A SYSTEMIC COMPARISON OF DIFFERENT CHIMERIC ANTIGEN RECEPTOR (CAR) DESIGNS FOR RETARGETING OF NK-92 CELLS AGAINST TUMOR ANTIGENS

by

ELIF ÇELIK

Submitted to the Graduate School of Engineering and Natural Sciences

in partial fulfillment of

the requirements for the degree of

Master of Science

Sabancı University

July 2019

A SYSTEMIC COMPARISON OF DIFFERENT CHIMERIC ANTIGEN RECEPTOR (CAR) DESIGNS FOR RETARGETING OF NK-92 CELLS AGAINST TUMOR ANTIGENS

APPROVED BY:

Asst. Prof. Dr. Tolga Sütlü (Thesis Supervisor) .....

Prof. Dr. Batu Erman .....

Asst. Prof. Dr. Emre Deniz .....

DATE OF APPROVAL: 18/07/2019

ABSTRACT

A SYSTEMIC COMPARISON OF DIFFERENT CHIMERIC ANTIGEN RECEPTOR (CAR) DESIGNS FOR RETARGETING OF NK-92 CELLS AGAINST TUMOR ANTIGENS

Elif Çelik

Biological Sciences and Bioengineering, M.Sc. Thesis, 2019

Thesis Supervisor: Tolga Sütlü

Keywords: natural killer cells, immunotherapy, chimeric antigen receptor

Cancer immunotherapies focus on the power of the immune system to attack tumor cells. Recently, Chimeric Antigen Receptors expressing T cells (CAR-T cells) have received clinical approval for antigen-specific adoptive immunotherapy against CD19 in B cell malignancies. CAR vector designs have dramatically developed since their initial discovery and now include first-generation CARs (CD3ζ-based CAR), second-generation CARs with additional costimulatory domains such as CD28 or CD137 and third generation CARs (CD3ζ with two costimulatory domains) and recently fourth generation CAR with a transgene for cytokine stimulation.

Natural Killer (NK) cells have ability recognize the tumor cells by their native receptors and have grown to be promising candidates for adoptive immunotherapy of cancer. CAR expression in NK cells is also clinically tested and carries the potential to translate into clinical application but the majority of literature on CAR vector design relies on observations from T cells.

This thesis aims to use NK-92 cells for evaluation of different designs in order to optimize a CAR vector that could be efficiently used to retarget NK cells against tumor antigens. CAR transgenes comprising identical antigen binding domains that target CD19, combined with different intracellular signaling domains (CD3ζ , CD28 and CD137) are transferred to NK-92 cells via the use of lentiviral vectors. Cytotoxic activity and antigen-specificity of CAR-NK-92 cells are evaluated against the CD19- classical NK cell target K562 cell line and the CD19+ cell line Daudi and Namalwa by analysis of

degranulation and cytokine secretion. Our results provide valuable data for optimal CAR vector design in NK cells.

ÖZET

NK-92 HÜCRELERİNİN TÜMÖR ANTİJENLERİNE YÖNLENDİRİLMİŞ FARKLI KİMERİK ANİTJEN RESEPTÖR TASARIMLARININ SİSTEMİK KARŞILAŞTIRILMASI

Elif Çelik

Biyoloji Bilimleri ve Biyomühendislik, Yüksek Lisans Tezi, 2019

Tez Danışmanı: Tolga Sütlü

Anahtar kelimeler: doğal öldürücü hücreler, immünoterapi, kimerik antijen reseptör

Kanser immünoterapileri, bağışıklık sistemindeki hücrelerin tümörlere karşı olan saldırma gücüne odaklanır. Yakın geçmişte, B hücresi malignitelerinde bulunan CD19'a karşı Kimerik Antijen Reseptörleri ile modifiye T hücreleri (CAR-T hücreleri), klinik immünoterapi uygulamaları için onay almıştır. CAR vektör tasarımları, ilk keşiflerinden bu yana çarpıcı biçimde gelişmektedir. Birinci nesil CAR'ları (CD3ζ tabanlı), CD28 veya CD137 gibi ek sinyal bölgelerinden birini içeren ikinci nesil CAR'lar ve CD3ζ'nın yanında iki sinyal bölgesi daha içeren üçüncü nesil CAR'lar takip etmiş, ayrıca bunların yanına sitokin stimülasyonu için bir genin eklenmesiyle elde edilen dördüncü nesil CAR vektörleri de geliştirilmektedir.

Doğal Öldürücü (NK) hücreler, kendi doğal reseptörleri tarafından tümör hücrelerini tanıyabilmektedir ve bu sebeple adoptif immünoterapi için umut vadeden yeni adaylar olarak öne çıkmaktadır. CAR ifade eden NK hücreleri de klinik olarak test edilmekte ve klinik uygulamaya çevrilme potansiyelini taşımaktadır fakat CAR vektör tasarımlarına dair literatürün büyük çoğunluğunu T hücreleri üzerinden yapılan gözlemler oluşturmaktadır.

Bu tezde NK hücrelerinin tümör antijenlerine yönlendirilmesi için kullanılabilecek bir CAR vektörü tasarımının optimizasyonu için NK-92 hücrelerinin kullanılması amaçlanmıştır. CD19 antijenine karşı aynı antijen bağlanma bölgesine sahip fakat farklı hücre içi sinyal bölgelerinin (CD3ζ, CD28 ve CD137) kullanıldığı CAR genleri lentiviral vektörler aracılığıyla NK-92 hücrelerine aktarılmıştır. CD19+ hücre hatları Namalwa ve Daudi'nin yanı sıra CD19- hücre hattı K562 kullanılarak CAR-NK-

92 hücrelerinin degranülasyon ve sitokin salımı aktiviteleri ile antijen özgüllükleri değerlendirilmiştir. Sonuçlar NK hücrelerine özel CAR vektörü tasarımları için önemli veriler sağlamaktadır.

To my mother, grandmother, and grandfather…

Canım anneme, anneanneme ve büyükbabama…

ACKNOWLEDGEMENTS

I would first like to start by showing my thankfulness to my thesis advisor Dr. Tolga Sütlü for his infinite support, the patience he showed me and his guidance since 2016. I cannot tell how lucky I am to work in his lab. I always feel his support in my personal life and my academic life. His office was always open whenever I had a question or a trouble for my life or my master thesis. He strives for me to gain good ethical perception and to be a good scientist. Thank you for everything especially for introducing me to immunotherapy!

I would also like to thank Prof. Dr. Batu Erman. I feel very fortunate and privileged to able to attend scientific discussions with him and to take his classes. It was a great pleasure to work with him and his lab. I am forever grateful for his knowledge and guidance.

I would like to express my gratitude to Asst. Prof. Dr. Emre Deniz for accepting to be on my thesis committee and giving time and advice about my thesis.

I am indebted to Dr. Winfried S. Wels, he gifted us the CAR plasmids. Based on these plasmids we gathered a lot of data and experience.

I am thankful to Prof. Dr. İhsan Gürsel for the target cells, Sera Kadıgil Sütlü and Eren Atik for transportation of cells. If they had not helped, I wouldn't be able to complete this thesis or get any results to make a conclusion.

I would like to thank Dr. Ahsen Morva Yılmaz, İsmail Yılmaz and Sercan Keskin for cell sorting. Their hospitality at TUBİTAK-MAM was a great help.

I would like to thank our collaborator Duru Lab. I first met Dr. Adil Doğanay Duru at a Skype interview and after which he introduced me to Dr. Tolga Sütlü and shaped my academic career without knowing. One of my special thanks for Dr. Ece Canan

Sayitoğlu, she was a good teacher during my internship after that she was always supportive of me.

I would like to also thank the past and present members of Erman Lab.: Hakan Taşkıran, Liyne Noğay, Melike Gezen, Nazife Tolay, Ronay Çetin, Sanem Sarıyar, Sarah Barakat, Sinem Usluer, Sofia Piepoli. It was amazing to work at Erman Lab with you guys!

When I was an undergraduate student, I came to Sütlü Lab and met the great family! I will always be proud of this family. I would like to thank the past and present members of Sütlü Lab for giving me great memories: Alp Ertunga Eyüpoğlu, Ayhan Parlar, Aydan Saraç Derdiyok, Dr. Başak Özata, Cevriye Pamukcu, Didem Özkazanç Ünsal, Lolai Ikromazada, Mertkaya Aras, Pegah Zahedimaram, Seden Bedir. I would especially like to show my appreciation to Didem and Ayhan for teaching me everything since my first day as an intern to now and future. Didem is my sister whom I can call whenever I have trouble. Last one month was challenging for me, Didem, Başak, and Cevriye spent many hours trying to help me finish this thesis. You are the BEST! All of Sütlü Lab. members are always a part of my family. Thank you for everything!

I would like to thank my best friends Görkem Gençer and Pelin Karaturhan who have been my friends for 10 years. They have been always with me in my worst and most depressive times and of course most beautiful memories. Thank you for enduring me!

Finally, my biggest Thanks to My Family! I want to start with my grandmother Ayhan Özarabacı and my grandfather İbrahim Özarabacı, I always wish I had my grandfather with me, but I'm sure he's proud of me. I thank you both for your support and unconditional love. I hope, I have become a worthy grandchild for you. My dearest mother, Nuran Özarabacı. She always dreamed of her children getting a master’s degree. I hope you will be happy when you read these lines. Thank you for raising us like this. The biggest chances of my life are my big brother Onur Çelik and My twin sister Deniz Çelik. My brother has always been unconditional support and he is the dream brother for the little sisters. Thank you for being such a great brother. I would also like to thank Simge İplikçi for providing me with a home environment in Istanbul. My dear twin sister Deniz, I do not know how to thank you. You have always been with me for 24 years. You made me feel special and strong. I could not graduate without you. You are the best twin sister in the world. I love you!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
κ	Kappa
μ	Micro
μL	Microliter
μM	Micromolar
ADCC	Antibody-dependent cellular cytotoxicity
AKT	Activating Receptor tyrosine kinase
ALL	Acute Lymphocytic Leukemia
APC	Allophycocyanin
BCMA	B-cell maturation antigen
bp	Base pair
BV	Brilliant Violet
CAR	Chimeric Antigen Receptor
CIAP	Calf Intestine Alkaline Phosphatase
CD	Cell differentiation
CTLA-4	cytotoxic T-lymphocyte associated protein 4
CO_2	Carbon dioxide
CSR	Cytokine release syndrome
DC	Dendritic Cell
ddH2O	Distilled water
DLBCL	Diffuse large B-cell Lymphoma
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxade
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
EPCAM	Epithelial cell adhesion molecule
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GFP	Green Fluorescent Protein
Gly	Glycine

GvHD	Graft versus host disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human epidermal growth factor 2
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
HVEM	Herpesvirus entry mediator
ICOS	Inducible T-cell co-stimulator
IFN	Interferon
IL	Interleukin
IgG	Immunoglobulin G
IRES	Internal Ribosome entry site
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JNK	C-Jun N-terminal kinase
Kd	kilodalton
KIR	Killer-cell Immunoglobulin-like Receptor
LAK	Lymphokine-activated killer cell
LB	Luria Broth
mAb	Monoclonal antibody
MEM	Minimum Essential Media
MHC	Major Histocompatibility Complex
MOI	Multiplicity of Infection
NCR	Natural cytotoxicity receptors
NEAA	Non-essential Amino Acid
OXO	(5Z)-7-Oxozeaenol
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death protein 1
PI	Propidium Iodide
PI3K-AKT	Phosphoinositide-3-kinase-protein
PIPES	piperazine-N,N′-bis (2-ethanesulfonic acid)
PSMA	Prostate-specific membrane antigen
Puro	Puromycin
RPMI	Roswell Park Memorial Institute
Rpm	Round per minute
RT	Room Temperature
SEM	Standard Error of Mean
Ser	Serine
SFFV	Spleen Focus Forming Virus
SLAM	Signaling lymphocytic activation molecule
Syk	Spleen tyrosine kinase
TAA	Tumor-associated antigen
TCR	T-cell Receptor
TNF	Tumor Necrosis Factor
TRAIL	TNFα-Related Apoptosis-Inducing Ligand
VSV-G	Vesicular stomatitis virus G
WT	Wild Type
Zap-70	zeta-associated of 70 Kd tyrosine kinase

1. INTRODUCTION

1.1. Natural Killer Cells of The Immune System

1.1.1. Description and origin

The immune system consists of different cell types and soluble factors that are responsible for defending against intrinsic or extrinsic threats. The immune system is mainly separated into two branches as innate and adaptive immunity (Figure 1.1). Innate immunity is the first barrier in the body to show response to non-self-invaders and danger-associated molecular patterns (Medzhitov and Janeway 2000). Adaptive immunity responses come secondary to innate immunity and are responsible for recognizing and distinguishing specific molecules on pathogens (Alberts et al. 2002).

Natural killer (NK) cells are members of innate immunity but come from the lymphoid lineage and they are first described as a type of lymphocyte in mice simultaneously by two groups in 1975 (Herberman et al., 1975; Kiessling et al., 1975). They are derived from $CD34^+$ hematopoietic progenitor cells (Raulet and Vance 2006). One of the milestones of NK cells is the recognition of the 'missing-self' that seems to act as a safety switch for attempts of escape from T cell-mediated immunity by means of MHC downregulation (Ljunggren & Kärre, 1990).

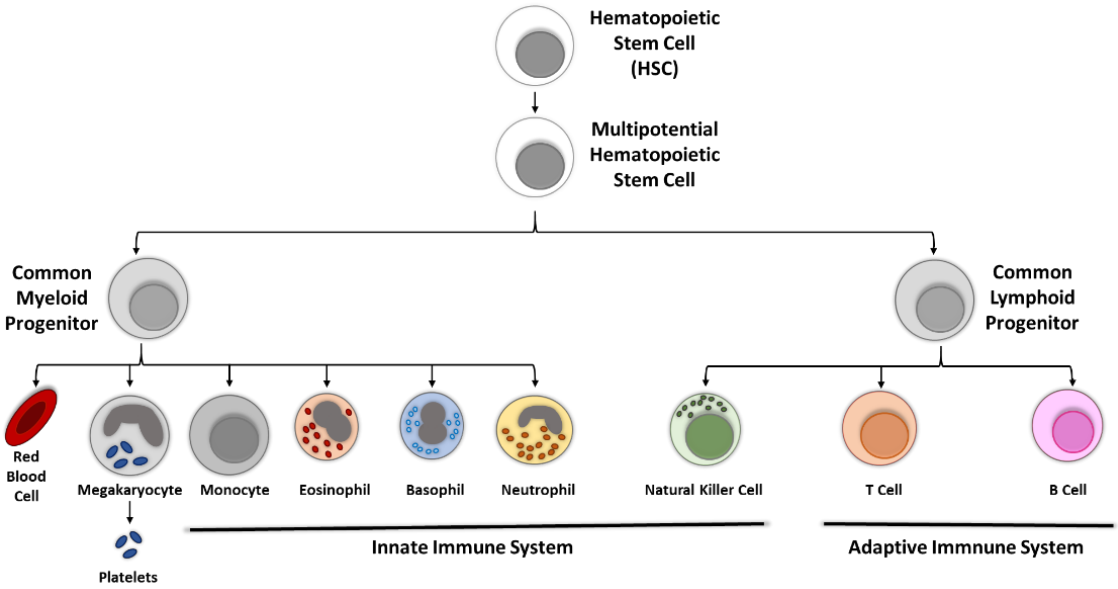

Figure 1.1. Hematopoiesis and branching of the immune system. Hematopoietic Stem Cells (HSCs) are found in the bone marrow and generate the two major cell types, myeloid and lymphoid progenitors. Common myeloid progenitors differentiate into red blood cells, platelets, monocytes and granulocytes (eosinophils, basophils and neutrophils) which are members of the innate immune system. NK cells are classified as members of the innate immune system but they are derived from the common lymphoid progenitor which also gives rise to the development of adaptive immunity members T and B cells.

1.1.2. Subtypes

Human NK cells are identified as $CD3^-$ $CD56^+$ lymphocytes that developed in the bone marrow and are found in the blood, skin, lungs, liver, spleen, and lymph nodes (Grégoire et al. 2007). Human peripheral blood contains 5-10% NK cells. In general, there are two types of pf NK cells with specific roles (Figure 1.2.).

The majority of NK cells in human peripheral blood (about 90%) have low levels of CD56 ($CD56^{dim}$) and a small number (about 10%) has high CD56 ($CD56^{bright}$) expression (Vivier et al. 2008). $CD56^{dim}$ cells express high levels of CD16 (FcRIII) but not the high-affinity IL-2 receptor alpha chain (CD25) (Chan et al. 2007). The upregulation of CD25 under IL-2 stimulation helps NK cells provide lymphokine-activated killer (LAK) activity resulting in higher cytotoxic activity (Muralikrishna, Varalakshmi, and Khar 1997). $CD56^{dim}CD16^{bright}CD25^{neg}$ cells have effector functions such as natural cytotoxicity or

antibody-dependent cellular cytotoxicity (ADCC). They have higher cytosolic activity and show high expression of inhibitory killer cell immunoglobulin-like receptor (KIRs) whereas CD56^{bright} NK cells have important regulatory roles (Lanier 2004).

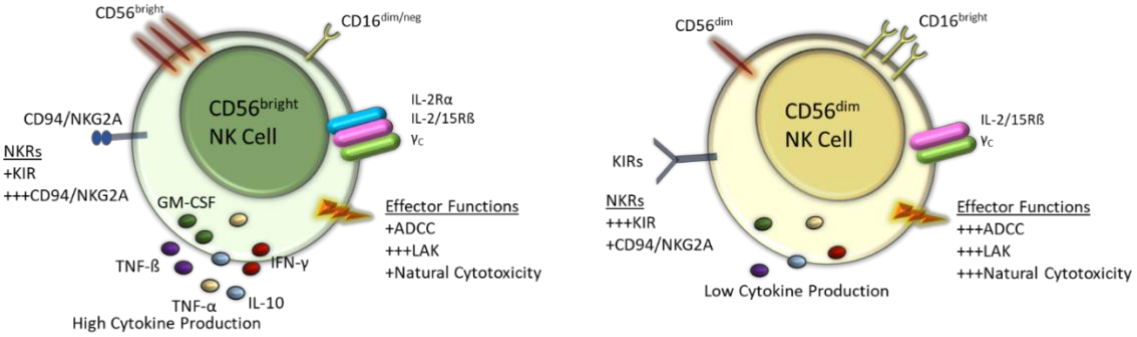

Figure 1.2. Phenotypic and functional comparison of CD56^{bright} and CD56^{dim} NK cells. CD56^{bright} NK cells express high levels of the IL-2 receptor, lack CD16 expression and produce high-level immunoregulatory cytokines. CD56^{dim} NK cells have KIR expression and high levels of cytotoxic activity, mainly responsible for natural cytotoxicity.

CD56^{bright} cells do not show any cytotoxic activity act as more regulatory cells (Ferlazzo et al. 2004). They have CD94 / NKG2A but lack expression of KIR, however secrete various cytokines such as Interferon-gamma (IFN-γ), Tumor Necrosis Factor-alpha (TNF-α), IL-10, IL-13, and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) there make a link between innate and adaptive immune response (De Maria et al. 2011). If CD56^{bright}CD16$^{dim/neg}$ cells interact with fibroblasts, they may differentiate into CD56^{dim}CD16$^{dim/neg}$ cells with high cytolytic activity and express the CD25 (Chan et al. 2007).

1.1.3. Effector mechanisms

The combination of activating and inhibitory receptors is responsible for the mechanism of action of the NK cell. NK cells distinguish the fate of the target cell according to the interactions of these receptors with their cognate ligands on the target cell (Figure 1.3.). Since the ligands for inhibitory receptors are generally MHC-I molecules, this makes NK cells able to recognize when MHC-I expression is lost on the target cell. This phenomenon is called “missing-self recognition” and it is the reigning model of NK cell activation, stating that NK cells mediate lysis of cells that do not express normal levels of self MHC ligands (Ljunggren and Kärre 1990). The activating signals and the inhibitory signals can

be considered as on a balance. As a results of viral infections or cellular stress; the cells can lose MHC-I expression on the cell surface and cause unbalance and NK cell activation occurs (Sentman, Olsson, and Kärre 1995).

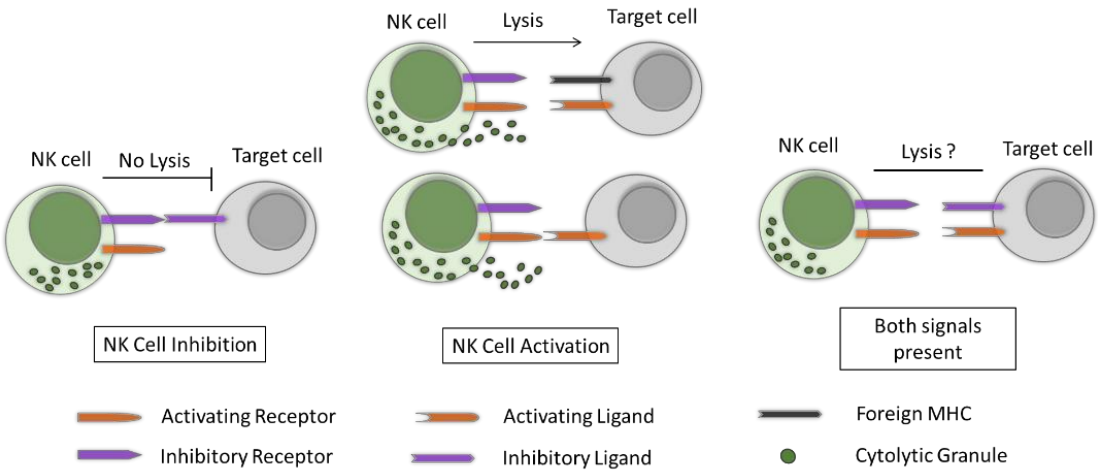

Figure 1.3. NK cell effector mechanisms. When NK cell faces with a target and if the only inhibitory ligand (mostly self-MHC molecules) engagement to inhibitory receptors, the target is protected from lysis, it is called NK Cell inhibition. If there is a low level of MHC molecules on the surface, and NK cells recognizing MHC low/absent cells is known as 'the missing-self recognition'. In NK cell Activation the inhibitory signal is missing, or target cells have a non-self MHC molecule so there is an activating signal, the target cell is killed with cytolytic granule. When both signals are present, the fate of the target cell is determined by the dominant signal (right-most).

The signals from these two groups of receptors are integrated intracellularly and the net result of this signaling dictates the NK cell to kill or spare the target cell.

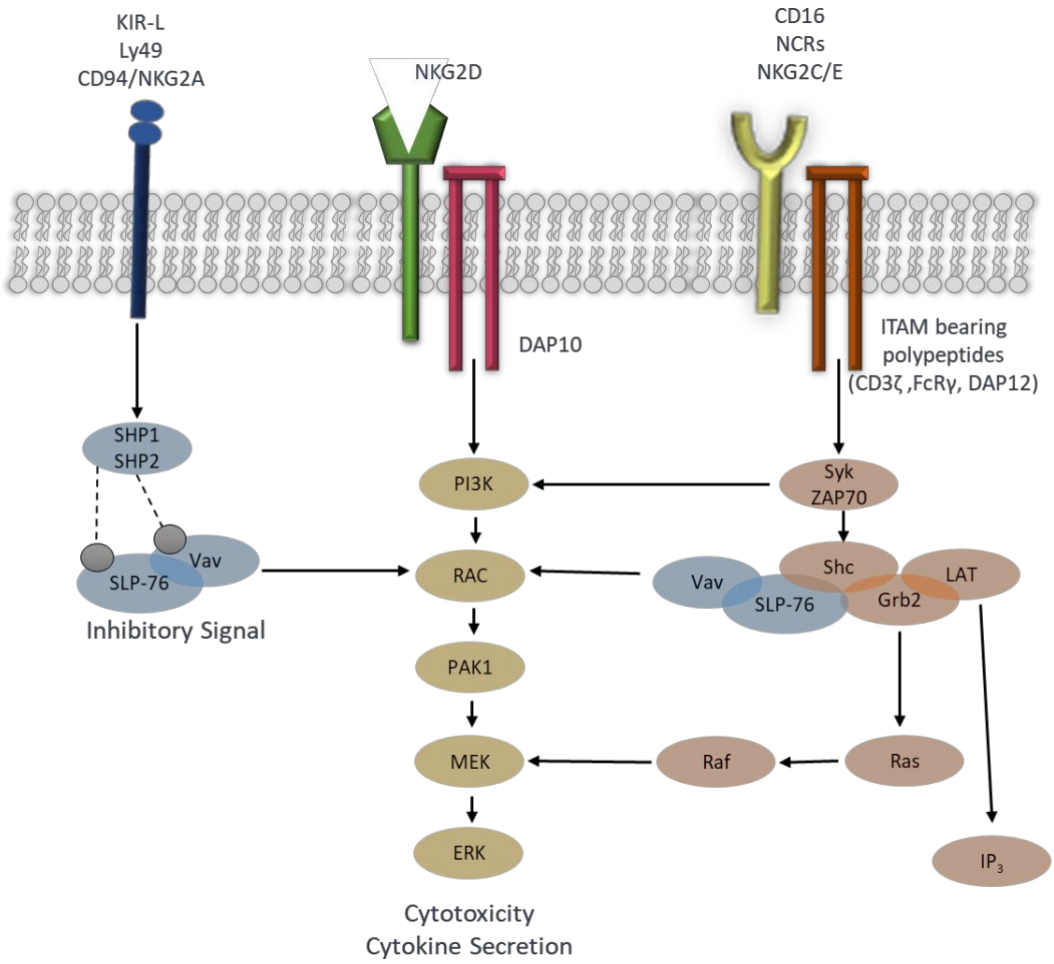

Figure 1.4. The integration of activating and inhibitory signals in NK cells. Figure adapted from (Vivier, Nunès, and Vély 2004).

Activating receptors such as CD16, the natural cytotoxicity receptors (NKp30, NKp44, NKp46), NKp80, and CD160 associate with (ITAM)-bearing molecules DAP12 and CD3ζ through their intracellular domains. Other ones such as NKG2D, 2B4, CD2 and DNAM-1 signal through non-ITAM-bearing DAP10 (Pegram et al. 2011). Upon the activation, ZAP70/SYK or PI3K pathways are turned on to lead the activation of the NK cell (Smyth et al. 2005). On the contrary, Inhibitory receptors have cytoplasmic immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic tails (Krzewski and Strominger 2008) (Figure 1.4.). The binding of inhibitory receptors to classical MHC I ligands (HLA-A, -B, -C) induces signals via SHP-1 and SHP-2 (Yusa, Catina, and Campbell 2002) that dephosphorylate the same intermediates as the activating signals are trying to phosphorylate. The net result of these phosphorylation/dephosphorylation events helps to quantify the extent of

inhibitory and activating signals received by the cell. If the activating signals are more dominant the perforin and granzyme containing granules begin to polarize towards the target cell and get secreted at the immunological synapse, causing apoptosis of the targeted cell (Bryceson et al. 2006).

NK cells also kill by death receptors Fas-ligands (FasL) and TNFα-related apoptosis-inducing ligand (TRAIL) which both induce apoptosis in target cells (Medvedev et al. 1997).

1.2. NK-92 Cell Line and its clinical applications

Currently, 10 different NK cells lines are known. NK3.3., YT cells (Yodoi et al. n.d.), NKL Cells (Robertson et al. n.d.), HANK1 cells (Kagami et al. 1998), NK-YS Cells (Tsuchiyama et al., n.d.), KHYG-1 Cells (Yagita et al. 2000), SNK-6 and SNT Cells (Nagata et al. 2001), IMC-1 Cells (I. M. Chen et al. 2004) and NK-92 cells (Gong, Maki, and Klingemann 1994). The first described cell line is NK3.3. which a normal NK-derived cell line obtained by Kornbluth in 1982 (Kornbluth 1982). Among these cell lines, however, NK-92 stands out with its high cytotoxic activity and wide clinical applicability.

NK-92 is an immortal natural killer cell line which is isolated from 50 years old male non-Hodgkin's lymphoma patient in 1992 (Gong, Maki, and Klingemann 1994). NK-92 cells are negative for CD3, CD4, CD8, and CD16, therefore, cannot mediate ADCC (HG Klingemann and Miyagawa 1996). The cell line is bound to IL-2 to survive however NK-92MI and NK-92 CI cells that are derived from NK-92 cells and have similar biological properties, but they are IL-2 independent (Tam et al. 1999). In the event of deprivation of IL-2 in the culture/medium, NK-92 cells lose the ability to form colonies and start to die (Gong, Maki, and Klingemann 1994). NK-92 cells express CD2 and CD56 but lack the inhibitory KIR receptor family members (except for low levels of KIR2DL4) and therefore are not subject to MHC-mediated inhibition of cytotoxic activity (Suck et al. 2016). NK-92 cells express high levels of Perforin and Granzyme B and are highly cytotoxic against tumor cells with low MHC expression such as human chronic myeloid leukemia cell line K562 (Boyiadzis et al. 2017). NK-92 express a relatively large number

of activation receptors such as NKp30, NKp46, 2B4 (also inhibitory), NK2GD, NKG2E and a few inhibitory receptors NKG2A and NKG2B (Maki et al. 2001).

Efficient isolation and expansion of primary NK cells from peripheral blood mononuclear cells (PBMCs) still poses problems and it is even more problematic to genetically modify them. Nevertheless, immunotherapy approaches involving primary NK cells continue to show high promise (Sutlu et al. 2012; Miller and Lanier 2019). NK-92 cells appear to be an alternative way for clinical application. There are phase 1 and 2 studies that use NK-92 cells in cancer immunotherapy (Tonn et al. 2013; Boyiadzis et al. 2017). NK-92 cells have been shown to exert cytotoxic activity against multiple myeloma cells (Swift et al. 2012). NK-92 infusions were applied in lung, kidney cancer, and melanoma patients and successful results were obtained (Arai et al. 2008; Tonn et al. 2013). However, NK-92 cells are IL-2 dependent and this may cause unexpected effects due to the IL-2 infusions that must accompany NK-92 infusions. In order to use NK-92 cells without IL-2 infusions, NK-92 -IL-2ER cells which express an endoplasmic reticulum-retained version of IL-2 were generated and shown to have high cytotoxicity as wild type NK-92 cells (Konstantinidis et al. 2005). More recently, NK-92 cells modified to express a functional TCR (Parlar et al. 2019; Mensali et al. 2019) and Chimeric Antigen Receptor modified NK-92 (Hans Klingemann, Boissel, and Toneguzzo 2016) cells show promising outcomes.

1.3. Immunotherapy

Traditional treatments like surgical excision is used in solid tumors for patients only in early stages of cancer and it rapidly loses its effect once the malignancy becomes metastatic. Chemotherapeutic agents and radiation provide great survival benefit for patients but can damage healthy tissue due to toxicities (Pabla and Dong 2012). Immunotherapy, on the other hand, has been a great hope for many of the cancer patients (Pardoll 2013) due to its promise of high efficiency coupled with tumor specificity. Cancer immunotherapy can basically be explained as the use of a patients' own immune system attack to selectively destroy tumor cells.

Cancer immunotherapy strategies can be categorized into different approaches. Molecular therapy (cytokine infusions and anti-immune checkpoint molecules; anti-CTLA-4, anti-PD-1), cellular therapy (e.g. CAR-T cells, TCR-T Cells) and vaccination therapy (e.g. Human papillomavirus (HPV)) (M. Liu and Guo 2018).

1.3.1. Cellular Immunotherapy

Cellular immunotherapy (also called adoptive immunotherapy) consists of transferring autologous or allogeneic immune cells for therapeutic purposes. Generally, the transferred cells are genetically modified or at least *ex vivo* expanded. Adoptive T cell therapy has shown a potentially powerful approach to cancer treatment (Yee et al. 2002) and seems to be among the most effective methods in cancer immunotherapy today (Hinrichs and Rosenberg 2014). NK cells play an important role in immunity against tumor cells thanks to their potent cytotoxicity function (Evren Alici et al. 2007). Novel NK-cell based therapeutic strategies in cellular immunotherapy provide a new set of tools that could be used to complement or replace many T cell based treatments (E. Alici and Sutlu 2009). Allogeneic NK cell products have been used in the treatment of a range of malignancies such as; leukemia, renal cell carcinoma, leukemia, colorectal cancer, hepatocellular cancer, lymphoma and melanoma (Geller and Miller 2011; Rizzieri et al. 2010)

1.3.1.1. Antigen-Specific Immunotherapy

Adaptive immunity is essential to produce antigen-specific B and T cells by random recombination of genomic loci (Pardoll 2013). Specific responses shown by B and T cells have the capacity to differentiate between self and non-self-antigens.

T cells require 3 distinct signals to get activated; i) antigen-specific interaction of its T cell receptor (TCR) with MHC-I, ii) co-stimulatory signaling through receptor such as CD28 and CD40L and iii) instructive cytokines secreted by the antigen presenting cell (Figure 1.5.) (Pross 2007).

In cytotoxic T cells, The TCR is a heterodimer consisting of two chains (TCR α and TCRβ) that can recognize antigenic peptides presented on MHC. TCR heterodimers combine with the CD3 complex: CD3δ, CD3γ, CD3ε, and CD3ζ which contains ITAMs (Samelson 2011). This complex is responsible for intracellular signal transduction events leading to T cell activation. As mentioned above, CD3ζ is also expressed in NK cells and

works as a signaling adaptor for activating receptors. Co-receptors such as CD4 and CD8 facilitate the TCR signaling (Lin and Weiss 2001).

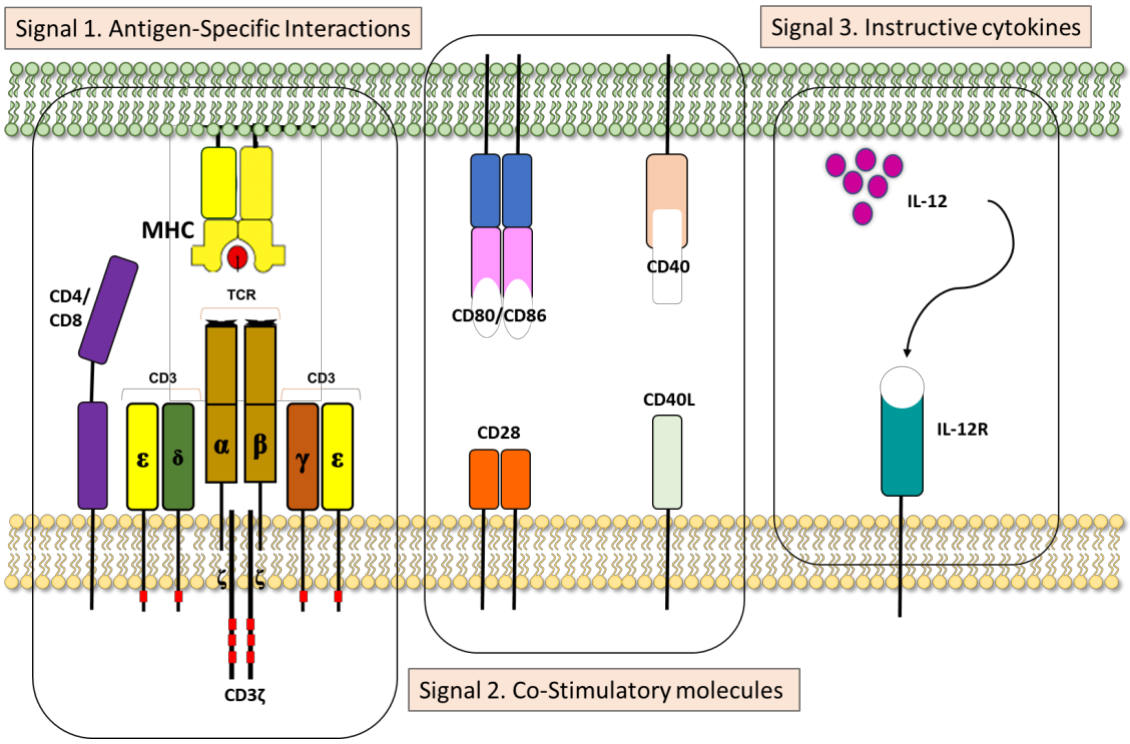

Figure 1.5. Activation of the T cell. T cells require 3 distinct signals for their activation. Signal 1 is the T cell Receptor (TCR) engagement of the peptide-MHC complex, Signal 2 is the engagement co-stimulatory receptors (CD28, CD40, etc.) and integrin molecules that contribute to signal transduction by modulating the response threshold. Signal 3 is the inflammatory cytokines including IL-12 or Type 1 IFN. All the above components along with accessory proteins essential for MHC are a part of the immunological synapse that initiates T-cell activation.

Costimulatory molecules like CD28 and cytotoxic T-lymphocyte associated protein 4 (CTLA-4) provide the second signal to enhance TCR signaling in the cytoplasm. The phenomenon of co-stimulation and the two signal model was initially discovered through CD28 signaling in 1987 (June et al. 1987). Since then, various co-stimulatory receptors (ICOS, CD27, OX-40, 4-1BB, HVEM, CD40, CD30, PD-1) have been identified (L. Chen and Flies 2013), and efficiently used to modify T cell activity especially in the field of immunotherapy (such as immune checkpoint inhibition therapies with anti-CTLA-4 or

anti-PD1 specific antibody). Signaling domains on co-stimulatory molecules are responsible for T cell growth, migration, differentiation and survival.

A third signal is still required for optimum T cell response and memory which is mostly shaped by the cytokine milieu during antigen presentation especially interleukin-2 (IL-2), IL-1 and IL-15 which are essential for proliferation of T cells (Raeber et al. 2018).

Antigen-specific immunotherapy aims to direct the immune response against specific molecules that could distinguish tumor cells from healthy cells (Hoffmann and Schuler 2013). These antigens are traditionally classified into two: tumor specific antigens (TSA) whose expression is restricted to the tumors, and the more commonly found tumor-associated antigens (TAA) which may also be expressed in some healthy tissues but to a much lower extent compared to the tumor. Genetic modification of T cells or NK cells for targeting these antigens rely on the transfer of either TCR genes or chimeric antigen receptor (CAR) genes. For antigen-specific immunotherapy, genetically modified NK or T cells are given to a patient to help the fight cancer (Figure 1.6.). (Rosenberg and Restifo 2015).

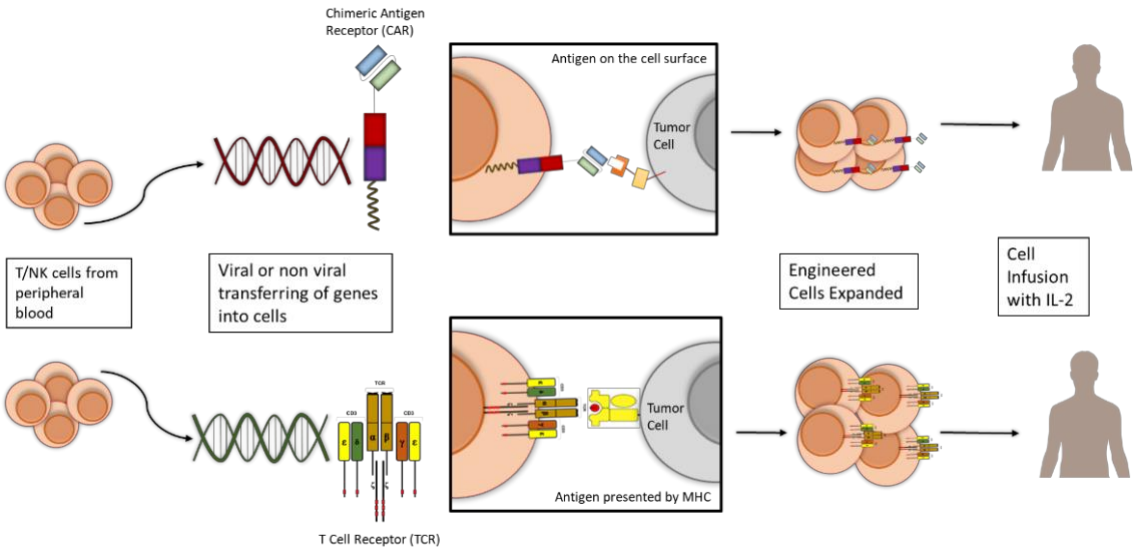

Figure 1.6. Antigen-specific immunotherapy. Antigen-specific immunotherapy is used for cancer treatment. CAR-T/NK cells and TCR-T/NK cells are engineered to produce special receptors on their surface. They are expanded in the laboratory and returned to the patient.

1.3.1.2.Chimeric Antigen Receptors

Chimeric antigen receptors (CAR) are also known as chimeric immunoreceptors and they can be designed to recognize specific antigens by the use of a single chain variable fragment (scFv) sequence derived from a specific antibody (Srivastava and Riddell 2015) (Figure 1.7.). scFv sequences are derived by fusion of the heavy and light chain variable fragments (VH-VL) of an antibody with a specific linker (Figure1.6.)(Haber et al. 2006). scFv designs were initially described in 1988, and the most commonly used linker sequences since have been the pentapeptide GGGGS (Gly4Ser) such as (G4S)3, (G4S)4 (Huston et al. 1988; Benhar and Reiter 2004; Andris-Widhopf et al. 2001). Other extracellular domains have been based on NK receptors such as CD16, DNAM1, or NKG2D that are used to target their cognate ligands (T. Zhang, Barber, and Sentman 2006; Wu et al. 2015; Lehner et al. 2012; Clémenceau et al. 2006).

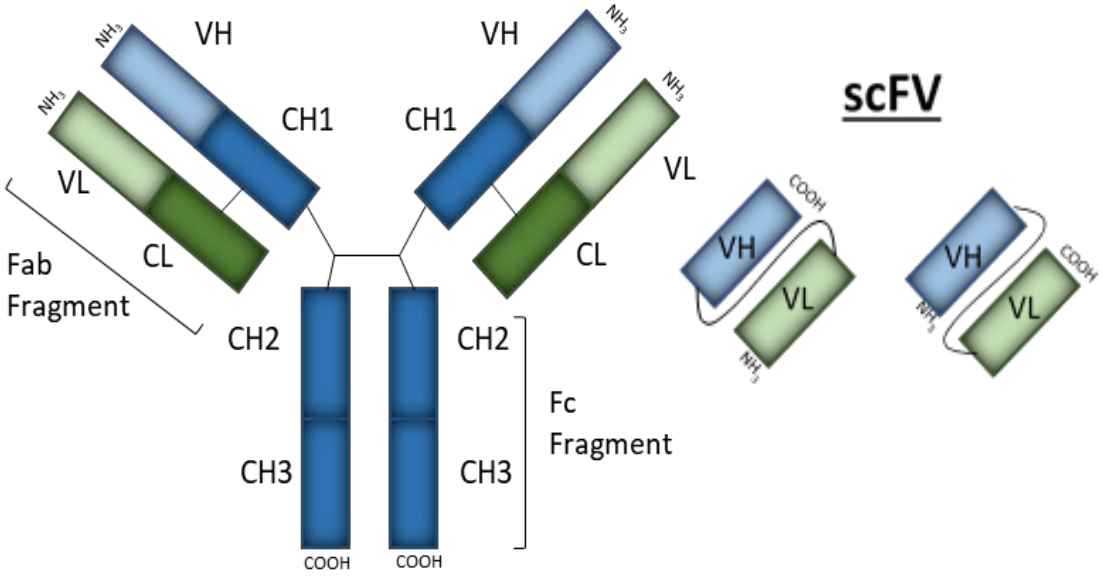

Figure 1.7. Antibody and single chain variable fragment (scFv) structure. Antibody consists of two heavy chains and two light chains. scFV is a fusion protein of variable regions of the heavy (VH) and light chains (VL) of antibody that combine with 10-25 amino acids short linker peptide. The left picture is an antibody, right pictures are possible scFv chains.

CAR-cells (NK or T cells) can target antigens on the tumor cell surface independent of MHC presentation. Intracellular domains of CAR designs are based on T-cell activation principles which are critical for initiation and regulation of the immune response. The binding of CAR to its ligand on the target cell through the scFv sequences initiates signal transduction in the intracellular domains and activates the T or NK cell to induce cytotoxic activity, proliferate and secrete cytokines.

Besides the extracellular binding domain that is generally an scFv sequence, CARs comprise a hinge domain that connects the scFv to a transmembrane domain and intracellular signaling domains (Gacerez, Arellano, and Sentman 2016).

Hinge is the non-antigen binding part of the extracellular domain. The most commonly preferred sequences is the CD8α hinge domain that provides both flexibility and stability to the designed receptor and can improve the capacity of the intracellular signaling domain (Lipowska-Bhalla et al. 2012).

The intracellular domains provide signaling which can be used to classify the different generations of CAR design. The first generation CARs have only signal 1 (CD3zeta signaling), second-generation CARs combine signal 1 and signal 2 (co-stimulatory domain) , third generation CARs combine signal 1 and two different signal 2, fourth generation CARs are combine signal 1,2 and 3 (Petersen and Krenciute 2019) (Figure 1.8.) .

First Generation CARs used only CD3ζ which is primary for T cell activation due to its ITAM signaling. In CAR-NK cells, first-generation CARs can also use DAP10 or DAP12 (Li et al. 2018). Second generation CARs use a co-stimulatory domain which provides the co-stimulatory signal to allow proliferation, memory and changes the cytokine/chemokine secretion profile of the modified cell. Most commonly used in T cells are CD28, 4-1BB (CD137) and OX-40. CD28 results in cytokine secretion such as IL-2, IL-10, IFN-γ (M Chmielewski, Hombach, and Abken 2011); 4-1BB induces IL-4 and resistance to activation-induced cell death (AICD) (Myers and Vella 2005); OX-40 does not induce IFN-γ but induces a similar amount of proliferation with 4-1BB (Hombach et al. 2012). Third generation CARs contain two different signaling domains that can drives the cells to specific differentiation or proliferation and provides more co-stimulatory signaling. This provides a more reliable and more lasting treatment when evaluated over

a long period of time (Enblad et al. 2015). Fourth Generation CARs (also referred to as "TRUCK") (Markus Chmielewski and Abken 2015) is the next and last generation CARs that include another transgene expression besides the CAR and possibly multi-antigen targeting. In this approach, extra transgenes for T or NK cell stimulation can be added to the second or third generation CARs and optimized for autocrine cytokine secretion such as IL-12 and IL-15 (Petersen and Krenciute 2019). The CAR domains used in the literature are listed in Table 1 by type and task (Weinkove et al. 2019; Kuhn et al. 2019; McNerney, Lee, and Kumar 2005; Xianghong Chen et al. 2009; Vinay and Kwon 2014; Fedorov, Themeli, and Sadelain 2013).

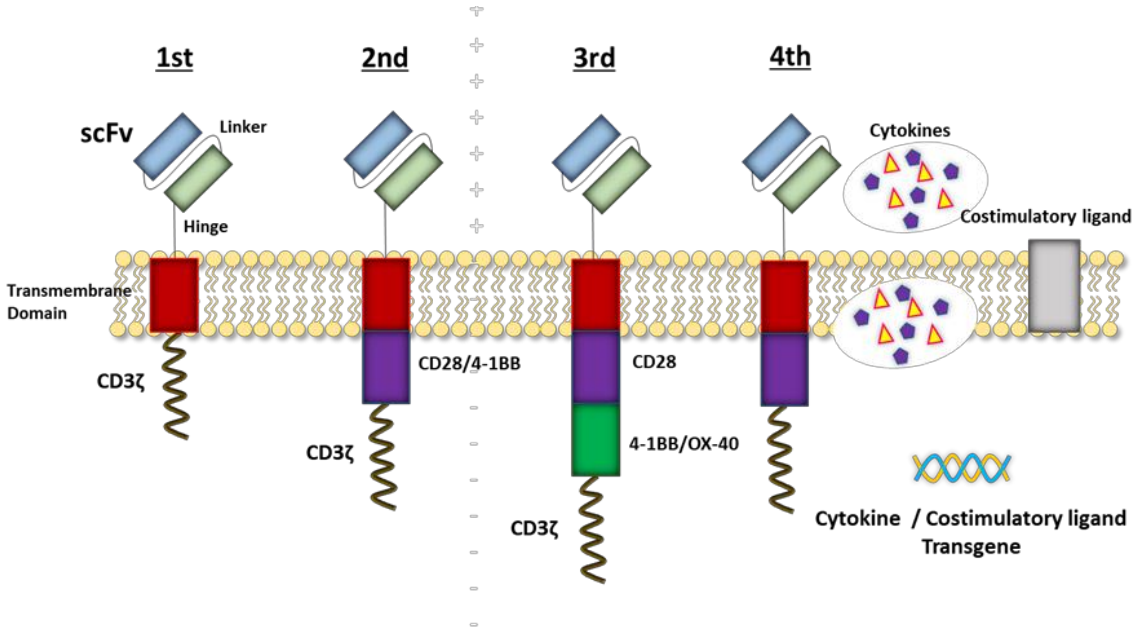

Figure 1.8. Generations of Chimeric Antigen Receptors. 1st generation CAR describes as a fusion of an extracellular single variable chain fragment with the CD3ζ intracellular signaling domain from TCR. If 1st Generation CAR includes co-stimulatory intracellular domains such as CD28/4-1BB, named 2nd Generation CAR. 3rd Generation CAR consists of two co-stimulatory intracellular domains. Recently, 4th Generation CAR was described, that combines 2nd generation CAR with cytokine or co-stimulatory ligands transgene.

Recent years have witnessed the introduction of CAR-T cells into clinical practice. The U.S. Food and Drug Administration (FDA) has approved KYMRIAH ™ (tisangenlecleucel) for Adult Refractory diffuse large B-cell Lymphoma (DLBCL) and young adult acute lymphoblastic leukemia (ALL) as well as YESCARTA™

(axicabtangene ciloleucel) for a certain type of B-cell lymphoma. UPMC Hillman Cancer Center is the creator of both ("First-Ever CAR T-Cell Therapy Approved in U.S." 2017; "FDA Approves Second CAR T-Cell Therapy" 2018). They are genetically modified autologous T-cells modified with CD19 specific CAR. In this treatment (Figure 1.9), peripheral blood T cells are collected from the patient, then genetically modified in the laboratory and expanded for about 2-3 weeks. In the meantime, patients receive chemotherapy. CAR-T cells are infused back to the patient and patients are monitored for side effects 2-3 months because treatment has the potential to cause severe side effects such as cytokine release syndrome (CRS), neurological events, infections, decreased oxygen level and %5-20 of patients are faced with acute Graft versus host disease (GVHD) (Brudno and Kochenderfer 2016; Jacoby et al. 2014).

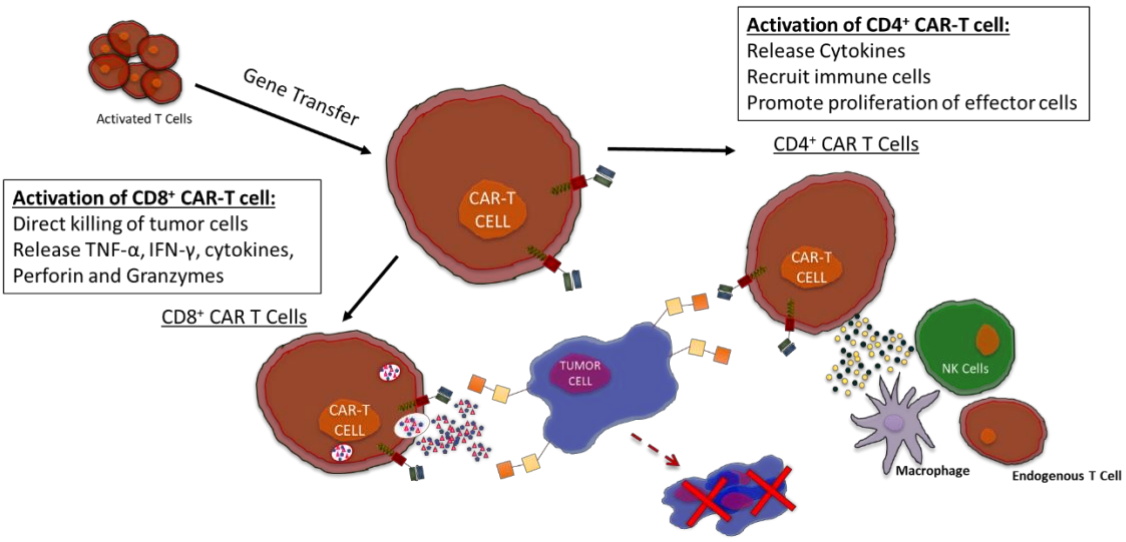

Figure 1.9. The use of CAR-T cells. CAR genes can be transferred to CD8 or CD4 T cells. If the CAR gene transfer to CD8 cytotoxic T cells enables them to recognize specific antigens on the target cell surface. This triggers the direct killing of antigen-positive target cells. The same approach can also be used in CD4 T cells in order to trigger the lease of cytokines and recruitment of other immune cells. Since antigen-specific cytotoxic activity is generally preferred in these treatments, most of CAR-T cell research is carried on CD8 T cells.

Table 1.1. CAR domains that used in NK/T cells

CAR Domain	Parts of the CAR	Family /Source	Ligands/Binding Part	Cell Expression	Functional Characteristic
CD28	Transmembrane and Intracellular Signaling Domain	Ig Family	CD80/86	Resting and activated T cells	IL-2 production, CD4+ T cell expansion
CD278 (ICOS)	Intracellular Signaling Domain		CD275 (ICOS-L)	Activated T cells, especially Tfh and Th17 cells	Th1 and Th17 polarisation
CD137 (4-1BB)	Transmembrane and Intracellular Signaling Domain	Tumor necrosis factor receptor superfamily	-	Memory CD8+ T cells, only the activation period of CD4+ T cells	co-stimulatory immune checkpoint molecule
OX40 (CD134)	Intracellular Signaling Domain		OX40L (CD252)	Activated T cells	Suppresses Treg development
CD27	Intracellular Signaling Domain		CD70		Increase proliferation and secretion of cytokines
CD40	Intracellular Signaling Domain		CD40L (CD154)		Increase proliferation and secretion of cytokines
CD244 (2B4)	Intracellular Signaling Domain	Signaling Lymphocyte Activation Molecule Family	CD48	Memory CD8+ T cells and NK cells	Activation and inhibitory function for NK cells
DAP10	Intracellular Signaling Domain	Hematopoietic cell signal	-	CD8+ T cells and NK cells	Intracellular signaling functions for receptors

DAP12	Intracellular Signaling Domain	transducer/DNAX-activating proteins	-		protein tyrosine kinase binding protein for receptors
CD3ζ	Transmembrane and Intracellular Signaling Domain	immunoglobulin superfamily containing a single extracellular immunoglobulin domain	-	Chains associate with T-cell receptor and some NK cells receptors	generate activation signal in T cells
CD8α	Transmembrane Domain	T cell Membrane Glycoprotein	MHC-I	CD8+ T cells	Recognize MHC-I class
KIR2DS2	Transmembrane and a cytoplasmic domain	Killer cell immunoglobulin-like receptor CD158 antigen-like family member	β2-Microglobulin–Independent Ligand	NK Cells	Receptor on natural killer (NK) cells for HLA-C alleles
CTLA4	Intracellular Signaling Domain	Immunoinhibitory receptors	B7	Activated T cells	Regulation of immune activation and immune checkpoints
PD-1	Intracellular Signaling Domain	Immunoinhibitory receptors	PDL-1	Activated T cells	Regulation of immune activation and immune checkpoints

1.4. Chimeric Antigen Receptor-Natural Killer Cells

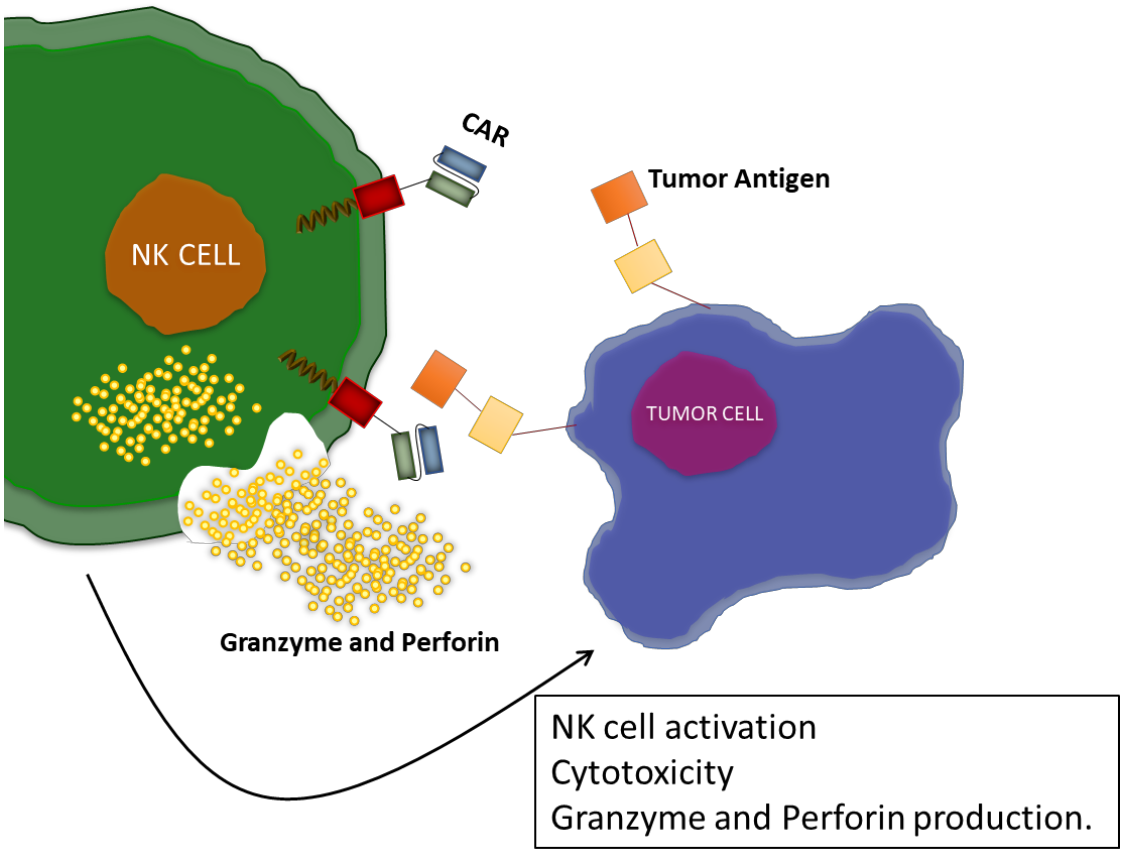

Figure 1.10. CAR-NK cells. CAR NK cells also have the capacity to recognize and kill tumor cells through their native activating and inhibitory receptors, making the escape of tumor cells through downregulation of the CAR target antigen less likely.

CAR-NK cell binding to specific antigen on target cells activates the signaling pathways to release cytotoxic granules containing perforin and granzyme B as well as secretion of cytokines (Oberschmidt, Kloess, and Koehl 2017) (Figure 1.10.). As stated above, natural cytotoxicity receptors (NCRs) in NK cells signal through CD3ζ that has ITAM domains. First generation of CAR vectors also rely on CD3ζ so it has been possible to use these vectors to activate (Eshhar et al. 1993) NK cells through the regular Syk-Zap70 pathway (Moretta et al. 2002). Second generation CARs also have co-stimulatory domains alongside CD3ζ. Depending on which co-stimulatory domain is used, different signaling pathways such as JNK or PI3K are activated in NK cells (Watzl and Long 2010). CD28 co-stimulatory domain in CAR-NK cells results in PI3K activation leading to IL-2

production (Kowolik et al. 2006). 4-1 BB improves NK cell cytotoxicity due to IFN-γ production but second generation 4-1BB- CD3ζ was found to be less effective compared to CD28-CD3ζ in NK-92 cells (Tonn et al. 2016). In NK cells, instead of CD3ζ, DAP12 or DAP10 was also tested as intracellular signaling domains. DAP12 increased the IFN γ release in CAR-NK cells compared to CD3ζ (Töpfer et al. 2015). These results suggest that vectors optimized in T cells may not always be directly applicable in NK cells and NK cell-oriented CAR designs can help to increase the efficiency of CAR-NK cells. Current pre-clinical trials of different designs in CAR-NK cells are listed in Table 1.2 with all domains, genetic modification methods and cell lines tested.

Comparison of the clinical and pre-clinical studies of CAR-NK and CAR-T cells reveals certain advantages of using NK cells for CAR therapy. These advantages are particularly due to the natural killing mechanism of NK cells and have drawn attention to CAR-NK clinical trials. Human NK cells used for CAR treatments can be derived from different origins, such as the NK cell line NK-92, primary cord blood and peripheral blood, all of which have recently been used effectively in clinical trials (Table 1.3.). Primary NK cells are easily isolated from patients and have relatively short life span (Koehl et al. 2016). Therefore, the risk of overexpansion and exhaustion of the cells is high. If NK-92 cells are used, they can be cultured long-term *in vitro* with a uniform phenotype and can reduce the cost (Glienke et al. 2015). More importantly, cytokine secretion profile of NK cells seems more safe than CAR-T cells in terms of IFN-γ and GM-CSF (Hans Klingemann 2014). This profile renders NK cell treatments less susceptible to adverse effects such as CRS and GvHD (Domogala, Madrigal, and Saudemont 2015).

Last and most important is the presence of the natural killing mechanism of NK cells. CAR-NK cells trigger the death of target cells in both CAR-dependent and CAR-independent ways because they have native activating receptors on the cell surface. If the target antigen is lost, NK cells can continue to be kill via their activation receptors (Mehta and Rezvani 2018). As clinal trials continue (Table 1.3.), CAR-NK based immunotherapies continue to deliver the are promise of better cancer treatment.

Table 1.2. List of Pre-Clinical Studies of NK cells

Disease	**Target**	**Hinge**	**TM**	**Intracellular Signal Domain**	**Genetic Modification Method**	**NK Cell Source**	**References**
Multiple myeloma	CD138	CD8	CD3ζ	CD3ζ	Lentiviral Vector	NK-92 (IL-2 independent)	(Jiang et al. 2014)
B-cell malignancies	CD19	CD8	Unknown	CD3ζ	Retroviral Vector	NK-92	(Romanski et al. 2016)
B-cell malignancies	CD19	CD8	CD28	CD3ζ	Lentiviral Vector	NK-92	(Tonn et al. 2016)
			CD28 CD137	CD28-CD3ζ CD137-CD3ζ			
Chronic lymphocytic leukemia	CD19	CD8	CD3ζ	CD3ζ	Electroporation	NK-92	(Boissel et al. 2009)
Acute/Chronic lymphocytic leukemia	CD19/CD20	Unknown	Unknown	CD3ζ	Lentiviral Vector	NK-92	(Boissel et al. 2013)
B-cell malignancies	CD20	CD8	CD3ζ	CD3ζ	Retroviral Vector	NK-92	(Müller et al. 2008)
Prostate cancer	EpCAM	CD8	CD3ζ	CD3ζ	Retroviral Vector	NK-92	(Daldrup-Link et al. 2009)
Prostate cancer	EpCAM	CD8	CD3ζ	CD3ζ	Retroviral Vector	NK-92	(Meier et al. 2011)
Colorectal cancer	EpCAM	CD9	CD3ζ	CD137-CD3ζ	Lentiviral Vector	NK-92	(Nowakowska et al. 2018)
Breast Cancer	EpCAM	CD8	CD28	CD28-CD3ζ	Lentiviral Vector	NK-92	(Sahm, Schönfeld, and Wels 2012)

Neuroblastoma	GD2	CD8	CD3ζ	CD3ζ	Retroviral Vector	NK-92	(Seidel et al. 2015; Esser et al. 2012)
Melanoma	GPA7	Unknown	HLA-A2	CD3ζ	Electroporation	NK-92 (IL-2 independent)	(G. Zhang et al. n.d.)
Brain Metastasis	HER2	CD8	CD3ζ	CD3ζ	Retroviral Vector	NK-92	(Alkins et al. 2013, 2016)
Breast Cancer	HER2	CD8	CD3ζ	CD3ζ	Retroviral Vector	NK-92	(Daldrup-Link et al. 2005; Meier et al. 2008)
Breast / ovarian cancer	HER2	CD8	CD3ζ	CD3ζ	Retroviral Vector	NK-92	(Uherek et al. 2002)
Breast / ovarian cancer, Melanoma, Renal cell carcinoma	HER2	CD8	CD3ζ	CD3ζ	Lentiviral Vector	NK-92	(Schönfeld et al. 2015)
			CD28 CD137	CD28-CD3ζ CD137-CD3ζ			
Glioblastoma	HER2	CD8	CD28	CD28-CD3ζ	Lentiviral Vector	NK-92	(C. Zhang et al. 2016)
Breast Cancer	HER2	CD8	CD28	CD28-CD3ζ	Electroporation	NK-92	(H. Liu et al. 2015)
Multiple myeloma	CS1	Unknown	Unknown	CD28-CD3ζ	Lentiviral Vector	NK-92	(Killer et al. 2014)
EBV^+	EBNA3C	Unknown	Unknown	CD137-CD3ζ	Retroviral Vector	NK-92 (IL-2 independent)	(Tassev, Cheng, and Cheung 2012)
Glioblastoma	EGFR/EGFRvIII	CD8	CD28	CD28-CD3ζ	Lentiviral Vector	NK-92	(Han et al. 2015; Genßler et al. 2016)

Brain Metastasis	EGFR	Unknown	Unknown	CD28-CD3ζ	Lentiviral Vector	NK-92	(Xilin Chen et al. 2016)
Mesothelin-expressing tumors	Mesothelin	CD8	NKG2D	CD3ζ	Transposon plasmids	iPSC-NK	(Li et al. 2018)
			CD16	2B4-CD3ζ			
			NKp44	DAP10-CD3ζ			
				2B4-CD3ζ			
			NKG2D	2B4-CD3ζ			
				CD137-CD3ζ			
				2B4-DAP12-CD3ζ			
				2B4-DAP10-CD3ζ			
				CD137-2B4-CD3ζ			
			CD28	CD28-CD137-CD3ζ			
Agressive T cell malignancies	CD3	CD8	CD8	CD28-CD137-CD3ζ	Lentiviral Vector	NK-92	(Kevin H. Chen et al. 2016)
	CD5						(K H Chen et al. 2017)

Table 1.3. Clinical Trials of CAR-NK (https://clinicaltrials.gov/ , update date: 1.07.2019)

NCT Number	Target	Disease/Conditions	NK Cell Source	Phase	Status	Location
NCT03056339	CD19	B-Lymphoid Malignancies, Lymphoma and Leukemia	Umbilical Cord Blood	Phase 1 and 2	Recruiting	Houston, Texas, United States,
NCT02892695	CD19	B cell Lymphoma and Leukemia	NK-92	Phase 1 and 2	Recruiting	Suzhou, Jiangsu, China
NCT01974479	CD19	B-cell Acute Lymphoblastic Leukemia	Haploidentical donor NK cells	Phase 1	Suspended	Singapore, Singapore
NCT00995137	CD19	B-cell Acute Lymphoblastic Leukemia	Haploidentical donor NK cells	Phase 1	Completed	Memphis, Tennessee, United States
NCT03579927	CD19	B cell Lymphoma	Umbilical Cord Blood	Phase 1 and 2	Not yet recruiting	Houston, Texas, United States
NCT03690310	CD19	Refractory B cell lymphoma	Not specify	Early Phase 1	Not yet recruiting	Allife Medical Science and Technology Co., Ltd.
NCT03824964	CD19/CD22	Refractory B cell lymphoma	Not specify	Early Phase 1	Not yet recruiting	Allife Medical Science and Technology Co., Ltd.
NCT03692767	CD22	Refractory B cell lymphoma	Not specify	Early Phase 1	Not yet recruiting	Allife Medical Science and Technology Co., Ltd.

NCT02742727	CD7	Lymphoma and Leukemia	NK-92	Phase 1 and 2	Unknown	Suzhou, Jiangsu, China
NCT02944162	CD33	Acute Myeloid Leukemia	NK-92	Phase 1 and 2	Unknown	Suzhou, Jiangsu, China
NCT02839954	MUC1	Carcinoma	Not specify	Phase 1 and 2	Unknown	Suzhou, Jiangsu, China
NCT03383978	HER2	Glioblastoma	NK-92	Phase 1	Recruiting	Frankfurt, Germany
NCT03415100	NKG2D	Solid Tumor	Autologous or allogeneic NK cells	Phase 1	Recruiting	Guangzhou, Guangdong, China
NCT03656705	-	Non-small Cell Lung Cancer	Costimulatory Converting Receptor (CCCR)-Modified NK92 Cells	Phase 1	Recruiting	Xinxiang, Henan, China
NCT03692637	Mesothelin	Epithelial Ovarian Cancer	peripheral blood	Early Phase 1	Not yet recruiting	Allife Medical Science and Technology Co., Ltd.
NCT03692663	PSMA	Castration-resistant Prostate Cancer	Not specify	Early Phase 1	Not yet recruiting	Allife Medical Science and Technology Co., Ltd.
NCT03940820	ROBO1	Solid Tumor	Not specify	Phase 1 and 2	Recruiting	Suzhou, Jiangsu, China
NCT03941457	ROBO1	Pancreatic Cancer	Not specify	Phase 1 and 2	Recruiting	Shanghai, China
NCT03931720	ROBO1	Malignant Tumor	Not specify	Phase 1 and 2	Recruiting	Suzhou, Jiangsu, China
NCT03940833	BCMA	Multiple Myeloma	NK-92	Phase 1 and 2	Recruiting	Wuxi, Jiangsu, China

2. AIM OF THE STUDY

CAR mediated targeting of tumor antigens is a promising approach for adoptive immunotherapy of cancer. Moreover, CAR-T cells have recently received clinical approval for antigen-specific adoptive immunotherapy against CD19 in B cell malignancies. CAR expression in NK cells is also clinically tested and carries the potential to translate into clinical application. CAR-NK cells trigger the death of target cells in both CAR-dependent and CAR-independent ways due to their natural killing mechanisms. Comparison of the pre-clinical studies of CAR-NK and CAR-T shows using NK cells for CAR therapy may be preferable due to safety and efficiency concerns.

This thesis investigates NK-92 cells as the effectors and evaluates CAR vectors that could be efficiently used to retarget NK cells, primarily focusing on different generations of CAR vectors for retargeting NK-92 cells towards B cell malignancies., CAR transgenes comprising identical antigen binding domains that target CD19, combined with different intracellular signaling domains (CD3ζ , CD28 and CD137) are transferred to NK-92 cells via the use of lentiviral vectors and the efficiency of CAR-NK-92 cells is tested *in vitro*.

Thus, this study aims:

I. To setup and optimize production of CAR NK-92 cells with anti-CD19 scFv
II. To evaluate the effect of different intracellular signaling domains in retargeting NK-92 cell mediated cytotoxicity
III. To develop novel and practical CAR vector designs optimized for NK cells

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

All the chemicals used in this thesis are listed in Appendix A.

3.1.2. Equipment

All the equipment used in this thesis are listed in Appendix B.

3.1.3. Buffers and solutions

Calcium Chloride ($CaCl_2$) Solution: 60mM $CaCl_2$ (diluted from 1M stock), 15% Glycerol, 10mM PIPES (pH 7.00) were mixed and sterilized by autoclaving at 121^oC for 15 minutes and stored at 4^oC.

Agarose Gel: For 100 ml 1% w/v gel, 1 g of agarose powder was dissolved in 100 ml 0.5X TBE buffer by heating. 0.01% (v/v) ethidium bromide was added to the solution.

Tris-Borate-EDTA (TBE) Buffer: For 1 L 5X stock solution, 54 g Tris-base, 27.5 g boric acid, and 20 ml 0.5M EDTA (pH 8.00) were dissolved in 1 L of ddH_2O. The solution is stored at room temperature (RT) and diluted 1 to 10 with ddH_2O for working solution of 0.5X TBE.

HBS solution (2X): 280 mM NaCl, 50mM HEPES, and 1.5 Mm Na_2HPO_4 adjust pH to 7.1 with 10 M NaOH, sterilize by passing through with 0.22 µm filter. Store at -20^oC.

3.1.4. Growth media

Luria Broth (LB): For 1 L 1X LB media, 20 g LB powder was dissolved in 1 L ddH2O and then autoclaved at 121°C for 15 minutes. For puromycin selection, at a final concentration of 100 µg/ml ampicillin was added to liquid medium just before use.

LB-Agar: For 1X agar medium in 400 ml in 1 L glass bottle, add 8 g LB powder and 6 g bacterial agar powder were dissolved in 400 ml ddH2O and then autoclaved at 121°C for 15 minutes. Then, autoclaved LB agar is mixed with antibiotic of interest at a desired ratio onto sterile Petri dishes. Sterile agar plates were kept at 4°C.

Complete DMEM: HEK 293FT cells were maintained in culture in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2mM L-Glutamine, 1mM Sodium Pyruvate, 0.1mM MEM Non-essential amino acid solution, and 25mM HEPES solution.

Complete RPMI 1640: NK-92 cell line is maintained in culture in RPMI1640 supplemented with 20% heat-inactivated fetal bovine serum, 1000 U/ml Interleukin-2 is culture every 2 daysç 25mM HEPES, 2mM L-Glutamine, 1X MEM vitamins, 0.1mM MEM Non-essential amino acid solution, 1mM Sodium Pyruvate, and 0.1 mM 2-mercaptoethanol.

K562, DAUDI, NAMALWA cells are maintained in culture in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum, 25mM HEPES, 2mM L-Glutamine, 1X MEM vitamins, 0.1mM MEM Non-essential amino acid solution.

Freezing medium: All the cell lines were frozen in heat-inactivated fetal bovine serum containing 6% DMSO (v/v).

3.1.5. Commercial kits used in this study

Table 3.1. List of Commercial Kits

Commercial Kit	**Company**
PureLink™ Genomic DNA Mini Kit	Thermo Fisher, USA

Nucleo Spin® Gel and PCR Clean-up Kit	Macherey-Nagel, USA
Nucleo Spin® Plasmid Midiprep Kit	Macherey-Nagel, USA
Nucleo Spin® Plasmid Miniprep Kit	Macherey-Nagel, USA
RNA isolation kit	Zymo Research, USA
RvertAid First Strand cDNA Synthesis Kit	Thermo Fisher, USA

3.1.6. Enzymes

Table 3.2. List of Enzymes

Enzyme	**Company**
ApaI	New England Biolabs, USA
BamHI-HF	New England Biolabs, USA
BglII	New England Biolabs, USA
BstEII	New England Biolabs, USA
CIAP	Fermentas, USA New England Biolabs, USA
EcoRI-HF	New England Biolabs, USA
EcoRV-HF	New England Biolabs, USA
HindIII-HF	New England Biolabs, USA
NcoI	New England Biolabs, USA
NotI	New England Biolabs, USA
PvuII-HF	New England Biolabs, USA
Q5 Polymerase-HF	New England Biolabs, USA
SacII	New England Biolabs, USA

SalI	New England Biolabs, USA
SfiI	Fermentas, USA
SphI	New England Biolabs, USA
T4 Ligase	New England Biolabs, USA
XbaI	New England Biolabs, USA
XhoI	New England Biolabs, USA

3.1.7. Antibodies

Table 3.3. List of Antibodies

Antibody	**Company**
Anti-human CD56 (BV421 conjugated, clone:NCAM 16.2)	Biolegend,USA
Anti-human CD56 (APC conjugated, clone:NCAM 16.2)	Biolegend,USA
Anti-human CD107a (PE/Cy7 conjugated, clone:H4A3)	Biolegend,USA
Anti-human CD19 (Purified, clone: SJ25C1)	Biolegend,USA
Anti-human CD19 (Brillant Violet 510™, clone: SJ25C1)	Biolegend,USA
Anti-c-Myc (Alexa Fluor ®594 conjugated, human and fusion protein, clone: 9E10)	Biolegend, USA
Anti-human TNF-α (APC conjugated, clone: MAb11)	Biolegend, USA
Anti-human IFN-γ (APC/Cyanine 7, clone: B27)	Biolegend, USA
Anti-human NKG2D (Brilliant Violet 510™ clone:1D11)	Biolegend, USA
Anti-human CD2 (APC/Cy7, clone: RPA-2.10)	Biolegend,USA
Anti-human CD244 (2B4) (PE, clone: C1.7)	Biolegend,USA
Anti-human CD335 (NKp46) (BV510, clone: 9E.2)	Biolegend,USA
Anti-human CD336 (NKp44) (PE/Cy7, clone: p44-8)	Biolegend,USA
Anti-human CD160 (APC, clone: BY55)	Biolegend,USA

Anti-human CD337 (NKp,30) (APC, clone: P30-15)	Biolegend,USA
Anti-human NKp80 (PE, clone: 5D12)	Biolegend,USA

3.1.8. Bacterial strains

Escherichia coli (*E.coli*) Top10 strain is used for general plasmid amplifications and lentiviral construct amplifications.

3.1.9. Mammalian cell lines

HEK293FT: Human embryonic kidney 293 (HEK293) cell line derivative that stably expresses the large T antigen of SV40 virus and has fast-growing specificity (Invitrogen R70007).

NK-92: Human natural killer cell line isolated in the year 1992 from a non-Hodgkin's lymphoma patient (ATCC® CRL 2407™).

DAUDI: The Daudi cell line is isolated from 16-year old Black male Burkitt's lymphoma patients in the year 1967 (ATCC® CCL-213™).

NAMALWA: The Namalwa cell line was derived from 3 years old female Burkitt's lymphoma patients (ATCC® CRL-1432™).

K562: K562 is the first established human immortalized myelogenous leukemia line from a 53 years old female chronic myelogenous leukemia patient in blast crisis (ATCC® CCL-243™).

3.1.10. Plasmids and oligonucleotides

Table 3.4. List of Plasmids

Plasmids	Purpose	Source
PUC57.CAR.3.137	Expression of 3rd generation CAR (CD28, CD137, and CD3ζ)	Genscript (NJ, USA)
LeGO-G2	Lentiviral construct for GFP expression and Puromycin resistance gene	A kind gift from Prof. Boris Fehse of University Medical Center Hamburg-Eppendorf, Hamburg, Germany
LeGO-iG2puro	Lentiviral construct for GFP expression with IRES	A kind gift from Prof. Boris Fehse of University Medical Center Hamburg-Eppendorf, Hamburg, Germany
LeGO-iG2puro.CAR3.137	Lentiviral construct for expression of 3rd generation CAR (CD28, CD137, and CD3ζ) with GFP, IRES and Puromycin resistance genes	Lab construct
LeGO-iG2puro.CAR3.137-19	Lentiviral construct for expression of 3rd generation CAR (CD28, CD137, and CD3ζ with an scFv fragment of CD19-specific antibody FMC63) with GFP, IRES and Puromycin resistance genes	Lab construct
pMDLg/pRRE	Virus production/packaging plasmid (*Gag/Pol*)	Addgene (#12251)
pRSV-REV	Virus production/packaging plasmid (*Rev*)	Addgene (#12253)
pCMV-VSV-g	Virus production/packaging plasmid (*Env*)	Addgene (#8454)

pS-63.z-IEW	Expression of 1st Generation CAR (only CD3 ζ) with an scFv fragment of CD19-specific antibody FMC63 and inserted into lentiviral transfer plasmid pHR'SIN-cPPT-SIEW (pSIEW) upstream of IRES and EGFP sequences	A kind gift from Dr. Winfried S.Wels from Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt, Germany
pS-63.28.z-IEW	Expression of 2nd Generation CAR (CD28 and CD3 ζ) with an scFv fragment of CD19-specific antibody FMC63 and inserted into lentiviral transfer plasmid pHR'SIN-cPPT-SIEW (pSIEW) upstream of IRES and EGFP sequences	A kind gift from Dr. Winfried S.Wels from Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt, Germany
pS-63.137.z-IEW	Expression of 2nd Generation CAR (CD137 and CD3 ζ) with an scFv fragment of CD19-specific antibody FMC63 and inserted into lentiviral transfer plasmid pHR'SIN-cPPT-SIEW (pSIEW) upstream of IRES and EGFP sequences	A kind gift from Dr. Winfried S.Wels from Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt, Germany

Table 3.5. List of Oligonucleotides

Oligo Name	SEQUENCE (5'to 3')	Purpose of Use
19EcoRI_Foward	TGCCGAATTCGCCACCATGGATTGGATCT	PCR for isolation of scFv fragment of CD19-specific antibody FMC63 with forward EcoRI cut site from pS-63.z-IEW
19SalI_Reverse	TTCGGTCGACACGGTCACGGTGGT	PCR for isolation of scFv fragment of CD19-specific antibody FMC63 with reverse SalI

		cut site from pS-63.z-IEW
SFFV_Foward	TGCTTCTCGCTTCTGTTC	Sequencing of 3rd Generation CAR
IRES_Reverse	GCCCTCACATTGCCAAAA	Sequencing of 3rd Generation CAR
Signal Peptide_Forward	ATGGATTGGATCTGGCGGAT	Confirmation of NK-92 CAR cells
CD137_Reverse	CAGTTCACAGCCGCCTTC	Confirmation of NK-92 CAR_CD137_CD 3ζ cells
CD28_Reverse	CCTTGGGGTCATGTTCATGT	Confirmation of NK-92 CAR_CD28_CD3ζ cell line
CD3IC_Reverse	TTCATGCCGATCTCGCTGTA	Confirmation of NK-92 CAR cells

3.1.11. DNA ladder

the DNA ladder used in this thesis is showed in Appendix C.

3.1.12. DNA sequencing

Sequencing service was commercially provided by McLab (http://www.mclab.com/), CA, USA.

3.1.13. Software, computer-based programs, and websites

Table 3.6. List of Software and Websites

Software/Websites	**Purpose of Use**
CLC Bio	Constructing vector maps, restriction analysis, DNA sequencing analysis, DNA alignments, etc
Tree Star Inc. Flow Jo	Analyzing flow cytometer data
http://www.ensembl.org/index.html	Human genome sequence information
https://www.addgene.org/	Plasmid map and sequence information
GraphPad Software, Inc., San Diego, CA, USA	Data analysis, statistical analysis
https://primer3plus.com/	Designing oligonucleotides
http://cool.syncti.org/	Designing sequences for improved expression of the protein within a human
http://www.molbiotools.com/silentmutator.html	Choosing unique restriction enzyme to design vectors
https://www.uniprot.org/	Human genome sequence information

3.2. Methods

3.2.1. Bacterial cell culture

Bacterial culture growth: Top10 *E.coli* cells were cultured in LB media with Ampicillin and cultured at 37^{o}C, 16 hours and 220 rpm shaking. Cells were spread on Ampicillin Petri dishes by the use of glass beads and incubated for 16 hours at 37^{o}C . At the end of the incubation, single colonie were picked from the plates. For glycerol stocks of bacteria, a single colony was grown at 3 ml LB media at 37^{o}C with 220 rpm shaking overnight with 220 rpm shaking, end of the incubation culture was diluted 1:3 and culture at 37^{o}C, 3 hours with 220 rpm shaking. Bacteria were taken at the log phase of growth and mixed with glycerol in 1ml at final 10% (w/v) and preserved in cryotubes at -80^{o}C.

Preparation of competent bacteria: One tip from glycerol stocked of competent Top10 *E.coli* cells were incubated in 3 ml LB without any antibiotics at 37°C, with 220 rpm shaking. After 4 hours of culture, they were transferred into a 250ml-flask with 50 ml LB and grown overnight at 37°C with 220 rpm shaking. The following day, 4 ml of overnight-grown culture was added into 400 ml of LB without any antibiotics in a 2L-flask and incubated at 37°C with 220 rpm shaking until OD_{590} is around 0.375. The culture is aliquoted into eight 50ml-tubes and incubated on ice for 10 minutes. Cells were kept on ice and centrifuged at 1600g for 10 minutes at 4°C. The supernatant was discarded, and each pellet was resuspended in 10 ml of ice-cold $CaCl_2$ solution and centrifuged at 1100g for 5 minutes again at 4°C. The supernatant was discarded, and each pellet was resuspended in 2 ml of ice-cold $CaCl_2$ solution. Cells were kept on ice for 30 minutes and combined in one tube and distributed into 200 µl aliquots that were snap-frozen in liquid nitrogen and stored at -80°C.

Transformation of competent bacteria: Competent Top10 *E.coli* cells were kept in 200 µl aliquots at -80°C. For each transformation, plasmid DNA and competent *E.coli* cells were thaw on ice. Plasmid DNA was added to competent *E.coli* cells, cells were incubated on ice for 30 minutes. The cells were taken to heat block at 42°C and immediately heat shocked for 90 seconds on ice. 800 µl of LB was added to each tube and competent cells were incubated at 37°C water bath for 45 minutes. The cells were centrifuged at 13000 rpm for 1 minute and the pellet was resuspended in 100 µl LB to be spread on Petri dishes. Glass beads were placed on ampicillin-LB agar Petri dishes. Plates were incubated at 37°C without shaking overnight.

Plasmid DNA isolation: Macherey-Nagel Mini-Midiprep Kits were applied according to manufacturer's protocols. The final DNA concentration and purity were measured by a NanoDrop spectrophotometer.

3.2.2. Mammalian cell culture

Maintenance of cell lines: HEK293FT cells were maintained in complete DMEM medium in sterile tissue culture flasks with filtered caps at an incubator set 37°C with 5% CO_2. Cells were split when confluency was reached maximum %90. Cells were washed

with PBS and trypsin-EDTA (0.25%) was added to cell culture flasks and incubated at 37°C with 5% CO_2 for maximum 5 minutes. Then the cells were resuspended in complete DMEM and split at a 1:5 ratio and split every two days. NK-92 cells were maintained in complete RPMI1640 with 1000 U/ml human Interleukin-2 (IL-2) in sterile tissue culture flasks with filtered caps at an incubator set to 37°C with 5% CO_2. NK-92 cells were seeded in a density minimum 300,000 cells/ml with fresh IL-2 was added every 2 days. Target cells (K562, Daudi, Namalwa) were maintained in a different complete RPMI1640 medium and cells were kept density minimum 300,000 cells/ml to maximum 1,000,000 cells/ml in their complete RPMI1640in sterile tissue culture flasks with filtered caps at an incubator set to 37°C with 5% CO_2.

Cell freezing: Regardless of cell type, before one day cells were split to a concentration of 500,000 cells/ml for suspension cells and to a confluence of 30-40% for adherent cells. The next day, cells were counted and at least $3x10^6$ cells/ vial were frozen. Each vial has 1 ml volume, cells were centrifuged at 300g for 5 minutes where supernatant was discarded, and the pellet was resuspended in 0.5ml FBS and incubated on ice for 20 minutes. In the meantime, 0.5 ml FBS with 12% DMSO was prepared and incubated on ice. When the incubation was over, 0.5 ml cell suspension was mixed with 0.5 ml freezing medium to reach final concentration of 6% DMSO in 1 ml. Cells were stored in cryotubes first in -80°C for short term storage, and after that they were placed to liquid nitrogen for long term storage.

Cell thawing: Cells were preserved in cryotubes at the liquid nitrogen. 15 ml tubes were filled with 5 ml FBS for every vial. When the cell suspension was at RT, 1 ml frozen sample was pipetted very slowly into FBS then centrifuged at 300g for 5 minutes and the supernatant was discarded. Regardless of cell type, the cell pellet was resuspended with complete media.

3.2.3. Design and cloning of a new Chimeric Antigen Receptor Vector

Throughout this study, two types of cloning strategies were used: CAR3.137 was synthesized by Genscript, USA into PUC57 construct which is cloned into LeGO.iG2puro vector for GFP expression and puromycin (puro) antibiotic selection

marker and end of the first cloning we generated construct of LeGO-iG2puro.CAR3.137. Second cloning strategy was an scFv fragment of CD19-specific antibody from pS-63.z-IEW into LeGO-iG2puro.CAR3.137 for expression of CD19 specific 3rd generation CAR by lentiviral transduction method.

3rd Generation CAR design and codon optimization: CAR design was carried out using human genome sequences for required genes retrieved from various references (Table 3.1.) Required CAR domains (Table 3.1) sequences were uploaded on Silent mutator website (Figure 3.1.) for analysis and chosen from unique restriction enzyme which has not cut site on LeGO-iG2puro for each domain. For improved expression of the proteins within human, genes and possible restriction enzyme cut sites are codon-optimized by using cool.syncti.org.

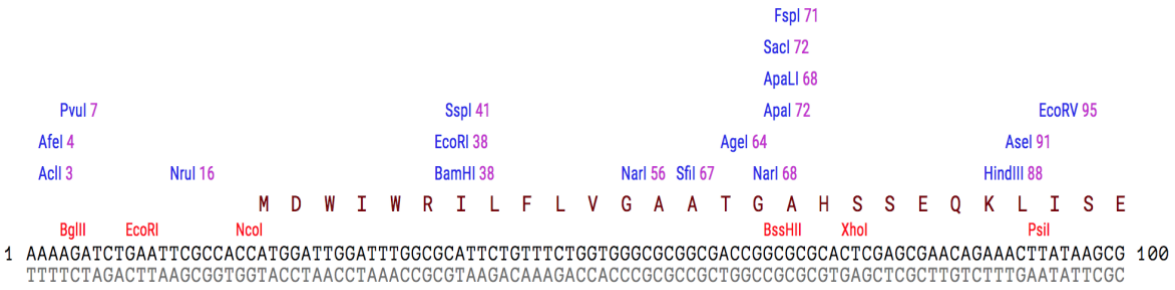

Figure 3.1. Example view of Silent Mutator website. Use to show all enzyme that we can use cut the Signal Peptide part of the Car Domain. AAAAGATCTGAATTCGCCACC sequence for BglII and EcoRI sites and Kozak sequence, the part of starting MDWI is the signal peptide part.

Digestion of LeGO.iG2puro vectors and CAR137-3 and ligation reactions: LeGO.iG2puro plasmids were used for production of lentivirus by $CaPO_4$ transfection methods on HEK293 FT cells. Backbone and insert CAR3.137 from PUC57 were digested and used in ligation reaction using the following digestion and ligation steps

Digestion of Backbone at 37°C, 1,5 hour

LeGO.iG2puro plasmid	5 µg
NEB BamHI-HF (10,000 U/ml)	1 µl
NEB NotI-HF (10,000 U/ml)	1 µl

NEB Cut Smart Buffer	3 µl
ddH_2O	Up to 30 µl
Total volume	30 µl

1st Digestion of insert at 37°C, 45 minutes

PUC57.CAR.3.137 plasmid	5 µg
NEB Bgl II (5,000 U/ml)	1,5 µl
NEB 3.1 Buffer	2 µl
ddH_2O	Up to 20 µl
Total volume	20 µl

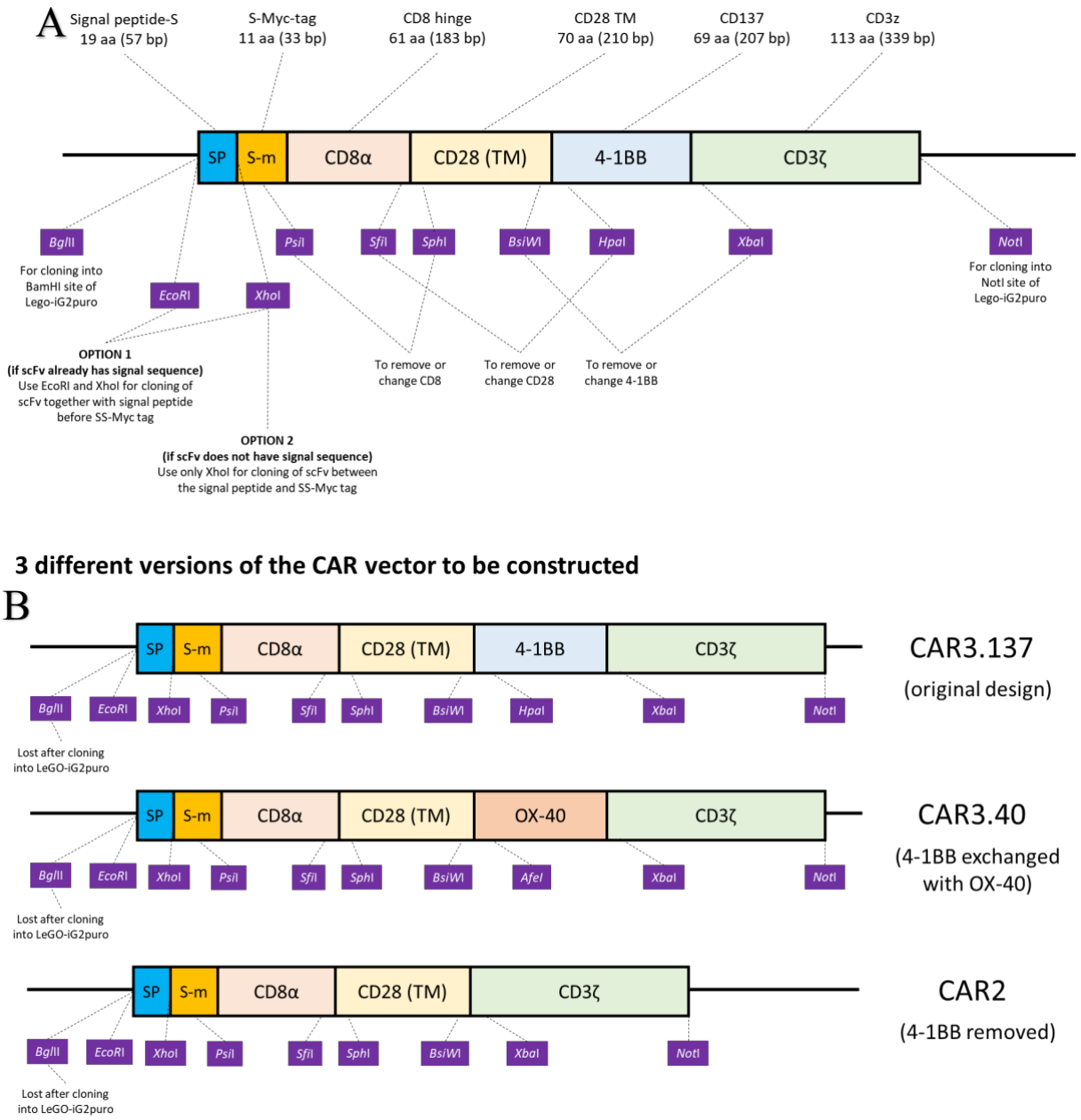

Figure 3.2. Development of novel CAR expression vectors. A) Cassette CAR Design. Design of our 3rd Generation CAR vector and restriction enzymes with the possibility of changing, the vector can be used for cloning full scFv sequence with (option 1) or without (option 2) the signal sequence directly before c-myc tag and can be used for making 3 different versions of the CAR vector B) constructed original design (CAR3.137), CD28-OX40-CD3ζ (CAR3.40) and 2nd generation CAR with CD28-CD3 ζ (CAR2).

Table 3.7. Chimeric Antigen Receptors Domains and Sequences

CAR Domains	**Full Name**	**Protein Sequences of Domains**	**Reference**
Signal Peptide	Human Immunoglobulin Heavy Chain Signal Peptide N-Terminal Signal Peptide VH 71-5'CL	MDWIWRILFLVGAATGAHS	("Sequences of Proteins of Immunological Interest" 1984)
c-Myc tag	-	S-EQKLISEEDL	(Killer et al. 2014)
CD8α (Hinge)	T-cell surface glycoprotein CD8 alpha chain Residues: 117-178 (Extracellular), UniProtKB - P01732 (CD8A_HUMAN)	ALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEASRPAAGGAVHTRGL	(Tonn et al. 2016)
CD28	T-cell-specific surface glycoprotein CD28 Residues: 151-220 (Extracellular-Helical-Cytoplasmic), UniProtKB - P10747 (CD28_HUMAN)	KPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	(Tonn et al. 2016)
CD137 (4-1BB)	Tumor necrosis factor receptor superfamily member 9 Residues: 187-255 (Helical-Cytoplasmic), UniProtKB - Q07011 (TNR9_HUMAN)	IISFFLALTSTALLFLLFFLTLRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEE GGCEL	(Tonn et al. 2016)
OX-40	A chimeric T cell antigen receptor that augments cytokine release and supports the clonal expansion of primary human T cells	RDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI	(Pulè et al. 2005)
CD3ζ	T-cell surface glycoprotein CD3 zeta chain UniProtKB - P20963 (CD3Z_HUMAN) Residues: 52-163 (Cytoplasmic)	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR*	(Tonn et al. 2016)

For preparing 2nd digestion of the insert, Nucleo Spin® Gel and PCR Clean-up Kit were used according to manufacturer's protocol, Resultant elution concentration containing the plasmid was measured by a NanoDrop spectrophotometer.

2nd Digestion of vector at 37°C, 45 minutes

1st Digestion PUC57.CAR.3.137 plasmid	Apx. 2 µg
NEB NotI-HF (10,000 U/ml)	1 µl
NEB Cut Smart Buffer	2 µl
ddH_2O	Up to 20 µl
Total volume	20 µl

After digestion reaction, the insert and backbone were loaded on %1 agarose gel which was prepared with 0.5 TBE for at 90 min, 100 V. Resultant elution concentration containing the plasmid was measured by a NanoDrop spectrophotometer.

CIP treatment of the backbone, 37°C, 30 minutes

All LeGO.iG2puro plasmid gel extraction product (except 200 ng which were used for ligation control)	30 µl
NEB CIP (10,000 U/ml	Depends ON the molarity of the DNA ends
NEB 10X Cut Smart Buffer	4 µl
ddH_2O	Up to 40 µl
Total volume	40 µl

To discard the CIP treatment, PCR Clean up (Nucleo Spin® Gel and PCR Clean-up Kit) was used. Ligation reaction of PCR-Clean up extracted backbone and Gel extract was carried out for 15 minutes at Room Temperature and 1 hour at 16 °C as follows:

CAR.3.137 gel extract	37,3 ng
PCR-Clean up product of CIP-LeGO.iG2puro plasmid	100 ng
NEB T4 DNA ligase	1,5 µl

NEB 10X T4 DNA ligase buffer	2 µl
ddH$_2$O	Up to 20 µl
Total volume	20 µl

Transformation and confirmation of positive colonies: For ligation samples, 10 µl of the above-described ligation reaction was used to transform 200 µl TOP10 competent *E.coli* cells. Next day, three colonies were picked from each transformation and miniprep cultures were started. Minipreps for plasmid DNA were carried out the following day with Macherey-Nagel commercial kit according to manufacturer's protocol. Completed minipreps were digested with a unique enzyme which is shown in Figure 1 A part.

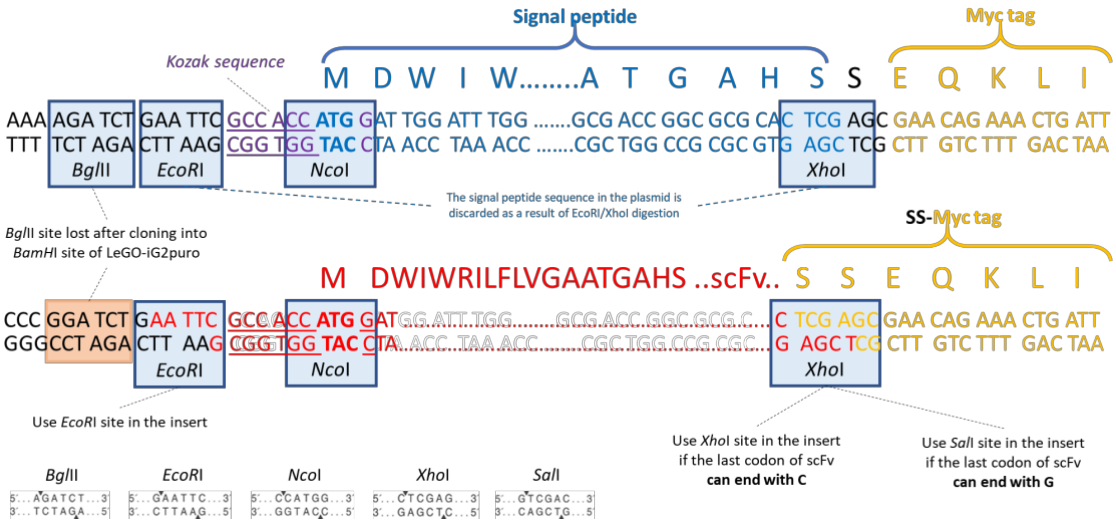

Figure 3.3. Schematic representation of cloning with scFv which have already signal peptide and LeGO.iG2puro.CAR3.137.

Polymerase Chain Reaction (PCR) of anti CD19 scFv from pS-63.z-IEW and Digestion of LeGO.iG2puro.CAR3.137 backbone and scFV insert and ligation reactions:

Our anti CD19 scFv were coming from pS-63.z-IEW (Oelsner, 2017) and the last codon of the scFv insert ends with G so we used SalI-EcoRI cloning.

PCR for anti CD19 scFv

pS-63.z-IEW	100 pg
10 mM NTPs	1 µl
NEB 5X Q5 Reaction Buffer	10 µl
10 µM 19EcoRI_Foward Primer	2.5 µl
10 µM 19SalI_Reverse Primer	2.5 µl
NEB Q5 High-Fidelity DNA Polymerase	0.5 µl
ddH_2O	Up to 50 µl
Total volume	50 µl

Condition of PCR

STEP	TEMPERATURE	TIME
Initial Denaturation	98 °C	30 seconds
30 Cycle	98 °C 66 °C 72 °C	10 seconds 30 seconds 20 seconds
Final Extension	72 °C	2 minutes
Hold	4 °C	

After PCR, the insert was tun on %1 agarose gel which was prepared with 0.5 TBE at 90 V, 1 hour. Gel Clean Up ws done using Nucleo Spin® Gel and PCR Clean-up Kit. Resultant elution concentration containing the plasmid was measured by a NanoDrop spectrophotometer.

Digestion of anti CD19 scFv insert at 37°C, 1,5 hour

PCR product of scFV	2000 ng
NEB EcoRI-HF (10,000 U/ml)	1 µl
NEB SalI (10,000 U/ml)	1 µl
NEB Cut Smart Buffer	3 µl
ddH_2O	Up to 30 µl
Total volume	30 µl

For preparing ligation of the insert, PCR-Clean up with using according to manufacturer's protocol Nucleo Spin® Gel and PCR Clean-up Kit, Resultant elution concentration containing the plasmid was measured by a NanoDrop spectrophotometer.

Digestion of LeGO.iG2puro.CAR3.137 backbone at at 37°C, 1,5 hour

LeGO.iG2puro.CAR3.137	5000 ng
NEB EcoRI-HF (10,000 U/ml)	1 µl
NEB XhoI (10,000 U/ml)	1 µl
NEB Cut Smart Buffer	3 µl

ddH_2O	Up to 30 µl
Total volume	30 µl

After digestion, run the backbone on %1 agarose gel which was prepared with 0.5 TBE. at 90 V, 1 hour. Gel Clean Up was done using Nucleo Spin® Gel and PCR Clean-up Kit. Resultant elution concentration containing the plasmid was measured by a NanoDrop spectrophotometer.

CIP treatment of the backbone, 37°C, 30 minutes

All LeGO.iG2puro.CAR3.137 plasmid gel extraction product (except 200 ng which were used for ligation control)	30 µl
NEB CIP (10,000 U/ml	Depends on the molarity of the DNA ends
NEB 10X Cut Smart Buffer	4 µl
ddH_2O	Up to 40 µl
Total volume	40 µl

To discard the CIP treatment PCR Clean up using Nucleo Spin® Gel and PCR Clean-up Kit was used

Ligation reaction of PCR-Clean up extracted backbone and Gel extracted insert was carried out for 16 hours at 16°C as follows:

Anti CD19 scFVgel extract	21,24 ng
PCR-Clean up product of CIP-LeGO.iG2puro.CAR3.137 plasmid	50 ng
NEB T4 DNA ligase	1,5 µl
NEB 10X T4 DNA ligase buffer	2 µl
ddH_2O	Up to 20 µl
Total volume	20 µl

Transformation and confirmation of positive colonies: For ligation samples, 10 µl of the above-described ligation reaction was used to transform 200 µl TopTen competent *E.coli* cells. Next day, three colonies were picked from each transformation and miniprep

cultures were started. Minipreps for plasmid DNA were carried out the following day with Macherey-Nagel commercial kit according to manufacturer's protocol.

3.2.4. Production of lentiviral vectors

For the production of VSV-G pseudotyped lentiviral vectors; first, 100 mm dishes were coated with 1ml Poly-L-Lysine solution (0.1% (w/v) in H_2O and incubated for 15 minutes, washed 2 times with 5 ml sterile dd H_2O and left with the lids open until dry. $5x10^6$ HEK293FT cells were seeded into poly-L-lysine coated 100 mm dishes. Next day, cells were transfected with 7.5 µg of vector plasmid (LeGO or pS-IEW vector), 3.75 µg of pMDLg/pRRE, 2.25 µg of pRSV-REV and 1.5 µg of phCMV-VSV-G using calcium phosphate transfection method. Plasmids were filled up to 450 µl dd H_2O and 2.5 M $CaCl_2$ was added. 500 µl 2X HBS and plasmid mix were mixed by bubbling and incubated 15 minutes. In the meantime, the media of the transfection plates were changed with fresh DMEM-Glutamax containing 25 µM Chloroquine. After 10 hours the medium was changed with DMEM Glutamax and thereafter virus-containing supernatant was collected at 24 hours and 36 hours, filtered with 0.45 µm filters and stored in -80°C until further use. A small aliquot from each production was used to determine viral titers by transduction of HEK293FT cells with serially diluted amounts of virus supernatant.

3.2.5. Lentiviral transduction of NK-92 cells

For each lentiviral transduction, 1 $x10^6$ / ml NK-92 cells were seeded in duplicates into T25 sterile tissue culture flasks with filtered caps and mixed with 5 ml virus-containing supernatant in the presence of 8 µg/ml of protamine sulfate and 1.5 µM (5Z)-7-Oxozeaenol in a final volume of 6 ml. The flasks were incubated at 37°C, 5% CO2 for overnight. At the end of the incubation, virus transduced cells were collected and spun down at 300xg for 5 minutes at room temperature after which the virus containing supernatant was removed and replaced with fresh medium. The cells were maintained in this medium for at least 3 days before the acquisition of gene expression was carried out.

For controlling of CAR expression, RT-PCR was carried out. GM NK-92 Cells were split 24 hours before to $5x10^5$/ml in 6 ml. Zymo Research RNA isolation Kits were applied according to manufacturer's protocols. The final RNA concentration and purity were measured by a NanoDrop spectrophotometer. RNA templates were used for the synthesis of cDNA which was used for PCR to control the expression different CAR signaling domains. RevertAid First Strand cDNA Synthesis Kit was applied according to manufacturer's protocols.

PCR:

cDNAs of CAR modified NK-92 cells	1:10 dilution
10 mM NTPs	0.5 µl
NEB 5X Q5 Reaction Buffer	5 µl
Signal Peptide Forward	1.25 µl
CD137 or CD28 or CD3IC Reverse Primer	1.25 µl
NEB Q5 High-Fidelity DNA Polymerase	0.25 µl
ddH_2O	Up to 25 µl
Total volume	25 µl

Condition of PCR

STEP	TEMPERATURE	TIME
Initial Denaturation	98 °C	30 seconds
30 Cycle	98 °C 63 °C 72 °C	10 seconds 30 seconds 20 seconds
Final Extension	72 °C	2 minutes
Hold	4 °C	

3.2.6. Flow cytometry

All antibody staining for flow cytometry was done according to the following protocol: For surface staining, the cells were washed once with PBS and incubated with appropriate amounts of antibody on ice for 30 min. Cells were washed PBS and carried on to analysis. The antibodies used for NK-92 cells were CD56 (clone: NCAM16.2), gating strategy was the first on single cells then to $CD56^{high}$ GFP^+ cell percentage was shown in results. For determination of possible target cells, CD19 (SJ25C1) was used. For checking chimeric antigen receptor expression, anti-c-Myc Antibody (clone: 9E10) was used.

GFP positive NK-92 Cells were sorted in $3x10^6$ cells/ml, %1.5 BSA PBS. Then incubated in RPMI 20%FBS with %1 Penicillin-Streptomycin.

For flow cytometer-based phenotyping of NK cell surface receptors, $2.5x10^5$ cells/tube were counted and incubated on ice for 30 minutes with MIXI or MIX II antibody mixture (Table 3.8.). In the meantime, $2.5x10^5$ cells/tube incubated with appropriate amounts of all fluorochrome isotype control. FACS tubes were washed with PBS and proceeded to acquisition in the flow cytometer.

Cells were acquired at LSR Fortessa, sorted at FACSAria Fusion and analyzed with FlowJo software.

Table 3.8. List of Antibodies that used for phenotyping analysis

Antibody	**Isotype**	**Volume**	**MIX TUBE**
Anti-human CD335 (NKp46) (BV510, clone: 9E.2)	BV510 Conjugated Mouse IgG1 κ	3 µl	MIX I
Anti-human CD336 (NKp44) (PE/Cy7, clone: p44-8)	PE/Cy7 Conjugated Mouse IgG1 κ	2 µl	MIX I
Anti-human CD337 (NKp,30) (APC, clone: P30-15)	(APC Conjugated Mouse IgG1 κ	3 µl	MIX I
Anti-human NKp80 (PE, clone: 5D12)	PE Conjugated Mouse IgG1 κ	3 µl	MIX I
Anti-human CD56 (BV421, clone: NCAM 16.2)	BV421 Conjugated Mouse IgG1 κ	1 µl	MIX I and MIX II
Anti-human CD160 (APC, clone: BY55)	APC Conjugated Mouse IgM κ	5 µl	MIX II
Anti-human CD244 (2B4) (PE, clone: C1.7)	PE Conjugated Mouse IgG1 κ	3 µl	MIX II
Anti-human NKG2D (Brilliant Violet 510™ clone:1D11)	BV510 Conjugated Mouse IgG1 κ	5 µl	MIX II

Anti-human CD2 (APC/Cy7, clone: RPA-2.10)	APC-Cy7 Conjugated Mouse IgG1 κ	2 µl	MIX II

3.2.7. Analysis of NK-92 cell degranulation

Effectors (GM NK-92 and WT NK-92) and Targets cells were seeded at $5x10^5$ cells/ml at 24 hours before the experiment. Cells were counted again at the experiment day and concentration was adjusted to $2.5x10^5$ cells/100 µl for each well of v-bottomed 96-well plates. Then, NK-92 cells were co-incubated with target cells at a ratio of 1:1 in a final volume of 200 µl in wells at 37°C and 5% CO2 for 1 hour. To block the CD19 on Target cells, purified anti CD19 antibody was used before the co-incubation. PE/Cy7-conjugated anti-CD107a mAb was added at the initiation of the assay and add Ionomycin (final concentration 0.25 µg/ml) and Phorbol 12-myristate 13-acetate (PMA)(Final concentration 1.25 µg/ml) for positive control wells. After 1 hour of coincubation, Monensin was added at a 1:100 dilution and the plates were incubated at 37°C and 5% CO2 for a further 3 hours. When incubation was finished, plates were taken out and centrifuged at 400g for 5 minutes at 4°C. Supernatant was discarded and stained with 1:50 dilution of APC-conjugated anti-CD56 mAb in PBS, resuspended and incubated on ice for 30 minured in dark. At the end of staining, cells were centrifuged and the supernatant discarded., Finally, they were resuspended in PBS and proceeded to acquisition in the flow cytometer

3.2.8. Intracellular TNF-α and IFN- γ staining

TNF-α and IFN-γ secretion was analyzed with the same protocol as degranulation except the addition of the anti-CD107a mAb at the initiation of the experiment. When incubation was finished, the plate were taken oyr and centrifuged at 400g for 5 minutes at 4°C. The supernatant was discarded and cells were stained with 1:50 dilution Fluorochrome-conjugated anti-CD56 mAb in PBS. For intracellular staining, cells were fixed and permeabilized in a solution containing 1% PFA containing 0.01% w/v Saponin in DPBS

and incubated on ice for 15 minutes, washed two times with Permeabilization Wash Buffer and incubated with appropriate amounts of antibody on ice for 30 min. The stained cells were then washed with Permeabilization Wash Buffer and data acquisition was carried out in flow cytometer.

3.2.9. Statistical analysis

For the preparation of graphs and statistical analysis, GraphPad Prism (GraphPad Software Inc. La Jolla, CA, USA) was used.

4. RESULTS

4.1.Cloning of the new Chimeric Antigen Receptors Vectors

For first ligation reaction, backbone LeGO.iG2puro (8445 bp) was digested by BamHI-HF and NotI-HF enzyme (Figure 4.1.A) and Insert CAR3.137 (1050 bp) was digested from PUC57 with NotI-HF and BglII enzyme (Figure 4.1.B).

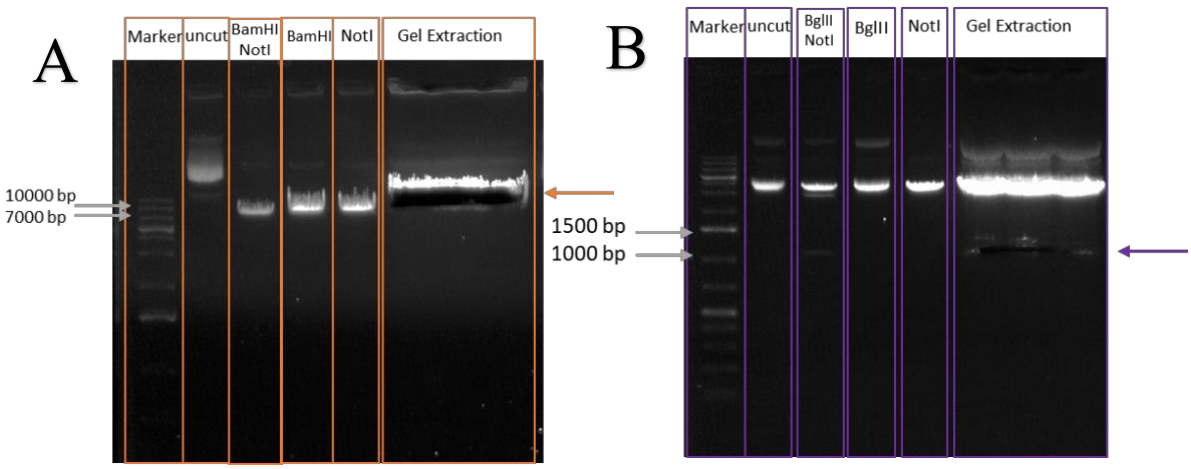

Figure 4.1. Gel image of Double Digestions of backbone LeGO.iG2puro and insert CAR1373. A) Digestion of LeGO.iG2puro with BamHI and NotI B) Digestion of insert CAR137.3 with BglII and NotI

After setting up the ligation as described in Materials and Methods, samples were used to transform *E. coli* cells. Next day, three colonies were picked from each transformation and miniprep cultures were started and controled with digestion.

For second ligation reaction, confirmed backbone LeGO.iG2puro.CAR (9431 bp) was digested by EcoRI-HF and XhoI enzyme (Figure 4.2) and insert scFV-antiCD19 (778 bp)

was generated with PCR from pS-63.z-IEW followed by digestion with EcoRI-HF and SalI enzyme (Figure 4.3.).

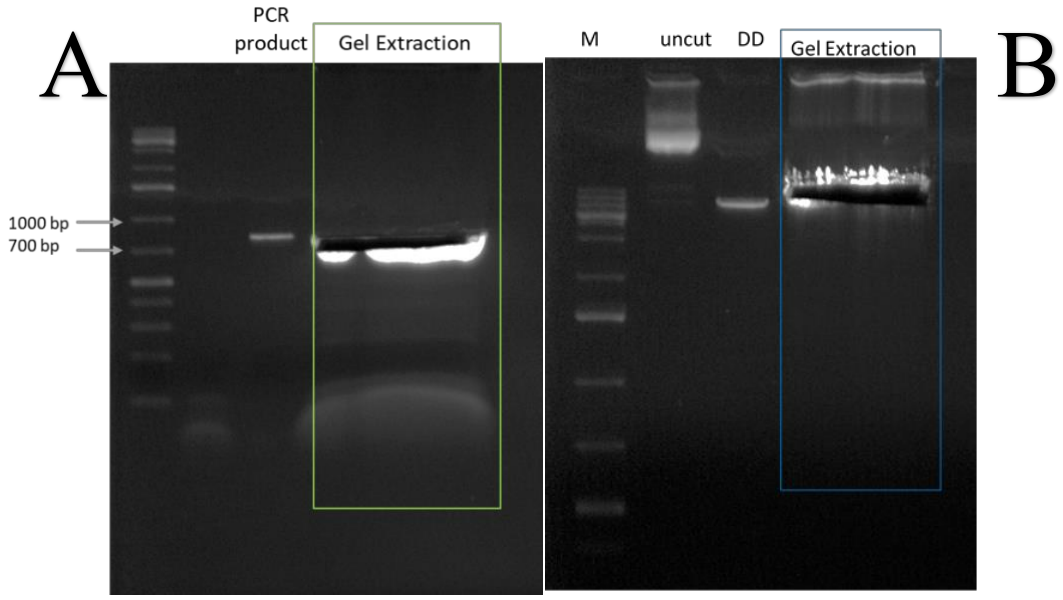

Figure 4.2. Gel image of Double Digestion of PCR product and LeGO.iG2puro.CAR **A**. PCR product (anti CD19 scFv with Signal peptide from pS-63.z-IEW plasmids), **B.**LeGO. iG2 puro

After setting up the ligation as described in Materials and Methods, samples were used to transform *E. coli* cells. Next day, three colonies were picked from each transformation and miniprep cultures were started and controled with digestion. Confirmed LeGO.iG2puro.CAR and LeGO.iG2puro.CAR-19 colonies were used for lentiviral vectors production (Figure 4.3.).

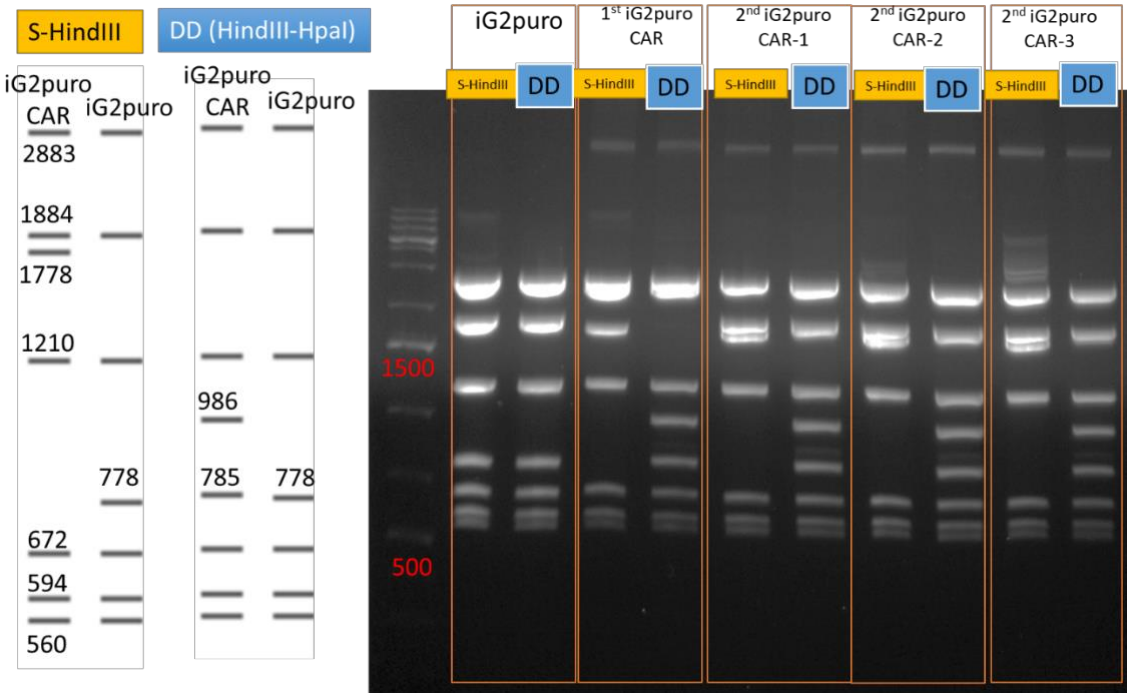

Figure 4.3. Examples of the control digestion of LeGO.iG2puro.CAR and LeGO.iG2puro. S is the single digestion with HindIII and DD is the double digestion with HindIII and HpaI. Left side is the expected band size. Right side is the gel image. We repeated cloning two times and did control digestion both of them (1st and 2nd LeGO.iG2puro.CAR)

4.2.Production of lentiviral Vectors

For comparison of different signaling domains on NK-92 cells, we used five different lentiviral constructs (Figure 4.4.). Codon optimized sequences of 3rd Generation CAR was cloned into LeGO backbone with eGFP. 1st and 2nd Generation CAR vectors are developed by Tonn et al. . For the production of VSV-G pseudotyped lentiviral vectors, we used HEK293FT cells and also determine viral titers by transduction of HEK293FT cells with serially diluted amounts of virus supernatant (Table 4.1.).

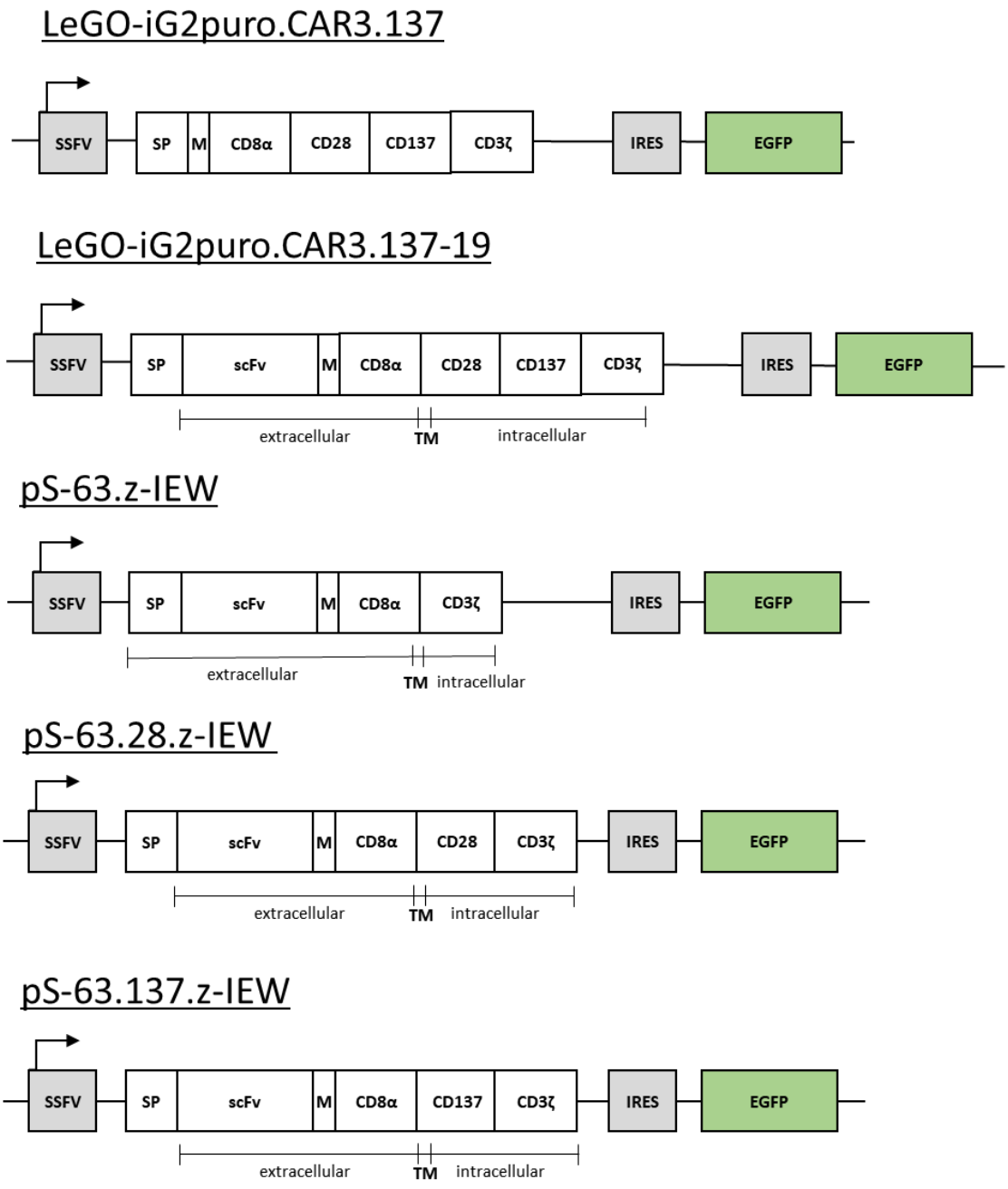

Figure 4.4. Lentiviral constructs. Lentiviral constructs were used for lentivirus. SFFV: Spleen focus-forming virus promoter, IRES: Internal ribosome entry site, SP: An immunoglobulin heavy chain signal peptide, M: Myc-tag, TM : Transmembrane, scFv: single chain variable fragment, EGFP: enhanced green fluorescent protein.

Table 4.1. Titration of Lentiviruses

Titration Table			
Number of Batch	Name of the Lentiviral Vector	Collection Time	Average number of infectionus particles x 10^6/ml
B1	LeGO-G2	24 Hour	3,82
B2	LeGO-G2	36 Hour	1,65
B3	LeGO-G2	36 Hour	2,1
B1	LeGO-iG2puro	24 Hour	0,52
B1	LeGO-iG2puro.CAR3.137	24 Hour	0,47
B1	LeGO-iG2puro.CAR3.137-19	24 Hour	0,54
B1	pS-63.z-IEW	24 Hour	0,77
B2	pS-63.z-IEW	36 Hour	0,23
B3	pS-63.z-IEW	24 Hour	0,61
B1	pS-63.28.z-IEW	24 Hour	0,35
B2	pS-63.28.z-IEW	36 Hour	0,172
B3	pS-63.28.z-IEW	24 Hour	0,52
B1	pS-63.137.z-IEW	24 Hour	0,9
B2	pS-63.137.z-IEW	36 Hour	0,37
B3	pS-63.137.z-IEW	24 Hour	1,31

4.3.Genetic Modification of NK-92 cells

Genetic Modification of NK-92 cells:

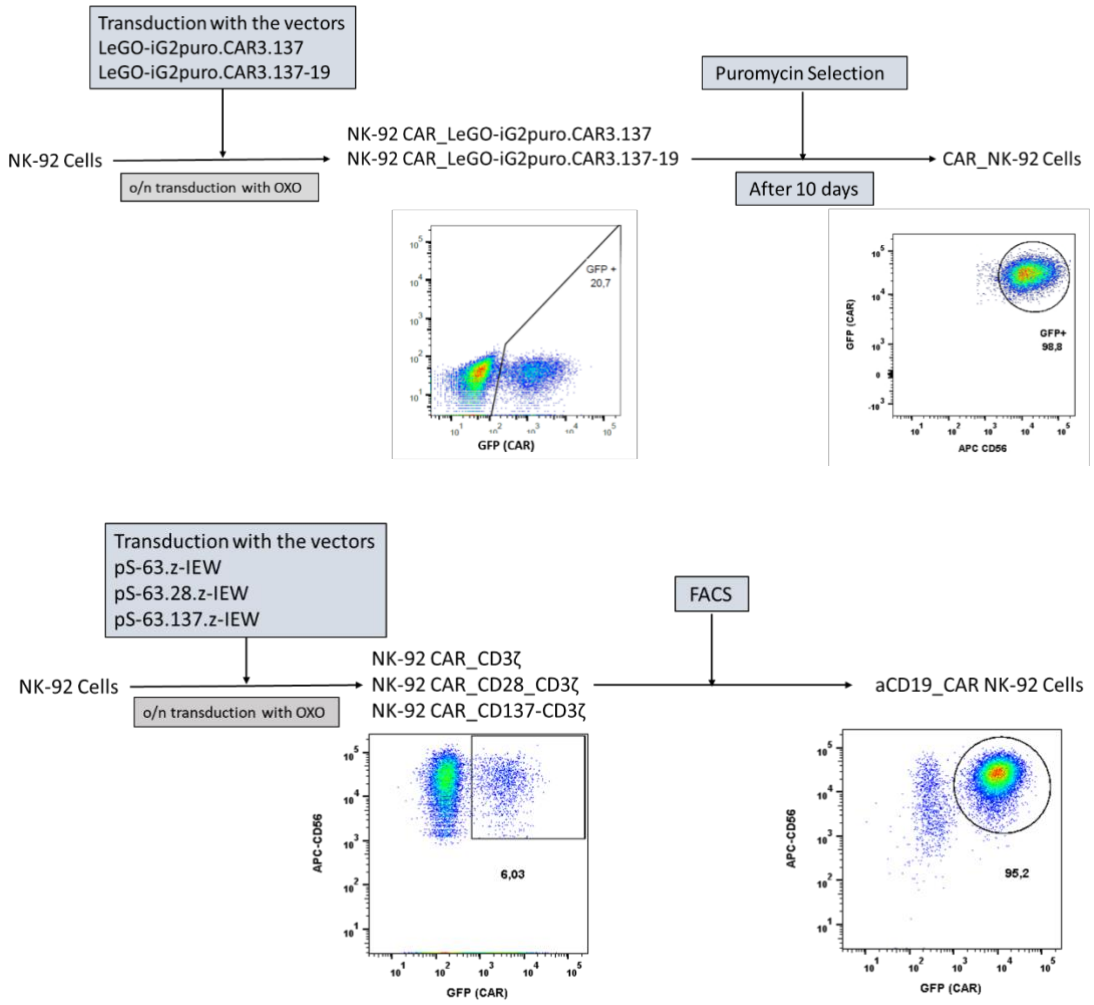

Figure 4.5. Genetic modification process of NK-92 with lentiviral vectors and sorted with puromycin selection or FACS.

In a standard transduction experiment, cells were incubated with lentiviral vector-containing supernatant for overnight and green fluorescence protein-positive (GFP^+) population was analyzed 3 days after transduction by flow cytometry (Figure 4.6). For flow cytometric analysis of NK-92 transductions, cells were stained with anti-CD56 antibody. After 3 days, we started selection with puromycin (Figure 4.6.) or cells were sorted by FACS. We used all produced viruses for NK-92 transduction and obtained different transduction results (Table 4.2.). When GFP expressing cells surpass 90%, we accepted CAR transduced cells are selected. Further experiments were used to confirm

CAR expression on cell surface and signaling domain expression. Transduction experiments were repeated 3 times (B1, B2 and B3) for controlled study.

Table 4.2. Results of +GFP the transduction and selection/sorting

Batch Number	Name of the Cells	MOI	Transduction Results (GFP+%)	After Selection or Sorting (GFP + %)	
B1	NK-92 iG2puro	2,5	25,4	99,7	Puromycin Selection
	NK-92 iG2puro.CAR3.137	2,5	18,7	98,1	Puromycin Selection
	NK-92 iG2puro.CAR3.137.19-1	2,5	22,5	98,5	Puromycin Selection
	NK-92 iG2puro.CAR3.137.19-2	2,5	20,7	98	Puromycin Selection
	NK-92 CAR_CD3ζ	3,5	9,2	94,6	FACS
	NK-92 CAR_CD28_CD3ζ	1,75	6,1	95,2	FACS
	NK-92 CAR_CD137_CD3ζ	4,5	10	93,4	FACS
B2	NK-92 G2	5	18	93,2	FACS
	NK-92 CAR_CD3ζ	1,1	1,8	93,7	FACS
	NK-92 CAR_CD28_CD3ζ	1	1,5	91,2	FACS
	NK-92 CAR_CD137_CD3ζ	1,8	1,5	94,5	FACS
B3	NK-92 G2	10	66	92	FACS
	NK-92 CAR_CD3ζ	3	8	90,7	FACS
	NK-92 CAR_CD28_CD3ζ	2,5	5	91,2	FACS
	NK-92 CAR_CD137_CD3ζ	6,5	18	91,8	FACS

Genetic modification of NK-92 cells with CAR vectors was efficient and enrichment of cells was feasible both by FACS and puromycin selection.

Confirmation of CAR Modified NK-92 Cells:

For detection of CAR expression on NK-92 cell surface, transduced NK-92 cells and wild type NK-92 cells were stained with anti-c-myc antibody then analyzed with flow cytometer (Figure 4.7.). Unfortunately, we observed that NK-92iG2puro.CAR3.137 and NK-92 iG2puro.CAR3.137.19 did not express c-myc tag on the cell surface. The plasmids were sent for sequencing.

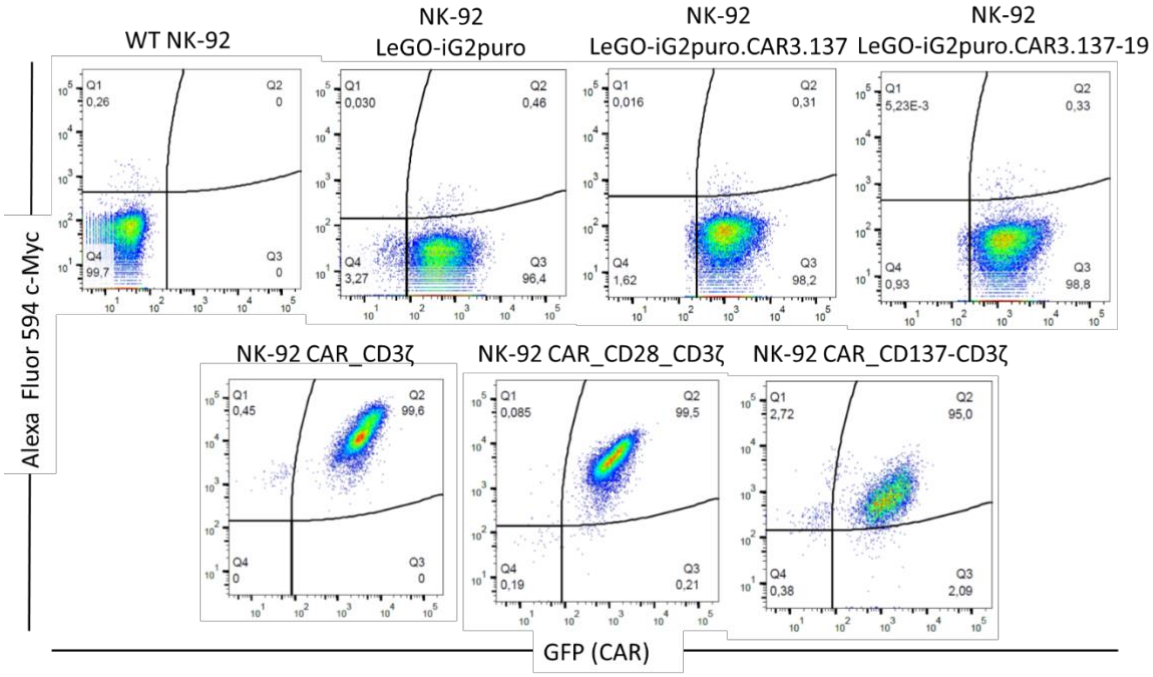

Figure 4.6. Confirmation of c-Myc expression on CAR NK-92 cell surface.

Three batches of transduced cells were prepared and analyzed by RT-PCR to confirm the proper expression of signaling domains (Figure 4.8.). The results showed that B1 and B3 cells show signs of cross-contamination while B2 cells were confirmed.

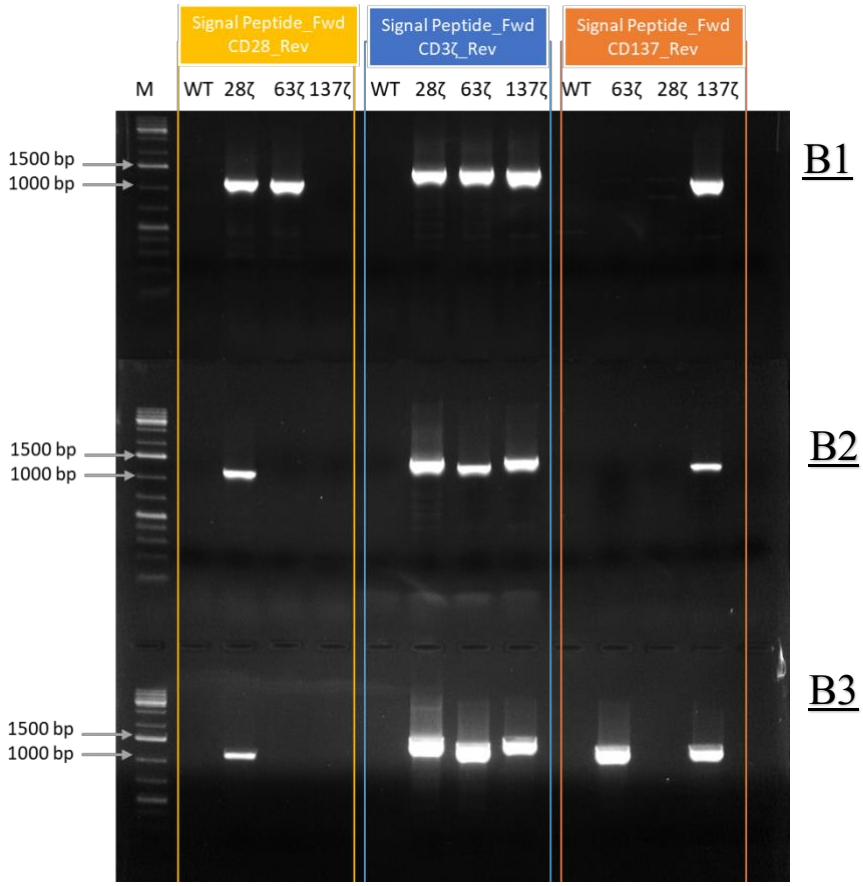

Figure 4.7. Confirmation of signaling domain expression on CAR NK-92 cells.

Flow cytometer-based Phenotyping Analysis:

Transduced NK-92 Cells were stained with different NK receptor specific antibodies (Figure 4.9.). The phenotyping results show that the use of different signaling domains in the vector influence the NK cell phenotype. We compared the results of receptor expression with isotype controls and observed significant change in the phenotype of CAR modified NK-92 cells (Figure 4.10.). For example, NKp30 and NKp80 receptors were shown to be downregulated in CD28-containing CAR while they were upregulated in CD137-containing CAR when compared to WT NK-92.

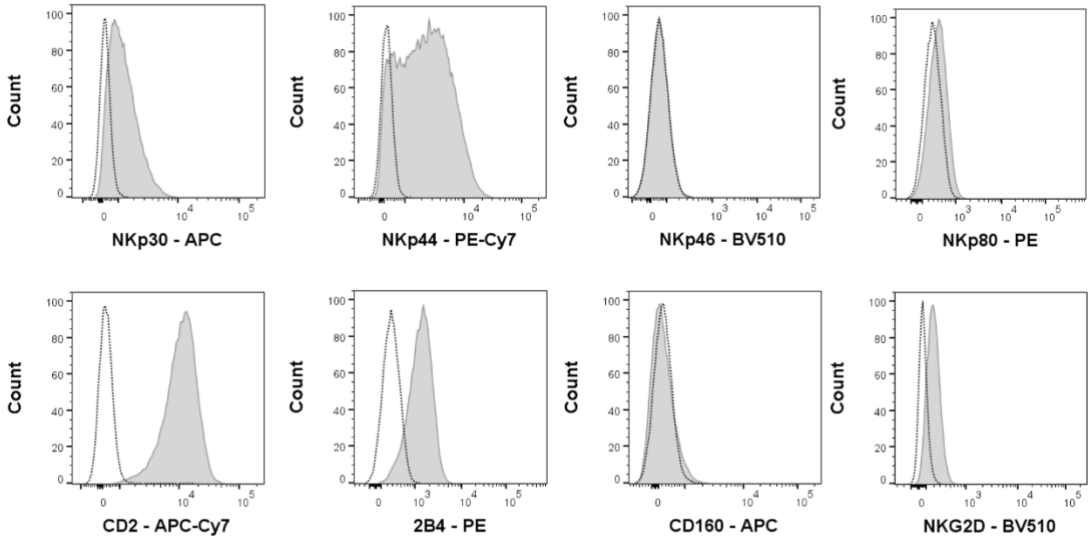

Figure 4.8. Examples data of the Phenotyping Analysis for NK92.28.CD3ζ cells

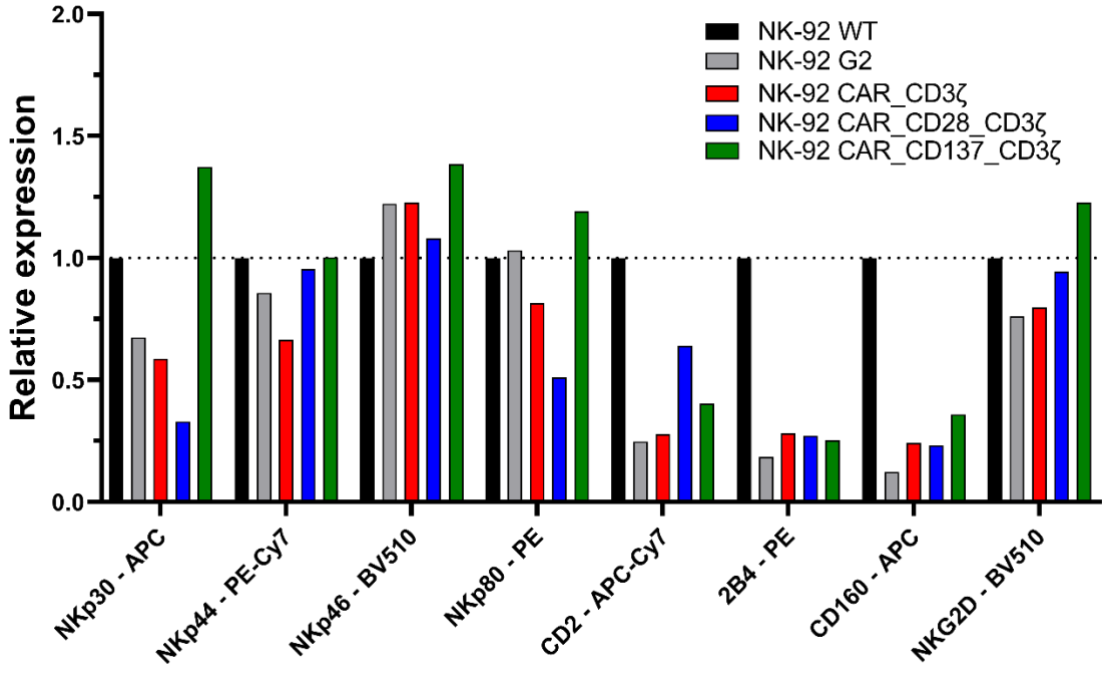

Figure 4.9. Mean fluorescence intensity (MFI) stained vs MFI isotype control

4.4.Functional Analysis of Genetically Modified NK-92 Cells

4.4.1. Degranulation of CAR-expressing NK-92 Cells

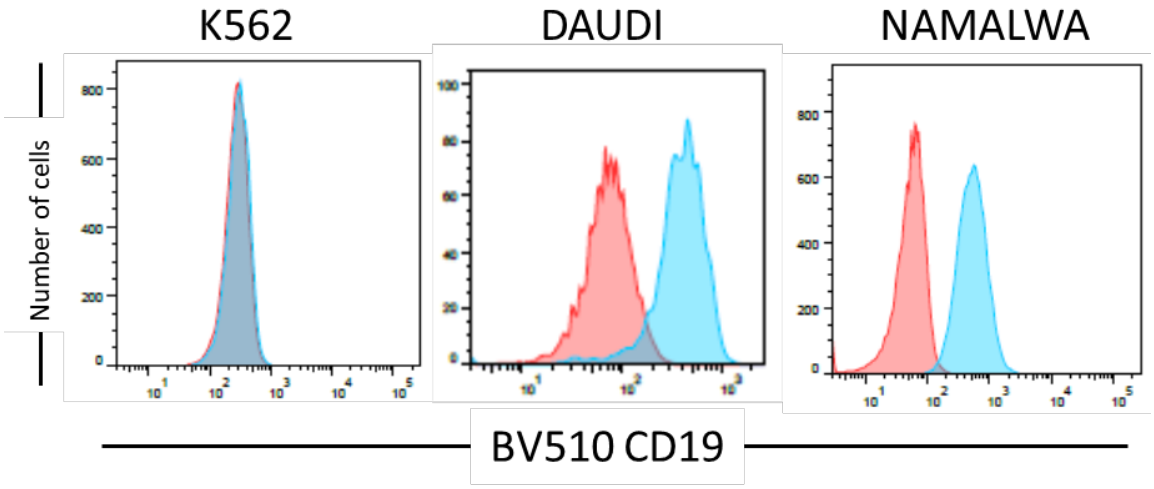

Figure 4.10. CD19 expression of Target Cells. Red line shows unstained and blue line shows CD19 expression levels.

To investigate the functionality of the CAR-modified NK-92 cells. We used CD19- K562 cell line and CD19+ Daudi and Namalwa cell lines (Figure 4.10) Recognition of CD19 is required for specific CAR cytotoxicity.

In flow cytometer-based degranulation assays (Figure 4.11) we used anti CD107a antibody to determine percentage of degranulation. Effectors and target cells ratio (E/T) is 1:1.

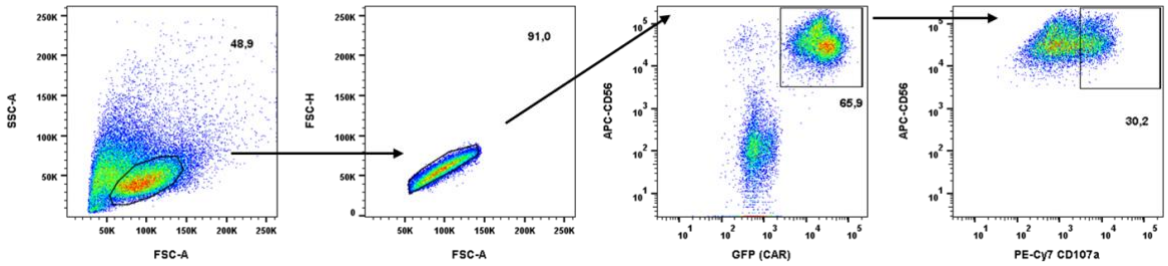

Figure 4.11. Degranulation examples of NK-92 cells

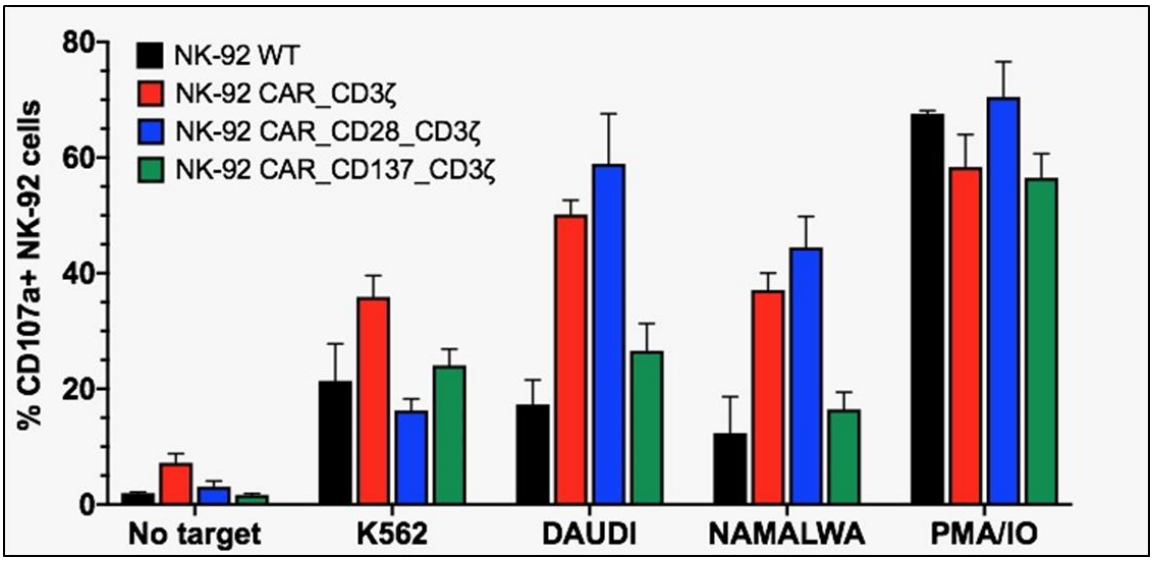

Figure 4.12. Degranulation results of 1st Batch CAR modified NK-92 Cells

We see a strong response against CD19+ cell lines Daudi and Namalwa by both the 1st gen vector as well as the CD28ζ vector while the CD137ζ vector fails to trigger any degranulation in this setting where Batch 1 CAR modified cells which showed signs of cross contamination were used (Figure 4.11).

In Batch 3 cells (also cross contamination) we see the strongest response against CD19+ cell lines Daudi and Namalwa 1st generation vector and second highest response in CD28ζ vector while the CD137ζ vector also trigger degranulation in this setting (Figure 4.13).

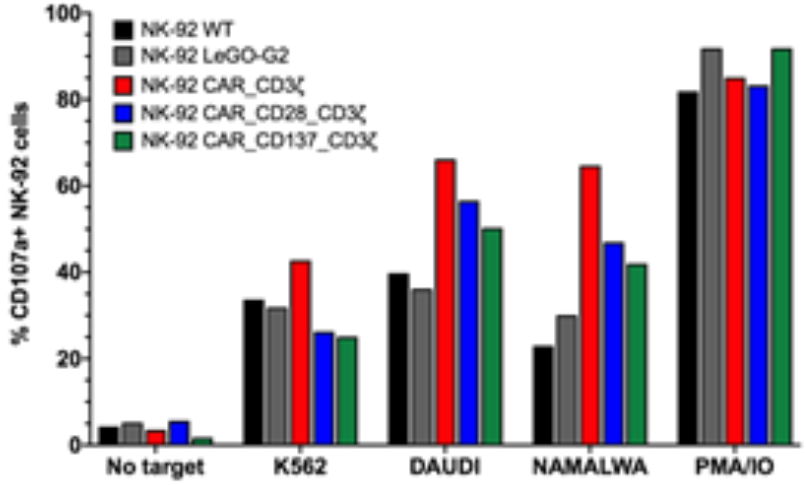

Figure 4.13. Degranulation results of 3rd Batch CAR modified NK-92 Cells

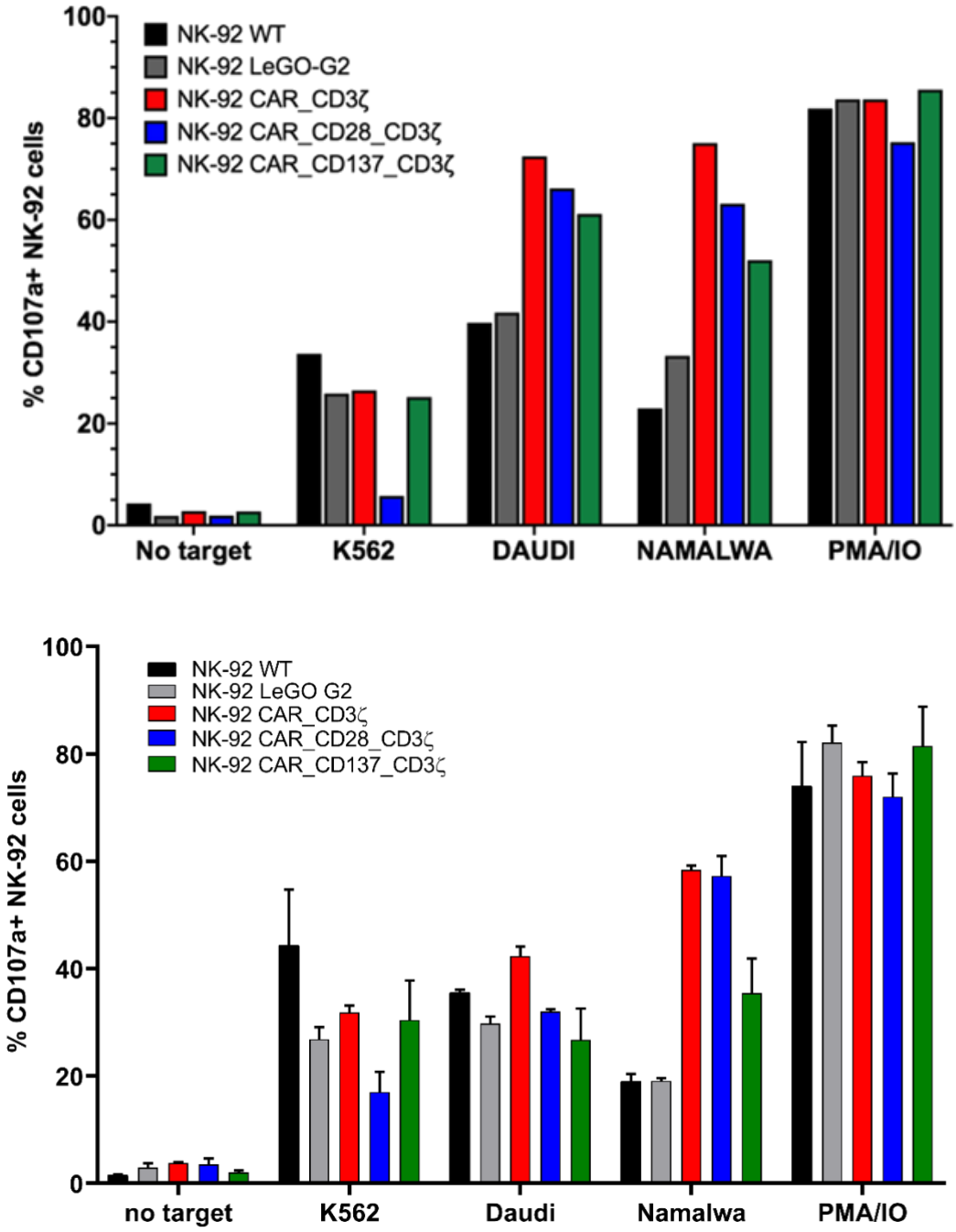

Figure 4.14. Degranulation results of 2nd Batch CAR modified NK-92 Cells. Top graphic is the first degranulation experiment, bottom graphic is the second degranulation experiments.

In Batch 2 cells, which are non-contaminated, we see a strong response against CD19+ cell line Namalwa by both the 1st gen vector as well as the CD28ζ vector and also to some extent by the CD137 vector (Figure 4.14). In Daudi cell lines, we observed that they lost CD19 expression during culture so they failed to trigger CAR-mediated responses.

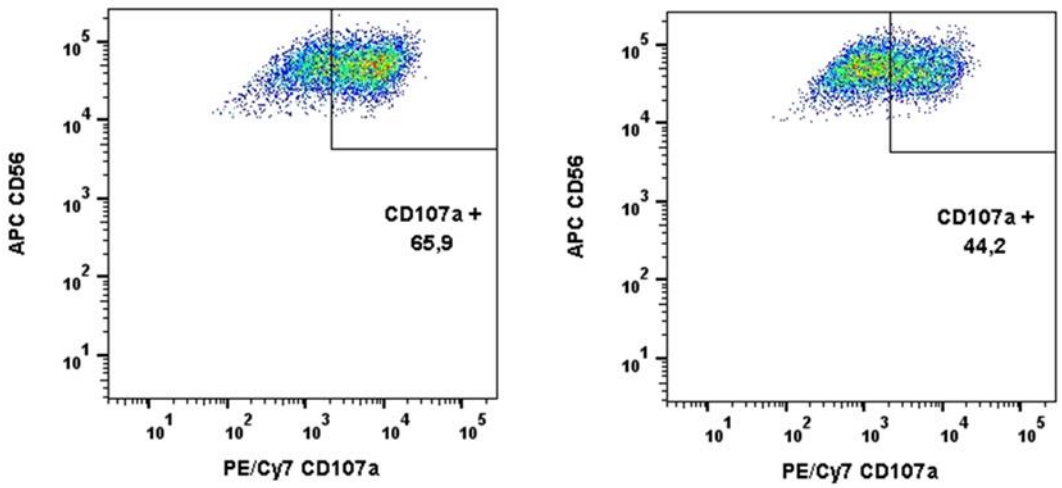

Figure 4.15. CD19 blocking Degranulation Results

To show CAR modified NK-92 specific activity against CD19, we did blocking degranulation experiments with Namalwa cell line and NK-92_CAR_CD28_CD3ζ.
The anti-CD19 antibody blocks CAR-NK-92 degranulation against CD19+ targets only to a certain extent (Figure 4.15). This could be due to the wrong choice of antibody clone for blocking experiments.

4.4.2. TNF-α and IFN- γ Secretion of CAR-expressing NK-92 Cells

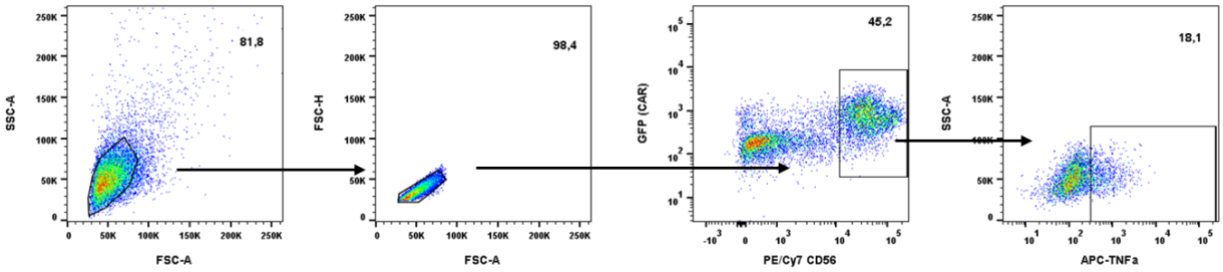

Figure 4.16. Examples of TNF-α Secretion of CAR-expressing NK-92 Cells

In flow cytometer-based cytokine secretion assays (Figure 4.16) we used anti TNF α antibody to determine percentage of TNF α secretion with intracellular stanning. Effectors and target cells ratio (E/T) are 1:1.

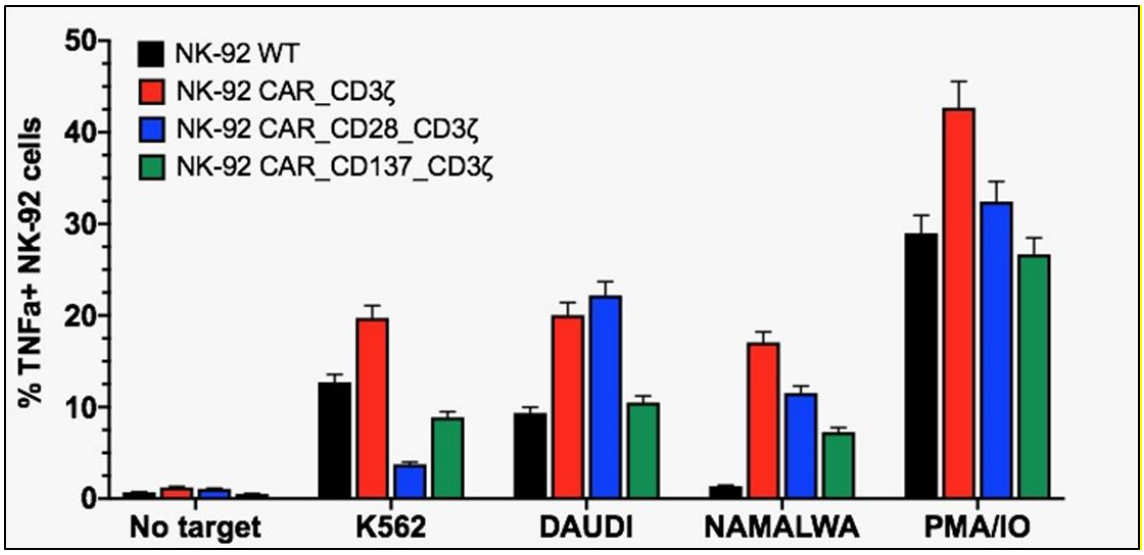

Figure 4.17. TNF-α Secretion of CAR-expressing NK-92 Cells

CAR vector containing CD137 intracellular domain fails to trigger TNFα secretion and CD28 does not increase the TNF α response compared to CD3ζ only (same as 1st batch's degranulation) (Figure 4.17.)

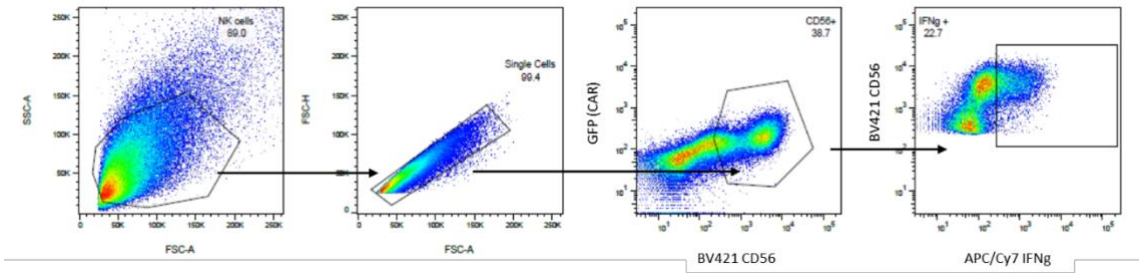

Figure 4.18. Examples IFN- γ of Secretion of CAR-expressing NK-92 Cells

In flow cytometer-based cytokine secretion assays (Figure 4.18) we used anti IFN- γ antibody to determine percentage of IFN- γ secretion with intracellular stanning. Effectors and target cells ratio (E/T) are 1:1.

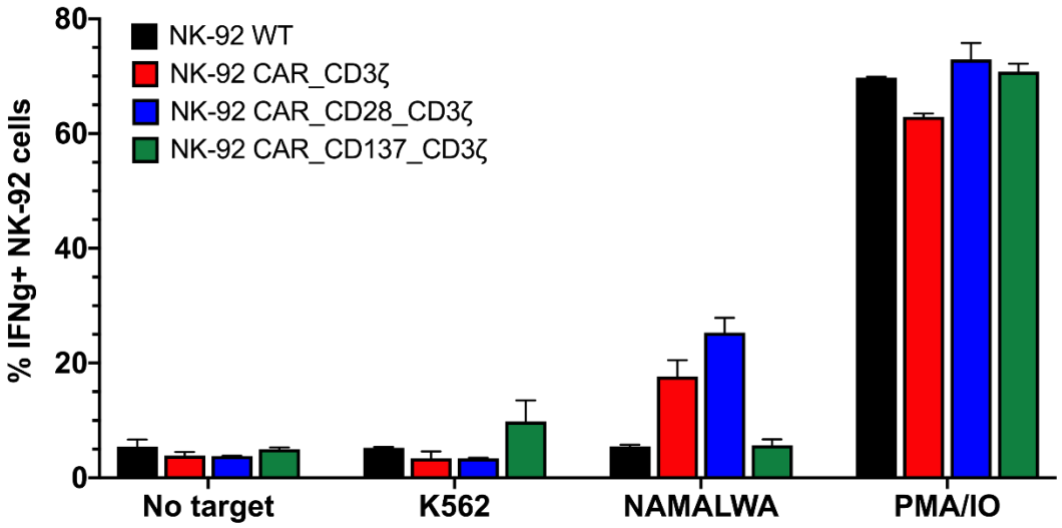

Figure 4.19. IFN-γ of Secretion of CAR-expressing NK-92 Cells

CAR vector containing CD137 intracellular domain fails to trigger IFN-γ secretion same as TNFα secretion (Figure 4.19). CD28 increases the IFN -γ response compared to CD3ζ only.

5. DISCUSSION & CONCLUSION

Cellular immunotherapy frequently relies on the transfer of autologous or allogeneic immune effector cells for therapeutic purposes. While these cells can simply be isolated and infused, efficient immunotherapy protocols also include certain steps where the effector cells are expanded *ex vivo* and/or genetically modified to increase their activity of specificity. Among the effectors used for cancer immunotherapy NK cells stand out with their high anti-tumor activity. While preclinical and clinical research on primary NK cells continues to deliver promising results, another source for NK cell-based immunotherapy stands out in the form of the NK-92 cell line that has many advantages compared to primary cells (Maki et al. 2001).

In this study, we used NK-92 cells engineered with different CARs targeting the same B-cell differentiation antigen (CD19) which is shown to be a clinically relevant target in B cell malignancies. CD19 is a tumor associated antigen which is already used for clinical CAR-T cell treatments and serves as a model in our research to optimize intracellular design of CAR vectors tailored for use in NK cells.

To investigate CAR-NK92 cells, CAR vector is consisted of CD19 specific scFv and CD8α hinge region either linked to CD3ζ or additionally CD28 or CD137 co-stimulatory domains. A survey of existing literature shows that CD28 and CD137 are frequently used in CAR designs. CAR-modified NK-92 cells are obtained with lentiviral transduction followed by puromycin selection or FACS sorting. We displayed stable CAR expression on cell surface following this procedure. To investigate the functionality of CAR modified NK-92 cells we used CD19 expressing B cell lymphoma cell lines and measured functionality with degranulation and cytokine secretion experiments.

Based on our findings, NK-92_CAR_CD137_CD3ζ cells were less effective than NK-92_CAR_CD3ζ and NK-92_CAR_CD28_CD3ζ in degranulation as well as TNFα and IFN-γ secretion. NK-92_CAR_CD3ζ cells showed slightly higher degranulation profile. This may be the result of differences in the CAR signaling depending on the internal design of the transgene. NK-92_CAR_CD3ζ has the intracellular signaling domain of CD3ζ which was shown to cause formation of disulfide links between CAR homodimers and heterodimers (Uherek et al. 2002; Schönfeld et al. 2015).

CAR_CD137_CD3ζ, CD28_CD3ζ, and only CD3ζ contain same anti CD19 scFv and same CD8α hinge region which plays a critical role in flexibility of the antigen binding domain that determines possible scFv-dependent effects. Also, target antigen plays a big role in activation of CAR (Hombach et al. 2007). Thus, the CAR vectors with different targets should be evaluated among themselves and direct comparison of internal vector designs can only be achieved by using the same antigen binding domain. Furthermore, it is possible that the antigen binding domain affects the intracellular design which means that the most effective intracellular design can be different for different antigens. For example, ErbB2-targeted CD28-CD3ζ and CD137-CD3ζ CARs displayed more certain cytotoxicity (Schönfeld et al. 2015).

Among the CAR-expressing NK cells obtained in this study, the first-generation vector seems to increase unspecific activity against CD19- K562 cells. The other vectors do not have this effect. We see a similar loss of specific activity when we overexpress CD3ζ in NK-92 cells for TCR expression (Parlar et al. 2019). Since CD3ζ is coupled to some NK cell activating receptors, we observe that its overexpression increases general activation of NK cells by affecting the cell surface levels of CD3ζ-coupled NK cell receptors. We suspect that we are seeing a similar effect here in the first-generation vector where the CD3ζ sequence utilized in the design is longer and includes the transmembrane domain that it uses to couple to NK receptor. In contrast, the other two vectors do not utilize CD3ζ transmembrane domains, hence are less likely to couple to NK cell receptors and affect their surface expression levels. For clinical use of these cells, strict characterization of these phenotypic effects on NK cells must be carried out for each vector.

Table 5.1. Comparison of different chimeric antigen receptors

		Vectors		
		CAR.3ζ	CAR.28.3ζ	CAR.137.3ζ
Parts of the CAR	Signal Peptide	+	+	+
	CD8a	+	+	+
	CD3ζ	+	+	+
	CD28	-	+	-
	CD137	-	-	+
T cell signaling		1	1,2	1,2
Degranulation	Daudi	+ + +	+ + +	++
	Namalwa	+ + +	+ +	++
	K562	+ +	+	++
TNF secretion	Daudi	+ + +	+ + +	+
	Namalwa	+ + +	+ +	+
	K562	+ + +	+	+ +
IFN secretion	Daudi			
	Namalwa	+ +	+ ++	+
	K562	+	+	+ +

When the data collected from different CAR designs are summarized, we can clearly see that the CD28ζ vector works better in NK-92 cells (Table 5.1). The first-generation vector seems to increase nonspecific activity in terms of both the degranulation and TNF secretion. The CD137ζ vector on the other hand does not seem to trigger high levels of antigen specific activation in NK-92 cells.

In Tonn et al. 2016, similar results were obtained also with primary NK cells and the authors reported that the 1st generation vector may be activated more rapidly in lower antigen densities than the others due to formation of disulfide bonds.

On the other hand, the newly designed CAR vectors failed to express c-myc-tag on the cell surface and degranulation is also failed. Our initial assessment reveals cloning steps during the preparation of the new vector may be a problem. Further studies to functionally clone the new vectors and use them for CAR expression in NK-92 cells are warranted.

In future works, we have in view to find and solve the problem of lab made constructs of 3rd Generation CAR vectors and also further characterize for effector functions especially by flow-cytometry based cytotoxicity assays. We plan to repeat TNF-α and IFN-γ secretion experiments with new methods and anti CD19 blocking experiments with a new antibody clone. Regarding further applications, other studies in our group will use the CAR vectors generated in this research to engineer new CAR NK-92 cells against various tumor antigens.

REFERENCES

Alberts, Bruce, Alexander Johnson, Julian Lewis, Martin Raff, Keith Roberts, and Peter Walter. 2002. "Lymphocytes and the Cellular Basis of Adaptive Immunity."

Alici, E., and T. Sutlu. 2009. "Natural Killer Cell-Based Immunotherapy in Cancer: Current Insights and Future Prospects." *Journal of Internal Medicine*. https://doi.org/10.1111/j.1365-2796.2009.02121.x.

Alici, Evren, Kyriakos V. Konstantinidis, Tolga Sutlu, Alar Aints, Gösta Gahrton, Hans Gustaf Ljunggren, and M. Sirac Dilber. 2007. "Anti-Myeloma Activity of Endogenous and Adoptively Transferred Activated Natural Killer Cells in Experimental Multiple Myeloma Model." *Experimental Hematology*. https://doi.org/10.1016/j.exphem.2007.08.006.

Alkins, Ryan, Alison Burgess, Milan Ganguly, Giulio Francia, Robert Kerbel, Winfried S. Wels, and Kullervo Hynynen. 2013. "Focused Ultrasound Delivers Targeted Immune Cells to Metastatic Brain Tumors." *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-12-2609.

Alkins, Ryan, Alison Burgess, Robert Kerbel, Winfried S. Wels, and Kullervo Hynynen. 2016. "Early Treatment of HER2-Amplified Brain Tumors with Targeted NK-92 Cells and Focused Ultrasound Improves Survival." *Neuro-Oncology*. https://doi.org/10.1093/neuonc/nov318.

Andris-Widhopf, J, P Steinberger, R Fuller, C Rader, and C F Barbas III. 2001. "Generation of Antibody Libraries: PCR Amplification and Assembly of Light-and Heavy-Chain Coding Sequences In: Phage Display Laboratory Manual." *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY*. https://doi.org/10.1101/pdb.prot065565.

Arai, S, R Meagher, M Swearingen, H Myint, E Rich, J Martinson, and H Klingemann. 2008. "Infusion of the Allogeneic Cell Line NK-92 in Patients with Advanced Renal Cell Cancer or Melanoma: A Phase I Trial." *Cytotherapy* 10 (6): 625–32. https://doi.org/10.1080/14653240802301872.

Benhar, Itai, and Yoram Reiter. 2004. "Phage Display of Single-Chain Antibody Constructs." In *Current Protocols in Immunology*. https://doi.org/10.1002/0471142735.im1019bs48.

Boissel, Laurent, Monica Betancur, Weiquan Lu, Daniela Krause, Richard Van Etten, Winfried Wels, and Hans Klingemann. 2013. "Retargeting NK-92 Cells by Means of CD19- and CD20-Specific Chimeric Antigen Receptors Compares Favorably

with Antibody-Dependent Cellular Cytotoxicity." *OncoImmunology* 2 (10): e26527. https://doi.org/10.4161/onci.26527.

Boissel, Laurent, Monica Betancur, Winfried S Wels, Hande Tuncer, and Hans Klingemann. 2009. "Transfection with MRNA for CD19 Specific Chimeric Antigen Receptor Restores NK Cell Mediated Killing of CLL Cells." *Leukemia Research* 33 (9): 1255–59. https://doi.org/10.1016/j.leukres.2008.11.024.

Boyiadzis, Michael, Mounzer Agha, Robert L Redner, Alison Sehgal, Annie Im, Jing-Zhou Hou, Rafic Farah, et al. 2017. "Phase 1 Clinical Trial of Adoptive Immunotherapy Using 'off-the-Shelf' Activated Natural Killer Cells in Patients with Refractory and Relapsed Acute Myeloid Leukemia." *Cytotherapy* 19 (10): 1225–32. https://doi.org/10.1016/j.jcyt.2017.07.008.

Brudno, Jennifer N., and James N. Kochenderfer. 2016. "Toxicities of Chimeric Antigen Receptor T Cells: Recognition and Management." *Blood*. https://doi.org/10.1182/blood-2016-04-703751.

Bryceson, Yenan T, Michael E March, Hans-Gustaf Ljunggren, and Eric O Long. 2006. "Synergy among Receptors on Resting NK Cells for the Activation of Natural Cytotoxicity and Cytokine Secretion." *Blood* 107 (1): 159–66. https://doi.org/10.1182/blood-2005-04-1351.

Chan, Antoni, Deng-Li Hong, Ann Atzberger, Simon Kollnberger, Andrew D Filer, Christopher D Buckley, Andrew McMichael, Tariq Enver, and Paul Bowness. 2007. "CD56bright Human NK Cells Differentiate into CD56dim Cells: Role of Contact with Peripheral Fibroblasts." *Journal of Immunology (Baltimore, Md. : 1950)*.

Chen, I. Ming, Margaret Whalen, Arthur Bankhurst, Cordelia E. Sever, Rashmi Doshi, David Hardekopf, Karen Montgomery, and Cheryl L. Willman. 2004. "A New Human Natural Killer Leukemia Cell Line, IMC-1. A Complex Chromosomal Rearrangement Defined by Spectral Karyotyping: Functional and Cytogenetic Characterization." *Leukemia Research*. https://doi.org/10.1016/S0145-2126(03)00254-6.

Chen, K H, M Wada, K G Pinz, H Liu, K-W Lin, A Jares, A E Firor, et al. 2017. "Preclinical Targeting of Aggressive T-Cell Malignancies Using Anti-CD5 Chimeric Antigen Receptor." *Leukemia* 31 (10): 2151–60. https://doi.org/10.1038/leu.2017.8.

Chen, Kevin H., Masayuki Wada, Amelia E. Firor, Kevin G. Pinz, Alexander Jares, Hua Liu, Huda Salman, et al. 2016. "Novel Anti-CD3 Chimeric Antigen Receptor Targeting of Aggressive T Cell Malignancies." *Oncotarget*. https://doi.org/10.18632/oncotarget.11019.

Chen, Lieping, and Dallas B. Flies. 2013. "Molecular Mechanisms of T Cell Co-Stimulation and Co-Inhibition." *Nature Reviews Immunology*. https://doi.org/10.1038/nri3405.

Chen, Xianghong, Fanqi Bai, Lubomir Sokol, Junmin Zhou, Amy Ren, Jeffrey S Painter, Jinhong Liu, et al. 2009. "A Critical Role for DAP10 and DAP12 in CD8+ T Cell-Mediated Tissue Damage in Large Granular Lymphocyte Leukemia." *Blood* 113 (14): 3226–34. https://doi.org/10.1182/blood-2008-07-168245.

Chen, Xilin, Jianfeng Han, Jianhong Chu, Lingling Zhang, Jianying Zhang, Charlie Chen, Luxi Chen, et al. 2016. "A Combinational Therapy of EGFR-CAR NK Cells and Oncolytic Herpes Simplex Virus 1 for Breast Cancer Brain Metastases." *Oncotarget*. https://doi.org/10.18632/oncotarget.8526.

Chmielewski, M, A A Hombach, and H Abken. 2011. "CD28 Cosignalling Does Not Affect the Activation Threshold in a Chimeric Antigen Receptor-Redirected T-Cell Attack." *Gene Therapy* 18 (1): 62–72. https://doi.org/10.1038/gt.2010.127.

Chmielewski, Markus, and Hinrich Abken. 2015. "TRUCKs: The Fourth Generation of CARs." *Expert Opinion on Biological Therapy* 15 (8): 1145–54. https://doi.org/10.1517/14712598.2015.1046430.

Clémenceau, Béatrice, Nicolas Congy-Jolivet, Géraldine Gallot, Régine Vivien, Joëlle Gaschet, Gilles Thibault, and Henri Vié. 2006. "Antibody-Dependent Cellular Cytotoxicity (ADCC) Is Mediated by Genetically Modified Antigen-Specific Human T Lymphocytes." *Blood* 107 (12): 4669–77. https://doi.org/10.1182/blood-2005-09-3775.

Daldrup-Link, Heike E., Sidhartha Tavri, Priyanka Jha, Reinhard Meier, Tobias D. Henning, Tina Muller, Daniel Hostetter, et al. 2009. "Optical Imaging of Cellular Immunotherapy against Prostate Cancer." *Molecular Imaging*. https://doi.org/10.2310/7290.2009.00002.

Daldrup-Link, Heike E, Reinhardt Meier, Martina Rudelius, Guido Piontek, Morand Piert, Stephan Metz, Marcus Settles, et al. 2005. "In Vivo Tracking of Genetically Engineered, Anti-HER2/Neu Directed Natural Killer Cells to HER2/Neu Positive Mammary Tumors with Magnetic Resonance Imaging." *European Radiology* 15 (1): 4–13. https://doi.org/10.1007/s00330-004-2526-7.

Domogala, Anna, J. Alejandro Madrigal, and Aurore Saudemont. 2015. "Natural Killer Cell Immunotherapy: From Bench to Bedside." *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2015.00264.

Enblad, Gunilla, Hannah Karlsson, Kristina Wikstrom, Magnus Essand, Barbara Savoldo, Malcolm K. Brenner, Gianpietro Dotti, et al. 2015. "Third Generation CD19-CAR T Cells for Relapsed and Refractory Lymphoma and Leukemia Report from the Swedish Phase I/IIa Trial." *Blood* 126 (23).

Eshhar, Z., T. Waks, G. Gross, and D. G. Schindler. 1993. "Specific Activation and Targeting of Cytotoxic Lymphocytes through Chimeric Single Chains Consisting of Antibody-Binding Domains and the Gamma or Zeta Subunits of the Immunoglobulin and T-Cell Receptors." *Proceedings of the National Academy of Sciences* 90 (2): 720–24. https://doi.org/10.1073/pnas.90.2.720.

Esser, Ruth, Tina Müller, Dörthe Stefes, Stephan Kloess, Diana Seidel, Stephen D. Gillies, Christel Aperlo-Iffland, et al. 2012. "NK Cells Engineered to Express a GD 2-Specific Antigen Receptor Display Built-in ADCC-like Activity against Tumour Cells of Neuroectodermal Origin." *Journal of Cellular and Molecular Medicine*. https://doi.org/10.1111/j.1582-4934.2011.01343.x.

"FDA Approves Second CAR T-Cell Therapy." 2018. *Cancer Discovery*. https://doi.org/10.1158/2159-8290.CD-NB2017-155.

Fedorov, Victor D., Maria Themeli, and Michel Sadelain. 2013. “PD-1- and CTLA-4-Based Inhibitory Chimeric Antigen Receptors (ICARs) Divert off-Target Immunotherapy Responses.” *Science Translational Medicine*. https://doi.org/10.1126/scitranslmed.3006597.

Ferlazzo, Guido, Dolca Thomas, Shao-Lee Lin, Kiera Goodman, Barbara Morandi, William A Muller, Alessandro Moretta, and Christian Münz. 2004. “The Abundant NK Cells in Human Secondary Lymphoid Tissues Require Activation to Express Killer Cell Ig-like Receptors and Become Cytolytic.” *Journal of Immunology (Baltimore, Md. : 1950)* 172 (3): 1455–62. https://doi.org/10.4049/jimmunol.172.3.1455.

“First-Ever CAR T-Cell Therapy Approved in U.S.” 2017. *Cancer Discovery*. https://doi.org/10.1158/2159-8290.cd-nb2017-126.

Gacerez, Albert T., Benjamine Arellano, and Charles L. Sentman. 2016. “How Chimeric Antigen Receptor Design Affects Adoptive T Cell Therapy.” *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.25419.

Geller, Melissa A, and Jeffrey S Miller. 2011. “Use of Allogeneic NK Cells for Cancer Immunotherapy.” *Immunotherapy* 3 (12): 1445–59. https://doi.org/10.2217/imt.11.131.

Genßler, Sabrina, Michael C Burger, Congcong Zhang, Sarah Oelsner, Iris Mildenberger, Marlies Wagner, Joachim P Steinbach, and Winfried S Wels. 2016. “Dual Targeting of Glioblastoma with Chimeric Antigen Receptor-Engineered Natural Killer Cells Overcomes Heterogeneity of Target Antigen Expression and Enhances Antitumor Activity and Survival.” *Oncoimmunology* 5 (4): e1119354. https://doi.org/10.1080/2162402X.2015.1119354.

Glienke, Wolfgang, Ruth Esser, Christoph Priesner, Julia D Suerth, Axel Schambach, Winfried S Wels, Manuel Grez, Stephan Kloess, Lubomir Arseniev, and Ulrike Koehl. 2015. “Advantages and Applications of CAR-Expressing Natural Killer Cells.” *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2015.00021.

Gong, J H, G Maki, and H G Klingemann. 1994. “Characterization of a Human Cell Line (NK-92) with Phenotypical and Functional Characteristics of Activated Natural Killer Cells.” *Leukemia*.

Grégoire, Claude, Claude Grégoire, Lionel Chasson, Lionel Chasson, Carmelo Luci, Carmelo Luci, Elena Tomasello, et al. 2007. “The Trafficking of Natural Killer Cells.” *Immunological Reviews*. https://doi.org/10.1111/j.1600-065X.2007.00563.x.

Haber, E., R. E. Bruccoleri, M. Mudgett-Hunter, D. Levinson, J. S. Huston, R. J. Ridge, M. N. Margolies, J. Novotny, M. S. Tai, and R. Crea. 2006. “Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain Fv Analogue Produced in Escherichia Coli.” *Proceedings of the National Academy of Sciences*. https://doi.org/10.1073/pnas.85.16.5879.

Han, Jianfeng, Jianhong Chu, Wing Keung Chan, Jianying Zhang, Youwei Wang, Justus B. Cohen, Aaron Victor, et al. 2015. “CAR-Engineered NK Cells Targeting Wild-Type EGFR and EGFRvIII Enhance Killing of Glioblastoma and Patient-Derived Glioblastoma Stem Cells.” *Scientific Reports*. https://doi.org/10.1038/srep11483.

Herberman, R., M E Nunn, and D H Lavrin. 1975. “Natural Cytotoxic Reactivity of Mouse Lymphoid Cells against Syngeneic Acid Allogeneic Tumors. I. Distribution of Reactivity and Specificity.” *International Journal of Cancer. Journal International Du Cancer* 16 (2): 216–29. https://doi.org/10.1002/ijc.2910160204.

Hinrichs, Christian S, and Steven A Rosenberg. 2014. “Exploiting the Curative Potential of Adoptive T-Cell Therapy for Cancer.” *Immunological Reviews* 257 (1): 56–71. https://doi.org/10.1111/imr.12132.

Hoffmann, Thomas K., and Patrick J. Schuler. 2013. “Antigen-Specific Immunotherapy in Head and Neck Cancer.” *Advances in Cellular and Molecular Otolaryngology*. https://doi.org/10.3402/acmo.v1i0.21758.

Hombach, Andreas A, Johannes Heiders, Marcel Foppe, Markus Chmielewski, and Hinrich Abken. 2012. “OX40 Costimulation by a Chimeric Antigen Receptor Abrogates CD28 and IL-2 Induced IL-10 Secretion by Redirected CD4(+) T Cells.” *Oncoimmunology* 1 (4): 458–66. https://doi.org/10.4161/onci.19855.

Hombach, Andreas A, Verena Schildgen, Claudia Heuser, Ricarda Finnern, David E Gilham, and Hinrich Abken. 2007. “T Cell Activation by Antibody-like Immunoreceptors: The Position of the Binding Epitope within the Target Molecule Determines the Efficiency of Activation of Redirected T Cells.” *Journal of Immunology (Baltimore, Md. : 1950)* 178 (7): 4650–57. https://doi.org/10.4049/jimmunol.178.7.4650.

Huston, J S, D Levinson, M Mudgett-Hunter, M S Tai, J Novotný, M N Margolies, R J Ridge, R E Bruccoleri, E Haber, and R Crea. 1988. “Protein Engineering of Antibody Binding Sites: Recovery of Specific Activity in an Anti-Digoxin Single-Chain Fv Analogue Produced in Escherichia Coli.” *Proceedings of the National Academy of Sciences of the United States of America*.

Jacoby, Elad, Haiying Qin, Yinmeng Yang, Christopher Daniel Chien, and Terry J Fry. 2014. “CD19 CAR T Cells Maintain Efficacy in the Allogeneic Environment but Mediate Acute Graft-Versus-Host-Disease Only in the Presence CD19+ Acute Lymphoblastic Leukemia.” *Blood* 124 (21).

Jiang, Hua, Wenhao Zhang, Peipei Shang, Hui Zhang, Weijun Fu, Fei Ye, Tianmei Zeng, et al. 2014. “Transfection of Chimeric Anti-CD138 Gene Enhances Natural Killer Cell Activation and Killing of Multiple Myeloma Cells.” *Molecular Oncology* 8 (2): 297–310. https://doi.org/10.1016/j.molonc.2013.12.001.

June, C H, J A Ledbetter, M M Gillespie, T Lindsten, and C B Thompson. 1987. “T-Cell Proliferation Involving the CD28 Pathway Is Associated with Cyclosporine-Resistant Interleukin 2 Gene Expression.” *Molecular and Cellular Biology*.

Kagami, Yoshitoyo, Shigeo Nakamura, Ritsurou Suzuki, Shinsuke Iida, Yasushi Yatabe, Yasutaka Okada, Tomoko Kobayashi, et al. 1998. “Establishment of an IL-2-Dependent Cell Line Derived from ‘nasal-Type’ NK/T-Cell Lymphoma of CD2 + , SCD3 - , CD3ε + , CD56 + Phenotype and Associated with the Epstein-Barr Virus.” *British Journal of Haematology*. https://doi.org/10.1046/j.1365-2141.1998.01029.x.

Kiessling, R., Eva Klein, and H Wigzell. 1975. “‘Natural’ Killer Cells in the Mouse.” *European Journal of Immunology*, no. 5: 112–17.

https://doi.org/10.1002/eji.1830050208.

Killer, Natural, Cells Enhance, In Vitro, In Vivo Anti-tumor, Activity Against, and Human Multiple. 2014. "CS1-Specific Chimeric Antigen Receptor (CAR)-Engineered Natural Killer Cells Enhance In Vitro and In Vivo Anti-Tumor Activity Against Human Multiple Myeloma." *Leukemia*. https://doi.org/10.1038/leu.2013.279.CS1-Specific.

Klingemann, Hans. 2014. "Are Natural Killer Cells Superior CAR Drivers?" *Oncoimmunology* 3: e28147. https://doi.org/10.4161/onci.28147.

Klingemann, Hans, Laurent Boissel, and Frances Toneguzzo. 2016. "Natural Killer Cells for Immunotherapy - Advantages of the NK-92 Cell Line over Blood NK Cells." *Frontiers in Immunology* 7: 91. https://doi.org/10.3389/fimmu.2016.00091.

Klingemann, HG, and B Miyagawa. 1996. "Purging of Malignant Cells from Blood after Short Ex Vivo Incubation with NK-92 Cells [Letter; Comment]." *Blood* 87 (11).

Koehl, U., C. Kalberer, J. Spanholtz, D. A. Lee, J. S. Miller, S. Cooley, M. Lowdell, et al. 2016. "Advances in Clinical NK Cell Studies: Donor Selection, Manufacturing and Quality Control." *OncoImmunology*. https://doi.org/10.1080/2162402X.2015.1115178.

Konstantinidis, Kyriakos V., Evren Alici, Alar Aints, Birger Christensson, Hans Gustaf Ljunggren, and M. Sirac Dilber. 2005. "Targeting IL-2 to the Endoplasmic Reticulum Confines Autocrine Growth Stimulation to NK-92 Cells." *Experimental Hematology*. https://doi.org/10.1016/j.exphem.2004.11.003.

Kornbluth, J. 1982. "Cell Surface Phenotype of a Cloned Line of Human Natural Killer Cells." *The Journal of Immunology*.

Kowolik, Claudia M, Max S Topp, Sergio Gonzalez, Timothy Pfeiffer, Simon Olivares, Nancy Gonzalez, David D Smith, Stephen J Forman, Michael C Jensen, and Laurence J N Cooper. 2006. "CD28 Costimulation Provided through a CD19-Specific Chimeric Antigen Receptor Enhances in Vivo Persistence and Antitumor Efficacy of Adoptively Transferred T Cells." *Cancer Research* 66 (22): 10995–4. https://doi.org/10.1158/0008-5472.CAN-06-0160.

Krzewski, Konrad, and Jack L Strominger. 2008. "The Killer's Kiss: The Many Functions of NK Cell Immunological Synapses." *Current Opinion in Cell Biology* 20 (5): 597–605. https://doi.org/10.1016/j.ceb.2008.05.006.

Kuhn, Nicholas F, Terence J Purdon, Dayenne G van Leeuwen, Andrea V Lopez, Kevin J Curran, Anthony F Daniyan, and Renier J Brentjens. 2019. "CD40 Ligand-Modified Chimeric Antigen Receptor T Cells Enhance Antitumor Function by Eliciting an Endogenous Antitumor Response." *Cancer Cell* 35 (3): 473-488.e6. https://doi.org/10.1016/j.ccell.2019.02.006.

Lanier, L. L. 2004. "Human Natural Killer Cells Isolated from Peripheral Blood Do Not Rearrange T Cell Antigen Receptor Beta Chain Genes." *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.163.1.209.

Lehner, Manfred, Gabriel Götz, Julia Proff, Niels Schaft, Jan Dörrie, Florian Full, Armin

Ensser, et al. 2012. “Redirecting T Cells to Ewing’s Sarcoma Family of Tumors by a Chimeric NKG2D Receptor Expressed by Lentiviral Transduction or MRNA Transfection.” *PloS One* 7 (2): e31210. https://doi.org/10.1371/journal.pone.0031210.

Li, Ye, David L. Hermanson, Branden S. Moriarity, and Dan S. Kaufman. 2018. “Human IPSC-Derived Natural Killer Cells Engineered with Chimeric Antigen Receptors Enhance Anti-Tumor Activity.” *Cell Stem Cell*. https://doi.org/10.1016/j.stem.2018.06.002.

Lin, J, and A Weiss. 2001. “T Cell Receptor Signalling.” *Journal of Cell Science*.

Lipowska-Bhalla, Grazyna, David E Gilham, Robert E Hawkins, and Dominic G Rothwell. 2012. “Targeted Immunotherapy of Cancer with CAR T Cells: Achievements and Challenges.” *Cancer Immunology, Immunotherapy : CII* 61 (7): 953–62. https://doi.org/10.1007/s00262-012-1254-0.

Liu, Hui, Bo Yang, Tingting Sun, Lin Lin, Yi Hu, Muhong Deng, Junlan Yang, et al. 2015. “Specific Growth Inhibition of ErbB2-Expressing Human Breast Cancer Cells by Genetically Modified NK-92 Cells.” *Oncology Reports*. https://doi.org/10.3892/or.2014.3548.

Liu, Ming, and Fukun Guo. 2018. “Recent Updates on Cancer Immunotherapy.” *Precision Clinical Medicine* 1 (2): 65–74. https://doi.org/10.1093/pcmedi/pby011.

Ljunggren, Hans Gustaf, and Klas Kärre. 1990. “In Search of the ‘Missing Self’: MHC Molecules and NK Cell Recognition.” *Immunology Today* 11 (C): 237–44. https://doi.org/10.1016/0167-5699(90)90097-S.

Maki, G, H G Klingemann, J A Martinson, and Y K Tam. 2001. “Factors Regulating the Cytotoxic Activity of the Human Natural Killer Cell Line, NK-92.” *Journal of Hematotherapy & Stem Cell Research* 10 (3): 369–83. https://doi.org/10.1089/152581601750288975.

Maria, Andrea De, Federica Bozzano, Claudia Cantoni, and Lorenzo Moretta. 2011. “Revisiting Human Natural Killer Cell Subset Function Revealed Cytolytic CD56(Dim)CD16+ NK Cells as Rapid Producers of Abundant IFN-Gamma on Activation.” *Proceedings of the National Academy of Sciences of the United States of America* 108 (2): 728–32. https://doi.org/10.1073/pnas.1012356108.

McNerney, Megan E, Kyung-Mi Lee, and Vinay Kumar. 2005. “2B4 (CD244) Is a Non-MHC Binding Receptor with Multiple Functions on Natural Killer Cells and CD8+ T Cells.” *Molecular Immunology* 42 (4): 489–94. https://doi.org/10.1016/j.molimm.2004.07.032.

Medvedev, A E, A C Johnsen, J Haux, B Steinkjer, K Egeberg, D H Lynch, A Sundan, and T Espevik. 1997. “Regulation of Fas and Fas-Ligand Expression in NK Cells by Cytokines and the Involvement of Fas-Ligand in NK/LAK Cell-Mediated Cytotoxicity.” *Cytokine* 9 (6): 394–404. https://doi.org/10.1006/cyto.1996.0181.

Medzhitov, Ruslan, and Charles Janeway. 2000. “Innate Immune Recognition: Mechanisms and Pathways - Medzhitov - 2002 - Immunological Reviews - Wiley Online Library.” *Immunological Reviews* 173: 89–97.

http://onlinelibrary.wiley.com.globalproxy.cvt.dk/doi/10.1034/j.1600-065X.2000.917309.x/pdf.

Mehta, Rohtesh S., and Katayoun Rezvani. 2018. "Chimeric Antigen Receptor Expressing Natural Killer Cells for the Immunotherapy of Cancer." *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2018.00283.

Meier, Reinhard, Daniel Golovko, Sidhartha Tavri, Tobias D. Henning, Christiane Knopp, Guido Piontek, Martina Rudelius, Petra Heinrich, Winfried S. Wels, and Heike Daldrup-Link. 2011. "Depicting Adoptive Immunotherapy for Prostate Cancer in an Animal Model with Magnetic Resonance Imaging." *Magnetic Resonance in Medicine*. https://doi.org/10.1002/mrm.22652.

Meier, Reinhard, Morand Piert, Guido Piontek, Martina Rudelius, Robert A. Oostendorp, Reingard Senekowitsch-Schmidtke, Tobias D. Henning, et al. 2008. "Tracking of [18F]FDG-Labeled Natural Killer Cells to HER2/Neu-Positive Tumors." *Nuclear Medicine and Biology*. https://doi.org/10.1016/j.nucmedbio.2008.02.006.

Mensali, Nadia, Pierre Dillard, Michael Hebeisen, Susanne Lorenz, Theodossis Theodossiou, Marit Renée Myhre, Anne Fåne, et al. 2019. "NK Cells Specifically TCR-Dressed to Kill Cancer Cells." *EBioMedicine* 40 (February): 106–17. https://doi.org/10.1016/j.ebiom.2019.01.031.

Miller, Jeffrey S., and Lewis L. Lanier. 2019. "Natural Killer Cells in Cancer Immunotherapy." *Annual Review of Cancer Biology*. https://doi.org/10.1146/annurev-cancerbio-030518-055653.

Moretta, Alessandro, Roberto Biassoni, Maria Cristina Mingari, Daniela Pende, Lorenzo Moretta, and Cristina Bottino. 2002. "Human Natural Killer Cells: Their Origin, Receptors and Function." *European Journal of Immunology*. https://doi.org/10.1002/1521-4141(200205)32:5<1205::aid-immu1205>3.0.co;2-y.

Müller, Tina, Christoph Uherek, Guitta Maki, Kai Uwe Chow, Annemarie Schimpf, Hans Georg Klingemann, Torsten Tonn, and Winfried S. Wels. 2008. "Expression of a CD20-Specific Chimeric Antigen Receptor Enhances Cytotoxic Activity of NK Cells and Overcomes NK-Resistance of Lymphoma and Leukemia Cells." *Cancer Immunology, Immunotherapy*. https://doi.org/10.1007/s00262-007-0383-3.

Muralikrishna, K, C Varalakshmi, and A Khar. 1997. "Differential Modulation of LAK and ADCC Functions of Natural Killer Cells from AK-5 Tumor-Bearing Rats by IL-2, IL-12 and IFN-Gamma." *Cytokines, Cellular & Molecular Therapy*.

Myers, Lara M, and Anthony T Vella. 2005. "Interfacing T-Cell Effector and Regulatory Function through CD137 (4-1BB) Co-Stimulation." *Trends in Immunology* 26 (8): 440–46. https://doi.org/10.1016/j.it.2005.06.003.

Nagata, H, A Konno, N Kimura, Y Zhang, M Kimura, A Demachi, T Sekine, K Yamamoto, and N Shimizu. 2001. "Characterization of Novel Natural Killer (NK)-Cell and Gammadelta T-Cell Lines Established from Primary Lesions of Nasal T/NK-Cell Lymphomas Associated with the Epstein-Barr Virus." *Blood* 97 (3): 708–13. https://doi.org/10.1182/blood.v97.3.708.

Nowakowska, Paulina, Annette Romanski, Nicole Miller, Marcus Odendahl, Halvard

Bonig, Congcong Zhang, Erhard Seifried, Winfried S. Wels, and Torsten Tonn. 2018. “Clinical Grade Manufacturing of Genetically Modified, CAR-Expressing NK-92 Cells for the Treatment of ErbB2-Positive Malignancies.” *Cancer Immunology, Immunotherapy* 67 (1): 25–38. https://doi.org/10.1007/s00262-017-2055-2.

Oberschmidt, Olaf, Stephan Kloess, and Ulrike Koehl. 2017. “Redirected Primary Human Chimeric Antigen Receptor Natural Killer Cells as an ‘off-the-Shelf Immunotherapy’ for Improvement in Cancer Treatment.” *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2017.00654.

Pabla, Navjotsingh, and Zheng Dong. 2012. “Curtailing Side Effects in Chemotherapy: A Tale of PKCδ in Cisplatin Treatment.” *Oncotarget*. https://doi.org/10.18632/oncotarget.439.

Pardoll, Drew. 2013. “06 Cancer Immunology.” In *Abeloff’s Clinical Oncology: Fifth Edition*. https://doi.org/10.1016/B978-1-4557-2865-7.00006-0.

Parlar, Ayhan, Ece Canan Sayitoglu, Didem Ozkazanc, Anna-Maria Georgoudaki, Cevriye Pamukcu, Mertkaya Aras, Benjamin J. Josey, et al. 2019. “Engineering Antigen-specific NK Cell Lines against the Melanoma-associated Antigen Tyrosinase via TCR Gene Transfer.” *European Journal of Immunology*. https://doi.org/10.1002/eji.201948140.

Pegram, Hollie J., Daniel M. Andrews, Mark J. Smyth, Phillip K. Darcy, and Michael H. Kershaw. 2011. “Activating and Inhibitory Receptors of Natural Killer Cells.” *Immunology and Cell Biology*. https://doi.org/10.1038/icb.2010.78.

Petersen, Christopher T., and Giedre Krenciute. 2019. “Next Generation CAR T Cells for the Immunotherapy of High-Grade Glioma.” *Frontiers in Oncology*. https://doi.org/10.3389/fonc.2019.00069.

Pross, Susan. 2007. “T-Cell Activation.” In *XPharm: The Comprehensive Pharmacology Reference*, 1–7. Elsevier. https://doi.org/10.1016/B978-008055232-3.60244-2.

Pulè, Martin A., Karin C. Straathof, Gianpietro Dotti, Helen E. Heslop, Cliona M. Rooney, and Malcolm K. Brenner. 2005. “A Chimeric T Cell Antigen Receptor That Augments Cytokine Release and Supports Clonal Expansion of Primary Human T Cells.” *Molecular Therapy*. https://doi.org/10.1016/j.ymthe.2005.04.016.

Raeber, Miro E, Yves Zurbuchen, Daniela Impellizzieri, and Onur Boyman. 2018. “The Role of Cytokines in T-Cell Memory in Health and Disease.” *Immunological Reviews* 283 (1): 176–93. https://doi.org/10.1111/imr.12644.

Raulet, David H, and Russell E Vance. 2006. “Self-Tolerance of Natural Killer Cells.” *Nature Reviews. Immunology* 6 (7): 520–31. https://doi.org/10.1038/nri1863.

Rizzieri, David A, Robert Storms, Dong-Feng Chen, Gwynn Long, Yiping Yang, Daniel A Nikcevich, Cristina Gasparetto, et al. 2010. “Natural Killer Cell-Enriched Donor Lymphocyte Infusions from A 3-6/6 HLA Matched Family Member Following Nonmyeloablative Allogeneic Stem Cell Transplantation.” *Biology of Blood and Marrow Transplantation : Journal of the American Society for Blood and Marrow Transplantation* 16 (8): 1107–14. https://doi.org/10.1016/j.bbmt.2010.02.018.

Robertson, MJ, KJ Cochran, … C Cameron - Experimental, and undefined 1996. n.d. "Characterization of a Cell Line, NKL, Derived from an Aggressive Human Natural Killer Cell Leukemia." *Europepmc.Org*. Accessed July 11, 2019. https://europepmc.org/abstract/med/8599969.

Romanski, Annette, Christoph Uherek, Gesine Bug, Erhard Seifried, Hans Klingemann, Winfried S. Wels, Oliver G. Ottmann, and Torsten Tonn. 2016. "CD19-CAR Engineered NK-92 Cells Are Sufficient to Overcome NK Cell Resistance in B-Cell Malignancies." *Journal of Cellular and Molecular Medicine*. https://doi.org/10.1111/jcmm.12810.

Rosenberg, Steven A., and Nicholas P. Restifo. 2015. "Adoptive Cell Transfer as Personalized Immunotherapy for Human Cancer." *Science*. https://doi.org/10.1126/science.aaa4967.

Sahm, Christiane, Kurt Schönfeld, and Winfried S. Wels. 2012. "Expression of IL-15 in NK Cells Results in Rapid Enrichment and Selective Cytotoxicity of Gene-ModiWed EVectors That Carry a Tumor-SpeciWc Antigen Receptor." *Cancer Immunology, Immunotherapy*. https://doi.org/10.1007/s00262-012-1212-x.

Samelson, Lawrence E. 2011. "Immunoreceptor Signaling." *Cold Spring Harbor Perspectives in Biology*. https://doi.org/10.1101/cshperspect.a011510.

Schönfeld, Kurt, Christiane Sahm, Congcong Zhang, Sonja Naundorf, Christian Brendel, Marcus Odendahl, Paulina Nowakowska, et al. 2015. "Selective Inhibition of Tumor Growth by Clonal NK Cells Expressing an ErbB2/HER2-Specific Chimeric Antigen Receptor." *Molecular Therapy*. https://doi.org/10.1038/mt.2014.219.

Seidel, Diana, Anastasia Shibina, Nikolai Siebert, Winfried S. Wels, C. Patrick Reynolds, Nicole Huebener, and Holger N. Lode. 2015. "Disialoganglioside-Specific Human Natural Killer Cells Are Effective against Drug-Resistant Neuroblastoma." *Cancer Immunology, Immunotherapy*. https://doi.org/10.1007/s00262-015-1669-5.

Sentman, Charles L., Mats Y. Olsson, and Klas Kärre. 1995. "Missing Self Recognition by Natural Killer Cells in Mhc Class i Transgenic Mice. a ‗receptor Calibration' Model for How Effector Cells Adapt to Self." *Seminars in Immunology*. https://doi.org/10.1006/smim.1995.0015.

"Sequences of Proteins of Immunological Interest." 1984. *Analytical Biochemistry*. https://doi.org/10.1016/0003-2697(84)90805-4.

Smyth, Mark J., Erika Cretney, Janice M. Kelly, Jennifer A. Westwood, Shayna E.A. Street, Hideo Yagita, Kazuyoshi Takeda, Serani L.H.Van Dommelen, Mariapia A. Degli-Esposti, and Yoshihiro Hayakawa. 2005. "Activation of NK Cell Cytotoxicity." *Molecular Immunology*. https://doi.org/10.1016/j.molimm.2004.07.034.

Srivastava, Shivani, and Stanley R. Riddell. 2015. "Engineering CAR-T Cells: Design Concepts." *Trends in Immunology*. https://doi.org/10.1016/j.it.2015.06.004.

Suck, Garnet, Marcus Odendahl, Paulina Nowakowska, Christian Seidl, Winfried S Wels, Hans G Klingemann, and Torsten Tonn. 2016. "NK-92: An 'off-the-Shelf Therapeutic' for Adoptive Natural Killer Cell-Based Cancer Immunotherapy."

Cancer Immunology, Immunotherapy : CII 65 (4): 485–92. https://doi.org/10.1007/s00262-015-1761-x.

Sutlu, Tolga, Sanna Nyström, Mari Gilljam, Birgitta Stellan, Steven E. Applequist, and Evren Alici. 2012. "Inhibition of Intracellular Antiviral Defense Mechanisms Augments Lentiviral Transduction of Human Natural Killer Cells: Implications for Gene Therapy." *Human Gene Therapy*. https://doi.org/10.1089/hum.2012.080.

Swift, Brenna E., Brent A. Williams, Yoko Kosaka, Xing Hua Wang, Jeffrey A. Medin, Sowmya Viswanathan, Joaquin Martinez-Lopez, and Armand Keating. 2012. "Natural Killer Cell Lines Preferentially Kill Clonogenic Multiple Myeloma Cells and Decrease Myeloma Engraftment in a Bioluminescent Xenograft Mouse Model." *Haematologica*. https://doi.org/10.3324/haematol.2011.054254.

Tam, Y K, G Maki, B Miyagawa, B Hennemann, T Tonn, and H G Klingemann. 1999. "Characterization of Genetically Altered, Interleukin 2-Independent Natural Killer Cell Lines Suitable for Adoptive Cellular Immunotherapy." *Human Gene Therapy* 10 (8): 1359–73. https://doi.org/10.1089/10430349950018030.

Tassev, D V, M Cheng, and N-K V Cheung. 2012. "Retargeting NK92 Cells Using an HLA-A2-Restricted, EBNA3C-Specific Chimeric Antigen Receptor." *Cancer Gene Therapy* 19 (2): 84–100. https://doi.org/10.1038/cgt.2011.66.

Tonn, Torsten, Hans Klingemann, Winfried S. Wels, Sarah Oelsner, Juliane Wagner, Miriam E. Friede, Susanne Badura, et al. 2016. "Continuously Expanding CAR NK-92 Cells Display Selective Cytotoxicity against B-Cell Leukemia and Lymphoma." *Cytotherapy*. https://doi.org/10.1016/j.jcyt.2016.10.009.

Tonn, Torsten, Dirk Schwabe, Hans G Klingemann, Sven Becker, Ruth Esser, Ulrike Koehl, Meinolf Suttorp, Erhard Seifried, Oliver G Ottmann, and Gesine Bug. 2013. "Treatment of Patients with Advanced Cancer with the Natural Killer Cell Line NK-92." *Cytotherapy* 15 (12): 1563–70. https://doi.org/10.1016/j.jcyt.2013.06.017.

Töpfer, Katrin, Marc Cartellieri, Susanne Michen, Ralf Wiedemuth, Nadja Müller, Dirk Lindemann, Michael Bachmann, Monika Füssel, Gabriele Schackert, and Achim Temme. 2015. "DAP12-Based Activating Chimeric Antigen Receptor for NK Cell Tumor Immunotherapy." *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1400330.

Tsuchiyama, Junjiro, Tadashi Yoshino, Masaharu Mori, Eisaku Kondoh, Takeshi Oka, Tadaatsu Akagi, Akio Hiraki, et al. n.d. "Characterization of a Novel Human Natural Killer-Cell Line (NK-YS) Established From Natural Killer Cell Lymphoma/Leukemia Associated With Epstein-Barr Virus Infection." www.bloodjournal.org.

Uherek, Christoph, Torsten Tonn, Barbara Uherek, Sven Becker, Barbara Schnierle, Hans-Georg Klingemann, and Winfried Wels. 2002. "Retargeting of Natural Killer-Cell Cytolytic Activity to ErbB2-Expressing Cancer Cells Results in Efficient and Selective Tumor Cell Destruction." *Blood*.

Vinay, Dass S., and Byoung S. Kwon. 2014. "4-1BB (CD137), an Inducible Costimulatory Receptor, as a Specific Target for Cancer Therapy." *BMB Reports*. https://doi.org/10.5483/BMBRep.2014.47.3.283.

Vivier, Eric, Jacques A. Nunès, and Frédéric Vély. 2004. “Natural Killer Cell Signaling Pathways.” *Science*. https://doi.org/10.1126/science.1103478.

Vivier, Eric, Elena Tomasello, Myriam Baratin, Thierry Walzer, and Sophie Ugolini. 2008. “Functions of Natural Killer Cells.” *Nature Immunology*. https://doi.org/10.1038/ni1582.

Watzl, Carsten, and Eric O. Long. 2010. “Signal Transduction During Activation and Inhibition of Natural Killer Cells.” In *Current Protocols in Immunology*. Hoboken, NJ, USA: John Wiley & Sons, Inc. https://doi.org/10.1002/0471142735.im1109bs90.

Weinkove, Robert, Philip George, Nathaniel Dasyam, and Alexander D Mclellan. 2019. “Selecting Costimulatory Domains for Chimeric Antigen Receptors: Functional and Clinical Considerations.” *Clinical & Translational Immunology* 8. https://doi.org/10.1002/cti2.1049.

Wu, Ming-Ru, Tong Zhang, Andre Alcon, and Charles L Sentman. 2015. “DNAM-1-Based Chimeric Antigen Receptors Enhance T Cell Effector Function and Exhibit in Vivo Efficacy against Melanoma.” *Cancer Immunology, Immunotherapy : CII* 64 (4): 409–18. https://doi.org/10.1007/s00262-014-1648-2.

Yagita, M, C L Huang, H Umehara, Y Matsuo, R Tabata, M Miyake, Y Konaka, and K Takatsuki. 2000. “A Novel Natural Killer Cell Line (KHYG-1) from a Patient with Aggressive Natural Killer Cell Leukemia Carrying a P53 Point Mutation.” *Leukemia* 14 (5): 922–30. http://www.ncbi.nlm.nih.gov/pubmed/10803526.

Yee, C, J A Thompson, D Byrd, S R Riddell, P Roche, E Celis, and P D Greenberg. 2002. “Adoptive T Cell Therapy Using Antigen-Specific CD8+ T Cell Clones for the Treatment of Patients with Metastatic Melanoma: In Vivo Persistence, Migration, and Antitumor Effect of Transferred T Cells.” *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.242600099.

Yodoi, J, K Teshigawara, … T Nikaido - The Journal of, and undefined 1985. n.d. “TCGF (IL 2)-Receptor Inducing Factor (s). I. Regulation of IL 2 Receptor on a Natural Killer-like Cell Line (YT Cells).” *Am Assoc Immnol*. Accessed July 11, 2019. https://www.jimmunol.org/content/134/3/1623.short.

Yusa, Sei-ichi, Tracey L Catina, and Kerry S Campbell. 2002. “SHP-1- and Phosphotyrosine-Independent Inhibitory Signaling by a Killer Cell Ig-like Receptor Cytoplasmic Domain in Human NK Cells.” *Journal of Immunology (Baltimore, Md. : 1950)* 168 (10): 5047–57. https://doi.org/10.4049/jimmunol.168.10.5047.

Zhang, Congcong, Michael C Burger, Lukas Jennewein, Sabrina Genßler, Kurt Schönfeld, Pia Zeiner, Elke Hattingen, et al. 2016. “ErbB2/HER2-Specific NK Cells for Targeted Therapy of Glioblastoma.” *Journal of the National Cancer Institute* 108 (5). https://doi.org/10.1093/jnci/djv375.

Zhang, Ge, Rongzhi Liu, Xuekai Zhu, Lei Wang, Juan Ma, Huamin Han, Xiaomin Wang, et al. n.d. “Retargeting NK-92 for Anti-Melanoma Activity by a TCR-like Single-Domain Antibody.” *Immunology and Cell Biology* 91 (10): 615–24. Accessed July 10, 2019. https://doi.org/10.1038/icb.2013.45.

Zhang, Tong, Amorette Barber, and Charles L Sentman. 2006. “Generation of Antitumor Responses by Genetic Modification of Primary Human T Cells with a Chimeric NKG2D Receptor.” *Cancer Research* 66 (11): 5927–33. https://doi.org/10.1158/0008-5472.CAN-06-0130.

APPENDIX A: Chemicals Used in This Study

Table A1. Chemicals used in this study.

Chemicals and Media Components	**Company**
Agar	Sigma, Germany
Agarose	Sigma, Germany
Ampicillin	GellGro, USA
Boric Acid	Sigma, Germany
Bovine Serum Albumin (BSA)	Sigma, Germany
Chloroquine	Sigma, Germany
Distilled Water	Merck Milipore, USA
DMEM	GIBCO, USA
DMSO	Sigma, Germany
DNA Gel Loading Dye, 6X	NEB, USA
DPBS	Sigma, Germany
EDTA	Applichem, Germany
Ethanol	Sigma, Germany
Ethidium Bromide	Sigma, Germany
Fetal Bovine Serum	GIBCO, USA
HEPES Solution, 1 M	Sigma, Germany
Interleukin-2	Proleukin, Novartis
Ionomycin from *Streptomyces conglobatus*	Sigma, Germany
Isopropanol	Sigma, Germany
LB Broth	Sigma, Germany
L-glutamine, 200 Mm	Sigma, Germany
MEM Vitamin Solution, 100X	GIBCO, USA
MEM Non-essential Amino Acid Solution	GIBCO, USA
2-Mercaptoethanol	Sigma, Germany
Methanol	Sigma, Germany
Monensin	Biolegend, USA
NaCl	Sigma, Germany
(5Z)-7-Oxozeaenol	Sigma, Germany
Penicillin-Streptomycin	Sigma, Germany
PFA	Biolegend, USA
Phorbol 12-myristate 13-acetate (PMA)	Sigma, Germany
PIPES	Sigma, Germany
Poly-L-Lysine	Sigma, Germany
Protamine Sulfate	GIBCO, USA
RPMI-1640	GIBCO, USA
Saponin	Sigma, Germany
Trizma	Sigma, Germany
Trypsin EDTA	GIBCO, USA

APPENDIX B: Equipment Used in This Study

Table BI. Equipment used in this study

Equipment	**Company**
Autoclave	Hirayama, HiClave HV-110, Japan
Balance	ISOLAB, 302.31.002, Germany
Centrifuge	Eppendorf, 5415D, Germany Eppendorf, 5702, Germany VWR, MegaStar 3.0R, USA Beckman Coulter, Allegra X-15R, USA
CO_2 Incubator	Thermo Fisher, Heracell Vios 160i, USA Binger, Germany
Automated Cell Counter	Thermo Fisher, Countess II FL, USA
Deep freezer	-80ºC, Forma,Thermo ElectronCorp.,USA -20ºC, Bosch, Turkey
Electrophoresis Apparatus	Biorad Inc., USA
Filters (0.22 mm and 0.45mm)	Merck Millipore, USA
Fluorescence-activated cell sorting	BD Aria Phusion, USA
Flow cytometer	BD LSR Fortessa, USA
Gel Documentation	Biorad, UV-Transilluminator 2000, USA
Heater Thermomixer Comfort	Eppendorf, Germany
Hemocytometer	ISOLAB, Neubauer, 075.03.001, Germany
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert Modell 300, Germany
Laminar Flow	Heraeus, HeraSafe HS 12, Germany Heraeus, HeraSafe KS, Germany
LightCycler® 480	Roche, Switzerland
Liquid Nitrogen Tank	Taylor-Wharton, 300RS, USA
Magnetic Stirrer	VELP Scientifica, Italy
Microliter Pipettes	Gilson, Pipetman, France Thermo Fisher Scientific, USA
Microscope	Zeiss, Primo Vert, Germany Zeiss Observer Z1, Germany
Microwave Oven	Bosch, Turkey
pH Meter	Mettler Toledo, USA
Refrigerator	Bosch, Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	New Brunswick Sci., USA NanoDrop 2000, Thermo Fischer Scientific, USA
Thermocycler	Eppendorf, Mastercycler, Germany
Vortex	VELP Scientifica, Italy

APPENDIX C: DNA Ladder Used in This Study

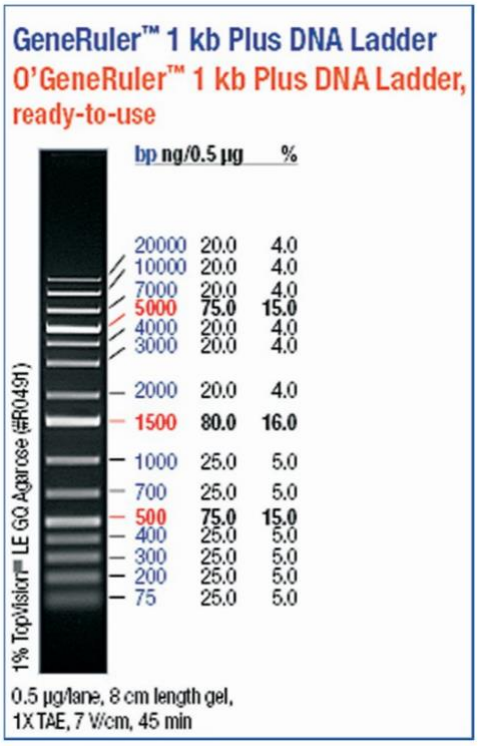

Figure C1. DNA ladder used in this study

APPENDIX D: Plasmid Maps

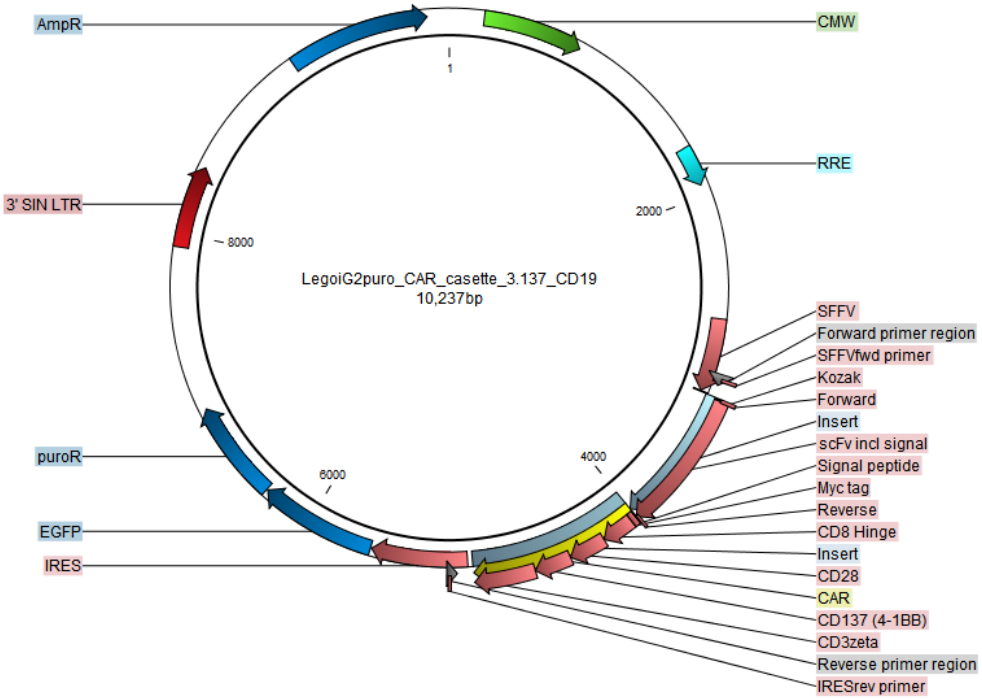

Figure D1. The vector map of LeGO-iG2puro.CAR3.137-19

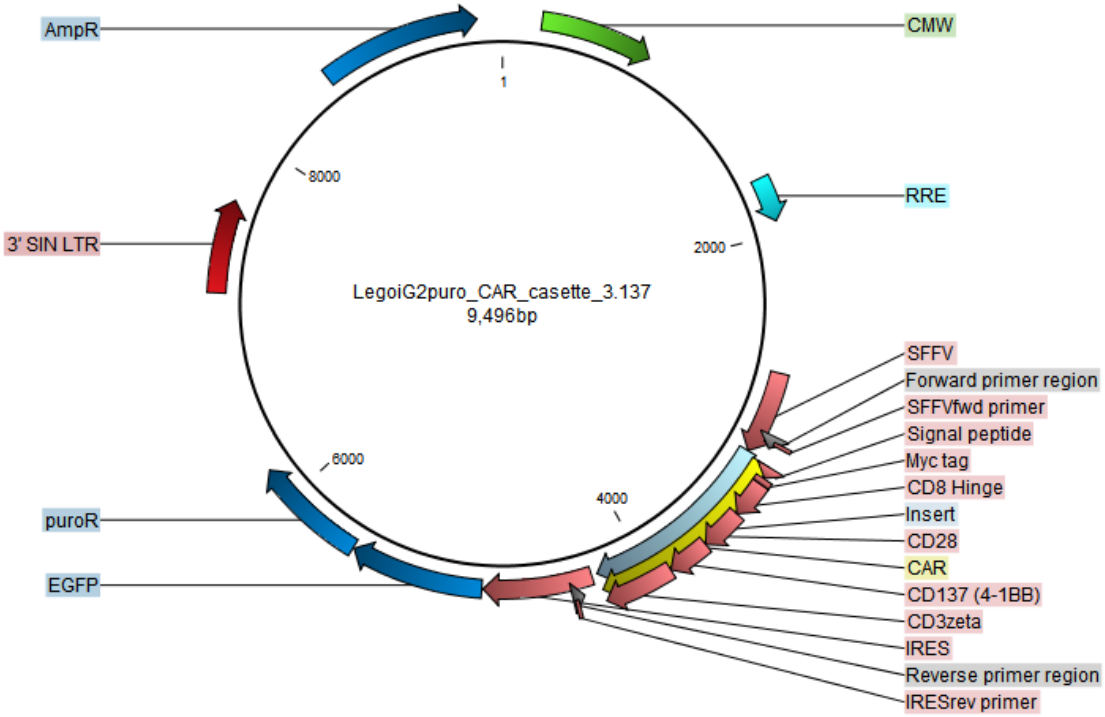

Figure D2. The vector map of LeGO-iG2puro.CAR3.137

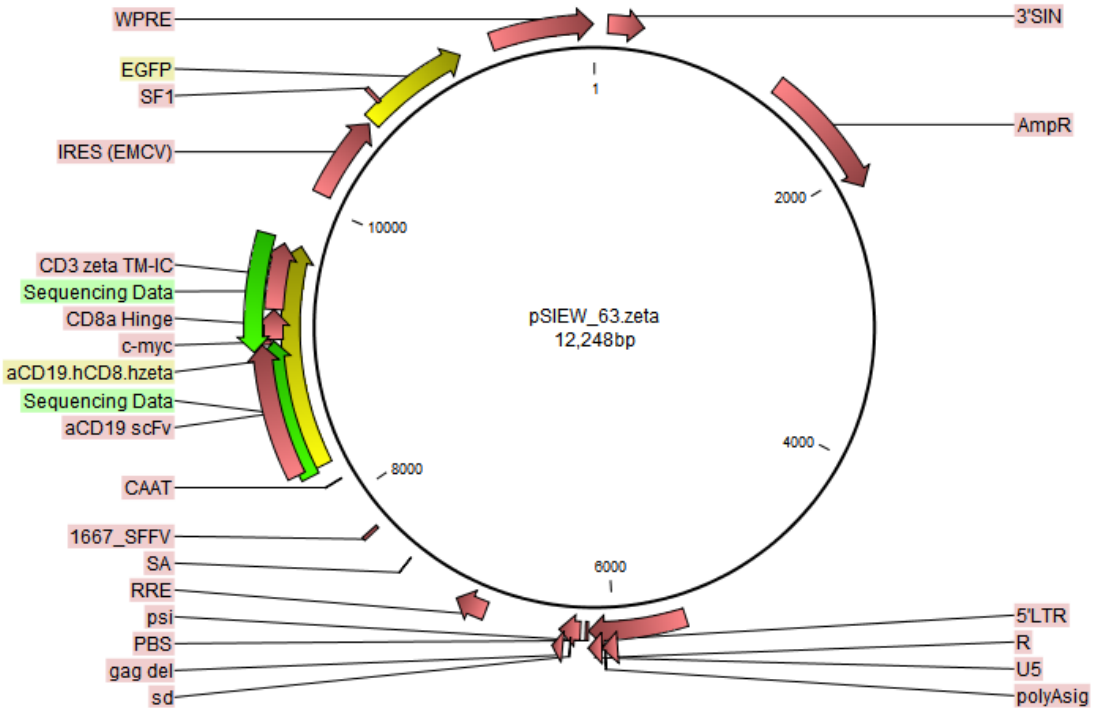

Figure D3. The vector map of pS-63.z-IEW

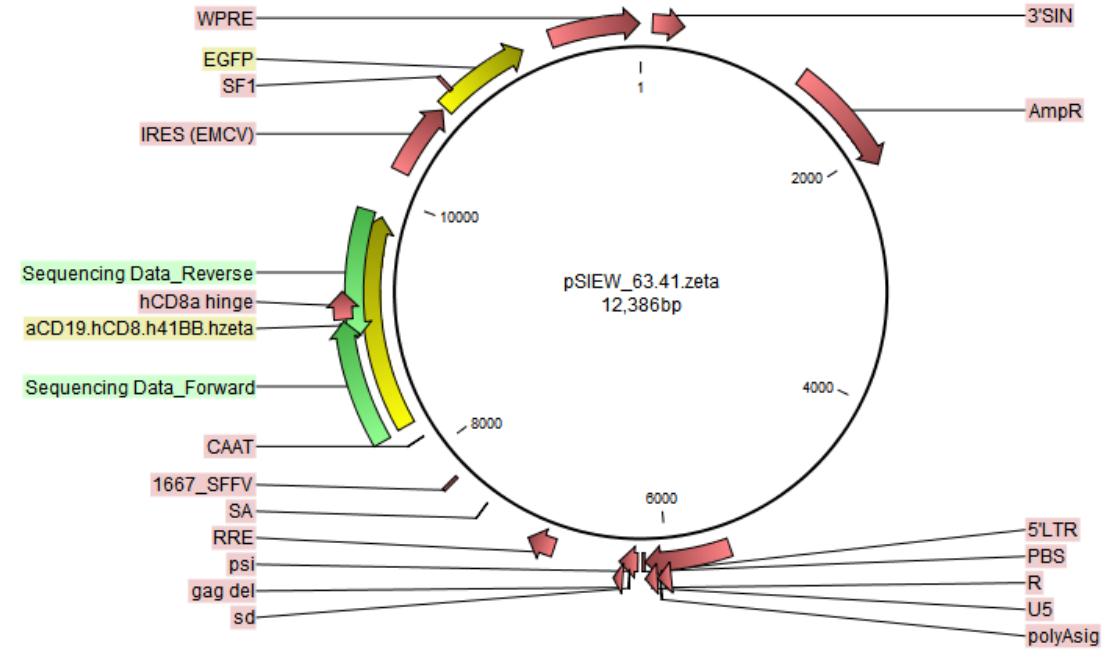

Figure D4. The vector map of pS-63.137.z-IEW

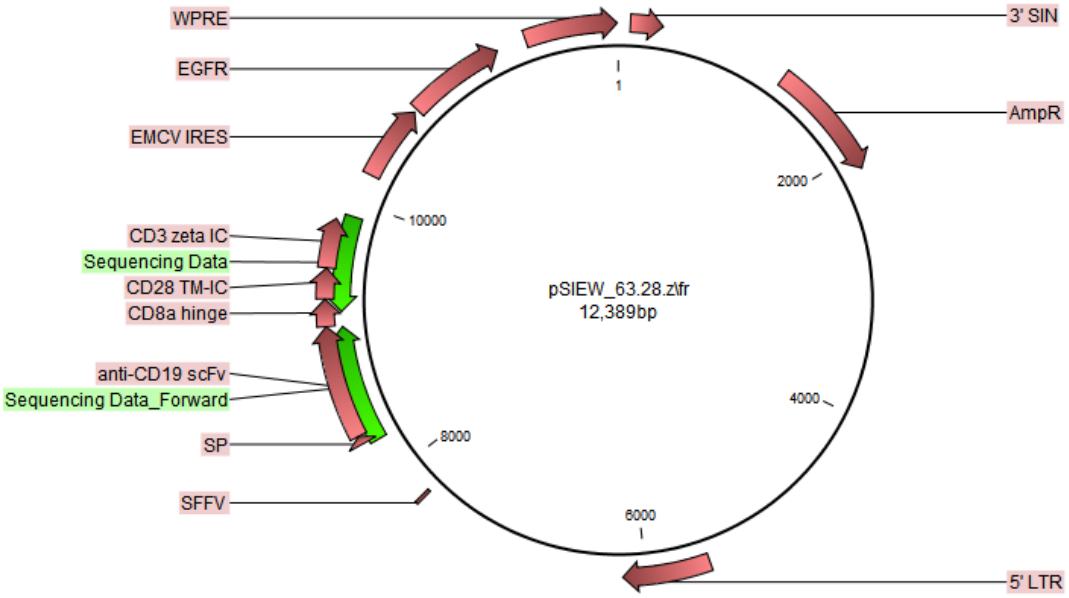

Figure D5. The vector map of pS-63.28.z-IEW

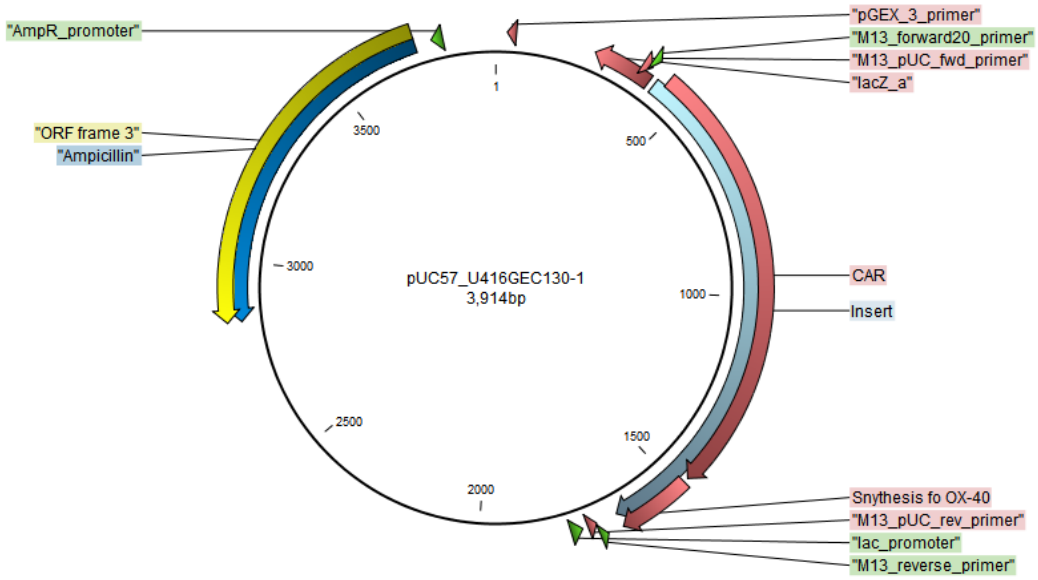

Figure D6. The vector map of PUC57.CAR.3.137

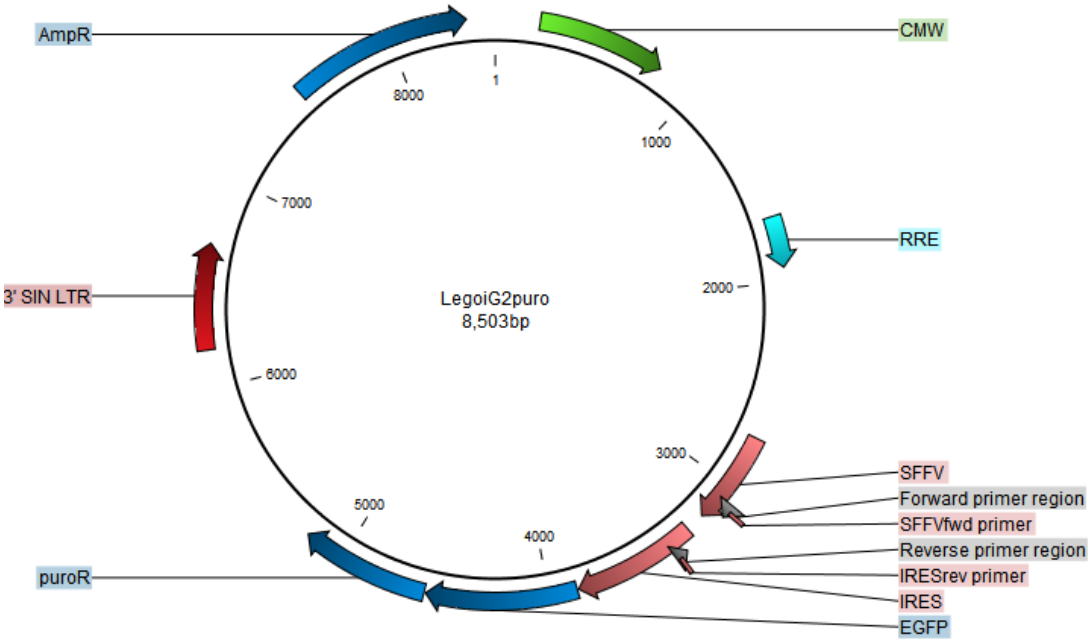

Figure D7. The vector map of LeGO-iG2puro

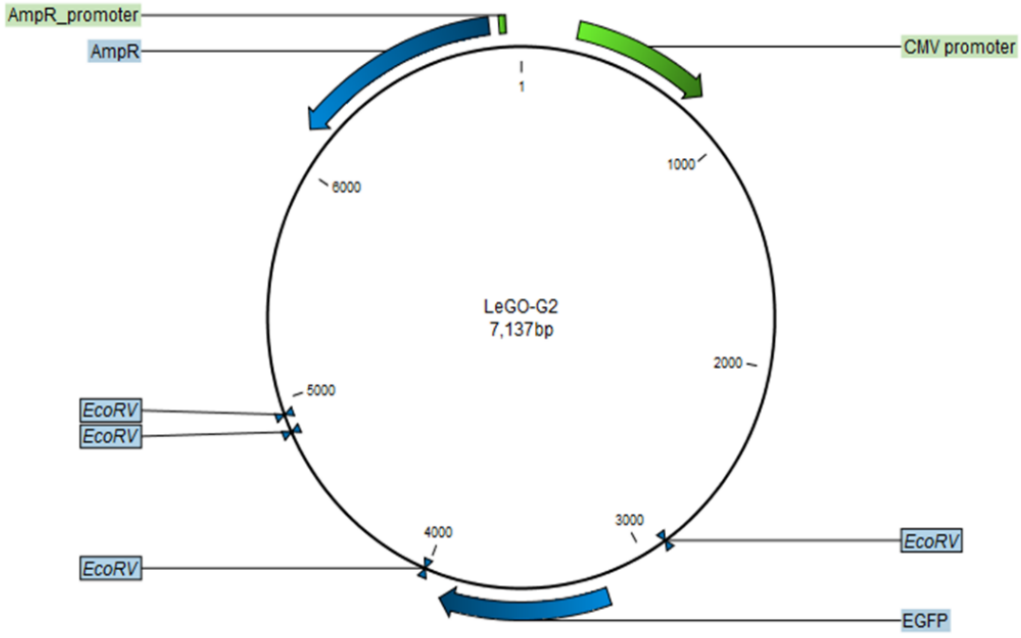

Figure D8. The vector map of LeGO-G2

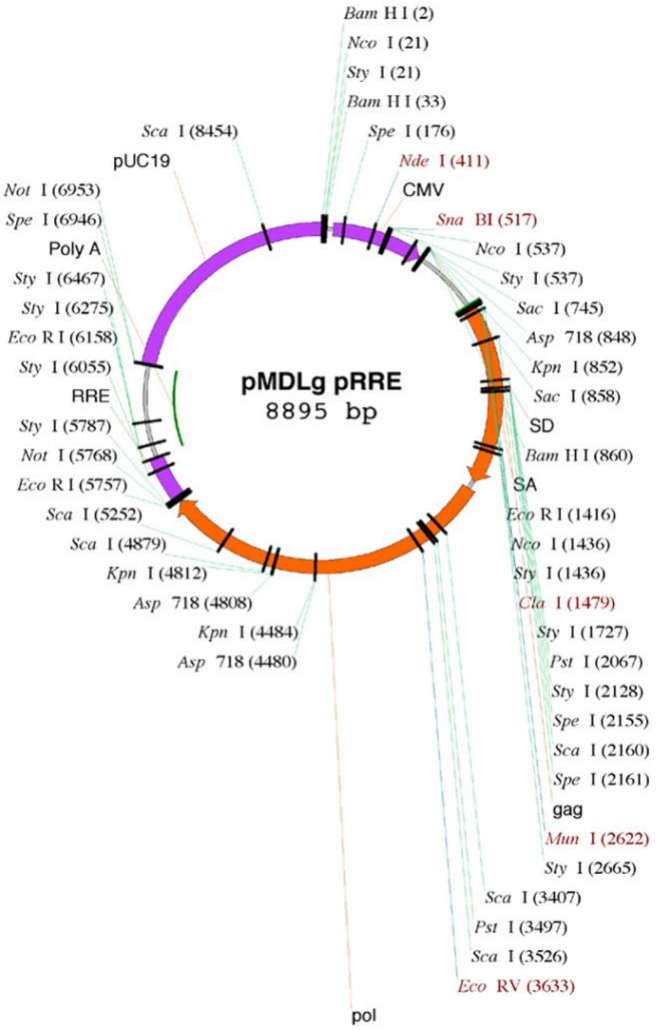

Figure D9. The vector map of pMDLg/pRRE

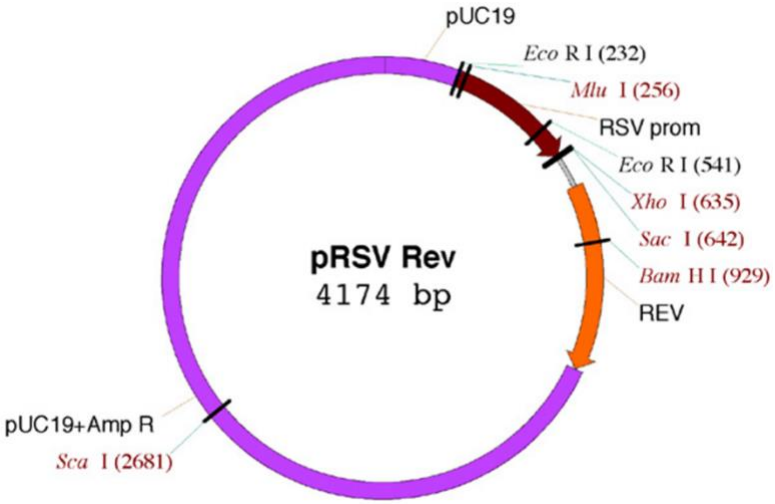

Figure D10. The vector map of pRSV-REV

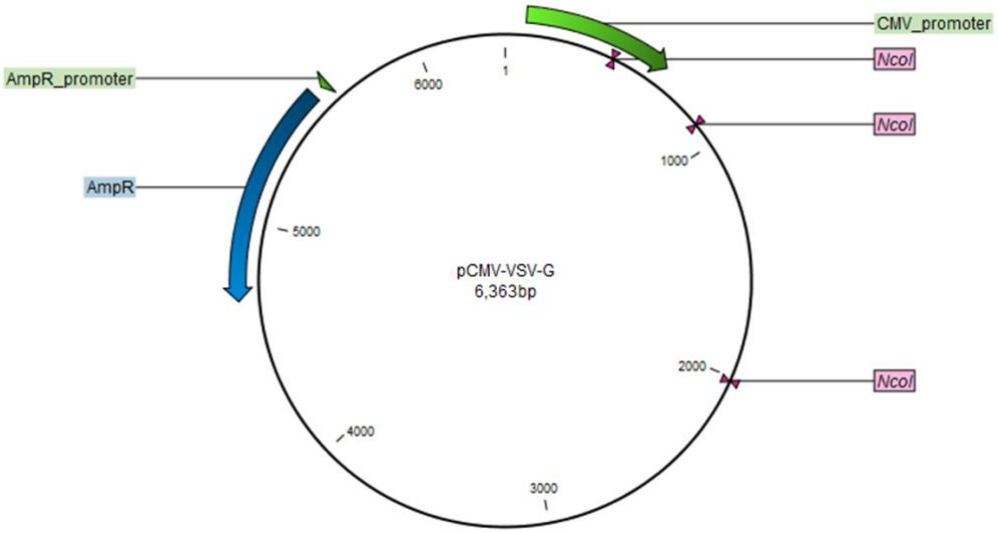

Figure D11. The vector map of pCMV-VSV-g

APPENDIX E: Sequencing Results

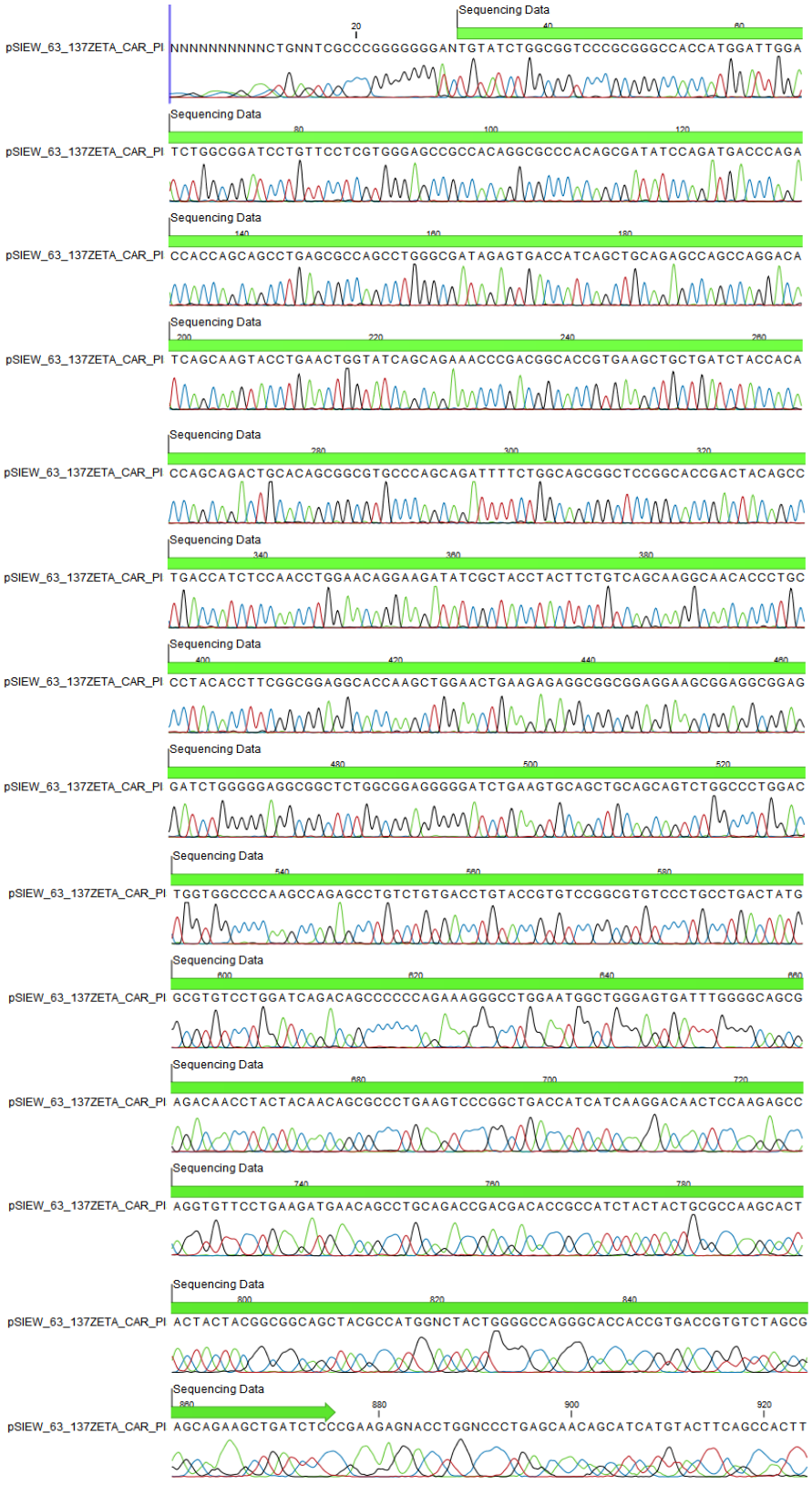

Figure E1. pS-63.137.z-IEW forward sequencing results

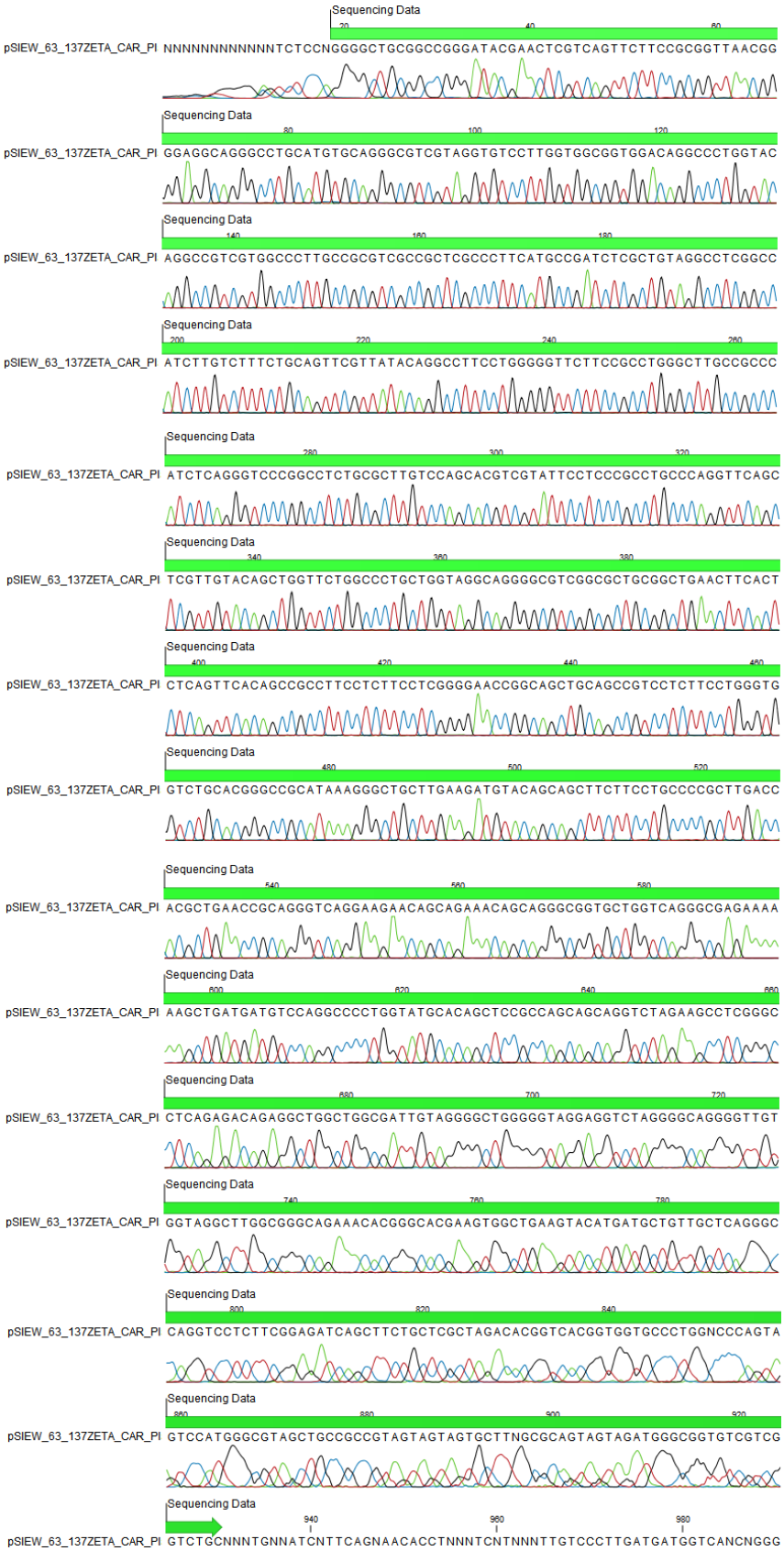

Figure E2. pS-63.137.z-IEW reverse sequencing results

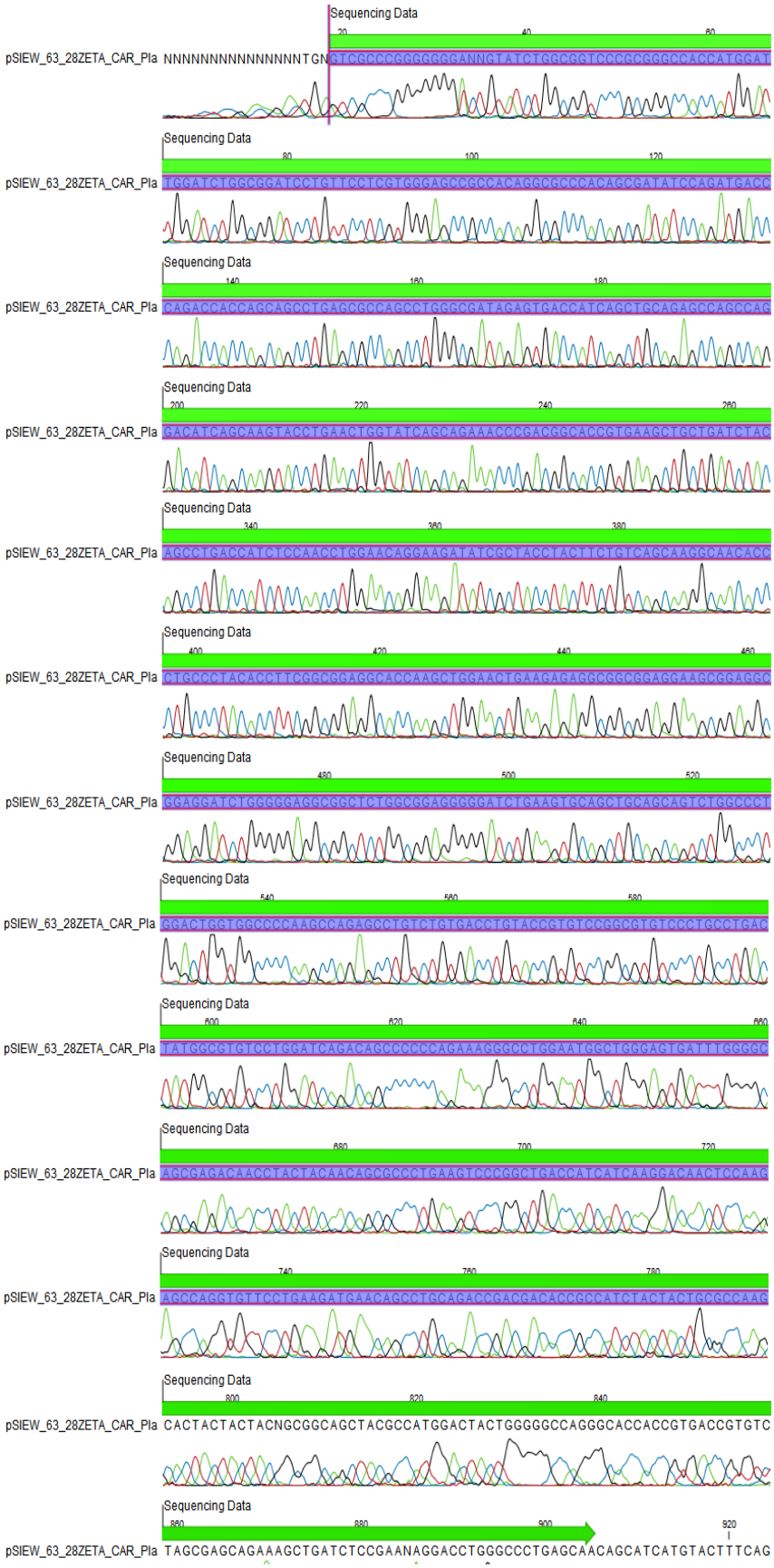

Figure E3. pS-63.28.z-IEW forward sequencing results

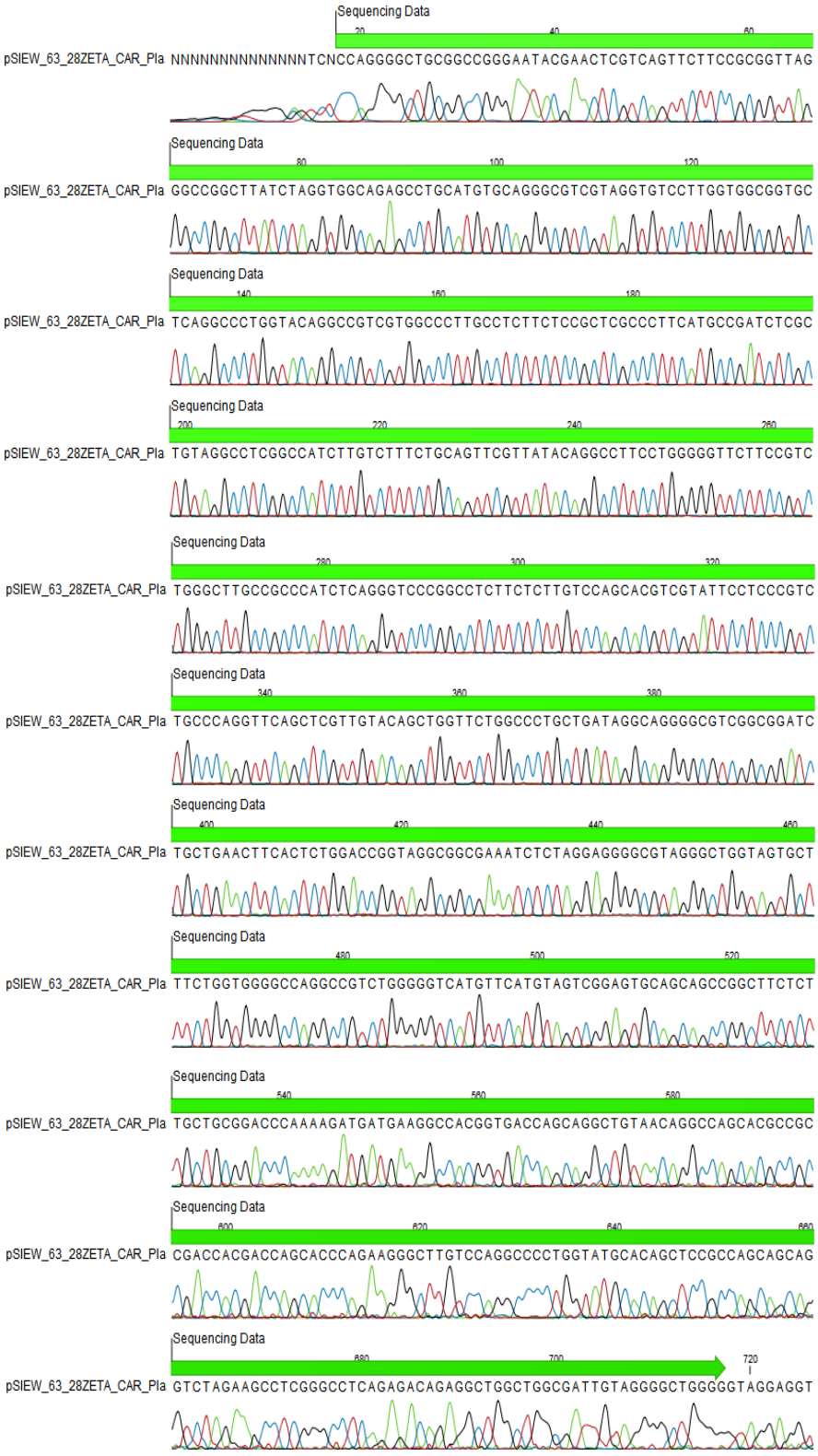

Figure E4. pS-63.28.z-IEW reverse sequencing results

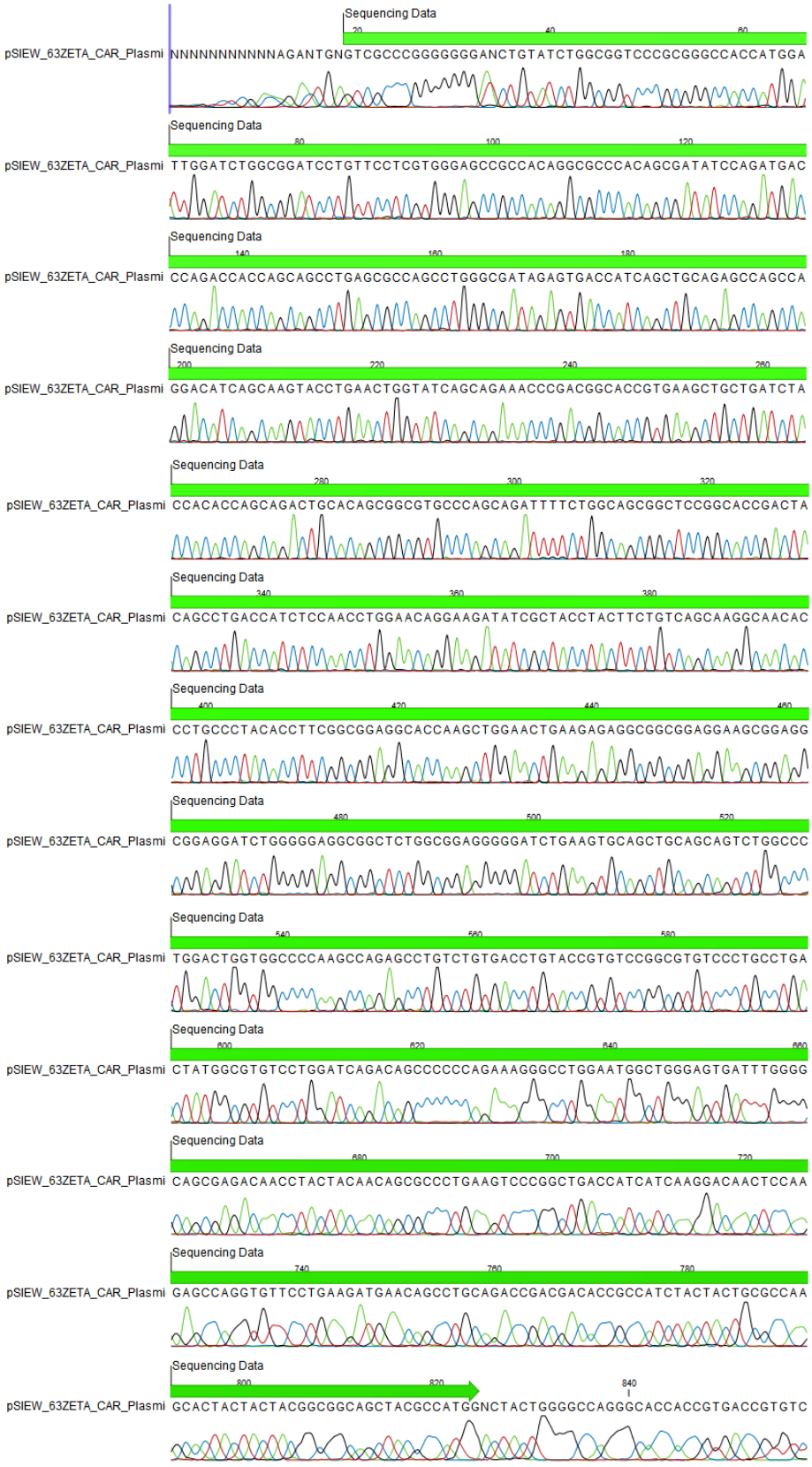

Figure E5. pS-63.z-IEW forward sequencing results

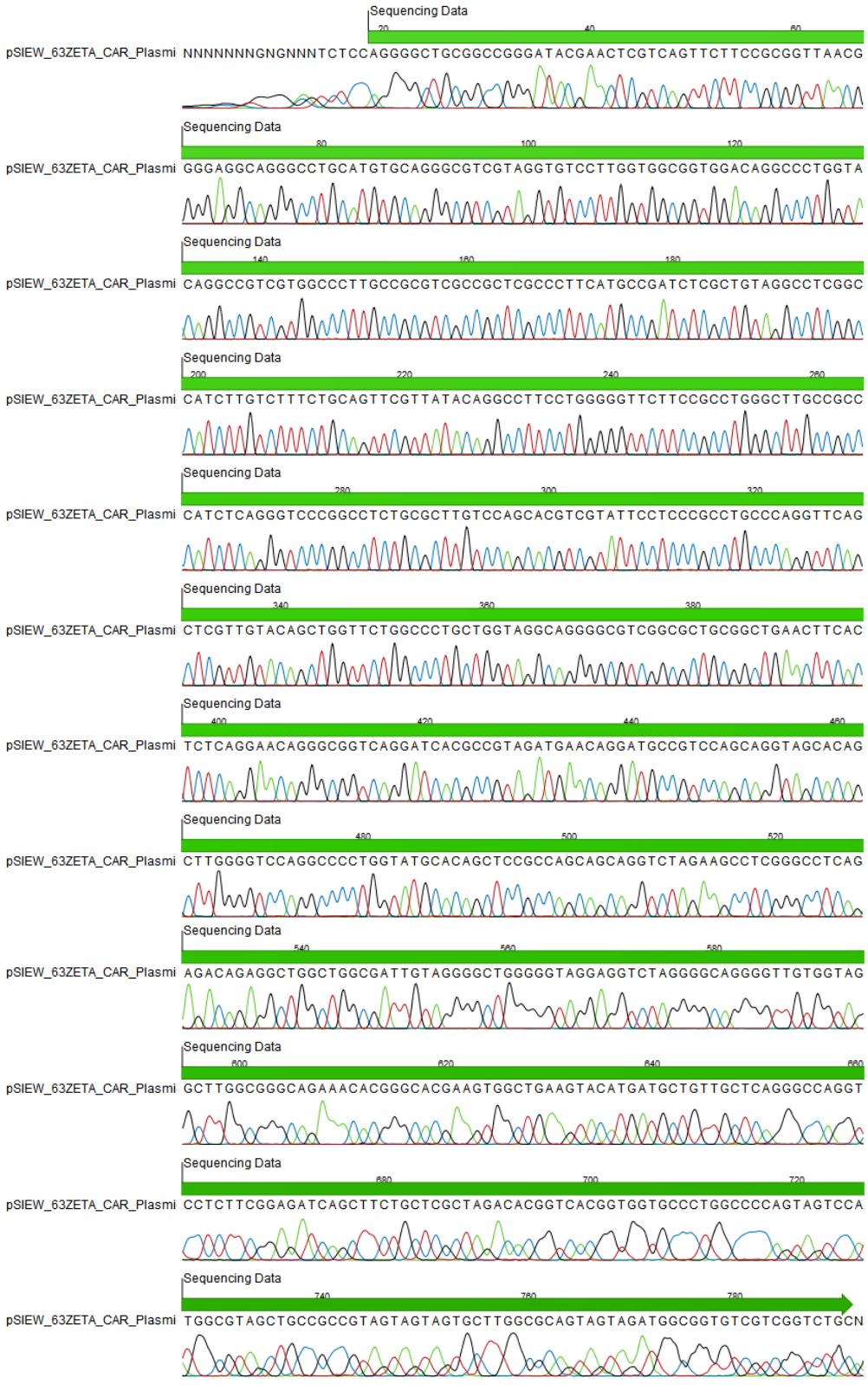

Figure E6. pS-63.z-IEW reverse sequencing results

APPENDIX F: Amino acids Letters

Amino acid	3 - letter code	1 - letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartate	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glycine	Gly	G
Glutamate	Glu	E
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Figure F1. List of amino acids