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Identification of Immunological Targets for Brain Cancer Immunotherapy

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Abstract

Background

Cancer immunotherapy has yielded many successes. Yet to some hard-to-treat brain tumors, such as glioblastoma multiforme（GBM) and diffuse intrinsic pontine glioma (DIPG), it still lacks substantial improvement. Neoantigens resulting from mutations in malignant cells are the key targets for employing adoptive cell therapies. A novel therapeutical strategy may be developed based on the identification of T cell receptors (TCRs) targeting specific neoantigens.

Methods

Previous work had been done to provide essential materials, including candidate neoantigen peptides, human leukocyte antigen (HLA) genotypes, and peripheral blood mononuclear cell (PBMCs) from patients and healthy donors (HDs). Autologous antigen-presenting cells (APCs) and T cells were isolated from PBMCs for in vitro assays. The activation of T cells against peptides was evaluated by the upregulation of 41BB utilizing flow cytometry (FACS). The cell populations with positive signals were sorted through FACS for TCR sequencing directly or after rapid cell expansion.

Results

T cells and APCs from 12 HDs were isolated. T cells from 10 HDs were analyzed after in vitro stimulation. T cells from HD30 showed reactions to several public neoantigens; while T cells from HD49 and HD53 showed reactions also to private neoantigens restricted in GBM patient C6.

Conclusion

The upregulation of 41BB indicated the activation of T cells and the existence of reactive TCRs against either public or private neoantigens in some HDs. Those reactive TCRs and their encoding sequences were the fundamentals of future works. Due to practical reasons, TCR sequencing cannot be done within this project. In future works, wildtype peptides will be included to further validate the results, ensuring identified TCRs recognize neoantigens specifically. Furthermore, the identified TCRs will be cloned and transferred to freshly isolated T cells to confirm their functionality.

Keywords

Cancer immunotherapy, brain cancer, neoantigen, MHC/HLA, TCR

Abbreviations

ACT – Adoptive cell therapy

APCs – Antigen-presenting cells

CAR-T therapy – Chimeric antigen receptor T cell therapy

CDRs – Complementarity-determining regions

CNS – Central nervous system

CRS – Cytokine release syndrome

DCM – Dead cell marker

DIPG – Diffuse intrinsic pontine glioma

DMSO – Dimethyl sulfoxide

FACS – Fluorescence-activated cell sorting

FBS – Fetal bovine serum

GBM – Glioblastoma multiforme

GMP – Good manufacturing practice

HD – Healthy donor

HLA – Human leukocyte antigen

MACS® – Magnetic-activated cell sorting

MHC – Major histocompatibility complex

NGS – Next generation sequencing

PBMC – Peripheral blood mononuclear cell

PBS – Phosphate-buffered saline

PI – Propidium iodide

TCR – T cell receptor

TCR-T therapy – T cell receptor-engineered T cell therapy

Popular Scientific Summary

A revolutionary treatment for brain cancer is on the way.

Cancer is a disease caused by the formation and spreading of aberrant cells. Most cancer cells carry mutations enabling them to behave differently from normal cells. As described in the central dogma of biology, proteins are encoded by genes. Thus, mutated genes can lead to mutated proteins. Normally, the human immune system is capable of removing abnormal cells in time to avoid the development of cancer. To do that, one of the most important steps is to recognize which cell is mutation-bearing. The immune cells, particularly B and T cells, circulate through the body every second as patrols. They use their receptors to inspect the protein expressed by other cells, to see if they have the trend to be rebellious.

However, devious cancer cells can also use special mechanisms to escape surveillance and undermine the function of the immune system. On the other hand, cancer-causing mutations may be shared among unrelated patients, but may also be unique to each patient. Both mutations and the receptors of immune cells are highly diverse, bringing more difficulties in treating patients efficiently.

Nowadays, with researchers gaining a better understanding of the immune system, and genetic engineering techniques becoming more sophisticated, cancer immunotherapies have emerged, offering new hopes to patients. In this project, we focused on two types of high-grade brain cancer: glioblastoma (GBM) and diffuse intrinsic pontine glioma (DIPG). The project aimed to improve a T cell receptor-based immunotherapy that could deliver efficient and precise treatment to patients with a matched genetic background.

Previous works had shortlisted specific mutations in GBM and DIPG. Peptides, a fragment of proteins, were synthesized to represent those mutations. T cells from healthy donors were loaded with the peptides to see if they could recognize the mutations, and therefore become activated. Through cutting-edge sequencing techniques, the reactive T cells were sorted to identify the matching receptors. Once the sequence of desired receptors is obtained, it can be reproduced in other T cells, enabling them to recognize the corresponding mutations.

Sequencing was included in the original plan, but it was not actually conducted due to limited time and practical reasons. In spite of this, the project had produced several accomplishments and resources that can be used in subsequent works. T cells from healthy donors did exhibit promising reactions after the peptide stimulation. Flaws in the validation assays were believed to be caused by exhaustion of the challenged T cells. Therefore, part of the subsequent works will focus on validating the results from this project.

It needs to be admitted that this approach is still a long way from being translated into actual clinical application. A series of necessary processes need to be finalized, including safety and efficacy examination and ethical review. If successful, it will provide a new, precise, safe, and efficient treatment strategy for suitable brain cancer patients with specified mutations, aiming to prolong patient survival, ameliorate prognosis and improve quality of life.

Introduction

The current situation in GBM and DIPG

A brain tumor is a collection of abnormal cells of the brain that grow and expand in an aberrant way. In the United States, about 70% of primary brain and other central nervous system (CNS) tumors are benign, with a growth rate and spreading tendency relatively lower than the malignant types. However, morbidity and mortality of benign brain tumors can vary in different patients depending on the subtypes of the tumor. The remaining 30% of malignant types, commonly known as brain cancers, are much more lethal as the mortality is nearly five-fold higher than the benign types. Notably, glioblastoma multiforme (GBM) accounts for nearly half of those malignant brain tumors[1]. As one of the most invasive types of brain tumor, GBM patients have poor prognoses. In a systemic review, the median age of diagnosis of GBM was about 60 years, while the five-year survival was less than 6%[2]. Although the incidence of tumors in children is relatively lower than in adults, brain cancer (27%) is remarkably as common as leukemia (28%) among childhood malignancies[3]. Diffuse intrinsic pontine glioma (DIPG) is one of the most representative cancer type that affect the brainstem with poor prognosis in children. Both GBM and DIPG are classified as WHO grade IV gliomas characterized by strong aggressiveness, rapid recurrence, and being necrosis-prone[4,5].

For high grade gliomas, conventional treatments, such as surgery, radio- and chemotherapy, showed limited efficiencies. To make matters worse, DIPG and other highly aggressive tumors affecting the brainstem are difficult to remove surgically. On the other hand, the development of next generation sequencing (NGS) technology immensely helped researchers reveal the underlying mechanisms of this category of hard-to-treat disease. Several genetic mutations have been identified through NGS technology and association analyses, for instance, mutated IDH1, EGFR, and PTEN, showing multiple connections to the genesis and progression of the disease[6]. Due to the codon degeneracy, single nucleotide substitution doesn't always lead to a change of the corresponding amino acid. But once the amino acid sequence of a protein has been altered, it is very likely to change its structure and function as well[7]. By targeting these non-synonymous mutations, various immunotherapies against high grade gliomas have been developed, including immune checkpoint inhibitors, adoptive cell transfers (ACTs), and therapeutical vaccinations[8]. But it is a pity that for high grade gliomas the clinical improvements from immunotherapeutic approaches remain marginal.

Mutations and neoantigens

According to the well-known "hallmarks of cancer" theory, cancerous cells' enabling characteristics include genome instability and mutations, and tumor-promoting inflammation, which further implies the emergency of mutations and malfunctioning of the immune system[9,10].

As a result of genomic alterations such as non-synonymous mutations in tumorous cells, new proteins termed neoantigens, are synthesized[11,12]. Recognition of neoantigens by immune cells was regarded as a pivotal step in cancer immunotherapy because it not only allows the immune cells to identify the cancerous cells but also reduces the risk of hazardous autoimmunity by specifically targeting neoantigens[13,14]. As mentioned above, certain types of tumors frequently have specific genetic mutations. Such mutations are often shared among individuals regardless of their heritage background. Thus, the corresponding neoantigens are termed "public neoantigens"[15]. Conversely, neoantigens resulting from mutations found uniquely in each

patient are defined as "private neoantigens"[16]. Peptides derived from neoantigens are restricted by common human leukocyte antigen (HLA) subtypes to start the antigen presentation process in the form of epitopes that have the potential to elicit T cell immune response[16].

Immunogenicity refers to the ability of a substance to evoke an immune response. In the case of exogenous protein antigens, the immunogenicity is mainly determined by their capability to produce peptides that bind to major histocompatibility complex (MHC) molecules through the process of antigenic protein degradation[17]. In contrast, the intensity of immunogenicity varies with respect to autoantigens in the body, particularly cancer rejection antigens[18]. Specifically, although tumor-associated antigens (TAAs) tend to be expressed in tumor tissue, they are encoded by the normal genome and are present in partly normal tissue, so that central and peripheral tolerance confers lower immunogenicity. Conversely, tumor-specific antigens (TSAs) are encoded by abnormal genes that are not present in normal tissues and are therefore recognized as highly immunogenic abnormal antigens, as mentioned earlier for neoantigens[18].

Antigen presentation to T cells through MHC molecules

Antigen-presenting cells (APCs), such as dendritic cells, macrophages, and B cells, are capable of presenting either internal or external antigens to the surface through MHC molecules, which are also called HLA in humans. On the other side, T cells are capable of recognizing presented antigens via their T cell receptors (TCRs) and trigger a cascade of immune responses.

In the simplified model, the MHC class I pathway mainly presents cytosolic antigens, while the MHC class II pathway presents antigens from extracellular environments after endocytosis. $CD8^+$ cytotoxic T cells and $CD4^+$ helper T cells bind to MHC class I and class II, respectively[19]. T cell activation and downstream signaling pathways were started by the interaction between the TCR and peptide-MHC complex. The majority of T cells express TCRs composed of one alpha (α) and one beta (β) chain, therefore called αβ T lymphocytes. Each TCR α and β chain consists of one variable (V) domain, one constant (C) domain, a transmembrane (TM) region, and a cytoplasmic (CYT) region. Each TCR α and β chain has 3 complementarity-determining regions (CDRs) on its variable domain, which are key elements to binding peptide-MHC complex[20]. In order to identify innumerable antigens, rearrangement of antigen receptor genes guarantees the polymorphism of T cells. The recombination of 3 gene segments, variable (V), diversity (D), and joining (J), can theoretically form up to 10^{16} αβ TCR repertoire[21,22]. In fact, only T cells that have passed both negative and positive selection and have proper affinity are retained to ensure their functionality and avoid harmful autoimmunity[17] (Figure 1).

Nevertheless, the immunogenicity to trigger the T cell immune responses varies in the context of different peptides. In addition to the source of the antigens as mentioned above, the other factor influencing immunogenicity include structural features, molecular size, antigen processing pathways, etc[23]. APCs may induce strong T cell responses by presenting the MHC-bound peptide after proteolysis in vivo. However, the same peptide may only elicit an inadequate response in vitro because of the change of antigen-presenting pathways[17,24].

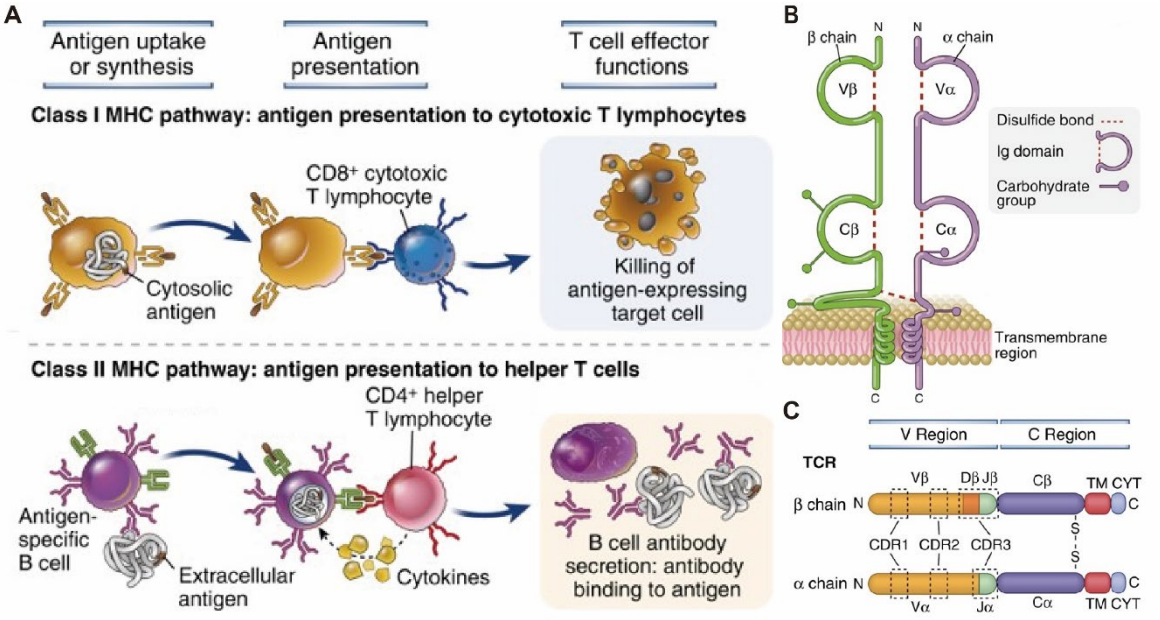

Figure 1. A. antigen presentation through MHC class I and class II pathway. Cytosolic antigens are presented to $CD8^+$ cytotoxic T lymphocytes, leading to the killing of the antigen-expressing cells. Extracellular antigens are presented by B cells to $CD4^+$ helper T cells, leading to activation of B cells to facilitate the elimination of extracellular antigens. B. Structure of the αβ T cell receptor. A typical αβ TCR is composed of an α chain and a β chain; each chain has a variable (V) domain and a constant (C) domain. C. Domains of TCR proteins. Three complementarity-determining regions locate in the variable domain of each chain. Rearrangements of V(D)J gene segments results in highly diverse TCRs. Figures adapted from Abbas, Abul K, Cellular and Molecular Immunology. Copyright 2022 Elsevier Inc.

Adoptive cell transfers in cancer immunotherapy

The "hallmarks of cancer" theory also characterized the cancerous cells by avoiding immune destruction[9,10]. Regarding this feature, immunotherapy of cancer aims at restoring the capabilities of the immune cells to recognize and eliminate the malignant cells[25]. For instance, adoptive cell transfer refers to a type of immunotherapy that use patients' autologous T cells to fight against cancerous cells after in vitro expansion with or without genetic engineering manipulations[26]. Briefly, mainstream ACTs with modified T cells can be grouped in two types based on the nature of the immune receptor that is used for the genetic manipulation: Chimeric antigen receptor T cell (CAR-T) and T cell receptor-engineered (TCR-T) cell therapy. CARs are usually single-chain receptors and can be assembled from molecules' domains from different signaling pathways. In contrast, TCRs consist of heterologous α and β subunits and can be formed naturally by somatic rearrangement[27] (Figure 2).

CAR-T therapy has yielded promising results, but is limited to a few specific types of cancers, particularly in hematopoietic malignancies, such as lymphoma and acute/chronic lymphocytic leukemia, since they mainly target CD19, CD20, or CD22 B lymphocytes antigens[28]. Similar therapeutical strategies against solid tumors have not shown the same promising clinical results so far[26]. Conversely, tumor regressions of melanoma and synovial sarcoma have been reported in clinical trials of TCR-T therapy, targeting MART-1 and NY-ESO-1, respectively[29–31].

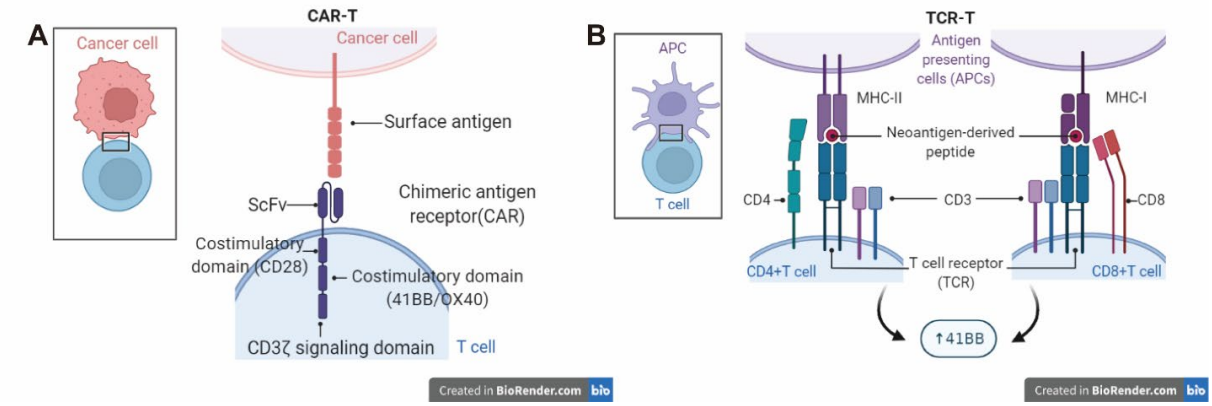

Figure 2. A schematic diagram of main ACTs. A. CAR-T cell therapy. Engineered T cells can directly recognize antigens expressed on the cell surface. B. TCR-T cell therapy. Engineered T cells can recognize antigens presented by APCs through either the MHC class I or class II pathway.

Applications of ACT in brain cancer

Historically, CNS was perceived as an "immune privileged" site mainly because the blood-brain barrier strictly limited the penetration of immune cells. The immune privilege was believed to play a protective role in CNS from the immune response-derived damage. Immune surveillance allows immune cells to monitor and eliminate the incipient tumor cells in most tissues[9]. However, the absence of immune surveillance leads to several adverse effects on the brain, including undermining the protective effects in tumorigenesis and development[32–34]. Despite the fact that recent discoveries of the presence of peripheral immune cells in CNS greatly challenged traditional views, the fact that the immune cells have difficulties infiltrating the tumor tissue in CNS has not changed[35].

Some published works suggested the feasibility of applying CAR-T cell therapy against GBM by targeting HER-2 or EGFR variant III antigen[36,37]. However, severe adverse effects and poor efficacies were also reported in other works: In a case report, the death of a colon cancer patient who received HER-2-directed CAR-T cells was suggested to be caused by non-specific reactivity in normal tissue, known as off-target toxicity[38,39]; In a translational clinical trial in GBM patients, the low persistence of EGFRvIII directed CAR-T cells was considered as one of the main reason for the hampered effectiveness[37]. In a study focusing on DIPG and other diffuse midline gliomas with mutated histone H3-K27M, researchers successfully induced antitumor effects by using anti-GD2 CAR-T cell therapy in lab animals[40]. A clinical trial was initiated based on this finding (NCT04099797).

By utilizing the autologous T cells with neoantigen-reactive TCRs, an ongoing clinical trial was launched to probe the possibility of TCR-T cell therapy in treating various metastatic cancers (NCT03412877)[41–43].

Limitations of ACTs

As a new emerging therapeutical strategy, ACTs for cancer are accompanied by a series of common adverse effects, mainly including cytokine release syndrome (CRS) and off-target toxicity. CRS, as known as "cytokine storm", can happen after the administration of engineered T cells to a patient, leading to fever, tachycardia, organ failure, and even death. IL-2 is suspected to be strongly associated with this side effect[44]. On the other hand, reinfused T cells may attack normal tissue due to the "off-target" autoimmunity, as exemplified by the neurotoxicity in anti-

CD19 CAR-T applications[45]. As an MHC-independent strategy, CAR-T cells are directly targeting the antigens expressed on the surface of cancerous cells. But the malignant cells can reduce the effectiveness of CAR-T cells by promoting antigen escape through unknown mechanism[46,47]. On the other hand, TCR-T cells are MHC-restricted, which means a tumor-specific TCR can only be applied in a patient that shares the same HLA allele with the source of the TCR[48]. In addition, due to the thymic selection to induce tolerance of self-antigen in T cells, TCRs recognizing autologous neoantigens usually have low affinity[48,49].

Significances

The previous works from the group provided a series of materials and results required for this project, including the candidate peptides encoding for neoantigens in GBM and DIPG, and peripheral blood samples donated by healthy individuals matching with HLA alleles of certain GBM and DIPG patients. In this project, we aimed to identify specific TCRs which can recognize the neoantigen-derived peptides. In vitro stimulation, flow cytometry, cell sorting, and TCR sequencing were the major approaches to achieve the results. 41BB (also known as CD137 or TNFRSF9) as an activation marker, was the key indicator in the analysis and selection for positive results[50]. If successful, the newly identified TCR sequence may promote the development of novel ACTs to target GBM and DIPG cancer cells expressing corresponding neoantigens.

Aims

The overall aim of this project was to improve the TCR-based ACT against GBM and DIPG through the identification of novel immunological targets and cognate T cell receptors. Prior to that, two respective aims ought to be achieved: 1) to evaluate the immunogenicity of predetermined peptides containing “hot-spot” non-synonymous point mutation in GBM and DIPG patients; 2) to screen specific TCRs that are capable of recognizing the mutated peptides to trigger T cell activation.

Materials and Methods

Study design

Due to the limitation of the time frame in this thesis project, my works consisted of the isolation of autologous APCs (B cells and dendritic cells) and T cells using previously prepared PBMCs from healthy donors, in vitro stimulation assays of T cells using previously produced candidate peptides, analytical screening using flow cytometry, and fluorescence-activated cell sorting. The previous work was briefly described to give a clear background of the study. Other team members will continue working on this project in the future.

Selection of candidate neoantigen peptides

To select hotspot somatic point mutations which represent public neoantigens in GBM and DIPG, previous analytical works were proceeded based on the Catalogue Of Somatic Mutations In Cancer (COSMIC) database collaborating with bioinformaticians at Karolinska Institutet. To identify individual private neoantigens, patients’ tumor samples were sent to Fulgent Genomics in California, USA to perform the whole exomic sequencing. Bioinformatic analysis and variant

calling to recognize point mutations in amino acid sequences were performed in collaboration with the research group of Hans Grönlund, CMM, Karolinska Institutet. Peptides of 25mers were synthesized according to the shortlisted mutations, and the mutation site was located in the center of the peptide sequence to represent neoantigens. Peptides were dissolved with DMSO at 10 mg/ml.

Patients' and healthy donors' blood processing

In previous works, samples from GBM and DIPG patients were isolated and processed for HLA haplotyping (Mutation Analysis Facility (MAF) at Karolinska Institutet, Stockholm). The nomenclature of HLA was following the international ImMunoGeneTics information system (IMGT). Peripheral blood samples from healthy donors with partly matching HLA were identified among samples already collected from the bloodbank at the Karolinska University Hospital, Huddinge. PBMCs separation and purification from the healthy donor blood samples were performed by Ficoll density separation. Prepared PBMCs were cryopreserved until further use.

Isolation of autologous APCs and T cells

PBMCs from a healthy donor were thawed and washed with sterile phosphate-buffered saline (PBS) buffer solution (homemade, Karolinska Institutet). According to the cell counting result, PBMCs concentration was adjusted to $5\text{-}10*10^6$ cells/ml and rested at $1*10^6$ cell/cm^2 approximately, with non-supplemented AIM-V medium in a plastic tissue culture flask of the proper size. Pulmozyme(GMP grade, 1 ug/ml) was used as DNAse to degrade DNA from dead cells and maintain good viability of other cells after thawing. Penicillin (100 U/ml) and streptomycin (100 ug/ml) were not recommended in the whole procedure, only used when necessary. After 1.5-2 hours of incubation at 37℃, 5% CO_2, the cells are roughly divided into two groups: non-adherent and adherent for the following isolation.

After the above-described incubation, the non-adherent cells in the supernatant were collected. The flasks were gently rinsed with AIM-V medium to collect the non-adherent cells again. Collected cells were prepared for magnetic-activated cell sorting (MACS®) using anti-human CD19 microbeads (Miltenyi Biotec, 130-050-301). The cells were split into two groups: $CD19^+$ B cells, and $CD19^-$ cells, which can broadly be considered as T cells. The positively selected B cells were then co-cultured with irradiated 3T3 cells expressing CD40L at approximately 1:1 ratio in B cell medium with 200 U/ml IL-4. CD40L is a costimulatory protein expressed by regulatory T cells (Treg) to trigger the activation and proliferation of B cells together with TCR-peptide-MHC interaction and several cytokines[17]. B cell medium consists of IMDM medium (Gibco) and 10% heat-inactivated human serum. On the other hand, the remaining T cells were cryopreserved until further use.

As for the adherent cells in the flask, which were considered as immature dendritic cells (DCs), the medium was replaced with DC medium with 800 IU/ml GM-CSF and 200 U/ml IL-4. DC medium consists of RPMI-1640 medium (Gibco), 5% heat-inactivated human serum, and 2 mM L-glutamine.

B cells and DCs were cultured for in vitro proliferation. On day 3, fresh medium and cytokine were added to both B cells and DCs. On day 5, DCs were harvested and cryopreserved until further use. On day 7, B cells were harvested. A portion of the B cells was kept for a second round of the same expansion till day 14, while most cells were cryopreserved until further use.

The harvesting time point may be slightly adjusted according to the proliferation rate of each individual. All of that cryopreservation used the same solution, which consisted of 90% fetal bovine serum (FBS, Sigma-Aldrich) and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich).

In vitro stimulation assays of T cells

Cells harvested in the previous steps were thawed and washed with PBS. T cells were resuspended in rapid expansion protocol (REP)-T medium at approximately $1*10^6$ cells/ml for resting and recovery overnight. REP-T medium consists of 45% RPMI-1640 medium, 45% AIM-V medium, 10% heat-inactivated human serum, and 25mM HEPES. B cells were thawed and resuspended in REP-T medium on the same day morning before co-culture with T cells. In each well of a 24-well plate, $0.25*10^6$ B cells were added with 1ml REP-T medium. Peptides were added in 10 ug/ml or 5 ug/ml for single or pool mutations, respectively. After 1.5-2 hours of incubation, the peptide-loaded B cells were washed once, then the $1*10^6$ T cells were added with IL-2 (3000 IU/ml) and IL-21 (30 ng/ml) for co-culture. The same stimulation was repeated for the same T cells in week 2 and week 3. A portion of T cells never stimulated with peptides were kept as the cell source for negative or positive controls.

Co-culture setup for analysis and cell sorting

After 3 rounds of stimulation, the T cells were ready for the first analysis to evaluate the extent of activation. In each well of a 96-well plate, $0.1*10^6$ newly thawed and washed B cells were added and stimulated with peptides as in the previous procedure. After 1.5-2 hours of incubation to load B cells with peptides, repeatedly stimulated T cells were added in 1:1 ratio to setup the analytical screening co-culture. In negative controls, T cells were co-cultured with non-peptide-loaded B cells. In positive controls, T cells were treated with bound anti-CD3 antibody (0.1ug/ml, clone OKT3, Miltenyi Biotec, 130-093-387).

Flow cytometry and FACS

The following day (at least 20 hours after the co-culture), the cells were stained with an antibody panel to be analyzed by flow cytometry. The staining panel was composed of the following antibodies: CD3-BV650 clone OKT3(Biolegend, 317324), CD8-BV570 clone RPA-T8(Biolegend, 301038), CD4-BV711 clone OKT4(Biolegend, 317440), 41BB-PE clone 4B4-1(Biolegend, 309804), dead cell marker (DCM)-infrared (ThermoFisher, L34976). The stained cells were fixed with BD Cytofix™ Fixation Buffer. BD LSR Fortessa was the cytometer used in most experiments.

Except for the fixation, the same operation and staining panel were applied for fluorescence-activated cell sorting (FACS). In addition, the DCM was replaced with propidium iodide (PI). The sorted T cells were cultured with 30 ng/ml anti-CD3 antibody (clone OKT3, Miltenyi Biotec, 130-093-387), 3000 U/ml IL-2, and excessive irradiated feeder cells in REP-T medium for expansion. Sony MA900 was the cell sorter used in all experiments. FCS files were generated and exported after every experiment. FlowJo (Treestar) V10.8 was used to analyze the data.

Sequencing for mutation-reactive TCRs

Due to the limited time and practical reasons, TCR sequencing was not conducted in this project. It was originally planned to sequence both alpha and beta chains of reactive TCR with commercial kit (Takara) by the Mutation Analysis Facility (MAF) at Karolinska Institutet.

Ethical considerations

All ethical permits needed for the project have already been obtained (Swedish Ethical Review Authority (Etikprövningsmyndigheten), diarienummer 2019-06326).

Results

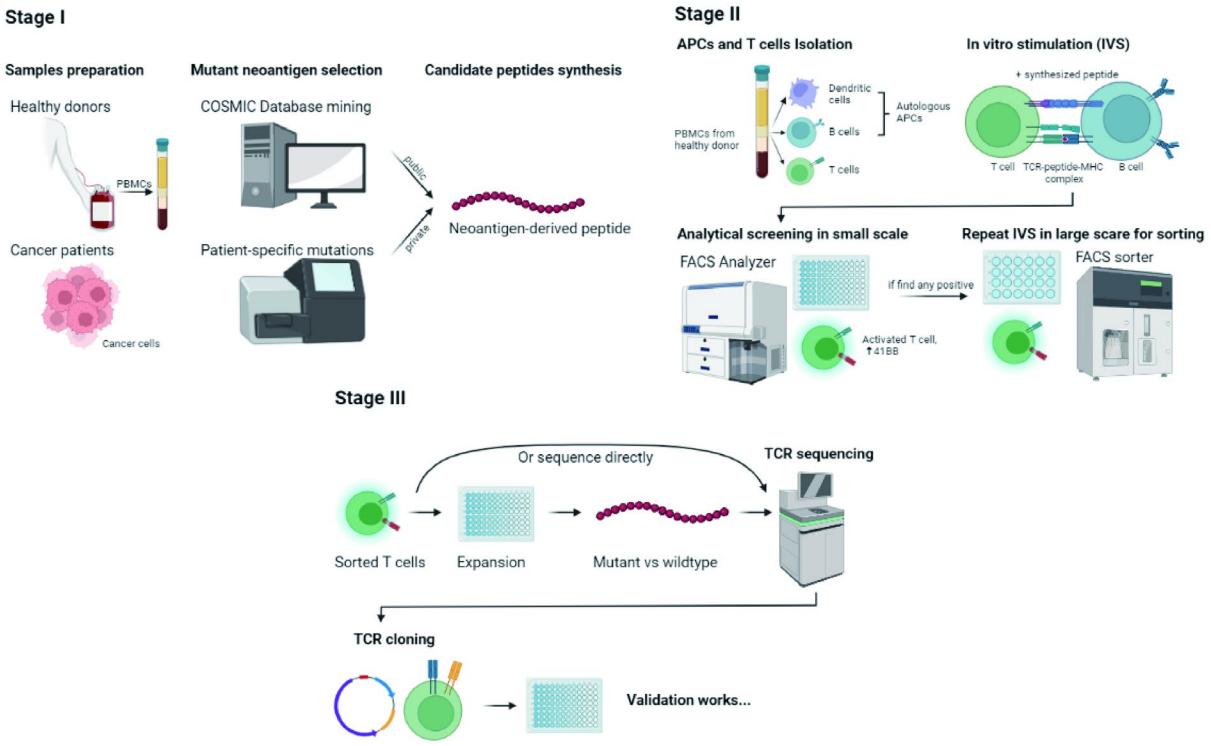

Figure 3. The overview of this experiment. Stage I. The previous works provided information and materials for this thesis project. Stage II. The major work of this thesis project was to screen reactive TCRs from healthy donors' T cells. The pipeline consisted of cell isolation, in vitro stimulation with peptides, analytical screening and cell sorting. Stage III. Future works will focus on validating the functionality and specificity of identified TCRs.

Identification of candidate mutated peptides

To identify potential targets for TCR-based immunotherapy, a library of peptides was established before the start of this project as described in Material and Method section and depicted in Figure 3. Candidate peptides encoding for public neoantigens selected from COSMIC database were listed in Table 1. This list comprised 21 point mutations in 9 different genes, including *ACVR1*, *BRAF*, *EGFR*, *H3F3A*, *HIST1H3B*, *IDH1*, *PIK3CA*, *PTEN*, and *TP53*. In some of these genes, only one mutation was selected, and they were tested as "single peptides" in the following works. On the other hand, more than one mutation was selected in other genes, and they were tested as "pool peptides". Table 2 is another list of candidate peptides but generated based on the exome sequencing (a method to obtain nucleotide sequence of protein-coding regions of genes) result of tumor sample from GBM patient C6 (HLA-A *02:01). Thus, this list represented private neoantigens, including 10 point mutations in 10 different genes. To maintain the screening efficiency, these 10 genes and corresponding peptides were divided into pool1 (*EGFR*, *TMEM126B*, *ITM2B*), pool2 (*DGUOK*, *FARS2*, *VCAM1*), and pool3 (*LCORL*, *POLI*, *SPANXB1*, *SLC22A5*). In addition, PIK3CA and TP53 peptides in Table 1 are also included as pool 4 and pool 5, respectively.

Table 1. List of candidate peptides selected from COSMIC public neoantigens

No.	Gene	Mutation	Mutant	Wildtype
1	*ACVR1*	p.G328V	LAHLHIEIFGTQ**V**KPAIAHRDLKSK	LAHLHIEIFGTQ**G**KPAIAHRDLKSK
2	*BRAF*	p.V600E	LTVKIGDFGLAT**E**KSRWSGSHQFEQ	LTVKIGDFGLAT**V**KSRWSGSHQFEQ
3	*EGFR*	p.A289V	MDVNPEGKYSFG**V**TCVKKCPRNYVV	MDVNPEGKYSFG**A**TCVKKCPRNYVV
	EGFR	p.G598V	IDGPHCVKTCPA**V**VMGENNTLVWKY	IDGPHCVKTCPA**G**VMGENNTLVWKY
4	*H3F3A*	p.G35R	TKAARKSAPSTG**R**VKKPHRYRPGTV	TKAARKSAPSTG**G**VKKPHRYRPGTV
	H3F3A	p.K28M	APRKQLATKAAR**M**SAPSTGGVKKPH	APRKQLATKAAR**K**SAPSTGGVKKPH
5	*HIST1H3B*	p.K28M	APRKQLATKAAR**M**SAPATGGVKKPH	APRKQLATKAAR**K**SAPATGGVKKPH
6	*IDH1*	p.R132C	LVSGWVKPIIIGC**H**AYGDQYRATDF	LVSGWVKPIIIGR**H**AYGDQYRATDF
	IDH1	p.R132H	LVSGWVKPIIIGH**H**AYGDQYRATDF	LVSGWVKPIIIGR**H**AYGDQYRATDF
7	*PIK3CA*	p.E542K	QLKAISTRDPLS**K**ITEQEKDFLWSH	QLKAISTRDPLS**E**ITEQEKDFLWSH
	PIK3CA	p.E545A	AISTRDPLSEIT**A**QEKDFLWSHRHY	AISTRDPLSEIT**E**QEKDFLWSHRHY
	PIK3CA	p.E545K	AISTRDPLSEIT**K**QEKDFLWSHRHY	AISTRDPLSEIT**E**QEKDFLWSHRHY
	PIK3CA	p.H1047R	ALEYFMKQMNDA**R**HGGWTTKMDWIF	ALEYFMKQMNDA**H**HGGWTTKMDWIF
	PIK3CA	p.R88Q	EAEREEFFDETR**Q**LCDLRLFQPFLK	EAEREEFFDETR**R**LCDLRLFQPFLK
8	*PTEN*	p.R173C	RDKKGVTIPSQR**C**YVYYYSYLLKNH	RDKKGVTIPSQR**R**YVYYYSYLLKNH
9	*TP53*	p.R175H	YKQSQHMTEVVR**H**CPHHERCSDSDG	YKQSQHMTEVVR**R**CPHHERCSDSDG
	TP53	p.R248Q	YMCNSSCMGGMN**Q**RPILTIITLEDS	YMCNSSCMGGMN**R**RPILTIITLEDS
	TP53	p.R248W	YMCNSSCMGGMN**W**RPILTIITLEDS	YMCNSSCMGGMN**R**RPILTIITLEDS
	TP53	p.R249S	MCNSSCMGGMNR**S**PILTIITLEDSS	MCNSSCMGGMNR**R**PILTIITLEDSS
	TP53	p.R273C	SGNLLGRNSFEV**C**VCACPGRDRRTE	SGNLLGRNSFEV**R**VCACPGRDRRTE
	TP53	p.V157F	LWVDSTPPPGTR**F**RAMAIYKQSQHM	LWVDSTPPPGTR**V**RAMAIYKQSQHM

Table 2. List of candidate peptides selected from GBM patient C6 private neoantigens

No.	Gene	Mutation	Mutant	Wildtype
10	*EGFR*	p.C219Y	RKFRDEATCKDT**Y**PPLMLYNPTTYQ	RKFRDEATCKDT**C**PPLMLYNPTTYQ
11	*TMEM126B*	p.I26L	RWELRKRPRTQN**L**YQMATFGTTAGF	RWELRKRPRTQN**L**YQMATFGTTAGF
12	*ITM2B*	p.E126V	TIEENIKIFEEE**V**VEFISVPVPEFA	TIEENIKIFEEE**E**VEFISVPVPEFA
13	*DGUOK*	p.R101W	AQSLGNLLDMMY**W**EPARWSYTFQTF	AQSLGNLLDMMY**R**EPARWSYTFQTF
14	*FARS2*	p.P269H	DELEIRWVDCYF**H**FTHPSFEMEINF	DELEIRWVDCYF**P**FTHPSFEMEINF
15	*VCAM1*	p.S730G	YFARKANMKGSY**G**LVEAQKSKV	YFARKANMKGSY**S**LVEAQKSKV
16	*LCORL*	p.R299H	SCPLSPIKMCLN**H**PIEWNLNLTTAS	SCPLSPIKMCLN**R**PIEWNLNLTTAS
17	*POLI*	p.C479R	FSKEKDINEFPL**R**SLPEGVDQEVFK	FSKEKDINEFPL**C**SLPEGVDQEVFK
18	*SPANXB1*	p.R63T	SESSTILVVRYR**T**NVKRTSPEELLN	SESSTILVVRYR**R**NVKRTSPEELLN
19	*SLC22A5*	p.V151M	DWKAPLTISLFF**M**GVLLGSFISGQL	DWKAPLTISLFF**V**GVLLGSFISGQL

Isolation of dendritic cells and B cells

PBMCs samples from 12 HDs were separated into T cells, DCs, and B cells. B cells and T cells from HDs were the main resources for downstream screening works. The HLA genotypes of HDs were already available from the previous work by other group members (Table 3).

Table 3. HLA alleles and cell isolation results of healthy donors

Heathy donors	HLA allele	Cell numbers (*10^6 cells)				
		PBMCs	T cells	DCs	B cells round1	B cells round2
HD5	A 03:01/32:01 B 40:01/57:01 C 03:04/06:02	200	74	28	53	50
HD8	A 02:01/30:01 B 13:02/44:02 C 05:01/06:02	165	74	6.9	179	71
HD19	A 01:01/23:01 B 13:02/44:03 C 04:01/06:02	314	85	35	122	37
HD30	A 11:01/23:01 B 13:02/35:01 C 04:01/06:02	180	100	13	123	67
HD33	A 01:01/02:01 B 27:05/51:01 C 01:02/02:02	292	115	41	120	67
HD35	A 01:01/02:01 B 08:01/44:02 C 05:01/07:01	145	58	20	50	76
HD44	A 02:01/02:08 B 18:01/51:01 C 04:01/12:03	138	51	15	36	62
HD45	A 02:01/03:01 B 07:01/X C 04:01/07:02	191	96	43	45	56
HD48	A 02:01/11:01 B 44:02/51:01 C 03:03/05:01	189	89	50	10	53
HD49	A 02:01/23:01 B 15:01/44:03 C 03:04/04:01	150	41	16	15	67
HD50	A 02:01/31:01 B 07:02/15:01 C 03:03/07:02	151	48	17	34	65
HD53	A 02:01/03:01 B 51:01/56:01 C 01:02/14:02	157	49	16	20	63

Cells isolated from HD5, 19, 30, 33 were tested for T cell reactions against public neoantigens (Table 1), while HD44, 44, 48, 49, 50, 53 were tested for private neoantigens specific to GBM patient C6 (Table 2).

T cell activation after in vitro stimulations and FACS

T cells' activation was indicated by upregulation of 41BB after rounds of in vitro stimulation, where T cells were cocultured and challenged with peptide-loaded B cells. Flow cytometry was the method to assess the T cell activation. By comparisons with negative controls, samples that had more than twice the percentage of $41BB^{+}$ cells were arbitrarily considered to have detectable activated populations. Samples that surpassed the threshold were prepared for FACS sorting to isolate T cells expected to have reactive TCRs.

For Table 1 public neoantigens, only T cells from HD30 showed reactivity to certain candidate peptides, including ACVR1, EGFR, IDH1, PIK3CA, PTEN, and TP53 (Figure 4, Figure S 2). To identify the exact specific neoantigens accurately, analytical screening was performed again with single peptides rather than pool peptides. $ACVR1^{G328V}$, $IDH1^{R132H}$, $PTEN^{R173C}$, $TP53^{R248W}$, $TP53^{R249S}$, $TP53^{R273C}$, and $TP53^{V175F}$ peptides were able to trigger the activation of T cells from HD30 (Figure 5A, Figure S 4). FACS cell sorting was conducted for both experiments according to the results (Figure 5B and C, Figure S 3 and Figure S 5).

Regarding private neoantigens specific to GBM patient C6, peptides in Table 2 (pool 1, 2, 3) plus PIK3CA and TP53 (pool 4, 5) were used to challenge T cells from HD44, 45, 48, 49, 50, 53. For the analyses in the last three HDs, the same stained samples were analyzed in different flow cytometers to assess the influence of different instruments on background signals. Peptides in pools 2, 3, 4, and 5 induced the activation of HD49's T cells (Figure 6A and D, Figure S 8 and 9). And T cells from HD53 were activated by peptides in pool3 (Figure 6C and F, Figure S 8 and 9). HD44, 45, 48, and 50 didn't show any significant response to neoantigen peptide challenges (Figure 6B, E, Figure 7, Figure S 8 to 10). Owing to time constraints, those reactive T cells were cryopreserved, and downstream analyses will be done by other team members in the future.

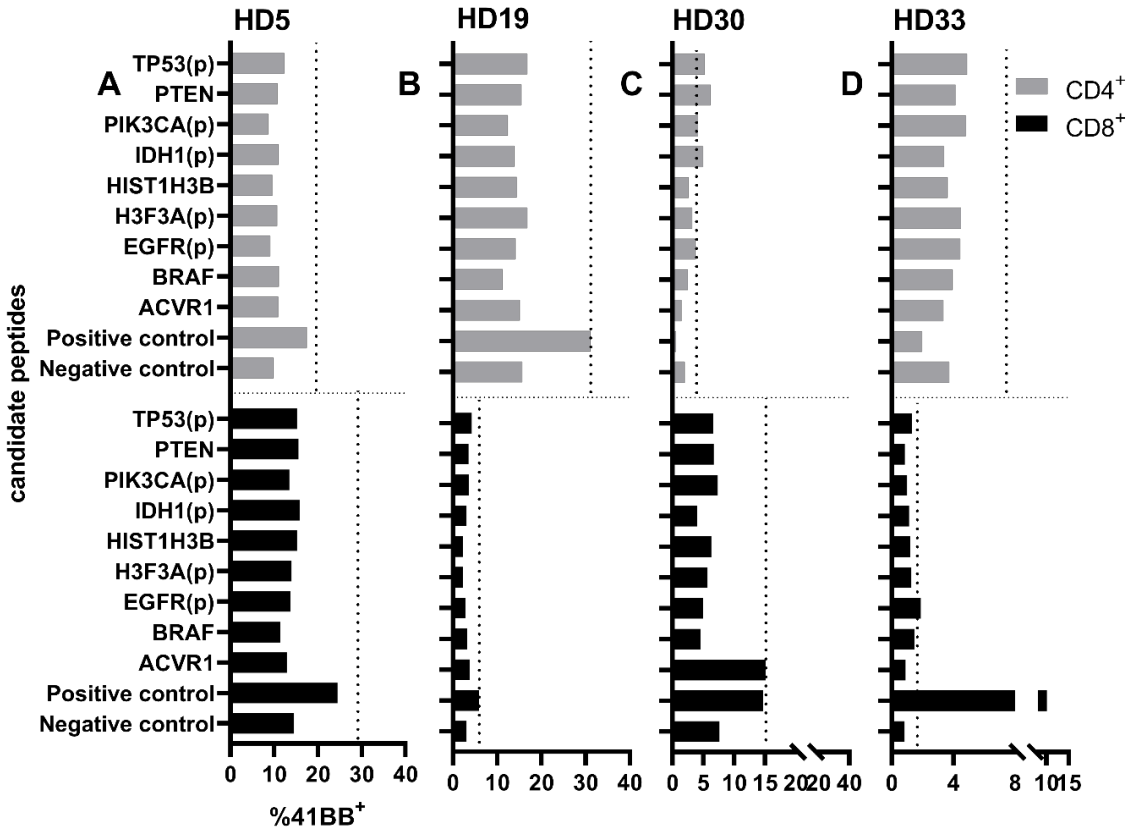

Figure 4. Analytical screening results of public neoantigens in 4 healthy donors. Peptides representing neoantigen ACVR1, BRAF, HIST1H3B, and PTEN were tested in the single way, while EGFR, H3F3A, IDH1, PIK3CA, and TP53 were tested in the pool way marked with (p). T cells from HD5(A), HD19(B), and HD33(D) didn't show reactions to any peptides. Only T cells from HD30(C) were arbitrarily considered to have reactions to certain peptides, including ACVR1, EGFR(p), IDH1(p), PIK3CA(p), PTEN(p), and TP53(p). The dashed lines indicate the threshold of reaction, which is double of $41BB^+$ percentage in negative controls.

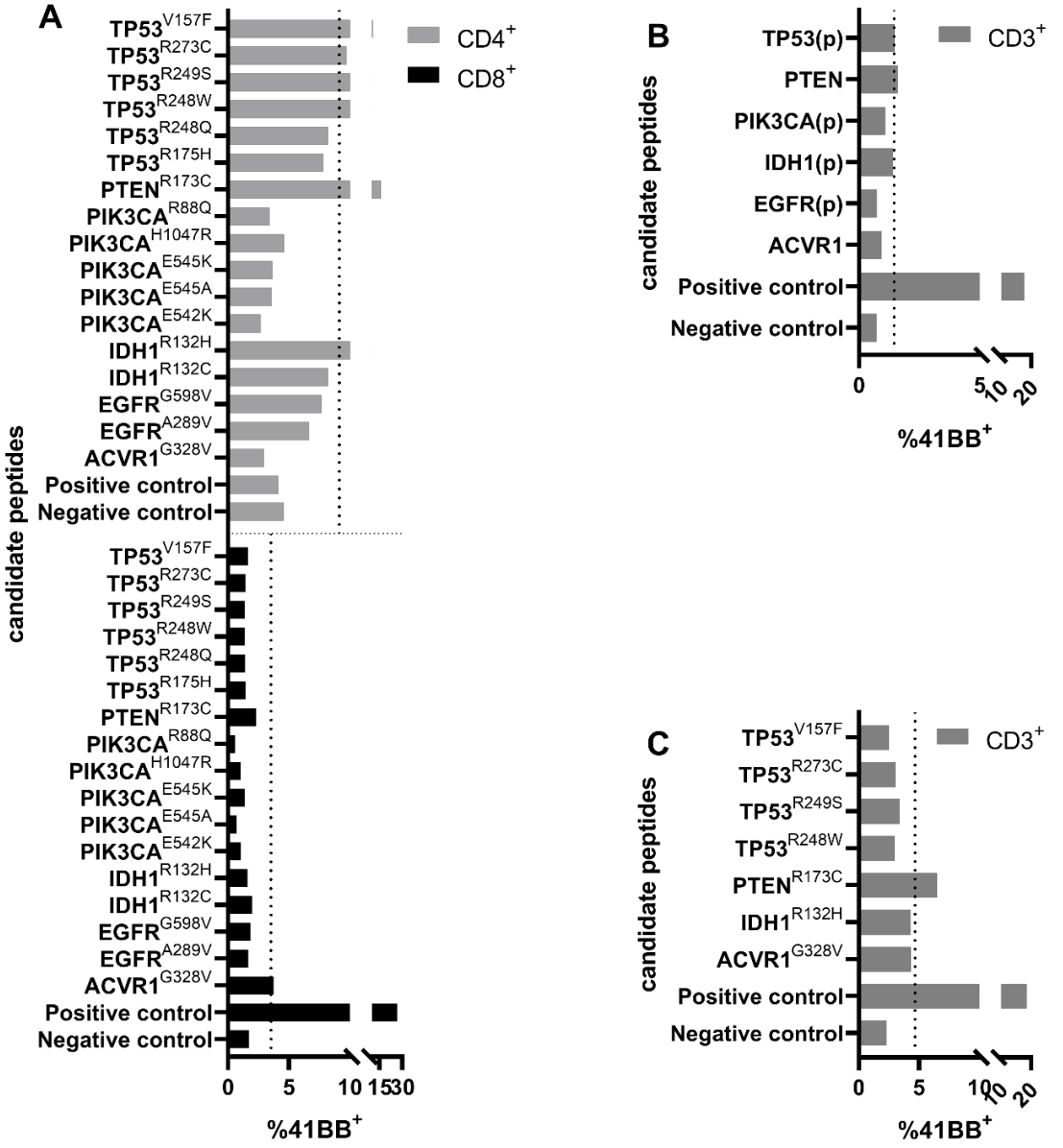

Figure 5. A. Analytical screening results of public neoantigens in HD30, using single rather than pool peptides. Shortlisted single peptides comprised ACVR1^{G328V}, IDH1^{R132H}, PTEN^{R173C}, TP53^{R248W}, TP53^{R249S}, TP53^{R273C}, and TP53^{V175F}. B and C. According to pool (Figure 4C) and single (Figure 5A) peptides analytical screening results, reactive T cells from HD30 were sorted by FACS. The dashed lines indicate the threshold of reaction, which is double of 41BB^{+} percentage in negative controls.

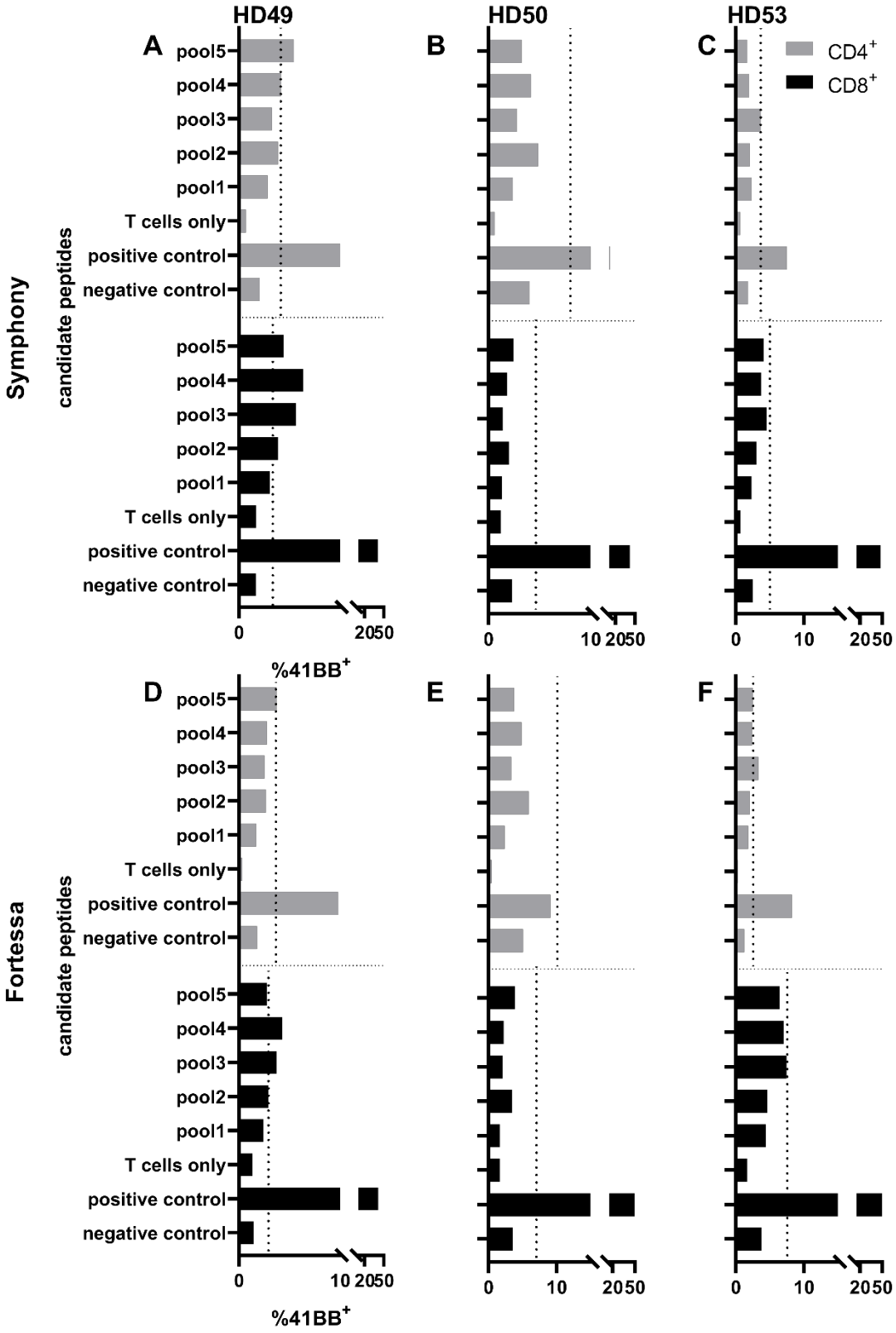

Figure 6. Analytical screening results for private neoantigens in HD49, 50, and 53. All these peptides were tested as pools. T cells from HD49 showed reaction to pool 2, 3 ,4 ,5 peptides (A, D); and T cells from HD53 showed

reaction to pool3 peptides (C, F). Same assays were analyzed in different flow cytometers (BD FACSymphony (A, B, C) and BD LSR Fortessa (D, E, F)) to compare the background signals. The dashed lines indicate the threshold of reaction, which is double of $41BB^+$ percentage in negative controls.

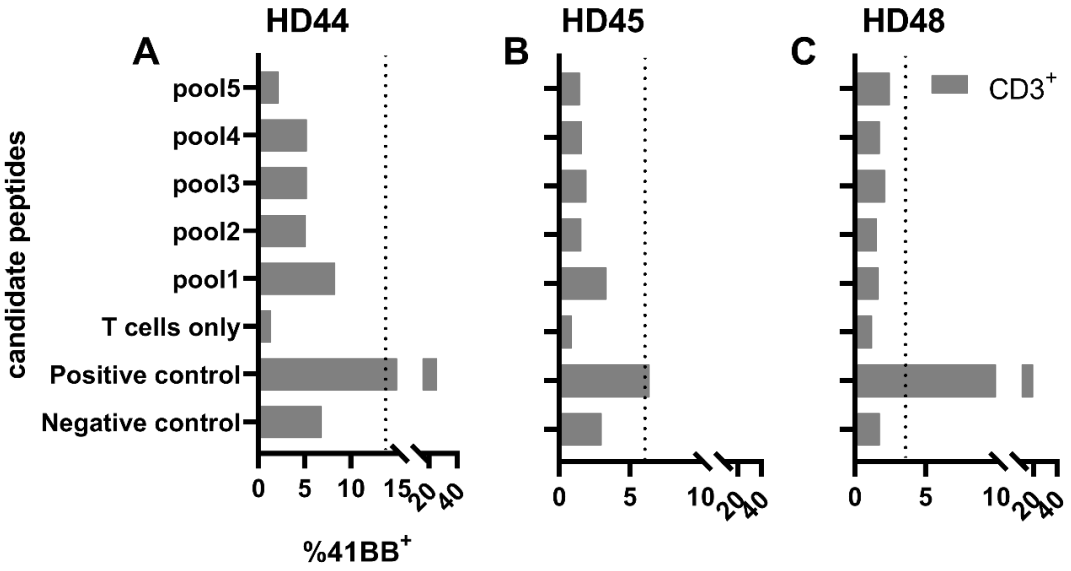

Figure 7. Analytical screening results for private neoantigens in HD44, 45, and 48. No peptides successfully induced any activations of T cells from those healthy donors. The dashed lines indicate the threshold of reaction, which is double of $41BB^+$ percentage in negative controls.

Only a few T cells survived after FACS sorting and lost their reactivity

In order to acquire a sufficient number of cells for downstream works, the sorted cells were cultured for rapid expansion, but only $IDH1^{R132H}$, $TP53^{R248W}$, and $TP53^{V157F}$ reactive T cells survived and proliferated in this process. After expansion, $TP53^{R248W}$ and $TP53^{V157F}$ "reactive" T cells were challenged by mutant and wildtype peptides to validate the observed result. However, neither mutant nor wildtype could successfully provoke the activation of those T cells again (Figure 8A, Figure S 6). $IDH1^{R132H}$ was not included in the comparison experiment because of lacking wildtype peptide. $TP53^{R248W}$ reactive T cells were used to conduct the titration test of peptides concentration, but no specific peptides concentration could contribute to revealing the difference between mutant and wildtype peptides treated T cells. The remaining T cells were cryopreserved to re-examine their reactivity with more sensitive methods (see Discussion) in the future.

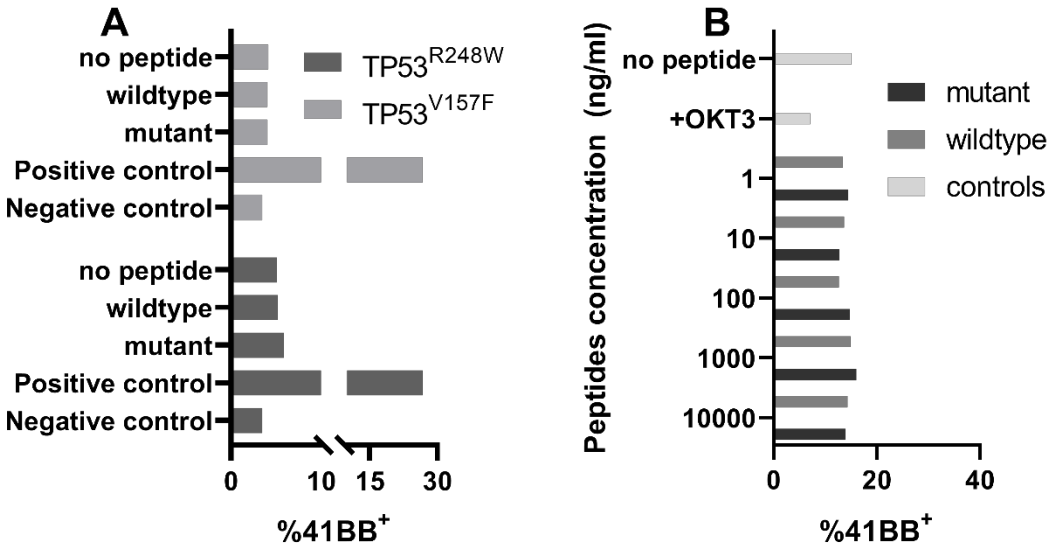

Figure 8. $TP53^{R248W}$ and $TP53^{V157F}$ reactive T cells from HD30 successfully survived and proliferated after sorting. A. Those cells were stimulated with either mutant or wildtype peptides to validate their reactions, but no significant difference was observed. B. No significant difference in $TP53^{R248W}$ reactive T cells was detected upon treatment by mutant and wildtype peptides in any concentration.

Discussion

Conclusions

In this study, we focused on characterizing the immunogenicity of possible mutated targets for certain brain cancer (GBM and DIPG). We stimulated healthy donors T cells with peptides encoding for neoantigens and we attempted to identify specific TCRs that could be used in the future for adoptive cell therapy. Overall, this is a complicated and long-term study involving newly established methodologies, therefore it was challenging to achieve meaningful results in a project of fewer than 6 months, such as obtaining sequences of TCR pairs that recognize a specific neoantigen. Although not all experiments were flawless, they have provided useful information and materials for downstream works for the group in the future.

Among the twelve prepared healthy donors' cells, ten of them were partly tested in this project. Most of them require further examinations and investigations. The remaining samples can also be allocated to similar studies in other cancer types.

With respect to public neoantigens commonly shared in GBM and DIPG patients, T cells from HD30 displayed reactions to $ACVR1^{G328V}$, $IDH1^{R132H}$, $PTEN^{R173C}$, $TP53^{R248W}$, $TP53^{R249S}$, $TP53^{R273C}$, and $TP53^{V175F}$ peptides. Corresponding reactive T cells were sorted and expanded in vitro. In the following validation experiment on T cells that survived and proliferated, no discrepancy was detected in terms of T cell activation upon stimulation with mutant or wildtype peptides.

On the other hand, private neoantigen pool3 selected from GBM patient C6 successfully activated the T cells from HD49 and 53. In addition, T cells from HD49 were also activated by neoantigen pools 2, 4, and 5.

Troubleshooting and improvements

In parallel to those promising outcomes mentioned above, some crucial issues affected the progress of the project.

First of all, the individual variations in different HDs impacted not only APC isolation but also subsequent in vitro stimulations. During APC isolation processes, healthy donors exhibited different composition proportions and proliferation rates. Cells that are relatively slow to proliferate require longer expansion time, along with the risk of lower viability. Additionally, the sensitivity of T cells from different HDs to peptides stimulation varies, resulting in various background signals. For instance, in the analytical screening for public neoantigens involving 4 HD, HD5 and HD19 had higher background signals (around 10%) in negative controls than HD30 and HD33 (around 5% and less) (Figure 4, Figure S 1 and 2). Instruments induced discrepancies had also been suspected to be the cause of high background signals. When testing T cells reactions against public neoantigen pools, the same samples were analyzed in two different flow cytometers: BD FACSymphony and BD LSR Fortessa. However no significant difference was shown between those two instruments (Figure 6, Figure S 8 and 9).

Appropriate setups of controls play a critical role in interpreting results generated after experiments and analysis. A fraction of T cells was never stimulated with peptides but co-cultured with autologous B cells in the in vitro stimulation process. Those T cells were the source of negative or positive control (see Materials and Methods). Oddly, positive controls sometimes failed in analytical screenings. For example, in the analytical screenings of HD30 and HD33, positive controls failed in $CD4^+$ T cells. Intriguingly, $CD8^+$ T cells in the same experiments still maintained the signals in positive controls (Figure 4C, D, and Figure 5A, Figure S 1 to 4). In addition, peculiar populations characterized as $CD3^+CD4^-CD8^-$ were detected when analyzing flow cytometry results. Surprisingly, positive control was restored in these “double negative” T cell populations. Taking the single peptides analysis with HD30 as an example (Figure 5A, Figure S 4), the 41BB upregulation signal was distinct in the $CD3^+CD4^-CD8^-$ population (Figure 9, Figure S 4). Regarding this issue, one possible explanation is that the $CD4^+$ T cells were overactivated, leading to the internalization of the receptor upon binding to ligands. Intracellular staining might be helpful to test this hypothesis, but inapplicable in actual experiments to ensure cells are alive for downstream procedures. Given this particular occurrence, a general, loose gating strategy was applied in downstream cell sorting operations; $CD3^+41BB^+$ cells were directly sorted from live cell population (Figure 5B, C, Figure S 3 and 5).

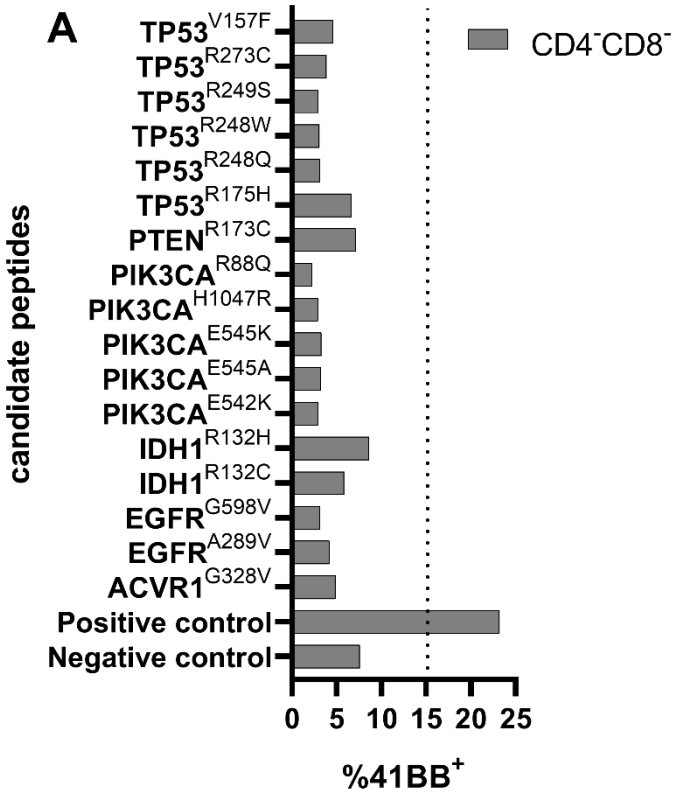

Figure 9. Analytical screening results of public neoantigens in HD30, using single peptides, same experiment as Figure 5A. The $CD3^{+}CD4^{-}CD8^{-}$ cells were successfully activated by bound CD3 antibody in the positive control.

After identifying single peptides that could activate T cells from HD30, sorted cells were expanded and challenged with mutant and wildtype peptides for comparison. The desired result should be that the mutant peptide-loaded groups should have higher 41BB expression levels than the wildtype groups. However, no significant discrepancies were observed in actual experiments. Inappropriate peptides concentration was suspected of causing these results (Figure 8A, Figure S 6). In this experiment, T cells never stimulated with peptides were used for positive controls just as in previous analytical screening experiments. Probably the same T cells that experienced sorting and expansion would have been a better and more proper source for positive control. This issue was solved by using expanded T cells in the following titration test, annotated as "+OKT3".

The titration test was performed to seek proper peptides concentration. But mutant or wildtype peptide-treated groups cannot differ from the group that didn't load any peptides in those sorted and expanded cells. In addition, the failed negative control in the titration test indicated possible exhaustion of those cells after such consecutive repetition of stimulation in the past weeks (Figure 8B, Figure S 7). Another important reason could be that more non-reactive T cell clones than reactive clones were obtained during sorting, and gradually become dominating in the subsequent expansion. In this case, assays with high sensitivity should be included to validate the reliability of the results from the previous experiments. For instance, ELISPOT can be employed to examine cytokine levels in cell culture medium after peptides stimulation. In fact,

it was originally planned that the sorted cells could be snap frozen and directly sent to the core facility for TCR sequencing. However, personnel in that facility were facing troubles with the sequencing kits. Even worse, the malfunction of the sorter resulted in a lower number of cells being obtained than indicated by the instrument. That could also be an explanation of why the cells could hardly survive after sorting.

Possible optimizations of current protocols

This project was performed based on previous similar projects conducted at the National Cancer Institute (NCI), NIH. Although many published studies applied similar methods, there is still much room for improvement in actual practice.

When analyzing flow cytometry results, a positive result was defined as having a 41BB fluorescence signal more than twice of the negative control. We need to acknowledge that such an arbitrary judgment criterion is prone to false positives and false negatives. Perhaps collaborations with statisticians could improve the judgement method.

The fluorescence conjugated antibodies panel employed in this project could also be refined to achieve better and more legible results for analysis. BD LSR Fortessa was the main flow cytometer used in this project. In some experiments, CD8-BV570 in the violet 585/42 channel could not be well compensated in that instrument, and causing inconvenience in analyzing works. The spillover could affect the result of the activation marker 41BB, whose antibody was conjugated with PE, detected in the yellow-green 586/15 channel. Therefore, the color scheme can be appropriately adapted to overcome the issue, whilst taking into account the compatibility with the instrument.

The high background signal issue mentioned above should also be improved. Besides using different flow cytometers, possible optimizations include shortening the length of peptides, decreasing the concentration and incubation time when loading peptides to APCs, and adjusting the frequency of peptides challenging during in vitro stimulation.

In parallel with this project, other researchers and students were also conducting similar experiments with different approaches. For example, they were trying to replace autologous B cells with DCs as APCs to present peptides to T cells; and to transfect APCs with neoantigen encoding DNA/RNA sequence to express the peptide internally. The merits of those various methods remain to be determined.

Future perspectives

The high standard was one of the most important features of this project. Most of the operations were carried out in the Pre-Good manufacturing practice (Pre-GMP) facility; GMP grade reagents were widely used; sterility was strictly required to avoid unnecessary antibiotic use. The reason for these is to ensure that the outcomes of the experiments can be reproduced and adapted to GMP and clinical standards. Thus, subsequent works should be conducted in Pre-GMP as much as possible.

For the finite results achieved in this project, further work will focus on validation to increase reproducibility. Assuming that after sorting, we successfully expanded and obtained a highly pure population of T cells with high specificity to corresponding mutant neoantigens. Then the cells can be sent to conduct TCR sequencing to identify both alpha and beta chains that compose a functional pair to recognize neoantigens.

Our group has established methods to re-examine the functionality of the obtained TCRs on fresh T cells to avoid exhaustion issues. Briefly, the variable domain of identified TCR will concatenate with the constant domain of a murine TCR, forming a murinized TCR. Murinization can not only avoid mispairing of endogenous and exogenous TCRs and allows the identification of correct TCR alpha/beta combination by multimer, but also improve the safety, functionality, and persistence of exogenous TCRs[27,51,52]. Fresh T cells will be transfected to reconstruct the hybrid TCR and stimulated with both mutant and wildtype neoantigen peptides to ensure the functionality and specificity of the identified TCR.

On the basis of identified TCRs, it is possible to conduct pre-clinical experiments when comprehensive safety and efficacy tests and ethical reviews are completed. Hopefully, the subsequent outcomes of this project will eventually be applied to appropriate patients with matching mutations, permitting precise, effective, safe, and reliable treatment.

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Supplementary Materials

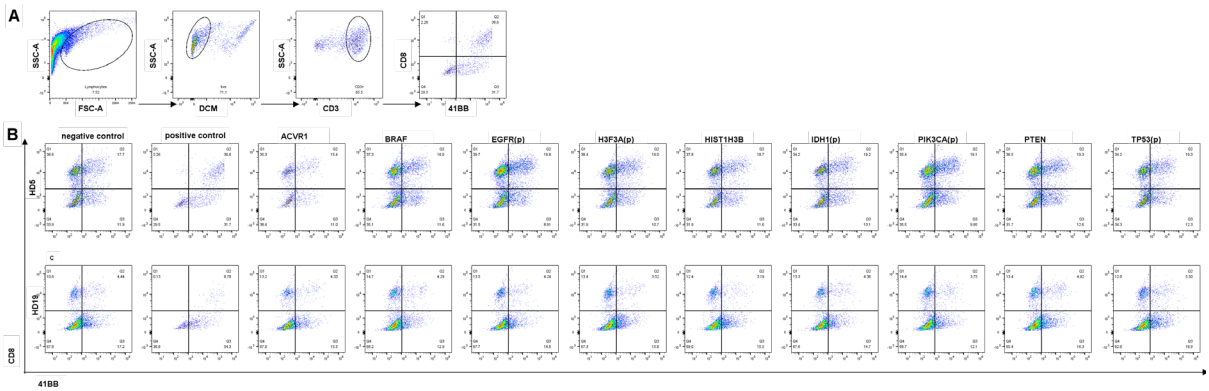


Figure S 1. The Analytical screening experiments for HD5 and HD19, stimulated with pool peptides. A. The gating strategy. B. The results of each tested pool peptide.

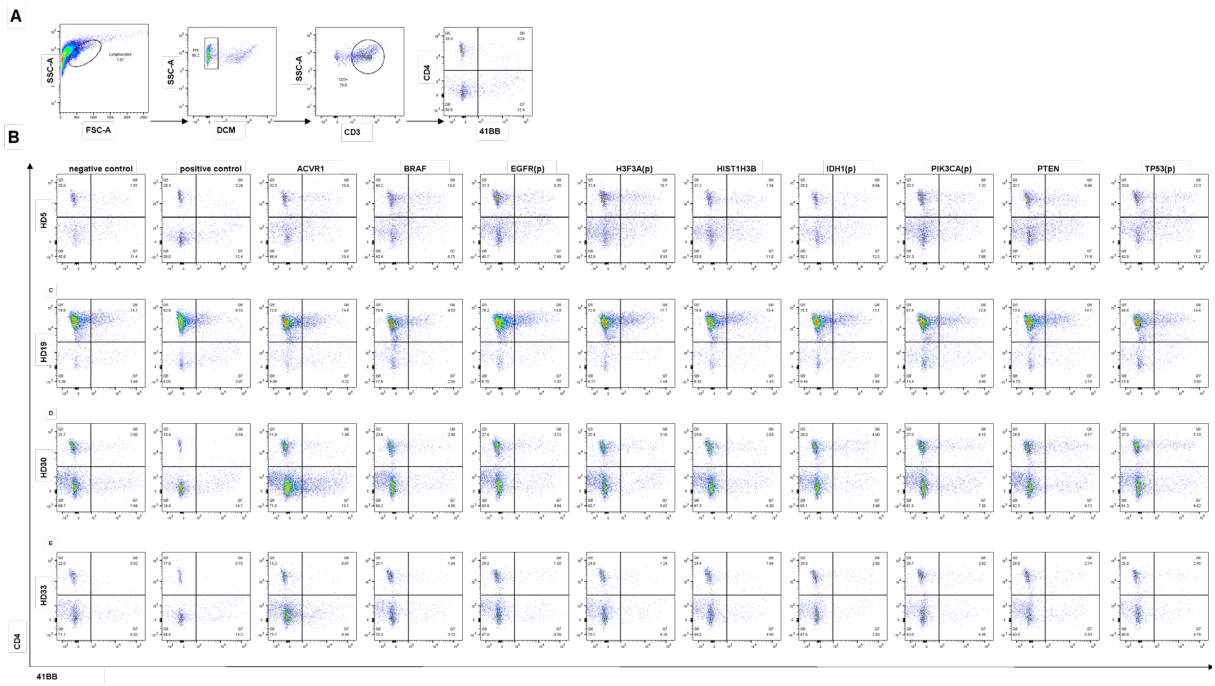


Figure S 2. The analytical screening experiments for HD5, 19, 30, and 33, stimulated with pool peptides. A. The gating strategy. B. The results of each tested pool peptides. HD5 and HD19 were tested again because of high background signal in the previous experiments.

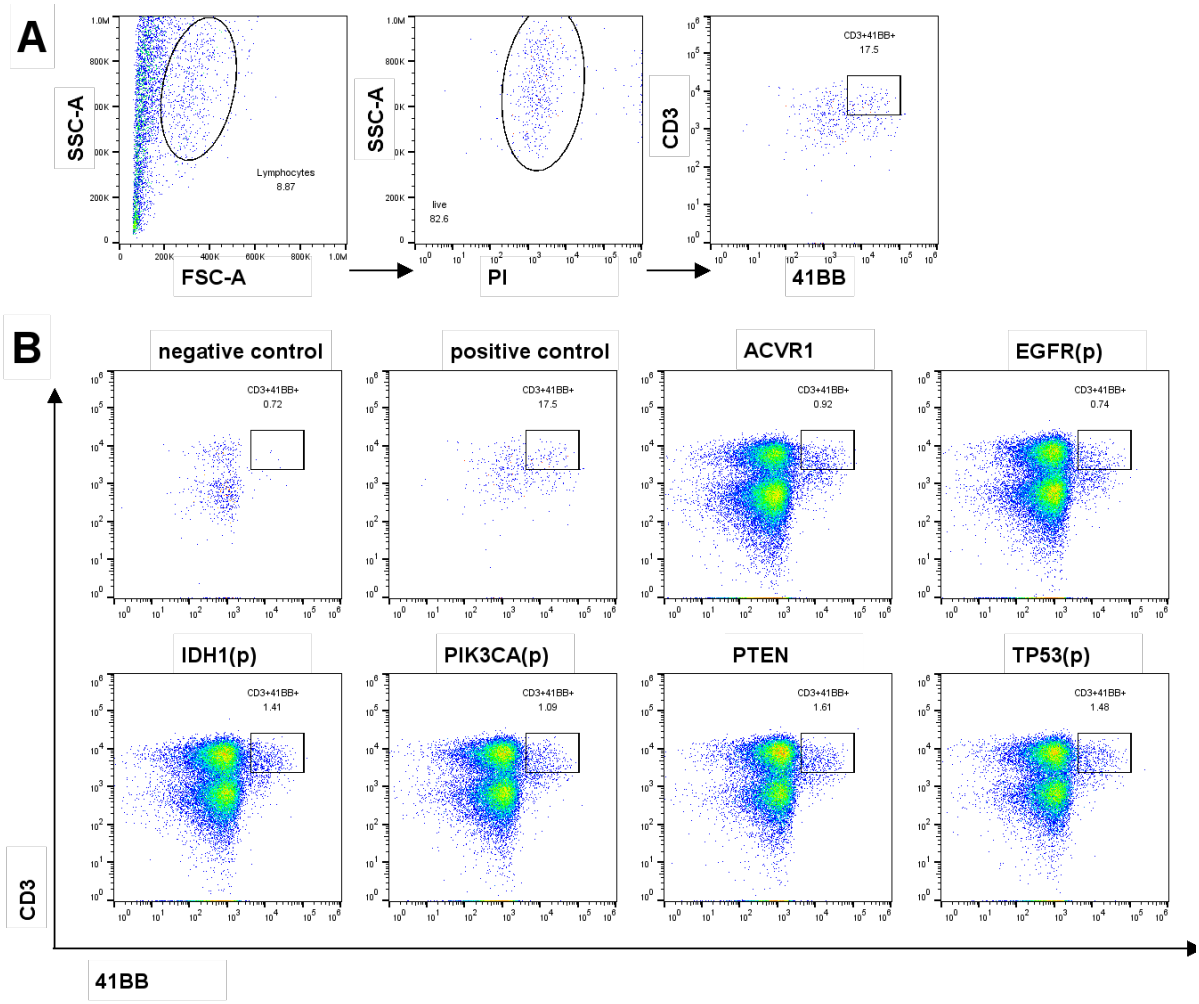
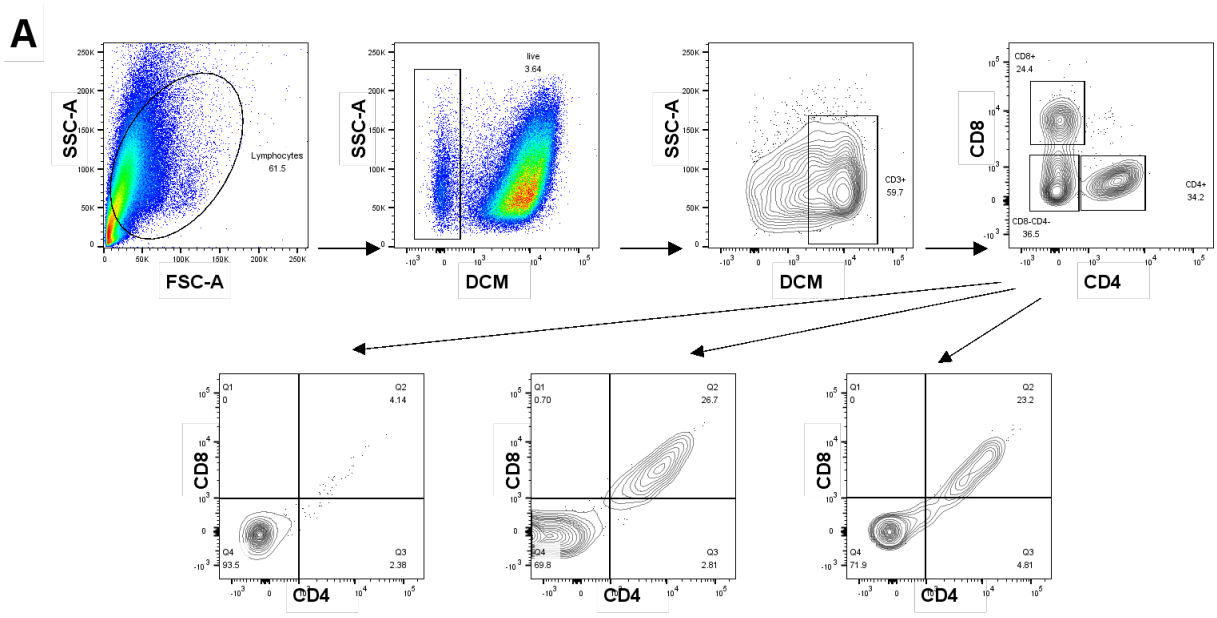
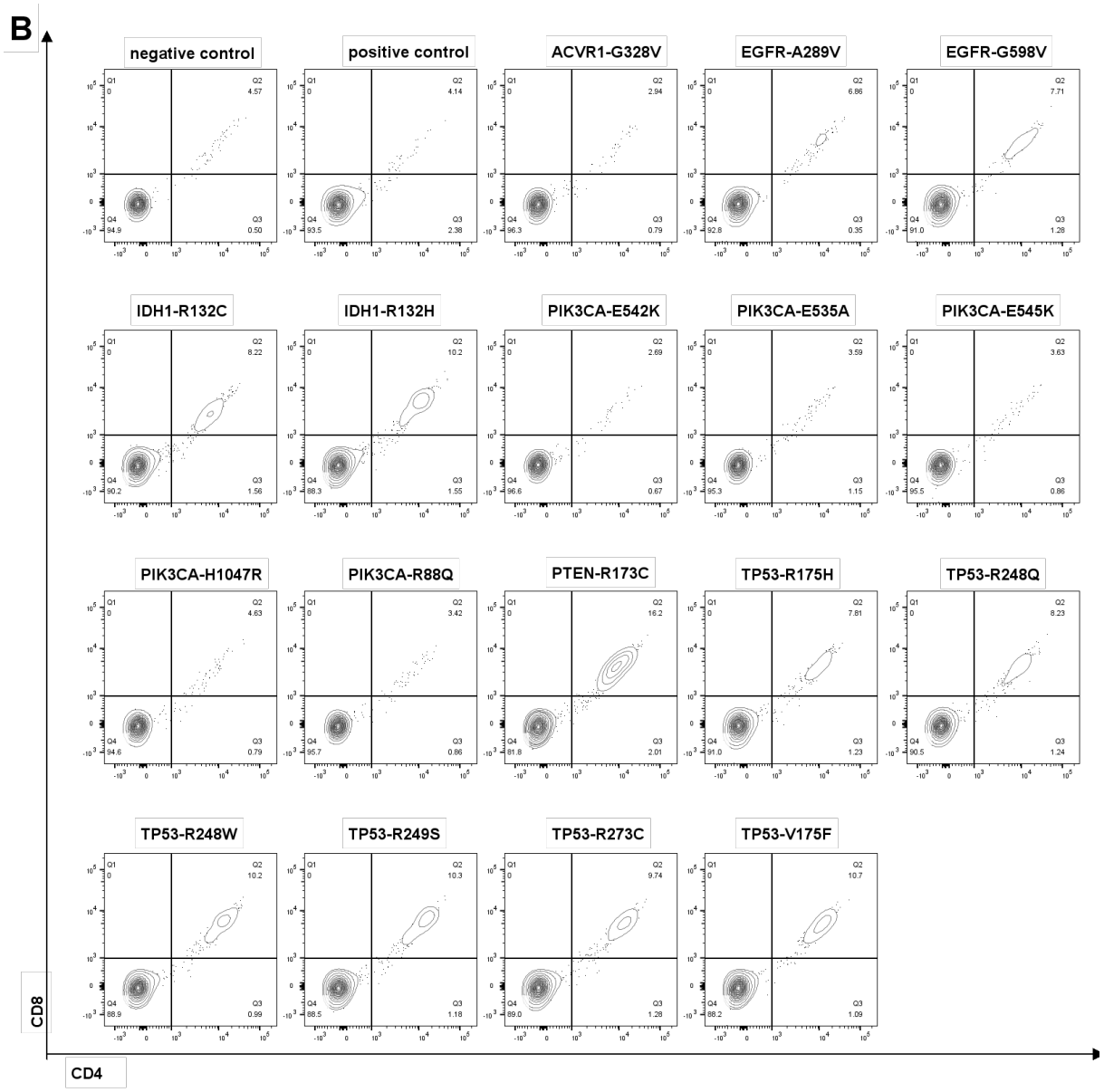
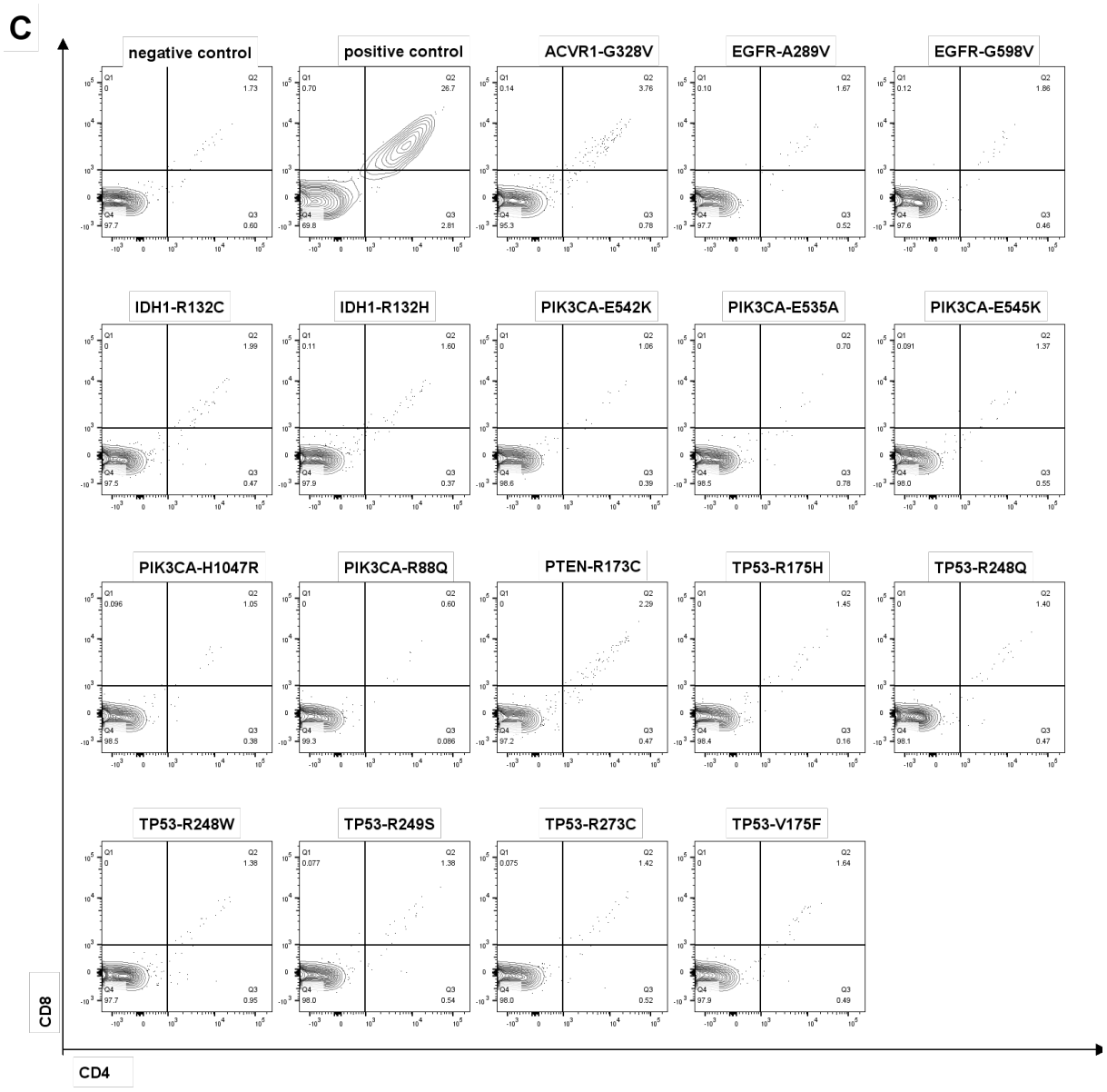


Figure S 3. The FACS cell sorting experiment for HD30 based on the previous analytical screening (Figure S 2), stimulated with pool peptides. A. The gating strategy. B. The results of each selected pool peptides.


A
SSC-A
FSC-A
Lymphocytes
61.5
live
3.64
DCM
CD3+
59.7
CD8+
24.4
CD8
CD4+
34.2
CD8-CD4-
36.5
CD4
Q1
0
Q2
4.14
Q4
93.5
Q3
2.38
0.70
26.7
69.8
2.81
23.2
71.9
4.81


B
negative control
positive control
ACVR1-G328V
EGFR-A289V
EGFR-G598V
IDH1-R132C
IDH1-R132H
PIK3CA-E542K
PIK3CA-E535A
PIK3CA-E545K
PIK3CA-H1047R
PIK3CA-R88Q
PTEN-R173C
TP53-R175H
TP53-R248Q
TP53-R248W
TP53-R249S
TP53-R273C
TP53-V175F
CD8
CD4


C
negative control
positive control
ACVR1-G328V
EGFR-A289V
EGFR-G598V
IDH1-R132C
IDH1-R132H
PIK3CA-E542K
PIK3CA-E535A
PIK3CA-E545K
PIK3CA-H1047R
PIK3CA-R88Q
PTEN-R173C
TP53-R175H
TP53-R248Q
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TP53-R249S
TP53-R273C
TP53-V175F
CD8
CD4

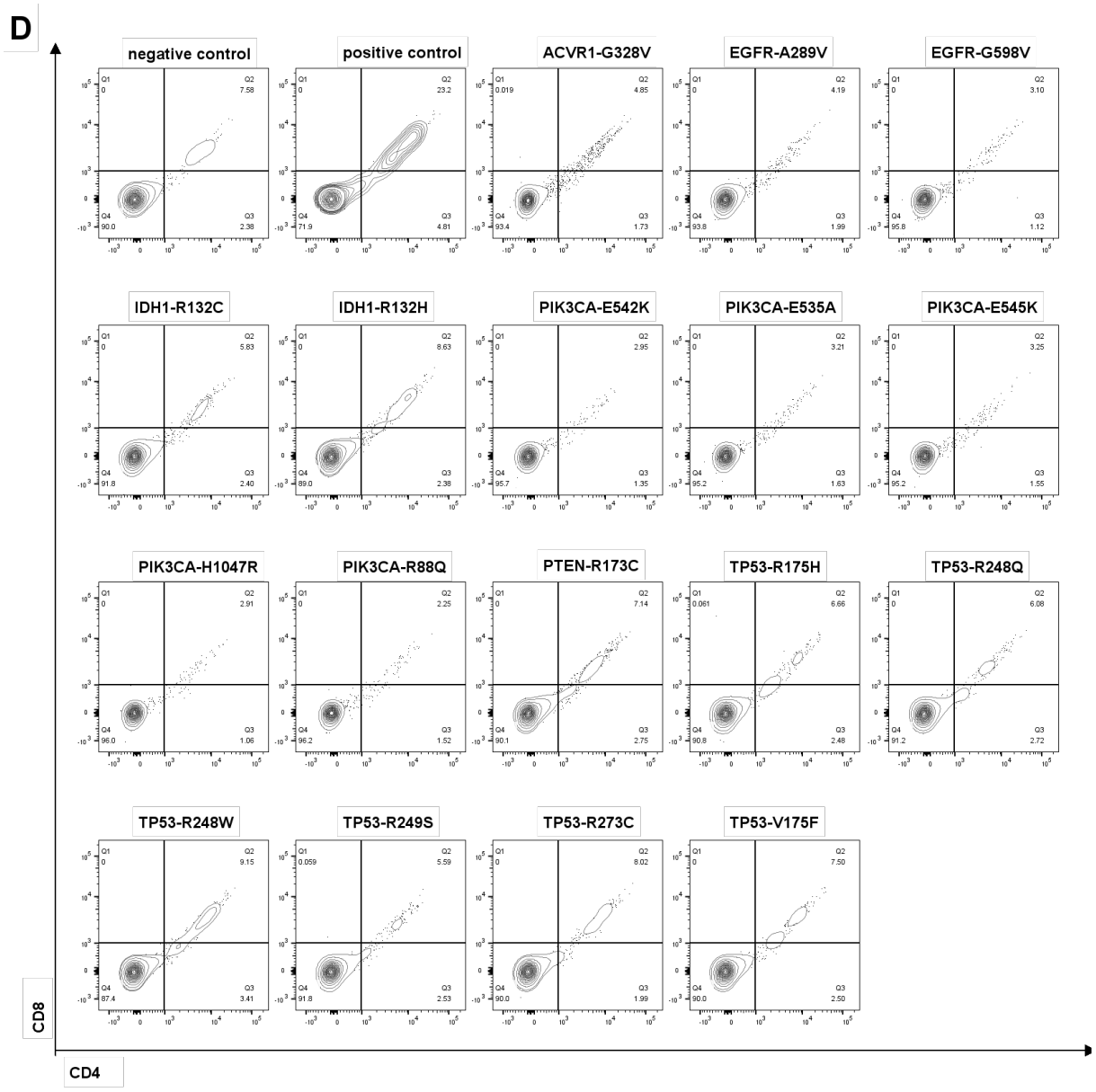


Figure S 4. The analytical screening experiment for HD30, stimulated with single peptides. A. The gating strategy. B-D. The results of each tested single peptides, in different T cells subpopulations. B. $CD4^+$ T cells. C. $CD8^+$ T cells. D. $CD4^-CD8^-$ "double negative" T cells.

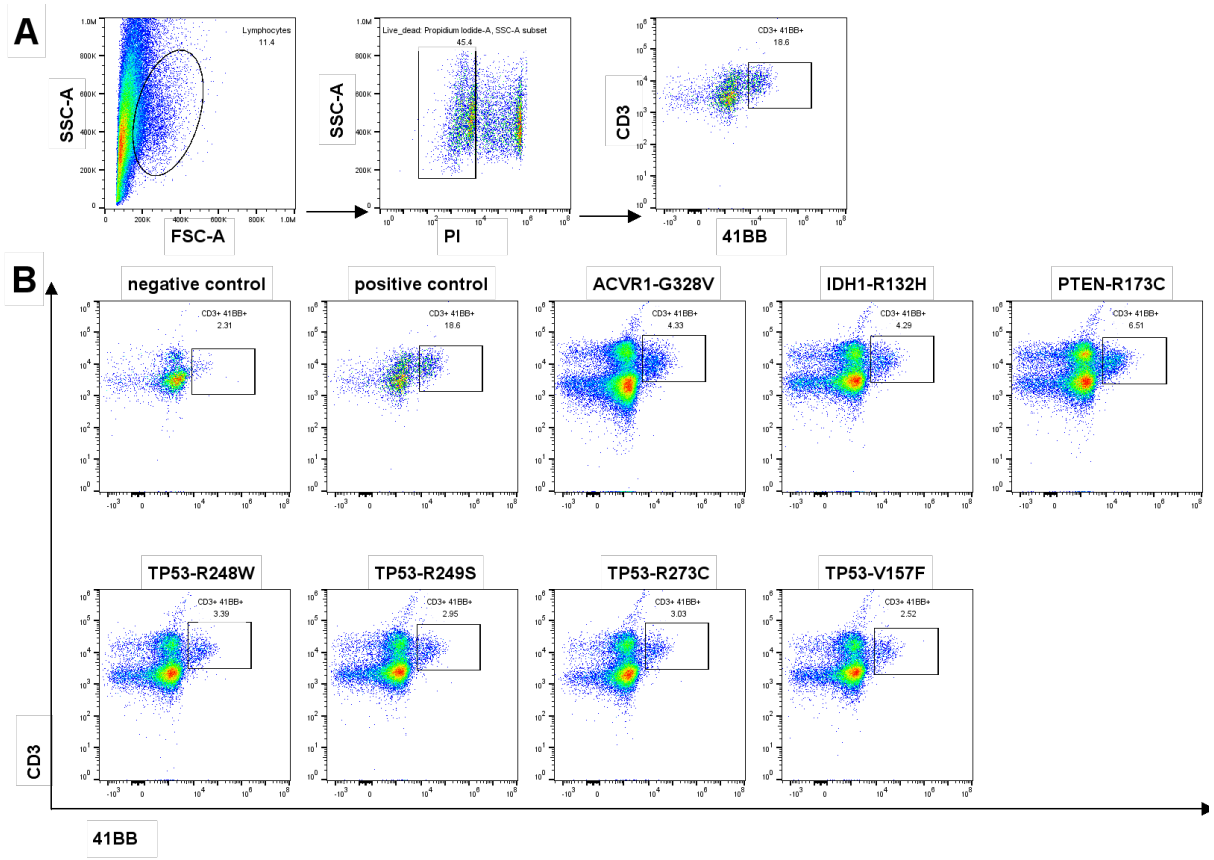


Figure S 5. The FACS cell sorting experiment for HD30 based on the previous analytical screening (Figure S 4), stimulated with single peptides. A. The gating strategy. B. The results of each selected single peptides.

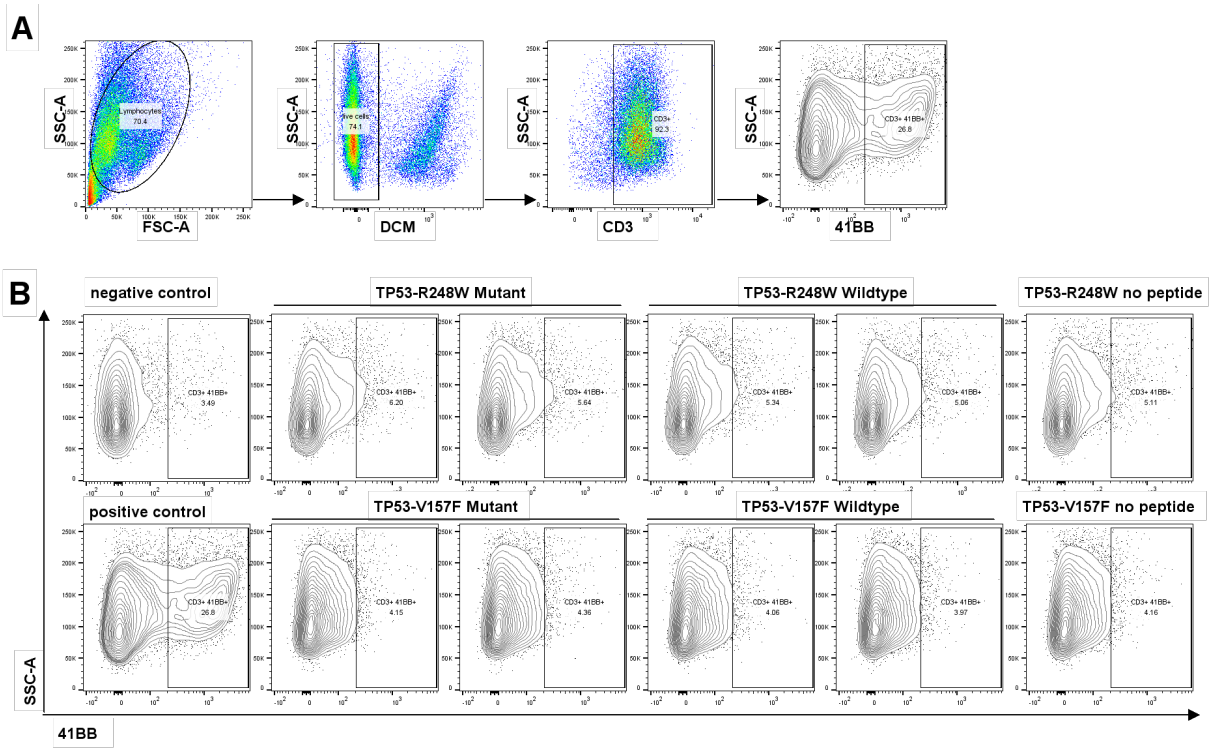


Figure S 6. The validating experiment to compare mutant and wildtype peptides in activating survived T cells from HD30. $TP53^{R248W}$ and $TP53^{V157F}$ reactive T cells were included. A. The gating strategy. B. The results of each condition. The negative control and positive control were using T cells that never stimulated with peptides as previous analyzing experiments.

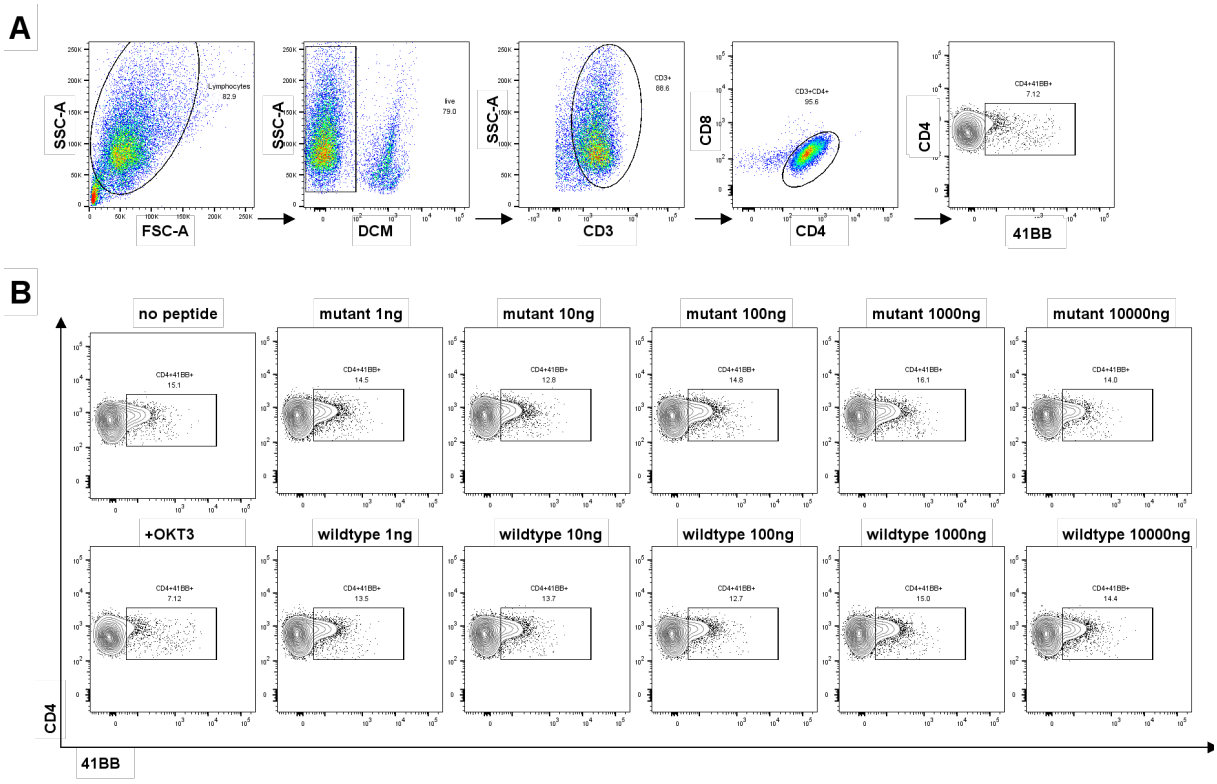
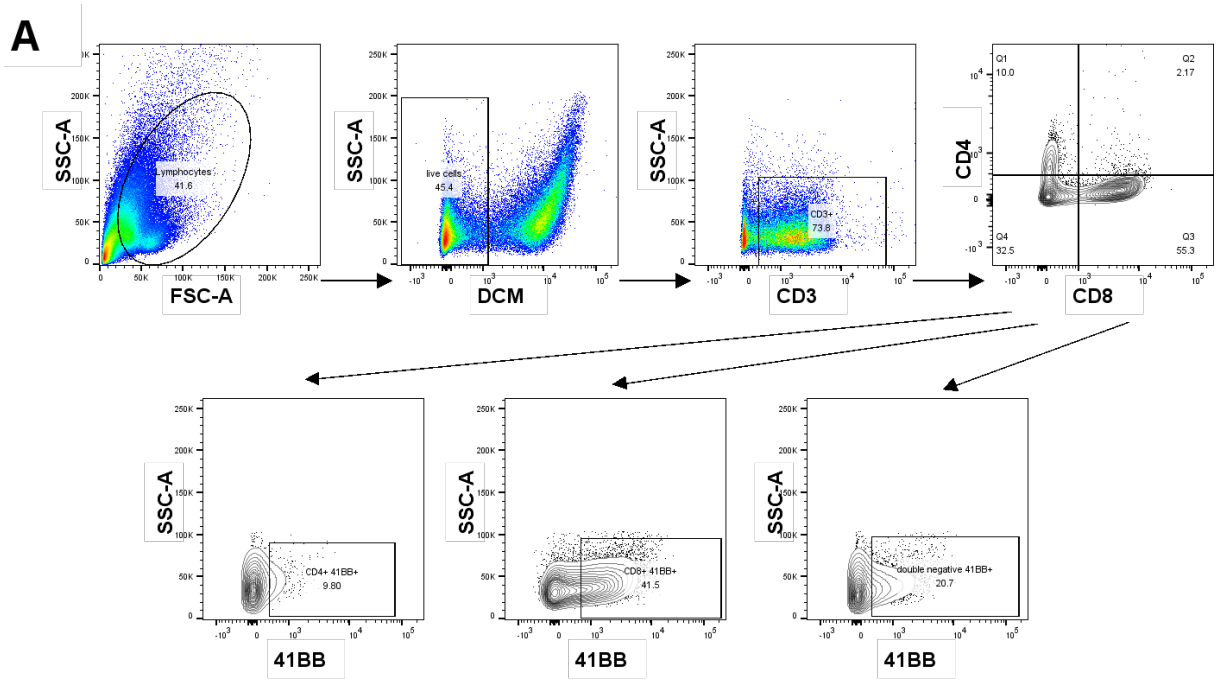


Figure S 7. The titration experiment to optimize peptide concentration for sorted cells, using TP53^{R248W} reactive T cells. A. The gating strategy. B. The results of each condition. Reactive sorted T cells were challenged by anti-CD3 antibody (clone OKT3) as a proper positive control, annotated as "+OKT3".



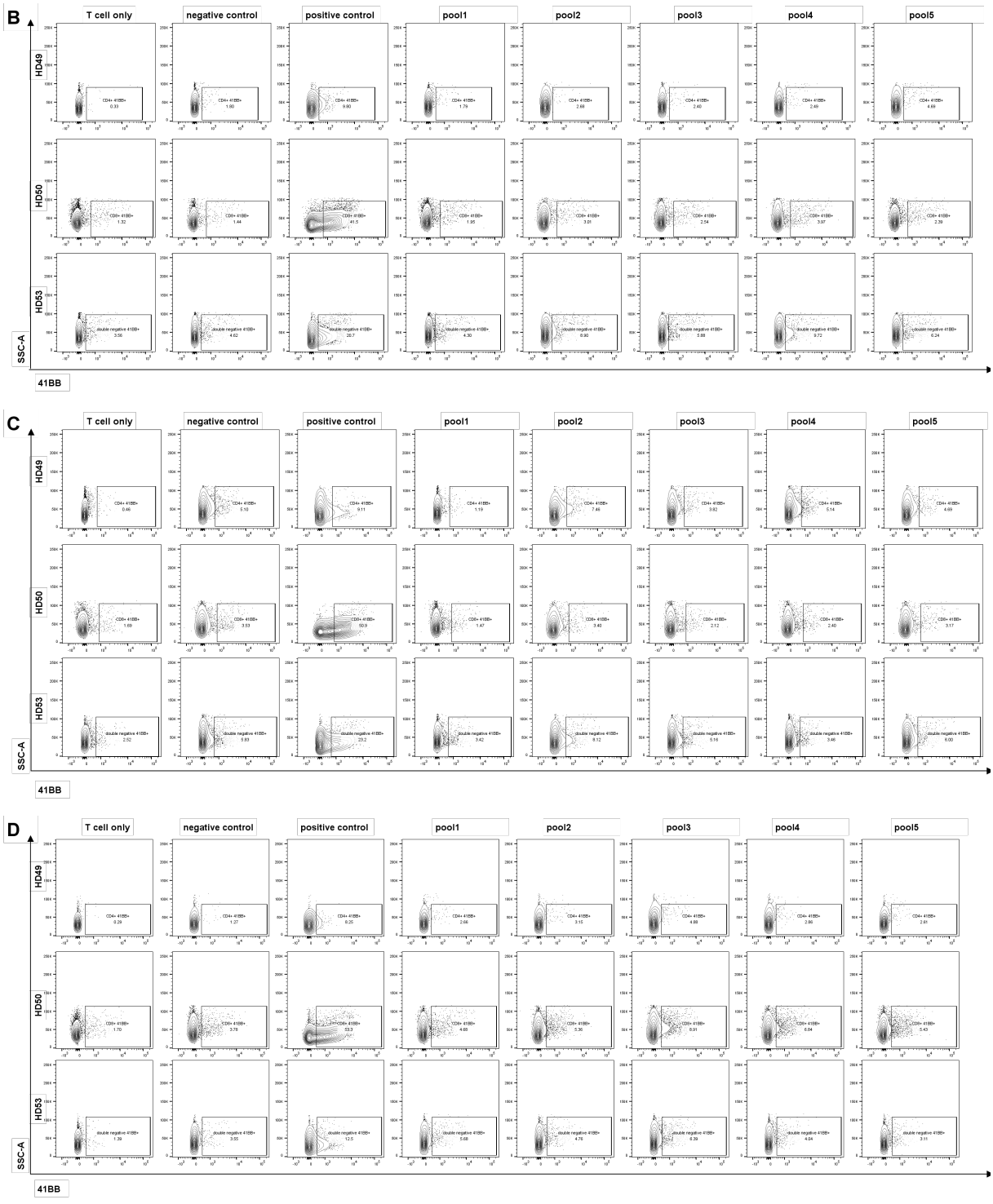
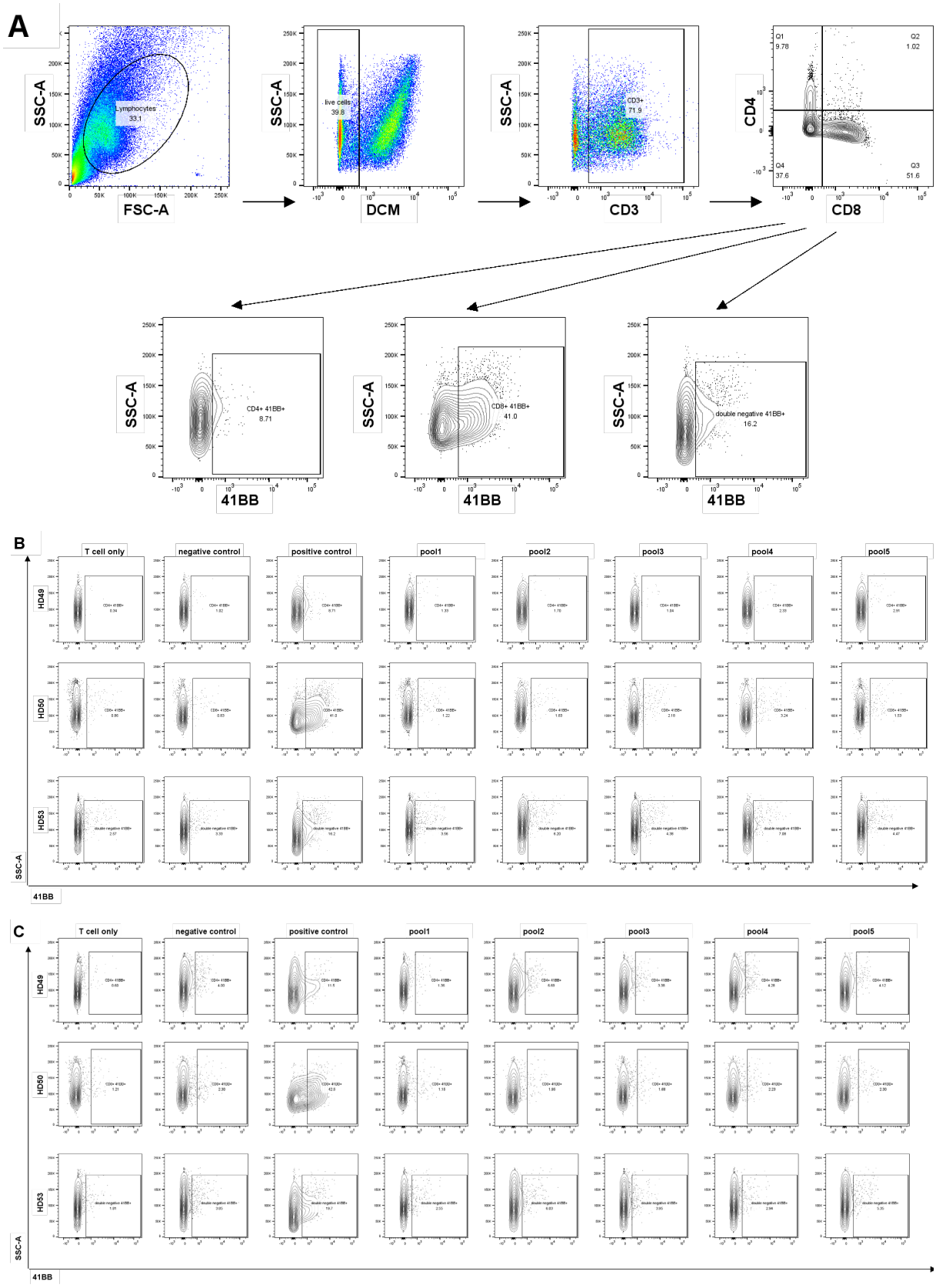


Figure S 8. The analytical screening experiments for HD49, 50, 53, stimulated with pool peptides, and analyzed in BD LSR Fortessa. A. The gating strategy. B-D. The results of each tested pool peptides, in different T cells subpopulations. B. $CD4^+$ T cells. C. $CD8^+$ T cells. D. $CD4^-CD8^-$ "double negative" T cells.


A
SSC-A
FSC-A
Lymphocytes
33.1
live cells
39.8
DCM
CD3+
71.9
CD3
Q1
9.78
Q2
1.02
Q4
37.6
Q3
51.6
CD4
CD8
CD4+ 41BB+
8.71
CD8+ 41BB+
41.0
double negative 41BB+
16.2
41BB
B
T cell only
negative control
positive control
pool1
pool2
pool3
pool4
pool5
HD49
HD50
HD53
SSC-A
41BB
C
T cell only
negative control
positive control
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pool4
pool5
HD49
HD50
HD53
SSC-A
41BB

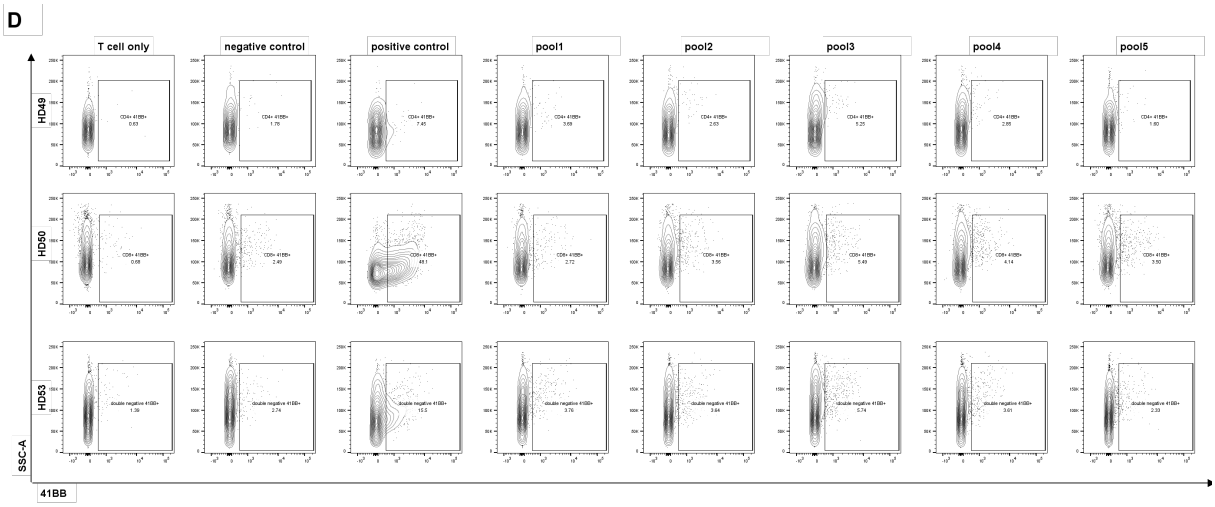

Figure S 9. The analytical screening experiments for HD49, 50, 53, stimulated with pool peptides, and analyzed in BD FACSymphony. A. The gating strategy. B-D. The results of each tested pool peptides, in different T cells subpopulations. B. $CD4^+$ T cells. C. $CD8^+$ T cells. D. $CD4^-CD8^-$ "double negative" T cells.

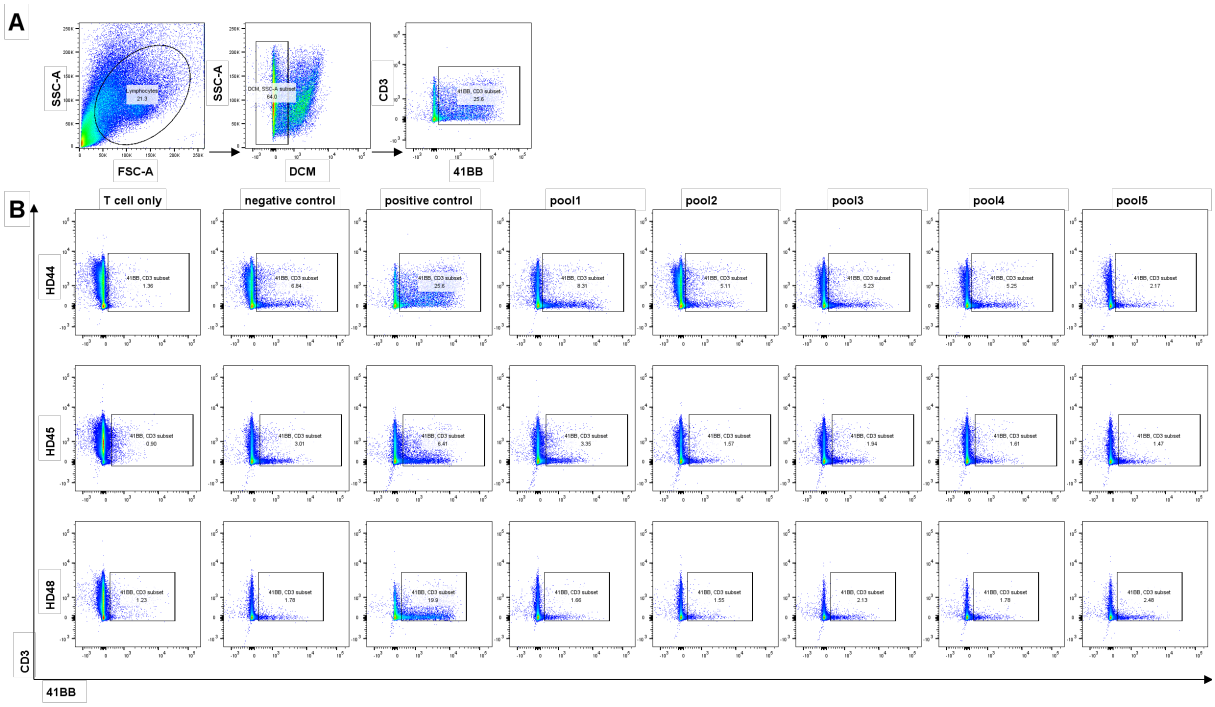

Figure S 10. The analytical screening experiments for HD44, 45, 48, stimulated with pool peptides, and analyzed in BD LSR Fortessa. A. The gating strategy. B. The results of each tested pool peptide.