BIOENGINEERED T CELLS FOR LEUKAEMIA AND LYMPHOMA

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Abstract

Cancer immunotherapy is a promising tool for treatment of malignancies. However, there are still hindrances that need to be overcome. Chimeric antigen receptors have the ability to direct immune cytotoxic cells towards tumour-associated antigens in major histocompatibility complex-independent manner. In this study 2 generations of such receptor-bearing T cells, against the CD19 B-cell marker, were investigated for treatment of chronic lymphocytic leukaemia. The 2nd generation of this genetically engineered T cell contains CD3ζ and CD28 intracellular domain, while the third generation has CD137 (4-1BB) in addition. Previous studies have demonstrated advantages of 2nd generation chimeric antigen receptor T cells compared with 1st generation. In this project the 2nd and 3rd generation T cells were compared for transduction efficiency, phenotype, proliferative capacity and cytotoxicity in response to antigen from a malignant B cell line.

The analysis of transduction showed similar transduction efficiency for both types of chimeric antigen receptor. However, the data from T cell phenotyping and cytotoxic analysis could not be used for drawing any conclusion, because of too little amount of samples and subsequently, lack of statistical analysis. Further, the proliferative capacity was similar between all transduced T-cell groups and did not give any conclusive data. The next step will be to stimulate the 2nd and 3rd generation T cells with autologous target cells and follow them for a longer time since allogeneic tumour cell lines trigger an alloreactivity that may mask the different activation states that may occur in the two T cell products.
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Abbreviations

APC – antigen presenting cell
AP-1 – activation protein 1
CAR – chimeric antigen receptor
CCR – CC chemokine receptor
CD – cluster of differentiation
CFSE – carboxyfluorescein diacetate succinimidyl ester
CLL – chronic lymphocytic leukaemia
CTL – cytotoxic T lymphocyte
DAG – diacylglycerol
DNA – deoxyribonucleic acid
FACS – fluorescence-activated cell sorting
FBS – foetal bovine serum
Fc – fragment crystallisable
FoxP3 – forkhead box protein 3
HC – healthy control
IKK – I kappa B kinase
IL – interleukin
IP₃ – inositol 1,4,5-trisphosphate
ITAM – immunoreceptor tyrosine-based activation motif
JNK – Jun N-terminal kinase
LS – large scale
MACS – magnetic activated cell sorting
MAPK – mitogen-activated protein kinase
MHC – major histocompatibility complex
MLV – murine leukaemia virus
NFAT – nuclear factor of activated T cells
NF-κB – nuclear factor kappa B
PBMC – peripheral blood mononuclear cell
PIP₂ – phosphatidylinositol 4,5-bisphosphate
PKC – protein kinase C
PLCγ1 – phospholipase Cγ1
SAPK – stress-activated protein kinase
scFv – single chain fragment variable
TAA – tumour associated antigen
Tc – T cytotoxic cell
T_{CM} – central memory T cell
TCR – T cell receptor
T_{EM} – effector memory T cell
TGF-β – transforming growth factor beta
Th – T helper cell
TNFR – tumour necrosis factor receptor
TRAF – tumour necrosis factor receptor-associated factor
ZAP70 – ζ chain-associated protein kinase 70
1 Introduction

The immune system is capable to distinguish malignant cells from healthy ones (Totterman et al., 2005; Seliger, 2008), and hence, has attracted attention of many scientists for cancer research. Tumour cells produce specific proteins called tumour-associated antigens (TAAs). These TAAs can be processed and presented by major histocompatibility complexes (MHC) of tumour cells or, when engulfed, on antigen presenting cells (APCs).

MHC molecules are membrane bound protein complexes which have a special groove for antigen binding and presentation to T cells. There are 3 classes of MHC molecules: class I, class II and class III. Only first 2 classes participate in antigen processing and presentation. Class I MHC molecules are expressed on almost all nucleated cells and carry out endogenous antigen processing pathway. They present antigen to cytotoxic T cells (Tc). Class II MHC molecules are located only on professional APCs and realise exogenous pathway of antigen processing and presentation. They present the antigen to helper T cells (Th). The T cells contact with the presented antigen via T cell receptors (TCRs) and together with help of co-receptors get activated and start to produce cytokines which further differentiate them and help B cells to produce antibodies (Abbas et al., 2010).

However, the tumour tissue has the ability to regulate the immune system and to remain in a shadow. Some of the mechanisms by which the malignant cells escape immune attack are as follows:

1. Down regulation of MHC molecules on the malignant cells and inhibition of TAA expression, processing and presentation. These events could happen on different levels: epigenetic, transcriptional or post-transcriptional, where some components of antigen processing and presentation machinery are not synthesised (Seliger, 2008).
2. Regulatory T cells (Tregs). The Tregs are characterised by expression of CD25 and transcription factor FoxP3. Normally, these cells are required for controlling immunity and protection of the organism from auto reactive T cells. Their production is stimulated by self-antigens (Nilsson et al., 2009). TAA-specific Tregs are found in tumour environment. Tregs suppress immunity via production of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β; Totterman et al., 2005).
3. Tumour microenvironment is enriched by Th2 cells which secret immune suppressive cytokines such as IL-10, IL-5 and IL-4. IL-10 suppresses expression of MHC class I molecules and might increase tolerance of the malignant cells towards apoptosis (Park et al., 2009; Totterman et al., 2005).
4. Decrease of expression of co-stimulatory molecules, which are important for T cells activation (Totterman et al., 2005).
1.1 Immunotherapy of Cancer

For successful immunotherapy of cancer at least some of these immunosuppressive mechanisms should be overcome. One of the approaches is to direct immune cells towards tumour in an MHC-independent manner. For this reason, T cells have been designed with a membrane bound antibody against the TAAs that are expressed on the surface of tumour cells in a non-MHC restricted manner. This antibody is called chimeric antigen receptor (CAR) and the T cells bearing it are called CAR T cells.

CARs are composed from extracellular antibody, transmembrane and intracellular signal transduction parts (fig. 1). The extracellular part consists of an antigen binding part, which is a murine single chain variable fragment (scFv) of both light and heavy chains and a sequence of amino acids, connecting the scFv part to transmembrane part. That sequence may be from hinge part of antibody giving flexibility to the scFv portion.

CARs are composed from extracellular antibody, transmembrane and intracellular signal transduction parts (fig. 1). The extracellular part consists of an antigen binding part, which is a murine single chain variable fragment (scFv) of both light and heavy chains and a sequence of amino acids, connecting the scFv part to transmembrane part. That sequence may be from hinge part of antibody giving flexibility to the scFv portion.

In this study B cell malignancy was investigated and the CAR T cells were designed against CD19, which is presented on the surface of all mature B cells, either normal or malignant (Kohn et al., 2011; Kalos et al., 2011). There are 3 generations of CAR T cells which differ one from another by their intracellular signalling domains (fig. 1). In our study the extracellular part consists of a murine single-chain fragment variable (scFv) connected to a transmembrane domain via a hinge region. The hinge is derived from a fragment crystallisable (Fc) portion of human immunoglobulin IgG1. The transmembrane domain is from CD28. The intracellular part of CAR from first generation of T cells is composed from ζ chain of CD3 molecule from TCR (fig.1A). The second generation CAR T cells have in addition signalling domain of CD28 molecule (fig 1B). The last is a co-stimulatory molecule that is normally found on the surface of T cells. During antigen presentation, interaction of the CD28 with B7 ligand on APC gives secondary co-stimulation for T cell activation. Many authors have shown that CAR T cells of second generation have better proliferation and anti-tumour effect compared with that of the first generation (Loskog et al., 2006; Cartellieri et al., 2010; Savoldo et
al., 2011). CAR T cells of the third generation have some additional signalling
domain from other stimulatory molecules; in our case it was CD137 (4-1BB; fig.
1C). The sequence of the intracellular domains linked to each other is exactly the
same as illustrated in fig. 1.

1.2 Signalling Transduction of T cell Co-Receptors CD3, CD28 and 4-1BB

Normally, TCR is complexed with CD3 as the last has longer cytoplasmic tail and
can initiate signalling transduction. Cytoplasmic part of ζ chain of CD3 molecule
contains 3 immunoreceptor tyrosine-based activation motifs (ITAMs). Activated
ITAMs induce signals for activation of certain genes via Ca-dependent, MAPK
and PKC (DAG) pathways (Abbas et al., 2010). After TCR-antigen/MHC binding,
ITAMs of ζ chain of CD3 get phosphorylated by tyrosine kinases and attract ζ
chain-associated protein kinase 70 (ZAP-70). Then, the activated ZAP-70
phosphorylates phospholipase Cγ1 (PLCγ1). This leads to hydrolysis of
phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into diacylglycerol (DAG) or/and
into inositol 1,4,5-trisphosphate (IP$_3$). The last leads to activation of nuclear factor
of activated T cells (NFAT) via Ca-dependent pathway. The DAG activates
protein kinase C (PKC), which leads to release of NF-κB from IκB. The free NF-
κB enters into nucleus. Both, NFAT and NF-κB activate genes which are
responsible for T cell proliferation and differentiation, for example IL-2. The other
pathway of T cell activation is via G protein Ras, which leads to mitogen-activated
protein kinase (MAPK) cascade and consequent fusion of transcriptional factors
Jun and Fos into activation protein 1 (AP-1) dimer, activator of many genes
including IL-2. Abbas et al., 2010; Cronin and Penninger, 2007).

Usually, the first signal that T cell gets from TCR-CD3 complex is not enough for
their further activation and proliferation. Second, so called co-stimulatory signal is
needed for final T cell response upon peptide antigen activation (Schwartz et al.,
1989). The main co-stimulator known up to date is CD28 molecule which gets
activated once bound to B7 molecule expressed on APCs. Signals from this
interaction lead to higher affinity binding of TCR with MHC/peptide complex and
induce T cell proliferation via IL-2 secretion and survival via anti-apoptotic Bcl-
X$_L$ molecule production Boomer and Green, 2010). However, the activation of
CD28 is possible also without participation of TCR (Luhder et al., 2003).

CD137 also called 4-1BB belongs to tumour necrosis factor receptor (TNFR)
superfamily and is a co-stimulatory molecule for T cells. It was shown that 4-1BB
deficient mice have decreased CTL activity and cytokine secretion (Kwon et al.,
2002). The CD137 provides long term survival signals for CD8+ T cells. The
intracellular part of 4-1BB have 2 binding sites for tumour necrosis factor
receptor-associated factor 2 (TRAF2). The binding of TRAF2 leads to activation
of JNK/SAPK pathway of AP-1 transcription factor activation and IL-2 synthesis.
The 4-1BB also leads to activation of NF-κB via IKK pathway. However, 4-1BB may have also the opposite effect via its death domain providing signal for apoptosis. 4-1BB may replace CD28 co-stimulation in the sense of IL-2 production, but apart from CD28, which gives induction of immune response, 4-1BB keeps the process ongoing giving survival signal to T lymphocytes. (Kwon et al., 2000). Melero et al. have shown that signals from 4-1BB lead to rapid tumour eradication (Melero et al., 1997).

The aim of this project was to compare CAR T cells from second and third generations. The specific aim was to create genetically engineered T cells of second (with CAR containing ζ chain of CD3 molecule and signalling domain of CD28), and third (containing signalling domain of CD137 in addition) generations using murine leukaemia viral (MLV) vector, to test the presence of the CARs by flow cytometry, to study different phenotype development during culture and proliferation and cytotoxic capacities. In the future it is planned to use these 2 generations for in vivo tumour eradication assessment on immunodeficient mouse model with q-PCR in the tumour site for the existence of CAR T cells.

2 Materials and Methods

2.1 Cells and Media

For propagation of plasmids carrying target gene and genes of MLV vector, OneShot® Stbl3™ Chemically Competent E. coli was used from Invitrogen™. For the experiments, following cell lines were used: human embryonic kidney cells (293T, ATCC®), malignant B cell line Karpas-422 (a kind gift from prof. K. Nilsson, Uppsala University) and CD19 negative K562 cell line (human bone marrow chronic myelogenous leukemia cell line, from ATCC®). Peripheral blood mononuclear cells (PBMCs) were isolated from 4 CLL patients (CLL W 84, CLL W 69, CLL W 74 and CLL M 61) and 4 healthy donors (HC M 61, HC W 70, HC W 72 and HC W 66) using Ficoll-Paque separation. Cells were frozen in freezing media containing 10% DMSO and they were stored at -80 °C until use. All the mentioned cells apart bacterial cells and 293T cell line were thawed and cultured in RPMI 1640 medium ((Cat.: 21875-034.) 500 ml. GIBCO®, Invitrogen™) supplemented with 10% of fetal bovine serum (FBS) and 100 U/ml penicillin and 100 µg/ml streptomycin. The 293T cell line was thawed and cultured in IMDM medium supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin. All the cells were cultured in 75 cm² (T75) flasks in 12 ml of the complete medium mentioned above. They were split 1:5 at each third day. Bacterial cells were maintained in LB medium containing 50 µg/ml ampicillin and in LB agar containing 100 µg/ml ampicillin (in-house product, Rudbeck core facility).
2.2 Plasmid Vector Propagation and Isolation

For production of murine leukaemia virus (MLV), 3 different plasmids were used: RDF114 plasmid containing gene for viral envelop, PegPam3 plasmid containing gene for gag-pol, and MLV vector with target gene (2\textsuperscript{nd} or 3\textsuperscript{rd} CAR) or empty vector (Mock). All the plasmids were a kind gift from prof. M.K. Brenner (Baylor College of Medicine, Houston, USA). The OneShot\textsuperscript{®} Stbl3\textsuperscript{™} Chemically Competent E. coli was transfected by these plasmids separately using heat-shock method and Super Optimal broth and Catabolite repression medium (SOC-medium). Then the transfected cells were selected on LB agar containing 100 µg/ml ampicillin overnight. Then one middle-size colony was incubated in 3 ml LB medium with 50 µg/ml ampicillin and cultured for 6-8 hours followed by reseeding 100 µl of the culture into 100 ml of new LB media and overnight growth in shaker at 37 °C. The plasmid DNA was isolated next day according QIAGEN\textsuperscript{®} Plasmid Plus Maxi Kit method. The concentration of the isolated plasmids was measured by NanoDrop 1000 Spectrophotometer and software ND 1000 version 3.7.0., from Thermo Scientific.

2.3 Production of MLV Retroviruses

The day before transfection of 293T cells, 2.5 x 10\(^6\) cells were seeded into 10 cm tissue culture Petri dishes in 11 ml of complete IMDM.

The transfection was done with the help of GeneJuice\textsuperscript{®} Transfection Reagent (Cat.: 70967-3, Novagen\textsuperscript{®}) according to the protocol. The plasmids were added according to the following proportion: 3.75 µg PegPam3 containing gag-pol genes, 2.5 µg RDF containing env gene and 3.75 µg Mock or CAR containing plasmid. After transfection, the 293T cells were cultured in incubator and supernatant containing viruses were collected on the second and third days after transfection. The supernatants were frozen in freezer at -80 °C and the cells were discarded in virkon solution.

2.4 CD19 Depletion and Stimulation of PBMCs

One day before PBMCs thawing, a non-tissue culture treated 24-well plate was coated with OKT-3 at 1µg/ml, 0.5 ml per well (LEAF\textsuperscript{™} Purified anti-human CD3. Clone: OKT-3, Cat.: 317304, Conc.: 1 mg/ml. BioLegend).

PBMCs, especially from CLL patients contain high level of B cells. In order to increase transduction efficiency and improve T cell expansion, the CD19 positive
cells should be depleted from the PBMCs before transduction. The PBMCs were thawed and washed and counted. The CD19 positive cells were depleted by means of anti-CD19 microbeads during magnetic activated cell sorting (MACS) from Miltenyi Biotec. LS columns were used for the separation. Cells stuck in LS columns were discarded and the collected fraction was washed, counted and seeded 2 ml per well into OKT-3 coated wells with cell concentration of 0.5 x 10^6/ml. Because of not enough amount of cells from CLL W 74 and CLL M 61 individuals, their PBMCs were not used further in experiments.

2.5 Transduction of PBMCs

The CD19 depleted and OKT-3 stimulated PBMCs were transduced with the MLV retrovirus carrying either gene for CAR of 2nd generation, or gene for CAR of 3rd generation, or Mock (for negative control). The transduction was done in retronectin (RetroNectin®, TAKARA) coated non-tissue culture treated 24-well plates. Initially, 0.5 ml viral supernatant was incubated per retronectin coated well for half an hour twice. Then in each well 0.5 x 10^6 PBMCs were plated in total 2 ml volume containing 1.5 ml retroviral vector supernatant. In the end 100 U/ml IL-2 (obtained from Apoteket AB) was added per well for stimulation of cell growth and the plates were spin down at 1000 x g for 5 min. The plates were kept in incubator at 37 °C.

2.6 CFSE Proliferation Assay

Transduced T cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). The staining was done according protocol from Molecular Probes™ (CellTrace™ CFSE Cell Proliferation Kit (C34554)). Cells from HC W 72 individual were not enough for the proliferation experiment and they were excluded. After staining, the cells were plated into 24-well plates for co-culturing for 5 days at 1:1 ratio with Karpas-422 as CD19^+ cells and with K562 as CD19^- cells. Also, they were plated into separate plate for culturing alone, without any target cell. All cells were stimulated with IL-2 at 50 U/ml concentration. After 5 days the cells’ proliferation was analysed by flow cytometry.

2.7 ^51^Cr Release Cytotoxic Assay

The cytotoxicity of the transduced cells towards CD19 target cells was analysed by radioactive ^51^Cr release assay. The ^51^Cr radioactive isotope in form of sodium chromate solution was obtained from Perkin Elmer, Boston, Mass (Cat.: NEZ030S005MC). Effector cells were the transduced T cells and target cells were Karpas-422 and K562 cells. The target cells were incubated in radioactive ^51^Cr solution for 2 hours with the dosage of 0.25 mCi per 10^6 cells. After the
incubation, target cells were washed 4 times and re-suspended at concentration of 10,000 cells/ml. The effector cells were co-cultured with target cells in triplicates in V-shaped 96-well plates at 30:1, 10:1, 3:1 and 1:1 ratios. In each plate in addition 6 wells of target cells were plated: in 3 of them Tween-20 (Triton® X-100 Merck KEBOLab Spånga, Cat.: 16559-1, UN 3082) was added to kill all the cells (for maximum release) and in the other 3 wells nothing was added (for spontaneous release, as negative control). Cells were co-cultured for 2.5 hours and supernatant was harvested into 96-well Isoplate™ Wallac, Turku, Finland. For analysis Optiphase Supermix scintillation fluid (Perkin Elmer) was added. The radioactivity was measured in 1450 MICROBETA Wallac Trilux liquid scintillation and luminescence counter (Perkin Elmer™ life sciences) with the help of software Wallac 1450 Microbeta Windows Workstation version 4.0.

The values from β-counter were converted into percentage of killed cells via the following formula:

\[
\%\text{cytotoxicity} = \frac{\text{sample value} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

### 2.8 Flow Cytometry

For flow cytometry phenotyping, the transduced PBMCs, Karpas-422 and K562 cell lines were used. The CAR was detected by goat anti-human scFv-Dylight649 antibodies from Jacksson ImmunoResearch Laboratories, Inc. Following mouse anti-human antibodies from (BioLegend or BD) conjugated with dyes were used:, CD3-PE, CD3-APC, CD3-PerCP, CD4-APC/Cy7, CD8-PE/Cy5, CD19-APC, CD45RA-APC/Cy7, CD27-PE, CD28-PE/Cy7, CCR7-PerCP/Cy5.5. As negative controls for non-specific binding the following γ-isotypes were used: γ-PE, γ-PE/Cy7, γ-PerCP/Cy5.5, γ-APC and γ-APC/Cy7. Cells were run in FACSCanto™ II cytometer, (BD) with BD FACSDivar software. The results were analysed with FlowJo 7.6.5 software.

A flowchart of the experiments is illustrated in figure 2.
3 Results

3.1 Phenotyping of Karpas-422 and K562 cell lines for CD19 expression

The Karpas-422 and K562 cell lines were used in these experiments as CD19 positive target cells and CD19 negative control cells respectively. The presence or absence of surface CD19 marker was confirmed by anti-CD19-APC antibody staining and flow cytometric analysis (fig. 3). They were checked also for CD3 expression (fig. 3B and C). Gating of the CD19 population was done according γ-APC isotype control. As can be seen from the figure only Karpas-422 cells stained with the anti-CD19 antibodies showed 98% expression of CD19 (fig. 3A). The isotype control staining of Karpas-422 or anti-CD19 staining of K562 cells did not detect CD19 population (fig. 3A and D). Both cell line were negative for CD3 marker, which was checked by anti-CD3-PE and γ-PE isotype control (fig. 3B and C).
3.2 Expression of Chimeric Antigen Receptor on the Transduced PBMCs and their Phenotypes

The CD19 depleted PBMCs were transduced with MLV virus containing CAR gene for 2nd or 3rd generations or with empty (mock) virus. The CAR expression was estimated by flow cytometry with the help of anti-human scFv-Dylight antibody 6 days after transduction (fig. 4.). As can be seen from the figure mock-transduced cells did not show antibody binding compared with 2nd and 3rd
generation CAR-transduced cells. The CAR-positive cells were gated out of CD3 population. Gating for CAR-positive population was done using mock transduced cells (fig. 4A). The transduction efficiency for 2\textsuperscript{nd} CAR-transduced cells was within the range of 72-92\% and for 3\textsuperscript{rd} CAR-transduced cells 72-82\% (fig. 4B). The exceptions are 2 individuals where the transduction efficiency for 3\textsuperscript{rd} generation CAR-transduced cells was 13\% and 10\% respectively.

**Figure 4. Phenotyping of CAR transduced T cells.** (A) Illustration of gating of CAR\textsuperscript{+} (blue and orange histograms) and CAR\textsuperscript{-} (red histogram) subsets from flow cytometry analysis of transduced PBMCs from HC W70 individual. The gating was done using the mock-transduced cells as negative control (red histogram). Blue and orange histograms represent T cells from 2\textsuperscript{nd} and 3\textsuperscript{rd} CAR transduced T cells, respectively. (B) Transduction efficiency of T cells in percentage from all 6 blood donors. Mock-transduced cells of individuals are lying on the horizontal axis, showing almost 0\% of CAR expression (black squares); 2\textsuperscript{nd} and 3\textsuperscript{rd} CAR transduced T cells (up-indicative and down-indicative black triangles respectively) have relatively similar CAR expression with the exception of 2 individuals transduced with 3\textsuperscript{rd} CAR gene.

The transduced cells were gated for live cells using FSC/SSC followed by CD3/CAR positive cells. The subpopulation was further phenotyped for CD4\textsuperscript{+} and CD8\textsuperscript{-} subsets (fig 5). As showed the analysis, the mean values for CD4 population in mock, 2\textsuperscript{nd} CAR and 3\textsuperscript{rd} CAR-transduced T cells was 53\%, 57\% and 51\% respectively. The CD8 population from the same transduced cells was 4.7\%, 28\% and 32\% respectively.
Figure 5. Proportion of CD4 and CD8 T cells within transduced population. The error bars represent mean values of CD4 and CD8 cells in percentage from mock, 2nd and 3rd CAR transduced T cells, (on 6th day after the transduction).

3.3 Two weeks culture of the CAR-transduced cells shifted the proportion of CD4/CD8 subsets towards CD8, but not in co-cultured cells

Eight days after transduction, the cells were stained with CFSE and some of them were co-cultured with CD19⁺ or CD19⁻ cells (Karpas-422 and K562 respectively) for 5 days and some of them were continued culturing without any target cell. After 5 days culture, the proliferation assay was done in flow cytometer, where, not only proliferation was measured, but also CD4/CD8 subpopulations were phenotyped (fig. 6).
The non-co-cultured 2nd and 3rd CAR-transduced cells had approximately 30% CD4 subset, while mean proportion of CD8 was around 60% (fig. 6, upper diagram). The second and third CAR T cells co-cultured with Karpas-422 or K562 had average 45% and 37% CD4 subpopulation and 24% and 28% CD8 subset respectively (fig. 6, lower 2 diagrams).

However, in the proliferation analysis no indication on a difference was detected between CD19+ and CD19− co-cultured transduced T cells. No consistent pattern was detected between mock, 2nd and 3rd CAR transduced cells co-cultured with the same cell type either (data not shown).
The mean value of CD4 and CD8 populations of 2nd CAR-transduced cells from CLL patients was approximately 45% each. The same subsets from 2nd CAR healthy controls (HC) were 55% and 25% respectively. The proportion of CD4 subset of 3rd CAR T cells from CLL patients and HC donors was around 28% and 55% respectively, while mean values of CD8 population from the same group was 48% and 30% respectively (HC; fig. 7).

![Figure 7](image)

**Figure 7. Proportion of CD4/CD8 CAR T cells from CLL patients and healthy donors.** The bar diagram indicates proportion of CD8+ and CD4+ T cells in chronic lymphocytic leukaemia patients (CLL) compared with that of healthy controls (HC). The data is taken from 2nd CAR transduced (light grey bars) and 3rd CAR transduced (dark grey bars) T cells. (The data is illustrated as mean values from all CLL (n=2) and all HC (n=4).)

### 3.4 Memory phenotype of the transduced population

The transduced cells (2nd and 3rd CAR expressing cells) were studied for naïve, central memory, effector memory and effector populations (fig. 8). For this study CCR7, CD45RA, CD27 and CD28 markers were tagged. The mean percentage of naïve population from 2nd and 3rd CAR transduced cells was approximately 13% and 14% respectively. They were detected as CD45RA+/CCR7high/CD27+/CD28+ population. Proportion of effector subset (CD45RA+/CCR7+/CD27+/CD28+) was below 2% for both transduced groups of T cells. The proportion of central memory end effector memory cells for 2nd transduced CAR T cells was 11% each and for 3rd CAR cells around 8-9% each (fig. 8, two middle bars for each transduced group). Central memory and effector memory cells were determined as CD45RA+/CCR7low/CD27mid/CD28+ and CD45RA+/CCR7/CD27low/CD28+ respectively.
Figure 8. Phenotyping of different memory and effector subsets among 2nd and 3rd CAR transduced T cells. The bar chart illustrates mean presence of naive, central memory, effector memory and effector T cells from all individuals in 2nd and 3rd CAR transduced cell groups.

3.5 \(^{51}\text{Cr} \) release cytotoxic assay analysis

The transduced T cells were co-cultured with the target cells Karpas-422 and K562 (as negative control) for detection of killing capability and difference between different transduced groups and between different co-culture groups. The killing ability was measured by radioactive \(^{51}\text{Cr} \) release assay. The results showed effector/target ratio dependent shift of cytotoxicity (fig. 9).

As can be seen from the figure, cytotoxicity of 2nd and 3rd CAR T cells from CLL W69 patient co-cultured with Karpas-422 cell line was below 50% and above 50% respectively at 30:1 effector/target ratio. Mock transduced cells’ cytotoxicity was around 18% for the same ratio. At 1:1 ratio the cytotoxic effect of 2nd and 3rd CAR cells was 22% and for mock transduced cells it was 5-6% (fig. 9A). For the same effector cells co-cultured with K562 cells at 30:1 and 1:1 ratio, cytotoxicity was approximately within the range of 35-39% and 10% respectively for all transduced groups (fig. 9C). Cytotoxicity of 3rd CAR, 2nd CAR and mock-transduced T cells from HC W70 individual co-cultured with Karpas-422 cells at 30:1 ratio was 42%, 28% and 5% respectively. At 1:1 ratio, cytotoxicity for the same cells was 18%, 17% and 1% respectively (fig. 9B). The same cells co-cultured with K562 cell line at 30:1 ratio had 22%, 18% and 17% cytotoxic effect respectively, while at 1:1 ratio the cytotoxicity was around 7% for all transduced groups (fig. 9D).
4 Discussion

The idea of adoptive cell transfer therapy has created a way to tag malignant cells in MHC independent manner. The T cells carrying chimeric antigen receptors against different tumour antigens have previously been evaluated. In this study CAR T cells against CD19 B cell marker were investigated for immunotherapy of CLL patients. In previous studies a comparison between first and second generation of CAR T cells (carrying ζ3-chain and ζ3-chain + CD28 signalling domain respectively) showed advantage of the 2nd CAR T cells in the sense of proliferation and survival in vitro and in vivo (Savoldo et al., 2011; Sadelain et al., 2009; Loskog et al., 2006).

We evaluated 2nd and 3rd generation CAR T cells carrying ζ3-chain + CD28 and ζ3-chain + CD28 + 4-1BB signalling domains respectively, for transduction efficiency, different phenotype development and cytotoxic effect during in vitro culture with target CD19+ cells. For the CAR transduction of T cells, mostly, lentiviral or retroviral vectors are used (Cartellieri et al., 2010). Lentiviral vectors are considered to be safer delivery system for T cell with higher efficiency of
transduction (Kalos et al., 2011; Porter et al., 2011). In our experiments MLV retroviral vector was used, and average 75% transduction efficiency was detected for both CAR genes. The exceptions were 2 individuals, whose T cells showed significantly lower expression of 3rd generation CAR (fig. 4B). The most probable reason for this is an error in transduction procedure or virus production. The transduction method and the vector itself should not be a problem, as the experiment was done even earlier repetitively (data not shown) and no significant difference was observed between 2nd and 3rd CAR transduction efficiency.

CAR CD4+ population from transduced PBMCs may provide Tregs which could not be beneficial for cancer immunotherapy (Kohn et al., 2010). However, Moeller and co-workers noticed that mixture of CD4 and CD8 populations have better tumour rejection effect, than used separately (Moeller et al., 2005). CAR transduced T cells develop more CD8 subset compared with CD4 (Neeson et al., 2010). We observed CD4 subset as majority CAR T cells phenotype on day 6 after transduction. However, on day 13 we observed more CD8+ population. In contrast, cells co-cultured with target cells (either CD19+ or CD19-) during 5 days showed similar CD4/CD8 proportion as it was on day 6. The amount of samples was not enough to draw statements out of the data. The interaction between transduced T cells and target cells should be studied in order to elucidate the consistent pattern governing development of CD4 and CD8 subsets of T cells. It is less plausible that CAR-CM19 interaction led to keeping of CD4 subset as majority, because the same result was detected in K562 co-cultured CAR T cells, and also in CAR negative T cells co-cultured with Karpas-422 cell line. The possible reason could be that T cells interacted via TCRs with target cells’ MHC molecules, which could lead to signals for CD4+ population proliferation. Commonly, CD3/IL-2 stimulation drives CD8+ T cell proliferation to a greater extent than it drives proliferation of CD4+ T cells (Lai et al., 2009), which reflects our data well.

For adoptive cell transfer the transduced cells should have high capacity of proliferation, resistance, and antitumour activity. These characteristics are determined by phenotyping of the cells for naïve, central memory (Tcm), effector memory (Tem) and effector subsets (Savoldo et al., 2011). Many studies have found that in vitro expansion of transduced T cells develops central memory and effector memory populations. (Savoldo et al., 2011; Barber et al., 2008; Sallusto et al., 1999; Lefrancois and Marzo, 2006). June and co-workers claim that in vitro and in vivo signalling activation via CD28 and 4-1BB domain also leads to generation of Tcm and Tem (June et al., 2009). However, Till and co-workers observed different data; in their experiments they conclude that majority of population is effector cell subset (Till et al., 2008). Naïve cells are rapidly dividing, but with the lowest antitumor ability, while effector cells have a very high cytotoxic capacity, but lack the proliferation. Tem have high cytotoxic effect, however, they are more sensitive compared with Tcm (Gattinoni et al., 2005; Klebanoff et al., 2005). In this experiments naïve T CAR cells were determined as CD45RA+/CCR7high/CD27+/CD28-, Tcm – as CD45RA+/CCR7low/CD27mod/CD28- Tem – CD45RA+/CCR7/CD27low/CD28+, and effector cells - CD45RA+/CCR7/CD27/CD28+. In our data the proportion of naïve phenotype
from both generation CAR cells was 13-14%, while the T<sub>CM</sub> and T<sub>EM</sub> phenotypes from 2<sup>nd</sup> generation CAR transduced T cells composed 11% each and from 3<sup>rd</sup> CAR cells 8-9% each. The effector T cells had negligible quantity (fig. 8).

K562 cell line is derived from lymphoblast of bone marrow of CML patient (ATCC). It was used as CD19 negative cells for control to CD19 positive target cells (Savoldo et al., 2011; Terakura et al., 2011). We confirmed their CD19 phenotype (fig. 3) and both Karpas-422 and K562 cells were co-cultured with transduced T cells during proliferation assay and cytotoxic assay. For proliferation assay the transduced T cells were stained with CFSE, which binds to membrane of the cells and can be detected in FITC channel. After each division of a cell, each daughter cell gets half of the CFSE amount, so that the detected light intensity will be reduced by half from generation to generation. In vitro co-culture of anti-LeY CAR T cells with target tumour cell line during 5 days showed proliferation of the T cells with the CFSE assay. In contrast, the mock transduced T cells did not respond to the target cells (Neeson et al., 2010). Cheadle et al. have also demonstrated antigen specific anti-CD19 CAR CD8<sup>+</sup> T cell (with CD3-ζ intracellular domain (1<sup>st</sup> generation of CAR T cell)) proliferation on 4<sup>th</sup> and 7<sup>th</sup> day of co-culture with normal B cells or malignant cells expressing CD19 (Cheadle et al., 2010). In this study the mock, 2<sup>nd</sup> and 3<sup>rd</sup> generation CAR transduced T cells were stained with CFSE and co-cultured with Karpas-422, K562 or cultured alone. After analysis of data, no significant difference was observed between proliferation histograms of CD19-positive or negative co-cultured or cultured alone cells (data not shown). No conclusion could be done in comparison of mock, 2<sup>nd</sup> and 3<sup>rd</sup> CAR T cells proliferation either. In their assay Neeson did not stimulate co-cultured T cells at all, in order to detect pure antigen-specific effect on proliferation. Cheadle and colleges have added 10U/ml of IL-2 for slightly stimulation of the T cells, but using CD19-mtm CAR T cells as negative control (mtm was used instead of CD3 ζ in intracellular domain as non-signalling domain) showed that the proliferation was not driven by the low dose of IL-2. In our study we have added 50 U/ml of IL-2 which could be a probable reason for general signal which was stronger than the one from antigen and initiated proliferation of all samples equally.

The antigen-specific cytotoxicity was determined by 51Cr release assay. The target cells, in our case Karpas-422 and K562, were incubated in solution of radioactive sodium chromate. After the incubation the target cells were washed and co-cultured with transduced T cells (effector cells) at 4 different ratios. In case of killing of target cells, they release absorbed 51Cr into the supernatant, which was collected and measured. The level of radioactivity was compared with wells, where Tween-20 was mixed for 100% killing, and with wells containing target cells alone as spontaneous dying value. Savoldo et. al has demonstrated higher cytotoxic effect of 2<sup>nd</sup> generation CAR T cells (anti-CD19-CD28Z) compared with 1<sup>st</sup> generation (Savoldo et al., 2011). Similar data was observed by Ahmed et al. in anti-HER2-CD28Z CAR cells cytotoxicity towards osteosarcoma cells and by Attianese et al. using 2<sup>nd</sup> generation anti-CD23 CAR T cells against CLL cells in vitro (Ahmed et al., 2009; Giordano Attianese et al., 2011). Interestingly,
Carpenito and colleges did not observe increase in cytolysis when compared 1\textsuperscript{st} (CD3Z) and 3\textsuperscript{rd} (CD3Z-CD28-CD137) generation CAR T cells in the Cr assay. Neither increased cytolysis was detected by 2\textsuperscript{nd} CAR T cells compared with 1\textsuperscript{st} CAR T cells by Hombach A. and co-workers (Hombach \textit{et al.}, 2001). In our experiments, from one CLL patient, we recorded 55% and 45% cytotoxicity of 3\textsuperscript{rd} and 2\textsuperscript{nd} CAR T cells respectively, when co-cultured with Karpas-422 at 30:1 ratio, while mock-transduced cells showed 18% cytolysis (fig 9A). In K562 co-culture, the cytotoxicity was 35-39% at 30:1 ratio and 10% at 1:1 ratio for all 3 transduced groups (fig 9C). Cytotoxicity of 3\textsuperscript{rd} CAR, 2\textsuperscript{nd} CAR and mock-transduced T cells from one healthy control co-cultured with Karpas-422 at 30:1 ratio was 42%, 28% and 5% respectively, while at 1:1 ratio it was 18%, 17% and 1% (fig. 9B). The same cells co-cultured with K562 cell line at 30:1 ratio showed 22%, 18% and 17% cytotoxic effect respectively, while at 1:1 ratio the cytotoxicity was around 7% for all transduced groups (fig. 9D). However, because of cell contamination in the harvested $^{51}$Cr supernatant, from the all 6 individuals only 2 were evaluated. The problem was too high values from certain wells, which were higher than those from maximum release wells. The reason for this was pipetting errors at some wells, when supernatant was collected for the assay. It is very important not to suck pellet while pipetting the supernatant, otherwise the cells will artificially increase the radioactive level in many dozens of times. So, because of too few samples no significant conclusion can be done.

In summary, the 2\textsuperscript{nd} and 3\textsuperscript{rd} CAR T cells were evaluated for transduction efficiency, different phenotype development, proliferation and cytotoxic capacities. The transduction was high enough for both genes. However, the phenotypes for different T cells and cytotoxic assay should be repeated as the amount of samples was not enough for statistical analysis. Especially more attention should be focused on CAR T cells from CLL patients. We noticed that CAR T cells from CLL patients give more CD8\textsuperscript{+} subset, than CD4\textsuperscript{+}, which is more favourable for immunotherapy, as CD8\textsuperscript{+} subset is more responsible for tumour cell elimination. The proliferation assay should be optimised for either low, or not at all IL-2 addition. In case of successful results with statistical significance, the test of these CAR T cells can be carried out in \textit{in vivo} study of tumour-bearing xenograft mouse model.

5 References


antitumor immunity compared with effecter memory T cells. Proc Natl Acad Sci USA, 102, (p. 9571-9576).


