IJRAR.ORG E-ISSN: 2348-1269, P-ISSN: 2349-5138

INTERNATIONAL JOURNAL OF RESEARCH AND ANALYTICAL REVIEWS (IJRAR) | IJRAR.ORG

An International Open Access, Peer-reviewed, Refereed Journal

In-Vivo Engineering of CAR-T cells Using Lentiviral Vector Platforms to Treat B-cell Malignancies

Abhishek Anand
MSc Cancer & Clinical Oncology
Barts Cancer Institute
Barts and The London School of Dentistry and Medicine
Queen Mary University of London

1. ABSTRACT:

BACKGROUND:

Chimeric Antigen Receptor-T cell immunotherapy is an innovative therapy which has revolutionized cancer treatment especially in the case of B-cell malignancies. However current ex-vivo manufacturing processes of CAR-T can be augmented and mechanisms to engineer CAR-T cell therapy in-vivo needs an addressal. In this regard, a VivoVec technology is developed which uses lentiviral vector platform to engineer CAR-T cells within the patient's own body making it an autologous cell immunotherapy. Other than this it will cut down on, time of manufacturing, cost and other logistical challenges like pre-treatment modalities and sample collection.

METHODOLOGY:

VivoVec technology is developed using a third-generation lentiviral vector which have better safety features. 11 major steps are involved in the production of UB-VV100 which lentiviral vector which have the specific payload in the form of genetic information. This payload is supposed to interact with the patient's immune cell and transduce it form functional CAR-T cells.

RESULTS:

UB-VV100 proved to stood out in all the experiments, validating its efficacy to target CD19+ B-cell malignancies. Six different studies were performed to test its specificity, efficacy and safety profile using animal models like humanized mice (NSG carrying Nalm-6 tumor cells) and canines (beagle dog) and in all 6 studies, UB-VV100 proved its potency to fight and kill tumor cells and leave the other tissues unharmed. All the studies were approved by IACUC which testifies its ethicality.

2 INTRODUCTION & REVIEW LITERATURE: BACKGROUND AND CURRENT SITUATION

2.1 ex-vivo CAR-T CELL PRODUCTION: Drawbacks & Challenges

2.1.1: LEUKAPHERESIS – *To Produce Autologous CAR-T Cells*

Chimeric Antigen Receptor-T (CAR-T) cell immunotherapy have proved to be quite potent in treating refractory/relapsed B cell malignancies which have paved the way for further investments to engineer efficient T cells which can give better results in terms of therapeutic delivery.[1] Currently, autologous (T cells withdrawn from patients' own body) CAR-T cells are produced by collecting blood from patients' own body through a process known as "leukapheresis" *(Figure 1),* which is followed by genetic modifications (transduction) and then its expansion to make it fit enough to be reinfused into the patient.

Thus, the whole process starting from collection of T-cells to its expansion and reinfusion takes almost 4-6 weeks. This makes CAR-T cell therapy unavailable in most of the cases. The patient needs to undergo a wait-period which can cause not only physiological complications because of the delay but also a mental breakdown due to anxiety and depression. Therefore, it becomes important to develop modalities incorporating allogenic CAR-T cell therapy, thereby skipping the process of leukapheresis.

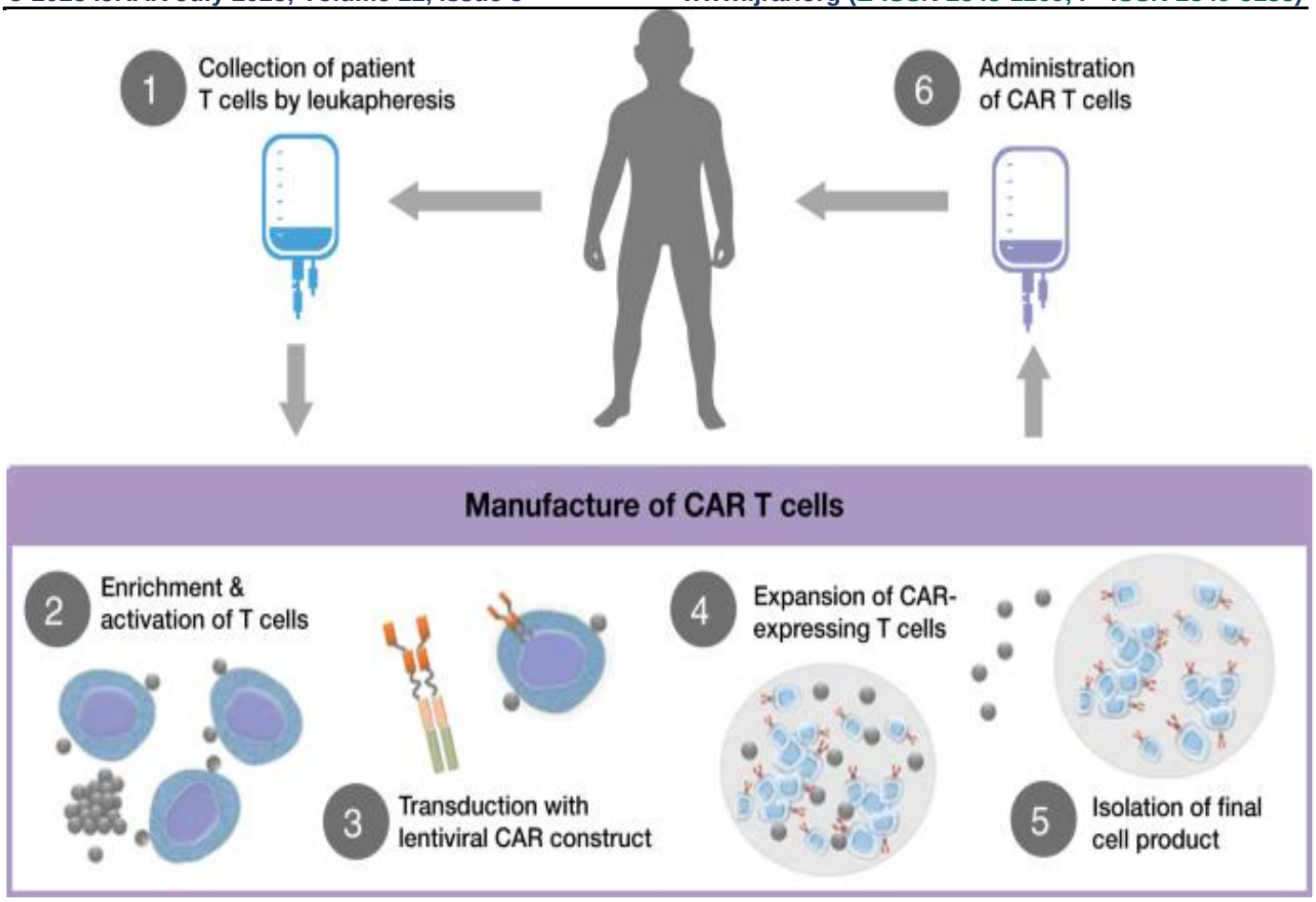


Figure 1: A schematic diagram depicting leukapheresis as the first step in CAR-T cell therapy followed by T-cell activation and transduction using lentiviral vector. After successful transduction expanded cells are isolated and reinfused into the patient's body.

2.1.2: LYMPHODEPLETION: *A Pre-treatment Chemotherapy*

Furthermore, the patient needs to undergo a pre-treatment before CAR-T cells are reinfused into the patient. This preparatory treatment is referred to as lymphodepleting chemotherapy which is meant to decrease the number of T cells and other immune cells in the patient's body[4]. Lymphodepletion is done to make sure that the infused CAR-T cells have the scope to engraft and expand to survive and proliferate in the patient's immunogenic environment. Without lymphodepletion, the existing lymphocytes such as T-cells, NK-cells etc., would compete with the infused CAR-T cells for the cytokines such as IL-7 and IL-15.[4] These cytokines play a major role in T-cell survival and its proliferation, which is important for CAR-T cell persistence for a prolonged period. However, lymphodepleting chemotherapy reduces T-cells in the patient's body, thereby increasing the risks associated with possibility of other infections caused by pathogens or any other agent.

2.1.3: SYSTEMIC CYTOKINE RELEASE: *Giving rise to toxicities*

The complexity of the manufacturing processes and risks associated with lymphodepleting chemotherapy followed by therapeutic delivery of CAR-T cells can result in severe cytotoxicity, labelled as SCR (Systemic Cytokine Release) which can give rise to physiological complications like CRS (Cytokine Release Syndrome) and MAS (Macrophage Activation Syndrome). A distinct neurotoxicity which is now referred to as ICANS (immune effector Cell associated neurotoxicity syndrome), characterized by the presence of toxic encephalopathy is observed, and is accompanied by mental deterioration like state of confusion and reading issues.[6] These complications pose a great set-back and discouragement in the usage of CAR-T cell therapy as a mainstream therapeutic modality. This therapy becomes unpredictable due to the risks of physiological and neurological complications making the whole therapeutic modality to have an uncertain prognostic outcome. Furthermore, the cost-effectiveness of CAR-T cell therapy implicating these complications makes the situation even worse. Therefore, to tackle with such logistical challenges, there is need to research on such mechanisms, which can ensure, CAR-T cell therapy availability, followed by optimizing its delivery, such that, it survives and persists for a prolonged period, without causing any severe physiological complications.

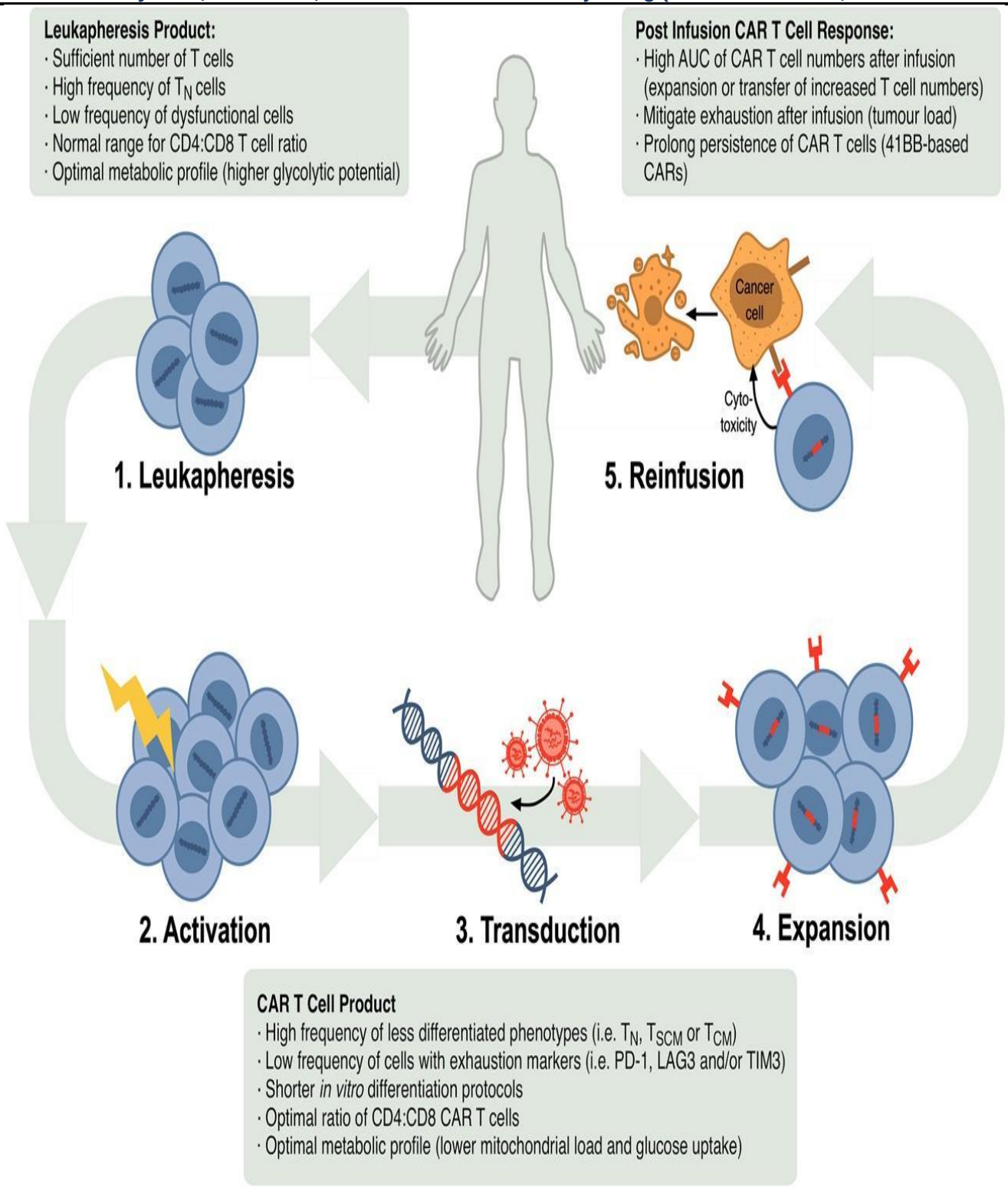


Figure 2: A schematic representation depicting the five major steps incorporated during the ex-vivo manufacturing processes of CAR-T cells.

2.2 VivoVec Technology: Unravelling Next Generation Therapeutic Strategies

2.2.1: Autologous '*off-the-shelf*' Cell Therapies: *Using Viral Vectors*

To overcome challenges as discussed in **2.1**, in-vivo engineering of CAR-T cells using a lentiviral vector (LVV) platform can be a potential solution to tackle with the drawbacks linked with the current ex-vivo manufacturing protocols which has been clearly demonstrated in various pre-clinical models (Figure 1). The use of viral vectors (for transducing desired gene) to engineer CAR-T cells ex-vivo is already in practice and can be further augmented to enable its direct administration to develop an in-vivo modality.[1] Viral vector are 'off-the-shelf' products, which means they are available for use whenever required.[1] The benefit of using viral vectors over other available 'off-the-shelf' cell therapy, is the production of autologous CAR-T cells which is usually allogenic in other cases. Allogenic cell therapies involve collection of T-cells from a healthy donor, and then preserving it for future use, making it available whenever required, but these allogenic therapies may not be a perfect match for the patient's immune system, which might cause various complications such as GVHD (Graft-versus-host-disease), inflammatory reactions etc. On the other hand, autologous cell therapies involve treating the patient with their own cells, which makes the process more feasible in terms of persistence and response.

Therefore, in this study, a third-generation vector UB-VV100 is developed to formulate a VivoVec technology, wherein, CAR-T cells can be produced in-vivo, within the patient's own body, thereby skipping the process of leukapheresis. This will save both time and cost which was previously, one of the major issues related to this therapy,[10] making it available in the form of autologous in-vivo 'off-the-shelf' CAR-T cell therapy.

2.2.2: UB-VV100: *An Overview*

A VivoVec technology is developed to enable engineering of CAR-T cells in-vivo with the help of a lentiviral vector platform which demonstrates positive outcomes giving a pre-clinical proof of its efficacy. Therefore, a surface-engineered third-generation self-inactivating replication-incompetent lentiviral vector (LVV) platform is designed which have the potential to manufacture CAR-T cells without an ex-vivo set-up which needs no pre-treatment in the form lymphodepletion.[1] When compared to previously used lentiviral vector platforms, these third-generation LVVs had many safety improvements such as separating the viral components across various plasmids, which eventually reduced the risk of generating replication-competent viruses. With respect to transcriptional control, certain regulatory elements were optimized in a way, to

improvise on precision and accuracy in delivering the therapeutic genes. To ensure self-inactivating (SIN) replication-incompetent LVV, the U3 region of the viral LTR (long terminal repeat) was deleted.[3] This helped in the integration of the vector into the host genome causing the LTR region to become non-functional, resulting in prevention of unwanted gene activation and the risk of activation of certain transcriptional factors present in the viral genome. Furthermore, it also reduced the chances of insertional mutagenesis which causes activation of oncogenes linked with mutation and consecutive silencing or downregulating the activities of tumor suppressor genes which plays a key role in curtailing the occurrence or spread of cancer after the gene therapy.[3] Hence, it was important, that the vector doesn't form any new viral particles, which is achieved by confining the vector delivery at the origin itself, and preventing its spread to other parts of the body. This was achieved by making the vector incompetent of replication which ensured that, not only the vector is specific but also non-infectious. These attributes made LVV a potential candidate which can be used for clinical purposes and trials.

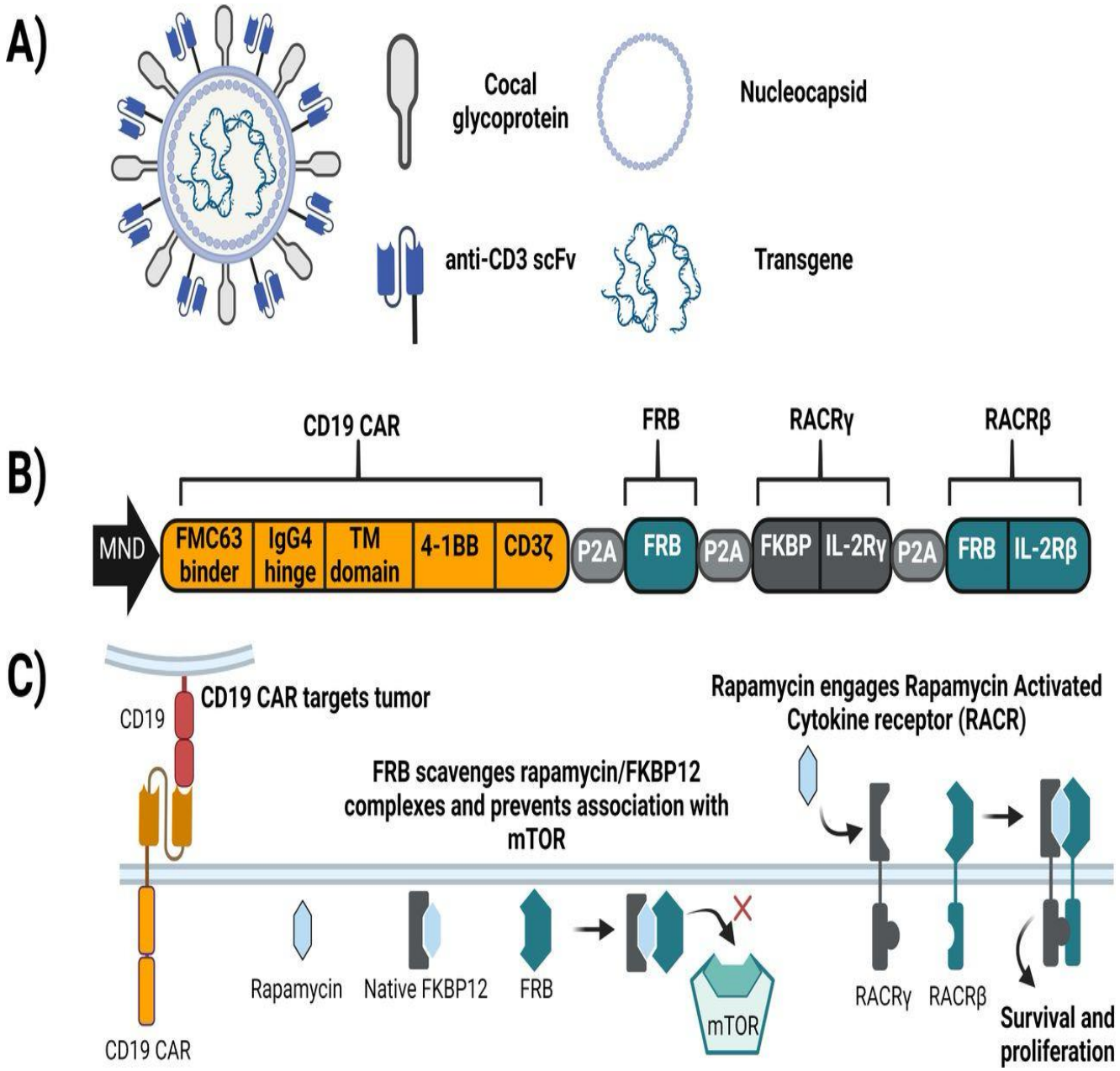


Figure 3: An overview of U-VV100 depicting its construct and attributes.
A) Diagrammatic representation of third-generation UB-VV100 viral vector; B) UB-VV100 viral vector driven by MND promoter and separated by P2A peptide fragment; C) Diagrammatic representation of components of UB-VV100 showcasing its ability to fight tumor cells. (sourced from https://jitc.bmj.com/content/11/3/e006292)

2.3.3: DESIGN Of VivoVec Vector: *UBVV100*

UB-VV100 is the first VivoVec platform-derived clinical candidate drug product, which targets CD19 for the treatment of B-cell malignancies (Figure 1A). CD19 is a type of glycoprotein which is mostly expressed in all kinds of B cells i.e., normal and malignant. It must be noted that CD19 is not present on any other hematopoietic cells solid tissues, making it a potential target to treat malignancies of B-cell origin.[11] These VivoVec particles carry a specific payload which is basically a therapeutic agent in the form of a genetic

material encapsulated in these particles. The encapsulated VivoVec particles are labelled as UB-VV100 which is a viral vector carrying a genetic code, targeted to deliver therapeutic genetic payload into the host cell to transfect it with the desired gene to reprogram T-Cells having potential to fight malignancies of B-cell origin.

LVVs are manufactured at clinical scale using suspension processes which basically refers to the production of viral particles using a cell suspension culture media. For clinical scale production, suspension processes are advantageous because of the scalable and reproducible characteristics of this bioprocess technique, enabling the manufacturer to control the scale-up, according to the needs and requirements. Moreover, the use of bioreactors in this process will ensure uniform growth, confirming consistent quality of the VivoVec particles, making it an ideal method which can be incorporated for clinical-scale production.[2] UB-VV100 particles are psuedotyped with the cocal fusion glycoprotein (a glycoprotein with a close structural relationship to that of VSV-G which have 71.5% shared amino acid sequence).[7] It must be noted that cocal is resistant to inactivation In humans, to ensure in-vivo persistence making it fit for direct administration. Serum constitutes various antibodies, having the ability to neutralize the viral particles which is referred to as serum inactivation, and it poses a great hurdle while using viral vectors as a therapeutic agent. Therefore, cocal glycoprotein coating will prevent UB-VV100 to undergo serum inactivation, thereby ensuring its effectiveness in terms of prolonged and consistent therapeutic stability.

UB-VV100 is designed to express an anti-CD3-scFv on the viral envelope to mediate T-cell binding, activation, and efficient T-cell transduction. T cells have CD3 protein on their surface which plays a crucial role in T cell activation, as it plays an important role in the signaling processes that enables T-cells to identify and respond to the antigens. Single chain variable fragments (scFv) are small antibody fragments which are engineered in such that it binds to CD3 specifically.[8] Therefore, the incorporation of anti-CD3-scFv on the viral envelope, will enable UB-VV100 to directly target and bind to T-cells. This is one of the steps, as the functionality of UB-VV100 depends on the fact that vector targets the right immune cells (T cells) and leave the other cells unharmed. Once UB-VV100 interacts and binds to T cells through CD3 protein, it will activate the T cells, making it functional and responsive to the therapeutic intervention. This will eventually activate the T cells which can now attack the cancer cells in accordance with the therapeutic modus operandi.

The process of transduction refers to the delivery of the genetic material (encapsulated in the vector) into the T cells. The presence of anti-CD3-scFv ensures efficient delivery by specifically interacting with the T cells and delivering its therapeutic payload into the cells. Once it has delivered the therapeutic agent inside the cell, the UB-VV100 can now reprogram the T cells in a way which can further aid in fighting the malignancy more efficiently.

2.2.4 UBVV100 Therapeutic Payload: *Mechanism of Action*

The payload encapsulated in UB-VV100 encodes for a polycistronic sequence (encoding for multiple protein) which is driven with the help of an MND promoter. The work-flow can be studied by categorising the functions of its sub-components as follows:[1]

1. **Polycistronic Sequence:** This sequence is responsible to drive the expression of the following four proteins under two major events:

a) Second generation anti-CD19 CAR activation (Figure 3B): The key components of the CAR are FMC63 scFv fragment, CD3-zeta signalling domain and a 4-1BB costimulatory domain.[1] FMC63 scFv (extracellular binding domain) is responsible to identify and bind to the CD19 antigen which is specific and mostly present on the surface of B-cell malignancies.[15] Therefore, FMC63 scFv is majorly responsible to bring the CAR-T cell towards the target. This makes FMC63 scFv a crucial component of the anti-CD19 CAR, which is responsible to enhance the specificity of the CAR-T cell. CD3-zeta (intracellular signalling domain) is made up of ITAMs (immunoreceptor tyrosine-based activation motifs) responsible to initiate an intracellular signalling cascade, once FMC63 scFv has activated CD3-zeta.[1,12] Lastly, the costimulatory domain, 4-1BB plays a supportive role in maintaining and amplifying the signals, thereby making the CAR-T cell therapy persistent. Hence, the combination of CD3-zeta and 4-1BB costimulatory domain will increase the chances of T cell survival and ensure efficient killing of the tumour cells. Therefore, anti-CD19 CAR activation is mediated with the help of two proteins i.e., FMC63 scFv protein and CD3-zeta protein.

b) Rapamycin activated cytokine receptor (RACR) System: The system is developed to resist rapamycin in order to increase the cell growth and survival. The RACR system is made up of two domains namely FKBP12- FRB domain and SCCR (synthetic chimeric cytokine receptor) domain. The FKBP12-FRB domain makes the CAR-T cells to function even in the presence of rapamycin, which instead is an immunosuppressive drug. When rapamycin binds with FKB12 protein (FK506-binding protein 12), it forms a complex, having the ability to downregulate and inhibit mTOR (mammalian target of rapamycin).[13] mTOR

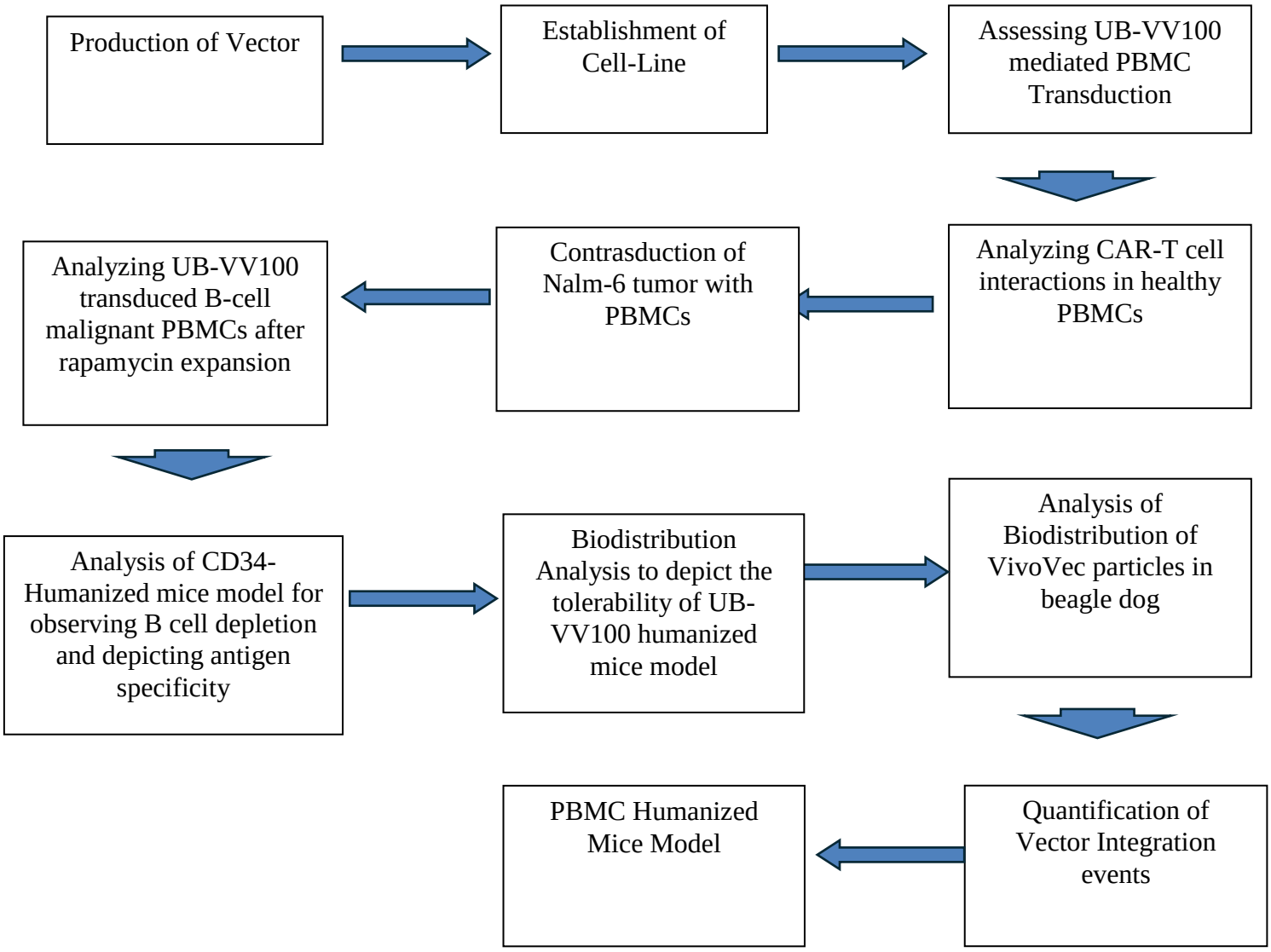
plays a major role in regulating cell growth and survival, and due to its consecutive inhibition, cells no longer have the ability to divide and proliferate, making rapamycin a drug involved in curtailing the growth of immune cells.[14] The FRB domain is the binding site for the FKBP12-rapamycin complex which is introduced as an augmentation in the CAR-T cells. Since the FRB domain can interact and bind with FKBP12-Rapamycin complex, it ensures mTOR expression throughout the therapeutic delivery. Therefore, in the presence of FRB-domain, rapamycin can no longer inhibit mTOR, and can be used in the treatment for other purposes. The SCCR domain, on the other hand, plays a key role in T-cell signalling, mediated with the help of FKBP12-IL-2R γ fusion, which constitutes for a fusion protein, formed as a result of linkage between FKBP domain and gamma-chain of the IL-2R.[13] The second component of SCCR is FRB-IL-2Rβ fusion, which implicates the fusion of FRB domain with the beta-chain of the IL-2R. In the presence of rapamycin, the two components of SCCR undergo heterodimerization (FRB-IL-2R γ + FRB-IL-2Rβ Dimer). The resultant heterodimer can initiate signalling cascades having functional attributes similar to IL-15. These characteristics of RACR system will eventually enable cellular growth followed by CAR-T cell survival, making the therapeutic intervention persistent, even in the presence of rapamycin.

2) P2A Sequences: These sequences are present in between the four proteins present in the anti-CD19 scFv and RACR system *(Figure 3B)*. As the polycistronic sequence is responsible for encoding these four proteins, it is important to prevent the fusion of these proteins into a single gene. The presence of P2A sequence will ensure that these proteins are encoded and produced as a single entity by preventing their fusion during translation process. P2A will ensure that each read (mRNA) is translated to give only one functional read product i.e. protein, making P2A sequence an important component of the payload.[16]

3) MND Promoter: This is a synthetic promoter, responsible to drive the expression of the genes present in the payload.[17] MND promoter will aid in constant expression of the polycistronic sequence, thereby ensuring persistent transcriptional activity, to form mRNA transcripts which can then be translated into the four proteins mentioned above.[1]

3. METHODOLOGY:

3.1: WORK-FLOW: *Generalized **Flowchart** (11 major steps)*



3.2: METHODOLOGY: PROCEDURE & PROTOCOLS STEPWISE

1. VECTOR PRODUCTION:

Lentiviral vector particles were engineered with the help of two main methods i.e., polyethlenimine-mediated transient transfection of in-house suspension adapted HEK293Ts or viral-production cells (Thermo Fisher Scientific) which was grown in a shaking flask incubator in FreeStyle or LV-Max medium respectively.[1] For large-scale production, these shaking flasks were replaced by bioreactors which enabled bulk production.[1] These viral vectors were further purified, concentrated and formulated with the help of a series of filtration processes which removed cell debris, host cell proteins, host cell DNA and residual plasmid DNA. The resultant viral particles were then sterilized via 0.2 µM filtration and were subsequently frozen. Furthermore, these lentiviral lots were tittered on SUP-T1 cells with the help of flow cytometry, performed against surface CAR expression and ddPCR against lentiviral Psi integration element using the

primer sets as: forward 5' -ACT TGA AAG CGA AAC- 3', reverse 5'-CACCCATCTCTCTCCTTCTAGCC- 3', TaqMan probe PSI-FAM: 5' -/56-FAM/ AGCTCTCTC/ZEN/GACGCAGGACTCGGC/3IABkFQ/-3'.[1] Finally the samples were analysed on the QX200 automated droplet generator and reader system. The formulations were examined and evaluated for particle count by p21 ELISA and western blot was performed to testify anti-CD3scFv incorporation. Vector formulations were confirmed and evaluated on the basis of particle count by p24 ELISA from ZeptoMetrix, USA. Furthermore, western blot technique was incorporated to confirm anti-CD3 scFv presence.

2. CELL LINES: *Five lines were used in this study which were purchased from different organizations.*

a) Peripheral Blood Mononuclear Cells (PBMCs): Cryopreserved PBMCs were purchased from AllCells located in California, USA and Bloodworks in Seattle, Washington, USA.

b) Nalm-6 and Raji Cell Lines: These cell lines were taken from Seattle Children Therapeutics and were modified to express specific genetic markers like mCherry and GFP::ffluc. These modifications can help to observe these cells in experiments, as the expressed genetic markers can fluorescence.[1]

c) CD19 Knockout (KO) Nalm-6 lines: This is also taken from Seattle Children Therapeutics. In these cell lines the CD19 gene encoding CD19 protein is knocked out.

d) HEK293T and SUP-T1 lines: These cell lines were obtained from ATCC (American Type Culture Collection). HEK293T (CRL-11268) is the most common type of cell line used in experiments involving is a process, such as transfection of a particular gene into the viral vector. SUP-T1 cells (CRL-1942) is a lymphoma cell line (T-cell).

e) Cryopreserved Human Patient Samples: These cell lines were obtained from ProteoGenex, California, USA.

3. ASSESSMENT OF UB-VV100 MEDIATED PBMC TRANSDUCTION:

The PBMCs were obtained from a donor and then cultured using a RPI 1640 Medium. The medium is treated with 10 % FBS (Fetal Bovine Serum) which is heat inactivated, to provide essential growth factors,[18] followed by cytokine supplementation (IL-2) at a concentration of 50 IU to promote T-cell growth. The transduction is done at a density of 2 million cells/ml combined with the vector (having desired payload), added directly into the wells containing PBMCs at an MOI (multiplicity of infection) kept at 5, meaning that each well will be infected by at least 5 viral particles over a span of 72 hours (3 days).[1] This is followed by rapamycin addition on the 3rd day at a concentration between 1-40 nM. The analysis is carried out with the

help of flow-cytometry on the 3rd and 7th day. FMC63 is used as a surface marker to identify CAR-T cells followed by a viability test to distinguish between dead and live cells carried out by staining of cells in a fixable viability dye. Cells were washed in a PBS buffer and then stained in FCAS buffer (PBS + 2% FBS) for a span of 30 minutes. The cells were then counted during flow cytometry by using CounBeads and the data was collected with the help of Cytoflex instrument.

4. **Analysing CAR-T cell Interactions in healthy PBMCs:**

To analyse the effect of transduced PBMCs tumour cells for the activation of functional CAR-T cells, healthy PBMCs were transduced with UB-VV100 and as control CD3/CD28 Dynabeads, obtained from Thermo Fischer Scientific were used which were not transduced with UB-VV100, but had the attributes similar to the antigen-presenting cells.[1] After 10 days of transduction, PBMCs were mixed with Nalm-6 tumour cells at varying degree of effector : target ratio (Transduced PBMCs: Tumour Cells). After 24 hours of co-culture, flow cytometry technique identified and distinguished live cells from dead cells.[1] The supernatant was collected and was used for cytokine analysis which was done with the help of Meso Scale Discovery Multiplex Detection System with the Proinflammatory Panel 1 (human) V-plex kit which have the ability to detect multiple cytokines in a single sample. Furthermore, the degranulation assay was done by culturing CAR-T cells and tumour cells in the ratio of 1:2 and incubated for four hours. The culture was treated with monensin and brefeldin for protein inhibition for easy detection of degranulation marker. CD107a (degranulation marker) was then stained with PE-labelled antibody diluted at 1:200 ratio. Finally, the level of CD107a, present on the surface of T cells, was measured depicting the effectiveness of CAR-T in killing the tumour cells.

5. **Contrasduction of Nalm-6 tumour with PBMCs:**

Cells from 8 healthy donor were collected and plated at a density of 500,000 cells per well, with an equal number of Nalm-6 cells (expressing GFP). The co-culture was then transduced with UB-VV100 which were analysed on the basis of presence of P2A sequence. Flow cytometry was used to detect the presence of CD19.

6. Analyzing UB-VV100 transduced B-cell malignant PBMCs after rapamycin expansion:

The PBMCs were collected from the patients suffering from B-ALL and R/R DLBCL. The sample was transduced with UB-VV100 which was followed by Rapamycin treatment starting from the 7th day to the 20th day after transduction. Transduced PBMCs were allowed to expand in the presence of 10nM rapamycin

and on the 20th day, the Rapamycin expanded CAR-T cells were analyzed for cytokine release as previously described.

7. Depletion of B cells in CD34 humanized NSG mice:

The study was approved by IACUC at the Fred Hutch Cancer Research Centre. The female mice (NSG) chosen was immunodeficient and was irradiated and then engrafted with CD34+ human haematopoietic stem cells at the Jackson Laboratories, Maine, USA. The vector was administered (thawed and treated with PBS) on the day of treatment via intraperitoneal injection (abdominal cavity) followed by blood sample collection using retro-orbital sinus (behind the eye) and stored in sodium-EDTA micro-containers to prevent them from coagulation. The blood sample was then centrifuged at 4000rpm for 10 minutes to separate plasma from the cells. For lysis of RBCs 1 * Pharmlyse (BD Biosciences) was used and then washed in PBS to remove the remaining debris. This was followed by staining (for viability) and flow cytometry (using 4-laser Cytoflex S flow cytometer from Beckman Coulter) and the data collected were analysed and processed using FlowJo V.10 software. Finally, the data was graphed on MS Excel and GraphPad Prism V.9 was used to plot the graphs.

8. **Biodistribution of UB-VV100 in CD34-humanized NCG mice:**

This study was conducted at Charles River Laboratories and was approved by IACUC protocol. A genetically modified female CD34-humanised mice was selected having humanization percentage of more than 45 % and was administered with UB-VV100 (thawed and treated with PBS) on the day of treatment via intraperitoneal injection (abdominal cavity) on the 1st day of the study. Blood samples were collected and assessed with the help of Flow Cytometry and FACS Lyric flow cytometer from BD Biosciences was used for data collection. For histopathological analysis FFPE (Formalin-Fixed, Paraffin-embedded) tissues were stained with H&E and were analysed by a board of pathologists at the Charles River Laboratories.

9. Biodistribution of αCD3 cocal pseudotyped vector encoding enhanced green fluorescent (eGFP) in canines:

The study was approved by IACUC and involved male and female beagles, aged 6-7 months (from Marshall Bioresources). The vector was administered to the beagles via intraperitoneal injection or inguinal lymph node injection. This was followed by FFPE

tissue staining and H&E staining. The stained tissue was analysed by pathologists at the Charles River Laboratory.

10. **Quantification of vector integration events and analysis of RNA expression:**

The DNA samples were extracted from flash-frozen whole blood and tissues by using Nucleospin Blood QuickPure Kit which from Macherey-Nagel), and on the other hand, for tissues DNeasy Blood and Tissue Kit was used which was bought from Qiagen. The extracted DNA was quantified using NanoDrop Lite Spectrophotometer (Thermo Fischer Scientific). For quantification of the vector qPCR was employed which was done in triplicate to make the whole process accurate and for the reaction, TaqPath ProAmp Master Mix bought from Applied Biosystems was used. 200ng of DNA was added to each well for the reaction to happen. The PCR reaction specifically focused on the viral PSI element of which is a part of the UB-VV100 paylaod and finally the PCR results were analysed by QuantStudio 7 Flex RT-PCR System which was from Applied Biosciences. RNA in-situ hybridization was performed to visualize and locate specific RNA sequences within the cells which employed RNAscope LS Multiplex Fluorescent Reagent Kit from the Advanced Cell Diagnostics. In this, 5µm thick FFPE sections were generated which was then pre-treated by applying heat, followed by protease to make them ready for hybridization, wherein oligonucleotide probes (target-specific) was used. Moreover, pre-amplifier and amplifier molecules were hybridized in such a manner, so as to, enhance the signal, which was detected with the help of TSA (tyramide signal amplification) reaction, which was used to attach a fluorophore. This fluorophore exhibits a fluorescence signal after getting attached with amplified sequence. These RNA signals were observed as small dots (fluorescent) within the cells, followed by its counterstaining them with DAPI, which was used to stain DNA and aided in the visualization of the nuclei. For imaging of these visuals, Panoramic Scan II digital slide scanner which was obtained from 3Dhistech was used and images were stored at a magnification of 40X. These data were manually examined under a microscope to identify the transduced cells and then quantify it.

11. Humanised Mice Model :

The study was approved by IACUC for the use of female mice (NSG DKO) which were purchased from Jackson Laboratories. The mice lacked the expression of MHC I and MHC II molecules which made them immunodeficient, thereby making them fit for the use of engraftment purpose in humans. The mice were then engrafted with 0.25E+06 Nalm-6 tumour cells which were injected via tail vein and these tumour cells were modified to make them express GFP and GFP::ffluc.[1] Then on day 4, animals were treated with 15mg/kg of XenoLight D-luceferin obtained from Perkin Elmer. This was again injected via tail vein and

reacted with firefly luciferase (ffluc) which was expressed by Nalm-6 cells. The reaction caused an emission of light which was detected. After 25 minutes of injecting D-Luciferin imaging was done by using an in-vivo imaging system (IVIS) Spectrum Instrument from Perkin Elmer. This aided in observing the tumour spread which was readily visible because of light emitted by the tumour cells. Humanization was done on the 1st day which was just a day before the treatment by injecting 20E+06 PBMCs through intraperitoneal route. This engrafted the animal with the human immune cells which was suitable enough to be used for comparative analysis to assess the human immune response. These animals on 0th day were divided into three groups and were injected accordingly. The first group was control followed by UB-VV100 group and then finally cocal control group. Lastly the blood samples were collected and analysed with the help of flow cytometry. The sole purpose of this study was to analyse the biodistribution of UB-VV100 vector in the mouse model having Nalm-6 tumour cells. Moreover, the study also aimed to depict the specificity and efficacy of UB-VV100, by analysing their interaction with controls incorporated.

4. KEY FINDINGS: *Critical Analysis*

4.1: Production of functional CAR-T cells: *Activation and transduction of T cells in a dose dependent manner*

- In this study the efficacy of UB-VV100 to activate and transduce T cells was analysed, by comparing genetic payload of UB-VV100 and a control containing the same payload but lacking anti-CD3-scFv (*Figure 4A)*. This experiment was done to assess the potency of UB-VV100 in activating T-cells and making them functional. The observations depicted UB-VV100 to activate and transduce CD4 and CD8 T cells in a dose-dependent manner. It implicated that, if we increase the concentration or level of UB-VV100, the activation of T-cells will be increased simultaneously. On the other hand, the control i.e., LVV was not able to activate and transduce the T Cells. This study also proves that unstimulated T cells cannot be transduced by lentiviral vectors pseudotyped with VSV-G or cocal glycoprotein *(Figure 4B).*
- It was also important to analyse whether UB-VV100 will interact and transduce CD3-negative population or not. It was found that UB-VV100 does not transduce CD3-negative population, confirming its specificity. Furthermore, vector particles without any genetic payload also showed no transduction, which confirmed that UB-VV100 is safe to use.

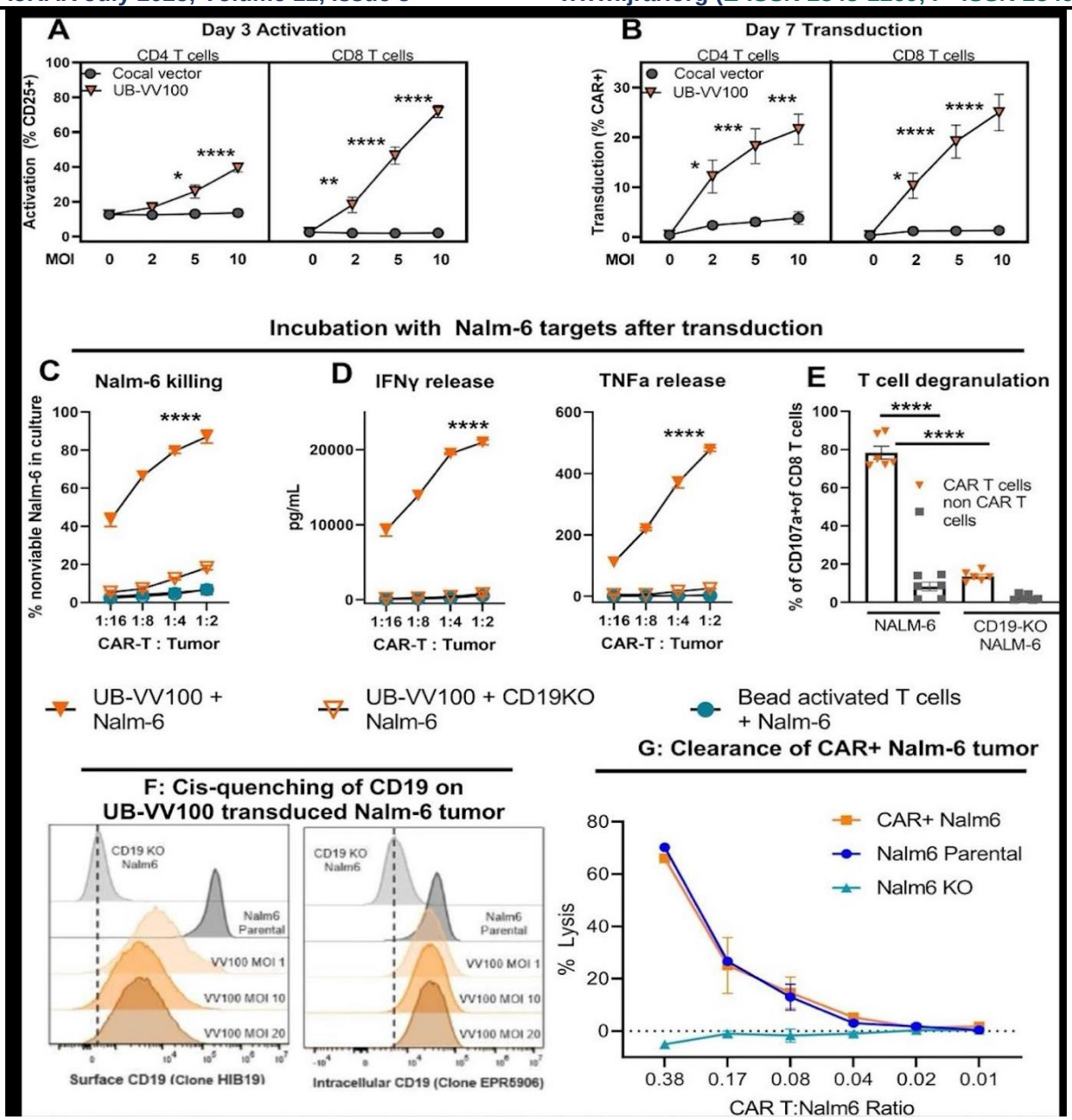


Figure 4: A) Graph depicting T-cell activation 3 days after vector incorporation which is measured by analyzing expression level of CD25 (IL-2 alpha receptor); B) Graph depicting transduction frequencies of CD4 and CD8 T cells 7 days after vector addition; C) Graph depicting a comparative analysis of killing Nalm-6 tumor cells; D) Graph depicting release of IFN gamma and TNF alpha during killing of tumor cells; E) Graph depicting T cell degranulation which is analyzed by flow cytometry; F) Graph depicting protein levels because of anti-CD19 antibody binding intracellularly to CD19 surface antigen. G) Graph depicting clearance of CAR + Nalm-6 tumor in the of % lysis. (image sourced from https://jitc.bmj.com/content/11/3/e006292#supplementary-materials).

- The next study was to assess the specificity of UB-VV100 transduced CAR-T cells to attack CD19+ cells and its effect on CD19-negative cells. For this purpose, few Nalm-6 tumour cells were subjected to knockout of CD19 gene encoding CD19 protein which is a specific antigen present on the surface of B-ALL. Therefore, two types of Nalm-6 cells were analysed i.e. CD19+ Nalm-6 cells and CD19KO Nalm-6 cells. It was observed that when they were incubated with UB-VV100 transduced CAR-T cells, the CD19+ Nalm-6 cells were lysed and killed *(Figure 4C)*, which was confirmed by the secretion of cytokines *(Figure 4D)*. However, CD19KO Nalm-6 cells were unaffected *(Figure 4C & 4D)*. Therefore, it was confirmed that payload of UB-VV100 is specific to CD19+ cells.

- To validate this observation, degranulation (cytotoxic granules) assessment was carried out. For this purpose, presence of CD107α (surface marker for these granules) was analysed. Significant degranulation was observed when CAR-T cells were co-cultured with CD19+ Nalm-6 cells confirming that anti-CD3-scFv-CAR-T cells effectively lyse CD19+ tumour cells *(Figure 4E)*. On contrary, CD19KO Nalm-6 cells did not exhibit any degranulation, thereby validating the specificity of UB-VV100 therapeutic payload.

- The study also addresses a potential risk of a phenomenon known as “epitope masking”, which was witnessed in a patient with paediatric B-ALL who was undergoing treatment with an *ex-vivo* manufactured anti-CD19 CAR-T cell therapy labelled as CTL019. The masking of CD19 epitope was because of the longer hinge region of the CAR-T cell receptor which made the therapy inefficient. In this regard, CAR+Nalm-6 cells with UB-VV100 were produced to analyse CD19 surface expression detection and to evaluate the risk of developing resistance against CAR-T cell cytotoxicity. Low MFI (median fluorescence intensity) depicted that surface detection of CD19 on CAR+Nalm-6 cells was reduced but, it was completely absent suggesting the presence of partial epitope masking. At the same time the amount of CD19 present in CAR+Nalm-6 cells was similar to that of non-transduced cells *(Figure 4G)*. The study also showcased the efficacy of anti-CD19 CAR-T, by establishing that CAR-T cells were able to lyse and kill the tumour cells in an antigen-dependent manner. The researchers predicted that, it is due to the short IgG4 hinge (14 amino acid) in CD19-CAR which was unable to mask the distal CD19 epitope, in contrast to CTL019 which encodes a longer CD8a hinge, having the

ability to mask CD19 epitope completely. Thus UB-VV100 proved to be efficient, even if epitope masking is witnessed, making it a better and a safer therapeutic agent to treat CD19+ cancers.

4.2: **RAPAMYCIN-INDUCED:** *RACR activation amplifies T-Cell Expansion*

- In this study the efficiency of the RACR system which was introduced as an innovation in UB-VV100 was analysed. The PBMCs were transduced with UB-V100 to produce CAR-T cells which were then subjected to rapamycin treatment at 0, 1, 4, 10 and 10 nM concentration to measure the extent of CAR-T cell expansion at varying concentrations. As a control, a culture was kept without any rapamycin treatment.

- It was observed that the CAR-T cell expansion was directly proportional to the concentration of rapamycin, thereby, confirming that CAR-T cell expansion in the presence of rapamycin is concentration-dependent, which means that by increasing the concentration of rapamycin, CAR-T cell expansion will also increase, making the therapy more robust and efficient. CAR+ CD4 and CD8 T-cells were increased and the maximum expansion was observed at 10nM in the majority of samples *(Figure 5A)*.

- Furthermore, it was observed that, the cultures without any rapamycin treatment (control) and CAR-negative cells (same well), did not match the extent of expansion when compared with cultures with rapamycin treatment. This confirmed that RACR system supports the growth and proliferation of CAR-T cells, which otherwise (in other cases) is reduced. This will aid in the use of rapamycin in our treatment (if needed specifically) without any concerns of immunosuppression.

- Moreover, it was established that T-cells thrived and survived in all the tests, making RACR system an important attribute of UB-VV100, which not only supports the growth and expansion, but also, enables T cell survival, making the therapeutic modality to persist for a prolonged period of time.

- Lastly, measurement of total number of CAR-T and their frequency in terms of percentage was calculated. Since, maximum expansion was observed at 10nM (in most of the cases), this study used samples from this segment of concentration only which were then observed over a period from the 7^{th} day – 21^{st} day. The results showcased the credibility of rapamycin at 10nM, as frequency and total number of cells increased significantly in the given time period *(Figure 5B)*.

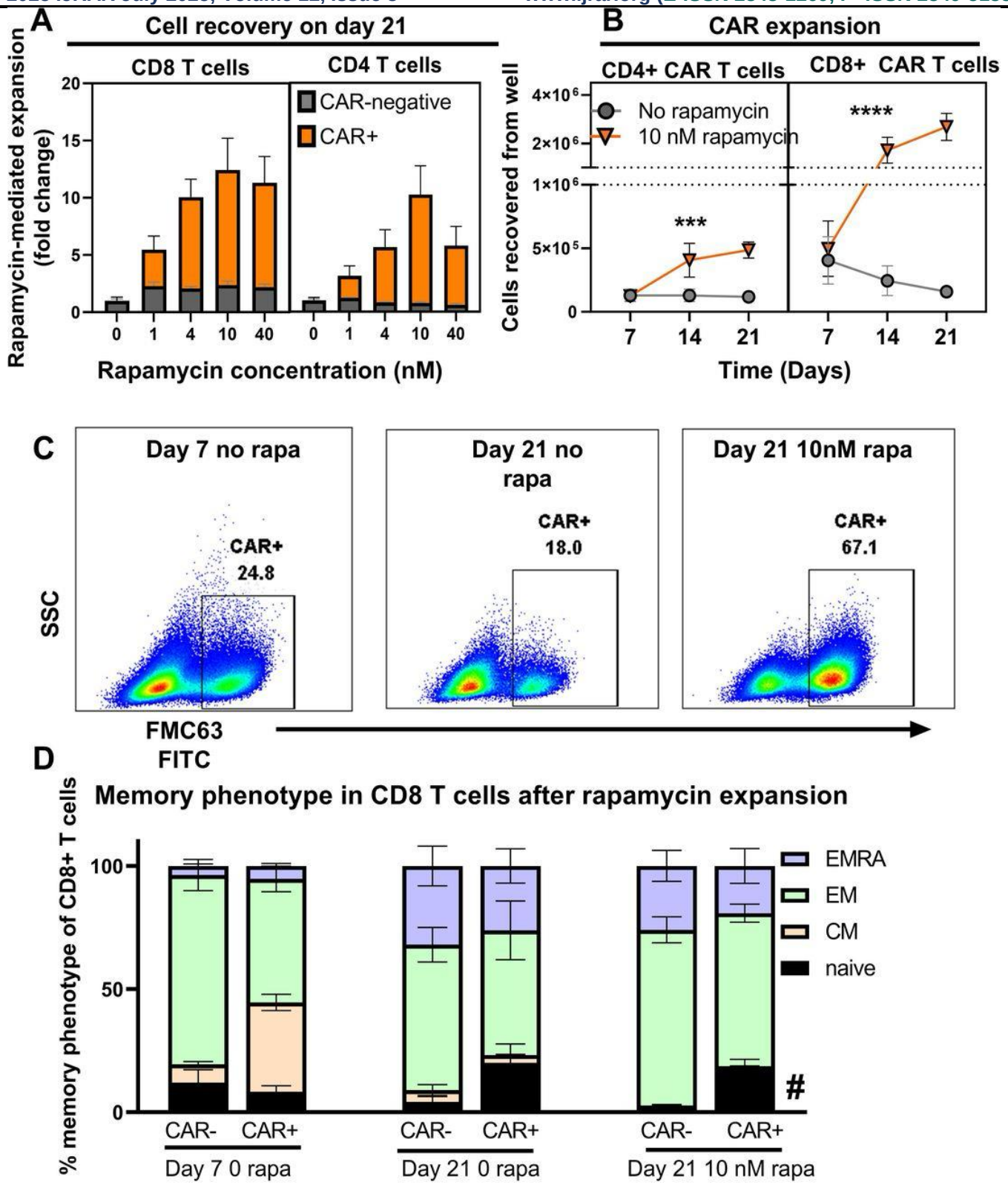


Figure 5: A) Graph depicting T cell expansion enumerated from flow cytometry and results were pooled based on three separate PBMC donors; B) Graph depicting peak expansion at 10nM rapamycin concentration; C) Representative flow staining of surface FMC63 detection and D) memory phenotype. (sourced from https://jitc.bmj.com/content/11/3/e006292)

- The next aim was to analyse the durability of the RACR signalling, stimulated and regulated by rapamycin and how it influences the memory differentiation state of CAR-T cells. For this purpose, rapamycin treatment was given at 10nM to CAR-T cells for a span of 18 days to ensure RACR system activation continuously, Then on the 21st day, analyzation of memory differentiation state was done for CAR-T cells and CAR-negative T cells (control). This was done by assessment of CD45RA expression and CCR7 expression.[21] Results showcased that after 18 days, CAR-T cells exhibited CD45RA and CCR7 naïve cells at higher frequency when compared with CAR-negative cells which was placed in the same well. Furthermore, it was observed that, despite constant RACR activation, there were no signs for terminal differentiation *(Figure 5D)* (T-cells losing their abilities/less effective T cells). Hence, it was deduced that, RACR system not only aids T cell expansion, but also ensures their maintenance, by preventing them to undergo terminal differentiation. To sum-up, RACR system plays a pivotal role in ensuring the persistence and consistency of the therapeutic intervention using UB-VV100.

4.3: FUNCTIONAL CAR-T CELLS: *In Pre-treated Patient Population*

- In this study the efficacy of UB-VV100 was analysed in terms of its ability to produce functional CAR-T cells in samples of PBMCs from patients suffering from B-cell malignancies such as B-ALL and R/R DLBCL.

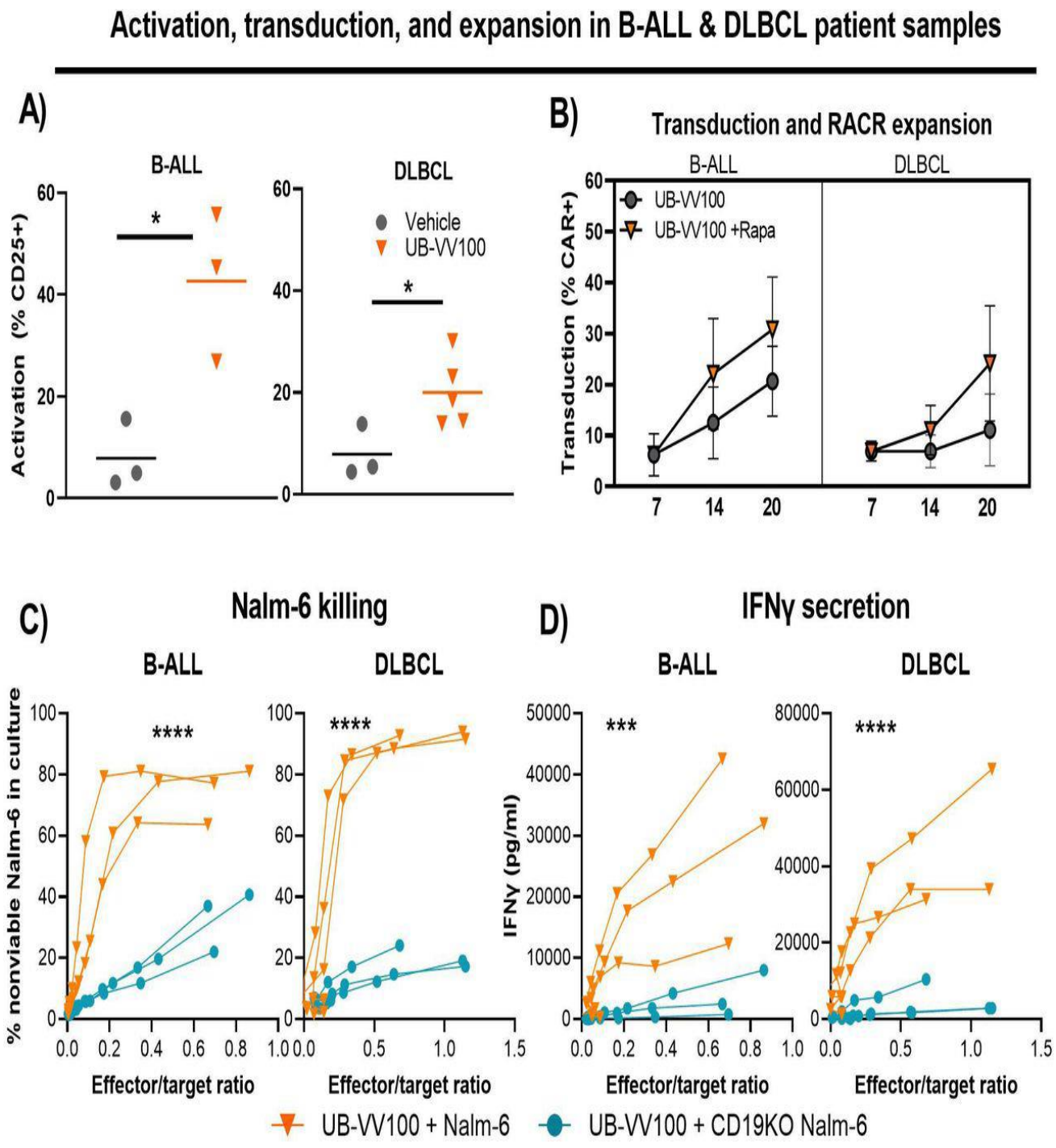


Figure 6: A) Graph depicting that in both the cases i.e. B-ALL and r/r DLBCL UB-VV100 mediated T cell activation and produced functional CAR-T cells; B) Graph depicting that treatment with rapamycin can give higher frequency of CAR-T cells by day 20; C) & D) Graphical representation depicting that rapamycin treated cultures can effectively kill tumor cells by cytokine release ability. (sourced from https://jitc.bmj.com/content/11/3/e006292)

- The challenge here were patients with B cell malignancies, having mostly higher inflammatory state compared with normal individuals. This inflammation is majorly due to disease and previously undertaken therapies, and can cause disparities in terms of CAR-T cell functioning and its expansion, while treating such patients with CAR-T cell therapy (this can be because of the abnormal cellular ratios e.g., CD4 : CD8 ratio).

- The PBMCs (from patients) of B-ALL (3 patients) and R/R DLBCL (5 patients) were transduced with UB-VV100 on day 0, with the aim to generate CAR-T cells. Following this, Rapamycin treatment was given on day 7 at a concentration of 10nM in order to amplify the growth of CAR-T cells. (control: without rapamycin)

- It was observed that UB-VV100 was able to generate CAR-T cells from both the samples of PBMCs *(Figure 6A).* This confirmed that UB-VV100 is even capable to generate CAR-T cells, even in an environment which is as challenging as it gets.

- As it was observed in the previous studies, rapamycin played an important role in this experimental set-up as well. Rapamycin activated RACR system, thereby **increasing the frequency of production of CAR-T cells, which was demonstrated** by samples having better frequency of CAR-T cells by day 20 when compared with samples without rapamycin treatment *(Figure 6B).*

- By analysing in-vitro model of Nalm-6 cells, it was confirmed that samples with rapamycin-enriched CAR-T cells were able to lyse cancer cells and secrete cytokines *(Figure 6C & 6D)*. To sum-up, UB-VV100 was able to kill cancer cells even in pre-treated patients.

4.4 SELECTIVE TRANSUCTION: *Implicated in Humanised Mouse Model*

- In this study humanised mice model having CD34+ haematopoietic stem cells (makes the immune system similar to that of humans) was incorporated to assess the biodistribution and the safety profile of UB-VV100. B-cell depletion was used as a marker to depict the activity of the therapeutic agent. Intranodal delivery of UB-VV100 is more beneficial as it will increase the chances of T cells getting exposed to the lymphoid tissues and consequently reduce the risk of systemic distribution of the viral vector. However, since mice models do not have a fully developed lymphatic system, the vector was injected through intraperitoneal site (abdominal cavity).

- The mice were treated with varying doses of viral vector as 0.4, 2 and 10 million transducing units (TU), which exhibits the functional genes present in the payload of UB-VV100. The results were observed by analysing the degree of depletion of B-cells in the mice.
- The study showcased that B-cell depletion occurs in accordance with the amount of dose injected i.e. dose-dependent depletion. At the highest (10 TU) dosage, complete eradication of B-cells was observed, known as B-cell aplasia, which confirmed a robust CAR-T cell activity.
- However, the control (mice without any doses of relevant CAR-T cells) was similar to the samples of untreated mice. Therefore, it was confirmed that B-cell depletion is majorly because of the activity of UB-VV100 *(Figure 7A)*.

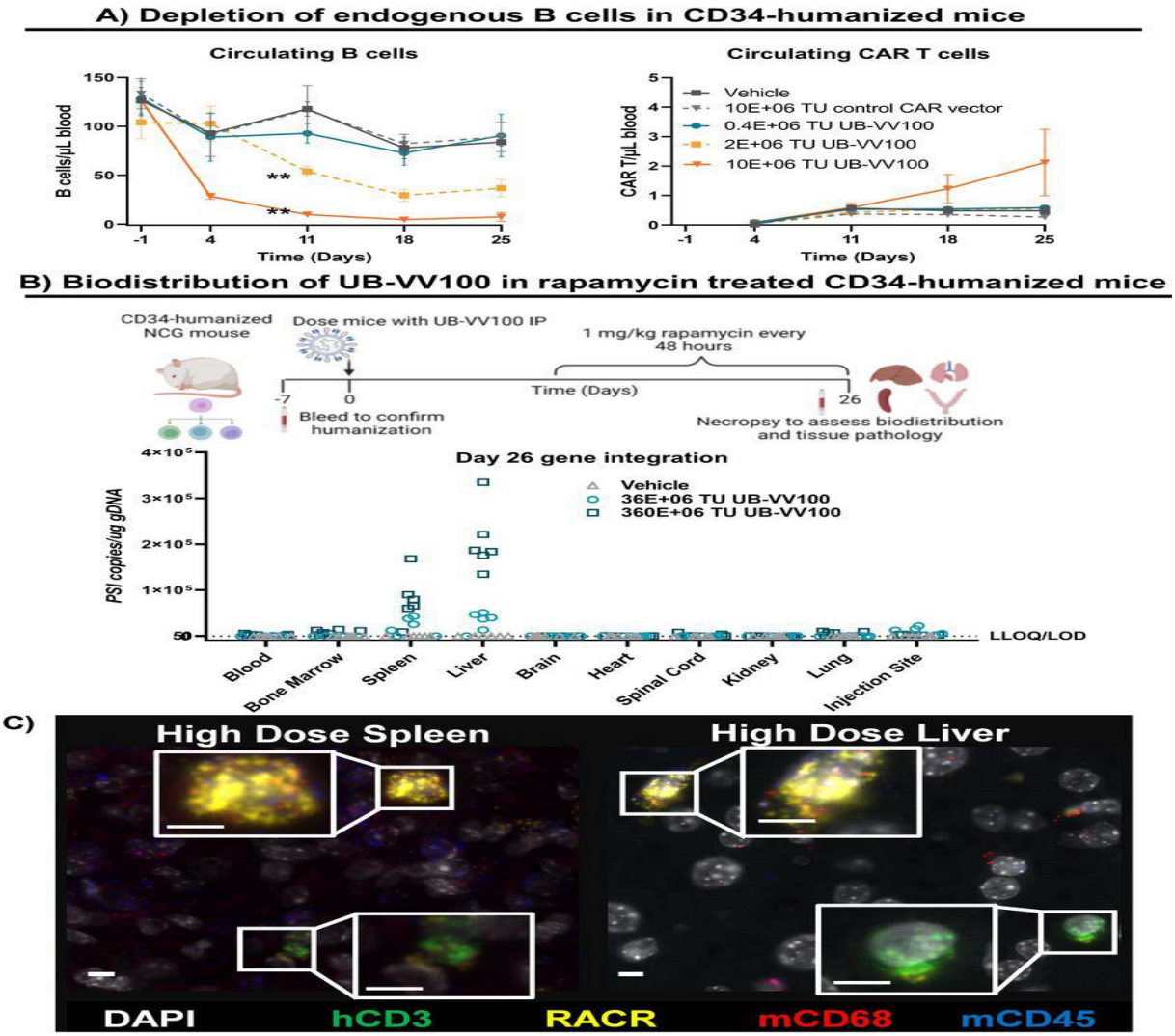


Figure 7: (A) Graph depicting the effect of UB-VV100 at varying dosage of TU i.e. at 0.4, 2 and 10 TU. The results show that B-Cell depletion occurs in a dose-dependent manner which means as the TU increases, B-cell depletion (killing) also increases. On the other hand, there was no such observations of depletion in the case of control. (B) q-PCR results showcased maximum integration of vector in organs like spleen and liver and minimum integration in organs like lungs, bone marrow and at the site of injection. (C) ISH results depicts that UB-VV100 interacts with a particular type of immune cells in spleen and liver with maximum being murine macrophages (mCD68) and nearly 0-10 % interaction with humanised CD3+. (sourced from https://jitc.bmj.com/content/11/3/e006292)

- Next, the circulating CAR-T cells in the blood was measured, and it was observed that concentration increased by increasing the dosage of UB-VV100. On the other hand, in the control group, there were no such observations, which confirmed CAR-T specificity based on the presence of the surface antigen i.e. CD19. This observation testifies that UB-VV100 is a safe therapeutic agent, which can be used to treat CD19-positive B-cell malignancies.

- The study also analysed the time lag between B-cell depletion and CAR-T cell detection, which gave some interesting outcomes. The B-cell depletion was witnessed on 11th day and the CAR-T cell detection was on the 18th day. One of the reasons for such observation, can be the activity of CAR-T cells in the tissues and not in the blood or elsewhere, it could be due to the interactions with their target antigens. To sum-up UB-VV100 is capable enough to deplete the B cells, and leave the other cells unharmed, thereby making it highly specific and safe.

- Now, the mice were given 36 or 360 TU of the viral vector UBVV-100 followed rapamycin dosage at alternate days starting from the 5th day of this study. It was found that mice were able to tolerate the given combined dosage without any complications, establishing the safety of the therapeutic modality (no negative reactions to the therapy) and as observed earlier, there was a dose-dependent depletion of B-cells which proved its efficacy as well. On completion of this study (28th day), necropsy was done for histopathological examination and, it was found that there was no significant difference between the animals treated with UB-VV100 and animals treated with control (vehicle) in terms of body weight and organ weight.

- Biodistribution of UB-VV100 was done with the help of q-PCR, to assess the presence of the vector in the tissues. It was found that the maximum integration of UB-VV100 was in the spleen and liver, and on the other hand, minimum integration was observed in the bone marrow, the site of injection and lungs *(Figure 7B)*. It depicted that UB-VV100 has a tendency to interact and integrate with cells of certain organs like liver and spleen. Now, to assess its capability to activate and integrate immune cells in the organs having maximum integration, in-situ hybridization (ISH) against the transgene was carried out. The results showed that the maximum number of cells which were transduced in these organs were murine macrophages which is a type of immune cells (91% - 100% murine CD68+) followed by a

smaller proportion of T-cells (0-10 % human CD3+) which were supposed to be from the humanized component of the mice *(Figure 7C).*

4.5: BIODISTRIBUTION IN CANINES: *Intranodal Delivery V/S Intraperitoneal Delivery*

- In this study biodistribution of the UB-VV100 in canines (beagle dog) was analysed in which the mode of delivery was through intranodal route (lymph nodes) or through the intraperitoneal route.
- As it was not possible in the NSG/NCG mice model to inject the vector via intranodal route, therefore canines were used to analyse whether this route of delivery is beneficial or not. For this purpose the UB-VV100 was subjected to genetic modification to carry a green fluorescent protein (EGFP) gene to track the transduction into the cells. However, the vector was designed for the purpose of targeting human T cells, there were chances that there would be no targeted activation of T cells.
- There were two routes selected for comparative analysis of the UB-VV100 interactions with the cells i.e. Intranodal delivery and intraperitoneal delivery. In the case of intranodal delivery, the vector was injected directly into the inguinal lymph nodes which is near to the groin region (T cells are concentrated) of the selected healthy beagle dogs by using ultrasound to guide the delivery appropriately *(Figure 8A)*. On the other hand, for intraperitoneal delivery, UB-VV100 was injected into the abdominal cavity.
- The results in the case of intranodal delivery depicted that the genetic payload was detected at maximum concentration at the site of injection i.e., inguinal lymph nodes. Furthermore, there was lower frequencies of transduction in the medial iliac and the lumbar lymph nodes, which are basically downstream lymph nodes and are a part of lymphatic drainage system. In the case of intraperitoneal delivery, the transduction was observed in maximum frequency in the blood and medial iliac and lymph nodes *(Figure 8B)*. The key finding of this study was that no genetic material of the vector was observed in any non-lymphoid organs depicting that, the therapeutic payload (genetic material encapsulated in UB-VV100) restricted to the organs linked with immune system (in both the cases), establishing and confirming that the vector targets specifically the tissue carrying immune cells.

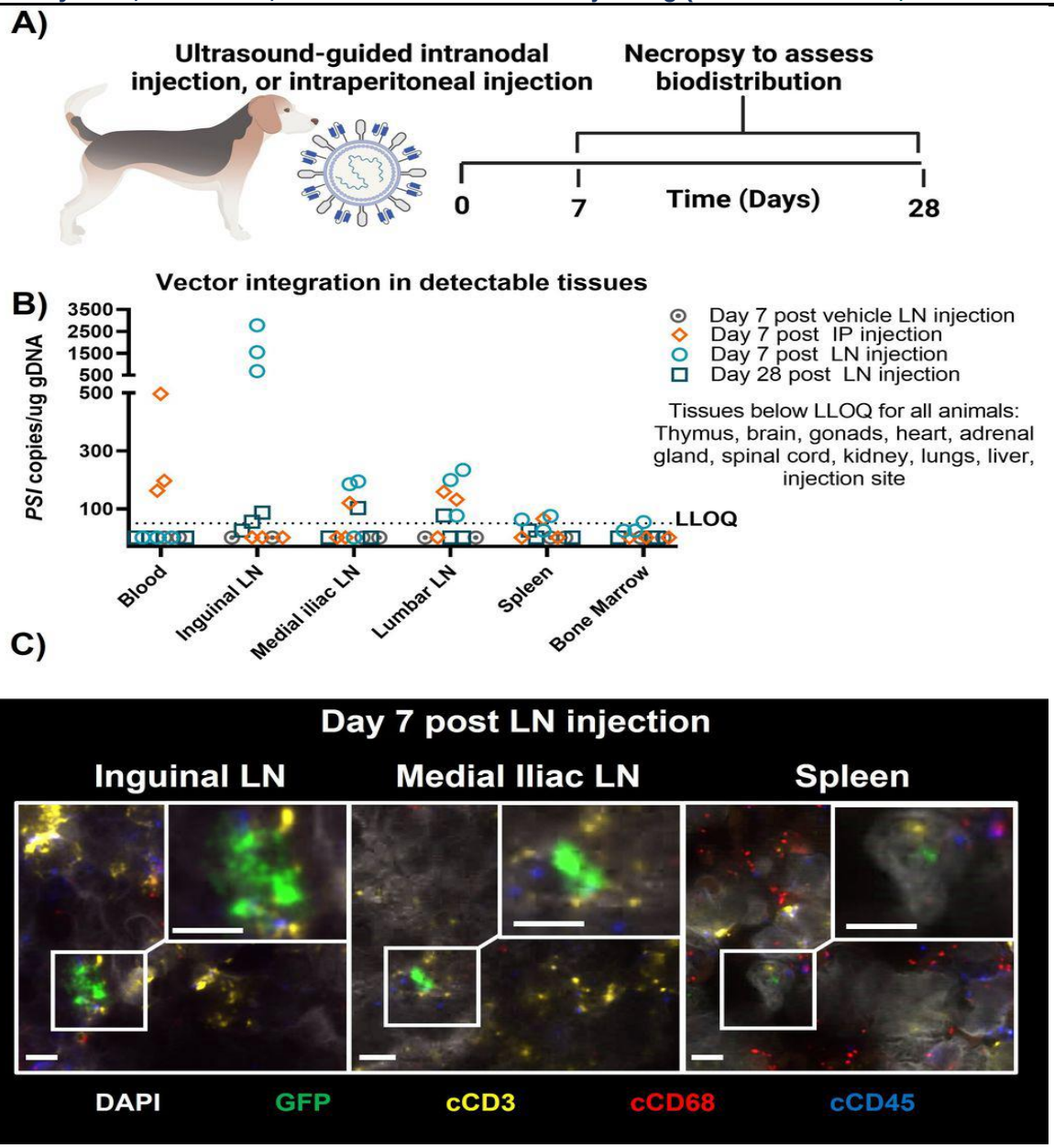


Figure 8: (A) Diagram depicting intranodal delivery of vector guided by ultrasound and the timescale of the study starting from 0th day to 28th day of the whole protocol. (B) Graphical representation exhibiting the transduced cells in various parts of canine. The graph depicts that via intranodal delivery maximum transduction was observed in the inguinal lymph node on the other hand, via intraperitoneal route, maximum transduction was in the blood. The graph also confirms that the transduction is restricted to the organs linked with immune system. (C) in situ fertilization images suggests that maximum transduction is in murine macrophages (green color due to expression of EGFP) and little transduction in canine CD3 cells (yellow color). (sourced from https://jitc.bmj.com/content/11/3/e006292)

● This study showed maximum frequency of transduction occurring in macrophages which was identified as Transgene-positive due to the expression of EGFP gene which was added to the vector and canine CD68+. On the other hand, T cells were identified as Transgene-positive and canine CD3+. The most astonishing observation was that, canine T cells were also transduced in the lymphoid regions of the dogs which were injected via intranodal delivery of the vector *(Figure 8C)*. This made intranodal delivery more effective comparted to intraperitoneal delivery in terms of targeting T- cells even in the absence of specific surface engineering.

4.6: UB-VV100 EFFICIENTLY KILLS TUMOR CELLS: *In Nalm-6 leukaemia mice model*

● In this study analysation of efficacy and potency of UB-VV100 to generate functional CAR-T cells in the case of systemic leukaemia by incorporating Nalm-6 leukaemia mice model was done. For this purpose, on 0^{th} day the NSG mice was subjected to intraperitoneal delivery (injection) of UB-VV100 at 27, 80 and 270 million transduction units (TU) *(Figure 9A)*. The mice was already knockout for MHC class I and MHC class II genes to avoid any chances of GVHD i.e. graft-versus-host-disease to aid in successful humanisation of mice model by introducing human PBMCs into the animal which was done just a day before UB-VV100 injection. On the 4^{th} day Nalm-6 cells which are human leukaemia cells were injected into the NSG mice via tail vein to stimulate systemic leukaemia.

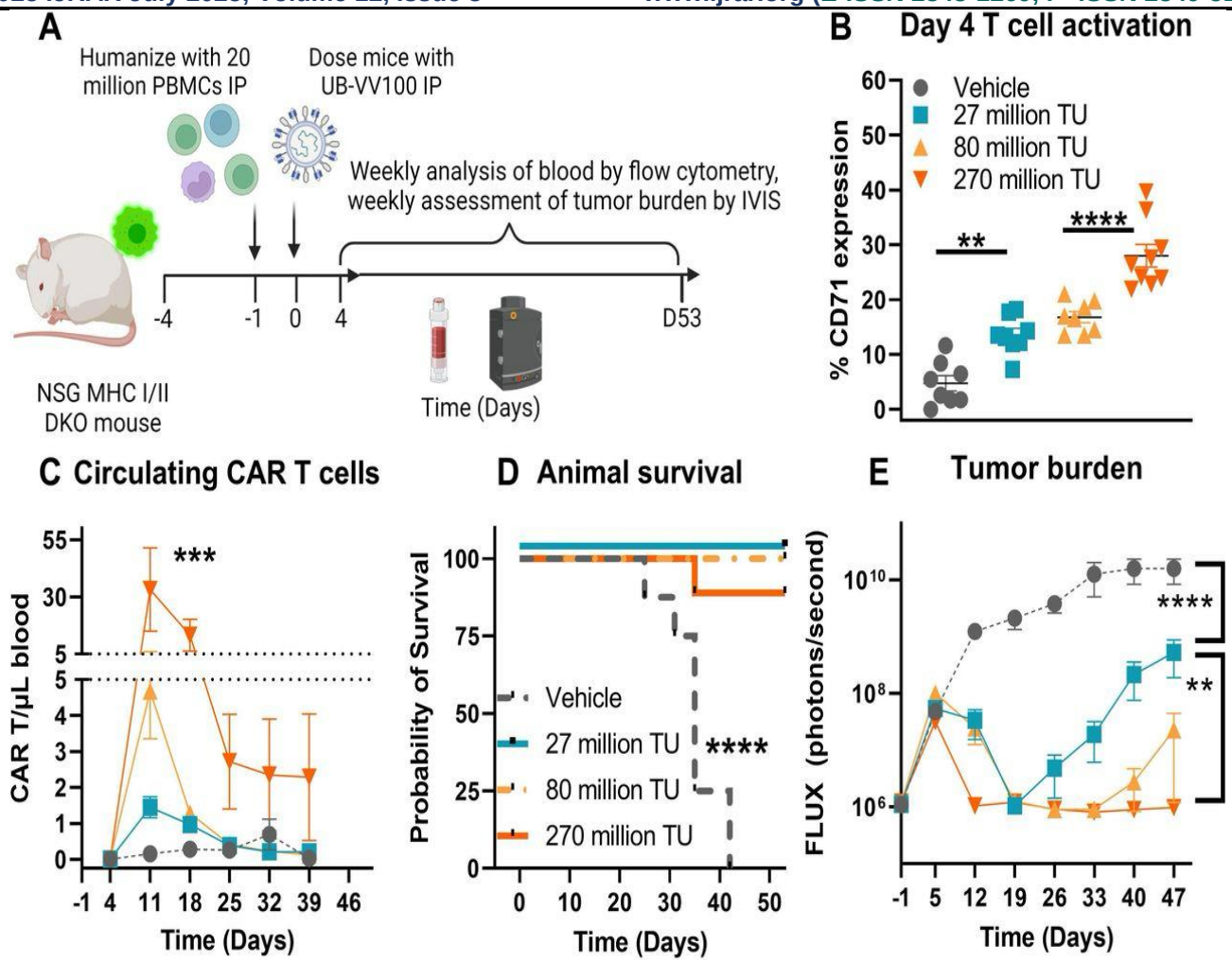


Figure 9: (A)Diagram depicting KO of MHC I & MHC II 4 days prior and getting humanized with 20 million PBMCs injected through intraperitoneal route 1-day prior UB-VV100 injection. Weekly analysis was done by to analyze tumor burden with the help of IVIS. (B) Graphical depicting dose-dependent increase in the expression of CD71on the dosage of UB-VV100 which confirms CAR-T cell expansion increases when doses are increased.
(C) On the 11th day CAR-T cells are detected, with highest frequency of CAR-T cells linked with the highest dosage of UB-VV100, implicating successful in-vivo production of CAR-T cells at higher doses. (D) Graph depicting that the models treated with UB-VV100 have better survival rates or probability when compared to those who were treated with vehicle or control, confirming that UB-VV100 increases OSR (overall survival rate). (E) Graph depicting complete absence of tumor cells at the highest dose of UB-VV100.
(sourced from https://jitc.bmj.com/content/11/3/e006292)

● The results depicted that UB-VV100 was able to generate functional CAR-T cells in-vivo which was measured by the activation of T cells (human). This was observed by the expression of CD71 recorded four days after the administration of UB-VV100. It was observed that there is a dose-dependent activation of T cells which was confirmed by CD71 expression, which increased with the concentration of the vector employed *(Figure 9B)*. Therefore, if the concentration of the vector is increased, there will a substantial increase in the activation of T-cells as well.

- On the 11th day of the study, CAR-T cells were now detectable in the blood-stream of the mice, confirming that in-vivo generation of CAR-T cells is achieved (dose-dependent manner) *(Figure 9C).*

- The next step was to analyse the efficacy of the generated CAR-T cells in lysing cancer cells. On the 12th day, the mice subjected to UB-VV100 showcased positive results, as there was a significant reduction in the population and density of tumour cells when compared to controls (vehicle with irrelevant CAR or without CAR). The most astonishing observation was the complete absence of tumour cells at the highest dosage *(Figure 9E).* This was validated and confirmed by the absence of any detectable traces (during terminal euthanasia) of Nalm-6 in the blood of models treated with UB-VV100, testifying its credibility to fight leukaemia efficiently. Furthermore, the survival index of the models with UB-VV100 was much higher when compared to the models treated with the vehicle (control) *(Figure 9D).*

- To confirm the specificity of UB-VV100, the results showed no observations of transduction of mCD45+ cells throughout the protocol. However, due to the absence of any tumour cells in UB-VV100 treated models, Nalm-6 transduction analysation was not carried out.

5. DISCUSSION:

We engineered lentiviral particles with cocal glycoprotein and anti-CD3 scFv, enabling them to target, activate, and transduce CD3+ T cells to express an anti-CD19 CAR and the RACR system. The resulting CAR T cells specifically engaged tumours and expanded more effectively in the presence of rapamycin compared to non-transduced cells. In animal models, a single dose of UB-VV100 led to T cell activation, CAR T-cell production, and the elimination of malignant B cells without causing significant tissue damage.

Normally, T cells in a resting state are not easily transduced by lentiviral vectors (LVVs) because they are in a dormant phase of the cell cycle. Previous research has focused on overcoming this challenge by modifying LVVs to improve their ability to transduce T cells, especially when combined with external stimuli like IL-7. In contrast, UB-VV100 achieved significant T-cell activation and CAR T-cell generation without additional stimulation, suggesting that further refining the vector's design could enhance its effectiveness.

Using LVVs in-vivo raises concerns about off-target effects and the broad distribution of the virus in the body. While our study did find widespread distribution of the transduced cells, they were mostly immune cells, such as T cells and macrophages. This finding aligns with previous studies showing LVVs' preference

for immune cells over other tissues. The transduction of macrophages, typically inefficient in lab conditions, may be due to their scavenging behaviour in the body, suggesting that CAR+ macrophages could help clear targeted cells.

Applying UB-VV100 in clinical settings presents unique challenges, particularly when dealing with patient-derived materials. Even so, UB-VV100 successfully generated functional CAR T cells from patient-derived cells with B-cell malignancies. One risk of in vivo CAR T-cell production is the potential for CAR transduction in malignant B cells, which could lead to immune evasion. However, our design of the CD19 CAR in UB-VV100 minimized this risk, and no immune escape was observed in our models.

In PBMC humanized mouse models, the use of rapamycin interfered with both humanization and tumour growth, making it difficult to study. However, our data suggest that pairing in vivo transduction with RACR-mediated expansion could address some of the effectiveness and safety issues seen with current CAR T-cell therapies. Expanding CAR T cells within the body might produce cells with better function and reduce the risk of cytokine release syndrome (CRS), a common side effect in these therapies. By avoiding lymphodepletion, a process that increases infection risk, UB-VV100 may offer a safer alternative, allowing rapamycin to protect nascent CAR T cells while supporting their growth.

In vivo CAR T-cell engineering could make these therapies more accessible by reducing costs, treatment wait times, and eliminating the need for chemotherapy, while also potentially improving both safety and effectiveness compared to current ex vivo CAR T-cell therapies.

FUTURE DIRECTION (in my opinion): in-vivo CAR-T cells optimised by AI tools can revolutionise cancer treatment not only limited to H.M but numerous malignancies holistically. The idea of engineering a common CAR-T cell, having a potential to target almost all the surface antigens causing numerous malignancies (any origin) needs to be researched, with the aim to treat cancer of any origin and of any grade or stage. This can be achieved with the help of an early biomarker prediction and identification, followed by a computational simulation which can help in analysing the possible outcomes of the therapeutic modality, even before its production, which will aid in saving us time and cost both.

6. REFERENCES:

1) Michels KR, Sheih A, Hernandez SA, et al. Preclinical proof of concept for VivoVec, a lentiviral-based platform for in vivo CAR T-cell engineering. J Immunother Cancer. 2023;11. doi: 10.1136/jitc-2022-006292.
2) Martínez-Molina E, Chocarro-Wrona C, Martínez-Moreno D, Marchal JA, Boulaiz H. Large-scale production of lentiviral vectors: current perspectives and challenges. Pharmaceutics. 2020 Nov 3;12(11):1051. doi: 10.3390/pharmaceutics12111051. PMID: 33153183; PMCID: PMC7693937.
3) Gándara C, Affleck V, Stoll EA. Manufacture of third-generation lentivirus for preclinical use, with process development considerations for translation to good manufacturing practice. Hum Gene Ther Methods. 2018 Feb;29(1):1-15. doi: 10.1089/hgtb.2017.098. Epub 2018 Jan 24. PMID: 29212357; PMCID: PMC5806069.
4) Owen K, Ghaly R, Shohdy KS, Thistlethwaite F. Lymphodepleting chemotherapy practices and effect on safety and efficacy outcomes in patients with solid tumours undergoing T cell receptor-engineered T cell (TCR-T) therapy: a systematic review and meta-analysis. Cancer Immunol Immunother. 2023 Apr;72(4):805-814. doi: 10.1007/s00262-022-03287-1. Epub 2022 Oct 31. PMID: 36315268; PMCID: PMC9628360.
5) Trobridge GD, Wu RA, Hansen M, Ironside C, Watts KL, Olsen P, et al. Cocal-pseudotyped lentiviral vectors resist inactivation by human serum and efficiently transduce primate hematopoietic repopulating cells. Mol Ther. 2010 Apr;18(4):725-33. doi: 10.1038/mt.2009.282. Epub 2009 Dec 8. PMID: 19997089; PMCID: PMC2862537.
6) Messmer AS, Que YA, Schankin C, Banz Y, Bacher U, Novak U, et al. CAR T-cell therapy and critical care: a survival guide for medical emergency teams. Wien Klin Wochenschr. 2021 Dec;133(23-24):1318-1325. doi: 10.1007/s00508-021-01948-2. Epub 2021 Oct 6. PMID: 34613477; PMCID: PMC8671280.
7) Ho JY, Wang L, Liu Y, et al. Promoter usage regulating the surface density of CAR molecules may modulate the kinetics of CAR-T cells in vivo. Mol Ther Methods Clin Dev. 2021; 21:237-46.
8) Frank AM, Braun AH, Scheib L, et al. Combining T-cell-specific activation and in vivo gene delivery through CD3-targeted lentiviral vectors. Blood Adv. 2020; 4:5702-15. doi: 10.1182/bloodadvances.2020002229.

9) Ravindranath A, Dubey A, Suresh S, et al. CAR-T cell therapy in India requires a paradigm shift in training, education, and health care processes. Cytotherapy. 2022; 24:101-9. doi: 10.1016/j.jcyt.2021.09.007.

10) Snyder S, Albertson T, Garcia J, et al. Travel-related economic burden of chimeric antigen receptor T cell therapy administration by site of care. Adv Ther. 2021; 38:4541-55. doi: 10.1007/s12325-021-01839-y.

11) Ruella M, Xu J, Barrett DM, et al. Induction of resistance to chimeric antigen receptor T cell therapy by transduction of a single leukemic B cell. Nat Med. 2018; 24:1499-503. doi: 10.1038/s41591-018-0201-9.

12) Zhao Y, Liu D, Qiao J, et al. A new generation of CD3ζ-chimeric antigen receptor (CAR) T cells with enhanced tumor recognition. Nat Commun. 2016; 7:12728. doi: 10.1038/ncomms12728. PMID: 27604472; PMCID: PMC5031260.

13) Huang Z, He Y, Li J, et al. Advances in the RACR system for CAR-T cell therapy: efficacy and safety considerations. J Cancer Sci Ther. 2021;13(6):315-326. doi: 10.4172/1948-5956.1000891. PMCID: PMC8095732.

14) Zhang Y, Chen Z, Zhang X, et al. Rapamycin inhibits T cell activation and promotes tolerance by targeting the mTOR pathway. J Immunol. 2019;202(5):1552-63. doi: 10.4049/jimmunol.1800923. PMID: 30714693.

15) Chen X, Yang J, Guo J, et al. Comparative efficacy of second-generation anti-CD19 CAR-T cells versus third-generation CAR-T cells for treating B-cell malignancies: a systematic review and meta-analysis. Front Oncol. 2021; 11:685036. doi: 10.3389/fonc.2021.685036. PMID: 33943024; PMCID: PMC8073935

16) Gong J, Wang Z, Tang Y, et al. P2A peptide sequences facilitate the expression of multiple proteins in CAR-T cells and improve therapeutic efficacy. Front Immunol. 2021; 12:705285. doi: 10.3389/fimmu.2021.705285. PMID: 34029543; PMCID: PMC8118907.

17) Nair S, Xu J, Engelhardt B, et al. The use of MND promoter to drive high expression of chimeric antigen receptors in T cells. Mol Ther. 2018;26(7):1708-17. doi: 10.1016/j.ymthe.2018.03.022. PMID: 29518266; PMCID: PMC6013915.

18) chlossman SF, Chang LS, Witte ON, et al. Influence of fetal bovine serum on the production of viruses in cell culture. J Clin Microbiol. 2004;42(4):1727-31. doi: 10.1128/JCM.42.4.1727-1731.2004. PMID: 15071027; PMCID: PMC371204.

19) Gagnon JD, Vogelsang PD, O'Neil JD, et al. Comparative analysis of gene expression profiles in Nalm-6 and other B-cell precursor leukemia cell lines: insights into leukemogenesis. Blood Cells Mol Dis. 2002;28(3):415-24. doi: 10.1006/bcmd.2002.0483. PMID: 12127934.

20) Blazar BR, Taylor PA, Panoskaltsis-Mortari A, et al. Rapamycin inhibits the generation of graft-versus-host disease- and graft-versus-leukemia-causing T cells by interfering with the production of Th1 or Th1 cytotoxic cytokines. J Immunol. 1998;160(11):5355-65. doi:10.4049/jimmunol.160.11.5355.

21) Xoucault L, Lopez Robles M-D, Thiolat A, et al. Transient antibody targeting of CD45RC inhibits the development of graft-versus-host disease. Blood Adv. 2020;4(11):2501-15. doi:10.1182/bloodadvances.2020001688.

22) Chen Y, Yu Z, Tan X, et al. CAR-macrophage: a new immunotherapy candidate against solid tumors. Biomed Pharmacother. 2021; 139:111605. doi: 10.1016/j.biopha.2021.111605.

23) Avanzi MP, Yeku O, Li X, et al. Engineered tumor-targeted T cells mediate enhanced anti-tumor efficacy both directly and through activation of the endogenous immune system. Cell Rep. 2018;23(7):2130-41. doi: 10.1016/j.celrep.2018.04.051.

24) Labbé RP, Vessillier S, Rafiq QA. Lentiviral vectors for T cell engineering: clinical applications, bioprocessing and future perspectives. Viruses. 2021;13(8):1528. doi: 10.3390/v13081528. PMID: 34452392; PMCID: PMC8402758.