A NOVEL APPROACH IN CANCER IMMUNOTHERAPY: NATURAL KILLER CELLS EXPRESSING T CELL RECEPTORS

by

AYHAN PARLAR

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NATURAL KILLER CELLS EXPRESSING T CELL RECEPTORS

APPROVED BY:

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

Prof. Batu Erman .....

Prof. Günnur Deniz .....

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ABSTRACT

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AYHAN PARLAR

Molecular Biology, Genetics and Bioengineering

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Thesis Supervisor: Tolga SÜTLÜ

Keywords: TCR gene therapy, NK cells, Cancer Immunotherapy

T cell receptor (TCR) gene therapy is developed to redirect cytotoxic T cells towards selected epitopes of tumor antigens. However, due to the heterodimeric nature of the TCR molecule, alpha and beta chains introduced by gene delivery have a risk of pairing with the endogenously expressed complementary alpha or beta chains in the T cell. This phenomenon named as “mispairing” gives rise to TCRs of unpredictable specificity and causes potentially lethal side effects.

Natural killer (NK) cells were discovered 40 years ago, by their ability to recognize and kill tumor cells without the requirement of prior immunization or stimulation. Since then, NK cells have grown to be promising agents for adoptive immunotherapy of cancer. In the last decade, several NK cell based anti-cancer products have been taken to clinical trials with promising results. However, to manufacture more efficient NK cell therapy products, it is essential to develop novel strategies to increase safety, efficiency and specificity with approaches such as retargeting NK cells against specific antigens which to date has only been possible with chimeric antigen receptors (CARs).

In this thesis, we propose to use NK cells for TCR gene therapy aiming to reprogram them to selectively target tumor or virus antigens in complex with major histocompatibility complex. Our results convincingly demonstrate that the introduction of a functional TCR complex to NK cells via lentiviral gene transfer dramatically enhances the efficiency to mount antigen-specific cytotoxic activity.

To our knowledge, the transfer of a TCR into an NK cell has never been reported before. Our strategy does not only have the potential to open up a whole new chapter in the field of cancer immunotherapy but also provides a final and definitive solution for the mispairing problem observed in TCR gene therapy.

ÖZET

KANSER İMMUNOTERAPİSİNDE ÖZGÜN BİR YAKLAŞIM:

T HÜCRE RESEPTÖRÜ SENTEZLEYEN DOĞAL ÖLDÜRÜCÜ HÜCRELER

AYHAN PARLAR

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Anahtar Kelimeler : TCR gen terapisi, NK hücreleri, Kanser immunoterapisi

T hücre reseptörü (TCR) gen terapisi T hücrelerinin sitotoksik aktivitesini belirli bir tümör antijeni epitopuna karşı yönlendirebilmek için geliştirilmiş bir yöntemdir. TCR molekülünün heterodimerik yapısı sebebiyle, dışarıdan verilen alfa ve beta zincirlerinin hücrenin içerisinde sentezlenen alfa ya da beta zincirleriyle eşleşme riski vardır. Bu olgu yanlış eşleşme problemi olarak adlandırılmakta ve özgüllüğü bilinmeyen, potansiyel olarak hayati yan etkileri olabilecek T hücre reseptörleri oluşturma riski taşımaktadır.

Öncesinde bir bağışıklamaya ve uyarılmaya gerek duymadan tümör hücrelerini tanıma ve öldürme yetenekleri ile bilinen Doğal Öldürücü (NK) hücreleri 40 yıl önce keşfedilmiştir. Bu tarihten günümüze, NK hücreleri adoptif kanser immünoterapisinde umut verici ajanlar olarak ön plana çıkmıştır. Son on yılda, birçok NK bazlı anti-kanser ürünleri klinik çalışmalarda denenmiş ve umut vadeden sonuçlar elde edilmiştir. Fakat daha etkili NK hücre terapisi ürünleri üretmek için, güvenlik, etkinlik ve belirli bir antijene karşı özgüllük gibi özellikleri artıran yeni yaklaşımlar geliştirmek gereklidir. Bu amaçla şimdiye kadar yapılan çalışmalarda uygulanan yöntem kimerik antijen reseptörleri (CAR) kullanılarak NK hücrelerini spesifik bir antijene karşı hedeflemek olmuştur.

Bu tezde, TCR gen terapisi için NK hücrelerinin kullanımı önerilmektedir. TCR geni transferiyle NK hücrelerinin tümör ve virüs antijenlerini seçici olarak tanımak için tekrar programlanması amaçlanmaktadır. Sonuçlarımız, lentiviral gen transferi aracılığıyla TCR kompleksini NK hücrelerinde ifade etmenin antijen-spesifik sitotoksisite aktivitesinin çarpıcı bir şekilde artırdığını göstermiştir.

Bildiğimiz kadarıyla NK hücresine TCR gen transferi çalışması daha önce hiç yapılmamıştır. Bulduğumuz bu yöntem kanser immunoterapisi alanında yeni bir yaklaşım olmakla kalmayıp aynı zamanda TCR gen terapisinde görülen yanlış eşleşme sorununun nihai ve kesin çözümüdür.

To my mother…

Canımdan çok sevdiğim anneme…

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
δ	Delta
κ	Kappa
μ	Micro
ACT	Adoptive cell transfer
ALL	Acute lymphoblastic leukemia
Amp	Ampicillin
APC	Antigen presenting cell
CAR	Chimeric Antigen Receptor
CD4	Cluster of differentiation 4
CD56	Cluster of differentiation 56
CD8	Cluster of differentiation 8
CI	Cell index
CTL	Cytotoxic T Lymphocyte
DAMPs	Damage associated molecular pattern
DC	Dendritic Cell
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	Food and Drug administration
GFP	Green Fluorescent Protein
GM-SF	Granulocyte macrophage colony stimulating factor
GOI	Gene of interest
GvHD	Graft versus host disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IDO	Indoleamine 2,3-dioxygenase
IL-	Interleukin-
Iono	Ionmycin
ITAM	Immunoreceptor tyrosine based activation motif
ITIM	Immunoreceptor tyrosine based inhibition motif
JNK	c-JUN terminal kinase
KIR	Killer immunoglobulin like receptor
LAK	Lymphokine activated killer
LB	Luria broth
LeGO	Lentiviral Gene Ontology vectors
LFA-1	Lymphocyte function-associated antigen 1
LTR	Long Terminal Repeat
mAb	Monoclonal Antibody
MHC	Major histocompatibility complex
MIC	MHC Class I related
NCAM	Neural cell adhesion molecule
NEAA	Non-essential Amino Acid
NFAT	Nuclear factor of activated T cells
NK	Natural Killer
NKT	Natural killer T
PAMPs	Pathogen associated molecular pattern
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PCR	Polymeric chain reaction
PFA	paraformaldehyde
PIPES	1,4-Piperazinediethanesulfonic acid
PLC-γ-1	Phospholipase gamma 1
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
Puro	Puromycin
PVDF	Polyvinylidene fluoride
RAG	Recombination activating gene
RNA	Ribonucleic acid
RT	Room temperature
RTCA	Real time cell analysis
SCGM	Stem Cell Growth Medium
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHP1	Src homology region 2 domain-containing phosphatase-1
siRNA	Small interfering RNA
SLAM	The signaling lymphocytic activation molecule
TAA	Tumor associated antigen
TAP	Transporter associated with antigen precessing
TBE	Tris boric acid
TCR	T-cell Receptor
tdTomato	Tandem dimer Tomato
TGF-β	Transforming growth factor

T_H	Helper T cell
TLR	Toll-like Receptor
TNFα	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis inducing ligand
Tyr	Tyrosinase
WB	Western blot
ZAP70	Zeta-chain-associated protein kinase 70

1. INTRODUCTION

1.1. The Immune System

The immune system is a complex of different cell types and soluble factors that together defend an organism against extrinsic and intrinsic threats ranging from pathogenic microorganisms to neoplasia. Typically, immune system is divided into two main branches that are named innate immunity and adaptive immunity.

Innate immunity is the first barrier in the body to respond against microbial attacks and tissue injury (damaged self). Innate immune system is composed of different cell types (dendritic cells, macrophages, neutrophils, mast cells, eosinophils, basophils and natural killer cells) as well as soluble factors (complement system, acute phase proteins, cytokines and chemokines) that together show a remarkably fast response against pathogenic invaders. The innate immune system has the capacity to recognize microorganisms and destroy them within minutes (Medzhitov & Janeway, 2000). Furthermore, it generates an inflammatory response and helps to activate the subsequent adaptive immune response. This prompt response by the innate immune system gives the organism time to constitute a more targeted immune response by antigen specific components of the adaptive immune system.

Innate immunity has a limited repertoire of receptors recognizing conserved microbial components shared by large groups of pathogens. The receptor repertoire of innate immunity has no genetic recombination. Genetically predetermined germline-encoded receptors recognize highly conserved and common structures among microbes or biologic consequences of infections. Therefore, innate immunity functions mostly rely on recognizing common molecules typical of microbes that are called Pathogen-associated Molecular Patterns (PAMPs) (Janeway Jr. & Medzhitov, 2002) or self-molecules grouped under the name Damage-associated Molecular Patterns (DAMPs) which are metabolic consequences of infection and inflammation (Bianchi, 2007). DAMPs are mostly biological molecules upregulated and released in the course of cell lysis and tissue damage. PAMPs and DAMPs are recognized by the germline encoded pattern recognition receptors (PRRs), which are expressed on innate immune cells and trigger an immune response to eliminate pathogens (Kawai & Akira, 2010; Takeuchi & Akira, 2010).

Although the innate immune system shows a rapid reaction to a wide range of pathogens, recognition of common PAMPs is limited. The massive variability of antigenic molecules has driven the evolution of the adaptive immune system (M. D. Cooper & Alder, 2006). In contrast to antigen detection by innate immunity whose receptors are encoded in fully functional form in the germline genome, adaptive immune recognition is based on receptors that undergo a process of somatic recombination in gene segments.

Adaptive immunity is specialized to recognize and distinguish self from non-self antigens. Another defining characteristic of adaptive immunity is the formation of memory, which means that after initial pathogen encounter, a secondary exposure to the same pathogen will trigger a more rapid response. The cells of the adaptive immune system include T and B lymphocytes that are central players in cellular immune responses. The recognition of antigens by both T and B cells rely on receptors that go through genetic recombination, which generates receptor diversity, enabling antigen-specific responses to be generated against any non-self entity. This thesis is mainly focused on advancing the power of adaptive immunity and innate immunity to target and eliminate cancer cells through the use of genetically modified cytotoxic lymphocytes. Thus, in the following section, I will be discussing the unique features of cytotoxic lymphocytes and their potential use in cancer immunotherapy.

1.2. Cytotoxic Lymphocytes of the Immune System

1.2.1. Natural Killer Cells

Natural killer cells are bone marrow-derived lymphocytes belonging to the innate immune system. They are derived from $CD34^+$ hematopoietic progenitor cells and need cytokines such as IL-15 to develop into mature NK cells (Di Santo, 2006; Yokoyama, Kim, & French, 2004). They are among the first responders to fight against pathogen invasion and malignant transformation. The percentage of NK cells among human peripheral blood lymphocytes is approximately 5-10% (Comans-Bitter et al., 1997; Shearer et al., 2003). NK cells were originally described in mice in 1975 as a type of lymphocyte that has the capacity to lyse target cells without prior stimulation or immunization (Waldhauer & Steinle, 2008). Today, the crucial role of NK cells in infection, autoimmunity and tumor immunosurveillance is well recognized (Caligiuri, 2008; Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008).

Human NK cells express the surface protein CD56 and lack the expression of CD3, which makes it possible to identify them as $CD56^+CD3^-$ lymphocytes. Two main types of NK cells which have distinctive functional roles are defined according to the density of CD56 expression on the cell surface **(Figure 1.1)**.

The majority of NK cells in the blood (90%) have low expression of CD56 ($CD56^{dim}$), whereas the remaining 10% express CD56 at a distinctively higher level ($CD56^{bright}$). $CD56^{bright}$ NK cells express the IL-2 receptor alpha chain (CD25) but not (or low levels of) FcRIII (CD16). Conversely, $CD56^{dim}$ NK cells exclusively express CD16 but lack expression of CD25. Early functional studies showed that $CD56^{dim}$ NK cells are proficient in effector functions such as natural cytotoxicity or antibody-dependent cellular cytotoxicity (ADCC) (L L Lanier, Le, Civin, Loken, & Phillips, 1986) while $CD56^{bright}$ NK cells have important roles as regulatory cells in the immune system and produce various cytokines such as Interferon-gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-

α), IL-10, IL-13 and Granulocyte Macrophafe Colony Stimulating Factor (GM-CSF) to act as a link between innate and adaptive immune responses (Perussia, 1991).

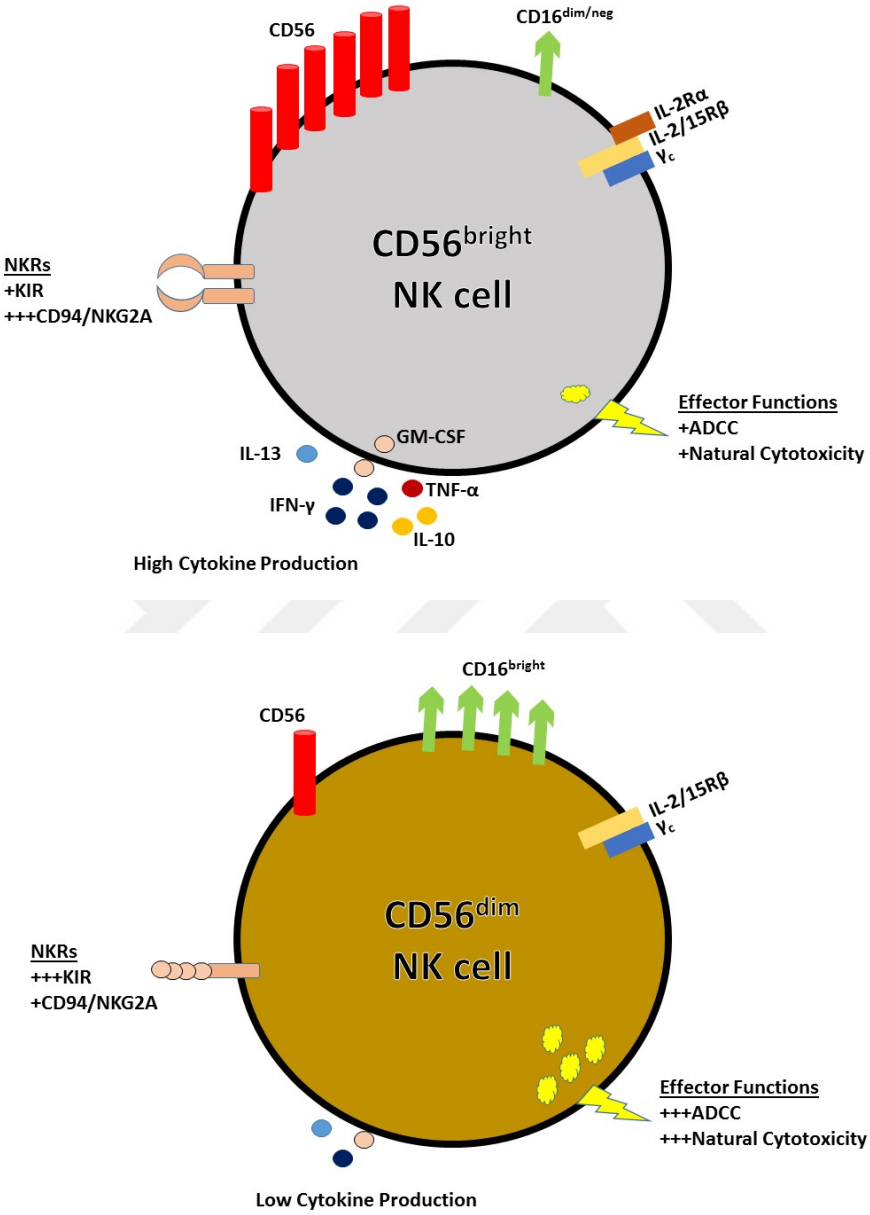

Figure 1.1 Phenotypic and functional comparison of CD56^{bright} and CD56^{dim} NK cells. CD56^{bright} NK cells express high levels of IL-2 receptor alpha chain (CD25) and are potent producers of immunoregulatory cytokines. Cytotoxic activity is mainly carried out by CD56^{dim} NK cells which are equipped with KIRs and high levels of CD16.

1.2.2. T cells

T cells are the central actors of the adaptive immune system. T cell-mediated immunity is a complex effort coordinated with other cells of the adaptive and innate immune systems. They develop antigen specific immune responses to eliminate invaders and mediate adaptive immunity against viral, bacterial or parasitic infections and tumor cells.

Development of T lymphocytes from $CD34^+$ hematopoietic stem cells takes place in many steps and crucially involves genomic DNA rearrangements in the T Cell Receptor (TCR) locus (Cobb, Oestreich, Osipovich, & Oltz, 2006). This rearrangement - known as V(D)J recombination- is the assembly of V (variable), D (diversity) and J (joining) gene segments of TCR chains that results in the expression of a unique TCR in each T cell, generating a tremendous receptor diversity **(Figure 1.2)**. The germline TCR loci are composed of many V, D and J gene segments which undergo rearrangements that lead to the expression of functional TCR genes.

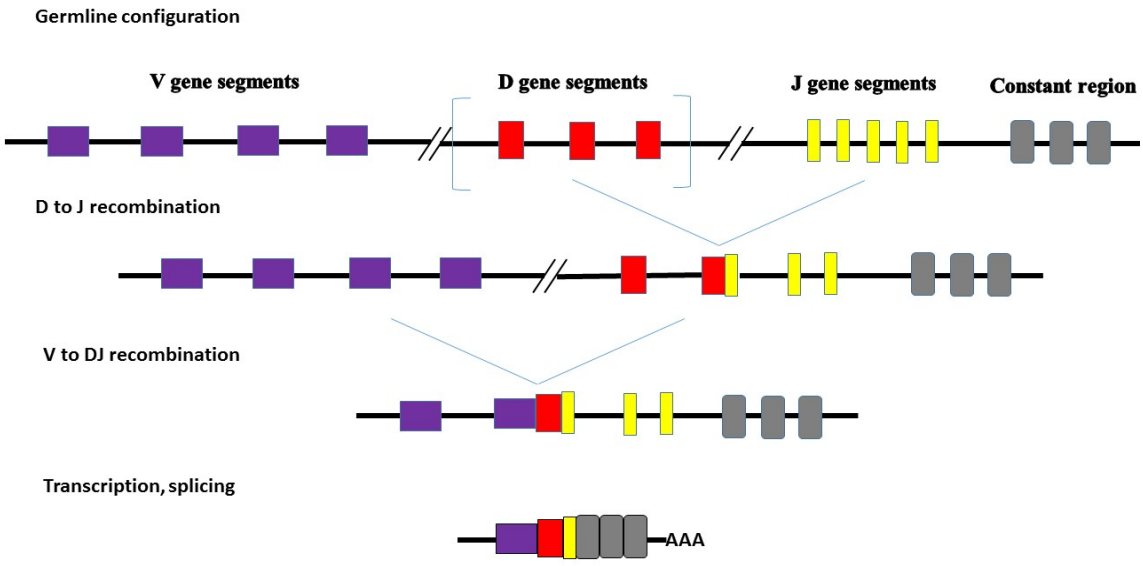

Figure 1.2: Schematic representation of V(D)J recombination. For the generation of a functional TCR gene, one segment from sets of V, D and J gene segments is brought together with sequences coding for the constant regions of the TCR.

Recombination activating gene (RAG)1 and RAG2 are responsible for V(D)J recombination. RAG1/2 complex forms a specific endonuclease in immature lymphoid cells and causes double-strand DNA cleavage at recombination signal sequences (RSS) that are located between V, D and J gene segments (Oltz & Osipovich, 2007). These breaks are then repaired by downstream DNA repair complexes that give rise to rearranged gene structure leading to the expression of a functional TCR gene. In each developing T cell, this process randomly chooses among a wide selection of different V, D and J segments to bring them together with constant region sequences for generation of functional TCR sequences. This random rearrangement lies at the heart of the tremendous diversity in TCR repertoire.

Phenotypically and functionally, T cells are divided into various subtypes. Among these the majority of T cells express alpha and beta chains, namely the α/β T cells, a small group of T cells expressing TCRs composed of γ and δ chains named as γ/δ T cells and a group of T cells expressing an invariant TCR (Vα24/Vβ11) along with the NK cell marker CD56 known as natural killer T (NKT) cells cover a gray zone in the link between innate and adaptive responses.

Conventional classification of T cells relies on the presence of the CD3/TCR complex on the cell surface along with one of the co-receptors CD4 or CD8. The largest group of T cells in humans is the $CD4^+$ αβ TCR-expressing helper T cells (T_H). These have a central role in stimulating response by innate and adaptive components of the immune system by secretion of cytokines. T_H cells have two main groups (T_H1 and T_H2) which have distinct cytokine secretion profiles. T_H1 cells are characterized to secrete mainly IFNγ and IL-2, evoking adaptive immune responses through mainly cytotoxic T cells. T_H2 cells are main producers of IL-4, IL-5, IL-10 and IL-13, enhancing antibody production and cell mediated response mainly through innate components of the immune system.

Another $CD4^+$ T cell group is regulatory T cells (T_{reg}) which constitutively express the transcription factor FoxP3 and high levels of the IL-2 receptor alpha chain, CD25. Regulatory T cells have a crucial role for maintenance of immune self-tolerance and homeostasis by suppressing aberrant or excessive immune responses harmful to host [96].

$CD8^+$ αβ TCR-expressing cytotoxic T lymphocytes (CTLs) are major actors showing cellular cytotoxicity against target cells harboring intracellular pathogens and

neoplastic cells. CTLs recognize target antigens presented in complex with classical MHC Class I molecules and kill target cells by means of contact-dependent mechanisms. After the recognition of antigenic peptide-loaded MHC class I through TCR, apoptotic cell death is activated in target cells due to the exocytosis of cytotoxic granules by T cells. The mechanisms of T cell recognition will be discussed further in the next chapter.

1.3. Recognition of Target Cells by Cytotoxic Lymphocytes

1.3.1. NK cell target recognition

Unlike T cells, NK cells do not go through genomic DNA rearrangement to express a variety of antigen-specific receptors on the cell surface. This constitutes a basis for classifying them into the innate immune system rather than the adaptive immune system. Instead of rearranged antigen-specific receptors, the function of NK cells are controlled by cooperating signals from two main sets of receptors that are germline-encoded: (i) activating receptors and (ii) inhibitory receptors (Biassoni, Bottino, Cantoni, & Moretta, 2002; Lewis L. Lanier, 2003). NK cell cytotoxicity depends on the balance between signals coming from inhibitory and activating receptors(L L Lanier, Corliss, & Phillips, 1997) (Lewis L. Lanier, 2003)(Bryceson, March, Ljunggren, & Long, 2006) (**Figure 1.3)**.

Recognition of a ligand by an activating receptor leads to signaling events that trigger the killing of the target cell (A. Moretta et al., 2001). Activating receptors either carry Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) (Lewis L Lanier, Corliss, Wu, Leong, & Phillips, 1998) in their cytoplasmic tail or interact with signaling adaptors that contain ITAMs. Activating ligand engagement leads to ITAM phosphorylation at the tyrosine residues and the Src homology 2 domain containing kinases such as Syk and ZAP70 are recruited for downstream signal transduction(Sutherland et al., 2002; Wu et al., 1999). These pathways trigger the polarization of cytotoxic granules to the interface between the NK cell and the target (the

immunological synapse) and their release towards the target cells (degranulation)(Tomasello, Blery, Vely, & Vivier, 2000).

Some examples of activating receptors are NKG2D (Zompi et al., 2003)(Billadeau, Upshaw, Schoon, Dick, & Leibson, 2003; Zompi et al., 2003), signaling lymphocytic activation molecule (SLAM) family molecule 2B4 (CD244), the natural cytotoxicity receptors NKp30, NKp44, NKp46 and NKp80 and the DNAX accessory molecule (DNAM-1, CD226). The engagement of activating receptors with their ligands leads to direct cellular cytotoxicity and triggers cytokine secretion.

These processes of activation are tightly controlled by inhibitory receptors whose ligands are mainly self MHC class I molecules. Killer cell immunoglobulin-like receptors (KIRs) are the main inhibitory receptor family on NK cells. KIR ligands are HLA-A, HLA-B and most importantly HLA-C molecules (L. Moretta & Moretta, 2004). Upon engagement to their ligands, they transmit a signal inhibiting the effects of activating receptors. Therefore, the presence of MHC class I on target cells is an inhibitory signal for NK cytotoxicity.

Inhibitory receptors signal through Immunoreceptor Tyrosine-based Inhibitory Motifs (ITIMs) in their cytoplasmic tails. Phosphorylation of tyrosine residues in ITIMs upon inhibitory ligand engagement leads to the recruitment Src homology 2 domain containing phosphatases (SHP1 or SHP2) (Tomasello et al., 2000) that dephosphorylate signaling intermediates shared with the activation pathways. This crosstalk leads to the integration of activation and inhibition signals since the kinases from activating receptor signaling and the phosphatases from inhibitory receptor signaling act on shared signaling intermediates (Biassoni et al., 2002; Lewis L Lanier, 2005, 2008; Yokoyama, 2005). If the inhibitory signals are dominant, triggering of cellular cytotoxicity cannot take place (Long, 2008; Veillette, Latour, & Davidson, 2002).

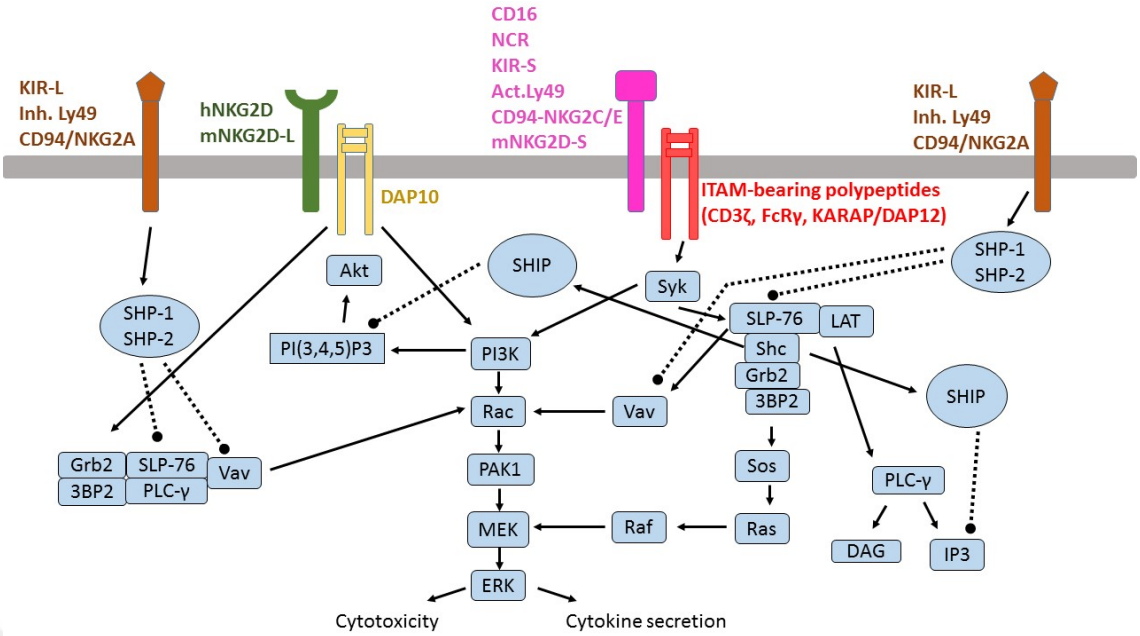

Figure 1.3 General overview of intracellular signaling pathways triggered by activating and inhibitory NK cell receptors.

Unlike T cells, NK cells do not recognize the identity of the peptide loaded onto the MHC molecule but rather sense the presence of a sufficient amount of MHC molecules on the target cell surface that is the indication of a healthy cell. In cases of viral infection or malignant transformation, the loss of MHC expression on the cell surface is a commonly encountered phenomenon. In such cases, NK cells have the capacity to detect the absence of MHC Class I on the target cell surface by a mechanism named as the "missing-self recognition" (Ljunggren & Karre, 1990)**(Figure 1.4)**.

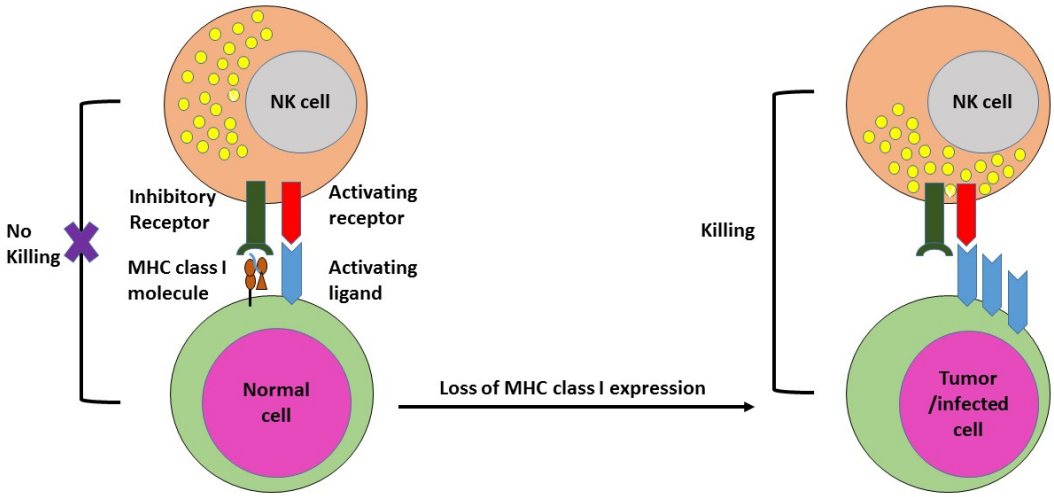

Figure 1.4: Missing-self recognition. The balance of inhibitory and activating signals received from a healthy cell keeps the NK cell at rest and no triggering of cytotoxic activity will occur. The loss of MHC-I expression on the target cell will free the engagement of inhibitory receptors on the NK cell, shifting the balance towards activation which will result in triggering of natural cytotoxicity.

According to this hypothesis, the presence of MHC class I molecules in normal target cells are inhibitory ligands for NK cells. If the target cell loses MHC class I expression on the cell surface as a result of infection or transformation, activating signals become dominant and NK cell activation occurs; resulting in target cell lysis. Thus, NK cells show cytotoxicity against the downregulation or loss of MHC in target cells due to tumoral, viral and other cellular stresses (Carbone et al., 2000; Uhrberg et al., 1997).

1.3.2. T cell target recognition

During the maturation process in the thymus, each T cell expresses a unique TCR recognizing a specific antigen on its cell surface. All T cells undergo a selection in the thymus to make sure this receptor has the ability to differentiate self from non-self. Therefore, T cells expressing TCRs against self-antigens are generally eliminated.

T lymphocytes that have not yet faced the antigen are called naïve T cells. They reside in lymphoid organs and circulate in the body via blood and the lymphatic system. For a naïve T cell to become activated, antigens have to be presented by other cells such as dendritic cells in secondary lymphoid organs. Dendritic cells are main antigen presenting cells (APCs) and on their cell surface, antigenic proteins are presented in complex with the major histocompatibility complex (MHC). APCs generate small peptides from pathogenic agents or self-proteins by antigen processing machinery **(Figure 1.5)**. These small peptides bind to MHC molecules in the endoplasmic reticulum and are presented on the cell surface in complex with MHC (Wucherpfennig et al., 2007).

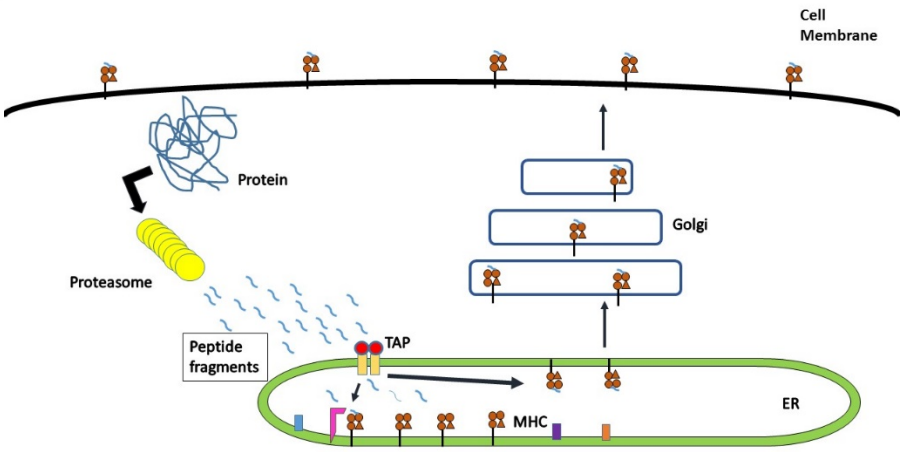

Figure 1.5: Antigen processing machinery.

The TCR recognizes a complex of the peptide and MHC (pMHC). Engagement of the TCR with pMHC triggers intracellular signaling events resulting in activation of T cell cytotoxicity. The activated T cells undergo clonal expansion and migrate into the tissues to kill target cells that present the specific pMHC complex.

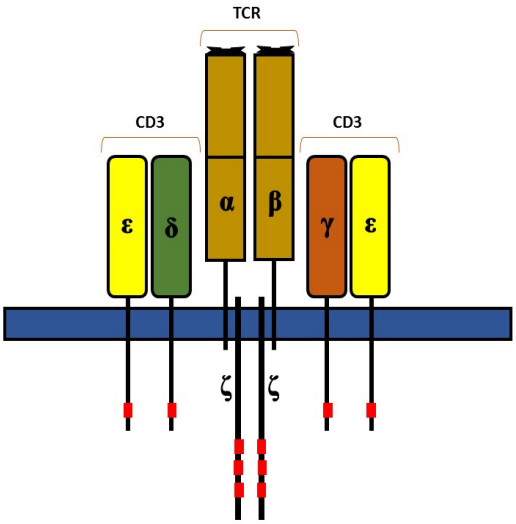

Figure 1.6: Structure of the CD3/TCR complex

The TCR is a heterodimer consisting of two chains: TCR α and β. TCR heterodimers come together with the CD3 complex that consists of CD3δ, CD3γ, CD3ε and CD3ζ **(Figure 1.6)**. This complex is responsible for intracellular signal transduction leading to T cell activation. All CD3 chains contain ITAMs in their cytoplasmic domains. Activation of naïve T cells requires the TCR complex, co-receptors and accessory molecules on the surface of T cells. Co-receptors such as CD4 and CD8 facilitate the TCR signaling. Costimulatory molecules like CD28 and CTLA-4 enhance TCR signaling in the cytoplasm. Also accessory proteins such as LFA-1 or CD2 provide extra stability for the interaction between the T cell and APC.

$CD4^+$ T cells interact with peptide-bearing MHC class II molecules (HLA-DR, HLA-DQ and HLA-DP). MHC class II molecules are expressed by professional APCs which are specialized to pick up and present antigens from their immediate environment. APCs generally localize at sites where pathogen encounter is most likely to occur and digest exogenous proteins by mechanism of phagocytosis and endocytosis in order to load these peptides onto MHC Class II molecules.

On the other hand, $CD8^+$ T cells recognize peptides (9-11 amino acids) loaded onto MHC class I molecules (HLA-A, HLA-B and HLA-C) that are expressed by almost all cell types in the body. These MHC class I restricted peptides come from proteins translated inside the cells which are generally endogenously expressed proteins but can also be pathogenic in case of intracellular infections such as viruses.

T cell activation is initiated, when a TCR binds to a pMHC complex on the target cell. The interaction between TCR and pMHC leads to the accumulation of TCR-associated molecules at the cell surface between T cell and APC in the so-called immunological synapse (Dustin, 2009) and triggers signal transduction cascades inside the T cell. This interaction causes biochemical changes in the cytoplasmic domain of CD3 complex that are essential for TCR signaling **(Figure 1.7)**. CD3 chains (γ, δ, ε and ζ) and TCR complex specifically bind to the pMHC and CD4 or CD8 co-receptors interact with MHC class II or MHC class I respectively to stabilize the TCR-pMHC interaction. CD4 and CD8 associated Src family protein tyrosine kinases Lck and Fyn (Weiss & Littman, 1994) phosphorylate tyrosine residues of the cytoplasmic ITAMs of TCR-associated CD3 chains. This phosphorylation leads to the recruitment of adaptor molecule, ζ-associated protein (ZAP70) which binds to ITAMs and phosphorylates downstream targets, leading to activation of effector T cell functions.

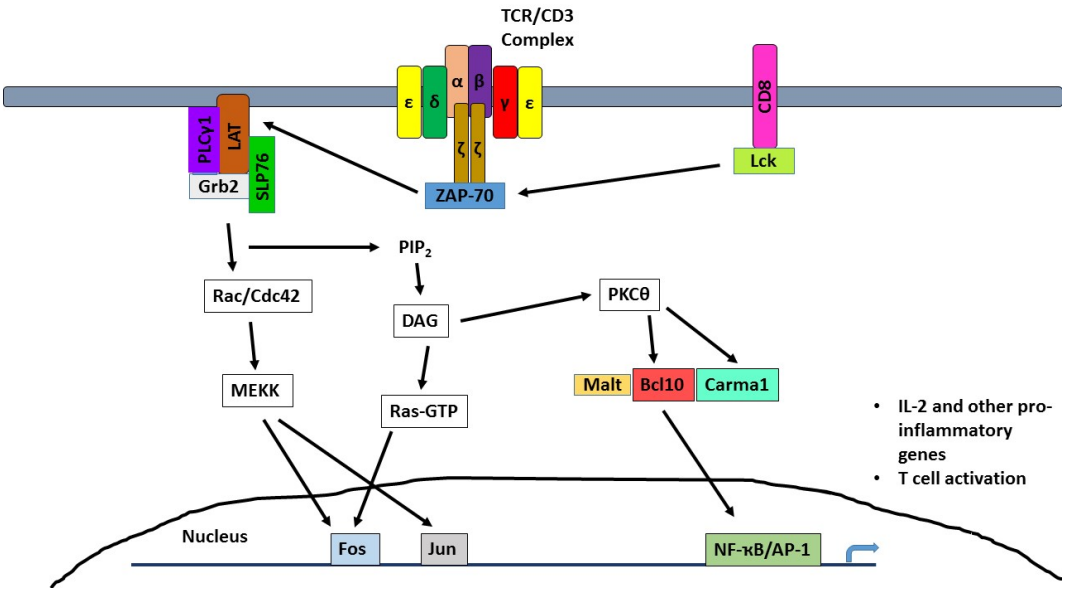

Figure 1.7: General overview of intracellular signaling pathways triggered by TCR engagement to pMHC.

1.4. Cellular Immunotherapy of Cancer

1.4.1. NK cells in cancer immunotherapy

NK cells are essential components of the innate immune system and play a critical role in host immunity against cancer by showing cytotoxicity function against various malignancies (Evren Alici et al., 2007). Recent progress in our understanding of NK cell immunobiology has paved the way for novel NK cell-based therapeutic strategies for the treatment of cancer(E. Alici & Sutlu, 2009).

Cancer cells can develop various mechanisms such as altering the expression of adhesion molecules, costimulatory ligands or activating ligands to escape immunosurveillance during tumor progression. Modulation of MHC class I expression, secretion of soluble MIC, FasL or, secreting immunosuppressive factors such as IL-10, TGF-β and indoleamine 2,3-dioxygense (IDO) and resisting Fas- or perforin-mediated apoptosis (Costello et al., 2002; Maki, Krystal, Dougherty, Takei, & Klingemann, 1998; Waldhauer & Steinle, 2008) are among other mechanisms that can lead to escape immunosurveillance. Moreover, in cancer patients, NK cell abnormalities have been observed; including decreased cytotoxicity, defective expression of activating receptors or intracellular signaling molecules, overexpression of inhibitory receptors, defective proliferation, decreased numbers in peripheral blood and in tumor infiltrate and defective cytokine production (Zitvogel, Tesniere, & Kroemer, 2006).

NK cell based immunotherapy focuses in improving antitumoral activity and promoting NK cell expansion. The first approach is modulation of endogenous NK cell activity in cancer patients by supplying extrinsic factors such as cytokines. interleukin 2 (IL-2) is the most important factor responsible for proliferation and expansion of NK cells (Becknell & Caligiuri, 2005; Sim & Radvanyi, 2014). IL-2 is a cytokine effecting many types of lymphocytes such as T_{reg} cells, CTLs, B cells and NK cells and has received FDA

approval for cancer treatment in 1992 (Margolin, 2008). IL-2 treatment increases *in vivo* activation of NK cell cytotoxicity depending on dose and schedule of administration (Gratama et al., 1993; Phillips, Gemlo, Myers, Rayner, & Lanier, 1987) and boosts the number of circulating NK cells (J S Miller et al., 1997). It is also shown to induce NK cell cytotoxicity against malignancies (Robinson & Morstyn, 1987; Rosenberg et al., 1987). IL-2 activation of NK cells *in vitro* demonstrates enhanced tumor killing (E Lotzová, Savary, & Herberman, 1987; Eva Lotzová, Savary, & Herberman, 1987).

NK cell based adoptive immunotherapy represents one of the most promising future strategies to combat cancer. To our knowledge, clinical trials using NK cells have clearly demonstrated a significant benefit in patients with various malignancies. However, none of the clinical products are routinely available or approved to be used in the clinic yet.

Allogeneic NK cell products have been used in treatment of a range of malignancies such as; leukemia, renal cell carcinoma, leukemia, colorectal cancer, hepatocellular cancer, lymphoma and melanoma (Geller & Miller, 2011; Jeffrey S. Miller et al., 2005; Rizzieri et al., 2010). In the first phase I clinical trial using the feeder-free *ex vivo*-expansion platform, adoptive transfer of NK cells from HLA identical siblings into patients with leukemia or carcinoma was well tolerated and safe alongside *in vivo* NK cell expansion, with only some infusion related complications (Barkholt et al., 2009).

Completed clinical trials with haploidentical donors are safe with only few reports of infusion-related complications such as dyspnea, nausea, hypertension, stroke, febrile reaction, and vomiting (Geller et al., 2011; Geller & Miller, 2011). So far, allogeneic NK cell transplantations derived from PBMCs or $CD34^+$ cells have shown promising results with engraftment, *in vivo* expansion of NK cells, complete remission, and a 100% 2-year event-free survival in one clinical trial by Rubnitz et al (Curti et al., 2011; Nguyen et al., 2011).

Several clinical studies have been performed with adoptive autologous NK cells in an attempt to target tumors such as; breast cancer, lymphoma, glioma renal cell carcinoma, non-small cell lung cancer and adenocarcinoma (Escudier et al., 1994; Lister et al., 1995). In general, autologous NK cell trials are safe with no known toxic side effect (Escudier et al., 1994; Krause et al., 2004; Lister et al., 1995).

Taken together, NK cell based medicinal products are safe to infuse with promising clinical benefits, however, there is still need for improvement in order to optimize

manufacturing for clinical applications and cost efficiency. There are numerous variables that may impact quality and quantity of NK cell products. Future pre-clinical research and results from more clinical trials will evaluate the contribution of each factor to the product purity, potency, and safety as well as assisting in acquiring NK cell products that can be manufactured reproducibly with the optimal safety and anti-tumor responses.

1.4.2. T cells in cancer immunotherapy

Up to now, there have been three types of treatment for cancer patients which are surgery, chemotherapy and radiotherapy. But recently, immunotherapy has started to become famous in this field. Adoptive T cell therapy has emerged as a potentially powerful approach to cancer treatment(Abad et al., 2008; Kessels, Wolkers, van den Boom, van der Valk, & Schumacher, 2001) (Yee et al., 2002). To date, adoptive T cell therapy has been shown to be the most effective method in cancer immunotherapy and has achieved promising results in cancer clinical trials (Cheadle et al., 2014; Dotti, Gottschalk, Savoldo, & Brenner, 2014; Hinrichs & Rosenberg, 2014; Jensen & Riddell, 2014; Ruella & Kalos, 2014).

The numerous advantages of T cells make them great candidates for cancer immunotherapy: they can distinguish foreign and self-antigens since they have specificity against a particular antigen. T cell activation leads to high clonal expansion after priming and because they have memory, after initial treatment, therapeutic effect goes on for many years.

T cells can recognize and target specific antigens expressed abundantly on the tumor cells but in low levels on healthy tissue. This kind of antigens are called tumor-associated antigens (TAAs). Lymphocytes, especially T cells, migrate into site of tumor and recognize them by TAA expression. These T cells fighting tumor cells are called tumor infiltrating lymphocytes (TILs) and have specificity against TAAs. In clinical trials, adoptive T cell therapy is currently used by targeting several TAAs, including

MART1, gb100 and tyrosinase in melanoma; however the number of targeted TAAs is dramatically increasing.

T cells reactive against any specific antigen isolated from the tumor tissues of cancer patients are grown and expanded to large numbers *in vitro*, and then infused back to the patient(de Witte et al., 2008). The potent antitumor effect of this therapy was shown clinically, with the example of melanoma (Yee et al., 2002) (**Figure 1.8)**.

Although this therapy approach has huge potential, it also has some factors that limit application. It is difficult to isolate and expand sufficient amount of TAA-specific T cells to reinfuse to patients.

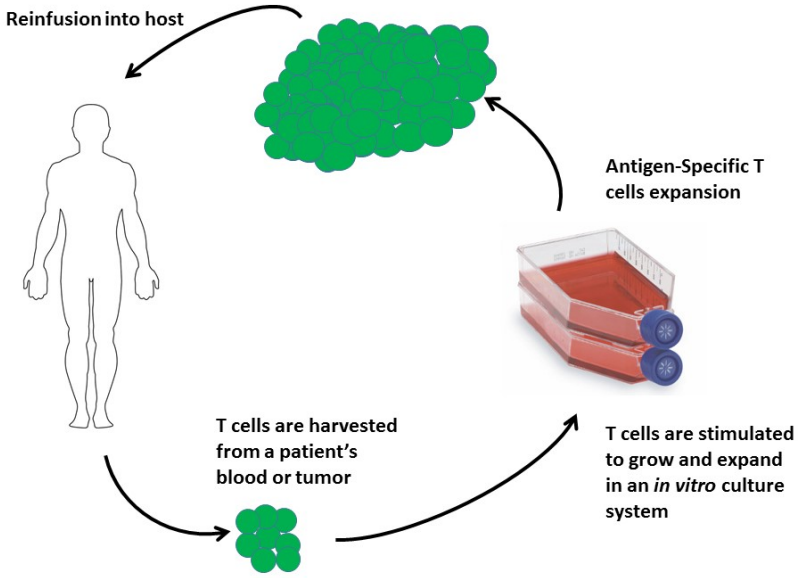

Figure 1.8 Schematic representation of adoptive T cell therapy

1.5. Genetic Modification of Lymphocytes for Cancer Immunotherapy

1.5.1. Gene delivery by lentiviral vectors

Gene therapy can be broadly defined as set of techniques that are based on the transfer of genetic material (DNA or RNA) into cells in an attempt to prevent or treat disease. Moreover, the ability of gene transfer into a cell surpasses the boundaries of this classical definition. The purpose and the exact technique of gene delivery vary greatly depending on the specific application. In the field of cancer immunotherapy, this ability has fueled applications based on the genetic modification of immune effector cells to use them as microscopic agents that have the capacity to find and destroy the malignant cells (Cusack & Tanabe, 2002). Traditionally, the focus of such applications has been T cells, but other players of the immune system such as NK cells also show promise in the development of cancer immunotherapy approaches.

The physical delivery of nucleic acid sequences carrying a certain gene-of-interest (GOI) into the cell constitutes a major hurdle in gene therapy. A commonly used approach is to use viruses as gene delivery vectors (Breyer et al., 2001). The natural life cycle of a virus depends on hijacking cellular defense mechanisms and carrying genetic information into the cell. Contrary to non-viral vectors, viral delivery of genes is generally highly efficient. This study makes use of HIV-1-based lentiviral vectors (Adamson & Jones, 2004) that belong to the Retroviridae family (Coffin, Hughes, & Varmus, 1997), consisting of single stranded RNA viruses with the capacity of reverse transcribing their genome into double stranded DNA and integrating into the host genome. Using integrating viral vectors also ensures stable integration of the transgene into the target cell genome and long-lasting expression.

1.5.2. TCR gene therapy

Recent decades have witnessed extraordinary improvements in the use of immunotherapy against malignancies and adoptive transfer of genetically modified

lymphocytes stands among promising tools in the fight against cancer. Harnessing the cytotoxic or immunomodulatory capacity of T cells while maintaining a minimal risk of serious side effects such as the graft-versus-host-disease (GvHD) has been a major goal for cancer immunotherapy (Uttenthal, Chua, Morris, & Stauss, 2012). A commonly used approach is the isolation and expansion of TAA-specific $CD8^+$ T cells for adoptive transfer(Dudley & Rosenberg, 2003) . The techniques of TCR gene therapy have improved this approach by supplying large populations of antigen-specific T cells generated by transduction with the genes for a TCR with high avidity for the target antigen (Johnson et al., 2006).

The first step of TCR gene therapy **(Figure 1.9)** is isolation of a T cell clone that has high affinity TCR against the target antigen. Generally, it is accomplished by culturing TILs. Another way is culturing peripheral blood mononuclear cells (PBMC) together with peptide-pulsed APCs. High affinity clones are selected by using MHC-peptide multimer staining (Clay et al., 1999; Morgan et al., 2003). After a clone has been selected, TCR α and β genes are identified and sequenced using molecular biology techniques. Then, these genes are used to design the expression construct promoting stable and high-level expression of TCR genes (L. Cooper, Kalos, Lewinsohn, Riddell, & Greenberg, 2000; Labrecque et al., 2001). For TCR gene delivery into a T cell, generally lentiviral and gammaretroviral vectors are used. Following genetic modification of the target T cell population purified from autologous patient lymphocytes, the genetically modified cells are expanded and reinfused into patient.

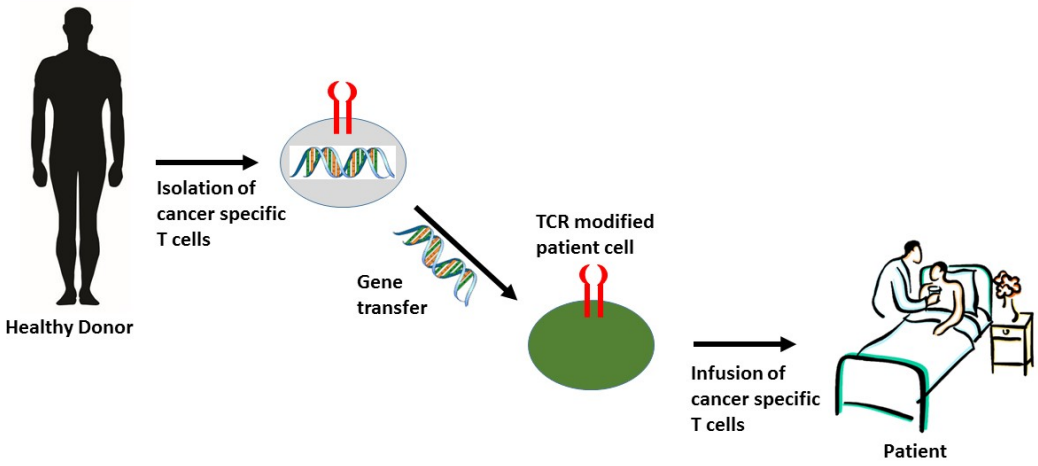

Figure 1.9. An outline of the TCR gene therapy approach for cancer immunotherapy

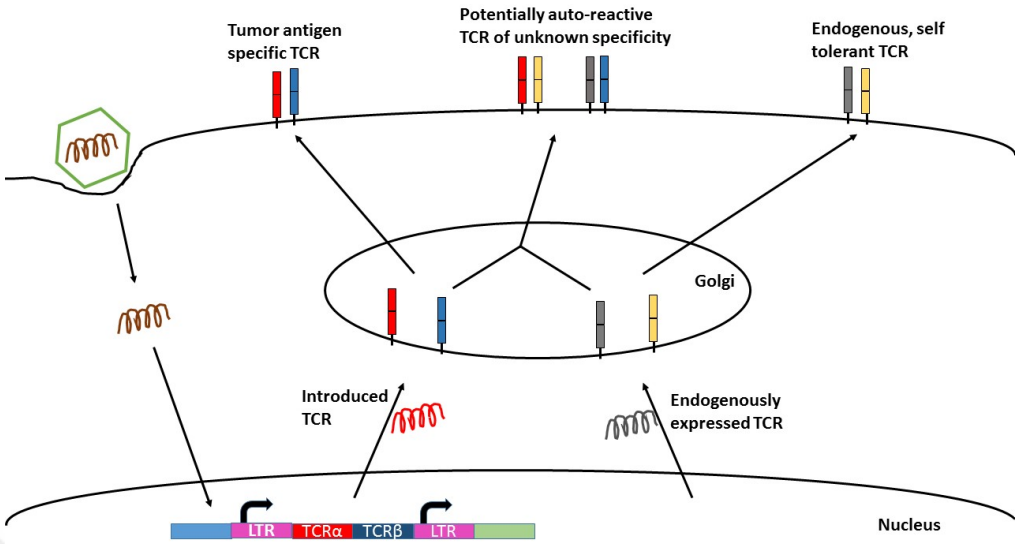

Figure 1.10. Mispairing problem in TCR gene therapy

The assembly and surface expression of the TCR introduced by gene delivery is a complex process, requiring pairing of the introduced α and β chains to form a heterodimer that then associates with the four CD3 chains, δ, γ, ε and ζ. The α and β chains introduced by gene delivery have a risk of pairing with the complementary β or α chains endogenously expressed in the genetically modified T cell (Coccoris et al., 2010). This phenomenon, called mispairing, has the potential to produce TCRs of unpredictable specificity that could be directed to self-antigens, generating autoreactive T cells that have not been subjected to central tolerance and may cause a lethal GvHD-like syndrome *in vivo* (Bendle et al., 2010).

Despite the promise of TCR gene therapy, the mispairing problem constitutes a bottleneck in the development of effective and safe therapies. Current approaches to minimizing mispairing during TCR gene transfer include modifications of the TCR sequence using constant region sequences from mouse(Cohen, Zhao, Zheng, Rosenberg, & Morgan, 2006) (Johnson et al., 2006; Stanislawski et al., 2001) but this approach imposes the risk of developing an immune response towards the mouse sequences used (Wucherpfennig et al., 2007) (Dembic Z et al., 1986) and eventual rejection of the

genetically modified cells. Other approaches include minimal structural modifications of the TCR in order to create additional disulphide linkages between the introduced α and β chains (Provasi et al., 2012) as well as optimizing equimolar translation of the introduced α and β chains through the use of 2A linkers in gene transfer vectors (Szymczak et al., 2004) in order to ensure that no excess TCR chain is available for mispairing. A variety of studies have focused on genetically linking TCR subunits to signaling chains in the CD3/TCR complex (Govers et al., 2014) as well as carrying out the genetic modification process in hematopoietic progenitors rather than mature T cells in order to exclude endogenous TCR expression (Vatakis et al., 2013) ; while others have focused on the detection(Shao et al., 2010) and elimination (Bendle et al., 2010)of T cells carrying mispaired TCR molecules after genetic modification. Besides, approaches based on siRNA technology or genome editing in order to knockout endogenous TCR expression (Okamoto et al., 2009; Provasi et al., 2012) are being widely investigated. Although successful to an extent, the majority of these procedures still carry a considerable risk of creating mispaired TCRs of unknown specificity. Therefore, mispairing stands out as one of the most crucial problems to be solved if safe and successful TCR gene therapy is to be achieved.

2. AIMS OF THIS STUDY

TCR gene therapy is a promising treatment method in cancer immunotherapy. But mispairing problem is a limiting factor in treatment success. Therefore, we have focused our efforts on developing a system that perfectly avoids the mispairing problem. In this study, we propose to use NK cells for TCR gene therapy in order to circumvent any risk of mispairing. The possibility of transferring TCR genes into an NK cell has, to our knowledge, not been reported before and carries the potential to transform the field by solving the major problem of mispairing in TCR gene therapy.

This study suggests an unconventional approach for the use of TCR gene therapy, where NK cells are used as the main effectors. TCR is a complex molecule, composed of six different chains: CD3 molecules (CD3γ, CD3δ, CD3ε, CD3ζ) assembled with the TCRα/β heterodimer. In order to ensure functional expression of a TCR by NK cells, a good starting point could be to investigate whether the molecules necessary for TCR assembly and signalling are readily present in NK cells. The table below presents an effort to characterize NK cells with regards to TCR related gene expression. As the table above clearly demonstrates, NK cells have almost all molecules necessary for TCR assembly and signalling readily available, with the exception of CD3γ and CD3δ. Lanier *et al.* has also demonstrated that human fetal liver NK cells express all CD3 chains and have cytoplasmic CD3γ/δ/ε complexes that cannot be transported to the cell surface. With this data in mind, we propose that the delivery of TCR α/β expressing vectors into NK cells, along with vector-based expression of the CD3 complex should be sufficient in order to observe TCR assembly on the NK cell surface.

Molecule	Role	Expression in NK cells	Reference
CD4	Co-receptor	+	Bernstein HB, et al. Journal of immunology. 2006;177:3669-76.
CD8	Co-receptor	+	Addison EG, et al. Immunology. 2005;116:354-61.
CD28	Co-stimulation	+	Hunter CA, et al. Journal of immunology. 1997;158:2285-93.
LFA-1	Adhesion	+	Barber DF, et al. Journal of immunology. 2004;173:3653-9.
CD3γ	TCR complex	-	Lanier LL, et al. Journal of immunology. 1992;149:1876-80.
CD3δ	TCR complex	-	Moingeon P, et al. European journal of immunology. 1990;20:1741-5. Lanier LL, et al. Journal of immunology. 1992;149:1876-80.
CD3ε	TCR complex	+ (?)	Lanier LL, et al. Journal of immunology. 1992;149:1876-80.
CD3ζ	TCR complex	+	Moingeon P, et al. European journal of immunology. 1990;20:1741-5.
Lck	TCR signaling	+	Biondi A, et al. European journal of immunology. 1991;21:843-6. Pignata C, et al. Cellular immunology. 1995;165:211-6.
Fyn	TCR signaling	+	Rabinowich H, et al. Journal of immunology. 1996;157:3860-8.
ZAP70	TCR signaling	+	Rabinowich H, et al. Journal of immunology. 1996;157:3860-8.
LAT	TCR signaling	+	Jevremovic D, et al. Journal of immunology. 1999;162:2453-6.
ERK	TCR signaling	+	Yu TK, et al. Journal of immunology. 2000;164:6244-51.
JNK	TCR signaling	+	Kumar D, et al. Journal of immunology. 2009;182:1011-20.
NFAT	TCR signaling	+	Fric J, et al. Blood. 2012;120:1380-9.
SLP-76	TCR signaling	+	Peterson EJ, et al. European journal of immunology. 1999;29:2223-32.
Vav-1	TCR signaling	+	Chan G, et al. European journal of immunology. 2001;31:2403-10.
PLC-γ-1	TCR signaling	+	Upshaw JL, et al. Journal of immunology. 2005;175:213-8.

Table 2.1. Expression of TCR-related genes in NK cells

The close developmental link between T and NK cells, the similarity of cellular signaling and cytotoxicity mechanisms as well the expression of the majority of TCR related genes in NK cells provides a firm basis for the feasibility of this approach.

This research primarily focuses on the use of TCR genes for the genetic modification and retargeting of NK cells towards TAAs, in an effort to develop a new approach in TCR gene therapy that circumvents the problems associated with mispairing of TCR chains. Specific aims to be addressed within the scope of this project are:

- Identification of the minimum requirements for the expression of functional TCRs on the NK cell surface;
- Targeting NK cell-mediated cellular cytotoxicity towards a certain TAA via the expression of TCRs on the NK cell surface.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

Chemicals and Media Components	Company
Agarose	Sigma, Germany
Ampicillin Sodium Salt	CellGro, USA
Boric Acid	Molekula, UK
Bradford Reagent	Sigma, Germany
BX795	Sigma, Germany
Chloroquine	Sigma, Germany
Cr51	PerkinElmer, USA
Distilled Water	Merck Millipore, USA
DMEM	GIBCO, USA
DMSO	Sigma, Germany
DNA Gel Loading Dye, 6X	NEB, USA
DPBS	Sigma, Germany
ECL	Sigma, Germany
EDTA	Applichem, Germany
Ethanol	Sigma, Germany
Ethidium Bromide	Sigma, Germany
Fetal Bovine Serum	ThermoScientific, USA
HEPES Solution, 1 M	Sigma, Germany
Hydrochloric Acid	Merck Millipore, USA
Interleukin2	Proleukin, Novartis,

Isopropanol	Sigma Germany
Kanamycin Sulfate	ThermoScientific, USA
LB Agar	BD,USA
LB Broth	BD,USA
L-glutamine, 200mM	Sigma, Germany
Mart-1 peptide fragment	Proimmune, UK
MEM Vitamin solution, 100X	Sigma, Germany
MEM Non-essential Amino Acid Solution	Sigma, Germany
2-Mercaptoethanol	Sigma, Germany
Methanol	Sigma, Germany
Monensin	Biolegend, USA
NaCI	Sigma, Germany
Permeabilization wash buffer	Biolegend, USA
PFA	Biolegend, USA
PIPES	Sigma, Germany
Poly-D-Lysine	BD, USA
Protamine Sulfate	Sigma, Germany
Protease inhibitors	Sigma, Germany
RPMI	GIBCO, USA
SCGM	CellGro, USA
SDS-PAGE	Sigma, Germany
Skim milk	Sigma, Germany
Solution, 100 mM	Sigma, Germany
Sodium Pyruvate	Sigma, Germany
Triton X-100	Sigma, Germany
Trizma	Sigma, Germany
Tyrosinase peptide fragment	Proimmune, UK

Table 3.1 List of chemicals used in this project

3.1.2. Equipment

Equipment	**Company**
Autoclave	Hirayama, HiClave HV-110, Japan
Balance	Sartorius, BP221S, Germany Schimadzu, Libror EB-3200 HU, Japan
Centrifuge	Eppendorf, 5415D, Germany Eppendorf, 5702, Germany

CO_2 incubator	Binder, Germany
Countess II FL automated cell counter	Thermo Fisher Scientific, USA
Deepfreezer	-80°C, Forma,Thermo ElectronCorp.,USA 20°C,Bosch,Turkey
Electrophoresis Apparatus	Biorad Inc., USA
Filters (0.22 μm and 0.45μm)	Merck Millipore, USA
Flow cytometer	BD FACScanto, USA BD LSR Fortessa USA ACEA NovoCyte 3000 USA
Gel Documentation	Biorad, UV-Transilluminator 2000, USA
Heater Thermomixer Comfort	Eppendorf, Germany
Hemocytometer	Hausser Scientific, Blue Bell Pa., USA
Ice Machine	Scotsman Inc., AF20, USA
Incubator	Memmert, Modell 300, Germany Memmert, Modell 600, Germany
Laminar Flow	Heraeus, HeraSafe HS12, Germany Heraeus, HeraSafe KS, Germany
Liquid Nitrogen Tank	Taylor-Wharton, 3000RS, USA
Luminescent Image Analyzer	GE, USA
Magnetic Stirrer	VELP Scientifica, Italy
Microliter Pipettes	Gilson, Pipetman, France Isolab, Germany Thermo Fisher Scientific, USA
Microscope	Zeiss, Primo Vert, Germany Zeiss Observer Z1, Germany Olympus IX70 inverted, USA
Microwave Oven	Bosch,Turkey
Packard Cobra Auto-Gamma 5000	GMI, Ramsey. MN
pH meter	WTW, pH540 GLP MultiCal, Germany
Refrigerator	Bosch, Turkey
Shaker Incubator	New Brunswick Sci., Innova 4330, USA
Spectrophotometer	Schimadzu, UV-1208, Japan Schimadzu, UV-3150, Japan
Thermocycler	Eppendorf, Mastercycler, Germany
xCELLigence RTCA	ACEA, USA
Vortex	Velp Scientifica, Italy

Table 3.2 The equipments used in this study

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°C for 15 minutes and stored at 4°C.

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.

Phosphate-buffered saline (PBS): For 1000 ml 1X solution, 100 ml 10X DPBS was added to 900 ml ddH_2O and the solution was filter-sterilized.

2% PFA in 1X permeabilization Wash Buffer

Lysis Buffer: 1%Triton X-100, 0.15M NaCI, 0.002M EDTA (pH 8.0) and 0.05 M Trizma (pH 7.4) and protease inhibitors.

ECL: 0.45 mM Luminol, 0.625 mM Comaric Acid, 0.07M Trizma [pH 8.8]

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.

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 selection, kanamycin at a final concentration of 50 µg/ml or ampicillin at a final concentration of 100 µg/ml was added to liquid medium just before use.

LB-Agar: For 1X agar medium in 1L, 20 g LB powder and 15 g bacterial agar powder were dissolved in 1 L ddH2O and then autoclaved at 121°C for 15 minutes. Then, autoclaved LB agar is mixed with antibiotic of interest at desired ratio. Kanamycin at a final concentration of 50 µg/ml or ampicillin at a final concentration of 100 µg/ml was added to prepared medium just before pouring onto sterile petri dishes. Sterile agar plates were kept at 4°C.

DMEM: 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.

RPMI: YTS cell line is maintained in culture in RPMI1640 supplemented with 20% heat-inactivated fetal bovine serum, 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. A375, A375 Tyr, T2 and K562 cell lines are maintained in culture in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum, 25mM HEPES, 2mM L-Glutamine, 0.1mM MEM Non-essential amino acid solution and 1mM Sodium Pyruvate.

SCGM: NK-92 cell line is maintained in culture in CellGro SCGM supplemented with 20% heat-inactivated fetal bovine serum. 1000 U/ml Interleukin-2 is added to culture every 48 hours. Also, NK-92 cell line is maintained in culture in RPMI1640 supplemented with 20% heat-inactivated fetal bovine serum, 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.

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

Commercial Kit	Company
Calcium Phosphate Transfection Kit	Sigma-Aldrich, USA
InsTAclone PCR Cloning Kit	Thermo, USA
NuceloSpin® Gel and PCR Clean up	Macherey-Nagel, USA
NuceloSpin® Plasmid Miniprep Kit	Macherey-Nagel, USA
NuceloSpin® Plasmid Midiprep Kit	Macherey-Nagel, USA
PureLink® HiPure Plasmid Midiprep Kit	Invitrogen, USA

Table 3.3 Commercial kits in this study

3.1.6. Enzymes

All of the restriction enzymes, polymerases and PCR reaction supplements are obtained from either Fermentas or New England Biolabs.

3.1.7. Antibodies

Antibody	**Company**
Mouse APC anti-CD56 (NCAM 16.2)	BD Biosciences, USA
APC mouse anti-human CD107a	BD Biosciences, USA
APC mouse anti-human TCRαβ	BD Biosciences, USA
APC mouse anti-human HLA-A2	BD Biosciences, USA
Human CD3ePerCP	BD Biosciences, USA
Mouse anti-CD247	BD Biosciences, USA
Rabbit pAb CD3δ	Abcam
Goat pAb CD3γ	Abcam
Rabbit anti-β actin pAb HRP conjugate	Cell signaling Technology
Donkey Anti-goat IgG-HRP	Santa Cruz Biotechnology
Rabbit Anti-mouse IgG-HRP	Sigma Aldrich
Goat Anti-Rabbit IgG-HRP	Cell signaling Technology
Tyrosinase (368-376)/HLA-A2 pentamer	Proimmune, UK

Table 3.4 The antibodies used in this study

3.1.8. Bacterial strains

Escherichia coli (*E.coli*) DH-5α strain is used for general plasmid amplifications and Top10 strain is used for lentiviral construct amplifications.

3.1.9. Mammalian cell lines

293FT: Human embryonic kidney 293 (HEK293) cell line derivative that stably express 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™).

YTS: Derivative of YT cell line that was originally from a 15-year old male with acute lymphoblastic leukemia (ALL) were TCR-negative cells with NK cell activity (DSMZ ACC 434).

T2 (174 x CEM.T2): T2 cell line is T cell leukemia which is characterized by deficient in transporter associated with antigen processing (TAP) protein which has an important role in antigen presenting machinery. This cell line is useful for studying and T cell recognition of class I major histocompatibility antigens (ATCC® CRL-1992™).

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

A375: A375 is a human skin malignant melanoma cell line which is isolated from 54-year-old female patient in 1973. This cell line is HLA-A2+ (ATCC® CRL-1619™).

A375 TyR: This cell line is Tyrosinase gene transduction form of A375 cell line. It overexpreses tyrosinase gene.

3.1.10. Plasmids

Plasmid	Purpose	Source
pcDNA 3.1(+) DGE	Expression of CD3**δ**, CD3ɣ and CD3***ε***	Genscript (NJ, USA)
pcDNA 3.1(+) DGE_P2Z	Expression of CD3**δ**, CD3ɣ, CD3***ε*** and CD3*ζ*	Genscript (NJ, USA).
Lego-iT2puro	Control vector expressing TdTomato	Gift from Boris Fehse
Lego-T2puro	Control vector expressing TdTomato	Gift from Boris Fehse
Lego-DGE-iT2puro	Expression of CD3**δ**, CD3ɣ and CD3***ε*** with dTomato	Lab construct
Lego-DGEZ-iT2 puro	Expression of CD3**δ**, CD3ɣ, CD3***ε*** and CD3*ζ* with tdTomato	Lab construct
TyrTCR-IRES-eGFP (TyrTCR)	Expression of TCR**α** and TCRβ chains specific for tyrosinase	Gift from Brusko *et al.*
pMDLg/pRRE	Virus production/packaging plasmid (*Gag/Pol*)	Addgene
pRSV-REV	Virus production/packaging plasmid (*Rev*)	Addgene
phCMV-VSVG-G	Virus production/packaging plasmid (*Env*)	Addgene

Table 3.5 The plasmids used in this study

3.1.11. DNA Ladder

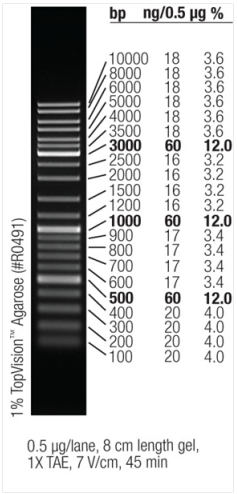

Figure 3.1 DNA ladder used in the study

3.1.12. DNA sequencing

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

3.1.13. Software, computer-based programs and websites

SOFTWARE, PROGRAM, WEBSITE NAME	COMPANY/ADDRESS	PURPOSE OF USE
FlowJo v10	Tree Star Inc.	Viewing and analyzing flow cytometry data
CLC Main Workbench v7.7	CLC bio	Constructing vector maps, restriction analysis, DNA sequencing analysis, DNA alignments, etc
Ensembl Genome Browser	http://www.ensembl.org/index.html	Human genome sequence information
GraphPad Prism v7	GraphPad Software, Inc., San Diego, CA, USA	Data analysis, statistical analysis
Addgene	https://www.addgene.org/	Plasmid map and sequence information, CRISPR design tool guidelines

Table 3.6. Complete list of software and programs.

3.2. Methods

3.2.1. Cell lines

293FT cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 0.1 mM non-essential amino acids, 6 mM L-glutamine, 1 mM sodium pyruvate and 20 mM HEPES. NK-92 cells were maintained in CellGro SCGM supplemented with 20% FBS and 1000 U/ml rhIL-2. T2, K562, A375 and A375Tyr cells were maintained in RPMI-1640 medium supplemented with 10% FBS. YTS cells were maintained in RPMI1640 supplemented with 20% heat-inactivated fetal bovine serum, 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.

3.2.2. Production of lentiviral vectors

For production of VSV-G pseudotyped lentiviral vectors, $14x10^6$ 293FT cells were plated into a poly-D-lysine coated 150 mm dish. Next day cells were transfected with 30 µg of vector plasmid (LeGO vectors courtesy of Prof. Boris Fehse, University Medical Center Hamburg-Eppendorf, Hamburg, Germany), 15 µg of pMDLg/pRRE, 10 µg of pRSV-REV and 5 µg of phCMV-VSV-G using calcium phosphate transfection kit in the presence of 25 µM Chloroquine. 10 hours after transfection, the medium was changed and thereafter virus containing supernatant was collected every 24 hours for 2 days and stored in -80°C until further use. A small aliquot from each production was used to determine viral titers by transduction of 293FT cells with serially diluted amounts of virus supernatant.

3.2.3. Lentiviral transduction of NK cells

For each lentiviral transduction, $0.25x10^6$ NK-92 or YTS cells per well were seeded in a 24-well plate and mixed with an appropriate amount of virus supernatant in the presence of 8 µg/ml of protamine sulfate and 3 µM BX795 in a final volume of no more than 1 ml. The plates were incubated at 37°C, 5% CO_2 for 6 hours. At the end of the incubation, cells were spinned down at 300xg for 5 minutes at room temperature after which the supernatants were removed from the wells and 1 ml of fresh growth medium per well was added. The cells were maintained in this medium for at least 3 days before acquisition of gene expression was carried out.

3.2.4. Flow cytometry

All antibody stainings for flow cytometry were done according to the following protocol: For surface stainings, the cells were washed once with PBS and incubated with appropriate amounts of antibody at 4°C for 30 min. The labeled cells were then washed with PBS and data acquisition was done. The antibodies used for NK cells were CD56 (NCAM16.2) and CD3e (UCHT1). For intracellular staining, cells were fixed and permeabilized for 15 minutes in a solution containing 2% PFA in 1X Permeabilization Wash Buffer, washed two times with Permeabilization Wash Buffer and incubated with appropriate amounts of antibody at 4°C for 30 min. The labeled cells were then washed with Permeabilization Wash Buffer and data acquisition was carried out. For data acquisition, FACSCanto, LSR Fortessa or NovoCyte 3000 instruments were used depending on availability. Data were analysed with the FlowJo software

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3.2.5. Western blot analysis

Cells were lysed in lysis buffer (1% Triton X-100, 0.15M NaCl, 0.002M EDTA (pH 8.0) and 0.05 M Trizma [pH 7.4]) and protease inhibitors. Protein content was measured by Bradford assay and analyzed under reducing conditions with 12% SDS-PAGE, followed by electroblotting on PVDF transfer membranes. The membranes were blocked with 5% skim milk. Membranes were incubated overnight at 4℃ with the indicated primary antibodies. The dilutions of antibodies were prepared as follows: mouse anti-CD 247 (51-6527GR; BD Pharmingen, 1:500), rabbit pAb CD3δ (ab103573, Abcam, 1:300), goat pAb CD3γ (ab200563, Abcam, 1:300), rabit anti β-actin pAb HRP conjugate (5125S, Cell Signaling Technology, 1:2000). After washing, membranes were treated with horseradish peroxidase-conjugated secondary antibodies, donkey Anti-goat IgG-HRP(sc-2033, Santa Cruz Biotechnology, 1:2500) incubated overnight at 4℃ , rabbit Anti-Mouse IgG-HRP (A9044, Sigma Aldrich, 1:80000) and goat Anti-Rabbit IgG-HRP (7074P2, Cell Signaling Technology, 1:20000) incubated 1 hour at room temperature. Then the bands were visualized using ECL (0.45 mM Luminol, 0.625 mM Comaric Acid, 0.07M Trizma [pH 8.8]) in Luminescent Image Analyzer. Images were quantified using ImageJ (https://imagej.nih.gov/ij/).

3.2.6. Peptides and HLA-A2 Pentamers

Tyrosinase$_{(368-379)}$ and Melan-A/Mart-1$_{(27-35)}$ peptides as well as APC-conjugated Tyrosinase$_{(368-379)}$/HLA-A2 pentamers were purchased from ProImmune Ltd (Oxford, UK). 0.5x10^6 NK-92 cells were washed once with PBS and incubated with 10 ul of Tyrosinase$_{(368-379)}$/HLA-A2 pentamers at 4°C for 30 min. The labeled cells were then washed PBS twice and data acquisition was carried out. Data were analysed with the FlowJo software.

3.2.7. Analysis of NK cell degranulation

TAP-deficient T2 cells were pulsed with different concentrations of indicated peptides in serum free RPMI medium at 26°C overnight in 5% CO_2. Cells were subsequently washed and incubated in RPMI medium at 37°C for 60 min. Then, NK-92 or YTS cells were co-incubated with T2 or A375 target cells at a ratio of 1:1 in a final volume of 200 µl in round-bottomed 96-well plates at 37°C and 5% CO_2 for 6 h. Fluorochrome-conjugated anti-CD107a mAb was added at the initiation of the assay. After 1 h of coincubation, Monensin was added at a 1:100 dilution. The cells were then washed, resuspended in ice-cold PBS and immediately analyzed by flow cytometry.

3.2.8. Analysis of NK cell cytotoxicity by Xcelligence RTCA

Real time cell viability experiments were performed by using xCELLigence RTCA DP device placed in a humidified incubator at 37 °C and 5% CO_2. The E-16 plates were incubated with 100µl of cell-free growth medium (10% FBS) at room temperature for 15 min. After incubation background impedance signal was measured to control all the connections. The target cells were seeded into E-16 plate as indicated concentrations which are $1x10^4$ in 100µl for A375 Tyr cells and $1,5x10^4$ in 100µl for A375 cells. The plates are mounted to device and incubated for 30 min before starting the experiment. The target cells were allowed to grow for about 16h before adding effector cells. The following day, the effector cells were added onto the target cells at an E:T ratio of 1:1. Real time measurements were performed by recording the Cell index (CI) every 15 min, for 40h. Data analysis was carried out with the RTCA software (version 1.2).

3.2.9. Analysis of NK cell cytotoxicity by ^{51}Cr release assay

NK-cell cytotoxicity was measured in a ^{51}Cr-release assay against K562, T2 and A375 cells. Briefly, cells were labeled with 100 uCi ^{51}Cr for 1 hour at 37°C, NK cells were mixed with the labeled cells at different effector:target ratios and incubated for 4

hours. Supernatants (70 µl) were transferred into 4 ml sample tubes and counted using a Packard Cobra Auto-Gamma 5000 Series Counting System (GMI, Ramsey, MN).

3.2.10. Live cell imaging

For live cell imaging $0.25x10^6$ A375 or A375(Tyr) cells were seeded in 6-well plates with glass coverslips (0.17 mm thickness, 25 mm diameter) at the bottom and left to grow overnight. The next day, coverslips were removed from the plates, rinsed once with PBS and mounted into an Attofluor Cell Chamber and 900 µl fresh growth medium was added into the chamber. Thereafter, the chamber was put in the microscope and $0.1x10^6$ effector cells in resuspended in 100 µl medium were added. After letting the effector cells settle at the bottom of the chamber for 5 minutes, images were recorded with 15 sec intervals for a total of 2 hours. For incubation and analysis, a ZEISS Observer Z1 fluorescent microscope equipped with a XLmulti S1 Incubator unit was used. Image analysis and video exporting was done using the ZenPRO software.

3.2.11. Statistical analysis

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

4. RESULTS

4.1. Cell Surface Assembly of TCR Complex in NK Cells

4.1.1. Genetic modification of NK-92 and YTS cell lines for TCR expression

TCR complex is composed of six different chains: CD3 molecules (CD3δ, CD3γ, CD3ε, CD3ζ) assembled with the TCR α/β heterodimer. Various studies have identified that NK cells have almost all molecules necessary for TCR assembly and signaling readily available, with the exception of CD3δ and CD3γ. Lanier et al. has also demonstrated that human fetal liver NK cells express all CD3 chains and have cytoplasmic CD3γ/δ/ε complexes that cannot be transported to the cell surface. With this data in mind, we delivered TCR α/β expressing vectors into NK cells, along with lentiviral vector-based expression of CDδ, CD3γ and CD3ε in the presence and absence of CD3ζ to observe TCR assembly on the NK cell surface.

For detailed analysis of factors effecting TCR assembly on the NK cell surface and optimization of TCR expression, we have used the NK cell lines NK-92 and YTS as model systems. For the expression of CD3 chains in NK cells we used two different lentiviral constructs either with or without the CD3ζ chain **(Figure 4.1)**. For CD3 chains, codon optimized sequences linked with 2A sequences were synthesized by Genscript (NJ, USA). These genes were cloned into LeGO backbone with eGFP or tdTomato fluorescent markers fused to puromycin resistance gene. For the expression of TCR sequences, we

used the lentiviral TyrTCR-IRES-eGFP (TyrTCR) vector developed by Brusko et al. that codes for a co-receptor-independent TCR against the melanoma antigen tyrosinase-derived peptide (368-379) YMDGTMSQV ($Tyr_{368-379}$) presented in the context of HLA-A*0201 [98,99].

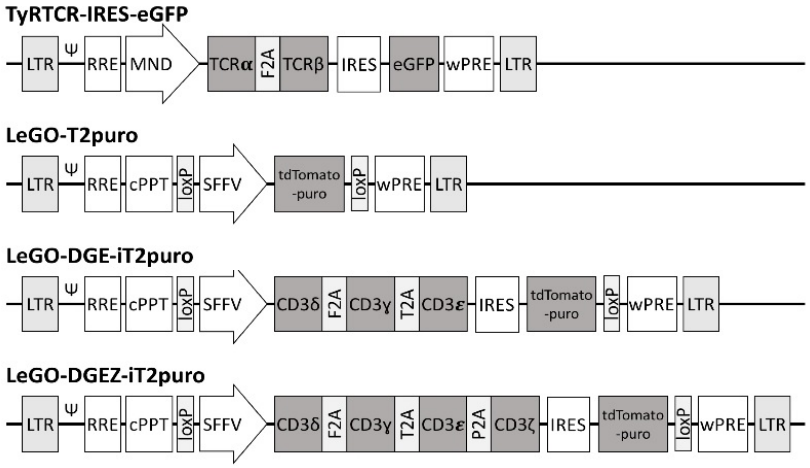


Figure 4.1. Lentiviral vector constructs designed and used to genetically modify NK cells.

For the expression of TCR in NK cells, we followed a 2-step genetic modification process as exemplified in **Figure 4.2**. NK cells were first transduced with the LeGO-DGE-iT2puro or LeGO-DGEZ-iT2puro constructs as well as the empty LeGO-T2puro vector as a control and enriched with puromycin selection. Then, each cell line (NK-92 T2puro, NK-92 DGE-iT2puro, NK-92 DGEZ-iT2puro) underwent a second transduction with the TyrTCR-IRES-GFP vector. In all cell lines, we observed approximately 10-20% $GFP^{+}tdTomato^{+}$ cells 72 hours post-transduction which were then subjected to fluorescence-activated cell sorting (FACS) for purification.

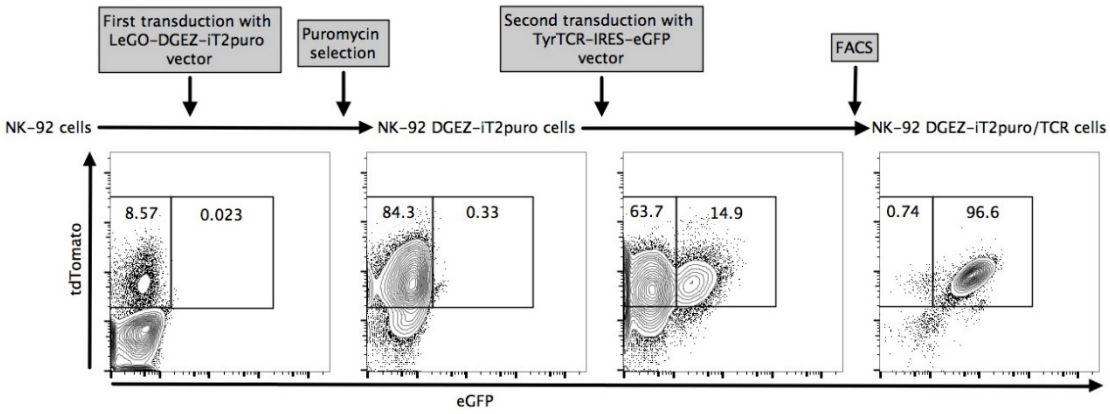


Figure 4.2. Representative example of the genetic modification process of NK cells. Firstly, NK cells were transduced with a vector expressing the CD3 subunits (either LeGO-DGE-iT2puro or LeGO-DGEZ-iT2 puro) and the genetically modified cells were enriched by puromycin selection. Second transduction was done on this enriched population with the TyrTCR-IRES-eGFP vector and the resulting $eGFP^+$ $tdTomato^+$ cells were selected with fluorescence-activated cell sorting (FACS).

4.1.2. Surface expression of CD3/TCR complex

Initially, we determined the expression levels of surface CD3ε on genetically modified NK-92 cells by flow cytometry. NK-92 cells do not express CD3ε molecule on the cell surface; however, in line Lanier et al., there is trace amounts of intracellular CD3ε expression on NK-92 cells. When NK-92 cells are transduced with DGE or DGEZ (NK-92-DGE-iT2puro and NK-92-DGEZ-iT2puro, respectively) surface expression of CD3ε was still absent as analyzed by flow cytometry. However, intracellular staining showed a high level of CD3ε expression in transduced cells compared to control NK-92 T2puro cells **(Figure 4.3)**.

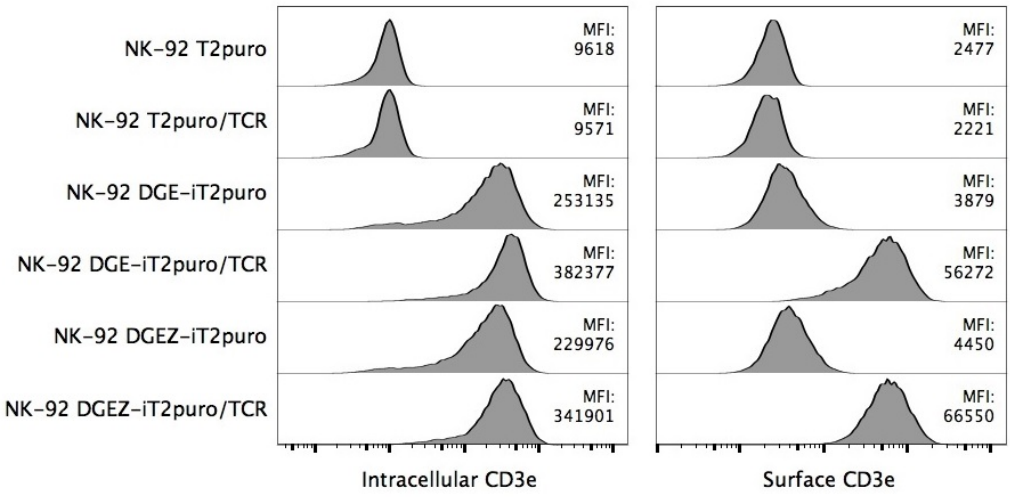


Figure 4.3 Flow cytometry-based cell surface and intracellular CD3e expression analysis in NK cells transduced with lentiviral vectors.

This suggests that despite high levels of vector-driven expression, CD3 chains of the TCR complex (or at least CD3ε) -similar to the case during T cell development- cannot be stably expressed on the surface of NK-92 cells in the absence of the TCR α/β chains. The expression of CD3δ, CD3γ, and CD3ζ in genetically modified NK-92 cells were also characterized with western blot analysis **(Figure 4.4)**.

As mentioned above TyrTCR is specific to tyrosinase protein-derived peptide (368-379) YMDGTMSQV ($Tyr_{368-379}$) in complex with HLA-A2. In order to determine cell surface expression of TyrTCR on NK92 cells and investigate whether or not TyrTCR retains its functionality and antigen specificity, we next stained NK-92 cells with $Tyr_{368-379}$/HLA-A2 pentamers.

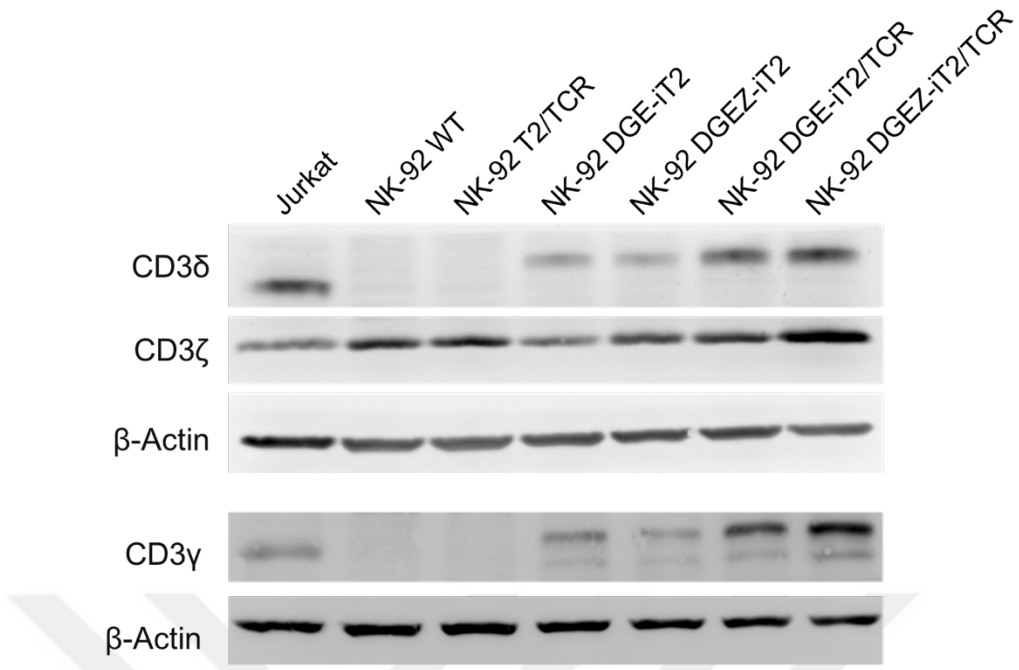


Figure 4.4. Western blot analysis of CD3δ, CD3γ and CD3ζ in genetically modified NK-92 cells.

In order to determine cell surface expression of TyrTCR on NK cells, we next stained genetically modified NK-92 and YTS cells with anti-TCR α/β antibody as well as $Tyr_{368-379}$/HLA-A2 pentamers (**Figure 4.5**). These results also confirmed that only NK cells expressing TCR α/β together with the CD3 chains could be efficiently stained with anti-TCR α/β antibody or $Tyr_{368-379}$/HLA-A2 pentamer.

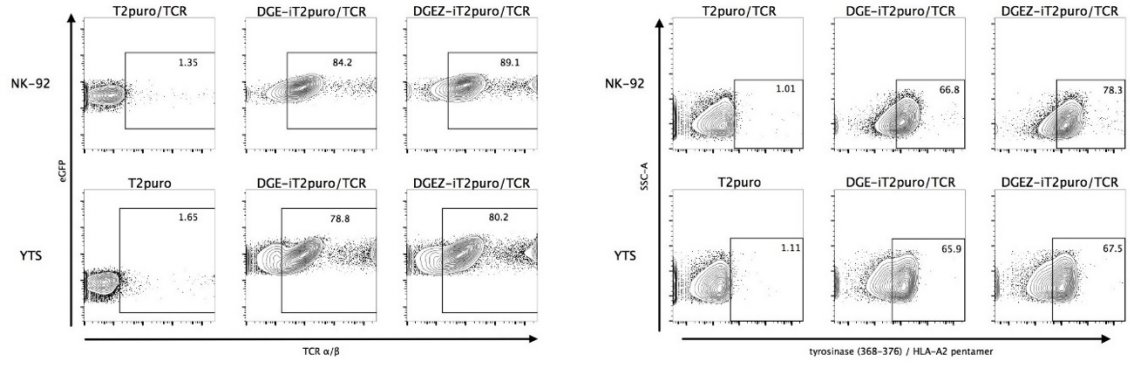


Figure 4.5 Flow cytometry-based analysis of anti-TCR staining and $tyrosinase_{(368-376)}$/HLA-A2 pentamer staining on genetically modified NK-92 cells.

The inclusion of ectopic CD3ζ expression appears to minimally increase cell surface staining of CD3ε as well as the percentage of anti-TCR α/β or $Tyr_{368-379}$/HLA-A2

pentamer staining. This suggests that, owing to the native CD3ζ expression in NK cells, the inclusion of CD3ζ ectopic expression is not crucial but an abundance of CD3ζ can increase the efficiency of TCR complex assembly at the cell surface. Taken together, these results clearly demonstrate that the ectopic expression of CD3δ, CD3γ, CD3ε and TCR α/β heterodimer but not CD3ζ is necessary for establishment of TCR complex on the surface of NK-92 and YTS cells.

In parallel with CD3ε cell surface expression patterns, only NK-92-DGE-iT2puro/TyrTCR and NK-92-DGEZ-iT2puro/TyrTCR cells could be detected by the cognate $Tyr_{368-379}$/HLA-A2 pentamer suggesting that expression and MHC binding of TyrTCR on the NK cell surface is dependent on the presence of the CD3 complex. Introduction of CD3 complex enables TCR trafficking to the cell surface and stable cell surface expression of TCR complex that results in antigen recognition.

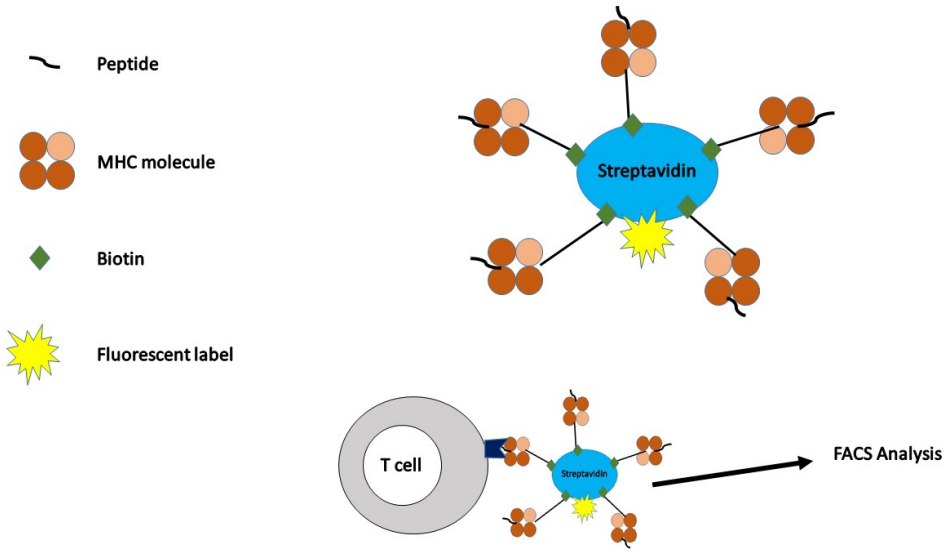


Figure 4.6 The structure of tyrosinase(368-376)/HLA-A2 pentamer

4.2. Triggering and specificity of TCR-expressing NK cells

4.2.1. Degranulation of TCR-expressing NK cells

To test for antigen-specific triggering, we primarily assessed degranulation capacity of TCR-expressing NK cells against the HLA-$A2^+$ T2 cell line (**Figure 4.7**). As the T2 cell line is TAP-deficient, cell surface expression of HLA-A2 is very low and cell surface HLA-A2 levels can be upregulated by providing exogenous peptides, which sets a platform to study peptide specific T cell responses. Thus, we investigated degranulation against T2 cells alone or loaded with $Tyr_{368-379}$ as well as with an HLA-A2 restricted tumor associated antigen (Mart-1)-derived epitope that should not be recognized by TyrTCR.

In the case of NK-92 cells, both DGE-iT2puro/TCR and DGEZ-iT2puro/TCR cells degranulated against non-loaded (DMSO) and Mart-1-loaded T2 cells similarly and at a slightly higher level than control cells expressing only T2puro or T2puro/TCR. On the other hand, T2 cells loaded with $Tyr_{368-379}$ peptide triggered degranulation of both NK cell lines at a significantly higher level.

With YTS cells, we were not able to observe any response to PMA/Ionomycin but a similar trend was seen against T2 targets where only background levels of degranulation were present against DMSO controls or Mart-1 peptide loaded cells while a high response to Tyr peptide loaded T2 cells were recorded in both DGE-iT2puro/TCR and DGEZ-iT2puro/TCR expressing cells.

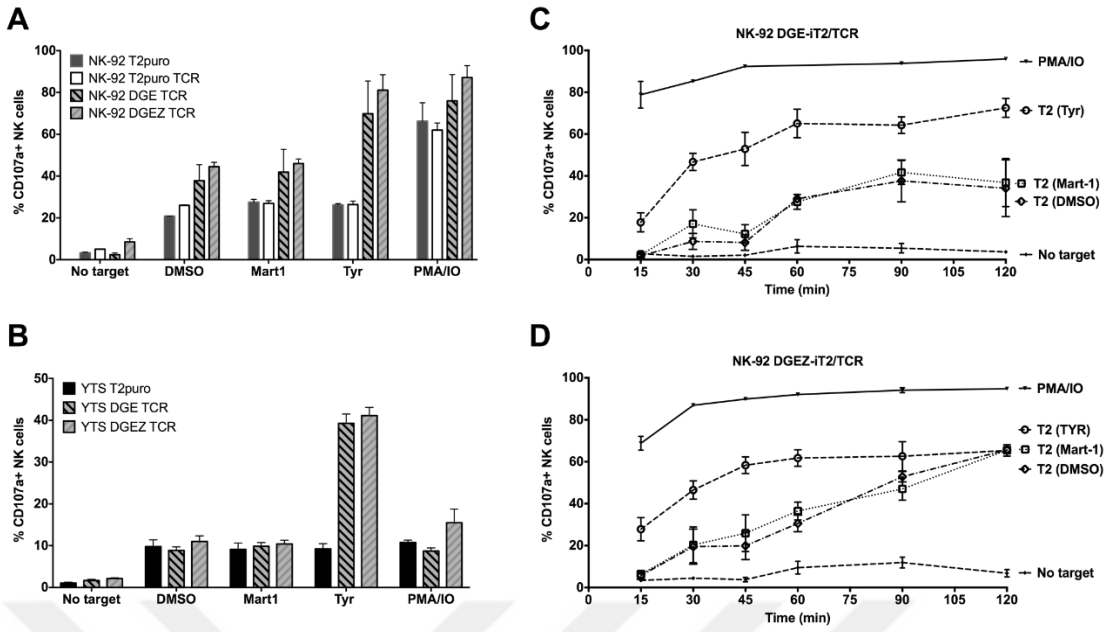


Figure 4.7. Degranulation of genetically modified NK cells against T2 targets. To assess NK cell degranulation capacity, cell surface expression levels of CD107a on NK cells is measured upon target cell exposure. T2 cells loaded with indicated peptides or DMSO (empty) is mixed with transgenic NK-92 cells (A) or YTS cells (B) at 1:1 ratio for 5 hours. After 1 hour of coincubation golgi stop was added. T2 cells loaded with indicated peptides or DMSO (empty) is mixed with NK-92 DGE-iT2/TCR cells (C) or NK-92 DGEZ-iT2/TCR cells (D) at 1:1 ratio. Cell surface expression of CD107a is measured at indicated time points with flow cytometry.

Analysis of the dynamics of degranulation during the first two hours of contact with peptide-loaded T2 target cells has shown that the TCR-triggered response of NK-92 cells is extremely fast with significant antigen-specific responses seen as early as 15 minutes (**Figures 4.6 C and D**). We have also noticed that the triggering of DGEZ-expressing cells seems to have lost a certain degree of specificity against Tyr peptide-loaded cells and shows higher activity against non-specific targets. We hypothesize that this could be due to the overexpression of CD3ζ chain effecting expression of NK cell activating receptors natively coupled with this signaling adaptor.

Besides investigating the triggering by peptide-loaded cells, we also sought to determine whether the TyrTCR introduced by genetic modification has the capacity to recognize endogenously processed peptides in complex with HLA-A2 (**Figure 4.8**). For this purpose, we used the HLA-A2^{+} melanoma cell line A375 and a version of the same cell line that overexpresses the tyrosinase protein A375(Tyr). Degranulation against A375 (Tyr) targets in both NK-92 and YTS cells were quite efficient and proved that the

introduced TCR can recognize endogenously processed epitopes. On the other hand, the background degranulation of DGEZ-iT2puro/TCR expressing cells against A375 targets that do not express the tyrosinase protein were elevated, correlating with results from degranulation against peptide loaded T2 cells and hinting at a loss of specificity when CD3ζ chain is overexpressed.

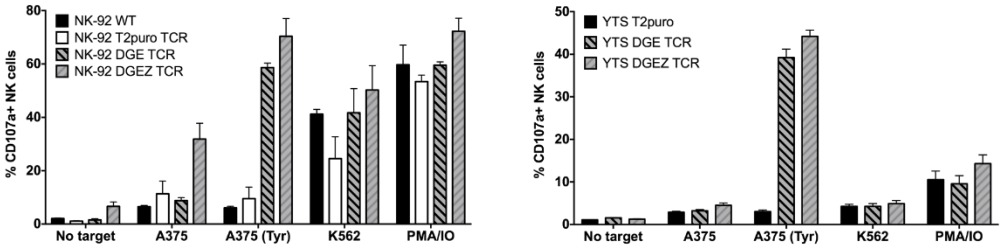


Figure 4.8. Degranulation of genetically modified NK cells against A375 and K562 targets. Target cells including A375, A375 (TYR) and K562 cells were coincubated with transgenic NK-92 cells (A) or YTS cells (B) at 1:1 ratio for 5 hours. Degranulation capacity of cells were assessed by flow cytometry.

Taken together, these data suggest that NK cells expressing CD3δ, CD3γ, CD3ε and TCRα/β on their cell surface could detect an antigenic peptide presented by MHC-I and selectively degranulate against these cells. The inclusion of CD3ζ in vector design does not seem to help increase antigen-specific triggering but on the contrary, might increase background activity against non-specific targets.

4.2.2. Cytotoxic activity of TCR-expressing NK cells

As the definite demonstration of antigen-specific triggering we analyzed cytotoxic activity of genetically modified NK-92 and YTS cells against A375 and A375(Tyr) targets. Electrical impedance-based analysis of A375 and A375(Tyr) cell growth using the XCelligence RTCA instrument in the presence/absence of effector cells has revealed that both NK-92 and YTS cells efficiently kill tyrosinase-expressing targets (**Figure 4.9 A**) upon specific TCR expression. Quantification of cytotoxic activity as measured by XCelligence RTCA at the 4h timepoint (**Figure 4.9 B**) shows high cytotoxic activity against A375(Tyr). Standart 4h ^{51}Cr-based cytotoxicity with NK-92 cells against A375 and A375(Tyr) also confirms these observations (**Figure 4.9 C**). DGE-iT2puro/TCR cells show higher antigen-specific cytotoxic activity than DGEZ-iT2puro/TCR cells despite

their slightly lower degranulation rate while DGEZ-iT2puro/TCR cells show signs of elevated activity against the background A375 cells without Tyr expression. These effects also seem to be reproducible in the case of YTS cells where the overexpression of CD3ζ negatively affects antigen-specificity of responses.

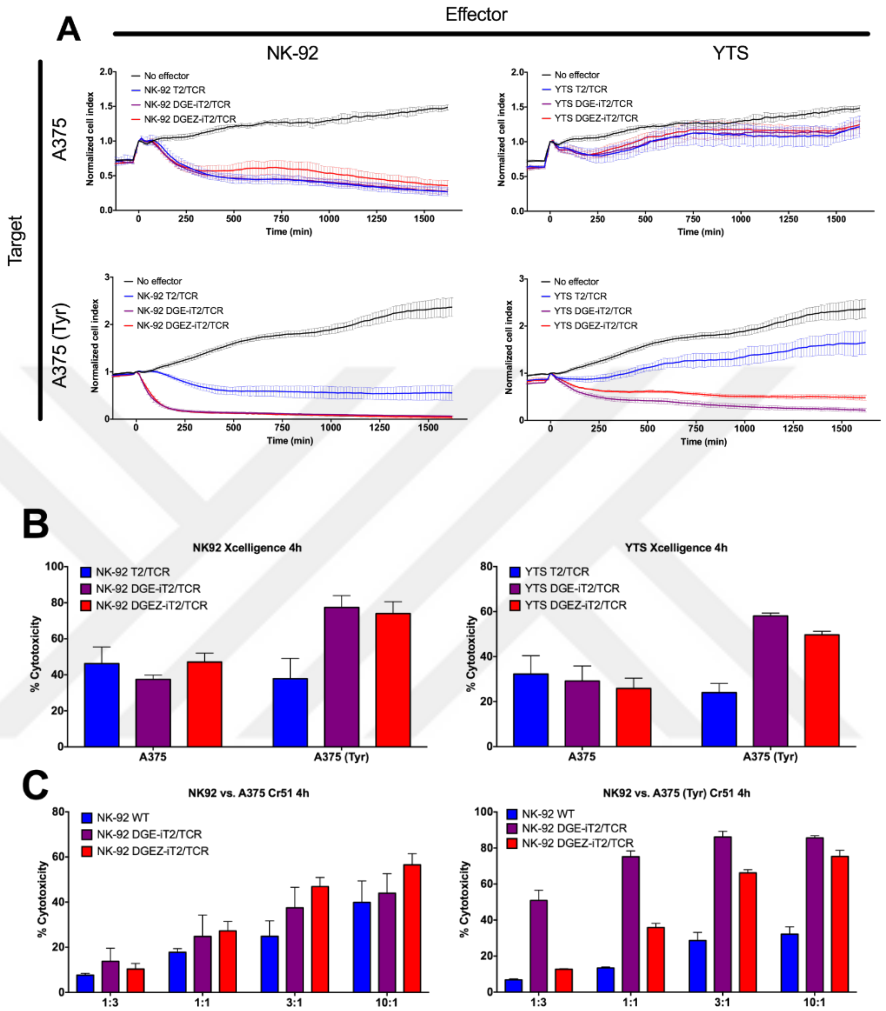
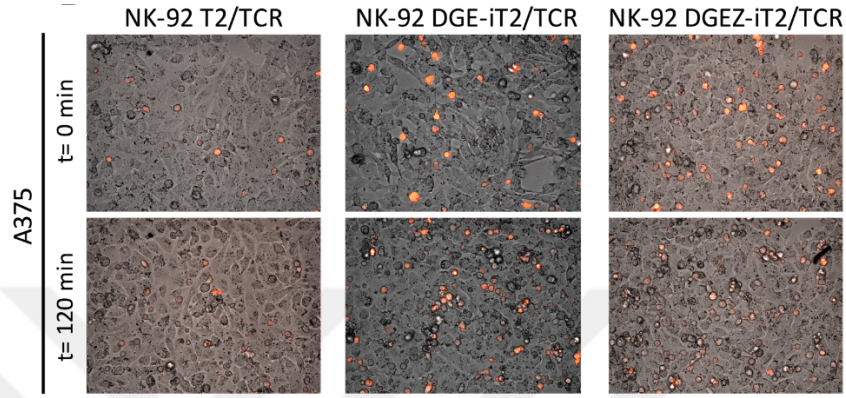


Figure 4.9. Cytotoxic activity of TCR-expressing NK cells. **(A-B)** A375 cells or A375 (Tyr) cells were seeded into E-16 plate. 16 hours later, transgenic NK-92 cells or YTS cells were added onto the target cells at an E:T ratio of 1:1. Real-time measurements were performed by recording the Cell index (CI) every 15 min, for 40h using *Xcelligence RTCA platform*. **(C)** Target cell killing is assessed by Cr^{51} release assay. Cr51 labelled A375 or A375 (Tyr) cells were coincubated with transgenic NK-92 cells at indicated ratios for 4 hours. Target cell killing is calculated through measuring radioactivity in the co-culture supernatant.

Finally, we have run live cell imaging experiments to record the antigen-specific cytotoxic activity of TCR gene modified NK-92 cells. In a two-hour imaging experiment, we were able to observe the rapid antigen-specific killing of A375(Tyr) cells by TCR-

expressing NK-92 cells while activity against the non-tyrosinase-expressing A375 cells was at a minimum. Images from the beginning and the end of the two-hour experiment with different targets and effectors are presented in **Figure 4.10** and the videos are available online.

A



B

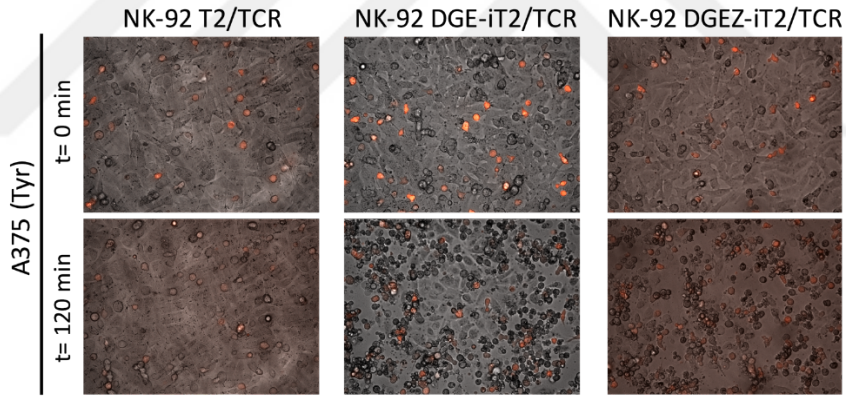


Figure 4.10. Live cell imaging of TCR expressing NK cells. Genetically modified NK-92 cells were mixed with A375 or A375 (Tyr) cells at 1:2.5 ratio and images were captured for 2 hours every 15 seconds. Representative images at the start of coincuboation (upper panel) and at the end of the experiment (lower panel).

5. DISCUSSION & CONCLUSION

According to latest statistics, newly diagnosed cases of cancer sum up to 1.6 million individuals in the US (2016 estimate - cancer.gov) and 174.000 in Turkey (2013 incidence - kanser.gov.tr). Reflecting the ageing population, this number is projected to grow dramatically in the near future. Besides the progress in the cure rate using better refined chemotherapeutics and targeted molecular therapies in low and middle risk patients; there seems to be no significant improvement in the high risk groups over the last two decades. For various malignancies such as high-risk leukaemia where patients relapse after stem cell transplantation, development of advanced therapies to fight minimal residual disease and recurrences are needed.

Adoptive immunotherapy is a rapidly growing field that presents promising novel approaches to cure a wide variety of cancers including solid tumors and hematological malignancies. Infusion of *ex vivo* activated and expanded NK cells, T cells (e.g. naïve, regulatory, antigen-specific), MSCs, DCs as well as gene-manipulated stem and effector cells are crucial personalized approaches in modern medicine. Recent years have seen a substantial increase in immunotherapy clinical trials making use of advanced therapy medicinal products (ATMPs) comprising *ex vivo* manipulated cells. Additionally, pre-clinical research is constantly aiming at better design and optimum preparation of such immunotherapy products with the ultimate goals of excellent cost-effectivity along with maximum therapeutic effect.

Expressing functional TCRs on NK cells stands out as a unique discovery combining robust and effective cytotoxic capacity of NK cells with exclusive antigen

specificity of T cells as a novel approach to develop cell-based immunotherapy of cancer and potentially viral infections such as HIV.

The two most common strategies for genetically targeting cytotoxic lymphocytes to specific antigens are the transfer of gene encoding TCR α/β heterodimers or CARs. While both approaches are being widely tested on T cells (Stauss, 2015), to our knowledge, TCR-mediated targeting of NK cells have been not reported so far. The transfer of CAR genes into NK cells and their use in adoptive immunotherapy has been primarily tested in animal models and more recently in various clinical trials with promising results (Dahlberg, Sarhan, Chrobok, Duru, & Alici, 2015). Zhang *et al.* has recently reported the redirecting of NK-92 cytotoxicity by genetic modification with a CAR derived from a TCR-like antibody against gp100/HLA-A2 complex (Zhang et al., 2013). While this study also claims to confer TCR-like specificity to NK cells, in essence the approach used is a CAR design where the antibody sequence is fused to CD3ζ intracellular domain to turn the antibody into a receptor and signaling is mediated through CD3ζ alone in a classical 1st generation CAR design.

Our approach provides a proof-of-principle for functional TCR expression on NK cells by making use of a TCR α/β heterodimer that functions independently of the co-receptors CD4 and CD8. While it remains unknown whether co-receptor dependent TCRs will work in the same manner by using endogenous CD4 or CD8 expression in NK cells, it is also possible to include CD4 or CD8 in the design of the genetic modification process should that be necessary. Testing this approach with different TCRs targeted against tumor-associated or viral antigens is warranted.

Another parameter to optimize in the case of TCR expression on NK cells is whether there will be mutual interference of this modification with the mechanisms of missing-self recognition (Ljunggren & Karre, 1990) in NK cells. In our experimental setting where T2 cells have almost no surface MHC expression due to TAP deficiency and NK-92 cells have no KIR expression except for low levels of KIR2DL4 (Maki, Klingemann, Martinson, & Tam, 2001), it is not possible to make any statements about missing-self recognition. Regardless, we see a high rate of degranulation when WT NK-92 cells are incubated with DMSO control T2 cells potentially due to CD40-CD40L interaction (Carbone et al., 1997; Maki et al., 2001). Despite this high background, TCR expression on NK-92 cells is still able to mount an impressive degranulation response against peptide-loaded T2 cells. In order to decipher the exact nature of the interactions

between MHC-restricted antigen-specific triggering of TCR expressing NK cells as shown here and the classical MHC-mediated inhibition of NK cells through KIR-ligand interactions, further investigations on KIR-expressing NK cells against MHC-expressing targets using genetically modified cell lines as well as primary NK cells are warranted.

The method presented here provides a proof-of-concept for functional TCR expression in NK cells. This is a very novel finding that has the potential to create a paradigm shift in the development of TCR gene therapy applications. TCR-modified NK cells have increased target cell killing (efficiency) and through specific interaction in between TCR and antigens, it provides accurate detection and targeting of tumor cells and virus infected cells. Most importantly, by using NK cells for TCR gene therapy, any risk of TCR mispairing will be avoided which makes the procedure safer. Detailed characterization of the effects of this genetic modification has to be carried out and compared in both safety and efficiency to the current approach of using T cells.

In conclusion, this study presents a novel approach in immunotherapy where we intend to overcome the major hurdle of "mispairing" in TCR gene therapy by turning our efforts towards using NK cells as a source that has similar cytotoxic capacity with T cells but present themselves baggage-free with no endogenous TCR expression. Further studies to better characterize the pros and cons of this approach compared to using T cells and direct comparison of the *in vivo* efficiencies are necessary.

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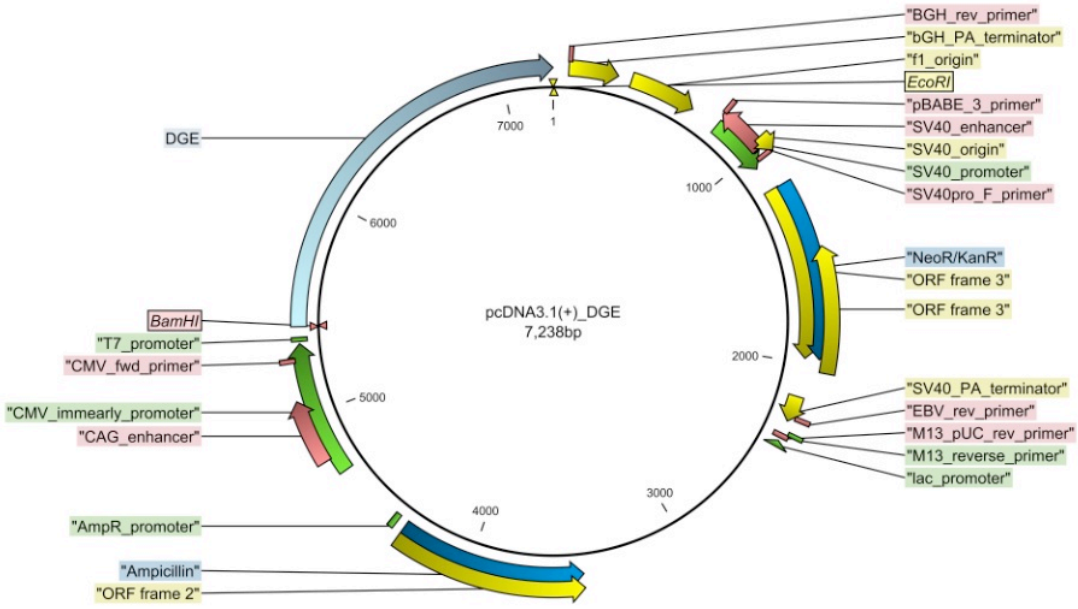
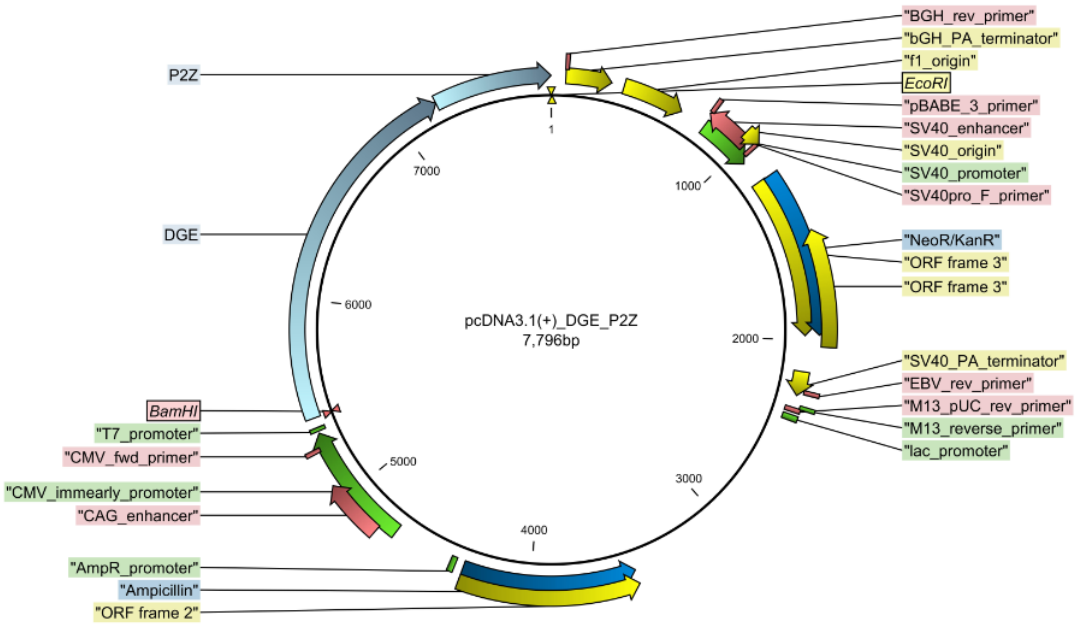
7. APPENDIX:

pcDNA3.1(+)DGE, pcDNA3.1(+)DGEZ, LegoiT2puro, Lego DGEiT2 puro, LegoDGEZiT2puro

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P2Z
DGE
"NeoR/KanR"
"ORF frame 3"
"ORF frame 3"
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"SV40_PA_terminator"
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"M13_pUC_rev_primer"
"M13_reverse_primer"
"lac_promoter"
BamHI
"T7_promoter"
"CMV_fwd_primer"
"CMV_immearly_promoter"
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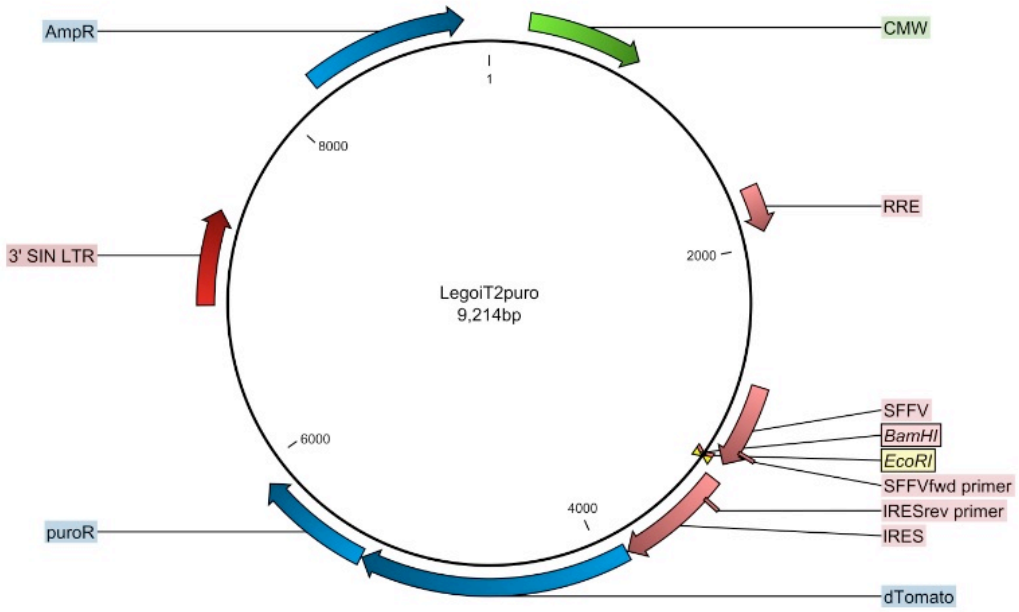
7000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IRES
puroR
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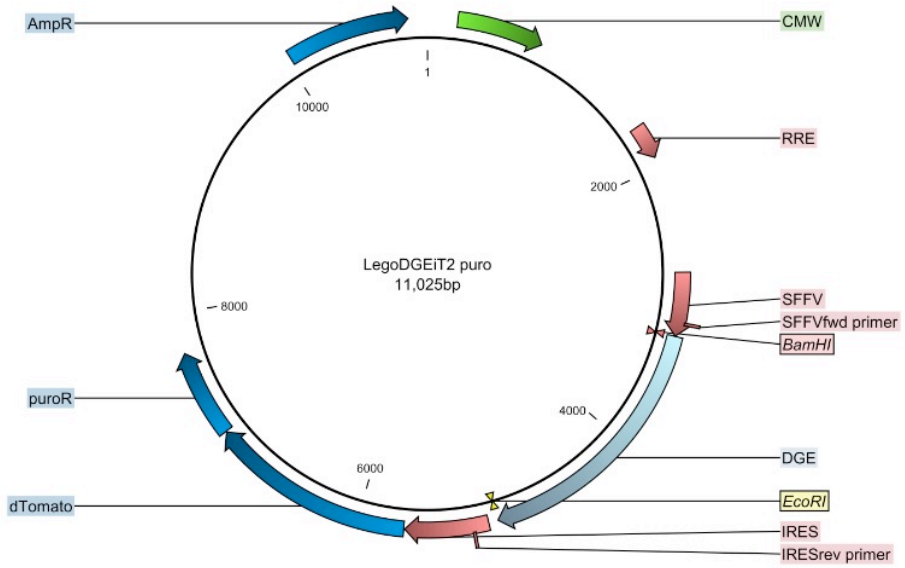
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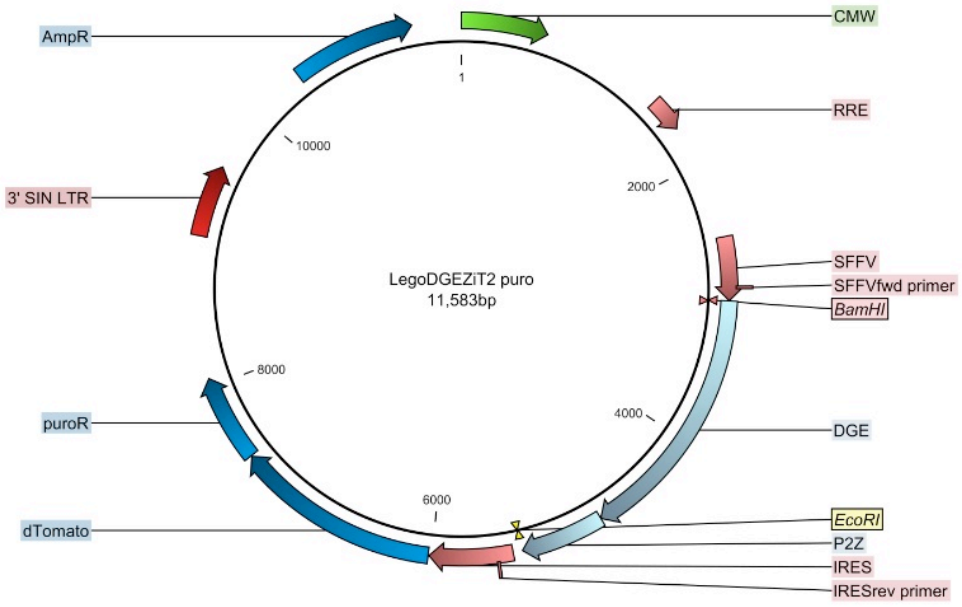
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