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TESI DI LAUREA

DEVELOPMENT OF A NOVEL PLATFORM FOR CD8$^+$ T CELL ANTIGEN DISCOVERY

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Table of contents

Abstract

Recognition of specific antigens by T cells is essential for initiating an efficient adaptive immune response to eliminate threats — such as tumors and pathogens. This process has been harnessed for therapeutic purposes, leading to the development of vaccines and cellular-based treatments against cancer. Currently, identification of clinically relevant antigen—T cell receptor (TCR) interactions is possible by screening libraries of peptides and immunoreceptors, but still requires foreknowledge of one of the two counterparts. This thesis focuses on the early development stages of a novel cell-based platform for CD8+ T cell antigen discovery. The proposed method exploits engineered antigen presenting cells (APCs) that conditionally express an anti-CD69 membrane-bound antibody upon interaction between their peptide-major histocompatibility complex (pMHC) with its cognate TCR. Binding to CD69, expressed on the activated T cell, will then induce formation of cell doublets, which can be sorted via flow cytometry and sequenced in order to simultaneously retrieve the TCR's and the antigen's sequences — even when both of them are unknown. This double-regulated circuit aims to fill the technological gaps in antigen discovery approaches, allowing the development of therapies to be used against a larger spectrum of infections, autoimmune diseases and cancers.

1. Introduction

T lymphocytes (T cells) play an essential role in adaptive immune responses, being involved in cancer and pathogen elimination, homeostasis maintenance and generation of the immunological memory, as well as B-cell activation. These functions can be exerted upon their activation, which occurs through recognition of specific antigens presented by major histocompatibility complex (MHC) proteins expressed on the membrane of an antigen presenting cells (APCs).

1.1 Antigen Presenting Cells

APCs' role in cell-mediated immunity is to process peptides and load them onto MHC molecules on their surface, allowing antigen recognition and activation of lymphocytes expressing cognate TCRs. Antigens can be distinguished – based on the source they originate from – in endogenous and exogenous:

- endogenous antigens derive from proteins generated within the cell. Their role in the immune response is critical in the context of infection by intracellular pathogens (viruses and intracellular bacteria, for instance) and cancer;
- exogenous antigens derive from the extracellular milieu and are uptaken by phagocytosis. Their role in the immune response is critical in the context of infection by extracellular pathogens – such as extracellular bacteria – or allergic reactions driven by allergens. They enter the body via inhalation, ingestion or injection. They can comprehend non-protein molecules, but these are not recognized by T cells.

While endogenous antigens can be presented by almost all types of cells, presentation of exogenous ones can only occur upon their internalization – which is a prerogative of professional antigen presenting cells. This category comprehends dendritic cells, macrophages and B cells: all of them share the ability to (i) express MHC-II molecules on their surface and to (ii) deliver costimulatory signals to naïve T helper cells. In particular:

- dendritic cells (DCs) have the most efficient antigen-presenting activity, due to the high constitutive expression levels of MHC-II and co-stimulatory molecules on their surface;
- macrophages express MHC-II and co-stimulatory molecules after being activated by phagocytosis of certain antigens;

- B cells' expression of MHC-II proteins is constitutive, but they need to be activated to express co-stimulatory molecules.

1.1.1 Major Histocompatibility Complexes

MHC proteins – also known as human leukocyte antigen (HLA) proteins, in humans – are required for specific engagement and proliferation of T cells in lymphoid tissues and successive establishment of an effective immune response. Specifically, this function is carried out by the two major groups of MHC molecules: class I and class II. Although their structure is quite similar, the molecular mechanisms underlying their effector functions and their molecular/cellular counterparts are different: MHC class I molecules present endogenous peptides to $CD8^+$ T cells, while MHC class II molecules present exogenous peptides to $CD4^+$ T cells. Moreover, their spatial expression is different: MHC-I molecules are present on the surface of all nucleated cells (and on platelets), whilst MHC-II proteins expression is restricted to professional antigen presenting cells.

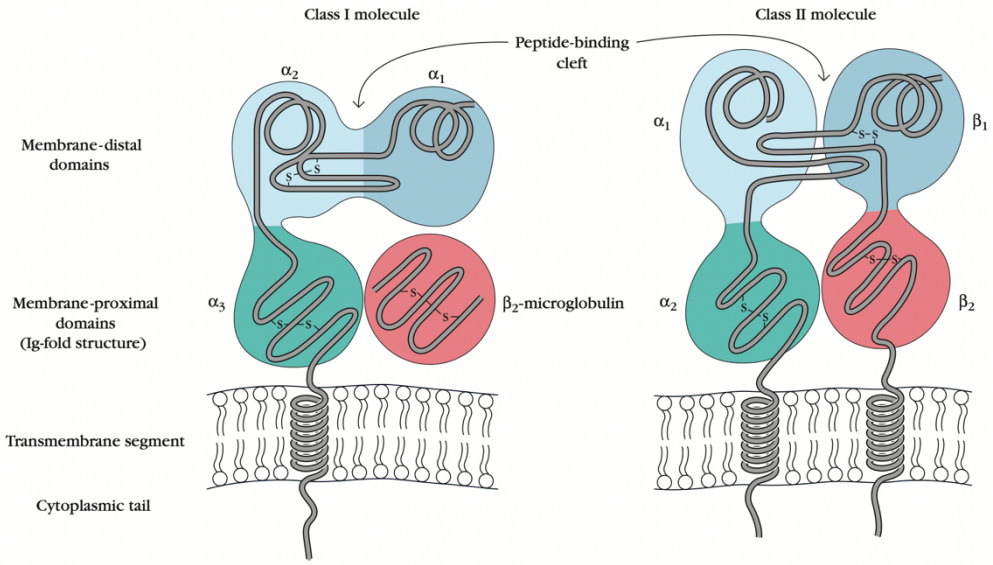


Figure 1 – **Structure of MHC molecules**. MHC-I (left) shows an unique a- chain composed of three domains, associated with B2-microglobulin domain (red); interaction between a-1 and a-2 domains generate the peptide-binding cleft in the membrane-distal portion of the molecule. MHC-II (right) derives from the association of two different chains (a and b); the peptide-binding groove is determined by interaction between the two membrane-distal domains (a1 and b1, in blue). Kuby Immunology 7th ed.

1.1.2 Major Histocompatibility Complex: Class I

The molecular structure of MHC-I (Figure 1, left) is characterized by an α-chain and a B2-microglobulin molecule – 45 kDa and 12 kDa, respectively – associated via non-covalent interactions. The α chain comprises three extracellular domains (α 1, 2, 3), a transmembrane domain and a cytoplasmic region. The interaction between α1 and α2 domains generates a cleft represented by eight antiparallel B-sheets flanked by two α-helixes: this structure is known as peptide-binding groove, is located on the top of the MHC molecule and its role is to bind endogenous peptides. The α3 domain is responsible for the interaction with CD8 molecules on $CD8^+$ T lymphocytes' surface.[1]

1.1.3 MHC-I antigen presentation: the cytosolic pathway

In order to be efficiently presented to T cells, antigens need to be processed into peptides of appropriate length and that hold specific biochemical features – otherwise, they cannot interact with the peptide-binding groove.

Dysfunctional intracellular proteins are marked for proteolysis by attachment of ubiquitin to a lysine residue, leading to proteasome-mediated degradation in the cytosol. Interaction with the peptide-binding groove is strongly enhanced for peptides of 8-10 amino acids length and containing hydrophobic or basic amino acid residues in the carboxyl-terminal positions – also known as anchor residues. These features allow said peptides to be transported by TAP (transporter associated with antigen presentation), a protein that spans the membrane of the rough endoplasmic reticulum (RER), into the RER's lumen. Here, a series of molecular chaperones promote correct peptides folding and MHC-I assembly. First, calnexin folds the α-chain and allows its association with β2-microglobulin; then, calnexin is released and MHC-I interacts with calreticulin and tapasin (TAP-associated protein). The latter mediates the interaction between TAP and MHC-I, so that high-affinity peptides can be loaded into the cleft – while those that don't bind to MHC-I are degraded. After peptide acquisition, the pMHC-I complex gains stability and, once dissociated from calnexin and tapasin, can exit RER and pass through Golgi apparatus before being transported to the cell's surface.[1,2]

1.1.4 Major Histocompatibility Complex: Class II

Structure of MHC class II (Figure 1, right) is formed by the non-covalent association of two different glycoproteic chains: α (33 kDa) and β (28 kDa). Both polypeptides comprise a transmembrane domain, as well as a cytoplasmic domain and two external regions (α1 and α2 domains in the α chain, β1 and β2 domains in the β chain). α1 and β1 domains interact to create the peptide-binding groove which, although sharing structural characteristics with the MHC-I's cleft, does not present conserved residues that bind to the terminal amino acids of short peptides, thus forming an open-ended groove and allowing the interaction with peptides longer than 11 amino acids (exogenous peptides).[3] The α2 and β2 membrane-proximal domains are responsible for the interaction of MHC-II with CD4 receptors on $CD4^+$ T cells. MHC-II molecules are expressed on the surface of professional APCs and also on thymic epithelial cells, where they mediate positive and negative selection of thymocytes.[1]

1.1.5 MHC-II antigen presentation: the endocytic pathway

Antigen internalization by professional APCs leads to its degradation inside compartments of the endocytic processing pathway. First, antigens are engulfed in early endosomes (pH = 6.0–6.5); then, they move to late endosomes (pH = 5.0–6.0) and finally to lysosomes, characterized by pH of 4.5–5.0 and by the presence of acid-dependent hydrolases. Antigens are degraded into peptides of 13-18 amino acids length and interact with the MHC-II's open-ended groove, as previously mentioned. Newly synthesized MHC-II complexes are associated with a protein called invariant chain (Ii, also named CD74) that interacts with the peptide-binding groove and prevents endogenous peptides to bind to class II MHC in the RER. MHC-II-Ii complexes are transported from the RER to the Golgi network and eventually to the endocytic pathway, which causes the partial degradation of the Ii and results in the formation of CLIP (class II-associated invariant chain peptide), a small peptide that sterically impairs premature bonding of peptides. Exchange of CLIP with the correct peptide is catalyzed by HLA-DM, a class II molecule expressed in the endosomal compartment. The peptide-MHC-II complex is, at this point, ready to be transported and displayed on professional APCs' surface, where it is strongly stabilized by neutral pH conditions.[1,2]

1.1.6 Polymorphism of MHC molecules

Human MHC-I α-chain is encoded by HLA-A, HLA-B and HLA-C genes, while MHC-II's α and β chains are encoded by two pairs of HLA-DR, HLA-DP and HLA-DQ genes – as shown in Figure 3. HLA loci are known to be highly polymorphic: the number of different MHC's allelic variants expressed in humans amounts to several hundreds. Studies show that the most polymorphic residues are localized in the peptide-binding groove, thus suggesting that the high allelic diversity reflects in the affinity of an MHC molecule for a given peptide.[4] One individual can only express up to six different HLA-I and twelve different HLA-II proteins, yet the relatively limited number of possible variants displayed must be capable of presenting the enormous landscape of antigens that the immune system needs to interface with. Indeed, peptide binding by MHC molecules is defined promiscuous: a specific MHC molecule can bind numerous different peptides and some peptides can bind to different MHC molecules.[1]

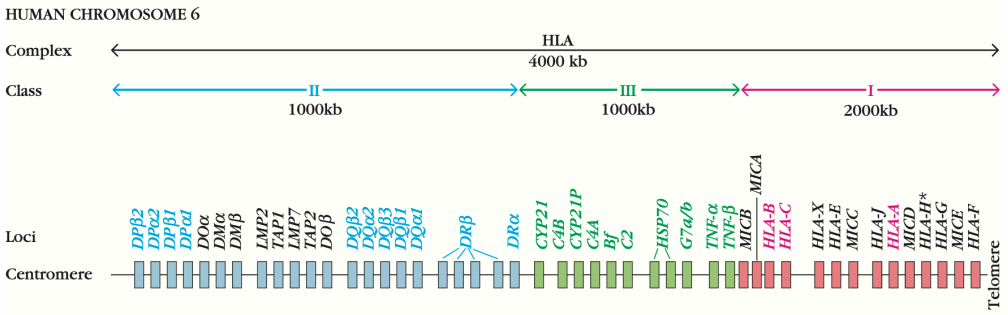


Figure 2 – Human HLA encoding region. Kuby Immunology 7th ed.

1.2 T lymphocytes

T lymphocytes (T cells) originate in the bone marrow from hematopoietic stem cells (HSCs), which differentiate into progenitors and migrate to the thymus – a primary lymphoid organ – where they undergo maturation, selection and successive export to peripheral tissues. Peripheric T lymphocytes are distinguished into several subsets – including naïve T cells, memory T cells and Treg lymphocytes. Naïve T cells haven't been activated yet, since they have not been in contact with cognate peptides. Memory T cells have been previously activated, are present in a quiescent state and can be re-activated in order to maintain long-term immunity. Tregs' function is to maintain the immunological

response in a physiological range and downregulate it when needed. T cells' activation is triggered by MHC-mediated antigen presentation: APCs display peptide-MHC complexes (pMHC) that interact with TCRs, expressed on T cells' surface.

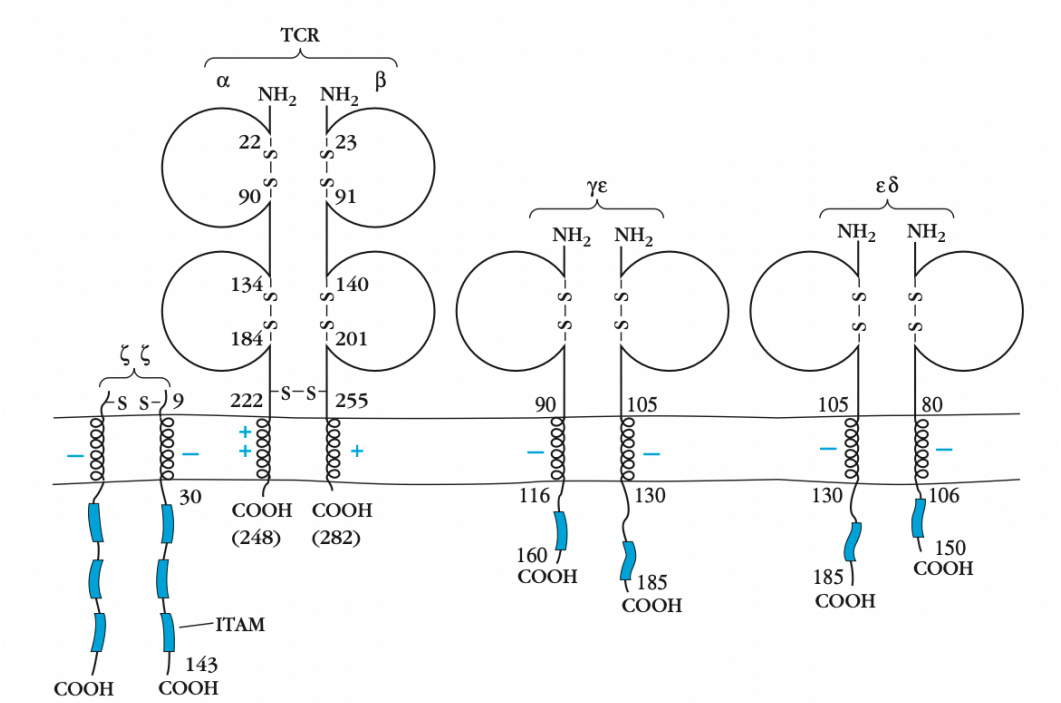


Figure 3 – Schematic representation of the structure of the TCR complex. Kuby Immunology 7th ed.

1.2.1 The T cell receptor complex

The TCR is a 450 kDa heterodimeric protein composed of two chains – α/β or δ/γ – both containing a constant and a variable region [Figure 4], delimited by an intrachain disulfide bond that spans 60-75 amino acids. The variable regions, located on the amino-terminal ends, comprehend three hypervariable domains called complementarity determining regions (CDRs) – CDR1, CDR2 and CDR3 – responsible for pMHC binding. TCR is non-covalently associated to **CD3** on cell's surface, creating the TCR complex [Figure 4]. CD3 is composed of three subunits formed by five conserved polypeptides that associate to form three trimers: γ-epsilon, δ-epsilon, ζ-ζ. On their intracellular region, these subunits present so-called immunoreceptor tyrosine-based activation motifs (ITAMs), which are essential for signal transduction and subsequent lymphocyte's activation. During

interaction with pMHC, the TCR complex clusters with so-called co-receptor molecules, namely **CD4** and **CD8**. CD4 is a 55 kDa molecule that binds to a conserved domain of the MHC-II molecule. Its structure is characterized by a cytoplasmic tail, a transmembrane region and four immunoglobulin-like domains on its extracellular portion: D1, D2, D3 and D4. CD8 binds to the invariant chain of MHC-I molecules and can be found either in the $\alpha\beta$-heterodimeric form or in the $\alpha\alpha$-homodimeric form. The α and β chains are 30 kDa glycoproteins that present an immunoglobulin-like domain on the extracellular domain, in addition to a transmembrane domain and a cytoplasmic tail. Both CD4's and CD8's cytoplasmic tails contain residues that can be phosphorylated – which is essential for the signal transduction that leads to T-cell's activation.

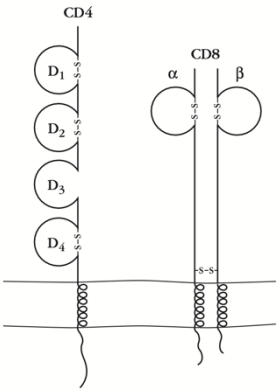


Figure 4 – Molecular structure of CD4 (left) and CD8 (right) co-receptors. Kuby Immunology 7th ed.

1.2.2 TCR diversity: somatic recombination

$\alpha\beta$TCR is genetically encoded by two loci: TCRα and TCR β. The former comprises the V and J segments, whilst the latter also includes the D segment. Stochastic rearrangement of V(D)J segments takes place in the thymus, during lymphocyte development. The genes are flanked by RRS (recombination signal sequences) that are formed by an heptameric sequence and a nonameric sequence separated by a spacer sequence of 12 or 23 bp. RSS are recognized by VDJ recombinase – a lymphoid tissue specific complex of enzymes that comprehends recombination activating genes 1 and 2 (RAG-1 and RAG-2) – which catalyzes DNA's cleavage, generating coding-ends and signal-ends. Nonhomologous end-joining (NHEJ) proteins carry out the joining phase: while signal-ends are precisely joined, coding-ends need to be modified through nucleotide excision or addition. V(D)J recombination leads to formation of a large number of random gene combinations for the TCR chains, which eventually results in the expression of the extensive landscape of different TCRs displayed by the immune system. For instance, combinatorial joining of 100 V_α and 50 J_α gene segments can lead to formation of 5000 possible sequences for the TCR α

chain, while arrangement of 25 V_β, 2 D_β and 12 J_β gene segments result in 600 different combinations. Furthermore, the random combination of α and β TCR variable regions can generate 3 x 10^6 different T-cell receptors, each with different epitope-binding specificities.[1,5,6]

1.2.3 T cell activation: the immunological synapse

The interaction between a TCR and the cognate pMHC is essential for the activation of the T cell, but not sufficient. In order to reach a complete and functional activation state, which leads to their proliferation and survival, lymphocytes need to further interact with the APC through co-stimulatory signals. Specifically, CD28 is a well characterized co-stimulatory molecule expressed on T cells' surface, that binds to its ligands CD80 (B7.1) and CD86 (B7.2) on the APC's membrane, upon specific interaction between the lymphocyte and the APC. A third signal is induced by IL-2's interaction with its receptor, leading to proliferation and differentiation of the lymphocyte. In absence of co-stimulation, lymphocytes undergo a process of inactivation that leads to anergy, a state in which they are functionally unable to exert their effector functions and that eventually results in apoptosis. When the peptide-MHC engages with a cognate TCR, either CD4 or CD8 co-receptor – after binding to the invariant region in the MHC molecule – are brought closer to the TCR complex. The tyrosine kinase Lck – associated with co-receptor's intracellular tail – phosphorylates ITAMs in ζ-chain's cytoplasmic region, leading to recruitment of ZAP-70 and subsequent phosphorylation of membrane-bound adaptor molecules, which activate several transduction pathways. Specifically:

- phospholipase C is activated by phosphorylation and cleaves membrane-associated phospholipids into inositol 1,4,5-triphosphate and diacylglycerol. The former leads to opening of Ca^{2+} channels, while the latter activates protein kinase C;
- Ca^{2+} influx from the endoplasmic reticulum and form the extracellular space leads to translocation of nuclear factor of activated T-cells (NFAT) into the nucleus, resulting in transcription of cytokines with growth-promoting functions (e.g. IL-2, IL-4);
- protein kinase C allows the NF-kB transcription factor to enter the nucleus, where it initiates expression of genes involved in T-cell activation and essential to provide survival signals.

1.2.4 Thymic selection

Before undergoing the process of maturation in the thymus, lymphocytes are double-positive for CD8 and CD4 glycoproteins – through which they interact with the constant region of class I and class II MHC molecules, respectively. During **positive selection**, $CD8^+$/$CD4^+$ T cells bind either MHC-I or MHC-II and this interaction determines their fate: lymphocytes that bind an antigen presented by MHC-I with high affinity will downregulate the expression of CD4 on their surface, therefore becoming $CD8^+$ cells; on the other hand, lymphocytes that interact with antigens presented by MHC-II will become $CD4^+$ cells. By the time they exit the thymus, T cells will be differentiated into two distinct subsets – $CD8^+$ and $CD4^+$ – characterized by different effector functions, as explained in the following paragraphs. The thymus is also the site of **negative selection**, which consists in the elimination of T-cells that have too high affinity for self-pMHC complexes, allowing the creation of a self-tolerant TCR repertoire in peripheral T-cells.

1.2.5 $CD4^+$ T cells

$CD4^+$ T lymphocytes, also known as T helper cells (Th cells), assist other immune cells in exerting their functions by secreting cytokines that allow and mediate the immune response. They are divided into several subsets, determined by the cytokine environment:

- **T_H1 subset** is determined by IL-12 and IFN-gamma. They are responsible for the upregulation of STAT4 (Signaling Transducer and Activator of Transcription) and STAT1, respectively, leading to expression of master transcription factor *T-bet*. This eventually determines the Th1 transcription profile and, consequently, production of IFN-gamma – which support the production of IgG antibodies and activation of $CD8^+$ T-cells;
- **T_H2 subset** is induced by IL-4, which leads to upregulation of STAT6 followed by expression of GATA3, the master transcription factor responsible for the Th2 signature. T_H2 cells produce IL-4 and IL-5, promoting isotypic switch of antibodies from IgM to IgE, mediating eosinophil activation and allergic reactions;
- **T_H17 subset** is the result of IL-6 and IL-23-mediated activation of STAT3, which leads to expression of ROR-γt. TH17 cells produce IL-17, IL-21 and IL-

22 in order to activate neutrophils and promote differentiation of follicular lymphocytes;

- **T_{reg}** subset differentiation is promoted by TGF-β, which leads to expression of FOXP3 through STAT-5 signaling and results in the production of TGF-β and IL-10. Their function is to modulate the immune response by downregulating T cells' activation, as well as maintaining self-tolerance and prevent autoimmune diseases.[1,7]

1.2.6 $CD8^+$ T cells

$CD8^+$ T lymphocytes have the role to kill dysfunctional cells – especially cancerogenic and infected ones – after recognizing specific antigens on their surface, presented by MHC-I molecules. Once activated into effector cells (also called cytotoxic T lymphocytes, CTLs) they proliferate and gain cytotoxic activity, which can be exerted via two pathways: the cytotoxic protein pathway and the Fas ligand pathway. The **cytotoxic protein pathway** is mediated by perforins and granzymes; perforins facilitate the entry of granzymes granules in the target cell, which are able to induce the endogenous apoptosis pathway, leading to cell death. On the other hand, the **Fas ligand pathway** consists in the interaction between Fas ligand on $CD8^+$ T cells' surface and Fas receptor expressed by the target cells; this interaction results in the activation of Caspase 1 protein and eventually in DNA fragmentation and apoptosis.

1.2.7 T cells' role in cancer suppression: the cancer-immunity cycle

Cancer development is caused by the accumulation of genetic mutations, some of them leading to dysregulation of physiological cellular processes. On a phenotypical level these alterations are observed in the form of neoantigens, which are presented on the surface of cells bound to class I MHC molecules. As mentioned in previous paragraphs, MHC-I-mediated antigen presentation enables recruitment of cytotoxic T-cells and eventually cancer cells' death by apoptosis. The succession of events that allow the adaptive immune system to eradicate cancer is known as cancer-immunity cycle and, in this context, two stages can be recognized – priming stage and effector stage. The first one involves a process known as cross-presentation, which is carried out by tumor-infiltrating DCs that are able to internalize neoantigens and present them on MHC-I on their surface. Following their migration in adjacent tumor-draining lymph

nodes, they interact with $CD8^+$ antigen-specific T-cells, inducing generation of cytotoxic T cells with effector functions. The priming phase is aided by $CD4^+$ T-cells, which are activated into effector T-helper cells after interacting with pMHC-II complexes presented by DCs. Their function is primarily mediated by cytokine production and leads to $CD8^+$ cells' efficient proliferation and activation. Chemokines release in the tumor tissue allows CTLs migration from lymph nodes to the tumor micro-environment (TME) in order to exert their neo-acquired effector functions. When T-cells enter the TME they are called tumor-infiltrating lymphocytes (TILs). The quantity of TILs in the TME is considered a prognostic marker for cancer, and tumors can be distinguished in "hot" or "cold", based on the presence (and localization) of a high or low number of immune cells, respectively. The formers are characterized by an Infiltrated-Inflamed TME – in which CTLs are localized in the tumor core region – and are more susceptible to CTLs-mediated suppression activity. On the other hand, cold tumors are characterized by an Infiltrated-Excluded TME – in which CTLs reside in the marginal region of the tumor – and are more prone to escape immune responses and grow. After death of cancer cells, more neoantigens are released in the TME, progressively reinforcing the response every time the cancer-immunity cycle is re-enacted.

2. T Cell Antigen Discovery

Antigen specificity is an essential feature of the adaptive immune system. Both B and T lymphocytes express antigen-specific receptors on their surface – BCR (B Cell Receptor) and TCR, respectively. Specifically, the latter allows T cells to recognize target MHC-associated peptides among the extensive landscape of pMHC (estimated around 100.000) presented by an APC.[8] The collection of BCRs and TCRs expressed in one individual is defined as adaptive immune receptor repertoire (AIRR). Characterization of the AIRR would be useful for the enhancement of immunotherapies and for the development of more effective and target-specific CAR-T cells, as well as gaining further knowledge on CTL's activity in tumor suppression. Hence, shedding light on the sophisticated TCR-pMHC interactions network currently stands out as one of the primary goals in cancer immunotherapy and related fields. Development of strategies that are able to systematically disclose and characterize such interactions – in unbiased and high-throughput fashion – is necessary. Although evolution of effective T cell antigen discovery approaches is hindered by several factors – (i) low affinity of pMHC-TCR interactions, (ii) MHC's polymorphism and TCR's diversity and (iii) the large number of possible peptides deriving from antigen processing – numerous strategies have been developed in the past years. Their advantages and limitations will be reviewed in the next paragraphs, focusing on $CD8^+$ T cells ligand discovery in the context of their antitumoral activity.[9]

2.1 Available methods

Currently available technologies will be here divided into two main categories, namely pMHC-driven and TCR-driven approaches. The first one includes methods that use known peptide sequences to identify responsive TCRs. Approaches belonging to the second category, instead, involve the use of known TCRs to identify cognate peptides in the context of antigenic libraries.

2.1.1 pMHC-driven approaches

Traditionally, antigen discovery has been performed by **functional assays** that involved co-culture of T cells with peptide-loaded APCs, followed by measurement of surface markers expression, lymphocytes proliferation, cytokines production and cytolytic activity. Therefore, activated T cells – expressing cognate

TCRs – could be identified. Such techniques, however, present important limitations: they are expensive and low throughput, thus requiring high numbers of T cells to obtain consistent data. Indeed, they are now considered obsolete, and they have been replaced by more efficient approaches. Substantial advancements in ligand discovery have been allowed by development of soluble **pMHC multimers**. Reported for the first time by Altman et al., pMHC tetramers – originated by multimerization of four pMHC-I complexes bound together via a fluorophore-labeled streptavidin molecule – are capable of interacting with more than one TCR on a specific T cell, therefore increasing avidity and binding stability. Hence, T cells expressing cognate TCR, stained by pMHC tetramers, are detectable through flow cytometry. An alternative is represented by the use of heavy metal ions to tag pMHC multimers and subsequent identification of antigen-specific T cells via time-of-flight mass cytometry (CyTOF). However, while using combinations of multiple pMHC multimers allows detection of a larger number of T cell specificities[10,11], the limited amount of fluorophore/heavy metal ion tags available limits the total number of epitopes that can be simultaneously screened (up to 109). In 2016, Bentzen et al.[12] introduced labeling of MHC multimers with DNA barcodes, which allows screening of >1000 peptides specificities in one single sample. This technique has been furtherly enhanced by Zhang et al., using **TetTCR-seq** (tetramer-associated T cell receptor sequencing)[13]; in this case, DNA-barcoded antigen tetramers were generated through in vitro transcription and translation (IVTT), allowing high throughput determination of TCRα and TCRβ sequences by next generation sequencing (NGS) after single-cell FACS-sorting, thus resulting in the detection of antigen-specific TILs even in small clinical samples.[8,9,14,15] Another way to employ MHC multimers is represented by the generation of **MHC microarrays**. In the first studies, antigen-specific $CD8^+$ T-cells were identified using polyacrylamide-coated microscope slides, which allowed parallel screening of multiple T-cell specificities. Thorough the years, the technique has been improved in terms of complexity (up to 30-40 T-cells specificities) and sensitivity. pMHC molecules on microarrays can be co-spotted with cytokine capture antibodies and co-stimulatory molecules to detect functional activation of T-cells. This kind of interaction, however, does not ensure activation of all specific $CD8^+$ cells.

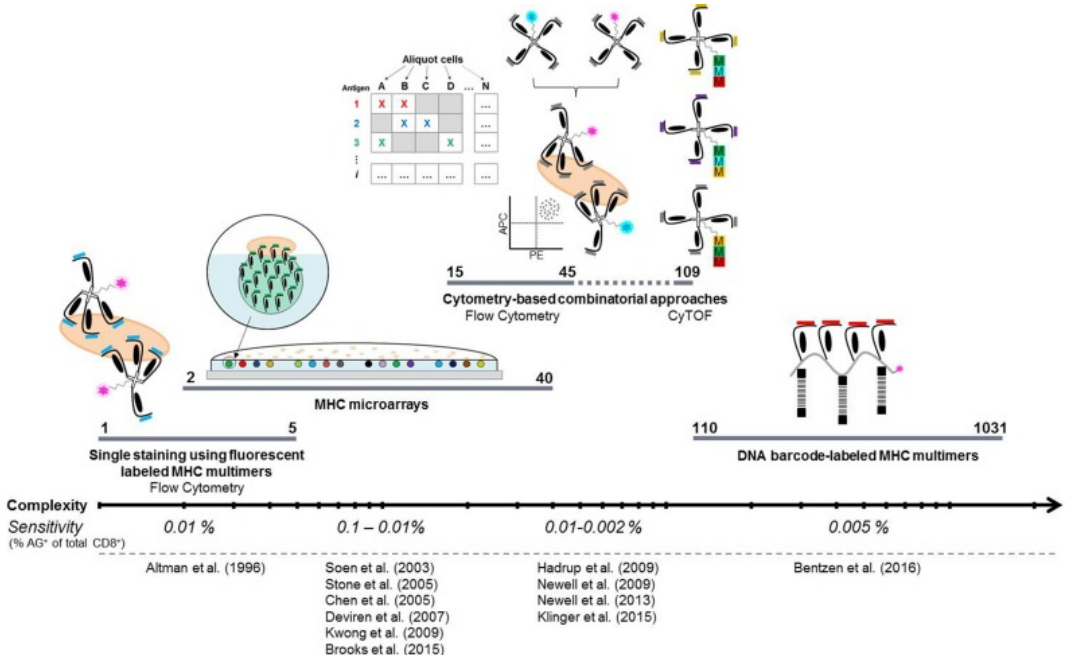


Figure 5 – pMHC multimers technology enhancements through the years. *Bentzen, A.K., Hadrup, S.R. Evolution of MHC-based technologies used for detection of antigen-responsive T cells.* Cancer Immunol Immunother ***66**, 657–666 (2017). https://doi.org/10.1007/s00262-017-1971-5*

In the past years, new opportunities have become possible thanks to the development of microfluidic devices. Microfluidic antigen-TCR engagement sequencing (**MATE-seq**), for example, enabled screening of DNA-barcoded pMHC-presenting nanoparticles (pNP) against CD8+ T-cells populations derived from donor's PBMCs.[16]

2.1.2 TCR-driven approaches

In order to identify cognate ligands, T cell clones can be screened against peptide libraries. Historically, peptide display on Escherichia coli's surface has been largely employed to express genetic libraries. However, being the peptide-MHC complex a multichain eukaryotic protein, E. coli does not represent a suitable platform for displaying antigenic libraries. Therefore, other strategies have been developed in order to display eukaryotic protein libraries – such as yeast- and baculovirus-based systems. **Yeast display** of pMHC complexes has been impaired by their low efficiency in terms of stability. Association of three polypeptides – heavy chain, β2-microglobulin and peptide – is required for their correct expression on a cell's surface. A strategy developed by Kranz et al., consisting in the expression of pMHC-I molecules in a single-chain form and fused

to Aga-2 protein – an α-agglutinin component linked to Aga1p, which is anchored to the cell-wall – permits their stable expression on *Saccaromices cerevisiae*'s surface. In the same study, it was proven that displayed complexes were able to bind TCRs and antibodies that were specific for a determined pMHC-I, thus allowing identification of yeast cells expressing the specific TCR target.[17] Developments of this technique have been used to define TCRs' cross-reactivity, specificities and mimotopes. For instance, Gee et al. applied an yeast display approach in order to identify reactive TCRs in TILs isolated from patients with colorectal adenocarcinoma.[18] **Baculoviral display** represent a similar approach. The advantage of using baculoviruses is their flexibility – proteins can be expressed either on the viral particle or on infected insect cells – and the fact that they are able to accept large constructs. The peptide is linked to the N-terminus of B2-microblobulin domain of the MHC-I molecule which, in turn, is attached to the gp64 baculoviral protein. Construction of these systems has been reported by Crawford et al. and Wang et al. in 2004 and 2005, respectively. Libraries can be obtained by homologous recombination or by direct cloning of PCR fragments in baculoviral DNA. Whilst the former leads to creation of 10^5 peptides libraries, the latter allows generation of much larger libraries (up to 10^8 peptides). Similarly to yeast display approches, soluble TCRs interact with cognate pMHC complexes and cells can be sorted via fluorescent-activated cell sorting (FACS).[19,20] Described techniques have the advantage of allowing screening of billions of pMHC complexes; unfortunately, soluble TCRs' generation process is non-robust and stands out as one of their main obstacles.[9] Furthermore, just as before-mentioned MHC multimers approaches, they rely solely on physical affinity between a TCR and the cognate pMHC; thus, they are not representative of physiological and productive interplays between T-cells and APCs.

To overcome this limitation, some research groups have focused their activity on developing cell-based technologies capable of recognizing interactions that induce T cell's activation. Joglekar et al., in 2019, proposed a T-cell antigen discovery strategy based on Signaling and Antigen-presenting Bifunctional Receptors (**SABRs**). These chimeric receptors comprehend (i) an extracellular domain represented by an HLA-A2 protein in the form of a single chain trimer (SCT) – namely a peptide, a β2-microglobulin domain and an heavy chain covalently linked together – connected to (ii) a CD3ζ intracellular domain and (iii)

a CD28 co-stimulatory domain. The construct was transduced into NFAT-GFP-Jurkat cells, which conditionally express GFP (green fluorescent protein) upon NFAT binding, as a result of productive interaction between the extracellular pMHC portion and another cell expressing its cognate TCR. This permits identification of interacting APCs expressing the cognate antigen, which are sorted via fluorescence activated cell sorting (FACS); their DNA is extracted and the epitope-encoding region is amplified and sequenced. Using this method, it is currently possible to screen a TCR against libraries of 10^6 peptides. An advantage of this method is that complicated and non-robust production of soluble TCRs – as well as synthesis of peptides and MHC molecules – is eschewd.[20] Another approach exploits the physiological phenomenon of **trogocytosis**, which consists in membrane-proteins transfer between two interacting cells. Specifically, when TCR-expressing Jurkat cells efficiently interact with SCT-expressing K562 cells, the TCR is transferred from Jurkat to K562 cells, marking them for isolation via FACS. The cognate epitope is then identified by next-generation sequencing (NGS). Sharma et al. developed a Foster resonance energy transfer (FRET)-based method to detect target cells. In this case, the antigen libraries have a size of about 10^6 peptides and are transduced into the cells in the form of minigenes.[22] **T-Scan**, developed by Kula et al., is a high-throughput method for systematic identification of cognate antigens. Lentiviral transduction allows target cells to express libraries of candidate antigens, which are endogenously processed and presented on MHC molecules. These cells are then co-cultured with $CD8^+$ T-cells, resulting in cytotoxic granules secretion from CTLs into cells expressing their cognate antigen. Target cells also express a reporter that becomes fluorescent after cleavage by granzyme B, allowing detection of cells that productively interacted with the TCR of interest. Cognate-antigen expressing cells are sorted via FACS and the epitope sequence is retrieved. Using this method, two novel CMV-derived antigens were identified. T-Scan allows TCR specificity determination of 10-100 T-cells simultaneously, since it can be multiplexed. Also, its unbiased nature permits detection of low-affinity interactions – which are reported to be essential in CTLs' tumor-suppressing activity[23] and would be missed by other approaches, e.g. MHC multimers. Finally, endogenous processing allows to consistently increase the range of physiologically-relevant peptides that are screened – including pre- and post-translational splicing products.[24]

2.1.3 Limitations of currently available approaches

Although great improvements have been made in the development of antigen discovery methods, some limitations are still present and need to be addressed in order to re-direct this research field in the upcoming years. The previously described technologies are limited to (i) analysis of small numbers of TCRs at time, (ii) use of primary human T cells is still poor and (iii) $CD4^+$ T cells antigen discovery has not been extensively studied, yet. In addition, none of these techniques allow simultaneous retrieval of interacting T-cell and APC.

3. Purpose of the project

In order broaden the spectrum of antigens that can be exploited for therapeutic ends, limitations presented by currently available technologies need to be overcome. Therefore, this project aims at the development of a novel approach for the discovery of T cells ligands, focusing on interactions between CD8+ T cells' receptors and MHC-I molecules. Theoretically, this platform will allow the simultaneous retrieval of the T cell and the APC presenting its cognate antigen, in the form of cell-doublets. Doublets sorting via flow cytometry will allow sequencing of both the TCR and the epitope at a single-cell level. Given its unbiased nature, this method could provide a tool for high-throughput screenings of peptide panels against immunoreceptors libraries, even without previous knowledge of one of the two counterparts.

3.1 Experimental Design

Interactions between TCRs and pMHC complexes are mediated mostly by H-bonds and Van der Waals forces. Their weak nature poses a problem for the retrieval of both the APC and the T cell while they are interacting. This approach aims at exploiting the high-affinity interaction between an antibody and its epitope – specifically, the binding of CD69 (expressed on the activated T cell's surface) to an anti-CD69 membrane-bound antibody (expressed on the cognate-antigen presenting cell's surface). To ensure that this method can discriminate between specific and non-specific interactions, a double-regulated circuit was designed, in which (i) the CD69 molecule is expressed on the T cell only after the recognition of the specific pMHC and (ii) the anti-CD69 antibody is conditionally displayed on the APC's surface only after the pMHC has interacted with its cognate TCR. While the former naturally occurs in physiological conditions, the latter needs to be implemented by genetically engineering the APC. Since MHC molecules do not comprehend, nor are associated with, a signal-transduction domain (unlike TCRs), two possible approaches have been taken into consideration:

1. Synthetic Notch receptors, developed for the first time by Morsut et al. [25], are constructs that mimic the activity of the endogenous Notch receptors. The endogenous Notch receptor comprehends an extracellular domain which, upon specific binding of the cognate ligand, induces proteolytical cleavage of the intracellular domain. Since the latter is represented by a

transcriptional activator, its translocation to the cell's nucleus initiates the expression of determinate genes. Synthetic Notch receptors are generated by maintaining the original notch-core (transmembrane region) and substituting the extracellular or the intracellular domains – or both. This approach allows scientists to engineer cell lines that can translate extracellular stimuli in intracellular signaling cascades that lead to conditional expression of user-defined genes. This approach could be useful to induce the expression of a surface-displayed anti-CD69 antibody upon specific interaction between the MHC-I molecule and its cognate TCR, making it possible to create a double-regulated circuit.

2. SABR constructs are composed of an SCT molecule as extracellular domain and a Z-domain of the CD3 complex as intracellular domain. The ζ-domain induces an intracellular activation cascade upon interaction of the SCT with its cognate TCR, ultimately resulting in the expression of NFAT. In the original setting, NFAT then binds to a reporter that presents multiple NFAT binding sites, allowing transcription of downstream encoded genes – namely, a GFP coding sequence.[21]

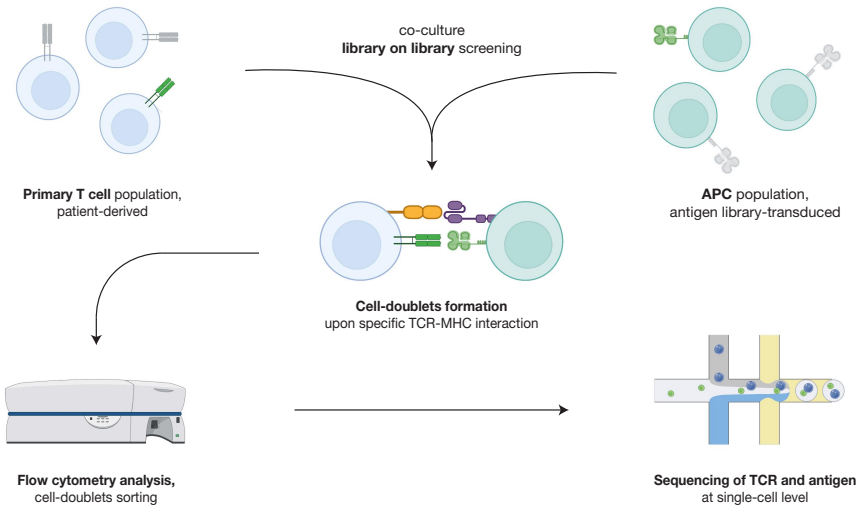


Figure 6 – Graphical overview of the proposed project's flow.

Libraries of peptides can be transduced in APCs, which are then co-coltured with $CD8^+$ T cells and, in case of antigen recognition, cell-doublets formation can be observed via flow cytometry. Doublets can be sorted and analyzed by single-cell sequencing to retrieve both the TCR's and the antigen's sequences from the two

interacting partners. Since T cells don't need to be genetically modified, this method can be used on directly on primary human T cells populations. Validation of the method could be carried out using primary $CD8^+$ TILs isolated from colon-rectal cancer human biopsies.

4. Materials and methods

4.1 Plasmids design and cloning

Plasmid maps, cloning strategies and PCR primers were designed using SeqBuilder Pro software (version 17.2.1). Original plasmids were purchased from Addgene. Cloning experiments were performed using different strategies:

a) Restriction-enzymes based cloning: backbone plasmid and insert plasmid were digested with the same restriction enzyme(s) to generate complementary sticky-ends. Digestions were carried out overnight by mixing 15 ug DNA, 1 µL of each restriction enzyme (NEB, Thermo Scientific) and the suitable at optimal temperature. Backbones were dephosphorylated using Calf Intestinal Alkaline Phosphatase (CIP) for 10' at 37 °C (followed by 2' at 80 °C for stopping the reaction) in order to increase cloning efficiency. Then, digested DNA was run on 1% agarose gel for quality control and selection of the desired band, which was extracted using ProMega Wizard® Genomic DNA Purification Kit, according to manufacturer's protocol. Extracted DNA was checked at NanoDrop for yield and purity determination. Ligation reaction was carried out overnight at 16 °C by mixing the digested backbone DNA and the digested insert DNA in a molar ratio of 1:3, in presence of T4 DNA ligase and T4 ligase buffer. Negative control reactions lacked the insert DNA. The day after, 6 µL of each ligation reaction were added to 50 uL of chemically competent E. coli (OneShot Stbl3, Invitrogen), which were thermically shocked and plated on dishes containing 12 mL LB (Luria Bertani) Agar additioned with ampicillin. Dishes were kept in incubator at 37 °C overnight. Colonies growth was checked the day after. They were picked and grown in 6 mL LB Broth (additioned with ampicillin) overnight at 37 °C in incubator with agitation for Miniprep extraction. DNA extracted via Miniprep (QIAprep® Spin Miniprep Kit) was digested with specific restriction enzymes that allowed to discriminate plasmids in which the insert was correctly cloned and those in which ligation wasn't successful. In some cases, Miniprep screening was performed vis Sanger sequencing. If the insert was present, the corresponding colony was expanded for Midiprep of Maxiprep DNA extraction (Qiagen Plasmid Midi/Maxi Kit). The resulting plasmidic DNA was stored at -20 °C for further use.

b) PCR cloning: PCR forward and revers primers were designed, flanking the sequence on template DNA that needed to be cloned into the new construct. Primers comprehended the same restriction sites present in the backbone. PCR products were run on 1% agarose gel to check for correct amplification. Amplicons were extracted from the gel using ProMega Wizard® Genomic DNA Purification Kit, according to manufacturer's protocol and were digested in parallel with the backbone, using the same restriction enzymes to generate complementary sticky-ends. Digestions were run on 1% agarose gel for quality control. Ligation reactions and further steps were performed as described in Paragraph 4.1.a.

c) Oligo-annealed cloning: forward and reverse synthetic single-stranded oligonucleotides were designed to include a complementary central region flanked by a 5' overhang (on the forward primer) and by a 3' overhang (on the reverse primer). Oligo annealing was performed by placing mixed oligos in thermocycler at 95 °C for 2', then gradually decreasing temperature to 25 °C over 45'. Products of the reaction, represented by double-strand DNA oligonucleotides with sticky-ends, were mixed with the desired backbone presenting complementary sticky-ends for ligation at RT for 1 hour. Transformation and following steps were performed as described in Paragraph 4.1.a.

4.2 Cell culture

293T cells were cultured in DMEM supplemented with 10% FBS, 1x PenStrep and 1x GlutaMax. Jurkat, 2D3 and K562 cells were cultured in RPMI 1640 supplemented with 10% FBS, 1x PenStrep and 1x GlutaMax. Cells were cultivated at 37°Cin a humidified atmosphere containing 5% CO_2.

4.3 Retroviral production

$1x10^6$ HEK-293T cells were resuspended in 10 mL of DMEM and plated in dishes pre-coated with 10 mL of poly-L-lysine solution (8 mL 0.1% Gelatin / 2 mL 0.01% poly-L-lysine). The day after, cells were transfected with 25 μg of lentiviral transfer vector, 15 μg of pD8.1 and 1 μg of pVSV-G, using Invitrogen's Calcium Phosphate Transfection Kit, according to manufacturer's protocol. After 12-14 hours, the medium was changed by removing all DMEM and adding 4 mL of RPMI 1640 to

the cells. Lentiviral supernatants were collected 30 hours after medium-change, filtered through 0.40 μm PES filters and used directly on cells for transduction or stored at -80 °C for further use.

4.4 Lentiviral production

$1x10^6$ HEK-293T cells were resuspended in 10 mL of DMEM and plated in dishes pre-coated with a poly-L-lysine solution (8 mL 0.1% Gelatin / 2 mL 0.01% poly-L-lysine). The day after, cells were transfected with 25 μg of lentiviral transfer vector, 15 μg of pD8.1 and 4 μg of pVSV-G, using Invitrogen's Calcium Phosphate Transfection Kit, according to manufacturer's protocol. After 12-14 hours, the medium was changed by removing all DMEM and adding 4 mL of RPMI 1640 to the cells. Lentiviral supernatants were collected 30 hours after medium-change, filtered through 0.40 μm PES filters and used directly on cells for transduction or stored at -80 °C for further use.

4.5 Cell transduction

Retroviral/lentiviral supernatants were used to culture $1x10^6$ target cells in 6-wells plates, in presence of 5 μg/mL Polybrene. Cells were then centrifuged (spinfection) for 90' at 2500 rpm. 2 mL of RPMI 1640 were added the day after. Transduced cells were checked at flow cytometer after 4 days.

4.6 Functional assays

4.6.1 SCT functionality test

70-80% confluent cells were stained with Trypan Blue Solution, counted at LUNA-II automatic cell counter and the desired amount was harvested and centrifuged. Pellets were resuspended in RPMI 1640 to reach a concentration of $1x10^5$ cells/100 μL. Each co-culture condition was performed by mixing 10^5 Jurkat cells with 10^5 K562/JY cells in a total volume of 200 μL RPMI 1640 per well. Single-culture conditions were performed by adding 10^5 Jurkat cells/well in 200 μL of RPMI 1640. Dynabeads were added at a ratio of 1:3 (T cells:Dynabeads) by mixing Jurkat cells a concentration of $1x10^5$ cells/100 μL RPMI 1640 with Dynabeads at a concentration of $3x10^5$ cells/100 μL RPMI. PMA/ionomycin treatment was performed at 18-hour time-point by seeding $2x10^5$ Jurkat cells per well in 200 μL RPMI 1640 in presence of 0.05 ng/mL phorbol myristate acetate (PMA) and 1

μg/mL ionomycin for 3 hours; then, they were pelleted and resuspended in 200 μL of RPMI 1640. All conditions, excluding the PMA/i treated cells, were added to separate wells of a 96-well U-bottom plate and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. Cells were then collected in FACS tubes and stained with anti-CD69-APC (Biolegend) and anti-HLA-A2-APC-Cy7 (Biolegend) for analysis at flow cytometer.

4.6.2 T cells' activation time-course

70-80% confluent cells were stained with Trypan Blue Solution, counted at LUNA-II automatic cell counter and the desired amount was harvested and centrifuged. Pellets were resuspended in RPMI 1640 to reach a concentration of $1.30x10^5$ Jurkat cells/100 μL and $1x10^5$ K562 cells/100 μL. In every co-culture condition, $1.30x10^5$ Jurkat cells and $1x10^5$ K562 cells were mixed and added to a single well in a total volume of 200 μL RPMI 1640. Single-culture conditions were prepared by seeding $1x10^5$ Jurkat cells per well in a total volume of 200 μL of RPMI 1640. PMA/ionomycin treatment was performed by seeding $1.3x10^5$ Jurkat cells per well in 200 μL RPMI 1640 in presence of 0.05 ng/mL PMA and 1 μg/mL ionomycin for 3 hours; after 3 hours, they were pelleted and resuspended in 200 μL of RPMI 1640. All conditions were added to separate wells in 96-well U-bottom plates and incubated at 37 °C in a 5% CO_2 incubator for a total 72-hours time-span. Cells were collected at multiple time-points (0.5, 1, 2, 3, 4, 5, 6, 8, 24, 30, 48 and 72 hours) in FACS tubes and stained with anti-CD69-APC (Biolegend) and anti-HLA-A2-APC-Cy7 (Biolegend) for analysis at flow cytometer.

4.6.3 Anti-CD69 scFv functional test

70-80% confluent K562 cells were stained with Trypan Blue Solution, counted with LUNA-II™ Automated Cell Counter and the desired amount was harvested and centrifuged. Pellets were resuspended in RPMI 1640 containing 2.5 μg/mL of CD69-HisTag soluble protein (Acro Biosystems #AAH07037), then added to a 96-well U-bottom plate at a concentration of $1x10^5$ cells/well. The plate was incubated at 37 °C in a 5% CO2 incubator for 2 hours, then cells were collected, washed two times with PBS and stained with anti-CD69-AlexaFluor647 antibody (Biolegend) for flow cytometry analysis.

4.6.4 Anti-CD69 scFv binding affinity determination

70-80% confluent K562 cells were stained with Trypan Blue Solution, counted with LUNA-II™ Automated Cell Counter and the desired amount was harvested and centrifuged. Pellets were resuspended in RPMI 1640 containing increasing concentrations of CD69-HisTag soluble protein (Acro Biosystems #AAH07037). The following protein concentrations were tested: 0, 0.125, 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 125 ug/mL. Conditions were added to a 96-well U-bottom plate at a concentration of $1x10^5$ cells/well. The plate was incubated at 37 °C in a 5% CO2 incubator for 2 hours, then cells were collected, washed two times with PBS and stained with anti-CD69-AlexaFluor647 antibody (Biolegend) for flow cytometry analysis.

4.6.5 SABR construct validation test

70-80% confluent cells were stained with Trypan Blue Solution, counted with LUNA-II™ Automated Cell Counter and the desired amount was harvested and centrifuged. Pellets were resuspended in RPMI 1640 at a final concentration of $1x10^5$ cells/100 mL. Co-culture conditions were prepared by mixing $1x10^5$ Jurkat cells with $1x10^5$ K562 cells or $SABR^+$ Jurkat cells and added to a 96-well plate in a final volume of 200 µL of RPMI 1640 per well. Single-culture conditions contained $1x10^5$ cells per well in 200 µL of RPMI 1640. PMA/ionomycin treatment was performed at by seeding 2x105 Jurkat cells per well in 200 µL RPMI 1640 in presence of 0.05 ng/mL PMA and 1 µg/mL ionomycin for 3 hours; after 3 hours, they were pelleted and resuspended in 200 µL of RPMI 1640. All conditions were added to separate wells in 96-well U-bottom plates and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. Cells were collected in FACS tubes and stained with anti-CD69-APC (Biolegend) and anti-HLA-A2-APC-Cy7 (Biolegend) for analysis at flow cytometer.

4.6.6 NFAT-GFP-antiCD69 reporter functionality test

70-80% confluent cells were stained with Trypan Blue Solution, counted with LUNA-II™ Automated Cell Counter and the desired amount was harvested and centrifuged. Pellets were resuspended in RPMI 1640 at a final concentration of $3.5x10^5$ cells/100 mL. Co-culture conditions were prepared by mixing $3.5x10^5$ TCR-transduced Jurkat cells with $3.5x10^5$ or $SABR^+$ Jurkat cells and added to a 96-well

plate in a final volume of 200 µL of RPMI 1640 per well. Single-culture conditions contained 3.5x10^5 cells per well in 200 µL of RPMI 1640. PMA/ionomycin treatment was performed at by seeding 3.5x10^5 Jurkat cells per well in 200 uL RPMI 1640 in presence of 0.05 ng/mL PMA and 1 µg/mL ionomycin for 3 hours; after 3 hours, they were pelleted and resuspended in 200 µL of RPMI 1640 containing CD69-HisTag soluble protein at a concentration of 50 µg/mL. All conditions were added to separate wells in 96-well U-bottom plates and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. Cells were collected in FACS tubes and analyzed at flow cytometer.

4.7 Flow cytometry analysis

Flow cytometry analysis were performed using LSR II cytometer, after staining the cells with fluorophore-conjugated anti-human antibodies. Collected cells were centrifuged for 3' at 2500 g, pellet was incubated with 1:200 antibody dilution in PBS at 4 °C for 15'. Then, cells were washed by centrifuging them for 3' at 2500 g and pellets were resuspended in 200 µL of PBS. Antibodies used for staining cells in the described experiments are the following:

Antigen	**Species**	**Fluorochrome**	**Manufacturer**
Single chain trimer (HLA-A*0201)	Human	APC-Cy7	Biolegend
HisTag	/	Alexa Fluor 647	Biolegend
CD69	Human	APC	Biolegend

Data collection was powered by FACSDiva software and subsequent plot analysis was performed using FlowJo 10.7.1 software.

4.8 Statistical analysis and data representation

Statistical analysis and relative graphs were realized using GraphPad Prism (v. 9.6.1). Data representation and figures were designed with BioRender and Adobe Illustrator 2023.

5. Results

5.1 Single chain trimer molecules trigger T cells' activation in vitro

Both synNotch and SABR strategies rely on the expression of pMHC complexes in the form of single chain trimers (SCTs). The correct expression and functionality of these molecules is essential for the efficient antigen-specific activation of T cells. Thus, the first step in the platform's set-up process was to test if SCT-expressing cells were able to interact with cognate T cells and trigger their activation. The HLA-A*02 SCT sequence was retrieved from Joglekar et al.'s construct (*pCCLc-MND_A0201_SABR_Backbone*, Addgene #119050) and cloned into a new plasmid. A WPRE (Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element) sequence was added downstream in order to improve the expression (Figure 7a), while a BsmBI restriction site allowed cloning of the desired antigen in the molecule. Three different SCT constructs were generated, each one containing a different antigen sequence (as reported in Table 1). Specifically:

- NY-ESO-1: epitope analogue of New York esophageal squamous cell carcinoma-1 (cancer testis);
- HPV16: epitope derived from the Human Papillomavirus type 16;
- MLANA: a fragment of the MART-1 (melanoma antigen recognized by T cells) protein that is found on the surface of malignant melanoma cells.

For all of them, the cognate TCRs' alpha and beta chains are known and reported in Table 1. The lentiviral vectors were then transduced into K562 cells, which lack an endogenous MHC-I molecule. Transduction efficiency was checked via flow cytometer analysis after staining with an anti-HLA-A2 antibody, as shown in Figure 7c.

Antigen	Antigen sequence	TCR
NY-ESO-1	SLLMWITQC	1G4
HPV16	TIHDIILECV	E6
MLANA	ELAGIGILTV	F5

Table 1 – List of antigens cloned into the SCT construct for validation experiments, their aminoacidic sequences and their cognate TCRs.

On the other hand, alpha and beta chains of the 1G4, E6 and F5 TCRs were cloned into three different retroviral constructs containing the dTomato sequence as constitutive reporter (Figure 8a). Vectors were then transduced into Jurkat cells (endogenous-TCR^{+}) and 2D3 cells (endogenous-TCR^{-}, CD8^{+} and NFAT-GFP^{+}). The exogenous TCR expression was checked via flow cytometry looking for dTomato fluorescence, as shown in Figure 8c.1 and 8c.2. Jurkat cells were also transduced with a vector containing eGFP as expression marker (Figure 8c.3).

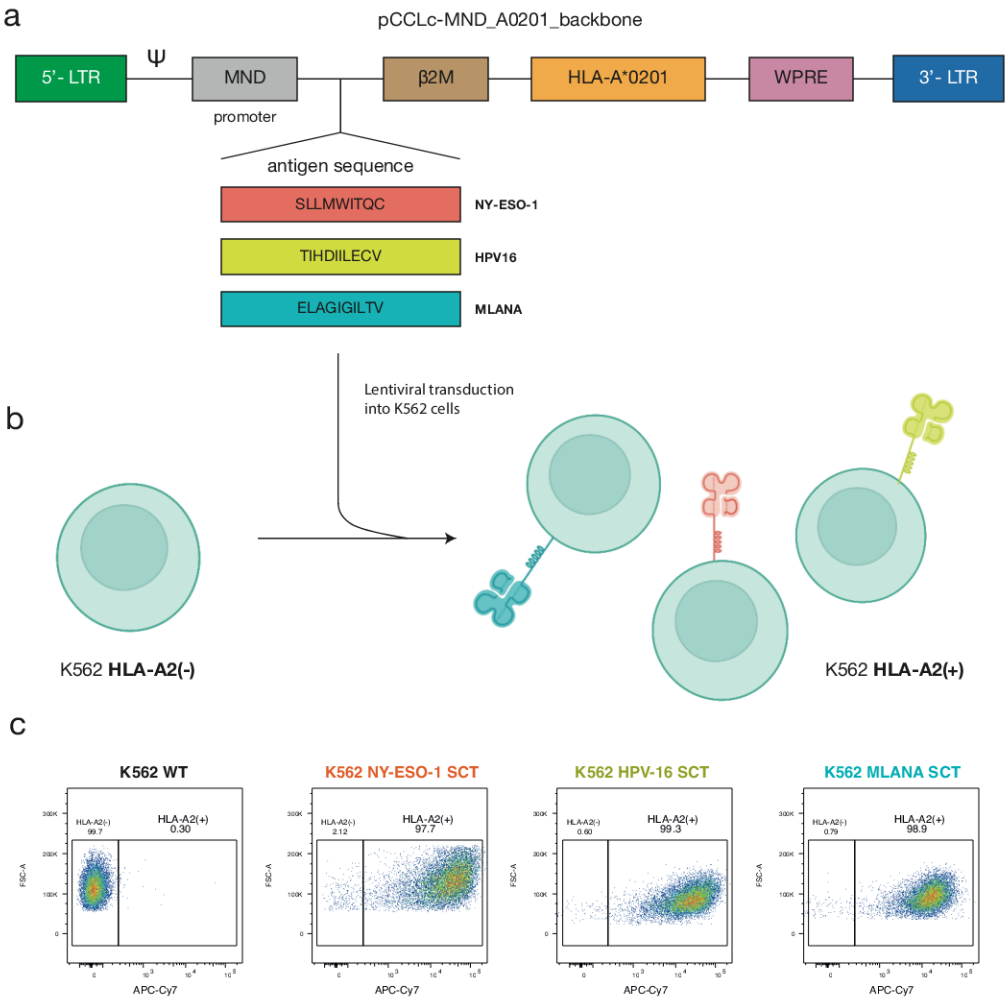


Figure 7 – **Generation of SCT^{+} K562 cell lines**. a) Map of lentiviral constructs used for the expression of SCT molecules on K562 cells with aminoacidic sequences of NY-ESO-1, HPV-16 and MLANA antigens. b) Schematic representation of K562 cells before and after transduction. c) Flow cytometry plots showing percentage of SCT-transduced cells, stained with anti-HLA-A2 APC-Cy7 antibody.

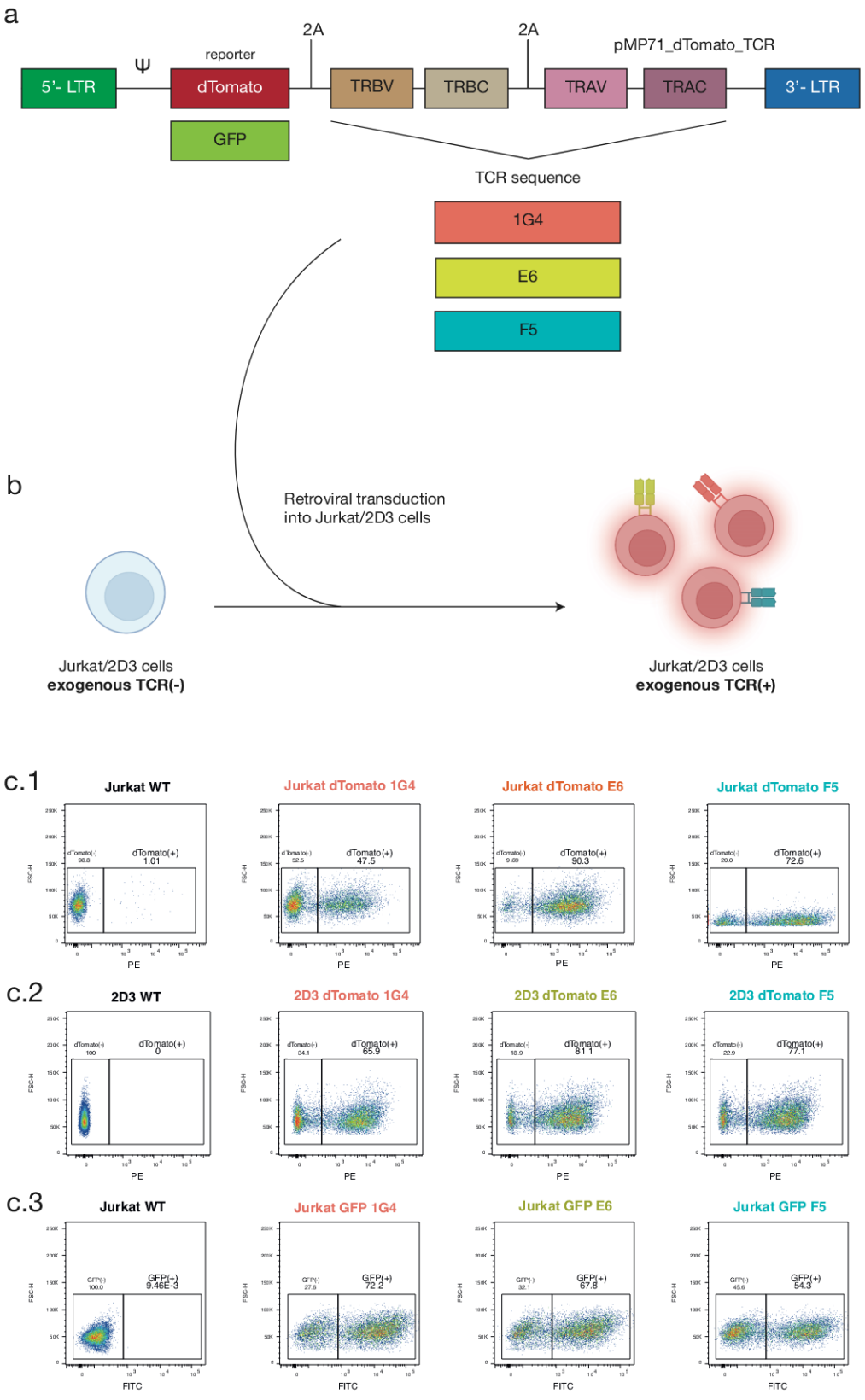


Figure 8 – **Generation of TCR-transduced T cell lines.** a) Map of retroviral constructs used for the transduction of Jurkat and 2D3 cell lines with 1G4, E6 and F5 TCRs. b) Schematic representation of Jurkat cells before and after transduction. c) Flow cytometry plots showing percentage of transduced cells: c.1) Jurkat cells expressing exogenous TCR with dTomato reporter; c.2) 2D3 cells

expressing exogenous TCR with dTomato reporter; c.3) Jurkat cells expressing exogenous TCR with GFP reporter.

Finally, the functionality of TCR-transduced Jurkat cells and SCT^+ K562 cells was assessed by performing a co-culture assay. As positive control of T cells' activation, JY cells have been used. Since they endogenously express the HLA-A*0201 molecule, the desired soluble peptide can be loaded onto the HLA protein by directly adding it to the culture medium. The subsequent formation of a pMHC complex on cells' surface allows JY to activate T cells expressing the cognate TCR. Other positive controls included the use of Dynabeads, which activate T cells in a TCR-dependent manner by presenting antibodies that bind to CD3 and CD28. In addition, TCR-independent activation of T cells was checked by treating them with PMA/ionomycin.

The following conditions were tested:

a) TCR-transduced T cells + cognate SCT^+ K562 cells;
b) TCR-transduced T cells + JY cells treated with cognate peptide (positive control);
c) Wild-type T cells + SCT^+ K562 cells (negative control);
d) TCR-transduced T cells alone (negative control);
e) Wild-type T cells alone (negative control);
f) TCR-transduced cells alone (negative control);
g) T cells treated with Dynabeads 1:3 (positive control);
h) T cells treated with PMA/ionomycin (positive control).

For every condition, both Jurkat and 2D3 engineered cell lines were tested. After 6 hours of incubation, they were analyzed via flow cytometry looking for CD69 expression (APC). As reported in Figure 8, T cells that interacted with their cognate SCT became $CD69^+$, in some cases even more than those co-cultured with peptide-treated JY cells. While CD69 signal was detected in positive controls (Dynabeads and PMA/i), it was absent in wild-type Jurkat and 2D3 cells, since they lack expression of the cognate TCR.

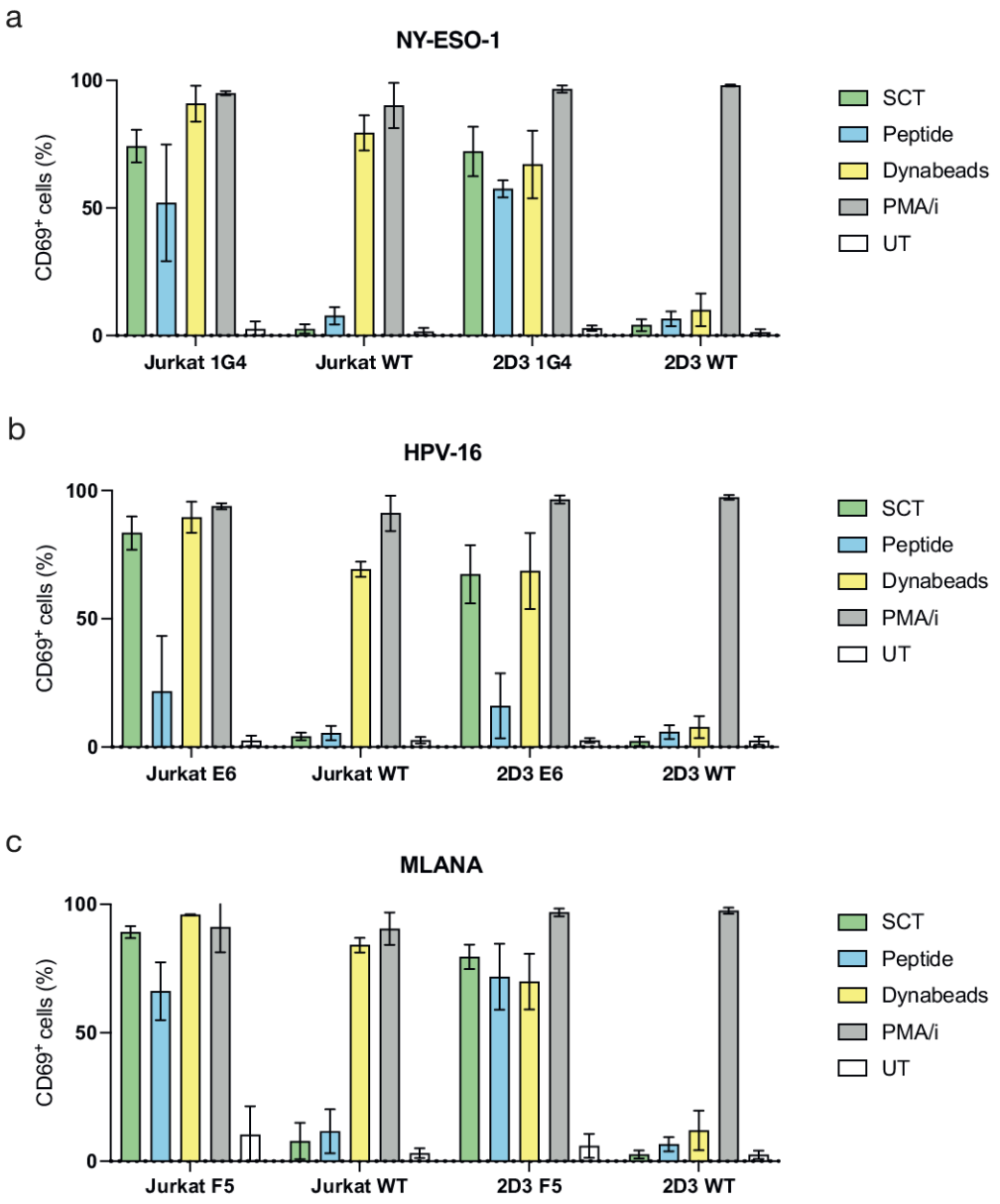
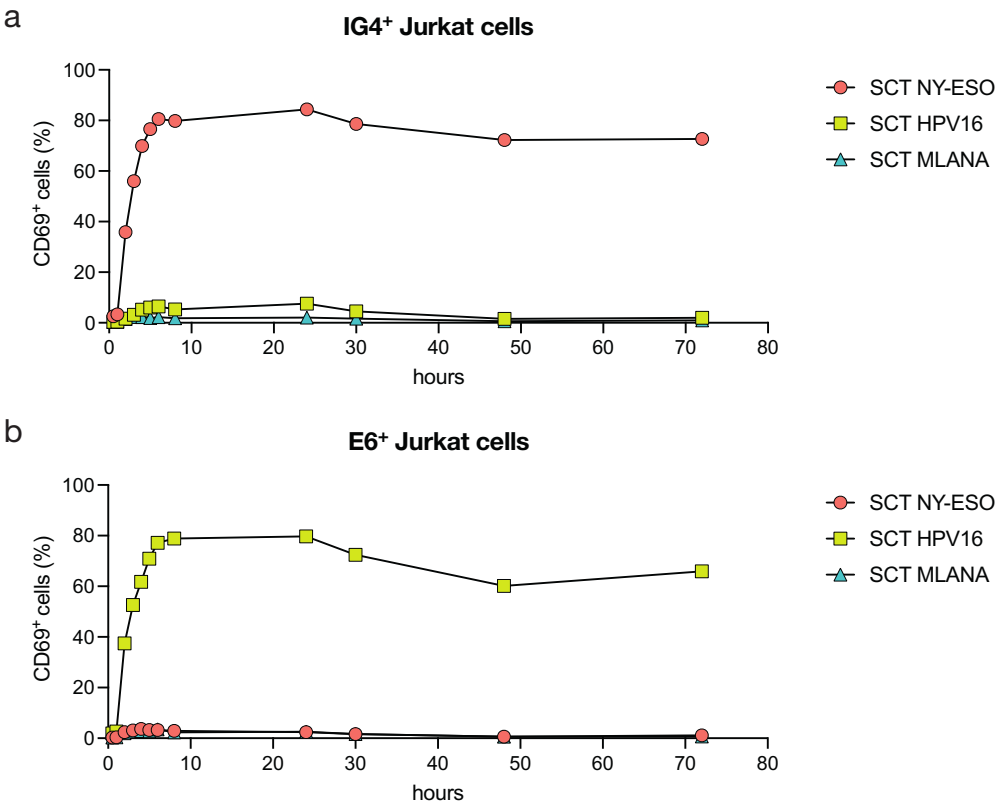


Figure 9 – Results of co-culture functional assay between SCT⁺ K562 and TCR-transduced Jurkat cells. Graphs show percentage of CD69⁺ of transduced and wild-type Jurkat and 2D3 cell lines after co-culture with cognate SCT (green), MHC-bound soluble peptide (light blue), activating Dynabeads (yellow), PMA/i treatment (grey) and in single-culture (white). Antigens tested: a) NY-ESO-1, b) HPV-16 and c) MLANA. Data shown as mean ± s.d. of n = 3 technical replicates.

Overall, these results show that the SCT construct is capable of efficiently activating both Jurkat and 2D3 TCR-transduced cells and, thus, shall be used in the following steps of the project.

5.2 Time-course of T cells' in-vitro activation

Since this approach relies on CD69's expression by T cells for the formation of cell-doublets, it is essential to assess the timepoint at which the expression of this activation marker is the highest in in-vitro conditions. To do so, TCR-transduced Jurkat cells were co-incubated either with the cognate or the non-cognate SCT-transduced K562 cells. T cells' activation was tested thorough multiple timepoints, covering a total 72 hours time-span, by looking for $CD69^+$ cells via flow cytometry. Results show that CD69 expression starts to significantly increase after 2 hours of co-culture and reaches its peak at 6 hours, which is maintained until the 24-hour time-point. Then, the percentage of $CD69^+$ Jurkat cells slowly but progressively decreases (Figure 10). The activation curve is conserved for all the three TCRs tested (namely 1G4, E6 and F5). Lack of activation in conditions where Jurkat cells were co-coltured with a non-cognate SCT confirms that the construct is able to activate T cells in an antigen-specific manner.



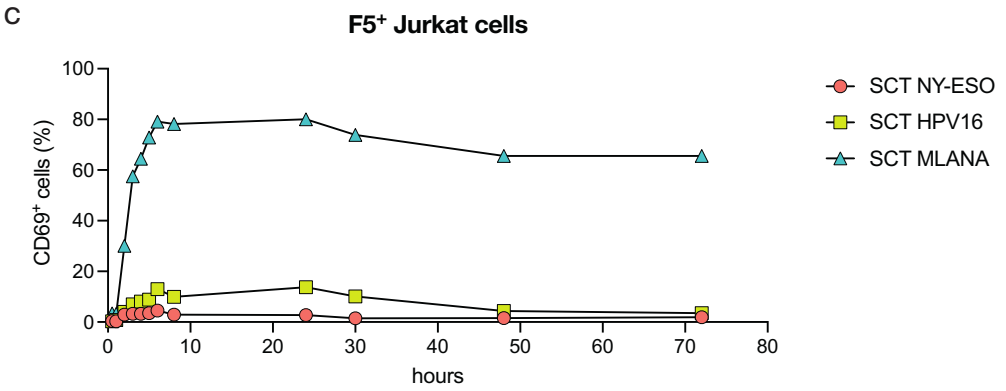


Figure 10 – Activation time-course of Jurkat cells transduced with 1G4 (a), E6 (b) and F5 (c) TCRs (GFP⁺). Activation rate is indicated as percentage of CD69⁺ cells, after staining with anti-CD69-APC antibody for flow cytometry analysis.

5.3 Constitutive expression and test of membrane-bound anti-CD69 scFv molecules on K562 cells

Single chain fragment variable (scFv) are –25 kDa recombinant antibodies containing immunoglobulins' variable light chain and variable heavy chain, connected by a linker region – providing the same antigen-binding capacities as their corresponding IgG antibodies. To achieve membrane-bound expression of anti-CD69 scFVs, their original structure needed to be modified to include:

- a transmembrane domain (TD), which allows the molecule to be anchored to the cell's surface;
- an hinge region (HR), that confers flexibility to the scFv and assured its correct positioning and binding to the epitope.

Schematic structure of the resulting membrane-bound scFv is shown in Figure 10a. Four different constructs were produced, each one expressing the variable light chain and variable heavy chain sequences of retrieved from different anti-CD69 antibody clones, re-adjusted in order to create different combinations, as reported in Table 2 and Figure 10b. In addition, a blue fluorescent protein (BFP) sequence was inserted before the scFv gene, serving as expression marker. The complete structure of the construct can be visualized in Figure 10c.

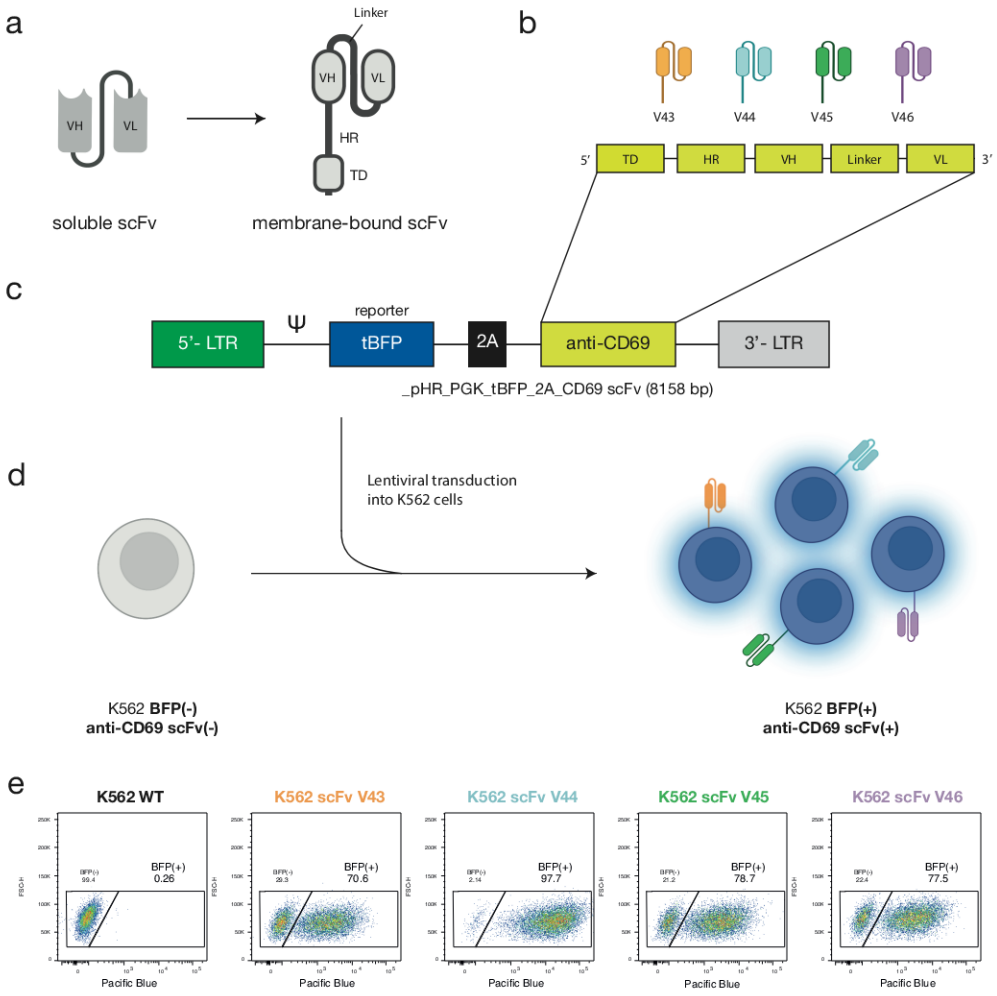


Figure 10 – **Generation of four different K562 cell lines expressing anti-CD69 scFv constructs**. a) Schematic illustrations of a soluble scFv structure (left) and membrane-bound scFv structure including a transmembrane domain (TD), an hinge region (HR), variable heavy (VH) and variably light (VL) sequences connected via a linker. b) Graphical representation of the four different anti-CD69 scFv constructs generated and their general sequence. c) Genetic map of the final construct for the constitutive expression of anti-CD69 membrane-bound scFv. d) Schematic representation of K562 cells before and after transduction with the lentiviral vectors. e) Flow cytometry plots showing percentage of transduced cells (BFP^+).

To test their ability to bind CD69 proteins, these constructs were transduced into K562 cell lines. Transduction efficiency was assessed via flow cytometry by determining the percentage of BFP^+ cells (Figure 10e). Then, the four anti-CD69 $scFV^+$ K562 cell lines were incubated with a soluble recombinant His-tagged CD69 protein (2.5 ug/mL), like shown in Figure 11a.

	VH	VL	Signal peptide	Hinge region
V43	160-c7	160-c7	IgK	CD28
V44	160-c76	234-61	CD28	CD8
V45	160-c76	234-83	IgK	CD8
V46	160-103	160-103	CD28	CD28

Table 2 – Combinations of VH, VL, signal peptide and hinge region sequences used to generate four different membrane-bound anti-CD69 scFv constructs. VH and VL sequences were retrieved from a published CD69 antibody patent.[26]

To test their ability to bind CD69 proteins, these constructs were transduced into K562 cell lines. Transduction efficiency was assessed via flow cytometry by determining the percentage of BFP$^+$ cells (Figure 10e). Then, the four anti-CD69 scFV$^+$ K562 cell lines were incubated with a soluble recombinant His-tagged CD69 protein (2.5 µg/mL), like shown in Figure 11a.

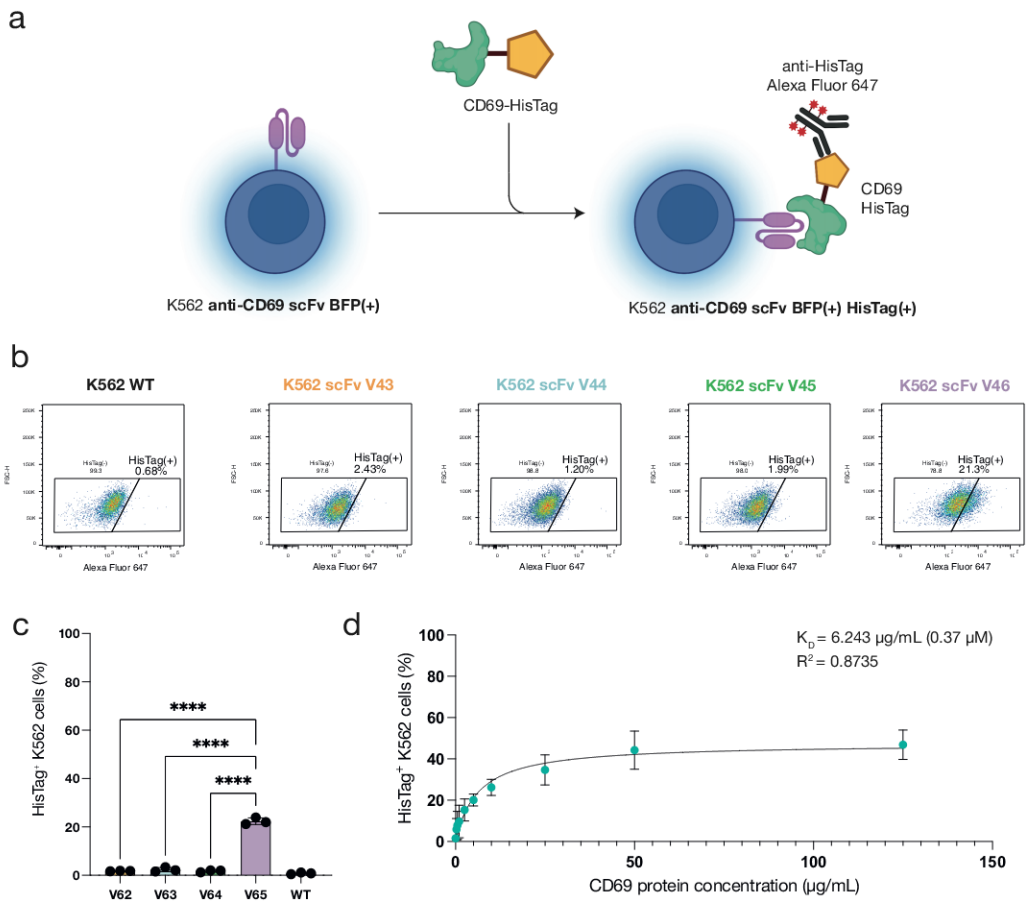


Figure 11 – Anti-CD69 scFv functional test. a) Schematic representation of the experiment used to assess the functionality of anti-CD69 membrane-bound scFv molecules. Transduced K562 are

co-cultured with soluble CD69-HisTag for 3 hours and stained with an anti-HisTag conjugated antibody for further flow cytometry analysis. b) Flow cytometry plots resulting from the experiment, gated on BFP^+ cells. c) Statistical analysis comparing HisTag positivity of the four different K562 populations, performed via one-way ANOVA. d) Non-linear regression between different concentration of CD69-HisTag protein and relative percentage of $HisTag^+$ cells. For c) and d) data are shown as mean ± s.d. of n = 3 technical replicates. **** $p \leq 0.0001$

Percentage of $HisTag^+$ cells was determined through flow cytometry analysis after staining with anti-HisTag Alexa Fluor 647, reflecting the ability of the four different anti-CD69 scFv constructs to bind and retain the soluble protein on the cell's surface. Results show that only one of the four anti-CD69 scFv was able to bind CD69 (Figure 11b and 11c). Therefore, this scFv (V46) was selected and used for further experiments.

5.4 Anti-CD69 scFv dissociation constant determination

To assess the affinity of the surface-displayed anti-CD69 scFv for its epitope, a titration experiment was performed. K562 cells expressing the anti-CD69 scFv were tested by treating them with different concentrations of soluble His-tagged CD69 protein – ranging from 0.125 μg/mL to 125 μg/mL. After analysis via flow-cytometry, the percentage of $HisTag^+$ cells were determined for each one of the CD69 concentrations tested and data were represented in a titration curve, as shown in Figure 11d. The dissociation constant (K_D) of the anti-CD69 scFv, expressed as:

$$K_D = [R][L] / [RL]$$

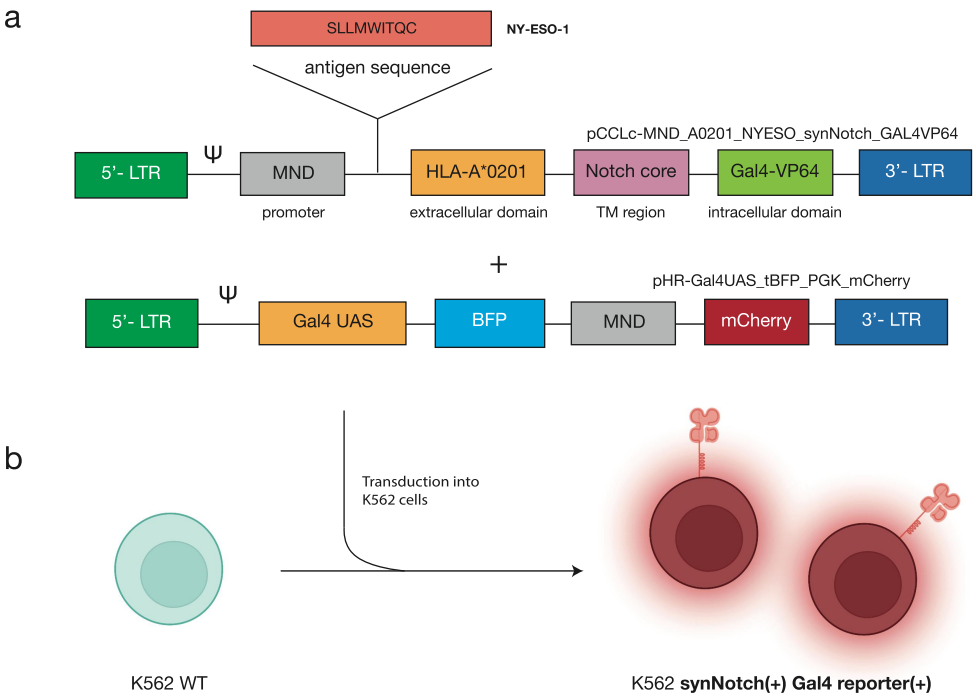
where:
[R] stands for Receptor concentration
[L] stands for Ligand concentration
[RL] expresses the concentration of Ligand bound to its Receptor

was calculated through Graph Prism software and is equal to 6.243 ug/mL, corresponding to 0.37 μM.[27,28]

5.5 Generation and functional test of synNotch construct

As explained in Paragraph 3.1, to generate a system that allows the inducible expression of the anti-CD69 scFv on K562 cells' surface, two approaches were taken into consideration. The first one involved the generation of a synNotch receptor. For this purpose, the synNotch construct reported in Morsut et al.'s

work was retrieved (*pHR_PGK_antiCD19_synNotch_Gal4VP64*, Addgene #79125) and genetically modified: the original extracellular domain, coding for an anti-CD19 scFv, was substituted with a SCT sequence containing the NY-ESO-1 antigen (Figure 12a). The intracellular domain is represented by a Gal4 transcriptional activator that, after specific interaction between the extracellular domain and its cognate TCR, initiates expression of genes coded by a reporter construct (*pHR_Gal4UAS_tBFP_PGK_mCherry*, Addgene #79130). This plasmid contains an upstream activating sequence (UAS), which allows Gal4's binding and subsequent transcription of BFP, and an mCherry sequence under the control of the constitutive PGK promoter, allowing detection of Gal4 reporter^{+} cells (Figure 12a). In the final setting, this reporter will be modified to contain the anti-CD69 scFv sequence. However, the efficiency of this inducible system needed to be ensured first. To do so, K562 cells were co-transduced with the synNotch and Gal4 reporter lentiviral vectors. Transduction efficiency was observed at flow-cytometer (Figure 12c). Specifically, K562 cells were stained with anti-HLA-A*0201 APC-Cy7 antibody, so that synNotch-transduced cells, which express an SCT on their surface, became APC-Cy7^{+}. Then, Gal4-reporter transduction was checked by looking for mCherry^{+} cells. Double-positive cells (APC-Cy7^{+} and mCherry^{+}) express both the synNotch construct and its reporter, as shown in Figure 12c (left plot).



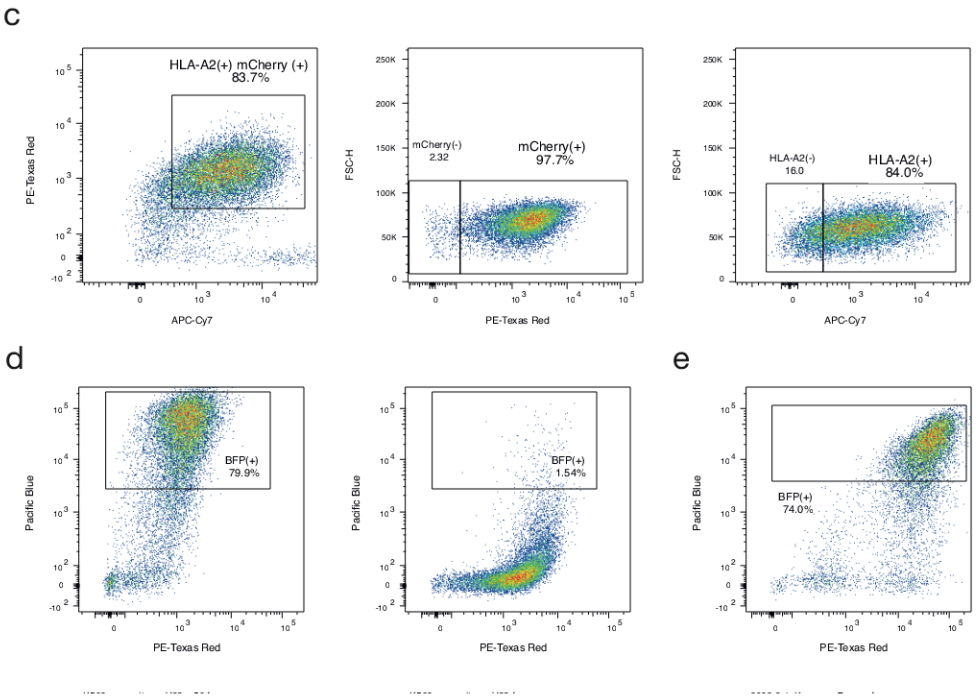


Figure 12 – SynNotch construct generation and functional test. a) Design of synNotch and synNotch reporter vectors. b) Schematic representation of K562 cells before and after transduction. c) Flow cytometry plots showing populations of K562 co-transduced with synNotch and Gal4-reporter (left), transduced with Gal4-reporter only (center) and synNotch construct only (right). d) Flow cytometry plots showing BFP positivity in single-coltured synNotch$^+$ Gal4-reporter$^+$ double-positive cells (left), but not in Gal4-reporter$^+$ cells (right). e) Flow cytometry plot showing BFP$^+$ RAM7 synNotch+ Gal4-reporter$^+$ cells.

Unfortunately, cells resulted also BFP$^+$ when co-transduced with synNotch and Gal4-reporter constructs, meaning that the intracellular Gal4 transcriptional activator cleavage took place even in absence of extracellular domain's ligand. This phenomenon, named ligand-independent activation (LIA), has been previously reported in literature by Yang et al.[29] Their results suggest that the addition of an hydrophobic aminoacidic sequence (*QHGQLWF*, named RAM7) at the interface between the notch-core and the intracellular domain can reduce synNotch's activation when the ligand is non-present or non-specific. Hence, the following step was cloning the RAM7 sequence into the synNotch construct. Unfortunately, as shown in Figure 12e, this enhanced version of the molecule did not reduce LIA and cells expressed BFP in a constitutive manner. Eventually, given the strong limitations presented by this approach, it was temporarily laid aside.

5.6 SABR construct allows inducible expression of GFP on APCs upon cognate interaction between TCR and SCT

Since the synNotch system's outcome wasn't satisfactory, an alternative has been tested. As previously described, in its original setting, the SABR construct allows expression of GFP in an antigen presenting cell, upon antigen-specific interaction between the extracellular SCT domain and its cognate TCR. To validate the functionality of this method, three different SABR constructs were produced.

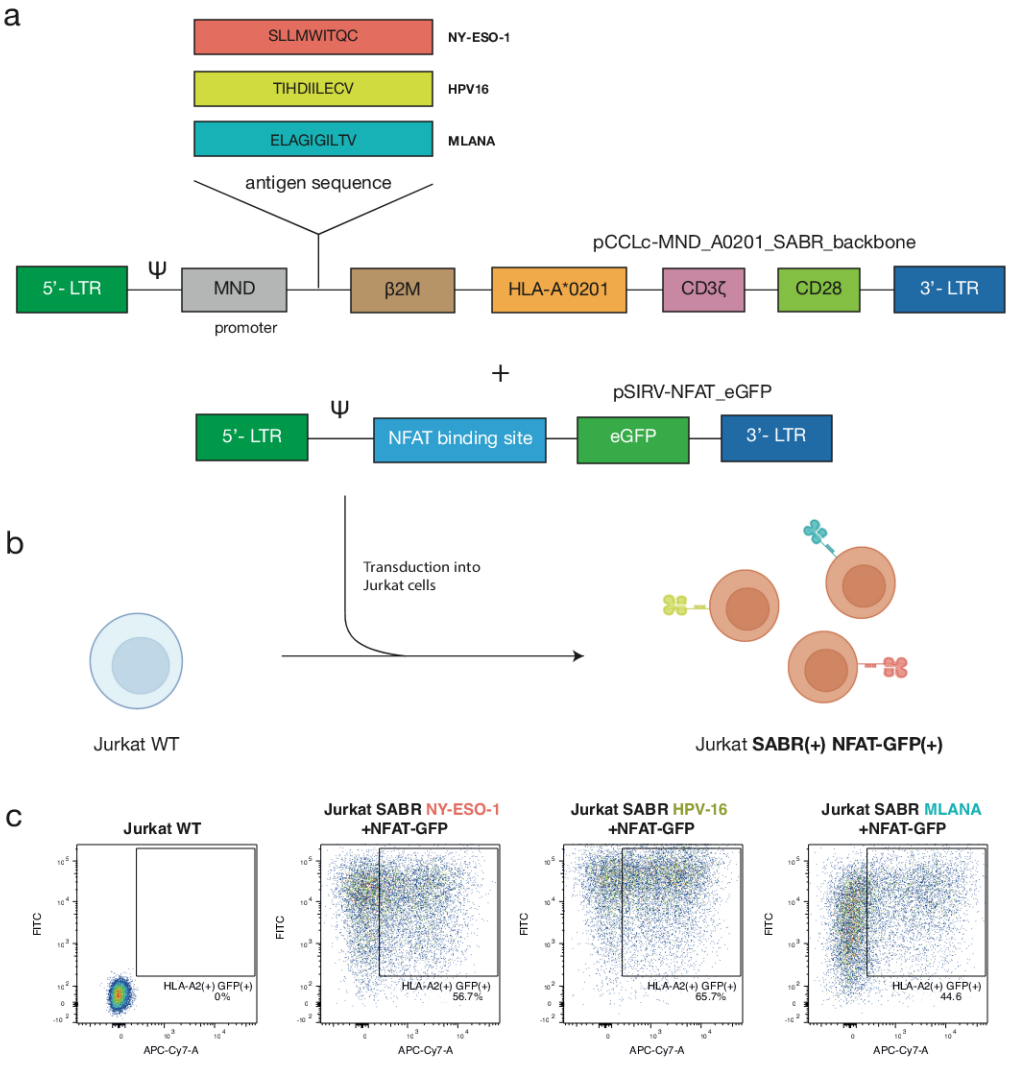


Figure 13 – SABR+ NFATGFP+ Jurkat cells generation. a) Plasmid maps of SABR construct and NFAT-GFP reporter. b) Schematic representation of Jurkat cells before and after transduction. c) Flow cytometry plots showing transduction efficiencies after staining with anti-HLA-A2 APC-Cy7.

Starting from the original plasmid (*pCCLc-MND_A0201_SABR_Backbone*, Addgene #119050), the previously used antigens – NY-ESO-1, HPV16 and

MLANA – were cloned into the SCT sequence. Jurkat cell lines were transduced with the NY-ESO-1 SABR, HPV16 SABR and MLANA SABR lentiviral vectors and the NFAT-GFP reporter (*pSIRV-NFAT_eGFP*, Addgene #118031), as shown in Figure 13.

Then, a co-culture assay was performed to test their functionality, which comprehended the following conditions:

a) SABR$^+$ Jurkat cells + cognate TCR-transduced dTomato$^+$ Jurkat cells;
b) SABR$^+$ Jurkat cells + non-cognate TCR-transduced dTomato$^+$ Jurkat cells;
c) SCT$^+$ K562 cells + cognate TCR-transduced dTomato$^+$ Jurkat cells (positive control);
d) SABR$^+$ Jurkat cells treated with PMA/ionomycin;
e) SABR$^+$ Jurkat cells (negative control);
f) TCR-transduced dTomato$^+$ Jurkat cells alone (negative control).

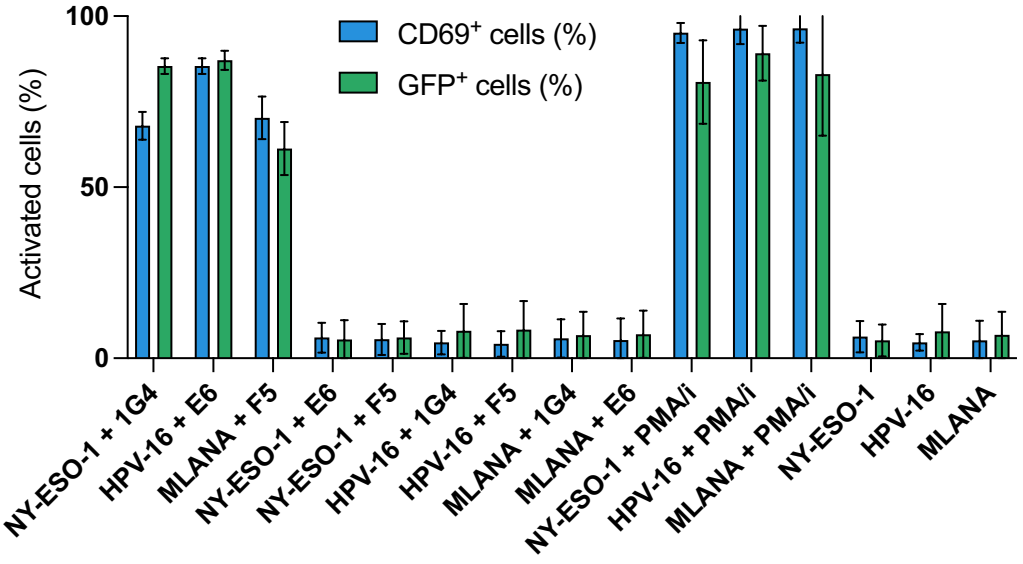


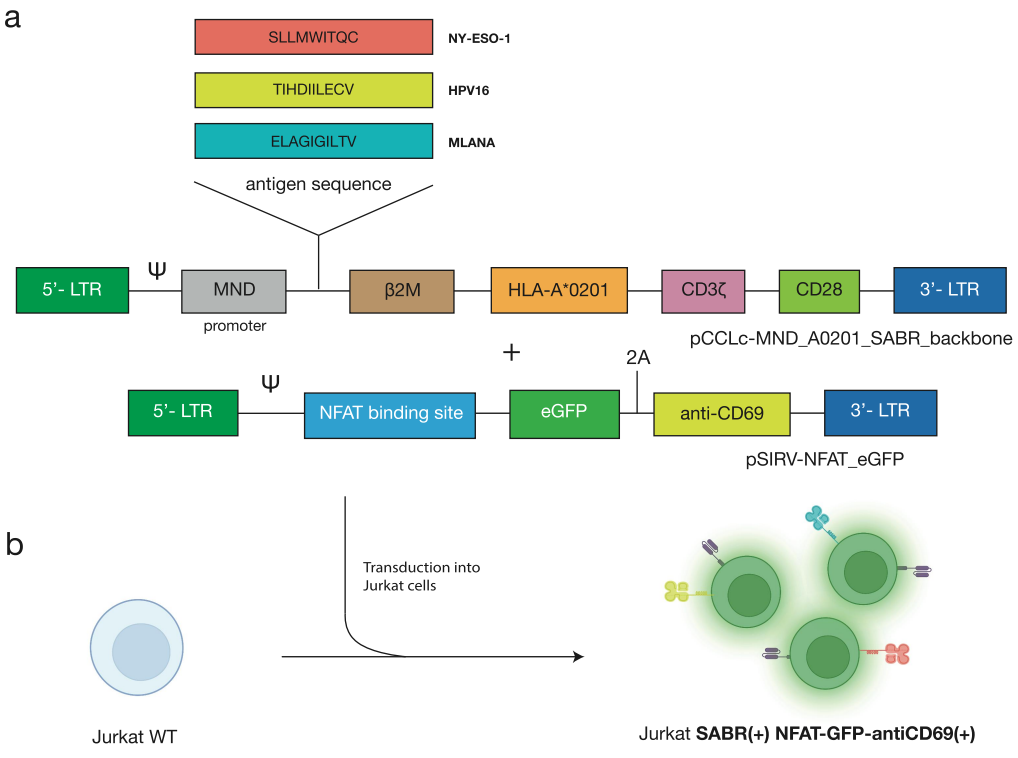
Figure 14 – Graphical representation of SABR validation test results. Percentage of activated cells is reported as CD69$^+$ (in blue) and GFP$^+$ (in green). Data shown as mean ± s.d. of n = 3 technical replicates.

Analysis by flow cytometry revealed that SABR$^+$ Jurkat cells and TCR-transduced Jurkat cells were both CD69$^+$ after 6 hours of co-incubation, in conditions where specific TCR-SCT interactions occurred. When activated, SABR$^+$ cells were also GFP$^+$, meaning that T cells' activation effectively leads to transcription and

expression of genes encoded by the reporter. CD69 and GFP expression was not detected in conditions where non-specific antigen-TCR interactions took place. Also, co-culture of SCT^+ K562 cells with their cognate TCR-transduced Jurkat cells was performed as positive control for the latter's activation. Treatment with PMA/i guaranteed a TCR-independent control for Jurkat cell's activation, while culturing of $SABR^+$ and TCR-transduced Jurkat cells alone served as negative control, indeed resulting in $CD69^-$ and GFP^- populations. Results shown in Figure 14 suggest that SABR is an efficient strategy to trigger inducible expression of desired genes upon specific recognition of the cognate TCR by an SCT molecule. Thus, it could be considered as a valid alternative for the creation a double-regulated circuit between interacting T cells and APCs.

5.7 Generation of NFAT-GFP-antiCD69 reporter allows expression of anti-CD69 scFv on APCs' surface upon cognate interaction

To obtain the inducible expression of the anti-CD69 scFv construct on APCs' surface, the NFAT-GFP reporter was genetically engineered. The anti-CD69 scFv sequence was cloned after the GFP gene, as shown in Figure 15a. $SABR^+$ K562 cells were transduced with the new NFAT-GFP-anti-CD69 reporter (Figure 15b) and tested to assess their functionality in a co-culture assay.



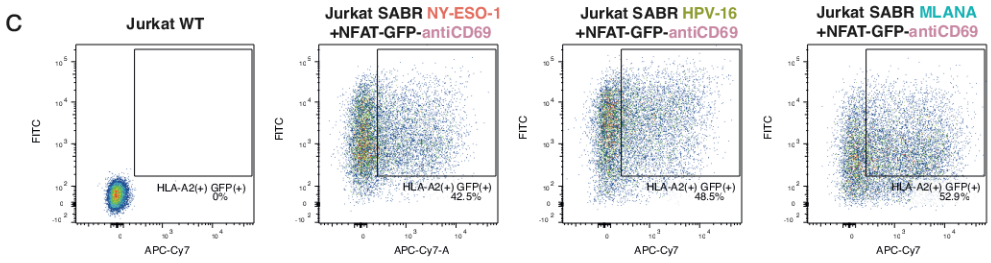
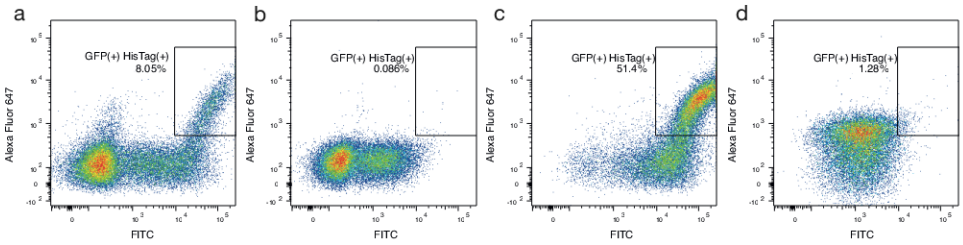


Figure 15 – Generation of NFAT-GFP-antiCD69⁺ SABR Jurkat cell lines. a) Design of SABR and NFAT-GFP-antiCD69 reporter constructs. B) Schematic representation of Jurkat cells before and after transduction. c) Flow cytometry plots showing efficiencies of transductions after staining with anti-HLA-A2 APC-Cy7 antibody.

Conditions were the following:

a) SABR⁺ NFAT-GFP-anti-CD69⁺ Jurkat cells + cognate TCR-transduced dTomato⁺ Jurkat cells;
b) SABR⁺ NFAT-GFP-anti-CD69⁺ Jurkat cells + non-cognate TCR-transduced dTomato⁺ Jurkat cells;
c) SABR⁺ NFAT-GFP-anti-CD69⁺ Jurkat cells treated with PMA/I (positive control);
d) SABR⁺ NFAT-GFP-anti-CD69⁺ Jurkat cells alone (negative control).

All co-cultures were treated with CD69-HisTag soluble protein at a concentration of 50 µg/mL. Staining with anti-HisTag AlexaFluor-647 shows that activated Jurkat cells (conditions a and c) are able to bind and retain the protein on their surface (HisTag⁺ and GFP⁺), while cells in conditions b and d show basal GFP expression levels (fluorescence intensity < 10^4) and no HisTag positivity.



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6. Conclusions

In the context of cell-mediated immune responses against threats, initiation of appropriate defense mechanisms strongly depends on specific interactions between TCRs and MHC-bound antigens. Identifying clinically relevant immunoreceptors and their cognate epitopes is crucial to gain further knowledge on immune-related diseases and to develop therapies for a larger plethora of patients. For instance, characterizing new targetable tumor antigens is crucial for the development of cell-based therapies like CAR-T cells and engineered TCR. In the past decades, considerable improvements in this field have been possible thanks to the development of more efficient and sensitive antigen discovery approaches – as described in Paragraph 2.1. Starting from traditional functional assays to the latest high-throughput cell-based systems (T-Scan, SABR and many more), scientists have managed to significantly broaden the spectrum of antigens and responsive TCRs to be used in the clinical practice. Specifically, pMHC-driven approaches involve screening of known antigen sequences against polyclonal T cell populations, while TCR-driven approaches allow screening of known TCRs against large panels of peptides. However, none of these methods allow to retrieve the sequences of the two interacting partners when both are unknown and, thus, library-on-library screening is still not possible.

A novel strategy for the discovery of MHC-I restricted antigens is hereby presented. This method relies on the formation of stable cell-doublets upon specific and productive interactions between APCs and T cells. Doublets can be physically sorted via flow cytometry and analyzed at single-cell level to retrieve the antigen and the TCR sequences. By not requiring foreknowledge of either one of the two, the aim is to fill the before-mentioned technological gap and allow screening of polyclonal TCR repertoires against antigen libraries. APCs are engineered in order to conditionally express, upon recognition of the cognate TCR by the pMHC complex, a membrane-anchored anti-CD69 scFv that binds to CD69, which is displayed on the T cells' surface once they are activated. This system acts as a double-regulated circuit that enables the stabilization of immunological synapses, discriminating productive interactions from non-productive ones. Patient-derived primary $CD8^+$ T cells can be directly used for

analysis, without needing to be genetically modified. Validation of this approach will be carried out by looking for clinically relevant interactions between TILs isolated from colon-rectal cancer samples and their cognate neoantigens. Neoantigens identified using this method could aid the development of cell-based immunotherapies.

The first stages of the development of this technology have been presented in Paragraph 5. K562 cells expressing SCT molecules were successfully generated and tested, while Jurkat cells and 2D3 cells were transduced with exogenous TCRs. SCT molecules are able to activate both Jurkat and 2D3 engineered cell lines in an antigen-specific manner – simulating the activation triggered by soluble peptides loaded onto endogenous HLA molecules. This first accomplishment suggests that the SCT constructs is a reliable approach for the transduction of genetically encoded antigen libraries into APCs. The key element of the whole system is the affinity-mediated stabilization of productive interactions, which employs the expression of an anti-CD69 scFv on the surface of APCs. Therefore, assessing its capacity to interact with CD69 and characterizing its binding affinity was necessary to proceed with further experiments. Out of four different scFv constructs produced and expressed on K562 cells, only one (named V65) was identified as capable of binding a soluble CD69-Histagged protein and, subsequently, its dissociation constant was calculated to be equal to 0.37 μM. This value is comparable to that of other scFv molecules, as reported in literature. Then, a mechanism for the conditional expression of the anti-CD69 scFv on APCs' surface was designed and implemented. Use of a synNotch receptor was impaired by its ligand-independent activation and even improved versions of the construct were not able to resolve the problem. Thus, the SABR approach described by Joglekar et al. was exploited, after validating its functionality. By adapting it to the present technology, some alterations were made. Specifically, the SABR reporter construct was genetically engineered to express the membrane-bound anti-CD69 scFv upon engagement of the extracellular SCT with a cognate TCR, and transduced into Jurkat cells. Co-culture assays were performed and shown that only activated cells – which showed an higher expression of GFP compared to non-activated ones – were able to bind and retain the CD69 soluble His-tagged protein on their surface, proving that membrane-anchored anti-CD69 scFv is expressed upon productive interaction between the

SCT and the cognate TCR. Further experiments are currently being performed to determine if Jurkat cells expressing the SABR construct and the NFAT-GFP-antiCD69 reporter are able, once activated by interaction with the cognate TCR or by PMA/i treatment, to bind to the CD69 protein displayed on activated TCR-transduced cells. If binding occurs, cell-doublets should form and they should be visible via flow cytometry analysis.

However, results reported in Fig(?) show a little percentage of HisTag$^+$ Jurkat cells upon pMHC-TCR cognate interaction. Being the two cell populations specific for each other, the expected rate of cells expressing the anti-CD69 scFv is higher than what has been observed. This poses some concerns about the sensitivity of this approach, which translates in its ability to capture all significative T cell-APC pairs. To increase its efficiency, further experiments shall be performed to improve the anti-CD69 scFv's affinity, perhaps by testing different combinations of hinge regions, transmembrane domains and V_H/V_L chains. Although the K_D calculated in Paragraph (?) is comparable to that of most previously reported scFv molecules, it might not be enough to crosslink cells. Thus, this aspect needs to be improved. Also, addition of an expression marker into the anti-CD69 scFv construct could allow determination of the actual ratio of cells that are expressing the scFv upon activation, since HisTag positivity is not a precise indicator of its presence on their surface. By looking for a specific expression marker, it could be possible to understand if the low HisTag$^+$ cell percentage is due to a lack of expression of the anti-CD69 scFv or if it's caused by insufficient binding affinity. In addition, Jurkat cells transduced with the NFAT-GFP reporter show a basal GFP fluorescence signal, which should be eliminated to improve the signal-to-noise ratio.

After appropriate optimization, further experiments will be carried out to determine if this method can be used in clinical contexts. These will involve the use of primary CD8$^+$ T lymphocytes, which are expected to exert a cytotoxic activity on APCs. To guarantee an efficient performance and reduce false-negative results, induction of the apoptotic process in SABR-expressing cells need to be attenuated.

Overall, the presented results offer an overview of the first stages of development of an alternative approach for CD8$^+$ T cell antigen discovery. Once finished, this

method could offer a way to identify relevant antigens and immunoreceptors by allowing library-on-library screening – which would stand out as a novelty in this context. As a result, newly discovered TCRs and target antigens could be exploited in translational research, with the ultimate aim of improving the immunological armamentarium against cancer and many other diseases.

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