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建立表達單一第一型白血球抗原的人造抗原呈現細胞以刺激 T 細胞及抗原表位定位

Establishment of the mono-allelic HLA class I expressing artificial antigen presenting cells for T cells stimulation and epitope mapping

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建立表達單一第一型白血球抗原的人造抗原呈現細胞以刺激T細胞及抗原表位定位

（論文中文題目）(Chinese title of Master's thesis)

Establishment of the mono-allelic HLA class I expressing artificial antigen presenting cells for T cells stimulation and epitope mapping

（論文英文題目）(English title of Master's thesis)

本論文係 趙佳儀 （姓名） R09445125 （學號）在國立臺灣大學 微生物學研究所 (系/所/學位學程)完成之碩士學位論文，於民國 111 年 8 月 2 日承下列考試委員審查通過及口試及格，特此證明。

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中文摘要

T 細胞在後天性免疫中扮演重要的角色，現今有許多癌症免疫療法，即是希望可藉由刺激更多的 T 細胞，使其活化、擴增，最後有效清除癌細胞而得到治療的成效。而刺激更多的 T 細胞有許多方法，其中一項就是分離人體內的樹突細胞，讓此抗原呈現細胞在體外受到抗原刺激，再將活化的抗原呈現細胞打回體內，因而可在體內達到刺激 T 細胞的效果，但此方法十分耗時且昂貴，因此，逐漸發展出利用人造的抗原呈現細胞在體外刺激 T 細胞。

而抗原呈現細胞刺激 T 細胞需要三種訊號，其一就是需要主要組織相容性複合體將抗原的抗原表位呈現給 T 細胞的受體；其二，需要一些協同刺激的分子放大刺激 T 細胞的訊號及延長刺激 T 細胞的時間，這些分子包含 CD86、CD70、4-1BBL 等等。其三，需要細胞激素如白血球介素-2 (IL-2)等的協助，以增加 T 細胞的擴增、活化。因此，本研究目的即是要做出一種人造的抗原呈現細胞，在人造的抗原呈現細胞上給與刺激 T 細胞所需的主要組織相容性複合體，並利用帶有 4-1BBL、CD86 等協同刺激分子的質體使此細胞過度表現這些分子，最後在體外給予細胞激素作為額外的刺激，期望能在體外更有效率、快速地刺激並活化 T 細胞。

除了好的抗原呈現細胞可以更有效率刺激 T 細胞外，一個好的抗原表位 (Epitope) 也很重要。抗原表位被 T 細胞受體辨識後即可活化 T 細胞，因此對於了解哪些抗原表位會引起 T 細胞反應是必要的。

目前較常使用 T2 細胞作為抗原表位定位的工具，由於此細胞缺乏抗原加工相關轉運體(transporter associated with antigen processing; TAP protein)而無法將內源性的胜肽運至表面與主要組織相容性複合體結合，因此可利用外加胜肽來測試此抗原表位是否會與細胞表面的主要組織相容性複合體結合而穩定其結構，若偵測到的主要組織相容性複合體表現量有上升則代表可成功結合。但 T2 細胞表面並不是表現單一等位基因的主要組織相容性複合體，因此即使胜肽結合上去使偵測到的

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主要組織相容性複合體表現量有升高，也不清楚此胜肽是與哪個主要組織相容性複合體的等位基因結合。基於此，我們利用群聚且有規律間隔的短回文重複序列(CRISPR)技術建立一株缺乏抗原加工相關轉運體的細胞，並只表達單一等位基因主要組織相容性複合體，相比於以往使用的 T2 細胞株，可以更精確知道此抗原表位會與哪個主要組織相容性複合體的等位基因結合，因此可使用此細胞株作為抗原表位定位的工具。

有了人造抗原呈現細胞可以更有效率地刺激毒殺性 T 細胞，再加上可以更精確知道抗原表位的主要組織相容性複合體基因型，對於未來免疫療法會有極大的貢獻，希冀可以造福更多需要被治療的病患們。

關鍵字：群聚且有規律間隔的短回文重複序列、主要組織相容性複合體、人造抗原呈現細胞、缺乏抗原加工相關轉運體、免疫療法

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英文摘要

T cells play a vital role in adaptive immunity. With enough functional T cells, cancer cells can be effectively eliminated to achieve cancer cure. One strategy to stimulate more functional T cells is to isolate patients' dendritic cells, a kind of antigen presenting cells, which are then activated *in vitro* and subsequently transferred to patients. However, this approach is very time-consuming and expensive; therefore, artificial antigen presenting cells (APCs) have gradually gained wide interest, and are also used to stimulate T cells *in vitro*.

APCs activate antigen-specific T cells require three distinct signals. Signal one is antigen-specific signaling mediated by T-cell receptor engagement of pathogenic peptides presented by major histocompatibility complex (MHC) molecules, also named human leukocyte antigens (HLA) in humans. Signal two is costimulatory molecules expressing on APCs, which mainly function to amplify signals and prolong the time of T cell stimulation and activation. Signal 3 is polarizing signaling mediated by various cytokine milieus to enhance T cell activation and expansion.

Accordingly, in my thesis study, the first research purpose is to establish an artificial APC with robust ability for T cell stimulation. First of all, we used the technique of clustered, regularly interspaced, short palindromic repeats (CRISPR) to knock out the endogenous class I MHC and then transferred mono-allelic one on this artificial APC.

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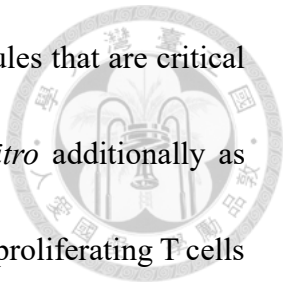

Second, we generated the cells overexpress the costimulatory molecules that are critical for T cell activation. Last, we supple the cells with cytokine *in vitro* additionally as another stimulator. The ability of this artificial APC in activating and proliferating T cells will be examined and optimized.

On the other hand, APCs can present epitopes to T cells and stimulate them. As a result, epitopes are important for stimulating T cells. However, it remains a time-consuming and labor-intensive work for epitope mapping. It is known that T2 cells lack the transporter associated with antigen processing (TAP) protein, so can be utilized for epitope mapping. Sometimes, the results of epitope mapping are ambiguous due to the presence of multiple HLA alleles. For the unambiguous identification of class I HLA-restricted epitopes, the cells expressing mono-allelic class I HLA that we established previously were used to knock out the TAP genes by CRISPR. After generating these mono-allelic HLA expressing TAP Knock out cell lines, we can precisely confirm the class I HLA-restricted epitopes to T cells. In the end, it is possible to develop powerful T cell-based immunotherapies against many diseases using epitope mapping and artificial APCs.

Key words: CRISPR, human leukocyte antigens, artificial APC, TAP deficient knock out, immunotherapy

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1. Introduction

1.1 Cancer immunotherapy

Immunotherapy is a type of cancer treatment enabling the immune system to fight the disease. The immune system is boosted via immunotherapy for immune surveillance and killing of cancer cells. A variety of immunotherapies are available against existing cancers, including stimulating effector mechanisms and counteracting inhibitory and suppressive mechanisms[1]. The effectiveness of active immunotherapy depends heavily on the efficient stimulation of antigen-specific immune cells, such as cytotoxic T cells[2, 3].

Adoptive T cell transfer is a promising immunotherapy[4, 5]. *In vivo* activation of T cells is highly dependent on interactions with professional antigen presenting cells (APCs), including dendritic cells (DCs), which present tumor-specific antigens. Thus, APCs are administered into cancer patients in order to induce *in vivo* T cell activation. It has been demonstrated that natural APCs, in particular DCs, can efficiently activate and expand tumor antigen-specific naive T cells, resulting in the induction of large numbers of antigen-specific T cells. However, it is difficult to ensure the injected APCs can migrate to the target sites or whether the cells can perform proper ability of stimulation.

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On the other hands, adoptive T cell therapy can be achieved by *ex vivo* stimulation of T cells and subsequent reinfusion into a cancer patient. The latest trend in adoptive T cell transfer takes advantage of molecular engineering of active T cells with higher affinities for target cancer cells and a longer persistence. In order to achieve these goals, T cells were engineered to express either transgenic T cell receptors (TCRs) with increased affinity for their cognate peptide-major histocompatibility complexes (MHC) complexes or chimeric antigen receptors (CARs) with antibodies capable of identifying antigens that are independent of MHC[6-9]. In addition to the engineered receptors, T cells can also be stimulated by APC to enhance cytotoxicity.

1.2 Benefits of artificial APCs

However, natural APCs such as DCs have been shown to have numerous serious limitations over the years. Clinical trials have shown mixed results due to the insufficient knowledge regarding optimal antigen-loaded DC and the negative effects of immunosuppressive factors in the tumor microenvironment. Furthermore, *in vitro* culture of DCs is time-consuming and costly, and the quality of the stimulated DCs varies[10]. Consequently, a robust and off-the-shelf artificial APC for T cell stimulation is highly desirable[11].

The development of artificial APCs is an alternative for stimulation T cells *in vivo* and *in vitro*[12]. In comparison to natural APCs, artificial APCs have a more powerful

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ability to control T cell signaling and facilitate the generation of effector T cells for adoptive immunotherapy. Besides performing their effector functions to induce tumor cell death, the stimulated effector T cells can also develop memory T cells that can prevent relapse. It has been proven that artificial APCs can expand T cells and drive them to their target sites. In addition, they can establish the memory function of T cells and induce their effector function[13]. Hence, artificial APCs have a promising development on immunotherapy.

There are several kinds of artificial APCs based on different backbone[14-16]. For instance, bead-based and cell-based are more often used[17]. An artificial APC based on beads can easily be prepared, and the expression levels of T cell-activating signals can be controlled precisely. Moreover, the surface can contain known epitopes of antigens that are of interest. Cell-based artificial APCs differ from bead-based artificial APCs in that they can be stored for extended periods and can therefore be accessed readily. Most importantly, these artificial APCs can present novel epitopes because they can process antigens on their own. Considering these advantages, we were interested in developing a new class of cell-based artificial APCs[18].

1.3 Cell-based APCs

The backbone of a cell-based artificial APC must meet certain requirements[19]. Firstly, the APCs should not induce allo-specific T-cell responses, so the cell backbone

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should not express any human leukocyte antigen (HLA) class I or II molecules. Second, the desired genes should be stable on the surface of the cells and easily manipulate. Furthermore, it is preferable to use a cell line that is not harmful to humans as a backbone[20].

At present, most artificial APCs are based on K562 cells, which are derived from a chronic myelogenous leukemia patient in blast crisis[21]. Despite not expressing HLA class I, II, or inhibitory molecules such as PDL1 (B7H1), these cells do express adhesion molecules ICAM-1 (CD54) and LFA-3 (CD58), which are essential for effective immunological interactions. They have been developed as artificial APC for satisfying the requirements mentioned before.

The K562 cells, however, are difficult to transfect. This makes it difficult for them to express exogenous antigens. As a result of the poor transfection efficiency, we use HEK 293T, a highly transfectable derivative of human embryonic kidney 293 cells, which contains the SV40 T-antigen as the backbone[22].

1.4 Signals required for T cell activation

After choosing the backbone of artificial APCs, we should equip some essential materials on their surface. It is important to have three signals to make an APC function properly and stimulate T cells.

Signal one is antigen-specific signaling mediated by T cell receptor (TCR)

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engagement of cognate peptides presented by MHC molecules. This signal can last for several hours, and is necessary for effective activation of nuclear transcription factors through signal transduction pathways.

Additionally, cytotoxic T cells require a number of secondary signals to become activated and respond to a threat. Signal two is a set of costimulatory molecules that are present on APCs that amplify signals and prolong T cell stimulation and activation. For example, CD86、CD70 and 4-1BB ligand are some of the best-characterized costimulatory molecules[23, 24]. Activation, survival, and the effector function of T cells can be achieved through different costimulatory molecules.

In addition to receiving the specific antigen signal one and the general signal two, T cells also receive cytokines as additional instructions[25]. Signal three is polarizing signaling mediated by various cytokine milieus such as IL-2 to enhance T cell activation and expansion[26].

1.5 Mono-allelic HLA-expressing HEK 293T

Moreover, it is possible to activate antigen-specific T cells more precisely by stimulating HLA-matched epitopes. It is therefore essential to make artificial APCs that express mono-allelic HLAs. Using the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) technology, we first converted HEK 293T cells into HLA-null cell lines. After generating the HLA-null cell lines, we further transduced the HLA-

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null cell lines with individual mono-allelic HLA molecules. Next, we further pulsed these cell lines with HLA-matched peptides as signal one, then transduced them with the desired costimulatory molecules as signal two. As signal three, we will also add cytokines when the artificial APCs are used to stimulate T cells. Finally, an artificial APC is developed.

1.6 Desired costimulatory molecules

The costimulatory molecules are heterogeneous cell surface molecules that affect T cell differentiation and fate by amplifying or counteracting the initial activating signals. T cells are activated by interactions between costimulatory ligands and their receptors. Among the costimulatory molecules, there are three major families: the immunoglobulin (Ig) superfamily, the tumor necrosis factor (TNF) superfamily, and the emerging T cell immunoglobulin and mucin (TIM) domain family. Furthermore, different superfamilies can induce different pathways to achieve the goal of activating or differentiating T cells. Our artificial APC expresses the following costimulatory molecules, which we believe will enhance the ability of APCs to stimulate cytotoxic T cells.

1.6.1 CD86 (B7.2)

The CD86 protein belongs to the immunoglobulin superfamily, has an extracellular V and C-like domain, and is constitutively expressed or upregulated following the activation of B cells, dendritic cells (DCs), or macrophages. A

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costimulatory signal is produced when CD86 binds to its receptor CD28 on T cells, leading to T cell proliferation and IL-2 production through a pathway involving inositol triphosphate, protein tyrosine phosphorylation, and calcium influx. This interaction between CD86 and CD28 is essential for T cell stimulation by an APC[27].

1.6.2 OX40 Ligand (CD252)

OX40L is a type II transmembrane protein belonging to the TNF superfamily, also known as gp34, CD252, CD134L or TuNFSF4. It is induced in the activated T cells, DCs, B cells, and macrophages[28]. Upon binding to OX40L, OX40 on T cells can promote the activation of the NF-κB, PI3K-Akt, and NFAT pathways in the antigen-primed T cells[29]. Cell survival as well as other functional activities of effector and memory T cells are controlled by the interactions between them.

1.6.3 4-1BB ligand (CD137L)

As a member of the TNF superfamily, 4-1BB ligand, also known as CD137L, is mainly expressed on APCs, activated B and T cells, and is upregulated during activation of these cells[30]. In response to TCR engagement, 4-1BBL signaling triggers the expansion, proliferation, and cytokine production of T cells. It is also possible for this signal to replace CD28 costimulation in the presence of strong antigenic stimuli. Recent studies have shown that this signal is also essential for

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reversing established T cell tolerance and anergy. Additionally, 4-1BBL facilitates bidirectional communication between monocytes and B cells[31]. Therefore, 4-1BBL is essential for effective cytotoxic T cell activation on APCs[32].

Our artificial APCs with the abovementioned three signals can be used to stimulate cytotoxic T cells more efficiently.

1.7 The importance of epitope identification

Activation of T cells requires both the stimulation of APCs and the knowledge of epitopes presented by cognate HLAs. T cell epitopes, or antigenic determinants, are peptides presented by APCs that stimulate cognate cytotoxic T cells. Several antigens possess distinct epitopes on their surfaces, and therefore knowing the epitope that can stimulate T cells is important.

1.8 Strategies for epitopes identification

Several methods can be used to identify epitopes[33, 34]. One method is to use bioinformatics tools to predict epitopes. In an online database, the algorithm is used to determine the prediction score for the peptide. NetMHCpan, for instance, is a prediction tool used by IEDB Analysis Resource database.

Immunopeptidomics based on mass spectrometry is another method. In addition to identifying epitopes, this method also has the potential to supplement the genomic-based

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approaches used to identify immunogenic proteins by using immunomics techniques[35]. The first step is to infect or overexpress the antigens in the cells for antigen processing on their own. The HLA-peptide complex will then be immunoprecipitated for High Performance Liquid Chromatography (HPLC) and Liquid Chromatograph/Mass Spectrometer (LC/MS) analysis for the HLA-restricted epitopes.

1.9 Peptide binding assay (MHC binding assay)

To determine whether epitopes are indeed presented by the particular HLAs, we will perform peptide binding assays after obtaining epitopes[36]. Currently, T2 cells are the most commonly used tool for peptide binding assays. The cell line is an Epstein-Barr Virus (EBV) transformed lymphoblastoid deficient for an endogenous antigen processing protein, the transporter associated with antigen processing (TAP).

TAP protein delivers endogenous processed peptides to the endoplasmic reticulum (ER) for loading onto class I MHC molecules. In the following step, the peptide-MHC class I complexes leave the ER to display their antigenic cargo on the surface of the cell to cytotoxic T cells. In the absence of TAP protein, endogenous processed antigens cannot load onto the class I MHC complexes and cannot be stabilized, resulting in low or undetectable levels of MHC expression on the cell surface. By detecting the MHC expression levels, we can determine whether the previous identified peptides can bind to the MHC complexes by adding these peptides extracellularly. It is therefore possible to

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study antigen processing and T cell recognition of class I MHC antigens by using T2 cell lines.

1.10 Generate TAP genes knock out cell lines

Since T2 cells express HLA-A2, HLA-B51, and HLA-Cw1 on their surface, it can be used to identify HLA-A2, B51 and Cw1 restricted epitopes only[37]. However, it is hard to know the epitopes binding with which HLA-allele. Therefore, it is inconvenient to examine other identified HLA allele-restricted epitopes using T2 cells. Therefore, a cell line expressing other individual HLA alleles with deficiency of TAP protein is required for peptide binding assays. Using the previously generated mono-allelic HLA-expressing HEK 293T as a backbone, we will knock out TAP genes using CRISPR to generate a new cell line for peptide binding assays[38]. Combining our artificial APCs with epitopes that are presented to T cells could potentially increase the efficacy of T cell adoptive immunotherapies.

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2 Specific aim

Aim 1

To establish mono-allelic HLA class I expressing artificial antigen presenting cells to effectively activate $CD8^+$ T cell

Aim 2

To establish of the TAP-deficient mono-allelic HLA expressing cells for epitope mapping

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3 Materials and methods

3.1 Plasmids

The plasmids containing costimulatory molecules pLVX-EF1α-OX40L-T2A-4-1BBL-P2A-CD86, were constructed on lentivirus backbone with EF1α promoter. Costimulatory molecules OX40L sequence were derived from pcDNA3.1(-)-OX40L generated from Genomics), 4-1BBL sequence were amplified from cDNA of LCL48, CD86 sequence were derived from pCD86-EGFP obtained from Addgene (Cambridge, MA, USA).

The plasmids containing HLA genes, pLVX-A*11:01-β2m, pLVX-B*40:01-β2m and pLVX-C*07:02-β2m were constructed by cloning ORFs of class I HLA genes, including HLA-A*11:01, B*40:01, and C*07:02, and β2m into pLVX-del AcGFP-N1 (site-mutagenesis product, from Chih-Chiang Wang) derived from pLVX-AcGFP-N1 (catalog no.632154, Clontech) and possessing puromycin-resistant gene. The human codon-optimized Cas9 (hCas9) expression vector as well as the pLenti-U6-gRNA cloning vector were obtained from Addgene (Cambridge, MA, USA). This pLenti-hCas9-puro vector was constructed by combination of hCas9 and pLVX-AcGFP-N1 (Catalog no.632154, Clontech). By combining U6-BsmBI-gRNA scaffold with the blasticidin-resistant gene developed from pLVX-AccGFP-N1, pLenti-U6-

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gRNA-BSD was created. Afterwards, the gRNAs were cloned into pLenti-U6-gRNA-BSD.

The lentiviral production plasmids, p8.91 and pMD.G, were obtained from Academia Sinica, Taiwan. Agar plates containing ampicillin were used for the plating of all constructs transformed into DH5α competent cells. Single colonies grew in LB broth with Ampicillin and extracted the plasmid by using the Geneaid plasmid purification kit.

3.2 Cell lines and culture conditions

HLA-A*11:01-293T was generated by transduction of class I HLA-ABC null HEK 293T (#54-1-4) with pLVX-A*11:01-β2m. HLA-A*11:01-costimulatory molecules-293T was generated by transduction of HLA-ABC null HEK 293T (#54-1-4) with pLVX-A*11:01-β2m and pLVX-EF1α-OX40L-T2A-4-1BBL-P2A-CD86. HEK 293T, HLA-ABC null 293T (#54-1-4), HLA-A*11:01-293T and HLA-A*11:01-costiumulatory molecules 293T were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 292 μg/mL Glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin (100X GPS, Gibco) at 37°C and 5% CO_2. Additionally, 1.5 mg/mL puromycin was added to HLA-A*11:01-293T, HLA-A*11:01-costimulatory molecules 293T. Moreover, 250 μg/mL hygromycin was added to HLA-A*11:01 costimulatory molecules 293T.

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HLA-A*11:01-K562, HLA-B*40:01-K562 and HLA-C*07:02-K562 were generated by transduction of K562 with pLVX-A*11:01/ B*40:01/ C*07:02-β2m. HLA-A*11:01-costimulatory molecules-K562 was generated by transduction of K562 with pLVX-A*11:01 and pLVX-EF1α-OX40L-T2A-4-1BBL-P2A-CD86. K562, HLA-A*11:01-K562, HLA-B*40:01-K562, HLA-C*07:02-K562 and HLA-A*11:01-costimulatory molecules-K562 were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (100X PS, Gibco) at 37°C and 5% CO_2. Additionally, 2 mg/mL puromycin was added to HLA-A*11:01-K562, HLA-B*40:01-K562, HLA-C*07:02-K562 and HLA-A*11:01-costimulatory molecules-K562. Moreover, 200 μg/mL hygromycin was added to HLA-A*11:01-costimulatory molecules-K562.

T2 and T2-A11 were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (FBS), 292 μg/mL Glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin (100X PS, Gibco) at 37°C and 5% CO2.

LCL48 and human Peripheral Blood Mononuclear Cells (PBMCs) were maintained in T cell growth medium, which is Roswell Park Memorial Institute (RPMI 1640, Gibco) supplemented with 10% fetal bovine serum (FBS), 292 μg/mL Glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin (100X GPS, Gibco) at 37°C and 5% CO2.

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3.3 HLA gRNA / TAP genes gRNA design and cloning

HLA-gRNAs (upstream 5'-NGG) design that can target HLA-ABC at the same time were referred to the IPD-IMGT/HLA database (https://www.ebi.ac.uk/ipd/imgt/hla/allele.html). The gRNA was cloned into pLenti-U6-gRNA-BSD plasmid by ligating annealed oligo duplexes into *BsmBI* cassette. First, using T4 PNK (NEB, final 5 U) and T4 ligase buffer (NEB) to add 5'phosphates to the two ordered oligos (final 10 μM of each). Thermocycler conditions were adjusted to 37 °C for 30 min, 95 °C for 5 min, followed by a -0.1 °C/sec ramp down to 25 °C. Next, the digestion with *BsmBI* and ligation of annealed- phosphorylated oligos into vector were combined in one step. Reaction contained vector (final 50 ng), annealed-oligonucleotide diluted 1:250 (final 3-5 ng), *BsmBI* (Thermo), T7 DNA ligase (NEB), ATP (final 1 mM), DDT (final 1 mM), and 10x FastDigest Buffer (NEB), following 6 cycles of 37℃ for 5 min and 23℃ for 5 min. Finally, linear DNA was digested with PlasmidSafe exonuclease (Lucigen).

The sequences of TAP 1 and TAP 2 genes were gained from National Center for Biotechnology Information (NCBI). Next, TAP-gRNAs were designed by using the CRISPR/Cas9 target online predictor (CCTop) (https://cctop.cos.uni-heidelberg.de:8043/index.html). The gRNAs were cloned into pLenti-U6-gRNA-BSD plasmid by ligating annealed oligo duplexes into *BsmBI* cassette. The cloning steps and

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conditions were same with HLA-gRNAs cloning. The constructs were transformed into DH5α competent cells.

3.4 DNA transfection

Using Lipofectamine™ 3000, DNA transfection was performed according to the manufacturer's protocol. In brief, HEK 293T cells were seeded at a density of 1×10^6 per well in a 6-well plate. We diluted DNA and 5 μl p3000 reagent within 125 μl Opti-MEM™ I Reduced Serum Medium (Opti-MEM medium, Gibco) and mixed them with diluted Lipofectamine 3000 reagent (7.5 μl within 125 μl Opti-MEM™). Incubated at room temperature for 15 minutes, the mixture was added to cells drop by drop. We changed the medium to complete DMEM medium after 5-6 hours. A complete DMEM medium with antibiotic selection was changed after 48 hours.

We co-transfected the expression vectors containing pLenti-hCas9-puro and the gRNAs at a ratio of 3:2 with HEK 293T cells to achieve CRISPR knock outs. Cells were selected 72 hours after transfection using antibiotics, then analyzed by targeted polymerase chain reaction (PCR) using specific forward (F') and reverse (R') primer pairs (**Supplementary table 2**).

3.5 Gibson Assembly

pLVX-EF1α-OX40L-T2A-4-1BBL-P2A-CD86 was constructed by ligation of one

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vector and three fragments. They were ligated by NEBuilder® HiFi DNA Assembly Master Mix (Catalog No. E2621, New England Biolab) following the manufacture's protocol. In brief, the molecular ratio of the vector and each individual fragment was 1:1:1 (maximum 0.5 pmole for total). The mixture was then mixed with 10μL NEBuilder® HiFi DNA Assembly Master Mix for total 20μL mixture. Then, incubated the mixture at 50°C for 1 hr, and hold at 4°C for at least 10 minutes. The Gibson product was transformed 2μL into the competent cell DH5α and incubated at 37°C for 16 to18 hours. Plasmids were extracted by FavorPrepTM Plasmid DNA Extraction Mini Kit (Catalog No. FAPDE 300, FAVORGEN). Sequences were analyzed by Sanger sequencing (by Genomics) using the sequencing primer.

3.6 Lentivirus production

HEK 293T cells were seeded at a density of 1×10^6 per well in a 6-well plate. Then, 1.5μg of target plasmid, 0.5μg of pMD.G, 1μg of pCMV-dR8.91 and 6 μl p3000 reagent were diluted with 250 μl Opti-MEM medium, and then mixed with diluted Lipofectamine 3000 reagent (7 μl with 250 μl Opti-MEM medium). After incubation at room temperature for 10 minutes, the mixture was added into wells drop by drop. After 6 hours, Opti-MEM medium was replaced with 2 ml complete DMEM medium. 48 and 72 hours after transfection, 2 and 1.5 ml of lentiviral supernatant were collected per well. The collected supernatant then filtered by 0.45 μm filter and aliquot 500 μl per eppendorf tube. Aliquot

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virions were stored at -80 °C.

3.7 Lentiviral transduction of HEK 293T and K562 cells

HLA-A*11:01-293T, HLA-A*11:01-costiumlatory molecules-293T, HLA-A*11:01-K562, HLA-B*40:01-K562, HLA-C*07:02-K562 and HLA-A*11:01-costiumlatory molecules-K562 were generated by using lentiviral transduction.

One day before lentiviral transduction, HEK 293T-based cells were seeded in 12-well plates. On the day of infection, 500 μl of virions were thawed and mixed with polybrene (final concentration: 8 μg/mL). The virions were added into cells and centrifuged for 60 minutes at 1250g 32℃. After incubating at 37 °C overnight, the virion was replaced with selected culture medium by 1.5 μg/mL of puromycin or with 250 μg/mL hygromycin.

Regarding K562 cells, on the day of transduction, 4×10^5 cells were collected in 50 mL centrifuge tube. then, 500 μl of virions were thawed and mixed with polybrene (final concentration: 8 μg/mL). The virions were used to resuspend cells and centrifuge for 60 minutes at 800g 32℃. After incubating at 37 °C overnight, the virions were replaced with selected culture medium by 1.5 μg/mL of puromycin or with 200 μg/mL hygromycin.

3.8 RNA extraction and RT-qPCR assay

First, total RNA was extracted from cells using TRIzol™ Reagent (Catalog No.

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15596026, Thermo Fisher Scientific). Next, cDNA was synthesized using SuperScript™ III First-Strand Synthesis System (Catalog No. 18080051, Invitrogen). Following the manufacturer's instructions, RT-qPCR was performed with SYBR Green (MorreBio) and the 7500 Real-Time PCR System (Applied Biosystems™, ThermoFisher). *GAPDH* endogenous control gene was used to normalize all gene expression levels.

3.9 Intracellular cytokine staining

The target cells were stained with fluorescent-labeled anti-human surface antibodies for 30 minutes at 4 °C in dark, then cells were washed three times by wash buffer (PBS containing 2 % FBS). Next, 200 μl of fixation and permeabilization solution (BD Cytofix/Cytoperm™) were added to the sample for 20 minutes at room temperature. In the dark, the cells were stained with specific intracellular cytokine antibody after being washed three times with its specific wash buffer (10X dilution with injection water). A FACsCalibur flow cytometer (BD Biosciences) and Flowjo software (version 10.7.1) were used to analyze stained cells.

3.10 Flow cytometry

Target cells were harvested and stained with fluorescent-labeled anti-human antibodies for 30 minutes at 4 °C in dark as follows. The following antibodies were used: anti-HLA-ABC-APC Ab (G46-2.6; BD Biosciences), FITC anti-human CD86 Ab (BU63;

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BioLegend), PE/Cyanine7 anti-human CD137L (4-1BB Ligand) Ab (5F4; BioLegend) , PE anti-human CD252 (OX40L) Ab (11C3.1; BioLegend), PerCP/Cyanine5.5 anti-human CD8 Ab (SK1; BioLegend), Brilliant Violet 421™ anti-human IFN-γ Antibody (4S.B3; BioLegend) and Brilliant Violet 421™ anti-human CD19 Ab (HIB19; BioLegend) . Stained cells were analyzed using a FACsCalibur flow cytometer (BD Biosciences) and Flowjo software (version 10.7.1).

3.11 FACS sorting

For establishment of an HLA-ABC-null cell line, cells co-transfected with 3 plasmids (pLenti-hCas9-puro, pLenti-U6-g953-BSD and pLenti-U6-g1455-BSD) were harvested in the sorting buffer (final 1x PBS, 5mM EDTA, 25mM HEPES, 2% FBS) and stained with anti-HLA-ABC-APC Ab (G46-2.6; BD Biosciences) for 30 minutes at 4°C. Live, HLA-ABC-negative 293T cells were sorted to 15 mL centrifuge tubes using FACSDiva 8.0.1 sorter. After HLA-ABC-negative 293T cells were sorted for the second time, single cells were seeded in 10 cm culture dishes or 96-well plates. At 2 to 3 weeks after seeding, single cell clones were established.

For transduction of mono-allelic HLA-expressing cell lines, class I HLA-null cell line (HEK 293T #54-1-4) and K562 cells were transduced with mono-allelic HLA expressing plasmid. Cells were harvested in sorting buffer (final 1x PBS, 5 mM EDTA, 25 mM HEPES, 2% FBS) and stained with anti-HLA-ABC-APC Ab (G46-2.6; BD

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Biosciences) for 30 minutes at 4°C. Live, HLA-ABC positive cells were sorted to 15 mL centrifuge tubes using FACSDiva 8.0.1 sorter. Then the sorted cells were deeded with limiting dilution, after 2 to 3 weeks the single clones were established.

For TAP genes knock out experiments, HEK 293T #54-1-4 with TAP knock out and further overexpressed with HLA-A*11:01 were added A*11:01 restricted peptide and β2m to pulse these cells. These cells were also harvested in sorting buffer (final 1x PBS, 5 mM EDTA, 25 mM HEPES, 2% FBS) and stained with anti-HLA-ABC- APC Ab (G46-2.6; BD Biosciences) for 30 minutes at 4°C. Then, Live, HLA-ABC positive cells were sorted to 15 mL centrifuge tubes using FACSDiva 8.0.1 sorter. Then the sorted cells were deeded with limiting dilution, after 2 to 3 weeks the single clones were established.

3.12 Polymerase chain reaction (PCR) and sequencing for Single-cell clones

At 2 to 3 weeks after sorting, HLA-ABC-negative single-cell clones were established and cultured in 12-well plates. Clonal genomic DNA was isolated from each clone using a DNeasy Blood & Tissue Kits (QIAGEN) and MagNA Pure LC DNA Isolation Kit I (Roche) according to the manufacturer's instructions. Amplification of each target regions was carried out using PCR with forward and reverse primers specific to HLA-A, HLA-B, and HLA-C exons 2 and 3 and standard protocols. The thermocycler setting consisted of 1 cycle of 98°C for 20 seconds, 7 cycles of 98°C for 5 seconds, 65°C for 30 seconds, and 72°C for 2 minutes, and 31 cycles of 98°C for 5 seconds, 60°C for 30

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seconds, and 72°C for 2 minutes, and 1 cycle of 72°C for 5 minutes. PCR products were analyzed with 1% agarose gels, Taq DNA Polymerase Master Mix RED (AMPLIQON), and a RTUTM Mass 100 DNA Ladder for screening of predicted large deletion clones. PCR products of selected clones were analyzed by Sanger sequencing (by Genomics) using the same primers.

3.13 Peptide binding assay

For T2 or T2/A11 cells, we added 100 μg/mL peptide (A3CON1, HBc 141 and Pol 664; from Chih-Chiang Wang) and 20 μg/mL β2m (from Chih-Chiang Wang) into 10^6 cells in 96-well plates. A flow cytometry analysis was done following overnight incubation at 37°C and 5% CO_2 to detect changes in HLA expression by using HLA-ABC-APC (G46-2.6; BD Biosciences).

For HEK 293T #54-1-4 TAP KO A1101, we seeded 10^6 cells into 6-well plates before adding peptide. Following overnight incubation of cells with 100 μg/mL peptide and 20 μg/mL β2m, the cells were stained for HLA expression using HLA-ABC-APC (G46-2.6; BD Biosciences). HLA-A*11:01-293T was generated by transduction of HLA-ABC null HEK 293T (#54-1-4) with pLVX-A*11:01-β2m. HLA-A*11:01-costimulatory molecules-293T was generated by transduction of HLA-ABC null HEK 293T (#54-1-4) with pLVX-A*11:01-β2m and pLVX-EF1α-OX40L-T2A-4-1BBL-P2A-CD86. HEK 293T, HLA-ABC null 293T (#54-1-4), HLA-A*11:01-293T and HLA-A*11:01-

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costiumulatory molecules 293T were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 292 μg/mL Glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin (100X GPS, Gibco) at 37°C and 5% CO_2. Additionally, 1.5 mg/mL puromycin was added to HLA-A*11:01-293T, HLA-A*11:01-costimulatory molecules 293T. Moreover, 250 μg/mL hygromycin was added to HLA-A*11:01 costimulatory molecules 293T.

HLA-A*11:01-K562, HLA-B*40:01-K562 and HLA-C*07:02-K562 were generated by transduction of K562 with pLVX-A*11:01/ B*40:01/ C*07:02-β2m. HLA-A*11:01-costimulatory molecules-K562 was generated by transduction of K562 with pLVX-A*11:01 and pLVX-EF1α-OX40L-T2A-4-1BBL-P2A-CD86. K562, HLA-A*11:01-K562, HLA-B*40:01-K562, HLA-C*07:02-K562 and HLA-A*11:01-costimulatory molecules-K562 were maintained in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (100X PS, Gibco) at 37°C and 5% CO_2. Additionally, 2 mg/mL puromycin was added to HLA-A*11:01-K562, HLA-B*40:01-K562, HLA-C*07:02-K562 and HLA-A*11:01-costimulatory molecules-K562. Moreover, 200 μg/mL hygromycin was added to HLA-A*11:01-costimulatory molecules-K562.

T2 and T2-A11 were maintained in Iscove's Modified Dulbecco's Medium (IMDM,

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Gibco) supplemented with 10% fetal bovine serum (FBS), 292 μg/mL Glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin (100X PS, Gibco) at 37°C and 5% CO_2.

LCL48 and human Peripheral Blood Mononuclear Cells (PBMCs) were maintained in T cell growth medium, which is Roswell Park Memorial Institute (RPMI 1640, Gibco) supplemented with 10% fetal bovine serum (FBS), 292 μg/mL Glutamin, 100 U/mL penicillin, and 100 μg/mL streptomycin (100X GPS, Gibco) at 37°C and 5% CO_2.

3.14 Peripheral blood mononuclear cells (PBMCs) isolation

A donor who recovered from covid-19 donated PBMCs with the following HLA genotypes: HLA-A*11:01, HLA-B*40:01 and HLA-C*07:02. Three months after recovering from COVID-19, blood samples were collected in EDTA tubes. The PBMCs were isolated by standard procedure of Ficoll gradient centrifugation (Ficoll-Paque^{TM} PLUS; Cytiva, 17144003).

3.15 aAPC functional test

We seeded 2×10^5 HEK 293T-based aAPCs in 48-well plates before stimulation. Next, 1 μg/mL HLA-A*11:01-specific covid-19 peptide (KTFPPTEPK; synthesized by Kelowna company) or 2 μg/mL covid-19 overlapping peptide (Synthesized by JPT Peptide Technologies) was added into the wells. In addition, frozen PBMCs were thawed in 6-well plates containing complete RPMI. The plates were then incubated overnight at

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37°C with 5% CO_2. In the following day, peptide-pulsed aAPCs were washed twice in PBS and 2×10^5 PBMCs were added into these wells for 2 hours. We then added 1.5 μl of 10X Golgi-Stop (BD GolgiStop^{TM}) to each well and incubated them for 5 hours. After 7 hours of stimulation, the PBMCs were collected and used the method of intracellular cytokine staining to analyze the proportion of IFN-γ secretion $CD8^+$ T cells by flow cytometry.

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4 Results

Aim 1

4.1 Establishment of an HLA-null cell line by CRISPR gene editing

In order to develop an artificial APC for stimulating cytotoxic T cells, we first generated a class I HLA-null cell line, which was then transduced with mono-allelic class I HLAs along with the required costimulatory molecules. In the end, an artificial APC was generated. Furthermore, to test the functionality of our artificial APC, we stimulated $CD8^+$ T cells from healthy donors with HLA-matched epitopes. **Figure 1** illustrates this procedure.

First, we used the technique of CRISPR gene editing to knock out the endogenous class I HLAs (HLA-A, B and C) in HEK 293T, as shown in **Figure 2**. HEK 293T cells were chosen as a suitable model for our approach because of their high transfection efficiency, exceptionally high protein production, and homologous haplotypes of HLA class I (A*02:01, B*07:02, and C*07:02). Two gRNAs, g953 and g1455, which target HLA-A, B, and C genes simultaneously (**Supplementary table 1**), and lenti-hCas9-puro plasmid that encodes Cas9 protein were used to knock out endogenous HLA class I molecules. Then, we co-transfected these plasmids into HEK 293T cells. After transfection, we stained the transfected HEK 293T cells with HLA-ABC antibody (HLA-

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ABC-APC, G46-2.6; BD Bioscience). The stained cells were separated into two groups, HLA-positive and HLA-negative expressing cells. We then sorted HLA class I-negative cells into 10-cm plates. After clonal expansion of the sorted cells, the proportion of HLA class I-negative cells progressively predominated. Next, we performed second-time sorting, and most of the cells were HLA class I-negative (**Figure 3A**). We sorted the portion of HLA class I-negative cells into 10-cm plates (HLA class I negative pool).

At 2–3 weeks after sorting, cell clones were cultured and analyzed for the genotypes and expression of HLA class I genes. Then the single clones were analyzed by PCR-based genotyping using specific primer pairs for gRNA target regions in exons 2 and 3 of each HLA class I gene for further validation of the HLA expression (**Figure 3B**).

4.2 Genetic analysis of HLA class I-negative cell clones

We discerned deletions, insertions, or lack of amplification based on the size of PCR product derived from HEK 293T WT genomes (control cell). The PCR products were further detected as single or double bands, indicating the presence of homozygous or heterozygous alleles at class I HLA loci. In the absence of amplification, homozygous large deletions were assumed to be responsible. It is considered the presence of wild-type (WT) class I HLA genes when the PCR product size was the same or similar to that of the control cells; these sequences probably contained no mutations, substitutions, insertions, or deletions.

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Among the clones, #54 was the most promising one in terms of successful deletion of the HLA genes. At the HLA-B and HLA-C loci, this clone showed detectable changes (**Figure 3B**). In the HLA-A loci there was a faint WT PCR product that suggested mixing with non-edited clones. Therefore, we performed a limiting dilution on #54 to further generate subclones. To detect editing, we used the HLA-A exon 1 forward primer and HLA-A reverse primer (**Supplementary table 2**) and found a weak PCR product with the same size as the WT cell (**Figure 3C**). To edit #54-1, we used two other gRNAs, gAE2 and gAE3, in the second round of CRISPR editing.

After generation of the subclones of #54-1, PCR-based genotyping was used to detect the editing. With the HLA-A exon 1 forward primer and HLA-A exon 7 reverse primer, the three clones #54-1-4, #54-1-5, and #54-1-7 demonstrated detectable changes in PCR products (**Figure 3D**). There was a large deletion between HLA-A2 and HLA-H, which was a pseudogene, according to the sequencing results. Further, homologous recombination between the HLA-H and HLA-A2 loci was confirmed by a pair of HLA-H-specific primer and HLA-A2-specific primer (**Supplementary figure 1**). We also performed Western blotting to detect the HLA expression. Based on the results, we further confirmed that the HLA-A, B and C had been deleted (**Figure 3E**). Then the deletion of HLA-A, HLA-B, and HLA-C genes was also validated using Sanger sequencing (**Figure 4**)

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4.3 Establishment of a mono-allelic HLA expressing cell line

Having generated the class I HLA-null cell line (#54-1-4), we transduced the cells with pLVX-A*11:01-β2m to express HLA-A*11:01, which were then selected by puromycin-containing DMEM. Once the population of cells was large enough for sorting, the cells were stained with HLA-ABC-APC (G46-2.6; BD Biosciences) and then sorted for those positive with HLA (**Figure 5A**). By performing the limiting dilution and splitting the cells into 10-cm dishes, we were able to obtain single clones of these cells.

Using home-made anti-HLA Class I antibody (W6/32), we stained the single clones for 30 minutes at 4 °C, and then stained them with second antibody FITC-conjugated-anti-mouse IgG. To evaluate the HLA expression level, we used flow cytometry to detect FITC expression (**Figure 5B**). Clones #9、#50、#52、#54 were those with higher mean fluorescence intensity (MFI) of FITC, so we used HLA -ABC-APC (G46-2.6; BD Biosciences) to stain these clones for a more precise detection of HLA expression. For our artificial APC, clones #9 and #50 were chosen because they exhibited higher MFIs than WT HEK 293T cells by two times (**Figure 5C**).

Additionally, we generated K562-based mono-allelic HLA expressing cells for comparison with HEK 293T-based APC. As K562 cells do not express any HLA molecules on their surface, we only need to transduce them with mono-allelic HLAs. We then used the same protocol as HEK 293T for selecting clones with higher levels of HLA

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expression after transducing K562 cells with pLVX-A*11:01-β2m, pLVX-B*40:01-β2m, and pLVX-C*07:02-β2m. As a result, we generated K562-A*11:01, K562-B*40:01, and K562-C*07:02 (**Supplementary figure 2**).

4.4 Generation of a mono-allelic HLA-expressing artificial APC

Our artificial APC was constructed using HEK 293T #54-1-4 A1101 #50 as the backbone after mono-allelic HLA expressing cell lines were established. Now that our APC had signal 1 already, we needed to add costimulatory molecules for signal 2. The first constructed a plasmid containing CD86, OX40L, and 4-1BBL, three important costimulatory molecules for APC. HEK 293T #54-1-4 A1101 #50 was then transduced with these three molecules through the lentiviral vectors. FITC anti-human CD86 Ab (BU63; BioLegend), PE anti-human CD137L (4-1BB Ligand) Ab (5F4; BioLegend), and PE anti-human CD252 (OX40L) Ab (11C3.1; BioLegend) were used to stain the cells after selection, then the triple-positive expressing cells were sorted (**Figure 5D**).

4.5 Functional test of HEK 293T-based artificial APC

To evaluate the function of the artificial APC, we used covid-19 peptide-pulsed aAPC to stimulate the PBMCs from a donor who recovered from covid-19. Intracellular cytokine staining was performed after PBMCs were stimulated with peptide-pulsed aAPC for 7 hours. Lastly, we analyzed the data using flow cytometry. Data analysis revealed

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that the positive control of anti-CD3/CD28 treatment only produced 5.75% IFN-γ secreting $CD8^+$ T cells, which was lower than normal stimulation. A set of confirmed covid-19 spike overlapping peptides which can stimulate T cells was also used as a positive control, but the stimulated cells were only 0.16%. PBMCs stimulated with HLA-A*11:01 covid-19 peptide-pulsed aAPC with or without costimulatory molecules only produced 0.25 % IFN-γ secreting $CD8^+$ T cells (**Figure 6**).

Consequently, we could not determine whether our artificial APC were functional based on these data. It is necessary to troubleshoot this experiment to determine why stimulation efficiency was so low. Having identified the causes that influence the results, we can test the artificial APC again.

Aim 2

4.6 Establishment of a TAP genes knock out cell line

To create a new tool for epitope mapping, we used the CRISPR gene editing to knock out the TAP genes in the HLA-null cells (HEK 293T #54-1-4) as a backbone. The cells were then transduced with mono-allelic HLA. Lastly, we can perform peptide binding assays using the HLA-restricted peptides (**Figure 7**).

The first step was to generate several gRNAs targeting exons 8 to 11 of the TAP gene. Two gRNAs were transfected into HEK 293T WT cells to test their efficiency. We then

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reversely transcribed the RNA from the transfected cells into cDNA. Finally, we examined the efficiency of gRNAs using RT-qPCR. According to the results, g10933 combined with g11779 had the highest editing rate (**Figure 8A**). Additionally, we extracted genomic DNA from cells and used PCR-based genotyping to confirm the deletion of TAP gene. The truncated PCR product was also detected in g10933/g11779 treated cells (**Figure 8B**). Thus, we selected these two gRNAs for editing HLA null HEK 293T #54-1-4 cells.

After transfecting HLA null HEK 293T #54-1-4 with g10933 and g11779, we selected cells using the selection marker. Following selection, we performed limiting dilution to generate single clones of TAP genes knock out cells. We detected TAP-knock out cells using PCR-based genotyping. As a result, we found truncated PCR products of both TAP 1 and TAP 2 genes in clone #11 (**Figure 9A**). Sanger sequencing was also used to confirm the deletion (**Figure 9B**). In the end, we generated the TAP-deficient HLA-null HEK 293T cells.

4.7 Peptide binding assay of TAP knock out cell line

Then, we transduced the TAP-deficient HLA-null HEK 293T cells with pLVX-A*11:01-β2m. To determine whether TAP-deficient cell lines indeed lose the antigen presentation ability, we performed peptide binding assays after transduction of monoallelic HLAs. We first seeded the TAP-deficient HEK 293T-A*11:01 cells in 6-well

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plates. The next day, we added 100 μg/mL HLA-A*11:01-restricted peptides and 20 μg/mL β2m to the cells, and incubated them for one more day. The cells were then stained with HLA-ABC-APC (G46-2.6; BD Biosciences) to detect HLA expression. According to the results, HLA expression was increased in the cells after adding cognate HLA-restricted peptides (**Figure 10**). The TAP-deficient HEK 293T-A*11:01 cell can therefore be used to perform peptide binding assays. We will generate single clones of these cells in the future for more stable peptide binding assays and for epitope mapping.

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5 Discussion

Aim 1

5.1 Generation of HLA class I-null expressing HEK 293T cells by CRISPR editing and its application

HEK 293T cells were completely eliminated of HLA class I genes using the CRISPR/Cas9 system. Since monoclonal antibodies that are specific for the individual HLA alleles are not always effective and available, screening class I HLA (HLA-A, B and C)-negative cells can be challenging by mono-allelic HLA class I gene editing. Thus, we designed gRNAs that induced large deletions at the HLA-A, HLA-B, and HLA-C loci simultaneously and screened them using PCR with gene-specific primers. Furthermore, we used pan-HLA-ABC monoclonal antibodies, which can recognize all HLA class I molecules, to analyze the expression of HLA class I molecules. In spite of this, this strategy was less efficient due to the fact that the multiple editing of six individual HLA class I gene regions. The advantage of this strategy is that it not only eliminates all HLA class I genes, but also restores the expression of specific alleles and antigen-presenting function instead of inhibiting β2M molecules.

Moreover, as HEK 293T cells can be edited more efficiently through gene delivery or mRNA transfer, HLA class I null-HEK 293T cells can be edited to serve as artificial

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APCs by transferring single alleles of HLA genes. Adoptive cell therapies have been studied with artificial APCs in HLA-deficient cells such as Drosophila cells, mouse fibroblasts, or human leukemia cells. A nonspecific immune response may, however, be induced by xenogeneic or tumor-derived cells, but not by our HEK 293T cells. As a consequence, our mono-allelic HLA class I-expressing HEK 293T cells might be used in place of this system, and offers beneficial characteristics like high transfection efficiency and low background of immunogenicity. In addition, mono-allelic HLA class I-expressing HEK 293T cells combined with immunopeptidomics approaches can provide insight into peptide-based vaccines and universal immunotherapy.

5.2 The homologous recombination occurs in HLA-A of HLA-null expressing HEK 293T cells

Our method for generating HLA-null expressing HEK 293T cells involved the use of CRISPR editing twice to knock out the HLA-A gene, and we found that recombination of HLA-A2 and HLA-H occurred finally. We encountered the CRISPR-induced homologous recombination (HR) situation, which is unusual. To clarify whether this condition occurred, we first used Sanger sequencing (by Genomics) to check whether the PCR products amplified from clone #54-1 were truly identical to WT. This clone had the same PCR product size as HEK 293T WT, so we thought it would not be edited successfully at that time (**Figure 3C**). The sequencing results revealed that the PCR

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product we saw was not HLA-A2 genes, but HLA-K, which is a pseudogene. HLA-K and HLA-A2 have a high level of similarity, so the primer we used for detecting HLA-A2's deletion may also target HLA-K. As a result, the first CRISPR knockout had already succeeded, but we were unaware of it at the time. By the time we performed the second round of CRISPR editing with another two gRNAs targeting HLA-A2, the targets of these gRNAs had already been deleted, so they targeted another HLA allele instead. gRNAs were finally targeted on HLA-H due to the similarity in sequence between HLA-H and HLA-A2 (**Supplementary figure3**) and the distance between them is just about 50K. To conclude, the first CRISPR editing edited HLA-A2, and the second CRISPR editing edited HLA-H as well. Therefore, there were large deletions between them, followed by HR, resulting in a combination of HLA-A2 and HLA-H.

Considering the high similarity between HLA sequences, primers and gRNAs need to be carefully designated to target only the appropriate alleles, otherwise the primers and gRNAs would target other sites, resulting in difficulties interpreting the results.

5.3 HEK 293T-based and K562-based mono-allelic HLA expressing artificial APC

In addition to HEK 293T-based mono-allelic HLA expressing artificial APCs, K562-based mono-allelic HLA expressing artificial APCs were also generated. Our next step will be to test the function of both artificial APCs and compare their stimulation efficacy. A previous study published in 2017 compared HEK 293T-based APC with K562-based

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APC, and found that HEK 293T-based artificial APCs stimulated lower T cell proliferation, but exhibited higher frequency of antigen-specific T cells. The lower T cell proliferation may be due to the different costimulatory molecules, so we hope that our HEK 293T-based artificial APC could yield higher levels of T cell proliferation than K562-based ones by adding other costimulatory molecules.

Moreover, we observed that HLA expression levels gradually decrease when mono-allelic HLA expressing K562 cells are cultivated (**Supplementary figure 4**). Using the same clone, we observed a gradual decrease in MFI. Within a week, the intensity would be 1.5 log lower. We concluded from this phenomenon that when using K562-based APCs to stimulate T cells, we need to use freshly thawed cells or freshly transduce them with mono-allelic HLA. This increases the inconvenience of using K562-based APCs. The mono-allelic HLA and costimulatory molecules of our APCs derived from HEK 293T are stable, so they can overcome this disadvantage. Artificial APCs made from HEK 293T therefore have promising applications in cytotoxic $CD8^+$ T cell activation.

5.4 Functional test of HEK 293T-based artificial APC

According to our results, only 5.75 % of IFN-γ secreting $CD8^+$ T cells were detected by anti-CD3/CD28 stimulation, while few cells were detected by other groups of stimulation. The first reason for low stimulation efficiency may be due to the poor quality of PBMCs. The viability of PBMCs was less than 95%. Second, PBMCs from the donor

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were collected three months after recovering from covid-19, and the frequency of SARS-CoV-2 specific memory T cells might decline at this time point. As a final point, we did not validate the authenticity and activity of the synthesized peptide, so unsuccessful synthesis of functional peptides cannot be excluded. For testing the function of our artificial APC, it is important to ensure all of the points mentioned above.

5.5 Future application of artificial APC

Besides efficiently enriching antigen-specific $CD8^+$ T cells, another purpose of this aAPC is to reverse and stimulate exhausted T cells in chronic diseases. Perhaps we can modify signal 3 to improve and optimize the stimulatory function of this aAPC. Since TCR cloning is difficult, our artificial APC will be beneficial to patients with cancer or chronic viral diseases if we are able to effectively stimulate and expand exhausted antigen-specific T cells.

Aim 2

5.6 Establishment of a TAP genes knock out cell line

As a heterodimer, TAP protein contains two half-transporters, TAP1 and TAP2, with a typical ABC transporter core that consists of two nucleotide binding domains and two transmembrane domains. According to reports, the TAP 2 protein will slowly degrade as the TAP 1 protein is destroyed. As a result, we can design gRNAs that only target TAP 1

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protein. (However, one of the gRNAs we used can target on both TAP 1 and TAP 2 protein). Researchers have previously developed gRNAs targeting the transmembrane domain of TAP 1 protein to inhibit its function. As part of our research, we designed gRNAs targeting exons 8 to 11 of the nucleotide domain of TAP 1 protein. These exons encode sections of the ATP-binding domain containing Walker A and Walker B motifs, which coordinate ATP binding and hydrolysis. Therefore, gRNAs designed to target exons 8 to 11 of TAP1 protein can also ruin its function. Our successful peptide binding assay confirmed that our TAP-deficient mono-allelic HLA expressing cells could indeed destroy and be used to test peptide binding for further research (**Figure 10**).

5.7 TAP-deficient mono-allelic HLA expressing HEK 293T cells

In recent years, T2 cells have become the most widely used tool for peptide binding assays. In spite of this, T2 cells express HLA-A2, HLA-B51, and HLA-Cw1 on their surface, which makes it difficult to determine which epitope is associated with which HLA allele on its surface. It is therefore necessary for peptide binding assays to use cells expressing mono-allelic HLA that lack TAP protein.

In 2021, a team used human B cell line 721.221 that were considered HLA-null expressing as the backbone, then performed sequential transductions to knock out TAP genes and transferred mono-allelic HLA to the cells. 721.221 cells, however, were recently found to not be HLA-null cells; instead, they also express few HLA class I

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molecules. As a result, our HEK 293T cells expressing mono-allelic HLA have the advantage that they express mono-allelic HLA only, and can map epitopes more precisely.

For peptide binding assays, we knocked out the TAP genes on HEK 293T using CRISPR/Cas9 system. A TAP-deficient HEK 293T-based cells can easily be manipulated and edited. In this way, the desired mono-allelic HLA can be transferred on the surface of the cells and peptide binding assays can then be performed with the identified peptides. Even though the experiment scale of HEK 293T cells is larger than the size of T2 cells, which can carry out experiments in 96-well plates, and the amount of peptide be used is also larger, HEK 293T cells can identify any HLA-restricted peptides if we transfer this HLA allele. With this advantage, the generation of this cell line is expected to increase the accuracy of epitope mapping.

5.8 Future application of TAP-deficient mono-allelic HLA expressing HEK 293T cells

T-cell epitope mapping is clinically important, particularly for patients with cancer or chronic viral diseases, but it is usually labor- and time-consuming. MS-based immunopeptidomics in combination with mono-allelic TAP-deficient cell lines will be a robust platform for unbiasedly discovery and validation of HLA-restricted T cell epitopes.

In conclusion, we have generated a novel platform with artificial APC and TAP-deficient cells, which can be used to increase the effectiveness of T cell stimulation and

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epitope mapping, thereby providing a promising strategy to enhance the efficacy of T cell-based immunotherapy in the future.

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6 Figures

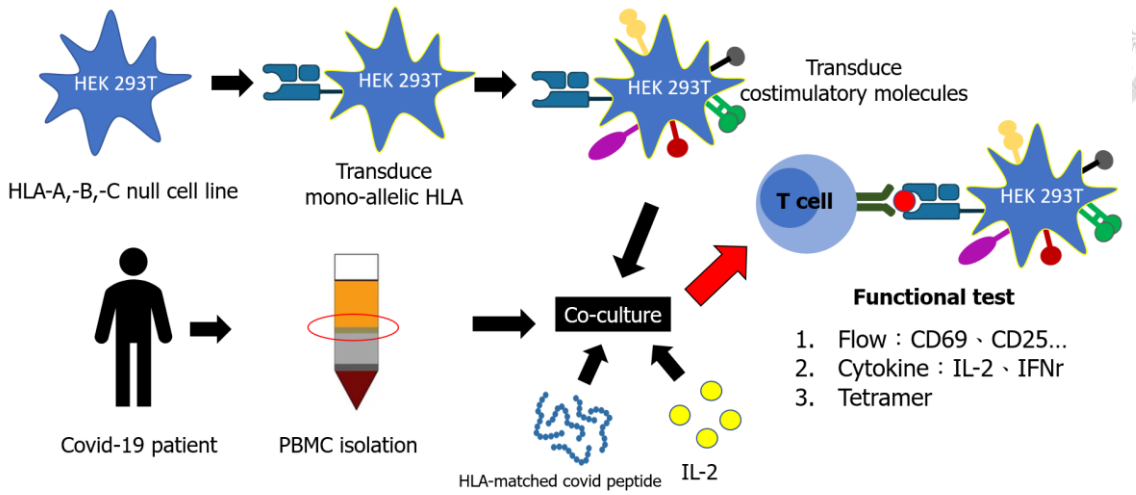

Figure 1. Schematic illustration of the flowchart for generation of a functional artificial APC

To generate HLA-null cell lines, we knocked out the endogenous class I HLAs of HEK 293T cells using the CRISPR gene editing strategy. The artificial APCs are generated by transducing HLA-null cells with individual mono-allelic HLAs and the required costimulatory molecules. With the artificial APCs pulsed with an HLA-matched covid-19 peptide and some cytokines, we will stimulate PBMCs isolated from covid-19 recovered patient. To evaluate the function of the artificial APCs, we can detect activation markers and cytokine secretion of T cells by flow cytometry.

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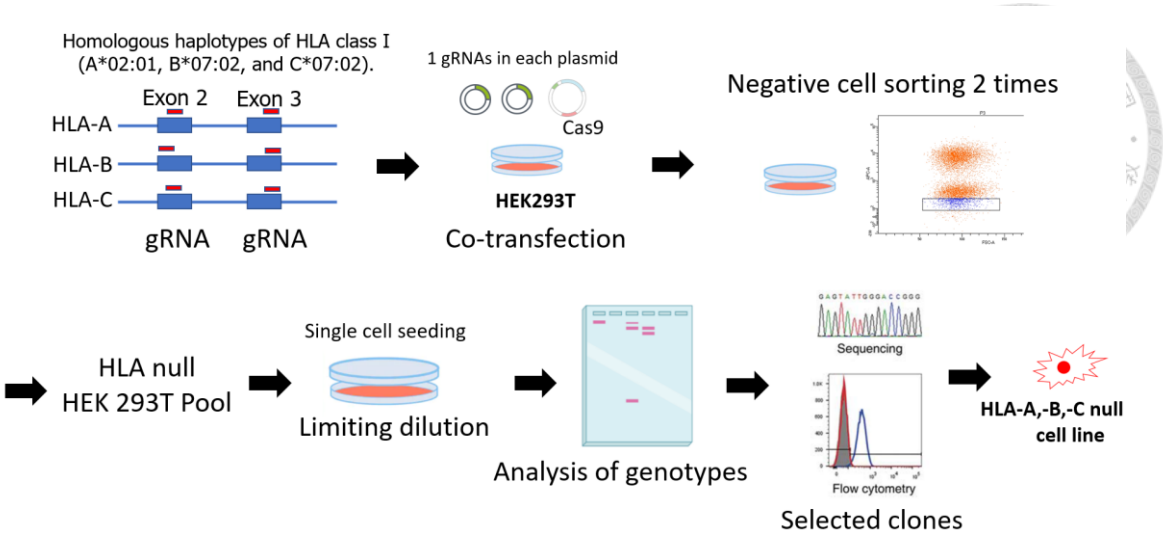

Figure 2. Schematic illustration of the flowchart for generation of HLA-null HEK 293T cells

We designed gRNAs targeting exon 2 and exon 3 of HLA-A, B, and C to generate HLA-null HEK 293T cells. Following co-transfection of the gRNAs and Cas9 plasmids, we stained the cells with HLA-ABC-APC and then carried out two rounds of HLA negative sorting. The genotypes of clones were then determined by PCR after single cell seeding. The deletion of HLA was also confirmed by Sanger sequencing.

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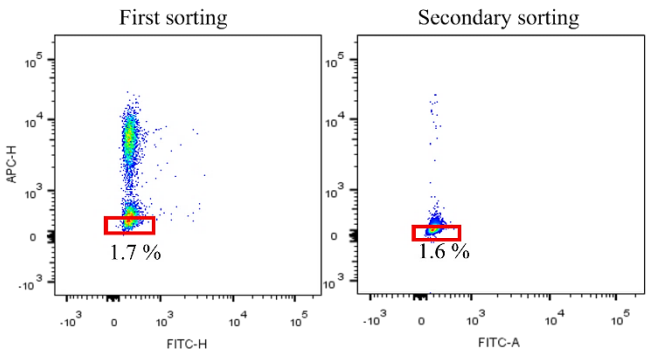
A.



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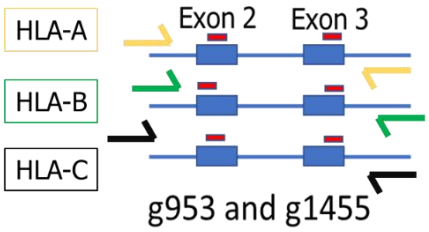
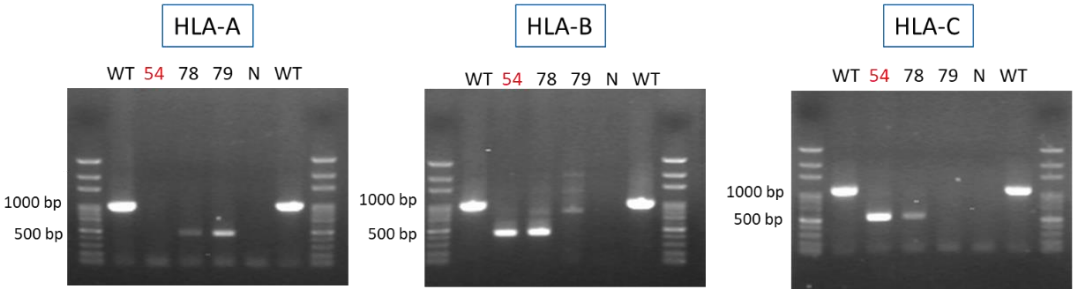


	WT size	Deleted size	Predicted size
HLA-A	986 bp	513 bp	473 bp
HLA-B	943 bp	517 bp	426 bp
HLA-C	1035 bp	522 bp	513 bp



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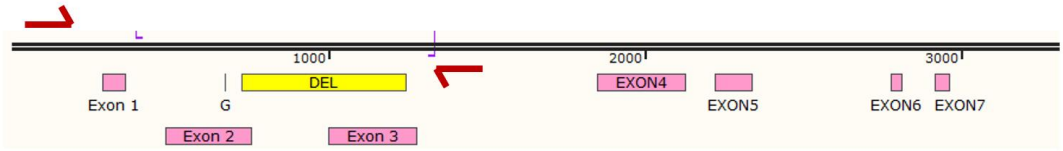

Exon1 F' + Exon 3 R'

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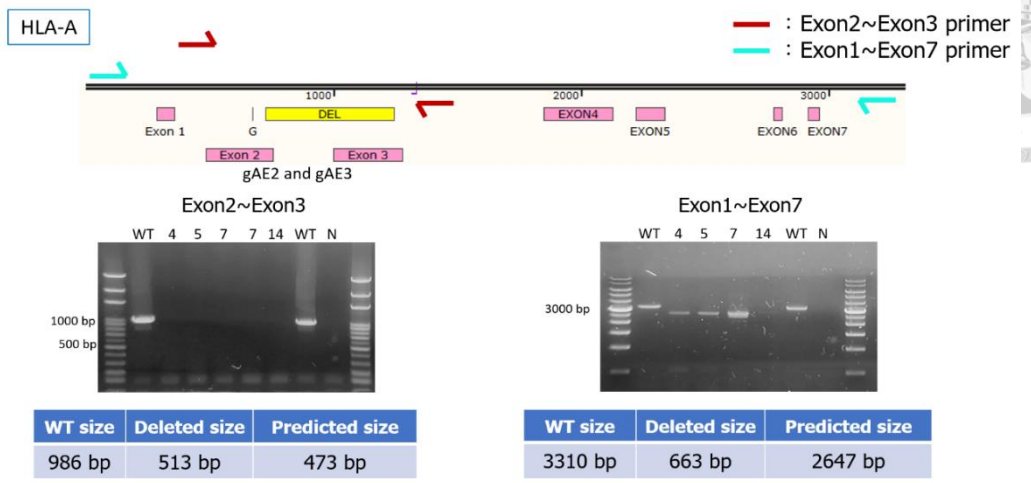
D.



WT size	Deleted size	Predicted size
986 bp	513 bp	473 bp

WT size	Deleted size	Predicted size
3310 bp	663 bp	2647 bp

E.

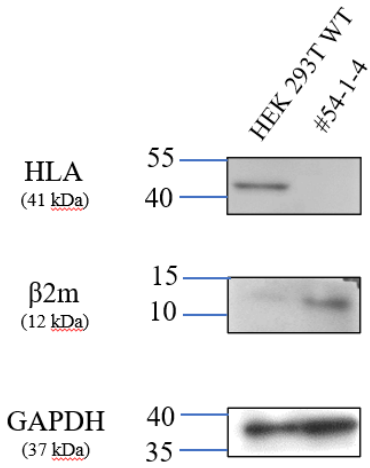


Figure 3. Screening and identification of class I HLA-negative HEK 293T cell lines

A. After transfection of gRNAs, the expression of HLA class I molecules was analyzed by staining with HLA-ABC antibody and fluorescence-activated cell sorting. The proportion of HLA class I-negative cells was predominate after second time sorting.

B. Following clonal separation, targeted PCR was used to analyze the genotypes of the single clones. According to the results, clone #54 may be the most promising for becoming HLA-null.

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C. Clone #54 was re-seeded to eliminate the possibility of HLA-null cells mixing with WT cells. Additionally, we carried out PCR-based genotyping in order to detect the expression of HLA. Weak PCR products with a size close to WT were found in the results.

D. We used another gRNAs (gAE2 and gAE3) to edit exon 2 and exon 3 of HLAs, then using the same protocol to generate subclones. After genotyping the subclones by PCR, we used the primers to amplify the exon 1 to exon 7 could detect deletion PCR product in #54-1-4, #54-1-5 and #54-1-7.

E. We carried out Western blotting to confirm no HLA expression in our #54-1-4 clone.

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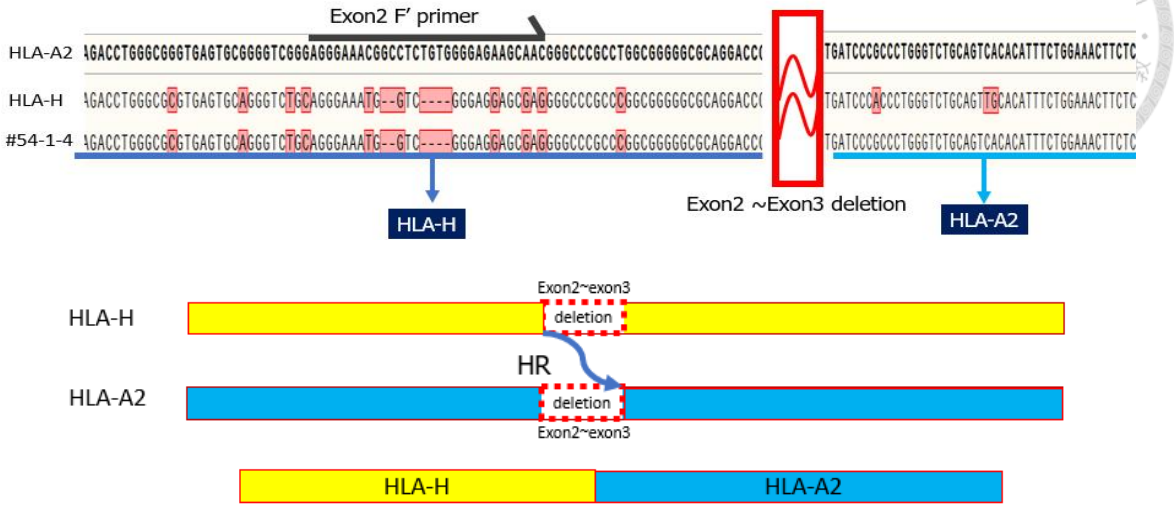
A.



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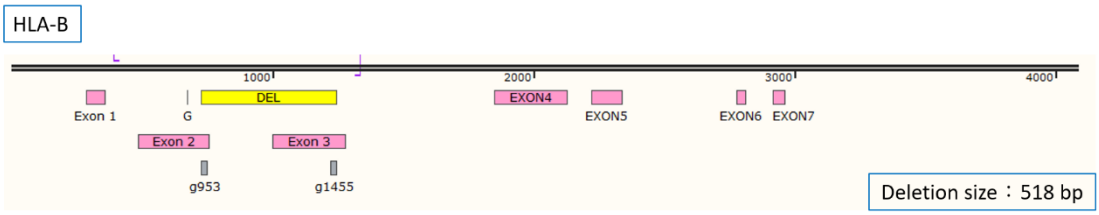

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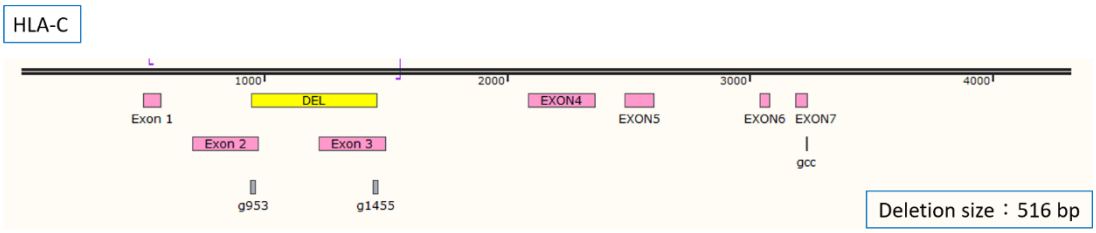

Figure 4. Mapping of the CRISPR-induced deletion of the HLA-A locus in HLA null #54-1-4

A. We used Sanger sequencing to confirm the deletion condition of HLA-A, and found that there were large deletion and homologous recombination occurring between the HLA-A2 and HLA-H loci.

B. Confirmation of the deletion condition of HLA-B by Sanger sequencing.

C. Confirmation of the deletion condition of HLA-C by Sanger sequencing.

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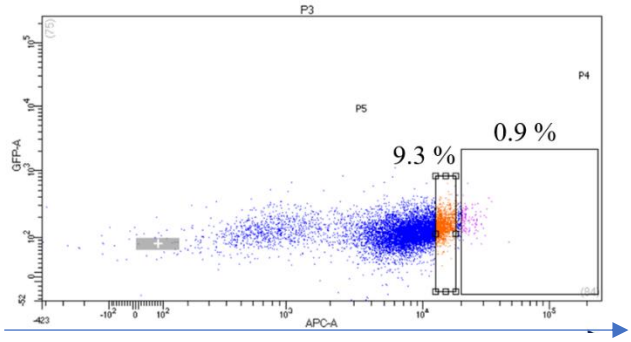
A.



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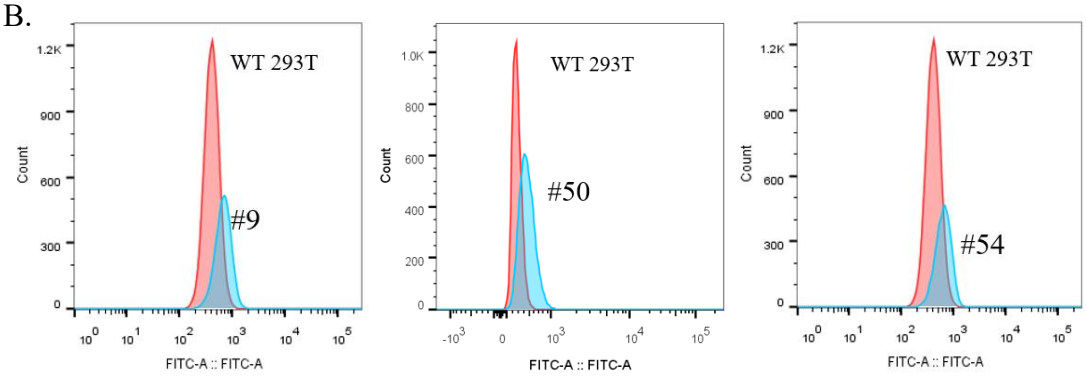
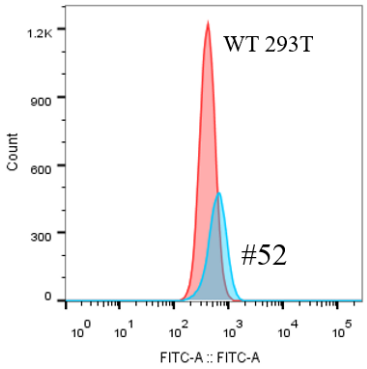
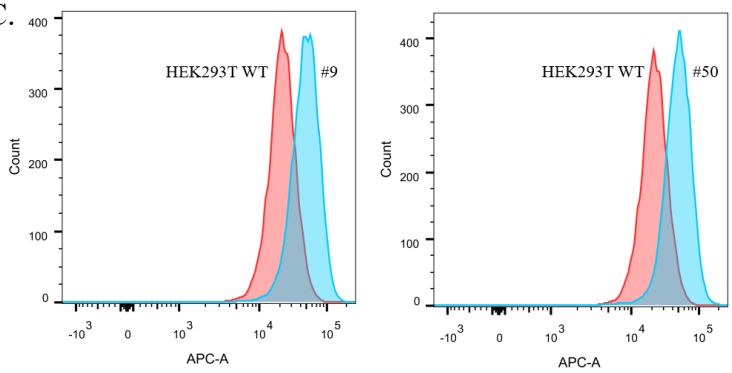




Clone number	MFI of clone / MFI of WT
#9	1.65
#50	1.88
#52	1.53
#54	1.56

C.



Clone number	MFI of clone / MFI of WT
#9	2.22
#50	2.25

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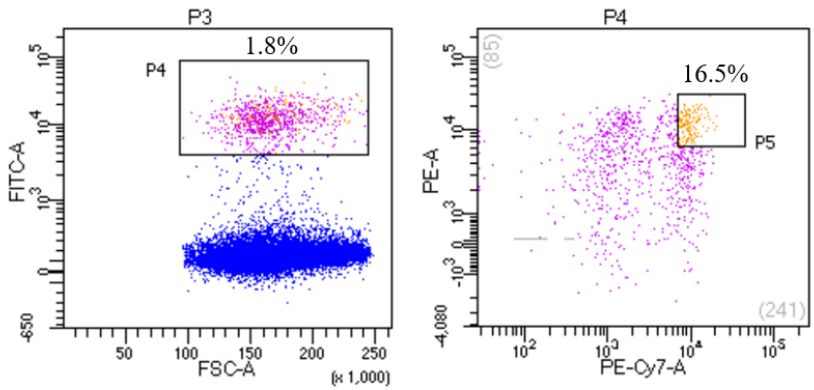

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Figure 5. Selecting mono-allelic HLA expressing HEK 293T #54-1-4

A. We transduced HLA-A*11:01 gene to the HLA-null cell line #54-1-4, and then stained the cells with HLA-ABC-APC to sort HLA-positive cells. We sorted two groups of cells with different HLA expression levels, and then performed the limiting dilution to generate single clones.

B. The HLA expression level was evaluated by home-made anti-HLA Class I antibody (W6/32) in combination with the second antibody FITC-conjugated-anti-mouse IgG. Clones #9、#50、#52、#54 were those with higher mean fluorescence intensity of FITC.

C. We used HLA -ABC-APC (G46-2.6; BD Biosciences) to stain these clones for a more precise detection of HLA expression. In the artificial APC, clones #9 and #50 were selected because their MFIs were higher than HEK 293T WT's by two times.

D. We transduced clone #50 with pLVX-EF1α-OX40L-T2A-4-1BBL-P2A-CD86 to express CD86, OX40L and 4-1BBL, which are important costimulatory molecules for APCs. We then stained the cells with FITC-conjugated anti-human CD86 Ab (BU63; BioLegend), PE anti-human CD137L (4-1BB Ligand) Ab (5F4; BioLegend), and PE anti-human CD252 (OX40L) Ab (11C3.1; BioLegend), then sorted triple-positive expressing cells.

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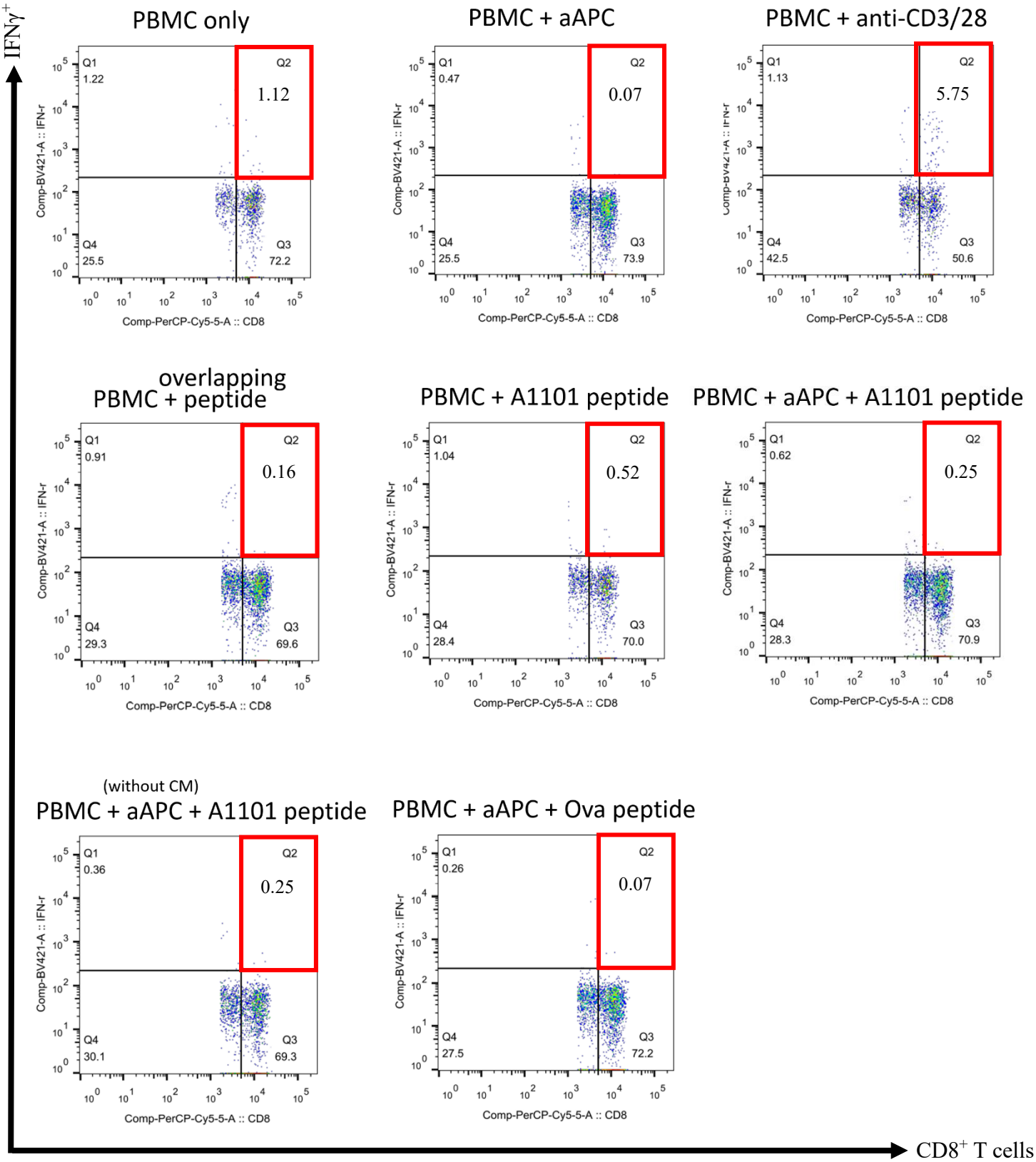

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Figure 6. Functional test of HEK 293T-based aAPC

PBMCs were stimulated with peptide-pulsed aAPC for 7 hours, then intracellular cytokine staining was performed to detect IFN-γ secreting $CD8^+$ T cells. Finally, analyze the data using flow cytometry. Data revealed that the positive control of anti-CD3/CD28 treatment produced 5.75 % IFN-γ secreting $CD8^+$ T cells, and another positive control using covid-19 overlapping peptide triggered 0.16 %. The unstimulated group of PBMC only, PBMC with aAPC and PBMC with non-HLA-A*11:01 Ova peptide-pulsed aAPC were detected 1.12 %, 0.07 % and 0.07 % respectively. The stimulated group of PBMC with covid-19 HLA-A*11:01 peptide stimulate 0.52 % IFN-γ secreting $CD8^+$ T cells, however, our covid-19 HLA-A*11:01 peptide-pulsed aAPC with and without costimulatory molecules triggered 0.25 %.

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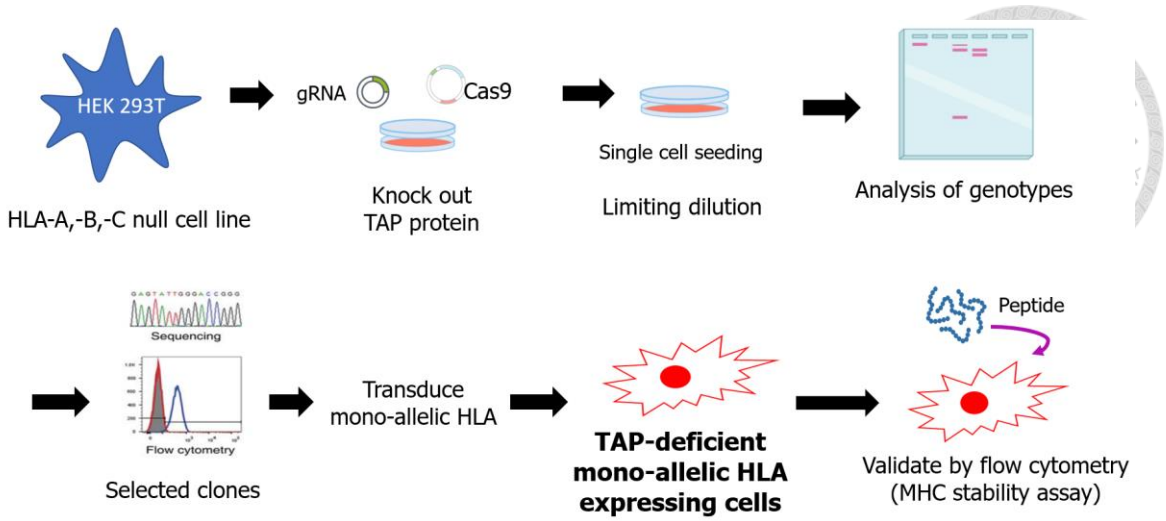

Figure 7. Schematic illustration of the flowchart for generation of TAP-deficient mono-allelic HLA expressing HEK 293T cells.

We used CRISPR to knock out the TAP genes in HLA-null cells (HEK 293T #54-1-4). After single cell seeding to generate single clones, we then used PCR-based genotype to detect the TAP genes knock out condition. After generating the TAP-deficient cells, the cells were then transduced with mono-allelic HLA. In the end, we carried out the peptide binding assay using the HLA-restricted peptide to test our TAP-deficient cell line whether can be used to epitope mapping.

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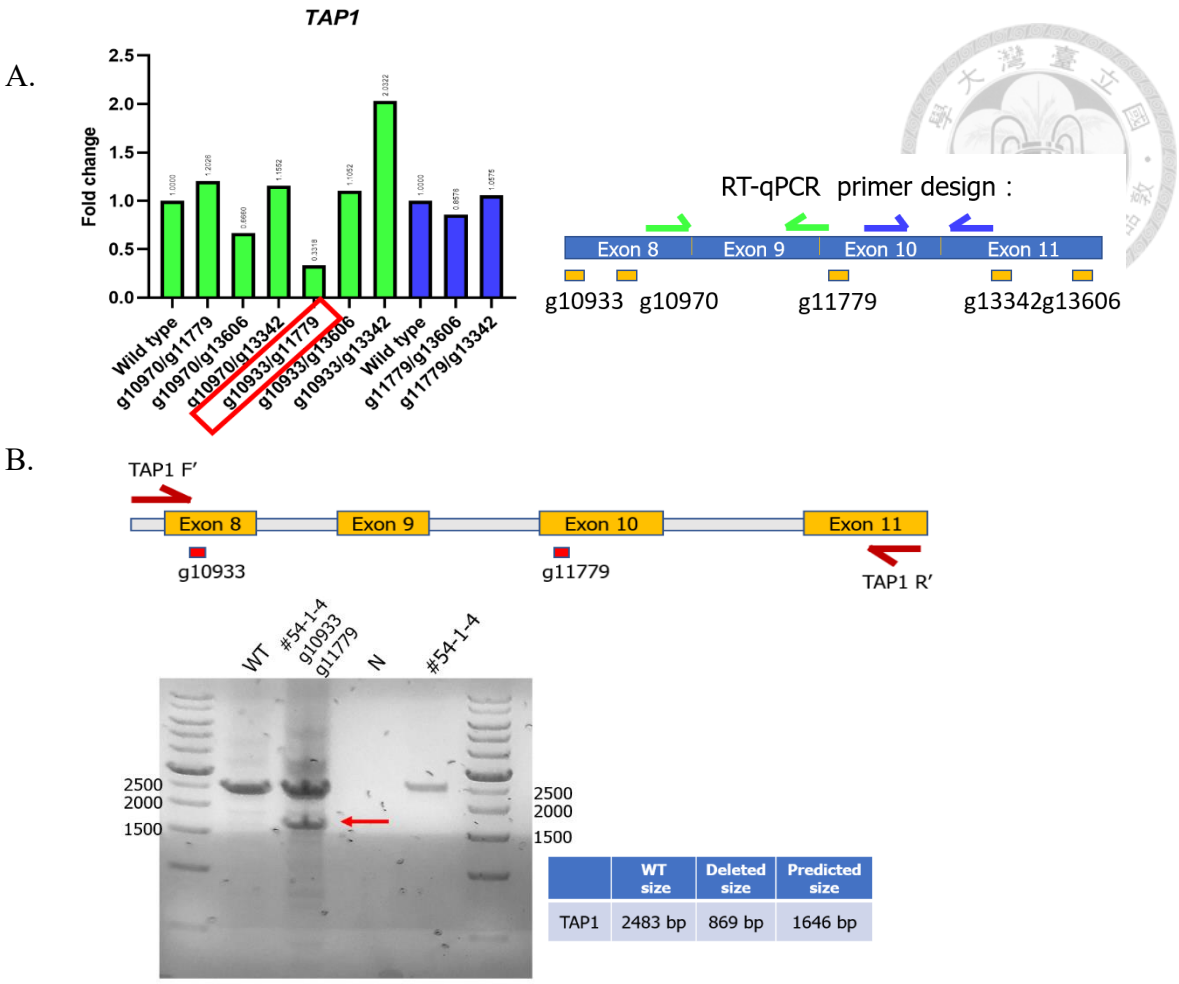

Figure 8. gRNA editing efficiency to knock out TAP genes by RT-qPCR.

A. We generated several gRNAs target on exon 8 to exon 11 of TAP protein, then transfected HEK 293T to test its editing efficiency. We then reversely transcribed the RNA from the transfected cells into cDNA and examined the efficiency of gRNAs using RT-qPCR. According to the results, g10933 combined with g11779 had the highest editing rate.

B. We extracted genomic DNA from cells and used PCR-based genotyping to confirm the deletion of TAP gene. A deletion PCR product was also detected in g10933/g11779-treated cells.

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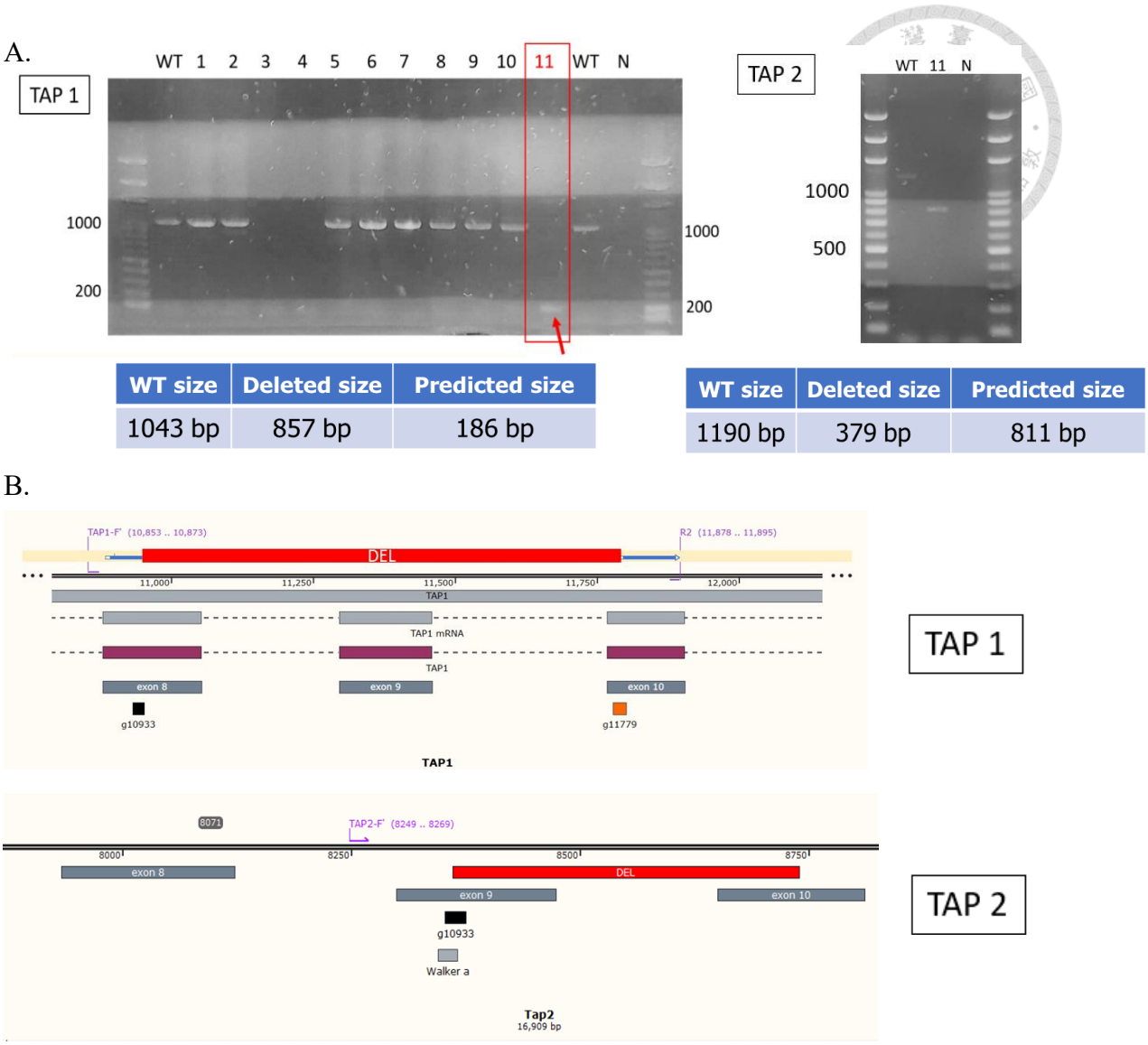

WT size	Deleted size	Predicted size
1043 bp	857 bp	186 bp

WT size	Deleted size	Predicted size
1190 bp	379 bp	811 bp

Figure 9. Screening and identification of TAP-knockout HEK 293T #54-1-4 cells.

A. We transfected HLA-null HEK 293T #54-1-4 with g10933 and g11779, used selection marker to select cells, and then performed limiting dilution to generate single clones of TAP-knockout cells. We detected TAP-knockout cells using PCR-based genotyping, and identified the deletion PCR products of TAP 1 and TAP 2 proteins in clone #11.

B. Sanger sequencing was carried out to confirm the deletion condition of TAP 1 and TAP 2 protein.

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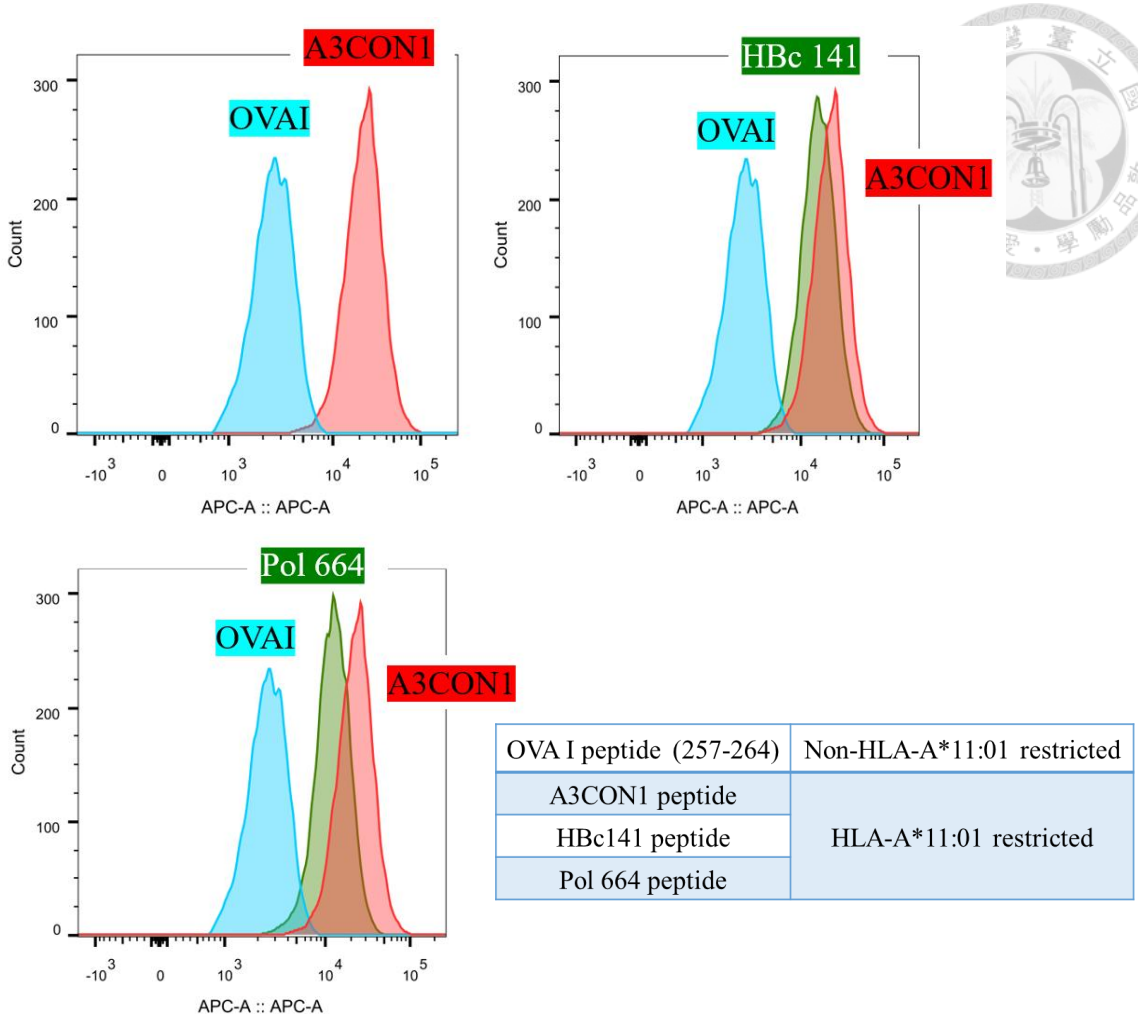

OVA I peptide (257-264)	Non-HLA-A*11:01 restricted
A3CON1 peptide	HLA-A*11:01 restricted
HBc141 peptide	
Pol 664 peptide	

Figure 10. Peptide binding assay of HLA-A*11:01 HEK 293T TAP-knockout cells

We used the TAP-deficient mono-allelic HLA expressing cells for peptide binding assay. Three HLA-A*11:01-restricted peptides, A3CON1, HBc 141 and HBV Pol 664 peptides, were used to stabilize the structure of class I HLA. Additionally, a non-relevant peptide OVA I was used as a negative control because it is not an HLA-A*11:01 restricted-peptide. HLA expression was determined by staining of HLA-ABC-APC antibody and analysis with flow cytometry.

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8 Supplementary information

gRNA ID	gRNA Sequences with PAM
g953	AGACCAACATCATCGGCGCGTCC
g1455	GGAGTGGCTCCGCAGATACCTGG
gAE2	GAGCCAGAGGATGGAGCCGCGGG
gAE3	GAAGGAGACGCTGCAGCGCACGG

Supplementary table 1. gRNA sequences for HLA-A, B, C target editing

Primer ID	Primer Sequence (5' to 3')
HLA-A forward	GAAACSGCCTCTGYGGGGAGAAGCAA
HLA-A reverse	TGTTGGTCCCAATTGTCTCCCCTC
HLA-B forward	GGGAGGAGCGAGGGGACCSCAG
HLA-B reverse	GGAGGCCATCCCCGGCGACCTAT
HLA-C forward	CTCMTCCTGCTGCTCTCGGGA
HLA-C reverse	TGGGAGGCCATSCCGGGAGAT
HLA-A exon 1 forward	CCCGAAGGCGGTGTATGGAT
HLA-A exon 7 reverse	TGGGGGTGGGGTGGTCTCC
HLA-B exon 1 forward	GAGGGCCGCGTCTGCAAT
HLA-B exon 7 reverse	AGAACAGTCCTTGATGACGGGT
HLA-B exon 1 forward	CCTGAGGTGAGGTAAGGTAAGGC
HLA-B exon 7 reverse	GAAGGAACAATTCTAGACTATGGACCC
HLA-H forward	GCAGGGTCTGCAGGGAAATGG
HLA-A2 reverse	CACTGCCTGGGGTAGAACAAAAACAGA

Supplementary table 2. Sequences of HLA-A, HLA-B, HLA-C, HLA-H specific primers

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Primer ID	Primer Sequence (5' to 3')
TAP1-g5526 forward	CACCGCTGGGCCGTGCTCTGGCTG
TAP1-g5526 reverse	AAACCAGCCAGAGCACGGCCCAGC
TAP1-g9893 forward	CACCGGTGGGCAGCTGGTGACCAG
TAP1-g9893 reverse	AAACCTGGTCACCAGCTGCCCACC
TAP1,2-g10933 forward	CACCGTGGGTCTGGGAAGAGCACAG
TAP1,2-g10933 reverse	AAACCTGTGCTCTTCCCAGACCCAC
TAP1-g10970 forward	CACCGAATCTGTACCAGCCCACCGG
TAP1-g10970 reverse	AAACCCGGTGGGCTGGTACAGATTC
TAP1-g11779 forward	CACCGCTGGGAGCCAGCTGTCAGG
TAP1-g11779 reverse	AAACCCTGACAGCTGGCTCCCAGC
TAP1-g13606 forward	CACCGCCAGGGCTGATGCTTTGGTG
TAP1-g13606 reverse	AAACCACCAAAGCATCAGCCCTGGC
TAP1-g13342 forward	CACCGAAAGGGGTGCTACTGGGCCA
TAP1-g13342 reverse	AAACTGGCCCAGTAGCACCCCTTTC

Supplementary table 3. gRNA primers design

gRNA ID	gRNA Sequences with PAM
g5526	GCTGGGCCGTGCTCTGGCTGGGG
g9893	GGTGGGCAGCTGGTGACCAGTGG
g10970	AATCTGTACCAGCCCACCGGGGG
g11779	GCTGGGAGCCAGCTGTCAGGGGG
g13606	CCAGGGCTGATGCTTTGGTGTGG
g13342	AAAGGGGTGCTACTGGGCCATGG
g10933	TGGGTCTGGGAAGAGCACAGTGG

Supplementary table 4. gRNA sequences for TAP genes knock out

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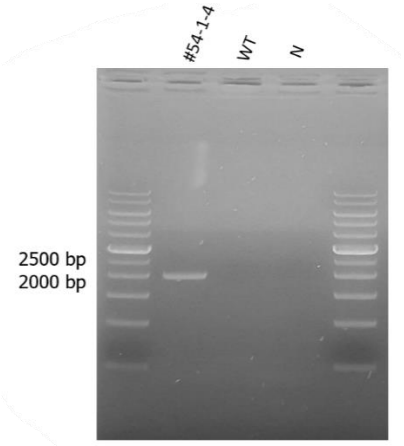
Primer ID	Primer Sequence (5' to 3')
TAP1-qPCR-F1	CCCAGGCTGTGGAGGTAC
TAP1-qPCR-R1	CACCAGCGCCGTCAC
TAP1-qPCR-F2	GCACCGCTACCTGCACA
TAP1-qPCR-R2	CCCTGAGGGAGTCCAGAGA
TAP1-qPCR-F3	CGAGCATTGATCCGGAAACC
TAP1-qPCR-R3	ATGAGCTGCTGGTGGGTTC
TAP2-long-F'	AGAGCCGGTAGCGAGGT
TAP2-long-R'	AGCAACAGCAAAAGACTTCCA
TAP2-F'	GGATTTTGAGGGAGAAGGGGC
TAP2-R'	AGGTCCTTCGTCCTCCCT
TAP1-long-F'	GAGGACTCGGCGGTACC
TAP1-F'	CTTATCGTGTGCTTCTCTGGC
TAP1-R'	GCTGAAAAGTGCGCTGCAA
TAP1-R2	GCTGTTTGCATCCAGGGC

Supplementary table 5. Sequences of TAP1 and TAP2 specific primers



Supplementary figure 1. Confirmation of homologous recombination (HR) in #54-1-4

We designed primers one is specific to HLA-H and one is to HLA-A2 to confirm whether

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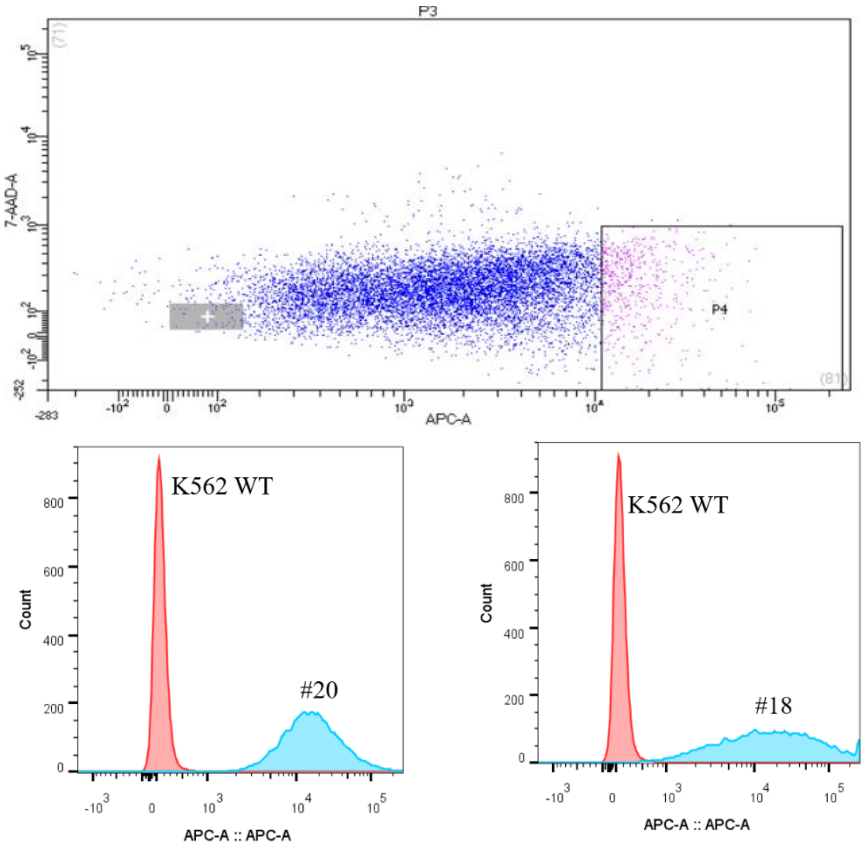
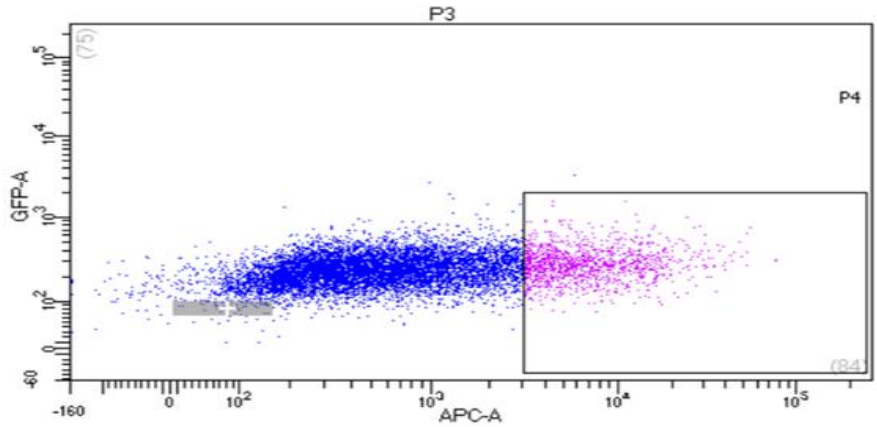
our clone is a recombinant of HLA-A2 and HLA-H. WT cells is not the recombinant, so we cannot amplify PCR product by these two primers; however, our clone can. Hence, we can confirm that there were large deletion and homologous recombination occurred in our clone.

A.



B.



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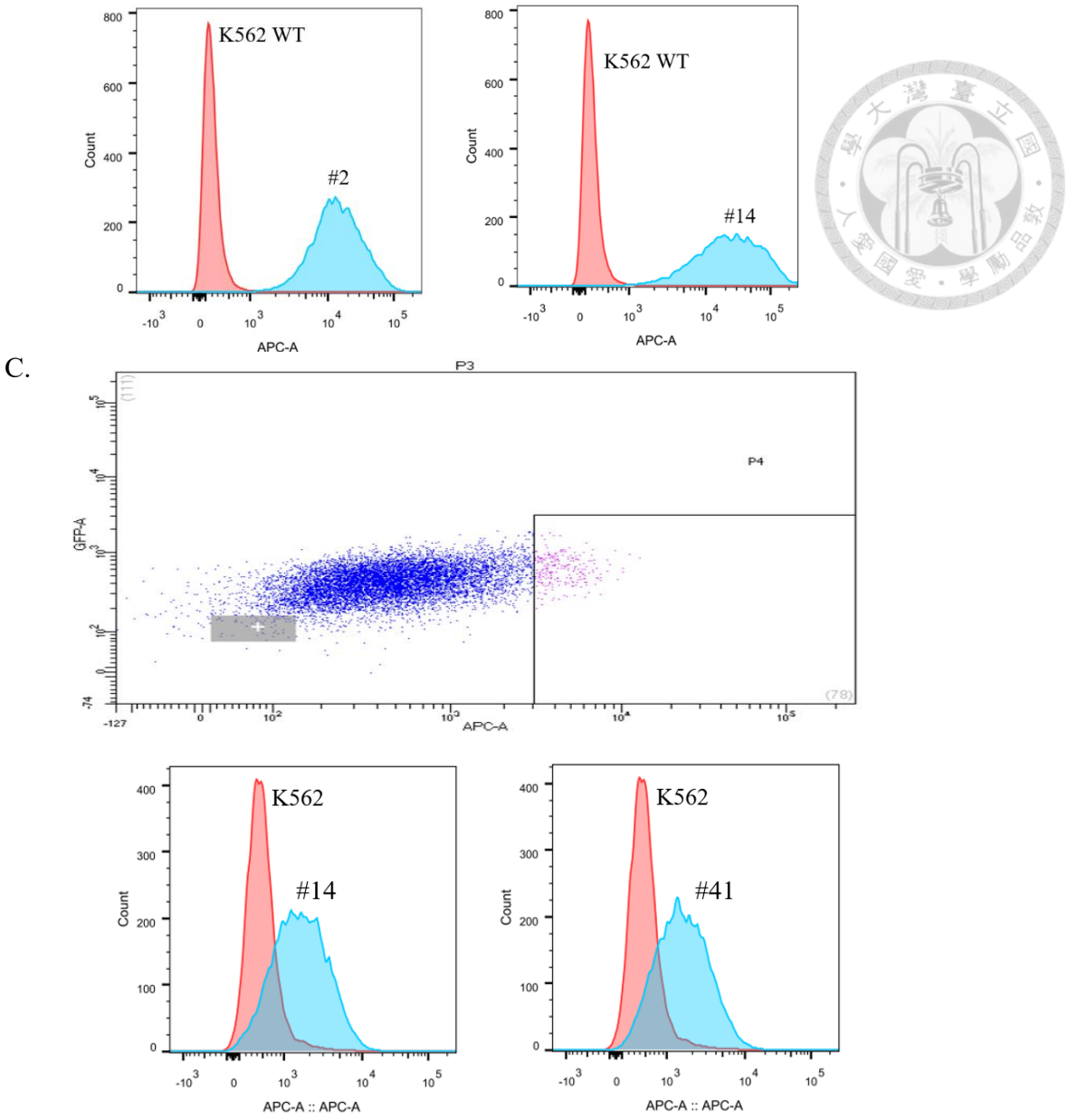

Supplementary figure 2. Mono-allelic HLA expressing K562 cells

A. The results of sorting K562-A*11:01 pooled and the HLA expression condition of selected clones.

B. The results of sorting K562-B*40:01 pooled and the HLA expression condition of selected clones.

C. The results of sorting K562-C*07:02 pooled and the HLA expression condition of selected clones.

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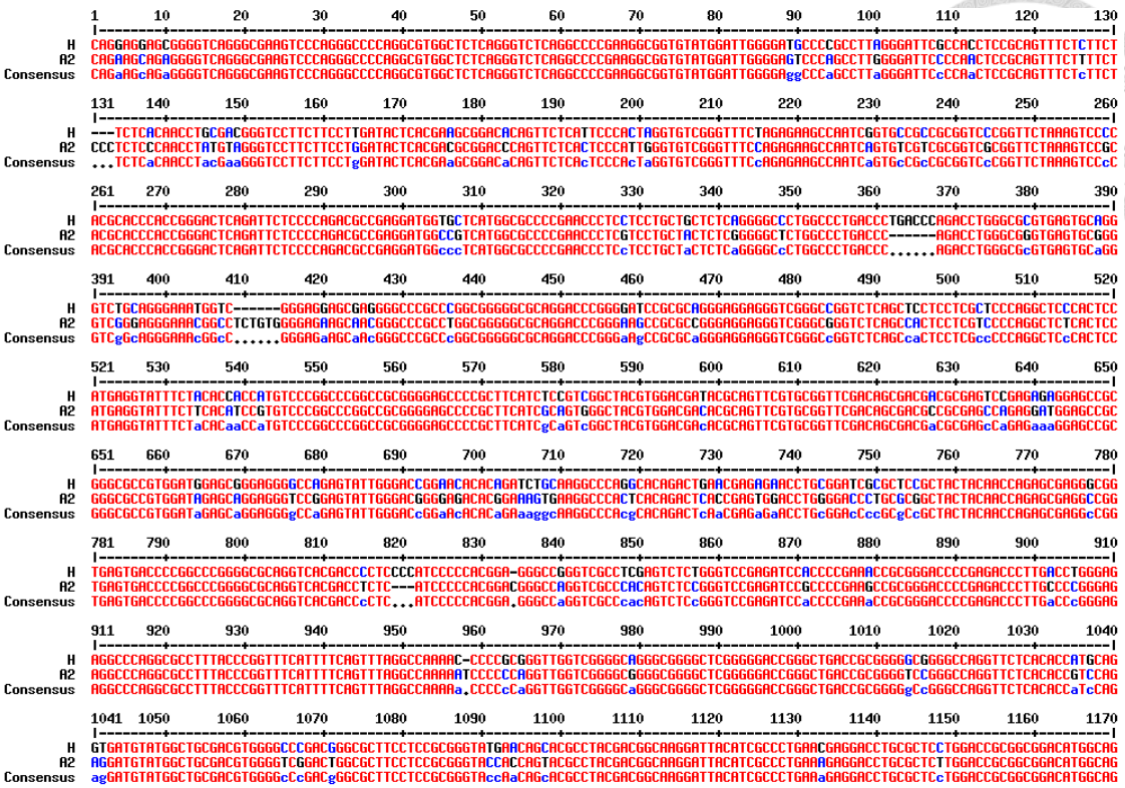

Supplementary figure 3. Alignment between HLA-H and HLA-A2

The alignment between HLA-H (first line) and HLA-A2(second line) showed the sequence of them are too similar. Red represents the same, while blue represent different sequences.

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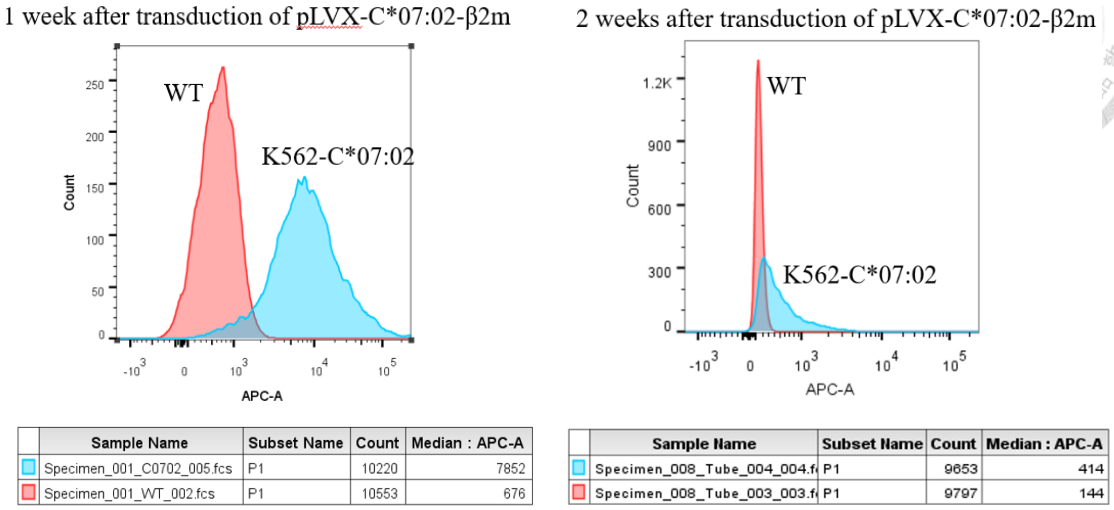
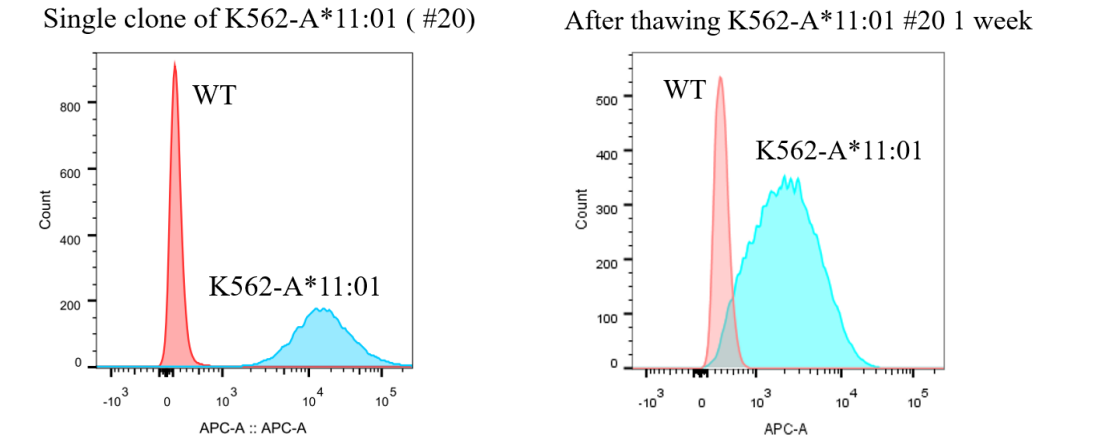
A.



Sample Name	Subset Name	Count	Median : APC-A
Specimen_001_C0702_005.fcs	P1	10220	7852
Specimen_001_WT_002.fcs	P1	10553	676

Sample Name	Subset Name	Count	Median : APC-A
Specimen_008_Tube_004_004.f	P1	9653	414
Specimen_008_Tube_003_003.f	P1	9797	144

B.



Sample Name	Subset Name	Count	Median : APC-A
20220628 20.fcs	P1	9998	16192
20220628 WT (APC).fcs	P1	10144	151

Sample Name	Subset Name	Count	Median : APC-A
Specimen_001_Tube_003_003.f	P1	7905	326
Specimen_001_Tube_004_004.f	P1	25377	2185

Supplementary figure 4. HLA expression level of K562-based artificial APC will decrease

A. The left figure represented the HLA expression level of K562 cells 1 week after pLVX-C*07:02-β2m transduction. The right figure represented the HLA expression

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level of K562 cells 2 weeks after transduction. We could find out the MFI of K562-C*07:02 decreased in one week compared to K562 WT cells. The HLA expression was detected by staining HLA-ABC-APC.

B. The left figure represented the HLA expression level of single clones of K562-A*11:01 #20. The right figure represented the HLA expression level of K562-A*11:01 clone #20 1 weeks after thawing. We could find out the MFI of K562-A*11:01 decreased in one week compared to previous MFI of clone #20. The HLA expression was detected by staining HLA-ABC-APC.

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