



Chapter 15

The Generation and Use of Allergen-Specific TCR Transgenic Animals

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Abstract

The generation of allergen-specific TCR transgenic animals allows for the characterization of allergen-specific T-cell responses in vivo and in vitro and is a powerful tool to study adaptive immunity to allergens. Here we describe an approach starting from the isolation of antigen-specific T-cell hybridomas and using PCR, flow cytometric, and co-culture methods to obtain antigen-specific MHC class II-restricted CD4⁺ TCR transgenic mice on the *Rag2*^{-/-} background.

Key words Hybridoma, T-cell fusion, T-cell receptor, Allergen, Transgenic

1 Introduction

The generation of allergen-specific T-cell receptor (TCR) transgenic animals has made a tremendous impact on the field of immunology and has greatly enhanced the possibilities to investigate the role of antigen-specific T cells in (allergic) disease [1–3]. Recognition of the TCR in the context of MHC-peptide ligands is critical in developmental selection and peripheral activation of T cells. A greater understanding of the TCR-ligand interaction as well as the activation and differentiation of T cells in vivo is essential for our understanding of pathology during disease. The advent of TCR transgenic mice has proven to be a powerful tool that enables researchers to phenotypically monitor a cohort of T cells with defined antigen specificity. In this way, various TCR transgenic mouse models have been instrumental for elucidating the process of thymic selection, central and peripheral T-cell tolerance, and T-cell responsiveness to different antigens or allergens [2, 4–8].

To obtain antigen-specific clones, T-cell hybridomas were generated by fusing T-cell lymphoma cell line BW5147 with in vitro restimulated CD4⁺ spleen cells of C57BL/6 mice that received the

specific antigen *in vivo*. Antigen-specific clones were single cell sorted and expanded, and a single clone was used for RNA isolation and cDNA synthesis using random hexamer primers. cDNA was amplified using a PCR screening panel, and the amplified partial V α and V β products were sequenced and used to determine the full-length TCR. Next, antigen-specific hybridoma cDNA was used to amplify the full-length V α - and V β -chain with a proofreading enzyme using full-length primers. The amplified bands were cloned in the TCR expression vectors p428 and p783, co-injected in an equimolar concentration of 1 ng/ μ L into the pronuclei of fertilized C57BL/6 *Rag2*^{-/-} oocytes, and transferred to a foster mother. Founders were screened for TCR functionality by flow cytometry, and offspring was genotyped by PCR with primers specific for the V α - and V β -transgene.

Below, we describe a detailed protocol used for the generation of novel mouse models with MHC class II-restricted CD4⁺ TCR transgenic T cells.

2 Materials

2.1 Immunization

1. Antigen formulated on aluminum hydroxide: dilute the antigen in sterile PBS in a 15 mL conical tube. Shake the aluminum hydroxide (Imject Alum, Thermo Scientific) well before use, and add it dropwise to the antigen in a 1/20 dilution while gently vortexing. Vortex the solution for 1 h prior to injection. Inject 500 μ L intraperitoneally with a 26-gauge needle (*see Note 1*).

2.2 T-Cell Fusion [9]

2.2.1 Culture of the BW5147 Fusion Partner

1. BW5147 lymphoma cell line.
2. DMEM10: DMEM supplemented with 10% fetal calf serum (FCS), nonessential amino acids (NEAA, 100 $\times$ diluted from commercial stock), 50 μ g/mL gentamycin, 50 μ M beta-mercaptoethanol, and 1 mM sodium pyruvate (all Gibco).
3. 25cm² culture flask.

2.2.2 In Vitro Restimulation of Lymph Node Cells

1. 70 μ m cell strainer.
2. 96-well round bottom plate.
3. HBSS (Gibco).
4. RPMI5: RPMI supplemented with 5% FCS, 50 μ g/mL gentamycin, 50 μ M beta-mercaptoethanol, and 2 mM GlutaMAX (all Gibco).
5. Peptide solution: prepare 100 μ L peptide solution per well. Dilute the peptide to 10 μ g/mL in RPMI5 (final concentration of 5 μ g/mL in the well).

2.2.3 CD4⁺ T-Cell Isolation

1. MACS buffer: prepare a solution of 0.5% bovine serum albumin (BSA) and 2 mM EDTA in PBS.
2. CD4⁺ T-cell isolation kit, Miltenyi Biotech or equivalent.

2.2.4 T-Cell Fusion

1. Two 400 mL beakers and two 600 mL beakers.
2. DMEM (Gibco).
3. 50% polyethylene glycol (PEG), commercial solution of 50% (Roche).
4. DMEM20: DMEM supplemented with 20% FCS, NEAA (100× diluted from commercial stock), 50 µg/mL gentamycin, 50 µM beta-mercaptoethanol, and 1 mM sodium pyruvate (all Gibco).
5. 96-well flat bottom plate.

2.2.5 Culture of the Fused T Cells

1. 2× HAT medium: dilute 50× hypoxanthine/aminopterin/thymidine supplement (Gibco) to 2× with DMEM20 medium. Store up to 2 months at 4 °C.
2. 1× HT medium: dilute 100× hypoxanthine/thymidine supplement (Gibco) to 1× in DMEM20. Store up to 2 months at 4 °C.
3. 24-well plate.
4. DMEM20: DMEM supplemented with 20% FCS, NEAA (100× diluted from commercial stock), 50 µg/mL gentamycin, 50 µM beta-mercaptoethanol, and 1 mM sodium pyruvate (all Gibco).
5. DMEM10: DMEM supplemented with 10% FCS, NEAA (100× diluted from commercial stock), 50 µg/mL gentamycin, 50 µM beta-mercaptoethanol, and 1 mM sodium pyruvate (all Gibco).

2.3 Screening of the T-Cell Clones

2.3.1 Culture of Bone Marrow-Derived Dendritic Cells [10]

1. 70% ethanol.
2. RPMI (Gibco).
3. Mortar and pestle.
4. 70 µm cell strainer.
5. Osmotic lysis buffer: prepare a solution of 155 mM NH₄Cl, 1 mM KHCO₃, 100 µM EDTA in water. Adjust the pH to 7.1–7.4 using HEPES. Sterilize using a 0.22 µm bottle top filter.
6. 100 mm petri dish.
7. RPMI5: RPMI supplemented with 5% FCS, 50 µg/mL gentamycin, 50 µM beta-mercaptoethanol, and 2 mM GlutaMAX (all Gibco).

8. Recombinant mouse GM-CSF: dilute GM-CSF to a final concentration of 20 ng/mL in RPMI5.
9. PBS.

2.3.2 Initial Screening

1. 96-well round bottom plate.
2. DMEM10: DMEM supplemented with 10% FCS, NEAA (100× diluted from commercial stock), 50 µg/mL gentamycin, 50 µM beta-mercaptoethanol, and 1 mM sodium pyruvate (all Gibco).
3. Peptide solution: prepare 100 µL peptide solution per well. Dilute the peptide to 4 µg/mL in DMEM10 (final concentration of 1 µg/mL).
4. Mouse IL-2 ELISA ready-set-go (eBioscience).
5. Microtiter plate reader.

2.3.3 Confirmation Screening

1. Material and buffers as described in Subheading 2.3.2, initial screening, except for the peptide solution.
2. Peptide solution: dilute the peptide to 4 µg/mL in DMEM10 (final concentration of 1 µg/mL in the well), and prepare a 1 in 2 serial dilution. Do this by each time transferring half of the tube content to a new tube with an equal volume of DMEM10, for five steps in a row. Prepare 50 µL per sample (*see Note 14*).
3. Anti-mouse CD3 antibody, anti-mouse CD4 antibody, and anti-mouse CD69 antibody.
4. Fixable live/dead staining (e Bioscience).
5. Fc-blocking reagent.
6. FACS buffer: prepare a solution of 0.25% BSA, 1 mM EDTA, and 0.05% NaN₃ in PBS.
7. Compensation beads (e Bioscience).
8. Flow cytometer.

2.3.4 Evaluation of the TCR Subtype

1. Mouse Vβ TCR screening panel (Becton Dickinson).
2. Anti-mouse Vα2 antibody, anti-mouse Vα3.2 antibody, anti-mouse Vα8.3 antibody, anti-mouse Vα11.1,11.2 antibody, anti-mouse CD3 antibody, and anti-mouse CD4 antibody.
3. Material and buffers as described in Subheading 2.3.3 confirmation screening (*see items 4–8*).

2.3.5 Freezing of Peptide-Specific T-Cell Hybridomas

1. Freezing medium: DMEM supplemented with 20% FCS, NEAA (100× diluted from commercial stock), 50 µg/mL gentamycin, 50 µM beta-mercaptoethanol, 1 mM sodium pyruvate (all Gibco) and 10% DMSO (Invitrogen).

2. Freezing container.
3. Cryovials.
4. -70°C freezer.
5. -150°C freezer or liquid nitrogen.

2.4 Generation of Monoclonal Cells

1. Anti-mouse CD3 antibody, anti-mouse CD4 antibody, anti-body against the corresponding $V\alpha$ and $V\beta$.
2. Material and buffers as described in Subheading 2.3.3 confirmation screening (*see items 4–7*).
3. FACS ARIA cell sorter or equivalent.
4. DMEM20: DMEM supplemented with 20% FCS, NEAA (100 $\times$ diluted from commercial stock), 50 $\mu\text{g}/\text{mL}$ gentamycin, 50 μM beta-mercaptoethanol, and 1 mM sodium pyruvate (all Gibco).
5. 96-well round bottom plate.
6. 24-well plate.

2.5 Confirmation and Selection of the Monoclonal T-Cell Clones

1. Material and buffers as described in Subheading 2.3.3 confirmation screening.
2. Material and buffers as described in Subheading 2.3.5 freezing of peptide-specific T-cell hybridomas.

2.6 Cloning

2.6.1 RNA Preparation

1. RNase-free 1.5 mL tubes and tips.
2. TriPure reagent (Roche).
3. Chloroform.
4. Isopropanol.
5. 75% ethanol.
6. RNase-free water.

2.6.2 cDNA Preparation

1. SensiFAST cDNA synthesis kit (Bioline) or equivalent.

2.6.3 Determination of the T-Cell Receptor Subtype by PCR

1. Fast taq DNA polymerase and the corresponding buffer.
2. Deoxynucleotide triphosphate (dNTP).
3. TCR subtype primers (*see Tables 1 and 2*).
4. Thermal cycler.
5. 1 $\times$ TBE buffer: dilute 10 $\times$ TBE buffer (Roche) to 1 $\times$ in water.
6. 2% agarose gel: dissolve 2% agarose in 1 $\times$ TBE buffer and add 1/10,000 SYBR Safe DNA gel stain (Invitrogen). Boil and pour the solution in a gel casting system.
7. Power source.

Table 1
Overview of the correspondence between different nomenclatures in the TCR Vα PCR panels and TCR Vα antibodies

PCR TCR panel [11]		PCR TCR panel [12]		Flow cytometry
IMGT subgroup	Primer sequence	IMGT subgroup	Primer sequence	TCR Vα antibodies
TRAV01	TGGATGGTTTGAAGGACAGTG			
TRAV02	CTGTTTATCTCTGCTGACCGG	Vα12	GTGGCATCTCTGTTTATCTCTGCTGACCGG	
TRAV03	ACGAAGGACAAGGATTCACTGT	Vα5	AAGGTTTTCTCAAAGTACGGAAATAAACGAA	
TRAV04	CTGGAGGACTCAGGCACTTACT	Vα11	AATGGGAGGTAAAGTCAACATTCAATTCT	Anti-mouse Vα11.1/11.2
TRAV05		Vα13	CGTTCAAATATGGAAAGAAAGCAGACCCAA	
TRAV06	GGTACCCGACTCTTTTCTGTGT	Vα4	GGAAGCAGCAGAGGGTTTTTGAAAGCTACATAC	
TRAV06-4	ACCCCTTTCAGAAGATGACTTCC			
TRAV06-5	TTTAAAGTCCCAAAGGCCAA			
TRAV06-6	TCCTGAAAAGTCATTACGGCTG			
TRAV06-7	AGAGCCTCAAGGGACAAAGAG			
TRAV07	AGACTCCCAGCCCAGTGACT	Vα1	CAGCAGAGCCCAGAATCCCTC	
TRAV07-5	ACATCAGAGAGCCGCAACC			
TRAV08	CCCTGCCCAGCTAATCTTAAT			
TRAV09	CTGCAGCTGAGATGCAAGTATT	Vα3	CTCAAAGTACTATTCCGGAGAGACCCAGTGGTT	Anti-mouse Vα3.2
TRAV09-1	TCCTATGGTGGATCCATTIACC			
TRAV10/05-2	TGGACAGAAAACAGAGCCAA			

TRAV11	CAGGCAAAGGTCTTGTGTCC	V α 14	CTGGTTGACCAAAAAGACAAAACGTCAAAAT
TRAV12	ACGCCACTCTCCATAAGAGCA	V α 8	ACAGACAACAAGAGGACCGAGCACCAAGGG Anti-mouse V α 8.3
TRAV13	GCTCTTTGACACATTTCTCTCC	V α 10	CTGACATCCACCACAGTCACATAAGGAAAGT
TRAV14-1/14-2	TGCAGTTATGAGGACAGCACTT	V α 2	ACCITTCCTTCAATAAAAAGGAGAAAAAGCTC ^a Anti-mouse V α 2
TRAV14-3	CTGCAGTTATGAGAACAGTGTCTT		
TRAV15	CCAGACGATTTCGGGAAAGTA	V α 7	CGACAAAACGTCTTCTTCTTCTACTGCAAAAAGAG
TRAV16	TTCCATCGGACTCATCATCAC		
TRAV17	AACCTGAAAGAAATCCCCCAGC		
TRAV19	GGAAGACGGAAGATTACACATT		
TRAV20	ACGCTCCTAATAGACATTTCGCT		
TRAV21	GTTCCTCTTCAGGGTCCAGA	V α 6	AGTATGGCTTTCCTGGCTATTGCCTCTGAC
TRAC	CACCAGCAGGTTCTGGGTTC		
		V α 9	CAAAGAGCTGCGACGTTCCCTT
		NJ108	GGCCCCATTGCTCTTTGGAATC
		NJ109	CGGCACATTGATTTGGGAGTC
		NJ110	CAGGCAGAGGGTGTGTCC

^aV α 2 in this panel corresponds to both TRAV14-1, TRAV14-2, and TRAV14-3.

Table 2
Overview of the correspondence between different nomenclatures in the TCR Vβ PCR panels and TCR Vβ screening panel for flow cytometry

PCR TCR panel designed in the house		PCR TCR panel designed in house [11]		Flow cytometry
IMGT subgroup	Primer sequence	IMGT subgroup	Primer sequence	TCR panel
Vβ1 F	GTGGCAGTTTGTGCAATCTGTGCC	TRBV01	ACACGGGTCACTGATACGGA	Anti-mouse Vβ2
Vβ1 R	CCACTTGCAGCCTCAGCTCCG			
Vβ2 F	GGCCGTTTGTCTCTCTGGTGGC	TRBV02	ATGGACAATCAGACTGCCCTCA	Anti-mouse Vβ4
Vβ2 R	TGTGGCCGAGTCATCAGGCTT			
Vβ3 F	GGAAGCAGGACACACAGGACCCA	TRBV03	TCACTCTGAAAATCCAAACCCA	Anti-mouse Vβ10b
Vβ3 R	AGTCCTCCAGTGCTGTGGGTTGG			
Vβ4 F	GGCTCCTAAAGCTGTGTGGCCT	TRBV04	TAAACGAAACACAGTTCCAAGGC	
Vβ4 R	TACACAGCAGAGTCCTCCGGCTC			
Vβ5 F	AGCTGCAGGCTTCTCCTCTATGT	TRBV05	ACGGTGCCCACTCGTTTAT	
Vβ5 R	AACGACTGGGCACCGTCTCA			
Vβ6 F	TGTTACAGACATGGGACAGAATGTCA			
Vβ6 R	GCCAAAGCTGCTGGCATAACATAGT			
Vβ7 F	TGCTCTGTGTATGGCCCTTTGT			
Vβ7 R	ACAGTGCAGAGTCCTTTGGCTC			
Vβ8 F	GCAGTGTGCAATTTGTTTCCTGC			
Vβ8 R	TGTCTGGGAGGGAGAGGCCAA			
Vβ9 F	CCTCACTAGTGTGCTGAATTCCTCCAA			

Vβ9 R	ACATGGAGAAGGAGCCCTCGG				
Vβ10 F	TGGTGAATCAACCCAGACACCT				
Vβ10 R	GCTGGCCCAGAAAGTACATGGAGG				
Vβ11 F	GCCACGGACACCCAGGCACTTC				
Vβ11 R	AGGTGCTCTGTGCGAGTCCCGC				
Vβ12-1 F	CTCGCTGATTCTGCCTGGGGC	TRBV12-1	GGATTCCCTACCCAGCAGATTCT		Anti-mouse Vβ5.2 ^a
Vβ12-1 R	AGTCCTCCAGTTCCAAAGGCACTCA				
Vβ12-2 F	CTTCCCTGAOCCCGCCTGGAA	TRBV12-2	AGATAAAGGAAACCTGCCCCAG		Anti-mouse Vβ5.1 ^a
Vβ12-2 R	CGGCAGAGTCCTCTAGCTCCAAAGG				
Vβ12-3 F	CTGGGGCACCAACCCTGCTTTC				
Vβ12-3 R	CCTCTAGGTCCAAGGCCACTCAGG				
Vβ13-1 F	TGGGCTCCAGGCTCTTCTGTGT	TRBV13-1	CCAGAAACAACGCAAGAAGACT		Anti-mouse Vβ8.3
Vβ13-1 R	TGCGTTGTCTCTGGTGGCCTTGT				
Vβ13-2 F	GGCTCCAGGCTCTTCTTCGTGC	TRBV13-2	GGCTACCCCTCTCTCAGACAT		Anti-mouse Vβ8.2 ^b
Vβ13-2 R	TGGCTTGGTCTGGAGGCCCTTGT				
Vβ13-3 F	TGGGCTCCAGACTCTTCTTTGTGGT	TRBV13-3	TGGCTTCCCTTTTCTCAGACA		Anti-mouse Vβ8.1 ^b
Vβ13-3 R	TGGAGGCCTTGTACCCATCAGGG				
Vβ14 F	GCACCAGGCTTCTTGGCTGGG	TRBV14	GCGACACAGCCACCTATCTC		Anti-mouse Vβ13
Vβ14 R	GGCTGTGTCGCCCTGCTTTG				
Vβ15 F	TGGGCATCCAGACCCCTCTGTTG	TRBV15	CGCAGCAAGTCTCTTATGGAA		Anti-mouse Vβ12

(continued)

Table 2
(continued)

PCR TCR panel designed in the house			PCR TCR panel designed in house [11]		Flow cytometry	
IMGT subgroup	Primer sequence		IMGT subgroup	Primer sequence	TCR panel	
Vβ15 R	TGCTGGCACACAGATACACAGC					
Vβ16 F	GGCCCCCAGGCTCCTTTTCTG	TRBV16		ATAGATGATTCAAGGATGCCC	Anti-mouse Vβ11	
Vβ16 R	CCGCTGAGTCCTGGGGTTGC					
Vβ17 F	TGTGATCTTCTGTCTTCTTGCAGCC	TRBV17		TGAGAAAGTTCCAATCCAGTCG	Anti-mouse Vβ9	
Vβ17 R	AGGCCTGCAGAGCCCAATGTAG					
Vβ18 F	GGTGTACCCACGAACCTAAGATACA					
Vβ18 R	TTCCAGATCTGCTGGCCCCAC					
Vβ19 F	TGCTGGGTAAACCCTTTGTCTCCT	TRBV19		GAAAGGCTATGATGCGTCTCG	Anti-mouse Vβ6	
Vβ19 R	CGGCCATCTCGTTCTTCTGGGC					
Vβ20 F	ACTGCTTCTATTACTTCTGGGGCCT	TRBV20		TTCCCATCAGTCATCCCAAC		
Vβ20 R	AGGATAAGTTGGGATGACTGATGGGA					
Vβ21 F	TGCAAACTTTTCTACTGTGTGCCCT	TRBV21		AAAATGCCCTGCTAAGAAACC		
Vβ21 R	GCCTTGTGGACTGGATCTCTATGTT					
Vβ22 F	TGTGGCTTCTCTGGTATGTAGCCC					
Vβ22 R	AGCTGCAGGGTGAGCTTAAGGGA					

V β 23 F	GGGTGCACGGCTCATTTGCTA	TRBV23	CAGCCTGGGAATCAGAAAG	
V β 23 R	GTGAGCTGGAGGGGCACACAG			
V β 24 F	TGGGTGCAAGACTGCTCTGCTG	TRBV24	GCATCCTTGGAAATCCTATCCT	Anti-mouse V β 17a
V β 24 R	ACAGACTGCTGGCACAGAGCTACA			
V β 25 F	GTGATCACTTCGCAGGAAAACCTGA			
V β 25 R	AGAGCTGCTGGCACACAGAAAGTG			
V β 26 F	GGCTACAAGGCTCCTCTGTTACAC	TRBV26	AGTGTCTCTTCAAACTCACCTT	Anti-mouse V β 3
V β 26 R	CTGGATGTCCCTTTTCAGGGATACA			
V β 27 F	GCCAAAGTTCATGCAATTGCTCTGGCCT			
V β 27 R	AGGCAGGAGTGTGGAGGGCT			
V β 28 F	CCAGTCTCTCAAAGATGTGTGCTCCT			
V β 28 R	GCACACATGCCCTGGTCGATGCT			
V β 29 F	AGGCTCATCTCTGTGTGTGTGCT	TRBV29	AAAGGATACAGGGTCTCACGG	Anti-mouse V β 7
V β 29 R	CCCGCTTCTTCCGTGAGACCC			
V β 30 F	ACATTCTCTGCTACTTCTTTGGAGCC	TRBV30	GGACAAAGTTTCCAATCAGCCG	
V β 30 R	AGGTTTGGCCGGCTGATTGGA			
V β 31 F	CTCTCTCTTGCCCTTTCTCTCTGGGC	TRBV31	TTCATCCTAAGCACGGAGAAAG	Anti-mouse V β 14
V β 31 R	GTCGTCTCTTCGGCCTGGAAGC			
Cb1and2 F	TGGCCAGGGGCTTCTTCCCT			
Cb1and2 R	GGAAC TGCACTTGGCAGCGGA			

(continued)

Table 2
(continued)

PCR TCR panel designed in the house		PCR TCR panel designed in house [11]		Flow cytometry
IMGT subgroup	Primer sequence	IMGT subgroup	Primer sequence	TCR panel
Cb1 F	GTCAGCACGGACCCCTCAGGC			
Cb1 R	TGGTGGCAGACAAGACCCCTT			
Cb2 F	GGCCAGAGGGCTCACCCAAAC			
Cb2 R	TGGCCATCAGCACCCAGGCCAC			
		TRBC1	TGCAATCTCTGCTTTTGATGGCTC	

^aThe MR9-4 antibody reacts with the Vβ5.1 and 5.2 TCR

^bThe MR5-2 antibody reacts with the Vβ8.1 and 8.2 TCR

2.6.4 Subcloning the TCR in the Teasy Vector

1. Phusion® high-fidelity DNA polymerase (New England Biolabs) or equivalent.
2. Material and buffers for PCR described in Subheading 2.6.3 determination of the T-cell receptor subtype by PCR.
3. Agarose Gel DNA Extraction Kit (Roche) or equivalent.
4. pGEM®-T Easy Vector System (Promega).
5. Deoxyadenosine triphosphate (dATP).
6. Fast taq DNA polymerase and the corresponding buffer.
7. T4 DNA ligase and 10× T4 ligase buffer (Promega) or equivalent.
8. Bacterial LB/agar plates with ampicillin and blue/white selection: dissolve 10.3 g LB broth and 7.5 g agar in 500 mL Milli-Q water and autoclave. When cooled to approximately 37 °C, add 100 µg/mL ampicillin, 0.5 mM IPTG, and 80 µg/mL X-gal (stock dissolved in DMSO), and pour plates (10–15 mL per 100 mm petri dish).
9. DH10B electrocompetent cells.
10. Electroporation system.
11. 0.2 cm cuvettes.
12. SOC medium (Invitrogen) or equivalent.
13. LB liquid medium: dissolve 10.3 g LB broth in 500 mL Milli-Q water and autoclave. When cooled to approximately 37 °C, add 100 µg/mL ampicillin.
14. High pure plasmid isolation kit (Roche) or equivalent.
15. Restriction enzymes for restriction analysis and the corresponding restriction buffer.
16. 1× TBE buffer: dilute 10× TBE buffer (Roche) to 1× in water.
17. 2% agarose gel: dissolve 2% agarose in 1× TBE buffer, and add 1/10,000 SYBR Safe DNA gel stain (Invitrogen). Boil and pour the solution in a gel casting system.
18. Power source.

2.6.5 Cloning of the TCR in CD4 Expression Vector

1. Genopure plasmid maxi kit (Roche) or equivalent.
2. SalI and XhoI restriction enzyme and the corresponding restriction buffer.
3. Agarose Gel DNA Extraction Kit (Roche) or equivalent.
4. T4 DNA ligase and 10× T4 ligase buffer (Promega) or equivalent.
5. Material, cells, and buffers for transformation as described in Subheading 2.6.4 subcloning of the TCR in the Teasy vector (*see items 7–13*).
6. Material and buffers for restriction analysis as described in Subheading 2.6.4 subcloning of the TCR in the Teasy vector (*see items 14–17*).

2.6.6 Preparation of the Injection Fragments

1. Genopure plasmid maxi kit (Roche) or equivalent.
2. NotI restriction enzyme and the corresponding restriction buffer.
3. Agarose Gel DNA Extraction Kit (Roche) or equivalent.
4. Material and buffers for restriction analysis as described in Subheading 2.6.4 subcloning of the TCR in the Teasy vector (*see items 14–17*).
5. Injection buffer: prepare a solution of 10 mM tris, pH 7.5, 0.1 mM EDTA, and 100 mM NaCl in ultrapure water. Sterile filter this solution.
6. Eppendorf centrifuge.

2.7 Evaluation of the TCR Mice

2.7.1 Genotyping

1. Material and buffers for PCR described in Subheading 2.6.3 determination of the T-cell receptor subtype by PCR.

2.7.2 Analysis of the Presence of T Cells in Peripheral Blood

1. 10 mM EDTA: dilute 0.5 M commercial EDTA in PBS (20 mL EDTA per 1 L PBS).
2. PBS.
3. Lympholyte cell separation medium (Cedar lanes).
4. Anti-mouse CD3 antibody, anti-mouse CD4 antibody, and anti-mouse CD69 antibody.
5. Material and buffers as described in Subheading 2.3.3 confirmation screening (*see items 4–8*).

2.7.3 Evaluation of TCR Specificity in a DC-T-Cell Co-culture

1. Material and buffers as described in Subheading 2.3.1 culture of bone marrow-derived dendritic cells.
2. Material and buffers as described in Subheading 2.3.3 confirmation screening (*see items 4–8*).
3. 70 μ m cell strainer.
4. Osmotic lysis buffer: prepare a solution of 155 mM NH₄Cl, 1 mM KHCO₃, 100 μ M EDTA in water. Adjust the pH to 7.1–7.4 using HEPES. Sterilize using a 0.22 μ m bottle top filter.
5. PBS.
6. MACS buffer: prepare a solution of 0.5% BSA and 2 mM EDTA in PBS. Sterilize using a 0.22 μ m bottle top filter.
7. CD4⁺ T-cell isolation kit, Miltenyi Biotech or equivalent.
8. CellTrace CFSE cell proliferation kit for flow cytometry (eBioscience), or equivalent. Prepare a solution of 50 μ M CFSE by 1/100 dilution of the CFSE stock in RPMI.
9. RPMI.
10. RPMI5: RPMI supplemented with 5% FCS, 50 μ g/mL gentamycin, 50 μ M beta-mercaptoethanol, and 2 mM GlutaMAX (all Gibco).

11. Peptide solution: dilute the peptide to 4 $\mu\text{g}/\text{mL}$ in RPMI5 (final concentration of 1 $\mu\text{g}/\text{mL}$ in the well), and prepare a $\frac{1}{2}$ serial. Do this by each time transferring half of the tube content to a new tube with an equal volume of RPMI5, for five steps in a row. Prepare 50 μL per sample (*see Note 14*, use RPMI5 instead of DMEM10).
12. 96-well round bottom plate.

3 Methods

3.1 Immunization

1. Inject 10–15 mice intraperitoneally with 10 μg antigen formulated on aluminum hydroxide, twice with a 1-week interval (days 1 and 8). Ten days after the last injection, inject the mice on 3 consecutive days (days 18, 19, and 20) with an intranasal injection of 10 μg of non-formulated antigen. Sacrifice the mice 1 day after the last injection (day 21) (*see Notes 1 and 2*).

3.2 T-Cell Fusion

All centrifugation steps are performed in a cooled centrifuge, for 7 min at $400 \times g$, unless specified otherwise. Cells are cultured in a humidified incubator at 37 °C and 5% CO_2 .

All products that come into contact with the cells should be sterile. Sterile working procedures should be applied.

3.2.1 Culture of the BW5147 Fusion Partner

1. Start-up BW5147 cells by rapid thawing in a 37 °C water bath. Resuspend cells in 9 mL of pre-warmed DMEM10 medium in a 15 mL conical tube and centrifuge cells at room temperature.
2. Resuspend cells in 1 mL DMEM10. Count cells and dilute to 2×10^5 cells/mL in DMEM10 medium. Transfer 10 mL of the cell suspension into a 25cm² culture flask and culture for 2 weeks.
3. Passage cells every 2 or 3 days by seeding at 2×10^5 cells/mL or 10^5 cells/mL, respectively. Cell concentrations should not exceed 10^6 cells/mL.
4. Seed cells 1 day prior to T-cell fusion at 3×10^5 cells/mL. For optimal results, cell viability should be over 90%.

3.2.2 In Vitro Restimulation of Lymph Node Cells

1. Isolate mediastinal lymph nodes from immunized mice. Obtain a single-cell suspension by mashing all lymph nodes through a 70 μm cell strainer using 1 mL HBSS, and rinse the cell strainer with 2 mL HBSS. Centrifuge cell suspension, and resuspend the cell pellet in 10 mL RPMI5 (*see Note 3*).
2. Count cells and dilute to a concentration of 3×10^6 cells/mL in RPMI5. Seed 100 μL (3×10^5 cells) per well in a 96-well round bottom plate. Add 100 μL antigen-specific peptide

per well at a final concentration of 5 µg/mL in RMP15 (*see Note 4*).

3. Culture cells for 4 days without passaging.

3.2.3 CD4⁺ T-Cell Isolation

1. Harvest restimulated lymph node cells, and transfer cells to a 50 mL conical tube, centrifuge, and resuspend cells in 10 mL MACS buffer.
2. Pre-warm centrifuge to room temperature for T-cell fusion.
3. Isolate CD4⁺ T cells using Miltenyi Biotech CD4⁺ T cells isolation kit, according to manufacturer's instructions (*see Note 5*).

3.2.4 T-Cell Fusion

1. Pre-warm in a 37 °C warm water bath:
 - (a) Two 400 mL and two 600 mL beakers all with 75 mL water inside
 - (b) Four 50 mL tubes with DMEM inside
 - (c) 2 mL aliquot of 50% poly ethylene glycol (PEG)
 - (d) One 50 mL tube with DMEM20
2. Harvest BW5147 cells and wash cells twice using 40 mL DMEM. Centrifugation steps are performed at room temperature. Resuspend cells in 10 mL DMEM (*see Note 6*).
3. Wash the enriched T cells twice using 10 mL DMEM. Centrifugation steps are performed at room temperature. Resuspend cells in 1 mL DMEM in a 15 mL conical tube.
4. Count BW5147 and enriched T cells, and add an equal number of BW5147 cells to the enriched T-cell suspension.
5. Centrifuge 5' at $800 \times g$ at room temperature and aspirate the supernatant.
6. Place the 400 mL beakers with warm water (37 °C) in the 600 mL beakers with warm water (37 °C) in the laminar flow, in order to mimic two warm water baths. Place the tube with cells and medium in separate baths.
7. Slowly and dropwise add 1 mL of pre-warmed PEG using a 1 mL micropipette to the cells during 1 min while gently stirring the pellet with the pipet (*see Note 7*).
8. Slowly and dropwise add 2 mL of pre-warmed DMEM to the cells with a 2 mL pipet, while gently stirring. Continue stirring for a total of 2 min (*see Note 8*).
9. Slowly and dropwise add an additional 7 mL of pre-warmed DMEM with a 10 mL pipet over a 2–3 min period while gently stirring.
10. Centrifuge 5' $800 \times g$, at room temperature.

11. Aspirate supernatant and vigorously add 10 mL of DMEM20 to the pellet, as to release the pellet completely. However, do not pipet the suspension up and down.
12. Dilute cells between 10^5 and 10^6 cells/mL by gently adding the appropriate volume of DMEM20 while stirring, based on the total number of T cells used for fusion.
13. Plate the cells in a 96-well flat bottom plate, by pipetting two drops (~ 100 μ L) of cell suspension per well with a 10 mL pipet and culture for 1 day (*see Note 9*).

3.2.5 Culture of the Fused T Cells

1. After 1 day, gently add 100 μ L of $2\times$ HAT medium to each well (*see Note 10*).
2. After 6 or 7 days, remove the top half of the medium of each well using a multichannel pipet and gently add 100 μ L of fresh $2\times$ HAT medium to each well.
3. Monitor cells daily; cells should be expanded when medium becomes slightly yellow and expansion of the cells is clearly visible under the microscope (about 50% of the bottom of the well is covered with cells). Expand the cells by reseeding them in a 24-well plate containing 1 mL of $1\times$ HT plating medium.
4. After 1 day, add an additional 1 mL of $1\times$ HT plating medium.
5. Duplicate cultures of clones that are growing well, by transferring half of the cells to a fresh 24-well plate and adding 1 mL of fresh DMEM20 medium. In this way, one plate can be used for screening experiments, and one for culture. From now on, cells should be able to grow in lower FCS conditions and can thus be cultured in DMEM10 medium.
6. Monitor cells daily, and maintain them by reseeding 25% of the cells in DMEM10 in case the medium turns yellow (*see Note 11*).

3.3 Screening of the T-Cell Clones

3.3.1 Culture of Bone Marrow-Derived Dendritic Cells

1. Isolate both femurs from one mouse and sterilize in 70% ethanol for 5 min. Aspirate the ethanol and rinse the femurs twice with 10 mL of RPMI. Crush the bones in 10 mL RPMI in a mortar with a pestle and repeat twice; pass the suspension over a 70 μ m cell strainer and centrifuge.
2. Lyse the erythrocytes by resuspending the cell pellet in 2 mL osmotic lysis buffer and incubate for 4 min on ice. Add 10 mL of RPMI and centrifuge the cell suspension.
3. Count and plate 3×10^6 cells per 100 mm petri dish in 10 mL RPMI5 with 20 ng/mL recombinant GM-CSF. Culture the cells for 8–10 days. On day 3, add 10 mL of RPMI5 with 20 ng/mL recombinant GM-CSF. On days 6 and 8, replace the top half of the medium by 10 mL RPMI5 with 20 ng/mL recombinant GM-CSF.

4. Harvest the cells by resuspending with a 10 mL pipet, and rinse the plate with 10 mL ice-cold PBS. Centrifuge and count the cells.

3.3.2 Initial Screening

1. For every individual clone, start a co-culture by seeding 50 μ L of bone marrow dendritic cells (2×10^5 c/mL) in 2 wells of a 96-well round bottom plate (*see Note 12*). Add 50 μ L of 4 μ g/mL peptide solution to one well (final concentration of 1 μ g/mL) and 50 μ L of medium to the second well. Resuspend the hybridoma clone with a 1 mL micropipette, transfer 100 μ L of cells to each well, and culture overnight. All steps are performed in DMEM10.
2. After 1 day, centrifuge the plates and collect the supernatant. Test the supernatant in an IL-2 ELISA. Clones that are specific for the peptide will show high IL-2 production in the presence of the peptide. Clones not responding to antigen can be discarded (*see Note 13*).

3.3.3 Confirmation Screening

1. In order to validate and assess the specificity of the clones, a similar co-culture experiment is performed. Start a co-culture by seeding six wells with 50 μ L of bone marrow dendritic cells (2×10^5 c/mL) in a 96-well round bottom plate (*see Note 12*). Count and add 100 μ L of the hybridoma cells at 10^6 cells/mL. Prepare a serial dilution of the peptide in 50 μ L and add to the different wells (include a medium control) (*see Note 14*). All steps are performed in DMEM10.
2. Culture overnight and quantify IL-2 production in supernatant by ELISA (*see Note 13*).
3. Additionally, evaluate increased expression of CD69, on CD3⁺CD4⁺ T cells, after peptide stimulation using flow cytometry. In short, prepare a 50 μ L staining mix per sample including all required antibodies in the recommended dilution, Fc- blocking reagent and live/dead stain in PBS (*see Note 15*). Prepare compensation beads and add single stain reactions, containing each individual antibody and the live/dead stain separately, in order to set the voltages on the flow cytometer. Centrifuge and resuspend cells in 50 μ L staining buffer, and incubate cells and compensation beads at 4 °C in the dark. After 30 min, wash and resuspend samples in 150 μ L FACS buffer and analyze on a flow cytometer.
4. Clones with the highest specificity, e.g., the lowest peptide concentration that still induces upregulation of CD69 and production of IL-2, can be selected (*see Fig. 1*).

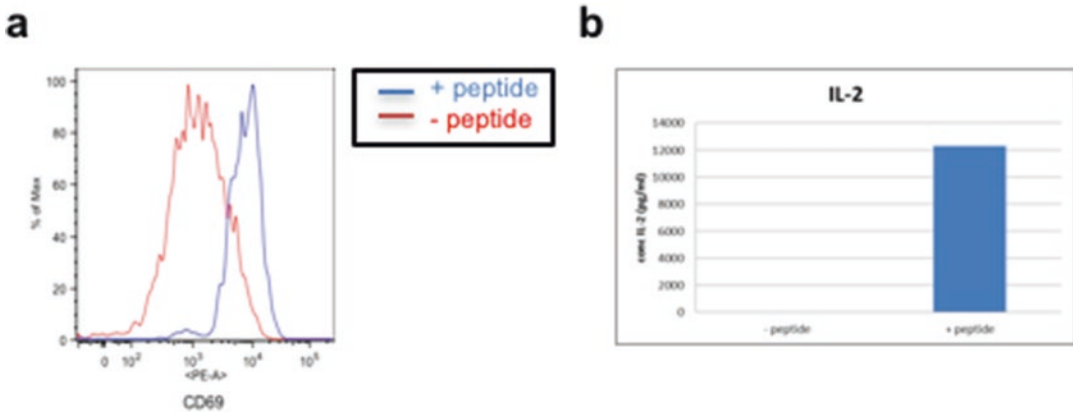


Fig. 1 Co-culture of a hybridoma clone with bone marrow-derived dendritic cells, in the presence or absence of antigen-specific peptide. Upregulation of CD69 (a) and induction of IL-2 (b) of the hybridoma clone after co-culture in the presence of peptide

3.3.4 Evaluation of the TCR Subtype

1. Evaluate the selected clones for their TCR variable domain by flow cytometry (as described above), using the mouse V β TCR screening panel (Becton Dickinson) and commercially available TCR V α antibodies (*see Note 16*).

3.3.5 Freezing of Peptide-Specific T-Cell Hybridomas

1. Select one clone based on all available screening data, and freeze all other specific clones as backup.
2. Collect T-cell hybridomas from their respective wells and transfer to a 15 mL conical tube (*see Note 17*). Centrifuge the cells, aspirate the supernatant, and resuspend the pellet in 1 mL of freezing medium (10% DMSO in DMEM20). Transfer the cell suspension into a cryovial and store it in a freezing container at -70°C . After 1 day cells can be transferred to liquid nitrogen for further storage.

3.4 Generation of Monoclonal Cells

1. Stain cells from the selected clone with anti-CD3, anti-CD4, and, if available, anti-TCR V α and -V β (as described before).
2. Single cell sort stained cells using fluorescence-activated cell sorting (FACS) in a 96-well round bottom plate with 200 μL DMEM10/well (*see Note 18*).
3. Culture sorted cells and feed weekly by replacing the top half of the medium with 100 μL /well DMEM10.
4. Expand growing cells of wells that become slightly yellow and where expansion of the cells is clearly visible under the microscope, by resuspending and transferring the cells to a 24-well plate with 1 mL of DMEM10.
5. Cells can be cultured as described before. Passage cells every 2 or 3 days by seeding at 2×10^5 cells/mL or 10^5 cells/mL, respectively. Cell concentrations should not exceed 10^6 cells/mL.

3.5 Confirmation and Selection of the Monoclonal T-Cell Clones

1. Screen monoclonal T-cell clones for their peptide specificity, by performing a co-culture using a peptide concentration range, as described before at “second confirmation screening” (*see* Subheading 3.3.3).
2. Select one clone with high peptide specificity, preferably with a TCR subtype to which antibodies are available. This clone will be used for cloning of the T-cell receptor.
3. Freeze cells of the selected clone as described in “freezing of peptide-specific T-cell hybridomas” (*see* Subheading 3.3.5). Prepare multiple vials of 10^6 – 2×10^6 cells. Preferentially, some peptide-specific clones can be frozen as a backup.
4. In addition, freeze cell pellets of the selected clone for RNA isolation by pelleting 10^6 cells by centrifugation. Aspirate supernatant and snap freeze the cell pellet on dry ice or in liquid nitrogen, finally store the pellets at -70°C .

3.6 Cloning

3.6.1 RNA Preparation

All centrifugation steps are performed in a cooled Eppendorf centrifuge. Cool centrifuge to 4°C prior to the experiment. RNA isolation should be performed using RNase-free materials and reagents.

1. Resuspend the cell pellet in 1 mL of TriPure reagent and vortex extensively. Add 200 μL chloroform, and vortex and incubate for 3 min at room temperature. Centrifuge for 15 min at $12,000 \times g$.
2. Carefully pipet the upper transparent layer into a new tube. Add 500 μL isopropanol, and vortex and incubate for 5 min at room temperature. Centrifuge for 10 min at $12,000 \times g$.
3. Remove the supernatant and add 1 mL of 75% ethanol. Vortex and centrifuge for 5 min at $7500 \times g$.
4. Remove supernatant and air-dry pellet. Resuspend pellet in 20 μL RNase-free water.

3.6.2 cDNA Preparation

1. Prepare cDNA using random hexamer primers, following the manufacturer’s instructions, using 1 μg RNA.

3.6.3 Determination of the T-Cell Receptor Subtype by PCR

1. Perform a PCR using a panel of TCR subtype primers (*see* Tables 1 and 2), with 0.1 μL of cDNA per reaction as a template (following the manufacturer’s instructions). The fusion partner BW5147 will give a positive reaction for TRAV20 and TRBV12-1. Other positive reactions are derived from the allergen-specific TCR and should be sequenced (*see* Note 19). Determine the correct $V\alpha$ and $V\beta$ subtype by blasting the sequence using the IMGT database (www.imgt.org). Design full-length primers in order to amplify the complete $V\alpha$ and $V\beta$ including a piece of the 5' UTR. The primers should contain SalI or XhoI restriction sites to allow subcloning the $V\alpha$

and V β into the TCR expression vectors p783 and p428, respectively.

3.6.4 Subcloning the TCR in the Teasy Vector

1. Amplify the V α and V β by performing a proofreading PCR using 2 μ L cDNA as a template and their full-length primers (following the manufacturer's instructions). Gel purify the amplicons using a gel isolation kit (following the manufacturer's instructions).
2. A-tail and subclone the amplicons using the T easy vector system (Promega). Transform the ligation reaction in high-efficiency competent cells applying blue-white selection screening for positive clones (following the manufacturer's instructions).
3. Grow the positive clones overnight in 5 mL liquid culture (LB + 100 μ g/mL ampicillin) at 37 °C while shaking and isolate DNA using a mini prep kit (following the manufacturer's instructions).
4. Confirm correct clones by using restriction analysis and sequencing.

3.6.5 Cloning of the TCR in CD4 Expression Vector

1. Prepare a maxi prep from one positive TCR V α and one TCR V β clone, following manufacturer's instructions.
2. Excise the inserts from the T easy vector by SalI or XhoI digestion. Incubate 10 μ g of DNA in 1 $\times$ restriction buffer with 1 μ L (10 units) of SalI or XhoI enzyme in a 50 μ L reaction, for a minimum of 3 h at 37 °C. Gel purify the inserts (following the manufacturer's instructions).
3. Linearize the CD4 expression vector p428 and p783 (*see* Fig. 2) with SalI. Incubate 10 μ g of vector with 1 μ L (10 units) of SalI enzyme in a 50 μ L reaction, for a minimum of 3 h at 37 °C. Gel purify the vectors (following the manufacturer's instructions).
4. Determine the concentration of isolated DNA and prepare a 10 μ L ligation reaction of maximum 100 ng total DNA, including insert and vector in a 3:1 molar ratio and 1 μ L (1 Unit) T4 DNA in 1 $\times$ T4 DNA ligase buffer. The V α should be cloned in the p783 and the V β in the p428 expression vector. Incubate for 30 minutes at room temperature, followed by overnight at 4 °C (*see* **Note 20**). Transform the ligation reaction in high-efficiency competent cells (following the manufacturer's instructions).
5. Positive colonies can be determined using a colony PCR. Single colonies are transferred to a 96-well PCR plate containing 25 μ L of Milli-Q water/well. Use 5 μ L in a colony PCR using the CD4 forward and reverse primer (CTCCTCGA CCCACTTCTGATG and GCTCAGATTCCCAACCAACAA, respectively) in combination with the reverse primer from the

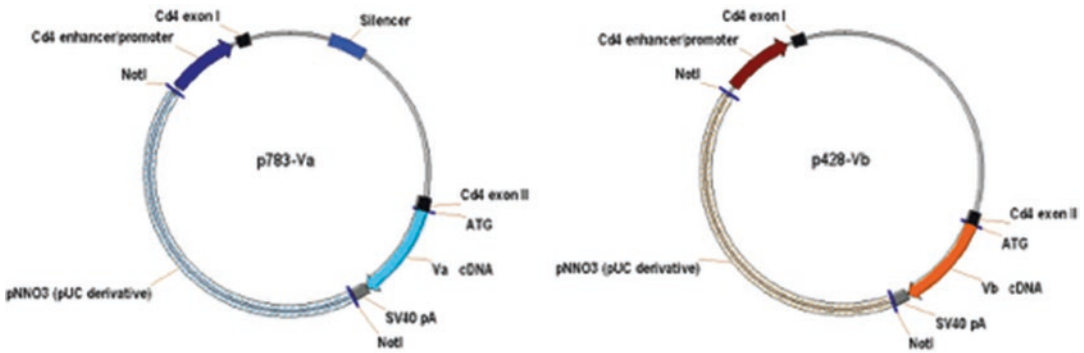


Fig. 2 Both TCR expression vectors p428 and p783 contain the mouse CD4 promoter and the mouse CD4 enhancer followed by a piece of mouse CD4 genomic sequence, a cloning site, and an SV40 polyA signal. The genomic sequence in both vectors contains mouse CD4 exon I, intron I containing different deletions in each vector followed by exon II up to the translation start site. The intron in plasmid p783 contains the transcriptional silencer element that is normally responsible for terminating CD4 expression in CD8 lineage cells. V α cDNA is cloned into the p783 and V β cDNA into the p428 expression vector. Injection fragments are excised from the pNN03 backbone by NotI digestion and injected into fertilized oocytes

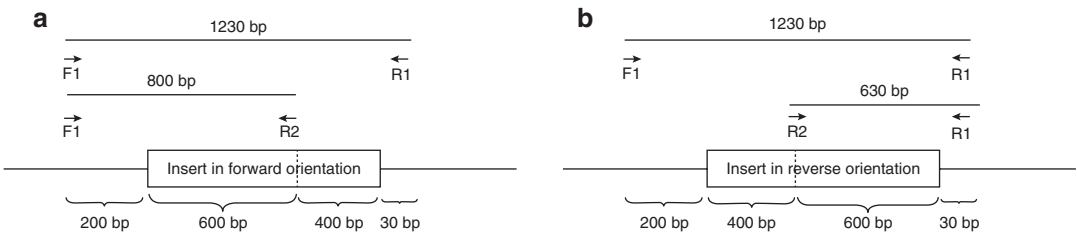


Fig. 3 Example of a colony PCR to evaluate the presence and orientation of the insert. A colony PCR is performed using CD4 forward (F1) and reverse (R1) primers in combination with the reverse primer from the TCR primer panel corresponding to the insert (R2). F1 and R1 are located on the TCR expression vectors ~200 bp upstream and ~30 bp downstream of the TCR cloning site Sall. In this example, R2 is located 600 bp in the insert. In correct orientation (**a**), the colony PCR will result in a product of ~800 bp (~200 bp upstream the insert +600 bp of insert). In reverse orientation (**b**), the colony PCR will result in a product of ~630 bp (600 bp of insert + ~30 bp downstream the insert). An additional product of ~1230 bp might be amplified in both orientations. In colonies where the vector re-ligated, not harboring any insert at all, a ~230 bp product is amplified

TCR primer panel corresponding to the insert. Analysis of the colony PCR on a 2% agarose gel will provide information on the presence and orientation of the insert (Fig. 3). Positive colonies are transferred to 5 mL liquid culture (LB + 100 μ g/mL ampicillin) and grown overnight at a 37 $^{\circ}$ C while shaking. Colonies are viable for a limited time and should be transferred to the medium within 8 h.

- Isolate DNA using a mini prep kit (following the manufacturer's instructions) and confirm correct clones by using restriction analysis and sequencing.

3.6.6 Preparation of the Injection Fragments

1. Prepare a maxi prep from one positive TCR V α and one TCR V β clone, following the manufacturer's instructions.
2. Prepare the injection fragments by NotI digestion of the construct. Incubate 20 μ g of maxi prep DNA in 1 $\times$ restriction buffer with 2 μ L (10 units) of NotI restriction enzyme in a 100 μ L reaction and incubate for a minimum of 3 h at 37 °C. Gel purify the fragments (following the manufacturer's instructions).
3. Determine the concentration of isolated DNA and confirm the fragments by restriction analysis. Incubate 100 ng of the fragments in 1 $\times$ restriction buffer with 0.5 μ L (5 units) of restriction enzyme in a 20 μ L reaction, for a minimum of 3 h at 37 °C. Analyze on an agarose gel.
4. Prepare an equimolar solution of 1 ng/ μ L of both TCR injection fragments (TCR V α and TCR V β) in 0.22 μ m pre-filtered injection buffer. Centrifuge for 30 minutes at 14,000 $\times g$ in a cooled centrifuge. Transfer the supernatant into a 1.5 mL tube and use this for microinjection in fertilized Rag2^{-/-} oocytes.

3.7 Evaluation of the TCR Mice

3.7.1 Genotyping

1. Perform a PCR using primers from the TCR PCR panel corresponding to the construct (TCR V α and V β). Analyze PCR products on a 2% agarose gel for presence of the transgene. Breed the transgenic founders with rag2^{-/-} mice to obtain germline transmission and characterize (3.7.2. and 3.7.3.) each line to select the best line for use in further experiment. Since the mice were generated via microinjection, the construct will be integrated multiple copy, randomly into the mouse genome. Each transgenic founder will have a different integration site and copy number and should be characterized separately as an independent line.

3.7.2 Analysis of the Presence of T Cells in Peripheral Blood

Microinjection was performed in Rag2^{-/-} oocytes. As Rag2-deficient animals fail to generate mature T or B lymphocytes, mature T cells will only be present in the peripheral blood when the inserted constructs provide a functional T-cell receptor. Therefore, assessing the presence of mature T cells in the peripheral blood will provide evidence of a functional TCR transgenic mouse (*see* Fig. 4).

1. Collect blood of transgenic animals in a 1.5 mL tube containing 100 μ L of 10 mM EDTA and add PBS to acquire a total volume of 800 μ L. Pipet 400 μ L lympholyte on the bottom of the tube, allowing it to form a layer under the diluted blood. Centrifuge at 600 $\times g$ for 20 min at room temperature, without brake (*see* Note 21).
2. Collect the interface with a 200 μ L pipet and transfer to a new 1.5 mL tube. Add FACS buffer to acquire a total volume of 1.2 mL and centrifuge. Stain cells with anti-CD3 and anti-CD4, as described before, and determine the presence of T cells.

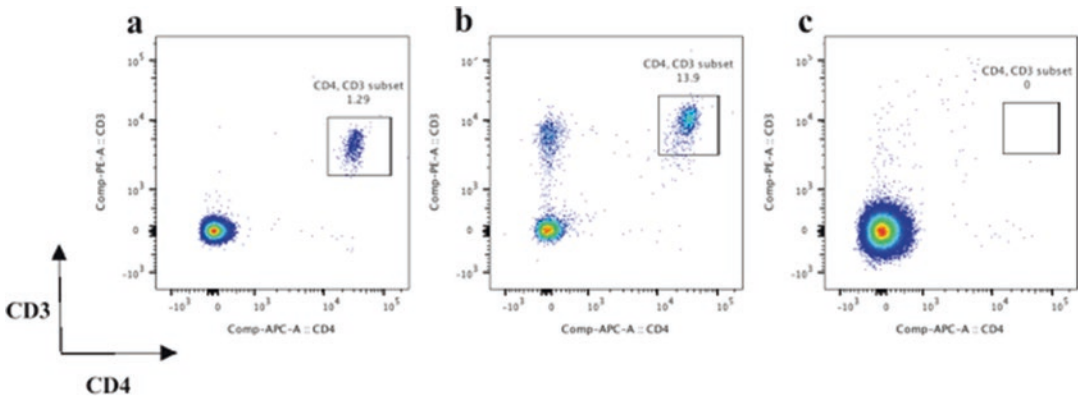


Fig. 4 Evaluation of the presence of CD3+CD4+ T cells in the peripheral blood of TCR transgenic (a), wild-type (b) and rag2^{-/-} (c) mice, analyzed by flow cytometry. Since TCR transgenic mice were generated by micro-injection in rag2^{-/-} oocytes, mature T cells will only be present when the inserted constructs provide a functional T-cell receptor

3.7.3 Evaluation of TCR Specificity in a DC-T-Cell Co-culture

1. One week prior to the experiment, prepare bone marrow dendritic cells, as described above.
2. Isolate the spleen and lymph nodes from a transgenic animal (*see Note 22*).
3. Obtain a single-cell suspension by mashing the spleen and lymph nodes through a 70 μ m cell strainer in 1 mL PBS and rinse with 2 mL PBS. Centrifuge the cell suspension and lyse the erythrocytes by resuspending the cell pellet in 1 mL osmotic lysis buffer, incubate for 4 min on ice, and centrifuge after adding 10 mL of PBS.
4. Isolate CD4⁺ T cells using Miltenyi Biotech CD4⁺ T cells isolation kit, according to manufacturer's instructions.
5. Wash the enriched CD4⁺ T cells twice in RPMI and label the cells with CFSE (*see Note 23*). Resuspend the cells in 1 mL of RPMI, dropwise add 110 μ L of 1/100 pre-diluted CFSE (5 μ M final concentration), while gently vortexing the cell suspension and incubate at 37 $^{\circ}$ C (*see Note 24*). After 10 min stop the CFSE staining by adding 10 mL of RPMI5, wash the cells twice in RPMI5 and count (*see Note 25*).
6. Start a co-culture by seeding 50 μ L of bone marrow-derived dendritic cells (2×10^5 cells/mL) in 6 wells of a 96-well round bottom plate. Add 100 μ L of the enriched CD4⁺ T cells at 10^6 cells/mL. Prepare a 1 in 2 serial dilution of the TCR-specific peptide in 50 μ L and add to the different wells (include a medium control), starting at 1 μ g/mL of peptide (*see Note 14*). All steps are performed in RPMI5. Culture the cells for 4 days.

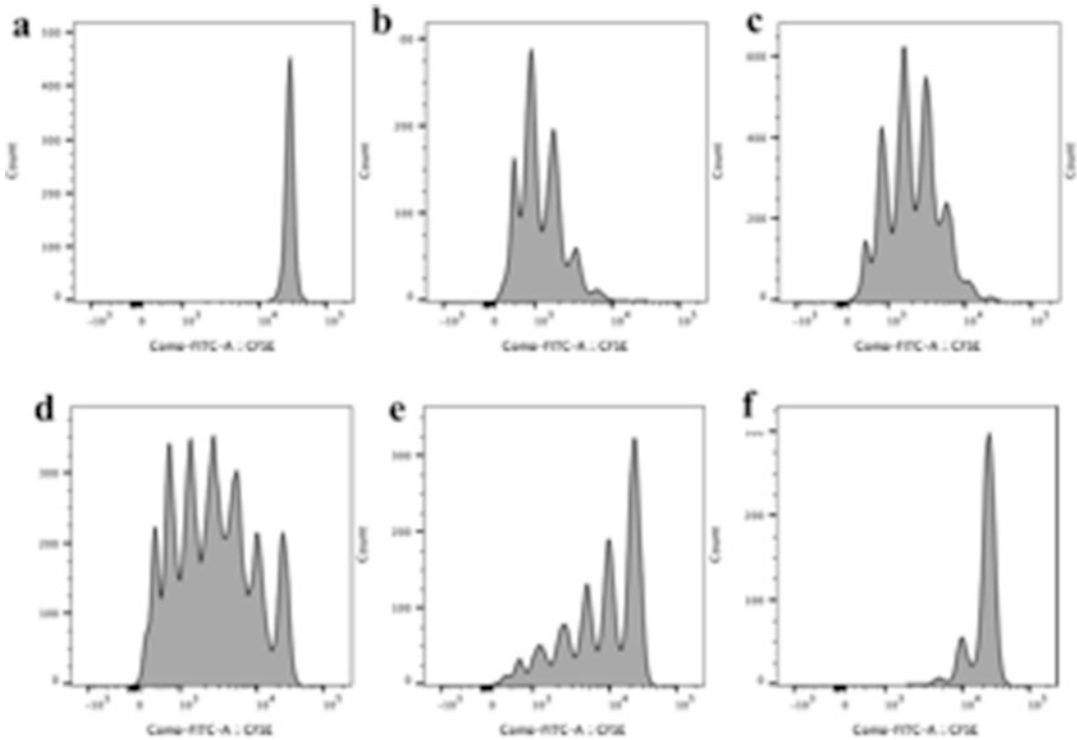


Fig. 5 Proliferation of CD3+CD4+ TCR transgenic T cells after a co-culture with bone marrow-derived dendritic cells in the absence (a) or presence of peptide in decreasing doses (b–f). Antigen-specific peptide was present in the culture at 1 $\mu\text{g/mL}$ (b), 0.2 $\mu\text{g/mL}$ (c), 0.04 $\mu\text{g/mL}$ (d), 0.008 $\mu\text{g/mL}$ (e), 0.0016 $\mu\text{g/mL}$ (f). CFSE content is decreased by half each time cells divide, resulting in one peak per generation of proliferating cells

7. Evaluate T-cell proliferation by assessing the CFSE profiles of CD3+CD4+ T cells by flow cytometry, as described before (see **Note 26**) (see Fig. 5).

4 Notes

1. As an example, for an antigen with a stock concentration of 200 $\mu\text{g/mL}$, 50 μL of antigen is diluted with 425 μL PBS, followed by dropwise addition of 25 μL aluminum hydroxide. Mice are injected intraperitoneally with 500 μL of this formulation.
2. The optimal immunization protocol is dependent on the antigen and should be optimized.
3. Activated T cells can be isolated from draining lymph nodes or spleen, depending on the route of antigen administration.
4. Depending on the peptide, incubation time and peptide dose can be different. This should be optimized before. The pep-

tide used can be a known immunogenic peptide, or immunogenicity can be predicted using IEDB database, and confirmed by in vitro experiments.

5. CD4⁺ T-cell isolation is described using the CD4⁺ T cell isolation kit from Miltenyi Biotech. However, isolation can also be performed by FACS or any other magnetic cell separation protocol.
6. It is important to wash the cells in medium without FCS or HEPES, as PEG will precipitate proteins, and HEPES can be toxic for the cells.
7. Pipetting the cells should be avoided from this step onwards, to avoid disrupting the fragile cell contacts.
8. Slow addition of the wash medium is necessary to gradually dilute the PEG, without lysing the cells. Small cells clumps will be visible.
9. Avoid using the outer wells of the plate.
10. HAT is added as a selection marker. It will kill the HAT sensitive unfused BW5147 cells, while fused cells will become HAT insensitive and survive.
11. Some clones will grow faster than others. Therefore, clones that become confluent very quickly after feeding can be reseeded at a lower density.
12. Here, we describe the use of bone marrow-derived dendritic cells as antigen-presenting cells. Alternatively, the syngeneic spleen cells can be used. In this case, plate 50 μL of the syngeneic spleen cells (2×10^6 c/mL).
13. Cytokine ELISA performed according to the manufacturer's instructions.
14. Preparation of a serial dilution with peptide. Prepare a stock of 4 $\mu\text{g/mL}$ (final concentration 1 $\mu\text{g/mL}$) and dilute 1/2 in DMEM10 for several steps. Mix 100 μL of peptide stock with 100 μL of DMEM10 (final concentration of 0.5 $\mu\text{g/mL}$), mix well, and transfer 100 μL to a second tube with 100 μL DMEM10 (final concentration 0.25 $\mu\text{g/mL}$); repeat till the appropriate amount of dilutions is obtained. The immunogenicity of the peptide used will determine the concentrations that need to be tested; therefore the example provided above is merely an indication of the required concentrations.
15. A maximum of 10^6 cells is stained in 50 μL . Increase the staining volume accordingly if the cell number is higher.
16. Selecting clones that express TCR domains to which an antibody is available provides advantages for future detection of TCR transgenic cells in mice.

17. Ideally, cells are reseeded 1 day prior to freezing. Cells are then in log phase and grow exponentially, increasing recovery after freezing.
18. Single clones can also be obtained by other techniques such as limiting dilution.
19. There are different TCR screening panels available, and the nomenclature can be misleading. The numbering used in the different panels is not the same, and this should be taken into account when analyzing the TCR subtype. An overview of the correspondence between nomenclatures can be found in Tables 1 and 2.
20. p428 is a good choice for a TCR V β chain because of a deletion in a critical silencer region of the CD4 promotor, it is already expressed from double-negative thymocyte stage onwards. Consequently, a V β chain driven from this promotor gives a critically early and continued signal for T-cell development of the cells carrying the transgene. p783 is a good choice for expression of a class II-restricted V α chain because it is not expressed in double-negative thymocytes (due to reinsertion of the CD4 silencer region) and thus will not contribute to a signal that would favor the unwanted formation of aberrant gamma-delta-like cells, which are often a complication in TCR transgenic mice. On top, p783 is silenced in CD8 cells and thus will not favor their development.
21. Alternatively, peripheral blood can also be analyzed directly after several erythrocyte lysis steps with osmotic lysis buffer. However, we experience that T cells are very fragile at this stage and can suffer from the repeated osmotic lysis steps. Isolating the lymphocytes via a gradient as described in the protocol increases the recovery of these cells.
22. Before performing this assay, make sure that there is sufficient transgenic offspring, as to ensure the transgenic line.
23. It is important that no FCS is present in the medium, as this markedly reduces the CFSE labeling.
24. A maximum of 50×10^6 cells is labeled in 1 mL; increase the volume of RPMI and CFSE accordingly if the cell number is higher.
25. The presence of FCS in the medium for washing of the cells is essential, as FCS stops the labeling, eliminating the toxic effects of CFSE.
26. It is important to include a live/dead marker in the staining, as a lot of nonproliferating cells die during the culture and should be excluded from the analysis.

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