UMFVBT - The Faculty of Medicine
Department III - Functional Sciences
The Discipline of Immunology, Allergology and Biology
**Olteanu Gheorghe-Emilian**

UNIVERSITATEA
DE MEDICINĂ ȘI FARMACIE
VICTOR BABEȘ | TIMIȘOARA

# DOCTORAL THESIS

## DEVELOPMENT, ANALYSIS, AND EVOLUTION OF MULTISPECIFIC CAR-T LYMPHOCYTES IN THE IMMUNOTHERAPY OF CANCER

## ABSTRACT

Scientific coordinator
**PROF. UNIV. DR. VIRGIL PĂUNESCU**

# CONTENTS

**KEYWORDS:** anti-EGFR, chimeric antigen receptor, cytotoxicity, flow-cytometry, immunotherapy, lymphocytes, multispecific, NK cells, PBMCs, real-time cell analysis, TiNK cells, transduction, transfection.

## I. PURPOSE AND OBJECTIVES OF THE RESEARCH

The present doctoral thesis aimed to elaborate on the development, analysis and monitoring of the evolution of multispecific CAR-T lymphocytes for the treatment of human solid malignancies. The need for cell-based immunotherapy in solid cancers is of utmost importance and necessity. Furthermore, the inadequacy of classical CAR-T immunotherapy due to its myriad of side effects, and the overall absence of adequate cell-based solution in solid tumours, highlights the need for this present research.

At this present moment, no viable cell-based immunotherapy exists for the treatment of solid malignancies. Progress has been made in regards to monoclonal antibodies, especially those that target the immune checkpoints i.e. cytotoxic T lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) and PD-1 ligand (PD-L1) inhibitors. Unfortunately, these biological treatments have pleomorphic successes, with extreme responders to treatment and those that show no response to treatment.

**Shortly this thesis is formulated as follows:**

- Transfection of the 293T (ATCC® CRL-3216™) cell line with a lentiviral vector that encodes an anti-epidermal growth factor receptor (EGFR) CAR. The 293T cells represent a human cell line derivative of human embryonic kidney 293 cells, that are highly transfectable.
- Transduction of effector cells with anti-EGFR CAR, for specific recognition of EGFR positive solid tumours.
- Transduction efficiency analysis of transduced effector cells, for the evaluation of the percentage of GFP positive effector cells. Assessment of anti-EGFR CAR expression on transduced effector cells with biotinylated human EGFRvIII protein Avitag™, biotinylated recombinant protein L.
- In vitro, functional validation of cytotoxicity of anti-EGFR CAR transduced effector cells against EGFR expressing tumour target cells

From a national scientific research standpoint to our knowledge the research collective involved in the thesis represents the only one studying the development of CAR technology for the immunotherapy of solid tumours. Internationally the

research theme of this thesis is the ongoing research aim of many world-class research collectives, from Europe, the United States of America and Asia e.g. Khaldoun Almhanna's research team, Alexandra Flemming's research team, and Laurence Cooper's research team et cetera.

**The objectives of this thesis are:**

- Development of effective transfection methods for lentiviral production of anti-EGFR CAR.
- Transduction of anti-EGFR CAR in immune effector cells, that are actively involved in anti-tumour immunity, such as lymphocytes, natural killer, or derivatives.
- Functional testing of the anti-tumour capacity of transduced effector cells i.e. functional cytotoxicity testing of anti-EGFR CAR transduced effector cells against EGFR+ cancer cell lines i.e. the MDA-MB-468 (ATCC® HTB-132™), SK-BR-3 [SKBR3] (ATCC® HTB-30™), HT-29 (ATCC® HTB-38™).

The research method and the research methodology used in the experiments carried out in this doctoral thesis is in line with the leading edge of the immunotherapy research field. The tools of research used are state of the art and universally cited as the most innovative approaches in cell-based immune therapy of cancer. The analytical methods employed in the generation of experimental data are classified as prime candidates for the best quantitative and qualitative research output.

## II. DEVELOPMENT OF EFFECTIVE TRANSFECTION METHODS FOR LENTIVIRAL PRODUCTION OF ANTI-EGFR CAR.

For the experimental part, a 2nd-generation lentiviral system was used. The 2nd generation system contains one packaging plasmid, encoding the Gag, Pol, Rev, and Tat genes. The transfer plasmid contains the viral long terminal repeat (LTR) sequences and, the psi packaging signal. The envelope protein Env, here represented by VSV-G, is encoded by a third, envelope plasmid.

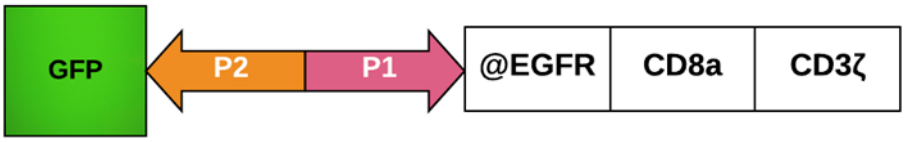


Fig. 1. Vector design from GEGTech.

## III. TRANSDUCTION OF ANTI-EGFR CAR IN IMMUNE EFFECTOR CELLS, THAT ARE ACTIVELY INVOLVED IN ANTI-TUMOUR IMMUNITY, SUCH AS LYMPHOCYTES, NATURAL KILLER, OR DERIVATIVES.

Once the protocol for transfection was established the next step was the transduction of PBMCs that were previously stimulated with Dynabeads CD3/CD28 T Cell Expander (ThermoFisher, cat. no. 111.31D), NK-92 cells, and T cells.

PBMCs were isolated from healthy donors and stimulated in effector cells with IL-2, IL-15, and IL-21 (cytokine stimulation) with or without CD3/CD28 T cell activation beads (ThermoFisher, cat.no. 11456D). After activation, the cells were transduced by spinoculation with subsequent 24 hours of incubation time with the virus. LV particles were used for the generation of anti-EGFR CAR effector cells. After 72 – 96 hours of monitoring, the intensity of GFP expression was evaluated by flow cytometry.

### III.1. Transduction efficiency analysis

GFP expression of anti-EGFR CAR transduced effector cells, was evaluated and analysed using the FACSverse flow cytometer (Becton, Dickinson and Company, New Jersey, USA).

GFP expression of transduced cells remains stable over a long period i.e. days analysed, this can be considered as successful transduction of anti-EGFR CAR. The percentage of GFP positive cells is >80% in the anti-EGFR CAR transduced TiNK effector cells, and >95% in the anti-EGFR CAR transduced stimulated and sorted PBMCs representing T cells. TiNK cells present a lower transduction rate probably due to lineage-specific NK resistance to viral infection, even if TiNK cells are not NK cells they behave like NK cells.

TiNK effector cells show an overall high percentage of GFP positivity denoting successful transduction.

## III.2. CAR EXPRESSION ANALYSIS

After the observation and analysis of GFP+ effector cells and confirmation of successful transduction. Anti-EGFR CAR analysis or CAR expression analysis was evaluated with Biotinylated human EGFRvIII protein, Avitag™, Biotinylated recombinant protein L from ACROBiosystems (Newark, Delaware, USA). The biotinylated recombinant protein L expression was evaluated by flow cytometry using the FACSverse (Becton, Dickinson and Company, New Jersey, USA).

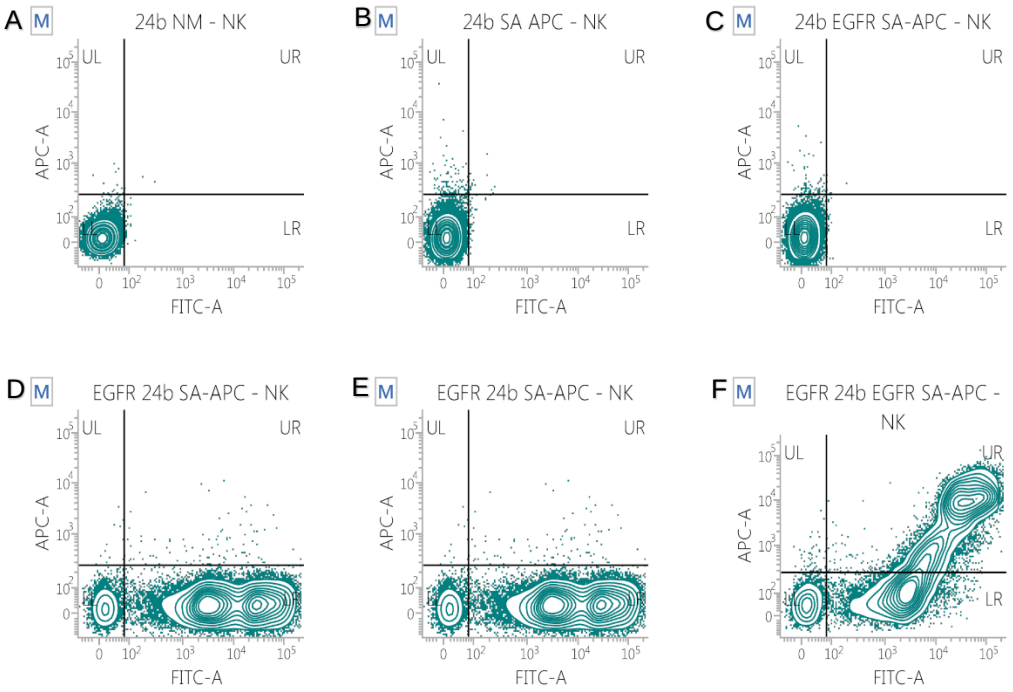


Fig. 2. Flow cytometry analysis of biotinylated EGFR + SA-APC. A, B, and C. Contour plots of A. un-transduced and un-stained NK effector cells, B. Streptavidin-APC stained un-transduced NK effector cells. C. EGFR Streptavidin-APC stained un-transduced NK effector cells. D, E, and F. Contour plots of D. anti-EGFR CAR transduced NK effector cells un-stained. E. anti-EGFR CAR transduced NK effector cells stained only with secondary antibody, streptavidin-APC. F. anti-EGFR CAR transduced NK effector cells stained fully, with EGFR streptavidin-APC, showing in UR – upper right CAR+ NK effector cells.

Surface expression of anti-EGFR-specific CAR on transduced effector cells ranged from 55-75% of all GFP positive cells analysed (transduced).

## IV. FUNCTIONAL TESTING OF THE ANTI-TUMOUR CAPACITY OF TRANSDUCED EFFECTOR CELLS I.E. FUNCTIONAL CYTOTOXICITY TESTING OF ANTI-EGFR CAR TRANSDUCED EFFECTOR CELLS AGAINST EGFR+ CANCER CELL LINES I.E. THE MDA-MB-468 (ATCC® HTB-132™), SK-BR-3 [SKBR3] (ATCC® HTB-30™), HT-29 (ATCC® HTB-38™).

Based on the RTCA results using anti-EGFR CAR transduced NK-92 cells and un-transduced NK-92 cells, different effector cells were selected for RTCA killing analysis of adherent tumour cell lines. After isolation and culture of PBMCs and subsequent isolation of T cells by immunomagnetic methods, PBMCs were stimulated with IL-2, IL-15, and IL-21. After 10 days of *in vitro* culture condition, the results highlighted a mixed phenotype of >70% $CD3^+$ and $CD56^+$ NKT cells, $CD3^-$ and $CD56^+$ NK cells. Immunomagnetic isolated T cells were enriched using the enrichment kit (STEMCELL Technologies Canada Inc.) described in the Materials and Methods section of the thesis. Using CD3/CD28 Dynabeads (Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation) to induce T cell activation and expansion the proliferation rate of activated cells was increased ~10 times. Moreover, the transduction rate was almost tripled from 30% to 90%.

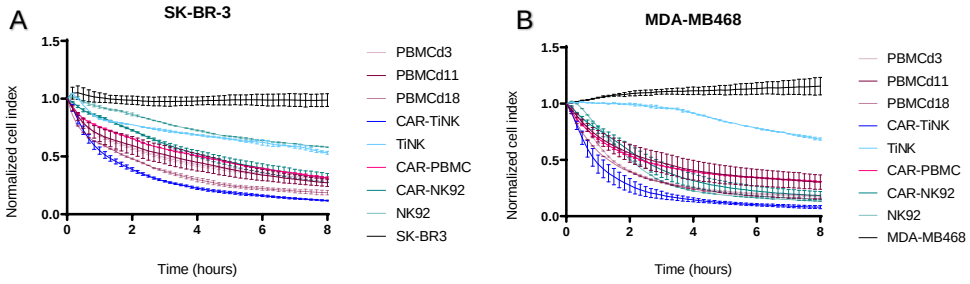


Fig. 3. Normalised NCI parameters of two RTCA experiments of NCI for a time frame of 8 hours after the addition of effector cells. A. Normalised NCI parameters of two RTCA experiments involving SKBR3 as target cells and anti-EGFR CAR transduced NK-92 cells, PBMC, and TiNK cells, and un-transduced NK-92 cells, TiNK cells, PBMC at day 3 of cell culture, PBMC at day 11 of cell culture, and PBMC at day 18 of cell culture as

effector cells. B. Normalised NCI parameters of two RTCA experiments involving MDA-MB-468 as target cells and the same effector cells used for the SKBR3 RTCA assay. A and B. The most efficient cytotoxic effector cells are anti-EGFR CAR transduced TiNK cells, their target cell killing aptness stems from their multispecific binding of target cells i.e. TCR receptors, NKp46, NKp30, and NKp44, NKG2D, and the artificially induced anti-EGFR CAR (for specificity).

The flow-cytometry analysis of the MICA/B expression of the adherent tumour cell lines, i.e. SKBR3, HT-29, and MDA-MB-468, showed a similar expression level in the SKBR3 and HT-29 adherent target cells, with the highest expression profile of MICA/B, found in the MDA-MB-468 adherent target cells. Apart from the HT-29 tumour cell line, the SKBR3 and MDA-MB-468 are tumour cell lines derived from metastatic sites, i.e. pleural effusions [129, 130], the HT-29 cell line is derived from a primary tumour.

The EGFR expression on the adherent tumour cell lines was highest in the MDA-MB-468 cells, with high expression profile in the HT-29 cells, and a moderate expression profile in the SKBR3 cells. EGFR is a transmembrane glycoprotein that is a member of the protein kinase superfamily and is highly expressed on cells of an epithelial lineage [152].

Considering the site of the derivation of the adherent target cells used, high EGFR expressions should be identified in the primary-tumour derived cell line with low expression profiles seen in metastatic derived tumour cell lines, the conceptual and factual evidence-based conclusion stems from the epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) hypothesis and loss or internalisation of epithelial lineage-specific markers, like EGFR [18].

Interestingly, the highest expression of EGFR was found to be in the MDA-MB-468 cells, a metastatic derived tumour cell line, although triple-negative breast carcinomas have high levels of EGFR expression [153].

Briefly, the specific lysis evaluated by flow cytometry was highest in the anti-EGFR CAR transduced TiNK effector cells, confirming the experimental observations made using the RTCA assay, comparative analysis of anti-EGFR CAR transduced NK-92 effector cells and anti-EGFR CAR transduced TiNK effector cells show an improved cytolytic activity of target cells by CAR transduced TiNK cells over CAR transduced NK-92 cells.

Finally, the multispecificity of effector type receptors and the addition of an anti-EGFR CAR receptor make TiNK effector cells a next possible tool in cancer immunotherapy.

Comparative analysis for spontaneous and specific lysis evaluated by flow cytometry, show an improved cytolytic activity of target cells by anti-EGFR CAR transduced TiNK effector cells. This improved effector activity is consistently higher and statistically significant than the spontaneous lysis seen in tumour cell lines with Welch's t-test p-value of $p = 0.020$.

## V. BIOSTATION FUNCTIONAL TEST – CYTOTOXICITY ASSAY

The morphology of the microscopic interactions of anti-EGFR CAR transduced effector cells i.e. PBMCs + beads + CK (chemokines – i.e. IL-2, IL-12, IL-15) representing TiNK cells and PBMCs + CK representing T cells, and the adherent target cells, (i.e. MDA-MB-468 and SKBR3 tumour cell lines), was evaluated using the live-cell recorded BioStation functional assay.

Cytolysis assessment was made by contact between effector cell and target cells and formation of large structures defined as clumps, between more than one effector cell and death or dying adherent target cells (with detachment from the bottom of the wells).

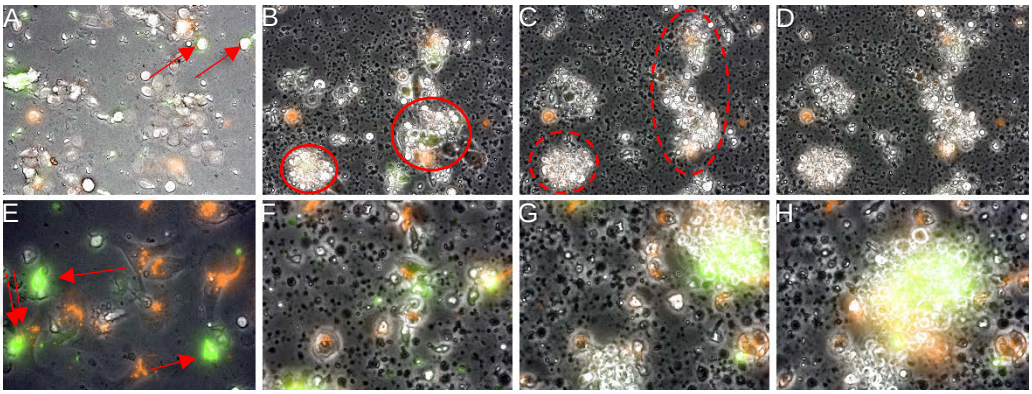


Fig. 4. Collage of captures from the BioStation cytotoxicity assay of MDA-MB-468 target cells and anti-EGFR CAR transduced TiNK cells. A, B, C, and D. x100 magnification of time points zero, 3.3 hours, 10 hours, the last time point in the assay representing 24 hours. Orange cells represent target cells stained with Lipophilic spDII tracer dye. FITC wavelength filter showing GFP+ transduced TiNK effector cells (red arrows). Cytolosis potency is seen early on in the monitoring time-frame of the assay (red ovals), and the formation of large clump structures are also observed (red dashed oval). E, F, G, and H. Represent x200 magnification of the same time points as in A, B, C, and D. Immune synapse formation is easily observed at this magnification (double red

arrows). As was the case for the SKBR3 adherent target cells, the MDA-MB-468 adherent target cells are anti-EGFR CAR transduced TiNK effector cells – sensitive and are rapidly killed by TiNK effector cells.

## VI. CONCLUSIONS

- Using CD3/CD28 Dynabeads (Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation) to induce T cell activation and expansion the proliferation rate of activated cells was increased ~10 times. Moreover, the transduction rate was almost tripled from 30% to 90%.

- Comparative analysis for spontaneous and specific lysis evaluated by flow cytometry showed an improved cytolytic activity of target cells by anti-EGFR CAR transduced TiNK effector cells. This improved effector activity is consistently higher and statistically significant than the spontaneous lysis seen in tumour cell lines - Welch's t-test $p = 0.020$.

- The most efficient cytotoxic effector cells are anti-EGFR CAR transduced TiNK cells, their target cell killing aptness stems from their multispecific binding of target cells i.e. TCR receptors, NKp46, NKp30, and NKp44, NKG2D, and the artificially induced anti-EGFR CAR (for specificity).

- The target cells used in the thesis experiments were adherent tumour cell lines MDA-MB-468, SKBR3, and HT-29, these were analysed for the expression of EGFR, and the expression of NKG2D ligand MICA/B by flow cytometry. Compressing the results of the experiments, anti-EGFR CAR transduced effector cells and transduced TiNK effector cells specifically present cytotoxicity against EGFR+ solid tumour targets.

## VII. PERSONAL CONTRIBUTIONS

- Development and generation of a functional 2nd-generation lentiviral system for expression of CAR EGFR ScFv.

- Successful implementations of 293T cell cultures for lentivirus production

- Establishment of TiNK effector cells. Highly cytolytic effector cell population generated from PBMCs isolated from healthy donors by combined IL-2, IL-15, and IL-21 cytokine stimulation, with CD3/CD28 T cell activation beads.

- Development of anti-EGFR CAR transduced TiNK effector cells for specific targeting of EGFR+ solid tumour targets

- Fundamental development of CARs for eventual translation to clinical immunotherapy in cancer.

- Elaboration of a hypothesis regarding the metastatic pathway based on the experimental observation documented while researching this thesis. The hypothesis was published (see list of published works) and subsequently cited in a high impact journal.

## VIII. FUTURE RESEARCH DIRECTIONS

- Implementation of a gene-editing tool like programmable nucleases, i.e. ZFNs, TALENs, and CRISPR/Cas9 associated nucleases for precise editing out of TCR from T cells thus eliminating the possibility of an allogeneic reaction.

- Improvement of transduction rate for TiNK effector cells.

- Implementation of ZFN, TALEN or CRISPR for precise editing out of various T cell receptors and/or NK receptors for functional analysis of their precious role in cytotoxicity and regulation of anti-tumour immune response.

- Development of a multispecific CAR that is anti-EGFR and anti-PD-L1 for the successful and specific target of solid tumour over-expressing EGFR and PD-L1.

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