Optimalization of a model system for T-cell receptor mediated activation

Towards predicting the process of antigen processing

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Layman's summary

Every day, the human body is tormented by numerous potential threats: bacteria, viruses and defects in cellular programming, which can injure cells and danger our well-being. That is why the adaptive immune system is essential for us to eradicate diseased cells. The MHC class I pathway is a part of the adaptive immune system that allows immune cells (cytotoxic T cells) to detect and eliminate diseased cells. So how does the MHC class I pathway work?

Inside every cell (except for reproductive cells and red cells), a specialized complex called the proteasome degrades proteins that are present in the cytosol, into smaller fragments: peptides. These peptides are transported into the Endoplasmic Reticulum (ER) and modified step by step to fit onto membrane-bound MHC class I molecules. These processes are performed by a series of proteins that form the antigen processing and presentation (APP) pathway. These APP proteins make sure that the peptide-MHC class I (pMHC) complex is transported to the cell surface for presentation. When foreign peptides are presented, for instance from malformed proteins or viral proteins, T cell receptors (TCRs) will recognize them and bind to the complex. Thereby, they activate the immune system, and assure that the cell is removed.

If we could predict which exact peptides are presented on a cell and evoke an immune response, we could apply this knowledge to battle tumors or develop vaccines. After all, we could then use viral peptides that we know cause a large immune response, to vaccinate people against that virus. Or we could boost someone's immune system with immunogenic peptides when it does not recognize tumor cells by itself. Unfortunately, the prediction of which peptides are immunogenic and highly presented by MHC class I is quite a challenge. In the APP pathway, the selection of which peptides are presented, and how frequent, is carefully controlled and depends on a lot of factors: availability and activity of APP proteins in the cell, chemical properties of the peptides and MHC class I molecules, and possible mutations in the peptides. These things vary considerably among different individuals.

We want to improve our understanding of the APP pathway and be able to predict the likelihood of a peptide to pass the APP steps. Mishto, our collaborator, is working on the development of a computational model based on quantitative data from *in vitro* and *in cellulo* experiments to make these predictions. In this research project, we set up the procedure for one of these *in cellulo* experiments. We present an assay in which we bring T cells containing an antigen-specific TCR in contact with antigen-presenting cells, and examine the T cell response by measuring the activation of three transcription factors involved in immune signaling using fluorescence. In the future, we can proceed by inhibiting the activity of APP proteins and look at the effect of each protein on T cell activation. This data can then be integrated in Mishto's computational model and contribute to our understanding of the MHC class I pathway and may lead to possible strategies for vaccine design.

Abstract

Major histocompatibility complex (MHC) class I antigen presentation is a vital process in every nucleated cell for adaptive immunity, and critical in defense against intracellular pathogens and tumor cells. MHC class I molecules present cellular or pathogen-derived peptides on the cell surface for epitope recognition by antigen-specific T cell receptors on cytotoxic CD8+ T cells. Preceding antigen presentation is the antigen processing pathway, where peptides are generated from proteins and modified for the formation of peptide-MHC class I (pMHC-I) complexes. The repertoire and expression of presented peptides varies among the human population and depends on a multitude of steps in the antigen processing and presentation (APP) pathway. Being able to predict which epitopes are presented by MHC class I molecules and elicit a high immune response could be beneficial for the identification of targets for immunotherapy and for vaccine design. However, the APP pathway is a complex regulated system, and therefore predictions of epitope recognition and immunogenicity can be challenging. To improve prediction of epitope immunogenicity, a collaboration was set up with the Mishto lab to develop a computational model for the APP pathway. In this research project, a T cell activation model was set up for the acquisition of quantitative data on the effect of APP protein inhibition on T cell activation. Here, we present a reproducible assay that quantitatively demonstrates T cell activation in response to melanoma gp100$_{209-217}$ epitope presentation, using the triple parameter reporter Jurkat 76 (J76 TPR) T cell line and measuring the activation of three reporter genes (NFAT-eGFP, NF-κB-CFP and AP-1-mCherry). This model can be used as the promising starting point for APP protein intervention assays of which data can be incorporated into Mishto's computational model for the prediction of epitope immunogenicity.

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Introduction

Major Histocompatibility Complex class I (MHC-I) proteins are vital players in the adaptive immune system. These proteins provide the ability for every nucleated cell to present peptides derived from intracellular proteins to immune cells and thereby offer a glimpse into the intracellular environment of the cell. Cells infected by intracellular pathogens present pathogen-derived peptides on the surface, leading to recognition by cytotoxic lymphocytes (CTL) or CD8+ T-cells caused by the interaction of T-cell receptors (TCR) to the peptide-MHC-I (pMHC-I) complex. In the same way, other types of malignant cells, e.g. tumor cells, are recognized by CTL through the presentation and recognition of unfamiliar peptides. TCR-mediated recognition leads to the activation of the CTL and through further signaling by cytokines, the adaptive immune system.[1]

MHC-I molecules, or human leukocyte antigens (HLA) in humans, are highly polymorphic and can bind a broad variety of peptides. These molecules are assembled in the ER from two polypeptide chains, an α-chain and β_2-microglobulin. The polymorphic α-chain, or heavy chain, contains one transmembrane helix, two membrane-distal domains, and one membrane-proximal immunoglobulin (Ig)-like structure (Figure 1). MHC-II molecules are made up of two anchored heavy chains with both an Ig-like subunit. In contrast, the MHC-I α-chain is non-covalently linked to the β_2-microglobulin (β_2m) which forms the second Ig-like structure. These domains support the peptide that binds in the space between the two α-helices. MHC-I heavy chains are encoded by three genes (HLA-A, HLA-B, HLA-C).[2] HLA alleles are polymorphic and extremely various among the human population. HLA polymorphism influences MHC-I assembly, expression, conformation and the peptide repertoire that is presented.[3] Whether a peptide can stably bind MHC-I molecules, depends on its structure and whether it matches the conformation of MHC-I, among others. The MHC-I binding groove is closed on the N- and C-terminal side, which makes its preferred peptide length specific and short, namely 8 to 10 amino acids. The binding groove possesses a conserved set of hydrogen bonds that bind the backbone of the peptide, and certain pockets that are occupied by peptide side chains. These pockets are numbered A to F and differ for each MHC-I allotype. Pocket B and F home the primary anchor residues of peptides (P2 and PΩ (last position)). These anchor residues are conserved between distinct peptides binding an MHC-I molecule.[4]

Prior to presentation on MHC-I molecules, proteins undergo a series of tightly regulated processing steps in order to generate peptides that are appropriate for loading onto MHC-I molecules and subsequent presentation (Figure 2Fout! Verwijzingsbron niet gevonden.), which we call the antigen processing and presentation (APP) pathway. Cytosolic proteins, either translated or transported from the endo-lysosomal vesicular system into the cytosol, are proteolyzed by (immuno)proteasomes into smaller peptides. These peptides are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), which is part of the protein loading complex (PLC).[5] In the ER, ER-resident aminopeptidases (ERAPs) perform N-terminal peptide processing and trim peptides to the appropriate size for MHC-I binding. Tapasin is associated to TAP and binds MHC-I molecules that are newly synthesized, thereby bringing the peptides and MHC-I molecules in close proximity. Together with other proteins of the PLC, like calreticulin (CRT), tapasin will then load the peptide onto MHC-I. In addition, tapasin has the ability to release sub-optimal peptides in favor of more stable peptides, thereby functioning as a quality control of the peptide repertoire.[4] Formation of pMHC-I causes a conformational change that leads to the release of the complex from tapasin. pMHC-I is transported to the Golgi complex and subsequentially to the cell surface for presentation to CD8+ T cells.[5,6] Recognition of epitopes by antigen-specific CD8+ T cells initiates immune signaling and results in activation of the adaptive immune system.[7]
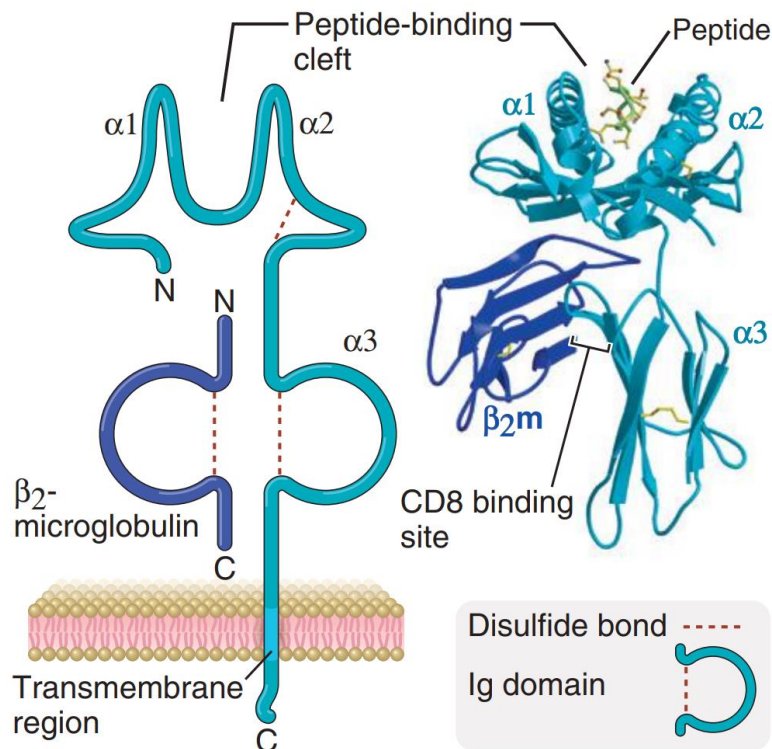
Figure 1: Schematic structure of an MHC-I molecule (from Abbas et al[1]).
MHC-I molecules consist of a polymorphic membrane-anchored α-chain and a non-covalently linked β2-microglobulin (left). The extracellular domains are shown in a ribbon diagram (right). Processed peptides bind MHC-I in-between the membrane-distal α-helices.

Hence, APP pathway activity can be regulated and changed on many levels. Nucleated cells have the ability of switching between different types of proteasome complexes, enabling them to alter catalytic activity and proteolytic dynamics. These diverse proteasome isoforms affect the cleavage sites of proteins and thereby immunogenicity of generated peptides. For instance, stimulatory cytokines can induce the formation of immunoproteasomes, which generated epitopes distinct from standard proteasomes.[8] In addition, the newly discovered importance of a mechanism called proteasome-catalyzed peptide splicing (PCPS) greatly enlarges the pool of possible presented epitopes. This process describes the ability of proteasomes to form novel peptide bonds during proteolysis in different ways (*trans*, *cis*, *and reverse cis*). It has been estimated that one-third of presented self-peptides is created by PCPS.[9] Together with HLA polymorphism, APP protein activity and availability, and other factors, PCPS makes that the pool of possible epitopes presented by MHC-I almost endless. A better comprehension of the processes that regulate which peptides are highly presented on MHC-I and elicit a large immune response, is decidedly advantageous for anticipating the most immunogenic epitopes.

Prediction of epitopes relevant in immune activation will be very useful for the selection of immunotherapy targets. For this major research project, a collaboration was initiated with Michele Mishto[9,10] to set up a computational model for the antigen processing pathway. The computational model will be based on *in vitro* and *in cellulo* data of the antigen processing pathway. In this research project, *in cellulo* data was obtained to match to the previously obtained *in vitro* data by the group of Michele Mishto. A T-cell activation assay was set up in order to obtain quantitative data about the effect of the expression levels of proteins in the APP-pathway on T-cell activation.
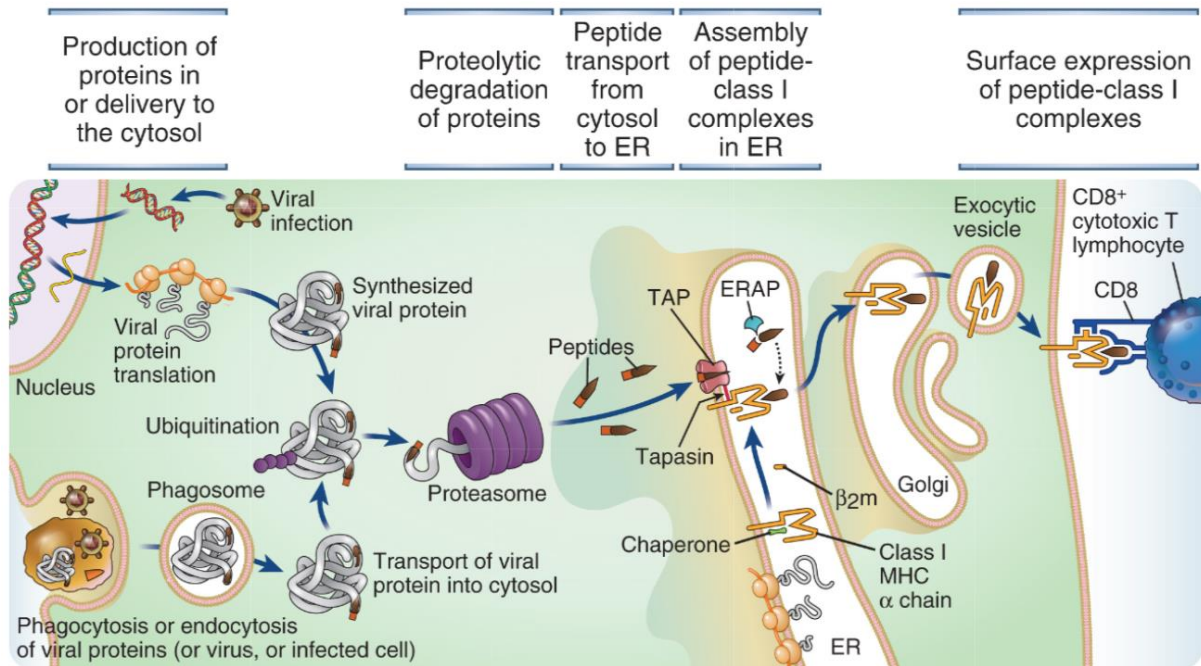
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Figure 2: A schematic overview of the MHC-I antigen processing and presentation pathway (from Abbas et al[1]).
Cellular proteins or proteins derived from intracellular pathogens undergo proteasomal proteolysis, followed by transport into the ER by TAP. Peptides are trimmed and modified for assembly of the pMHC-I complex. pMHC-I is transported to the cell membrane by exocytosis for antigen presentation, where antigen-specific CD8+ cytotoxic T cells can recognize the pMHC-I complex.

Here, we present a reproducible T-cell activation assay that quantitatively shows T cell activation in response to presentation of the melanoma gp100$_{209-217}$ epitope, using a triple parameter reporter Jurkat 76 (J76 TPR) T cell line. This model can be used as a starting point for a APP protein intervention assays of which data can be incorporated into Mishto's computational model of the prediction of epitope immunogenicity.

Materials & Methods

Plasmids

The γ-retroviral vector MP71-PRE encodes a TCR specific to the $gp100_{209-217}$ epitope, was received as a generous gift from the Mishto lab.[10] Plasmids were isolated using Quantum Prep® Plasmid Midiprep Kit (Bio-Rad).

Cell culture

293GALV packaging cells and cell lines for antigen presentation (Hela 33/2, Hela gp100 WT, Hela gp100 T210M, UKRV-Mel-15a) were cultured in Dulbecco's modified Eagle's medium (DMEM) GlutaMAX-I, supplemented with 10% FCS and 0,5% penicillin/streptomycin. Cells for antigen presentation were cultured in 6-well plates for maximal 18 passages. Jurkat 76 td CD8 (J76) and Jurkat 76 td CD8 triple parameter reporter (J76 TPR) cells were grown in IMDM (Iscove's Modified Dulbecco's Medium) GlutaMAX-I medium supplemented with 10% FCS and 0,5% penicillin/streptomycin. All cells were maintained at $37^{o}C$ and 5% CO_2. All tissue culture media were purchased from Gibco.

Antibodies and flow cytometry

The following antibodies were used for Western Blot: Anti-Melanoma gp100 (EP4863(2), Abcam), HRP-conjugated Beta-Tubulin (BT7R, Invitrogen), Swine Anti-Rabbit Immunoglobulins/HRP (polyclonal, Dako). The following monoclonal antibodies were used to confirm surface expression on transduced and non-transduced J76 and J76 TPR cell lines: mouse TCR β chain-PE (H57-597, BD Bioscience), CD8-VioBlue (BW135/80, Miltenyi), CD3-BV421 (SK7, BD Bioscience), CD3-PerCP (BW264/56, Miltenyi), CD3-APC-Cy7 (HIT3a, Biolegend). IFNγ-FITC (4S.B3, eBioscience), CD69-PE (TP1.55.3, Miltenyi), IL2-PerCPeFl710 (MQ1-17H12, eBioscience) were used for detecting intracellular cytokine expression. Flow cytometry analysis was performed using flow cytometers FACSCanto II (BD Bioscience) and Cytoflex LX (Beckman Coulter). FlowJo software (version 10) was used for data analysis.

Production of vector particles

293GALV cells were grown to 80% confluence in $10cm^2$ dishes for 24h at 37°C and 5% CO_2 in culture medium supplemented with 10 mM HEPES (from 1M, Gibco). The γ-retroviral vector MP71-PRE was transfected into the 293GALV cells by calcium phosphate precipitation. Medium was exchanged and virus supernatant was harvested after 48h and 72h. Viral medium was filtered through a 0.45-μm pore size filter and stored at -80°C. Alternatively, viral medium was filtered and concentrated by ultracentrifugation (25K, 2 hours), reconstituted and frozen in aliquots at -80°C .

Retroviral transduction of Jurkat cells

J76 andJ76 TPR cells were transduced with retrovirus containing retroviral vector MP71-PRE using spinoculation. Jurkat cells were seeded in 12-well plates at a concentration of $1x10^6$/mL in culture medium containing different virus concentrations and 10 μg/mL polybrene. Cells were centrifuged for 2 hr at 2000 RPM. After centrifugation, cells were incubated for 2 to 3 hours, and medium was replaced. After 24 hours, transduced cells were collected, counted and resuspended at $2,5x10^5$/mL in medium and incubated. After 48 hr of incubation, transduction efficiency was verified by mouse-TCRβ expression using the BD FACSCanto II instrument (BD Biosciences).

Co-culture experiments of gp100-expressing cell lines with gp100TCR-transduced Jurkat 76 cells

gp100-expressing cell lines (Hela gp100 WT, Hela gp100 T210M, UKRV-Mel-15a) were used for co-culture experiments. In addition, Hela 33/2 cells were loaded with $_{mut}gp100_{209-217}$ peptide (aa sequence: IMDQVPFSV) (ten-fold dilution series of 1 mM to 1 pM) or mock-treated for 1h before stimulation. gp100-expressing cell lines were co-cultured (5×10^4 or 1×10^5/well) with gp100TCR-transduced J76 cells (ratio 1:1, 1:5, and 1:10) in 96-well flat bottom plates in IMDM in a final volume of 200 μL. Cells were co-stimulated with 5 ng/mL PMA, 10 ng/mL PMA, anti-CD28, or not at all. Stimulation with 10 ng/mL PMA and 1 μM ionomycin was used as a positive control. After co-culturing for 16h, 24h, 48h,

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and 72h, medium was harvested and IFNγ release was measured using the Human IFN gamma Uncoated ELISA kit (Invitrogen). Alternatively, J76 cells and gp100-expressing cell lines were cocultured in presence of 10 µM monensin to inhibit vesicle excretion. After 6h, Jurkat cells were fixated and stained for CD69 or the intracellular cytokines IFNγ and IL2. Antibody levels were measured using the BD FACSCanto II instrument (BD Biosciences).

Cocultivation experiments of gp100-expressing cell lines with gp100TCR-transduced J76 TPR cells
The same gp100-expressing cell lines and Hela 33/2 cells loaded with exogenous peptide were used as for the co-culture experiments with J76 cells. gp100-expressing cell lines were cocultured (5×10^4/well) with J76 TPR cells ($2,5 \times 10^5$/well) in 96-well flat bottom plates in IMDM in a final volume of 200 µL. Stimulation with 5 ng/mL PMA and 400 nM ionomycin was used as positive control. Cells were harvested, stained for CD3, and fixated after 6h, 24h, and 48h of cultivation. Expression of the reporter genes was measured using a Cytoflex LX flow cytometer (Beckman Coulter).

Western blot
Hela 33/2 cells, Hela gp100 WT, Hela gp100 T210M, and UKRV-Mel-15a cells were harvested, washed 3 times with PBS and frozen at -80°C. Cells were thawed on ice and lysed using Pierce™ IP Lysis Buffer (Thermo Scientific). The tubes were centrifuged at 20.000x g and 4°C for 20 minutes. Laemmli Sample buffer (Bio-Rad) was added to supernatants and cleared lysate was heated at 85°C for 10 minutes. Samples were loaded onto a 4-20% TGX™ 15-well Precast Protein Gel (Biorad). Proteins were transferred to a PVDF membrane and blocked with 4% BSA PBS for at least 1 hour. Primary antibody staining was performed overnight at 4°C. The membrane was probed with secondary antibody for 1 hour at room temperature on a rocking platform, washed in PBS-T and subsequently PBS. Chemiluminescent detection was performed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific).

Results

Retroviral transduction using spinoculation is effective and reproducible for both the J76 cell line and the J76 TPR cell line

For generation of an antigen-specific cell line, human Jurkat T-cell clone 76 (J76) was obtained from the Heemskerk group (Leiden University Medical Center).[11] This cell line is deficient for both T-cell receptor (TCR) α and β chains. Therefore, upon transduction of a TCR, there will be no competition between TCRs or background activation from other TCRs. The aforementioned J76 clone was already transduced with CD8, since this cell line was originally also CD8^{-}. In addition, a J76 triple parameter reporter cell line (J76 TPR) was obtained from the Heemskerk group.[12] In this cell line, three reporter genes (NFAT-eGFP, NF-κB-CFP and AP-1-mCherry) were introduced with the purpose of visualizing transcriptional activation. In both cell lines, the aim was to introduce a TCR against the melanoma protein gp100$_{209-217}$ epitope (gp100TCR) that contains a mouse β chain constant region to enable the detection of the introduced TCR. For this, 293GALV cells were used as a packaging cell line for production of retrovirus containing a gp100TCR vector. Through retroviral transduction, gp100TCR was stably expressed on J76 and J76 TPR cells. In two separate experiments, virus particles containing gp100TCR were generated using calcium phosphate precipitation. Viral supernatant was collected and partially concentrated. J76 and J76 TPR were transduced with retroviral supernatant at increasing concentrations. An internal control for viral concentration was included by comparing unconcentrated viral supernatant with a dilution of the concentrated virus stock, which confirmed concentration percentages (Supplementary Figure 1A). Transduction efficiency was compared between the two virus stocks in the J76 cell line using flow cytometry (Figure 3A). Retroviral transduction with stock 2 shows a higher efficiency than with stock 1. The increase in gp100-TCR positive cells correlates with viral concentration, reaching a plateau of ±85% positive cells at a concentration of 8x the viral supernatant. This indicates that retroviral transduction efficiency increases logarithmically with viral concentration and that virus stock 2 has a higher virus concentration than virus stock 1. Stock 2 was used to transduce J76 TPR. Although less concentration conditions were included for this cell line, the same method was executed for retroviral transduction (Figure 3B). No significant differences were seen in transduction efficiency between cell lines. In both cell lines, a percentage of ±77% positive cells is reached at a concentration of 4x the viral supernatant, which was considered the most favorable condition because of the transduction efficiency in proportion to virus stock needed for retroviral transduction. In further experiments, TCR-expressing J76 and J76 TPR (J76 TCR and J76 TPR TCR) cells were used that were transduced with a viral concentration of 4x the viral supernatant.

CD3 is a reliable readout for TCR expression

During optimization of the retroviral transduction method, the association between CD3 expression and TCR expression was assessed (Figure 3C). A clear correlation was found between CD3 and TCR expression. Cells with a higher CD3 signal also have a higher TCR signal within the positive gate. This corresponds with the fact that TCR cell surface expression is necessary for CD3 expression as CD3 and TCR are presented as a complex on the cell surface. This raised the possibility of demonstrating TCR expression by measuring CD3 expression.
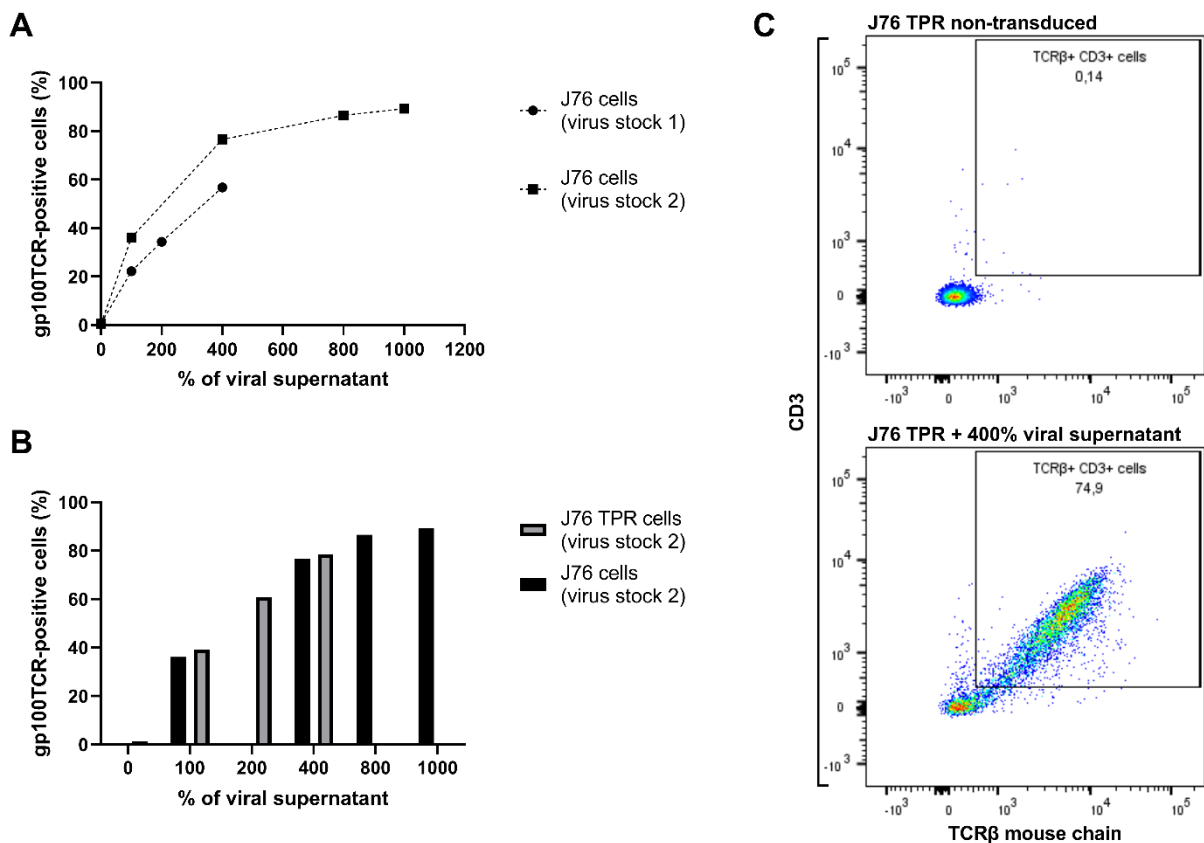
Figure 3: Retroviral transduction of J76 and J76 TPR cells with gp100TCR.
(A) Efficiency of retroviral transduction of J76 cells was analyzed using flow cytometry. Increasing concentrations of virus stocks from two independent experiments were applied. (B) Retroviral transduction efficiency was assessed in J76 TPR and J76 cells using flow cytometry with increasing concentrations of the same virus stock. (C) Co-expression of CD3 and TCRβ mouse chain was evaluated using flow cytometry. Non-transduced J76 TPR is shown as a negative control. Representative FACS plot is shown. Gating strategy is shown in Supplementary Figure 1B.

Expression of gp100 in antigen-presenting cells

For T-cell stimulation, multiple gp100 expressing cell lines were used for activation experiments. Before the start of this project, modified HeLa cells 33/2 expressing HLA-A*02:01 and immunoproteasome were transduced with the wildtype gp100 protein (gp100 WT) or the gp100 protein with a threonine to methionine mutation at position 210 (gp100 T210M). The T210M mutation has been shown to improve binding of gp100$_{201-230}$ to MHC-I molecules and positively affect the T-cell response.[10] In addition, melanoma UKRV-Mel-15a (URKV) cells were used, which endogenously express gp100. Percentage of gp100-expressing cells was assessed using flow cytometry (Figure 4A). gp100 expression levels were examined by calculating Mean Fluorescence Intensity (MFI) and western blot (Figure 4A and B). These experiments confirmed that all three cell lines ubiquitously express gp100. UKRV cells demonstrate the highest percentage of gp100-positive cells. Hela gp100 T210M cells show the highest gp100 expression and UKRV cells the lowest. The differences in gp100 expression are valuable for the analysis of the results from T-cell stimulation experiments.

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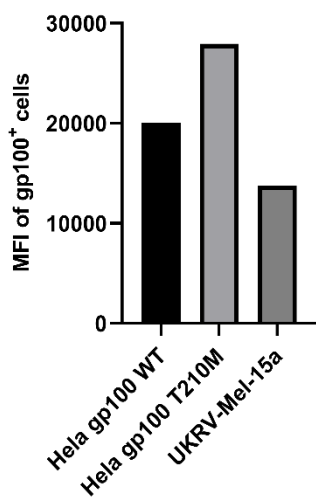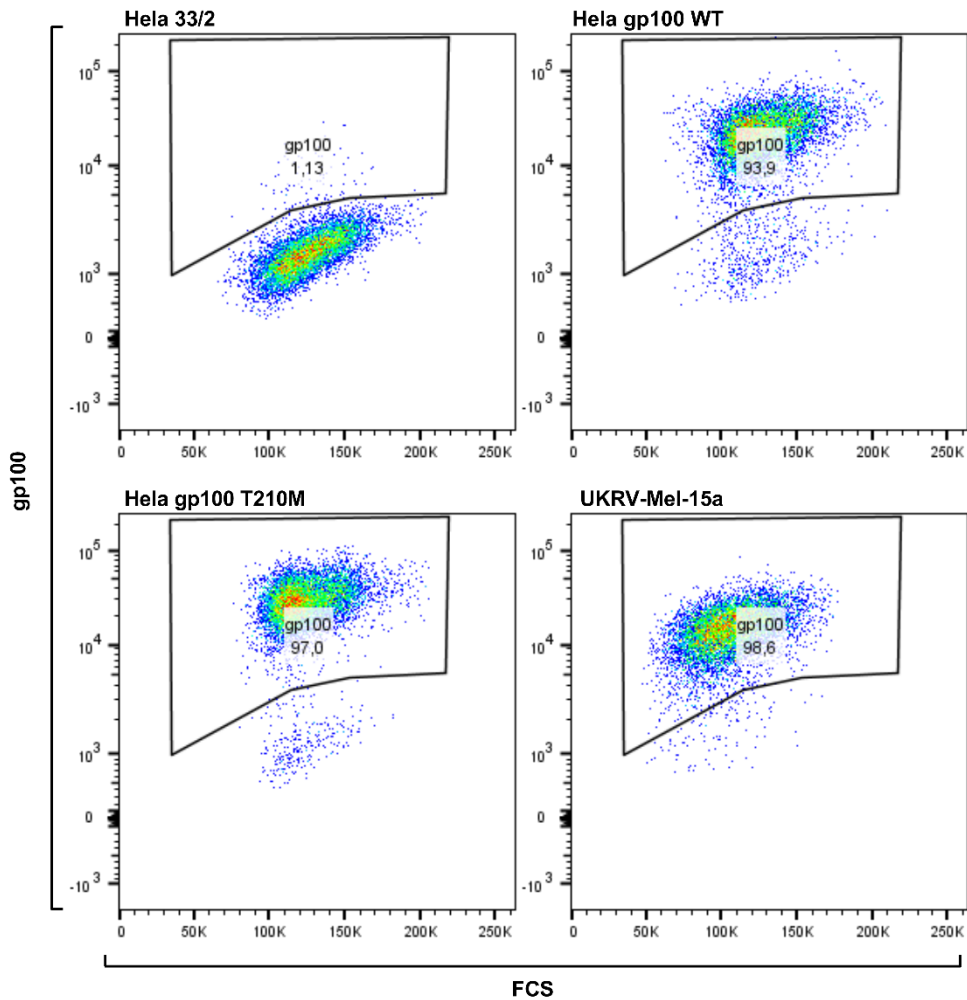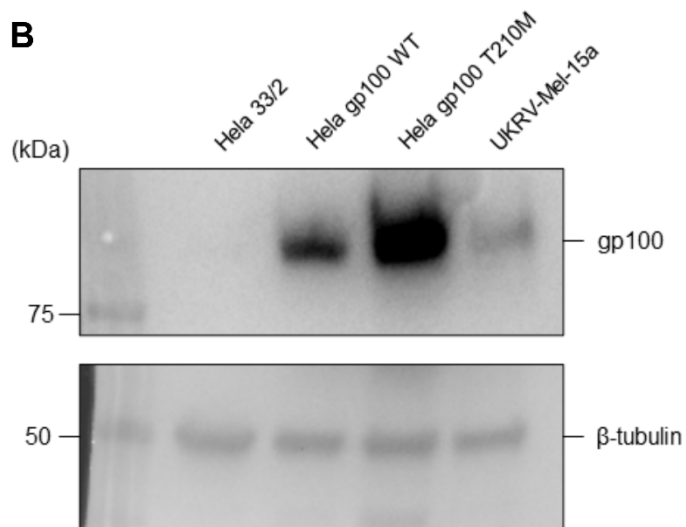
Figure 4: gp100-expression in antigen-presenting cells.
(A) Expression of gp100 in cell lysates of Hela gp100 WT, Hela gp100 T210M and UKRV-Mel-15a was assessed with flow cytometry. Non-transduced Hela 33/2 cells were used as a negative control. Mean Fluorescence Intensity (MFI) is calculated for the gp100-positive cells. (B) Western Blot analysis of gp100 expression in cell lysates. β-tubulin was used as a loading control. gp100 intensity is shown at 16.3s exposure. β-tubulin intensity is shown at 180s exposure.

Stimulating J76 cells by co-culture with antigen-presenting cells does not lead to a measurable increase in IFNγ or IL2 production or CD69 expression

Initially, the J76 TCR cell line was tested for its potential in a T-cell activation model. The aim was to obtain a T-cell line that is able to show a detectable antigen-specific immune response. To archive this, multiple co-culture experiments were set up in which J76 TCR cells were co-cultured for different durations with antigen-presenting cells (Hela gp100 WT, Hela gp100 T210M, UKRV-Mel-15a). Besides gp100-expressing cell lines, MHC-I molecules of non-transduced Hela 33/2 cells were preloaded with several concentrations of exogenous $_{mut}$gp100$_{209-217}$ peptide (hereinafter referred to as gp100 peptide), which contains the T210M substitution. T-cell activation was measured by IFNγ release. Unfortunately, no positive results were obtained after numerous alterations in cell concentrations, incubation time, and culture conditions. Lastly, to enhance the activation signal, co-stimulatory agents were added. Two concentrations of J76 TCR cells were applied and co-culture conditions and were combined with PMA (10 ng/mL or 50 ng/mL) or anti-CD28 stimulation. Ionomycin was used as a positive control. T-cell activation was assessed by measuring IFNγ release using ELISA (Figure 5A). In the positive controls, a clear increase in cytokine release was observed, with IFNγ concentrations reaching 480 pg/mL at the condition with highest concentrations of J76 TCR cells and PMA, demonstrating that J76 TCR cells can be stimulated to produce IFNγ. However, no significant differences were visible between co-culture conditions with and without antigen presentation. A slight increase was seen in conditions with PMA co-stimulation, but this effect was not specific for antigen presentation. In addition, the highest IFNγ signals (besides positive controls) were detected with J76 TCR cells that were not in co-culture. These observations led to the conclusion that J76 TCR cells do not release detectable amounts of IFNγ upon TCR-specific activation and IFNγ ELISA is no suitable readout for J76 TCR cell activation. In order to detect activated J76 TCR cells at a cell-specific level and to test for multiple activation markers, another co-culture method was set up where levels of T-cell activation markers (IFNγ, IL2, CD69) were measured using flow cytometry (Figure 5B). For co-stimulation, cells were cultured with 10 ng/mL PMA. Again, no significant increase in activation is seen as a result of antigen presentation. Moreover, CD69 was not detected at all, even after stimulation with ionomycin. In conditions using ionomycin, a relatively small percentage of J76 TCR cells showed measurable activation, with a maximum of 12%. After repeating both experiments several times and obtaining similar results, another cell line was selected for the T-cell activation system.

J76 TPR TCR cell line proves a reliable readout for T-cell activation in response to antigen presentation

After successfully transducing J76 TPR cells with gp100TCR, J76 TPR TCR cells were co-cultured with the same cell lines as described above. Additionally, Hela cells loaded with a ten-fold dilution series of gp100 peptide ranging from 1 pM to 1 mM were included in co-culture experiments. Co-culture experiments were performed in quadruplo without co-stimulation. Activation of the reporter genes was measured using flow cytometry (Figure 6, Supplementary Figure 3). One trial was excluded from the data analysis, due to lower activation outcomes possibly caused by experimental variations (see Conclusion & Discussion) (Supplementary Figure 4). In order to only include gp100TCR-specific T-cell activation, J76 TPR TCR cells were gated and only CD3-positive cells were analysed for reporter gene expression. Only conditions with gp100 peptide concentrations from 1 nm to 100 μM were repeated in triplicates (n=3) and were included in the analysis. gp100 peptide concentrations of 1 mM were shown for NF-κB-CFP in Figure 6B to show increase in activation. However, this condition was only incorporated once. Transcriptional activation was successfully shown in all three reporter parameters and specifically for stimulated cells, with increased reporter gene expression with longer cell stimulation (Figure 6A,B). Transcriptional reporter assays showed that J76 TPR TCR cells specifically responded to cells presenting gp100 peptide (Figure 6A). Hela gp100 T210M cells elicited the greatest response of all antigen-presenting cell lines. Interestingly, the responses of Hela gp100 WT cells and UKRV cells do not correlate with gp100 expression levels measured as determined above (Figure 4B).
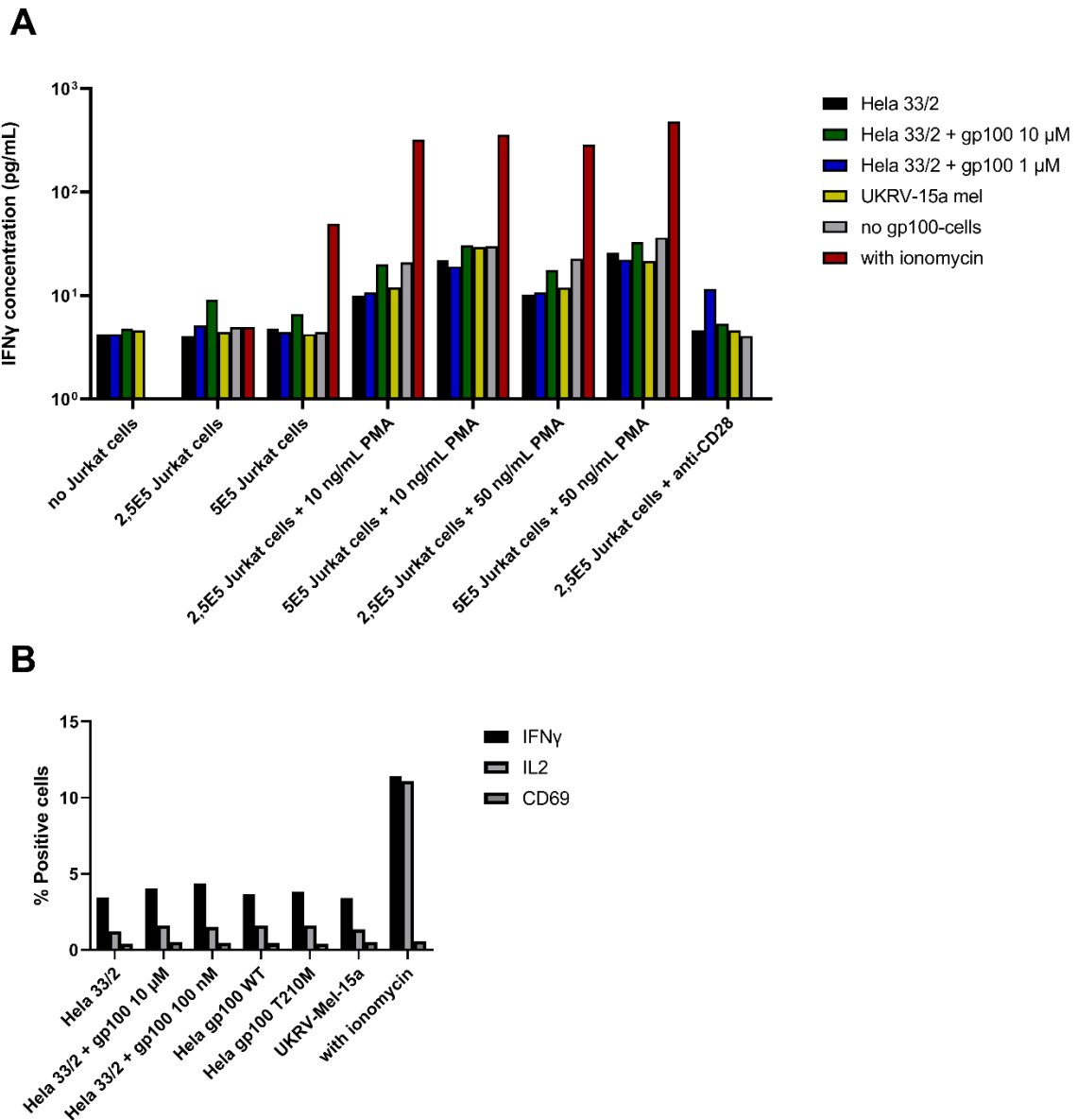
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Figure 5: Stimulating J76 TCR cells by co-culture with antigen-presenting cells does not change levels of IFNγ, IL2 or CD69 significantly.

(A) IFNγ release was measured by ELISA after T-cell stimulation by co-culture with antigen-presenting cells. PMA/ionomycin was included as positive control. Non-transduced Hela cells and single cell cultures were included as negative controls. (B) Assessment of T-cell activation markers using flow cytometry. IFNγ and IL2 were stained intracellularly, CD69 was stained on cell surface. Non-transduced Hela 33/2 cells were included as a negative control. Representative results are shown.

This indicates that T-cell activation is not only regulated by protein expression levels. Co-culture experiments with Hela 33/2 loaded with exogenous gp100 peptide resulted in dose-response curves that show concentration dependent activation of J76 TPR TCR cells (Figure 6B). Dose-response curves NFAT-eGFP and AP-1-mCherry are S-shaped, indicating in which range of peptide concentrations J76 TPR TCR response is detectable and exponential. This will be useful for setting up our model system for TCR-specific activation. NF-κB-CFP displays an exponential increase in activation upward of 1 mM peptide, suggesting that this reporter gene needs more stimulation by antigen presentation to be activated. J76 TPR TCR responses were compared with non-transduced J76 TPR cells, which were not activated in presence of presented gp100 peptide, confirming that J76 TPR TCR activation is mediated through the gp100TCR (Figure 6C).
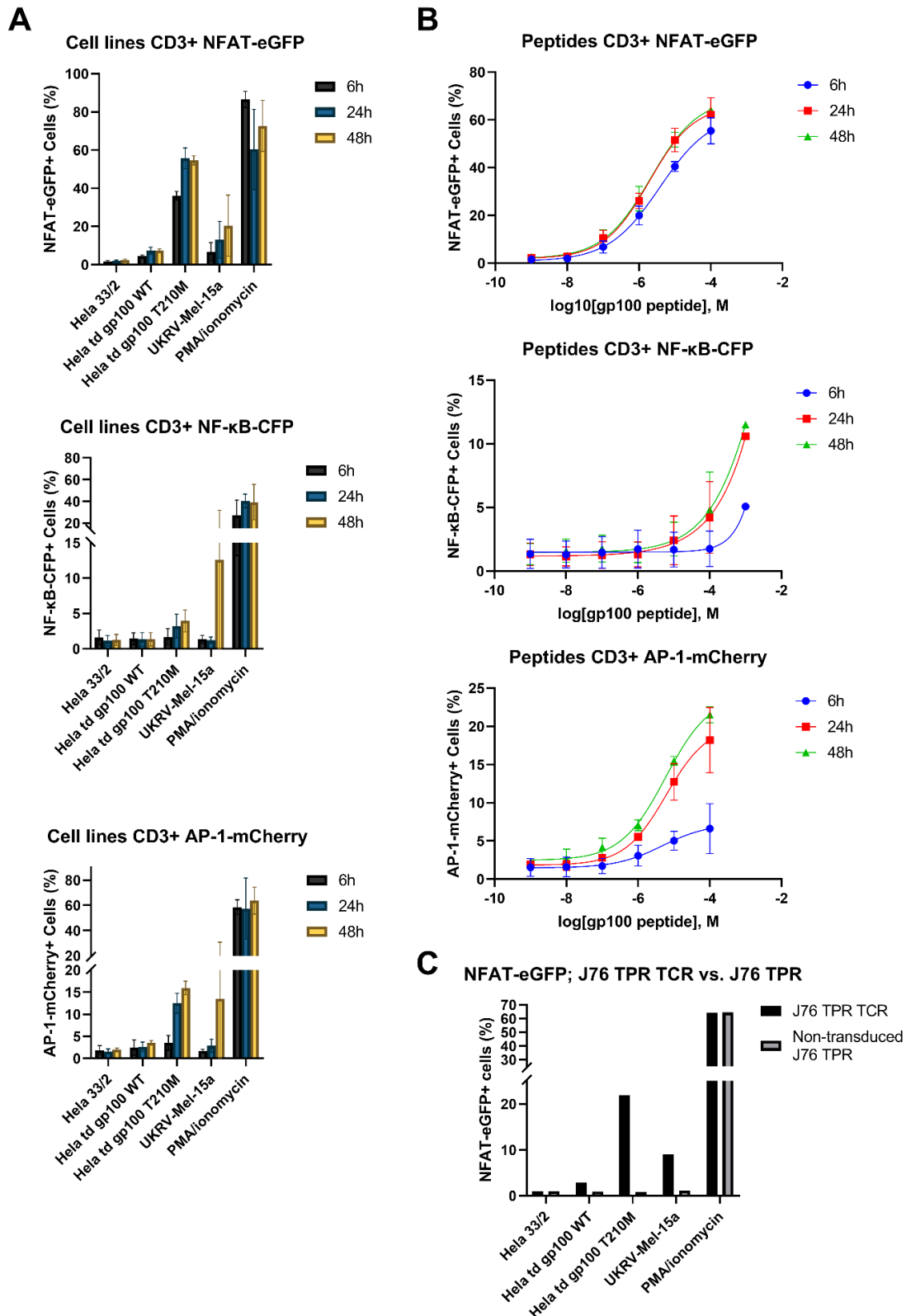
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Figure 6: Triple parameter reporters as a quantitative readout for antigen-specific activation of the J76 TPR TCR cell line.
(A) J76 TPR TCR cells were stimulated in co-culture with antigen-presenting cell lines (Hela gp100 WT, Hela gp100 T210M, UKRV-Mel-15a). Analysis of reporter gene expression by flow cytometry. Non-transduced Hela 33/2 cells were included as a negative control. PMA/ionomycin stimulation was included as a positive control. (B) J76 TPR TCR cells were stimulated in presence of Hela 33/2 cells with increasing concentrations of loaded exogenous gp100 peptide. Analysis of reporter gene expression by flow cytometry. (C) Comparison of reporter gene expression between antigen-presenting cell lines in co-culture with transduced and non-transduced J76 TPR cells. Results shown for 24h stimulation. Hela 33/2 cells were included as a negative control. PMA/ionomycin stimulation was included as positive control.

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Conclusion & Discussion

This major research project describes the optimization of a model system for T-cell receptor mediated activation. A method is introduced that quantitatively demonstrates T-cell activation in response to antigen presentation by transcriptional activation of three reporter genes: NFAT-eGFP, NF-κB-CFP and AP-1-mCherry. Control experiments confirmed that this activation was specific for the TCR-gp100/MHC-I interaction. By presenting a dose-response curve based on presented exogenous gp100 peptide, a solid base is provided for determining antigen presentation and T-cell activation, which can be useful for future intervention studies. The validity of the measurements is indicated by including cell lines with endogenous gp100-expression in this research.

In initial T-cell activation experiments with J76 TCR cells, IFNγ release was measured using ELISA and ConA was used as a positive control. After assessing gp100-expressing cell:J76 TCR cell ratios of 1:1, 1:5 and 1:10, using flat-bottom and round-bottom 96-well plates, and incubation times of 16h, 24h, 48h, and 72h, no positive results were obtained. IFNγ ELISAs only gave a positive result when using PMA/ionomycin as a positive control, indicating that J76 TCR cells could be activated to produce IFNγ. An explanation for undetectable IFNγ release could be that only a small percentage of J76 TCR cells had interacted with the MHC-I-gp100 peptide complex, resulting in IFNγ concentrations under the detectable threshold. Another explanation could be that these J76 TCR cells were activated by antigen presentation, but produced other cytokines than IFNγ. By performing flow cytometry on co-cultured J76 TCR cells and staining multiple activation markers (IFNγ, IL2, CD69), these theories were examined. All activation markers scored negative in co-culture conditions. Therefore, it remained uncertain whether gp100TCR interacted with the presented gp100 peptide on the adherent cells in co-culture. gp100 expression was verified by flow cytometry and Western Blot, but interaction of gp100TCR with the MHC-I-gp100 complex was not. Instead, we obtained a new J76 cell line with three parameter reporters, to assess T-cell activation on transcriptional level. Here we observed reproducible antigen-specific activation.

We performed the co-culture experiments with J76 TPR TCR cells four times. During N3, J76 TPR culture medium became infected. It was not clear if the cells in co-culture were also treated with this medium while it was infected, but no signs of infection were detected. Besides possible infection, co-culture was performed with estimated 4×10^4 Hela cells due to smaller availability. Additionally, the measurement of reporter gene activation and CD3 expression was delayed to the week after, which might have reduced fluorescence intensity. The results of this trial evidently showed lower levels of NF-κB-CFP and AP-1-mCherry compared to N1, N2 and N4 (Supplementary Figure 4). For these reasons, N3 was excluded from result analysis.

The majority of outcomes in our co-culture experiments show only minimal variation between the three included trials (N1, N2, N4). Nevertheless, in trial N1, UKRV cell numbers appeared lower than concentrations of other antigen-presenting cells under the microscope. As a result, NFAT-eGFP activation shows a large reduction at all three time points of co-culture. Furthermore, notable is that UKRV cells from N2 show reporter gene activation far exceeding those of N1 and N4. For all reporter genes, the difference between reporter gene activation was 20% between N2 and other trials after 48h co-culture with UKRV cells (Supplementary Figure 5). Because of these outlying results, co-culture of J76 TPR TCR cells and UKRV cells should be repeated when UKRV cells are used in sequential steps in this model system.

Our gp100 expression and T-cell activation assays demonstrate that responses of Hela gp100 WT cells and UKRV cells do not completely correlate with gp100 expression levels measured by flow cytometry and western blot. This indicates that T-cell activation is regulated on more levels than protein expression. Previous studies into proteasomal processing of the gp100$_{209\text{-}217}$ T210 epitope have stated

that the T210M mutation leads to a certain flexibility in its processing route which makes it possible for the peptide to be presented through both TAP-dependent, tapasin-dependent and TAP/tapasin-independent pathways,[13] which should be considered in future APP pathway intervention studies.

In general, our activation assays demonstrate the greatest transcriptional activation of the NFAT gene, which seems strongest at 24h. NFAT is activated by calcineurin, a phosphatase stimulated by the calcium-calmodulin pathway. This pathway is activated upon influx of cytosolic free calcium, a process that is induced almost instantly after T-cell activation. High cytosolic calcium levels are sustained for about 1 hour.[14] This explains why NFAT is the first transcription factor to be activated after antigen recognition by T-cells. An increase in AP-1 is visible over time, with the highest signal at 48h. Upon TCR signaling, NFAT interacts with AP-1 to induce transcription of a variety of cytokines and other immune-related proteins, like IFNγ and IL2.[14,15] On the other hand, when NFAT does not cooperate with AP-1, it contributes to downregulation of the immune response.[16] Therefore, and because IFNγ and IL2 cytokine production could not be detected in Jurkat J76 cells, a concurrent activation of NFAT and AP-1 should be detected over time, to conclude that T-cell activation took place. Activation of NF-κB remains quite low, with a maximal NF-κB-CFP-positive cell percentage of ±50% in cells stimulated with PMA/ionomycin and ±5% in J76 TPR TCR cells stimulated with 100 µM gp100 peptide. As NF-κB signaling plays an important role in the induction of cytokines like IFNγ,[17] the low NF-κB signal could explain why T-cell activation assays measuring cytokine release were unsuccessful. In conclusion, NFAT and AP-1 activation can be used in this model system as the main determinants of T-cell activation rather than NF-κB.

J76 cells are advantageous for researching TCRs due to the lack of alpha and beta TCR chains. However, demonstrating Jurkat activation has proven quite challenging as several activation markers were not upregulated. This was the case for this research project, but has also been stated before as a drawback for this cell line.[12] Our results using the J76 cell line have shown that readout of CD69 expression and IL2 and IFNγ production and release are inadequate for visualizing Jurkat activation. The J76 TPR cell line provides an efficient and cost-effective approach for quantitatively determining T-cell activation on transcriptional level for three major factors in T-cell activation. Sensitivity of this method could supposedly be adjusted by adapting ratios of co-cultured cell lines, longer incubation times or implementing costimulatory and coinhibitory signals, among others. For instance, Rosskopf et al introduced chimeric receptors in J76 TPR cells that consist of an intracellular CD28 domain and extracellular adhesion domains that serve as receptors to costimulatory ligands from target cells of J76 TPR cells. As activation of the intracellular CD28 domain leads to the induction of immune responses through intracellular signaling, introduction of these chimeric receptors boosted reporter sensitivity greatly.[12] Thus, a similar approach could be applied to increase reporter sensitivity in the J76 TPR T-cell activation assays.

The comparison of TCR activation by gp100 peptide in peripheral blood lymphocytes (PBLs) and Jurkat cells can provide an indication for the difference in sensitivity. Textoris-Taube et al performed coculture activation experiments using TCR transduced PBLs and K652 cells loaded with gp100 peptide. PBL activation was measured by IFNγ release.[10] The EC_{50} was established around 10 pM, which means a concentration of 10 pM of gp100 peptide was needed to trigger half of the maximal measured PBL response. In contrast, IFNγ release assays using J76 cells in this study resulted in too low IFNγ to establish the EC_{50}. For the TPR Jurkat cells, the EC_{50} lies between 1 and 10 µM when it is based on NFAT-eGFP activation (Figure 6B). That means that the TPR system is 1×10^6 times less sensitive than the PBL system. This could be explained by the fact that J76 TPR cells only express a limited set of costimulatory receptors compared to PBLs. Besides, PBLs show a higher sensitivity in IFNγ release assays and intracellular cytokine staining assays[18] than Jurkat cells. Therefore, it is reasonable to hypothesize that stimulation of transduced PBLs through antigen presentation will give a higher signal in transcriptional activation than stimulated J76 TPR TCR cells. Evaluating the function of TCR transduced PBL-derived T-

cells could be beneficial to have a better understanding of antigen-recognition by retroviral TCR constructs in more physiological conditions.

This is the first report describing wildtype and mutant gp100 as a subject for antigen presentation in co-culture with J76 TPR TCR cells, with the intent of evaluating steps in antigen processing. Using cell lines with endogenous gp100 (wildtype or mutant) expression, the next step of intervening in the APP pathway and assessing the effects on T-cell activation can be executed. Different concentrations of siRNAs against APP proteins can be applied to Hela 33/2 cells transduced with gp100 (WT or T210M) and melanoma cell lines to assess the effect of different degrees of inhibition on antigen processing, antigen presentation and T-cell activation.

The current approach for the development of vaccines against intracellular pathogens is to elicit T cell-mediated immune responses by presenting pathogen-derived antigens to CD8+ T cells. However, predicting which epitopes presented on the cell surface have a high immunogenicity in a large percentage of the population remains challenging. Together with other collaborators, the Mishto lab aims to create a computational model for the APP pathway in order to appreciate the impact of APP steps and predict the chances of an epitope passing the APP pathway. In combination with analyses of APP protein kinetics and HLA-I-peptide complex stability, our T-cell activation model will form the basis of this model. By grasping the rules for epitope processing and presentation, the selection of epitope candidates that are most relevant for immune activation will be improved, which opens up new opportunities for immunotherapies and vaccine design.

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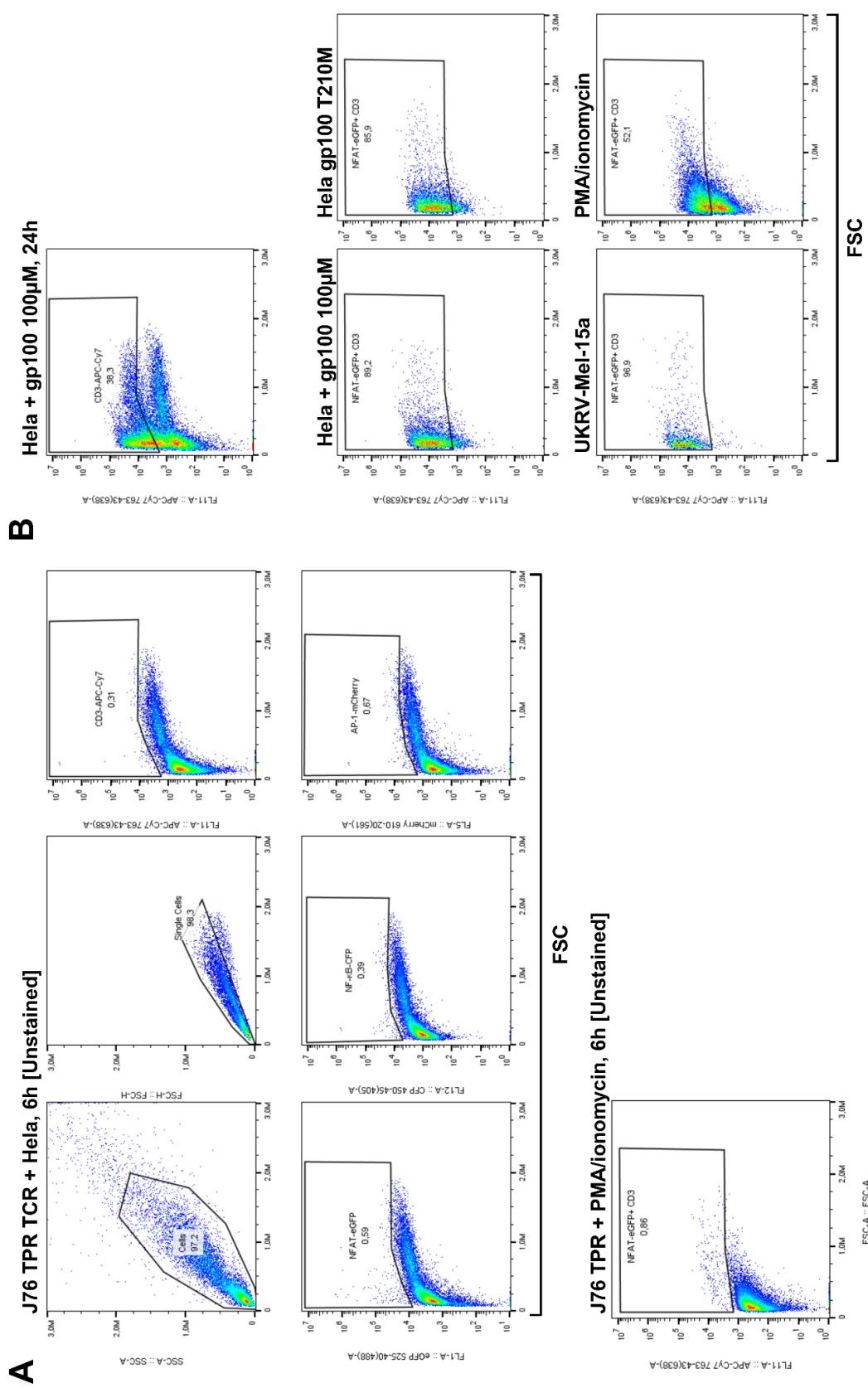
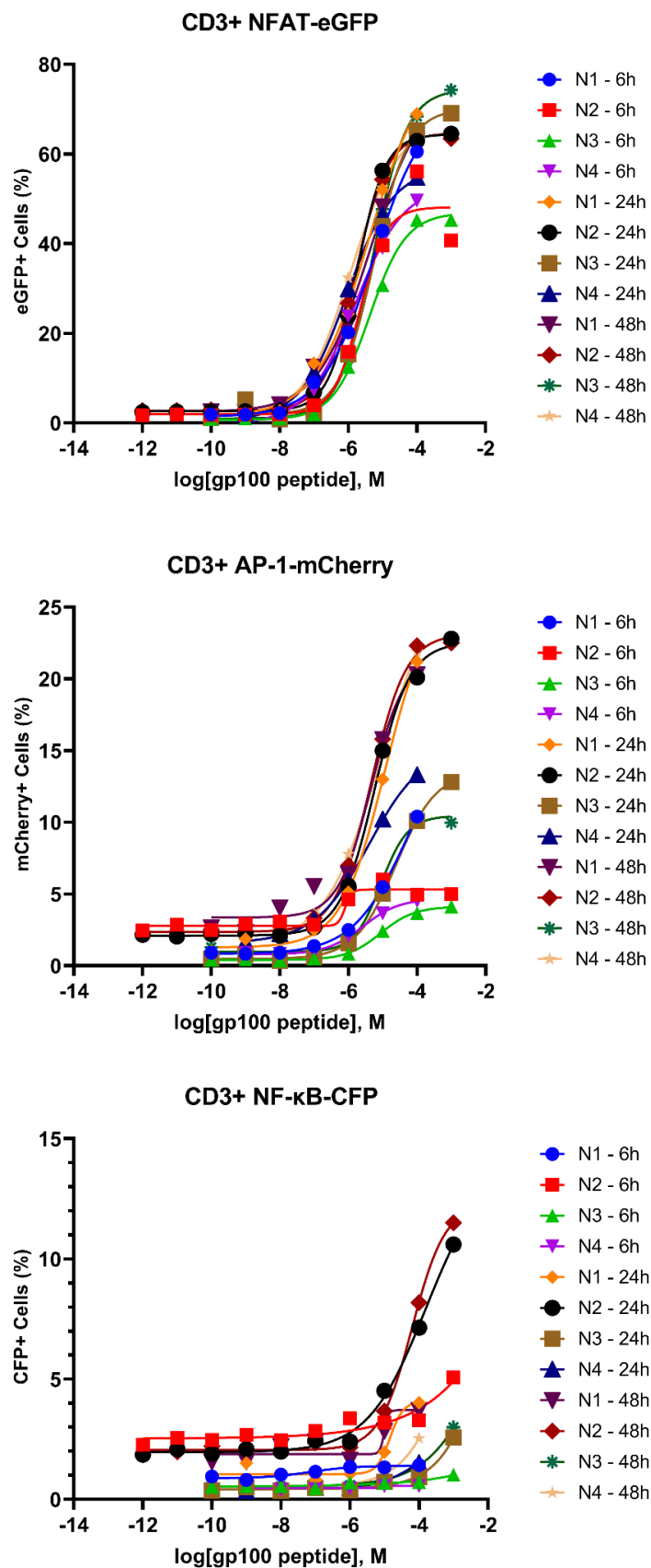# Supplementary data

A

B
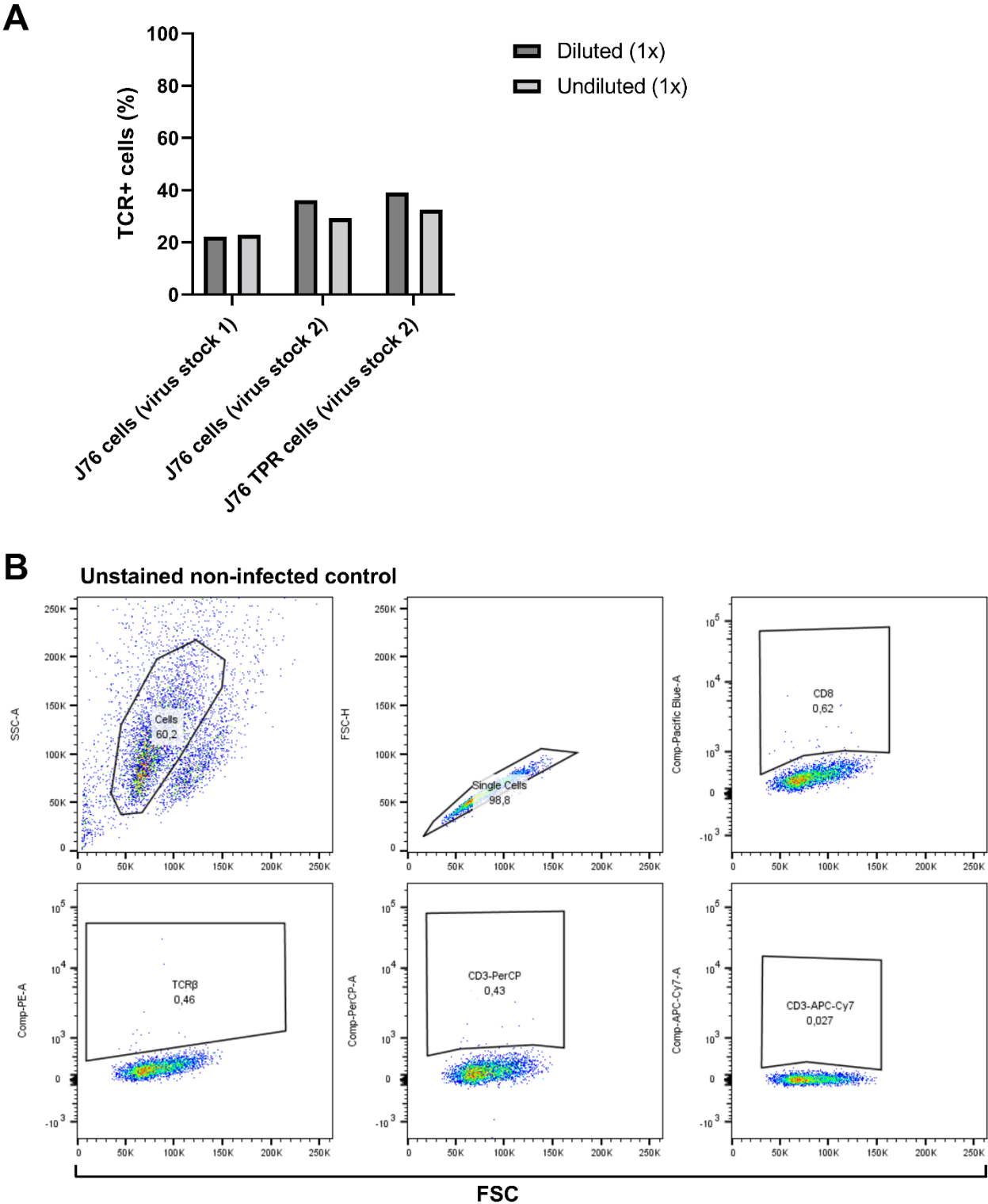
Supplementary Figure 1: Internal control and gating for J76 retroviral transduction experiments.
(A) Internal control for concentration of virus stock. Transduction efficiency of non-concentrated virus medium (Undiluted) is compared with that of concentrated medium diluted to its original concentration (Diluted). Measurements were performed by flow cytometry (B) Gating strategy for determining J76 TRP cells positive for CD8-VioBlue, TCRβ-PE, CD3-PerCP and CD3-APC-Cy7.
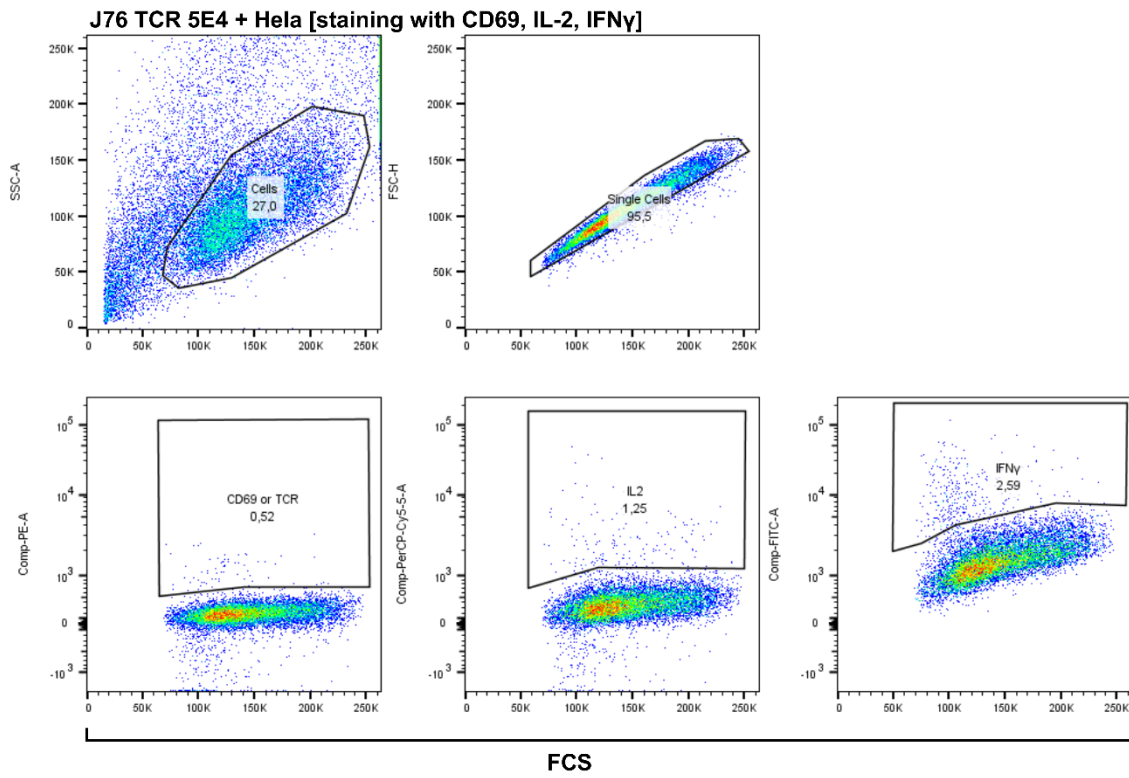
Supplementary Figure 2: Gating strategy for cytokine markers in flow cytometry analysis.
Gating strategy for determining J76 TRP cells positive for CD69-PE, TCRβ-PE, IL2- PerCPeFl710, and IFNγ-FITC.

Supplementary Figure 3: Gating strategies for J76 TPR TCR stimulation using flow cytometry.
(A) Gating strategies for Figure 4. CD3 expression in NFAT-eGFP-positive cells is gated in the positive control cells with PMA/ionomycin. (B) Demonstration of CD3 expression percentages (plots below) in NFAT-eGFP-positive compared to CD3 expression percentages in all J76 TPR TCR cells (plot above) shows that J76 TPR TCR activation is gp100TCR-specific. CD3 expression percentages in all J76 TPR TCR cells were similar in all conditions shown below. All cells are co-cultured for 24 hours.

Supplementary Figure 4: Individual results from J76 TPR TCR co-culture experiments performed in quadruplo.
Individual results of J76 TPR TCR cells co-cultured with Hela 33/2 cells loaded with exogenous gp100 peptide are shown, including peptide concentrations that were left out from Figure 6.

UKRV 48h trials N1-N4
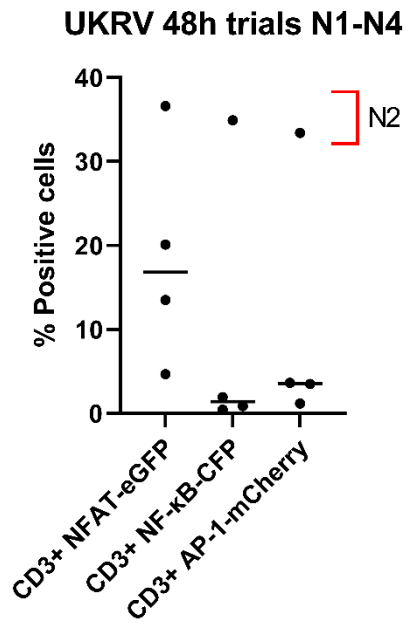
Supplementary Figure 5: Individual results from J76 TPR TCR co-culture experiments with UKRV-15a-Mel cells.
Individual results of J76 TPR TCR cells co-cultured with UKRV cells are shown. Measurements of J76 TPR TCR cell activation in trial 2 (N2) are indicated.