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Chimeric T cell receptors

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Abstract

The goal of effective immunotherapy is to restore the immune system's natural ability to recognize and eliminate transformed cells. The aim of this project was to develop a chimeric T cell receptor which would give the cytotoxic T lymphocytes a higher efficiency in destroying tumor cells. The chimeric T cell receptor was constructed by the fusion of the variable region of an antigen together with the intracellular part from an original T cell receptor.

Keywords

Bladder carcinoma, immune surveillance, immune escape, immunotherapy, chimeric T cell receptors, retroviral vector

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Chimeric T cell receptors

Sandra Andersson

Sammanfattning

I dagens svenska samhälle dör omkring 24% av befolkningen i cancer. Cancer är en sjukdom som inte kan botas på ett effektivt och för patienten skonsamt sätt. Även om man i vissa situationer lyckas bota cancer så söks fortfarande bättre behandlingsmetoder. Människans immunförsvar har förmågan att angripa de enskilda tumörcellerna och har därför potential att utrota cancer. Dessa immunattacker begränsas dock av tumörernas många sätt att skydda sig själva. Tumörceller uttrycker färre MHC klass I molekyler på sin cellyta, molekyler som är nödvändiga för att immunförsvarets T mördar celler skall känna igen dem. En annan försvarsmekanism är att de utsöndrar immunhämmande ämnen som kallas cytokiner. Detta gör att immuncellerna inaktiveras eller t.o.m. genomgår självdöd (apoptos).

Med immunterapi är målet att återställa immunförsvarets naturliga förmåga att känna igen och döda tumörceller genom att hjälpa immunförsvaret motstå tumörcellernas motattacker. I detta projekt konstruerades en specialiserad receptor till immunförsvarets T celler. Målet är att denna receptor ska kunna hjälpa T cellerna att känna igen tumörcellerna trots deras minskade uttryck av MHC klass I och att T cellerna bättre ska kunna undgå tumörernas hämmande cytokinutsläpp.

Examensarbete 20 p i Molekylär bioteknikprogrammet

Uppsala universitet februari 2006

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1 ABBREVIATIONS

amp	Ampicillin
APC	Antigen presenting cell
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DNA	Deoxyribonucleic acid
FACS	Flow cytometry
IL-1,2,3…	Interleukin 1, 2, 3…
LTR	Long term repeat
MACS	Magnetic cell sorting
MCS	Multiple cloning site
MHC	major histocompatibility complex
NK cells	Natural killer celler
PBMC	Peripheral blood mononuclear cell
TCR	T cell receptor
TGFβ	Transforming growth factor-β
T_H	T helper
T_K	T killer
T_{reg}	T regulatory

2 INTRODUCTION

2.1 Cancer

Cancer, a word that makes you react with a shiver. Almost everyone knows some individuals, friends or family members, who have been struck by the disease. The incidence of cancer rises every year. In the year of 2000, 24% of all deaths in Sweden were caused by cancer [1]. But what happens inside our bodies during an early stage of cancer? Is it possible that our own bodies have the ability to fight cancer? Research has shown that some patients' immune system can naturally recognize and eliminate tumor cells. By learning more about how this works, useful information can be gathered to develop effective immunotherapy for the treatment of cancer.

2.1.1 Classification

In the human body, millions of cells divide every minute. This happens normally in a predetermined restricted fashion, but mutations in the DNA may change this situation. A mutation is a permanent change in the DNA that can arise due to many different factors. One example is radiation, which can be absorbed by water molecules surrounding the DNA. When the electrons in the water get enough energy they will excite, leaving a free radical to attack the DNA.
Chemical mutagens can harm the DNA by interfering with correct nucleotide base pairing. When the mutation is located in genes controlling or affecting the cell cycle, the cycle can be disturbed and loose its ability to stop the proliferation when it is needed. The cell becomes malignant and starts dividing without restraint, thereby producing a tumor. The tumor will keep on growing, pushing the normal tissue aside [2, 3].

Cancer is classified in two different ways. It is either by the type of tissue it originates from or by the location in the body. There are five major groups from a histological point of view: carcinoma, sarcoma, myeloma, leukemia and lymphoma. Carcinoma, sarcoma etc. can further be located in many parts of the body such as the breast or bladder [4]. A carcinoma in the bladder is, hence, called bladder carcinoma. If the cancer form metastases, the second tumor bears the primary tumors name [5]. Today, this is the most common way of classifying cancer but the increasing knowledge of the disease makes way for new classification systems based on biological resemblance. For example, some tumors overexpress the Her2 receptor and independent of tumor origin the tumors can be called Her2-associated tumors.

2.1.2 Tumor immunology

Immune surveillance is the immune system's ability to detect and destroy tumor cells. It has been debated whether or not the immune system possesses such ability, but in recent years research is in favor of immune surveillance [6, 7]. But why do people get cancer then? Well, tumor cells can be very inventive when it comes to protecting themselves from the immune system. As described later, tumor cells can disguise themselves so they become invisible for the immune surveillance that patrols the human body.

The immune system reacts to danger signals from the human body and induces inflammation. The innate immune system is triggered with its effector cells such as macrophages and natural killer (NK) cells. The task of the effector cells is to recognize and kill cells that express "non-self" peptides. Effector cells destroy target cells either by engulfing them or by inducing apoptosis via death receptors. The dendritic cells (DCs) are also part of the innate immune system and function as professional antigen presenting cells (APC:s). As immature cells they have high phagocytic capacity, upon danger signals they mature and migrate to lymph nodes to trigger the adaptive immune system consisting of B- and T lymphocytes. The adaptive immune system can either be a cell mediated (T_H1) or a humoral (T_H2) response. [8, 9]. T_H1 and T_H2 are two antagonistic responses. The T_H1 response produces a cytokine profile that supports inflammation and cell mediated responses, while the T_H2 response on the other hand produces cytokines that mainly activate immune responses that depend on antibodies [10]. Effective anti-tumor responses are usually of TH1 type. The DCs present tumor peptides to T cells via MHC molecules and also give activation signals via costimulatory molecules. The T cells need two signals to be activated, one from the T cell receptor (TCR)/MHC interactions and the other from costimulatory molecules. The most important costimulatory signal is the crosslinking between CD28 and B7 molecules. T cells are divided into several subtypes. T killer (T_K) and T helper (T_H) cells utilize two different MHC molecules, class I or class II respectively. T_H cells are CD4 positive ($CD4^+$) and produce cytokines to enhance the T_H1 response when activated by the MHC class II, appropriate cytokines and costimulation. While the T_K cells are CD8 positive and get activated and differentiate into cytotoxic T lymphocytes (CTL) by TCR/MHC class I signaling. The CTLs will later expand and migrate to the tumor area where they can destroy the tumor cells. An immunological memory for the tumor-associated antigens may be formed during the response. In this way, the immune system will eliminate cancer progression in the future [9].

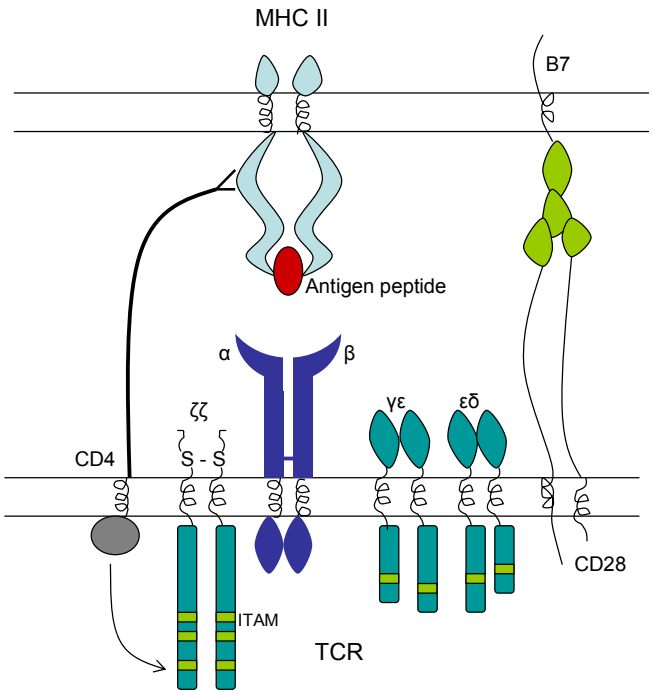

Figure 1. *The T cell receptor with its costimulatory signals. Interaction between the antigen peptide and the α and β chain of the TCR will induce an activation signal into the cell. The costimulatory signal, which in this case is represented by the interaction between B7 and CD28, will give the cell a second activation signal that is needed to fully activate the cell.*

However, the tumor cells can avoid detection by the immune system by several different strategies. The tumor cells are more genetically unstable compared to the rest of our normal cells, which means that a mutation in its DNA may cause the loss of ability to present antigens via MHC [7]. The processing and presentation of endogenous antigens is very important for the immune system to recognize transformed cells. The CTLs need the presentation of peptides from the MHC class I to recognize and, hence, destroy the tumor cell [10].

Cancer cells often have the ability to tilt the response towards T_H2 by making themselves and the cells in their surroundings produce cytokines such as IL10 and TGFβ. These cytokines have an overall negative impact on CTLs. The CTLs become anergic (nonresponsive) and without the CTLs the immune system fights in an inferior position [11]. IL10 and TGFβ also affect the T_H cells by turning them into T regulatory (T_{reg}) cells. The T_{reg} cells start to produce more IL10 and TGFβ, which will enhance the cytokine concentration even more. T_{reg} cells are $CD4^+$ and can be identified by the expression of Foxp3 and $CD25^{high}$.

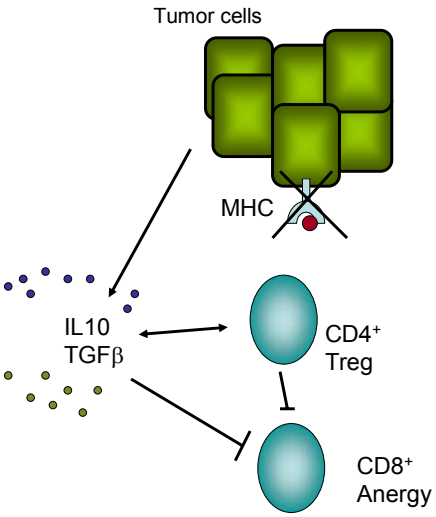

Figure 2. *Immune escape. Tumor cells downregulate MHC class I, which will cause a loss of antigen presentation. The downregulation will affect the T cells by making the recognition of tumor cells impossible. Production of IL10 and TGFβ will turn T helper cells into T_{reg} cells and also cause a state of anergy in CTLs.*

2.2 Immunotherapy

2.2.1 Clinical use

The goal of effective immunotherapy is to restore the immune system's natural ability to recognize and eliminate transformed cells by helping the system to overcome the tumor cells counterattack. Since the tumor cells have effective mechanisms for immune escape the immune system needs some help to recognize and kill tumor cells. Today, many different types of immunotherapy are being developed, such as cytokine therapy where stimulatory cytokines are administered systemically or into the tumor area. Cytokines of the immune system are particularly attractive candidates. Many cytokines either enhance or inhibit the T_H1 and T_H2 responses. Thus, the choice of cytokine can be based on the desired response to disfavor the disease [12]. Another method of immunotherapy utilizes monoclonal antibodies.

The purpose of this method is to select for a membrane molecule that is specific for certain tumor cells and create monoclonal antibodies for the molecule in question. It could for example be an anti-tumor monoclonal antibody for growth factor receptors. A monoclonal antibody for human epidermal growth factor receptor 2 (Her2), called Herceptin, is used as treatment today in patients with cancer of the breast [10, 13]. A third type of immunotherapy is accomplished by re-education and infusion of the patient's own CTLs. Blood is taken from the patient and sorted for T cells. The T cells are exposed to tumor antigen which will perhaps activate some of the cells. The cells that do react to the tumor antigen are isolated and expanded. The specialized T cells are then given back to the patient. If there are no T cells reacting to the antigens, the T cells can be genetically modified to express a tumor specific TCR. The genetically engineered T cell receptors are referred to as chimeric TCRs. When building a chimeric TCR, the intracellular endodomain of the TCR is joined to an antigen-recognizing ectodomain [14]. Chimeric TCRs will be described in more detail in the next section.

2.2.2 Chimeric TCR

2.2.2.1 Extracellular ectodomain

The ectodomain recognizes and binds the antigen. A natural TCR is composed of either α and β or γ and δ chains. These chains are responsible for the antigen specificity. The natural TCR ectodomain interacts with the MHC that in turn presents the antigenic peptide for the TCR. The interaction affinity is not as high as for antibody binding because of fewer combinations of gene rearrangements. In chimeric TCRs, the ectodomain is rebuilt to target a desired antigen. The ectodomain in a TCR can be replaced with the variable heavy- (V_H) and light (V_L) chains of an antibody, which would give the receptor higher affinity and selected specificity. When the ectodomain of the TCR is replaced with the single-chain variable (scFv) region of an antibody, the interaction with MHC is no longer necessary. However, the target antigen must be expressed on the tumor cell surface for CTL recognition and killing [14, 15].

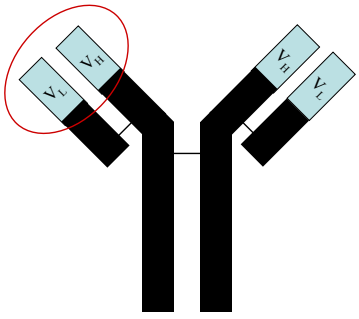

Figure 3. *An antibody with its variable chains encircled at the ends.*

2.2.2.2 Intracellular endodomain

Different intracellular endodomains can be utilized to ensure signaling into the T cell from the scFv. In this project, we decided to use the Zeta chain (see Figure 1, Zeta marked with the Greek letter ζ). When the ectodomain of the chimeric TCR is stimulated, the immunoreceptor tyrosine-based activation motif (ITAM) of the Zeta chain is phosphorylated by protein tyrosine kinases. This is the beginning of a chain reaction leading to cell activation and

proliferation. Transcription factors are activated and proteins will be produced, such as cytokines (IFNγ), death receptor ligands (FasL) and anti-apoptotic molecules needed for survival of the cell [10, 16].

2.2.2.3 Construction of a chimeric TCR

To construct a functional chimeric T-cell receptor, many different genes have to be fused together. All genes should be inserted one after another into a gene delivery vector without any stop codons except for after the last gene. Upon expression, the different genes will give rise to a fusion protein – the chimeric TCR (see Figure 4). The first gene in the vector construct is a signaling peptide. The signal peptide directs the receptor to the plasma membrane whereupon it is cleaved from the receptor. The second gene fragment is the gene encoding the antigen binding scFv. This gene determines the specificity of the chimeric TCR. Since the variable region consists of two chains, V_H and V_L, a linker is needed to keep them connected. In order to increase the receptor flexibility, a hinge region (also part of an antibody) is inserted. It is important that this part is neither too long nor too short, to maximize the flexibility. All these first genes are part of the exodomain, located outside the cell in the final receptor [14].
The gene encoding the Zeta chain is inserted after the hinge region. The Zeta chain has a transmembrane and an intracellular domain. To enhance signaling, the intracellular domain of the costimulatory molecule CD28 can be fused at the end of the chimeric TCR [17, 18]. Normally, there will be two signals activating the CTL, i.e. one from the natural TCR and the other from the costimulatory molecule CD28 (see Figure 1). The TCR and CD28 each initiate a specific activation pathway. In chimeric TCR signaling, hopefully only one ingoing signal is required to initiate the two activation pathways, see figure 4.

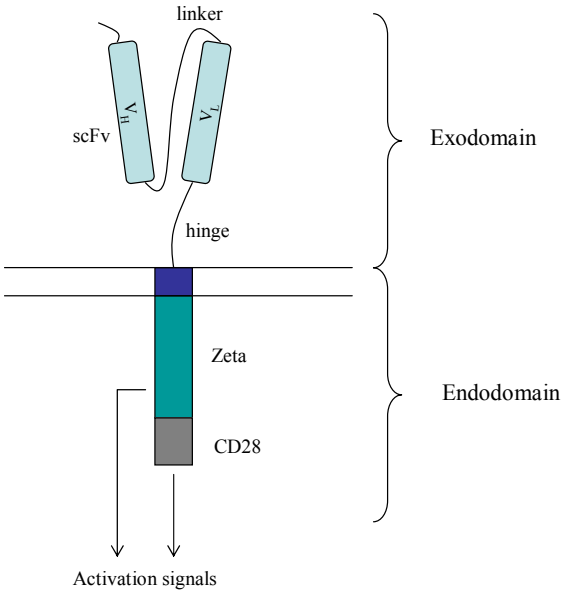

Figure 4. *A chimeric T cell receptor.*

2.3 Project description

2.3.1 Aim

This project consisted of two parts. The first part of the project was to construct a new chimeric TCR targeted for bladder carcinoma cells. The chimeric receptor was to be built to

recognize Her2, a surface protein expressed by bladder carcinoma cells. The construct for this chimeric TCR is in this report referred to as the Her2 vector. The second part of the project was to test the capacity of a previously constructed vector. This chimeric TCR was built to recognize a surface protein expressed on a subset of B cells named CD19. There are two variants of the CD19 receptor and the difference between these two was studied in this project.

In this project, all the genes required for a chimeric TCR needed to be inserted into a retroviral vector plasmid. This vector can be taken up by CTLs cells by virus transduction. The goal was then to have the CTLs start to express the chimeric TCR on their surface which will help the cells to recognize the tumor. The chimeric receptor included the variable chains of an antibody for binding to tumor cells, making MHC class I presentation unnecessary. The chimeric TCR can also be fused with the costimulatory molecule CD28, which may help the cell to withstand the suppressive effects of IL10 and TGFβ. The hope for this project was to show that the custom-made CTL will have the capacity to migrate to the tumor area and kill tumor cells.

2.3.1.1 The Her2 construction

The Her2 vector was designed to contain five genes. The first gene encodes a signal peptide which will direct the complex to the membrane. The ectodomain is taken from the variable region of an antibody against Her2, a cell surface protein expressed in bladder carcinoma. The hinge from an antibody is used to provide flexibility in all directions. The hinge can later be fused to a gene encoding the Zeta chain, a part taken from the original TCR. At the end of the chimera the gene for the costimulatory molecule CD28 will be inserted. The addition of CD28 helps T cells resist the IL10 and TGFβ secreted by tumor cells (Loskog, unpublished data). The stop codon for each gene except for CD28 should be removed.

The aim was to insert all the genes for the Her2 vector into a MLV backbone vector (Figure 5A). The MLV-backbone has an ampicillin (amp) resistance gene as well as two long terminal repeats (LTR) which contain the promoter region.

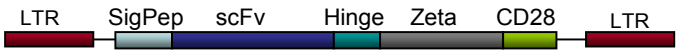

Figure 5A. *The Her2 vector, containing the five genes fused together. See text for further information.*

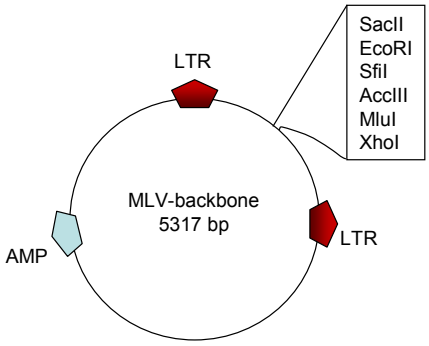

Figure 5B. *MLV backbone. Extra cloning sites have been added.*

Vector	Length (bp)	Restriction enzymes
Signal peptide	86	*Sac*II *Eco*RI
Heavy and light chain, scFv	700[1]	*Eco*RI *Sfi*I
Hinge region	83	*Sfi*I *Acc*III
Zeta chain	428	*Acc*III *Mlu*I
Costimulatory molecule CD28	137	*Mlu*I *Xho*I

Table 1. *The genes targeted for the construction have sites for restriction enzymes, which can be used for insertion into the MLV vector.*

2.3.1.2 The CD19 and CD19z28 vector

B cell malignancies are common in both childhood and adult life. A specific marker for B cells is CD19 which is a surface protein expressed on all B cells [19]. It is a good target molecule since it is expressed in both early and late stages of development. CD19 is important for B cell development, activation and differentiation [10]. If a CTL expressed a chimeric TCR directed towards the CD19 molecule all the B cell derived tumor cells could be eliminated. This would also mean that normal B cells also expressing CD19 would be affected by the treatment. However, since hematopoietic stem cells do not express CD19, they would survive and be able to produce new B cells after the treatment cycle.

At the Division of Clinical Immunology, Uppsala University, two CD19 directed chimeric vectors were already constructed at the start of this thesis project and they were ready for evaluation in vitro. The constructed vectors were similar to the Her2 vector except that the scFv region was taken from an antibody directed to the surface protein CD19 instead of Her2. The hinge region is a bit shorter than that in the Her2 construct, which may limit the flexibility of the molecule. The two versions of the CD19 construct differ in that one of them has the CD28 gene incorporated in the vector. In this report, the two vectors are referred to as the CD19 vector and the CD19z28 vector.

The aim of this project was to generate retroviral vectors coding the chimeric TCRs by a procedure described in more detail in a later section (2.3.1.3) and use them for transduction of CTLs. The chimeric T cells were evaluated for the expression of IL10 and TGFβ receptors. Since some results have indicated that chimeric T cells with the CD28 molecule resist the negative actions of IL10 and TGFβ, it was investigated whether the expression of IL10 and TGFβ receptors was downregulated in such T cells.

[1] May vary depending on target molecule.

2.3.1.3 Production of retroviral vectors

A relatively new method based on a three-plasmid system was used to produce retroviral vectors that could transfer the chimeric TCR genes into T cells. This method reduces the risk of unwanted recombination which may result in wild-type viruses spreading the chimeric TCR. The retroviruses are made from three plasmids; one containing the MLV-vector with the genes encoding the chimeric TCR, the second plasmid contains *gag* and *pol* and the last plasmid carries the *env* gene. The genes *gag, pol* and *env* are all essential to make a retrovirus. The *gag* gene encodes core and structural proteins while the *pol* delivers the reverse transcriptase, proteases and integrases. The ENV protein will provide the retroviral coat protein, which will build the envelope of the virus [20]. The three plasmids are cotransfected into 293T cells, in which the virus will be produced. The vector is harvested days 3 and 4. The virus is thereafter ready for transduction of T cells.

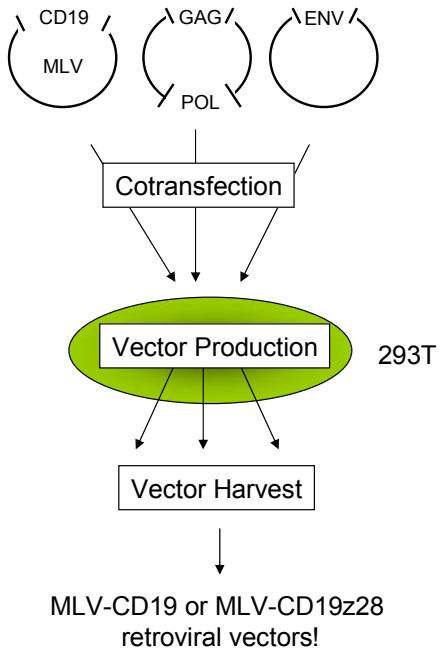

Figure 6. *Retroviral vector production using a three-plasmid system.*

3 METHODS

3.1 Construction of the vector

The MLV backbone, 3517 bp, was used to clone the chimeric TCR genes. All genes were taken from a cDNA library previously constructed in the laboratory. ScFv was cloned from hybridoma cells, the signal peptide and the hinge region from B cells (LCL), and the remaining genes from PBMCs. To insert each gene into the MLV backbone vector, the gene and vector was cut with the two restriction enzymes matching the gene's ends (see Table 2). If the two restriction enzyme buffers and preferred working temperature could be matched the two digestions were performed at the same time. If not, the digestion had to be divided in two steps with a DNA extraction in between using Gel extraction Kit, Omega Bio-tek.1.5 µl enzyme (BioLabs, Ipswich, MA) and 2 µl buffer were mixed with the DNA in a total volume of 20µl. The digestion reactions were incubated for one hour at the temperatures given in Table 2. The cleaved DNA was loaded onto a 1% agarose, Et-Br gel and run at 65V. The gel was analyzed in a UV light chamber.

Vector	**Size bp)**	**Restriction enzymes**	**Buffer**[2]	**Buffer temperature (°C)**	**Annealing PCR temperature (°C)**
Signal peptide	86	SacII *Eco*RI	4 1,2,3,4	37 37	58
Heavy and light chain, scFv	700[3]	*Eco*RI *Sfi*I	1,2,3,4 2,4	37 50	62
Hinge region	83	*Sfi*I *Acc*III	2,4 F	50 65	66
Zeta chain	428	*Acc*III *Mlu*I	F 3	65 37	62
iCD28	137	*Mlu*I *Xho*I	3 2,3,4 +BSA	37 37	65

Table 2. *Reaction conditions for different restriction enzymes and different DNA targets.*

Gel extraction

Bands of expected sizes were cut out from the gel and the DNA was extracted, using E.S.N.A.® Gel extraction Kit, according to the protocol provided by the company (Omega Bio-tek, Doraville, GA).

Ligation

The gene insert and the vector were ligated using 2 µl 5* T4 DNA Ligase Buffer and 2 µl T4 DNA Ligase (Invitrogen, Paisley, UK). The mixture was incubated 16 hours at 16°C.

[2] Recommended by the company (BioLabs, Ipswich, MA).
[3] May vary depending on target molecule.

Transformation of bacteria
GC5™ Chemically Competent Cells (Sigma, St. Louis, MO) were mixed with 4 µl of the ligated product from the previous step. The bacteria were incubated for 30 minutes on ice, followed by heat shock for 45 seconds at 42°C. The product was allowed to cool down on ice two minutes before 1 ml S.O.C. medium (Invitrogen, Paisley, UK) was added. The cells were incubated while shaking one hour at 37°C. 200 µl of the final solution was spread on agar plates containing LB+agar and 50ug/ml ampicillin. Plates were incubated over night at 37°C.

PCR Screening
Colonies from agar plates were screened for the desired gene. A solution of 0.15 µl Taq DNA polymerase (Invitrogen Paisley, UK), 2.5 µl 10* PCR reaction buffer (Invitrogen, Paisley, UK), 1.5 µl 50 mM $MgCl_2$ (Invitrogen Paisley, UK), 2 µl 5 mM dNTP, 12.35 µl H_2O, 2 µl of forward (Fw) and reverse (Rv) primer (cybergene, Huddinge, Sweden) and 2.5 µl DNA was run in a PCR machine. Annealing temperature was set according to the primers used, referred by the company, see Table 2.

Miniprep
Colonies which were positive in the screening, were taken to grow in liquid media (shaking at 37°C), in a solution of 2 ml LB media and 50ug/ml amp. The plasmids were extracted using Gen Elute™ Plasmid Miniprep Kit provided by SIGMA according company protocol (Sigma, St. Louis, MO).

Maxiprep
Vectors containing the right genes were amplified using EZNA® Plasmid Maxiprep Kit provided by Omega Bio-tek according company protocol (Omega Bio-tek, Doraville, GA).

3.2 PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (provided by the Uppsala University Hospital blood center). The buffy coat was diluted 1:1 with PBS and centrifuged 20 minutes at 1500 rpm without brakes on top of 10 ml Ficoll Paque (Amersham Bioscience AB, Uppsala, Sweden). The white fraction consisting of mononuclear blood cells was extracted, washed with PBS and centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded and the cells diluted with RPMI medium (Appendix 1). Cells were frozen at -80°C in freezing media (Appendix 1).

3.3 MACS sorting

Magnetic cell sorting (MACS) was performed on human lymphocytes according to company protocol. The selection was based on a system using Microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The magnetic beads were connected to αCD4- and αCD8 antibodies that isolated the desirable population through magnetic force. Cells were rinsed with MACS wash (Appendix 1) for higher purity. Once the desired population was obtained, human cells were diluted in RPMI medium (solution composition given in Appendix 1).

3.4 Cell culture

Frozen, sorted PBMCs from buffy coats, obtained in section 3.2, were thawed. Five million cells were diluted in 10 ml RPMI medium (Appendix1) and stimulated with OKT3 (Apoteket, Uppsala, Sweden) the first day. Cells were divided in three wells in a 12 well plate for incubation at 37°C. On day 3, 80 u/ml hIL-2 was added to the cells. At day 5, the T cells were ready for retroviral transduction, see section 3.6.

3.5 Virus-vector

1.5 million 293T cells were cultured in 10 ml DMEM medium (Appendix 1) in a Petri dish over night. 470 µl DMEM and 30 µl GeneJuice (provided by Novagene) was added to the three plasmid mixture. The plasmid mix consisted of 3.75 µg retrovector plasmid (MLV-bb-CD19 or MLV-bb-CD19z28), 2.5 µg PegPam containing the viral genes *gag-pol*, and 3.75ug of *env* encoding the viral gene envelope. For transduction of human cells, envelope RDF114 was used. The solution containing the three plasmids was dripped on 293T cells. 48 hours later, the first harvest was accomplished and new DMEM medium was added. After another 24 hours, a second harvest was done. Viral vectors were frozen at -80°C in aliquots of 3 ml.

3.6 Transduction

To prepare for the transduction, 1 ml PBS together with 25 µl RetroNectin (Göteborgs termofabrik, Göteborg, Sweden) was incubated at 4°C over night in 12-well plates. Next day, the vectors were thawed. 0.5 ml of virus solution was added to the PBS/RetroNectin and let to incubate half an hour at room temperature. The procedure was repeated once. The rest of the virus solution, 2 ml, was added together with the CD3 stimulated T cells. R10- or RPMI medium was added to a total volume of 3 ml. The plate was spun for 5 minutes to ensure contact between the RetroNectin, retroviral vectors and T cells.

3.7 FACS

200 000 T cells per FACS tube were used together with 2.5 µl of each antibody. CD4 and CD8 antibodies were marked with APC and IL10R with PE (BD Biosciences, San Jose, CA). The cells and antibodies were mixed and incubated at room temperature for 20 minutes. PBS was added and the cells were spun at 1500 rpm for 5 minutes. The supernatant was discarded and 250 µl FACS fix solution was added. The cells were analyzed by flow cytometry (FACSCalibur, BD Biosciences, San Jose, USA).

3.8 Staining

18 glass slides were prepared by cytospin, each containing 500 000 cells with either control (untransduced cells), CD19 or CD19z28 (transduced cells). Six glass slides per group represented TGFβRI, TGFβRI control, TGFβRII, TGFβRII control, TGFβRIII and TGFβRIII control. For TGFβRI, the antibody was diluted 1:200, TGFβRII 1:500 and TGFβRIII 1:100 as recommended by company protocols. Detection of the receptors was observed after the addition of the secondary antibody. For the TGFβRIII the secondary antibody step had to be divided into three parts, see Table 3.

Target	**Primary antibody Santa Cruz Biotechnology**	**Secondary antibody ENVISION**
TGFβRI	rabbit polyclonal IgG	goat anti-rabbit
TGFβRI control	PBS	goat anti-rabbit
TGFβRII	mouse monoclonal IgG	anti-mouse
TGFβRII control	PBS	anti-mouse
TGFβRIII	goat polyclonal IgG	rabbit anti-goat + rabbit serum, PAP
TGFβRIII control	PBS	rabbit anti-goat + rabbit serum, PAP

Table 3. *First- and secondary antibodies used in staining experiments with TGFβ receptors I-III.*

4 RESULTS

4.1 Results with Her2

4.1.1 Insertion of genes into the Her2 vector

After insertion of the Zeta gene into the MLV-backbone, the product was digested with the restriction enzymes *Acc*III and *Mlu*I. The results from the gel electrophoresis confirmed the presence of a band at 550 bp (Figure 7A-B). A second digestion with *Mlu*I and *Xho*I was performed for introduction of the iCD28 gene. Figure 7C demonstrates that during the *Mlu*I-*Xho*I digestion a molecule of 600 bp had been cleaved out from the MLV-backbone. The size of the molecule indicated that this was the Zeta gene. The MLV vector was extracted from the gel and run through a PCR with primers for the Zeta chain. The PCR results confirmed that the Zeta chain was no longer a part of the chimera. The procedure was performed a second time digesting the MLV-zeta product with *Sac*II and *Eco*RI. The results confirmed the previous results.

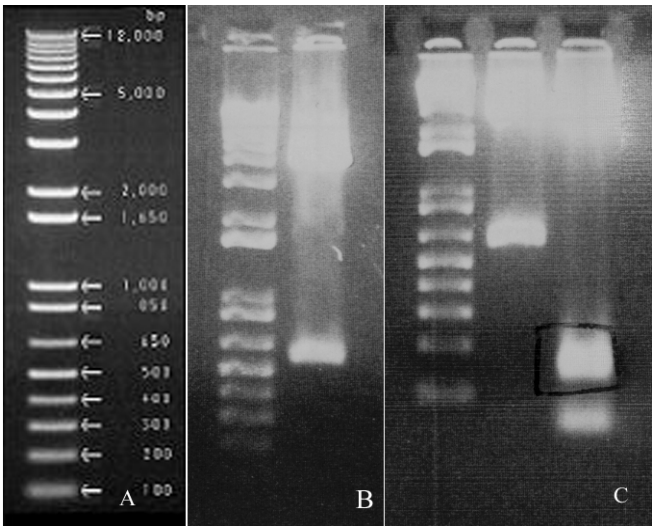

Figure 7. *A) 1 Kb Plus DNA Ladder, Invitrogen, Paisley, UK. B) Lane1: Ladder. Lane2: MLV-Zeta cleaved with AccIII and MluI. C) Lane1: Ladder. Lane2: MLV-Zeta cleaved with MluI and XhoI. Lane3: TOPO-CD28 cleaved with MluI and XhoI.*

4.2 Results with CD19 and CD19z28

4.2.1 Stimulation of PBMCs

In our experiments we wanted to introduce a chimeric TCR in CD8 positive T cells, therefore it was important to be able to culture high levels of $CD8^+$ cells. During cell cultures of PBMCs with OKT3/IL2 stimulation, the number of CD4 and CD8 positive cells were counted, see Figure 8. In a normal batch of PBMCs, about 30% are $CD4^+$ and 13% are $CD8^+$, making $CD4^+$ cells twice as common as $CD8^+$ cells. When stimulated with OKT3 and IL2 the $CD8^+$ population rises considerably, reaching a level of 90%. Thus, a selection for $CD8^+$ cells had been accomplished. After stimulation, cells were transduced with either CD19 or CD19z28, both encoding for the chimeric TCR. Cells transduced with the CD19 construct appear to lower the level of $CD8^+$ cells, noticeable but not significantly. The level of $CD8^+$ cells decreased further for cells transduced with CD19z28 but the decrease is not significantly different from the other two groups.

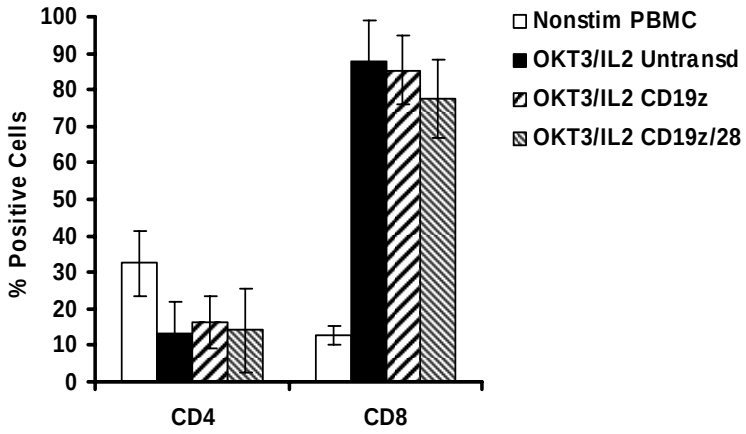

Figure 8. *Number of CD4 or CD8 positive cells before and after stimulation with OKT3 and IL2 and transduction of PBMC, measured by FACS.*

4.2.2 Transgene expression

Figure 8 demonstrates the transgene expression of the two vectors CD19 (A and B) and CD19z28 (C and D). The antibody used was specific for mouse IgG kappa of the light chain (B and D). Since the antibody detects mouse IgG kappa it demonstrated that the chimeric TCR had been correctly translated and assembled at the T cell's surface. Both vectors transduced around 25% of target cells.

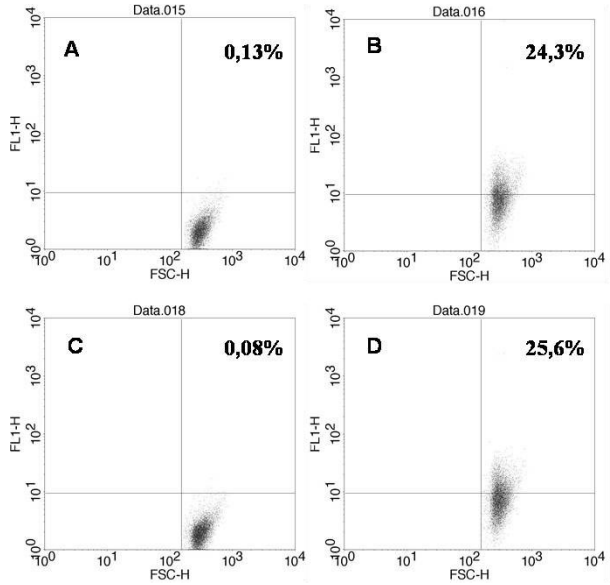

Figure 9. *Transgenic expression. A and B are CD19 transduced cells whereas C and D are CD19z28. A and C represent staining with negative control antibodies. B and D represent staining using mouse IgG kappa light chain directed antibody.*

4.2.3 Receptors for IL10 and TGFβ

By flow cytometry it was possible to display the IL10R expression of the cells. The histogram in Figure 10 shows the expression of IL10R in untransduced cells as the black solid line. The thin line represents the CD19 transduced cells while the dotted line correspond to the CD19z28 transduced cells. In the comparison between T cells transduced with either CD19 or CD19z28 no difference could be distinguished. Neither could a difference be seen between the untransduced and transduced cells.

T cells were also stained with antibodies detecting TGFβ receptor I-III, see Figure 11. All T cells were positive for TGFβRII and negative for TGFβRI and TGFβRIII. No difference in expression of the TGFβRII could be seen in transduced T cells.
A decrease in the amount of IL10 and TGFβ receptors in transduced cells could have explained why these cells seem to be less sensitive against tumor cells, but since no difference could be seen other possible explanations have to be invented and investigated.

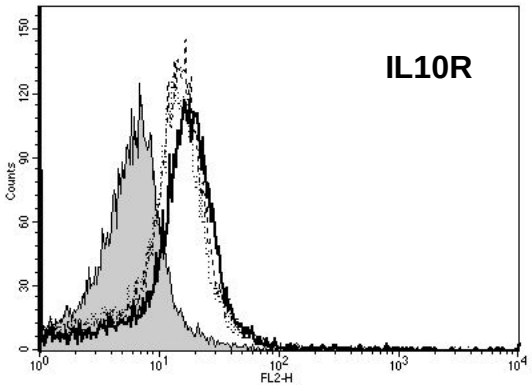

Figure 10. *FACS histogram demonstrating the IL10R expression in CD8+ cells. Black line showing untransduced cells, thin line CD19 transduced cells and the dotted line CD19z28 transduced cells.*

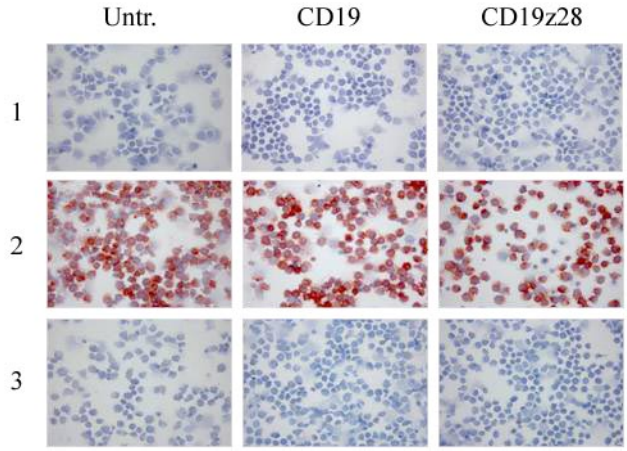

Figure 11. *Staining of T cells for TGFβR. Untransduced cells, CD19 transduced cells and CD19z28 transduced cells were stained with antibodies for TGFβ receptor I-III. All T cells were positive for TGFβRII and negative for TGFβRI and TGFβRIII. (Numbers 1-3 in the figure represent TGFβ receptors I-III.)*

4.2.4 Cytotoxicity study

A cytotoxicity study was performed with cells transduced with the two vectors CD19 and CD19z28. T cells with the two chimeric TCRs were tested for their ability to lyse $CD19^+$ Daudi cells (tumor cells). Figure 12 shows the efficiency in killing tumor cells with or without the presence of T_{reg} cells. The results indicate that CD19 T cells were greatly influenced by the presence of T_{reg} cells. In the presence of T_{reg} cells which produce IL10 and TGFβ, only 30% of the tumor cells were eliminated. T cells with the CD19z28 chimeric TCR however exerted their killing independently of T_{reg} cells.

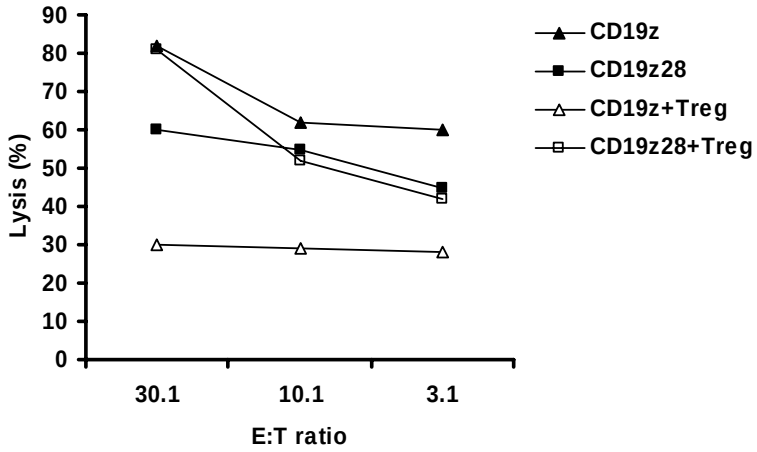

Figure 12. *Cytolytic function of T cells with two chimeric TCRs. The E:T ratio represents the number of T cells to Daudi cells quota.*

5 DISCUSSION

The Her2 vector is not yet ready for *in vitro* evaluation. The results obtained during the reconstruction have been contradictory, making progress difficult. The main struggle was to confirm the insertion of genes. At different occasions the MLV vector was sent for sequencing to the Uppsala Genome Center. The results never confirmed our own data from our experiments. The absence of uniform results slowed down the process considerably. In the end we decided to continue with the chimera without sending samples for sequencing, but instead trust the PCR screening results. The decision was also influenced by the fact that there were practical problems at the sequencing center at this time.

The decision to use a retroviral vector to transfer the chimeric TCR genes into T cells gave us some drawbacks. The multiple cloning site (MCS) can sometimes alter, making it hard to introduce new genes into the vector. Consequently, it was decided to move the MCS into another plasmid. An alternative way is to insert all the genes in the plasmid and later transfer the whole construct back to the MLV vector, a strategy which will be tried next in this project. Some experiments also indicated that the enzymes that have been used may not have worked properly. Due to these problems we decided to only use enzymes supplied by Promega and after that there have been no problems connected with the restriction cleavages.

Selecting for $CD8^+$ cells during cell culture with OKT3 and IL2 stimulation was feasible. Cells transduced with CD19z28 contained approximately 80% $CD8^+$ and 10% $CD4^+$ cells. Even though $CD8^+$ cells are desired, the presence of $CD4^+$ cells does not necessarily hamper the treatment. A small number of $CD4^+$ cells may have a positive influence in the destruction of tumor cells. $CD4^+$ cells with the chimeric TCR may interact with the tumor leading to an increased production of cytokines and enhancing the T_H1 response. The ratio of 10% CD4+ and 80%CD8+ cells may possibly be an advantageous distribution. FACS evaluation confirmed that the chimera was assembled and expressed at the surface of T cells. The chimeric TCR transgene expression was approximately 25%, which is an acceptable value after retroviral transduction. Nevertheless, the procedure will be modified in the future to achieve even higher transduction frequencies.

In the beginning of this project, we hoped to find an answer to why T cells transduced with the costimulatory molecule CD28 become less sensitive to IL10 and TGFβ produced by the tumor. A possible explanation could be a downregulation of the IL10R and TGFβR on the T cell. The results from the staining of CD19- and CD19z28 vector confirm the presence of TGFβRII is in all T cells, but not TGFβRI or TGFβRIII. The TGFβR is not well studied on cells of the immune system, so all observations are interesting. There was no obvious difference in the comparison between the expression of TGFβRII on CD19 and CD19z28 engineered T cells. Neither was the IL10R differently expressed by such T cells. It appears as if the decrease in response to IL10 and TGFβ is not caused by a downregulation of the IL10 or TGFβ receptors. Although no downregulation of the IL10 or TGFβ receptors could be established, the cytotoxicity study still demonstrated the lack of inhibition of CD19z28 CTLs in the presence of IL10 and TGFβ producing T_{reg} cells. This means that the CD19z28 CTLs protect themselves from T_{reg} cells by other means. It is possible that the activation status of the CTLs is increased by CD28 addition and that this generates T_{reg} cell resistant CTLs.

The results presented in this thesis are promising. This method, using chimeric TCRs, is a relatively tumor-specific treatment which yields an effective lysis of tumor cells (Figure 12). So far, no cancer therapy is 100% effective. Today, many different treatments using immunotherapy are developed and tested. A major concern is that immunotherapy is mostly tested in patients who have no other alternatives and represent end-stage disease. At this stage the immune system is severely impaired. The current approach to engineer and expand robust killer T cells in vitro for reinfusion to the patient may prove to be an effective strategy.

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7 APPENDIX

MACS
0.5g BSA
0.4ml EDTA (0.5M)
100ml PBS

RPMI medium
10% FBS
1% Pest
89% RPMI 1640, +L-Glutamine

DMEM medium
10% FBS
1% PEST
0.01% NaPyr
88.99% D-MEM, +4500 mg/L Glucose, +GlutaMAXTM 1, -Pyruvate

Freezing media
20% FBS
10% DMSO
70% RPMI

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