國立臺灣大學醫學院微生物學研究所

碩士論文

Graduate Institute of Microbiology

College of Medicine

National Taiwan University

Master Thesis

建立雙參數報告系統以有效篩選高功能性 T 細胞受體以應用於 T 細胞免疫療法

Development of a dual-parameter reporter system to facilitate the screening of highly functional T cell receptors for T cell-based immunotherapy

林家安

Chia-An Lin

指導教授：楊宏志 博士

Advisor : Hung-Chih Yang, Ph.D.

中華民國 112 年 7 月

July 2023

doi:10.6342/NTU202303793

國立臺灣大學碩士學位論文
口試委員會審定書
MASTER'S THESIS ACCEPTANCE CERTIFICATE
NATIONAL TAIWAN UNIVERSITY

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

建立雙參數報告系統以有效篩選高功能性T細胞受體以應用於T細胞免疫療法

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

Development of a dual-parameter reporter system to facilitate the screening of highly functional T cell receptors for T cell-based immunotherapy

本論文係 林家安 (姓名) R10445110 （學號）在國立臺灣大學 微生物學 (系/所/學位學程)完成之碩士學位論文，於民國 112 年 7 月 28 日承下列考試委員審查通過及口試及格，特此證明。

The undersigned, appointed by the Department / Institute of Microbiology on 28 (date) 7 (month) 2023 (year) have examined a Master's thesis entitled above presented by Chia-An Lin (name) R10445110 (student ID) candidate and hereby certify that it is worthy of acceptance.

口試委員 Oral examination committee:

楊宏志

（指導教授 Advisor）

系主任/所長 Director:

doi:10.6342/NTU202303793

致謝

轉眼間，兩年的碩士班生活就劃下了句點。我感到非常榮幸能夠加入由宏志老師帶領的R722大家庭。在這裡，我們不僅能夠盡情地學習知識，還能透過與實驗室的其他成員相處，學習團隊合作的重要性。回想起這段過程，充滿了豐富的收穫。

首先，我要感謝R722的大家長，和善又幽默的宏志老師。感謝您給予我這個學習的機會，也衷心感謝您即使在忙碌的時候仍會停下腳步，關心我們的學習狀況。在您身上，我不僅學到了無限的專業知識，也受到了您樂觀和上進的榜樣。我期許自己未來能夠像您一樣，正面迎接任何困難。同樣地，我要感謝牟昀老師、口試委員董馨蓮老師以及曾岱宗醫師。每次的口試中，老師們都給予我許多有益的建議，啟發我進一步思考。

此外，我還要感謝R722大家庭中的夥伴們。感謝品宏和慶輔學長，在我初次進入實驗室時，耐心地指導我，使我對動物實驗的技術操作和原理有了基本的認識。這段過程讓我受益匪淺。同樣地，我也要特別感謝活潑又細心的阿琦學姊，不僅在分生實驗與技術的教學上十分用心，也時常陪我聊天談心，在我遭遇瓶頸或是心情低落時成為支撐我走下去的力量，還會跟于嬋學姐一起關心我有沒有好好吃飯，真的謝謝妳們! 再來也很感謝婉婷學姊願意在我更換題目後帶著我做實驗，即使中途遭遇許多問題，也還是耐心的引導我，陪我釐清和找出解決問題的方法，我從妳身上學到好多。也謝謝欣妤、優秀的學弟妹宜美和得銨，總是面不改色地聽我胡言亂語，跟你們聊天歡笑是我很寶貴的時光，因為有你們的陪伴我才能走過這段不容易的日子。

在這兩年的時光，從對實驗室的一切感到緊張陌生，到後來的逐漸熟悉，除了對醫學研究領域有了更深的認識，更是了解了待人處事和團隊合作的重要。謝謝所有在這條路上支持著我的人，我會帶著這份力量，繼續勇敢地前行。

doi:10.6342/NTU202303793

摘要

T 細胞受體工程性 T 細胞 (engineered TCR-T cell)是一種應用於過繼性 T 細胞療法(adoptive T-cell therapy)的改造 T 細胞，對於治療人類癌症具有巨大的潛力。將患者周邊血中分離出自體 $CD8^+$ T 細胞，在體外將細胞進行培養放大後，透過基因編輯讓細胞表達對腫瘤具有特異性的 T 細胞受體，最後再重新輸注回患者體內，以更有效率地清除腫瘤細胞。

為了提升治療效率，測量這些 T 細胞受體工程性 T 細胞之效用功能的好壞至關重要。而其中，T 細胞受體的功能親和力 (functional avidity)就是決定 T 細胞功能的關鍵因素之一，它反映了 T 細胞對不同濃度的抗原之反應能力。因此，為了能夠有效地評估 T 細胞受體的功能親和力，我們利用缺乏內源性 T 細胞受體的 Jurkat 細胞株開發了一個雙參數報告系統 (dual-parameter reporter system)。

這個系統能夠藉由報告基因的表達，分析在 T 細胞活化中十分重要的轉錄因子 NFAT 和 NF-κB 的活性，來測量 T 細胞活化的程度。通過將具有腫瘤特異性的 TCR 轉導到這些報告細胞中，我們可以在較繁雜的 T 細胞功能測試前，就先快速地篩選出具有高功能親和力的候選 T 細胞受體。

此外，由於此系統能夠同時測量 NFAT 和 NF-κB 的活性，相較於評估單一的 T 細胞活化路徑，更能夠提高功能親和力的可靠性和參考價值，並且可以深入比較兩個路徑與 T 細胞功能之間的關聯性。

這個報告系統能夠加速用於癌症治療的 T 細胞受體工程性 T 細胞的篩選。透過測量候選 T 細胞受體的功能親和力，我們可以優先篩選出可能具有理想之效用功能的 T 細胞受體工程性 T 細胞，以此提高免疫細胞療法的效率。

關鍵詞：T 細胞受體、工程性 T 細胞、功能親和力、T 細胞活化、免疫細胞療法

doi:10.6342/NTU202303793

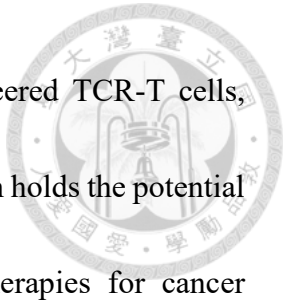
Abstract

T cell receptor (TCR)-engineered T cells (TCR-T) represent a promising approach for adoptive T cell immunotherapy (ACT) and hold great potential in the treatment of human cancer. Autologous $CD8^+$ T cells can be isolated from patients' peripheral blood, expanded in vitro, modified to express tumor-specific TCRs, and finally reinfused into patients' body to improve antitumor ability.

To optimize this treatment strategy, it is crucial to evaluate the efficacy of T cell effector functions. One key factor in determining T cell functionality is the functional avidity of the TCR, which reflects the T cell's response to varying antigen levels. To efficiently assess TCR functional avidity, we have developed a dual parameter reporter system utilizing the Jurkat cell line devoid of endogenous TCRs. This system allows for the analysis of NFAT and NF-κB activities, two critical transcription factors required for T cell activation. By transducing tumor-specific TCRs into these reporter cells, we can effectively select TCR candidates with enhanced effector functions before conducting functional assays on tumor-specific TCR-T cells.

Additionally, the system enables simultaneous measurement of the expression levels of NFAT and NF-κB, enhancing the reliability and reference value of the measured functional avidity. Furthermore, it enables a comparative analysis of the two TCR

doi:10.6342/NTU202303793



activation signaling pathways and the effector functions of engineered TCR-T cells, providing valuable insights into their interplay. Ultimately, this system holds the potential to expedite the development of effective T cell-based immunotherapies for cancer treatment.

Key words : T cell receptor, engineered T cells, functional avidity, T cell activation, adoptive cell therapy

doi:10.6342/NTU202303793

Content

doi:10.6342/NTU202303793

doi:10.6342/NTU202303793

1 Introduction

1.1 T cell-based immunotherapy in cancer

1.1.1 Adoptive cell therapy (ACT)

Cancer is a major public health challenge, impacting millions of individuals worldwide. Despite notable progress in cancer treatment, it remains a leading cause of death, resulting in approximately 10 million deaths each year. The immune system plays a significant role in protecting the host from malignant cells. Within the adaptive immune system, T cells exhibit a remarkable ability to identify and eliminate cancer cells, leading to significant advancements in cancer immunotherapy[1,34-36].

Adoptive cell therapy (ACT) involves the utilization of engineered T cells or tumor-infiltrating T cells (TILs) obtained from a patient's tumor or T cells isolated from the patient's peripheral blood. The engineered T cells are expanded in vitro and genetically modified to express either a T cell receptor (TCR) or a chimeric antigen receptor (CAR) which can recognize tumor-specific antigens. Subsequently, these genetically engineered T cells were re-infused into the patients to destroy tumor cells[2,3,37-39].

1.1.2 Distinctive features of TCR-T cell compared to CAR-T cell therapy

doi:10.6342/NTU202303793

While CAR-T cell therapy has shown impressive clinical outcomes in specific cases of B cell leukemia or lymphoma, many challenges still limit the therapeutic efficacy of CAR-T cells in treating solid tumors and hematological malignancies.

Structurally, CAR-T cells and TCR-engineered T cells differ. CAR-T cells consist of engineered intracellular and extracellular domains. The extracellular domain comprises a single-chain variable fragment (scFv), which serves as the antigen binding domain of the antibody, they can only recognize specific antigens on the surface of the cancer cells, which are commonly not expressed on solid tumors. In contrast, TCRs comprised most commonly of an α and β chain, which rely on peptide processing or presentation by major histocompatibility complex (MHC) molecules, allowing them to recognize peptides from both the intra- and extra-cellular compartments[3,4,40].

Based on current bioinformatic predictions, approximately 27% of the human proteome contains membrane-integral structures that could potentially be targeted by CAR therapies, which means many potential targets within cancer cells are inaccessible to most CAR-based treatments[41-43]. Unlike CAR T-cells, TCR-engineered T cells can recognize tumor antigens presented by MHC molecules from all cellular compartments, making them better suited for targeting solid

doi:10.6342/NTU202303793

tumors, which often express intracellular antigens. Therefore, TCR-T therapy may be more effective than CAR T-cell therapy for treating solid tumors, which make up the majority of all malignancies[5,41].

1.1.3 Clinical application of engineered TCR T-cell therapy

Historically, TCR-T cell therapy has targeted tumor-associated antigens such as carcinoembryonic antigen (CEA) in colorectal cancer, glycoprotein gp100 (PMEL) in melanoma, melanoma antigen recognized by T-cells 1 (MART-1) in melanoma, melanoma-associated antigen 3 (MAGE-A3) in melanoma/multiple myeloma, and New York esophageal squamous cell carcinoma 1 (NY-ESO-1) in melanoma[6,7]. Recently, TCR-T cells have also been designed to target neoantigens resulting from tumor-specific DNA mutations, which are more unique to individual tumors but less commonly shared among cancer patients[6, 8].

Evidently, TCR-T cell therapy holds great potential in the treatment of cancer. Future research aims to achieve two key objectives: personalized identification of tumor-specific TCRs for therapy and exploring potential modifications to enhance TCR signaling and effector function.

1.2 The overview of T cell antigen recognition and activation

doi:10.6342/NTU202303793

T cells play a crucial role in recognizing foreign antigens on target cells. This recognition occurs through the heterodimeric T-cell receptor (TCR), composed of α- and β-chains that can undergo random rearrangements of V (variable), D (diversity), and J (joining) gene segments, which allows for the specific recognition of a wide variety of antigens[10].

The T-cell receptor (TCR) initiates signal transduction upon binding to peptide-MHC complexes on antigen-presenting cells (APCs)[9,31]. The cytoplasmic domains of CD4 and CD8 co-receptors interact with the SRC family protein tyrosine kinase LCK, which is subsequently directed to the TCR through simultaneous binding of CD8 to MHC class I or CD4 to MHC class II complexes[32]. LCK phosphorylates the CD3 complex, facilitating ZAP70 activation. ZAP70, in turn, phosphorylates the transmembrane adaptor protein LAT, leading to the formation of the LAT signalosome along with adaptors and effectors[33]. Activation of LAT-associated effector molecules triggers the transmission of signals through three primary pathways: the Ca^{2+}-calcineurin, nuclear factor-κB (NF-κB), and mitogen-activated protein kinase (MAPK) pathways. These pathways drive the nuclear translocation and activation of nuclear factor of activated T cells (NFAT), NF-κB, and activator protein 1 (AP-1), which

doi:10.6342/NTU202303793

collectively promote T cell proliferation, migration, cytokine production, and effector functions[30].

1.3 Optimization of engineered TCR T-cell therapy

1.3.1 TCR affinity, avidity, and functional avidity

TCR affinity, TCR avidity, and functional avidity are the main factors that determine pMHC recognition and T-cell activation. TCR affinity refers to the strength of the interaction between a single TCR and the pMHC ligand, while TCR avidity measures the overall strength of multiple TCR-pMHC engagements. Functional avidity, on the other hand, represents T-cell activity at various peptide epitope concentrations. Mean functional avidity is typically described as the EC50 concentration, which indicates the peptide dose at which half-maximal activation of the T-cell population occurs[11,44,45].

1.3.2 Unexpected downsides of high-affinity TCRs

Most tumor-associated antigens recognized by $CD8^+$ T cells originate from autologous proteins and are protected by immune tolerance to prevent autoimmunity. [12] Due to thymic selection, the binding affinity of physiological TCR affinities are limited to ~1 to 100μM[13,14]. Thus, cells with down-regulated tumor antigen presentation may not be effectively targeted by TCR-T cells engineered with a wild-type TCR[15,16]. In order to enhance the

doi:10.6342/NTU202303793

effectiveness of TCR-T cells, it is crucial to predict the T cell response to tumor antigens. This is supported by several studies indicating a correlation between TCR affinity and T-cell functional activity[16-18, 48-50]. As a result, researchers have been developing modified TCRs with high affinity for use in clinical trials[19,20]. However, higher TCR affinity does not necessarily indicate better T cell function. Multiple studies have reported a plateau in T-cell functional activity beyond a specific affinity and avidity threshold[14,21,51,52]. Moreover, in a notable case, an affinity-enhanced TCR has resulted in treatment-induced patients' deaths during a cell therapy clinical trial due to unexpected cross-reactivity with a self-antigen, highlighting the potential safety concerns associated with high-affinity TCR-T cells[22,53,54].

1.3.3 Efficient and precise evaluation of TCR functional avidity

Based on previous research findings, it has been shown that the level of TCR affinity and avidity does not accurately represent the effectiveness of T cell immune response[21,23]. To evaluate TCR functional avidity in a more efficient and precise way, researchers have developed Jurkat-derived reporter cell lines to evaluate the functional avidity of cloned TCRs. These reporter cells allow for rapid and standardized assessment of TCR activation by measuring the expression of

doi:10.6342/NTU202303793

reporter genes driven by critical transcription factors in T cell activation, such as nuclear factor of activated T-cells (NFAT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and activator protein 1 (AP-1)[24,25]. Therefore, reporter cell lines can serve as valuable tools for evaluating the functional avidity of transduced TCRs and provide an efficient platform for predicting T-cell functions in the development of effective TCR T-cell immunotherapy in cancer treatments[46,47].

doi:10.6342/NTU202303793

2 Specific Aims

- Aim 1

  To efficiently screen and select highly functional T cells, a dual-parameter reporter system was established to evaluate TCR functional avidity.

- Aim 2

  To compare the differential expression of the NFAT and NF-κB pathways in the reporter assay, as well as their specificity differences in T cell activation.

doi:10.6342/NTU202303793

3 Materials and methods

3.1 Plasmid construction

The NFκB reporter plasmid, pLVX-$(NF\kappa B)_3$-miniP-mCherry-Fluc, contained tandem repeats of three NFκB binding sites upstream of a minimal CMV promoter that drove the expression of mCherry and firefly luciferase. The plasmid was constructed from PCR products of pLVX-$(NF\kappa B)_3$-miniP-mCherry (from Pei-Yi Wu), pUC19-T7-SARS-CoV-2 T2A-mCherry-P2A-PuroR DI replicon (from Chi) and pGL4.10 (Catalog No. E6651, Promega). The assembly was performed using the NEBuilder® HiFi DNA Assembly Master Mix (Catalog No. E2621, New England Biolab) through Gibson assembly method.

The plasmids used for lentivirus production, pCMV-dR8.91 and pMD.G, were obtained from the RNAi Core of Academia Sinica, Taiwan. These constructs were transformed into DH5α competent cells and plated on agar plates containing Ampicillin. Single colonies were selected and cultured in LB broth with Ampicillin. Subsequently, plasmid extraction was performed using the Geneaid plasmid purification kit.

3.2 Cell lines and culture conditions

The dual-parameter reporter Jurkat cells (clone #38.7.46.20) were generated by transducing $NFAT_{x7}$-AcGFP-hCD8-$TCR_{\alpha/\beta}^{-/-}$- Jurkat cells (clone 38.7.46 from Wan-Ting Hong) with pLVX-$(NF\kappa B)_3$-miniP-mCherry-Fluc. Jurkat cells were cultured in

doi:10.6342/NTU202303793

RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 292 μg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Similarly, HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 292 μg/mL glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). All cells were maintained at 37°C with 5% CO2.

3.3 Lentiviral transduction of Jurkat cells

The dual-parameter reporter Jurkat cells were generated through lentiviral transduction. Lentivirus packaging was carried out by DNA transfection using Lipofectamine™ 3000. HEK293T cells were seeded at a density of $1x10^6$ cells per well in a 6-well plate using lentivirus packaging medium (Opti-MEM supplemented with 5% FBS, 1% L-glutamine, and 0.5% sodium pyruvate) for 24 hours.

Next, 1.5 μg of the target plasmid, 0.5 μg of pMD.G, 1 μg of pCMV-dR8.91, and P3000™ reagent were diluted with Opti-MEM and mixed with diluted Lipofectamine™ 3000 reagent. After incubating the mixture at room temperature for 10 minutes, it was carefully added to the cells. After 6 hours, the medium was replaced with 2 mL of lentivirus-packaging medium. Subsequently, 2 mL and 1.5 mL of the supernatant were collected per well after 48 and 72 hours of transfection, respectively.

doi:10.6342/NTU202303793

The collected supernatant was then filtered using a 0.45 μm filter, aliquoted into 500 μL per Eppendorf tube, and stored at -80 °C.

On the day of lentivirus transduction, 4 x 10^5 Jurkat cells were collected in individual 50 mL centrifuge tubes. Subsequently, 500 μL of the prepared virus solution was thawed and mixed with polybrene (final concentration: 8 μg/mL). The cells were resuspended in the virus solution and centrifuged for 1 hour at 2000g and 32°C. After centrifugation, the cells were transferred to 12-well plates and incubated overnight at 37°C. The following day, the virus solution was replaced with RPMI culture medium.

3.4 Stimulation experiments and reporter assays

Multiple clones of Jurkat cells were seeded in 96-well plates at a density of 2x10^5 cells per well. Subsequently, the cells were either stimulated with anti-CD3 (UCHT1, BD Pharmingen) and CD28 antibodies (CD28.2, BD Pharmingen) for 24 hours or with PMA and ionomycin (Sigma Aldrich) for 6 or 24 hours. Following the stimulation, the cells were harvested and analyzed using flow cytometry and luciferase assay. The fluorescence expressions were analyzed using the LSR II Flow Cytometer (BD Biosciences) or the Attune NxT Flow Cytometer (Thermo Fisher Scientific). The luciferase assays were analyzed using the SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices).

doi:10.6342/NTU202303793

3.5 Establishment of the dual parameter reporter Jurkat cell line

To establish the dual-parameter reporter Jurkat cell line, we first transduced $NFAT_{x7}$-AcGFP-hCD8-$TCR_{\alpha/\beta}^{-/-}$- Jurkat cells (clone 38.7.46 from Wan-Ting Hong) with the NFκB reporter plasmid. Next, we used the FACSAria™ III cell sorter (BD Biosciences) to sort out cells that did not express NFκB, which were subsequently maintained. Then, these cells were limited diluted and seeded at a density of 0.5 cells per well in 96-well plates to generate single-cell clones.

Afterward, the clones that successfully expanded were stimulated with PMA (10 ng/mL) and ionomycin (800 ng/mL) for 24 hours. Using the Attune NxT Flow Cytometer (Thermo Fisher Scientific), we analyzed the expression of NFAT and NFκB in each clone. Among the clones, clone #38.7.46.20 exhibited low background and high inducibility of reporter gene expressions. As a result, it was selected as the optimal clone for the dual-parameter reporter Jurkat cell line, and was then utilized in the subsequent TCR transduction experiment.

doi:10.6342/NTU202303793

4 Results

4.1 The expression of NFAT reporter of TCR knockout Jurkat cells by different stimulation strategies

To facilitate my research, it was necessary to identify an optimal method for stimulating Jurkat cells. Stimulation with anti-CD3/CD28 antibodies and PMA/ionomycin have both been widely used in many studies as effective approaches for Jurkat cell activation. However, in order to avoid TCR mispairing issues, I aimed to establish a reporter cell system using a previously generated TCR knockout Jurkat cell line, which lacked endogenous TCR expression.

After TCR knockout, the expression of the CD3 complex is also disrupted, which theoretically renders antibody stimulation ineffective. Therefore, I conducted separate experiments to compare the effects of antibody stimulation and PMA/ionomycin stimulation on the TCR KO clone of NFAT reporter Jurkat cells (#38.41.19) with the non-KO clone (#38.41).

The results revealed that the non-KO cells demonstrated a strong response to the anti-CD3/CD28 Ab stimulation, while the TCR-KO clone showed no response **(Figure 1A)**. In contrast, when the same cells were stimulated with PMA/Ionomycin, both the wild-type and TCR-KO clone exhibited responsiveness **(Figure 1B)**.

doi:10.6342/NTU202303793

4.2 Establishment of the dual-parameter reporter Jurkat cells

4.2.1 Screening of the reporter Jurkat cells with low-background and high-inducibility of NF-κB expression

Next, I transduced the NFκB-responsive mCherry and Firefly luciferase reporter constructs into Jurkat clone #38.7.46 (obtained from Wan-Ting Hong), which already expressed the NFAT-GFP reporter, human CD8, and lacked the endogenous TCR expression **(Figure 2A)**.

NF-κB can be activated in response to diverse external stimuli related to immune response, oxidative stress, cell proliferation, differentiation, and survival [26]. As a result, NF-κB often displays elevated background activity. To obtain cells with reduced background expression and increased inducibility upon cell activation, two rounds of sorting were performed following the transduction.

After a resting period of 10 days following transduction, NF-κB-negative cells were isolated to obtain cells with reduced background expression **(Figure 2B)**. Subsequently, the collected cells were stimulated with PMA/ionomycin (10/800 ng/mL) for 24 hours. Nine days after the first sorting, the collected cells were stimulated with PMA/ionomycin (10/800 ng/mL) for 24 hours. A second round of sorting was then conducted to collect cells with the highest NF-κB expression, comprising approximately 10% of the total population. This enabled

doi:10.6342/NTU202303793

the acquisition of cells with the highest NF-κB expression levels post-stimulation, which were further sorted into single cells **(Figure 2C)**. The two rounds of sorting facilitated the screening of a cell population characterized by lower NF-κB background expression and enhanced inducibility.

4.2.2 Troubleshooting for the unexpected cell death caused by PMA and ionomycin stimulation

During the experiment, we encountered an issue where nearly all of the cells died approximately 3 days after the second round of sorting. Consequently, we decided to repeat the second round of sorting. Considering that fluorescence-activated cell sorting exposes cells to various stresses and forces, we reasoned that these conditions might contribute to the observed cell death.

After collecting the cells this time, we confirmed their viability using trypan blue staining, and indeed, the cells were all alive right after sorting. Therefore, we suspected that the cell death observed after the second sorting might also be attributed to apoptosis triggered by PMA/ionomycin stimulation.

Upon investigating previous literature, we found reports of cell death occurring with PMA/ionomycin stimulation[27], which led us to speculate whether the cell death following the second sorting was caused by excessively

doi:10.6342/NTU202303793

high concentrations or prolonged exposure to PMA/ionomycin during the stimulation process.

Therefore, to address this issue, I made adjustments to the experimental approach. Following the first sorting, I employed a limiting dilution method, distributing cells at a concentration of 0.5 cells per well in a 96-well plate. This approach allowed me to minimize potential stress caused by the cell sorter. Subsequently, I cultured these single cells and utilized only a portion of the cells from the grown clones for stimulation and analysis **(Figure 3)**. By adopting this strategy, I could identify the clone with the highest NF-κB expression without inducing widespread cell death caused by PMA/ionomycin stimulation.

These adjustments improved the reliability and reproducibility of the experimental results, and enabled the selection of the most promising NF-κB-expressing clone for further investigation.

4.2.3 Optimal selection of the ideal clone of the established dual-parameter reporter Jurkat cells

Finally, 24 single clones were successfully maintained. After stimulation with PMA/ionomycin and subsequent analysis using flow cytometry, I selected seven clones that exhibited higher median fluorescence intensity (MFI) values for NFAT-GFP and NF-κB-mCherry expression **(Figure 4A, 4B)**. These selected clones

doi:10.6342/NTU202303793

underwent further experiments to compare their responses under both unstimulated and stimulated conditions **(Figures 4C-4E)**.

In this design of dual-parameter reporter Jurkat cells, NF-κB is equipped with both mCherry fluorescent protein and firefly luciferase reporter. Luciferase offers advantages over fluorescent reporters, as it avoids autofluorescence issues and provides lower background expression in typical cells. With these characteristics, we expect to achieve a wider dynamic range for more accurate quantification of the target reporter signal.

Among the seven selected clones, we chose clone #20 and #24 for further evaluation in the firefly luciferase assay due to their lower background and higher inducibility of the fluorescence reporter. The results indicate that clone #20 displayed a broader dynamic range between the unstimulated and stimulated groups compared to clone #24, with induction folds of 15 and 7.4, respectively. **(Figure 4E)**. Therefore, for subsequent TCR transduction experiments, we will utilize Jurkat #38.7.46.20 as the preferred dual-parameter reporter Jurkat cell.

doi:10.6342/NTU202303793

5 Discussion

5.1 Advantages and limitations of utilizing TCR-KO Jurkat cells to create reporter systems

In the process of establishing the reporter system, we chose to use Jurkat cells that had already undergone knockout of their endogenous TCR. This decision was driven by the need to avoid TCR mispairing issues when introducing the TCR of interest into the reporter cells in future experiments.

Specifically, when both the original TCRs and the target TCR are present in the same cell, there is a possibility of cross-pairing during the formation of alpha and beta TCR chains. This could result in TCR chain mispairing, leading to the generation of unintended specificities and potentially limiting the expression of the desired TCRs [28, 29]. By using previously established TCR-KO Jurkat cells generated through CRISPR-Cas9 gene editing, we can ensure specific expression of the target TCR without unintended mispairing.

However, our results also showed that TCR-KO Jurkat cells did not respond to anti-CD3/CD28 antibody stimulation but did respond to PMA/ionomycin stimulation. This caused a difficulty when we generated NF-κB reporter cell line. During the sorting process of this study, we encountered cell death after PMA/ionomycin stimulation, revealing that high concentrations and prolonged exposure to PMA/ionomycin could

doi:10.6342/NTU202303793

trigger excessive apoptosis. These experiments highlight the importance of choosing suitable stimulation strategies and validating experimental protocols to avoid potential confounding factors when studying T cell activation in different experimental contexts.

5.2 The importance of NFAT and NF-κB dual-parameter reporter system

Upon TCR recognition of MHC-presented peptides, the CD3 complex initiates a signaling cascade, activating various effector molecules and triggering three main downstream signaling pathways. The first pathway involves the activation of AP-1 through the MAPK pathway. The second pathway involves NF-κB, activated downstream of PKC. The third pathway involves NFAT, which is activated through the calcium ion pathway[30].

Among these pathways, NFAT is the most specific for T cell activation, while NF-κB and AP-1 can also be activated by cytokine stimulation or cellular damage, in addition to TCR signaling. Besides, complete T cell activation requires the collaboration of these three transcription factors, leading to robust proliferation and activation of T cells.

To comprehensively dissect the intricate signaling cascades governing T cell activation and function, a more holistic approach can be achieved by combining multiple reporters in a single Jurkat cell line. We hypothesize that T cell function could

doi:10.6342/NTU202303793

be influenced differently by different signaling pathways, NF-kB and NFAT. However, this hasn't been proven by previous studies.

After successfully establishing the dual-parameter reporter cells, the next step involves introducing the cloned antigen-specific TCR construct into these reporter cells, thereby enabling the expression of the targeted TCR. Subsequently, upon stimulation with the corresponding peptide and APC, the fluorescence signals of NFAT and NF-kB can be measured using flow cytometry. Additionally, the luciferase expression level is observed, facilitating the identification of TCR variants exhibiting elevated activity. Lastly, the selected TCR candidates are then transferred into primary human T cells to conduct comprehensive functional tests. This process aims to verify the utility of our dual-parameter reporter as an effective tool for screening highly functional TCRs. Furthermore, it provides the opportunity to simultaneously observe the impact of activating both NFAT and NF-kB pathways on T cell function.

This strategy allows us to observe and study how different signaling pathways correlate with T cell activation and function. By integrating various reporter systems, we can gain a deeper understanding of the complex interplay between these pathways and their impact on T cell responses. Such an approach holds great promise for advancing our knowledge of T cell biology and immune regulation.

doi:10.6342/NTU202303793

5.3 The application and future potential of the dual-parameter reporter system in TCR-T cell immunotherapy.

After establishing this reporter system, we expect to efficiently screen potentially well-functioning TCRs by introducing the target TCR and measuring the reporter's expression. This approach overcomes the issue of disparity between measuring affinity and avidity and the actual T cell function. Instead, it directly measures functional avidity, which is theoretically more closely related to T cell function. By focusing on functional avidity measurement through the dual-parameter system, we can prioritize TCRs with higher potential for effective target cell recognition and tumor elimination.

Compared to laborious T cell functional tests that require primary human T cells, the dual-parameter system offers a simpler and faster way to screen and identify TCRs with desired functionality.

By rapidly identifying TCRs with high functional avidity using the dual-parameter system, we can significantly improve the success rate of TCR-T cell therapies, leading to better clinical outcomes for patients battling cancer. Moreover, the insights gained from this approach can contribute to the ongoing refinement and optimization of TCR engineering strategies, ultimately advancing the field of cancer immunotherapy.

doi:10.6342/NTU202303793

6 Figures

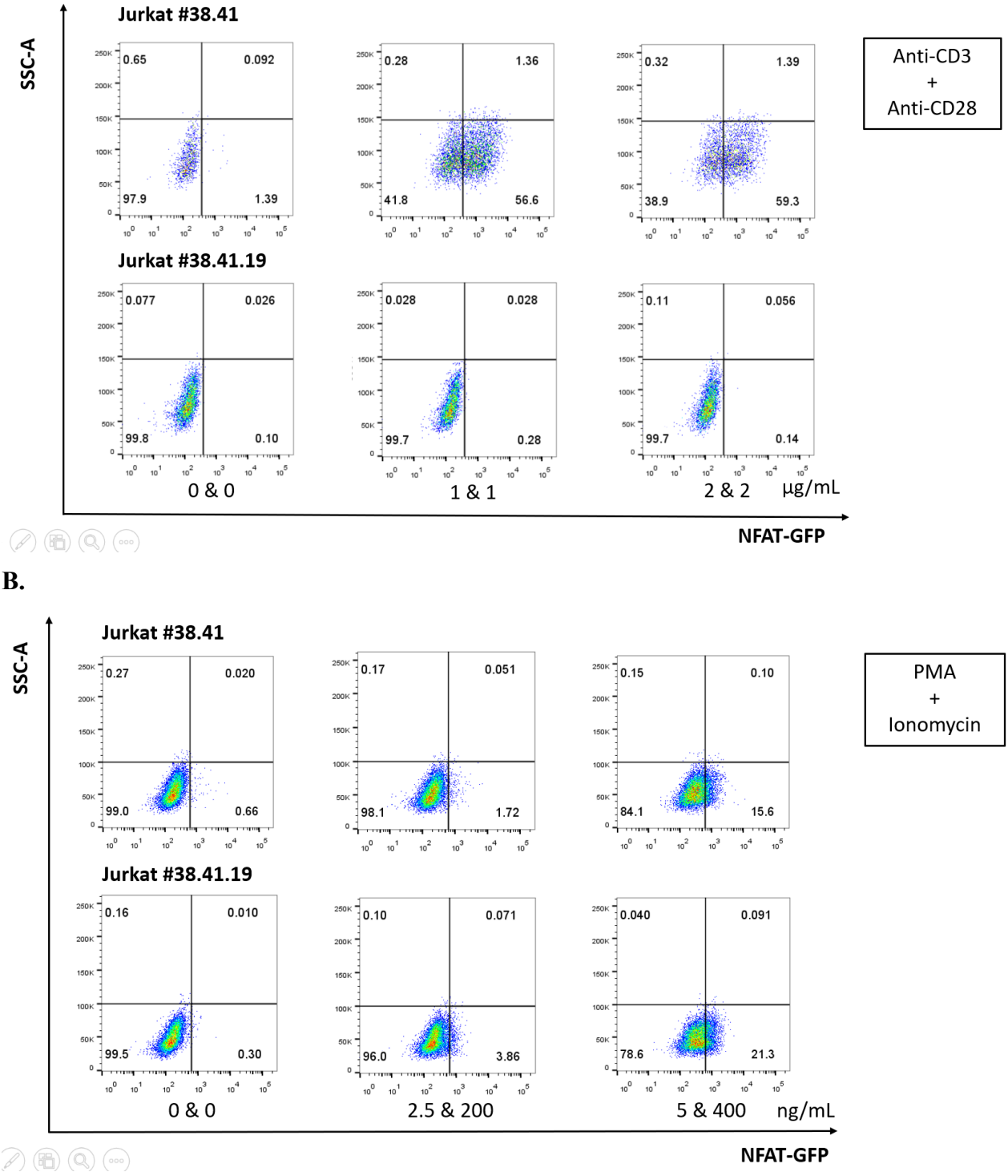


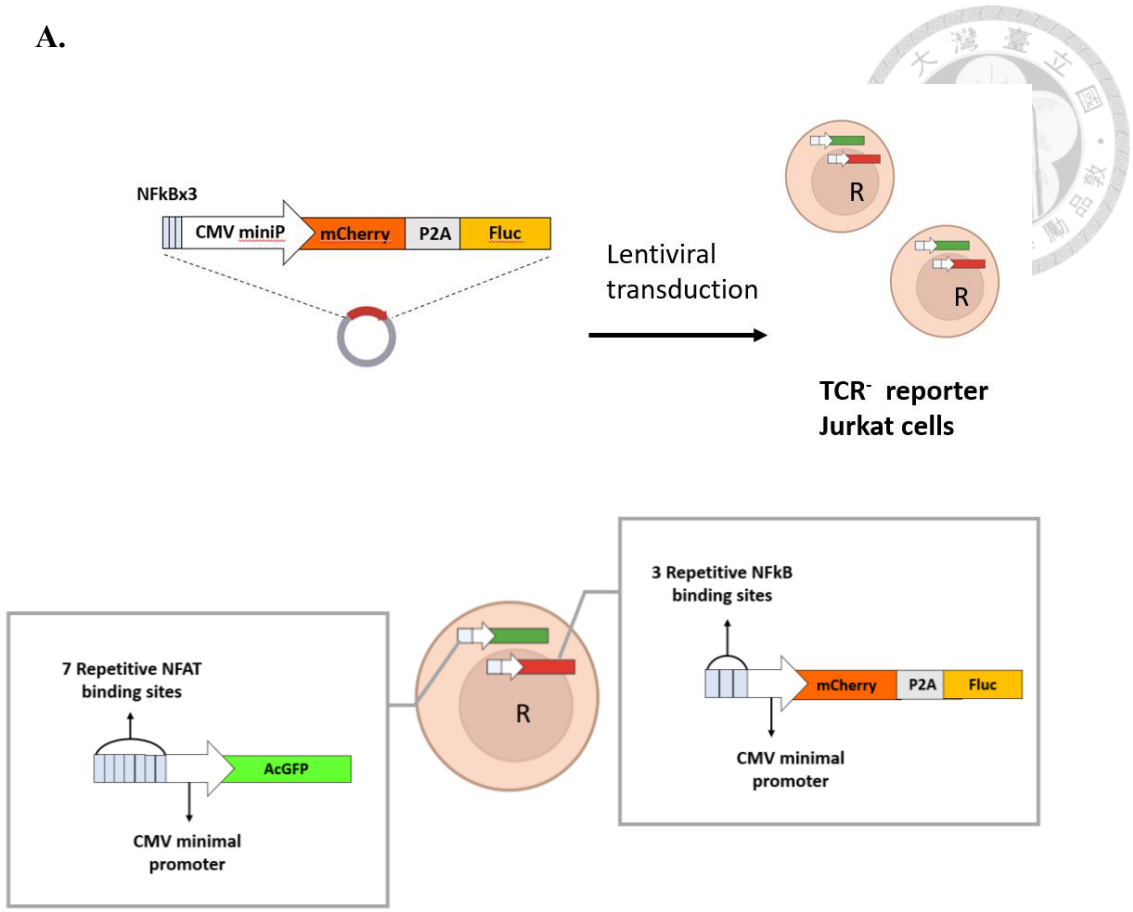
Figure 1. Stimulation responses of TCR KO and non-KO Jurkat cells.

doi:10.6342/NTU202303793

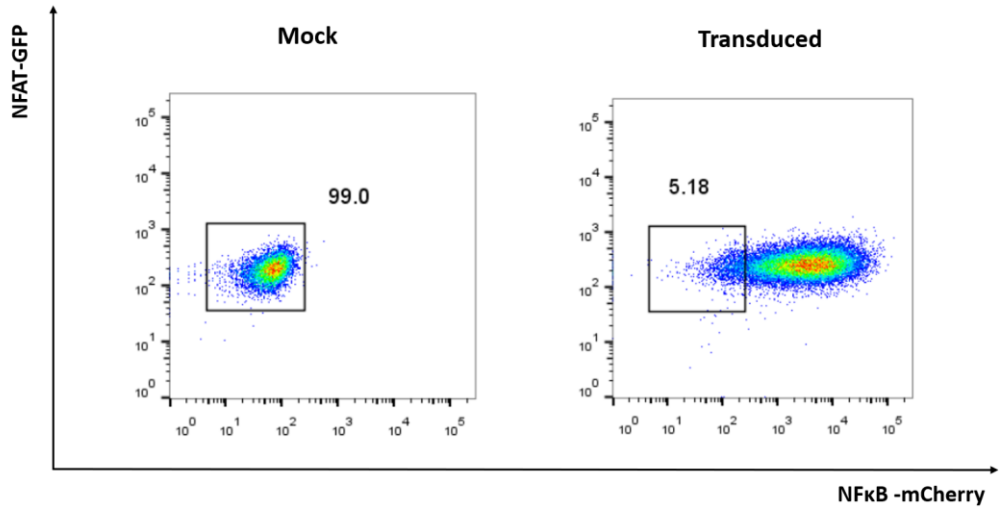
(A) Jurkat clones #38.41 and #38.41.19 were either unstimulated or stimulated with anti-CD3/CD28 antibodies at concentrations of 1/1 μg/mL and 2/2 μg/mL for 24 hours. **(B)** Jurkat clones #38.41 and #38.41.19 were either unstimulated or stimulated with PMA/ionomycin at concentrations of 2.5/200 ng/mL and 5/400 μg/mL for 6 hours. All cells were analyzed by flow cytometry.

doi:10.6342/NTU202303793

A.



B.



doi:10.6342/NTU202303793

C.

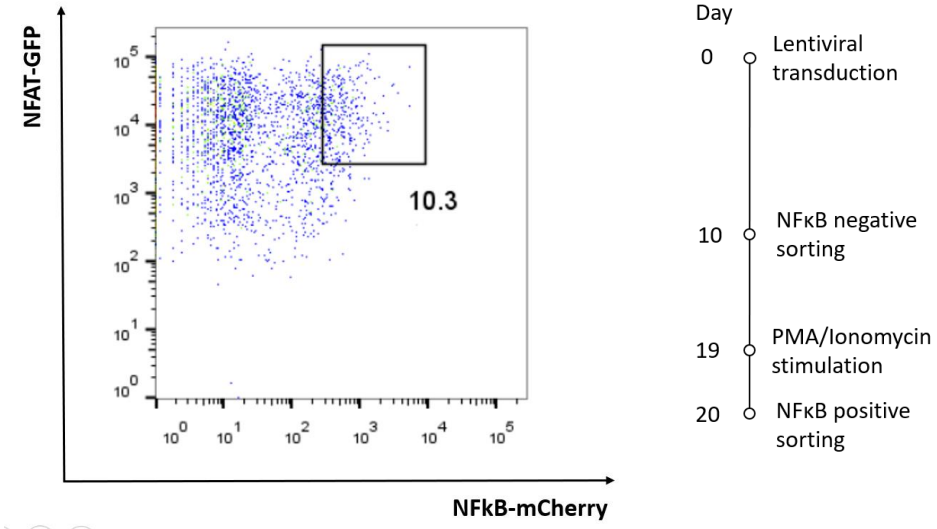


Figure 2. Isolation of transduced reporter Jurkat cells with low background expression and high inducibility of NF-κB.

(A) Schematic illustration of generation of dual-parameter reporter cells through transduction of Jurkat #38.7.46 with NF-κB reporter construct. The NFAT reporter gene consists of repetitive NFAT binding sites, a CMV minimal promoter, and an AcGFP reporter. The NF-κB reporter gene consists of repetitive NF-κB binding sites, a CMV minimal promoter, and reporters for mCherry and Firefly luciferase. **(B)** Cell sorting of the reporter Jurkat cells with negative NF-κB expression 10 days post-transduction. **(C)** Cell sorting after stimulation with PMA/ionomycin (10/800 ng/mL) for 24 hours to collect cells with the highest NF-κB expression.

doi:10.6342/NTU202303793

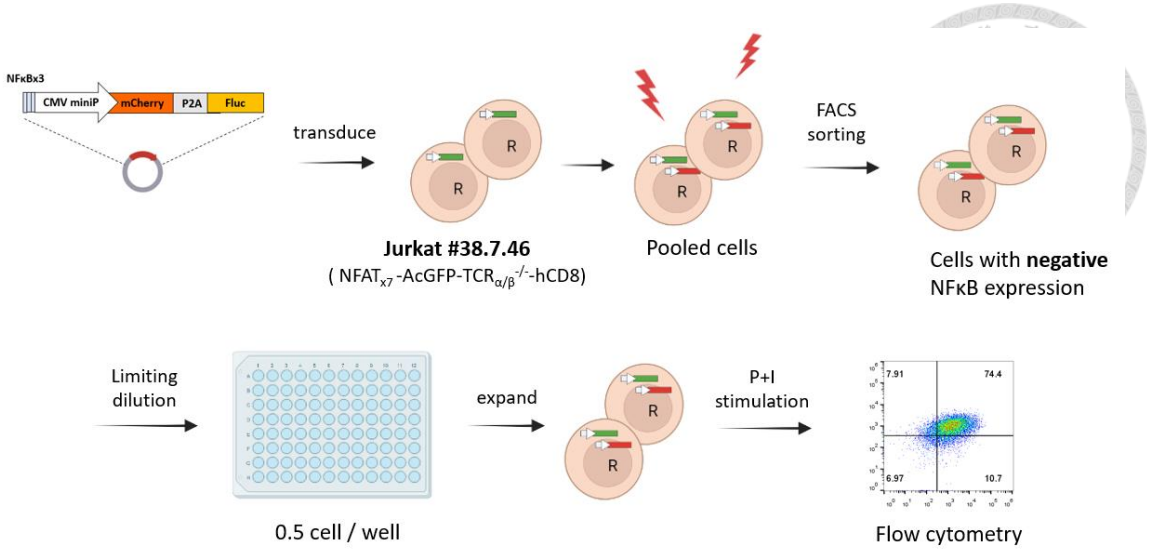
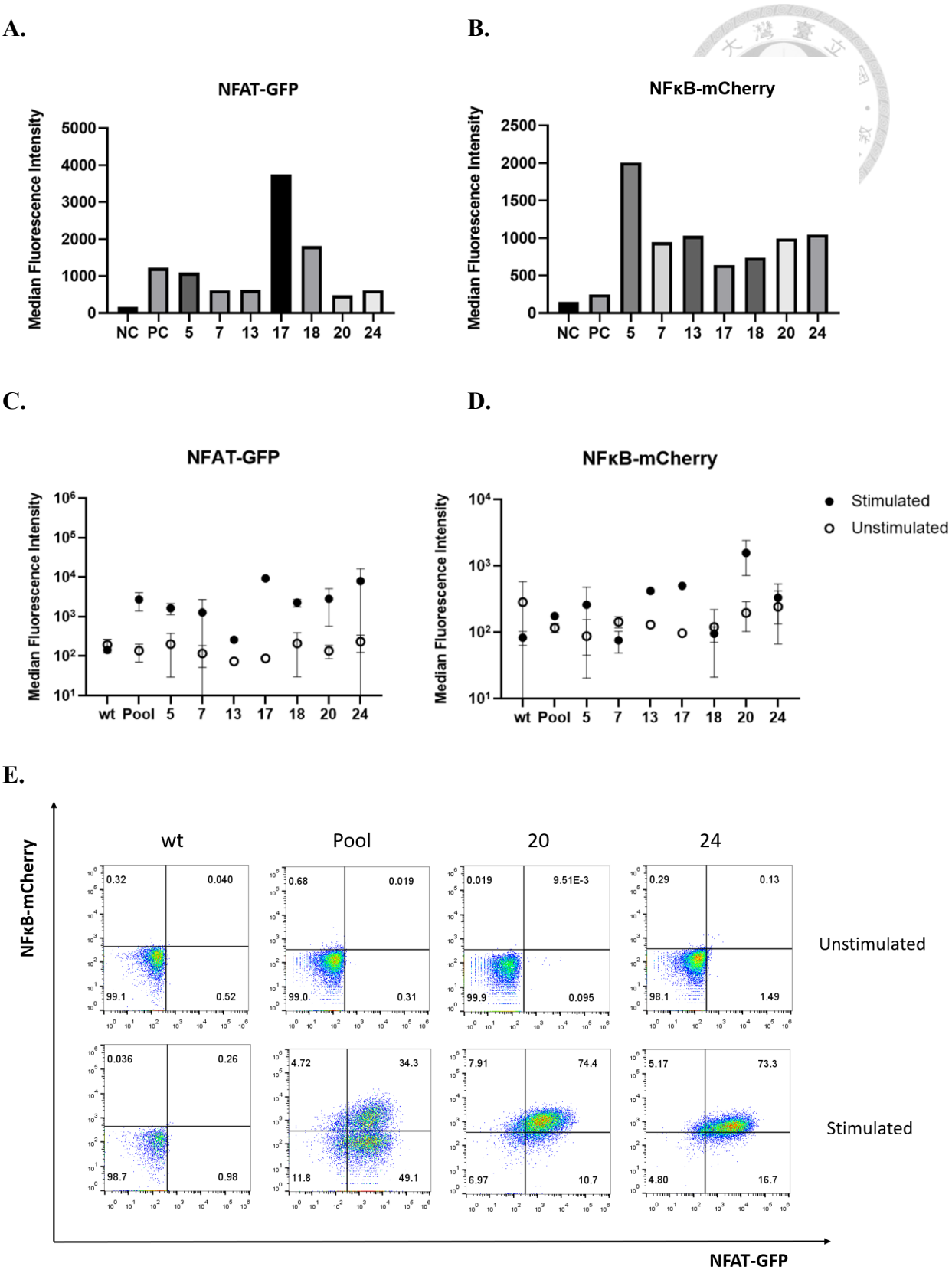


Figure 3. Experimental adjustments to improve cell viability in the screening process of reporter cells.

Schematic illustration of the optimized process for screening cells with lower NF-κB background and enhanced inducibility.

doi:10.6342/NTU202303793



doi:10.6342/NTU202303793

F.

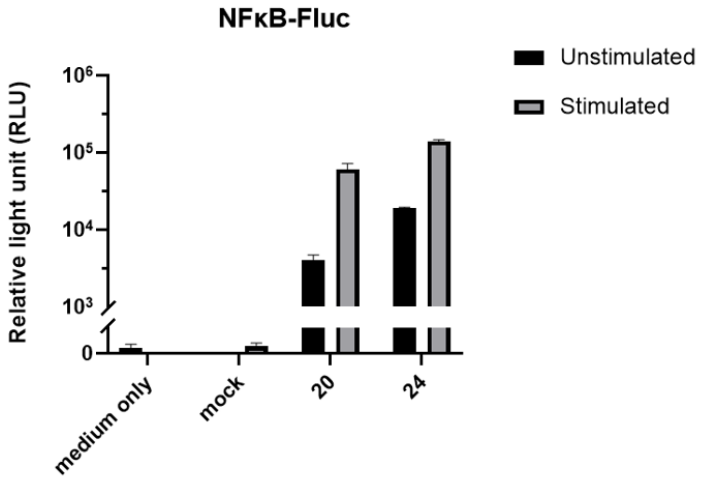


Figure. 4 Selection of the ideal clone from the established dual-parameter reporter Jurkat cells.

(A, B) Summary bar graph depicting the median fluorescence intensity (MFI) of seven clones that exhibited higher (A) NFAT-GFP and (B) NF-κB-mCherry expression after stimulation with PMA/ionomycin (10/800 ng/mL) for 24 hours.

(C, D) Summary bar graph depicting the median fluorescence intensity (MFI) of (A) NFAT-GFP and (B) NF-κB-mCherry expression in selected individual reporter Jurkat cell clones either unstimulated or after stimulation with PMA/ionomycin (10/800 ng/mL) for 24 hours. **(E)** Flow cytometry graph of NFAT-GFP and NF-κB-mCherry expression of the reporter Jurkat cell clone #20 and #24 **(F)** Summary bar graph showing the relative light unit (RLU) of NF-κB-firefly luciferase expression in clone #20 and #24 either unstimulated or after stimulation with PMA/ionomycin (10/800 ng/mL) for 24 hours. NC: unstimulated pooled cells Jurkat #38.7.46 transduced with

doi:10.6342/NTU202303793

NF-κB reporter construct. PC: stimulated pooled cells Jurkat #38.7.46 transduced with NF-κB reporter construct. wt : wild type Jurkat cells. Pool : pooled cells Jurkat #38.7.46 transduced with NF-κB reporter construct. Mock : Jurkat #38.7.46 (without NF-κB reporter).

doi:10.6342/NTU202303793

7 References

1. Want, M. Y., Bashir, Z., & Najar, R. A. (2023). T Cell Based Immunotherapy for Cancer: Approaches and Strategies. *Vaccines (Basel)*, *11*(4).
2. Met, O., Jensen, K. M., Chamberlain, C. A., Donia, M., & Svane, I. M. (2019). Principles of adoptive T cell therapy in cancer. *Semin Immunopathol*, *41*(1), 49-58.
3. Gaissmaier, L., Elshiaty, M., & Christopoulos, P. (2020). Breaking Bottlenecks for the TCR Therapy of Cancer. *Cells*, *9*(9).
4. Jacoby, E., Shahani, S. A., & Shah, N. N. (2019). Updates on CAR T-cell therapy in B-cell malignancies. *Immunol Rev*, *290*(1), 39-59.
5. Chandran, S. S., & Klebanoff, C. A. (2019). T cell receptor-based cancer immunotherapy: Emerging efficacy and pathways of resistance. *Immunol Rev*, *290*(1), 127-147.
6. Sun, Y., Li, F., Sonnemann, H., Jackson, K. R., Talukder, A. H., Katailiha, A. S., & Lizee, G. (2021). Evolution of CD8(+) T Cell Receptor (TCR) Engineered Therapies for the Treatment of Cancer. *Cells*, *10*(9).
7. Zhao, L., & Cao, Y. J. (2019). Engineered T Cell Therapy for Cancer in the Clinic. *Front Immunol*, *10*, 2250.
8. Garber, K. (2018). Driving T-cell immunotherapy to solid tumors. *Nat Biotechnol*, *36*(3), 215-219.
9. Davis, M. M., Boniface, J. J., Reich, Z., Lyons, D., Hampl, J., Arden, B., & Chien, Y.-h. (1998). Ligand recognition by αβ T cell receptors. *Annual review of immunology*, *16*(1), 523-544.
10. Scaviner, D., & Lefranc, M.-P. (2000). The human T cell receptor alpha variable (TRAV) genes. *Experimental and clinical immunogenetics*, *17*(2), 83-96.
11. Campillo-Davo, D., Flumens, D., & Lion, E. (2020). The Quest for the Best: How TCR Affinity, Avidity, and Functional Avidity Affect TCR-Engineered T-Cell Antitumor Responses. *Cells*, *9*(7).
12. Boon, T., Coulie, P. G., Eynde, B. J. V. d., & Bruggen, P. v. d. (2006). Human T cell responses against melanoma. *Annu. Rev. Immunol.*, *24*, 175-208.

doi:10.6342/NTU202303793

13. Tan, M. P., Gerry, A. B., Brewer, J. E., Melchiori, L., Bridgeman, J. S., Bennett, A. D., Pumphrey, N. J., Jakobsen, B. K., Price, D. A., Ladell, K., & Sewell, A. K. (2015). T cell receptor binding affinity governs the functional profile of cancer-specific CD8+ T cells. *Clin Exp Immunol*, *180*(2), 255-270.
14. Zhong, S., Malecek, K., Johnson, L. A., Yu, Z., Vega-Saenz de Miera, E., Darvishian, F., McGary, K., Huang, K., Boyer, J., & Corse, E. (2013). T-cell receptor affinity and avidity defines antitumor response and autoimmunity in T-cell immunotherapy. *Proceedings of the National Academy of Sciences*, *110*(17), 6973-6978.
15. Rapoport, A. P., Stadtmauer, E. A., Binder-Scholl, G. K., Goloubeva, O., Vogl, D. T., Lacey, S. F., Badros, A. Z., Garfall, A., Weiss, B., & Finklestein, J. (2015). NY-ESO-1–specific TCR–engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nature medicine*, *21*(8), 914-921.
16. Liu, Q., Tian, Y., Li, Y., Zhang, W., Cai, W., Liu, Y., Ren, Y., Liang, Z., Zhou, P., & Zhang, Y. (2020). In vivo therapeutic effects of affinity-improved-TCR engineered T-cells on HBV-related hepatocellular carcinoma. *Journal for Immunotherapy of Cancer*, *8*(2).
17. Holler, P. D., & Kranz, D. M. (2003). Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation. *Immunity*, *18*(2), 255-264.
18. Schmid, D. A., Irving, M. B., Posevitz, V., Hebeisen, M., Posevitz-Fejfar, A., Sarria, J., Gomez-Eerland, R., Thome, M., Schumacher, T. N., & Romero, P. (2010). Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. *The Journal of Immunology*, *184*(9), 4936-4946.
19. Robbins, P. F., Morgan, R. A., Feldman, S. A., Yang, J. C., Sherry, R. M., Dudley, M. E., Wunderlich, J. R., Nahvi, A. V., Helman, L. J., & Mackall, C. L. (2011). Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *Journal of Clinical Oncology*, *29*(7), 917.
20. Chinnasamy, N., Wargo, J. A., Yu, Z., Rao, M., Frankel, T. L., Riley, J. P., Hong, J. J., Parkhurst, M. R., Feldman, S. A., & Schrump, D. S. (2011). A TCR targeting the HLA-A* 0201–restricted epitope of MAGE-A3 recognizes multiple epitopes of the MAGE-A antigen superfamily in several types of cancer. *The Journal of Immunology*, *186*(2), 685-696.

doi:10.6342/NTU202303793

21. Schmid, D. A., Irving, M. B., Posevitz, V., Hebeisen, M., Posevitz-Fejfar, A., Sarria, J., Gomez-Eerland, R., Thome, M., Schumacher, T. N., & Romero, P. (2010). Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. *The Journal of Immunology*, *184*(9), 4936-4946.
22. Cameron, B. J., Gerry, A. B., Dukes, J., Harper, J. V., Kannan, V., Bianchi, F. C., Grand, F., Brewer, J. E., Gupta, M., & Plesa, G. (2013). Identification of a titin-derived HLA-A1–presented peptide as a cross-reactive target for engineered MAGE A3–directed T cells. *Science translational medicine*, *5*(197), 197ra103-197ra103.
23. Rius, C., Attaf, M., Tungatt, K., Bianchi, V., Legut, M., Bovay, A., Donia, M., Peakman, M., Svane, I. M., & Ott, S. (2018). Peptide–MHC class I tetramers can fail to detect relevant functional T cell clonotypes and underestimate antigen-reactive T cell populations. *The Journal of Immunology*, *200*(7), 2263-2279.
24. Morimoto, S., Fujiki, F., Kondo, K., Nakajima, H., Kobayashi, Y., Inatome, M., Aoyama, N., Nishida, Y., Tsuboi, A., & Oka, Y. (2018). Establishment of a novel platform cell line for efficient and precise evaluation of T cell receptor functional avidity. *Oncotarget*, *9*(75), 34132.
25. Rosskopf, S., Leitner, J., Paster, W., Morton, L. T., Hagedoorn, R. S., Steinberger, P., & Heemskerk, M. H. (2018). A Jurkat 76 based triple parameter reporter system to evaluate TCR functions and adoptive T cell strategies. *Oncotarget*, *9*(25), 17608.
26. Yu, H., Lin, L., Zhang, Z., Zhang, H., & Hu, H. (2020). Targeting NF-kappaB pathway for the therapy of diseases: mechanism and clinical study. *Signal Transduct Target Ther*, *5*(1), 209.
27. Catala-Rabasa, A., Ndagire, D., Sabio, J. M., Fedetz, M., Matesanz, F., & Alcina, A. (2011). High ACSL5 transcript levels associate with systemic lupus erythematosus and apoptosis in Jurkat T lymphocytes and peripheral blood cells. *PloS one*, *6*(12), e28591.
28. Rego, R. T., Morris, E. C., & Lowdell, M. W. (2019). T-cell receptor gene-modified cells: past promises, present methodologies and future challenges. *Cytotherapy*, *21*(3), 341-357.
29. Thomas, S., Stauss, H. J., & Morris, E. C. (2010). Molecular immunology lessons from therapeutic T-cell receptor gene transfer. *Immunology*, *129*(2), 170-177.
30. Gaud, G., Lesourne, R., & Love, P. E. (2018). Regulatory mechanisms in T cell receptor signalling. *Nature Reviews Immunology*, *18*(8), 485-497.
31. Huppa, J. B., & Davis, M. M. (2003). T-cell-antigen recognition and the

doi:10.6342/NTU202303793

immunological synapse. *Nature Reviews Immunology*, *3*(12), 973-983.
32. Love, P. E., & Hayes, S. M. (2010). ITAM-mediated signaling by the T-cell antigen receptor. *Cold Spring Harbor perspectives in biology*, *2*(6), a002485.
33. Hořejší, V., Zhang, W., & Schraven, B. (2004). Transmembrane adaptor proteins: organizers of immunoreceptor signalling. *Nature Reviews Immunology*, *4*(8), 603-616.
34. Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., & Schreiber, R. D. (2001). IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature*, *410*(6832), 1107-1111.
35. van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., ... & Boon, T. (1991). A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science*, *254*(5038), 1643-1647.
36. Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., & Schreiber, R. D. (2002). Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology*, *3*(11), 991-998.
37. Tsuji, T., Yasukawa, M., Matsuzaki, J., Ohkuri, T., Chamoto, K., Wakita, D., ... & Nishimura, T. (2005). Generation of tumor-specific, HLA class I–restricted human Th1 and Tc1 cells by cell engineering with tumor peptide–specific T-cell receptor genes. *Blood*, *106*(2), 470-476.
38. Morgan, R. A., Dudley, M. E., Wunderlich, J. R., Hughes, M. S., Yang, J. C., Sherry, R. M., ... & Rosenberg, S. A. (2006). Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science*, *314*(5796), 126-129.
39. Davila, M. L., Riviere, I., Wang, X., Bartido, S., Park, J., Curran, K., ... & Brentjens, R. (2014). Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Science translational medicine*, *6*(224), 224ra25-224ra25.
40. Chang, Z. L., Lorenzini, M. H., Chen, X., Tran, U., Bangayan, N. J., & Chen, Y. Y. (2018). Rewiring T-cell responses to soluble factors with chimeric antigen receptors. *Nature chemical biology*, *14*(3), 317-324.
41. Weiner, L. M., Murray, J. C., & Shuptrine, C. W. (2012). Antibody-based immunotherapy of cancer. *Cell*, *148*(6), 1081-1084.
42. Fagerberg, L., Jonasson, K., von Heijne, G., Uhlén, M., & Berglund, L. (2010). Prediction of the human membrane proteome. *Proteomics*, *10*(6), 1141-1149.
43. Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P.,

doi:10.6342/NTU202303793

Mardinoglu, A., ... & Pontén, F. (2015). Tissue-based map of the human proteome. *Science*, *347*(6220), 1260419.

44. Tan, M. P., Gerry, A. B., Brewer, J. E., Melchiori, L., Bridgeman, J. S., Bennett, A. D., ... & Sewell, A. K. (2015). T cell receptor binding affinity governs the functional profile of cancer-specific CD8+ T cells. *Clinical & Experimental Immunology*, *180*(2), 255-270.
45. Campillo-Davo, D., Versteven, M., Roex, G., De Reu, H., van der Heijden, S., Anguille, S., ... & Lion, E. (2020). Rapid assessment of functional avidity of tumor-specific T cell receptors using an antigen-presenting tumor cell line electroporated with full-length tumor antigen mRNA. *Cancers*, *12*(2), 256.
46. Jutz, S., Leitner, J., Schmetterer, K., Doel-Perez, I., Majdic, O., Grabmeier-Pfistershammer, K., ... & Steinberger, P. (2016). Assessment of costimulation and coinhibition in a triple parameter T cell reporter line: Simultaneous measurement of NF-κB, NFAT and AP-1. *Journal of immunological methods*, *430*, 10-20.
47. Müller, T. R., Schuler, C., Hammel, M., Köhler, A., Jutz, S., Leitner, J., ... & Steinberger, P. (2020). AT-cell reporter platform for high-throughput and reliable investigation of TCR function and biology. *Clinical & Translational Immunology*, *9*(11), e1216.
48. Tian, S., Maile, R., Collins, E. J., & Frelinger, J. A. (2007). CD8+ T cell activation is governed by TCR-peptide/MHC affinity, not dissociation rate. *The Journal of Immunology*, *179*(5), 2952-2960.
49. Corse, E., Gottschalk, R. A., & Allison, J. P. (2011). Strength of TCR–peptide/MHC interactions and in vivo T cell responses. *The Journal of Immunology*, *186*(9), 5039-5045.
50. Krogsgaard, M., Prado, N., Adams, E. J., He, X. L., Chow, D. C., Wilson, D. B., ... & Davis, M. M. (2003). Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. *Molecular cell*, *12*(6), 1367-1378.
51. Laugel, B., van den Berg, H. A., Gostick, E., Cole, D. K., Wooldridge, L., Boulter, J., ... & Sewell, A. K. (2007). Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties. *Journal of Biological Chemistry*, *282*(33), 23799-23810.
52. Schmid, D. A., Irving, M. B., Posevitz, V., Hebeisen, M., Posevitz-Fejfar, A., Sarria, J. C., ... & Rufer, N. (2010). Evidence for a TCR affinity threshold delimiting maximal CD8 T cell function. *The Journal of Immunology*, *184*(9), 4936-4946.

doi:10.6342/NTU202303793

53. Amos, S. M., Duong, C. P., Westwood, J. A., Ritchie, D. S., Junghans, R. P., Darcy, P. K., & Kershaw, M. H. (2011). Autoimmunity associated with immunotherapy of cancer. *Blood, The Journal of the American Society of Hematology*, *118*(3), 499-509.
54. Johnson, L. A., Morgan, R. A., Dudley, M. E., Cassard, L., Yang, J. C., Hughes, M. S., ... & Rosenberg, S. A. (2009). Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. *Blood, The Journal of the American Society of Hematology*, *114*(3), 535-546.

doi:10.6342/NTU202303793