Recombinant T-cell receptors from HPA-1a specific T-cells

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Abstract

Fetal- and neonatal alloimmune thrombocytopenia is a condition that can cause hemorrhage and potential brain damage or death in neonates. It is most often a result of maternal alloantibodies specific for fetal Human Platelet antigen 1a (HPA-1a) that destroy fetal platelets. This condition can occur when women homozygous for the amino acid proline in position 33 on β3-integrin (HPA-1b) are pregnant with a fetus that has a paternally inherited leucine at position 33 on the β3-integrin (Leu33). Nearly all of these women express the class II molecule MHC DRA/DRβ3*01:01. The recognition of the HPA-1a peptide-MHC complex by T-cells involve a peculiar form of T-cell receptor-peptide-MHC interaction in which the Leu33 residue is essential for T-cell stimulation, not by being directly presented to the TCR, but by acting as an anchor residue and increasing peptide binding and stability of the peptide-MHC complex, compared to the Pro33 allovariant. Studies of immunized women have been found to involve a diverse set of T-cells with varying responsiveness.

To build a solid platform in which to study the peptide-MHC interactions involved in this condition, the goal of this project was to attempt to produce soluble, recombinant T-cell receptors from an HPA-1a-specific T cell line (D7T4) characterized from an alloimmunized woman. Developing such a method would not only allow for the study of mechanisms involved in this condition, but also to study peptide-MHC TCR interactions in general, as well as potentially allowing for the production of diagnostic and therapeutic products. To achieve this, the sequence encoding the D7T4 T-cell was engineered and constructs containing D7T4-TCRα and D7T4-TCRβ subunits prepared in cloning vectors. These D7T4-TCRαβ inserts were transferred into expression vectors used in multigene baculoviral vectors, and subsequently recombined and transferred to a bacmid. Baculovirus stocks were produced by transfection of the Sf9 insect cell line (*Spodoptera frugiperda)* and protein expression cultures were set up by virus infection of the H5 insect cell line (*Trichoplusia ni).* Proteins from the culture supernatant, were found to be of the expected size, analyzed by SDS-PAGE. By isolation of protein by the poly-histidine tail of D7T4-TCRβ and subsequent staining of the Twin-Strep-Tag on D7T4-TCRα, the presence of D7T4-TCRαβ in a dimeric form was confirmed by flow cytometry and western blot. However, by testing for D7T4-TCRαβ functionality by T cell proliferation assay, the protein was not found to competitively inhibit the stimulation of T-cells in culture, indicating that it may not have correct conformation and not binding the L33-DRA/DRβ3*01:01. In addition, it was not stained by general anti-TCR antibodies analyzed by flow cytometry.

The cause of the protein not functioning was not discovered, but a range of possibilities were considered. While it is possible that the protein did not function due to faulty conformation, it could also be caused by too low affinity for stable binding of TCR monomeric units. Future designs could

benefit from compensating for this by increasing avidity through creation of multimers. Multiple more recent studies have found that the most effective way of increasing soluble TCR expression and function is to increase stability and/or avidity. The introduction of disulfide-bridges is a common method for increasing stability, but the most promising strategy thus far seems to be the fusion of an Fc-region to the protein. An iterative process of development, involving multiple steps of affinity-increasing modifications and identification of stability increasing variants and mutations was considered the most promising continuation of this project.

Introduction

Fetal- and neonatal alloimmune thrombocytopenia (FNAIT) is most often caused by formation of maternal alloantibodies against the fetal Human Platelet Antigen 1a (HPA-1a), defined by a single amino acid difference on β3-integrin (1). Immunization of pregnant women can occur if they are homozygous for the amino acid proline in position 33 on β3-integrin (HPA-1b) and the fetus has a paternally inherited leucine at position 33 (HPA-1a). In addition, a majority of immunized women are carriers of the DRβ3*01:01 MHC class II complex (2, 3). Maternal IgG specific for HPA-1a can cross the placenta and bind fetal platelets, resulting in platelet destruction and potentially thrombocytopenia. While rare, this condition can cause intracranial hemorrhage and risk of brain damage or death (3).

T-cells specific for HPA-1a are essential in inducing a response against HPA-1a, and further investigation of the mechanisms involved are important for further understanding of FNAIT, prediction and prevention, and for development of potential therapeutic targets. The interactions of T-cell recognition and HPA-1a- DRA/DRβ3*01:01 MHC class II complex formation have previously been investigated thoroughly. It has been found to involve a peculiar form of T-cell allorecognition in which the stimulatory Leu33 amino acid (L33) is buried in the peptide-binding groove of DRA/DRβ3*01:01 MHC class II complex, rather than being directly presented for recognition (4, 5). Studies of this interaction, substituting L33 for other small hydrophobic amino acids, also causes T-cell stimulation, indicating that rather than the change in the recognized epitope, it is the increased stability of peptide-MHC that is the cause of its immunogenicity (2).

Considering that the stability of peptide-MHC is vital in the immunogenicity, the authors discuss that the immunogenicity is most likely dependent upon the HPA-1a specific T-cells. In the studies identifying this peculiar allorecognition, a panel of clonal HPA-1a-specific, HLA-DRβ3*01:01-restricted $CD4^+$ T-cell lines were isolated and used. In isolation of these T-cells from blood samples from women that gave birth to children with clinically manifested FNAIT, numerous clones were found, indicative of a diverse response. These were also found to have high diversity in responsiveness when

stimulated with DRA/DRβ3*01:01 positive antigen presenting cells (APCs) presenting modified peptides (2). Following this, the major goal of this study was to investigate and test methods for the creation of vectors to express recombinant T-cell receptors (TCRs) from these clones, for further studies into the underlying mechanisms.

Methods

Donors and isolation of T-cell receptor sequences

In this study, the TCR sequence from the T-cell clone D7T4 was used (2), isolated from an HPA-1b homozygous, HPA-1a-immunized woman, carrying a *DRB3*01:01* allele. Their study was approved by the Regional Committee for Medical Research Ethics, North Norway, and blood samples were obtained after written informed consent, in accordance with the Declaration of Helsinki. These sequences have been published prior to this study, and the research group still have approval for working with genetic material isolated from these women.

Gene constructs for desired TCRs were designed using these TCR sequences in GeneArt (Thermo Fisher Scientific). These were customized to contain restriction sites at strategic sites and antibiotic resistance genes for isolation and validation. All constructs were codon optimized for improved gene expression in *Trichoplusia ni* ovarian cells (High Five/H5). For isolation and analysis of D7T4-TCRαβ subunits and later, validation of dimerization, the D7T4-TCRα subunit was designed with a Twin-Strep-Tag, while D7T4-TCRβ contains a poly-histidine tail (6xHis).

Amplification and purification of expression vectors, D7T4-TCRα and D7T4-TCRβ inserts

10 ng of D7T4-TCRα construct components (D7T4-TCRα insert and expression vector pUCDM (EMBL Grenoble) and D7T4-TCRβ construct components (D7T4-TCRβ and expression vector pFL (EMBL Grenoble)) were separately transformed according to manufacturer instructions into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen) and One Shot PIR1 chemically competent *E. coli* (Invitrogen) respectively. Transformed cells were incubated at 37°C overnight on LB medium plates containing appropriate antibacterial resistance for each component, followed by preculturing of colonies in 3 mL LB-medium overnight incubation at 37°C at 150 rpm. 40 µL of preculture were transferred to 20 mL (1:500) antibiotic-containing LB-medium and incubated overnight at 37°C at 150 rpm. After centrifugation of cultures at 3000 x g for 10 minutes, solutions of purified plasmid for each component were prepared with QIAprep Spin Miniprep Kit (QIAGEN) according to manufacturer instructions. Quantification and evaluation of plasmid DNA purity was measured with NanoDrop

1000 (ThermoFisher). A 260/280 nm absorbance ratio of >1.8 was considered to be sufficient purity in samples.

Transfer of inserts from cloning vectors to expression vectors

Plasmid DNA pD7T4-TCRα, pD7T4-TCRβ, pUCDM and pFL (2 µg) were separately digested by 1 µl (20 U/µL) restriction enzymes (New England Biolabs; NEB) as per manufacturer instructions, in appropriate buffers and adjusted to 30 µL reactions with nuclease-free water. BamHI and XbaI were used for D7T4-TCRα and pUCDM, and XhoI and NheI for D7T4-TCRβ and pFL. Restriction digestions were performed at 4°C for 2 minutes, 37°C for 12 hours, 65°C for 20 minutes and 4°C hold. Gel-electrophoresis with a 0.9% agarose gel in 1xTAE buffer at 65V for 50 minutes followed by 10-minute staining with GelRed Nucleic Acid Gel stain (Biotium) was performed for all four samples. *In silico* simulations of expected cut segment placement were performed, and bands for cut inserts and vectors, at expected locations, were visualized with UV light and removed by scalpel. DNA was extracted from gel with Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) per manufacturer instructions. Quantification and evaluation of DNA purity was measured with NanoDrop 1000(ThermoFisher).

Ligation of inserts into expression vectors, D7T4-TCRα into pUCDM and D7T4-TCRβ into pFL, was performed in a 5:1 ratio for insert and vector in 10 µL reactions with T4 DNA ligase and T4 buffer (Invitrogen) followed by a cycling program with 16°C for 35 minutes, 65°C for 15 minutes and 4°C hold.

After ligation, pUCDM-D7T4-TCRα was transformed into One Shot PIR1 Chemically competent *E. coli* (Invitrogen) and incubated on LB-plates with chloramphenicol. pFL-D7T4-TCRβ was transformed into One Shot TOP10 chemically competent *E. coli* (Invitrogen) incubated on LB-plates with ampicillin. Both were amplified in corresponding broths, and DNA purified as described above for inserts and vectors.

PCR testing / Confirmation of ligation result

PCR reactions with primers specific for sequences in on each side of the inserts (pUCDM/pFL forward: TATTGCCGTCATAGCGCG, pUCDM reverse: CAGGGGGAGGTGTGGGAG and pFL reverse: CCGGAGTAGGTCGCGAATC).

These were performed in 25 µL reactions. Using 1 µL 20 ng/µL plasmid with 2.5 µL 10x PCR buffer, 0.5 µL dNTP, 0.25 µL HotStarTaq (QIAGEN) and nuclease-free water. PCR cycle: Hold 95°C for 15 minutes; 35 cycles of 94°C for 1 min, 54°C for 30 seconds, 72°C for 1 minute; followed by a final 72°C for 10 minutes. PCR products were analyzed by gel-electrophoresis and confirmation of ligation

success by expected insert-vector length. (~550 bp between primers if vector without insert and ~1400-1500 bp between for vector-insert).

CRE-recombination and transformation of reaction products into competent E. Coli

For CRE-recombination, 500 ng vector with 300 ng insert, 1 µL 10x Cre reaction buffer (NEB), 1 µL Cre recombinase (NEB) and 4 µL nuclease-free water for 10 µL reactions were used. Samples were cycled at 30°C for 30 minutes, 70°C for 10 minutes, before being held at 4°C continuously until storage and use for transformation.

Reaction products were transformed into TOP10 *E. coli* cells using 5 µL recombination mix added to 50 µL competent TOP10 *E. coli* (ThermoFisher). The mix was incubated on ice for 30 minutes before 30 second 42°C heat-shock and a further 5-minute incubation on ice. 400 µL S.O.C. medium (ThermoFisher) was added before incubation at 37°C and 150 rpm for 120 minutes. 100 µL mix was plated on LB-plates with ampicillin and chloramphenicol and incubated overnight at 37°C. Colonies were inoculated in 3 mL LB-medium containing ampicillin and chloramphenicol overnight. Cultures were centrifuged at 3000g and purified by QIAprep Spin Miniprep Kit (QIAGEN) according to manufacturer instructions. Plasmid concentration and purity measured by NanoDrop 1000spectrophotometer (ThermoFisher).

Confirmation of desired Cre-recombination product by restriction cutting

Using the Cre-ACEMBLER software (Christian Becke, Imre Berger, Mathias Hafke), *in-silico* Cre-recombination of donor and acceptor vectors were performed to generate final plasmid product. This sequence was used in SnapGene (Insightful Science; available at snapgene.com) for simulations of restriction cutting and expected digestion products. Selected restriction enzymes resulted in multiple segments and non-similar lengths after digestion, simplifying identification of desired plasmid containing only one acceptor and donor vector. Restriction enzymes SalI, NcoI, HindIII and MscI (all from NEB) were selected.

Products were digested in 15 µL reactions, using 1.5 µL BSA (10X), 1.5 µLbuffer, 1.0 µL restriction enzyme and adjusted to 10 µL with dH_2O before adding 5 µL recombined plasmid. Cutting was performed at 4°C for 2 minutes, 37°C for 12 hours, 65°C for 20 minutes and 4°C for 2 minutes and products analyzed on 65V for 50 minutes in 0.9% agarose gel in 1xTAE buffer followed by 10-minute staining with GelRed Nucleic Acid Gel stain (Biotium) visualized by UV transilluminator.

Transformation of MAX Efficiency DH10Bac Competent cells (EMBacY) and purification and isolation of bacmid from EMBacY

10 ng of recombined plasmid DNA was added to 50 µL solution of competent EMBacY and incubated on ice for 30 minutes before 45 second heat-shock at 42°C before another 5-minute incubation period on ice. 500 µL SOC medium was supplied before incubation for 4-6 hours. 20 minutes before plating, 40 µL IPTG and 17 µL X-Gal (Sigma Aldrich) were spread on LB-plates with kanamycin, tetramycin and gentamycin for blue-white screening and selection of desired colonies.

Fresh white colonies were incubated overnight in 5 mL kanamycin, tetracycline and gentamycin-containing LB-medium at 37°C. After incubation, cells were centrifuged at 3000 x g and purified using buffers from QIAprep Spin Miniprep Kit (QIAGEN). For DNA precipitation after purification, 600 µL isopropanol was added to 800 µL of purified bacmid in N3 neutralization buffer and frozen for 1 hour at -70°C before centrifugation at 13000 rpm for five minutes at 4°C, followed by two rounds of washing, first using 200 µL 70% ethanol before centrifugation, then 50 µL 70% ethanol and finally resuspension of DNA-containing pellet in 30 µL sterile water. Bacmid concentration and purity was measured by NanoDrop 1000 spectrophotometer (ThermoFisher).

Culturing of insect cells

Cells from *Spodoptera frugiperda* (Sf9) and *Trichoplusia ni* (High Five (H5), ThermoFisher) were used, as recommended by Fitzgerald et al. (6). Cells were initially grown as adherent cultures in cell culture flasks (Nunc, EasYFlask Cell Culture Flasks, ThermoFisher) before transfer to Erlenmeyer shake flasks in suspension culture for increased volume production. Sf9 was grown in Sf-900 II serum-free medium (ThermoFisher, Cat. # 11496015) and H5 in Express Five Medium (ThermoFisher, Cat. # B85502). In adherent cultures, cells were passaged upon 90 % confluency. Suspension cultures were passaged when reaching a concentration of 2-4 x 10^6/mL, Sf9 was passaged to 1.0x10^6/mL, and H5 to 0.8 x 10^6/mL. Cultures were considered viable and healthy when approximately doubling or more every ~24 hours. Cell viability was also measured by Trypan blue staining, expecting more than 90% live cells in healthy cultures.

Transfection of insect cells with bacmid

Before transfection, 1 mL of culture (0.8x10^6/mL for H5 and 1.0x10^6/ml for Sf9), with expected doubling time and high cell viability were seeded to 6-well tissue culture plates (Nunc Cell-Culture Treated Multidishes, ThermoFisher) and incubated for approximately one hour at 27°C. While incubating, lipid-complexes with bacmid DNA for efficient transfection was prepared for each well. For these, 200 µL cell medium (Sf-900 II for Sf9/Express Five for H5) was added to 3-6 µg of bacmid before adding 10 µL PLUS Reagent (ThermoFisher) and incubating for 20 minutes. At the same time,

a mix of 200 µL medium and 16 µL of CellFectin-II (ThermoFisher) was prepared and incubated. After incubation, the two transfection-mixes were mixed and incubated a further 20 minutes before adding 200 µL transfection-mix to each well. Cultures were incubated in darkness for 48-60 hours, before harvest, centrifuging at 3000 x g for 4 minutes and storing supernatant (V_0). 3 mL of fresh medium was added to wells for further incubation for protein expression testing.

Virus amplification

After initial transfection, 0.5 mL of V_0 was added to 20 mL of culture containing $1.0x10^6$/mL Sf9 cells. These were incubated at 27°C at 90 rpm and split every 24 hours to $1.0x10^6$/mL until proliferation arrest. After approximately 48 hours of proliferation arrest, cultures were centrifuged at 500 x g for 4 minutes before transfer of supernatant to new tube and centrifugation at 3000 x g for 4 minutes followed by sterile filtration of supernatant (V_1).

Titration of bacmid concentration for transfection and transfection rate measurements

Experiments evaluating the optimal viral volume were performed by transfection of 25 mL suspension cultures of H5 in 250 mL in Erlenmeyer shake flasks at an initial concentration of $0.8x10^6$/mL, adding 12.5 µL V1 (0.05% of total volume) and 62.5 µL V1 (0.25%) and assessing differences in growth after 24, 48, 72 and 96 hours by flow cytometry on FacsCanto (BD Biosciences) and flow cytometry data analyzed with FlowJo (BD Life Sciences). After assessing optimal bacmid volumes, an addition of 0.5% of total culture volume as bacmid was assumed to be adequate for achieving both longer viable lifespan of cultures as well as having a high enough viral load for high-volume protein production.

Analysis of protein produced from bacmid-transfected cells

Filtering, concentrating and purifying recombinant protein-containing cell culture

For filtration and increasing concentration of cell culture supernatant containing D7T4-TCRαβ, the Masterflex L/S standard digital pump system was used with the Sartorius Vivaflow 200 Crossflow Casette (VWR) following manufacturer protocol.

Purification of recombinant protein solution from concentrated and filtered cell culture was performed with the Strep-Tactin XT High-Capacity resin and accompanying buffer solutions (IBA GmbH, Goettingen, Germany) in an Econo-Column chromatography column (Bio-Rad). Preparation was performed as per manufacturer instructions, and resin buffer washed out of column by two washing steps using Buffer W (IBA GmbH, Goettingen, Germany). The Strep-Tactin resin is documented as having a 5 mg/mL capacity for binding the Strep-tag II on the D7T4-TCRα subunit of the recombinant protein, and is delivered in a 50% suspension. 2 mL resin was used in the column,

for a potential yield of 5 mg of recombinant protein. After flow-through of all protein solution, the column was again washed with Buffer W, before using Buffer BXT 1x (IBA GmbH, Goettingen, Germany) for elution. Protein concentration and purity was measured by NanoDrop 1000 spectrophotometer (ThermoFisher).

Isolation and antibody coating of protein for analysis

Dynabeads His-Tag Isolation and Pulldown (ThermoFisher) are magnetic beads highly selective for the poly-histidine (6xHis) tail on the D7T4-TCRβ subunit. Due to their magnetic properties, these can bind protein in solution and allows for retaining protein-bound beads by magnetism while changing the solution they are in, simplifying isolation, washing and staining procedures. 50 mL PBSA with 5 µL Tween20 (Sigma-Aldrich) and 5 µL beads per sample were prepared before adding 1 mL of sample from incubated insect cell cultures. After two hours incubation, mixtures were washed three times by addition and mixing of 1 mL of PBSA-Tween before removing supernatant while sample tubes were mounted on magnet. Protein-coated beads were resuspended in 100 µL PBS after washing.

SDS-PAGE using protein-antibody coated dynabeads

40 µL of sample with protein-coated beads were washed twice in PBS-Tween before resuspension in 10 µL PBS-Tween. 3.8 µL 4x Nupage LDS-reagent (Life Technologies) and 1.5 µL NuPAGE Sample Reducing agent (Life Technologies), per manufacturer instructions, was added to samples, for total reaction volume of 15 µL. To release protein from beads, samples were heated at 76°C for 10 minutes before transferring supernatant to gel (without beads).

Samples were run on NuPAGE Novex 4-12% Bis-Tris Gels, 1.5 mm, 15 well gel (Life Technologies) with 600 mL 1x NuPAGE Running Buffer (30 mL 20X buffer (Life Technologies) + 570 mL MQ H2O) supplied with 500 µL NuPAGE Antioxidant (Life Technologies), using 12 µL samples and 7 µL Novex Sharp Pre-stained protein standard ladder. After electrophoresis, the gel was stained using SimplyBlue Safestain (ThermoFisher) as described by manufacturer.

Analysis of D7T4-TCRαβ dimerization

D7T4-TCRαβ bound to Dynabeads His-Tag Isolation and Pulldown (ThermoFisher) was used and stained with StrepMAB-Classic (Beckman Coulter), a mouse anti-Strep-tag antibody, using 0.25 µg/reaction and 10 µL Dynabeads-D7T4-TCRαβ in 50 µL reaction volume. These were incubated at 150 rpm for 20 minutes, before washing with 150 µL PBS and resuspending in 150 µL PBS in FACS Flow tubes. This was followed by a secondary stain with Alexa 488-conjugated goat anti-mouse IgG (Invitrogen) using the same volumes and procedure. Samples were analyzed on FacsCanto (BD Biosciences) and flow cytometry data analyzed with FlowJo (BD Life Sciences). Samples were also stained with anti-TCR PAN α/β-PE antibodies (Beckman Coulter) for flow cytometry.

Protein dimer confirmation by western blot

For primary staining of the poly-histidine tail on D7T4-TCRβ, mouse anti-Histidine antibody (Merck) was used. Mouse anti-Strep Tag II antibody (Beckman Coulter) was used for staining of the Strep-tag II on D7T4-TCRα. Horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Sigma Aldrich) was used as a secondary antibody for detection in western blot on all samples. A polyvinylidene difluoride (PVDF) membrane was prepared by immersion for 3 seconds in methanol, 10 seconds in Milli-Q water (Sigma Aldrich) and 5 minutes in 1x NuPAGE transfer buffer (Thermo Scientific) before placement between a polyacrylamide gel and filter paper enveloped by two 1x NuPAGE transfer buffer-soaked sponges on either side. The inner chamber was filled with transfer buffer and the outer with water for cooling, before one hour electrophoresis. After electrophoresis, the membrane was washed twice for 5 minutes in washing buffer (PBS with 0.05% Tween20). 5 mL blocking buffer (Superblock blocking buffer (Life Technologies) with 0.05% Tween20) and 10 µL goat IgG (Thermo scientific) was used for blocking, incubating the membrane in the solution for 1 hour before washing twice with washing buffer for 5 minutes. For primary staining, the membrane was incubated with primary antibodies in concentrations according to manufacturer recommendations, in 5 mL blocking buffer overnight at 4°C before washing three times with washing buffer. For secondary staining, the membrane was incubated for 1 hour in 5 mL blocking buffer with 5 µL HRP-conjugated goat anti-mouse IgG (Sigma Aldrich) and washed three times in washing buffer. The Supersignal west femto maximum sensitivity substrate reagents (Thermo Scientific) were used in equal proportions in a 3 mL solution, and the membrane incubated in darkness for 5 minutes in the solution before sealing it in plastic and initiating imaging on ImageQuant LAS 4000 (GE Healthcare). The machine settings were: Exposure type: Increment, Interval time: 10 seconds, Sensitivity: High, and set for chemiluminescence at -30°C. Images were analyzed and adjusted in ImageQuant LAS 4000 manufacturer software.

Functionality testing of D7T4-TCRαβ by proliferation assay

HPA-1a-specific T-cells (clone D8T104) were washed twice in PBS and resuspended in 0.1% PBSA. 2 µL 250 µM CFSE stock was diluted in 0.5 PBS and immediately added to the tube containing T-cells before transferring tube to ice and adding 5 mL ice cold Iscove's modified dulbeccos medium (IMDM) 10% FBS medium and incubating on ice for 5 minutes. After centrifugation for 5 minutes at 350 x g, cells were washed 3 times in 0.2% PBSA and resuspended in T-cell medium. After counting cells, cultures were diluted for a concentration of $1.0x10^6$/mL and stored on ice.

For peptide-pulsing of B-cells (EBV-transformed D18BL, expressing DRA/*DRB3**01:01), high viability cultures were prepared at $1.0x10^6$/mL in IMDM. Peptide stock of L33 and LolP1 (DRA/*DRB3**01:01

restricted synthetic peptides, L33: VSPMCAWCSDEALP**L**GSPRC, LolP1: ESWGAVWRIDTPDK**L**TGPFT (2)) in PBS was sterile filtered and were added to separate vials of 1 mL cell culture for a concentration of 4 µM, in two parallels for each before incubation at 37°C in 7.5% CO_2 atmosphere for 1 hour. After incubation, tubes were irradiated in two cycles, for a total of approximately 8000 rad followed by washing in 0.2% PBSA and resuspension in T-cell medium at $1.0x10^6$/mL.

Cells were plated in 96-well round bottom assay plates, with two parallels for each sample. Two wells were plated with 50 µL L33-pulsed B-cells and 50 µL D7T4-TCRαβ-bound beads, two with 50 µL L33-pulsed B-cells and 50 µL T-cell medium and two with 50 µL LolP1-pulsed B-cells and 50 µL T-cell medium. The plate was centrifuged at 350g for 1 minute before incubation at 37°C in 7.5% CO_2-atmosphere. 50 µL CFSE-labelled T-cells were added to all wells after incubation, before centrifuging at 350g for 1 minute and incubation ad 37°C in 7.5% CO_2 atmosphere for 4 days. 100 U/mL IL-2 was added after 24 hours.

For flow cytometry, PE-Alexa 610-conjugated anti-CD4 antibody (Caltag), with 3 mL 0.2% PBSA and 30 µL antibody, was used to label T-cells for gating and analysis. Unstained T-cells, CFSE-labelled T-cells and PE-labelled T-cells in 0.2% PBSA was used for compensation controls. For all samples, 50 µL of resuspended cells was collected from each well and mixed with 50 µL anti-CD4 stock before incubation on ice for 20 minutes. Samples were washed in 0.2% PBSA and resuspended in 200 µL 0.2% PBSA and CFSE intensity analyzed on FACSCanto (BD Biosciences). Data was analyzed by FlowJo (BD Life Sciences).

Results

To express soluble, recombinant HPA-1a specific TCRs in insect cells, the sequence for the TCRα and TCRβ in an HPA-1a specific T cell clone, D7T4, was used. These sequences were determined a by Ahlen et al. (2). Prior to initiation of this study, the sequence was modified *in-silico*, removing the connecting, transmembrane and cytoplasmic regions and adding sequences encoding specific restriction sites, linker regions, dimerization domains and tags for further purification and validation (a Twin-Strep-Tag (SAWSHPQFEKGGGSGGGSGGSAWSHPQFEK) on D7T4-TCRα and a poly-histidine tail (6xHis) on D7T4-TCRβ) (Figure 1A). TCR inserts (hereafter referred to as “inserts”) were delivered in cloning vectors that could be amplified in *E. coli.* Additionally, two expression vectors used in the baculovirus expression system (6), pUCDM and pFL (hereafter referred to as “vectors”), were similarly amplified. TCRα and -β genes from these vectors were ultimately inserted into in a single baculovirus construct (Fig. 1) for use in transfection of insect cells for D7T4-TCRαβ protein production.

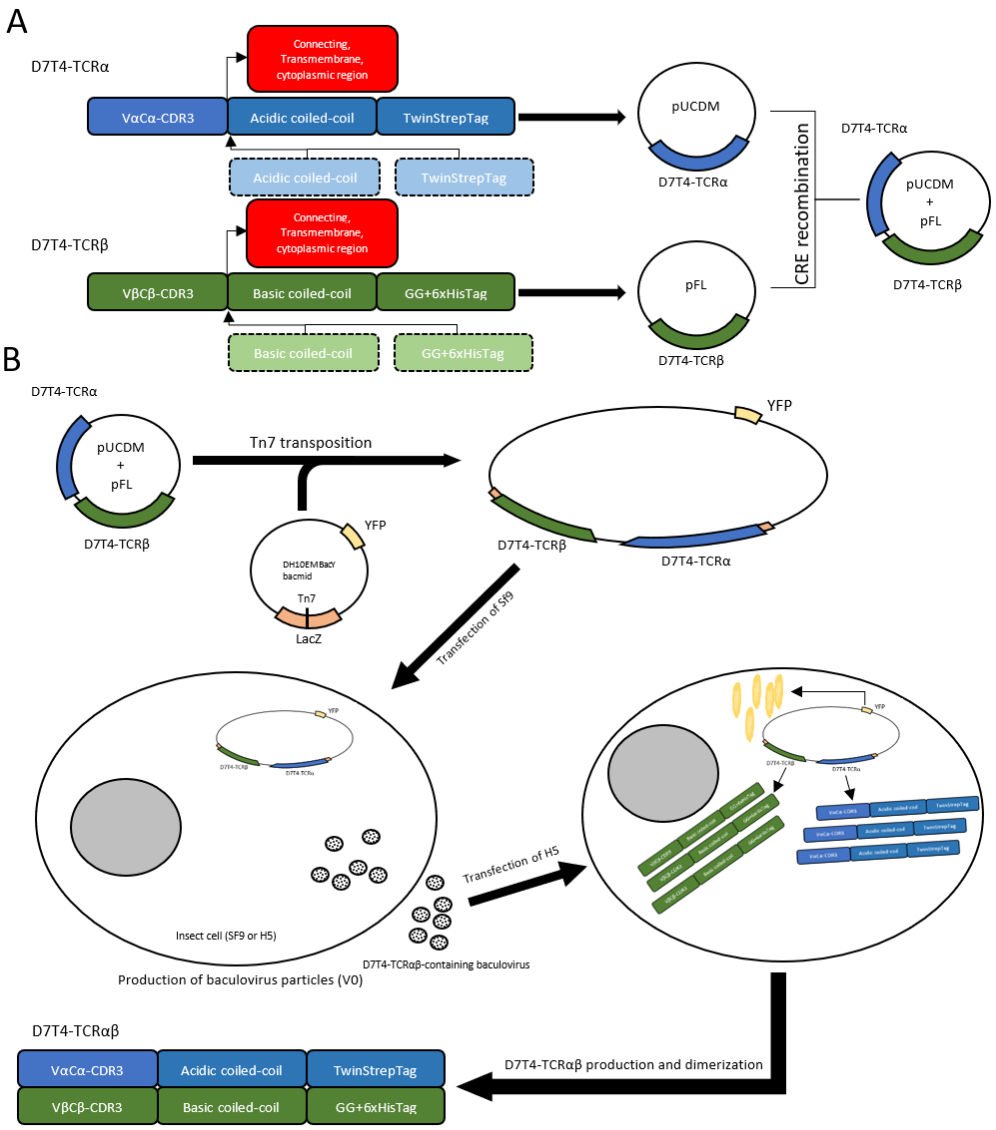


Figure 1. Schematic overview of study methods. (A) Constructs of modified TCRα and TCRβ subunits of the HPA-1a specific T-cell clone D7T4 (isolated and sequenced by Ahlen et al. (2)) were transformed into *E. coli* for amplification and cloning before insertion into the baculovirus expression system vectors pFL and pUCDM. These were recombined by CRE recombination. (B) After recombination, the plasmid was transformed into E. coli containing the DH10EMBacY bacmid. In this process, the Tn7 transposon located on pFL facilitated insertion of the insert-vector complex pFL-pUCDM-D7T4-TCRαβ into the modified baculovirus vector DH10EMBacY. Insertion that disrupts the LacZ gene inhibits a process making blue color, allowing for selection of colonies with correctly inserted genes by selecting only white colonies. Plasmid isolated from these colonies were used in transfection of insect cells. When transfected with this baculovirus, they produce both baculovirus containing D7T4-TCRαβ-genes, as well as producing D7T4-TCRαβ protein and yellow fluorescent protein (YFP), used in monitoring transfection rates by flow cytometry. D7T4-TCRαβ proteins are isolated at peak levels of YFP fluorescence.

Transfer of inserts from cloning vector to expression vector

All four vectors were cut with restriction enzymes as described in the methods chapter, and vector fragments were separated by gel electrophoresis (Fig. 2B and C). Expected fragment sizes and

locations on the gel matched a computer-generated virtual image of the gel (Fig. 2A). Uncut fragments did not match simulations well. This is due to software placing bands at locations based on plasmid length, and not accounting for uncut plasmid being circular and supercoiled, limiting movement in gel.

For verification of correct size of inserts and vectors before ligation, D7T4-TCRαβ subunits, as well as vectors pUCDM and pFL were digested by restriction enzymes for cleavage at desired sites for correct ligation, and analyzed by gel-electrophoresis. This allowed us to investigate that all digestion products were of expected size and for isolation of desired segments of correct size. pUCDM/D7T4-TCRα and pFL/D7T4-TCRβ digestion products of expected length for vector and insert were excised from gel at the locations indicated in figure 2B and 2C, before purification of DNA followed by ligation. While the gel had smearing above the indicated areas, only the areas encircled in figure 2 were excised, due to bands in these areas clearly indicating expected size, as well as having the highest plasmid concentration.

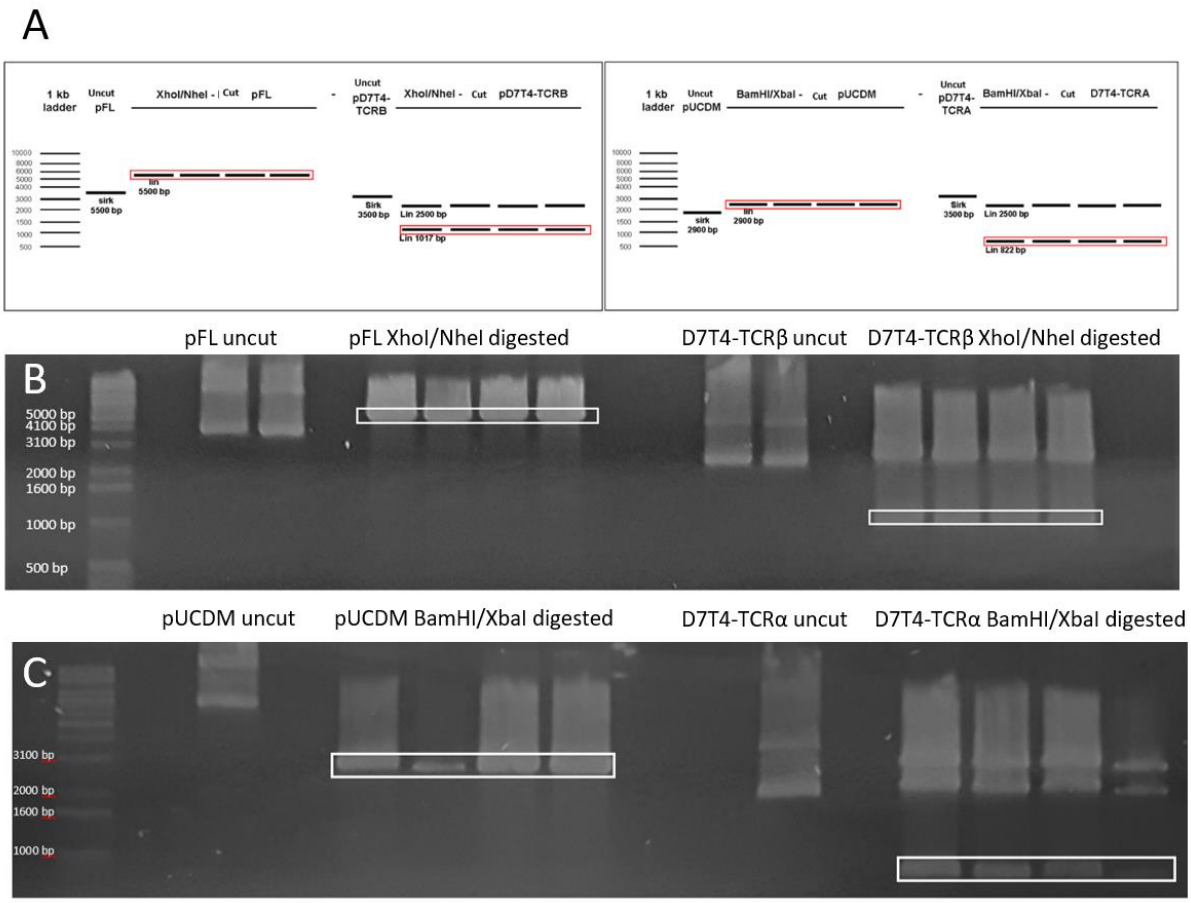


Figure 2: Restriction digestion of vectors and inserts produced bands with DNA of expected lengths. (A) *In-silico* simulations of gel using selected restriction enzymes. (B) pFL and D7T4-TCRβ at expected lengths of 5500 bp and 1017 bp, respectively, after digestion with XhoI and NheI. (C) pUCDM and D7T4-TCRα at expected lengths of 2900 bp and 822 bp, respectively after digestion with BamHI and XbaI.

Confirmation of correct ligation by PCR testing

To confirm that the ligation process had been successful, primers for each vector-insert pair was used in PCR testing of candidates. The primer binding sites were located before and after the expected location of D7T4-TCRα/β-inserts, so that only samples with correct insertion would have amplification products of the expected length. These were expected to be approximately 500 bp for vector without insert, and 1400-1500 for vector-insert amplification products. Amplification products were analyzed by gel-electrophoresis after PCR for expected length (Figure 3.) Three ligated pUCDM-D7T4-TCRα (Fig. 3A, candidates 1, 3 and 4) and two pFL-D7T4-TCRβ (Fig. 3B) candidates were found to be of the expected length. Concentrations and purity were analyzed for selected candidates prior to recombination.

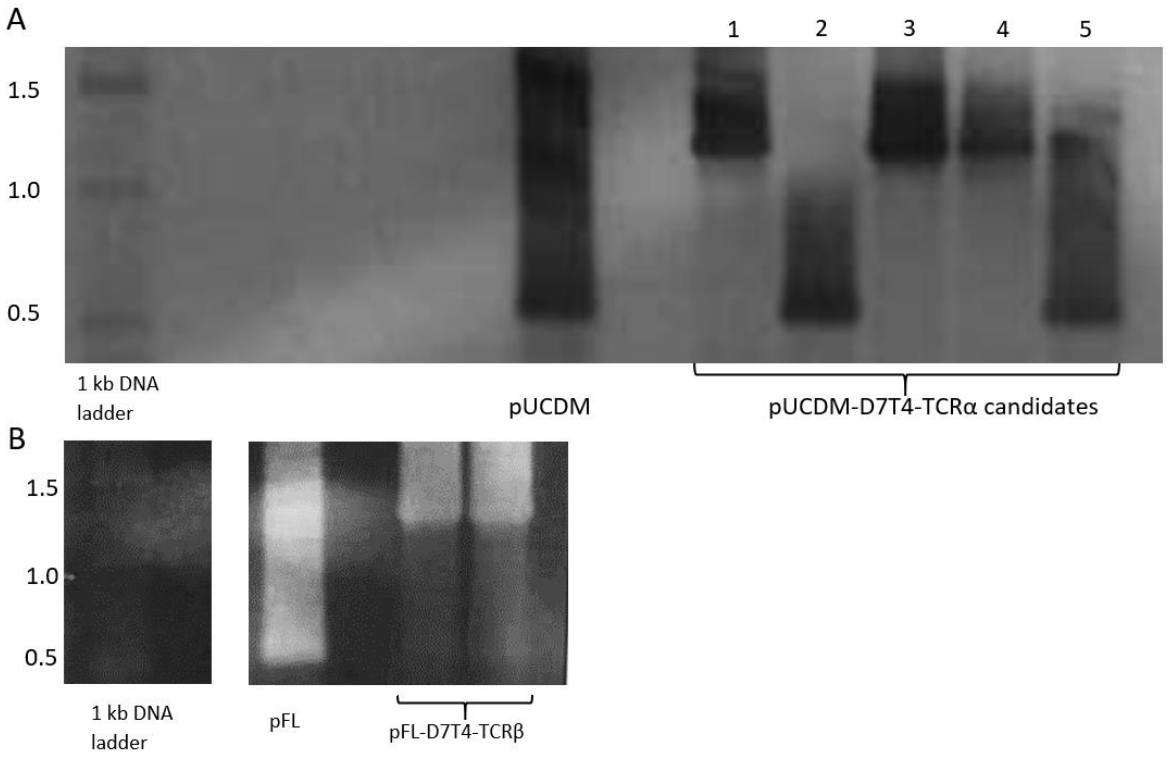


Figure 3: pUCDM-D7T4-TCRα and pFL-D7T4-TCRβ PCR amplicon with expected length on gel. (A) The expected length of the pUCDM-D7T4-TCRα amplicon was 1360 bp. Candidates 1, 3 and 4 were of the expected length. (B) The expected length of the pFL-D7T4-TCRβ amplicon was 1470 bp. Both tested candidates were of the expected length. All correct samples were analyzed for concentration and purity.

Confirmation of desired Cre-recombination product by restriction cutting

Having confirmed correct ligation of TCR inserts into baculovirus expression vectors, plasmid fusion, by insertion of pUCDM-D7T4-TCRα (containing the donor vector pUCDM) into pFL-D7T4-TCRβ (containing the acceptor vector pFL) was performed as described by Fitzgerald et al. (6). The acceptor vector pFL is the only vector that can be inserted into the bacmid EMBacY for production of

baculovirus, due to containing the Tn7 transposon. Both pUCDM and pFL contains LoxP sites, the binding site for Cre-recombinase, allowing for transposition of donor vector into the acceptor vector – ultimately allowing for insertion of D7T4-TCRαβ genes into the bacmid for transfection of insect cells for protein production.

The process of recombination was complicated by multiple issues. First, selection of cells that have acquired the recombined plasmid was achieved by antibiotic selection, with pFL containing ampicillin-resistance genes, while pUCDM has chloramphenicol resistance, allowing growth only for cells with the fused plasmid on plates containing both these antibiotics. In addition, fusion of multiple copies of vectors into one plasmid can occur. To recognize desired variants with one of each vector-insert pair, simulation of restriction enzyme digestion products was performed, and four enzymes that allow for a unique combination of product lengths were selected. Simulation prediction is visualized in figure 4A and with recombinant candidates shown in figure 4B**.** One candidate was found to be correctly recombined and used in transformation of DH10EMBacY and baculovirus production. Notably, digestion with MscI produces an extra band that was not expected from simulations. No combinations of vectors/inserts could produce the expected patterns with this restriction enzyme, but this issue was found to be due to methylations in the restriction sites impacting the predictions.

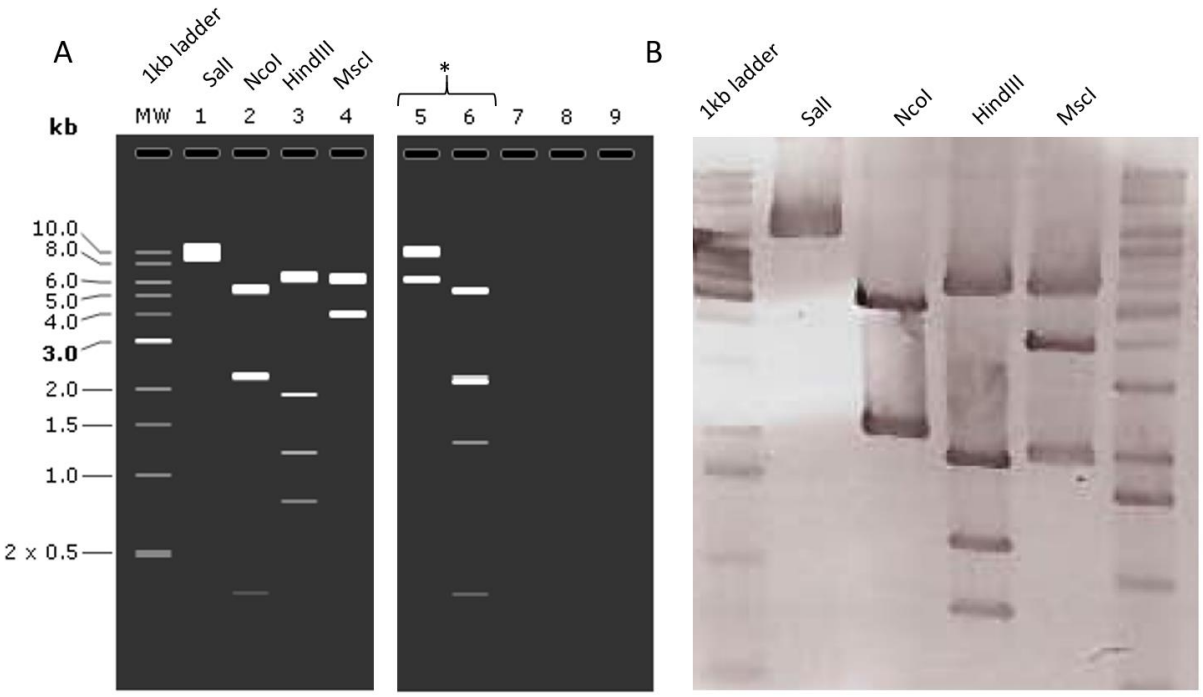


Figure 4. UV visualization of Cre-recombined candidates shows bands consistent with simulations. (A) Computer generated gel using Cre-recombined sequence of pUCDM-pFL-D7T4-TCRαβ and four restriction enzymes to uniquely identify recombination products in which only one of each vector and one of each insert is present. *Well 5 contains dual pFL-D7T4-TCRβ, and is identified by having two bands when digested with SalI. Well 6 contains dual pUCDM-D7T4-TCRα,

and is identified by having more bands when cut with NcoI. (B) Digestion products with correct bands for pUCDM-pFL-D7T4-TCRαβ. An extra band is seen with MscI cutting (well 4, annotated MscI) compared to predictions, however attributed to methylation in the restriction sites for this enzyme.

Transformation of MAX Efficiency DH10Bac Competent cells (EMBacY) bacmid isolation

After confirmation that the Cre-recombined plasmid contained both vectors pFL and pUCDM as well as D7T4-TCRαβ inserts, the plasmid was transformed into *E. coli* containing the DH10EMBacY bacmid (hereafter referred to as EMBacY). This is identical to the MultiBac bacmid described Fitzgerald et al. (6), except for including genes encoding yellow fluorescent protein (YFP). The pUCDM-pFL-D7T4-TCRαβ plasmid is inserted into the Tn7 cassette on EMBacY in this process. For selection of cells that had acquired the recombinant plasmid, two modes of selection were used. First, cells were grown on plates with medium containing kanamycin, tetracycline and gentamycin. Kanamycin/tetracycline resistance from EMBacY and gentamycin from the recombinant plasmid, allowing only cells with both EMBacY and plasmid to grow well. In addition, blue-white color screening of colonies was used for selection. This is possible due to the plasmid being inserted into the Tn7 cassette located in the middle of the LacZ gene of EMBacY. IPTG added to plates triggers transcription of LacZ, inducing expression of β-galactosidase, which hydrolyzes X-gal (also added to plates), causing blue coloration of colonies. The disruption of the LacZ gene by Tn7 transposition results in a lack of β-galactosidase, making colonies with plasmid insertion at the correct site white, and thus allows recognition of colonies with successful insertion of recombinant plasmid into EMBacY. Pure, high concentration samples were obtained from culturing of these colonies in medium and subsequent purification (Table 1).

Sample	Concentration (ng/µL)	A260/A280
D7T4-TCRαβ candidate #1	4152,24	1,84
D7T4-TCRαβ candidate #2	4519,31	1,79

Table 1: Bacmid concentration after isolation. High-concentration samples were acquired from DH10Bac competent cells. A A260/280 value of >1.8 are considered pure, so while candidate 2 was barely below, this was considered pure enough.

Transfection of insect cells for baculovirus amplification and protein production

After isolation of EMBacY-D7T4-TCRαβ bacmids from the transformed *E. coli*, these were used to transfect Sf9 insect cells. Following a low-volume transfection in 6-well plates for initial virus (V0) production, outlined in figure 5A, Sf9 was again used for higher-volume virus amplification (production of V1, figure 5B) due to their tendency to produce high yield of virus particles, but often

lacking high production of protein. As genes for yellow fluorescent protein (YFP) are encoded in EMBacY, analysis of transfection success was measured by counting the number of cells in culture expressing YFP by flow cytometry. Here we found high transfection rates using two EMBacY-D7T4-TCRαβ virus candidates for V1 production (Fig. 6).

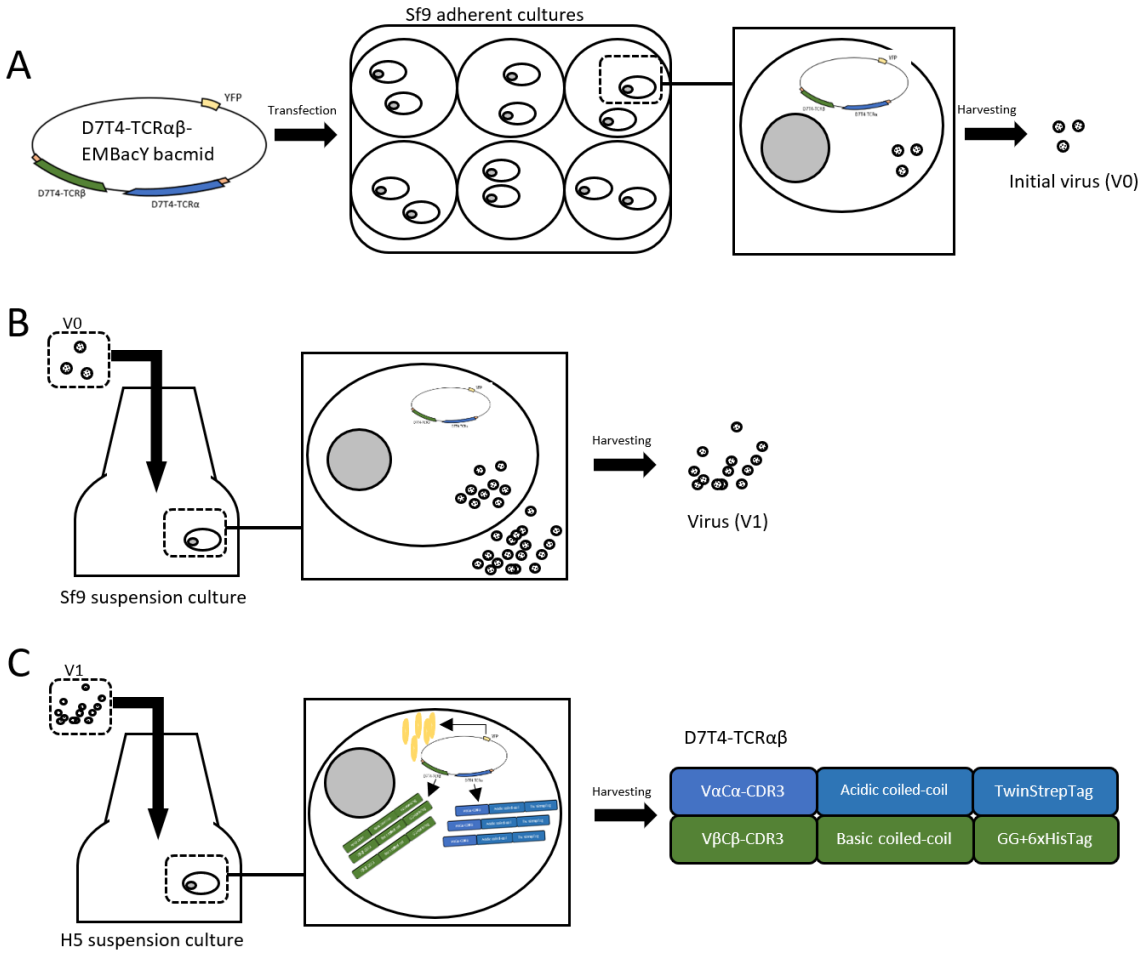


Figure 5. Schematic overview of methods used in baculovirus- and protein production. (A) Bacmid containing D7T4-TCRαβ inserts and EMBacY for transfection of insect cells. Initial virus production was done in 6-well plates with adherent Sf9 cultures. After incubation, initial virus particles were isolated. (B) Larger volume virus amplification was performed by transfection of Sf9 cells in suspension cultures, isolating greater numbers of virus particles (V1) after incubation. (C) Protein production was done with H5 cells transfected with V1 in appropriate concentrations. During incubation, D7T4-TCRαβ is produced and is expected to dimerize to form the desired D7T4-TCRαβ dimer.

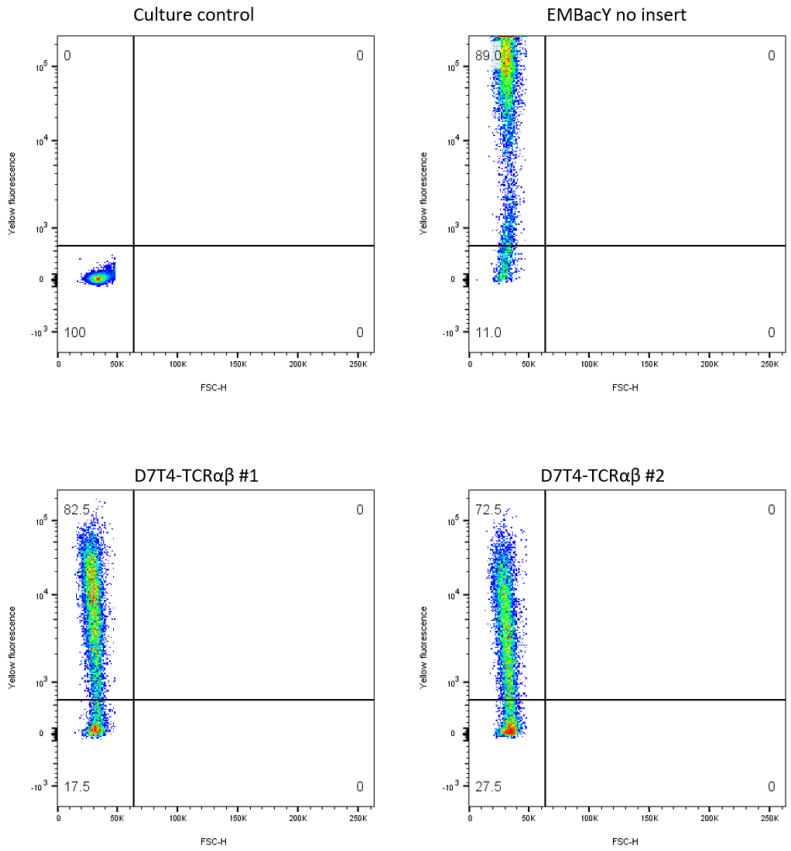
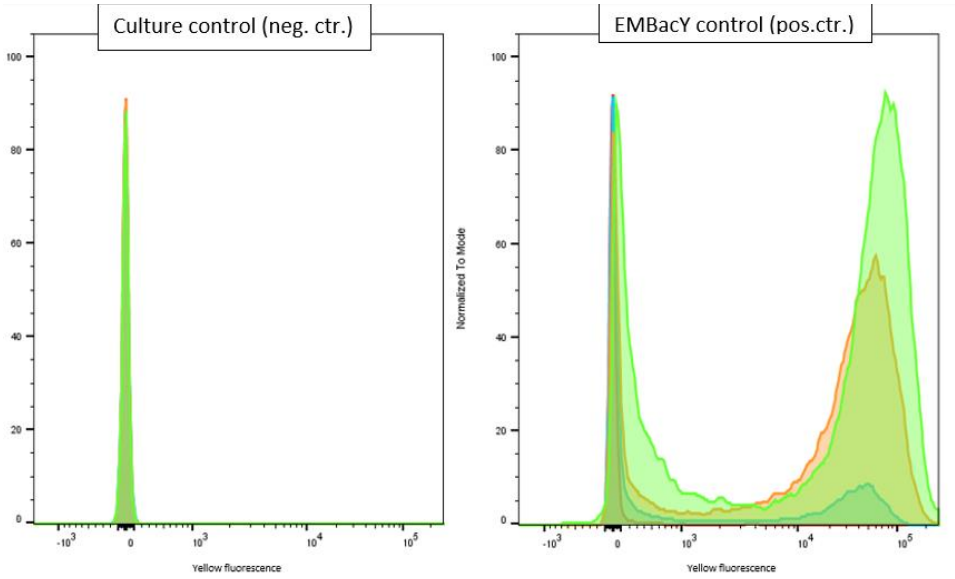


Figure 6. A majority of Sf9 cells in culture are successfully transfected by EMBacY-D7T4-TCRαβ baculovirus. As EMBacY contain genes for yellow fluorescent protein, the number of cells successfully transfected by baculovirus, and the relative amount of protein produced in the cells can be measured directly by flow cytometry. Here we compared two EMBacY-D7T4-TCRαβ candidates to the negative control using only Sf9 cell culture and a positive control, transfecting Sf9 with EMBacY without D7T4-TCRαβ inserted. Both candidates were found to have transfection rates comparable to the positive control.

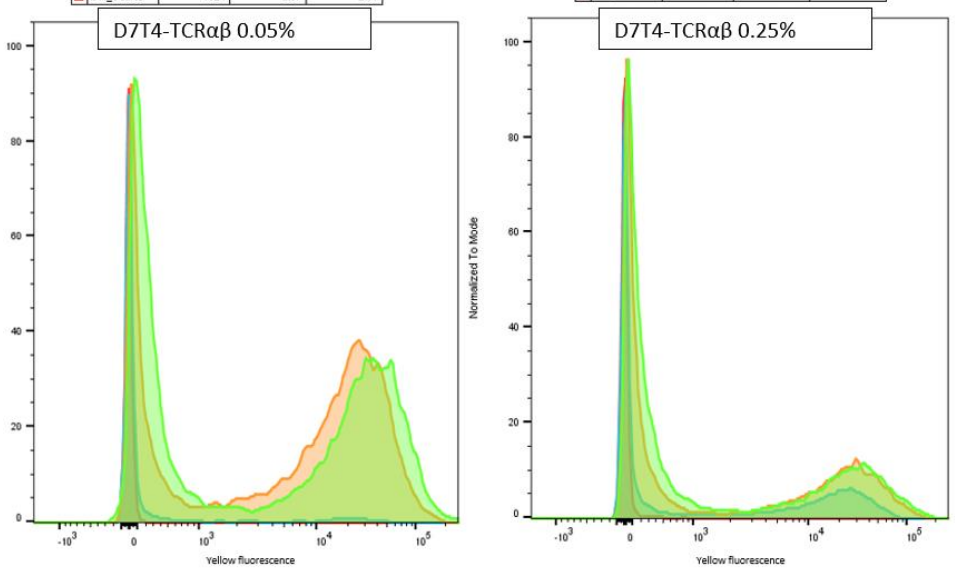
For protein production (figure 5C), the addition of large volumes of virus (V1) caused rapid deterioration of cell culture viability and cessation of culture growth, ultimately resulting in a lower maximum number of transfected cells and shorter timespan for optimal protein production. Initial gating in flow cytometry on viable cells based on FSC and SSC. On testing two virus candidates, titration experiments indicated that a bacmid concentration of 0.05% resulted in a slower initiation of transfection, but a higher overall number of transfected cells and a growth that is more similar to that of the positive control containing only EMBacY (Fig. 7). At both concentrations, the number of transfected cells reached a plateau after approximately 48 hours, resulting in a low total number of transfected cells as well as little time for protein production. Analysis of protein concentration and purity after isolation of protein from cultures held at this plateau-phase over time indicated that this strategy yielded both low concentrations and low production of the proteins of interest. Due to

storage of viral stocks over time in the project, later experiments (Fig. 8), showed that an increase of the bacmid concentration to 0.5% at initiation was suitable and more similar to the culture characteristics of the EMBacY control. At this concentration, many cells were rapidly transfected, and the number of remaining transfected cells after 70 hours of incubation were higher than those seen in titration experiments. At this point, a rapid increase in heterogenic and more granulated cells was seen on microscopic analysis (data not shown) as well as an increasing ratio of dead cells quantified by trypan blue-staining, and while it would be desirable to maintain this high number of transfected, protein producing cells for a longer period, the incubation was stopped at this point, to avoid the deteriorating culture viability seen when reaching a plateau phase in titration experiments.



Sample Name	Count	Median : FL1-A	Mean : FL1-A
96h_CC.fcs	6111	2.57	1.67
72h_CC.fcs	4694	2.57	2.37
48h_CC.fcs	2809	2.57	2.29
24h_CC.fcs	7924	2.57	2.08

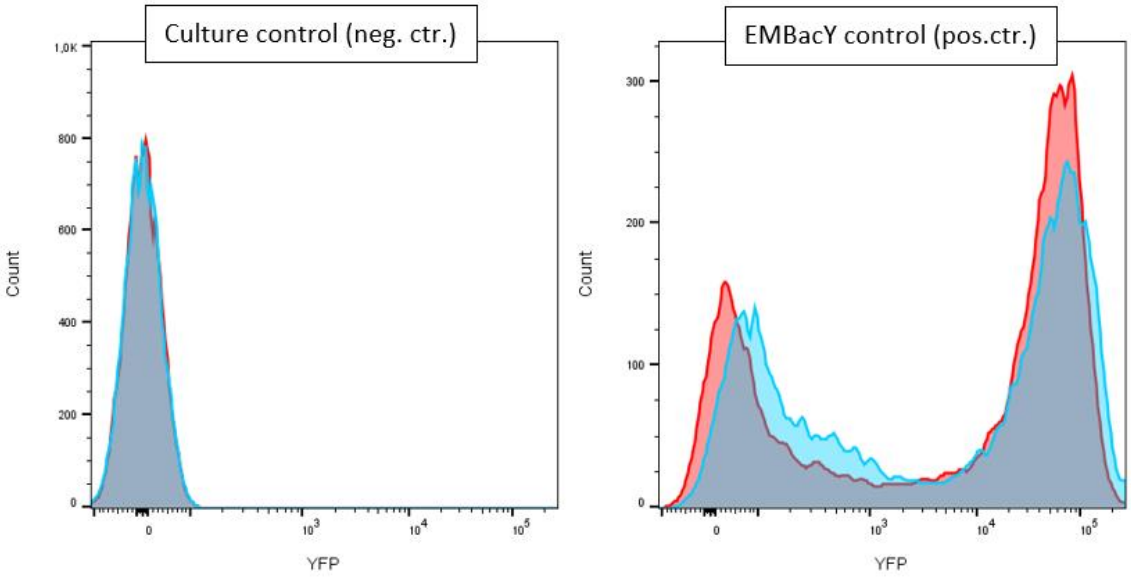
Sample Name	Count	Median : FL1-A	Mean : FL1-A
96h_E6.fcs	18311	40906	51314
72h_E6.fcs	17301	31869	39428
48h_E6.fcs	12249	125	14073
24h_E6.fcs	10183	6.42	189



Sample Name	Count	Median : FL1-A	Mean : FL1-A
96h_TCR10 - 0_05%.fcs	9402	5951	22000
72h_TCR10 - 0_05%.fcs	9021	10846	18789
48h_TCR10 - 0_05%.fcs	5329	19.3	2094
24h_TCR10 - 0_05%.fcs	8367	2.57	32.2

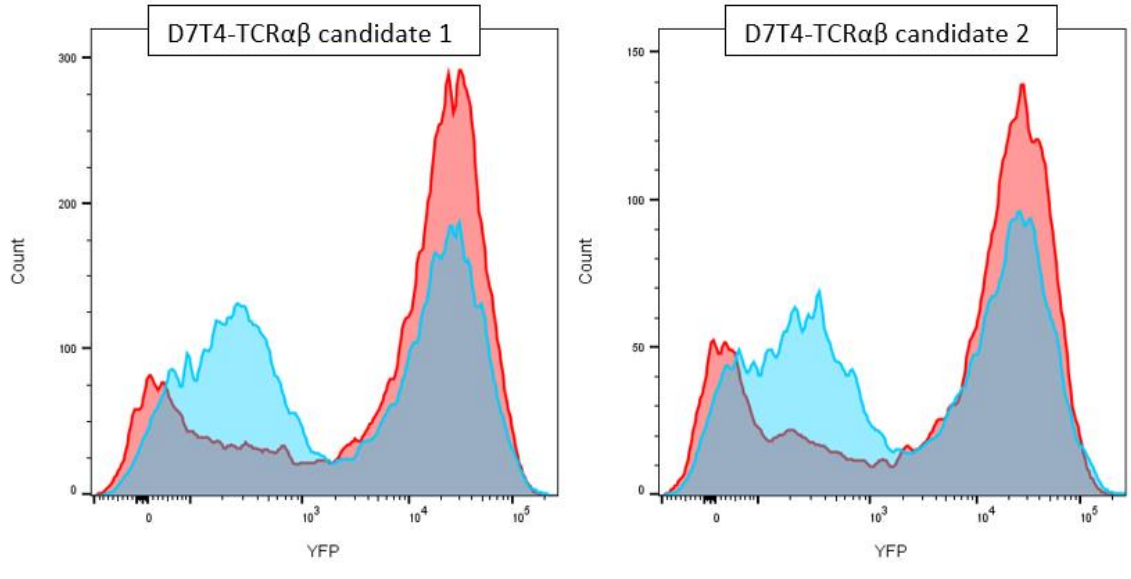
Sample Name	Count	Median : FL1-A	Mean : FL1-A
96h_TCR10 - 0_25%.fcs	18739	316	11917
72h_TCR10 - 0_25%.fcs	18248	312	12242
48h_TCR10 - 0_25%.fcs	11959	92.5	8292
24h_TCR10 - 0_25%.fcs	9969	6.42	117

Figure 7: Transfection of H5 with higher volume of D7T4-TCRαβ V1 virus containing bacmid causes an overall lower number of transfected cells due to rapid leveling and deterioration of cultures. Flow cytometry analysis after 24, 48, 72 and 96 hours after transfection indicates that a virus volume of 0.25% of initial total volume (lower right panel) reaches an early plateau before cessation of growth, and that a virus volume of 0.05% (lower left panel) is slower, but with overall higher yield. EMBacY without the added expense of producing TCR protein and bacmid (upper right panel) is assumed to be optimal growth, with increasing number of transfected cells over the entire timeframe of the experiment. Successful transfection is expected to be as similar to this growth pattern as possible. Negative control (pure H5 culture only) in upper left corner.



Sample Name	Count	Median : FL1-A	Mean : FL1-A
70h_CC.fcs	19825	1.30	2.29
48h_CC.fcs	19825	2.83	2.52

Sample Name	Count	Median : FL1-A	Mean : FL1-A
70h_E - 0_5%.fcs	19311	20886	41047
48h_E - 0_5%.fcs	19515	27486	37994



Sample Name	Count	Median : FL1-A	Mean : FL1-A
70h_TCR1-1 - 0_5%.fcs	18853	3012	15058
48h_TCR1-1 - 0_5%.fcs	19413	16078	21659

Sample Name	Count	Median : FL1-A	Mean : FL1-A
70h_TCR1-2 - 0_5%.fcs	10094	2606	15275
48h_TCR1-2 - 0_5%.fcs	9717	14467	20549

Figure 8: Confirmation of successful transfection of insect cells with D7T4-TCRαβ-containing V1. YFP expression in cells transfected with D7T4-TCRαβ-containing baculovirus is similar to the positive control with only EMBacY (upper right corner) after 48 (red) and 70 (blue) hours. The number of YFP-expressing cells is higher than in titration experiments at both time intervals, but the number of unhealthy cells (i.e. highly granular cells/unhealthy/dead cells) increases dramatically after 70 hours (causing a relative increase in YFP-negative cells). While the number of YFP-expressing cells at 70 hours indicates a high number of protein-producing cells, incubation was stopped to avoid potential alterations on culture and/or produced protein caused by this increase in abnormal or dead cells.

Analysis and purification of D7T4-TCRαβ

After confirmation of high-purity protein samples with good protein yield, we still did not know whether both subunits of D7T4-TCRαβ was produced and dimerized correctly. To address this, D7T4-TCRαβ protein from culture supernatant was isolated for analysis with cobalt-based magnetic beads. The beads bind selectively to the poly-histidine tail on the D7T4-TCRβ subunit, and it was therefore expected that it would also isolate D7T4-TCRα if the subunits were dimerized as expected (i.e. the D7T4-TCRαβ dimer). To this end, protein coated beads were used to isolate D7T4-TCRαβ from supernatant for analysis by SDS-PAGE. Based on the sequence of D7T4-TCRαβ, the expected molecular weights were 32.3 kDa for D7T4-TCRα and 35.2 kDa for D7T4-TCRβ. As such, bands at these weights were the expected outcome. Two parallel candidates were evaluated and compared to samples from cell cultures without added bacmid and with added bacmid without the D7T4-TCRαβ inserts. Both candidates had clear bands at expected sites, while no bands were seen in controls, indicating the presence of the D7T4-TCRαβ subunits (Figure 9). Negative EMBacY control also indicates that no protein of similar weight is found in cultures without D7T4-TCRαβ inserts.

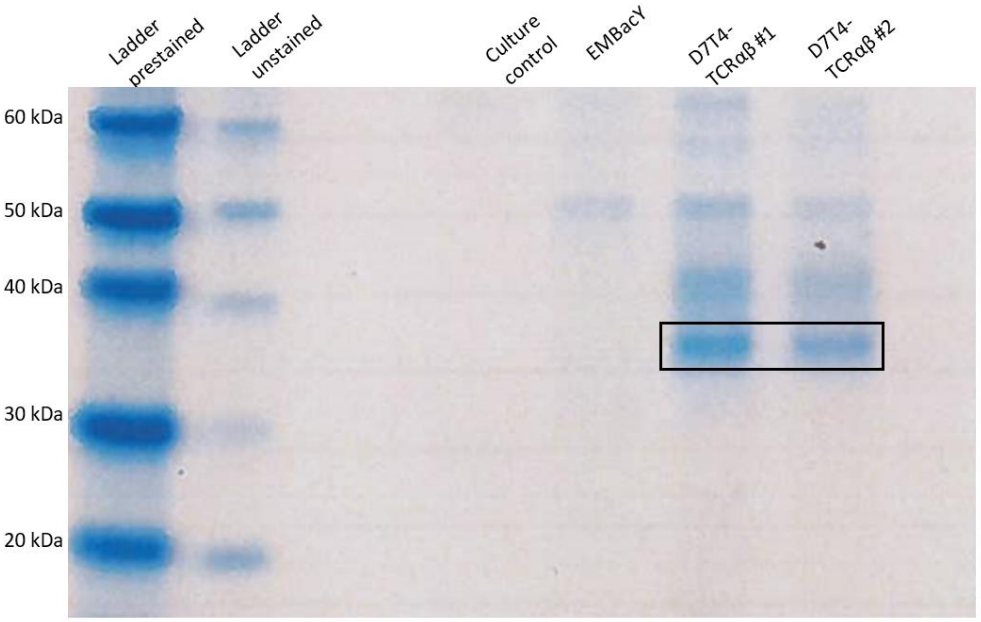


Figure 9: Bands for protein-containing samples on SDS-PAGE are of expected molecular weight and is not expressed in cells containing bacmid-backbone without D7T4-TCRαβ insert. Bands for TCRα and TCRβ subunits are expected to be 32.3 kDa and 35.2 kDa, respectively. On the gel, bands at approximately 30-35 kDa are seen clearly in sample wells (highlighted in square). Unclear bands at 60-70 kDa are seen in both TCR9 and 10, but possibly also in empty bacmid, so presence of D7T4-TCRαβ as a dimer is not clearly demonstrated. CC: Culture control, H5 cells without bacmid; E: H5 and bacmid without D7T4-TCRαβ insert.

After collecting supernatant from protein-producing cultures, the isolation of recombinant protein was performed by column chromatography using a resin for high-affinity binding to the StrepTag on D7T4-TCRα. After flow-through of supernatant and washing, the proteins were eluted. The elution buffer was continuously added, and eluate collected in 2 mL fractions until eight FACS flow tubes were filled with flow-through, to better capture those with high concentration and purity. After protein quantification, eluate from fractions with high concentration and high purity (Table 2) were pooled for storage and further analysis. E2 and E3 were of optimal purity and of high concentration, yielding approximately 1.7 mg of purified protein in total.

Sample	Protein (mg/mL)	A260/A280
Blank	-0,003	-3,009
E1	0,011	-11,911
E2	1,671	0,654
E3	0,462	0,649
E4	0,09	0,444
E5	0,015	-0,057
E6	0,005	2,146
E7	-0,004	1,831
E8	-0,008	0,873

Sample:	Protein (mg/mL)	A260/A280
Blank	-0.005	3.381
E2+E3	0.487	0.683
E4+E5	0	-15,849

Table 2: NanoDrop measurements of concentration after filtration and purification of cell culture indicated high yield of relatively pure protein. A) Concentration of protein in solution in all eight tubes filled with flow-through from column. Samples E2 and E3 were those of highest purity* and concentration. B) Having pooled the two eluates with the highest concentration, a yield of 3.5 mL of 0.487 mg/mL (approx. 1,7 mg of protein). *For protein, the ideal value for purity is A260/A280 = 0,6. A higher value indicating contamination with nucleic acid. A solution with 95% protein and 5% nucleic acid is expected to have an A260/A280 ratio of 1.06, so the samples ratio of 0.683 was considered pure.

Confirmation of D7T4-TCRαβ production and dimerization

Having shown that there are proteins of the expected molecular weight being produced, we needed to investigate whether these are the two monomers of the D7T4-TCRαβ-complex and if dimerization

had been successful. As the His-tag and pulldown dynabeads selectively binds the poly-histidine tail of D7T4-TCRβ, washing and then staining the same samples with antibodies specific for the Twin-Strep-tag on the D7T4-TCRα subunit would indicate if dimerization had been successful, and that the protein contained both subunits. As the anti-StrepTag antibody was not conjugated to a fluorochrome, a secondary anti-mouse antibody (binding to the Anti-StrepTag antibody) was used for staining and analysis. Flow cytometry analysis of these samples found a high (29236 median signal value) signal for the secondary antibody, indicating that many beads had been stained by anti-StrepTag antibodies (Fig. 10). A clear, narrow signal, similar to the negative controls also indicate little contamination or isolation of other proteins. This clear difference in fluorescence indicated the presence of D7T4-TCRαβ in its dimerized form. During these experiments, D7T4-TCRαβ bound to beads were also stained with PE-conjugated anti-TCR PAN α/β, recognizing a monomorphic determinant on human α- and β- chains, but this gave no signal on flow cytometry (data not shown), suggesting that while the D7T4-TCRαβ dimer is present, it is uncertain whether it exists in a recognizable conformation.

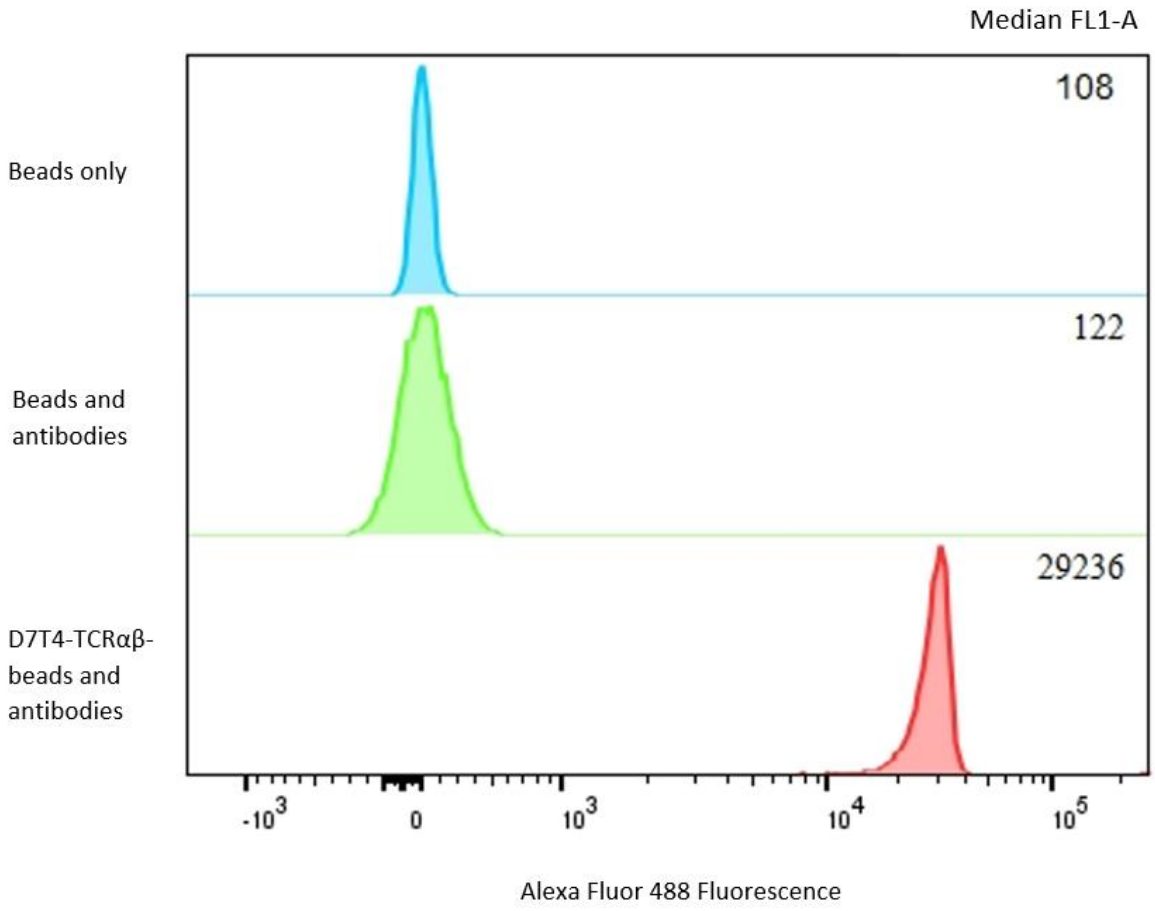


Figure 10: Flow cytometry indicates D7T4-TCRαβ expressed as a dimer. Antibody-staining of purified D7T4-TCRα on samples isolated via the poly-histidine tail on D7T4-TCRβ results in high-intensity signal on flow cytometry. Signal intensity for samples containing isolated D7T4-TCRαβ stained with anti-Strep-Tag antibody and secondary Alexa 488-conjugated antibodies are multiple orders of magnitude higher than the fluorescence of Dynabeads with same staining.

Further investigation of the dimerization was done by western blot. Like flow cytometry, this was done by isolation using structures on one subunit, then staining of a structure on the other, this experiment followed the same principle, using beads for isolation by the D7T4-TCRβ subunit, then staining of the Twin-Strep-Tag on the D7T4-TCRα subunit, confirming successful production of a D7T4-TCRαβ dimer if clear confirmation of D7T4-TCRα. The membrane was clearly stained at sites with D7T4 isolated by binding of the D7T4-TCRβ poly-histidine tail and stained with antibodies specific for the Twin-Strep-Tag on the D7T4-TCRα subunit (Fig. 11). Staining of pure D7T4-TCRαβ without beads was successful for both anti-strep antibodies and anti-6xHis antibodies, while no stain is seen on EMBacY without the D7T4-TCRαβ insert, indicating that the antibodies does bind to their respective targets on protein and that there is no interference from other potential products from the EMBacY backbone. This experiment combined with SDS-PAGE confirmation of subunit weights as well as those from flow-cytometry, were considered confirmation of successful D7T4-TCRαβ dimerization.

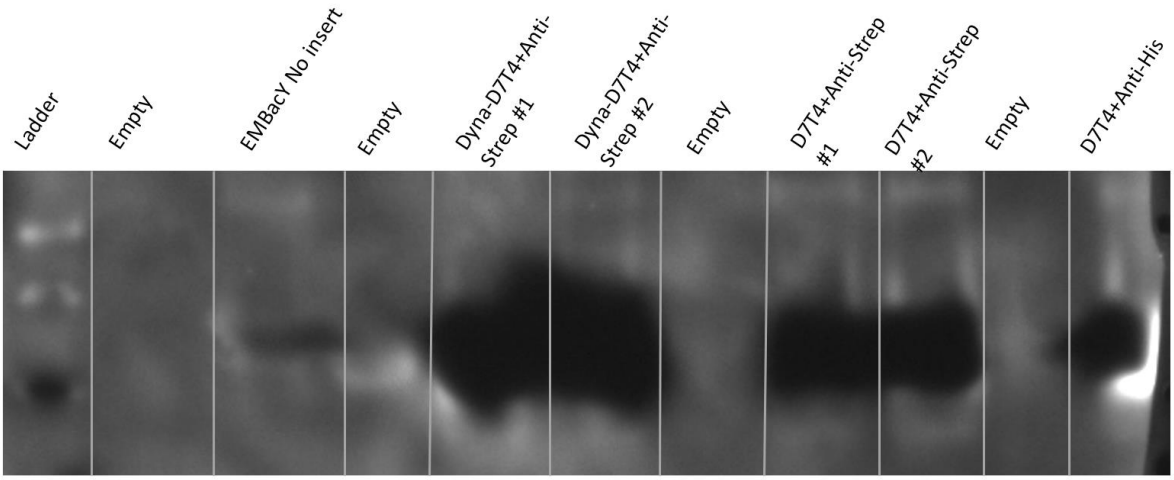


Figure 11: Confirmation of D7T4-TCRαβ dimer expression. D7T4-TCRαβ was isolated from culture supernatant using dynabeads selectively binding the poly-histidine tag on D7T4-TCRβ and later stained with antibodies specific for the Strep-tag II on D7T4-TCRα, demonstrating that the expressed protein exists in the expected dimer form. Positive controls for each subunit was also included, staining D7T4-TCRαβ without beads with anti-Strep and anti-His in separate samples. A negative control using supernatant from cultures with EMBacY without D7T4-TCRαβ insert was included to ensure that reactivity was specific, and that no EMBacY products resulted in positive signal.

Functionality testing

As experiments had shown that the produced recombinant protein contains both D7T4-TCRαβ subunits as expected, and that they are of the expected molecular weight, the next step in the process was to determine if it functions as a T-cell receptor. A proliferation assay was determined as a productive way to test this. In this assay, D7T4-TCRαβ-bound beads used in the previous assay was combined with cultures of peptide-pulsed B-cells from the in-house cell lines, the D18BL cell line as well as CFSE-labeled HPA-1a-specific T-cells (7). These T-cells are specific for HPA-1a (L33) peptide bound to DRA/DRβ3*01:01 MHC class II complex and proliferate in response to L33-peptide pulsed B-cell stimulation. CFSE binds intracellularly and is divided in daughter cells, and was used a dye for expressing degree of proliferation, as each daughter cell will have halved its fluorescence. So if T-cells were stimulated to proliferate, the median CFSE expression would be lower, but should the recombinant D7T4-TCRαβ interact with the peptide-MHC class II complex (HPA-1a peptide in DRA/DRβ3*01:01), it would compete with T-cells for binding and inhibit proliferation, and thus result in a higher median CFSE. LolP1 also binds to DRA/DRβ3*01:01 MHC class II complex, but does not stimulate proliferation, and was therefore used as a negative control.

Using two parallels for all samples, flow cytometry data (Fig. 12) indicate proliferation of T-cells stimulated with L33-pulsed B-cells with a median CFSE intensity of 14.6-16.2, while T-cells alone or those stimulated with LolP1-pulsed B-cells had a median intensity of approximately 30. As D7T4-TCRαβ was expected to compete for binding to DRA/DRβ3*01:01 MHC class II complex, the median CFSE intensity was expected to be higher than that of L33-stimulated cells, but no significant difference in these were found.

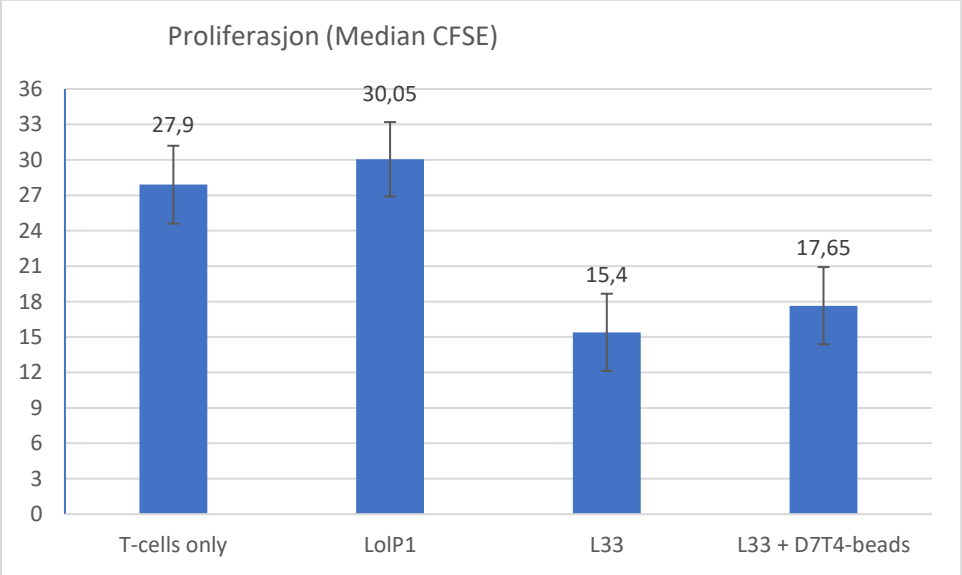


Figure 12: Proliferation of stimulated HPA-1a specific T-cells is not inhibited by incubation in presence of recombinant D7T4-TCRαβ. HPA-1a specific T-cells were cultured with peptide-pulsed B-cells, pulsed with either the stimulatory L33 peptide, or the non-stimulatory peptide LolP1. Cultures where recombinant D7T4-TCRαβ was added was expected to have lower proliferation due to it competing with T-cells for L33-MHC II complexes on the B-cells. The bars show average values of two parallel measurements and their standard deviation.

Discussion

The mechanism of alloimmunization causing FNAIT involves a unique form of T-cell recognition of HPA-1a on DRA/DRβ3*01:01 MHC class II complex. The single amino acid substitution causes stable binding of HPA-1a to DRA/DRβ3*01:01 MHC class II complex, rather than changes in the T-cell epitope (2, 4, 5). Expression of soluble, recombinant TCRs would create a solid platform for further investigation of the TCR-peptide-MHC complex mechanisms, structure as well as any potential diagnostic and therapeutic use, without the added complexity of culturing T-cells. Therefore, the primary goal of this project was the expression of soluble, recombinant T-cell receptors from the HPA-1a specific T-cell line D7T4-TCRαβ, through the use of multigene baculoviral vectors, and as a secondary goal, the examination of properties of the D7T4-TCRαβ peptide-DRA/DRβ3*01:01 MHC class II complex.

Through cloning and recombination of D7T4-TCRα and D7T4-TCRβ plasmids and vectors used in multigene baculoviral vectors, and their fusion, baculovirus vectors for transfection and expression of D7T4-TCRαβ in insect cells were produced. With these methods, expression of D7T4-TCRαβ in the expected dimer form was achieved. Through proliferation assays using D7T4-TCRαβ as a non-stimulatory competitor for T-cells in culture and expecting functional TCRs to reduce proliferation compared to positive controls, the expressed TCR was found to not reduce proliferation.

The cause for these receptors being non-functional is yet unknown, and a number of factors could be responsible. In all major steps of the project, confirmation of desired outcome was performed. In the first major phase, in creating vector-insert plasmids, most steps were clearly visualized to be correct by gel-electrophoresis, ensuring that all plasmids were of correct length in every step, further demonstrated by production of expected amplicon length after PCR with specific primers.

The process of Cre-recombination and EMBacY transformation was laborious due to the uncertainty that was involved in recombination, where multiple vector-insert copies could be incorporated in candidates. In this phase, multiple mechanisms for selection were involved. Ensuring that correct recombination had occurred with restriction enzyme digestion could be a source of error. At this stage, selection of enzyme combinations producing unique band patterns proved to be complicated, and small errors like not factoring for DNA methylation in some software could cause errors that

results in use of candidates with multiple vector-inserts, potentially increasing the cost of replication. For transformation of EMBacY, it is possible that colonies without recombinant plasmid could grow despite antibiotic selection, but the addition of blue-white screening makes it highly likely that recombinant protein was acquired and inserted at the right site.

If not for possible faults in original constructs, human/technical error or otherwise planning-based errors, all experiments using insect cells were the most likely candidates for errors. While Sf9 and H5 are relatively unproblematic to maintain, transfection with bacmid was highly unpredictable. Transfection performed with same candidates on cultures split at the moment of transfection and incubation initiation often produced different results. In the process of investigating optimal bacmid concentration at initiation, some cultures performed well for low concentrations and vice-versa, so the final protein-producing culture with 0.5% bacmid was more of a brute-forcing of protein production than an optimization of the procedure. Still, later confirmation of subunit size by SDS-page, analysis of dimerization by flow-cytometry and western blot seemed to indicate that the expected product had been made. One weakness in the last phase of the project is that the proliferation assay could/should be repeated with enough samples and parallels to potentially discover significant differences that was not seen in the performed experiment. This experiment should also include a variety of D7T4-TCRαβ concentrations, as the binding affinity of TCRs are known to be relatively low compared to many other protein-protein interactions (8). In physiological conditions, the relatively low affinity of TCRs is compensated by TCR avidity, the strength of multiple TCR-peptide-MHC interactions as well as TCR co-receptors (9). Further testing of soluble TCRs or creation of new variants could utilize this by binding of multiple D7T4-TCRαβ to a carrier, imitating the effects on avidity from multiple TCRs on cells, a process shown to improve binding characteristics close to that of monoclonal antibodies (10).

Recombinant TCRs are difficult to express and engineer, lacking persistently successful strategies, and seemingly requiring engineering specific for each unique TCR to achieve stable expression and functional conformation (8, 11). In the present study, D7T4-TCRαβ was engineered with this in mind, including the use of a highly stable acid-base coiled-coil, known for its high stability in dimerization (12), still this did not seem to result in a clearly functional protein. Wagner et. Al. (8) present a modernized method for expression of highly modified TCR sequences. They use an iterative approach involving multiple steps aimed at producing variants with exceptionally high target affinity. For generation of a library of high-affinity TCRα and -β variable region variants, they use a mammalian display system for identification of such variants, and subsequently combine these pairwise for investigation of combinations that yield both high affinity and high surface display on mammalian cells, commenting that surface display levels are useful as a predictor of expression yields of soluble

protein. In addition, they note that fusion of an antibody Fc domain to a poorly expressed protein can increase expression, so they use a modified TCR-Fc plasmid to express their selected TCR variant, testing multiple variants of subunit combinations transfected into Chinese hamster ovary cells. They found that the highest expression of protein was found when using plasmids with α- and β-subunits separate (similar to this study), with the Fc-region connected to the α-chain and addition of two disulfide bonds on the TCR constant domains for strong bonding of the base of the recombinant TCR. Using this method, they produce a clearly functional soluble TCR with a 60-fold increase in affinity compared to wild-type protein while also demonstrating functionality by using them for detection of cytomegalovirus-positive cells.

While the produced TCR in this study was not proven to be functional, the cause is not clear, and perhaps the fault lies in technical procedures, experiment design or simply that the protein had a non-functional conformation. It is also possible that the protein was functional and that the combination of affinity and concentration of the monomeric soluble TCR was too low to compete with T-cells for binding to peptide-MHC complexes on APCs. The strategies presented by Wagner et al. (8) adds interesting new strategies for solving these issues. Among these, Fc-fusion was considered to be the most interesting new method to improve upon the methods in the present study. Using immunoglobulin Fc-domains directly linked to a protein not only increases stability and solubility, but it also increases plasma half-life as well as serving as a method of purification. Due to this, it is an increasingly utilized method in both basic science as well as therapeutic development (13). Introduction of new disulfide bonds does increase stability (14), but not universally for all TCRs (15), and can also be a source of error in subunit pairing in dimerization as well reducing protein yield compared to other stabilizing methods (16). Wagner et al. (8) found that addition of a second disulfide bond increased melting point (i.e. stability) by 1.6°C (66.3-67.9°C) and reduced levels of free β-chain, but with similar binding affinity and specificity to variants lacking this, so the effects of this modification is not as certain as that of Fc-fusion to D7T4-TCRαβ. Another interesting approach in continuation of this project is stabilization through computationally predicted stability-enhancing mutations. Using the molecular modeling software Rosetta (17), Froning et al. (18) identified predicted stabilizing constant-domain mutations that increased stability and expression of TCRs, introducing yet another method for potentially improving yield and function of D7T4-TCRαβ in future studies.

While it is unclear why D7T4-TCRαβ was not functional in this study, the project has highlighted a number of issues in the production of soluble, recombinant TCRs. Multiple approaches for improving upon this method has also been found and discussed in this article, the increase of stability of D7T4-TCRαβ seems most promising for increasing expression and function, clearly demonstrated by

multiple studies discussed herein (8, 14, 16, 18). The potential utility of soluble TCRs presented in these studies also confirmed that the reasoning for initiating this project was sound, and that solving the issues encountered herein should be a priority onward.

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