

Real-Time Specificity and Potency Assessment of Human Papilloma Virus Specific Engineered T Cells

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Introduction

Human Papilloma Virus (HPV) is the causative agent for several cancers including cervical, anogenital, and a subset of head and neck squamous cell carcinomas (HNSCC). While HPV vaccines have proved very efficacious in protecting against cervical and other HPV-related cancer in unexposed individuals, it is not effective in infected individuals and therefore alternative approaches are warranted. The HPV E6 and E7 oncoproteins can be ideal targets for vaccine-based therapy in tumors where HPV is deemed to be the causative agent. These proteins are constitutively expressed and important for the survival of HPV cancers. However, despite exhaustive efforts these vaccines have failed to generate an effective immune response against HPV-based tumors in the clinic.^{1,2}

Adoptive T cell transfer (ACT) is a promising approach for cancer immunotherapy. Isolation of tumor infiltrating lymphocytes (TILs) from tumor biopsies, followed by *ex vivo* expansion and re-infusion, has shown regression of tumor types including melanomas and cervical cancers.³ However, this treatment is restricted to only a handful of tumors, especially those with higher neoantigen burden. Combining ACT together with cellular engineering approaches such as the introduction of chimeric antigen receptors or recombinant T cell receptors (TCR) selective for an antigen of interest has the potential to expand the utility of ACT for a wide range of cancers.⁴ In this report, the TCR of specific TIL clones which displayed selectivity for HPV E6 oncoprotein were identified, cloned and re-introduced into native T cells. The Agilent xCELLigence RTCA system was used to assess both the selectivity and potency of the TCR-engineered TILs toward HLA restricted cells.

Experimental

A noninvasive, label-free, and real-time cellular impedance monitoring technology (xCELLigence RTCA, Figure 1A) was adopted to measure the potency of biologics and immune cell-mediated cytotoxicity. The xCELLigence platform uses gold biosensors embedded in the bottom of microtiter wells to monitor the status of adherent cells, or suspension cells which have been tethered to the plate bottom (Figure 1B). The basic assay principle is based on impedance measurements through the surface of gold electrodes where attached cells act as insulators, impeding the flow of an alternating microampere electric current between electrodes. This impedance signal is measured automatically, at a frequency defined by the user (every 10 seconds, once per hour, etc.), and provides an extremely sensitive readout of cell number, cell size, and cell-substrate attachment strength. In contrast to surface-attached cancer cell targets, immune effector cells are non-adherent and therefore do not directly affect the impedance signal; however their cytotoxic activity can be detected through the reduction of the number of target cancer cells. Because of this property, the cytolytic activity of T cells, NK cells, CARTs, oncolytic virus, checkpoint inhibitors, bispecific antibodies, BiTEs, etc. can be selectively monitored in real time (Figure 1C).⁵



Figure 1A. The Agilent xCELLigence RTCA MP instrument monitors cell proliferation, morphology change, and attachment quality in a label-free, real-time manner. The MP (multiple plate) model can host up to six 96-well E-Plates. The instrument is placed in a standard CO₂ cell culture incubator and interfaces via a cable with analysis and control units that are housed outside the incubator.

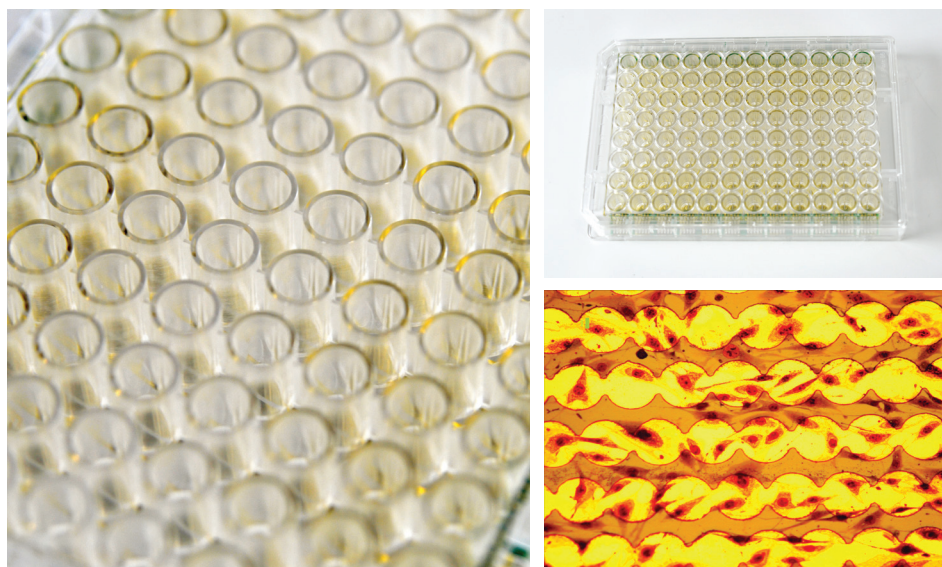


Figure 1B. Agilent xCELLigence real-time cell analysis (RTCA) instruments use patented microtiter plates (E-Plates), which contain gold biosensors embedded in the bottom of each well. These biosensors continuously and noninvasively monitor changes in cell number, cell size, and cell-substrate attachment quality. Close up of impedance biosensors on Agilent E-plates (bottom right image).

Methods and materials

For this study, CD8 T cells were positively selected from the blood of healthy donors (StemCell) and were stimulated *in vitro* with anti-CD3/CD28 beads and IL-2, IL-7, and IL-15 for two days. On the second day the T cells were electroporated using the MaxCyte with ribonucleoproteins (RNP) specific for the TRAC gene. Cells were placed back in culture with IL-2, and on the following day, the T cells were transduced with a lentiviral vector (LV) containing the neoantigen-specific TCR. The transduction was repeated the following day and the cells were expanded in IL-2-supplemented media for up to 14 days. T cells expressing the neoantigen TCR were enriched using a positive selection kit for CD34 (StemCell) prior to performing assays on xCELLigence system.

The xCELLigence RTCA MP instrument was used for all impedance experiments. First, 50 μL of target cancer cell culturing media was added to each well of 96 well E-Plates (Agilent Technologies) and the background impedance was measured and displayed as Cell Index. Dissociated adherent target cells were seeded at a density of 5000 cells/well of the E-Plate in a volume of 100 μL and allowed to passively adhere on the electrode surface. Post seeding, the E-Plate was kept at ambient temperature inside a laminar flow hood for 30 minutes and then transferred to the RTCA MP instrument inside a cell culture incubator. Data recording was initiated immediately at 15-minute intervals for the entire duration of the experiment. At the time treatment was applied, data acquisition was paused, 100 μL of media was removed from each well and effector cells were added at different effector to target (E:T) ratios ranging from 20:1 to 1:1, in a volume of 100 μL . In the experiments where Cisplatin were used, 10 μL of 10 mM Cisplatin stock solution was added to the appropriate wells. The

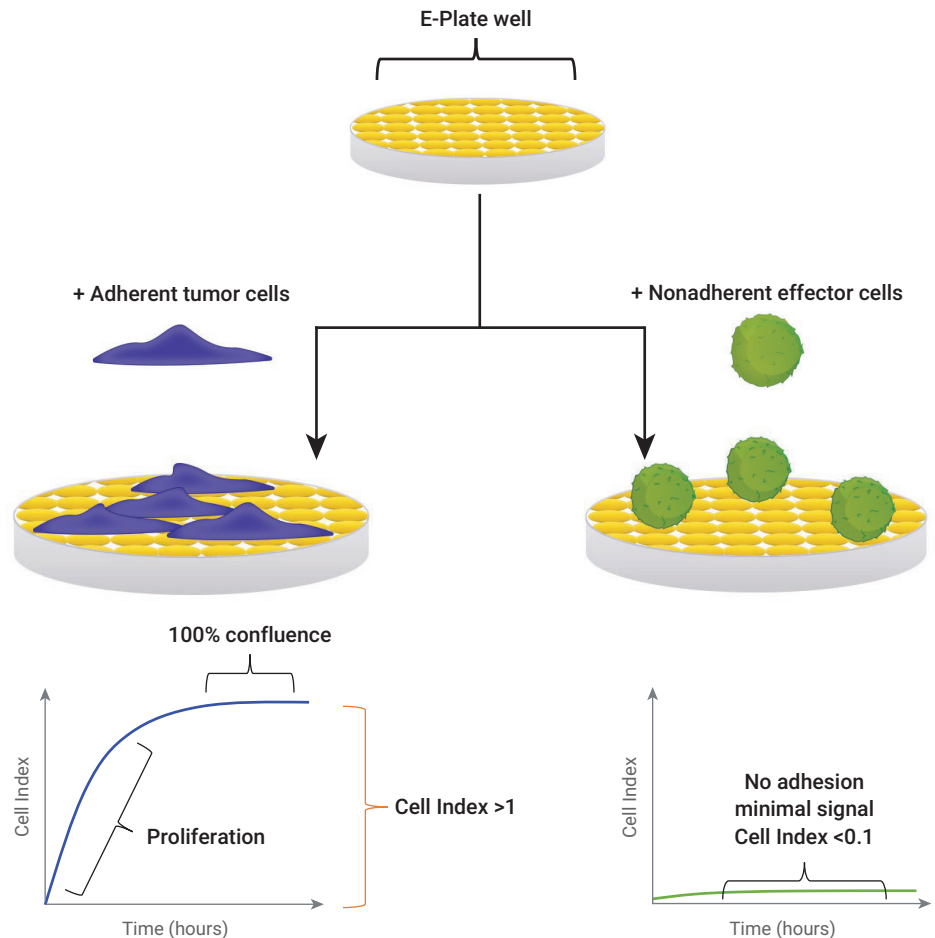


Figure 1C. Measuring tumor cell killing without labels. Adherent tumor cells easily attach to E-plates. At 100% adherent tumor cell confluence, the cell index is typically >1. Correspondingly, most effector cell types such as T cells and NK cells are nonadherent, and produce a low cell index, typically less than 0.1. Typically, there is an approximately 10-fold difference between the cell index of the two cell types. When two cell types are mixed, the decrease in signal corresponding to cytolysis is attributed to the tumor cell lysis.

E-Plate was kept in the laminar flow hood for 30 minutes before resuming the experiment. Changes in impedance were reported as Cell Index (CI) and Normalized Cell Index (NCI), which is derived by dividing the Cell Index value at each time point by the Cell Index at time before effector cell addition.

From the tumor infiltrating lymphocytes (TIL) of a head and neck squamous cell carcinoma (HNSCC) individual, a dominant T cell clone was identified with specificity against the E6 protein of HPV. Using single cell transcriptomics, the gene encoding for the T cell receptor (TCR α and β) was sequenced and subsequently engineered into lentiviral vector (LV) and transduced into J76 Jurkat T cells. The specificity of the engineered TCR was confirmed with HLA-A*02:01 matched antigen presenting cells (APCs). The genes encoding for the TCR α and TCR β of the E6-specific clone was then introduced into primary human T cells using a two-step protocol. First, the endogenous TCR α constant region (TRAC) of the primary T cell was disrupted to minimize potential nonproductive TCR α - β pairings. Second, the E6-specific TCR α and TCR β chains were introduced along with a truncated CD34 (tCD34) reporter gene using standard LV vectors.

In another experiment, a dominant T cell clone specific for the neoantigen: RPS2 V205I was identified from the tumor infiltrating lymphocytes (TIL) of a head and neck squamous cell carcinoma (HNSCC) individual. Using single cell transcriptomics, the TCR was sequenced, cloned into lentiviral vector and engineered into J76 Jurkat cells and its specificity was confirmed for an HLA-B*07:02 matched antigen presenting cells (APCs).

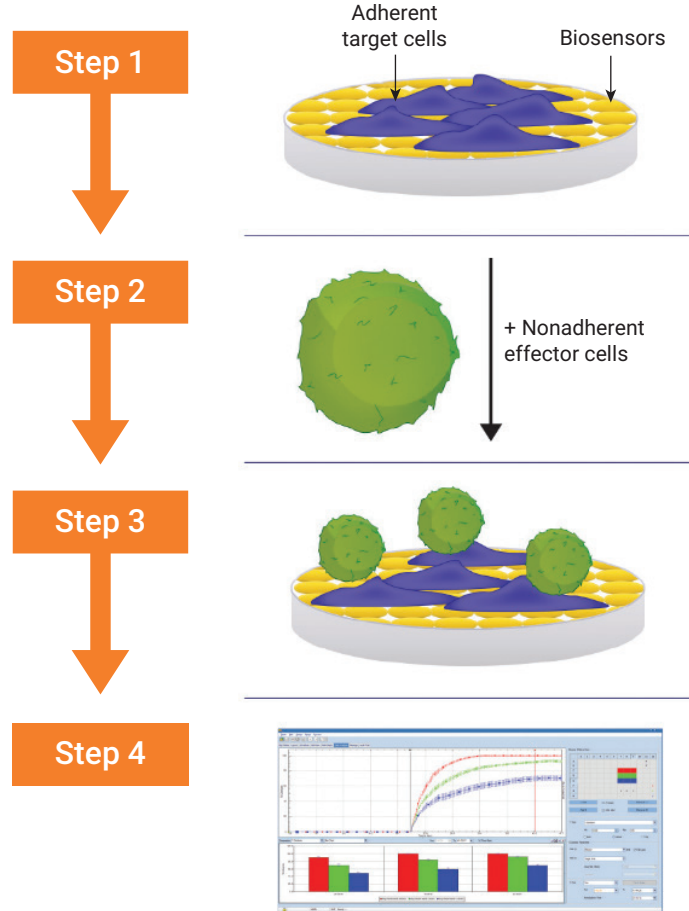


Figure 1D. Agilent xCELLigence RTCA technology offers a high throughput and easy workflow. Step 1: Adherent target cells (i.e. tumor cells) are seeded in E-Plate wells. The biosensor signal, also known as "Cell Index," increases as cells attach and proliferate and then plateaus as cells approach 100% confluence. Step 2: When added subsequently, nonadherent effector cells (i.e. immune cells) in suspension do not cause signal changes due to lack of adherence to the gold biosensors. Step 3: If effector cells induce the destruction of the target adherent tumor cells, the corresponding cytolytic activity can be sensitively and precisely detected. Step 4: Using an intuitive graphical interface designed specifically for the immuno-oncologist, RTCA Software Pro monitors cell killing in real time.

Results and discussion

The E6-specific engineered T cells were expanded *ex vivo* as described in the Material and Methods section. In order to test the specificity and potency of the E6 specific engineered T cells the xCELLigence real-time cell analysis (RTCA) system was used to perform the killing assays. Either E6+HLA-A*02:01⁺ cancer cell line (Figure 2A) and another E6+HLA-A*02:01⁺ tumor line (Caski, Figure 2B) was used as target cells and seeded in the wells of the E-Plate. The wells containing the cells showed robust attachment and growth of the indicated cells (Figures 2A and 2B, green curve). At approximately 20 hours after cell seeding, 10:1 effector to target (E:T) ratio of the TCR engineered T cells specific for HPV E6 protein were added to the wells (blue curve).

To assess nonspecific alloreactivity by the CD8 T cells, CD8 T cells that were stimulated and expanded but not subjected to CRISPR or LV editing (purple curve) were also added to separate wells at E:T ratio of 10:1 to assess background killing activity of the T cells. Additionally, as a positive control, the cells were treated with 10 μ M Cisplatin (red curve).

The data clearly demonstrate that TCR engineered T cells specific for HPV E6 protein display preferential cytolytic activity for both E6+ cells and for Caski cells which are E6 positive within the context of HLA-A*02:01⁺.

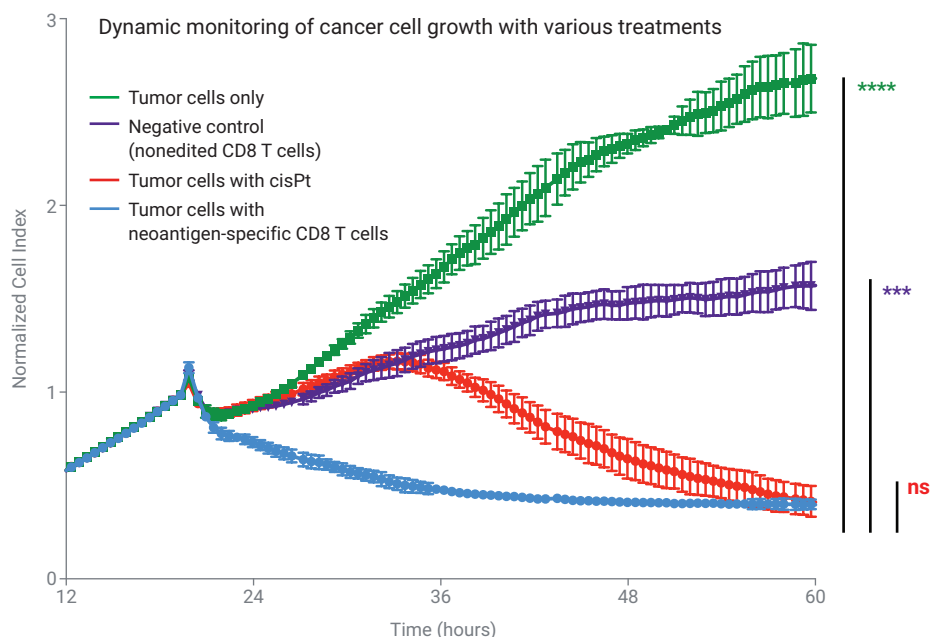


Figure 2A. Dynamic monitoring of a cancer line to assess the capacity of the TCR to specifically kill tumor lines expressing the HPV E6 protein at 10:1 effector to target ratio. The green curve shows the natural growth of the tumor cells with no added effector cells. The red curve demonstrates that the chemotherapeutic, cisPt, can kill these lines within 48 hours. To assess non-specific (alloreactivity) background killing by the CD8 T cells, the growth of CD8 T cells that were stimulated and expanded but not subjected to CRISPR or LV editing are represented by the purple curve. The neoantigen-engineered CD8 T cells (blue line) shows superior killing capacity than even chemotherapy at very early time points. Reprinted with permission of Schoenberger Lab 2020.

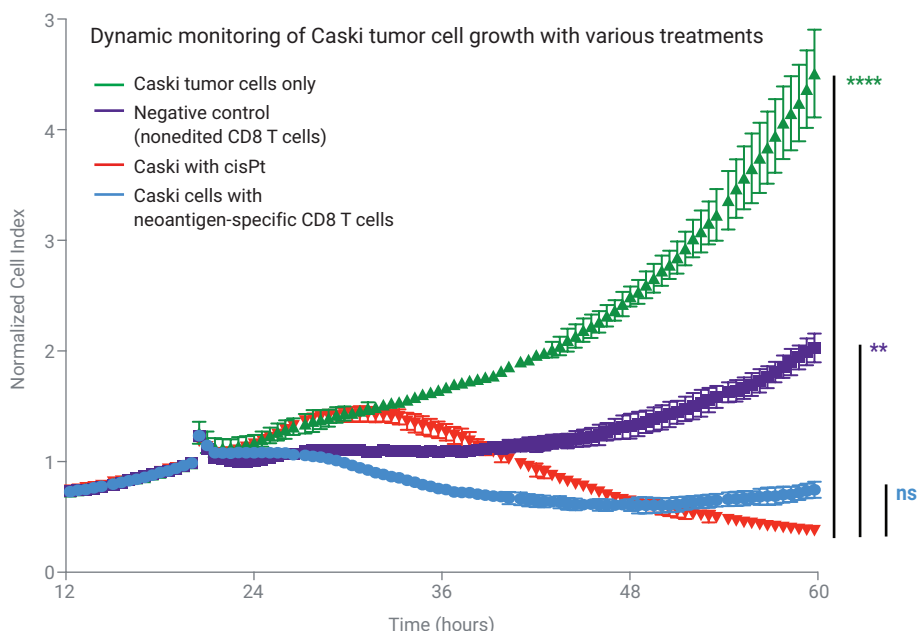


Figure 2B. Dynamic monitoring of E6+HLA-A*02:01⁺ tumor line (Caski) to assess the capacity of the engineered TCR to specifically kill tumor lines expressing the HPV E6 protein at 10:1 effector to target ratio. The green curve shows the natural growth the tumor cells with no added effector cells. The red curve demonstrates that the chemotherapeutic, cisPt, can kill the Caski cells within 48 hours. To assess nonspecific (alloreactivity) background killing by the CD8 T cells, the growth of CD8 T cells that were stimulated and expanded but NOT subjected to CRISPR or LV editing are represented by the purple curve. The neoantigen-engineered CD8 T cells (blue line) shows superior killing capacity than even chemotherapy at very early time points. Reprinted with permission of Schoenberger Lab 2020.

Additionally, the ability of a RPS2-V205I reactive TCR to recognize naturally processed and presented neoantigens on the surface of tumor cells was assessed (Figure 3). The original tumor line was established from the same individual from which the TCR was identified and lost expression of the restricting HLA-B*07:02 allele.

To assess the capacity of the TIL to specifically recognize and kill tumor lines, HLA-B*07:02⁺ and HLA-B*07:02⁻ cancer cells were seeded in the wells of the E-Plates and monitored as described above. The gray and black curves in Figure 3 show the growth of untreated HLA-B*07:02⁻ and HLA-B*07:02⁺ cells, respectively. The addition of RPS2 V205I specific TILs to HLA-B*07:02⁻ cells at different E:T ratios did not have any significant impact on the viability of HLA-B*07:02⁻ cells (gray curves). On the other hand, addition of RPS2 V205I specific TILs to HLA-B*07:02⁺ at different E:T ratio displayed a density dependent decrease in the viability of HLA-B*07:02⁺ cells. Addition of Cisplatin as a positive control showed robust and time-dependent killing of HLA-B*07:02⁻ cells (red curve).

Taken together, the data clearly illustrate the specificity, time, and density dependent killing of RPS2 V205I targeted TILs.

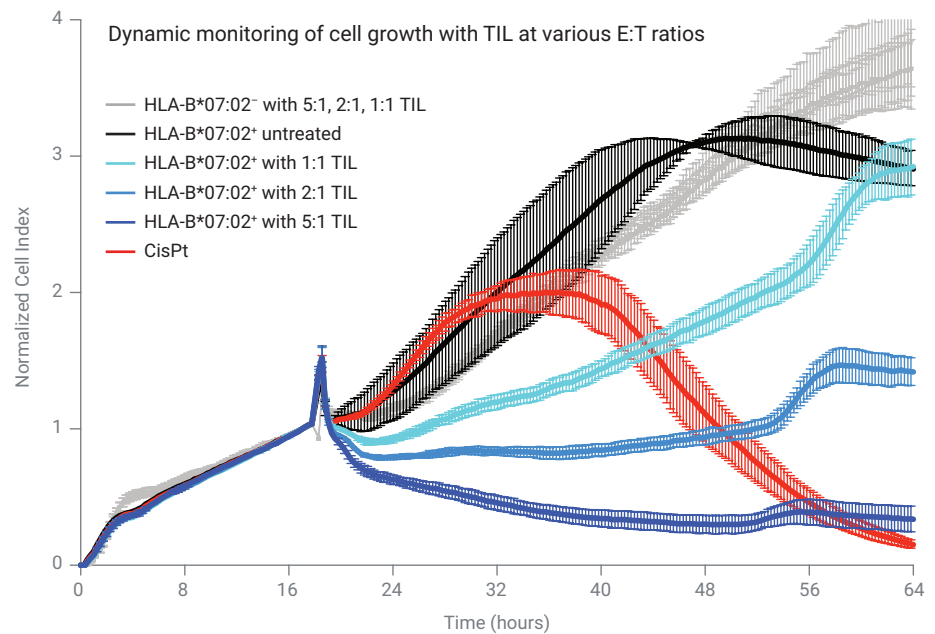


Figure 3. To assess the capacity of the TIL clone specific for the neoantigen RPS2 V205I to recognize and kill tumor lines, we monitored the growth of both HLA-B*07:02⁺ or HLA-B*07:02⁻ cancer cells. The black curve shows the natural growth of this cancer line with no intervention. The red curve demonstrates that the chemotherapeutic, cisPt, can kill this line within 60 hours. The original cell line (grey curves) was unresponsive to any of the TIL at various effector-to-target ratios (5:1, 2:1, or 1:1). Once the restricting HLA-B*07:02 allele was reintroduced, the blue curves indicated that the TIL identified from the same individual can now kill the tumor. Reprinted with permission of Schoenberger Lab 2020.

Conclusion

We have demonstrated the utility of the xCELLigence RTCA to evaluate the selectivity and potency of engineered neoantigen-specific T cells towards HLA restricted cells. These data demonstrate that any CD8 T cell engineered with this E6-reactive engineered TCR can recognize and specifically kill a target cell that expresses the E6 protein and appropriate restricting HLA allele.

These data also confirm that restoring the HLA-B*07:02 allele permits the rapidly expanded TIL to recognize and kill the tumor.

In summary, the xCELLigence RTCA potency assay can be used to easily evaluate the selectivity and potency of engineered neoantigen-specific T cells. Target tumor cell lines were simply seeded in an E-Plate, after which engineered effector T cells were added, and the kinetics of tumor cell destruction was noninvasively monitored over the course of days. Data acquisition was continuous and automatic. The quantitative and real-time nature of the impedance data made it easy to compare the potency between treatments.

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