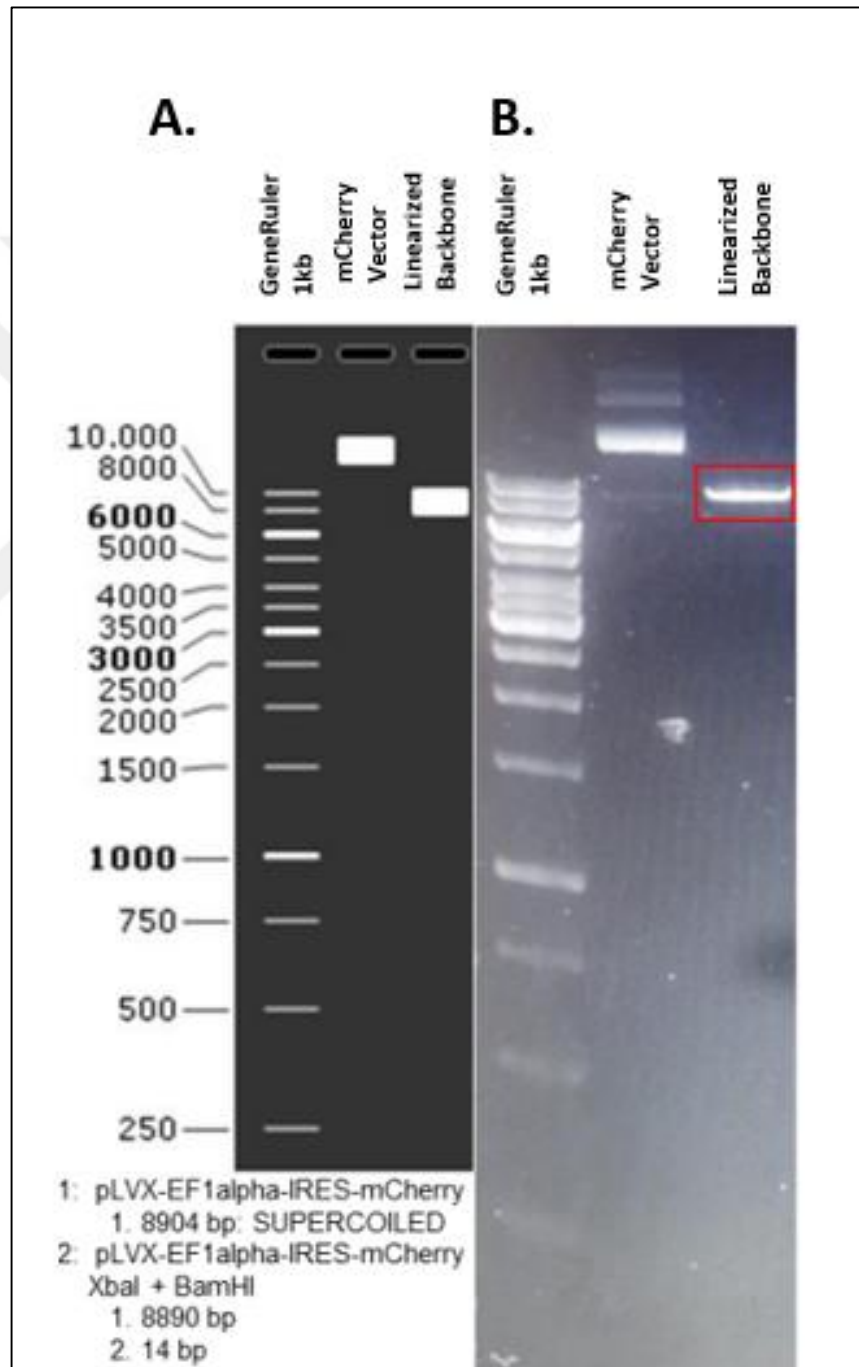


Figure 3.10 pLVX-EF1alpha-IRES-mCherry vector linearized with XbaI and BamHI restriction enzymes. A. The expected band appearance of the linearized backbone in 1.2% agarose gel. B. The band appearance of the linearized backbone in 1.2% agarose gel.

Linearized backbone and PDGFR- α insert were ligated and the self-ligated PDGFR- α CAR vector was constructed. The related map is presented in figure 3.8.

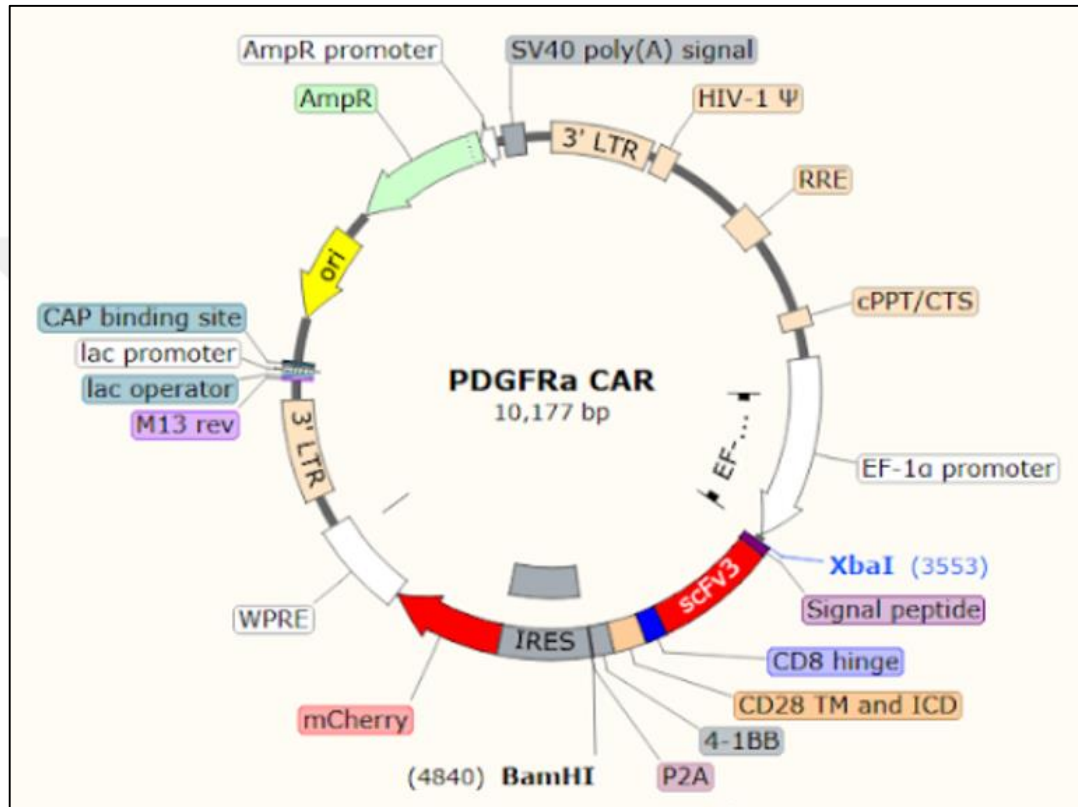


Figure 3.11 Self-ligated PDGFR- α CAR construct map.

3.2. PLASMID PRODUCTION AND CONTROL

3.2.1. Self-ligated PDGFR- α CAR

All ligation conditions succeeded in bacterial transformation, as shown in figure 3.8A. In each condition, single-cell colonies were isolated and restricted with SmaI enzyme for the preliminary confirmation of proper ligation. Except the colony 3 (Figure 3.9B), all plasmids resulted in band appearances at the expected size. The confirmed bands further proceeded to the sanger sequencing stage.

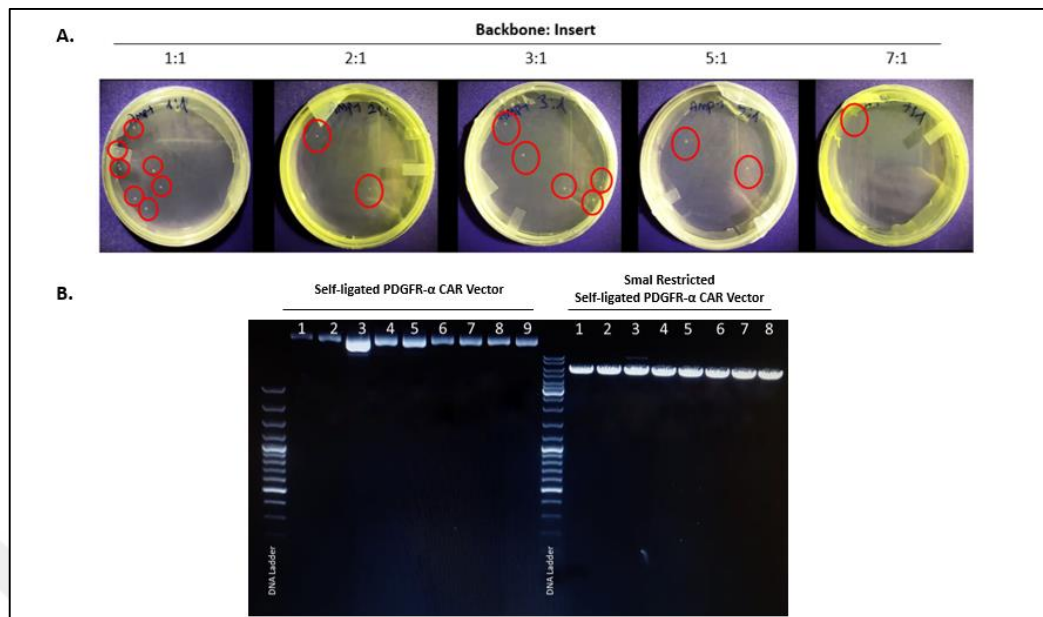


Figure 3.12 Bacterial Transformants and identity control of Self-ligated PDGFR- α CAR. A. Different backbone to insert ratios has varying transformation efficiency with positivity in each condition. B. Isolated DNA from the transformants pruned to restriction with SmaI.

3.2.2. Viral Packaging Plasmids

Viral packaging plasmids were successfully transformed into bacteria. Transformants were efficiently grown on ampicillin-containing agar plates due to the transmission of ampicillin resistance gene by the vectors.

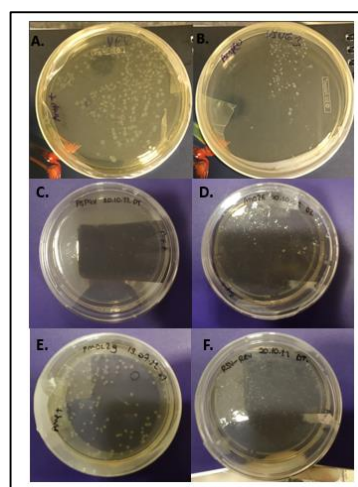


Figure 3.13 Bacterial transformation of viral packaging plasmids resulted in efficient colony formation. A. Transformants of pCMV-dR8.2 dvpr. B. Transformants of pCMV

VSV-G. C. Transformants of psPAX2. D. Transformants of pMD2.G. E. Transformants of pMDLg/pRRE. F. Transformants of pRSV-Rev.

Plasmids were confirmed before proceeding to the viral packaging step. The expected band sizes after being digested with restriction enzyme sizes are given in table 3.1.

Table 3.1 Lentiviral packaging plasmids and the expected band sizes

Plasmids	Expected Band Sizes (bp)
pCMV-dR8.2 dvpr	9567, 3485
pCMV-VSV-G	4815, 1692
psPAX2	7511, 2154, 1044
pMD2.G	3671, 1534
pMDLg/pRRE	5692, 2154
pRSV-Rev	2840, 1340

The expected band sizes were obtained for both the pCMV-dR8.2 dvpr and pCMV-VSV-G plasmids (Figure 3.14).

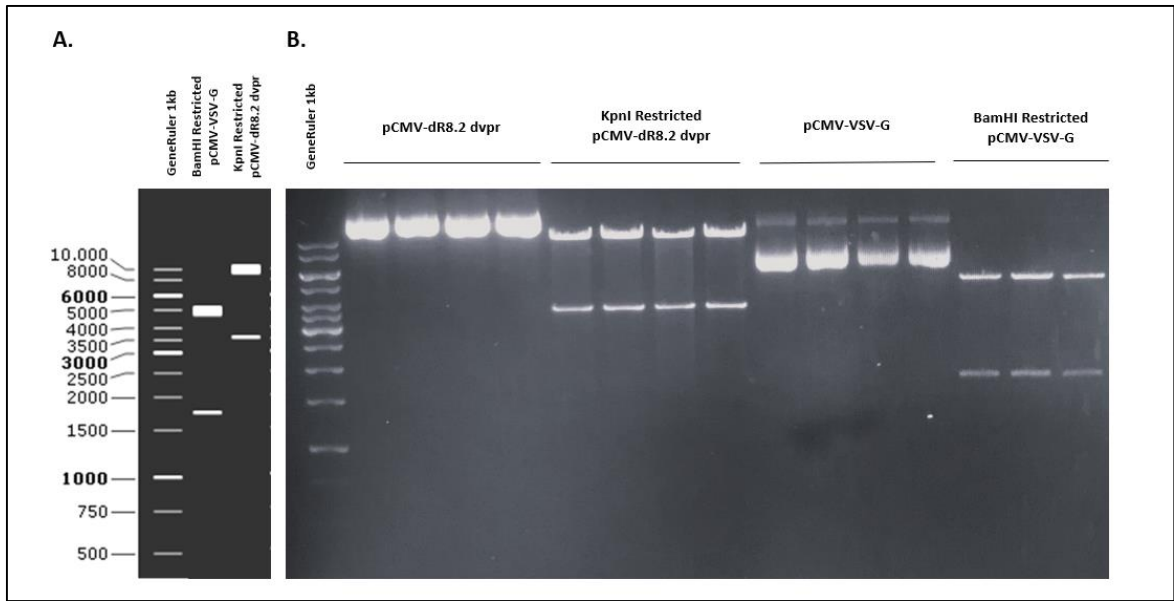


Figure 3.14 Gel images of pCMV-dR8.2 dvpr and pCMV-VSV-G plasmids after digestion with Kpn1 and BamHI, respectively. A. The expected band appearance of the restricted plasmids in 1.2% agarose gel. B. The band appearance of the restricted plasmids in 1.2% agarose gel.

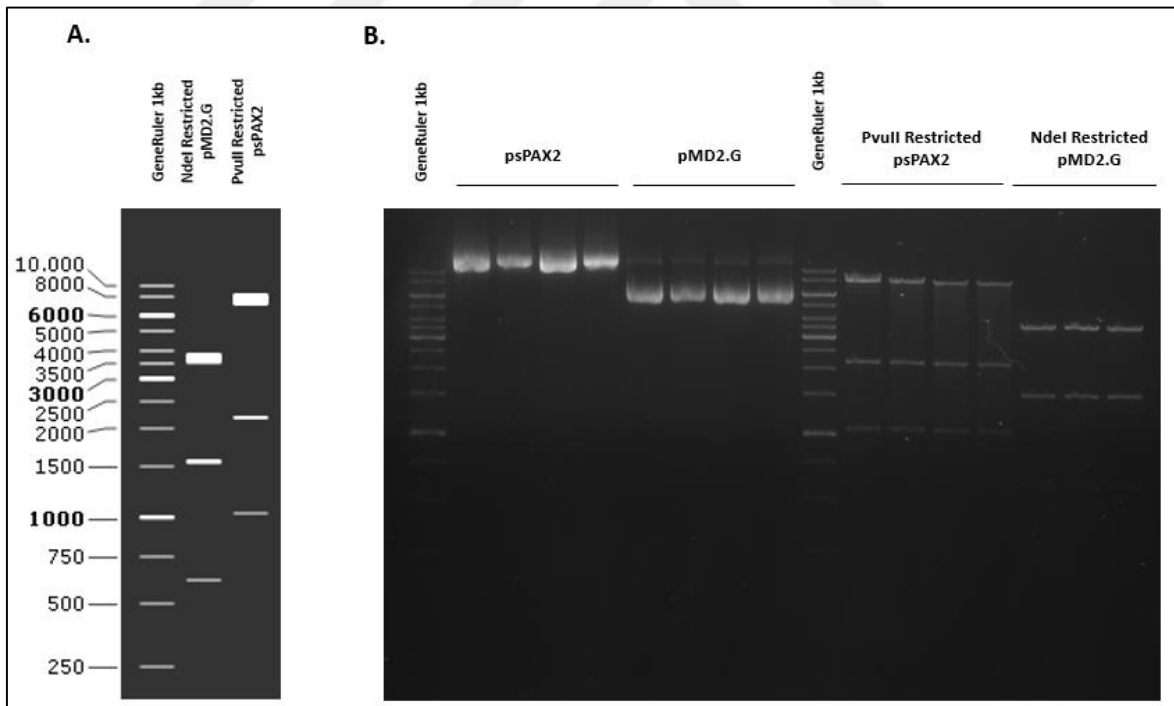


Figure 3.15 Gel images of psPAX2 and pMD2.G plasmids after digestion with PvuII and NdeI, respectively. A. The expected band appearance of the restricted plasmids in 1.2% agarose gel. B. The band appearance of the restricted plasmids in 1.2% agarose gel.

The expected band sizes were obtained for psPAX2, pMD2.G, pMDLg/pRRE and RSV-Rev plasmids.

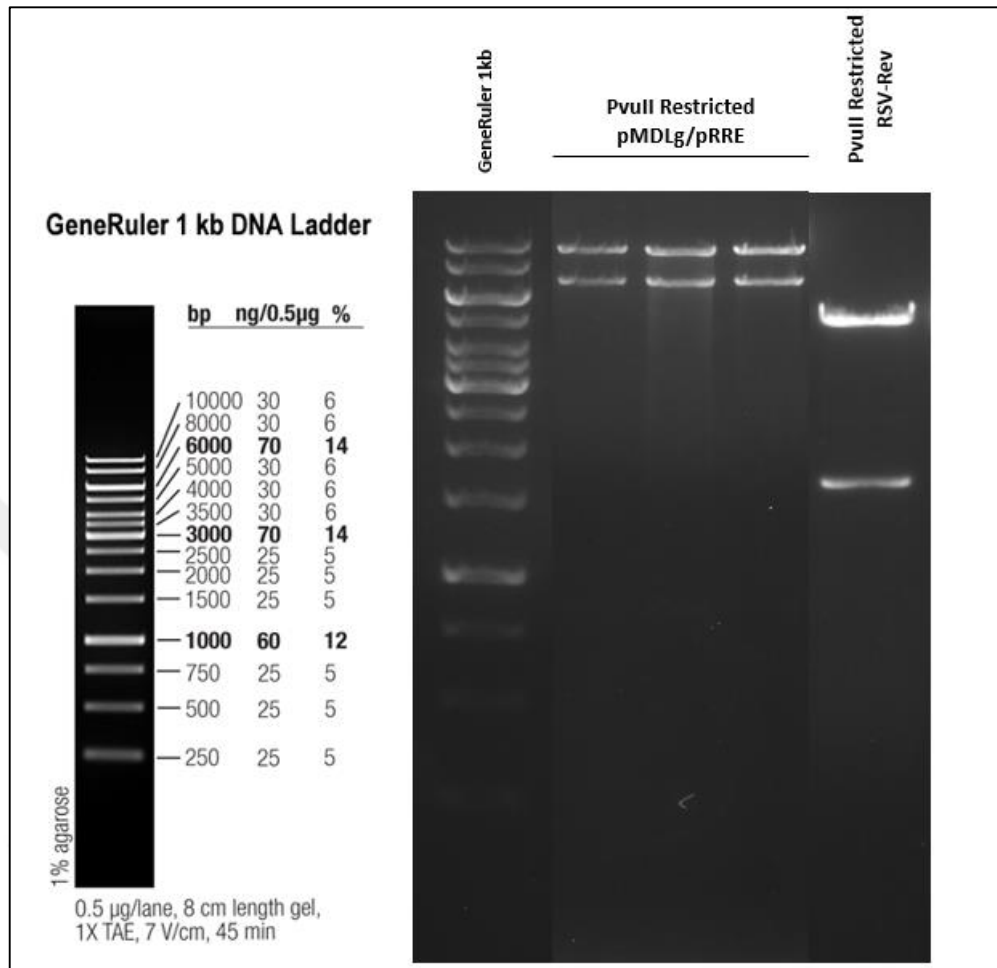


Figure 3.16 Gel images of pMDLg/pRRE and RSV-Rev plasmids after digestion with PvuII in 1.2% agarose gel.

3.2.3. CAR Vectors

Table 3.2 The expected band sizes for dualCAR, PD-L1 CAR, PDGFR- α CAR, SBCAR and SB100X plasmids enzymatic restrictions

Plasmids	Expected Band Sizes (bp)
dualCAR Vector	6860, 3606, 1380
PD-L1 CAR Vector	7213, 3356
PDGFR-α CAR Vector	7315, 3354
SBCAR Vector	5748, 4186
SB100X	3019, 962

The expected band sizes were obtained for dualCAR, PD-L1 CAR, PDGFR- α CAR, SBCAR and SB100X plasmids.

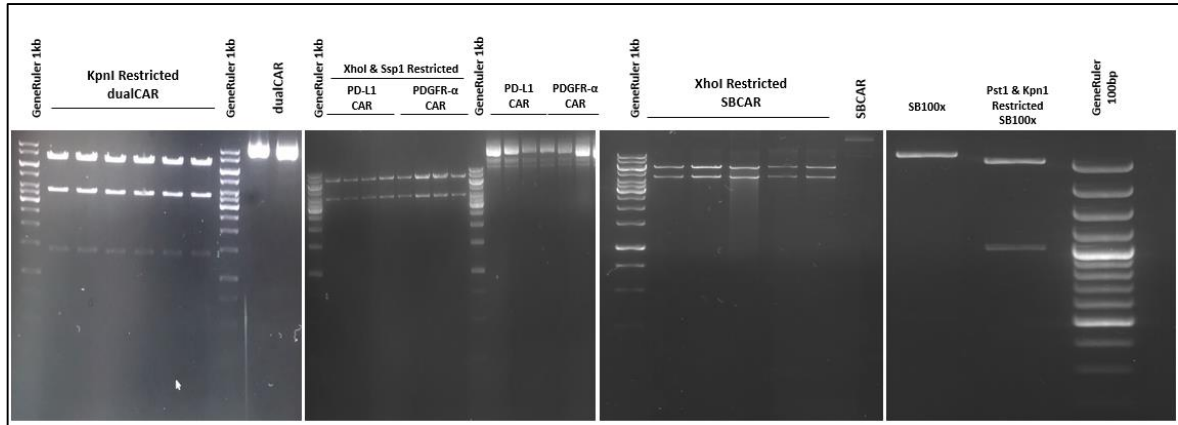


Figure 3.17 Gel images of dualCAR, PD-L1 CAR, PDGFR- α CAR, SBCAR and SB100X plasmids after digestion in 1.2% agarose gel.

3.2.4. Other Vectors

Table 3.3 The expected band sizes for pLV-EF1 α -IRES-mCherry, pLenti CMV eGFP Puro, and pLV-EF1 α -IRES-ZsGreen1 plasmids enzymatic restriction

Plasmids	Expected Band Sizes (bp)
pLVX-EF1 α -IRES-mCherry	7323, 1581
pLenti CMV eGFP Puro	5330, 2513, 776
pLVX-EF1 α -IRES-ZsGreen1	6785, 2092

pLV-EF1 α -IRES-mCherry, pLenti CMV eGFP Puro, and pLV-EF1 α -IRES-ZsGreen1 plasmids were run into in 1.2% agarose gel after digestion. The expected band sizes were obtained and vector identities were confirmed.

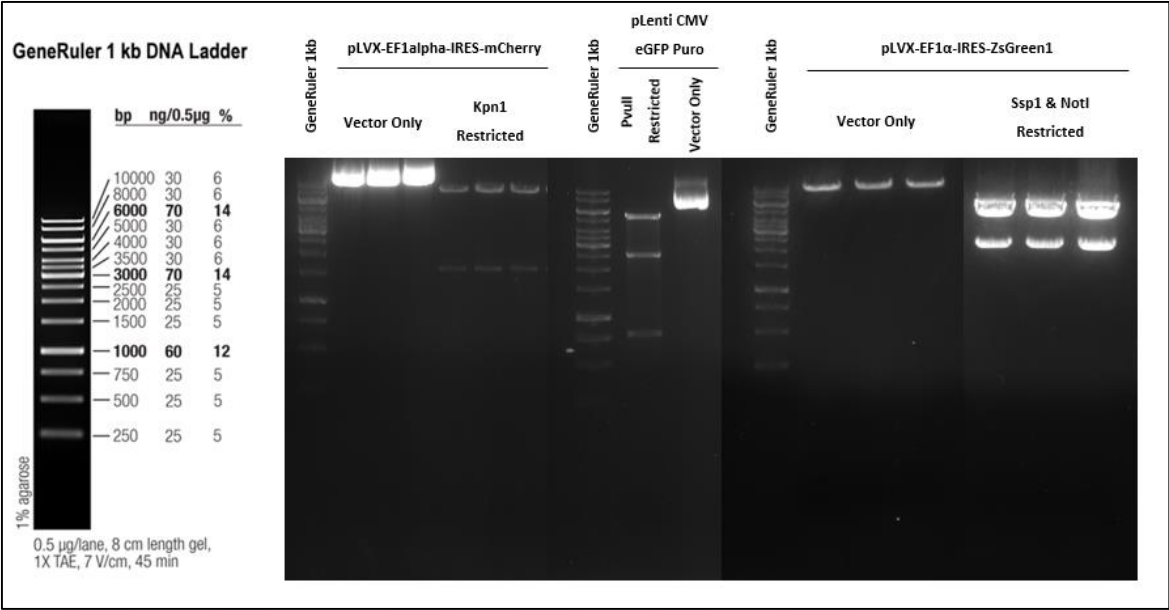


Figure 3.18 Gel images of pLV-EF1alpha-IRES-mCherry, pLenti CMV eGFP Puro and pLV-EF1a-Ires-ZsGreen1 plasmids after digestion in 1.2% agarose gel.

3.3. SANGER SEQUENCING

3.3.1. Sanger Sequencing of dualCAR Vector

ScFv regions of dualCAR vector were sequenced, and in-house sequence results were aligned with the sequence information provided by the manufacturer. The multiple alignments of sequences yielded 100% similarity.

3.3.2. Sanger Sequencing of Self-ligated PDGFR- α CAR Vector

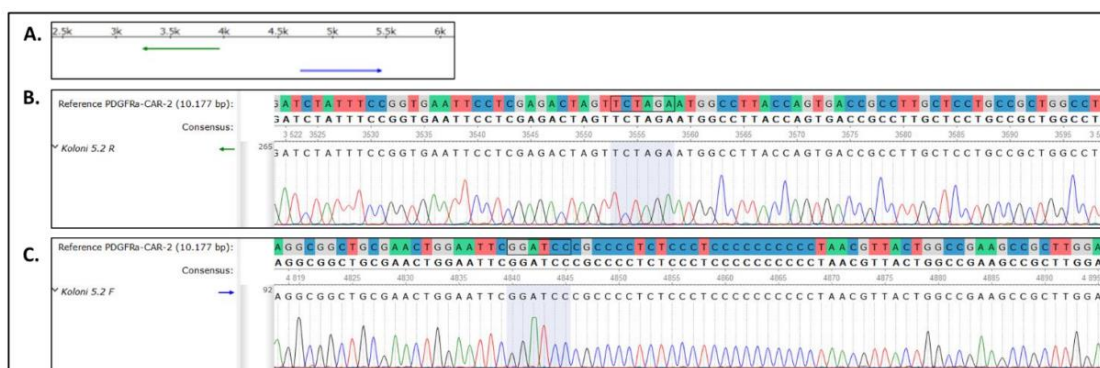


Figure 3.20 Chromatogram of Self-ligated PDGFR- α CAR obtained by sanger sequencing. A. Sequenced regions and directions shown on vector map. B. Chromatogram of XbaI restriction site directed sequencing. C. Chromatogram of BamHI restriction site directed sequencing.

Self-ligated PDGFR- α CAR was sequenced to confirm the compatibility of restriction and subsequent ligation. According to sequencing results, either base deletion or addition was not detected at the restriction/ligation sites. Results show that ligation occurred adequately.

3.4. OPTIMIZATION OF VIRAL PACKAGING

3.4.1. Efficiency Comparision of HEK293T and HEK293FT Cells in Viral Particule Production

HEK293T cells are derived from the human embryonic kidney cell line HEK293 by forced expression of SV40 T antigen. SV40 T expression provides the replicative advantage of SV40 replication origin-containing vectors while enhancing protein production. HEK293FT cells are a fast-dividing variant of HEK293T cells and were intentionally produced for viral packing (253–255). HEK293T and HEK293FT cells were transfected by using 2nd generation lentiviral packaging plasmids and dualCAR vector. Transfection efficiency was observed under fluorescent microscope and viral titers were measured by Lenti-X Gostix Plus. Fluorescent microscope images do not provide information about the produced viral particles, but information about CAR vectors transfected into cells can be obtained. All vectors must be transfected within the cell for an efficient viral packaging process. However,

the packaging plasmids do not have marker genes for this evaluation. Therefore, the efficiency of CAR vector transfection can not be correlated with viral production efficacy.

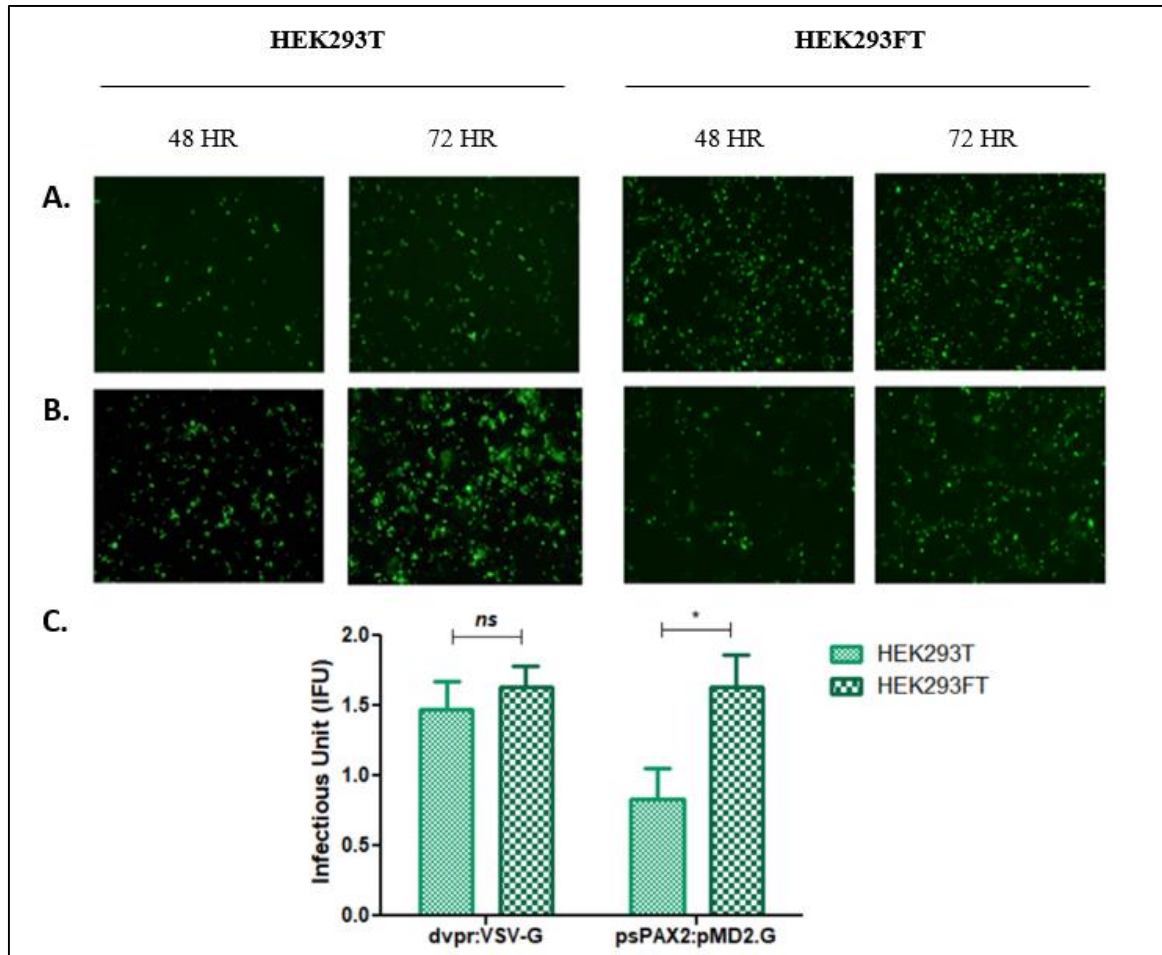


Figure 3.21 Comparison of HEK293T and HEK293FT cells in viral packaging efficiency. A. Cells were transfected with dvpr:VSV-G:dualCAR. B. Cells were transfected with psPAX2:pMD2.G. C. Lentiviral titer was calculated based on Lenti-X Gostix Plus measurement.

Transfection efficiencies vary between both the cell lines and the packaging plasmid combinations. HEK293T cells were more efficient in psPAX2:pMD2.G:dualCAR transfection while HEK293T cells were more efficient in dvpr:VSV-G:dualCAR transfection. However, viral titers were no significantly different dvpr:VSV-G mediated packaging while a significant difference were measured in psPAX2:pMD2.G mediated packaging. Though, the produced viral titer was extremely low in both cell lines. Nevertheless, the viral titer do not offer an evidence regarding the functionality of the produced viral particles.

3.4.2. Transfection optimization of HEK293FT Cell Line

HEK293FT cells were transfected with dvpr: VSV-G in combination with pLenti CMV eGFP Puro or dualCAR plasmids. Different transfection reagents were used during transfections to achieve the highest transfection efficiency. Also, viral titers were measured. Results indicate that lipofectamine 3000 provides the best transduction efficiency, though no difference in viral titer was obtained. pLenti CMV eGFP Puro is more efficiently transfected to HEK293FT cells than dualCAR vector. Significantly, Viafect and CaCl₂-mediated transfections yielded very low transfection efficiency. In viral titer measurements, a highly weak test band was observed in those conditions when used for dualCAR vector transfection.

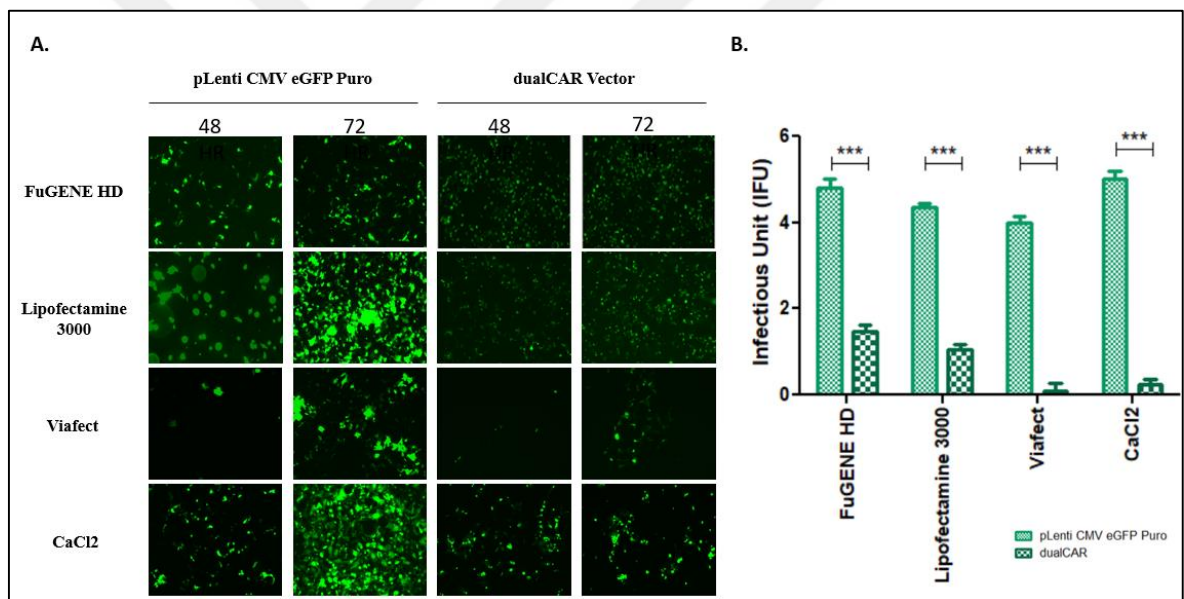


Figure 3.22 HEK293FT cells were transfected using dvpr:VSV-G in combination with pLenti CMV eGFP Puro or dualCAR and fluorescence microscope images were taken.

Transfection efficiencies and viral titer measurements were evaluated for both of the plasmids. FuGENE HD was selected as the optimal transfection reagent. Transduction experiments were conducted with HEK293FT cells and FuGENE HD for following experiments.

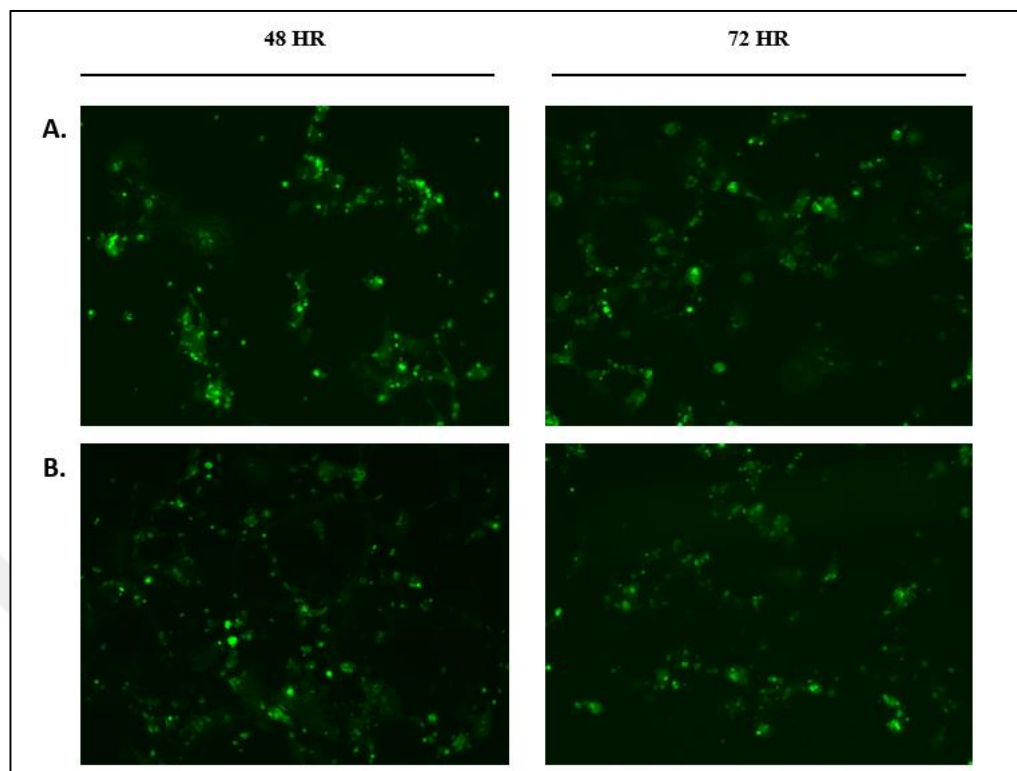


Figure 3.23 Fluorescence microscope images of HEK293FT cells during third generation lentiviral packaging A. HEK293FT cells transfected with pMDLg/pRRE, RSV-Rev and pCMV-VSV-G plasmids. B. HEK293FT cells transfected with pMDLg/pRRE, RSV-Rev and pMD2.G plasmids.

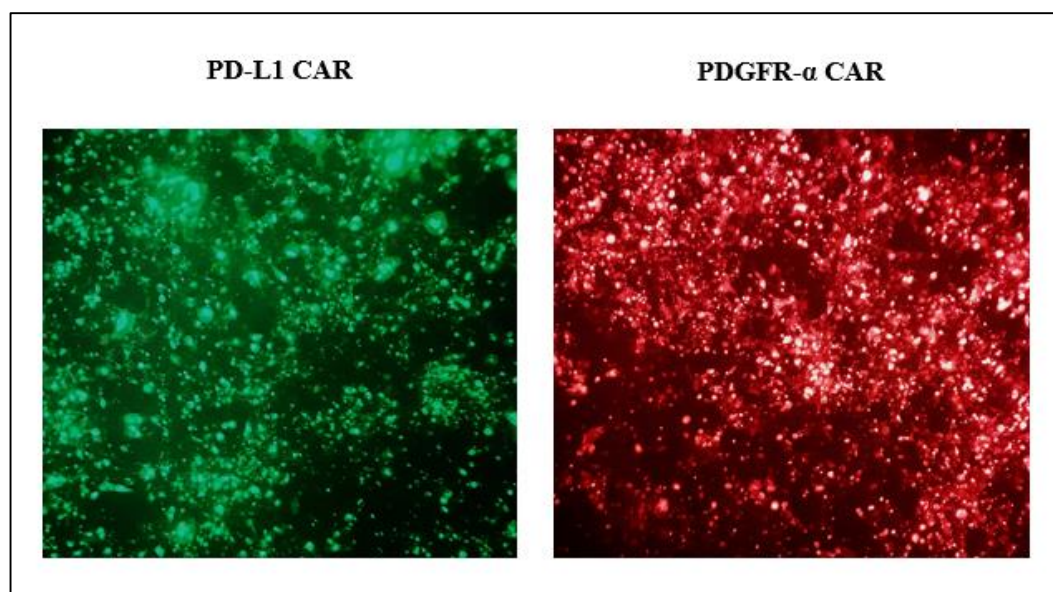


Figure 3.24 Fluorescence microscope images of HEK293FT cells transfected using dvpr:VSV-G in combination with PD-L1 CAR or PDGFR- α CAR.

3.5. OPTIMIZATION OF TRASDUCTION METHODS

Optimal medium conditions for efficient transduction were investigated. Transductions were performed by spinoculation method using 8 $\mu\text{g/mL}$ polybrene. Firstly, Medium A and Medium B were tested with pLV-EF1 α -IRES-mCherry carrying viral particles. It was found that low serum and IL-2-deprived condition impairs the transduction process of NK-92. Contrary, keeping the serum levels at normal culture conditions but increasing the IL-2 concentration by two-fold enhanced transduction efficiency. This result indicates IL-2 dependence on an efficient transduction process for NK-92 cells.

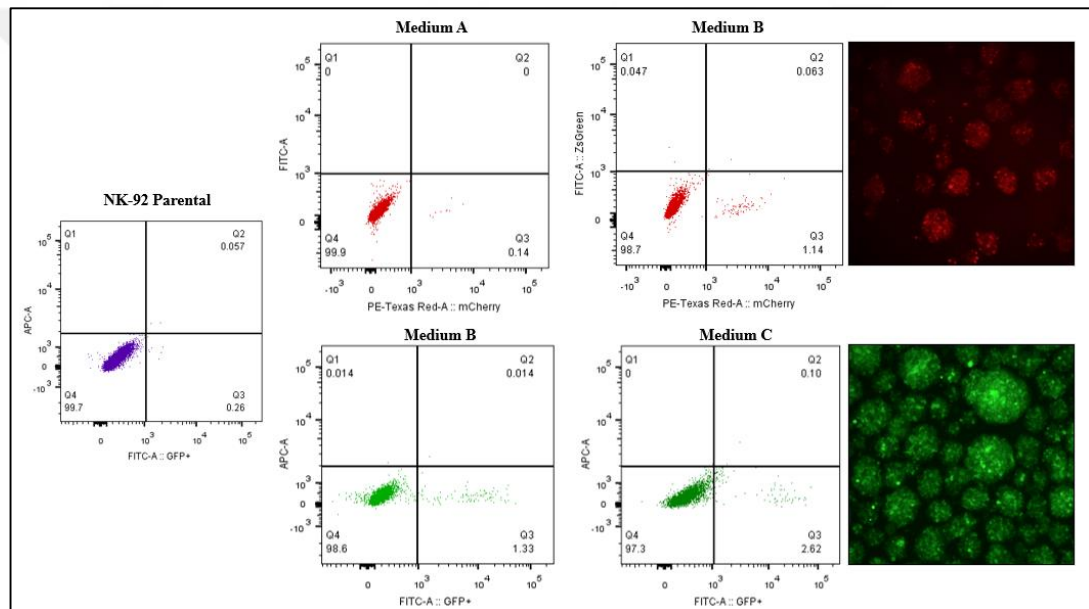


Figure 3.25 Different medium conditions effect NK-92 cell transduction efficiency.

Then, Medium B was compared with Medium C by transducing the NK-92 cells with pLenti CMV eGFP Puro carrying viral particles. Medium B efficiency was constant between different plasmids. Medium C, on the other hand, yielded the highest efficiency in transduction. Transduction protocol optimizations were conducted with Medium C. Positive cell populations were sorted. Fluorescence microscope images were taken after the enrichment by cell sorting.

Next, it was aimed to identify the best packaging condition and the method. Transduction efficiencies were checked by flow cytometry after 5 days from the experiment.

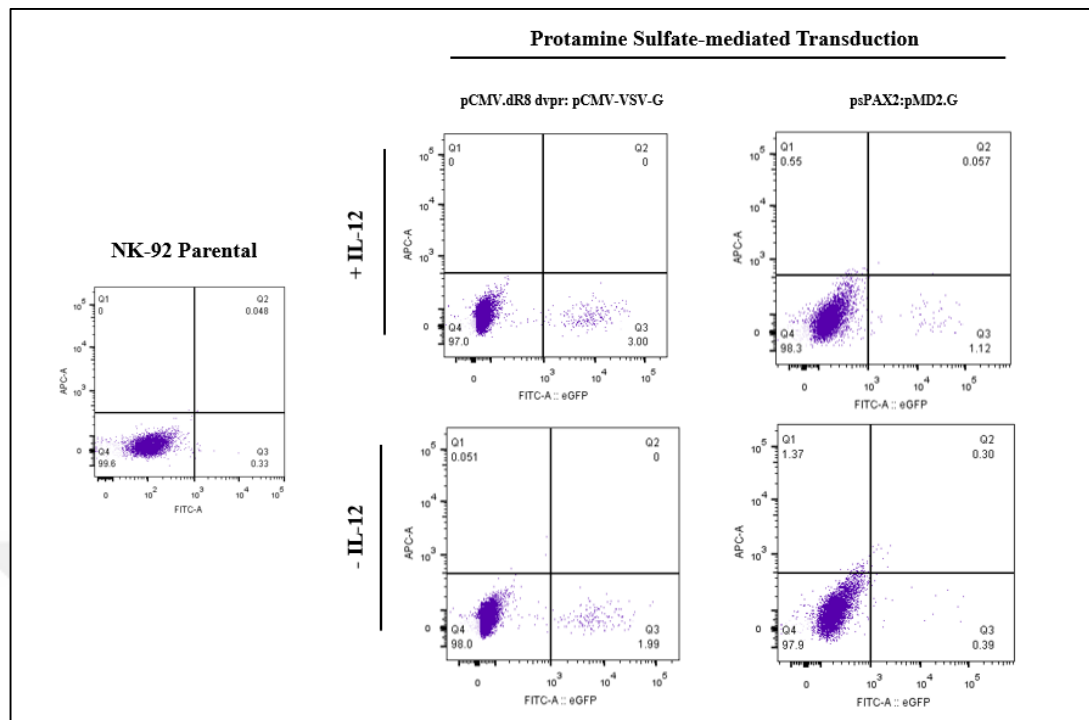


Figure 3.26 Protamine sulfate mediated transduction with or without IL-12 pre-incubation.

NK-92 cells were transduced by pCMV.dR8.2 dvpr: pCMV-VSV-G packaged pLenti CMV eGFP Puro or transduced by psPAX2: pMD2.G packaged pLenti CMV eGFP Puro.

There was a significant difference in transduction efficiency between packaging plasmids and with or without pre-incubation of IL-12. Protamine sulfate-mediated transduction is most suitable for pCMV.dR8.2 dvpr: pCMV-VSV-G packaged pLenti CMV eGFP Puro when NK-92 cells were pre-incubated with IL-12.

Dextran-mediated transduction yielded similar results but a lower efficiency compared to protamine sulfate.

For RetroNectin-mediated transduction, firstly, NK-92 cells were transduced with pCMV.dR8.2 dvpr: pCMV-VSV-G packaged pLenti CMV eGFP Puro either once or twice. It was observed that repeated transduction two times yields the highest efficiency among all methods. Compatible with the other methods, transduction efficiency is increased with IL-12 pre-incubation.

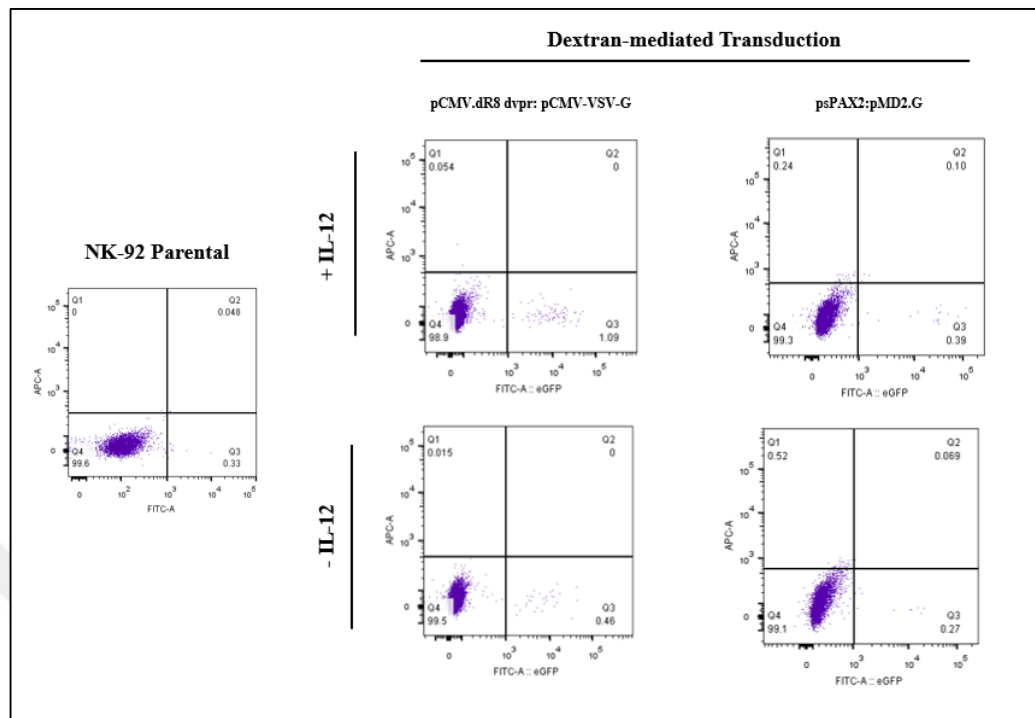


Figure 3.27 Dextran mediated transduction with or without IL-12 pre-incubation. NK-92 cells were transduced by pCMV.dR8.2 dvpr: pCMV-VSV-G packaged pLenti CMV eGFP Puro or transduced by psPAX2: pMD2.G packaged pLenti CMV eGFP Puro.

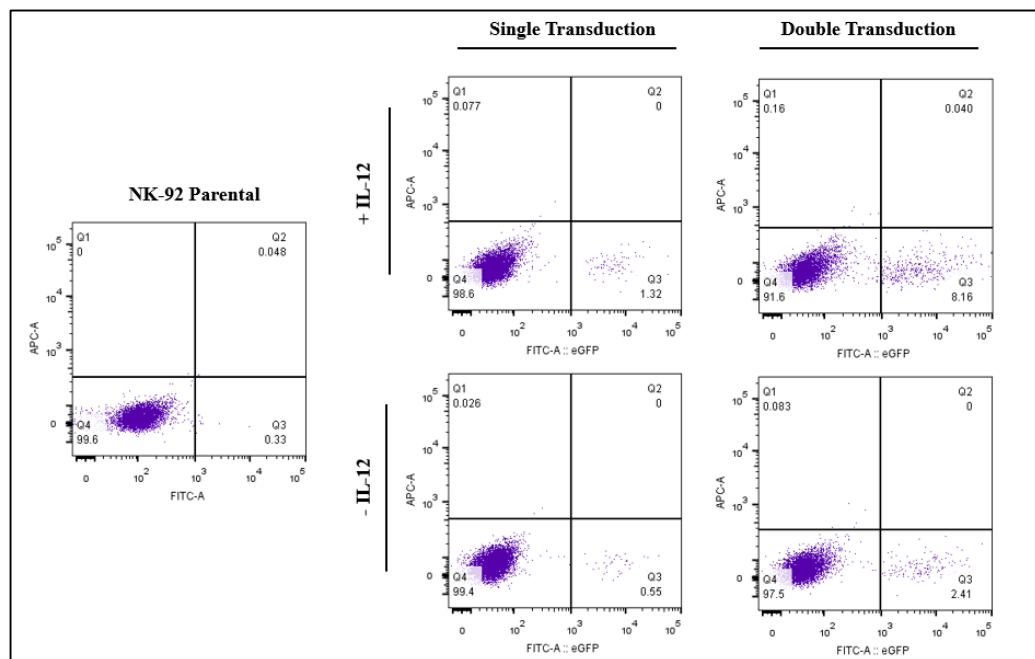


Figure 3.28 RetroNectin mediated transduction of pCMV.dR8.2 dvpr: pCMV-VSV-G packaged pLenti CMV eGFP Puro with or without IL-12 pre-incubation performed once or twice.

Hence the double transduction yielded the highest efficiency achieved, psPAX2: pMD2.G packaged pLenti CMV eGFP Puro was transduced twice with or without IL-12 pre-incubation.

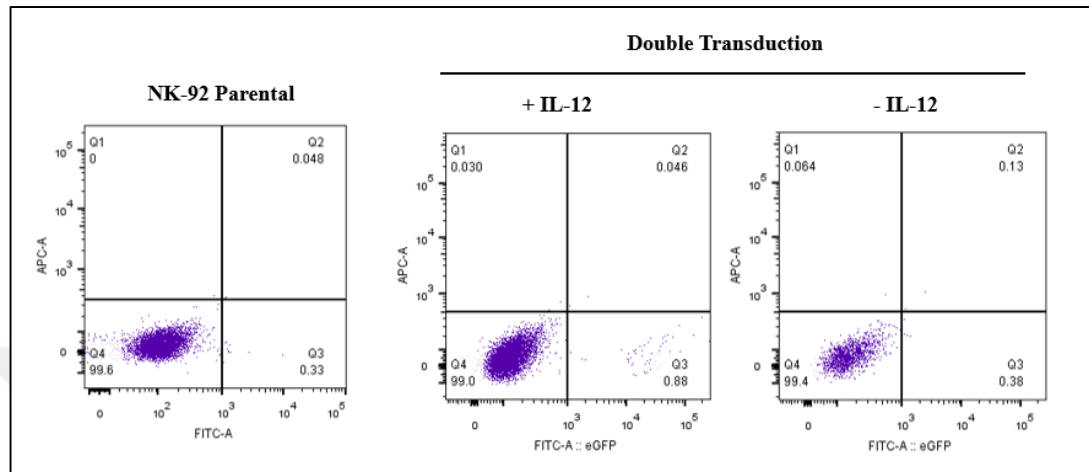


Figure 3.29 RetroNectin mediated transduction of psPAX2: pMD2.G packaged pLenti CMV eGFP Puro with or without IL-12 pre-incubation.

Regardless of the high efficiency obtained with pCMV.dR8.2 dvpr: pCMV-VSV-G packaged pLenti CMV eGFP Puro, psPAX2: pMD2.G mediated transduction yielded a very low transduction efficiency. Results were evaluated and determined that pCMV.dR8.2 dvpr: pCMV-VSV-G is the best packaging condition when NK-92 cell transduction is planned. Among all the methods, RetroNectin yielded the highest efficiency with a significant difference compared to other methods. Additionally, compatibility of the procedure with KHYG-1 NK cell line was analyzed, and similar efficiency was obtained. NK-92, and KHYG-1 transductions were proceeded with double transduction using RetroNectin and pre-IL12 incubation.

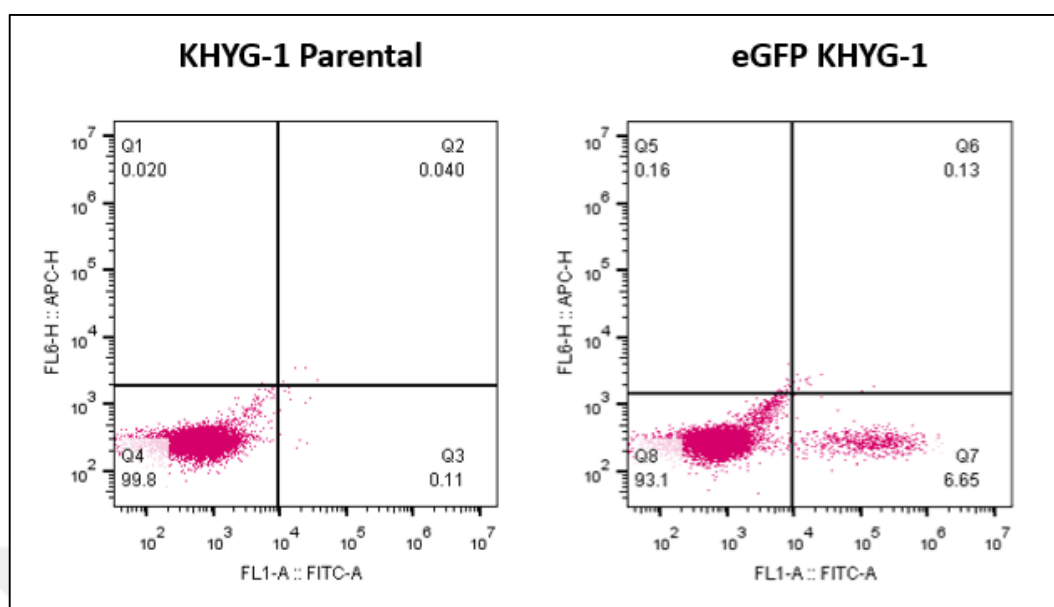


Figure 3.30 RetroNectin mediated transduction of pCMV.dR8.2 dvpr: pCMV-VSV-G packaged pLenti CMV eGFP Puro with IL-12 pre-incubation.

3.6. CAR VECTOR TRANSFER

3.6.1. dualCAR Vector

dualCAR vector was transduced to NK-92 and KHYG-1 cell lines. Accordingly to optimization studies transduction was applied by RetroNectin-mediated procedure. Even the best packaging plasmid combination identified as pCMV.dR8.2 dvpr:VSV-G, dualCAR vector transduction was applied with all the packaging combinations. Transduction efficiencies were analyzed by flow cytometry 5 days after the transduction. However, none of the conditions yielded positive results in any NK cell line. Obtained results do not indicate a low efficiency in the transduction process but a failure.

In order to eliminate any question mark regarding the results were caused by inconvenient method of transduction for NK cells, an “easy-to-transfect” cell line human immortalized astrocytes were transduced with dualCAR vector. As a positive control astrocytes were also transduced with pLenti CMV eGFP Puro.

Received results were similar to those of NK cells indicating the problem is not caused by “hard-to-transfect” characteristics of NK cells but the viral particle itself. It was suggested

that functional viral production containing the dualCAR vector was impaired. Experiments were proceeded to other CAR vector designs.

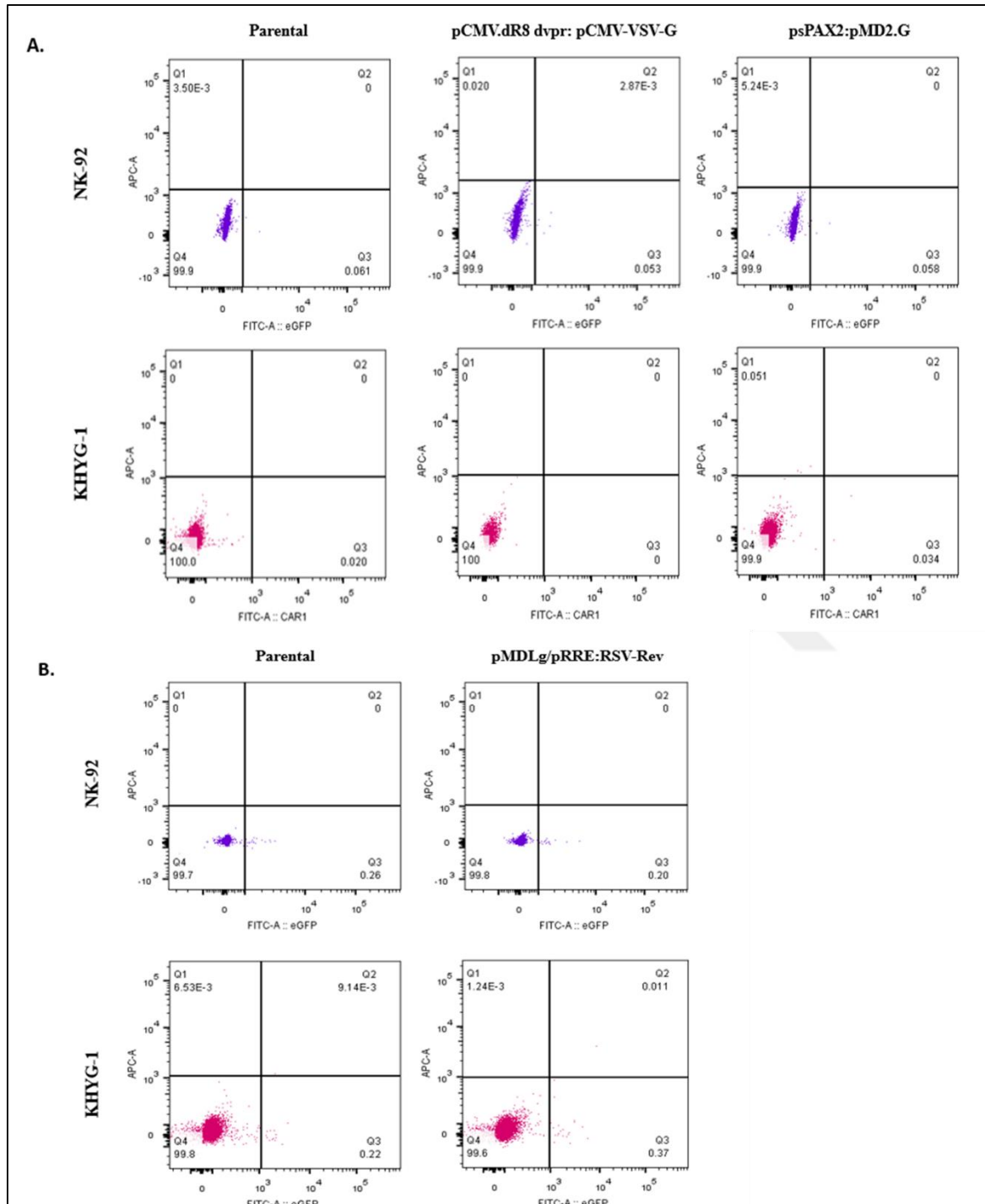


Figure 3.31 NK cell lines were transduced with dualCAR packaged lentiviral particles. A. NK-92 and KHYG-1 cell lines were transduced with dualCAR vector packaged with second generation packaging plasmids. B. NK-92 and KHYG-1 cell lines were transduced with dualCAR.

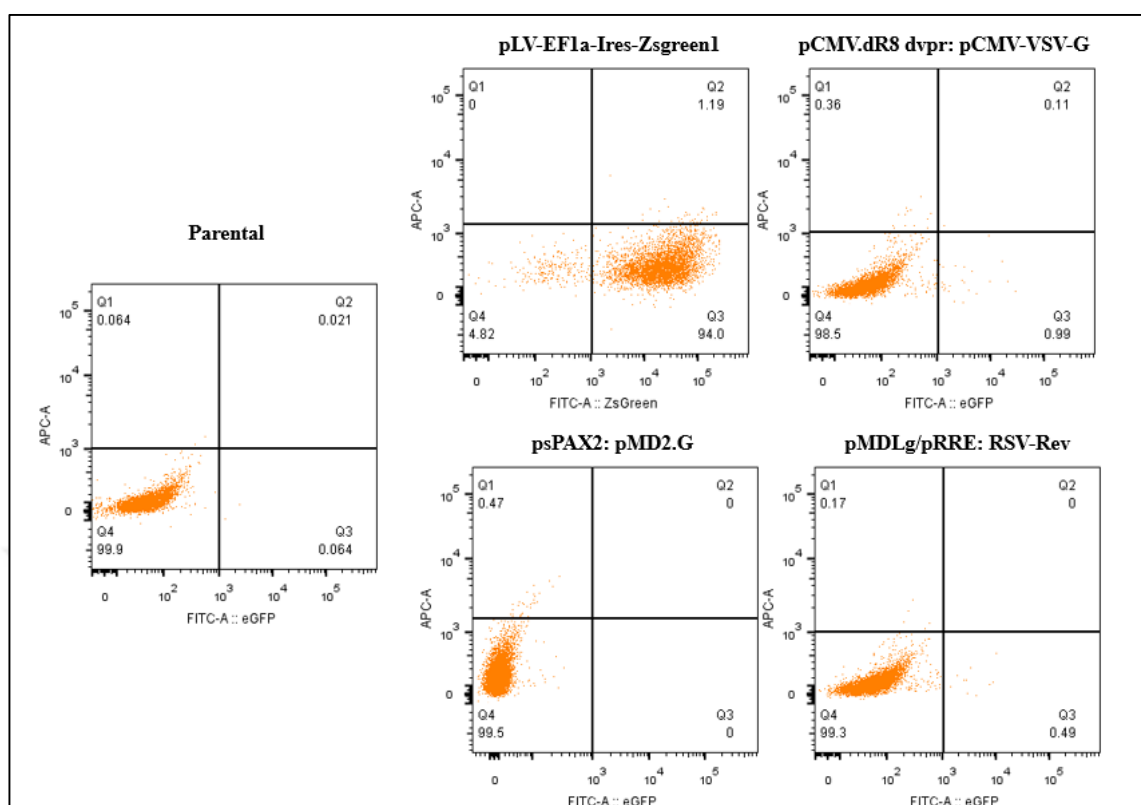


Figure 3.32 Human immortalized astrocyte cell line was transduced with dualCAR containing viral particles. pLV-EF1a-IRES-ZsGreen1.

3.6.2. Self-ligated PDGFR- α CAR

Impairment suggestion of viral production step is suspected because of the large plasmid size of dualCAR vector. Therefore, self-ligated PDGFR- α CAR plasmid were designed which would be in smaller size. Firstly, easy-to-transfect astrocytes was transduced to check the viral particle functionality. Cells were transduced with viral particles obtained from both HEK293T and HEK293FT. Also, second generation packaging plasmids were used. self-ligated PDGFR- α CAR was successfully transduced to astrocytes.

After confirming functional viral particle presence, NK cell lines were transduced with self-ligated PDGFR- α CAR. Distinct populations carrying the CAR vector was detected by flow cytometry. Though, the transduction efficiency was low.

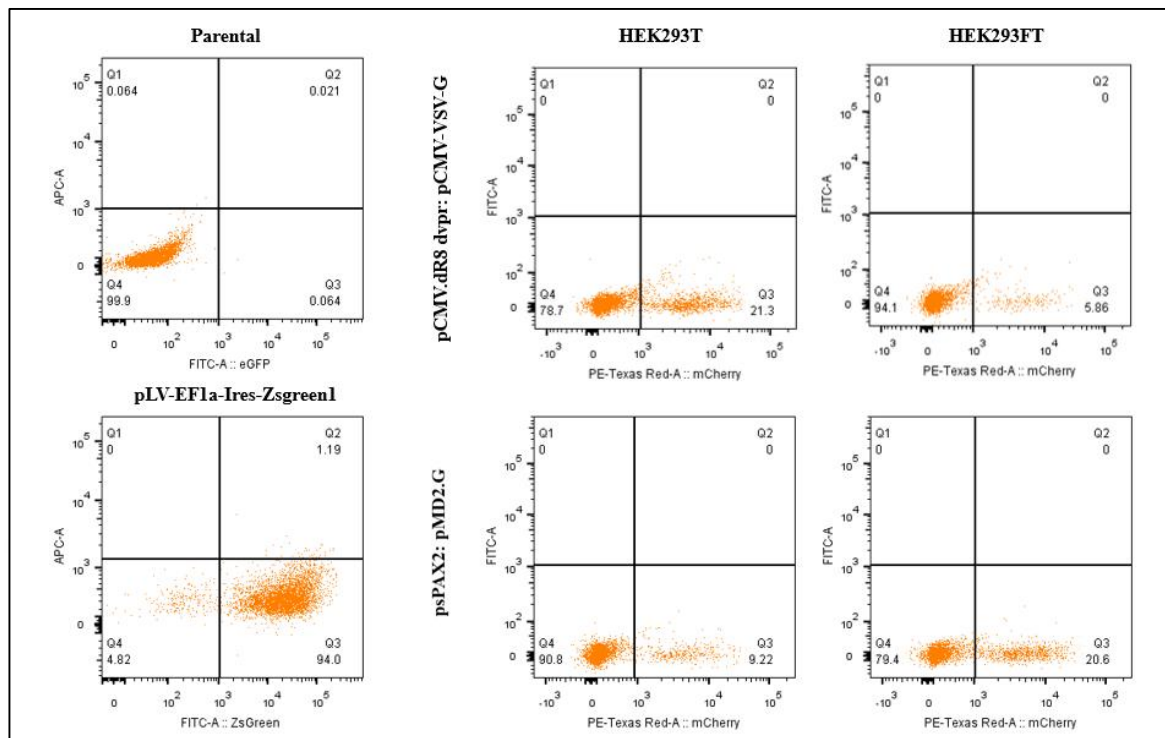


Figure 3.33 Human immortalized astrocytes were transduced with self-ligated PDGFR- α CAR. Viral packaging were performed both in HEK293T and HEK293FT cells.

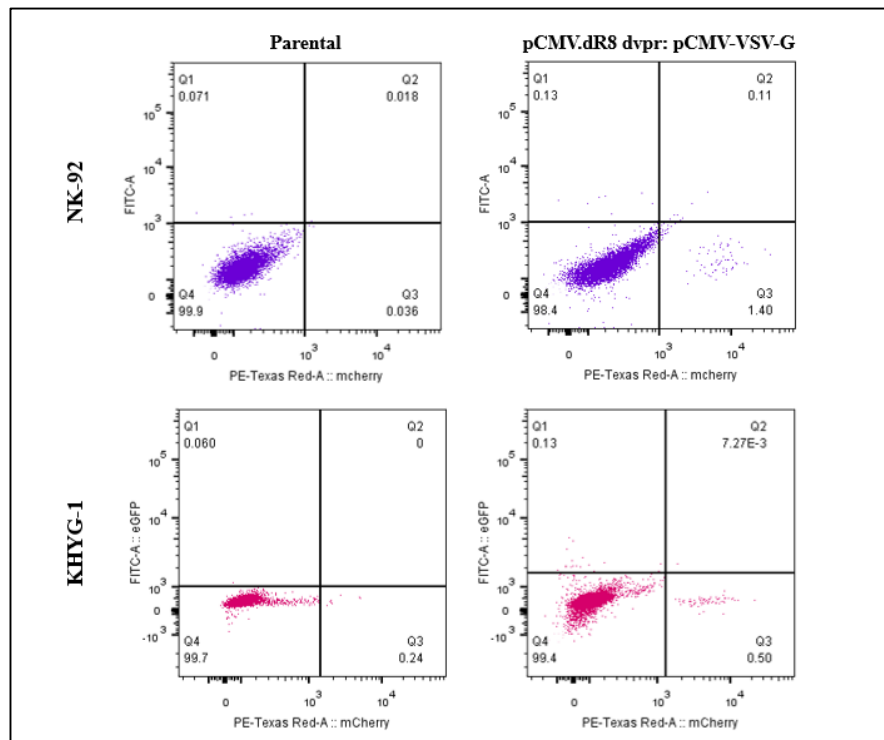


Figure 3.34 NK cell lines were transduced with self-ligated PDGFR- α CAR.

3.6.3. Divided CAR

Firstly, divided CAR plasmids were tested in Jurkat cells. Jurkat cells are similar to NK cells in transduction efficiency, both hard-to-transfect cell lines. Divided CAR plasmids' functionality were controlled by transducing and proceeding to expression analysis in Jurkat cells. Jurkat cells were successfully transduced and enriched populations were obtained by puromycin and gentamicin selection. However, NK cell lines could not be transduced with divided CAR plasmids, yet.

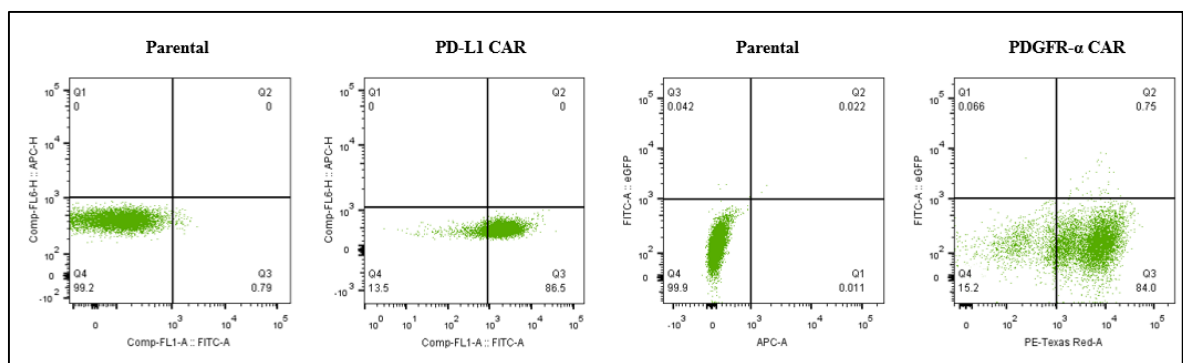


Figure 3.35 Jurkat cell line successfully transduced with divided CAR plasmids, and selected yielding high positivity detected by flow cytometry.

3.6.4. SBCAR

SBCAR plasmids were transduced to HEK293T, Jurkat and NK cell lines. Transfections were successful except the NK-92 cell line. HEK293T cells were easily transfected via liposome-mediated transfection method. However, the most efficient and stable transfection was achieved via Nucleofection in KHYG-1 cell line. NK-92 cells do not result in a stable transfection either with liposomal-mediated transfection method or Nucleofection. Jurkat cells are stably transduced with SBCAR vector via Nucleofection. Transfected HEK293T, KHYG-1, and Jurkat cells were referred to as HEK-SBCAR; KHYG-1-SBCAR; Jurkat-SBCAR. Positive cell populations were enriched either by cell sorting or puromycin selection for further experiments.

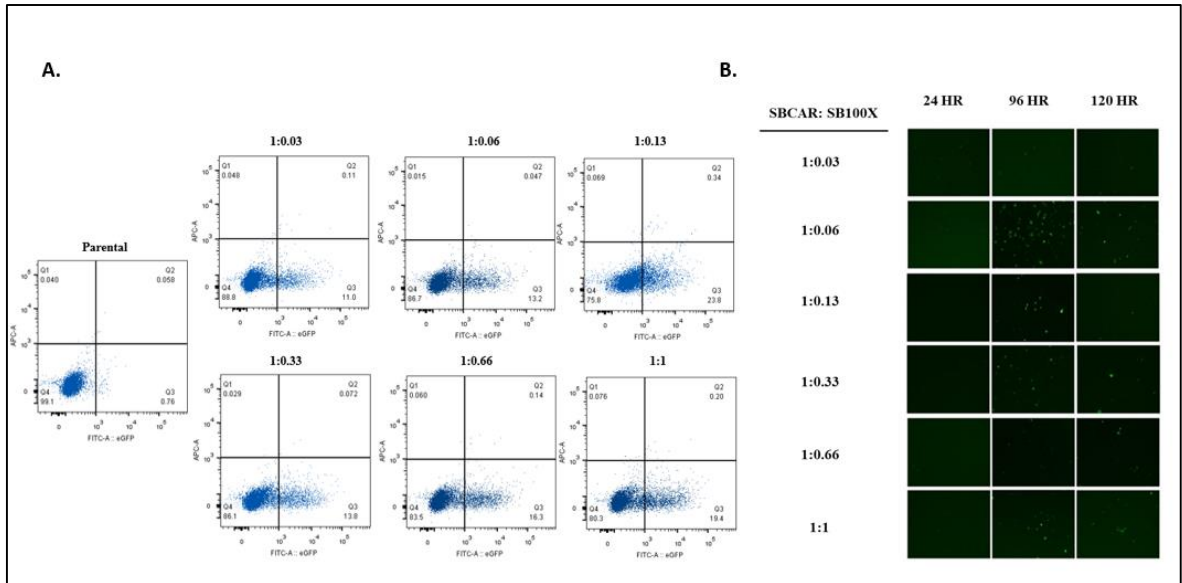


Figure 3.36 HEK293T cells were transfected with SBCAR. A. Transduction efficiency is controlled by flow cytometry. B. Fluorescence images of transfected HEK293T cells.

Nucleofection optimization studies of KHYG-1 cells provided the selection of the condition that exerts “highest viability”. It was determined that P3 primary cell kit of nucleofactor was the best solution protecting the viability. Also, it was determined that the most suitable nucleofection program is CA137 for NK-92 cell line and CM137 for KHYG-1.

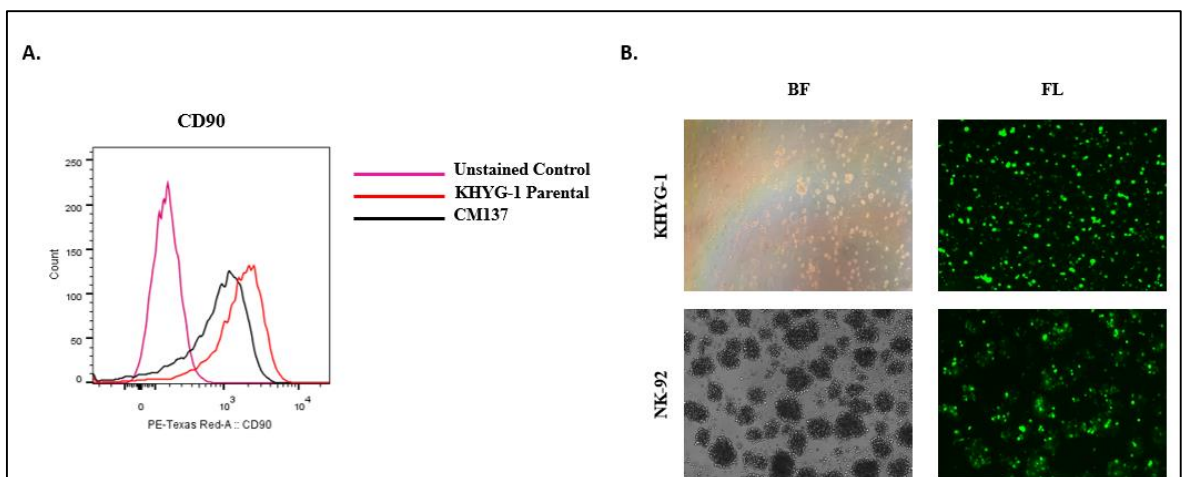


Figure 3.37 Nucleofactor optimization of KHYG-1 and NK92 cell lines. A. CD90 KO CRISPR plasmid was used in KHYG-1 optimization studies. CD90 expression was successfully knocked out in a minor population while preserving the cell viability with CM137 program of nucleofactor. B. KHYG-1 and NK-92 cell lines were transfected transiently with eGFP plasmid with CM137, and CA137, programs, respectively.

SBCAR transfection was successful in KHYG-1 cells when SBCAR: SB100X ratio was 1:0.06 and 1:0.66.

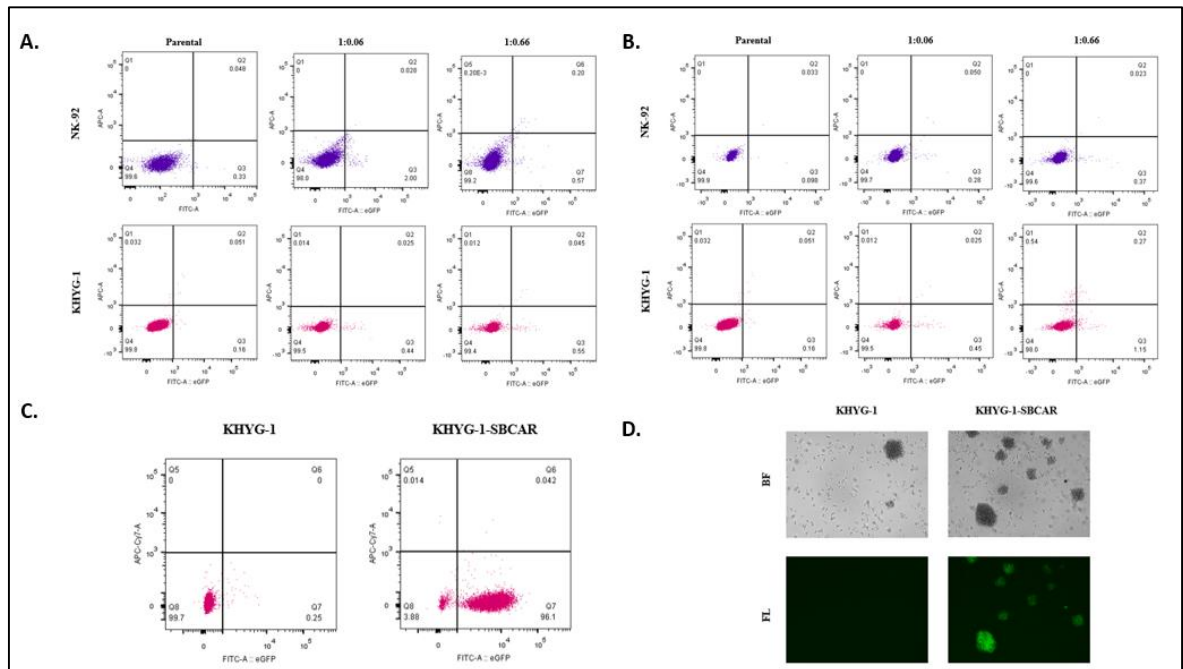


Figure 3.38 NK-92 and KHYG-1 cell line was transfected with SBCAR vector. A. NK-92 and KHYG-1 cell line was transfected with SBCAR vector via FuGENE HD-mediated transfection protocol. B. NK-92 and KHYG-1 cell line was transfected with SBCAR vector via Nucleofactor. C. Nucleofactor transfected KHYG-1 cells were exposed to 0.5 μ M puromycin selection and eGFP positive cell population percentage was checked by flow cytometry. D. Images of KHYG-1 and KHYG-1-SBCAR cells after puromycin selection.

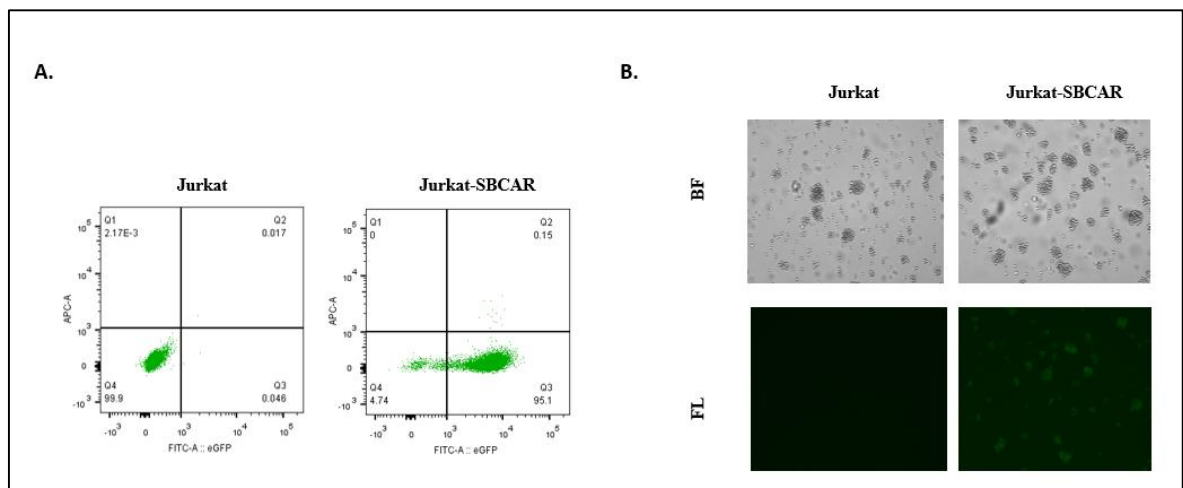


Figure 3.39 Jurkat cell line was transfected with SBCAR: SB100x vectors at 1:0.66 ratio. A. Nucleofactor transfected Jurkat cells were exposed to 0.5 μ M puromycin selection and eGFP positive cell population percentage was checked by flow cytometry. B. Images of Jurkat and Jurkat-SBCAR cells after puromycin selection.

3.7. CAR EXPRESSION ANALYSIS

CAR plasmids genomic integration is confirmed by PCR reaction using puromycin specific primers. Since parental cells do not have puromycin resistance gene no amplification is expected but if genomic integration of the transferred plasmids occurs then amplification is expected. Accordingly, all the cells positive for eGFP expression had amplified puromycin resistance gene. PCR results provide a preliminary data for genomic integration.

Additionally, chimeric antigen receptor expression was confirmed at protein level. Western blot is used to detect the CD3 ζ chain of CAR receptor. Normally, naive CD3 ζ , ~20kDa, is expressed in NK cells and T-cells. When CD3 ζ expressed within CAR structure ~70 kDa protein detection is expected. However, CD3 ζ expression do not confirm if CAR structure is expressed at the cell membrane. For the detection of CAR expression at cell surface protein L staining is used. CAR- CD3 ζ was detected in HEK-SB cells but could not be detected in KHYG-1-SBCAR cells despite the puromycin resistance gene presence and stable expression of eGFP.

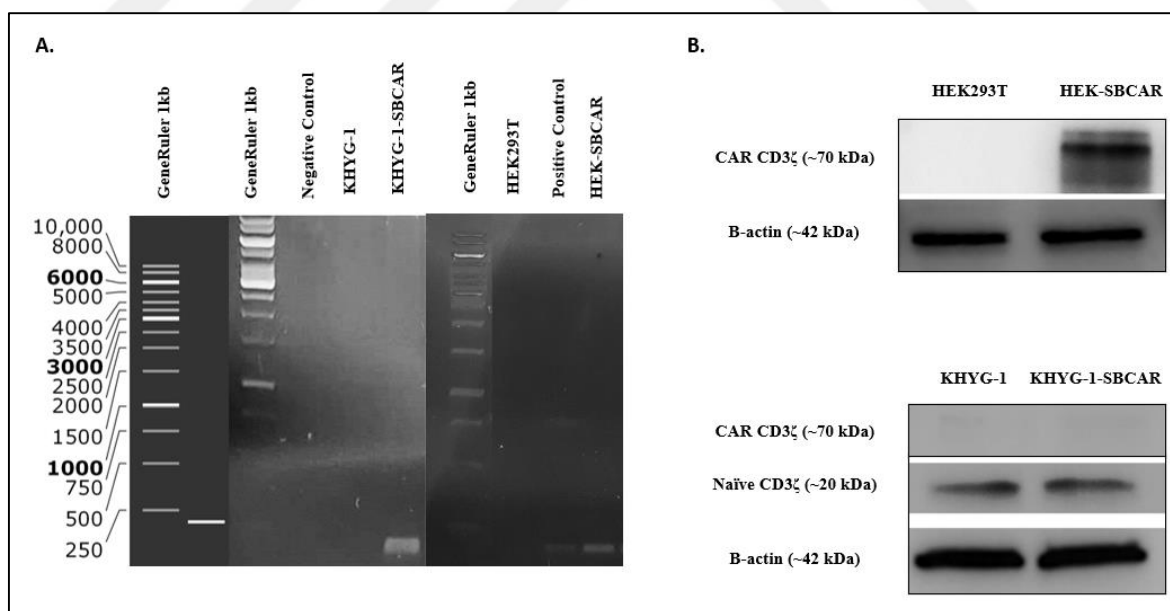


Figure 3.40 Confirmation of HEK-SBCAR and KHYG-1-SBCAR cells. A. HEK-SBCAR and KHYG-1-SBCAR cells express puromycin resistance gene within their genome. B. CAR- CD3 ζ expression is confirmed for HEK-SBCAR cells but not for KHYG-1-SBCAR cells.

Protein L binds immunoglobulin light chains, scFV regions and Fab fragments (256). In case of CAR expression, enhanced binding of protein- L in flow cytometric analysis is

expected. HEK-SBCAR cells stably express CAR structure (82%) on surface. However, KHYG-1-SBCAR cells did not exhibit a distinct CAR expressing cell population (22.4%) as HEK-SBCAR cells. Besides, the detected CAR surface expression was lost within time.

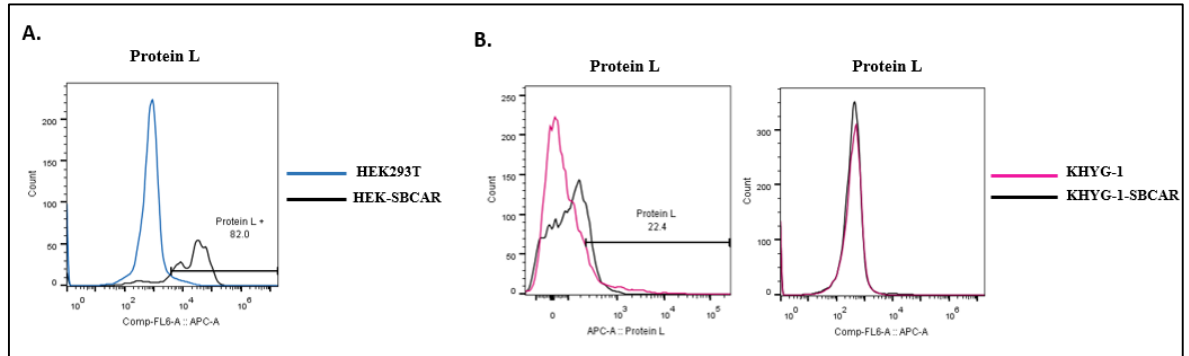


Figure 3.41 CAR surface expression was evaluated by protein L staining in HEK-SBCAR and KHYG-1-SBCAR cells A. HEK-SB cells high amounts of CAR on their surface as shown by protein L staining. B. KHYG-1-SBCAR cells initially found to be express low amount of CAR on their cell surface and the expression was lost within time.

Except the Jurkat-PDGFR- α cells surface CAR expression could not be detected. The cell surface expression detected in Jurkat-PDGFR- α cells was compatible with the literature examples.

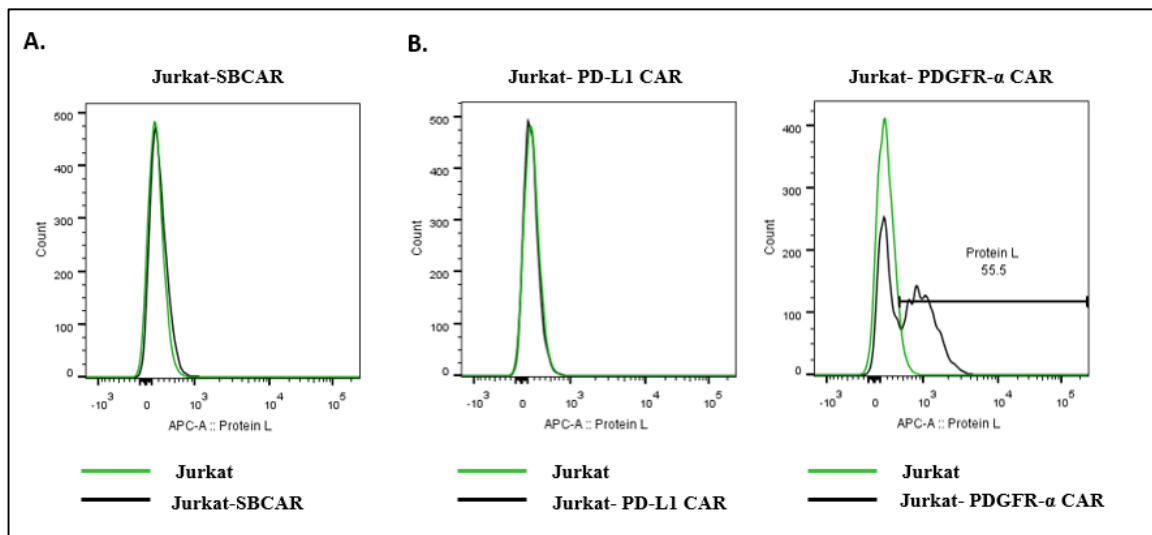


Figure 3.42 CAR surface expression was evaluated by protein L staining in Jurkat cells. A. Jurkat cells that were constantly positive for eGFP stained with protein L for cell surface CAR expression detection. B. Jurkat-PD-L1 and Jurkat-PDGFR- α cells that were constantly positive for eGFP stained with protein L for cell surface CAR expression detection.

3.8. CHARACTERIZATION OF JURKAT-SBCAR, JURKAT-PD-L1 CAR & JURKAT PDGFR-A CAR

Basal expression of PD-L1 and PDGFR- α proteins were evaluated in Jurkat cells. Later, PD-L1 and PDGFR- α expressions were evaluated in CAR vector transfected Jurkat cells. Only Jurkat-PDGFR- α CAR cells demonstrated upregulation of both proteins.

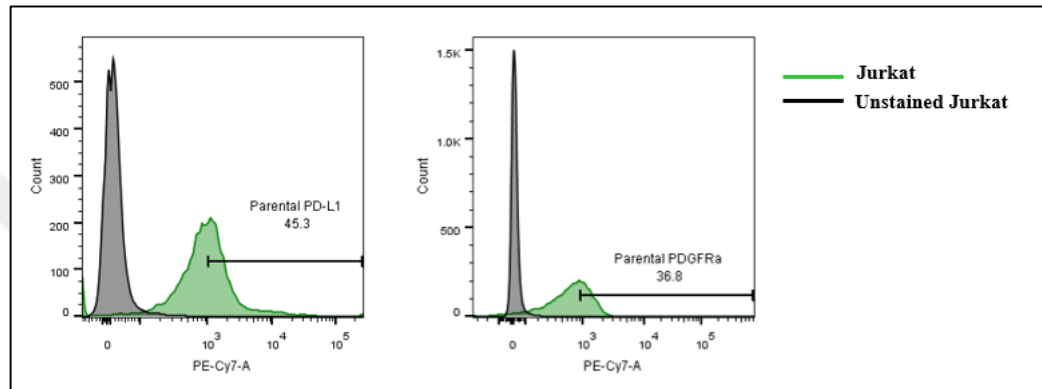


Figure 3.43 Basal PD-L1 and PDGFR- α cell surface expression of Jurkat cells.

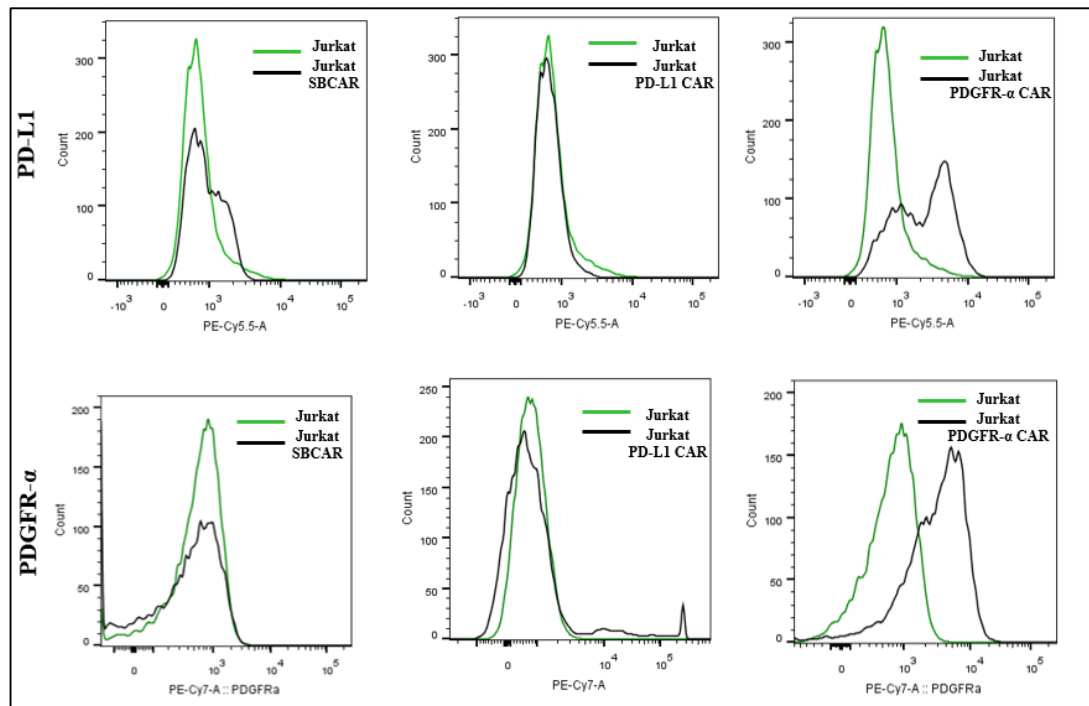


Figure 3.44 PD-L1 and PDGFR- α cell surface expression of SBCAR and divided CAR vector transfected Jurkat cells.

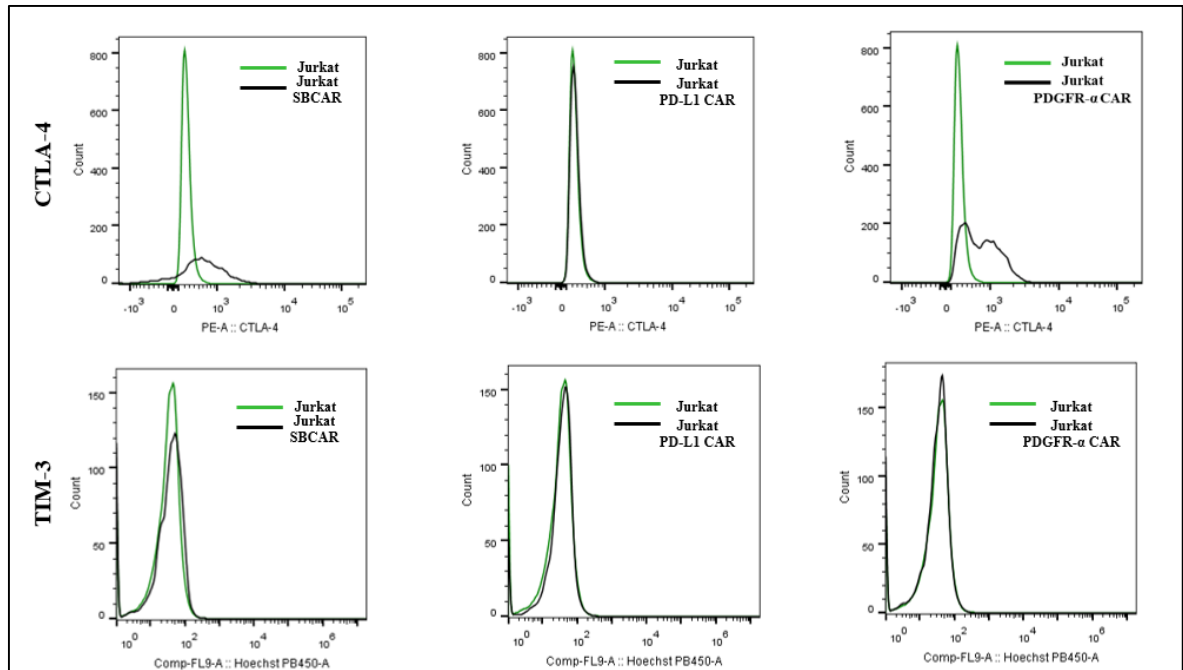


Figure 3.45 CTLA-4 and TIM-3 cell surface expression of SBCAR and divided CAR vector transfected Jurkat cells.

T-cell activation (CTLA-4) and exhaustion (TIM-3) markers were evaluated in CAR vector transfected Jurkat cells. Jurkat PD-L1-CAR cells do not express any of the markers while Jurkat-PDGFR- α CAR and Jurkat-SBCAR cells demonstrated upregulation of CTLA-4. None of the cells exhibit TIM-3 positivity.

4. DISCUSSION

An efficacious CAR-ACT rely on the appropriate design since the details and even the minor changes in CAR structure lead the fate of the application proficiency. It was reported that 72 CAR-NK cell line studies and 35 CAR- primary NK cell preclinical studies present in PubMed and Global Data research using the key words, “chimerical antigen receptor and natural killer cells” until March 2021 (257). However, the major limitation in CAR designs for NK cells is the CAR-T based knowledge. The NK- specific designs are limited (258). Therefore, the CAR design strategy that implicated in this thesis also benefit from accumulated knowledge on CAR-T generation.

The scFV selection is the primary step of CAR design. scFV regions for the selected targets are derived from antibodies. However, the binding affinity varies between the antibodies. Additionally, even the high affinity sequences are chosen, disfunction of CAR-cells still a possibility. So that high binding affinity do not correlate with the function, emphasizing the necessity of scFV optimization. Current strategies in scFV optimizations involves the production of CAR-cells and further assessing the suitability which is time consuming and labor intensive. Besides, the effectiveness is questionable of this empirical method (259–264). scFV library display is being investigated for the optimization studies and demonstrated promising results such as enhanced proliferation capacity, prolonged persistence, and higher reactivity against the target (264). Yet, the scFV library screens are at experimental stage obligating the “do-it, test-it” method. The selected targets in the CAR-NK design needs to be tested functionally once the CAR-NK cells are generated. In this thesis, multitarget strategy is established offering a high specificity against the chordoma cells. Multitargeting function in either and-gate or or-gate approaches. Dual targeting might be implicated as expressing two different CAR in one single cell (dual CARs, or tandem expression of two scFV domain in one CAR structure (tandem CAR). Both the dual and tandem CAR function in and-gate logic. Dual-CARs are cloned into a bicistronic vector to provide the expression of both CAR structure in a single cell. Though, dual expression plasmids require codon optimization since they are prone to DNA recombination due to the repeated regions. Also, dual expression in one vector enlarge the plasmid size limiting the viral packaging if viral backbone is used. Tandem CARs overcome the size limitation since two scFV expressed along one CAR. However, the design stage is much more challenging.

scFV conformations, spacer and linker sequence and length are crucial in optimal CAR expressing cell activation. And-gate logic stimulates the cytotoxicity when one or another scFV domain binds to target. Thus, any of the scFV domain binding is sufficient to exert cytotoxic function. However, this might result in a major drawback which is the increased toxicity risk (247,265–267). Therefore, in this thesis, a combinatorial CAR design is implicated. Combinatorial CARs function in or-gate logic, where two CARs are expressed in one cell. However, only one has the ITAM-containing domain, CD3 ζ , and the other contains the co-stimulatory domains. This ensures that a robust cytotoxic response is initiated only if two scFV binds to the target. The primary aim of choosing the combinatorial CAR design is to minimize the possible toxic side effect to surrounding healthy cells while maximizing the specificity against chordoma cells.

Previous studies demonstrated the PD-1 and PD-L1 expression in chordoma cells and chordoma infiltrating lymphocytes revealing a prognostic significance. Indeed, not all chordoma patients express high levels of PD-1 and PD-L1 and the expression of those proteins are dynamic due to such conditions as inflammatory state or exposure to ICI therapy. Migliorini et. al. suggested that PD1/PD-L1 expression might not be an indication of effective immunotherapy response in chordoma patients (228). It was reported that high target expressing cells are more promising against targeted therapies. However, in chordoma low PD-1/PD-L1 expressing cell response against PD-1/PD-L1 targeting is not known. A recent study generated a CAR structure targeting PD-L1 (scFV derived from atezolizumab). The CAR structure were implicated to T-cells, NK-cells and NK-92 cell line. CAR expressing cell efficiencies were evaluated in PD-L1^{high} MDA-MB-231 cells, and in PD-L1^{low} MCF-7 breast cancer cell lines. Degranulation and cytokine production was not responsive in MCF-7 cell line. However, long term killing assays exerted a delayed cytotoxicity compared with MDA-MB-231 cells and interestingly MCF-7 cell co-culture with activated PD-L1–CAR cells upregulated the PD-L1 expression on MCF-7. This results indicates an important possibility of PD-L1 targeting CAR to exert a self-amplifying effect on low target expressing cells. Besides, HER2-CAR cells also induced PD-L1 expression on MCF-7 cells enhancing the PD-L1–CAR function (268). Therefore, in this thesis, it is aimed to provide a CAR design to question that if dual targeting of PDGFR- α along with PD-L1 could exert an synergistic effect in a similar manner. Indeed, an enhanced activity is expected due to increasing the specificity by dual targeting. Though, an additional question

is asked if dual targeting strategy exert its synergistic effect by self-amplification of target antigens on chordoma cells. Future perspective of this study is the co-culture of CAR-NK cells with chordoma cells and following *in vivo* experiments performed by patient-derived xenograft (PDX) models. Chordoma cell lines readily have low PD-L1 and PDGFR- α expressions enabling the evaluation on low target expressing chordoma samples. In contrast, PDX models will enable the evaluation of both low and high target expressing chordoma samples *in vivo*. To test this hypothesis a preliminary data is obtained using Jurkat- PDGFR- α -CAR cells. Jurkat T-cell line have the basal expression of PD-L1 (45.3%) and PDGFR- α (36.8%). Jurkat- PDGFR- α -CAR cells demonstrated upregulated expression of both the PD-L1 and PDGFR- α which might be a clue of self-amplifying effect. Also, it could be envisioned that PDGFR- α targeting may enhance PD-L1 expression further improving the CAR-mediated cytotoxicity. Though, this effect might be a two-edged sword since the amplification effects might be observed on the CAR-cells itself. The increased PD-L1 expression on Jurkat- PDGFR- α - CAR cells may exert neutralizing or inhibiting function which must be evaluated in detail.

Stem cells eradicated by NK cells as demonstrated by allogeneic hematopoietic stem cell rejection but not solid tissue grafts. Additionally, NK cells can potentially kill non-proliferating cells (269). Thereby, NK cells have great potency in cancer stem cell (CSC) targeting and may be used as a second-line therapy following the anti-proliferative therapies. In breast, glioma, pancreatic, breast, and sarcoma tumor cell lines' CSC population selectively killed by NK cells. Also, primary cells obtained from fresh tissue of liposarcoma, Ewing's sarcoma and pancreatic cancer were co-cultured with NK cells. Correlated with the cell line results, fresh tissue samples also exhibit decreased CSC population upon NK exposure. The CSC-specific targeting of NK cells linked with the NKG2D and its ligand interaction (270). Pietra et. al. performed CD133 (melanoma CSC marker) cell enrichment, tumorsphere formation and radioresistance to provide CSC enriched models of melanoma. All methods results in selective NK-cell mediated cell death through the interaction of NKG2D receptor and its ligands (271). Accordingly, NK cell therapy is suggested to present unique advantages to eradicate those (CSCs) recurrence-related, drug and radioresistant subpopulations of cancer cells. Also, CSC killing potential of NK cells were evaluated in chordoma. Researchers applied a combinatory treatment consisting of anti-PD-L1 antibody, N-601, IL-15 superagonist complex (N-803) and PD-L1-targeted chimeric antigen receptor

high affinity NK cells. Results revealed preferential killing of chordoma CSCs due to the increased PD-L1 expression in this subgroup (272). Therefore, it was questioned if selected targets for chordoma cells might be an targeting strategy for chordoma CSC population as well. To elucidate this question, the previously performed and processed microarray data of our research group investigating the differential gene expressions between chordoma tumorspheres and parental chordoma cells is used. Depending on the fold changes it was demonstrated that PD-L1 expression is not differential between tumorspheres and parental chordoma cells. In the previous study, PD-L1 expression was found to be upregulated in CD133⁺/CD15⁺ CSC population of chordoma. However, the transcriptome results revealed no difference in PD-L1 expression. This could be influenced by the difference in methodology used to enrich the CSC population. PDGFR- α yielded statistical significance in fold change between tumorspheres and parental chordoma cells. Thus, selective targeting of CSC population of chordoma cells is a possibility to be investigated which may be beneficial to prolong the recurrence free survival of chordoma.

Hinge region provides the stability and necessary flexibility to access the target antigen. Length of hinge region should be designed depending on the antigen distance to the membrane. For example, long or complex antigens (e.g., glycosylated antigens) should be fused to long sequences of hinge region while membrane proximal antigens should be fused to short hinge sequences. In turn, the relation between scFV region and hinge domain determine the immunological synapse formation between the effector and target cell (273). Generally, immunoglobulin (Ig)-like domains are used in hinge regions but those regions may interfere with Fc gamma receptor (Fc γ R) causing off-target effects (274). Nevertheless, steric hinderence of MUC-1 recognition was reported to be better targeted when IgD hinge region used rather than CD28 (275). Though, the off-target effects are considered to be eliminated, and CD28, and CD8 α hinge regions become more prominent. The use of CD28 and CD8 α are further encouraged by the implication of these regions into FDA-approved immunotherapeutics, Yescarta and Kymriah (276–278). CAR structure, presented in this thesis, designed base on the FDA-approved therapeutics and literature examples. The design of hinge regions depend on the antigen recognition criteria but the physiological effect on T or NK cells remains unknown (273).

CAR design is focused on the combinatorial CAR in this thesis. Thus, a single cell should compromise two different CAR structure. To ensure the receipt of two parts of CAR by the

same cell the dual CAR structure is cloned into a single vector in dualCAR design. Thus, the CAR sequences should be divided and this is provided by ribosomal skipping proteins. In this design, self-cleaving peptides T2A and P2A are used. T2A and P2A polypeptides are intentionally chosen since the internal ribosome entry site (IRES), another ribosomal skipping protein, might decrease the expression of the gene that located after itself (279). Additionally, IRES may influence viral DNA transcription during lentiviral particle production limiting the produced titer and/or integration to target cell genome (280,281).

Optimal expression of CAR structure requires fine-tuning that can be controlled by promoter choice. High expression of CAR may trigger ligand-independent activation of CAR-T cells and consequent exhaustion state and decreased cytotoxic effect or off-target effects (277,282). Yet, the direct comparison of promoter choice is not fully elucidated for the long mRNA sequences of multitargeting CAR designs. Current FDA-approved CAR-T cells either second or third generation but encodes only one scFV. Therefore, the promoter requirement for long sequences is not known and suggested to differ from the requirements that drive short sequences (283). One of the most broad in spectrum study compared four different promoter (CMV, EF-1a, hPGK and RPBSA) in short (~2.4 kb) and long (~6.8 kb) sequences to be synthesized. CMV and EF1-a is found the strongest promoters both in the short and long sequences. None of the promoter was found to interfere with the viral DNA transcription or integration into genome. Promoter regions contains CpG islands that are prone to methylation and subsequent silencing of the promoter. Silencing probability is highest in the CMV promoter while EF-1a maintain the gene expression and stability. Nevertheless, CAR expression may be downregulated upon T cell exhaustion and this is more potent than the promoter methylation (284,283). Such comprehensive study encompassing CAR structure do not exist for NK cells. Just as different strength of promoters between long and short sequence synthesis, the cellular requirements may also differ between NK and T cells. In primary NK cells, transduction efficiency and transgene expression is compared between EFS, PGK, SV40, CMV, CBH, EF1a, UBC, and CAG promoters. EF1a provided the second highest transgene expression. Though, the transduction efficiency ~30% while EFS promoter provided ~40% efficiency. The stability of transgene demonstrated no difference between promoters (285). Even, this study helps to see the effect of promoters in NK cells, it do not provide information about CAR expression

which might be more complex, especially when the next generation CAR structures are used, than GFP expression. In the designed CAR vectors EF-1a promoter is used.

The initial results in dualCAR vector transduction in NK cell lines resulted in failure. Therefore, it was questioned that if the problem related to viral packaging or NK cell lines are hard to transduce. Indeed, NK cells are hard to transduce cell lines greatly limiting their use in CAR technologies but it was only expected to have a low transduction efficacy. Firstly, the produced viral particles were used to transduce human immortalized astrocyte cell line. This cell line is easily transduced by zsGreen transduction yielding 94% transgene positive population. However, no efficiency was obtained when dualCAR vector is used. Therefore, it is assumed that due to the large size (~12 kb) of the dualCAR vector viral packaging stage is impaired. Theoretically, lentiviral vectors up to ~9 kb is efficiently packaged. Anyhow, when the vector sizes approach to physical limit the packaging capacity is decreased (286). Also, it was demonstrated that if HIV-1 backbone is used in lentiviral vectors, then the every 1 kb increase in size mitigates the three times drop of viral titer (287). To test if the problem is caused by viral particle production, a divided CAR strategy is designed. Divided CAR plasmids were synthesized by Creative Biolabs to ensure the plasmid integrity and codon optimization but prior to commercial attainment the theory was tested using the self-ligated PDGFR- α CAR. Results demonstrated that self-ligated PDGFR- α CAR is efficiently packaged as they are successful in transducing the human immortalized astrocyte cells (efficiency ~5-25%) and NK cells (with a low efficiency ~0.50-1.40%). Right after the divided CAR plasmids provided they are also controlled by transducing Jurkat cell line which exerts similar difficulty in transduction such as NK cells. It was confirmed that both parts of CAR encoding plasmids were successfully packaged and a sufficient viral titer is obtained.

SBCAR plasmids were initially transfected to HEK293T cells to check if the plasmids are functional. Stable expression of eGFP is observed. Also, stable and high CAR expression on the cell surface was detected. However, both the KHYG-1 and Jurkat cells obtained stable eGFP expression, CAR expression could not be detected. Initially, KHYG-1 cells expressed the CAR on the cell surface but lost within time and the initial expression was too low. Promoter strength is not enough by itself to explain this outcome. Though, eGFP is the most distal transgene in the plasmid design. So, if the problem caused by promoter strength, then low to no eGFP expression is expected. Interestingly, Jurkat-PD-L1 CAR cells also did not

express CAR structure despite the eGFP expression. The expected (depending on the literature examples) CAR surface expression profile only provided by Jurkat- PDGFR- α CAR cells (256,288). When PD-L1 CAR and PDGFR- α CAR plasmids compared the only difference is the reporter gene choice (PD-L1 CAR- eGFP; PDGFR- α CAR mcherry). In this case, the eGFP expression is suspected to interfere with the CAR expression. One opinion is the use of reporter genes like GFP might overestimate the CAR expression. Indeed, the results indicate that eGFP expression is not an indicative of CAR expression in cells (289). There are several evidences that GFP expression may have an influence on gene expression profile of the host cell. For example, GFP sequence was used as a non-target control in a RNA interference (RNAi) study on *Nasonia vitripennis*. Transcriptomic analysis showed that 3.4% of total genes were differentially expressed in response to GFP RNAi. Authors proposed that the reason might be non-specific activation of the RNAi machinery and advised to use a different control sequence (290). Another study revealed that eGFP and eGFP fusion proteins polyubiquitination. Polyubiquitination, posttranslational modification, functions in kinase signaling activation and protein degradation processes within the cell. This study shows that protein machinery may be influenced by eGFP expression (291). Since, the genomic integration is occurred within transduced cells, the protein synthesis machinery may be interfered. Proteomic effects of stable GFP expression in breast cancer cell line, MDA-MB-231, is investigated. GFP expression is found to be associated with the expressions of proteins such as protein folding, and cytoskeletal organisation-related proteins (292). However, none of these studies provide a mechanistic answer how eGFP could interfere with the gene expression or in which conditions. The molecular mechanisms underlying the GFP-related changes in cellular physiology remains to be unknown. On the other hand, the CAR expression on HEK293T cells transduced with SBCAR vector might indicate a cell type dependent reaction to eGFP induced cellular changes if it takes place in NK and T-cells as well.

For many years, one of the biggest challenge is the persistence of the immune cells in CAR-ACT therapies. Many structural modification (e.g., addition of co-stimulatory domains) has been tested to copy the physiological conditions driving the full T-cell activation. Still, CAR degradation upon T-cell activation is a major drawback of *in vivo* studies. Recently, it was discovered that CAR-T cells may be activated in an antigen-independent manner and influence a tonic signaling which in turn activates the T-cell. Subsequent outcome of T-cell

activation is the exhaustion. The activation and exhaustion cycle of CAR-T cells before encountering the target cells compose the major reason of *in vivo* failure. The tonic signalling is triggered by the self-aggregation of CAR. The extracellular domain design has the huge impact on tonic signaling. The hinge regions based on IgG is more potent to induce this signaling than CD8 and CD28 (274,293–295). Additionally, CAR cells might be activated *in vitro* by self expressed antigens. It was shown that CD33-CAR-NK cells had CD33 expression 0.45% while non-transduced NK-cells have 54.95%. This shows that CAR-cells might target themselves and eradicate the targeted population (296). This is a major disadvantage since it might fasten the cell exhaustion. In order to access if self-targeting-associated exhaustion could be the reason of non-detected CAR expression TIM-3, a T-cell exhaustion marker is examined. None of the CAR transduced Jurkat cell exhibited TIM-3 positivity. Also, CTLA-4 expression was evaluated. CTLA-4 is expressed upon T-cell activation and its degradation is decreased (297). Jurkat PDGFR- α CAR cells exhibited enhanced CTLA-4 expression which might be an indicative of tonic signaling or self-antigen mediated activation. Though, further analysis is required for an definitive information. Besides, PD-L1 and PDGFR- α expressing populations of Jurkat cells is not eliminated after CAR transduction. Depending on the results the CAR expression is not thought to be downregulated due to exhaustion. Anyhow, the reason of why CAR structure is not expressed in cells transduced with PD-L1 CAR and SBCAR vectors remains unknown.

5. CONCLUSION

Chimeric antigen receptor-based therapeutics are the prominent components of immuno-oncology. However, despite the promising outcomes and advanced genetic engineering techniques, many obstacles still need to be overcome in the structural design and generation of chimeric antigen receptor-carrying cells. Moreover, several hindrances (e.g., tumor escape, tonic signaling, exhaustion state) must be solved to improve clinical efficiency. Additionally, exploration and detailed examination of the properties of CAR designs is obligatory since not all gene combinations or CAR structure-related parts are combinatorial. The selected sequences (e.g., signaling domains, co-stimulatory domains) should be wisely chosen since a minor change could influence significant functional differences. Also, numerous studies demonstrated that chordoma might be a good candidate for immunotherapeutic approaches. The chordoma-specific targeting strategies will allow individualized patient care and provide an alternative way to treat standard therapy-resistant patients. Therefore, chordoma-based basic research is warranted.

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