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Analysis of Disease-Related T Cell Receptors and their Antigens in $GABA_A$ Receptor Encephalitis

Dissertation

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Abstract

$GABA_A$ receptor encephalitis is a recently described form of inflammatory encephalitis, caused by antibodies against the $GABA_A$ receptors. The antibodies induce internalization of the receptor, resulting in a synaptic reduction of $GABA_A$ receptors. Thereby, the inhibitory receptor function is lost, and patients suffer from refractory seizures and status epilepticus. Even though auto-antibodies and their epitopes were described, nothing is known about the role of T cells in this disease, although CD4+ Th response is necessary for a humoral immune response. Further, it is known that CD8+ T cells often play key roles in encephalitis.

For a better understanding of the role of T cells in $GABA_A$ receptor encephalitis, the T cell repertoire in the cerebrospinal fluid (CSF) as well as in inflamed brain regions of an encephalitis patient (termed Index patient 2 (IP2)) were analysed.

Whereas the CD4+ T cell repertoire in CSF was polyclonal, one CD8+ T cell receptor, named IP2-8S1, was expanded in the CSF. The same clone was also detected in 11 % of isolated single CD8+ T cells from the hippocampus. Immunohistochemical stainings of brain biopsy indicated an obvious infiltration of activated, perforin expressing CD8+ T cells into the brain, whereas CD4+ T cells were found very rarely. These findings indicate a key role of IP2-8S1 in the disease etiology. Furthermore, it denotes the expression of an autoantigen in the CNS, recognised by IP2-8S1.

To identify the antigen of IP2-8S1 and to analyse its HLA-restriction, the TCR was transferred into mouse hybridoma cells which express sGFP under the NFAT enhancer as a reporter. Possible peptide candidates from the $GABA_A$ receptors α1 subunit were analysed for their ability to activate the hybridoma cells. Additionally, antigen screening experiments using plasmid-encoded combinatorial peptide libraries (PECPL) were performed.

Based on the presence of autoreactive antibodies in IP2, we initially expected to detect expanded CD4+ T cell clones, mediating humoral immune response in $GABA_A$ receptor encephalitis. To detect possible antigens of CD4+ T cell, the established method for identifying CD8+ T cell antigens with PECPL was modified to allow the screening of CD4+ T cell antigens. To this end, new libraries were designed and generated, where randomised nucleotide sequences were inserted into the invariant chain. Thus, randomized peptides are presented on MHC class-II molecules. As proof of concept, an auto-reactive TCR that recognises a known peptide presented by HLA-DR1 and HLA-DR2a was used. The positive control construct was characterized regarding the length of the sequence encoding the antigen as well as the influence of the signal sequence of the invariant chain. It was shown that by expression of antigenic peptides integrated into the invariant chain it is possible to load peptides with very low affinity onto MHC-II molecules and to activate TCRs.

Zusammenfassung

$GABA_A$ Rezeptor Enzephalitis ist eine erst kürzlich beschriebene Form der inflammatorischen Enzephalitiden, welche hervorgerufen wird durch Antikörper gegen den $GABA_A$ Rezeptor. Die Antikörper induzieren die Internalisierung des Rezeptors, was eine Reduktion der synaptischen Rezeptoren zur Folge hat. Die inhibitorische Funktion des Rezeptors geht dadurch verloren, und die Patienten leiden an für Medikamente unempfängliche Krampfanfällen als auch an Status epilepticus. Wenngleich Autoantikörpern als auch deren Antigene bereits beschrieben sind, weiß man bisher nichts über die Rolle von T Zellen in der Krankheit. Dies ist bemerkenswert, da CD4+ T-Helferzellen unabdingbar sind für eine humorale Immunantwort und CD8+ T-Zellen oft eine Schlüsselrolle in Enzephalitiden spielen. Für ein besseres Verständnis der Rolle von T Zellen in $GABA_A$ Rezeptor Enzephalitis, wurde deshalb das T Zell Repertoire der Zerebrospinalflüssigkeit (ZSF) als auch entzündeten Hirn-Regionen eines Patienten mit $GABA_A$-Rezeptor Enzephalitis ("Index Patient 2" (IP2)) untersucht.

Während das CD4+ T-Zell-Repertoire im ZSF sich als polyklonal herausstellte, wurde ein expandierter, CD8+ T Zell Rezeptor, genannt IP2-8S1, im ZSF identifiziert. Derselbe Klon wurde in 11 % aller Einzelzellen, die aus dem Hippocampus isoliert wurden, wiedergefunden. Immunhistochemische Färbungen der Gehirnbiopsie zeigten eine starke Infiltration von aktivierten, Perforin exprimierenden CD8+ T-Zellen, während CD4+ T-Zellen im Gehirn nur selten anzutreffen waren. Diese Ergebnisse zeigen, dass CD8+ T-Zellen im Krankheitsverlauf eine Schlüsselrolle einnehmen. Des Weiteren deuten die Ergebnisse eine Expression des Autoantigens, welches von IP2-8S1 erkannt wird, im zentralen Nervensystem an.

Um das Antigen von IP2-8S1 und seine HLA-Restriktion zu identifizieren, wurde der T-Zell-Rezeptor in Maus Hybridomzellen, welche sGFP unter der Kontrolle des NFAT-enhancers exprimieren, eingebracht. Mögliche Kandidaten-Peptide aus der $GABA_A$ Rezeptor α1 Untereinheit wurden hinsichtlich ihrer Fähigkeit, diese Hybridomzellen zu aktivieren, untersucht. Des Weiteren wurde versucht, Antigene mit Hilfe von Plasmid-kodierten kombinatorischen Peptid-Bibliotheken (PECPL) zu identifizieren.

Basierend auf dem Vorhandensein autoreaktiver Antikörper in IP2, haben wir erwartet, expandierte CD4+ T-Zell-Klone zu finden, welche die humorale Immunantwort vermitteln. Für die Detektion von CD4+ T-Zell-Antigenen wurde die Methode zur Identifikation von CD8+ T-Zell-Antigenen mit PECPL so modifiziert, dass sie die Charakterisierung von CD4+ T-Zell-Antigenen erlaubt. Hierfür wurden neue Bibliotheken entworfen und hergestellt. Dazu wurde eine randomisierte Nucleotidsequenz in die invariante Kette integriert, so dass eine zufällige Aminosäuresequenz von Klasse-II MHC Molekülen präsentiert wird. Als Nachweis der Machbarkeit wurde ein auto-reaktiver T-Zell-Rezeptor, der ein bekanntes Peptid auf HLA-DR1

und HLA-DR2a erkennt, verwendet. Die Positivkontrolle wurde hinsichtlich der Länge der Peptidsequenz als auch hinsichtlich des Einflusses der Signalsequenz der Invarianten Kette, analysiert. Es konnte gezeigt werden, dass es durch die Expression von Antigen Peptiden integriert in der Invarianten Kette, möglich ist, Peptide mit sehr geringer Affinität auf MHC-II Moleküle zu laden und T-Zell-Rezeptoren zu aktivieren.

Table of content

Abbreviation

AA	amino acid
amp	Ampicillin
APC	allophycocyanin
APCs	antigen presenting cells
bls	blasticidin
bp	base pairs
CD	cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary desoxyribonucleoic acid
CLIP	Class-II associated invariant chain peptides
CNS	central nervous system
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocyte
DAPI	4′,6-Diamidin-2-phenylindol
DC	dendtritic cells
ddH_2O	double-distilled water
DEPC	Diethyl pyrocarbonate
DNA	desoxyribonucleoic acid
DTT	Dithiothreitol
EB	Elution buffe
EDTA	Ethylenediaminetetraacetic acid
ER	endoplasmatic reticulum
ERAP1	ER-aminopeptidase 1
F	phenylalanine
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
h	hour
HC	hippocampus
HIER	heat-induced epitope retrieval
HLA	human leucocyte antigen
HLA-DR1	HLA-DRB1*01:01
HLA-DR2a	HLA-DR5*01:01
HLA-DR2b	HLA-DRB1*15:01
hMBP	human myelin basic protein
His	histidine
hygro	hygromycin
Ii	invariant chain
Ii-ECPL	invariant chain encoded combinatorial peptide library
IP2	Index patient 2
IP2-8S1	expanded CD8+ T cell clone of IP2
IP2-AV8S1	α-chain of IP2-8S1
IP2-BV8S1	α-chain of IP2-8S1
ITAM	immunoreceptor tyrosine-based activation motifs
L	leucine
LB	lysogeny broth
LCL	B-Lymphoblastoid cell line
LPS	lipopolysaccharides
LS	lateral sulcus
LMD	Laser microdissection
m	mouse
M	methionine
MAIT	Mucosal-associated T
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHC-I	MHC class I
MHC-II	MHC class II
MIIC	MHC-II compartment
Min	minutes
MR1	MHC-related protein 1
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
n	number of cells
neo	neomycin
NFAT	Nuclear factor of activated t cells
NGS	next generation sequencing
NMDA	N-methyl-D-aspartate

PAGE	polyacrylamide gel electrophiresis
PCR	Polymerase chain reaction
PE	phycoerythrin
PECPL	plasmid encoded peptide library
PI	propidium iodide
PLC	peptide loading complex
PND	paraneoplastic neurological disorders
PVDF	polyfinylidenfluorid
qPCR	quantitative polymerase chain reaction
RPMI	Roswell Park Memorial Institute
RT	reverse transcriptase
s	seconds
sc	single cell
SCLC	small cell lung cancer
SDS	sodium dodecylsulfate
sGFP	super green fluorescent protein
Th cell	T helper cell
TBE	Tris-Borate EDTA-Buffer
TCR	T cell receptor
TM	transmembrane
T_M	melting temperature
U	units
UMI	unique molecular identifier
UV	ultra violett
wt	wild type
w/v	weight per volume
v/v	volume per volume

1 Introduction

1.1 The immune system

The immune system protects the body from pathogens like viruses, bacteria, fungi and parasites as well as from altered cells. This complex system enables a differentiation of self and non-self, to eliminate infected or altered cells.

To perform this task, the immune system is composed of two parts, the innate and the adaptive immune response. The innate immune system is quite unspecific and thereby fast and versatile. It consists of barriers, the complement system and immune cells (granulocytes, monocytes and natural killer cells).

The selective and specific immune response, including the development of an immunological memory, is mediated by the adaptive immune system. This highly specialised system is an interaction of humoral and cellular immunity. The humoral immunity is mediated by antibodies (AB). B cells secrete ABs after activation by antigen recognition. AB can mark pathogens for elimination by the complement system or neutralize soluble antigens, like toxins, by binding. T cells represent the cellular immunity. They can induce apoptosis of infected cells or produce cytokines to enhance the immune response. In contrast to AB, T cell receptors (TCRs) do not recognize native antigens. For recognition by the TCRs, antigens are intracellularly degraded and the originated peptides are presented by major histocompatibility complexes (MHC) to TCR.

Both, the humoral and the cellular immunity of the adaptive immune system are antigen specific. After an infection memory cells are formed. Thus a reinfection can be eliminated faster and more efficient.

1.2 T lymphocytes

T cells mature in the thymus from lymphoid progenitor cells. During the maturation, TCR rearrangement is performed and autoreactive T cells are eliminated. This ensures diversity of functional TCRs and prevents autoimmunity.

One T cell carries around 30,000 TCR on the cell surface. Most TCRs are composed of one α and one β-chain. However, a minority of T cells also expresses TCR composed of one γ and one δ chain (Brenner *et al.,* 1986).

T lymphocytes can be subclassified into two main groups, depending on the expression of different co-receptors.

CD8+ T cells, express the co-receptor CD8. They recognize peptides presented on major histocompatibility complex class I (MHC-I), which is expressed on all nucleated cells in the body. CD8+ T cells eliminate viral infected cells as well as tumor cells by the release of

effectorproteins like granzyme B and perforin or by inducing apoptosis in target cells via the expression of Fas ligand (Berke 1995).

CD4 + T cells express the co-receptor CD4. They recognize peptides presented on major histocompatibility complex class II (MHC-II) of antigen presenting cells (APC). They play a crucial role in the regulation of the immune system by the production of cytokines and transcription factors (Nakayamada *et al.,* 2012). Furthermore, CD4+ T cells induce humoral immunity by the recognition of the peptide-MHC-II-complex on B cells and additional binding of the CD40 Ligand (CD40L) to CD40 on B cells (Parker 1993).

1.2.1 Structure of αβ T cell receptors

The α- and β-chain of a classical T cell receptor are connected via a disulfide bridge. The extracellular part of both chains consists of a variable region (v), a joining segment (j) and a constant region (c). The chains are anchored in the membrane via a hydrophobic transmembrane domain, which ends in a short cytoplasmic tail (Kronenberg *et al.,* 1986). This cytoplasmic tail of the TCR is too short for signal transduction into the cell. Therefore, the TCR assembles with accessory polypeptide chains, which form a CD3 complex. The complex is constructed by two heterodimeric chains (CD3 γ-ξ and CD3 δ-ξ) and a homodimeric ζ-chain (Figure 1-1 (A), Clevers *et al.,* 1988). CD3 is important for correct assembly and surface expression of the TCR complex, as well as for the signal transmission into the cell. The intracellular parts of the CD3 complex contain immunoreceptor tyrosine-based activation motifs (ITAMs). After the binding of the TCR to a peptide-MHC-complex, the ITAMs get phosphorylated by receptor associated phosphotyrosine kinases of the Src-family kinases (Smith-Garvin *et al.,* 2009). This phosphorylation activates a signal cascade whose actions include triggering Ca^{2+} influx into the cell. The increased Ca^{2+} concentration activates the phosphatase Calcineurin. The transcription factor nuclear factor of activated T cells (NFAT) gets activated by dephosphorylation via calcineurin. The activated NFAT gets translocated into the nucleus, where it activates the expression of specific genes, like interleukin-2 (IL-2).

IL-2 regulates the proliferation and differentiation of T cells. It can be used as an activation marker of T cells. The expression of reporter genes, like lacZ or super green fluorescence protein (sGFP), under the control of the NFAT promoter can also be used as tool for the detection of T cell activation (Karttunen and Shastri 1991).

For increased signal transduction, the co-receptors CD4 and CD8 bind to non-polymorphic regions of the MHC molecules. CD4 is a single polypeptide, composed of four domains (D1-D4), whereas CD8 is a heterodimer with one α-chain and one β-chain (Figure 1-1 (B)). However, CD8 can also be formed as CD8αα homodimer (Zamoyska 1998). By binding to MHC, the co-receptor associated Src family kinase Lck gets activated and also phosphorylates

the ITAMs of the CD3 complex (Barber *et al.,* 1989). Thereby, the signal transduction is increased.

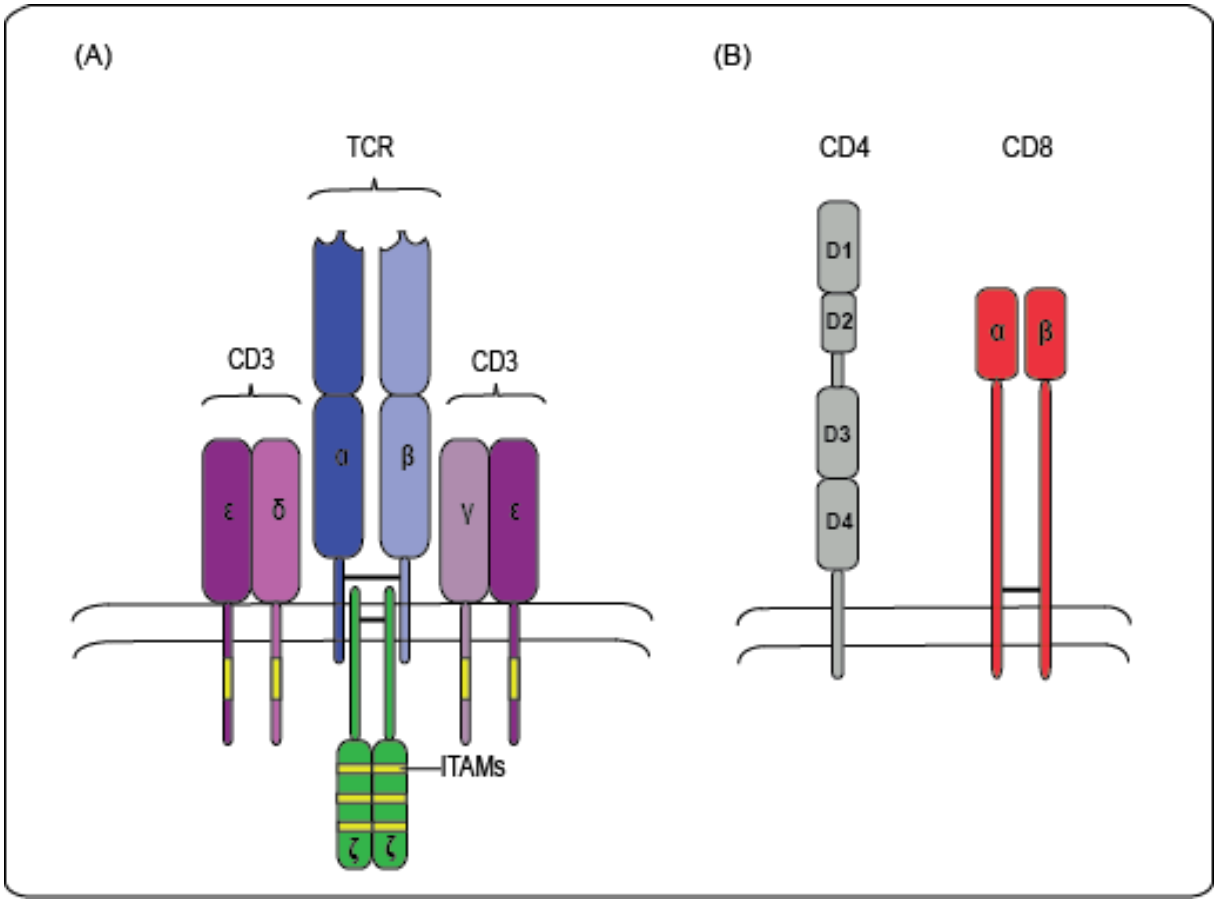

Figure 1-1 Structure of TCR complex and co-receptors. (A) Graphic of the TCR complex. The TCR is composed of one α and one β-chain connected via a disulfide bridge. This heterodimer is associated with CD3, which is formed by δξ and γξ and ζ, which is involved in the signal transduction. (B) The co-receptors CD4 and CD8 of T lymphocytes. CD4 is formed by 4 domains (D1-D4), whereas CD8 is formed by two chains (red) connected by a disulfide bridge. CD8 can be a heterodimer formed by one α and one β-chain, as well as homodimer formed by two α-chains (modified from Janeway 2002).

1.2.2 Diversity of T cell receptors

T cell receptors cover a broad spectrum of antigens recognition. This receptor diversity is developed by gene recombination in the thymus. During T cell maturation, first the β-chain is reassembled. After successfully rearrangement, the α-chain also undergoes recombination. The recombination is illustrated in Figure 1-2.

The alpha gene locus contains 54 TRAV genes, 61 TRAJ genes and one constant region. The beta gene locus contains 64-67 TRBV genes, followed by two gene groups, each composed of one TRBD (diversity, D_1 or D_2) followed by 13 TRBJ (TRBJ1.1-1.6 and TRBJ2.1-2.7) and one constant region (TRBC1 and TRBC2) (Arden *et al.,* 1995, Rowen *et al.,* 1996). During the rearrangement, a random V-gene is combined with a J-gene (α-chain) or with a D- and J-gene (β-chain). Additionally, palindromic (p) and non-germline (n) nucleotides are added between

the V- and J-region (α-chain) or between V-D and D-J (β-chain). The chains are transcribed to messenger RNA (mRNA), and the intron separating J- and C region is eliminated by post-transcriptional splicing. By this recombination, every cell contains an individual TCR. The highly variable regions in the chains form three complementary determining regions (CDR) CDR1, CDR2 and CDR3. The variable region of both chains mainly code for the CDR1 and CDR2 regions. The CDR3 regions, which has the highest diversity, is formed by the N (α-chain) and the NDN (β-chain).

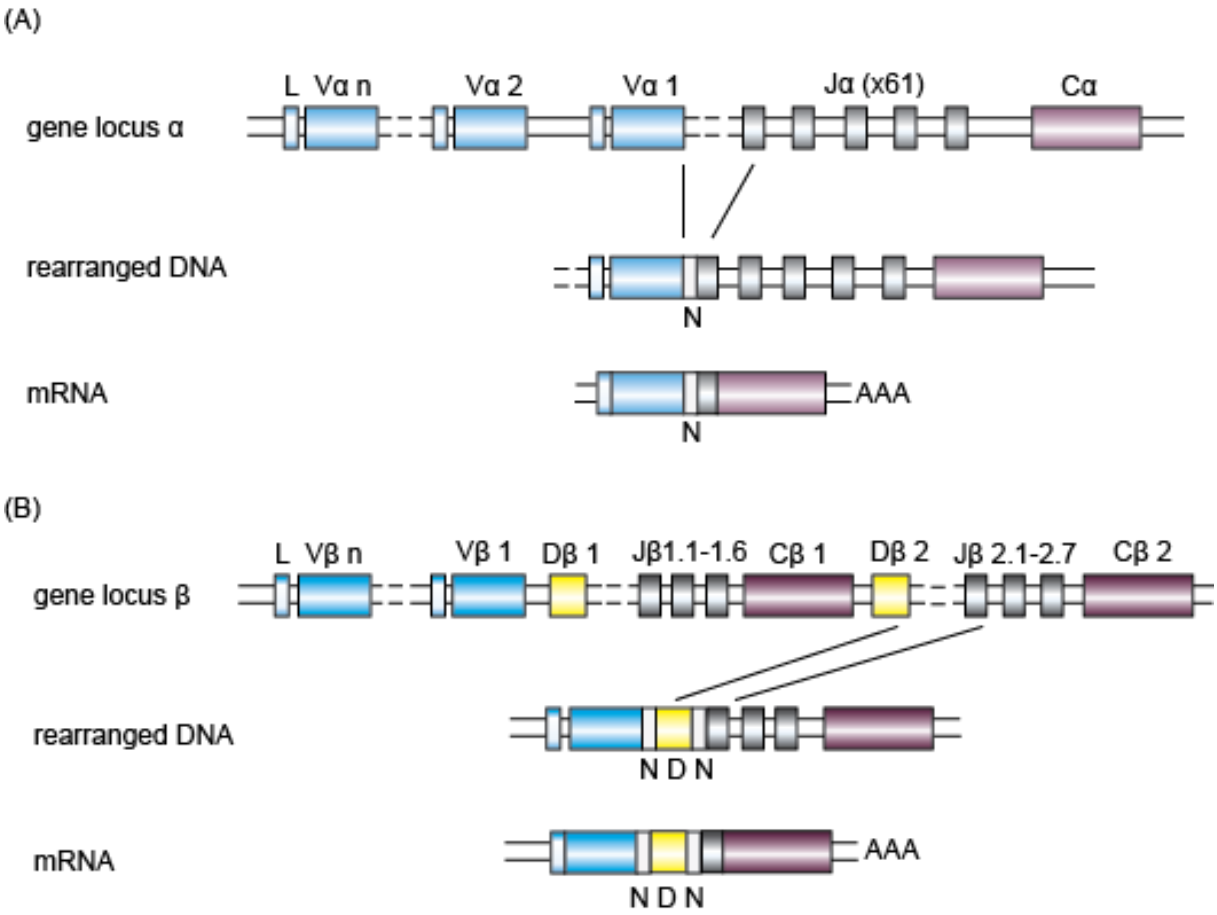

Figure 1-2 Rearrangement of TCR α- and β-chain. T cell diversity is generated by V-J (alpha chain, A) and V-D-J (β-chain, B) rearrangement. Some nucleotides are added randomly (N) to increase TCR diversity. The intron between J and the constant region C is removed after transcription by splicing (modified from Kronenberg 1986).

1.2.3 T cell maturation

T cell maturation in the thymus ensures that only functional, non-autoreactive T cells leave the thymus. The thymic seeding progenitors do not express TCRα and TCRβ and are double negative for CD4 and CD8. In the first step, the β-chain is rearranged. If the rearrangement was successful, a pre-TCR, composed of CD3, β-chain and an invariant pre-TCR α-chain is expressed and pre-TCR signalling is initiated (Böhmer 2005). The cells start to express CD4 and CD8 (double positive T cells) and the α-chain is rearranged. Mature T cells are selected

for the recognition of self-peptide-MHC complexes presented on cortical thymic epithelial cells (cTEC, positive selection). Missing interactions induce death by neglect. However, a high affinity of the TCR induces apoptosis, to prevent autoreactivity (negative selection). Only T cells with intermediate affinity differentiate to mature, single positive T cells (Klein *et al.,* 2014).

1.2.4 Antigen recognition of T cells

T cells can only recognize their antigens in association with MHC, in human called human leucocyte antigen (HLA). The highly variable CDR3 region of the TCR binds to the peptide presented on the MHC, and CDR1 and CDR2 mainly to the MHC (Rudolph *et al.,* 2006).
However, a single TCR is able to recognize more than one peptide-MHC-complex. Thus it is assumed that one TCR can recognize up to 1.3×10^6 peptides (Wooldridge *et al.,* 2012). The highly degeneracy of TCRs is called cross-reactivity. It ensures that most possible peptides presented by MHC are covered.
Additionally, one in 10^3-10^4 T cells are able to recognize peptides presented on allogeneic MHC (Felix and Allen 2007). During T cell development in the thymus, T cells cannot be selected for their ability to bind to all HLAs which are not expressed by the individual. Alloreactivity especially plays a role after transplantations and bone marrow transplantation. In this so called graft-versus-host disease, donor T cells recognize peptides presented on recipient cells.

1.3 Major Histocompatibility complex

MHC play an important role in the adaptive immune system as well as in the recognition of self and foreign molecules.
MHC-I molecules are expressed on all nucleated cells in the body. They are composed of a MHC-I heavy chain and an invariant subunit, the β-2-microglobulin (β_2m). The heavy chain is encoded by three different polymorphic genes: human leukocyte antigen HLA-A, -B and -C. The heavy chain forms a binding groove, where cytosolic peptides bind and get presented on the cell surface to CD8+ T cells (Neefjes *et al.,* 2011).
In contrast to MHC-I, MHC-II molecules are only expressed on APCs (B cells, dendritic cells (DC) and macrophages). They are composed of one α and one β-chain, which forms a binding groove (Cresswell *et al.,* 1987). These chains are encoded by three polymorphic genes, HLA-DP, -DQ and -DR. APCs take up extracellular proteins by endocytosis. These proteins are degraded in the lysosome, loaded onto MHC-II molecules and presented to CD4+ T cells (Neefjes *et al.,* 2011).

1.3.1 Comparison of MHC-I and MHC-II peptide loading

Due to their different function, MHC-I and MHC-II molecules are loaded with their peptides in different cell compartments, illustrated in Figure 1-3.

MHC-I molecules are folded in the endoplasmic reticulum (ER) by chaperons. The chaperone calnexin (CNX) performs the early folding of MHC-I molecules and binding of β_2m. Calreticulin (CRT) stabilizes MHC-I molecules while ERp57 mediates the disulfide bond formation (Ellgaard and Frickel 2003).

MHC-I molecules are loaded with cytosolic peptides, which have been degraded by the proteasome. After proteasomal cleavage, the C-terminus of the peptides are compatible with MHC-I binding, however the peptides are N-extended (Cascio *et al.,* 2001). These precursors get trimmed in the ER by the ER aminopeptidase 1 (ERAP1, Saric *et al.,* 2002).

For peptide loading to MHC-I, it binds to the peptide loading complex (PLC). In the complex MHC-I/β_2m is stabilized by CRT, ERp57 and Tapasin. Tapasin interacts with TAP1 and TAP2, which transport peptides of a length of 8-12 amino acids (AA) from the cytosol into the ER (Abele and Tampe 2004). After peptide binding, the peptide-MHC-I complex leaves the ER for presentation at the cell surface.

Additionally, APCs, especially DCs, have the ability to uptake and present extracellular antigens on MHC-I. This presentation of non-cytosolic peptides on MHC-I is called cross-presentation. This pathway is important for defence against tumor cells and viruses, which do not infect APCs (Kurts *et al.,* 2010).

The presentation of MHC-I can be modulated by Interferon gamma (INF-γ). INF-γ increases the expression of MHC-I, TAP, ERAP1 and the subunits of the immunoproteasome (Cascio *et al.,* 2001).

In contrast to MHC-I, MHC-II molecules are loaded with extracellular peptides in the MHC-II compartment (MIIC). To avoid peptide loading in the ER, the α- and β-chain of MHC-II associates with the invariant chain (Ii) in the ER (Cresswell *et al.,* 1987). Ii is needed for the assembly of α and β-chain in the ER (Kvist *et al.,* 1982). It blocks the binding groove of MHC-II, and thereby prevents binding of cytosolic peptides (Roche and Cresswell 1990). Additionally, it targets the MHC-II to the MIIC by a targeting signal in the cytoplasmic tail (Lotteau *et al.,* 1990). The targeting signal is recognized by the sorting adapters AP1, which is a *trans*-Golgi network adapter, and AP2, which is a plasma membrane adapter. This allows the complex to be targeted directly to the MIIC or it is targeted to the plasma membrane from where it is internalized by endocytosis and directed to MIIC (Neefjes *et al.,* 2011).

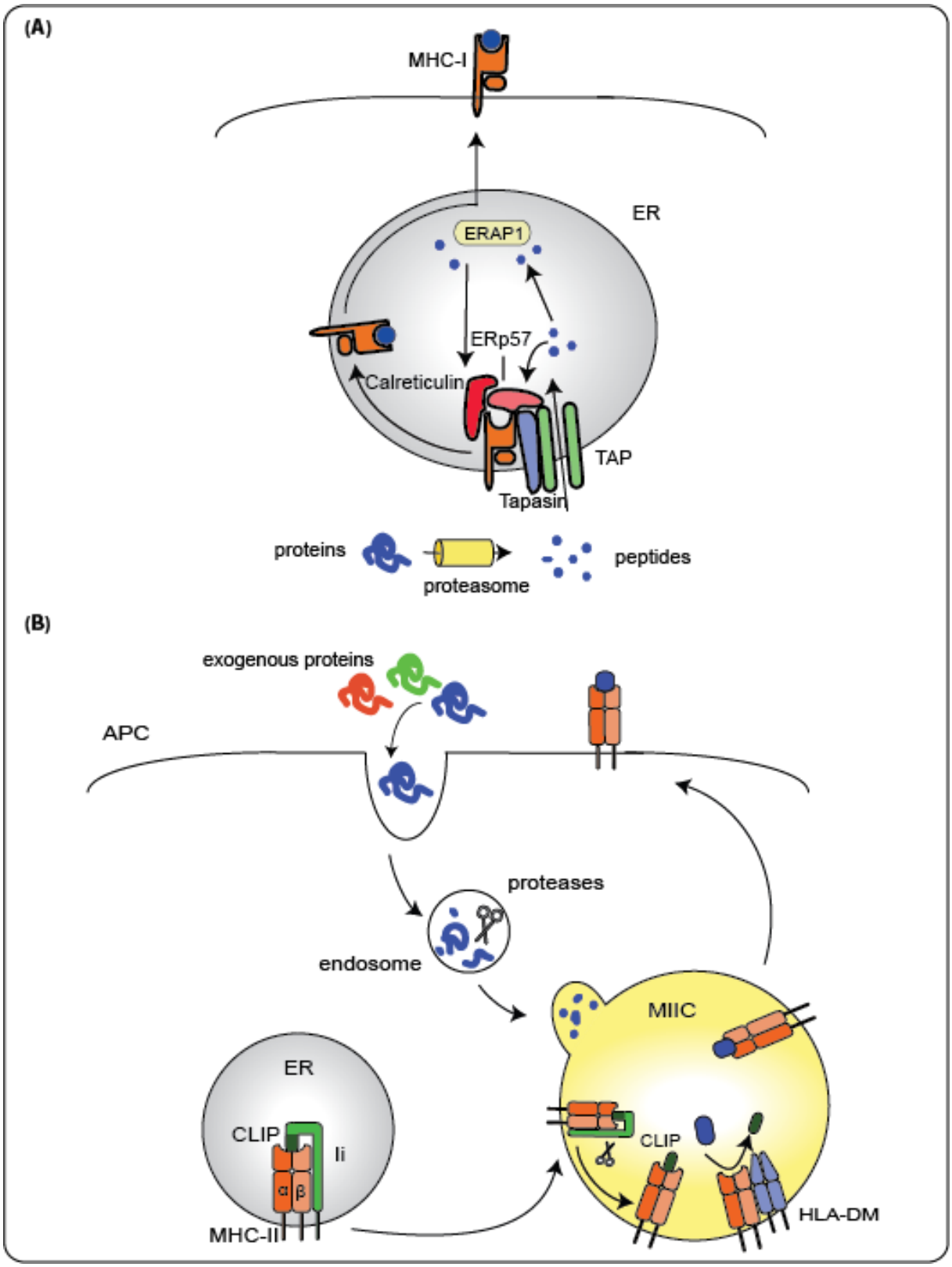

Figure 1-3 Comparison of peptide loading on MHC molecules. (A) Loading of MHC-I with cytosolic peptides in the ER. The proteasome degrades cytosolic proteins. The peptides are transported via TAP into the ER where they are loaded onto MHC-I with the help of several chaperones. (B) The invariant chain (Ii) binds to MHC-II in the ER. The complex is transported to the MHC-II compartment (MIIC). Ii is degraded but CLIP still blocks the binding groove. HLA-DM exchanges CLIP with compatible peptides, which are degraded in the endosome by proteases (Modified from Neefjes 2011).

In the MIIC Ii is degraded by the proteases Cathepsin S and Cathepsin L, however the class-II associated invariant chain peptide (CLIP) remains in the binding groove (Hsing *et al.,* 2005). The binding of HLA-DM to MHC-II stabilizes MHC-II and prevents degradation. Additionally, HLA-DM catalyses the replacement of CLIP with exogenously supplied peptides. The dissociation of CLIP occurs best at a pH of 5.0 (Sloan 1995). HLA-DM acts as an editor. If the peptide-MHC complex is instable, the peptide is exchanged (Kropshofer *et al.,* 1996). Only stable peptide-MHC-II-complexes are released for presentation on the cell surface.
In contrast to MHC-I, the peptides that bind to MHC-II can protrude out of the binding groove at the N- as well as at the C-terminus (Rammensee 1995). Thereby, the size of the bound peptides is typically 13-17 AA (Rudensky *et al.,* 1991). Shorter peptides can also bind to MHC-II, however peptides shorter than 11 AA are removed by HLA-DM (Kropshofer *et al.,* 1996). However, the part of the peptide in the binding groove, which interacts with the TCR, is usually 9 AA long, with preferred anchor residues at position P1, P4, P6 and P9 (Rammensee 1995). Anchor positions of MHC-II as well of MHC-I can be used for T cell epitope predictions.

1.4 Autoimmune diseases

The immune system established several mechanisms to avoid autoreactivity, like clonal deletion of T cell clones as well as clonal anergy in the absence of a costimulation after the recognition of a peptide-MHC complex. Despite these mechanisms some self-reactive T cells can escape the control mechanisms (Wekerle 1992). However, potentially autoreactive lymphocytes can exist simultaneously without causing problems, if the antigen is not accessible, described as immunological ignorance. Additionally, they are controlled by regulatory T cells (T_{reg}), which prevent their activation. However, if the self-tolerance controls fail, it leads to autoimmune diseases, whereby healthy tissue is attacked and destroyed.

1.4.1 Multiple Sclerosis

Multiple sclerosis (MS) is one of the most frequently neurological diseases in young adults. The disease is characterized by a chronic, inflammatory demyelination of the central nervous system (CNS) (Hartung *et al.,* 2014). Although the etiology is not definite, MS is most likely an autoimmune disease. Lymphocytes pass the distorted blood-brain barrier and infiltrate into the brain and spinal cord, leading to demyelination, axon damage and neurological dysfunction (Vries *et al.,* 2012). At the beginning, inflammation is transient in most patients and remyelination takes place. Later on, this relapse and remitting disease course changes to secondary progressive MS, whereby the disabilities accumulate (Compston and Coles 2008). Histological analysis of MS brains shows that MS can be differentiated into four patterns, with the common characteristic of inflammatory plaques (Lucchinetti *et al.,* 2000).

CD4+ and CD8+ T cells, B cells, plasma cells, macrophages and activated microglia accumulate in several focal lesions, whereby the infiltration is often dominated by CD8+ T cells (Hohlfeld 2015). CD8+ as well as CD4+ T cells injure oligodendrocytes and neurons (Antel *et al.,* 1994, Giuliani *et al.,* 2003). A key player in animal models of MS are myelin specific CD4+ T cells (Hohlfeld *et al*., 2015). Often unconventional T cells are involved in MS. T cell repertoire analysis could show an expansion of a mucosal-associated semi-invariant T (MAIT) cells-related TCR Vβ1+ T cell clone in active brain lesions at disease onset (Hohlfeld *et al*., 2016). Clonally expanded B cells and intrathecal expression of auto-antibodies can also be found in MS patients (von Büdingen *et al.,* 2001, Obermeier *et al.,* 2008, Brändle *et al.,* 2016).

Even though the cause of MS remains unclear, genetics and environmental factors are thought to trigger MS. The recurrence in families is higher than in the common population, especially for monozygotic twins (Compston 2008). Additionally, the expression of different HLA alleles influences the disease. The risk of MS is increased by the expression of different MHC-II molecules, like HLA-DRB1*15:01. On the other hand, the expression of protective class I alleles, like HLA-A*02:01, reduces the risk (Moutsianas 2015). Environmental factors, like lifestyle and infection with Epstein-Barr virus increase the risk for MS (Compston and Coles 2008, Levin *et al.,* 2003). A reason therefore might be molecular mimicry, whereby viral antigens share sequences with self-antigens in the CNS, and thereby trigger autoimmunity (Cusick *et al.,* 2012, Hohlfeld *et al*., 2015).

1.4.2 Paraneoplastic neurological diseases

Paraneoplastic neurological diseases (PND) are a subgroup of encephalitis. Encephalitis are acute inflammations of the brain associated with neurological dysfunctions. The disease is often caused by viral (e.g. herpes simplex virus) or other infections. It can also be antibody-associated, as it is the case for PNDs. However, often the encephalitis is idiopathic (Tunkel *et al*., 2008, Granerod *et al*., 2010).

PNDs occur as a result of tumor immunity, whereby anti-neuronal antibodies are found in the serum and/or in the CSF of the patient. The disease is often associated with specific tumors, like small-cell-lung cancer (SCLC), thymoma, lymphoma, breast- and ovarian cancer and neuroendocrine tumors (Höftberger *et al.,* 2015). The antibodies are specific for neuronal antigens, which are also expressed in the tumor. PNDs can be classified into two sub-groups: antibody-mediated PND and T cell mediated PND.

In the antibody-mediated PND, the antibodies target cell-surface antigens, like N-methyl-D-aspartate (NMDA) receptors or γ-aminobutyric acid type B ($GABA_B$) receptors. Thus they are directly pathogenic. Although the expression of the antibodies is often associated with tumors, they can also occur without cancer (Dalmau and Rosenfeld 2008).

In the T cell mediated PND, the expressed antibodies are targeted against intra-cellular antigens. The disease is mediated by T cells which are probably targeted against the same antigens, which are recognized by the antibodies, as it was observed in patients with anti-Yo (also called cdr2) and anti-Hu antibodies (Albert *et al.,* 2000, Benyahia *et al.,* 1999). Additionally, a high infiltration of oligoclonal cytotoxic T cells (CTL) is found in the brain as well as in the tumor and the treatment of only the humoral immunity is not promising (Dalmau and Rosenfeld 2008).

The diagnosis of PND is difficult, because the first symptoms often occur before cancer is diagnosed. The disease can be classified by classic syndromes combined with the expression of paraneoplastic antibodies. Additionally, inflammation of the CSF can be observed. However, atypical symptomes, the presence of poorly characterized antibodies and the missing determination of a tumor can impede the diagnosis (Dalmau and Rosenfeld 2008).

The comparison of the TCR repertoire in the tumor and the CNS can deliver insights into the pathology of the disease. Pellkofer *et al.* (2009) could detect identical T cell clones in the tumor, CSF and the blood of a patient with paraneoplastic encephalomyelitis with adrenal neuroblastoma. This indicates a shared antigen between the tumor and the affected CNS tissue, opening new strategies for therapies. PNDs are usually treated by removing the tumor in combination with immunotherapy and plasmaphereses, to eliminate the antibodies (Höftberger *et al.,* 2015).

1.4.3 $GABA_A$ receptors in diseases

The $GABA_A$ receptor is involved in various diseases. Differences in the expression pattern of $GABA_A$ receptors subunits and point mutations in the different subunits play a role in different types of epilepsy, anxiety and schizophrenia (Moehler 2006).

A new disease pattern was first described by Petit-Pedrol *at al.,* 2014. They detected antibodies against $GABA_A$ receptor α1 and β3 subunits in the serum and CSF of patients who suffer from encephalitis with refractory seizures and status epilepticus. The antibodies selectively reduce the expression of $GABA_A$ receptor clusters at synapses, which causes refractory seizures. Patients with high antibody titres showed behaviour changes and cognitive deficits combined with extensive cortical and subcortical brain MRI abnormalities. The seizures were refractory to anti-epileptic treatment, however some patients responded to immunotherapy. Anti-$GABA_A$ antibodies can appear in combination with antibodies against intracellular antigens, like glutamic acid decarboxylase 65 (GAD65), as well as antibodies against surface antigens, like NMDA or $GABA_B$ receptors (Petit-Pedrol *et al.,* 2014, Ohkawa *et al.,* 2014, Pettingill *et al.,* 2015).

In another study by Pettingill *et al,* 2015 IgG1 antibodies against the α1 and γ2 subunits were identified. Additionally, IgM antibodies against $GABA_A$ receptor without subclass specificity

were found in patients with low antibody titres. Only 47 % of the patients suffered from seizures, however other symptoms like memory impairment (47 %), hallucinations (33 %) and anxiety (20 %) were observed (Pettingill *et al.,* 2015).

Even though the disease has similarities with PNDs, an underlying tumor was not frequently found. In the study of Petit-Pedrol *at al.,* 2014 one patient out of 18 was observed with a thymoma. Ohkawa *et al.*, 2014 also described two patients with invasive thymoma in combination with the expression of antibodies against $GABA_A$ receptor β3 subunit. Simabukuro *et al.,* 2015 could even show by staining of tissue sections that the tumor tissue of a patient, with thymoma and $GABA_A$ receptor antibodies, expresses $GABA_A$ receptors. These findings indicate a linkage of $GABA_A$ receptor associated encephalitis and thymoma. This linkage shows that the disease presumably is paraneoplastic. However, because the disease pattern is quite new, more patients need to be screened for antibodies against $GABA_A$ receptor to verify the linkage.

1.4.3.1 Diagnosis Index patient 2

The case Index patient 2 (IP2) was one of the index patients described by Petit-Pedrol *et al.,* 2014. IP2 was a 51-year-old male, in whose serum and CSF antibodies against α1, β3 subunit of $GABA_A$ receptor were detected. The patient showed behavioural changes, depressions and psychosis, followed by clonical seizures and epilepsy, resulting in status epilepticus. The patient was treated with anticonvulsants and immunosuppressants, however after 10 weeks the status epilepticus persisted and the patient died of a sepsis (Petit-Pedrol *et al.,* 2014).

E. Beltrán (Institut für klinische Neuroimmunologie, Ludwig-Maximilians-Universität München, AG Dornmair) cloned the antibody from CSF and could show, in cooperation with J. Dalmau (August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain) that it binds to the α1 subunit (unpublished data). IP2 suffered from a synaptic reduction of $GABA_A$ receptors, caused by antibody-mediated internalization of the receptor.

Even though the availability of auto-antibodies and their antigens are described, the pathogenesis of the disease is still unclear. Is the disease paraneoplastic, associated with a thymoma? And which role do the T cells play in the diasease? The fact that antibodies against $GABA_A$ α1 subunit were found in the patient, already indicates an involvement of CD4+ T cells, which are crucial for a humoral immune response. For other PNDs it was shown that CD8+ T cells have a key role, with the presence of cross-reactive T cells in the tumor tissue as well as in the CSF, indicating a shared antigen (Pellkofer *at al.,* 2009). Which role do CD8+ and CD4+ T cells play in $GABA_A$ receptor encephalitis?

Thus, it is indispensable to uncover disease related T cells and their antigens for a better understanding of the disease pattern, as well as for a specific therapy.

1.4.3.2 Structure and function of γ-Aminobutyric acid type A receptor

γ-Aminobutyric acid type A ($GABA_A$) receptors are ligand gated ion channels, which belong to the Cys-loop superfamily (Connolly and Wafford 2004). They are responsible for fast inhibitory transmission in the CNS (Schofield *et al.,* 1987). The heteropentameric structure is formed by a combination of different subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3) (Barnard *et al.,* 1998, Mehta and Ticku 1999), however a combination composed of two α_1, two β_2 and one γ_2 subunits is most abundant (Sieghart and Sperk 2002). One subunit consists of an extracellular domain and four transmembrane (TM) domains connected by an intracellular loop between TM3 and TM4 (Karlin and Akabas 1995, Figure 1-4). The subunit composition determines the properties of the receptor, such as the affinity for ligands, it's conductance as well as the kinetics (Gonzalez 2013).

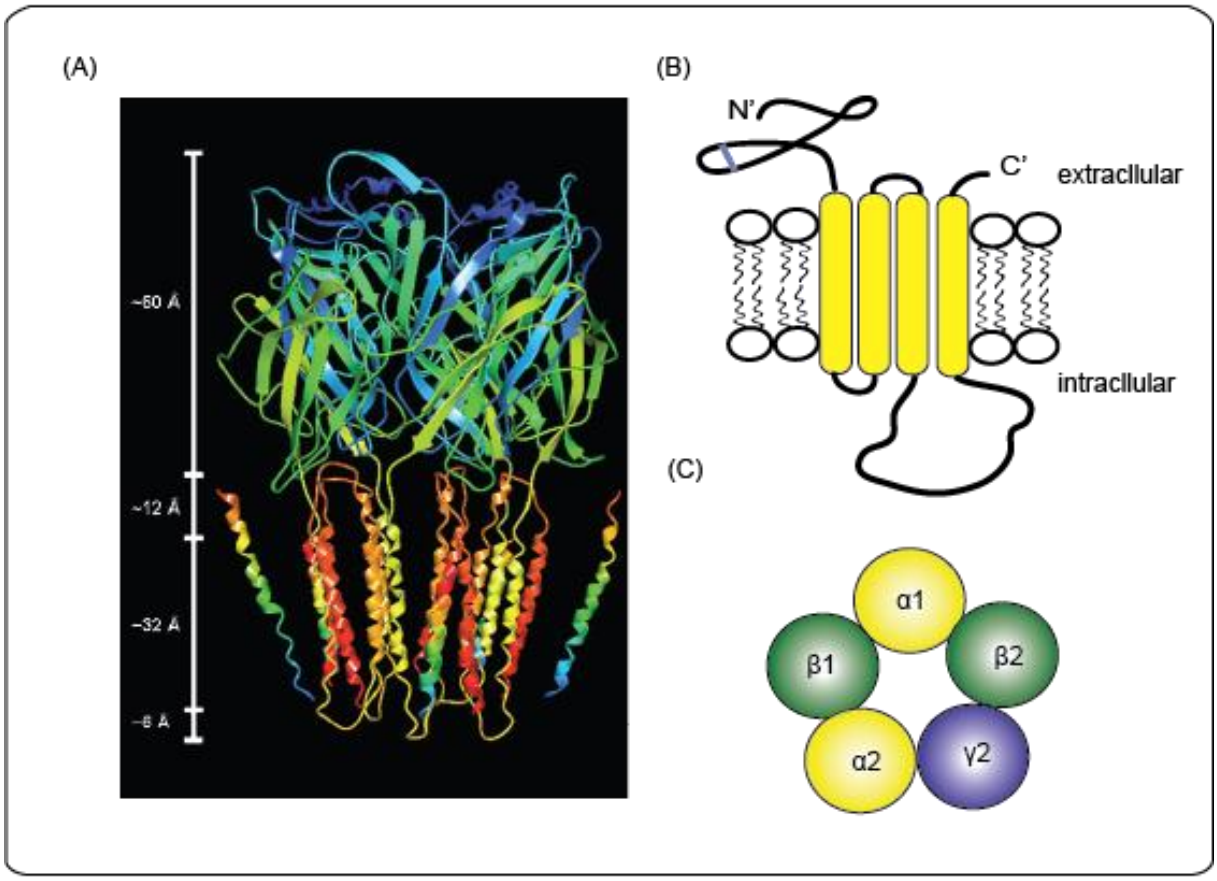

Figure 1-4 Illustration of $GABA_A$ receptor. (A) Model of the side view of $GABA_A$ receptor (adopted from Campagna-Slater 2007), illustrating the transmembrane domain (bottom) and the extracellular domain (top). (B) Model of one subunit of $GABA_A$ receptor, composed of an N-terminal extracellular domain with disulfide bridge and four transmembrane domains, connected with an intracellular loop between TM3 and TM4. (C) Illustration of the formation of a $GABA_A$ receptor by 5 subunits forming a pore in the membrane.

$GABA_A$ receptors contain two GABA binding sites located at the interface of α and β subunits (Sigel *et al.,* 1992, Amin and Weiss 1993). Binding of agonistic GABA leads to a conformational change, which results in an opening of the pore and, thereby, chloride and bicarbonate ions

can pass through the pore (Kash *et al.,* 2004). The influx of Cl^- results in an inhibitory hyperpolarization (Goetz *et al.,* 2007).

As all members of the Cys-loop superfamily, $GABA_A$ receptors can be allosterically modulated by zinc ions. Zinc ions can bind to three allosteric sites, one site in the ion channel and two sites at the α and β interface, and thereby inhibit the receptor function. However, the zinc sensitivity depends on the subunit composition of the receptor (Hosie *et al.,* 2003). Additionally, pharmaceutical drugs, like benzodiazepines, barbiturates, picrotoxin and anesthetical steroids can modulate receptor activity, by binding to allosterical binding sites (Olsen and DeLorey 1997).

$GABA_A$ receptors are localized at inhibitory synapses as well as outside of synapses (Somogyi *et al.,* 1989) in different regions of the brain, including the hippocampus (Sieghart and Sperk 2002).

Although the $GABA_A$ receptor is most abundant in the brain, other peripheral tissues as well as the immune system, also express $GABA_A$ receptors (Akinci and Schofield 1999, Alam *et al.,* 2006).

1.5 Identification of disease related TCR

Autoreactive T cells play a key role in autoimmune diseases. In psoriasis it was shown that an antigen expressed in melanocytes triggers CD8+ T cell response (Arakawa 2015). In several autoimmune diseases, like MS and $GABA_B$ receptor limbic encephalitis, an infiltration of lymphocytes is observed (Hohlfeld *et al.,* 2016, Golombeck *et al.,* 2016). However often the T cell repertoire, their antigens and their role in the disease remain unknown. For the better understanding of the pathogenesis of autoimmune diseases, as well as for the development of convenient therapies, it is essential to identify disease related TCR and their antigens.

To distinguish disease relevant T cells from bystander T cells, the morphology of the T cells, especially their location in the lesion is important. Close contact with target cells is a possible hint for autoreactivity. Secondly, the expression of activation markers and oriented expression of lytic granular, like perforin and granzyme B, indicate disease related T cells in the lesion. Last but not least the analysis of the TCR repertoire can show if the T cell population is polymorphic or if a clonal expansion of one or more T cell clones is present in the lesion, indicating a proliferation after antigen stimulation (Dornmair *et al.,* 2003).

The TCR α- and β-chains can be identified by a clone-specific approach, combining CDR3 spectratyping of the β-chain, staining of tissue sections for corresponding TCR Vβ regions and laser microdissection of the cells. After RT-reaction and pre-amplification of the chains, the β-chain is amplified with clone-specific primers and the α-chain with a universal primer set (Seitz *et al.*, 2006). However, this technique is limited to the availability of antibodies for the Vβ-chains. An improved approach by Kim *et al.* (2012) allows the analysis of both, the α-chain as

well as the corresponding β-chain at once. This unbiased multiplex RT-PCR combines the described universal primer set for the amplification of the α-chain (Seitz *et al.*, 2006) with an unbiased primer set to amplify all possible β-chains. This allows analysis of both, the α-chain as well as the corresponding β-chain of a single cell (Kim *et al.*, 2012).

A new strategy to analyse the T cell repertoire is by next generation sequencing (NGS). This technique allows thousands of sequences to be obtained in a single run (Ruggiero *et al.,* 2015, Held *et al.,* 2015). By the usage of a unique molecular identifier (UMI), expanded T cells clones can be distinguished from PCR amplificates, and thereby a quantification of the T cell clones is possible (Shugay *et al.,* 2014). However, NGS only shows the expansion of different α- and β-chains, but the matching chains cannot be assigned. Only because one α-chain shows up for example 20 times, it is still not proven, that it pairs with the β-chain which also shows up 20 times. Thus, to concomitantly identify pairs of α- and β-chains, single cell analysis is still required.

1.6 Identification of TCR antigens

The antigen identification of disease related TCR is challenging. For the correct presentation of peptides, the proteins have to be processed correctly in the cell and loaded onto MHC moleucles. Additionally, TCRs have a low affinity for peptide-MHC-complexes. Thereby, the usual biochemical methods, which are based on affinity and are applied in the antigen search of antibodies, like immunoprecipitation, are out of the question (Dornmair *et al.,* 2003).

The usage of cDNA libraries from affected tissue, presented on melanoma cells can be used for the antigen screening (van der Bruggen *et al.,* 1991). This approach assumes the availability of patient material, which is not always the case. Furthermore, a correct antigen processing is required.

Antigen screening with synthetic peptide libraries (Gundlach *et al.,* 1996) can overcome the antigen processing problems. However, the more complex these libraries are, the more diluted is the correct antigen, and might not activate the TCR anymore. Additionally, they cannot be recovered by cloning (Siewert *et al.,* 2012).

Moreover, the MHC repertoire in one individual is polymorph as well as polygenic (Neefjes *et al.,* 2011). Usually, one individual is heterozygote for MHC alleles. Thereby, different MHC molecules are expressed on the cell surface and the MHC-restriction of the disease related TCR is unknown.

The detection of cytokines, like INF-γ, by enzyme linked immunosorbent assay (ELISA) are often used as read-out for T cell activation (Fujii *et al.*, 2001). However, this read-out is not sensitive enough for antigen screenings with libraries.

1.6.1 Methods for the Identification of CD8+ T cell receptor mimotopes

To overcome these problems, Siewert *et al.,* 2012 designed an unbiased method for the identification of CD8+ T cell mimotopes with plasmid-encoded combinatorial peptide libraries (PECPL). Mimotopes are peptides, which mimic the antigen. Thereby they are recognised by the immune system.

For the screening of CD8+ T cell antigens, COS-7 cells are co-transfected with plasmids coding for MHC-I and PECPL. The short peptides are loaded onto MHC-I molecules and get presented on the cell surface. These cells are overlaid with $58^{-/-}$ cells, which are transfected with hCD8αβ and the TCR α and β-chain of interest. The advantage of $58^{-/-}$ cells is, that they are negative for TCR α- and β-chain. Thereby they do not express a TCR on the cell surface but possess the signalling machinery for T cell activation. Thereby, they do not form chimeric TCR with the transfected chains (Blank *et al.*, 1993). The transfected α- and β-chain associate with the murine CD3 and show up on the cell surface (Figure 1-5). Additionally, the cells are transfected with sGFP under the control of the NFAT-enhancer. After the binding of the TCR to the correct peptide-MHC complex, the signal cascade triggers sGFP expression causing the cell to fluorescence green (Figure 1-5).

By transfection with PECPL, no antigen processing is required. Short peptides are directly expressed and loaded onto MHC-I molecules. In addition, no knowledge about the possible antigen is necessary. The cells are kept in physical contact by gravity, thus high affinity is not needed. The detection system, based on single cell activation is highly sensitive, and the correct peptides can be detected among millions of irrelevant peptides. In contrast to peptide libraries, the plasmids can be easily recovered by PCR (Siewert *et al.,* 2012).

New candidate antigens of a presumably autoreactive, CD8+ TCR from a MS patient were identified using this screening approach (Rühl *et al.,* 2016). Moreover, melanocytes expressing ADAMTS-like protein 5 (ADAMTSL5) were identified as a target of a psoriatric, autoreactive TCR (Arakawa *et al.,* 2015).

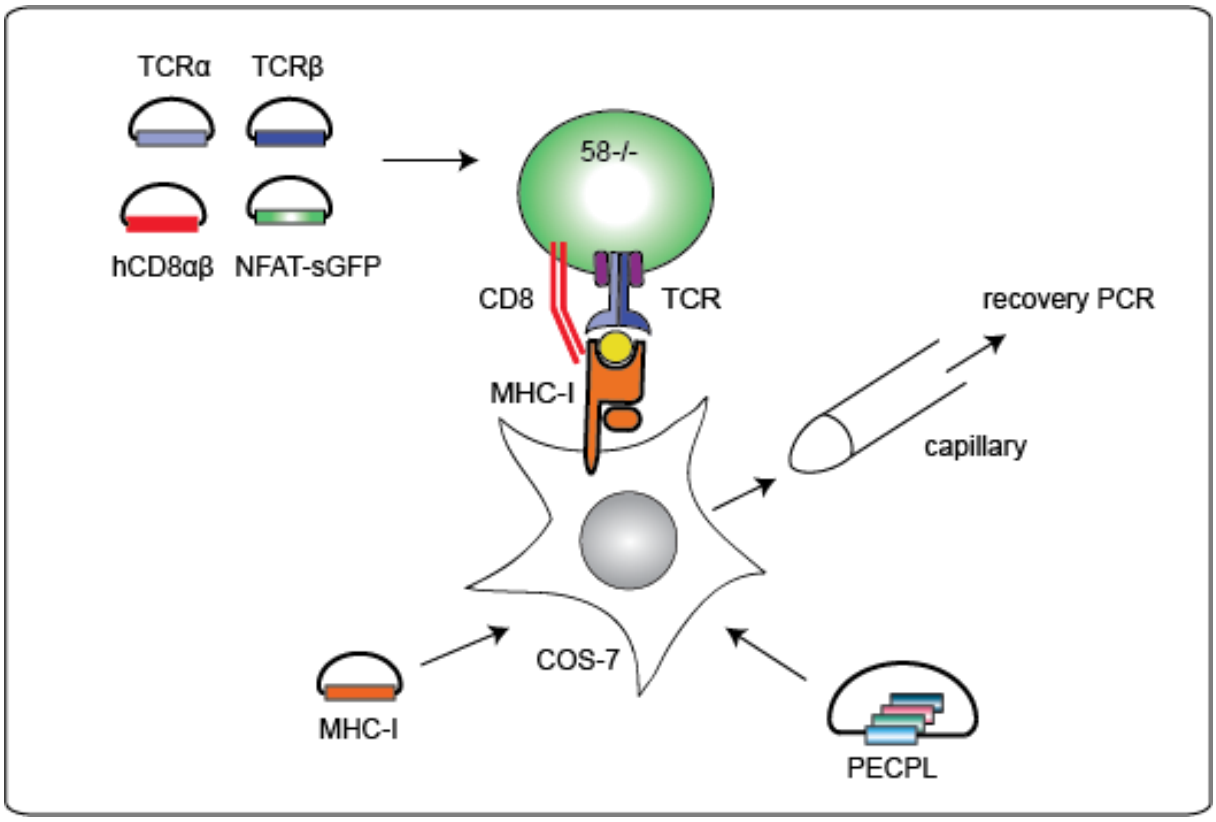

Figure 1-5 Unbiased identification of CD8+ T cell antigens with plasmid-encoded combinatorial peptide libraries. A eukaryotic cell detection system is used for the antigen identification of the TCR of interest. $58^{-/-}$ T hybridoma cells are transfected with TCRα and TCRβ-chains. Additionally, hCD8αβ and sGFP under the NFAT-enhancer are transfected. In parallel, COS-7 cells are transfected with MHC-I and a plasmid-encoded combinatorial peptide library (PECPL). COS-7 cells express the peptide library, which are loaded onto MHC-I molecules and presented on the cell surface. The two cell lines are cocultivated for 16 h. After binding of the TCR of $58^{-/-}$ to the correct peptide-MHC-complex, sGFP synthesis is triggered. The underlying COS-7 can be isolated with a capillary via a micromanipulator and recovery PCR is performed. Modified from Siewert *et al.* 2012.

1.6.2 Methods for the identification of CD4+ TCR mimotopes

The method established by K. Siewert *et al.* (2012) is only useable for the detection of CD8+ T cell antigens. Other trials were made for the identification of CD4+ t cell antigens. Sanderson *et al.* demonstrated 1995 that fusion proteins with the invariant chain successfully enter the MHC-II presentation pathway, and hence are efficiently presented by MHC-II molecules on the cell surface. These Ii derivates showed an enhanced T cell stimulation compared to native or recombinant proteins (Malcherek *et al.,* 1998). However, comparable to CD8+ T cell antigens, the cells have to uptake the proteins, process them and load them correctly onto MHC-II molecules.

For library screening, fibroblast cells expressing library peptides covalently fused to the N-terminus of the β-chain of MHC-II can be used and cocultivated with hybridoma cells expressing the TCR (Boen *et al.,* 2000). However, new libraries have to be generated for every HLA.

Another possibility is the application of a vector, coding for the invariant chain, in which CLIP can be substituted by antigenic peptides or a library (Fujii *et al.,* 2001). However, the read-out system with the detection of INF-γ in the supernatant of the cells is not sensitive enough for library screening

In this study the detection system established by K. Siewert *et al.* (2012) for the identification of CD8+ T cell antigens is modified to allow the detection of CD4+ T cell antigens. Some parts of the detection method can be adopted, like the detection system. Other parts, like the libraries, have to be designed for the usage for the identification of CD4+ T cell antigens.

In the CSF of IP2 antibodies against the $GABA_A$ receptor were detected. This indicates an involvement of CD4+ T cells in the disease, because CD4+ T cells are crucial for most humoral immune responses. The new established antigen screening system for CD4+ T cell antigens could be applied to possible disease related CD4+ T cells in $GABA_A$ receptor encephalitis. The identification of CD4+ T cell antigens could contribute to a better understanding of the pathology of $GABA_A$ receptor encephalitis.

Moreover, the new screening system for the detection of CD4+ T cell antigens could also be applied for other autoimmune diseases with involved CD4+ T cells, like MS. The knowledge of the antigens does not only contribute to a better understanding of the diseases, but also opens new ways for possible therapies.

1.7 Aim of this study

Patients with $GABA_A$ receptor encephalitis suffer from a synaptic reduction of $GABA_A$ receptors, caused by antibody-mediated internalization of the receptor. Even though auto-antibodies and their antigens were described, nothing is known about the role of T cells in these patients. Thus, for a better understanding of the disease pathogenesis, as well as for a future specific therapy, it is indispensable to uncover disease related T cells and their antigens. The presence of antibodies against $GABA_A$ α1 subunit already indicate the existence of CD4+ T cells, which are crucial for most humoral immune responses. Moreover, CD8+ T cells often play a key role in autoimmune diseases.

For the better understanding of the role of T cells in the disease, disease related T cells have to be distinguished from bystander T cells in the TCR repertoire. For interesting, expanded clones, the disease related TCR, their HLA-restriction, and antigenic peptides must then be analysed. Although a sensitive method is available for the identification of CD8+ T cell antigens, no method is available to identify CD4+ T cell antigens among millions of bystanders.

The aim of this study was therefore focused on:

The CD4+ and CD8+ TCR repertoire of IP2 should be analysed in the CSF, the hippocampus and the lateral sulcus. To concomitantly identify matching α- and β-chains of the TCR, single cells are analysed. The repertoire is analysed using the following methods:

- Identification of matching α- and β-chains from single cells of the CSF of IP2
- Immunohistochemistry (IHC) for the infiltration of CD8+ and CD4+ T cells in the brain
- Single cell analysis of TCRs from brain sections isolated by laser microdissection
- Bulk cell analysis by a clone-specific approach and by next generation sequencing in cooperation with E. Beltrán

The identified disease related, presumably auto-reactive TCR of IP2 should then be analysed for their antigens. To this end, expanded T cell clones should be expressed *in vitro* and possible candidate antigens should be screened for T cell activation. Additionally, possible expanded CD8+ T cell clones should be analysed by the application of the unbiased PECPL method. Based on the expression of autoreactive antibodies in IP2, we expect to also identify expanded CD4+ T cell clones. To identify antigens of such possible CD4+ T cell candidates, the PECPL method should be modified to allow screening of MHC-II peptides. Following points should be established in parallel to the repertoire studies for the screening of CD4+ T cell antigens:

- Design and generation of new libraries
- Confirmation of the functionality of the system by the application of the well characterised, MS derived model T cell clone “Bbc9” as proof of concept

2 Material and Methods

2.1 Material

2.1.1 Instruments

Balance	Entris (Sartorius, Göttingen, Germany)
	Pioneer™ PA214CM (Ohaus, Greifensee, Switzerland)
Centrifuges	Sprout (Heathrow Scientific LLC, Illinois USA)
	5417 R (Eppendorf, Hamburg, Germany)
	Haraeus™ Multifuge™ X3R (Thermo Scientific, Waltham, Massachusetts, USA)
	Avanti JXN-26 Rotor JA10 (Beckman Coulter, Krefeld, Germany)
Cell counting chamber	Neubauer (Carl Roth GmbH, Karlsruhe, Germany)
Cryotom	CM 1950 (Leica, Wetzlar, Germany)
Electroporation	Gene Pulser (Bio-Rad, Munich, Germany)
	4D Nucleofector™ Core Unit (Lonza, Basel, Switzerland)
	4D Nucleofector™ X Unit (Lonza)
ELISA reader	Wallac VICTOR² 1420 Multilabel Counter, (Perkin Elmer, Waltham, Massachusetts, USA).
Flow Cytometer	BD FACSVerse™ (Beckton Dickinson, Franklin Lakes, New Jersey, USA)
	BD FACSAria ™ Ilu (Beckton Dickinson)
Incubator	APT.Line™ BD (E2) (Binder, Tuttlingen, Germany)
	New Brunswick™ Innova 44 (Eppendorf)
	New Brunswick™ Galaxy 170S and 170R (Eppendorf)
Imager	AlphaIager™ (Biozym Scientific, Oldendorf, Germany)
	Odyssey Fc (LI-COR, Lincoln, Nebraska, USA)
Laser	CryLaS FTSS 355-50 (Zeiss, Oberkochen, Germany)
Microscope	Wilovert S (Wilhelm Will KG, Wetzlar-Nauborn, Germany)
	DM IL LED (Leica)
	Axiovert 200 M (Zeiss)
Objectives	Plan-Apochochromat® 5x/0.16; ∞/0.17 (Zeiss)
	Plan-Apochochromat® 10x/0.45 (Zeiss)
	Plan-Apochochromat® 20x/0.8 (Zeiss)
Filter	sGFP: 472/30 nm, 520/35 (BrightLine Flourescence Filter, Semrock) and Cy3: 545/25 nm and 605/70 nm (Zeiss)
CCD Camera	CoolSNAP HQ2 (Photometrics, Tucson, AZ, USA)
Lamp	Fluoreszenz lamp HXP 120 (Visitron, Puchheim, Germany)

	AxioObserver.Z1 (Zeiss)
Objectives	Plan-Apochromat® 5x/0.16 (Zeiss)
	Plan-Apochromat® 10x/0.45 (Zeiss)
	EC Plan-Neofluar® 20x/0.5 (Zeiss)
	EC Plan-Neofluar® 40x/0.75 (Zeiss)
Filter	sGFP: ET525/50M and ET470/40; Cy3: ET630/75M and ET560/40X; (CHROMA, Olching, Germany)
CCD Camera	CoolSNAP HQ2 (Photometrics)
Lamp	Sola-SM Light Engines® (Lumencor, Inc., Beaverton, Oregon, USA)
	Axiovert 200 M (Lasermicrodissection)
Objectives	LD Plan-Neofluar 20x/0.40 Korr (Zeiss)
	LD Plan-Neofluar 40x/0.60 Korr (Zeiss)
Filter	10x basic-Z FITC/GFP BP450-490 and BP515-565; Cy3: 20x basic BP546/12 and BP575-640 (Zeiss)
CCD Camera	HV-D30 (Hitachi KokusaiElectric America, Woodbury, NY, USA)
Lamp	Fluoreszenz lamp HXP 120 (Visitron)
Microinjector	Cell Tram Vario (Eppendorf)
Micromanipulator	LN25 Mini (Luigs und Neumann, Ratingen, Germany)
pH meter	pH521 (WTW, Weilheim, Germany)
Pipetting Robot	Janus Automated Workstation (Perkin Elmer, Waltham, Massachusetts, USA)
Power Supply	EPS 3501 XL (Amersham Pharamacia Biotech, Freiburg, Germany)
	LKB ECPS 3000/150 (Amersham Pharamacia Biotech)
	PowerPac300 (Bio-Rad, Munich, Germany)
Robo Mover	PALM Microlaser Technologies (Bernried, Germany)
Spectrophotometer	NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA)
	UV-1600 PC Photospectrometer (VWR International GmbH, Darmstadt, Germany)
Sterile Bench	FlowSafe B-[MaxPro]^2-160 (Berner, Elmshorn, Germany)
Thermocycler	T3 Thermocycler (Biometra, Göttingen, Germany)
	Bioer Genetouch Thermal Cycler (Biozym)
	Mastercycler gradient (Eppendorf)
	Master cycler pro vapo protect (Eppendorf)
Water preparation	MilliQ Advantage (Merck Millipore, Billerica, Massachusetts, USA)

2.1.2 Plasmids

Plasmid	Size	Resistance Gene	Reference
pHSE3'-HLA-DRA*	9.5 kb	amp^R, hygro^R	Section 2.4.5.5
pHSE3'-HLA-DR1*	10.0 kb	amp^R, neo^R	Section 2.4.5.5

pHSE3'-HLA-DR2a*	10.0 kb	amp^R, neo^R	Section 2.4.5.5
pHSE3'-HLA-DR2b*	10.0 kb	amp^R, neo^R	Section 2.4.5.5
pHSE3'-HLA-A*02:01*	10.2 kb	amp^R, neo^R	Section 2.4.5.2
pHSE3'-HLA-A*23:01*	10.2 kb	amp^R, neo^R	Section 2.4.5.2
pHSE3'-HLA-B*35:01*	10.2 kb	amp^R, neo^R	Section 2.4.5.2
pHSE3'-HLA-B*44:03*	10.2 kb	amp^R, neo^R	Section 2.4.5.2
pHSE3'-HLA-C*04:01*	10.2 kb	amp^R, neo^R	Section 2.4.5.2
pHSE3'-HLA-C*16:01*	10.2 kb	amp^R, neo^R	Section 2.4.5.2
pHSE3'-Blasticidin*	8.2 kb	amp^R, bls^R	Section 2.4.5.8
pHSE3'-Blasticidin-largeT antigen*	10.3 kb	amp^R, bls^R	Section 2.4.5.8
pRSV-AV8S1*	6.1 kb	amp^R, $hygro^R$	Section 2.4.5.3
pRSV-BV8S1*	6.1 kb	amp^R, neo^R	Section 2.4.5.3
pRSV-AV22S1	6.1 kb	amp^R, $hygro^R$	G. Giegerich, K-Dornmair
pRSV-BV9.1	6.1 kb	amp^R, neo^R	G. Giegerich, K-Dornmair
pcDNA6/V5-HisA	5.1 kb	amp^R, bls^R	Invitrogen
pcDNA-NFAT-sGFP	7.4 kb	amp^R, bls^R	K. Siewert, K. Dornmair
pcDNArc-spacer	7.6 kb	amp^R, bls^R	K. Siewert, G. Rühl
pcDNA-candidates IP2-8S1*	5.1 kb	amp^R, bls^R	Section 2.4.5.4
pcDNA-N27	5.1 kb	amp^R, bls^R	G. Rühl
pc-DNA-Ii-wt*	5.7 kb	ampR, blsR	Section 2.4.5.6
pcDNA-delta-Ii*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-Ii-hMBP141-149*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-Ii-hMBP141-150*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-Ii-hMBP141-151*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-Ii-hMBP139-151*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-delta-Ii-hMBP141-149*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-delta-Ii-hMBP141-150*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-delta-Ii-hMBP141-151*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-delta-Ii-hMBP139-151*	5.7 kb	amp^R, bls^R	Section 2.4.5.6
pcDNA-Ii-ECPL-N27*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N27-M1,9*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N33*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N33-M2,10*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N36-C*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N36-N*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N36-C-M2,10*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N36-N-M3,11*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N39*	5.7 kb	amp^R, bls^R	Section 2.4.5.7
pcDNA-Ii-ECPL-N39-M3, 11*	5.7 kb	amp^R, bls^R	Section 2.4.5.7

***** constructed during this study

2.1.2.1 Plasmids expressing possible IP2-8S1 antigen-candidates

Plasmid	Size	Resistance Gene	Reference
pcDNA-CLWAWILLL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-GLGERVTEV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-GLGERVTEV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-ILSQVSFWL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-TAMDWFIAV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-FIAVCYAFV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-LLFGIFNLV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-LLYTMRLTV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-YLPCIMTVI*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-IMTVILSQV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-YAWDGKSVV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-RLSRIAFPL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-IAFPLLFGI*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-PLLFGIFNL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-GIFNLVYWA*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-NLVYWATYL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-TMPNKLLRI*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-HLEDFPMDA*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-VLTMTTLSI*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-AMDWFIAVC*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-EYVVMTTHF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-TYLPCIMTV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-WFIAVCYAF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-CYAFVFSAL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-FVFSALIEF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-IVQSSTGEY*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-MDWFIAVCY*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-KPKKVKDPL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-PVSDHDMEY*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-DAHACPLKF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-LPCIMTVIL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-WFIAVCYAF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-IAVCYAFVF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-MEYTIDVFF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-IEFATVNYF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-LEDFPMDAH*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-GEYVVMTTH*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-LLDGYDNRL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4

pcDNA-VFTRILDRL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-KTDIFVTSF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-PMDAHACPL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-QYDLLGQTV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-GYFVIQTYL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-KPPEPKKTF*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-TYLNREPQL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-MTVLRLNNL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-YAYTRAEVV*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-FHLKRKIGY*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4
pcDNA-TATSYTPNL*	5.1 kb	amp^R, $hygro^R$	Section 2.4.5.4

***** constructed during this study

2.1.3 Primer

All primers were ordered at Metabion (Planegg, Germany) dissolved as 100 µM solution. For the synthesis of libraries, RT reaction and the touch down PCR of the multiplex single cell TCR PCR, primers were ordered HPLC purified. For other purposes primers were ordered desalted. Primer sequences see Section 5.1.

2.1.4 Buffers and solutions

DNA loading buffer (6×)	10 mM Tris-HCl pH 7.5, 50 % (v/v) glycerin, 0.02 % (m/v) bromphenol blue, 0.02 & (m/v) xylenblue
FACS Buffer	PBS (pH 7.2), 1 % FBS
Laemmli sample buffer (reducing, 3×)	0.15 M tris-HCL pH 6.8, 7.5 % (w/v) SDS, 45 % glycerol, 0.01 % (w/v) bromphenol blue, 6 % (v/v) β-mercaptoethanol
ELISA Wash	PBS + 0.05 % (v/v) Tween 20
MACS Buffer	PBS (pH 7.2), 2 mM EDTA, 0,5 % FBS
SDS running buffer	0.1 % (w/v) SDS, 0.02 M tris, 0.2 M glycine
TBE Buffer	90 mM Tris-HCl, pH 8.0, 90 mM boric acid, 2 mM EDTA
Tris-EDTA Buffer	10 mM Trizma base, 1 mM Titriplex, 0.05 % Tween-20
Western Blot blocking buffer	PBS + 5 % (w/v) Blotting grade blocker (Bio-Rad, Munich, Germany)
Western Blot buffer anode	50 mM boric acid, 20 % (v/v) methanol, pH 9
Western Blot buffer cathode	50 mM boric acid, 5 % (v/v) methanol, pH 9
Western Blot washing buffer	PBS + 0.05 % (v/v) Tween 20

2.1.5 Media and additives

Ampicillin	0.1 mg/ml (Sigma, St. Louis, Missouri, USA)
Blasticidin	Invitrogen, Carlsbad, USA

Freezing media	FBS + 10 % (v/v) DMSO
Geneticin (G418) Sulfate	Santa Cruz, Dallas, Texas, USA
Hygromycin B	50 mg/ml (Invitrogen)
Lysogeny broth media (LB)	0.1 % (w/v) Bacto Tryptone (Becton Dickinson, Franklin Lakes, New Jersey, USA) 0.05 % (w/v) yeast extract (Becton Dickinson) 0.1 % (w/v) NaCl (Sigma-Aldrich, St. Louis, Missouri, USA)
LB plates	LB media + 1.5 % Bacto Agar (Becton Dickinson)
Puromycin	Biomol, Hamburg, Germany
RPMI complete	RPMI 1640 (Sigma-Aldrich) 10 % FBS (Biochmrom GmbH, Berlin, Germany)) 100 U/ml Penicillin (Invitrogen) 100 µg/ml Streptomycin (Invitrogen) 1× MEM non-essential amino acids (Invitrogen) 1 mM MEM sodium pyruvate (Invitrogen) 2 mM L-Glutamin (Sigma-Aldrich)
Trypsin	Trypsin-EDTA Solution: 0.05 % Trypsin, 0.02 % EDTA (Sigma-Aldrich)

2.1.6 Enzymes, Kits and Reagents

Cloning Kits	TOPO® TA-Cloning (Invitrogen) pcDNA™ 3.1 Directional TOPO® Expression Kit (Invitrogen)
Co-precipitant	Pellet Paint (Merk Millipore)
DNA isolation kit	QIAamp DNA Micro Kit (Qiagen GmbH, Hilden, Germany)
Gel Extraction Kit	MinElute (Qiagen GmbH) QIAquick (Qiagen GmbH)
Glycogen for mol. Bio.	20 mg/ml (Roche, Mannheim, Germany)
iProof™	High-Fidelity DNA Polymerase 2 U/µl (Bio-Rad)
Nucleofection	Amaxan™ SE Cell Line 4D-Nucleofector™ X Kit (Lonza)
PeqGreen	DNA/RNA Dye (Peqlab Biotechnologie GmbH, Erlangen, Germany)
PCR Purification Kit	MinElute (Qiagen GmbH, Hilden, Germany) QIAquick (Qiagen GmbH)
Plasmid purification	QIAprep Spin MiniPrep Kit (Qiagen) HiSpeed Plasmid Maxi Kit (Qiagen) Pure Yield™ Plasmid Miniprep System (Promega, Madison, WI, USA)
Rapid DNA Dephosphorilation and Ligation Kit	Roche

restriction enzymes	Aat-II, AgeI-HF, AscI, BamHI, KasI, NotI-HF (New England Biolabs, Ipswich, MA, USA)
RT-PCR	OneStep RT PCR Kit (Qiagen GmbH, Hilden, Germany) Superskript III, 200 U/µl (Invitrogen, Carlsbad, USA)
T4 DNA Ligase	1 U/µl (Invitrogen)
Taq DNA Polymerase	2,5 U/µl (Roche)
Transfection Reagents	FuGENE® HD Transfection Reagent (Promega, Madison, WI, USA)

2.1.7 Antibodies

Table 2-1 Primary antibodies

Specificity	Clone (Isotype)	Lable	Concentration [mg/ml]	Company
hCD3	Polyclonal (rabbit)	-	0.6	Dako, Glostrup, Denmark
mCD3e	145-2C11 (Hamster IgG)	-	0.5	eBioscience, San Diego, USA
mCD3e	145-2C11 (Hamster IgG)	APC	0.2	eBioscience
mCD3e	145-2C11 (Hamster IgG)	FITC	0.5	eBioscience
hCD4	RPA-T4 (mouse IgG1, κ)	AF-488	-	Becton Dickinson, Franklin Lakes, New Jersey, USA
hCD4	4B12 (mouse IgG1, κ)	-	244	Dako
mCD4	GK1.5 (Rat IgG2b, κ)	PE	0.2	eBioscience
hCD8	SK1 (mouse IgG1, κ)	APC	-	BD Bioscience
hCD8α	LT8 (mouse IgG1)	Cy3 (in-house)	1	AbD Serotec, Puchheim, Germany
hCD8α	C8/144B (mouse IgG1, κ)		157	Dako
hCD74	PIN.1 (mouse IgG1)	-	1	Abcam, Cambridge, UK
hHLA-DR	L243 (mouse IgG2a, κ)	FITC	0.012	eBioscience
hPerforin	B-D48 (mouse IgG1)	-	1	Abcam

Table 2-2 Secondary antibodies

Specificity	origin	Lable	Concentration [mg/ml]	Company
mouse IgG	goat	AF488	2	life technologies (Carlsbad, Carlifornia, USA)
mouse IgG1	goat	AF488	2	life technologies
rabbit IgG	goat	AF594	2	life technologies
mouse IgG (H+L)	donkey	IRDye800CW	1	LI-COR
mouse Immunglobulins	goat F(ab')2	FITC	0.4	Dako

Table 2-3 Isotype controls

origin	Lable	Concentration [mg/ml]	Company
mouse IgG1	-	0.05	BD Bioscience
mouse IgG1	-	0.5	life technologies
mouse IgG2a κ	FITC	0.2	eBioscience
armenian hamster IgG	APC	0.2	eBioscience
hamster IgG2, λ1	FITC	0.5	BD Pharmingen
mouse IgG1, κ	APC	0.5	BD Pharmingen
rabbit IgG	-	0.5	BD Pharmingen
mouse IgG1 κ	-	0.5	BD Pharmingen
mouse IgG1	Cy3 (in house)	-	BD Pharmingen

2.1.8 Patient sample: Index patient 2

Antibodies against the α1 subunit of $GABA_A$ receptor were detected in the CSF of IP2. We received a small amount of CSF of the patient and additionally frozen and paraffin embedded biopsy sections of the hippocampus (HC) and the lateral sulcus (LS) from our Spanish cooperation partern Dr. Josep Dalmau.

2.1.9 Eucaryotic cell lines

Cell line	origin	Media	source
Cos-7	African green monkey kidney fibroblast-like cell line	RPMI complete	ATCC
$58^{-/-}$ Hybridoma-cells	TCR deficient mouse T hybridoma	RPMI complete	K. Dornmair (originated from H. von Böhmer and U. Welzien)

LTK$^-$	mouse fibroblasts	RPMI complete	ATCC
LCL	B-lymphoblastoid cell line	RPMI complete	J. Mautner (Helmholtz Zentrum München)
EBV B cells	patient derived	RPMI complete	J. Mautner

2.1.9.1 HLA-typing of LCL and EBV B cells

Cell line	HLA-A	HLA-B	HLA-Cw
17490	02:01, A03:01	35:01, B40:01	03; 04:01
#28	29:02, 30:01	42:01, 44:03	16:01, 17:01
#36	24:03, 29:02	18:01, 44:03	12:03, 16:01
#43	02:01, 23:01	18:01, 44:03	04:01, 07:01
IBi	11:01, 24:02	18:01, 51:01	04:01, 07:01
JM	02:01, 03:01	15:29,51:01	12:03,14:02
KK	01:01, 23:01	50:01, 57:02	06:02, 18
TG	23:01, 24:02	07:02, 07:05	07:02, 15:05
Fe	01:01	08:01	07:01

2.2 Isolation of T cells

2.2.1 MACS Purification of CSF

Cells from the CSF of patient IP2 were purified via MACS according to manufacturer's instructions, briefly described below. After positive selection of CD4+ T cells, the flow-through was used to purify CD8+ T cells.

In the first step, CSF was centrifuged for 10 minutes at 300x g at 4 °C. The supernatant was discarded, the cells washed twice with 1 ml MACS Buffer and resuspended in 80 µl MACS Buffer. 20 µl CD4 Micro Beads, human (Milteny Biotec) were added, mixed well and incubated for 15 min at 4 °C. After adding 2 ml MACS Buffer and centrifugation at 300x g at 4 °C, cells were washed with 2 ml MACS Buffer. The supernatant was discarded, cells resuspended in 500 µl MACS buffer, and magnetic separation was performed with a MS column. The flow-through was collected for further purification for CD8+ T cells. After washing 3 times with 500 µl MACS buffer, the column was removed from the magnet and CD4+ T cell were eluted with 1 ml MACS buffer.

The flow-through was purified with CD8 MicroBeads, human (Milteny Biotec), as described. All steps were perfomed on ice.

2.2.2 Single Cell sorting of purified lymphocytes

Single cells were isolated from the purified CD4+ and CD8+ T cells with a 20 µl pipette in a microscope. To this end, the cells were diluted with MACS Buffer in a 3.5 cm dish and the cells picked in 2 µl MACS Buffer into a PCR tube on dry ice.

2.2.3 Isolation of single cells from cryosections by laser microdissection

1.0 PET membrane slides (Zeiss) were prepared for cryosections. After baking the slides for 4.5 hours at 180 °C, slides ware sprayed with RNaseZAP (Sigma-Aldrich) to remove RNase, and washed with H_2O/DEPC. Slides were coated for one hour in a wet-chamber with poly-L-lysine-hydrobromide (2 µg/µl, Sigma-Aldrich). Residuals were removed with H_2O/DEPC and slides UV-irradiated for 30 minutes. 10 µm hippocampal sections and sections of the lateral sulcus of patient IP2 were cut in a cryotome and mounted on 1.0 PET foiled slides.
To avoid mRNA degradation in the samples, laser microdissection (LMD) was performed in an UV-irradiated room, wearing protective clothing and all staining steps were performed in the presence of 1 U/µl Protector RNAse Inhibitor (Roche).
10 µm hippocampal sections and sections of the lateral sulcus of patient IP2 on 1.0 PET foiled slides were used for laser microdissection. CD8+ and CD4+ T cells in the brain sections were stained in a short protocol. Thus slides stored in -80 °C were dried for one minute in an exsiccator and fixed with acetone. After washing with 100 µl DEPC H_2O, cells were stained with 100 µl anti-CD4-AF488 (BD Bioscience, 1:100) for 5 minutes. After removing the first antibody by washing with DEPC H_2O cells were stained with 100 µl anti-CD8-Cy3 (AbD Serotec, 1:50 in DEPC H_2O) for 3 minutes. After washing, the tissue was covered with 250 µl 2-propanol. The tissue was screened for 30 minutes with the Axiovert 200 M microscope for CD4+ and CD8+ T cells. Positive cells were marked in the program PALM Robo Software V3.2.0.11 (PALM Microlase Technologies). After 30 minutes the cells were cut out of the dried tissue via laser and collected in the oil covered lid of single PCR tubes. Tubes were stored on dry ice until RT-reaction was performed.
The RT-reaction master mix was pipetted into the lid of the PCR tubes and tubes centrifuged for 3 minutes at 6,000×g. Unbiased single cell TCR PCR was performed (2.2.2).

2.3 Immunohistochemistry

For staining of frozen tissue sections, the slides, which were stored at -20 °C were dried for 10 min at room temperature. Afterwards the tissue was fixed for 10 min in acetone. The sections were washed 3 times for 5 min with PBS and blocked by covering the tissue with 100 µl of 2 % (m/v) BSA in PBS. 100 µl primary antibody (polyclonal anti-hCD3 (1:500), anti-hCD8α-Cy3 (1:50), anti-hCD4-AF488 (1:100) or anti-hPerforin (1:100) were added and incubated for one

hour at room temperature. Double staining was performed by combining two antibodies. After washing three times for 5 min with PBS, the secondary antibody (goat anti-rabbit IgG AF488, 1:1000 or anti-mouse IgG1 AF488) was added and incubated for 30 min at room temperature. The tissue was washed 3 times, whereby 4′,6-Diamidin-2-phenylindol (DAPI, 1:1000) was added in the third washing step. The tissue was embedded in Darko Flourescence embedding media and sealed with a cover glass.

Paraffin sections were incubated twice for 10 min in Xylene for deparaffinization. Rehydration of the tissue was performed stepwise, started with incubation for 5 min in 50 % Xylene and 50 % Ethanol, followed by 100 % Ethanol, 95 % Ethanol, 70 % Ethanol and 50 % Ethanol, each step incubated for 5 min. Afterwards, the slides were put in running cold tap water and remained in tap water until heat-induced epitope retrieval (HIER). HIER was performed in a steamer at 95 °C for 30 min in Tris-EDTA Buffer. After steaming, the slides were cooled down to room temperature in the Buffer for 20 min. The tissue was permeabilised for 5 min with PBS + 0,05 % Triton and unspecific binding sides blocked for 1 h with PBS containing 1 % (m/v) BSA and 10 % (v/v) FBS. 200 µl primary antibody (polyclonal rabbit anti-hCD3 (1:50), anti-hCD8 (Clone C8/144B, 1:50) or mouse anti-hCD4 (Clone 4B12) in PBS containing 1 % (m/v) BSA were incubated for 1 hour at room temperature. Double staining was performed by combining two antibodies. After washing three times with PBS, the secondary antibodies (mouse IgG1 κ and rabbit IgG) were incubated for 30 min at room temperature. The tissue was washed 3 times, whereby DAPI (1:1000) was added in the third washing step. The tissue was embedded in Darko Flourescence embedding media and a cover glass added.

2.4 Molecularbiological Methods

2.4.1 Unbiased multiplex single cell TCR PCR

An unbiased protocol for single cell TCR polymerase chain reaction (PCR), based on Seitz *et al.,* 2006 and Kim *et al.,* 2012 was used to identify the T cell receptor α- and β-chains. The protocol was further improved by Q. Zhou (Institut für klinische Neuroimmunologie, Ludwig-Maximilians-Universität München, AG Dornmair) during her PhD thesis (unpublished data) and utilized with her kindly permission in this study.

In the first step, cDNA was created by reverse transcriptase (RT), from mRNA in the T cells. The RT reaction was followed by a touchdown PCR with primers for α- and β-chain of the TCR. Afterwards, for the β-chain, an anchor (UP) was added in a runoff reaction, followed by an anchor PCR. Alpha PCR was performed with the PCR product of the touch-down PCR and five different primer sets, covering all different Vα-chains. Primer sequences see Section 5.1.

2.4.1.1 RT-reaction

The master mix for the RT-reaction was added to the picked single cells.

5× one step RT-PCR buffer	4 µl
dNTP (10 mM each)	0.8 µl
Cα-RT-imp (100 µM)	0.06 µl (0.3 µM)
Cβ-RT-2 (100 µM)	0.06 µl (0.3 µM)
Enzyme Mix	0.8 µl
RNase OUT	0.5 µl (20 units)
H_2O	13.78 µl
	Σ20 µl/tube

RT-reaction was performed at 50 °C for 30 minutes with a lid temperature of 52 °C. The product was stored at 4 °C for further usage.

2.4.1.2 Touch-down PCR

The master mix for the touch-down PCR was added to the RT-reaction.

5× One step RT-PCR buffer	1 µl	
dNTP (10 mM each)	0.2 µl	
Vp-primer (3 µM each)	0.6 µl	(0.072 µM)
Cα-out Cβ-mid4 pool (10 µM each)	0.2 µl	(0.08 µM)
Enzyme Mix	0.2 µl	(when RT was frozen, add 1.0 µl)
H_2O	2.8 µl	
	Σ 5 µl	

After activation of HotStar Taq DNA Polymerase at 95 °C for 15 min, Touch-down PCR was performed with 4 different annealing temperatures. Four cycles at 61 °C, 58 °C and 56 °C and forty cycles at 53 °C, followed by a final elongation step for 10 min.

Activation of HotStar Taq DNA Polymerase	95 °C	15 min
Denaturation	94°C	1 min
Annealing	61 °C/ 58 °C / 56 °C/ 53 °C	1 min
Extension	72 °C	1 min
Final Extension	72 °C	10 min

2.4.1.3 Run-off reaction

Run-off reaction was used to add an anchor (UP new) ahead of the Vβ-region. In the next step the anchor can be used as a universal primer for all TCRβ V chains.

10× buffer	2 µl	
dNTP	0.4 µl	
Vp+ pool (11.1 µM each)	0.2 µl	(0.11 µM)
Cβ-in (100µM)	0.02µl	(0.1 µM)
Taq DNA Polymerase	0.1 µl	
H_2O	16.28 µl	
Product touch-down PCR	1 µl	
	Σ 20 µl	

Initial Denaturation	94 °C	2 min	
Denaturation	94°C	30 sec	
Annealing	56 °C	2 min 30 sec	3 cyles
Extension	72 °C	15 min	
Final Extension	72 °C	10 min	

UP new-anchor was infixed in 3 cycles of denaturation, annealing and extension, followed by a final extension for 10 min.

2.4.1.4 Anchor PCR

All TCRβ-chains were amplified in 30 cycles of anchor PCR with the primers Cβ-in and UP new.

10× buffer	2 µl
dNTP	0.4 µl
Cβ-in (100 µM)	0.1 µl (0.5 µM)
UP new (100 µM)	0.1 µl (0.5 µM)
Taq DNA Polymerase	0.1 µl
H_2O	16.3 µl
Product run-off reaction	1 µl
	Σ 20 µl

Initial Denaturation	94 °C	2 min	
Denaturation	94°C	30 sec	
Annealing	58 °C	1 min	30 cycles
Extension	72 °C	1 min	
Final Extension	72 °C	10 min	

2.4.1.5 Alpha PCR

For the αTCR PCR five different primer sets were used in a touch down PCR (2.4.1.2), with an initial denaturation of 2 minutes.

10× buffer	2 µl	
dNTP (10 mM each)	0.2 µl	
Cα-rev-in (100 µM)	0.1 µl	(0.5µM)
Vα-for-in pool (10 µM each)	1 µl	(0.5µM)
Taq	0.2 µl	
H_2O	15.5 µl	
Product touch-down PCR	1 µl	
	Σ 20 µl	

2.4.2 Clone-specific TCR PCR

Clone-specific PCR for the clone IP2-8S1 was performed in three steps as nested PCR.
Clone-specific PCR 1:

5× One step RT-PCR buffer	1 µl
dNTP (10 mM each)	0.2 µl
Primer for (10 µM)	1 µl
Primer rev (10 µM each)	1 µl
Enzyme Mix	0.2 µl
H_2O	2.4 µl
	Σ 5 µl

For the clone-specific PCR1 the primers Vα-8,21-for-out and Cα-out were used for the α-chain, or VP7 and Cβ-mid4 for the beta chain. 5 µl Master Mix were added after the RT-reaction. PCR protocol (Section 2.4.1.4).

Clone-specific PCR 2/3:

10× buffer	2 µl
dNTP	0.4 µl
Primer-for (100 µM)	0.1 µl
Primer-rev (100 µM)	0.1µl
Taq DNA Polymerase	0.25 µl
H_2O	16.15 µl
Product PCR 1/2	1 µl
	Σ 20 µl

For clone-specific alpha PCR 2, the primers Vα8/1-for-in and Cα-rev-in were used. For clone-specific alpha PCR 3 N05-13 Vα-8.1-spec-for and N05-13 Vα8.1-spec-rev.
Clone-specific beta chain PCR2 was run as a semi-nested PCR with the primers VP7 and Cβ-in. Afterwards a nested PCR with N05-13 Vβ-8.1-spec-for and N05-13 Vβ8.1-spec-rev was performed. All three clone-specific PCRs were run under the same conditions (Section 2.4.1.4).

2.4.3 Sample preparation for NGS

NGS was performed in cooperation with E. Beltrán as described by Gerdes *et al.,* 2016. For the quantification of the TCR repertoire, UMIs were added. (Shugay *et al.,* 2014). cDNA was synthesized from frozen biopsy sections of the hippocampus and the lateral sulcus of IP2 with the SmartScribe Kit (Clontech Laboratories, Mountain View, CA). cDNA was incubated with uracil-DNA glycosylase (New England BioLabs, Ipswich, MA), followed by two nested PCRs with 27 cycles each. The used primers are listed in Section 5.1. The Ovation Ultralow System V2 (NuGENE, San carlos, CA, USA) was used to add adaptors and barcodes. The samples were analysed at IMGM Laboratories GmbH (Planegg, Germany).

2.4.4 Standardising single cell PCR with the Janus pipetting robot

The Janus pipetting robot was used to standardise single cell PCR (scPCR). Protocols were designed for the run-off reaction, the anchor PCR as well as the alpha PCR with the Janus pipetting robot. To avoid contaminations, the robot was UV-radiated for 30 min prior application. Additionally, it was UV-radiated after the run for another 30 min.
The deck of the robot was arranged as shown in Figure 2-1. For the run-off reaction, as well as for the anchor PCR and clone-specific PCR2 and 3, 19 µl Master Mix were pipetted into a 96 well plate on a plate adapter support tile. For the pipetting 175 µl conductive tips, for liquid tracking, were used. 8× dispense per aspirate and all four tips of the tip adapter were used. Tips were changed after pipetting 32 samples. The waste mode was used for pipetting. The

performance files were set for high sensitivity of liquid level sense, with an aspirate speed of 10 µl/s, an aspirate delay of 200 ms, a dispense speed of 5 µl/s and a dispense delay of 1000 ms. No waste volume was used. A transport airgap of 3 µl was used, and a system air gap of 15 µl. The slope was set to 1.011 µl/µl and the offset to -0.633 µl. Best settings were evaluated by pipetting and measuring of the tubes with a balance. With these values, the offset and slope were calculated according to manufacturer's instructions, for precise pipetting. After pipetting the master mix into the well, the Tip Dip/Touch mode was used, to ensure that no liquid stays at the tip. The Tip Dip/touch was performed 15 mm above the well bottom and with an offset of 25 % of the well width. Thereby, drops which were outside at the tip go into the well. This step was important for precise pipetting of the master mix.

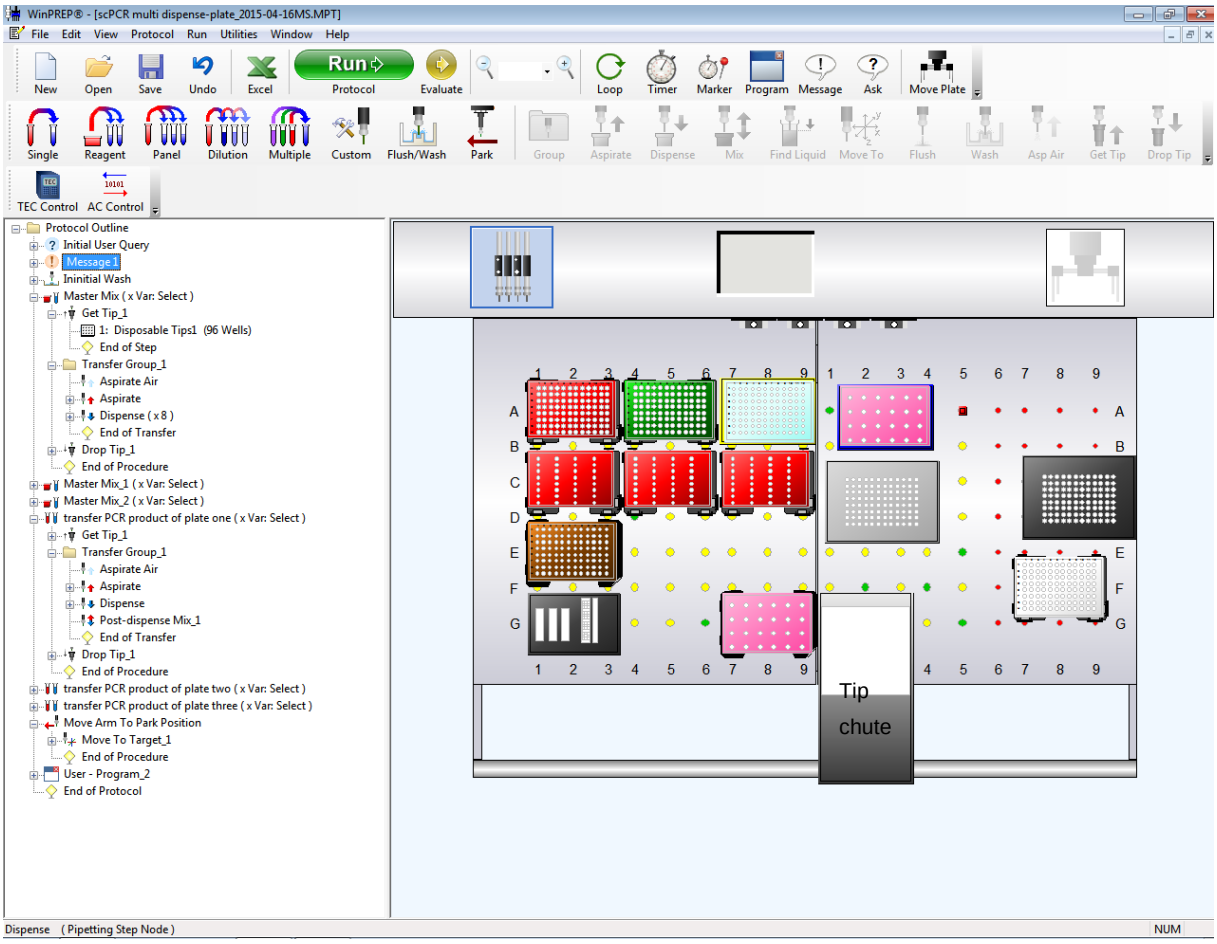

Figure 2-1 Janus deck arrangement for pipetting the run-off reaction. The program was designed in WinPREP. On the left side all pipetting steps are shown. On the right side the arrangement on the deck of the robot is shown. On the top the tips (25 µl non-conductive, 175 µl conductive and 1 ml non-conductive tips) were placed. In second row the PCR productes (stripes) were placed. The Master Mix was pipetted from a 1.5 ml tube in position G7 to a 96 well plate (row 3), and 1 µl of PCR product was transferred from the 8-stripes to the 96-well plate.

Afterwards the PCR products were transferred into the master mix, either from 8-stripes on top of rack on a plate adapter support tile (run-off reaction) or from 96-well plate on plate adapter support tile (anchor PCR). 1 µl dispense volume was transferred in 25 µl non-conductive tips.

To ensure no PCR product remain in the tips, blow out mode was used, with a blow out volume of 20 µl. All 4 tips of the adapter were used. The performance file was set to an aspirate speed of 100 µl/s with an aspirate delay of 200 ms and a dispense speed of 20 µl/s and a delay of 200 ms. A transport air gap of 3 µl was used, to ensure, no liquid is lost during the moving of the tip adapter, and thereby avoid contaminations. The slope was set to 0.940 µl/µl and the offset to -0.740. A post-dispense mix with 10 µl for three times was added. After the pipetting, the tips were disposed at the tip chute.
All plates stand on racks filled with ice, to avoid a warming of the master mix. After the start, the user was requested to fill all tip boxes with new tips. Additionally, here the user can select how many samples the robot should pipette.
The designed programs were used to perform scPCR of the samples of LMD of the lateral sulcus.

2.4.5 Cloning strategies

2.4.5.1 TOPO-TA cloning

TOPO-TA cloning was used for the cloning of small amounts of PCR products. The TOPO-TA cloning kit (Invitrogen) was used according to manufacturer's instructions. Positive clones were selected by blue-white screening and colony PCR (Section 2.4.12).

2.4.5.2 Cloning MHC-I alleles of IP2

All MHC-I alleles of IP2 were cloned from mRNA of 2 different B-lymphoblastoid cell lines (LCL, from Josef Mautner, Helmholtz Zentrum München): LCL #28 and LCL #43. For this purpose, cDNA was synthesized from isolated mRNA (Section 0 and 2.4.7) and PCR performed with primers for HLA-A, -B and –C to add SalI and BamHI cleavage sites. The PCR products were cloned into pHSE3'-neo, as described for MHC-II constructs (Section 2.4.5.5).

2.4.5.3 Cloning of TCR chains

DNA encoding the α-chain (IP2-AV8S1) and the β-chain (IP2-BV8S1) of IP2-8S1, without the constant regions, were ordered at GeneArt (Invitrogen) in pMA-T. The sequences were ordered as non-optimized and flanked with the cleavage sites SalI and PvuII for the α-chain, and SalI and AvaI for the β-chain. Additionally, a silent mutation was added in the N(D)N region in the β-chain, to distinguish between contaminations and real PCR products in the repertoir studies. The cassette vectors pRSV-hygro and pRSV-neo, which already contain the constant regions for TCR α- or TCR β-chain, were used for cloning.

For the α-chain the PCR products and pRSV-hygro were cleaved with SalI and PvuII and ligated (Section 2.4.9 and 2.4.11). The β-chain was cloned into pRSV-neo by restriction digestion with SalI and AvaI.

2.4.5.4 Cloning of possible antigen-candidates of $GABA_A$-receptor alpha 1 subunit

Candidate antigens of the $GABA_A$ receptor α1 subunit for IP2-8S1 were selected using databases, which comprise HLA epitopes (NetMHC 4.0 Server, Andreatta and Nielsen 2016) and proteasomal cleavage predictions and TAP transport efficiency (IEDB NetCTL Prediction, Vita 2015), for HLA-A and HLA-B of IP2. To identify possible candidates of HLA-C*04:01 and HLA-C*16:01, for which no database exists, the sequence was analysed for anchor positions described by Rasmussen *et al.* 2014.

Oligonucleotide linkers coding for candidate antigens of $GABA_A$-receptor α1 subunit (for-linker) and the complementary reverse oligonucleotide linkers (rev-linker) were ordered at Metabion, so that after annealing the candidates have sticky ends, as after digestion with AscI and NotI (sequences see Section 5.1). For annealing 5 µM of the for-linker and of the rev-linker in 100 µl were incubated at 95 °C for 5 min, followed by 75 °C for 5 min and room temperature for 5 min. 1 µl of the insert was ligated with 52 ng of AscI/NotI digested pcDNA-V5HisA vector in 1× ligation buffer containing 1 U of T4 DNA ligase (Invitrogen) in a total volume of 20 µl. After 30 min incubation at room temperature, heat shock (Section 2.5.2.1) was performed with 1 µl of the ligation product and 20 µl of TOP10 in 96 v-well plates. A list of all cloned plasmids expressing possible antigen-candidates are listed in Section 2.1.2.1.

2.4.5.5 Cloning MHC-II chains in pHSE3'

MHC-II chains were cloned from pRSV as templates (Klaus Dornmair) into pHSE3'. pHSE3' does not exhibit a SV40 origin, hence it will not get amplified in COS-7 cells in contrast to the library which are expressed in pcDNA.

The α-chain HLA-DRA*0101, which is in common for all HLA-DR, was cloned into pHSE3'-hygro, and the β-chains HLA-DRB1*010101 (DRB1), HLA-DRB1*15010101 (DR2b) and HLA-DRB5*010101 (DR2a) were cloned into pHSE3'-neo.

PCR was performed to add the cleavage sites SalI and BamHI to all constructs. PCR products and vectors were cleaved with SalI and BamHI and ligated (Section 2.4.11).

2.4.5.6 Cloning of the invariant chain and positive controls

The invariant chain wild-type (Ii-wt) was ordered at GeneArt (Invitrogen) in pMA-T, flanked by the restriction digestion sites AscI and BspeEI. However, restriction digestion with BspEI was blocked by methylated DNA. Thereby PCR with p33-wt-AscI-for and p33-wt-BspEI-rev was

performed (Section 2.4.8) and the PCR product digested with AscI and BspEI. pcDNArc-spacer (rc: “rare cutter”, cloned by Katherina Siewert) was digested with AscI and AgeI. AgeI and BspEI have the same sticky-end overhangs, thereby after ligation, the cleavage site is silent. This is important for further cloning of the libraries with AgeI and KasI into Ii-wt.

The nucleotide sequences coding for different length of hMBP in the invariant chain were ordered as positive control at GeneArt (Invitrogen) in pMA-T. Following sequences were ordered: Ii-hMBP(141-149), Ii-hMBP(141-150), Ii-hMBP(141-151) and Ii-hMBP(139-151). To avoid contaminations, the constructs were ordered at GeneArt instead of cloning them in house. Additionally, silent mutations were added, where the primer for the library screening usually anneals. Thereby the constructs will not be amplified by PCR when the libraries are screened (Section 2.9.2). The constructs were cloned into pcDNA as described above for Ii-wt.

The signal sequence was removed by restriction digestions of pcDNA-Ii-wt, and the positive controls, with AscI and ApaI. The oligonucleotides delta-p33-for and delta-p33-rev-new were annealed (2.4.5.4) and the sticky ends ligated with the digested vector (Section 2.4.11).

2.4.5.7 Cloning of Invariant chain encoded combinatorial peptide libraries

For the identification of CD4+ T cell antigens, libraries which are expressed in the invariant chain, were used. To this end, CLIP of Ii was exchanged by combinatorial peptide libraries encoded by different length of nucleotides with and without anchor positions. The resulting libraries are called Ii-encoded combinatorial peptide libraries (Ii-ECPL)

The libraries were prepared of HPLC purified, single strand oligonucleotides (Metabion), flanked by restriction sites AgeI and KasI and filled up with CLIP-AgeI to double strands. Therefor 5 µM of oligonucleotide (CLIP-N27-AgeI/KasI, CLIP-M1,9-AgeI/KasI, CLIP-N33-AgeI/KasI, CLIP-N33-Anker-M-AgeI/KasI, CLIP-N39-AgeI/KasI, CLIP-N39-Anker-M-AgeI/KasI, CLIP-N36-C-AgeI/KasI, CLIP-N36-C-Anker-M-AgeI/KasI, CLIP-N36-AgeI/KasI, CLIP-N36-Anker-M-AgeI/Kas; sequences are listed in Section 5.1 in Table 5-17) and 5 µM CLIP-AgeI-for (for CLIP-N27-AgeI/KasI, CLIP-M1,9-AgeI/KasI) or CLIP-KasI-rev (rest) in 1x PCR buffer in 100 µl were denaturated for 5 min at 99 °C. After cooling down, 100 µl of 1x PCR buffer containing 400 µM dNTPs and 10 U Taq DNA Polymerase (Roche) were added. To generate double strands, the mixture was gradually heated at 60 °C for 4 min, 63 °C for 4 min, 65 °C for 4 min and a final step at 68 °C for one hour.

The products were precipitated (2.4.12) and resuspended in 50 µl EB (Qiagen). The constructs and the vector pcDNA-V5His-A-Ii-ECPL-wt were sequentially digested with AgeI-HF and KasI. After dephosphorylation of the vector the constructs were ligated at 42 °C for 16 h (2.4.11), preceptitated and *E. coli* transformed by electroporation (Section 2.5.2.2).

2.4.5.8 Cloning pHSE3'-Blasticidin-large T antigen

The gene for Blasticidin resistence was cloned from pcDNA via PCR and introduced into pHSE3' by digestion with AatII and NdeI followed by ligation (2.4.11). Afterwards the gene for large T antigen was amplified from mRNA of COS-7 cells by PCR. The PCR product was integrated into pHSE3'-Blasticidin by restriction digestions with SalI and BamHI and ligation (Section 2.4.9 and 2.4.11).

2.4.6 Isolation of mRNA

Messenger RNA (mRNA) was isolated from B-lymphoblastoid cell lines (LCL, from Josef Mautner) with TRIzol (Thermo Fisher Scientific). 15 ml cell suspension was centrifuged at 300× g for 5 min. The cell pellet was resuspended in 750 µl TRIzol and incubated for 5 min at room temperature. 750 µl chloroform were added, mixed, incubated for 5 min at room temperature and centrifuged at full speed (5417 R, Eppendorf) for 15 min. The upper, aqueous phase was transferred into a new 1.5 ml tube. 10 µl glycogen were added and vortexed. Afterwards 700 µl of 70 % isopropanol were added, vortexed and incubated at -20 °C for 20 min. After centrifugation at full speed (5417 R, Eppendorf) for 20 min, the pellet was washed with 1 ml of 80 % ethanol, and centrifuged again for 5 min at full speed. The supernatant was discarded and the pellet dried for 30 min. The pellet was resolubilized in 50 µl DEPC-H_2O and stored at -80 °C.

2.4.7 cDNA synthesis from mRNA

Super Skript III reverse transcriptase (Invitrogen) was used for the synthesis of complementary DNA (cDNA) from mRNA.

1 µg of RNA and 1 µl oligo (dt) primer (50 µM, Invitrogen) were filled up to a final volume of 12 µl with ddH_2O. The primer was annealed to the poly-a tail of the mRNA at 65 °C for 10 min. 1 µl of dNTPs (10 mM each, Qiagen), 2 µl of dithiothreitol (DTT,100 mM) and 5 µl of 5× FS Buffer were added and incubated at 42 °C for 2 min. cDNA was synthesized at 42 °C for one hour after adding 1 µl of Super Script III (200 U/µl, Invitrogen). cDNA was stored at -20 °C.

2.4.8 PCR

Polymerase chain reaction was used for the amplification of DNA fragments. The PCR mix was prepared as followed:

10× buffer	10 µl
dNTP	2 µl
Primer-for (100 µM)	0.5 µl
Primer-rev (100 µM)	0.5 µl
Taq DNA Polymerase	1 µl
Template	1 µl
ddH_2O	85 µl
	∑ 100 µl

PCR was performed in 30 cycles of denaturation, primer annealing and amplification of the DNA. The annealing temperature was calculated depending on the GC/AT content of the primers. 4 °C were calculated for GC-pairs, 2 °C for AT-pairs. Elongation time depends on the length of the expected PCR product. Taq DNA Polymerse transcribes 1.000 nucleotides per minute.

Initial Denaturation	94 °C	2 min	
Denaturation	94°C	30 sec	
Annealing	calculated	30 sec	30 cycles
Extension	72 °C	calculated	
Final Extension	72 °C	10 min	

2.4.9 Restriction Digestion

Restriction digestion of plasmids and PCR products was performed with restriction enzymes, purchased from New England Biolabs, under manufacture recommended conditions at 37 °C for 1 h to overnight digestion. Total glycerol concentration in the digestion mix was kept under 5 % to avoid star activity. Double digestion was performed if the same buffer was compatible for both enzymes, and the restriction sites were more than 100 bp apart from each other. For sequential double digestion the first enzyme was heat inactivated for 20 min at 65 °C before adding the second enzyme, if the same buffer could be used. Otherwise ethanol precepitaion was performed (2.4.15) to change the buffer.

For electroporation of eukaryotic cells, the plasmids were linearised, either with AatII (pcDNA-NFAT-sGFP, pHSE3'-HLA-DRβ), NdeI (pRSV-AV8S1, pRSV-BV8S1) or XmnI (pHSE3'-HLA-DRA). Linearised plasmids were ethanol precipitated (Section 2.4.15).

2.4.10 Agarose gel electrophoresis

DNA fragments were separated according to size by agarose gel electrophoresis. Depending on the size of the DNA fragment either 1 % (w/v) or 2 % (w/v) agarose in TBE Buffer with 1 µg/ml Ethidumbromid or 1:25000 peqGreen (Peqlab) were used. Samples were mixed with the appropriate amount of 6× loading buffer and loaded on the gel. Additionally, either 5 µl of 1 kb ladder (New England Biolabs) or 50 bp DNA Ladder (Peqlab) were loaded. The gel was run for 30 minutes at 120 V in TBE buffer. DNA fragments were visualized under UV-light. Isolated DNA fragments were purified with the QIAquick® Gel Extraction Kit (Qiagen) and eluted in 50 µl Elutionbuffer (EB) or with the MinElute® Gelextraction kit (Qiagen) and eluted in 12 µl EB.

2.4.11 Dephosphoylation and Ligation

To avoid religation, the digested vectors were dephosphorylated with the Rapid DNA Dephosphorilation and Ligation Kit (Roche) according to manufacturer's instructions.
For ligation T4 DNA Ligase (1 U/µl, Roche) was used. Ligation was performed in 20 µl reaction volume, containing 1 U T4 DNA ligase and a molar ration of 1:5 of DNA insert and vector. After ligation at room temperature for one hour or 16-48 h at 16 °C, depending on the acquired number of *E. coli* colonies. The ligation mix was ethanol precipitated before transfection (Section 2.4.15).

2.4.12 Colony screening

Colony PCR was performed with the grown bacteria to select for the positive clones. To this end, PCR with the clones as templates, was performed. In parallel the colonies were grown over night in 1 ml LB media containing 0.1 mg/ml ampicillin.

2.4.13 Plasmid Preparation

Plasmids were isolated from *E. coli* suspension cultures. For small scales of Plasmids, mini preparation was performed. To this end, 3 ml LB media containing 0.1 mg/ml ampicillin were inoculated with a positive clone and incubate over night at 37 °C and 150 rpm. Plasmids were isolated of 1-2 ml bacteria suspension with the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. DNA was eluted in 35 µl EB Buffer and stored at -20 °C.
For higher DNA yields, HiSpeed Plasmid Maxi Kit (Qiagen) was used. To this end 250 ml LB media containing 0.1 mg/ml ampicillin were inoculated and grown over night at 37 °C and 150 rpm. The next day DNA was isolated according to manufacturer's instructions.

2.4.14 Sequencing

DNA sequencing was performed at the Sequencing Service of Ludwig-Maximilians-Universität München (LMU). 5-10 ng DNA (100-200 bp), 10-40 ng DNA (200-500 bp) or 150-300 ng DNA (plasmids) were send for sequencing in a total volume of 7 µl containing 3,3 µM of primer. Sequences were analysed with Chromas.

2.4.15 Ethanol precipitation of DNA

For DNA purification ethanol precipitation was used. For this purpose, 10 % (v/v) NaAcetate (pH 5.2) was added to the DNA and vortexed briefly. Afterwards 250 % (v/v) Ethanol absolute was added and vortexed again. After incubation for > 1 h at – 80 °C the precipitated DNA is centrifuged for 1 h at 20,817x g and 4 °C. The supernatant was discarded and the pellet washed twice with 200 µl of 80 % (v/v) ethanol. The pellet was dried and solubilized in the desired volume of elution buffer. For the construction of libraries and for the library screening, full recovery of DNA is needed. Hence 2 µl of Pellet Paint (Merck) and 1 µl of Glycerol (20 mg/ml, Roche) were added as carrier DNA.

2.4.16 DNA Isolation of tissue

Genomic DNA of IP2 was isolated with the QIAamp DNA Micro Kit (Qiagen). A 10 µm brain section was put in a 1.5 ml tube. The tissue was lysed and DNA isolated by manufacturer's instructions for the isolation of genomic DNA from tissue.

2.4.17 HLA-typing

HLA typing was performed by the "Labor für Immungenetik und Diagnostik" at the Klinikum der Universität München. To this end 200 µl of DNA with a concentration of 27 ng/µl was send for HLA typing.

2.5 Handling of prokaryotic cells

2.5.1 Cultivation and freezing of *E. coli* cultures

E. coli were cultivated at 37 °C either in LB media or on LB plates containing 0.1 mg/ml ampicillin (LB^{Amp}). Cells were cultivated in flasks with 5-times the culture volume and with 180 rpm for optimal aeration. Cell number was determined by spectrophotometry. To this end, OD_{600} was measured in the UV-1600 PC Photospectrometer (VWR), after blanking with LB^{Amp}. For optimal measurement the OD_{600} should be between 0.1-0.5. If necessary, the samples were diluted. A bacteria suspension of OD_{600}=1 is assumed to contain1×10^6 bacteria per µl.

However, for exact colony numbers, a defined volume was spread on LB agar plates, grown at 37 °C and counted the next day.
For glycerol stocks 50 % (v/v) autoclaved glycerol was added to the bacteria suspension, aliquoted into cryo tubes and frozen at -80 °C.

2.5.2 Transformation of prokaryotic cells

2.5.2.1 Transformation of *E. coli* by heat shock

Chemical competent *E. coli* TOP10 were transformed by heat shock. For this purpose, the bacteria, which are kept at -80 °C, were thawed on ice. 1 µl plasmid or 10 µl ligation mix were added and incubated on ice for 30 min. The heat shock was performed at 42 °C for 30 seconds. To recover the cells were put on ice for 2 min. 250 µl SOC medium (Invitrogen) were added and the bacteria incubated for 1 h at 37 °C and 150 rpm shacking to produce β-Lactamase for ampicillin resistance.
Afterwards 200 µl, 50 µl and the remains were streaked out onto agar plates containing 0.1 mg/ml ampicillin. For blue-white screening the plates were pre-coated with 40 µl X-galactose. The plates were incubated over night at 37 °C.

2.5.2.2 Transformation of *E. coli* by electroporation

For the cloning of libraries and library screening high yields are essential, hence electroporation was used. 2 µl of ligation mix were pipetted in prechilled tubes. ElectroMAX™ DH10B™ T1 (Invitrogen) were thawed on ice and 20 µl (library screening) or 50 µl (cloning of libraries) bacteria were added to the ligation mix and transferred into a prechilled 0.1 cm electrode cuvette (Bio-Rad). Cells were electroporated with the Gene Pulser (Bio-Rad) with 2,0 kV, 25 µF and 200 Ω. 1 ml SOC medium was added and incubated for 1 h at 37 °C and 150 rpm shacking.
For the production of libraries, 200 ml LB^{Amp} were inoculated with the 1 ml bacteria. Serial dilutions were plated onto agar plates, to estimate the magnitude of the libraries.
For the library screening, 50 µl of a 1:50 dilution was plated onto LB^{Amp} agar plates to calculate the number of different plasmids of the library. 20 ml LB^{Amp} were inoculated with the rest of bacteria.

2.6 Handling of eukaryotic cells

2.6.1 Cultivation and freezing of eukaryotic cells

All eukaryotic cell lines were cultivated in different tissue culture vessels in RPMI complete media at 37 °C and 5 % CO_2. For stable transfected cells the appropriate amount of antibiotic

was added. At a density of 80 % cells were splitted. To this end, adherent cells were washed twice with PBS and incubated for 5-10 min with Trypsin-EDTA (Invitrogen). The reaction was stopped by adding RPMI + 10 % FBS. Detached cells were centrifuged at 300× g for 5 min, resuspended in 10 ml media and seeded as required.

Suspension cells were splitted by centrifuging them at 300× g for 5 min, resuspending the cells in 10 media and seed them as required.

The cell number per milliliter was calculated in a Neubauer counting chamber. Therefor, 20 µl of cell suspension was diluted with 20 µl trypanblue, for the staining of dead cells. 4 big squares of the Neubauer counting chamber were counted and the number of cells per milliliter calculated be the average cell number (n) per square multiplied be the dilution coefficient (dc) and 10^4.

$$c_{cells} = n^x dc^x 10^4$$

For freezing the centrifuged cells were resuspended in FBS containing 10 % DMSO. The cryovials were frozen in boxes containing isopropanol for slowly freezing to -80 °C.

2.6.2 Transfection of eukaryotic cells by FuGENE®

For library screening 500.000 Cos-7 cells were seeded in 3 ml RPMI complete in a 3.5 cm dish. After the cells adhered they were transfected. For this purpose, 2 µg DNA in 100 µl RPMI were mixed with 7 µl pre-warmed FuGENE® (Promega, Madison, Wisconsin, USA). The mix was vortexed and incubated for 10 min at room temperature. The mix was dropped onto the cells. The transfected cells were further incubated at 37 °C and 5% CO_2 until needed.

For screening of subpools 150,000 were seeded in 1 ml RPMI complete in a 24-well plate. 500 µg DNA were filled up to a final volume of 25 µl with RPMI. 1,75 µl pre-warmed FuGENE® was added and transfection proceeded as described above.

The amount of DNA was used half for the transfection of the library or positive control and half for the transfection of HLA, if there were no stabile transfected COS-7-HLA available.

2.6.3 Transfection of eukaryotic cells by GenaxxoFect-plus

Mouse fibroblast cells were transfected via GenaxxoFect-plus (Genaxxon bioscience GmbH, Ulm, Germany). 1 µl of GenaxxoFect-plus was diluteted in 40 µl Dilution Buffer. 300 ng DNA were filled up with Dilution Buffer to 40 µl. Both liquids were combined and mixed by pipetting several times. After incubation for 20 min at room temperature, 150.000 cells in 350 µl RPMI complete without Pen/Strep were added. The mix was seeded into one well of a 24-well plate and incubated under usual conditions.

2.6.4 Transfection of eukaryotic cells by nucleofection

For nucleofection 1×10^6 Cos-7 cells were centrifuged for 10 min at 100× g. The supernatant was discarded and the cells resuspended in 100 µl SE solution (Lonza) containing 82 µl solution, 18 µl of supplement and 1 µg DNA. Cos-7 cells were pulsed with the program CM130. The pulsed cells were incubated 10 min at room temperature. 500 µl pre-warmed RPMI complete was added, incubated for 10 min at room temperature and the cells transferred in 3.5 cm dishes containing 2.5 ml pre-warmed RPMI compete. The transfected cells were further incubated at 37 °C and 5 % CO_2.

2.6.5 Transfection of eukaryotic cells by electroporation

For stabile transfection of eukaryotic cells electroporation was used. $4\text{-}7 \times 10^6$ cells were centrifuged and washed twice with RPMI. The cells were resuspended in 800 µl RPMI and transferred into a pre-cooled 4 mm electroporation cuvette (Eppendorf). 30 µg linearized and precipitated DNA (2.4.9 and 2.4.15) was resuspended in 60 µl RPMI and added to the cuvette. After incubation for 10 min on ice cells were pulsed with 280 V and 960 µF (Bio-Rad Genepulser). The cells were put on ice for 10 min and afterwards incubated in 10 ml pre-warmed RPMI complete for 30 min at room temperature. Subsequently the cells were transferred into a 175 cm^2 cell culture bottle and filled up to 70 ml with RPMI complete. The cells were incubated at 37 °C and 5 % CO_2 for two days, before continuing with antibiotic selection.

2.6.6 Single clone selection of stabile transfected cells

48 h after electroporation, the transfected cells were set on antibiotic selection. For this purpose, cells were seeded in 24-well plates in half conditioned and half fresh RPMI complete containing three different antibiotic concentrations (Table 2-4).

Table 2-4 Overview of antibiotic concentrations for the selection of transfected cell lines

Cell line	Plasmid	Antibiotic	Stock [mg/ml]	Selection [µg/µl]	Maintenance [µg/ml]
58$^{-/-}$	pcDNA-NFAT-sGFP	Blasticidin	10	3-5	3
	pRSVhygro TRAV	Hygromycin	50	300-500	300
	pRSV.neo TRBV	G418	100	2000-3000	1500
Cos-7	pHSE3' HLA-DRα	Hygromycin	50	300-500	300
	pHSE3' HLA-DRβ	G418	100	2000-3000	1500
LTK$^-$	pHSE3' HLA-DRα	Hygromycin	50	150-300	150
	pHSE3' HLA-DRβ	G418	100	300-500	300

To reduce the amount of spontaneously activated hybridoma cells and to get a clone with high sGFP expression, single cell dilution of stable clones of $58^{-/-}$ TCRαβ-CD8-NFAT-sGFP cells and $58^{-/-}$ TCRαβ-NFAT-sGFP cells was performed. An average of 0.3 cells/well were seeded in 200 µl into a 96-well vessels, wrapped into cling wrap to avoid evaporation and incubated at 37 °C, 5 % CO_2. 10-14 days later single clones were screened for CD3 activation and spontaneously activated cells (Section 2.8.1).

Table 2-5 Stable transfected cell lines

Cell Line	Transfected plasmids	Application
$58^{-/-}$		
IP2-8S1	pRSV-IP2-AV8S1	TCR expression for antigen screening
	pRSV-IP2-BV8S1	
	pcDNA-NFAT-sGFP	
	pLPC-hCD8α-IRES2-hCD8β	
Bbc9	pRSV-AV22S1	TCR expression for antigen screening
	pRSV-BV9.1	
	pcDNA-NFAT-sGFP	
COS-7		
COS-7-DR1	pHSE3'-HLA-DRA	Antigen presenting cell
	pHSE3'-HLA-DRB1	
COS-7-DR2a	pHSE3'-HLA-DRA	Antigen presenting cell
	pHSE3'-HLA-DR2a	
COS-7-DR2b	pHSE3'-HLA-DRA	Antigen presenting cell
	pHSE3'-HLA-DR2b	
LTK$^-$		
LTK-DR1	pHSE3'-HLA-DRA	Antigen presenting cell
	pHSE3'-HLA-DRB1	
LTK-DR2a	pHSE3'-HLA-DRA	Antigen presenting cell
	pHSE3'-HLA-DR2a	
LTK-DR2b	pHSE3'-HLA-DRA	Antigen presenting cell
	pHSE3'-HLA-DR2b	

2.6.7 MACS purification of stable transfected cells

After stable transfection, some cells may be antibiotic resistant but do not express the protein of interest. To eliminate these cells, MACS purification was used. To purify CD8-transfected $58^{-/-}$ hybridoma cells, CD8 MicroBeads, human (Milteny Biotec GmbH, Bergisch Gladbach, Germany) were used. To increase the expression of HLA-DR of stable transfected COS-7 cells, Anti-HLA-DR MicroBeads, human (Milteny Biotec GmbH) were used.

For the purification, the cells were trypsinised if necessary (COS-7) and centrifuged at 300× for 10 min. The cells were resuspended in 10 ml MACS Buffer and counted in a Neubauer counting chamber (2.6.1). After centrifugation, the cells were resuspended in the required amount of MACS Buffer, the appropriate amount of MACS Beads (20µl/10^7 cells) was added and the purification proceeded according to manufacturer's instructions.

2.7 Flow cytometry

2.7.1 Detection of surface expression

Flow cytometry was used to verify the expression of surface proteins on different cell lines. 250,000 cells were washed twice with FACS Buffer. Surface proteins were detected with -FITC, -PE or –APC-labelled antibodies (Section 2.1.7). Antibodies were diluted 1:50 in FACS buffer, added to the cells and incubated for 30 minutes on ice in the dark. Isotype controls were added in the same concentration as the antibodies and proceeded in the same way. After washing twice, the cells were resuspended in FACS Buffer + Topro (1:6000) or FACS Buffer + propidium iodide (PI, 1:500). Results were analysed with FlowJo V9 (Tree Star, Ashland, USA).

2.7.2 Fluorescence-activated cells sorting

To increase the surface expression of HLA-DR on stable transfected COS-7 cells, fluorescence-activated cell sorting (FACS) was used. Cells were labelled with a FITC-labled antibody against human HLA-DR as described in section 2.7.1. FACS was performed with the FACSAria (BD) at the Max Planck Institute of Biochemistry by Dr. Markus Moser. The best 10 % of the population expressing HLA-DR was sorted and cultivated for further experiments.

2.7.3 Detection of intracellular expression

The expression of the invariant chain (Ii, CD74) and MHC-II libraries based on the invariant chain, were detected by intracellular flow cytometry. All steps were performed at room temperature.

Transient transfected Cos-7-DRB1 were trypsinised and centrifuged for 5 min at 300xg. The cells were fixed in 80 % (v/v) methanol for 5 min. After washing with PBS, cells were permeabilized with PBS + 0.01 % (v/v) Tween for 20 min and unspecific binding sites were blocked with PBS containing 10 % (v/v) goat serum and 0.3 % (m/v) glycine for 15 min. The cells were centrifuged at 300× g for 5' and resuspended in 50 µl of 20 µg/ml anti-CD74 (abcam) or equivalent amount of isotype control. The antibody was incubated for 30 min. Successive the cells were washed twice with PBS and resuspended in 50 µl of 0.08 µg/µl polyclonal goat anti-mouse Immunoglobolins/FITC Goat F(ab')2 (Dako, 0,4 g/l). Before FACS analysis the

cells were washed twice with PBS to remove unbound secondary antibody. Results were analysed with FlowJo V9 (Tree Star, Ashland, USA).

2.8 T hybridoma activation assay

As readout for T cell activation, sGFP-expression was observed by fluorescence microscopy or flow cytometry (Section 2.8.5). Additionally, IL-2 release was measured by ELISA (2.8.6).

2.8.1 CD3 activation of T hybridoma cells

CD3 activation assay of T hybridoma cells served as a positive control. 150 µl of anti-mCD3ξ (1 µg/ml in PBS, clone 145-2C11, eBioscience) was coated on a 96-well microtiter plate. After 3 h of incubation at 37 °C the solution was removed, 150 µl of T hybridoma cells added and incubated for 16 h.

2.8.2 Activation of T hybridoma cells by synthetic peptides

Synthetic peptides were dissolved in ddH_2O with a concentration of 10 mM. 1 µl of the peptide solution was added to 30,000 stable transfected COS-7 cells expressing HLA-DR in 96-well plate and incubated for 3 h prior adding 50,000 T hybridoma cells. The T hybridoma cells were incubated for 16 h.

2.8.3 Activation of Bbc9 T hybridoma cells by Ii encoded positive control

Bbc9 T hybridoma cells were activated with Ii encoded positive controls (Ii-hMBP), to test, if the screening-system with libraries expressed in the invariant chain instead of CLIP is working for MHC-II library screening. To this end, COS-7 stably expressing HLA-DR were transfected with pcDNA-Ii-hMBP (141-149, 141-150, 141-151 and 139-151, 2.4.5.6) and pcDNA-delta-Ii-hMBP (141-149, 141-150, 141-151 and 139-151, 2.4.5.6) in 24-well plates (Section 2.6.2). 250,000 Bbc9 T hybridoma cells were overlaid and incubated for 16 h at 37 °C and 5 % CO_2.

2.8.4 Cocultivation with LCL cells

150,000 LCL cells (Fe, 17490, #28, #36, #43, AJo, IBi, JM, KK, TG) in 100 µl RPMI complete in a 96-round bottom plate were stimulated with 100 U/ml and 500 U/ml of INF-γ. After incubation for 24 h, 50,000 IP2-8S1 T hybridoma cells were added, centrifuged shortly, so that the cells accumulate at the bottom, and incubated for 16 h. Activation of hybridoma cells was screened by FACS (Section 2.8.5).

2.8.5 Detection of sGFP expression in $58^{-/-}$ αβ cells after activation

As a read out of activation of hybridoma cells, the cells were stably transfected with pcDNA-NFAT-sGFP. Thereby after activation the cells express sGFP. Fluorescence can be detected either by fluorescence microscope, or by flow cytometry.
For flow cytometry the cells were transferred into a v-bottom plate. After centrifugation for 5' at 300× g the cells were washed twice with PBS. If necessary, cells were additionally stained for CD3+ (Section 2.7.1). Finally, resuspended in 150 µl FACS Buffer + Topro (1: 6,000). Results were analysed with FlowJo V9 (Tree Star, Ashland, USA).

2.8.6 IL-2 ELISA

IL-2 ELISA was used as a second read out for the activation of hybridoma cells. The murine IL-2 in the supernatant of activated cells was detected with the IL-2 ELISA Ready-SET-Go kit of eBioscience (San Diego, USA). ELISA was performed on 96-well MaxiSorb Nunc Immuno-plates (Thermo Scientific) according to manufacturer's instructions. Standard curves were performed with IL-2 in a range between 2 pg/ml and 200 pg/ml. Absorption was measured at 450 nm in the Wallac VICTOR^{2}1420 Multilabel Counter (Perkin Elmer).

2.9 Unbiased identification of TCR antigens

2.9.1 Mimotope screening with plasmid-encoded combinatorial peptide libraries

Library screening for CD8+ TCR antigens with PECPL was performed as described by Siewert *et al.* 2012. The workflow is illustrated in Figure 2-2. Adherent Cos-7 cells, expressing HLA molecules and PECPL, are overlaid with soluble $58^{-/-}$ expressing the TCR of interest and sGFP under the control of the NFAT-enhancer as read out. After activation, $58^{-/-}$ turn green, and the underlying COS-7 cell can be isolated. The plasmids are recovered by PCR and screened for reactivation.

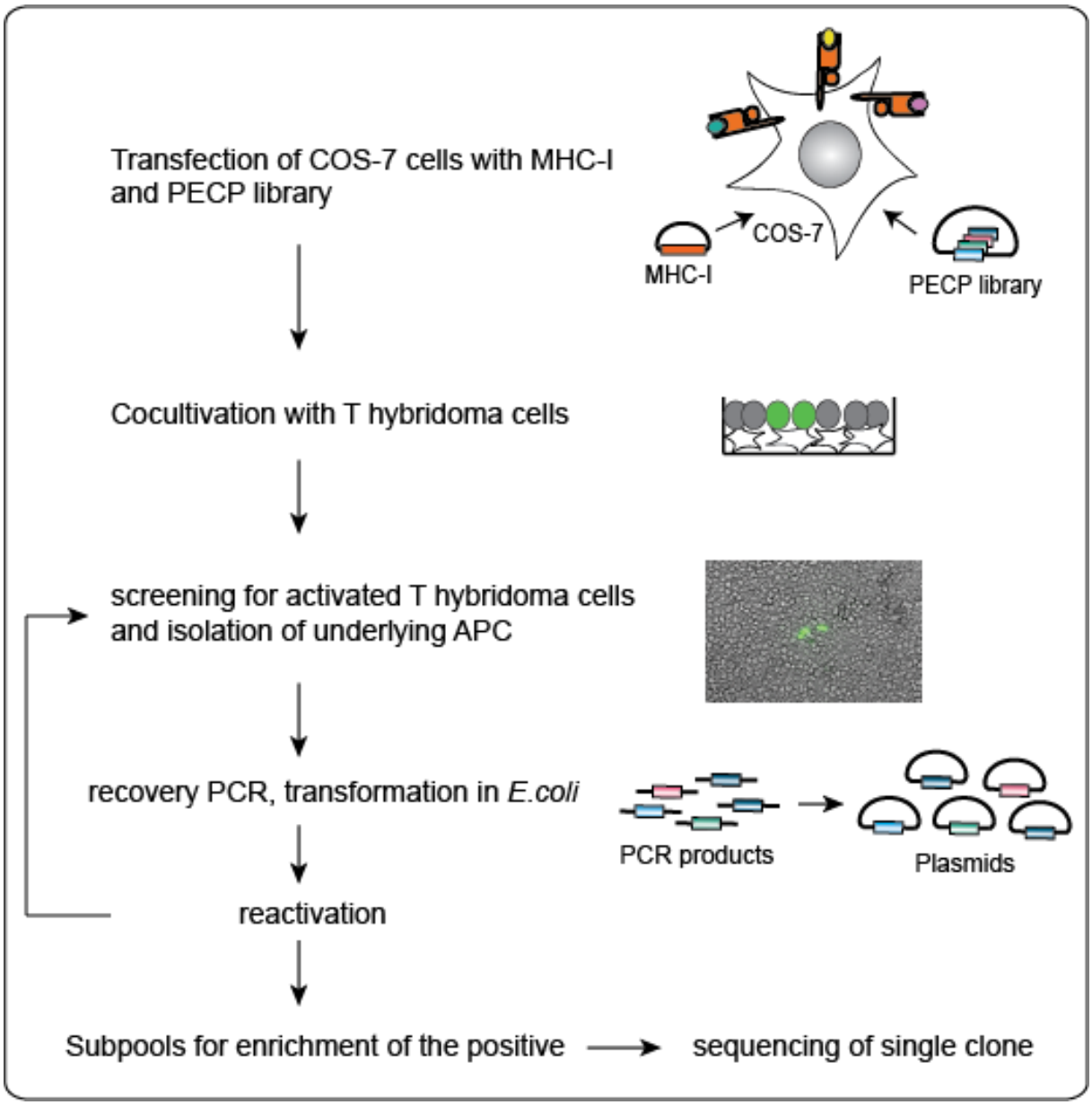

Figure 2-2 Workflow unbiased Identification of CD8+ T cell antigens. For the library screening, COS-7 cells are transfected with a plasmid coding for MHC-I and PECP library and cocultivated with T hybridoma cells. The cells are screened with a fluorescence microscope for the expression of sGFP. The underlying COS-7 cells are isolated with a micromanipulator, recovery PCR is performed and the PCR products cloned into pcDNA3.1™/V5-His-TOPO®. *E. coli* are transformed with the plasmids, the plasmids isolated and screened for reactivation. Positive clones are enriched in several steps of subpooling. In the last step, positive single clones are sequenced.

For the library screening COS-7 cells, which served as APC were co-transfected with a plasmid coding for HLA and PECPL via FuGENE® transfection (Section 2.6.2) or nucleofection (Section 2.6.4) and incubated at 37 °C and 5 % CO_2. 32 h or 56 h after transfection, the cells were over-laid with 1.5×10^6 58-IP2-8S1 cells. After cocultivation for 16, the cells were screened for sGFP expression.

If clusters of ≥ 2 activated 58-IP2-8S1 cells were detected, the underlying COS-7 cells was isolated with the help of a micromanipulator and a 14 µm capillary. The isolated cell was

transferred into a prechilled 200 µl PCR tube containing 7 µl H_2O/DEPC. The tube was kept on ice until the plasmids were recovered by PCR.

The peptide-encoding region of the plasmids of the picked COS-7 cell were amplified by PCR. 50 µl of 2x iProof™ High Fidelity Master Mix, 0.5 µl pcDNA-2nd-for-TOPO (100 µM) and 0.5 µl pcDNA-rev3 (100 µM) were added to the picked cell and filled up to 100 µl with H_2O/DEPC.

After an initial denaturation at 98 °C for 3 min, PCR was performed in 40 cycles of denaturation for 20 s at 98 °C, annealing for 20 s at 56 °C and elongation for 30 s at 72 °C, followed by a final elongation at 72 °C for 10 min. The PCR products were kept at -20 °C.

The PCR products were purified with the QIAquick® PCR purification kit (Qiagen), diluted to 1 ng/µl and cloned into pcDNA3.1™D/V5-His-TOPO® with the pcDNA™ 3.1 Directional TOPO® Expression Kit (Invitrogen) according to manufacturer's instructions. The samples were ligated for 30 min at room temperature followed by 16 °C over night. The DNA was precipitated (Section 2.4.15) and solubilized in 2 µl Elution Buffer (EB, Qiagen). *E. coli* Electro Max DH10B (Invitrogen) were transformed with the ligation constructs by electroporation (Section 2.5.2.2). The next day, the number of different plasmids were calculated from the agar plates, and DNA was isolated.

2.9.2 Mimotope screening with Invariant chain-encoded combinatorial peptide libraries

For the identification of CD4+ T cell mimotopes with Ii-ECPL, COS-7 cells stably expressing HLA-DR1 or HLA-DR2a molecules were employed as APC. The cells were transfected with Ii-ECPL of different length via FuGENE® transfection (Section 2.6.2) or nucleofection (Section 2.6.4) and incubated for 32 h or 56 h. The cells were overlaid with 1.5×10^6 58-Bbc9 cells and incubated for 16 h. The cells were screened for activation and the underlying COS-7 cell picked as described in section 2.9.1.

Recovery PCR was performed with p33-lib-for and p33-lib-rev, using standard PCR protocol (Section 2.4.8) running 40 cycles with an annealing temperature of 58 °C and an elongation time of 45 s. PCR products were kept at - 20 °C.

PCR products were purified with the QIAquick® PCR purification kit (Qiagen). Afterwards the PCR products and pcDNA-Ii-33 were digested with AgeI and KasI (Section 2.4.9). The digested vector was dephosphorylated and 100 ng of dephosphorylated vector was ligated with the PCR products at 16 °C over night (Section 2.4.11). After precipitation (Section 2.4.15), the DNA was resolubilized in 2 µl EB and transformed into *E. coli* Electro Max DH10B (Invitrogen) by electroporation (Section 2.5.2.2). The next day, the number of different plasmids were calculated from the agar plates, and plasmids isolated.

2.9.3 Reactivation and enrichment by subpooling

COS-7 cells were seeded in 24 well plates and transfected in duplicate with the purified plasmids (Section 2.9.1 and 2.9.2) by FuGENE® (Section 2.6.2). Either COS-7 cells stably expressing the appropriate MHC molecules were used, or plasmids coding for MHC alleles were co-transfected. 32 h and 56 h after transfected, the cells are overlaid with 250,000 T hybridoma cells and cocultered for 16 h. The wells were screened in a fluorescence microscope by hand, or by using the scanning program ZEN Pro (Zeiss), establishes by G. Rühl (Institut für klinische Neuroimmunologie, Ludwig-Maximilians-Universität München, AG Dornmair). The bacteria stock of the positive well contains the positive clone besides the calculated number of bystander plasmids (Section 2.5.2.2). For subpooling, the original stock was diluted 1/10 and 30 x 1.25 ml LB^{Amp} were inoculated with the bacteria. The bacteria were grown overnight and plasmids were purified the next day. The samples were screened for reactivation as described before.
Positive reactivation was also used for a second round of picking, to enrich the positive clone.

2.10 SDS-Page

Sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western Blot was used to analyse if the transfected Cos-7 cells express the invariant chain Ii-ECPL-wt with the correct size.
For SDS-Page 8-20 % Tris-Glycine gels (NuPage) were used. 50,000 cells were resuspended in 75 µl 1× Laemmli sample buffer (reducing). The proteins were denatured at 95 °C for 5 min. 25 µl of the samples and 7.5 µl of full range rainbow marker (Amersham) were loaded onto the gel. The gel was run with a constant voltage of 130 V for 90 min. Afterwards the gel was used for Western Blot (Section 2.11)

2.11 Western Blot

Western Blot was used to detect CD74 expression of the transient transfected cells.
For this purpose, the proteins which were separated by SDS-Page were blotted onto a polyfinylidenfluorid (PVDF) membrane in a semi-dry blotter. Whatman paper were saturated with anode or cathode buffer and the PVDF membrane activated with methanol. The gel and the membrane were assembled in the transfer cell between 3 Whatman papers. The proteins were blotted with a constant current of 35 mA per gel for 3 h. Unspecific binding sides were blocked with blocking buffer for 1 h at room temperature or over night at 4 °C. For protein detection anti-CD74 [PIN.1] (abcam) was diluted 1:1,000 in blocking buffer, added to the membrane and incubated for one hour at room temperature in the dark. After washing the membrane 3 times for 5 min with washing buffer the detection antibody donkey anti-mouse

IRDye800CW (1:10,000 LI-COR, Lincoln, NE, USA) was added and incubated for one hour at room temperature. The membrane was washed again 3 times for 5 min. Signals were detected with the Odyssey Fc (LI-COR). The marker was detected at 700 nm for 0.5 min, the detection antibody at 800 nm for 10 min.

3 Results

3.1 IP2: Identification of disease relevant T cell receptors

$GABA_A$ receptor encephalitis is a new autoimmune disease pattern, first described by Petid-Pedrol *et al.* 2014. Patients indicate high titre serum antibodies against the α1/β3 subunits, causing a reduction of synaptic $GABA_A$ receptors. Subsequently the patients develop seizures or refractory status epilepticus (Petit-Pedrol et al., 2014). Although, the antibody derived immunity is described, it is not known which role T cells play in the disease. However, T cells often have a key role in autoimmune diseases, like Psoriasis, MS or inflammatory myopathies, by infiltration of autoreactive T cells into the target tissue. Additionally, the presence of antibodies against $GABA_A$ α1 subunit indicate the existence of CD4+ T cells, which are crucial for most humoral immune responses

To analyse the role of T cells in $GABA_A$ encephalitis, the T cell repertoire in the CSF (Section 3.1.1), the hippocampus (Section 3.1.2) and the lateral sulcus (Section 3.1.3) of IP2 was characterised with different methods like IHC, LMD and NGS. All isolated TCR were designated according to Arden (Arden 1995).

3.1.1 T cell repertoire from the CSF of IP2

The T cell repertoire of the CSF of IP2 was analysed by an unbiased multiplex single cell TCR PCR (Section 2.4.1), which allows to analyse the matching α- and β- chain of single T cells. To this end, a universal α- and β-chain primer pool (Seitz *et al.,* 2006; Kim *et al.,* 2012), which was further improved by Q. Zhou (personal communication), was used.

250 µl CSF were sorted via MACS beads (Section 2.2.1). Firstly, the CSF was positively sorted for CD4+ T cells, followed by a positive selection for CD8+ T cells of the flow-through. Single cells were isolated with a pipette at the microscope. RT-reaction and unbiased TCR PCR were performed.

40 CD4+ T cells and 48 CD8+ T cells were isolated from the CSF of IP2. 15 β-chains of the 40 CD4+ T cells were obtained. Hereof 12 corresponding α-chains could be identified, including one cell which expresses two different α-chains, but only one β-chain (Table 3-1).

The identified TCR of the CD4+ T cells in CSF of IP2 show a high variety. Every cell is unique. There is no clonal expansion of any CD4+ T cell in the CSF. The CD4+ T cells in the CSF of IP2 are polyclonal.

Table 3-1 TCR chains of single CD4+ T cells from CSF of IP2. TCR β-chains and if detected, corresponding α-chains are listed, with their TRV and TRJ chains, their peptide sequence of the V-CDR3-Joining regions, and the frequency, how often the TCR was detected in CSF.

TCR no.	Chain	TRV	TRJ	Peptide Sequence V-CDR3-Joining	Frequency (Total: 15)
1	β	7S3	1-1	CASS-LKTGGYL-NTEAFFGQG	1
	α	18S1	45	CAF-MG-GGGADGLTFGKG	1
2	β	17S1	1-1	CASS-WPGQG-NTEAFFGQG	1
	α	23S1	58	CAVR-VV-ETSGSRLTFGEG	1
	α	3S1	12	CATD-Q-MDSSYKLIFGSG	1
3	β	13S3	1-1	CASS-SLQGSPRG-NTEAFFGQG	1
	α	2S3	44	CAVV-SP-TGTASKLTFGTG	1
4	β	13S1	1-2	CAS-RSGD-NYGYTFGSG	1
	α	18S1	41	CA-FPGR-SGYALNFGKG	1
5	β	6S2	2-1	CASS-IKNP-YNEQFFGPG	1
	α	1S5	53	CAV-SAR-GGSNYKLTFGKG	1
6	β	5S1	1-1	CASS-FGAR-TEAFFGQG	1
7	β	9S1	1-1	CASS-QGSWARM-NTEAFFGQG	1
	α	2S1	54	CAM-RL-QGAQKLVFGQG	1
8	β	3S1	1-5	CASS-IEG-NQPQHFGDG	1
	α	8S2	4	CAEN-P-GGYNKLILEQGPG	1
9	β	8S1	2-5	CAS-TLRGT-ETQYFGPG	1
10	β	8S1	2-1	CASS-FRLAGY-YNEQFFGPG	1
11	β	25S1	1-4	CASSQ-PLAGS-TEKLFFGSG	1
	α	14S1	27	CAYRS-GP-AGKSTFGDG	1
12	β	13S6	1-2	CASS-YTEAS-YGYTFGSG	1
	α	4S2	20	CIV-SV-SNDYKLSFGAG	1
13	β	8S1	2-3	CASS-WGV-TDTQYFGPG	1
	α	2S2	21	YLC-VMRG-NFNKFYFGSG	1
14	β	3S1	1-2	CAS-TLRDS-YGYTFGSG	1
15	β	13S1	1-2	CAS-TDSTGTGA-NYGYTFG	1
	α	2S1	49	CAV-TP-NTGNQFYFGTG	1

Furthermore, the β-chains of 13 cells of the 48 picked CD8+ T cells were identified in total. Six of these 13 cells expresses the same β-chain.

The matching α-chains of 11 cells were obtained, whereof one cell expressed two functional α-chains. All of the six CD8+ T cells with the same β-chain, also expresses the same α-chain (Table 3-2).

In contrast to the CD4+ T cells, which are all unique in CSF, the accumulation of one CD8+ T cell clone indicates that the CSF contains an expanded CD8+ T cell clone. The β-chain of this

expanded clone is expressed in 46 % of all β-chains of CD8+ T cells found in CSF. The expanded clone (TCR no. 16, Table 3-2) was termed IP2-8S1.

Table 3-2 TCR chains of single CD8+ T cells from CSF of IP2. TCR β-chains and if detected, corresponding α-chains are listed, with their TRV and TRJ chains, their peptide sequence of the V-CDR3-Joining regions, and the frequency, how often the TCR was detected in CSF.

TCR no.	Chain	TRV	TRJ	Peptide Sequence V-CDR3-Joining	Frequency (Total: 13)
16 (=IP2-8S1)	β	8S1	2-3	CASS-AGG-DTQYFGPG	6
	α	8S1-A1	49	CAAS-WG-TGNQFYFGTG	6
17	β	8S1	1-6	CASS-FLDK-NSPLHFGNG	1
	α	19S1	48	CAV-IGRGP-NFNEKLTFGTG	1
18	β	5S1	1-5	CASS-QGPQ-SNQPQHFGDG	1
	α	24S1	5	CAVVS-AE-DTGRRALTFGSG	1
19	β	6S4	1-4	CAS-TVA-TNEKLFFGSG	1
	α	2S2	20	CAMS-VD-DYKLSFGAG	1
20	β	6S5	2-5	CASS-PGQG-QETQYFGP	1
21	β	6S2	1-5	CASSL-AEGY-SNQPQHFGDG	1
22	β	8S2	2-6	CASS-LSSD-GANVLTFGAG	1
	α	1S4	30	CAVGA-P-NRDDKIIFGKG	1
23	β	9S1	1-6	CASSQ-DTGR-SPLHFGNG	1
	α	2S1	4	CAVN-SQ-GGYNKLIFGAG	1
	α	25S1	53	CA-AS-SGGSNYKLTFGKG	1

3.1.2 T cell repertoire analysis of the hippocampus of IP2

3.1.2.1 Analysis of infiltrated T cells in the hippocampus by IHC

IP2-8S1 was clonally expanded in the CSF of IP2 (Section 3.1.1). Biopsy sections of the hippocampus of IP2 were used to analyse, whether there is also an infiltration of T cells into the brain. Furthermore, it was analysed, if the infiltrated T cells are also activated.

First, IHC for CD3, CD4 and CD8 was performed with paraffin-embedded biopsy sections of the hippocampus. Staining of the hippocampus demonstrated a much higher infiltration of CD8+ T cells, compared to CD4+ T cells (Figure 3-1). An average of 77 CD3+ T cells were detected per cm^2 in the hippocampal region, from which 13 % were double positive for CD3 and CD4 and the remaining 87 % double positive for CD3 and CD8. IHC showed that CD8+ T cells in the hippocampus were predominantly parenchymal, whereas CD4+ T cells were mostly perivascular (Figure 3-1). In the hippocampus regions with an infiltration of many CD8+ T cells were found and less infiltration of CD4+ T cells. Thereby, the disease exhibit great analogies

to other immune diseases, like MS, where also CD8+ T Cells were far numerous compared to CD4+ T cells in the analysed MS lesions (Babbe *et al.,* 2000).

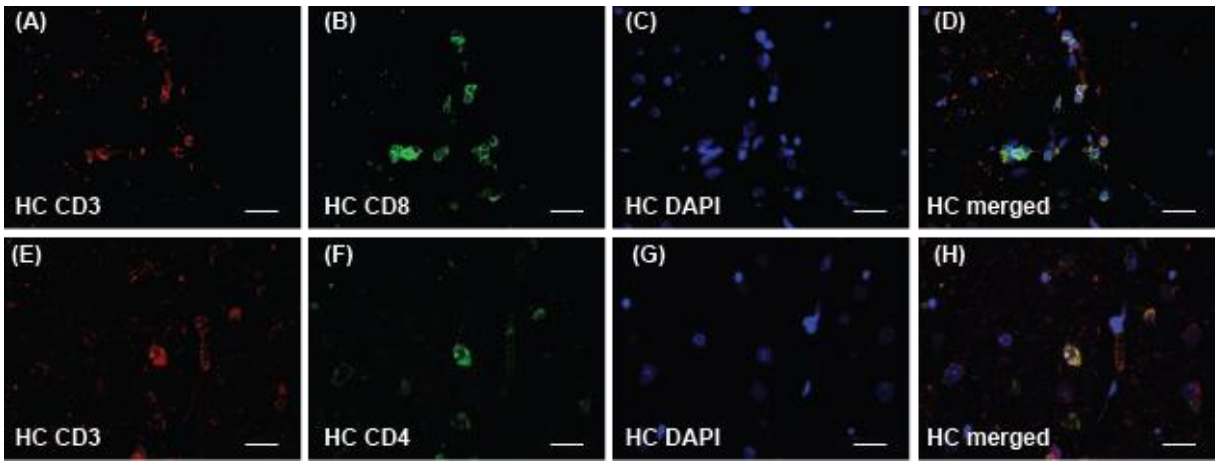

Figure 3-1 Infiltration of T cells in the hippocampus (HC) of patient IP2. Hippocampal paraffin sections of brain biopsy of IP2 were stained for CD3 (red, A, E), CD8 (green B), DAPI (blue, C; G) and CD4 (green, F), merged (D, H) represent matched colors. (A-D) CD8+ T cells in the HC. (E-H) CD4+ T cells in the HC. Scale bar: 25 µm.

To distinguish between bystander CD8+ T cells, which are attracted by cytokines, and activated CD8+ T cells, IHC for activation markers were used. Activated CD8+ T cells express cytotoxic granules, composed of perforin and granzymes (Pipkin and Lieberman 2007). For this purpose, staining of CD8, perforin and DAPI of frozen brain sections was performed and visualized by fluorescence microscopy (Figure 3-2).
This IHC showed that activated CD8+ T cells are present in the hippocampus. Around 50 % of the CD8+ T cells express perforin.

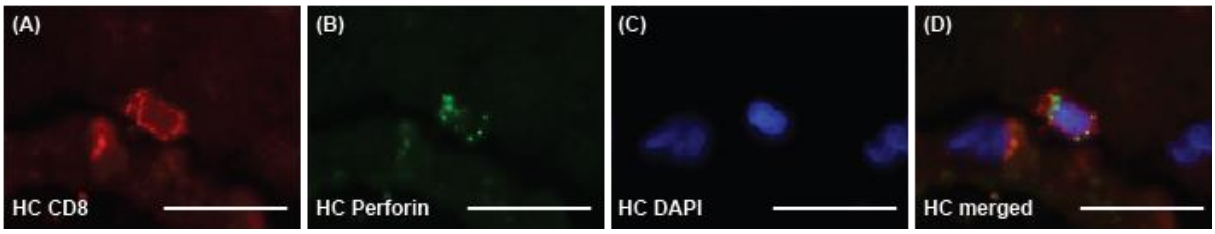

Figure 3-2 Potential pathogenic role of CD8+ T cells in the hippocampus (HC) by the expression of activation markers Sections of the HC were costained for the expression of CD8 (red, A), perforin (green, B) and DAPI (C) and afterwards merged (D) The staining demonstrates expression of perforin, and thereby the presence of activated CD8+ T cells in infiltrated regions of the hippocampus. Scale bar: 20 µm.

3.1.2.2 T cell repertoire of the hippocampus

For the analysis of the TCRs in the brain, firstly bulk cells of the hippocampus were used to isolate mRNA, transcribe it to cDNA and perform a clone specific PCR for the expanded T cell clone IP2-8S1. The presence of the α-chain IP2-AV8S1 of the expanded T cell clone was

verified in the hippocampus of IP2 by PCR. This suggests an infiltration of the expanded CD8+ T cells to the brain. The β-chain IP2-BV8S1 was not detected.

Due to the fact that IP2-BV8S1 did not show up during bulk cell analysis, and that bulk cell analysis does not show how often the expanded clone occurs in the inflammatory brain regions of IP2, single cells were isolated by LMD from hippocampal sections. The cells were analysed in parallel by unbiased multiplex TCR PCR and by clone-specific PCR.

179 single T cells were isolated by LMD of the hippocampus (Figure 3-3). 177 of them were positive for CD8+ and two of them were positive for CD4+. The isolated cells were analysed by unbiased multiplex TCR PCR. No TCR chain was identified of the CD4+ T cells. However, three other β-chains of CD8+ T cells were identified in the hippocampus, which were not detected in CSF. One of them was detected in three CD8+ T cells of the hippocampus (TCR no. 24). No corresponding α-chain was identified (Table 3-3).

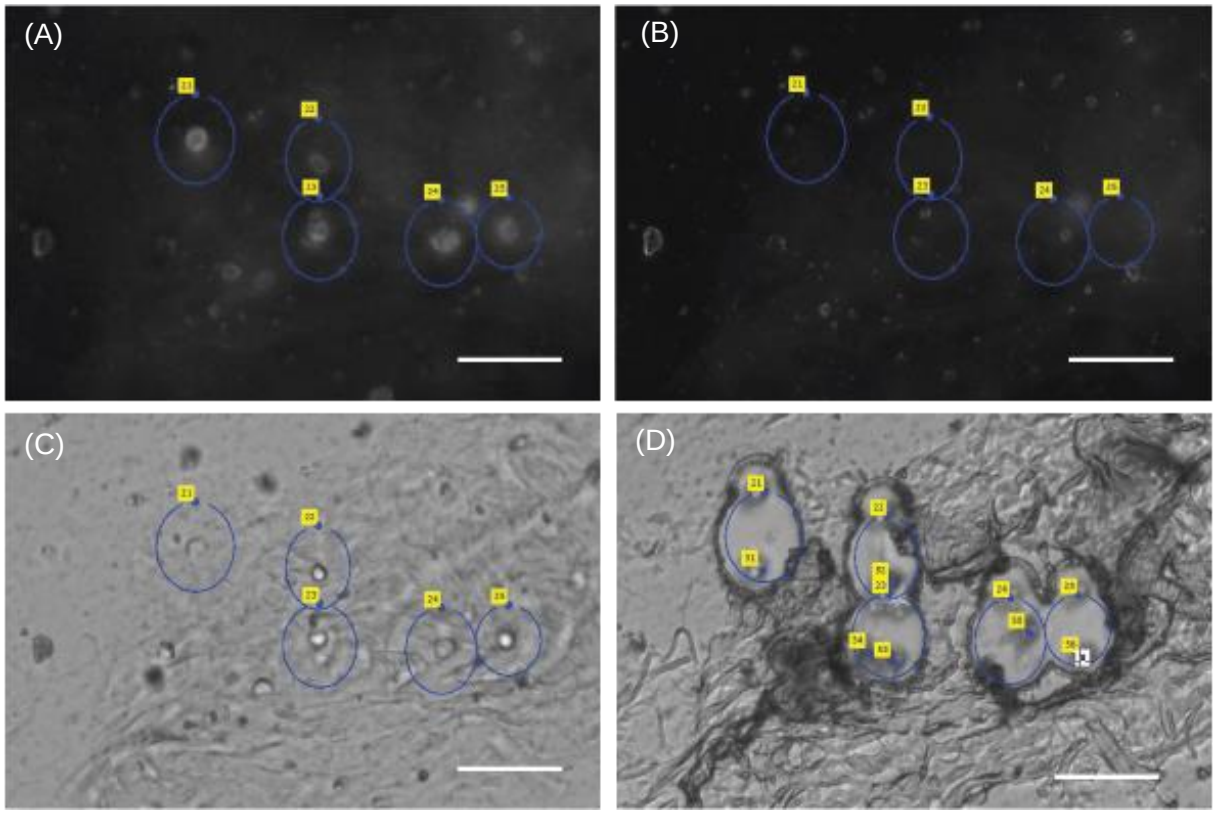

Figure 3-3 Laser microdissection of single cells in HC sections of brain biopsy. 10 µm hippocampal sections on PET foil were stained with a Cy-3 labeled antibody against human CD8 (A) and a AF488 labeled antibody against CD4 (B). Positive cells were marked and controlled in transmitted light (C). Single cells were isolated by LMD (D). Scale bar: 25 µm.

In parallel, clone-specific PCR was performed with the samples of the isolated CD8+ T cells. The β-chain IP2-BV8S1 (TCR no. 16), which was clonally expanded in CSF, was detected in 22 CD8+ T cells from the hippocampus by clone-specific PCR The corresponding α-chain IP2-AV8S1 was found in 19 of the 22 cells (Table 3-3).

This provides clear evidence that IP2-8S1 is expanded in the CSF of IP2 and that it is also present in infiltrated brain regions. Furthermore, it is also strongly expanded in the hippocampus. 19 cells out of 177 of all isolated CD8+ T cells from the hippocampus (11 %) express IP2-8S1, and 22 out of 27 β-chains in the hippocampus (81 %) are IP2-BV8S1. Additionally, CD8+ T cells also express perforin, indicating the presence of activated CD8+ T cells in the hippocampus (Section 3.1.2.1).

Table 3-3 TCR chains of single CD8+ T cells of the hippocampus of IP2. TCR β-chains and if detected, corresponding α-chains are listed, with their TRV and TRJ chains, their peptide sequence of the V-CDR3-Joining regions, and the frequency, how often the TCR was detected in CSF.

TCR no.	Chain	TRV	TRJ	Peptide Sequence V-CDR3-Joining	Frequency (Total: 27)
16 (= IP2-8S1)	β	8S1	2-3	CASS-AGG-DTQYFGPG	22
	α	8S1	49	CAAS-WG-TGNQFYFGTG	19
24	β	6S3	2-7	CASSL-NGIS-SYEQYFGPG	3
25	β	6S2	2-2	CASSL-APN-GELFFGEG	1
26	β	8S1	1-6	CAS-RRA-NSPLHFGNG	1

3.1.2.3 Validation of IP2-8S1 in the hippocampus by NGS

Next generation sequencing (NGS) studies were performed in cooperation with E. Beltrán and the sequencing was performed at IMGM Laboratories GmbH (Planegg, Germany) to validated the presence of IP2-8S1 in the brain. Samples were prepared from bulk cells. To distinguish between PCR amplification and clonal expansion, a unique molecular identifier (UMI) was added during PCR. Only sequences with different UMI were counted for the quantification.
IP2-AV8S1 was detected 236 times in the hippocampus. Together with one other α-chain, IP2-AV8S1 was one of the most expanded α-chain (Table 3-4). The NGS reaction for the β-chains provided less chains than expected. Only three different beta chains were detected, not including IP2-BV8S1. Due to the low yield of beta chains in the hippocampus, the NGS sequencing reaction is repeated currently by E. Beltrán. The α-chain was detected in the same rate in the hippocampus by LMD compared to NGS. Thus we expect to detect IP2-BV8S1 with the same frequency in the next NGS reaction.
Although NGS reaction for the β-chain is currently repeated, the results of the hippocampus validate the data of LMD, where it was shown that the expanded clone also shows up frequently in the infiltrated regions of the hippocampus (Table 3-4).

Table 3-4 Comparison of T cell repertoir analysis LMD and NGS of the HC. The quantity of detected IP2-AV8S1 and IP2-BV8S1 in the HC detected in LMD and NGS are listed. (*) NGS reaction of the β-chain is repeated at the moment, due to low yields in the first experiment.

TCR no.	Chain	TRV	TRJ	Peptide Sequence V-CDR3-Joining	LMD HC	NGS HC
16 (= IP2-8S1)	β	8S1	2-3	CASS-AGG-DTQYFGPG	22	n.a.*
	α	8S1	49	CAAS-WG-TGNQFYFGTG	19	236

3.1.3 T cell repertoire analysis of the lateral sulcus of IP2

3.1.3.1 Analysis of infiltrated T cells in lateral sulcus by IHC

IHC of the hippocampus of IP2 showed a strongly infiltration of CD8+ T cells into the brain, as well as the presence of the expanded CD8+ T cell clone IP2-8S1 in the hippocampus. The lateral sulcus of IP2 was also inflamed. Biopsy sections of the lateral sulcus of IP2 were analysed by IHC. The TCR repertoire was analysed in bulk cells, single cells and by NGS.
The T cell infiltration of the lateral sulcus of IP2 was analysed by IHC of paraffin sections for CD3, CD4 and CD8.The same infiltration pattern as in the hippocampus was also observed in the lateral sulcus (Figure 3-4).

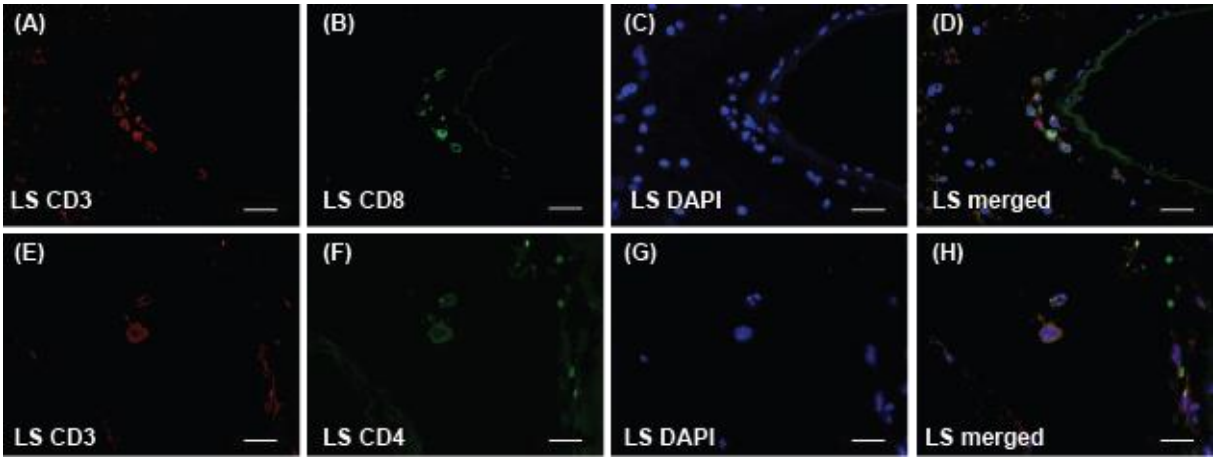

Figure 3-4 Infiltration of T cells in the lateral sulcus (LS) of patient IP2. Paraffin sections of the lateral sulcus of IP2 were stained for CD3 (red, A, E), CD8 (green B), DAPI (blue, C; G) and CD4 (green, F), merged (D, H) represent matched colors. (A-D) CD8+ T cells in the LS. (E-H) CD4+ T cells in the LS. Scale bar: 25 µm.

147 CD3+ T cells were detected per cm^2. 80 % of them were CD8+ T cells, and 20 % CD4+ T cells. In contrast to the hippocampus, where many CD8+ T cells were parenchymal, CD8+ T cells in the lateral sulcus were predominantly perivascular. Comparable to hippocampal staining, CD4+ T cells were mostly perivascular.

Staining for the expression of activation markers in CD8+ T cells was used to distinguish between activated CD8+ T cells and bystanders in the lateral sulcus. Frozen biopsy sections were stained for CD8, perforin, and DAPI and visualized by fluorescence microscopy (Figure 3-5).

IHC for perforin showed that activated CD8+ T cells are also available in the lateral sulcus. Around 80 % of the infiltrated CD8+ T cells express the activation marker perforin.

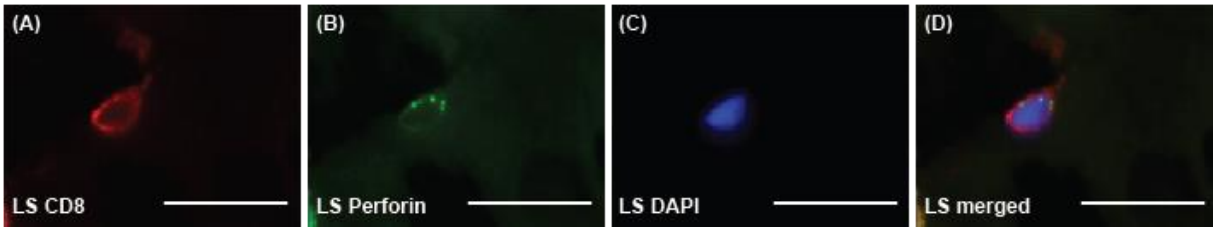

Figure 3-5 Potential pathogenic role of CD8+ T cells in the lateral sulcus (LS) by the expression of activation markers Sections of the LS were costained for the expression of CD8 (red, A) perforin (green, B), and DAPI (C) and afterwards merged (D) The staining demonstrates expression of perforin, and thereby the presence of activated CD8+ T cells in infiltrated regions of the lateral sulcus. Scale bar: 20 µm.

3.1.3.2 T cell repertoire of the lateral sulcus

Staining of the lateral sulcus also showed inflamed regions. To analyse if the expanded clone IP2-8S1 also occurs in the lateral sulcus, first bulk cells of frozen biopsy sections of the lateral sulcus were analysed by clone-specific PCR for IP2-8S1. For this purpose, mRNA was isolated from the biopsy sections, transcribed to cDNA and clone specific PCR for the expanded T cell clone IP2-8S1 was performed. Comparable to the hippocampus, IP2-AV8S1 was verified in the lateral sulcus of IP2 by PCR. This presumes an infiltration of the expanded CD8+ T cells to the lateral sulcus. IP2-BV8S1 was not detected.

To analyse the T cell receptor repertoire of the lateral sulcus, single cells were isolated by LMD from frozen sections and unbiased as well as clone-specific PCR for the detection of IP2-8S1 was performed in parallel. For this purpose, 10 µm sections of the lateral sulcus were stained for CD4 and CD8. Single T cells were isolated by LMD (Figure 3-6) and analysed by unbiased and clone-specific PCR. Pipetting of the PCR was performed with the Janus pipetting robot (Section 3.1.4).

In total 111 T cells were isolated by LMD of the lateral sulcus. All isolated T cells were detected as CD8+. None of them were positive for CD4.

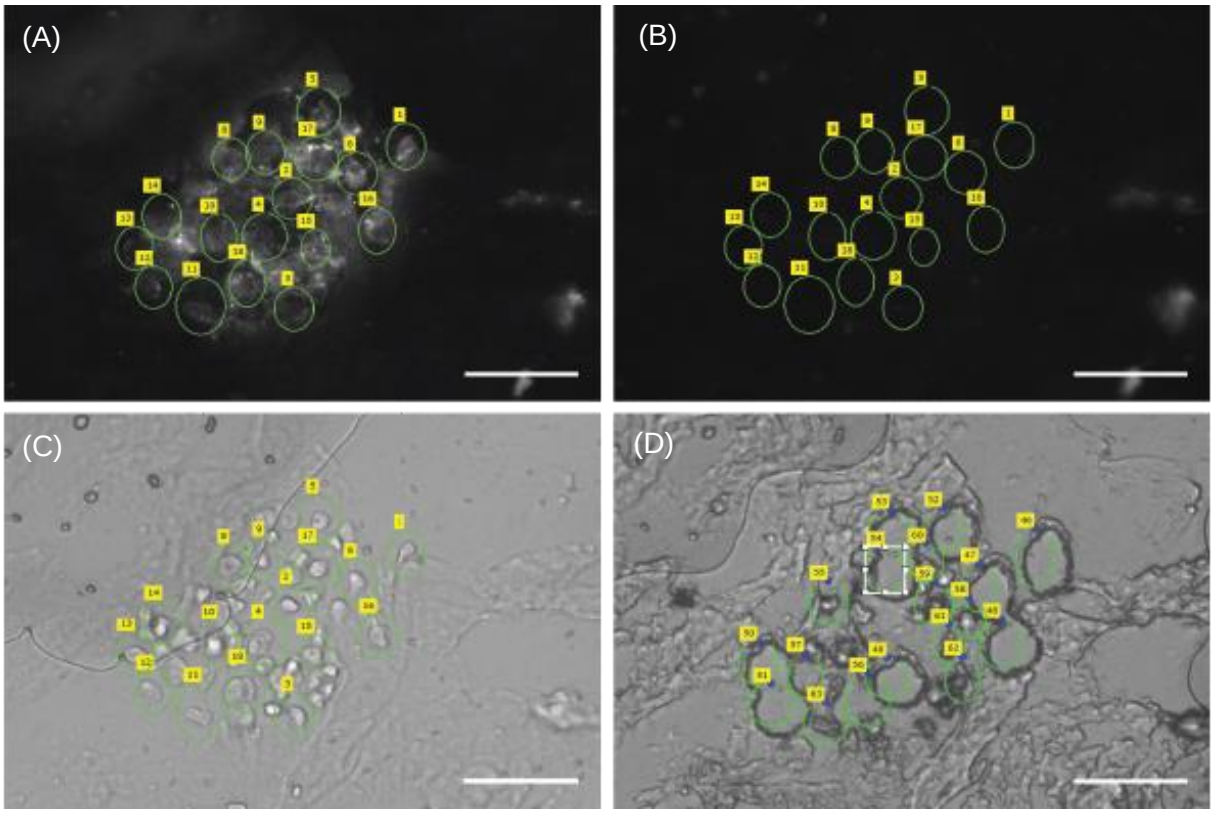

Figure 3-6 Laser microdissection of single cells in LS sections of brain biopsy. 10 µm sections of the lateral sulcus of IP2 on PET slides were stained with Cy3-labeled antibody against human CD8 (A) and AF488 labeled antibody against human CD4 (B). Stained cells were marked and controlled in transmitted light (C). LS section after the isolation of single cells LMD (D). Scale bar: 25 µm.

No TCR chains were detected in the isolated single cells by unbiased multiplex TCR PCR and IP2-8S1 was not detected by clone-specific TCR PCR. However, two TCR β-chain were identified by clone-specific TCR PCR from the lateral sulcus. One of them showed up in two single isolated CD8+ T cells (Table 3-5). Both β-chain are also TRV-B8S1, but their CDR3 region differs from IP2-8S1. Both chains were neither found in CSF nor in the hippocampus of IP2. The corresponding α-chains were not detected.

Table 3-5 TCR chains of single CD8+ T cells from the lateral sulcus of IP2. TCR β-chains are listed, with their TRV and TRJ chains, peptide sequence of the V-CDR3-Joining regions, and the frequency, how often the TCR was detected in CSF.

TCR no.		TRV	TRJ	Peptide Sequence V-CDR3-Joining	Frequency (Total: 2)
27	β	8S1	2-1	CASS-PGLAMD-YNEQFFGPG	2
28	β	8S1	1.4	CAS-AG-TNEKLFFGSGT	1

3.1.3.3 Validation of IP2-8S1 in the lateral sulcus by NGS

NGS studies of the lateral sulcus were performed in cooperation with E. Beltrán and run at IMGM Laboratories GmbH (Planegg, Germany), as described before (Section 2.4.3 and 3.1.2.3), to validated the presence of IP2-8S1 in the lateral sulcus. Samples were prepared from bulk cells.

IP2-AV8S1 was detected one time in the lateral sulcus and IP2-BV8S1 three times (Table 3-6). NGS validates the infiltration of IP2-8S1 in the lateral sulcus, shown by clone-specific PCR of bulk cells (Section 3.1.3.2). In comparison to the hippocampus, the infiltration of IP2-8S1 is less.

Table 3-6 Comparison of T cell repertoir analysis LMD and NGS of the HC and the LS

TCR no.	Chain	TRV	TRJ	Peptide Sequence V-CDR3-Joining	LMD LS	NGS NGS
16 (= IP2-8S1)	β	8S1	2-3	CASS-AGG-DTQYFGPG	-	3
	α	8S1	49	CAAS-WG-TGNQFYFGTG	-	1

3.1.4 Standardizing single cell PCR with Janus Pipetting Robot

The Janus Pipetting Robot was used to standardize single cell PCR (sc PCR). To this end, two programs were designed during this thesis together with G. Rühl, and further adapted by M. Schönwetter (Institut für klinische Neuroimmunologie, Ludwig-Maximilians-Universität München, AG Dornmair). The protocols were optimized for perfect pipetting by adjusting the performance files for pipetting, like aspiration and dispension speed and so forth (Section 2.4.4).

The advantages in using the pipetting robot are, that the pipetting is standardised and it can be used for high-throughput experiments. Additionally, the robot was placed inside a box in a clean room. This reduces the risk of contaminations during sc PCR. The room as well as the inside of the box can be UV-irradiated, which further reduces the risk of contaminations.

With these optimized protocols, it is now possible to use the robot for the pipetting of the run-off reaction, the anchor PCR, as well as the alpha PCR (Section 2.4.1). Furthermore, it is also ready for pipetting of clone-specific PCR2 and 3 (Section 2.4.2). Precise and contamination-free pipetting were tested for several PCRs. During this study, it was successfully utilized for pipetting of the PCRs of LMD samples of the lateral sulcus.

3.2 IP2-8S1 antigen search

Clone IP2-8S1 could be detected not only in the CSF of IP2, but also in the hippocampus and the lateral sulcus. Additionally, the clone showed up several times, which is ascribed to a clonal expansion of CD8+ T cells. The clonal expanded CD8+ T cell also infiltrated the brain. This indicates an important role of IP2-8S1 in the disease. However, it is not known which antigen is recognized by the TCR.

Several approaches were used to identify the HLA restriction of IP2-8S1 and to find the antigen of the receptor. Hybridoma cells expressing sGFP under control of the NFAT enhance and IP2-8S1 (58-IP2-8S1), were cocultivated with LCLs expressing the HLA alleles of IP2 to test if the TCR recognises an endogenous peptide (Section 3.2.3.2). Additionally, possible antigen candidates of the $GABA_A$ receptor α1 subunit were tested for their ability to activate 58-IP2-8S1 cells (Section 3.2.4). Furthermore, library screening with PECPL and 58-IP2-8S1 cells was performed (Section 3.2.5).

3.2.1 *In vitro* expression of recombinant IP2-8S1 T cell receptor

IP2-AV8S1 and IP2-BV8S1, coding for the variable, N(D)N and joining region of the α- and β-chain of IP2-8S1 were ordered at Geneart and cloned into pRSV that already contained the constant region of the TCR. The expression vectors coding for the α- and β-chain were cotransfected into $58^{-/-}$-CD8+-NFAT-sGFP cells (produced by K. Siewert and G. Rühl) by electroporation and set on antibiotics for the selection of clones stably expressing IP2-8S1 (Section 2.6.6)

3.2.2 Detection of the expression of TCR, CD8 and NFAT-sGFP

IP2-8S1 was successfully transfected into $58^{-/-}$ hybridoma cells. Expression of murine CD3 and human CD8 in the received clones was detected by flow cytometry (Figure 3-7). The clone, showing highest expression levels was kept and used for further studies.

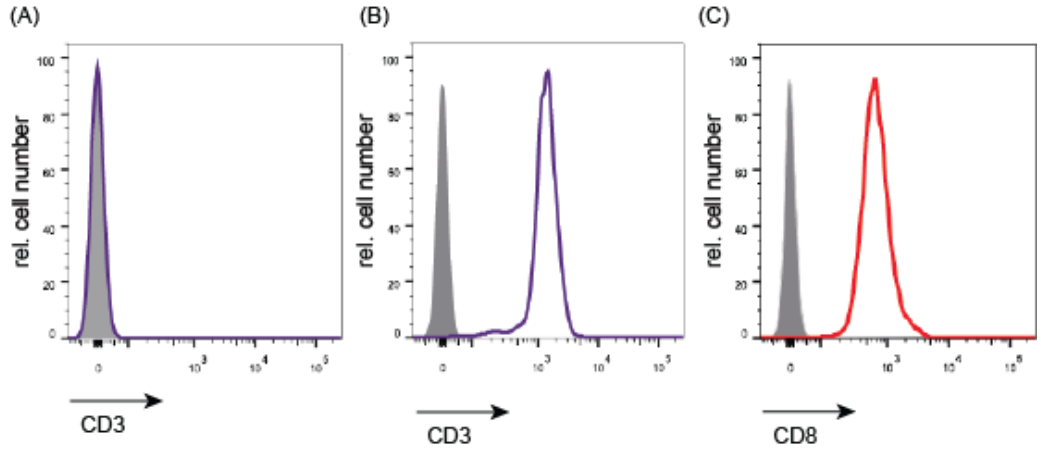

Figure 3-7 Detection of surface expression of TCR and CD8 of transfected 58$^{-/-}$ hybridoma cells by flow cytometry. The expression of TCR in 58$^{-/-}$ transfected with IP2-AV8S1 and IP2-BV8S1 was detected with a FITC-labeled antibody against murine CD3ξ (purple). 58$^{-/-}$ do not express CD3 (A). After transfection with IP-AV8S1 and IP-BV8S1 CD3 expression of the cells is detected by flow cytometry (B). The expression of CD8 was detected with an APC-labeled antibody against human CD8 (C).

The expression of sGFP under the control of the NFAT-enhancer in hybridoma cells is used as a reporter for the mimotope screening. The cells were activated *in vitro* by CD3 cross-linking via a murine anti-CD3 antibody, to test their ability to express sGFP after activation. sGFP expression was detected by flow cytometry as well as by microscopy (Figure 3-8).

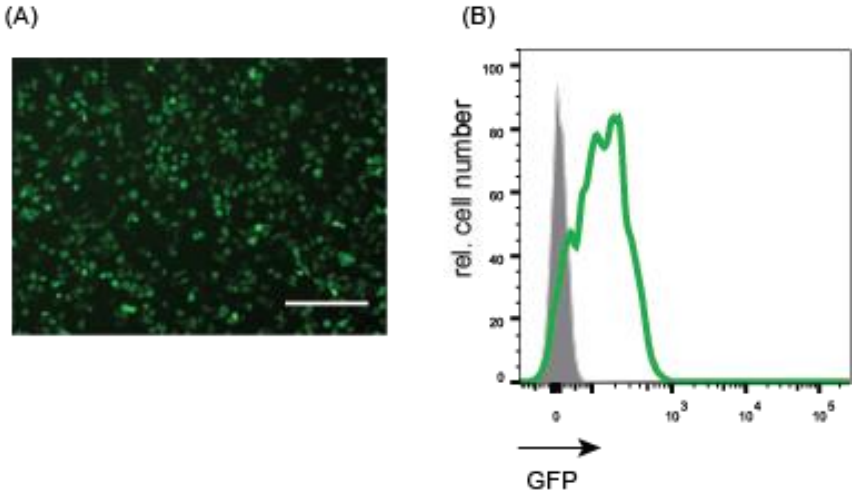

Figure 3-8 Expression of sGFP in 58-IP2-8S1 cells after CD3 activation. (A) Fluorescence microscopy of CD3 activated 58-IP2-8S1, scale bar 200 µm. (B) Flow cytometry of CD3 activated 58-IP2-8S1 cells, gated on living cell population.

3.2.3 HLA restriction of IP2-8S1

TCRs are HLA restricted in their antigen recognition (Zinkernagel and Doherty 1974). For the library screening of IP2-8S1 it is indispensable to know which HLA molecules were expressed by IP2.

3.2.3.1 HLA typing of IP2-8S1

For HLA typing, genomic DNA was isolated of a 10 µm hippocampal section of IP2 with the QIAamp DNA micro kit (Section 2.4.16). The isolated DNA was send to the “Labor für Immungenetik und Diagnostik” at the Klinikum der Universität München to perform HLA typing of IP2.

IP2 is heterozygote in the expression of MHC-I and MHC-II alleles. The expressed HLA alleles are displayed in Table 3-7.

Table 3-7 HLA typing of IP2

MHC class	Gene	Allele 1	Allele 2
MHC-I	HLA-A	*02:01	*23:01
	HLA-B	*35:01	*44:03
	HLA-C	*04:01	*16:01
MHC-II	HLA-DQB1	*02:02	*05:03
	HLA-DPB1	*02:01	*04
	HLA-DRB1	*07:01	*14:54/14:01

3.2.3.2 Cocultivation of IP2-8S1 with LCL

One method to identify the MHC-restriction of TCRs is to incubate them with LCL expressing different HLA alleles. In case the TCR recognizes endogenous peptides, presented by the correct HLA molecule, the TCR gets activated. It was not possible to establish patient relevant LCL. Thus, LCL of other patients, covering all HLA alleles of IP2 were used for cocultivation. HLA typing of the different LCL are listed in Section 2.1.9.1. Cocultivated 58-IP2-8S1 cells were analysed for activation by flow cytometry, gated on living, CD3+ cells. It was not possible to analyse the cells by microscopy, because the cells are cultivated in round-bottom vessel, to enhance their contact. Thus, it cannot be focused well in the microscope. In addition, LCLs express sGFP as reporter gene, which was introduced during the generation of LCLs. Thereby, in the microscope it cannot be distinguished between sGFP-expressing LCLs and activated hybridoma cells.

EBV B-cells from patient Fe and only the hybridoma cells IP2-8S1 were used as negative control. To enhance the peptide presentation in LCL cell lines, they were stimulated with different concentrations of INF-γ (100 Units (U) and 500 U, Section 1.3.1).

Flow cytometry did not show an activation of IP2-8S1 by the cocultivation with LCL. The percentage of activated IP2-8S1 varied between 0.2-1.9%, which is only background. Also stimulation with INF-γ did not increase activation of IP2-8S1. As positive control, 58-IP2-8S1 cells were activated by cross-linkage via anti-CD3 antibodies (Figure 3-9).

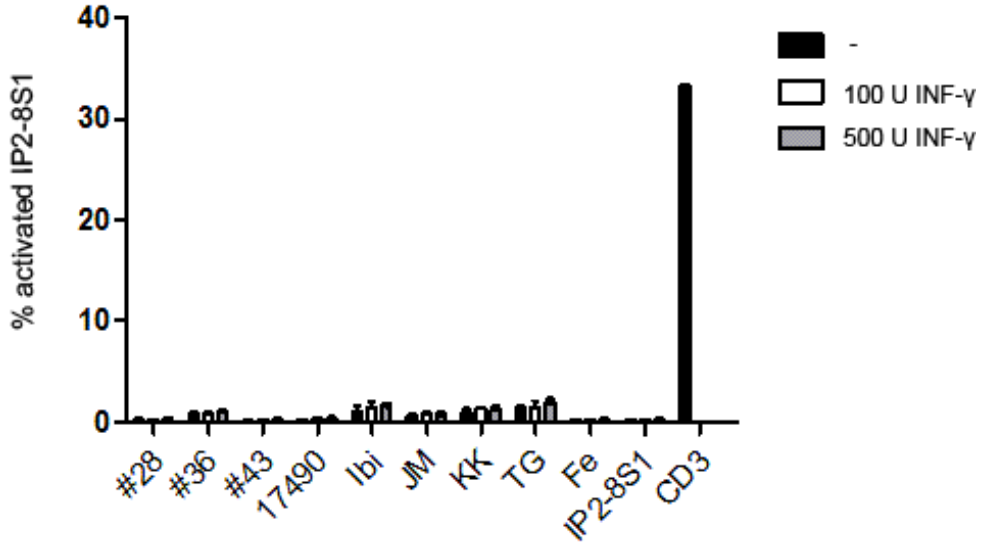

Figure 3-9 Cocultivation of IP2-8S1 with LCL. 58-IP2-8S1 cells were cocultivated with different LCLs without and after stimulation with 100 Units (U) and 500 U of INF-γ. IP2-8S1 alone or **c**ocultivated with Fe served as negative controls. Activation of 58-IP2-8S1 cells via CD3 antibody was used as positive control. Activation varied between 0.2-1.9 %, which represent background.

3.2.4 Analysis of possible antigen-candidates from $GABA_A$ receptor of HLA-dependent epitope binding

In the CSF of IP2, antibodies which recognizes the $GABA_A$ receptor α-1 subunit were identified. The antibody was cloned from B cells of the CSF. *In vitro* studies confirmed the binding to the $GABA_A$ receptor α-1 subunit (E. Beltrán, J. Dalmau, unpublished data). The $GABA_A$ receptor is expressed in the hippocampus for mediating inhibitory synaptic transmission (Houser 1988, Schoch 1985). Simultaneously, an infiltration of the expanded CD8+ T cell IP2-8S1 was found in the hippocampus (Section 3.1.2) and the lateral sulcus (Section 3.1.3). This might indicate that the expanded TCR clone IP2-8S1 recognize an antigen of $GABA_A$ receptor α-1 subunit as well. To select possible antigen-candidates, databases, which comprise HLA epitopes (NetMHC 4.0 Server, Andreatta and Nielsen 2016) but also proteasomal cleavage predictions and TAP transport efficiency (IEDB NetCTL Prediction, Vita 2015) were used. Although the binding patterns and affinities for some HLA alleles of IP2 are characterized in detail, the databases did not include information for all MHC-I alleles of IP2, in particular for HLA-C*04:01 and HLA-C*16:01. Therefore, databases analysis was only performed for HLA-A and -B with the amino acid (AA) sequence of $GABA_A$ receptor α-1 subunit. 9-meric peptides were analysed for their HLA-binding affinities, for their transport into the ER by TAP, and additionally for their potential proteasomal cleavage. Antigen-candidates which fulfil these criterial were selected for the screening. To identify some candidates presented by HLA-C*04:01 and HLA-C*16:01, for which no database search exists, the sequence was analysed for anchor positions, described by Rasmussen *et al.* 2014. For HLA-C*04:01 they described a preferred aspartic acid at position three and a leucine, isoleucine or phenylalanine at position nine. For HLA-

C*16:01 an alanine at position 2, an arginine at position 5 and also a leucine, isoleucine or tyrosine at position 9 is preferred.
DNA coding for 48 antigen-candidate peptides (Section 2.1.2.1) were cloned (Section 2.4.5.4), and co-transfected into COS-7 cells together with plasmids coding for the HLA alleles. 58-IP2-8S1 cells were overlaid, and after 16 h screened for activation in the fluorescence microscope, using the scanning system (Section 2.9.3). Every candidate peptide was tested with all six different HLA alleles. However, no activation was found with any candidate. Thereby, HLA-restriction of IP2-8S1 and its peptide antigens are still unknown.

3.2.5 Library Screening IP2-8S1 with PECPL

To identify possible antigens of IP2-8S1 PECPL-N27 was used. PECPL-N27 is coding for a random, nine AA long peptide. For the screening of possible antigens COS-7 cells were cotransfected with PECPL-N27 and HLA alleles, either by FuGENE® transfection or nucleofection. The screening was performed under different conditions. Either all six HLAs of IP2 were screened individually, or HLA-A (HLA-A*02:01 and HLA-A*23:01), HLA-B (HLA-B*35:01 and HLA-B*44:03), HLA-C (HLA-C*04:01 and HLA-C*16:01) or all six HLA alleles were pooled. Additionally, either 1 µg or 0.1 µg of PECPL were transfected.
Furthermore, a HLA-specific PECPL (A2-269) was used for the screening with HLA-A*02:01. The library expresses fixed anchor positions, composed of an isoleucine at position two, a valine at position six and a leucine at position nine. The anchor positions are specific for HLA-A*02:01 whereby the binding to the HLA molecule is facilitated.
The screened areas, the number of detected clusters and the number of activated 58-IP2-8S1 cells (= cluster size) using the different screening approaches are listed in Table 3-8. In general, K. Siewert, G. Rühl and A. Niedl (Institut für klinische Neuroimmunologie, Ludwig-Maximilians-Universität München, AG Dornmair) observed that the presence of activated clusters indicates the presence of the TCR antigen in the transfected COS-7 cell (Siewert *et al.,* 2012, Rühl *et al.,* 2016). In clusters composed of a higher number of activated hybridoma cells, the probability of antigen-specific activation is increased. Thereby, variations in the cluster size and cluster frequency in the different approaches, can also give a hint for HLA-restriction.
For the screening of all HLA alleles individually two clusters of the size of four activated 58-IP2-8S1 cells were detected with HLA-C*04:01 (Table 3-8 and Figure 3-10 (D)). With the other HLA alleles, no activated 58-IP2-8S1 cells were detected.
In the approach, where all six HLAs were pooled, no cluster of activated 58-IP2-8S1 was detected (Table 3-8).

Table 3-8 Overview library screening IP2-8S1. For the library screening, two different libraries were analysed. Different amounts of the library were transfected, in combination with IP2-specific HLA alleles. The area which was screened, the number of detected clusters and the cluster size of n activated 58-IP2-8S1 cells are listed.

Library	Transfected amount [µg]	HLA	Area [cm^2]	Cluster	Cluster size [n activated cells]
N27	1	A*02:01	93.6	-	-
N27	1	A*23:01	93.6	-	-
N27	1	B*35:01	93.6	-	-
N27	1	B*44:03	93.6	-	-
N27	1	C*04:01	93.6	2	4
N27	1	C*16:01	93.6	-	-
N27	1	A*02:01 and A*23:01	140.4	4	3-4
N27	1	B*35:01 and B44*03	140.4	3	3-4
N27	1	C*04:01 and C*16:01	152.1	4	3-5
N27	1	all six HLAs	46.8	-	-
A02-269	1	A*02:01	23.4	-	-
N27	0.1	A*02:01	23.4	-	-
N27	0.1	A*23:01	23.4	-	-
N27	0.1	B*35:01	23.4	-	-
N27	0.1	B*44:03	23.4	-	-
N27	0.1	C*04:01	23.4	-	-
N27	0.1	C*16:01	23.4	-	-

In the approaches where HLA-A, -B or -C were pooled, clusters of activated 58-IP2-8S1 cells were detected in all samples expressing different HLA alleles (Table 3-8 and Figure 3-10 A-C). The clusters were detected with the same frequency and the same cluster size of activated 58-IP2-8S1 cells. No differences were observed concerning the different HLA-pools. Thereby, HLA restriction could not be resolved.

For all detected clusters, the underlying transfected COS-7 cell were picked, plasmid recovery PCR was performed and cloned into pcDNA3.1D/V5-His-TOPO (Section 2.9.1).

For one sample of the HLA-A pool, one sample of the HLA-C pool and one sample of the HLA-C*04:01, reactivation was observed. However, the reactivation signal was not reproducible during the subpooling procedure. Thus, the underlying COS-7 after reactivation was isolated again, and proceeded as described before. However, no reactivation was observed anymore.

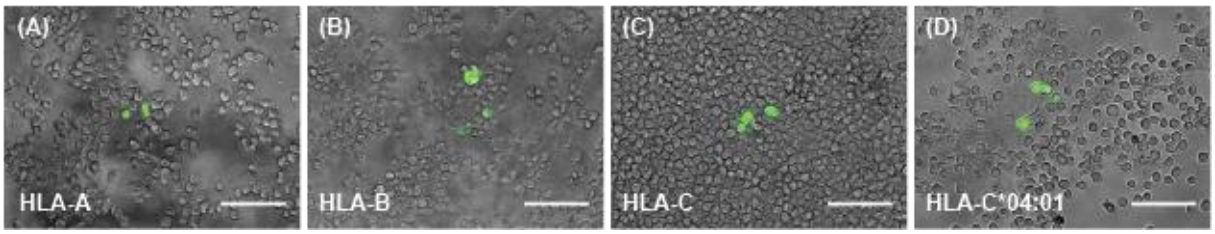

Figure 3-10 Cluster of activated 58-IP2-8S1 cells. COS-7 cells were transfected with PECPL N27 and pooled HLA-A (A), pooled HLA-B (B), pooled HLA-C (C) of IP2 and HLA-C*04:01 (D). The transfected cells were cocultivated with 58-IP2-8S1 cells. The green cells represent 58-IP2-8S1 cells, expressing sGFP after activation, indicating the presence of an antigen recognized by 58-IP2-8S1. Scale Bar: 100 µm.

3.3 Identification of MHC-II mimotopes

We expected to detect an expanded CD4+ T cell clone in IP2, mediating CD4+ T cell help for the humoral immunity in $GABA_A$ receptor encephalitis. Although we then detected a CD8+ T cell clone, we wanted to create the opportunity to screen for CD4+ T cell antigens. Therefor, as a second project, a method to identify MHC-II mimotopes was established. To this end, the method to identify CD8+ T cell antigens of K. Siewert *et al.* (2012) was modified to allow the identification of CD4+ T cell antigens.

As described in Section 1.3.1, the peptide loading of MHC-I and MHC-II molecules is different. Therefore, new libraries for MHC-II were designed (Section 3.3.1). The functionality of the system was tested with the model TCR Bbc9, for which the HLA restriction and the peptide antigen are known. Stable COS-7 cells expressing HLA-DR and 58-Bbc9 hybridoma cells expressing the TCR Bbc9 and sGFP under the control of the NFAT-enhancer were generated (Section 3.3.3 and Section 3.3.4). The ability to activate 58-Bbc9 cells in a HLA-restricted manner was analysed with hMBP-derived peptides of different length. The ability to express Ii-ECPL in COS-7 cells was determined by Western Blot and intracellular flow cytometry (Section 3.3.5). Furthermore, the functionality of the system was confirmed with the positive control Ii-hMBP, expressing hMBP-derived peptides of different length in Ii (Section 3.3.7). In addition, 58-Bbc9 cells were screened for antigens with different Ii-ECPL presented by HLA-DR1 and HLA-DR2a (Section 3.3.9).

An overview over the established system for the identification of MHC-II mimotopes is depicted in Figure 3-11. For the MHC-II libraries, CLIP of Ii-wt was changed to a randomised library sequence (Figure 3-11 A). These Ii-encoded combinatorial peptide libraries (Ii-ECPL) are cotransfected with pHSE3' expressing HLA-DRα and HLA-DRβ into COS-7 cells (Figure 3-11 B). COS-7 cells expressing HLA-DR molecules, which are complexed in the ER with the Ii-ECPL. Thus MHC-II molecules are already loaded in the ER with the library peptides (Figure 3-11 C). Depending on the signal sequence, these complexes are either targeted directly to the cell surface, or to the MIIC by the N-terminal signal sequence of the Ii-ECPL. Here Ii is

degraded, the peptides of the library remain in the binding groove and the complex gets transported to the cell surface, where the peptides of the library are presented to 58$^{-/-}$ hybridoma cells. As a read out for T cell activation, the same detection system as for MHC-I library screening is used. The expression of sGFP under the control of the NFAT-enhancer in hybridoma cells is supposed to be more sensitive than the measurement of the cytokines in the supernatant by ELISA.

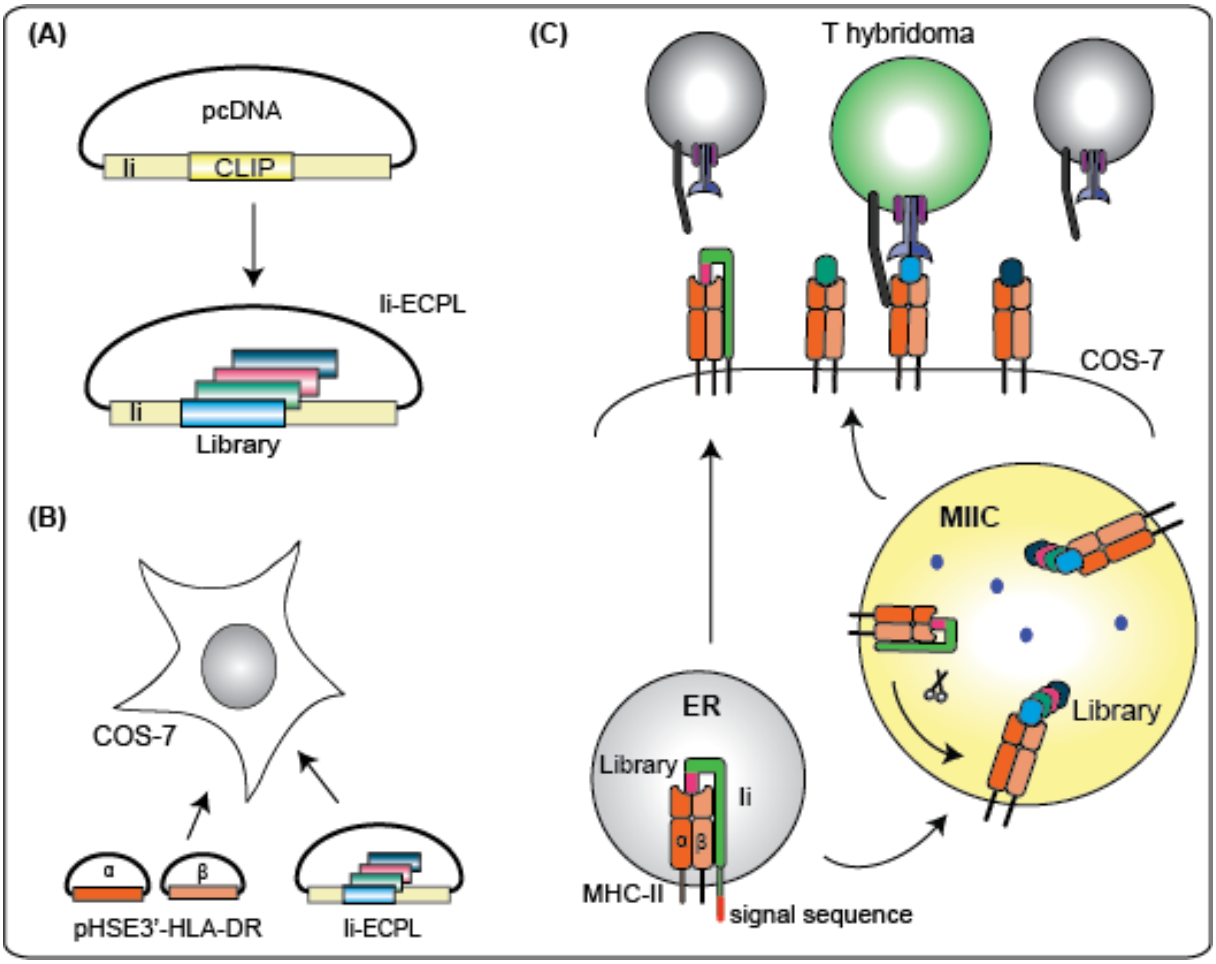

Figure 3-11 Library presentation on MHC-II molecules. (A) For Ii-ECPL, CLIP of Ii is exchanged by a random library. (B) pHSE3' encoding HLA-DRα and –DRβ and pcDNA-Ii-ECPL are co-transfected into COS-7 cells. (C) COS-7 cells express HLA-DR molecules which are loaded in the ER with the library expressed in Ii. Depending on the N-terminal signal sequence of Ii, the complex can either be transported directly to the cell surface or to MIIC, where the invariant chain is degraded and the library loaded on MHC-II molecules is transported to the cell surface. At the cell surface, the library is presented by HLA-DR molecules to hybridoma cells which express TCR and sGFP under the NFAT-enhancer for the detection of activation.

3.3.1 Design of MHC-II Libraries

The design of MHC-II libraries is illustrated in Figure 3-12. MHC-II libraries are cloned in pcDNA-Ii-wt. Hereby CLIP of Ii is replaced by $(NNN)_{9\text{-}13}$ or by $(NNK)_{9\text{-}13}$. N stands for a random nucleotide, K for a nucleotide of the keto-group (guanine or thymin). The application of K at the

third position reduces the risk of Stop-codons in the library. X represents a random AA in the library. Libraries with a methionine (M) at position X and X+8 as anchors for binding at MHC-II molecules, were also designed. The libraries were named by the length of the random nucleotide sequence (N) and the position of the methionine-anchor in the peptide (M, Table 3-9). An overview of the cloned Ii-ECPL is shown in (Table 3-9).

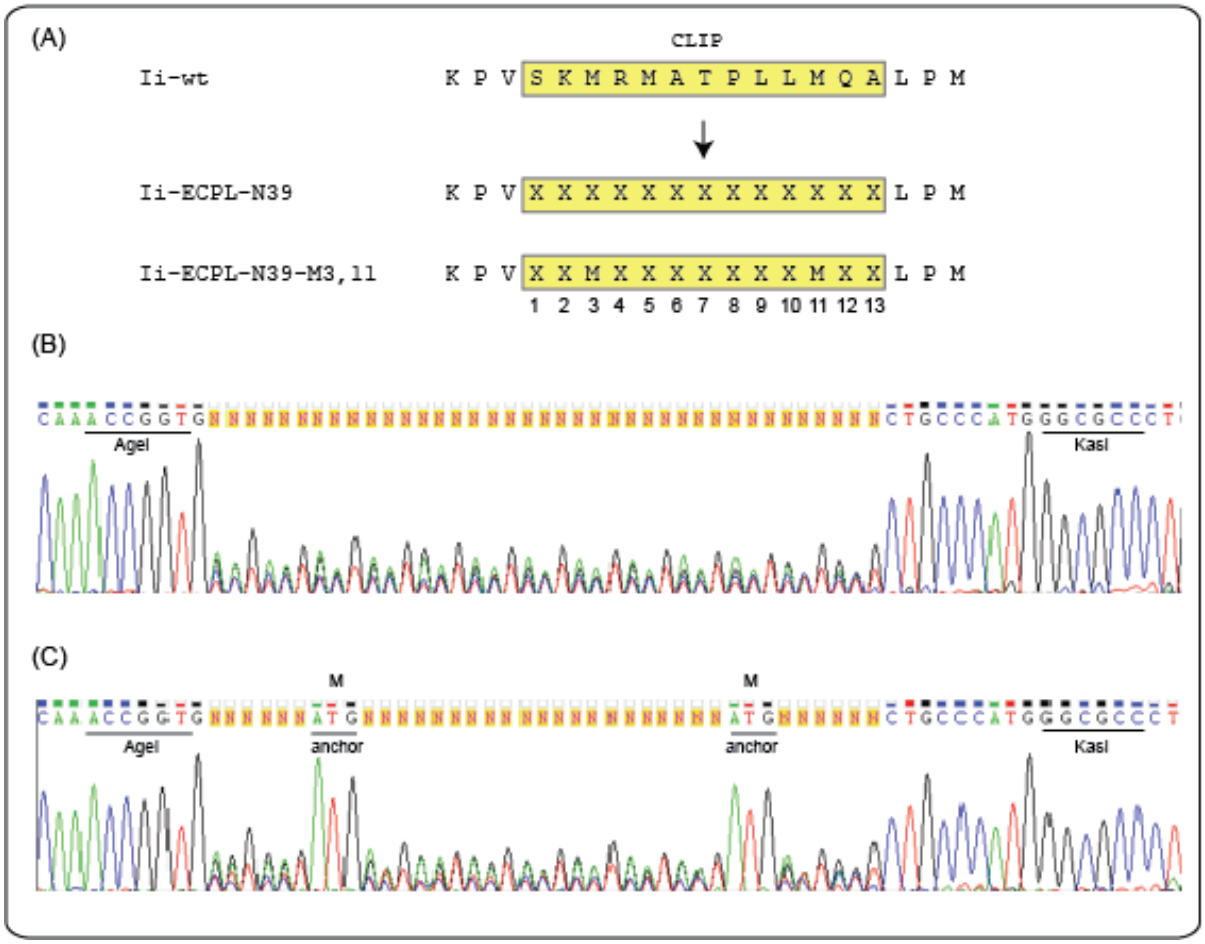

Figure 3-12 Design of MHC-II libraries. For the MHC-II libraries CLIP in the invariant chain (Ii) was exchanged by a random sequence (A). Electropherogramm of Ii-ECPL-N39 (B) and Ii-ECPL-N39-M3,11, which codes for methionine anchors at AA position 3 and 11 (C).

The number of different plasmids of the libraries vary between 7.5×10^7 - 7×10^9 clones (Table 3-9).

30 single clones were picked and send for sequencing. 50 - 70 % of the sequences code for a correct library without stop codon. Around 30 % of the sequences contain stop-codons. For some sequences, Ii had a deletion after the restriction site KasI, whereby Ii was not coded correctly anymore after KasI, due to the restriction digestion. The remaining number of different plasmids cover a broad spectrum of possible peptides.

Table 3-9 Overview of the cloned Ii-ECPL and the number of different plasmids of the libraries. CLIP of Ii was replaced by different library constructs to receive Ii-encoded combinatorial peptide libraries (Ii-ECPL). N describes the nucleotide length of the library, M the position of the methionine in the peptide.

Library	Aminoacid sequence	No. of different plasmids
Ii-ECPL-N27	-PVSKXXXXXXXXXQALP-	$7^{x}10^{9}$
Ii-ECPL-N27-M1,9	-PVSKMXXXXXXXMQALP-	$7.7^{x}10^{8}$
Ii-ECPL-N33	-PVSXXXXXXXXXXXXALP-	$2^{x}10^{8}$
Ii-ECPL-N33-M2,10	-PVSXMXXXXXXXMXALP-	$5.5^{x}10^{8}$
Ii-ECPL-N36-C	-PVSXXXXXXXXXXXXLP-	$2^{x}10^{8}$
Ii-ECPL-N36-N	-PVXXXXXXXXXXXXALP-	$7.5^{x}10^{7}$
Ii-ECPL-N36-C-M2,10	-PVSXMXXXXXXXMXXLP-	$1.4^{x}10^{8}$
Ii-ECPL-N36-N-M3,11	-PVXXMXXXXXXXMXALP-	$2,9^{x}10^{8}$
Ii-ECPL-N39	-PVXXXXXXXXXXXXLP-	$3.6^{x}10^{9}$
Ii-ECPL-N39-M3,11	-PVXXMXXXXXXMXXLP-	$3^{x}10^{8}$

3.3.2 Bbc9 as proof of concept

For the establishment of a system to identify CD4+ T cell antigens, the T cell receptor Bbc9 was used as a proof of concept. It recognizes hMBP139-153 presented by HLA-DRB1*01:01 (DR1) and HLA-DRB5*01:01 (DR2a), however it does not recognize the same peptide presented by HLA-DRB1*15:01 (DR2b) (Giegerich *et al.,* 1992). Because the HLA restrictions and the antigen of the TCR Bbc9 are described well, it can be used as a proof of concept for the MHC-II library screening system.

3.3.3 Expression of TCR, CD4 and NFAT-sGFP in $58^{-/-}$ T hybridoma cell line

For the library screening $58\alpha^{-}\beta^{-}$ T hybridoma cells were transfected with the α- and β-chain of Bbc9 in pRSV (Giegerich *et al.,* 1992). The TCR α- and β-chain of Bbc9 associate with the murine CD3 and thereby shows up as a chimeric TCR on the cell surface (Blank *et al.*, 1993). $58^{-/-}$ cells also express human CD4 (Blank *et al.*, 1993). Surface expression of murine CD3 and human CD4 was analysed by flow cytometry (Figure 3-13 A, B).

Clones of 58-Bbc9 cells with strong surface CD3 expression were additionally transfected with pcDNA-NFAT-sGFP, coding for sGFP under the NFAT-enhancer (Siewert *et al.,* 2012). Upon activation the cells express sGFP (Figure 3-13 C, D). Stable transfected clones were screened for highest level of sGFP expression after CD3 activation and lowest rate of spontaneously activated cells.

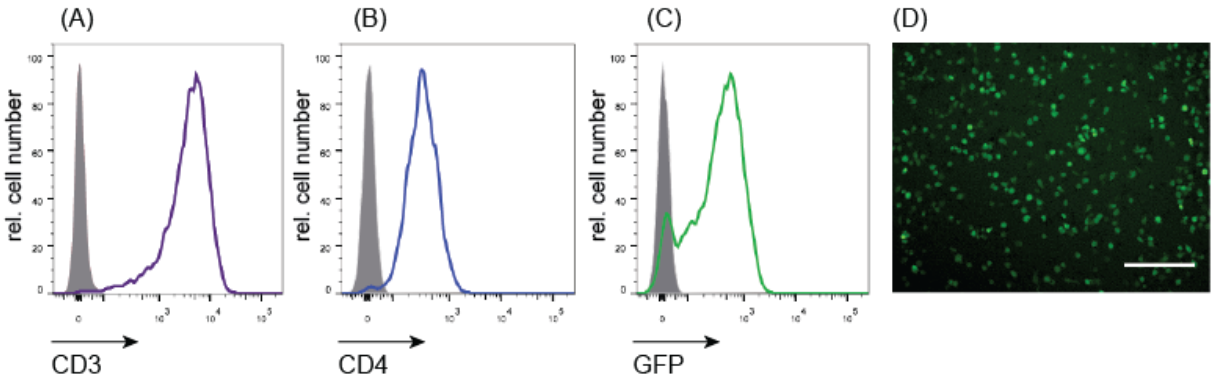

Figure 3-13 Flow cytometry and fluorescence microscopy of 58-Bbc9 cells. The expression of TCR in stable transfected 58-Bbc9 cells was detected with an APC-labeled antibody against murine CD3ξ (A) and the expression of CD4 with a PE-labeled antibody against human CD4 (B). The expression of sGFP in 58-Bbc9 cells after CD3-linkage, was detected by flow cytometry, gated for living cells (C), and by fluorescence microscopy (D). Scale bar: 200 µm.

3.3.4 Expression of HLA-DR molecules in COS-7 cells

COS-7 cells were used for the presentation of Ii-ECPL on HLA-DR. To this end, HLA-DRA, DRB1, DR2a and DR2b coding sequences were cloned into pHSE3'. In contrast to pRSV, pHSE3' does not contain an SV40 origin. Thus it does not get amplified in COS-7 cells.

COS-7 cells, which do not express HLA-DR (Figure 3-14), were cotransfected with pHSE3'-HLA-DRA and pHSE3'-HLA-DR1 or pHSE3'-HLA-DR2a or pHSE3'-HLA-DR2b. After single cell isolation, almost all cells of COS-7-DR1 expressed HLA-DR. However, the expression of COS-7-DR2a was low and almost no cells expressed HLA-DR2b (Figure 3-14). To increase the amount of HLA-DR+ cells, the cells were purified via MACS Beads for HLA-DR (COS-7-DR2a and COS-7-DR2b) and sorted by FACS (COS-7-DR1 and COS-7-DR2a). For FACS, the best 10 % of the population were sorted and cultivated further. Comparison of HLA-DR expression of the cells before and after FACS, showed only a little increase of HLA-DR expression in COS-7-DR1. However, the expression of HLA-DR2a in COS-7-DR2a cells was increased. The surface expression of HLA-DR2b was increased successfully by several rounds of MACS purification (Figure 3-14).

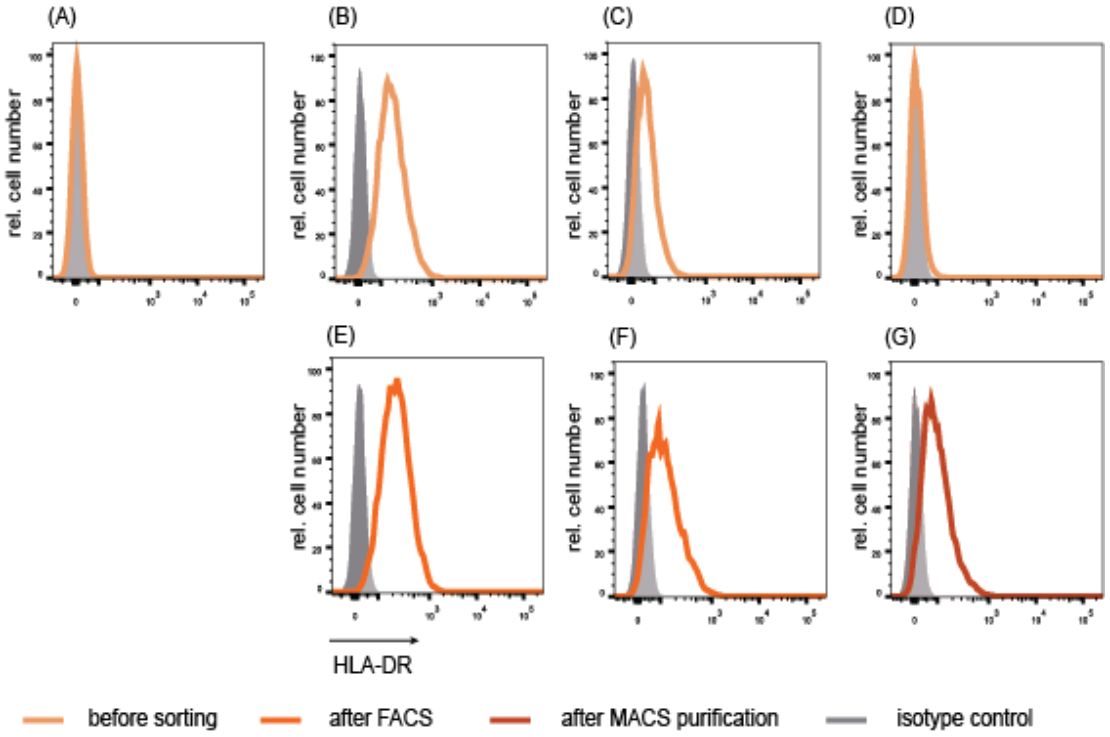

Figure 3-14 Expression of HLA-DR in stable transfected COS-7 cells. Expression of HLA-DR in COS-7 cells was detected by flow cytometry with a FITC-labeled anti-hHLA-DR antibody. Non-transfected COS-7 cells do not express HLA-DR (A). Expression of HLA-DR in COS-7 cells co-transfected with pHSE3'-HLA-DRA and pHSE3'-HLA-DR1 (B), pHSE3'-HLA-DR2a (C) and pHSE3'-HLA-DR2b (D). To increase the expression of HLA-DR, COS-7-DR1 (E) and COS-7-DR2a (F) were purified via FACS and COS-7-DR2b purified via MACS beads (G).

3.3.5 Detection of Ii-ECPL expression in COS-7-DR1 cells

To detect whether COS-7 cells express Ii-ECPL, the cells were transfected with pcDNA-Ii-ECPL-N39. Ii-ECPL was detected with an antibody against the N-terminal signal sequence of Ii (CD74). Detection was visualised by Western Blot and intracellular flow cytometry.

3.3.5.1 Detection of Ii-ECPL expression by Western Blot

Expression of Ii-ECPL was detected by Western Blot. COS-7-DR1 cells were transfected with 1 µg pcDNA-Ii-N39 by nucleofection and the cells were harvested after 48 h. Non-transfected COS-7-DR1 cells served as negative control.

Untransfected COS-7-DR1 did not show a signal for CD74 in Western Blot (Figure 3-15 lane 1), which proves that COS-7 cells do not express the invariant chain. After transfection of COS-7-DR1 cells with Ii-ECPL, a strong signal showed up in Western Blot, with the molecular weight of Ii (33 kDa, Figure 3-15 lane 2). This shows that Ii-ECPL is expressed in COS-7 cells after transfection

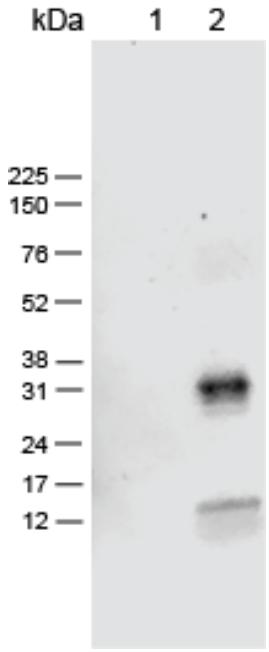

Figure 3-15 Western Blot showing expression of Ii-N39 in COS-7-DR1 cells transfected with pcDNA-Ii-N39. Lane 1: untransfected COS-7-DR1 cells. Lane 2: COS-7-DR1 cells transfected with 1 µg pcDNA-Ii-N39 by nucleofection. The invariant chain was detected with an antibody specific for CD74 (PIN.1).

3.3.5.2 Detection of Ii-ECPL expression in by intracellular flow cytometry

To find the best transfection conditions for the expression of Ii-ECPL in COS-7 cells, intracellular flow cytometry was performed. COS-7-DR1 cells were transfected by nucelofection and by FuGENE® with two different amounts (1 µg and 100 ng) of pcDNA-Ii-ECPL-N39, whereby the total transfected DNA amount was kept constant at 1 µg by adding fish sperm DNA. Intracellular FACS was performed 48 h and 72 h after transfection. COS-7-DR1 cells transfected with 1 µg fish sperm DNA served as negative control.

COS-7-DR1 cells only transfected with fish sperm DNA, did not express CD74 (Figure 3-16). After transfection, either with FuGENE® or by nucleofection, COS-7-DR1 expressed Ii-ECPL. The expression level for both transfection methods are higher for 1 µg of transfected pcDNA-Ii-ECPL compared to the transfection of 100 ng. The expression level after nucleofection is much higher compared to FuGENE® transfection, especially for the transfection of small amounts of DNA. However, the expression level 72 h after nucleofection is lower than after 48 h, presumably explained by degradation of DNA (Figure 3-16). For FuGENE® transfection no difference in the expression level is observed 48 h and 72 h after transfection (Figure 3-16).

The results show that nucleofection should be the method of choice for the library screening.

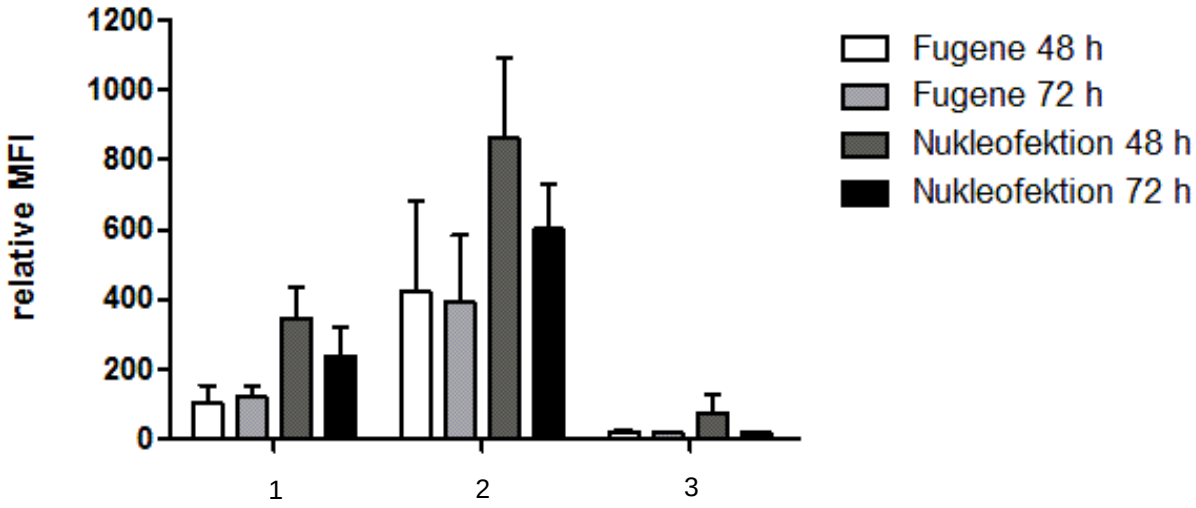

Figure 3-16 Expression of the invariant chain library in COS-7-DR1. COS-7-DR1 cells were transfected with 0.1 µg Ii-ECPL-N39 + 0.9 µg fish sperm DNA (1), 1 µg Ii-ECPL-N39 (2) or with 1 µg fish sperm DNA (3). Intracellular FACS for CD74 was performed 48 h and 72 h after transfection.

3.3.6 Activation of Bbc9 by synthetic peptides

In the first step, 58-Bbc9 cells were activated with diverse length of hMBP-derived synthetic peptides presented on COS-7 expressing HLA-DR. The synthetic peptides and their AA sequences are shown in Table 3-10.

Table 3-10 Overview peptide sequences of hMBP-derived synthetic peptides

peptide	AA sequence	source
hMBP(141-149)	FKGVDAQGT	K. Dornmair
hMBP(140-149)	GFKGVDAQGT	K. Dornmair
hMBP(141-150)	FKGVDAQGTL	K. Dornmair
hMBP(140-150)	GFKGVDAQGTL	K. Dornmair
hMBP(139-153)	KGFKGVDAQGTLSKI	K. Dornmair

The activation with synthetic peptides were performed, to prove, if 58-Bbc9 cells are activated by peptides presented by HLA molecules on COS-7 cells as APC. It also shows which peptide can be used as a positive control expressed in Ii. Furthermore, it provides an evidence, which library length is reasonable for the identification of mimotopes. The vehicle (H_2O) as well as hMBP(50-69, GAPKRGSGKDSHHPARTAHY) served as negative control. COS7-DR2b cells, expressing the irrelevant HLA-DR2b were additionally used as negative control. Activation of 58-Bbc9 cells was detected by fluorescence microscopy (Figure 3-17 A-U), flow cytometry (Figure 3-17, V) and IL2-ELISA (Figure 3-17, W).

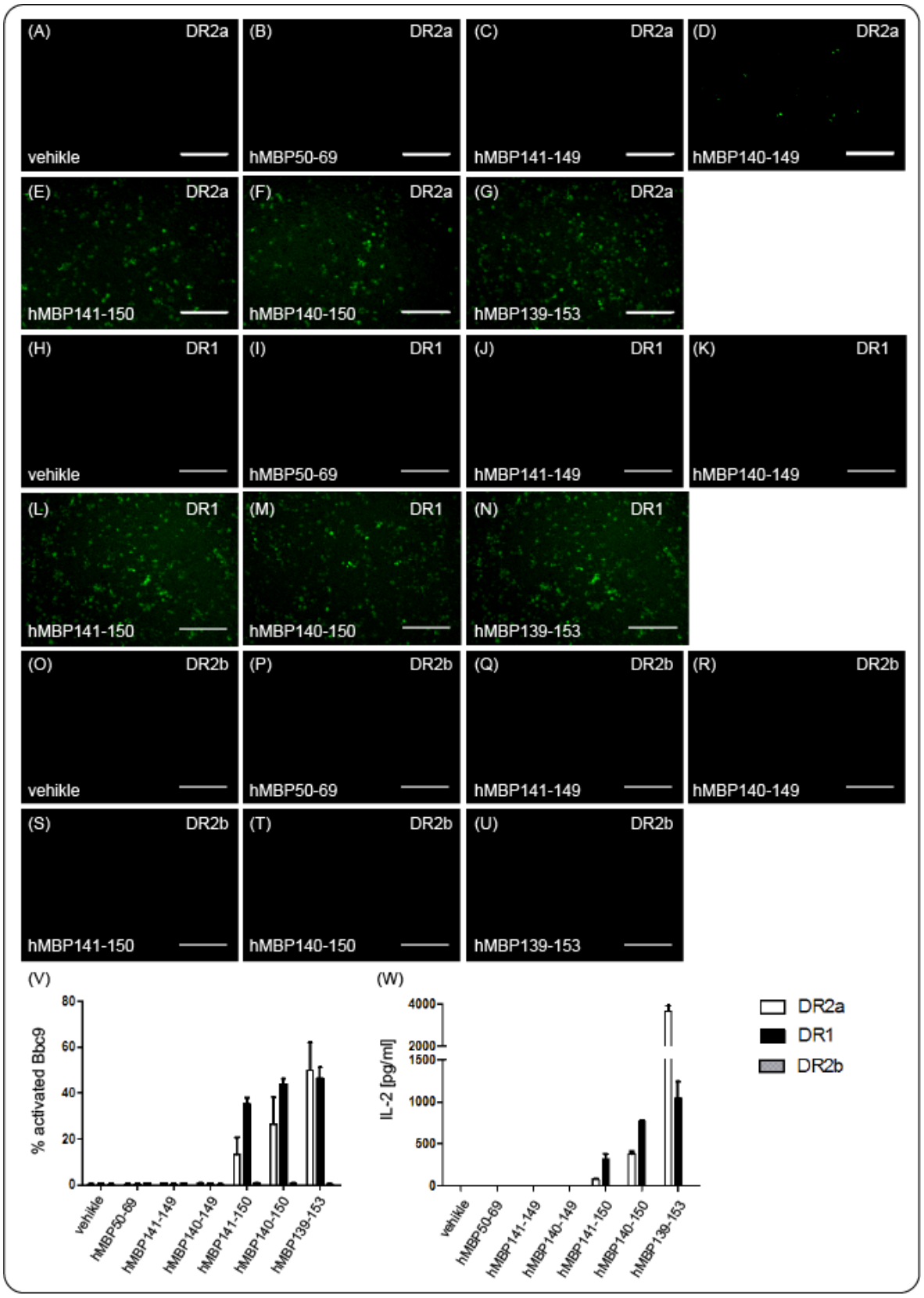

Figure 3-17 Activation of Bbc9 by variations of hMBP-derived peptides presented by HLA-DR. hMBP50-69 (B, I, P), hMBP141-149 (C, J, Q), hMBP140-149 (D, K, R), hMBP141-150 (E, L, S), hMBP140-150 (F, M, T), hMBP139-151 (G, N, U) or only the vehicle were added to COS-7 cells, stably expressing HLA-DR2a (A-G), HLA-DR1 (H-N) or HLA-DR2b (O-U). After 3 h incubation 58-Bbc9 cells were added and cocultivated for 16 h. The expression of sGFP was analysed by fluorescence microscopy (A-U). Scale bar: 200 µm. Additionally, the percentage of activated Bbc9 was determined by flow cytometry (V) and the IL-2 concentration in the supernatant was measured by IL-2 ELISA.

58-Bbc9 cells were strongly activated by hMBP(141-150), hMBP(140-150) and hMBP(139-153), presented on COS-7-DR2a cells. Less activation was observed with the construct hMBP(140-149). No activation was observed by the shorter construct hMBP(141-149). Both negative controls did not activate Bbc9 (Figure 3-17). The activation of Bbc9 was additionally validated by the detection of sGFP expression by flow cytometry (Figure 3-17 V) and the detection of IL-2 in the supernatant by ELISA (Figure 3-17 W). Comparison of the sensitivity of the three detection methods showed that fluorescence microscopy is the most sensitive detection method. Clusters with only several activated 58-Bbc9 cells were visible by the presentation of hMBP140-149 by COS-7-DR2a cells (Figure 3-17 D), however no activation was detected neither in flow cytometry nor by ELISA (Figure 3-17 V, W).

Almost the same activation pattern was observed for the synthetic peptides presented by COS-7-DR1 cells, however, no activation at all was detected for hMBP140-149 (Figure 3-17, H-N).

No activation was observed by any of the peptides presented on HLA-DR2b, neither in fluorescence microscopy nor by flow cytometry or Il-2 ELISA (Figure 3-17 O-W).

The activation with the peptides hMBP(141-150) and hMBP(140-150) is stronger when presented by HLA-DR1 molecules compared to HLA-DR2a. However, for the longest peptide hMBP(139-153) the activation is more intense presented by HLA-DR2a molecules compared to HLA-DR1 (Figure 3-17 V, W).

The results show that it is possible to specifically activate 58-Bbc9 cells with the correct peptide presented by the matching HLA-DR molecules on COS7 cells. The results also confirm the restriction of Bbc9 to HLA-DR2a and DR1, as observed before (Giegerich *et al.,* 1992). Additionally, Bbc9 specifically recognizes an at least 10 AA long peptide of hMBP, which must include the phenylalanine (position 141) and leucine (position 150) for optimal activation. These results are in accordance with previous experiments, where also different length of peptides presented by HLA-DR1 and HLA-DR2a molecules were used to activate Bbc9 (unpublished data K. Dornmair, E. Meinl, Institut für klinische Neuroimmunologie, Ludwig-Maximilians-Universität München) detected with T cell proliferation as read-out (Meinl *et al*., 1993).

3.3.7 Activation of Bbc9 by hMBP-derived peptides expressed in Ii

A positive control construct (Ii-hMBP) in pcDNA was cloned to test if 58-Bbc9 cells can be activated by the correct peptide expressed in the invariant chain in pcDNA. For this purpose, CLIP was replaced on DNA level by sequences coding for four hMBP-derived peptides of different length (hMBP(141-149), hMBP(141-150), hMBP(141-151) and hMBP(139-151)). The length was varied to test which construct can activate 58-Bbc9 cells most efficient. This evidence can be used for the decision which library length are useful for CD4+ T cell antigen screening. The transfected amounts of DNA were varied, to find the best concentration for

activating 58-Bbc9 cells and low cell death ratio of COS7-DR cells after transfection concomitantly.

Additionally, the N-terminal signal sequence in the four constructs was removed (delta-Ii constructs). COS-7 cells are no professional APCs. Therefore, it is not known whether they may recognize the N-terminal signal sequence of the invariant chain (Figure 3-11). Ii-hMBP and delta-Ii-hMBP constructs were compared in matters of cellular viability after transfection and activation efficiency of 58-Bbc9 cells.

After FuGENE® transfection of 500 ng pcDNA-Ii-hMBP(139-151) and pcDNA-delta-Ii-hMBP(139-151) cellular viability of COS-7-HLA-DR cells was only 50 % after 48 h and 40 % after 72 h. Cellular viability is increased to 60 % (48 h) and 50 % (72 h) for the transfection of 250 ng and to 80 % (48 h) and 70 % (72 h) for 100 ng (Figure 3-18). Cellular viability is decreased for all constructs 72 h after transfection compared to 48 h. However, there is no difference in cellular viability comparing the constructs with and without N'-terminal signal sequence (Figure 3-18).

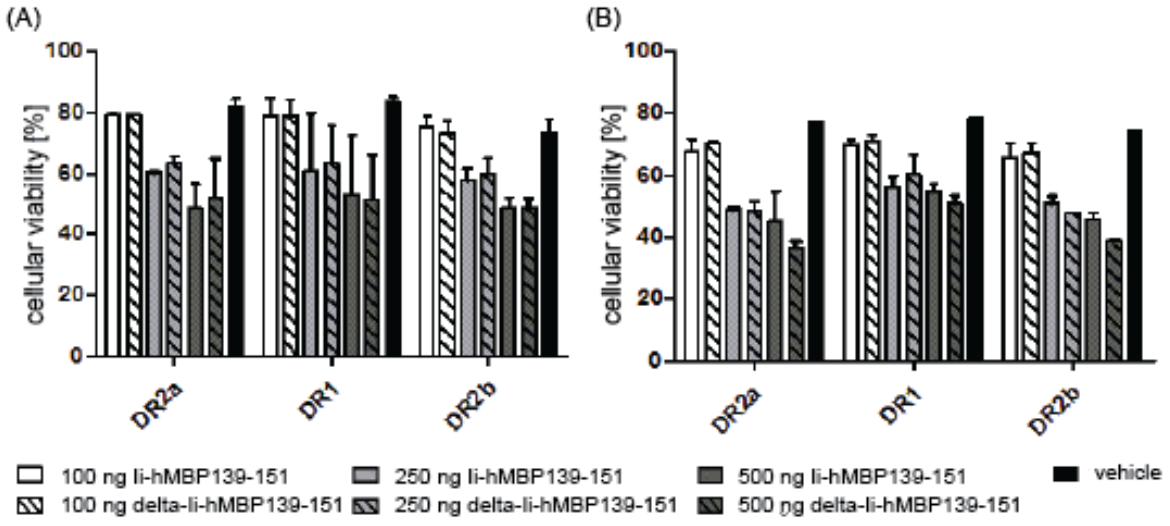

Figure 3-18 Cellular viability of COS-7-HLA-DR cells 48 h (A) and 72 h (B) after transfection with pcDNA-Ii-hMBP(139-151) compared with pcDNA-delta-Ii-hMBP(139-151). COS-7-DR1 cells were transfected with 100 ng, 250 ng or 500 ng of pcDNA-Ii-hMBP(139-151) (plain) or pcDNA-delta-Ii-HMBP139-151 (hatched). Cellular viability was detected by flow cytometry.

The results of activating 58-Bbc9 cells with the different pcDNA-Ii-hMBP and pcDNA-delta-Ii-hMBP constructs, transfected by FuGENE®, are presented in Figure 3-19 and Figure 3-20. For fluorescence microscopy activation of 58-Bbc9 cells by Cos7-DR1 cells with six different constructs are exemplarily presented in Figure 3-19. Results of the activation of 58-Bbc9 cells by all constructs presented on COS-7 cells expressing different HLA-DR molecules detected by flow cytometry and IL-2 ELISA are presented in Figure 3-20.

Activation of 58-Bbc9 cells was observed by Ii-hMBP constructs, presented on COS-7-DR2a and COS-7-DR1 (Figure 3-19 and Figure 3-20). No activation was observed with the negative

controls (pcDNA-Ii-wt and pcDNA-delta-Ii), nor by presentation of Ii-hMBP constructs by COS-7-DR2b cells. This verify that the system for the presentation of CD4+ T cell antigens is functional. MHC-II molecules are loaded correctly with Ii including the peptide, and the complex is transported to the cell surface, where the antigen is presented to hybridoma cells. The activation of 58-Bbc9 cells by constructs with the N'-terminal signal sequence compared to the constructs without, showed higher activation levels (Figure 3-19 and Figure 3-20). This effect cannot be ascribed to differences in cellular viability after transfection (Figure 3-18). Presumably the transportation to the cell surface is more efficient for constructs with signal sequence.

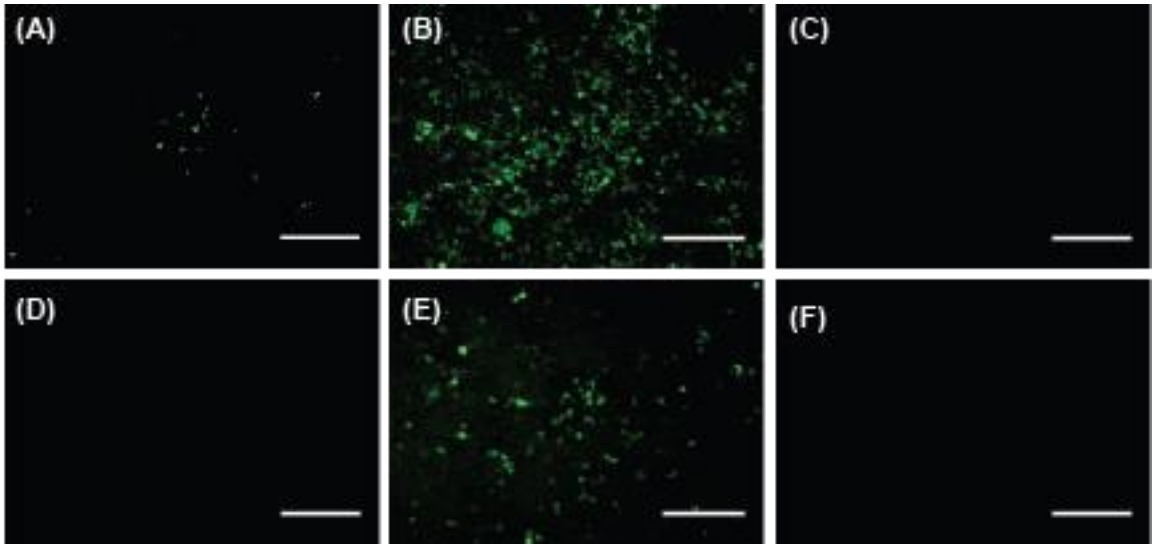

Figure 3-19 Activation of Bbc9 by Ii-hMBP constructs presented on COS7-DR1 cells. COS-7-DR1 cells were transfected with 250 ng of the following constructs: pcDNA-Ii-hMBP(141-149) (A), pcDNA-Ii-hMBP(139-151) (B), pcDNA-Ii-wt (C), pcDNA-delta-Ii-hMBP(141-149) (D), pcDNA-delta-Ii-hMBP(139-151) (E) and pcDNA-delta-Ii using FuGENE® transfection. Scale Bar: 200 µm.

The highest activation level of 58-Bbc9 cells was observed with the longest construct, expressing 13 AA in the invariant chain (Ii-hMBP(139-151)), followed by Ii-hMBP(141-151) and Ii-hMBP(141-150). Interestingly the nine AA construct expressed in the invariant chain Ii-hMBP(141-149) also showed low activation of 58-Bbc9 cells. The synthetic peptide of the same length did not show any activation (Section 3.3.5).

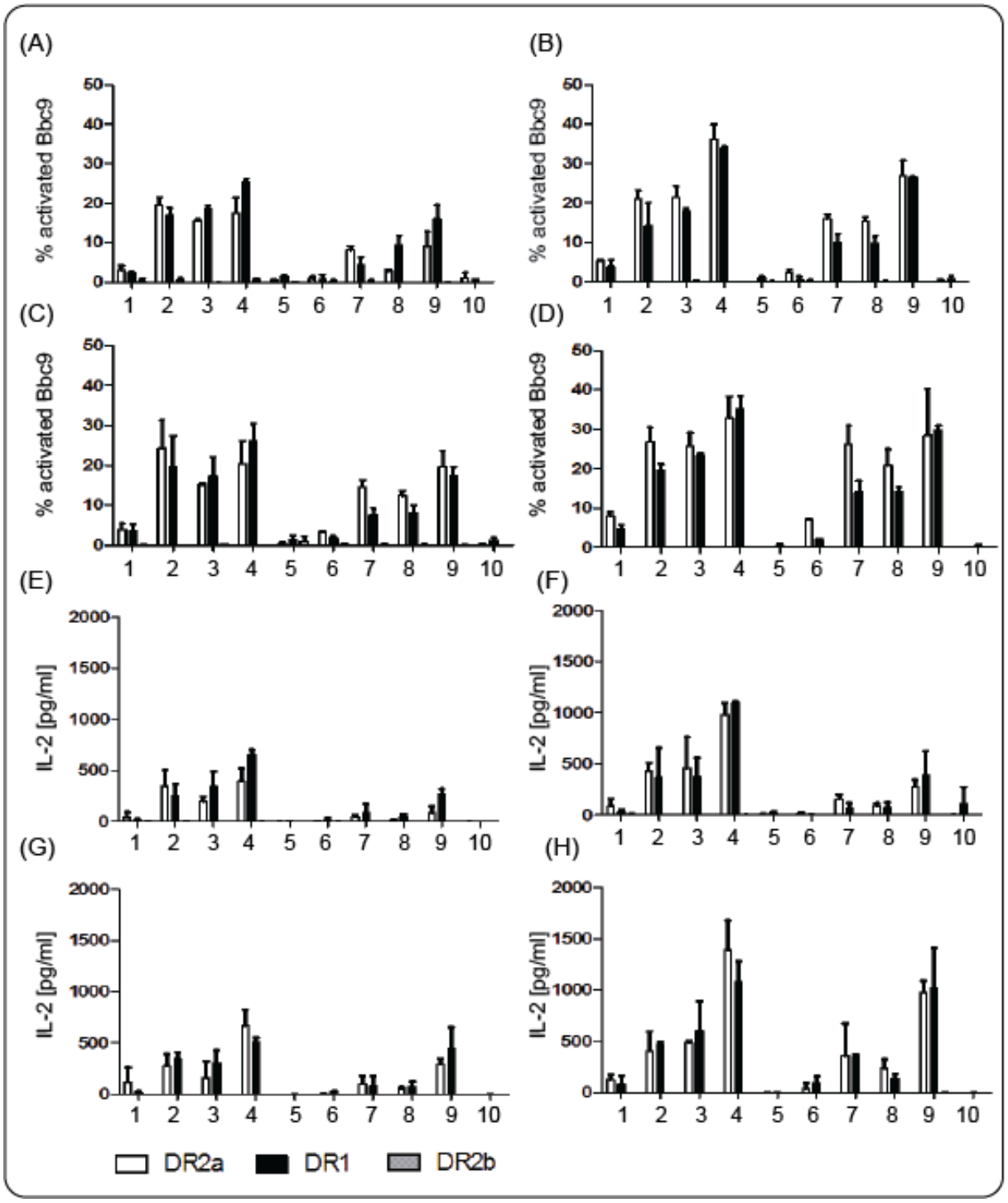

Figure 3-20 Activation of 58-Bbc9 cells by Ii-encoded positive control (Ii-hMBP) presented on COS-7-HLA-DR. COS-7-HLA-DR cells were transfected with 100 ng (A, C, E, G) or 250 ng (B, D, F, H) pcDNA-Ii-constructs using FuGENE® transfection and analysed after 48 h (A, B, E, F) and 72 h (C, D, G, H). Activated 58-Bbc9 cells were analysed by flow cytometry, gated for living, CD3+ cells (A-D). The supernatant was tested for IL-2 concentration by ELISA (E-H). COS-7-HLA-DR cells were transfected with following constructs: pcDNA-Ii-hMBP(141-149) (1), pcDNA-Ii-hMBP(141-150) (2), pcDNA-Ii-hMBP(141-151) (3), pcDNA-Ii-hMBP(139-151) (4), pcDNA-Ii-wt (5), pcDNA-delta-Ii-hMBP(141-149) (6), pcDNA-delta-Ii-hMBP(141-150) (7), pcDNA-delta-Ii-hMBP(141-151) (8), pcDNA-delta-Ii-hMBP(139-151) (9) and pcDNA-delta-Ii (10).

3.3.8 Comparison of the sensitivity using different detection methods

To analyse until which dilution the correct antigen is still detectable, and which detection method (fluorescence microscopy, flow cytometry or IL-2 ELISA) is most sensitive, a titration of pcDNA-Ii-hMBP(139-151) diluted in pcDNA-Ii-wt was performed. Therefore, COS-7-DR1 cells, seeded in 24-well plates were transfected with the titration preparations by FuGENE® (Section 2.6.2). The cells were always transfected with 250 ng DNA, whereas the amount of pcDNA-Ii-hMBP(139-151) was reduced in a dilution series from 1:10 to $1:10^6$. The transfected DNA was kept constant with 250 ng per sample by adding pcDNA-Ii-wt. The cells were incubated for 32 h and 56 h before adding 58-Bbc9 cells. The hybridoma cells were cocultivated with the transfected COS7-DR1 for 16 h. Afterwards, the cells were analysed by fluorescence microscopy for activated 58-Bbc9 cells. Additionally, the cells were analysed by flow cytometry. For this purpose, the cells were stained with an APC-labeled AB against CD3 and PI for counterstaining. It was gated on living, CD3+ cells. Further, concentration of IL-2 in the supernatant was detected by ELISA.

The analysis by fluorescence microscopy showed that until a dilution of $1:10^3$ there were still several clusters composed of nine activated 58-Bbc9 cells visible (Figure 3-21 C, J). At a dilution of $1:10^4$ at least one cluster composed of four (48 h) or seven (72 h) activated 58-Bbc9 cells per well was detected. This also shows that the cluster size is enlarged for 72 h preparation compared to 48 h (Figure 3-21 D, K). At a dilution of $1:10^5$ and $1:10^6$ clusters of two activated 58-Bbc9 cells or no activated cells were visible (Figure 3-21 E, F, L, M). In the negative control, transfected with pcDNA-Ii-wt, no activation was visible.

In flow cytometry, activated cells were still detectable until a dilution of $1:10^3$ (Figure 3-21 O). For higher dilutions it was not possible to distinguish between background and activated cells, even there were still some clusters visible in the microscope.

The expression of IL-2 by ELISA was also detectable until a dilution of $1:10^3$ (Figure 3-21 P). For higher dilutions, the ELISA, which has a detection limit of 2 pg/ml, is not sensitive enough.

The titration showed that compared to flow cytometry and IL-2 ELISA, fluorescence microscopy was the most sensitive method to detect activated 58-Bbc9 cells. Clusters of activated 58-Bbc9 cells were visible as immediate neighbours, indicating a specific activation. This close contact of a few activated cells can neither be visualized by flow cytometry nor detected by IL-2 ELISA. Activated 58-Bbc9 cells were still detectable until dilutions of $1:10^4$-$1:10^5$ by fluorescence microscopy in 24-well format. This should be sufficient enough to detect mimotopes, after the first isolation of cells from a 3.5 cm dish, to enrich the positive candidate. After cloning, it must be ensured that not more than 10^4-10^5 clones are screened per well of a 24-well plate, otherwise, the positive clone is too diluted and thereby not detectable anymore.

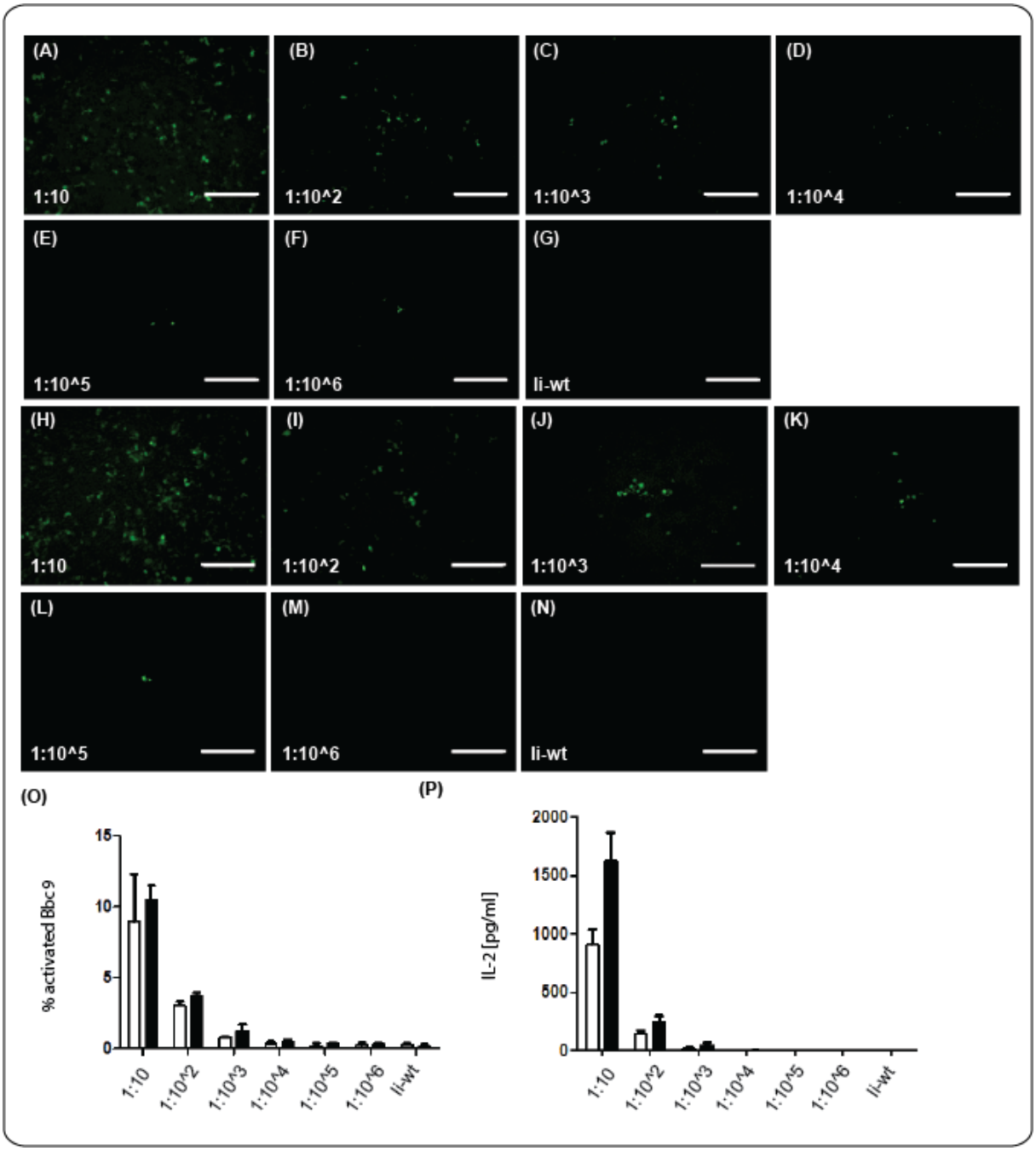

Figure 3-21 Comparison of the sensitivity of different detection methods. COS-7-DR1 cells were transfected with a dilution series of pcDNA-Ii-hMBP(139-153) diluted in pcDNA-p33-wt. 58-Bbc9 cells were added 32 h or 56 h after transfection. The cells were screened by fluorescence microscopy 48 h (A-G) and 72 h (H-N) after transfection. Scale bar: 200 µm. 58-Bbc9 cells were additionally analysed by FACS, gated on CD3+, living cells (O) and the supernatant was analysed by ELISA (P).

3.3.9 Library Screening 58-Bbc9 cells with Ii-ECPL

The experiments with Ii-hMBP constructs showed that peptides expressed in the invariant chain are loaded to MHC-II molecules in the ER and hybridoma cells can be activated by the presentation on the cell surface (Section 3.3.7). 58-Bbc9 cells were screened with different MHC-II libraries and different MHC-II alleles (HLA-DR1 and HLA-DR2a) for activation. Different transfection methods and DNA concentrations (Table 3-11) were used, to determine

differences in the frequency of the presence of activated 58-Bbc9 cells as well as differences in the number of activated cells per cluster.

Table 3-11 Library Screening Bbc9. Bbc9 was screened with Ii-ECPL expressed in COS-7 cells expressing either transiently (t) or stably (s) HLA-DR2a or HLA-DR1 molecules. Different Libraries were transfected with different amount of DNA and by FuGENE® (F) or by nucleofection (N). The number of clusters and the cluster sizes, by the number of activated 58-Bbc9 cells in the cluster, detected in the viewed area are displayed.

Library (pcDNA)	Transfected library [µg]	HLA	Area [cm^2]	Cluster	Cluster size [n act. cells]
Ii-ECPL-N27	1 µg, F	DR1 (t)	140.4	16	2-4
Ii-ECPL-N27	1 µg, F	DR2a (t)	110	-	-
Ii-ECPL-N33-M2,10	2 µg, F	DR2a (s)	374.4	15	3-4
Ii-ECPL-N33-M2,10	0.1 µg, N	DR2a (s)	70.2	-	-
Ii-ECPL-N33	2 µg, F	DR1 (s)	140.4	4	2-3
Ii-ECPL-N33	1 µg, N	DR1 (s)	140.4	3	15-17
Ii-ECPL-N39	2 µg, F	DR1 (s)	351	18	2-5
Ii-ECPL-N39	1 µg, N	DR1 (s)	257.4	12	3-17
Ii-ECPL-N39	0.1 µg, N	DR1 (s)	198.9	2	5-7
Ii-ECPL-N39	1 µg, N	DR2a (s)	11.7	-	-
Ii-ECPL-N39	0.1 µg, N	DR2a (s)	11.7	-	-
Ii-ECPL-N39-M3,11	2 µg, F	DR2a (s)	163.8	-	-

In contrast to FuGENE® transfection, a higher cellular viability was determined by nucleofection, also after the transfection of high amounts of DNA, visualised in a transmitted microscope.

Both, for HLA-DR1, as well as for HLA-DR2a clusters of activated 58-Bbc9 cells were detected by fluorescence microscopy. However, the experiments showed a high variability in the cluster size of activated 58-Bbc9 cells, depending on the length of the library and the transfection method. Clusters of 2 to 4 activated Bbc9 were observed with the short library Ii-ECPL-N27 and by transfection with FuGENE®. Larger cluster sizes were observed with longer libraries. The biggest clusters were found after nucleofection of pcDNA-Ii-ECPL-N33 and pcDNA-Ii-ECPL-N39 with up to 17 activated 58-Bbc9 cells (Table 3-11 and Figure 3-22).

The underlying COS-7 cells were isolated with a micromanipulator and plasmids recovered by PCR. Samples were analysed for reactivation.

For some isolated samples of all Ii-ECPL reactivation was observed. Further subpooling did not lead to a mimotope of Bbc9. The reactivation of 58-Bbc9 cells during the subpooling was not reproducible. During the subpooling procedure the COS-7-HLA-DR cells often died after transfection with FuGENE®. However, nucleofection, where the cells had a higher viability was not usable for many samples, due to low DNA quality and high costs. A second round of library

screening followed by DNA isolation and recovery, using the plasmids of the first round, was performed to futher enrich the positive clone. However, no reactivation of 58-Bbc9 cells was observed after cloning.

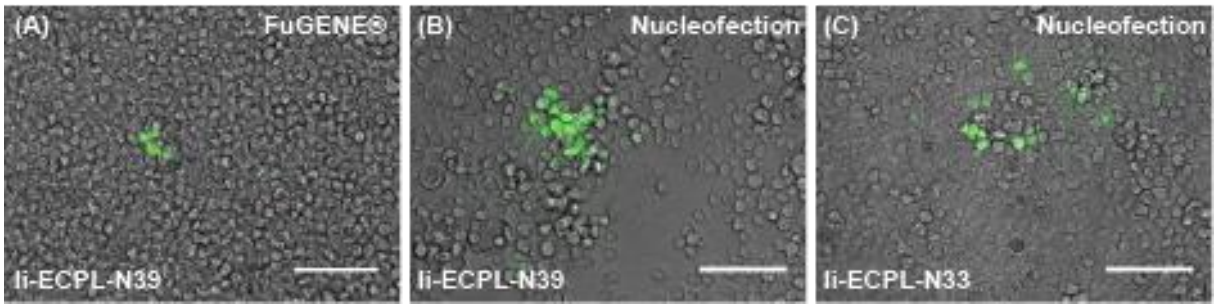

Figure 3-22 Comparison clusters of activated Bbc9 using different libraries and transfection methods. COS-7-DR1 were transfected with Ii-ECPL-N39 (A, B) or Ii-ECPL-N33 (C) using FuGENE® transfection (A) or Nucleofection (B, C).

3.3.10 LTK⁻ cells as APCs as alternatives to COS-7 cells

It was possible to activate 58-Bbc9 cells with Ii-hMBP constructs expressed in COS-7-HLA-DR cells (Section 3.3.7). However, it was tested, if the mouse fibroblast cell line LTK⁻ might be more suitable for CD4+ TCR antigen screening, and thereby it can be used as alternative APCs to substitute COS-7 cells.

LTK⁻ cells were co-transfected with pHSE3'-HLA-DRA and pHSE3'-HLA-DR1/DR2a/DR2b by electroporation. Single clones were analysed for the expression of HLA-DR molecules on the cell surface by flow cytometry (Figure 3-23). Clones with highest expression levels were kept for further studies.

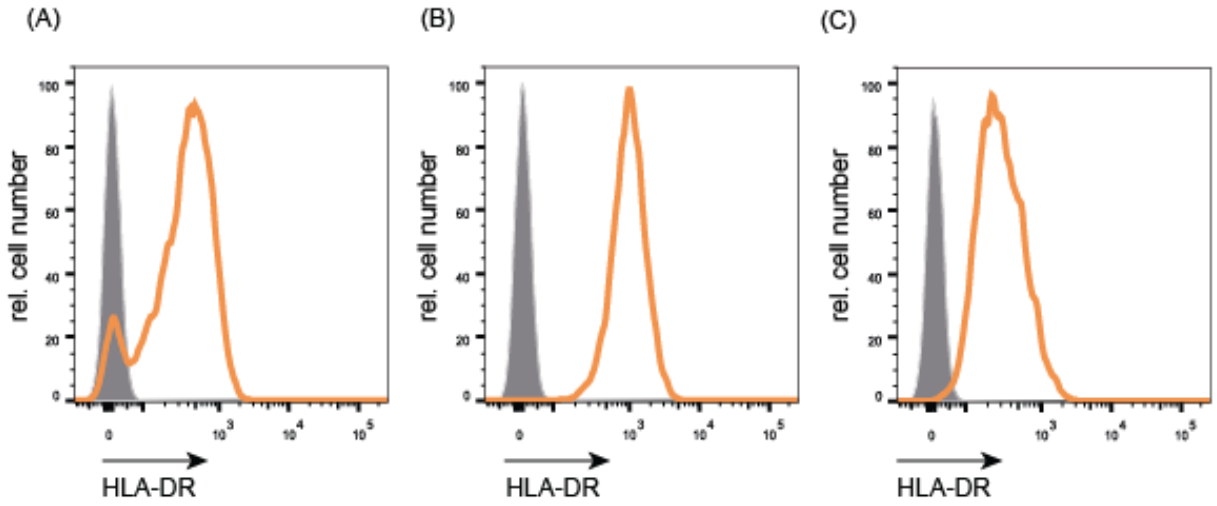

Figure 3-23 Expression of HLA-DR on LTK⁻ cells. The expression of HLA-DR molecules in LTK⁻ cells was analysed by flow cytometry with a FITC-labeled antibody against human HLA-DR. LTK⁻ cells were cotransfected with pHSE3'-HLA-DRA and pHSE3'-HLA-DR2a (A), pHSE3'-HLA-DR1 (B) and pHSE3'-HLA-DR2b (C).

Transfection efficiency of LTK$^-$ cells with FuGENE® was very low. Thus, only 2 % of the cells expressed sGFP after the transfection of pcDNA-sGFP. Thereby a new transfection method, using GenaxxoFect-Plus (Genaxxon bioscience GmbH, Ulm, Germany), was established together with M. Schönwetter to improve the transfection efficiency.

Additionally, it was observed that the transfection is much more efficient, if the cells are splitted the day before transfection. Thereby, the cells are in a good growth phase, and thereby the uptake of DNA is more efficient. Thus, it was possible to increase transfection efficiency over 50 %.

Cocultivation of 58-Bbc9 cells with LTK-HLA-DR2a cells and LTK-HLA-DR1 cells transfected with pcDNA-Ii-hMBP(139-151) and pcDNA-delta-Ii-hMBP(139-151) constructs, showed activation of 58-Bbc9 cells. In contrast to COS-7 cells, no difference in activation was observed comparing the constructs with N'-terminal signal sequence pcDNA-Ii-hMBP(139-151) and pcDNA-delta-Ii-hMBP(139-151), where the signal sequence was delted. The negative controls pcDNA-Ii-wt and pcDNA-delta-Ii did not show any activation of 58-Bbc9 cells, neither cocultivation with LTK$^-$ cells expressing HLA-DR2b (Figure 3-24).

The activation of 58-Bbc9 cells by Ii-hMBP(139-151) and delta-Ii-hMBP(139-151) presented on LTK$^-$ cells show that LTK$^-$ cells are a possible alternative to COS-7 cells as APCs in screening of CD4+ T cell antigens.

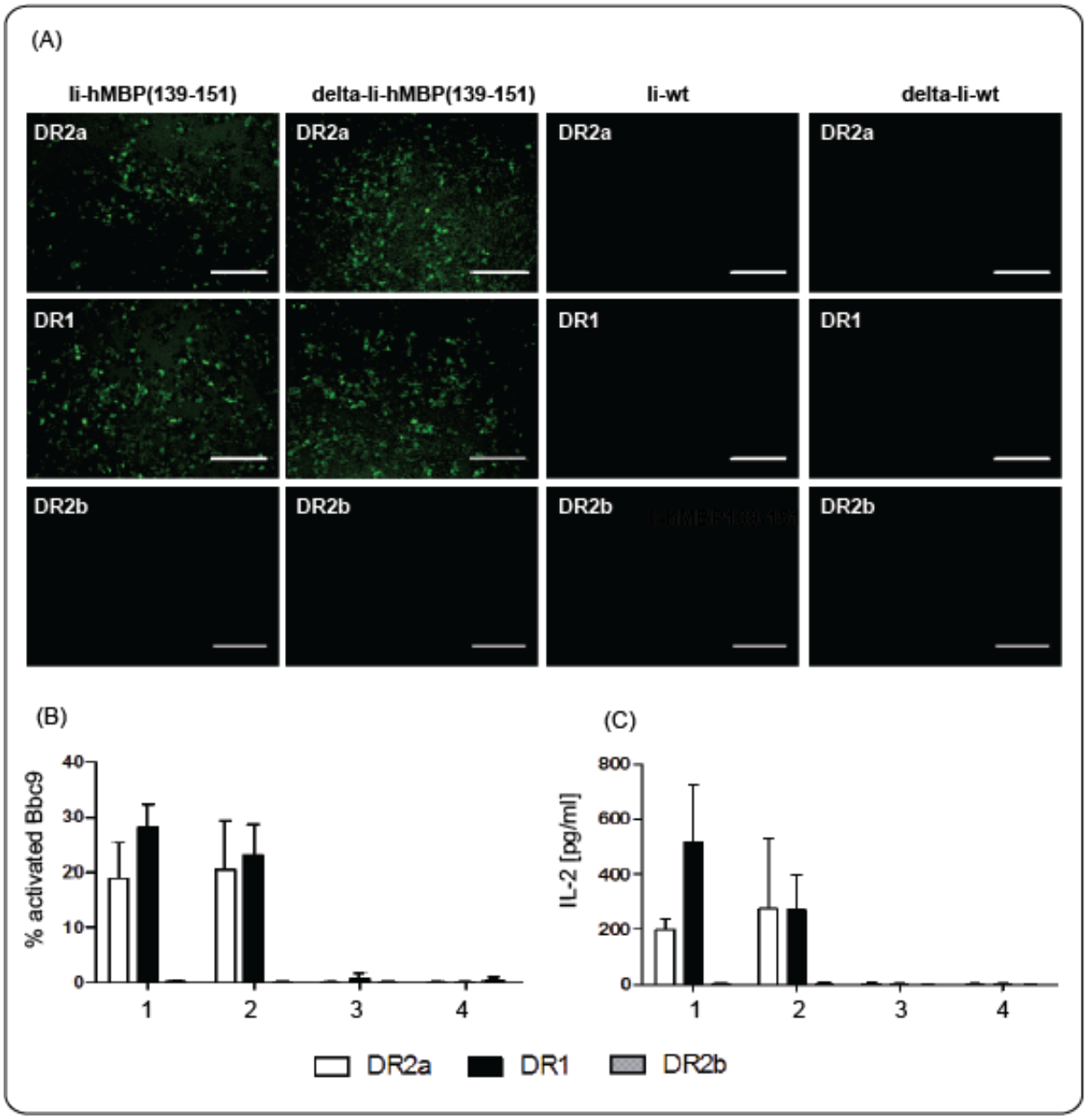

Figure 3-24 Activation of Bbc9 by Ii-hMBP presented on LTK$^-$ cells. LTK$^-$ cells expressing HLA-DR2a, -DR1 or -DR2b were transfected with pcDNA-Ii-hMBP(139-151) (1), pcDNA-delta-Ii-hMBP(139-151) (2) and cocultivated with 58-Bbc9 cells. pcDNA-Ii-wt (3) and pcDNA-delta-Ii (4) served as negative controls. Activation was first detected by fluorescence microscopy. Scale bar is 200 µm (A). The percentage of activated 58-Bbc9 cells was measured by flow cytometry, gated on CD3+, living cells (B). The concentration of IL-2 in the supernatant was measured by ELISA (C).

4 Discussion

$GABA_A$ receptor encephalitis is recently described disease pattern, whereby patients suffer from antibodies against the $GABA_A$ receptor, which causes refractory seizures (Petit-Pedrol 2014). The antibodies bind specifically different subunits of the $GABA_A$ receptor, which causes an internalisation of the receptor, and thereby the inhibitory receptor function is lost, causing neurological and psychological symptoms. It is still unknown whether the disease belong to paraneoplastic neurological disorders, although association to thymomas was found in some patients (Ohkawa 2014, Simabukuro 2015). Further, similarities to neurological surface receptors, which are often involved in PNDs were found (Section 1.4.2).
Even though the humoral immunity is described, nothing is known about the role of T cells in the disease. Uncovering the influence of T cells, their pathogenicity as well as their antigens, would give insights into the etiopathology of the disease. Additionally, it could smooth the way for new treatment options. Three connected questions were adduced. First, the T cell repertoire of IP2 was analysed, and the disease related CD8+ T cell clone IP2-8S1 was detected. IP2-8S1 was used to screen for possible antigens of the TCR. Additionally, CD4+ T cells are also involved in $GABA_A$ receptor encephalitis to perform CD4+ T cell help for the production of antibodies. To analyse antigens of CD4+ T cells, a new screening system was established during this study. Therefore, the antigen screening for CD8+ T cells (K. Siewert *et al.,* 2012) was modified to allow the detection of CD4+ T cell antigens.

4.1 Index patient 2

To uncover the role of T cells in $GABA_A$ receptor encephalitis, the T cell repertoire of the CSF, the hippocampus and the lateral sulcus of IP2 (Petit-Pedrol *et al.,* 2014) was analysed (Section 4.1.1). Furthermore, the expanded clone IP2-8S1 was successfully cloned and analysed for HLA-restriction (Section 4.1.2) and antigen recognition (Section 4.1.3).

4.1.1 T cell repertoire

4.1.1.1 IHC of inflamed brain regions of IP2

IHC analysis of brain sections showed a high infiltration of CD8+ T cells in the hippocampus and the lateral sulcus of IP2. In contrast to CD8+ T cells, CD4+ T cells were very rarely found in both areas. Such low numbers are surprising, since CD4+ T cells are involved in the antibody response and are found frequently in infiltrated brain regions in autoimmune diseases. For example, in acute lesions of relapsing and remitting MS CD4+ T cells are predominant (Raine 1994).

Stainings for perforin proved that the CD8+ T cells are not only infiltrated into the brain, but they are also activated. The infiltration of CD8+ T cells in combinations with the expression of cytolytic granules prove an effector function of CD8+ T cells. The cells infiltrate into the brain, where they recognize their antigen and get activated. However, in IHC the target cell of the CD8+ T cells was not visible (Section 3.1.2.1, Figure 3-2 and Section 3.1.3.1, Figure 3-5). The nucleus of the target cell can be in a different layer of the biopsy section, and thereby it is not visible. Furthermore, the target could be localised on the axons of neurons, thus, the nucleus could be far away. To identify the target cell, costaining for different cell types in combination with staining for CD8 and perforin could be performed. An orientation of perforin to one cell type could indicate a possible target cell (Goebels *et al*., 1996). The stained target cells could eventually be isolated by laser microdissection and quantitative PCR (qPCR) with cell line specific primers could be performed, to identify the target cell.

4.1.1.2 T cell repertoire of the CSF of IP2

T cell analysis of the CSF of IP2 indicated about equal numbers of CD4+ and CD8+ T cells in the CSF. 40 CD4+ and 48 CD8+ T cells were isolated from 250 µl CSF.

Single cell analysis of the T cells isolated from CSF of IP2 showed a polyclonal distribution of CD4+ T cells in the CSF. The β-chain of 15 CD4+ T cells were identified, additionally 11 corresponding α-chains. Each TCR showed up only once. This polyclonal infiltration let suggest that the CD4+ T cells are only bystanders. Additionally, almost no CD4+ T cells were found in the inflamed region of the brain. Due to the humoral immunity with specific antibodies against the $GABA_A$ receptor α1 subunit, we expected to detect a clonal expansion of one or fewer CD4+ T cell clones, which might have been involved in the activation of B cells. CD4+ T cells are inevitable for the activation of B cells. They stimulation B cells for proliferation, somatic hypermutation as well as immunoglobulin class switching. However, in contrast to CD8+ T cells, CD4+ T cells do not need to be on-site of inflammation, for activation of B cells. The activation occurs in lymph nodes (Garside *et al.,* 1998). Thereby, the analysis of the CD4+ T cell in CSF and brain sections is not sufficient for a complete repertoire analysis. For the detection of possible disease related CD4+ T cell clones, the T cells in lymph nodes and in the blood have to be analysed. This was not possible during this thesis, because of the lack of biopsy material.

In contrast to CD4+ T cells, the TCR repertoire of CD8+ T cells in the CSF of IP2 had a strong bias to one particular clone. The TCR from six CD8+ T cells out of eleven identified TCRs were identical. All other TCRs showed up only once. Thereby, T cell repertoire studies for CD8+ T cells of the CSF of IP2 showed a clonal expansion of one TCR, which was called IP2-8S1. This clonal expansion indicates an effector function of the expanded clone, and thereby it could play a key role in the disease.

4.1.1.3 T cell repertoire of inflamed brain regions of IP2

Bulk cell analysis of the hippocampus and the lateral sulcus of IP2 by clone-specific PCR showed the presence of IP2-AV8S1 in both infiltrated brain regions. IP2-BV8S1 was not detected. However, in sc PCR both chains were detected. These results indicate problems during the clone-specific PCR of the β-chain during bulk cell analysis. In contrast to sc PCR, higher cDNA concentrations are used. Probably the β-chain primers of the clone-specific PCR1 bind unspecifically to a different DNA region, and thereby the primers for clone-specific PCR2 cannot bind anymore to the PCR product and cannot amplify IP2-BV8S1.

PCR on bulk cells already indicated the presence of IP2-AV8S1 in the brain. To further analyse the TCR repertoire in the brain, to quantify the presence of IP2-8S1 in the brain, and also to detect IP2-BV8S1, single cells were isolated by LMD of both infiltrated brain areas. The α- and β-chain were analysed by unbiased multiplex TCR PCR and in parallel by clone-specific TCR PCR.

As already shown by IHC, almost no CD4+ T cells were detected in both brain areas, thereby, only two CD4 + T cells were isolated from the hippocampus, and none of the lateral sulcus. However, no TCR sequence was received from PCR. These findings are in accordance with the repertoire analysis of the CSF. Presumably the disease relevant CD4+ T cells are not in the CSF, but probably present in the lymph nodes, where they activate relevant B cells to produce antibodies against the $GABA_A$ receptor α1 subunit.

Four different β-chains were identified from the 177 isolated CD8+ T cells of the hippocampus (Table 3-3). Two of the β-chains only showed up once and the third chains (clone 24) showed up three times in the hippocampus. However, the corresponding α-chains were not identified. The forth identified β-chain was the chain of the clonal expanded T cell from CSF: IP2-8S1. The β-chain of this clone was detected 22 times in the hippocampus. Furthermore, the corresponding α-chain of IP2-8S1 was identified in 19 of the 22 cells. IP2-8S1 was identified with clone-specific primers but not with the unbiased multiplex TCR PCR. This is due to the higher efficiency of clone-specific primers compared to the pool of the unbiased method (Dornmair 2003). The unbiased approach contains a lot of different primers covering all TCR chains. The primers have different PCR efficiency and might also interfere with each other. Thereby, the approach with clone-specific primers, where in each PCR step only one primer pair is used, is more efficient. The infiltration of IP2-8S1 was additionally validated by NGS, where it was also shown, that IP2-8S1 is expanded in the hippocampus (E. Beltrán).

The presence of many CD8+ T cells which express the TCR IP2-8S1 in the inflamed region of the hippocampus shows that IP2-8S1 is not only expanded in the CSF, but also in the brain. The clonal expansion of IP2-8S1 in the CSF and the hippocampus set the clone apart from bystander T cells. It highly indicates the presence of the target antigen in the CNS. Furthermore, it ascribes a key role of IP2-8S1 in the disease.

Although bulk cell analysis proved the presence of IP2-AV8S1 in the lateral sulcus, the clone couldn't be detected by single cell analysis of 111 CD8+ T cells, isolated by LMD. Only one new β-chain, but not the corresponding α-chain, was found in the lateral sulcus (Table 3-5). This result does not mean that IP2-8S1 is not present in the lateral sulcus. In fact, it shows that more single cells have to be isolated and analysed. Bulk cell analysis as well as NGS definitely proved the presence in the lateral sulcus. However, NGS showed that the rate of IP2-8S1 in the lateral sulcus is much lower compared to the hippocampus. The lower rates indicate, that the levels of the autoantigen of IP2-8S1 is increased in the hippocampus compared to the lateral sulcus. The lower rates also indicate that a lot more CD8+ T cells have to be isolated, to detect IP2-8S1 in the lateral sulcus. Additionally, the isolated single cells could have been derived from the wrong area in the biopsy section. Analysis of more single cells would validate the findings from bulk cell PCR and NGS. However, due to time limitations, it was not possible to isolate further cells during this thesis.

The achievement to get PCR products from LMD, especially depends on the mRNA quality of the tissue. The sample is always stored at -80 °C, thawing is avoided and RNase inhibitors are used while staining and LMD. Although, a RNase inhibitor is used during LMD, the screening for T cells in the biopsy and their isolation by LMD is restricted to 30 min for marking cells and 30 min for cell isolation. Otherwise the mRNA will be degraded by RNase. Thus, mRNA quality is not sufficient for RT-PCR. One method to overcome this problem would be to run the RT-PCR *in situ* and isolate the cells afterwards (Bagasra 2007). Thereby, mRNA would already be transcribed to cDNA in the tissue. Afterwards the tissue can be stained and all T cells can be isolated by LMD without time pressure. However, the major problem could be the digestion of the tissue with proteinase K. This step is required for the digestions of membrane proteins, to facilitate the PCR master mix to penetrate the cells (Bagasra 2007). The digestion could destroy the antigens for IHC. However, the digestion could be adapted to the tissue, to avoid antigen destruction. Thereby it would be possible to isolate all the T cells from one brain section without wasting any of the valuable biopsy material. Additionally, with one staining a lot more T cells could be isolated, which would also safe time and costs for chemicals, like RNase inhibitors and ABs.

Afterwards unbiased multiplex TCR PCR can be performed high throughput by the Janus pipetting robot. The robot was programmed during this thesis for the protocols for the run-off reaction, the anchor PCR, the alpha PCR as well as for clone-specific PCR. The protocols of the robot were adjusted for precise pipetting. The robot was put into a separated room and additionally into a box to avoid contaminations. UV radiation of the robot pre- and after usage lowers the contamination risk additionally. No contaminations were observed during the testing of the robot. Furthermore, it was successfully employed for the unbiased multiplex TCR PCR and the clone-specific PCR of samples isolated from the lateral sulcus.

By the application of the robot, the PCRs are standardized and optimized. Thereby the robot gives us the possibility for high throughput repertoire analysis of FACS sorted blood cells or CSF samples.

4.1.2 HLA restriction IP2-8S1

To analyse the disease relevant TCR IP2-8S1, it was successfully cloned into expression plasmids, which already contain the constant regions for TCR α- or TCR β-chain. To distinguish between contaminations and real PCR products in the repertoire studies silent mutations were added in the N(D)N region in the β-chain and in the α-chain. The plasmids coding for IP2-AV8S1 and IP2-BV8S1, were stably transfected into hybridoma cells expressing CD8. Furthermore, as activation marker, sGFP under the control of the NFAT-enhancer, was transfected. Single cell dilution was performed to isolate the best clone, expressing high levels of sGFP after activation, and with only little spontaneous activation to reduce the background. The thereby generated cell lines specifically express the TCR IP2-8S1 and sGFP as well as IL-2 after activation. Both activation markers can be used for the detection of T cell activation in further experiments.

To identify possible antigens of IP2-8S1 it would be helpful, to know the HLA-restriction of the T cell receptor.

HLA sequencing of IP2 showed that the patient expressed six different MHC-I alleles as well as six different MHC-II alleles (Table 3-7). Cocultivation of 58-IP2-8S1 cells with LCL, covering all MHC-I alleles, did not lead to an activation of IP2-8S1. Consequently, IP2-8S1 does not recognise an endogenously presented antigen on MHC-I molecules.

The antigens of the antibodies found in IP2 recognise an epitope of the $GABA_A$ receptor α1 subunit as their antigen. Thereby it was presumed that IP2-8S1 might also recognise a $GABA_A$ receptor α1 subunit derived peptide presented on MHC-I. Possible candidates of the $GABA_A$ receptor α1 subunit were selected by their predicted affinity to HLA alleles, TAP transport and proteasomal degradation, and tested for their ability to activate IP2-8S1. However, none of the 48 tested candidates was recognised by IP2-8S1. This absence of activation does not exclude the $GABA_A$ receptor α1 subunit as possible target, since only 48 candidates of the receptor were tested. The probability of a peptide to be presented on MHC-I molecules and thereby be recognised by a TCR is increased for peptides with high affinity for the MHC molecule, a high transport rate by TAP and the existence of large hydrophobic or basic residues for optimal cleavage after the residues by the immunoproteasome (Cascio *et al.,* 2001). However, peptides with less optimal amino acids composition for all these criteria are also presented by MHC-I molecules, and thereby they could be a possible antigen of IP2-8S1. To exclude the $GABA_A$ receptor α1 subunit as possible antigen, all possible peptides have to be screened. However, this approach would be very time-consuming and expensive.

Another possibility would be to produce the $GABA_A$ receptor α1 subunit recombinantly and digest it with consumable 20S proteasome (Chen *et al.,* 2013). Afterwards the digested protein can be added to COS-7 cells expressing MHC-I. After incubation, 58-IP2-8S1 cells can be added, cocultivated for 16 h and screened for T cell activation by fluorescence microscopy, flow cytometry or IL-2 ELISA. By the cleavage with the 20S proteasome, the subunit would be cleaved comparable to the natural cleavage in the body. This approach would be easier and cheaper for testing $GABA_A$ receptor α1 subunit as a possible antigen of IP2-8S1 than cloning all possible candidates or synthesising every peptide.

4.1.3 Library Screening IP2-8S1

The HLA-restriction could not be revealed. Thus, library screening for IP2-8S1 was performed with all six MHC-I alleles.

In one set of experiments, all HLA alleles were screened individually with a N27 library. Only in the samples with HLA-C*04:01 two clusters were found. However, further subpooling of the recovered plasmids did not result in a mimotope, because no reactivation was visible. The fact that only in the samples with HLA-C*04:01 clusters were found, could indicate a restriction to HLA-C*04:01. However, in another set of experiments, HLA-A, HLA-B or HLA-C were pooled and cotransfected for library screening. For all the pooled HLA alleles, clusters of activated 58-IP2-8S1 cells were found. The size and frequency of the clusters were comparable for all HLA-pools. No obvious HLA-restriction, where the clusters were only found by the screening with one HLA-pool, was visible. Additionally, clusters were found with the same frequency in all samples. The cluster sizes were almost in the same ratio between 3 to 5 activated 58-IP2-8S1 cells (Table 3-8). Thereby, HLA-restriction cannot be distinguished by the accumulation of clusters in one pool, compared to the others. If one pool would always show an increased cluster size compared to the other pools, it would indicate the HLA-restriction of IP2-8S1. Subpooling of the recovered plasmids did not lead to a mimotope, because either no reactivation was observed, or the reactivation was lost during the subpooling procedure.

For finding a mimotope of IP2-8S1, larger areas have to be screened for each HLA allele. Useful for future experiments would be to generate COS-7 cells which stably express the different HLA alleles. Thereby, HLA molecules are always expressed and do not depend on the transfection efficiency of each experiment. Besides this, the cells are less stressed, if only the library is transfected and has to be expressed additionally. However, the problem is that a specific antibody is needed for the selection of transfected cells, because COS-7 cells also express MHC-I molecules. A specific antibody is only available for HLA-A*02:01.

Library screening would be further eased with the application of HLA-specific libraries, as they were already used for HLA-A*02:01 (Section 3.2.5). MHC-I binding prediction tools can be used for the design of HLA-specific libraries (Rasmussen *et al.,* 2014, Vita *et al.,* 2015,

Andreatta and Nielson 2016). By the usage of these libraries it is ensured that the peptides have optimal binding properties for their MHC molecule. Thereby the presentation on the cell surface as well as the probability to find a mimotope is enhanced. Additionally, the libraries can be varied in the length between 8-11 AA for the antigen screening. It might be possible that longer or shorter peptides are recognized easier by IP2-8S1.

If the target cell of the TCR IP2-8S1 could be identified by IHC and qPCR (Section 4.1.2), cell specific cDNA libraries could be designed for the library screening, comparable to cDNA libraries from tumor tissue (van der Bruggen *et al.,* 1991). This would scale down the size of the library to peptides, which are expressed and presented by the target cell. A smaller area has to be screened to identify the antigen of IP2-8S1.

Furthermore, it is still unsolved if $GABA_A$ receptor encephalitis is a PND. Often the disease is associated with thymoma (Ohkawa *et al.,* 2014, Simabukuro *et al.,* 2015). However, IP2 was not screened for a tumor. Additionally, in other PNDs tumors are also not always detectable, because the tumors can be tiny and thereby, they are easily missed (Dalmau and Rosenfeld 2008). The analysis of the TCR repertoire of additional patients with thymoma could be helpful to solve this question. Possible expanded T cell clones could be screened for their antigens with tumor specific cDNA libraries. It would be interesting to see, if the target antigen of the expanded TCR is present in the tumor as well as in the CNS.

The detection of mimotopes with PECPL established by K. Siewert *et al*. 2012 is still a new method. Although it was validated with two additional TCR (Rühl *et al.,* 2016, Arakawa *et al.,* 2015), the method needs further optimization for higher efficiency. Already in the first step, the transfection of COS-7 cells with the library, an optimization could increase the efficiency of activated clusters and the following steps of subpooling. The transfection efficiency does not only depend on the cell cycle and condition of the cells, but also on the transfection method, as well as the amount of transfected DNA per cell. A quantification of the numbers of plasmids, which goes into one cell has to be adapted for the library screening. In the case that one cell takes up many different plasmids at once, the correct antigen might be too diluted to activate the TCR. In the other case, if only one or two plasmids go into the cell and are amplified by the large T antigen via the SV40 origin, the density of the correct antigen on the cell surface is high. However, a huge area has to be screened to find the correct antigen in a library of $> 10^7$ clones. Thereby the plasmid number has to be optimized and standardized to a median, so that the signal can still be detected, and the areas, which have to be screened are not too huge.

One explanation why sometimes no reactivation after plasmid recovery is observed, is that even though the method does not depend on high affinities of the TCR to the peptide-MHC-complex, because the cells are kept together my gravity (Siewert *et al.,* 2012), it might still happen that the hybridoma cells move after activation. Thereby, the activated cells might not

be directly superposed to the activating APC. This would lead to the isolation of the wrong COS-7 cell and thereby no reactivation will be visible after plasmid recovery. For the isolation of the correct cell, it would be helpful, if the COS-7 cell would also light up after activation.
Another explanation that no reactivation is observed, is that the COS-7 cells can burst during the picking procedure. Thus, the plasmids can stick to the capillary, and the positive signal is lost.
The signal loss of both, by picking the wrong COS-7 and by the burst of the cell, can be avoided by changing the dimension for the library screening from 3.5 cm dishes to 384 well format (G. Rühl, personal communication). The transfected cells can be pipetted into the 384 wells by the Janus pipetting robot, and the wells screened for activation by the scanning program “Zen pro”. All COS-7 cells of the activated well, can be transferred to a PCR tube after detaching the cells for 5 min on ice. To make sure, no cell is lost, the well can be additionally washed with the PCR master mix. By this procedure, a loss of the correct plasmid by the movement of the hybridoma cells, as well as by problems during the picking procedure, is avoided.
Furthermore, the detection could be changed from the expression of sGFP controlled by the NFAT-enhance to the expression of luciferase under the control of the NFAT-enhancer. After the addition of the substrate luciferol to the media and activation of the hybridoma cells, the bioluminescence can be detected by an ELISA reader in 384 well plates. This would additionally safe a lot of time, because one plate can be detected much faster by an ELISA reader compared to the screening by fluorescence microscopy.
Although single cell dilution of 58-IP2-8S1 cells was performed to reduce the background of spontaneously activated hybridoma cells, spontaneous activation cannot be completely excluded. The expression of sGFP under the control of the NFAT-enhancer is triggered by increased Ca^{2+} concentration in the cell. However, Ca^{2+} elevation is not only related to antigen recognition. T cells, which are not exposed to their antigens, also show short-lasting Ca^{2+} peaks (Mues *et al.*, 2013). These Ca^{2+} peaks highly likely induce spontaneous activation of the hybridoma cells. Thereby it might happen that the COS-7 cell, which was isolated, does not carry the plasmid expressing the correct peptide. Furthermore, spontaneous activation can falsely be interpreted as positive signal. Thereby, in the next round, the signal seems to be lost, even though the signal was always just background. The background of spontaneous activated cells can be reduced, by single cell dilution and by keeping the cells in culture not longer than six weeks. After this time period a new vial should be thawed from the liquid nitrogen storage.
Another reason, why no reactivation is visible, might be that the correct peptide is lost during cloning. Furthermore, the number of clones screened for reactivation might be too low, whereby the correct clone is not screened. The area which is screened to detect reactivation

in the subpools might also be not sufficient enough. By the optimization with the transfection of a definite number of plasmids per cell, this problem could be avoided.
Another possibility why the correct antigens cannot be found, might be that the correct peptide is degraded in the cell by proteases. Thereby only a small amount of the correct peptide will be presented by MHC-I molecules on the cell surface. Depending on the degradation, the amount can be enough for T cell activation. However, the next time it is screened, the amount of remaining peptide is not enough anymore for T cell activation, and thereby, a once positive signal is lost. The same might be observed, after the formation of aptamers with the peptides in the cell. This formation leads to less available free peptide in the cell, which can be loaded on MHC molecules. Consequently, the correct peptide cannot be presented on the cell surface, resulting in a missing activation signal.
Another possibility is that the correct peptide is in some way toxic for the cell. This results in a dying of COS-7 cells expressing the desired peptide. The correct peptide cannot be presented on the cell surface anymore, and no activation signal can be observed.
These challenges, where the peptides are degraded, form peptide-aptamers in the cell or when the peptide is toxic for the cell, are not easy to solve. One possibility would be to replace COS-7 cells as APC by another cell line. Other cell lines, especially derived from different species, have a different proteome. Thereby, there is the chance that the protease, which degrades the peptide, is not present. Hence, a T cell activation should be detectable.
The method to identify TCR mimotopes by PECPL is limited to peptides as possible antigens of the TCR. However, there are several T cell subtypes which do not recognise peptides as antigens. Mucosal-associated invariant T (MAIT) cells, for example, recognise vitamin B derivatives presented by MHC-related protein 1 (MR1, Kjer-Nielsen *et al.,* 2012). Another example is γδ TCRs, which can recognise conformational epitopes, lipids, carbohydrates or small molecules, alone or bound to CD1 (Dornmair *et al.,* 2003). Hence the premise of the method is that the TCR recognises peptides presented by MHC-I molecules.
Altogether, HLA-restriction and the target antigen of the expanded CD8+ T cell clone IP2-8S1 is still unknown. By the optimization and standardization of the detection methods, and further screenings for antigens with additional libraries, it should be possible to identify the target antigen and thereby solve the role of T cells in $GABA_A$ receptor encephalitis.

4.2 Detection of MHC-II mimotopes

The identification of CD4+ T cell antigens is fundamental for a better understanding of autoimmune diseases and the interaction of the immune system. Additionally, the knowledge of CD4+ T cell antigens would lead the way for new therapeutic options of tumors, autoimmune diseases as well as vaccination strategies. In IP2, antibodies against the $GABA_A$ receptor were detected. CD4+ are crucial for most humoral immune response. Thus we expected to identify

an expanded CD4+ T cell clone in IP2. However, to identify the antigen of CD4+ T cells, a new screening system was required. With the so far establishes unbiased method, based on the screening for antigens with PECPL, it is only possible to identify CD8+ T cell antigens. Thus, the method was modified to allow screening of CD4+ T cell antigens. Though, in IP2 an expanded CD8+ T cell clone was then detected and not, as expected an expanded CD4+ T cell clone. However, the new established method can be used to identify antigens of expanded CD4+ T cell clones in other diseases.

4.2.1 MHC-II libraries

Due to the different loading of MHC-I und MHC-II (Section 1.3.1) new libraries, expressed in Ii, were designed for the screening of CD4+ T cell antigens. Ii-ECPL of different library length were successfully cloned and produced in *E. coli*. The number of different plasmids of the libraries varied between 7.5×10^7 - 7×10^9 clones. This yield of different plasmids per library is very good. However, it must be pointed out that around 30% of the clones contain a STOP codon in the library and are thereby not useful. The remaining number of different plasmids are nevertheless very high and cover a broad spectrum of possible antigens.

By Western Blotting it was shown that empty COS-7 cells do not express Ii, but after transfection of pcDNA-Ii-ECPL, the cells express the libraries in Ii. The expression of Ii-ECPL in COS-7 cells was the first premise for further experiments. Intracellular FACS for CD74 after the transfection of different DNA concentrations with different transfection methods showed that nucleofection is the method of choice for library screening. In contrast to FuGENE® transfection, the expression of Ii-ECPL is much higher, especially for low DNA concentrations. The higher expression of CD74 after nucleofection compared to FuGENE® transfection is due to higher transfection efficiency by nucleofection. However, after 72 h the level of CD74 decreases, especially after the transfection of higher DNA concentrations. This effect can be ascribed due to DNA degradation in the cell. Thereby, picking of activated clusters should be performed between 48-72 h.

4.2.2 Bbc9 as proof of concept

The well characterised TCR Bbc9 was chosen as proof of concept. Due to the knowledge of HLA-restriction, as well as the peptide specificity of the TCR, Bbc9 is well suitable as positive control, to test, whether the new established system for the identification of CD4+ T cell antigens is working accurately (Section 3.3.2).

The α- and β-chain of Bbc9 were successfully transfected into $58^{-/-}$ hybridoma cells, which already express human CD4 (Blank *et al*., 1993). Furthermore, pcDNA-NFAT-sGFP was transfected, and the clone expressing highest level of sGFP and lowest amount of spontaneously activated cells, was chosen for further experiments.

The specificity of the 58-Bbc9 cells to HLA-DR1 and -DR2a was proven by incubation of 58-Bbc9 cells with hMBP-derived synthetic peptides presented on COS-7 cells stably expressing HLA-DR1, -DR2a and as negative control HLA-DR2b. A specific activation with an at least 10 AA long peptide, including a phenylalanine (F) at position 1 (hMBP(141), F141) and a leucine (L) at position 10 (hMBP(150), L150), presented on HLA-DR1 and -DR2a was verified. The specificity of the system was confirmed by presentation of the peptides by an irrelevant HLA allele (HLA-DR2b) and by the presentation of an irrelevant peptide (hMBP(50-69)) by the correct HLA alleles. No signal was observed in any of the negative controls. The results are confirmed by fluorescence microscopy, flow cytometry as well as by IL-2 ELISA, all three methods reflecting the same specificity of Bb9.

The peptides must have a minimum length of ten AA for activating Bbc9. Longer peptides (hMBP(139-153)) activated Bbc9 more efficiently. Natural peptides loaded on MHC-II are 13-17 AA long (Rudensky *et al.,* 1991). Shorter peptides than eleven AA are usually exchanged by HLA-DM (Kropshofer *et al.,* 1996). Furthermore, the AA F141 and L150 are essential for the recognition by Bbc9. The 10 AA long peptide hMBP(140-149) only activates Bbc9 a little presented on HLA-DR2a but not at all on HLA-DR1, and less strong, than the also 10 AA long peptide hMBP(141-150). This shows how important the AA F141 and L150 are for an optimal activation of Bbc9.

The experiments show that Bbc9 was specifically activated by hMBP. Furthermore, HLA-restriction was ensured. No unspecific activation was detected. Thereby, the cell-based assay can be applied for CD4+ T cells for the measuring of TCR activation by the different synthetic peptides.

Plasmids, coding for Ii-hMBP with diverse length of hMBP-derived peptides, demonstrated that an activation of 58-Bbc9 cells is also possible with these constructs. No activation was observed by the presentation of the constructs on HLA-DR2b molecules, proving that the HLA-restriction is preserved with Ii-hMBP constructs. The activation of 58-Bbc9 cells with the Ii-hMBP constructs, but not with the negative control Ii-p33-wt, presented by HLA-DR1 as well as by HLA-DR2a show a specific activation of Bbc9 by the Ii-hMBP constructs, restricted to the hMBP peptide. The invariant chain itself does not generate an unspecific activation of the TCR. Although no activation of 58-Bbc9 cells was observed with 9 AA long synthetic peptide hMBP(141-149), Ii-hMBP(141-149) showed weak activation of 58-Bbc9 cells. This activation can be ascribed to the binding properties of Ii. Although, nine AA long peptides, or even shorter, can also bind successfully to MHC-II molecules, natural bound peptides are usually longer (Rammensee 1995). The binding properties of a nine AA long peptide to MHC-II molecules are not good. However, expressed in the invariant chain, the peptide takes advantage of the AA residues of Ii, which bind to MHC-II molecules by non-specific interactions (Rammensee 1995). Thereby, Ii-hMBP(141-149) can be loaded onto MHC-II molecules and can be

presented on the cell surface, hence it is anchored by the invariant chain to MHC-II. However, activation is still lower, compared to longer constructs, because L150 is missing.
The expression of peptides with low affinity to MHC-II molecules in Ii can provide an opportunity to present these peptides on the cell surface and induce T cell activation, however with lower efficiency.
The comparison in inducing activation in Bbc9 using Ii-hMBP constructs with and without N-terminal signal sequence of Ii, showed that Ii-hMBP constructs activate Bbc9 more efficiently than delta-Ii-hMBP constructs. The more intense activation of Ii-hMBP constructs cannot be ascribed to differences in cellular viability after transfection, due to the fact that no differences in cellular viability were observed (Figure 3-18). Thus, the signal sequence must be recognised by COS-7 cells. The loaded MHC-II complex is targeted to the cell surface, where it can activate the TCR on hybridoma cells. All designed libraries contain the full length Ii chain, with signal sequence, due to the fact that the activation by Ii-hMBP constructs was much stronger with signal sequence, compared to constructs without.
The experiments with Ii-hMBP constructs demonstrate that our designed system for the identification of MHC-II antigens is functional. COS-7 cells successfully expressed Ii-hMBP constructs. The constructs are loaded onto MHC-II molecules and are presented on the cell surface to TCR-expressing hybridoma cells. The TCR of these hybridoma cells can specifically and in an HLA-restricted manner be activated. The activation can be detected with different methods, using fluorescence microscopy, flow cytometry as well as IL-2 ELISA. Thereby, the system is ready to use for the screening of CD4+ T cell antigens.

4.2.3 Comparison of the sensitivity of different detection methods

Our cellular detection system enables us to screen TCR activation by three different ways: by fluorescence microscopy, by flow cytometry as well as by IL-2 ELISA. Siewert *et al.* 2012 were using fluorescence microscopy as method of choice, because it was the most sensitive method for the detection of CD8+ T cell antigens. The titration of pcDNA-Ii-hMBP(139-151) in pcDBA-p33-wt showed that fluorescence microscopy is also the best choice for the detection of CD4+ T cell antigens. In titration, clusters with several activated 58-Bbc9 cells were still visible at dilutions of $1:10^4$ in 24-well plates. In contrast, flow cytometry and IL-2 ELISA were only sensitive enough to detect dilution of $1:10^3$. The difference is that in fluorescence microscopy, clusters of activated 58-Bbc9 cells can be visible close to each other. Even if no other cells are activated in the well, this close contact of a few activated cells indicate recognition of their antigen, and thereby presence of the plasmid expressing the correct antigen. However, in flow cytometry and IL-2 ELISA all cells, or the supernatant is analysed. The number of activated cells thereby must be much higher to yield an activation signal above background. This is further proved by the fact that the weak activation of hMBP(140-149) presented by HLA-DR2a

molecules was only detectable by fluorescence microscope. No activation was observed by flow cytometry neither by ELISA. Fujii *et al.* (2001) also designed a method for the detection of CD4+ T cell antigens, however, they were only able to identify the correct antigen out of 1,000 irrelevant, due to the fact that INF-γ ELISA was used as read-out system.

The number of different plasmids of Ii-ECPL vary between 7.5×10^7 to 7×10^9 clones, depending on the used library (Table 3-9). The plasmid expressing the correct antigen is enriched by isolating the underlying COS-7 cells and recovering the plasmids of the library by PCR. For reactivation it must be ensured that the number of different plasmids is not exceeding 10^4 - 10^5 clones. Otherwise no reactivation can be detected in a 24-well plate, because the correct clone will be too diluted. However, if larger areas are screened, reactivation will still be detectable. Consequently, the area to be screened for the reactivation, is adjusted to the number of different plasmids after picking.

4.2.4 COS-7 cells as antigen presenting cells

COS-7 cells were chosen as antigen presenting cells, because they are easy to culture, the transfection efficiencies are high, they are adherent cells and because they worked well for the detection of CD8+ T cell antigens.

However, COS-7 cells were not efficiently expressing HLA-DR molecules. Only after several transfection and several rounds of MACS purification and FACS, clones, with stable expression of HLA-DR molecules were received. Furthermore, COS-7-DR cells often died after the transfection of Ii-constructs. Both let suggest that COS-7 cells are overstrained with the presentation of MHC-II libraries. COS-7 cells are kidney cells, and thereby no professional APCs. One reason for the cell dying after transfection might be that COS-7 cells do not recognize the N-terminal signal sequence. Thereby the Ii-ECPL-loaded MHC-II molecules would get stucked in the ER and block the excretion machinery of the cell. However, the comparison of cellular viability after transfection of constructs with and without signal sequence did not differ. Cellular viability was comparable for both variants.

Transfection of COS-7-DR cells with Ii-ECPL by nucleofection showed higher cellular viability as compared to FuGENE® transfection. Nucleofection is more gentle to the cells compared to FuGENE® transfection. Furthermore, the transfection efficiency is increased and thereby also the expression levels of the library. Hence, nucleofection was the method of choice for the library screening.

Although cell death is reduced after nucleofection of Ii-ECPL, during the subpooling procedure, DNA quality of the isolated plasmids is not sufficient to use nucleofection. Bacterial lipopolysaccharides (LPS), which are toxic for the cells, remain in the isolated DNA from *E. coli*. These LPS can penetrate the cells by nucleofection and thereby induce cell death. This can be avoided by using FuGENE® transfection. Another possibility would be to use a plasmid

isolation kit with additional endotoxin removal buffer, as it is provided by promega. Plasmid preparation with this kit and following nucleofection reduced the cell death remarkably. However, the costs for nucleofection of many subpooling samples must be considered. Although, nucleofection yields increased cellular viability after transfection, after reducing the transfected DNA amount from 500 ng to 250 ng, cellular viability was also increased with FuGENE® transfection (Figure 3-18), and the signal was still very good, which was shown with Ii-hMBP constructs (Figure 3-19 and Figure 3-20).

Hence, nucleofection is the method of choice for the initial library screening and picking of cells. However, for all following transfections FuGENE® transfection is sufficient.

As an alternative to COS-7 cells, it was shown that LTK$^-$ cells also have the ability to activate 58-Bbc9 cells after transfection of Ii-hMBP(139-151). LTK$^-$ cells also fulfil all criteria for the application in the library screening. The cells are easy to culture, they can be transfected and they are adherent. LTK$^-$ cells were successfully stably transfected with HLA-DR1, -DR2a or -DR2b. The expression levels of HLA-DR were higher as compared to stable COS-7 cells expressing HLA-DR, even without further purification via MACS beads or FACS.

Although stable transfection with plasmids coding HLA-DR was successful, the transient transfection with FuGENE® was challenging. The transfection efficiencies were very low. However, transfection was improved by the usage of GenaxxoFect-Plus as transfection reagent and by splitting the cells the day before transfection. With this new established transfection method, it was possible to activate 58-Bbc9 cells with the positive control Ii-hMBP(139-151). Comparable to usage of COS-7 cells as APCs, no activation was observed with the negative controls Ii-wt or delta-Ii. Furthermore, HLA-restriction is conserved and no activation was observed with the irrelevant HLA-DR2b allele.

The experiments show that LTK$^-$ cells are a possible alternative to COS-7 cells as APCs for the library screening. Specific and HLA-restricted activation of TCRs is ensured. Furthermore, mouse fibroblast might even be more efficient than COS-7 cells for the activation of murine hybridoma cells. It was shown that 58$^{-/-}$ cells can be activated more efficiently by mouse fibroblasts than TCR-transfected human cell lines, presumably traced back to the expression of murine costimulatory factors (Dornmair *et al.,* 2003). However, this has to be tested in further experiments.

Another possibility would be to use a cell line which is derived from a professional APC, best from murine origin, because the used hybridoma cells are also from mouse. Possible cell lines would be the mouse microglia cell line SIM-A9 or mouse microglia cell line C8-B4. Both cell lines originated from spontaneous transformation and are adherent cell lines (Alliot *et al.,* 1996, Nagamoto-Combs *et al.,* 2014). The advantage of these cell lines compared to COS-7 cells would be that they are both of murine origin. Thereby, they express appropriate costimulating factors for the hybridoma cells. This could increase the activation of hybridoma cells during the

library screening. Additionally, they originated from professional APCs. The whole machinery of the expression of MHC-II molecules, the targeting to the MIIC as well as the loading with possible peptides and the presentation on the cell surface is optimized in these cell lines. Thereby, they might not be overtaxed after the transfection of Ii-ECPL, as it is the case for COS-7 cells.

It is possible to use COS-7 cells for the detection of CD4+ T cell antigens, however, there might be other cell lines such as LTK^- cells which are more appropriate for this purpose. Further cell lines have to be tested in further studies.

4.2.5 Library screening Bbc9

Library screening for the TCR Bbc9 was performed with two different HLA alleles, HLA-DR1 and HLA-DR2a. Both HLA alleles are able to activate Bbc9, whereas the focus in this work was pointed to HLA-DR1. Comparison of the ability to activate 58-Bbc9 cells with different libraries, varying in the length, showed that the cluster sizes of activated 58-Bbc9 cells were bigger for longer libraries (Table 3-11). This result is in good accordance to the results of activation by Ii-hMBP constructs expressed in pcDNA (Figure 3-20). The best activation was also observed with the longest construct Ii-hMBP(139-151) expressed in pcDNA, which would be the equivalent to the Ii-N39 library. However, almost only low activation was found with Ii-hMBP(141-149) expressed in pcDNA, which would be the equivalent to the Ii-N27 library. Both results show that library screening for CD4+ T cell antigens will be more efficient for longer libraries. According to the results of Ii-hMBP constructs the random sequence of Ii-ECPL should be at least 30 nucleotides (10 AA) long.

COS-7 cells stably expressing HLA-DR molecules were transfected with Ii-ECPL by two different methods. On the one hand via nucleofection, and on the other hand by FuGENE® transfection. Comparison of the sizes of the activated clusters showed that after nucleofection up to 17 activated 58-Bbc9 cells can be found in one cluster, whereas after FuGENE® transfection only clusters of maximum five activated Bbc9 were found. One reason therefore is that after nucleofection the cellular viability is better. Thus cells will start growing earlier and more rapidly. Furthermore, the expression levels of Ii-ECPL are higher after nucleofection (Figure 3-16). Thus, more library-loaded MHC-II molecules are presented on the cell surface, and 58-Bbc9 cells can be activated more efficient.

After transfection of less DNA per cell, the number of different plasmids per cell is reduced. Thereby, the cell can amplify each plasmid more often, and consequently, the correct antigen is presented more efficiently on the cell surface. Although, it was expected that the cluster sizes might increase after the transfection of less amount of DNA per cell, the cluster sizes were in fact smaller. After the nucleofection of 1 µg of pcDNA-Ii-N39 per $1^{x}10^{6}$ COS-7 cells the clusters had a size of up to 17 activated 58-Bbc9 cells. In comparison, after the transfection of

0,1 µg of the same library per $1^{x}10^{6}$ COS-7 cells, the size of the activated clusters was only composed of up to seven activated 58-Bbc9 cells. Although the number of activated 58-Bbc9 cells is not increased after transfection of less DNA, the correct plasmid is amplified via the SV40 origin in higher copy number in the COS-7 cells. Thereby, the following steps of subpooling are facilitated. However, by the transfection of less DNA each cell expresses a lower number of different plasmids. Thereby, huge areas have to be screened, which is very time consuming. A balance has to be found. The change to 384-well plates instead of 3.5 cm dishes, as well as the change to the expression of luciferase under the control of the NFAT-enhancer, already discussed in Section 4.1.3, can improve the library screening. The same amount of transfected cells can be screened for activation much faster. Thereby, the number of plasmids per cell can be adjusted much better, without a time loss.

Although big clusters of up to 17 activated 58-Bbc9 cells were detected and isolated from the Ii-ECPL screening, no mimotope was detected jet. Often no reactivation was observed after the cloning of the PCR products back into Ii in pcDNA. All the reasons described in Section 4.1.3 for MHC-I antigen search, like the bursting of the COS-7 cell during the isolation procedure, are also a challenge for the screening of CD4+ T cell antigens.

Furthermore, the PCR products have to be digested with the restriction enzymes AgeI and KasI for cloning it into Ii. For the identification of CD8+ T cell antigens, no restriction digestion is necessary. The PCR product is straightforwardly cloned via the pcDNA™ 3.1 Directional TOPO® Expression Kit (Invitrogen). By digestion with restriction enzymes, it might happen that the PCR product, coding for the positive antigen, also gets digested, and thereby it is not available anymore in the pool. Thus, no reactivation may be detected.

Additional steps of DNA purification are required after the restriction digestion, compared to the cloning procedure for MHC-I antigen search. Each purification step increases the risk of losing the sequence coding for the positive candidate, leading to a lack of reactivation signal. In addition, the religation efficiency of the customised TOPO ligation kit might be higher and more convenient compared to the conventional cloning.

Another challenge was the restriction digestions of the cloning vector pcDNA-Ii-wt, into which the PCR products were cloned. The vector has to be serial digested with AgeI and KasI. However, the insert is very small (55 bp). Thus, it is not trivial to separate completely digested vector from only partially digested vector. The undigested vectors may religate and increased the background. Thus, larger areas have to be screened for reactivation. One way to minimise the background would be to insert a bigger spacer into pcDNA-Ii-wt via AgeI and KasI (pcDNA-Ii-spacer), comparable to pcDNArc-spacer, used for the cloning of PECPL. Another possibility to decrease the background would be to use different restriction sites, like AscI in combination with KasI. The only difficulty would be the increased error rate of the polymerase for the

generation of the longer PCR product. However, a proof-reading polymerase would reduce this risk.

The calculation of how many clones have to be screened, to detect one mimotope is based on statistics. This calculation was established for CD8+ T cell antigens. However, CD4+ T cells have an even lower affinity to the peptide-MHC complex than CD8+ T cells (Cole *et al*., 2007). This lower affinity makes it more challenging to identify a CD4+ T cell antigen. No mimotope was detected jet for CD4+ T cell antigens by the new established method based on Ii-ECPL. One reason might be that the statistic might not be transferable to the screening of CD4+ T cell antigens. It might be that an exceeding number of clones and a larger area have to be screened for the CD4+ library screening.

To facilitate the recognition of a possible mimotope by Bbc9, clone-specific libraries could be designed, composed of a random NNK-sequence, amplified by adding the anchors F and L for example at AA position 1 and 10 of the random peptide sequence, resulting in the library Ii-ECPL-N30-F1,L10. The possibility of recognition by Bbc9 will be increase by adding these anchors, and thereby the library screening will be more efficient.

The results show that clusters of specifically activated 58-Bbc9 cells were detected, using different Ii-ECPL. With further improvement of the library screening system, as well as the cloning procedure for CD4+ T cell antigens, new mimotopes will presumably be found soon. The detection of new mimotopes will validate that the Ii-ECPLs are working for the detection of new CD4+ T cell antigens, as it was already shown with the Ii-hMBP constructs.

4.3 Outlook

This study contributes to the characterisation of idioplastic encephalitis with presumably paraneoplastic background. It is the first study analysing the role of T cells as well as the TCR repertoire of a patient with $GABA_A$ receptor encephalitis. This strategy of analysing the TCR repertoire in inflamed tissues is transferable to several other diseases, especially auto-immune diseases. Thereby the role of T cells in the etiology of many diseases can be revealed.

The analysis of the TCR repertoire in other patients with antibodies against the $GABA_A$ receptor is inevitable to confirm the cytotoxic role of autoaggressive CD8+ T cells in the disease. Furthermore, the TCR repertoire in the tumor tissue and CNS in patients with $GABA_A$ receptor encephalitis and thymoma have to be analysed. It would be interesting, if the same expanded and activated CD8+ T cells are present in the CNS as well as in the tumor, indicating the presence of the antigen in both tissues.

However, the study clearly showed that CD8+ T cells play a key role in the disease. This knowledge can be useful for the treatment of patients with $GABA_A$ receptor encephalitis.

After improving the library screening of CD8+ T cell antigens, like the usage of 384 well plates as described in Section 4.1.3, it should be possible to also identify the antigen of the expanded

CD8+ T cell clone IP2-8S1. This will be a big step forward for the understanding of the role of T cells in $GABA_A$ receptor encephalitis.

CD4+ T cells play an important role in many auto-immune diseases, like myelin-specific CD4+ T cells in MS (Hohlfeld *et al.,* 2015). To identify CD4+ T cell antigens, the detection method for the identification of CD8+ T cells (Siewert *et al.,* 2012) was transferred to the screening of CD4+ T cell antigens. In general, the identification of CD4+ T cells antigens is even more challenging than the identification of CD8+ T cell antigens, due to their lower affinity (Cole *et al.,* 2007). During this thesis, several technical aspects, like the transfection method, were improved and it was shown that the method is working for the detection of CD4+ T cell antigens. However, there are still some aspects which need to be adapted for the screening of CD4+ T cell antigens, like the calculation of how many clones have to be screened during the subpooling procedure as described in Section 4.2.5. Nevertheless, the new established system can be applied for other TCR of CD4+ T cell antigens, from any disease, like auto-immune diseases and tumors.

The identification of the T cell target does not only maintain to a better understanding for the etiology of the disease, however it also opens the floodgates for new treatment opportunities, like new vaccination strategies, immune therapies and the application as biomarkers of diseases.

5 Supplement

5.1 Primer and Oligonucleotides

Table 5-1 Primer for TCR RT-reaction

Label	Sequence	T_M
Cα-RT-imp	5' GCCACAGCACTGTTGC 3'	52 °C
Cβ-RT-2	5' GWAGAAGCCTGTGGCC 3'	52 °C

Table 5-2 Primer for TCR Touch-Down PCR

Label	Sequence
Cα-out	5' GCAGACAGACTTGTCACTGG 3'
Cβ-mid-4	5' TGGGTGTGGGAGATCTCTG 3'
VP1	5' TSYTTTGTCTCCTGGGAGCA 3'
VP2	5' CCTGAAGTCGCCCAGACTCC 3'
VP3	5' GTCATSCAGAACCCAAGAYACC 3'
VP4	5' GGWTATCTGTMAGMGTGGAACCTC 3'
VP5	5' ATGTACTGGTATCGACAAGAYC 3'
VP6	5' CACTGTGGAAGGAACATCAAACC 3'
VP7	5' TCTCCACTCTSAAGATCCAGC 3'
VP8	5' CAGRATGTARATYTCAGGTGTGATCC 3'
VP9	5' CCAGACWCCAARAYACCTGGTCA 3'
Vα-1^{14}-for-out	5' AGSAGCCTCACTGGAGTTG 3'
Vα-1^{235}-for-out	5' CTGAGGTGCAACTACTCATC 3'
Vα-2-for-out	5' CARTGTTCCAGAGGGAGCC 3'
Vα-3,25-for-out	5' GAARATGYCWCCATGAACTGC 3'
Vα-4,20-for-out	5' WTGCTAAGACCACCCAGCC 3'
Vα-5-for-out	5' AGATAGAACAGAATTCCGAGG 3'
Vα-6,14-for-out	5' RYTGCACATATGACACCAGTG 3'
Vα-7-for-out	5' CACGTACCAGACATCTGGG 3'
Vα-8,21-for-out	5' CCTGAGYGTCCAGGARGG 3'
Vα-9-for-out	5' GTGCAACTATTCCTATTCTGG 3'
Vα-10,24-for-out	5' ASTGGAGCAGAGYCCTCAG 3'
Vα-11-for-out	5' TCTTCAGAGGGAGCTGTGG 3'
Vα-12-for-out	5' GGTGGAGAAGGAGGATGTG 3'
Vα-13,19,26-for-out	5' SAASTGGAGCAGAGTCCTC 3'
Vα-15-for-out	5' CCTGAGTGTCCGAGAGGG 3'
Vα-16-for-out	5' ATGCACCTATTCAGTCTCTGG 3'
Vα-17-for-out	5' TGATAGTCCAGAAAGGAGGG 3'

Vα-18-for-out	5‘ GTCACTGCATGTTCAGGAGG 3‘
Vα-22,31-for-out	5‘ CCCTWCCCTTTTCTGGTATG 3‘
Vα-23,30-for-out	5‘ GGCARGAYCCTGGGAAAGG 3‘
Vα-27-for-out	5‘ CTGTTCCTGAGCATGCAGG 3‘
Vα-28-for-out	5‘ AGACAAGGTGGTACAAAGCC 3‘
Vα-29-for-out	5‘ CAACCAGTGCAGAGTCCTC 3‘
Vα-32-for-out	5‘ GCATGTACAAGAAGGAGAGG 3‘

Table 5-3 Primer for TCR Run-off reaction

Label	Sequence
VP1+	5‘ ACAGCACGACTTCCAAGACTCACYTTTGTCTCCTGGGAGCA 3‘’
VP2+	5‘ ACAGCACGACTTCCAAGACTCACCTGATGTCGCCCAGACTCC- 3‘’
VP3+	5‘ ACAGCACGACTTCCAAGACTCAGTCATSCAGAACCCAAGAYACC 3‘
VP4+	5‘ ACAGCACGACTTCCAAGACTCAGGWTATCTGTMAGMGTGGAACCTC 3‘’
VP5+	5‘ ACAGCACGACTTCCAAGACTCAATGTACTGGTATCGACAAGAYC 3‘’
VP6+	5‘ ACAGCACGACTTCCAAGACTCACACTGTGGAAGGAACATCAAACC 3‘
VP7+	5‘ ACAGCACGACTTCCAAGACTCATCTCCACTCTSAAGATCCAGC 3‘
VP8+	5‘ ACAGCACGACTTCCAAGACTCACAGRATGTARATYTCAGGTGTGATCC 3‘
VP9+	5‘ ACAGCACGACTTCCAAGACTCATCAGACWCCAARAYACCTGGTCA 3‘

Table 5-4 Primer for TCR Anchor PCR

Label	Sequence
UP-new	5‘ AGCACGACTTCCAAGACTCA 3‘
Cβ-in	5‘ TCTGATGGCTCAAACACAGC 3‘

Table 5-5 Primer-Sets for Alpha PCR

Set	Label	Sequence
	Cα-rev-in	5’-AGTCTCTCAGCTGGTACACG-3’
#1	Vα-4/1-for-in	5’-ACAGAAGACAGAAAGTCCAGC-3’
	Vα-4/2-for-in	5’-GTCCAGTACCTTGATCCTGC-3’
	Vα-6-for-in	5’-GCAAAATGCAACAGAAGGTCG-3’
	Vα-8/1-for-in	5’-CAGTGCCTCAAACTACTTCC-3’
	Vα-8/2-for-in	5’-GCCTCAGACTACTTCATTTGG-3’
	Vα-14-for-in	5’-ACAGAATGCAACGGAGAATCG-3’
	Vα-24-for-in	5’-CCTTCAGCAACTTAAGGTGG-3’
	Vα-28-for-in	5’-TCTCTGGTTGTCCACGAGG-3’
#2	Vα-2/1-for-in	5’-TGGAAGGTTTACAGCACAGC-3’

	Vα-2/2-for-in	5′-TGGAAGGTTTACAGCACAGG-3′
	Vα-5-for-in	5′-CAGCATACTTACAGTGGTACC-3′
	Vα-10-for-in	5′-TCACTGTGTACTGCAACTCC-3′
	Vα-12-for-in	5′-TACAAGCAACCACCAAGTGG-3′
	Vα-22-for-in	5′-AGGCTGATGACAAGGGAAGC-3′
	Vα-31-for-in	5′-GTGGAATACCCCAGCAAACC-3′
#3	Vα-7-for-in	5′-CTCCAGATGAAAGACTCTGC-3′
	Vα-13-for-in	5′-TTAAGCGCCACGACTGTCG-3′
	Vα-17-for-in	5′-CTGTGCTTATGAGAACACTGC-3′
	Vα-18-for-in	5′-CCTTACACTGGTACAGATGG-3′
	Vα-21-for-in	5′-TGCTGAAGGTCCTACATTCC-3′
	Vα-23-for-in	5′-GTGGAAGACTTAATGCCTCG-3′
	Vα-32-for-in	5′-TCACCACGTACTGCAATTCC-3′
#4	Vα-3-for-in	5′-TTCAGGTAGAGGCCTTGTCC-3′
	Vα-11-for-in	5′-AGGGACGATACAACATGACC-3′
	Vα-15-for-in	5′-CCTCCACCTACTTATACTGG-3′
	Vα-19-for-in	5′-CCTGCACATCACAGCCTCC-3′
	Vα-25-for-in	5′-AGACTGACTGCTCAGTTTGG-3′
	Vα-26-for-in	5′-CCTGCATATCACAGCCTCC-3′
	Vα-29-for-in	5′-ACTGCAGTTCCTCCAAGGC-3′
#5	Vα-1^{235}-for-in	5′-AAGGCATCAACGGTTTTGAGG-3′
	Vα-1^{14}-for-in	5′-CTGAGGAAACCCTCTGTGC-3′
	Vα-9-for-in	5′-ATCTTTCCACCTGAAGAAACC-3′
	Vα-16-for-in	5′-TCCTTCCACCTGAAGAAACC-3′
	Vα-20-for-in	5′-ACGTGGTACCAACAGTTTCC-3′
	Vα-27-for-in	5′-ACTTCAGACAGACTGTATTGG-3′
	Vα-30-for-in	5′-CTCTTCACCCTGTATTCAGC-3′

Table 5-6 Primer for Clone-specific PCR IP2-8S1

Label	**Sequence**	**T_M**
N05-13_Vα8.1-spec-for	5‘ AGACATTCGTTCAAATGTGGG 3‘	60 °C
N05-13_Vα8.1-spec-rev	5‘ ATAGAACTGGTTACCaGTgCC 3‘	60 °C
N05-13_Vβ8.1-spec-for	5‘ AGATCCAGCCCTCAGAACC 3‘	60 °C
N05-13_Vβ8.1-spec-rev	5‘GCCAAAATACTGCGTcTCtCC 3‘	60 °C

Table 5-7 Primers fo the preparation of NGS samples

Label	Sequence
reverse transcription	
TSO	TCGUCGGCAGCGTCAGAUGTGTAUAAGAGACAGNNNNUNNNNUNNNNUCTT(rG)$_4$
TRA_RT	AGTCTCTCAGCTGGTAC
TRB_RT	TCTGATGGCTCAAACAC
PCR1	
TSO_PCR	TCGTCGGCAGCGTCAGATG
TRA_OUT	AGTCTCTCAGCTGGTACACG
TRB_OUT	TCTGATGGCTCAAACACAGC
PCR2	
TSO_PCR(N)	(N)$_{2-4}$GCAGACAGACTTGTCACTGG
TRA_IN	(N)$_{2-4}$GGGTCAGGGTTCTGGATAT
TRBJ1.1	ACTGTGAGTCTGGTGCCTTGT
TRBJ1.2	ACAACGGTTAACCTGGTCCCCGAA
TRBJ1.3	GGTCCTCTACAACAGTGAGCCAAC
TRBJ1.4	AAGACAGAGAGCTGGGTTCCACTG
TRBJ1.5	GGAGAGTCGAGTCCCATCA
TRBJ1.6	TGTCACAGTGAGCCTGGTCCCATT
TRBJ2.1	CCTGGCCCGAAGAACTGCTCA
TRBJ2.2	GTCCTCCAGTACGGTCAGCCTAGA
TRBJ2.3	TGCCTGGGCCAAAATACTGCG
TRBJ2.4	TCCCGGCGCCGAAGTACTGAA
TRBJ2.5	TGACCGTGAGCCTGGTGCCCG
TRBJ2.6	TCGAGCACCAGGAGCCGC
TRBJ2.7	CTGCTGCCGGCCCCGAAAGTC

Table 5-8 Primer for cloning MHC-I alleles and recovery PCR

Label	Sequence	T_M
HLA-A0101-Lead	5‘ CTCGTCGACATGGCCGTCATGGCGCCC 3‘	62 °C
HLA-A0101-end	5‘ TACGGATCCTCACACTTTACAAGCTGTGAG 3‘	60 °C
HLA-B3501-Lead	5‘ CTCGTCGACATGCGGGTCACGGCGCC 3‘	60 °C
HLA-B0801-end	5‘ TACGGATCCTCAAGCTGTGAGAGACACATC ‘	62 °C
HLAC_for_Sal	5‘ ACACGTCGACATGCGGGTCATGGCGCC 3‘	58 °C
HLA-Cw1203-end	5‘ TACGGATCCTCAGGCTTTACAAGCGATGAG 3‘	62 °C
pcDNA 2nd for TOPO	5‘ CACCTCCGGCGCGCCACCATG 3‘	74 °c
rev3	5‘ TGGTGATGGTGATGATGACC 3‘	60 °c

Table 5-9 Oligonucelotides Candidates IP2-8S1 for HLA-A*02:01

Label	Sequence
CLWAWILLL-for	5′CGCGCCACCATGTGTCTTTGGGCCTGGATCCTCCTTCTGTGAGC 3′
CLWAWILLL-rev	5′GGCCGCTCACAGAAGGAGGATCCAGGCCCAAAGACACATGGTGG 3′
GLGERVTEV-for	5′CGCGCCACCATGGGATTGGGAGAGCGTGTAACCGAAGTGTGAGC 3′
GLGERVTEV-rev	5′GGCCGCTCACACTTCGGTTACACGCTCTCCCAATCCCATGGTGG 3′
ILSQVSFWL-for	5′CGCGCCACCATGATTCTCTCACAAGTCTCCTTCTGGCTCTGAGC 3′
ILSQVSFWL-rev	5′GGCCGCTCAGAGCCAGAAGGAGACTTGTGAGAGAATCATGGTGG 3′
TAMDWFIAV-for	5′CGCGCCACCATGACAGCTATGGATTGGTTTATTGCCGTGTGAGC 3′
TAMDWFIAV-rev	5′GGCCGCTCACACGGCAATAAACCAATCCATAGCTGTCATGGTGG 3′
FIAVCYAFV-for	5′CGCGCCACCATGTTTATTGCCGTGTGCTATGCCTTTGTGTGAGC 3′
FIAVCYAFV-rev	5′GGCCGCTCACACAAAGGCATAGCACACGGCAATAAACATGGTGG 3′
LLFGIFNLV-for	5′CGCGCCACCATGCTGCTATTTGGAATCTTTAACTTAGTCTGAGC 3′
LLFGIFNLV-rev	5′GGCCGCTCAGACTAAGTTAAAGATTCCAAATAGCAGCATGGTGG 3′
LLYTMRLTV-for	5′CGCGCCACCATGTTGCTGTACACCATGAGGCTGACAGTGTGAGC 3′
LLYTMRLTV-rev	5′GGCCGCTCACACTGTCAGCCTCATGGTGTACAGCAACATGGTGG 3′
YLPCIMTVI-for	5′CGCGCCACCATGTACCTGCCATGCATAATGACAGTGATTTGAGC 3′
YLPCIMTVI-rev	5′GGCCGCTCAAATCACTGTCATTATGCATGGCAGGTACATGGTGG 3′
IMTVILSQV-for	5′CGCGCCACCATGATAATGACAGTGATTCTCTCACAAGTCTGAGC 3′
IMTVILSQV-rev	5′GGCCGCTCAGACTTGTGAGAGAATCACTGTCATTATCATGGTGG 3′
YAWDGKSVV-for	5′CGCGCCACCATGTATGCATGGGATGGCAAAAGTGTGGTTTGAGC 3′
YAWDGKSVV-rev	5′GGCCGCTCAAACCACACTTTTGCCATCCCATGCATACATGGTGG 3′
RLSRIAFPL-for	5′CGCGCCACCATGCGACTGTCAAGAATAGCCTTCCCGCTGTGAGC 3′
RLSRIAFPL-rev	5′GGCCGCTCACAGCGGGAAGGCTATTCTTGACAGTCGCATGGTGG 3′
IAFPLLFGI-for	5′CGCGCCACCATGATAGCCTTCCCGCTGCTATTTGGAATCTGAGC 3′
IAFPLLFGI-rev	5′GGCCGCTCAGATTCCAAATAGCAGCGGGAAGGCTATCATGGTGG 3′
PLLFGIFNL-for	5′CGCGCCACCATGCCGCTGCTATTTGGAATCTTTAACTTATGAGC 3′
PLLFGIFNL-rev	5′GGCCGCTCATAAGTTAAAGATTCCAAATAGCAGCGGCATGGTGG 3′
GIFNLVYWA-for	5′CGCGCCACCATGGGAATCTTTAACTTAGTCTACTGGGCTTGAGC 3′
GIFNLVYWA-rev	5′GGCCGCTCAAGCCCAGTAGACTAAGTTAAAGATTCCCATGGTGG 3′
NLVYWATYL-for	5′CGCGCCACCATGAACTTAGTCTACTGGGCTACGTATTTATGAGC 3′
NLVYWATYL-rev	5′GGCCGCTCATAAATACGTAGCCCAGTAGACTAAGTTCATGGTGG 3′
TMPNKLLRI-for	5′CGCGCCACCATGACCATGCCCAACAAACTCCTGCGGATCTGAGC 3′
TMPNKLLRI-rev	5′GGCCGCTCAGATCCGCAGGAGTTTGTTGGGCATGGTCATGGTGG 3′
HLEDFPMDA-for	5′CGCGCCACCATGCATTTGGAGGACTTCCCTATGGATGCCTGAGC 3′
HLEDFPMDA-rev	5′GGCCGCTCAGGCATCCATAGGGAAGTCCTCCAAATGCATGGTGG 3′
VLTMTTLSI-for	5′CGCGCCACCATGGTGCTCACCATGACAACATTGAGCATCTGAGC 3′
VLTMTTLSI-rev	5′GGCCGCTCAGATGCTCAATGTTGTCATGGTGAGCACCATGGTGG 3′
AMDWFIAVC-for	5′CGCGCCACCATGGCTATGGATTGGTTTATTGCCGTGTGCTGAGC 3′
AMDWFIAVC-rev	5′GGCCGCTCAGCACACGGCAATAAACCAATCCATAGCCATGGTGG 3′

Table 5-10 Oligonucelotides Candidates IP2-8S1 for HLA-A*23:01

Lable	Sequence
EYVVMTTHF-for	5’CGCGCCACCATGGAATATGTTGTTATGACCACTCATTTCTGAGC3’
EYVVMTTHF-rev	5’GGCCGCTCAGAAATGAGTGGTCATAACAACATATTCCATGGTGG3’
TYLPCIMTV-for	5’CGCGCCACCATGACATACCTGCCATGCATAATGACAGTGTGAGC3’
TYLPCIMTV-rev	5’GGCCGCTCACACTGTCATTATGCATGGCAGGTATGTCATGGTGG3’
WFIAVCYAF-for	5’CGCGCCACCATGTGGTTTATTGCCGTGTGCTATGCCTTTTGAGC3’
WFIAVCYAF-rev	5’GGCCGCTCAAAAGGCATAGCACACGGCAATAAACCACATGGTGG3’
CYAFVFSAL-for	5’CGCGCCACCATGTGCTATGCCTTTGTGTTCTCAGCTCTGTGAGC3’
CYAFVFSAL-rev	5’GGCCGCTCACAGAGCTGAGAACACAAAGGCATAGCACATGGTGG3’
FVFSALIEF-for	5’CGCGCCACCATGTTTGTGTTCTCAGCTCTGATTGAGTTTTGAGC3’
FVFSALIEF-rev	5’GGCCGCTCAAAACTCAATCAGAGCTGAGAACACAAACATGGTGG3’
IVQSSTGEY-for	5’CGCGCCACCATGATTGTCCAGTCAAGTACAGGAGAATATTGAGC3’
IVQSSTGEY-rev	5’GGCCGCTCAATATTCTCCTGTACTTGACTGGACAATCATGGTGG3’
MDWFIAVCY-for	5’CGCGCCACCATGATGGATTGGTTTATTGCCGTGTGCTATTGAGC3’
MDWFIAVCY-rev	5’GGCCGCTCAATAGCACACGGCAATAAACCAATCCATCATGGTGG3’
KPKKVKDPL-for	5’CGCGCCACCATGAAGCCAAAGAAAGTAAAGGATCCTCTTTGAGC3’
KPKKVKDPL-rev	5’GGCCGCTCAAAGAGGATCCTTTACTTTCTTTGGCTTCATGGTGG3’

Table 5-11 Oligonucelotides Candidates IP2-8S1 for HLA-B*35:01

Lable	Sequence
PVSDHDMEY-for	5’CGCGCCACCATGCCCGTTTCAGACCATGATATGGAATATTGAGC3’
PVSDHDMEY-rev	5’GGCCGCTCAATATTCCATATCATGGTCTGAAACGGGCATGGTGG3’
DAHACPLKF-for	5’CGCGCCACCATGGATGCCCATGCTTGCCCACTAAAATTTTGAGC3’
DAHACPLKF-rev	5’GGCCGCTCAAAATTTTAGTGGGCAAGCATGGGCATCCATGGTGG3’
LPCIMTVIL-for	5’CGCGCCACCATGCTGCCATGCATAATGACAGTGATTCTCTGAGC3’
LPCIMTVIL-rev	5’GGCCGCTCAGAGAATCACTGTCATTATGCATGGCAGCATGGTGG3’
WFIAVCYAF-for	5’CGCGCCACCATGTGGTTTATTGCCGTGTGCTATGCCTTTTGAGC3’
WFIAVCYAF-rev	5’GGCCGCTCAAAAGGCATAGCACACGGCAATAAACCACATGGTGG3’
IAVCYAFVF-for	5’CGCGCCACCATGATTGCCGTGTGCTATGCCTTTGTGTTCTGAGC3’
IAVCYAFVF-rev	5’GGCCGCTCAGAACACAAAGGCATAGCACACGGCAATCATGGTGG3’

Table 5-12 Oligonucelotides Candidates IP2-8S1 for HLA-B*44:03

Lable	Sequence
MEYTIDVFF-for	5’CGCGCCACCATGATGGAATATACAATAGATGTATTTTTCTGAGC3’

MEYTIDVFF-rev	5’GGCCGCTCAGAAAAATACATCTATTGTATATTCCATCATGGTGG3’
IEFATVNYF-for	5’CGCGCCACCATGATTGAGTTTGCCACAGTAAACTATTTCTGAGC3’
IEFATVNYF-rev	5’GGCCGCTCAGAAATAGTTTACTGTGGCAAACTCAATCATGGTGG3’
LEDFPMDAH-for	5’CGCGCCACCATGTTGGAGGACTTCCCTATGGATGCCCATTGAGC3’
LEDFPMDAH-rev	5’GGCCGCTCAATGGGCATCCATAGGGAAGTCCTCCAACATGGTGG3’
GEYVVMTTH-for	5’CGCGCCACCATGGGAGAATATGTTGTTATGACCACTCATTGAGC3’
GEYVVMTTH-rev	5’GGCCGCTCAATGAGTGGTCATAACAACATATTCTCCCATGGTGG3’

Table 5-13 Oligonucelotides Candidates IP2-8S1 for HLA-C*04:01

Lable	Sequence
LLDGYDNRL-for	5’CGCGCCACCATGCTCCTAGATGGCTATGACAATCGCCTGTGAGC3’
LLDGYDNRL-rev	5’GGCCGCTCACAGGCGATTGTCATAGCCATCTAGGAGCATGGTGG3’
VFTRILDRL-for	5’CGCGCCACCATGGTCTTCACCAGGATTTTGGACAGACTCTGAGC3’
VFTRILDRL-rev	5’GGCCGCTCAGAGTCTGTCCAAAATCCTGGTGAAGACCATGGTGG3’
KTDIFVTSF-for	5’CGCGCCACCATGAAGACTGATATCTTCGTCACCAGTTTCTGAGC3’
KTDIFVTSF-rev	5’GGCCGCTCAGAAACTGGTGACGAAGATATCAGTCTTCATGGTGG3’
PMDAHACPL-for	5’CGCGCCACCATGCCTATGGATGCCCATGCTTGCCCACTATGAGC3’
PMDAHACPL-rev	5’GGCCGCTCATAGTGGGCAAGCATGGGCATCCATAGGCATGGTGG3’
QYDLLGQTV-for	5’CGCGCCACCATGCAGTATGACCTTCTTGGACAAACAGTATGAGC3’
QYDLLGQTV-rev	5’GGCCGCTCATACTGTTTGTCCAAGAAGGTCATACTGCATGGTGG3’
GYFVIQTYL-for	5’CGCGCCACCATGGGCTACTTTGTTATTCAAACATACCTGTGAGC3’
GYFVIQTYL-rev	5’GGCCGCTCACAGGTATGTTTGAATAACAAAGTAGCCCATGGTGG3’
KPPEPKKTF-for	5’CGCGCCACCATGAAACCACCAGAACCCAAGAAAACCTTTTGAGC3’
KPPEPKKTF-rev	5’GGCCGCTCAAAAGGTTTTCTTGGGTTCTGGTGGTTTCATGGTGG3’

Table 5-14 Oligonucelotides Candidates IP2-8S1 for HLA-C*16:01

Lable	Sequence
TYLNREPQL-for	5’CGCGCCACCATGACGTATTTAAACAGAGAGCCTCAGCTATGAGC3’
TYLNREPQL-rev	5’GGCCGCTCATAGCTGAGGCTCTCTGTTTAAATACGTCATGGTGG3’
MTVLRLNNL-for	5’CGCGCCACCATGATGACAGTCCTCCGGTTAAATAACCTATGAGC3’
MTVLRLNNL-rev	5’GGCCGCTCATAGGTTATTTAACCGGAGGACTGTCATCATGGTGG3’
YAYTRAEVV-for	5’CGCGCCACCATGTATGCTTATACAAGAGCAGAAGTTGTTTGAGC3’
YAYTRAEVV-rev	5’GGCCGCTCAAACAACTTCTGCTCTTGTATAAGCATACATGGTGG3’
FHLKRKIGY-for	5’CGCGCCACCATGTTCCACTTGAAGAGAAAGATTGGCTACTGAGC3’
FHLKRKIGY-rev	5’GGCCGCTCAGTAGCCAATCTTTCTCTTCAAGTGGAACATGGTGG3’

TATSYTPNL-for	5’CGCGCCACCATGACAGCAACCAGCTACACCCCTAATTTGTGAGC3’
TATSYTPNL-rev	5’GGCCGCTCACAAATTAGGGGTGTAGCTGGTTGCTGTCATGGTGG3’

Table 5-15 Primers for cloning MHC-II chains

Label	Sequence	T_M
DRA*01-SalI-for	5‘ TATCGTCGACATGGCCATAAGTGGAGTCCC 3‘	62°C
DRBx-SalI-for	5‘ TATCGTCGACATGGTGTGTCTGAAGCTCCC 3‘	62°C
DRA*01-BamHI-rev	5‘ TATCGGATCCTTACAGAGGCCCCCTGCGT 3‘	62°C
DRB1-BamHI-rev	5‘ TATCGGATCCTCAGCTCAGGAATCCTGTTG 3‘	60°C
DRB5-BamHI-rev	5‘ TATCGGATCCTCAGCTCACGAGTCCTGTTG 3‘	62°C

Table 5-16 Primers for cloning the Invariant chain and MHC-II library screening

Label	Sequence	T_M
p33-wt-AscI-for	5‘ ATAGGCGCGCCATGGATGAC 3‘	58 °C
p33-wt-BspEI-rev	5‘ ATCATCCGGATCACATGGGGAC 3‘	58 °C
delta-p33-for	5‘cgcgccatgagctctggttcgtcaaagtgtagcagaggggcc3‘	-
delta-p33-rev-new	5‘ cctctgctacactttgacgaaccagagctcatgg3‘	-
p33-lib-for	5‘ TACACCGGCTTCAGCATCC 3‘	62 °C
p33-lib-rev	5‘ TCCAGTCGATGGTTTCCATG 3‘	60 °C

Table 5-17 Oligonucelotides for generation of Ii-ECPL

Label	Sequence
CLIP-AgeI-for	5‘ CCtCCCAAACCGGTGAGC 3‘
CLIP-n27-AgeI/KasI	5‘GCAGGGCGCCCATGGGCAaCGCCTGNNNNNNNNNNNNNNNNNNNNNNNNNNNCTTGCTCACCGGTTTGGGaGG 3‘
CLIP-M1,9-AgeI/KasI-for	5‘GCAGGGCGCCCATGGGCAaCGCCTGCATNNNNNNNNNNNNNNNNNNNNNCATCTTGCTCACCGGTTTGGGaGG 3‘
CLIP-KasI-rev	5‘ tatGGCGCCCATGGGCAG 3‘
CLIP-N33 AgeI/KasI	5‘agctACCGGTGAGCNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKGCGCTGCCCATGGGCGCCata 3‘
CLIP-N33 Anker M AgeI/KasI	5‘agctACCGGTGAGCNNKATGNNKNNKNNKNNKNNKNNKNNKATGNNKGCGCTGCCCATGGGCGCCata 3‘
CLIP-N39AgeI/KasI	5‘agctACCGGTGNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKCTGCCCATGGGCGCCata 3‘
CLIP-N39 Anker M AgeI/KasI	5‘agctACCGGTGNNKNNKATGNNKNNKNNKNNKNNKNNKNNKATGNNKNNKCTGCCCATGGGCGCCata 3‘

CLIP-N36-C	5'agctACCGGTGAGCNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKC
AgeI/KasI	TGCCCATGGGCGCCata 3'
CLIP-N36-C Anker M	5'agctACCGGTGAGCNNKATGNNKNNKNNKNNKNNKNNKNNKATGNNKNNKC
AgeI/KasI	TGCCCATGGGCGCCata 3'
CLIP-N36-N	5'agctACCGGTGNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKNNKGCGC
AgeI/KasI	TGCCCATGGGCGCCata 3'
CLIP-N36-N Anker M	5'agctACCGGTGNNKNNKATGNNKNNKNNKNNKNNKNNKNNKATGNNKGCGC
AgeI/KasI	TGCCCATGGGCGCCata 3'

Table 5-18 Primer for cloning pHSE3'-Blasticidin-large T

Label	Sequence	T_M
Blasticidin-Aat-II-for	5' AGCTGACGTCATGGCCAAGCCTTTGTCTCA 3'	60 °C
Blasticidn-NdeI-rev	5' GCTCATATGTTAGCCCTCCCACACATAAC 3'	60 °C
SalI-large_T-for	5' AGCGGTCGACATGGATAAAGTTTTAAACAGAGAG 3'	
BamHI-large_T-rev	5' AGCTGGATCCTTATGTTTCAGGTTCAGGGGG 3'	

5.2 Sequences

IP2-AV8S1

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  1  atg aca tcc att cga gct gta ttt ata ttc ctg tgg ctg cag ttg gac ttg
       m   t   s   i   r   a   v   f   i   f   l   w   l   q   l   d   l

 52  gtg aat gga gag aat gtg gag cag cat cct tca acc ctg agt gtc cag gag
       v   n   g   e   n   v   e   q   h   p   s   t   l   s   v   q   e

103  gga gac agc gct gtt atc aag tgt act tat tca gac agt gcc tca aac tac
       g   d   s   a   v   i   k   c   t   y   s   d   s   a   s   n   y

154  ttt cct tgg tat aag caa gaa ctt gga aaa aga cct cag ctt att ata gac
       f   p   w   y   k   q   e   l   g   k   r   p   q   l   i   i   d

205  att cgt tca aat gtg ggc gaa aag aaa gac caa cga att gct gtt aca ttg
       i   r   s   n   v   g   e   k   k   d   q   r   i   a   v   t   l

256  aac aag aca gcc aaa cat ttc tcc ctg cac atc aca gag acc caa cct gaa
       n   k   t   a   k   h   f   s   l   h   i   t   e   t   q   p   e

307  gac tcg gct gtc tac ttc tgt gca gca agt tgg ggt acc ggt aac cag ttc
       d   s   a   v   y   f   c   a   a   s   w   g   t   g   n   q   f

358  tat ttt ggg aca ggg aca agt ttg acg gtc att cca aat atc cag aac cct
       y   f   g   t   g   t   s   l   t   v   i   p   n   i   q   n   p

                          PvuII
                         ----+--
409  gac cct gcc gtg tac cag ctg aga gac tct aaa tcc agt gac aag tct gtc
       d   p   a   v   y   q   l   r   d   s   k   s   s   d   k   s   v
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460  tgc cta ttc acc gat ttt gat tct caa aca aat gtg tca caa agt aag gat
       c   l   f   t   d   f   d   s   q   t   n   v   s   q   s   k   d

511  tct gat gtg tat atc aca gac aaa act gtg cta gac atg agg tct atg gac
       s   d   v   y   i   t   d   k   t   v   l   d   m   r   s   m   d

562  ttc aag agc aac agt gct gtg gcc tgg agc aac aaa tct gac ttt gca tgt
       f   k   s   n   s   a   v   a   w   s   n   k   s   d   f   a   c

613  gca aac gcc ttc aac aac agc att att cca gaa gac acc ttc ttc ccc agc
       a   n   a   f   n   n   s   i   i   p   e   d   t   f   f   p   s

664  cca gaa agt tcc tgt gat gtc aag ctg gtc gag aaa agc ttt gaa aca gat
       p   e   s   s   c   d   v   k   l   v   e   k   s   f   e   t   d

715  acg aac cta aac ttt caa aac ctg tca gtg att ggg ttc cga atc ctc ctc
       t   n   l   n   f   q   n   l   s   v   i   g   f   r   i   l   l

766  ctg aaa gtg gcc ggg ttt aat ctg ctc atg acg ctg cgg ctg tgg tcc agt
       l   k   v   a   g   f   n   l   l   m   t   l   r   l   w   s   s

817  tga
       -
```

IP2-BV8S1

```
  1  atg gac tcc tgg acc ttc tgc tgt gtg tcc ctt tgc atc ctg gta gcg aag
       m   d   s   w   t   f   c   c   v   s   l   c   i   l   v   a   k

 52  cat aca gat gct gga gtt atc cag tca ccc cgc cat gag gtg aca gag atg
       h   t   d   a   g   v   i   q   s   p   r   h   e   v   t   e   m

103  gga caa gaa gtg act ctg aga tgt aaa cca att tca ggc cac aac tcc ctt
       g   q   e   v   t   l   r   c   k   p   i   s   g   h   n   s   l

154  ttc tgg tac aga cag acc atg atg cgg gga ctg gag ttg ctc att tac ttt
       f   w   y   r   q   t   m   m   r   g   l   e   l   l   i   y   f

205  aac aac aac gtt ccg ata gat gat tca ggg atg cct gag gat cga ttc tca
       n   n   n   v   p   i   d   d   s   g   m   p   e   d   r   f   s

256  gct aag atg cct aat gca tca ttc tcc act ctg aag atc caa ccc tca gaa
       a   k   m   p   n   a   s   f   s   t   l   k   i   q   p   s   e

307  ccc agg gac tca gct gtg tac ttc tgt gcc agc agc gca ggg gga gat acg
       p   r   d   s   a   v   y   f   c   a   s   s   a   g   g   d   t

358  cag tat ttt ggc cca ggc acc cgg ctg aca gtg cta gag gac ctg aaa aac
       q   y   f   g   p   g   t   r   l   t   v   l   e   d   l   k   n

                  AvaI
                 -+-----
409  gtg ttc cca ccc gag gtc gct gtg ttt gag cca tca gaa gca gag atc tcc
       v   f   p   p   e   v   a   v   f   e   p   s   e   a   e   i   s

460  cac acc caa aag gcc aca ctg gtg tgc ctg gcc aca ggc ttc ttc cct gac
       h   t   q   k   a   t   l   v   c   l   a   t   g   f   f   p   d

511  cac gtg gag ctg agc tgg tgg gtg aat ggg aag gag gtg cac agt ggg gtc
       h   v   e   l   s   w   w   v   n   g   k   e   v   h   s   g   v
```

```
562  agc acg gac ccg cag ccc ctc aag gag cag ccc gcc ctc aat gac tcc aga
       s   t   d   p   q   p   l   k   e   q   p   a   l   n   d   s   r

613  tac tgc ctg agc agc cgc ctg agg gtc tcg gcc acc ttc tgg cag aac ccc
       y   c   l   s   s   r   l   r   v   s   a   t   f   w   q   n   p

664  cgc aac cac ttc cgc tgt caa gtc cag ttc tac ggg ctc tcg gag aat gac
       r   n   h   f   r   c   q   v   q   f   y   g   l   s   e   n   d

715  gag tgg acc cag gat agg gcc aaa ccc gtc acc cag atc gtc agc gcc gag
       e   w   t   q   d   r   a   k   p   v   t   q   i   v   s   a   e

766  gcc tgg ggt aga gca gac tgt ggc ttt acc tcg gtg tcc tac cag caa ggg
       a   w   g   r   a   d   c   g   f   t   s   v   s   y   q   q   g

817  gtc ctg tct gcc acc atc ctc tat gag atc ctg cta ggg aag gcc acc ctg
       v   l   s   a   t   i   l   y   e   i   l   l   g   k   a   t   l

868  tat gct gtg ctg gtc agc gcc ctt gtg ttg atg gcc atg gtc aag aga aag
       y   a   v   l   v   s   a   l   v   l   m   a   m   v   k   r   k

919  gat ttc tga
       d   f   -
```

HLA-A*02:01

```
  1  atg gcc gtc atg gcg ccc cga acc ctc gtc ctg cta ctc tcg ggg gct ctg
       m   a   v   m   a   p   r   t   l   v   l   l   l   s   g   a   l

 52  gcc ctg acc cag acc tgg gcg ggc tct cac tcc atg agg tat ttc ttc aca
       a   l   t   q   t   w   a   g   s   h   s   m   r   y   f   f   t

103  tcc gtg tcc cgg ccc ggc cgc ggg gag ccc cgc ttc atc gca gtg ggc tac
       s   v   s   r   p   g   r   g   e   p   r   f   i   a   v   g   y

154  gtg gac gac acg cag ttc gtg cgg ttc gac agc gac gcc gcg agc cag agg
       v   d   d   t   q   f   v   r   f   d   s   d   a   a   s   q   r

205  atg gag ccg cgg gcg ccg tgg ata gag cag gag ggt ccg gag tat tgg gac
       m   e   p   r   a   p   w   i   e   q   e   g   p   e   y   w   d

256  ggg gag aca cgg aaa gtg aag gcc cac tca cag act cac cga gtg gac ctg
       g   e   t   r   k   v   k   a   h   s   q   t   h   r   v   d   l

307  ggg acc ctg cgc ggc tac tac aac cag agc gag gcc ggt tct cac acc gtc
       g   t   l   r   g   y   y   n   q   s   e   a   g   s   h   t   v

358  cag agg atg tat ggc tgc gac gtg ggg tcg gac tgg cgc ttc ctc cgc ggg
       q   r   m   y   g   c   d   v   g   s   d   w   r   f   l   r   g

409  tac cac cag tac gcc tac gac ggc aag gat tac atc gcc ctg aaa gag gac
       y   h   q   y   a   y   d   g   k   d   y   i   a   l   k   e   d

460  ctg cgc tct tgg acc gcg gcg gac atg gca gct cag acc acc aag cac aag
       l   r   s   w   t   a   a   d   m   a   a   q   t   t   k   h   k

511  tgg gag gcg gcc cat gtg gcg gag cag ttg aga gcc tac ctg gag ggc acg
       w   e   a   a   h   v   a   e   q   l   r   a   y   l   e   g   t
```

```
 562  tgc gtg gag tgg ctc cgc aga tac ctg gag aac ggg aag gag acg ctg cag
        c   v   e   w   l   r   r   y   l   e   n   g   k   e   t   l   q

 613  cgc acg gac gcc ccc aaa acg cat atg act cac cac gct gtc tct gac cat
        r   t   d   a   p   k   t   h   m   t   h   h   a   v   s   d   h

 664  gaa gcc acc ctg agg tgc tgg gcc ctg agc ttc tac cct gcg gag atc aca
        e   a   t   l   r   c   w   a   l   s   f   y   p   a   e   i   t

 715  ctg acc tgg cag cgg gat ggg gag gac cag acc cag gac acg gag ctc gtg
        l   t   w   q   r   d   g   e   d   q   t   q   d   t   e   l   v

 766  gag acc agg cct gca ggg gat gga acc ttc cag aag tgg gcg gct gtg gtg
        e   t   r   p   a   g   d   g   t   f   q   k   w   a   a   v   v

 817  gtg cct tct gga cag gag cag aga tac acc tgc cat gtg cag cat gag ggt
        v   p   s   g   q   e   q   r   y   t   c   h   v   q   h   e   g

 868  ttg ccc aag ccc ctc acc ctg aga tgg gag ccg tct tcc cag ccc acc atc
        l   p   k   p   l   t   l   r   w   e   p   s   s   q   p   t   i

 919  ccc atc gtg ggc atc att gct ggc ctg gtt ctc ttt gga gct gtg atc act
        p   i   v   g   i   i   a   g   l   v   l   f   g   a   v   i   t

 970  gga gct gtg gtc gct gct gtg atg tgg agg agg aag agc tca gat aga aaa
        g   a   v   v   a   a   v   m   w   r   r   k   s   s   d   r   k

1021  gga ggg agc tac tct cag gct gca agc agt gac agt gcc cag ggc tct gat
        g   g   s   y   s   q   a   a   s   s   d   s   a   q   g   s   d

1072  gtg tct ctc aca gct tgt aaa gtg tga
        v   s   l   t   a   c   k   v   -
```

HLA-A*23:01

```
   1  atg gcc gtc atg gcg ccc cga acc ctc gtc ctg cta ctc tcg ggg gcc ctg
        m   a   v   m   a   p   r   t   l   v   l   l   l   s   g   a   l

  52  gcc ctg acc cag acc tgg gca ggc tcc cac tcc atg agg tat ttc tcc aca
        a   l   t   q   t   w   a   g   s   h   s   m   r   y   f   s   t

 103  tcc gtg tcc cgg ccc ggc cgc ggg gag ccc cgc ttc atc gcc gtg ggc tac
        s   v   s   r   p   g   r   g   e   p   r   f   i   a   v   g   y

 154  gtg gac gac acg cag ttc gtg cgg ttc gac agc gac gcc gcg agc cag agg
        v   d   d   t   q   f   v   r   f   d   s   d   a   a   s   q   r

 205  atg gag ccg cgg gcg ccg tgg ata gag cag gag ggg ccg gag tat tgg gac
        m   e   p   r   a   p   w   i   e   q   e   g   p   e   y   w   d

 256  gag gag aca ggg aaa gtg aag gcc cac tca cag act gac cga gag aac ctg
        e   e   t   g   k   v   k   a   h   s   q   t   d   r   e   n   l

 307  cgg atc gcg ctc cgc tac tac aac cag agc gag gcc ggt tct cac acc ctc
        r   i   a   l   r   y   y   n   q   s   e   a   g   s   h   t   l

 358  cag atg atg ttt ggc tgc gac gtg ggg tcg gac ggg cgc ttc ctc cgc ggg
        q   m   m   f   g   c   d   v   g   s   d   g   r   f   l   r   g

 409  tac cac cag tac gcc tac gac ggc aag gat tac atc gcc ctg aaa gag gac
        y   h   q   y   a   y   d   g   k   d   y   i   a   l   k   e   d
```

```
 460  ctg cgc tct tgg acc gcg gcg gac atg gcg gct cag atc acc cag cgc aag
        l   r   s   w   t   a   a   d   m   a   a   q   i   t   q   r   k

 511  tgg gag gcg gcc cgt gtg gcg gag cag ttg aga gcc tac ctg gag ggc acg
        w   e   a   a   r   v   a   e   q   l   r   a   y   l   e   g   t

 562  tgc gtg gac ggg ctc cgc aga tac ctg gag aac ggg aag gag acg ctg cag
        c   v   d   g   l   r   r   y   l   e   n   g   k   e   t   l   q

 613  cgc acg gac ccc ccc aag aca cat atg acc cac cac ccc atc tct gac cat
        r   t   d   p   p   k   t   h   m   t   h   h   p   i   s   d   h

 664  gag gcc act ctg aga tgc tgg gcc ctg ggc ttc tac cct gcg gag atc aca
        e   a   t   l   r   c   w   a   l   g   f   y   p   a   e   i   t

 715  ctg acc tgg cag cgg gat ggg gag gac cag acc cag gac acg gag ctt gtg
        l   t   w   q   r   d   g   e   d   q   t   q   d   t   e   l   v

 766  gag acc agg cct gca ggg gat gga acc ttc cag aag tgg gca gct gtg gtg
        e   t   r   p   a   g   d   g   t   f   q   k   w   a   a   v   v

 817  gta cct tct gga gag gag cag aga tac acc tgc cat gtg cag cat gag ggt
        v   p   s   g   e   e   q   r   y   t   c   h   v   q   h   e   g

 868  ctg ccc aag ccc ctc acc ctg aga tgg gag cca tct tcc cag ccc acc gtc
        l   p   k   p   l   t   l   r   w   e   p   s   s   q   p   t   v

 919  cac atc gtg ggc atc att gct ggc ctg gtt ctc ctt gga gct gtg atc act
        h   i   v   g   i   i   a   g   l   v   l   l   g   a   v   i   t

 970  gga gct gtg gtc gct gct gtg atg tgg agg agg aac agc tca gat aga aaa
        g   a   v   v   a   a   v   m   w   r   r   n   s   s   d   r   k

1021  gga ggg agc tac tct cag gct gca agc agt gac agt gcc cag ggc tct gat
        g   g   s   y   s   q   a   a   s   s   d   s   a   q   g   s   d

1072  gtg tct ctc aca gct tgt aaa gtg tga
        v   s   l   t   a   c   k   v   -
```

HLA-A35:01

```
   1  atg cgg gtc acg gcg ccc cga acc gtc ctc ctg ctg ctc tgg ggg gca gtg
        m   r   v   t   a   p   r   t   v   l   l   l   l   w   g   a   v

  52  gcc ctg acc gag acc tgg gcc ggc tcc cac tcc atg agg tat ttc tac acc
        a   l   t   e   t   w   a   g   s   h   s   m   r   y   f   y   t

 103  gcc atg tcc cgg ccc ggc cgc ggg gag ccc cgc ttc atc gca gtg ggc tac
        a   m   s   r   p   g   r   g   e   p   r   f   i   a   v   g   y

 154  gtg gac gac acc cag ttc gtg agg ttc gac agc gac gcc gcg agt ccg agg
        v   d   d   t   q   f   v   r   f   d   s   d   a   a   s   p   r

 205  acg gag ccc cgg gcg cca tgg ata gag cag gag ggg ccg gag tat tgg gac
        t   e   p   r   a   p   w   i   e   q   e   g   p   e   y   w   d

 256  cgg aac aca cag atc ttc aag acc aac aca cag act tac cga gag agc ctg
        r   n   t   q   i   f   k   t   n   t   q   t   y   r   e   s   l

 307  cgg aac ctg cgc ggc tac tac aac cag agc gag gcc ggg tct cac atc atc
```

```
        r   n   l   r   g   y   y   n   q   s   e   a   g   s   h   i   i

  358   cag agg atg tat ggc tgc gac ctg ggg ccc gac ggg cgc ctc ctc cgc ggg
          q   r   m   y   g   c   d   l   g   p   d   g   r   l   l   r   g

  409   cat gac cag tcc gcc tac gac ggc aag gat tac atc gcc ctg aac gag gac
          h   d   q   s   a   y   d   g   k   d   y   i   a   l   n   e   d

  460   ctg agc tcc tgg acc gcg gcg gac acc gcg gct cag atc acc cag cgc aag
          l   s   s   w   t   a   a   d   t   a   a   q   i   t   q   r   k

  511   tgg gag gcg gcc cgt gtg gcg gag cag ctg aga gcc tac ctg gag ggc ctg
          w   e   a   a   r   v   a   e   q   l   r   a   y   l   e   g   l

  562   tgc gtg gag tgg ctc cgc aga tac ctg gag aac ggg aag gag acg ctg cag
          c   v   e   w   l   r   r   y   l   e   n   g   k   e   t   l   q

  613   cgc gcg gac ccc cca aag aca cac gtg acc cac cac ccc gtc tct gac cat
          r   a   d   p   p   k   t   h   v   t   h   h   p   v   s   d   h

  664   gag gcc acc ctg agg tgc tgg gcc ctg ggc ttc tac cct gcg gag atc aca
          e   a   t   l   r   c   w   a   l   g   f   y   p   a   e   i   t

  715   ctg acc tgg cag cgg gat ggc gag gac caa act cag gac act gag ctt gtg
          l   t   w   q   r   d   g   e   d   q   t   q   d   t   e   l   v

  766   gag acc aga cca gca gga gat aga acc ttc cag aag tgg gca gct gtg gtg
          e   t   r   p   a   g   d   r   t   f   q   k   w   a   a   v   v

  817   gtg cct tct gga gaa gag cag aga tac aca tgc cat gta cag cat gag ggg
          v   p   s   g   e   e   q   r   y   t   c   h   v   q   h   e   g

  868   ctg ccg aag ccc ctc acc ctg aga tgg gag cca tct tcc cag tcc acc atc
          l   p   k   p   l   t   l   r   w   e   p   s   s   q   s   t   i

  919   ccc atc gtg ggc att gtt gct ggc ctg gct gtc cta gca gtt gtg gtc atc
          p   i   v   g   i   v   a   g   l   a   v   l   a   v   v   v   i

  970   gga gct gtg gtc gct act gtg atg tgt agg agg aag agc tca ggt gga aaa
          g   a   v   v   a   t   v   m   c   r   r   k   s   s   g   g   k

 1021   gga ggg agc tac tct cag gct gcg tcc agc gac agt gcc cag ggc tct gat
          g   g   s   y   s   q   a   a   s   s   d   s   a   q   g   s   d

 1072   gtg tct ctc aca gct tga
          v   s   l   t   a   -
```

HLA-B44:03

```
    1   atg cgg gtc acg gcg ccc cga acc ctc ctc ctg ctg ctc tgg ggg gca gtg
          m   r   v   t   a   p   r   t   l   l   l   l   l   w   g   a   v

   52   gcc ctg acc gag acc tgg gcc ggc tcc cac tcc atg agg tat ttc tac acc
          a   l   t   e   t   w   a   g   s   h   s   m   r   y   f   y   t

  103   gcc atg tcc cgg ccc ggc cgc ggg gag ccc cgc ttc atc acc gtg ggc tac
          a   m   s   r   p   g   r   g   e   p   r   f   i   t   v   g   y

  154   gtg gac gac acg ctg ttc gtg agg ttc gac agc gac gcc acg agt ccg agg
          v   d   d   t   l   f   v   r   f   d   s   d   a   t   s   p   r
```

```
 205  aag gag ccg cgg gcg cca tgg ata gag cag gag ggg ccg gag tat tgg gac
        k   e   p   r   a   p   w   i   e   q   e   g   p   e   y   w   d

 256  cgg gag aca cag atc tcc aag acc aac aca cag act tac cga gag aac ctg
        r   e   t   q   i   s   k   t   n   t   q   t   y   r   e   n   l

 307  cgc acc gcg ctc cgc tac tac aac cag agc gag gcc ggg tct cac atc atc
        r   t   a   l   r   y   y   n   q   s   e   a   g   s   h   i   i

 358  cag agg atg tac ggc tgc gac gtg ggg ccg gac ggg cgc ctc ctc cgc ggg
        q   r   m   y   g   c   d   v   g   p   d   g   r   l   l   r   g

 409  tat gac cag gac gcc tac gac ggc aag gat tac atc gcc ctg aac gag gac
        y   d   q   d   a   y   d   g   k   d   y   i   a   l   n   e   d

 460  ctg agc tcc tgg acc gcg gcg gac acc gcg gct cag atc acc cag cgc aag
        l   s   s   w   t   a   a   d   t   a   a   q   i   t   q   r   k

 511  tgg gag gcg gcc cgt gtg gcg gag cag ctg aga gcc tac ctg gag ggc ctg
        w   e   a   a   r   v   a   e   q   l   r   a   y   l   e   g   l

 562  tgc gtg gag tcg ctc cgc aga tac ctg gag aac ggg aag gag acg ctg cag
        c   v   e   s   l   r   r   y   l   e   n   g   k   e   t   l   q

 613  cgc gcg gac ccc cca aag aca cat gtg acc cac cac ccc atc tct gac cat
        r   a   d   p   p   k   t   h   v   t   h   h   p   i   s   d   h

 664  gag gtc acc ctg agg tgc tgg gcc ctg ggc ttc tac cct gcg gag atc aca
        e   v   t   l   r   c   w   a   l   g   f   y   p   a   e   i   t

 715  ctg acc tgg cag cgg gat ggc gag gac caa act cag gac acc gag ctt gtg
        l   t   w   q   r   d   g   e   d   q   t   q   d   t   e   l   v

 766  gag acc aga cca gca gga gat aga acc ttc cag aag tgg gca gct gtg gtg
        e   t   r   p   a   g   d   r   t   f   q   k   w   a   a   v   v

 817  gtg cct tct gga gaa gag cag aga tac aca tgc cat gta cag cat gag ggg
        v   p   s   g   e   e   q   r   y   t   c   h   v   q   h   e   g

 868  ctg ccg aag ccc ctc acc ctg aga tgg gag ccg tct tcc cag tcc acc gtc
        l   p   k   p   l   t   l   r   w   e   p   s   s   q   s   t   v

 919  ccc atc gtg ggc att gtt gct ggc ctg gct gtc cta gca gtt gtg gtc atc
        p   i   v   g   i   v   a   g   l   a   v   l   a   v   v   v   i

 970  gga gct gtg gtc gct gct gtg atg tgt agg agg aag agc tca ggt gga aaa
        g   a   v   v   a   a   v   m   c   r   r   k   s   s   g   g   k

1021  gga ggg agc tac tct cag gct gcg tgc agc gac agt gcc cag ggc tct gat
        g   g   s   y   s   q   a   a   c   s   d   s   a   q   g   s   d

1072  gtg tct ctc aca gct tga
        v   s   l   t   a   -
```

HLA-C*04:01

```
   1  atg cgg gtc atg gcg ccc cga acc ctc atc ctg ctg ctc tcg gga gcc ctg
        m   r   v   m   a   p   r   t   l   i   l   l   l   s   g   a   l

  52  gcc ctg acc gag acc tgg gcc ggc tcc cac tcc atg agg tat ttc tcc aca
        a   l   t   e   t   w   a   g   s   h   s   m   r   y   f   s   t
```

```
 103  tcc gtg tcc tgg ccc ggc cgc ggg gag ccc cgc ttc atc gca gtg ggc tac
        s   v   s   w   p   g   r   g   e   p   r   f   i   a   v   g   y

 154  gtg gac gac acg cag ttc gtg cgg ttc gac agc gac gcc gcg agt cca aga
        v   d   d   t   q   f   v   r   f   d   s   d   a   a   s   p   r

 205  ggg gag ccg cgg gag ccg tgg gtg gag cag gag ggg ccg gag tat tgg gac
        g   e   p   r   e   p   w   v   e   q   e   g   p   e   y   w   d

 256  cgg gag aca cag aag tac aag cgc cag gca cag gct gac cga gtg aac ctg
        r   e   t   q   k   y   k   r   q   a   q   a   d   r   v   n   l

 307  cgg aaa ctg cgc ggc tac tac aac cag agc gag gac ggg tct cac acc ctc
        r   k   l   r   g   y   y   n   q   s   e   d   g   s   h   t   l

 358  cag agg atg ttt ggc tgc gac ctg ggg ccg gac ggg cgc ctc ctc cgc ggg
        q   r   m   f   g   c   d   l   g   p   d   g   r   l   l   r   g

 409  tat aac cag ttc gcc tac gac ggc aag gat tac atc gcc ctg aac gag gat
        y   n   q   f   a   y   d   g   k   d   y   i   a   l   n   e   d

 460  ctg cgc tcc tgg acc gcc gcg gac acg gcg gct cag atc acc cag cgc aag
        l   r   s   w   t   a   a   d   t   a   a   q   i   t   q   r   k

 511  tgg gag gcg gcc cgt gag gcg gag cag cgg aga gcc tac ctg gag ggc acg
        w   e   a   a   r   e   a   e   q   r   r   a   y   l   e   g   t

 562  tgc gtg gag tgg ctc cgc aga tac ctg gag aac ggg aag gag acg ctg cag
        c   v   e   w   l   r   r   y   l   e   n   g   k   e   t   l   q

 613  cgc gcg gaa cac cca aag aca cac gtg acc cac cat ccc gtc tct gac cat
        r   a   e   h   p   k   t   h   v   t   h   h   p   v   s   d   h

 664  gag gcc acc ctg agg tgc tgg gcc ctg ggc ttc tac cct gcg gag atc aca
        e   a   t   l   r   c   w   a   l   g   f   y   p   a   e   i   t

 715  ctg acc tgg cag tgg gat ggg gag gac caa act cag gac acc gag ctt gtg
        l   t   w   q   w   d   g   e   d   q   t   q   d   t   e   l   v

 766  gag acc agg cca gca gga gat gga acc ttc cag aag tgg gca gct gtg gtg
        e   t   r   p   a   g   d   g   t   f   q   k   w   a   a   v   v

 817  gtg cct tct gga gaa gag cag aga tac acg tgc cat gtt cag cac gag ggg
        v   p   s   g   e   e   q   r   y   t   c   h   v   q   h   e   g

 868  ctg ccg gag ccc ctc acc ctg aga tgg aag ccg tct tcc cag ccc acc atc
        l   p   e   p   l   t   l   r   w   k   p   s   s   q   p   t   i

 919  ccc atc gtg ggc atc gtt gct ggc ctg gct gtc ctg gct gtc cta gct gtc
        p   i   v   g   i   v   a   g   l   a   v   l   a   v   l   a   v

 970  cta gga gct atg gtg gct gtt gtg atg tgt agg agg aag agc tca ggt gga
        l   g   a   m   v   a   v   v   m   c   r   r   k   s   s   g   g

1021  aaa gga ggg agc tgc tct cag gct gcg tcc agc aac agt gcc cag ggc tct
        k   g   g   s   c   s   q   a   a   s   s   n   s   a   q   g   s

1072  gat gag tct ctc atc gct tgt aaa gcc tga
        d   e   s   l   i   a   c   k   a   -
```

HLA-C*16:01

```
  1  atg cgg gtc atg gcg ccc cga acc ctc atc ctg ctg ctc tcg gga gcc ctg
       m   r   v   m   a   p   r   t   l   i   l   l   l   s   g   a   l

 52  gcc ctg acc gag acc tgg gcc tgc tcc cac tcc atg agg tat ttc tac acc
       a   l   t   e   t   w   a   c   s   h   s   m   r   y   f   y   t

103  gcc gtg tcc cgg ccc ggc cgc gga gag ccc cgc ttc atc gca gtg ggc tac
       a   v   s   r   p   g   r   g   e   p   r   f   i   a   v   g   y

154  gtg gac gac acg cag ttc gtg cgg ttc gac agc gac gcc gcg agt cca aga
       v   d   d   t   q   f   v   r   f   d   s   d   a   a   s   p   r

205  ggg gag ccg cgg gcg ccg tgg gtg gag cag gag ggg ccg gag tat tgg gac
       g   e   p   r   a   p   w   v   e   q   e   g   p   e   y   w   d

256  cgg gag aca cag aag tac aag cgc cag gca cag act gac cga gtg agc ctg
       r   e   t   q   k   y   k   r   q   a   q   t   d   r   v   s   l

307  cgg aac ctg cgc ggc tac tac aac cag agc gag gcc ggg tct cac acc ctc
       r   n   l   r   g   y   y   n   q   s   e   a   g   s   h   t   l

358  cag tgg atg tat ggc tgc gac ctg ggg ccc gac ggg cgc ctc ctc cgc ggg
       q   w   m   y   g   c   d   l   g   p   d   g   r   l   l   r   g

409  tat gac cag tcc gcc tac gac ggc aag gat tac atc gcc ctg aac gag gac
       y   d   q   s   a   y   d   g   k   d   y   i   a   l   n   e   d

460  ctg cgc tcc tgg acc gcc gcg gac acg gcg gct cag atc acc cag cgc aag
       l   r   s   w   t   a   a   d   t   a   a   q   i   t   q   r   k

511  tgg gag gcg gcc cgt gcg gcg gag cag cag aga gcc tac ctg gag ggc acg
       w   e   a   a   r   a   a   e   q   q   r   a   y   l   e   g   t

562  tgc gtg gag tgg ctc cgc aga tac ctg gag aac ggg aag gag acg ctg cag
       c   v   e   w   l   r   r   y   l   e   n   g   k   e   t   l   q

613  cgc gcg gaa cac cca aag aca cac gtg acc cac cat ctc gtc tct gac cat
       r   a   e   h   p   k   t   h   v   t   h   h   l   v   s   d   h

664  gag gcc acc ctg agg tgc tgg gcc ctg ggc ttc tac cct gcg gag atc aca
       e   a   t   l   r   c   w   a   l   g   f   y   p   a   e   i   t

715  ctg acc tgg cag cgg gat ggc gag gac caa act cag gac acc gag ctt gtg
       l   t   w   q   r   d   g   e   d   q   t   q   d   t   e   l   v

766  gag acc agg cca gca gga gat gga acc ttc cag aag tgg gca gct gtg gtg
       e   t   r   p   a   g   d   g   t   f   q   k   w   a   a   v   v

817  gtg cct tct gga gaa gag cag aga tac acg tgc cat gtg cag cac gag ggg
       v   p   s   g   e   e   q   r   y   t   c   h   v   q   h   e   g

868  ctg ccg gag ccc ctc acc ctg aga tgg gag cca tct tcc cag ccc acc atc
       l   p   e   p   l   t   l   r   w   e   p   s   s   q   p   t   i

919  ccc atc gtg ggc atc gtt gct ggc ctg gct gtc ctg gct gtc cta gct gtc
       p   i   v   g   i   v   a   g   l   a   v   l   a   v   l   a   v

970  cta gga gct gtg gtg gct gtt gtt atg tgt agg agg aag agc tca ggt gga
       l   g   a   v   v   a   v   v   m   c   r   r   k   s   s   g   g
```

```
1021  aaa gga ggg agc tgc tct cag gct gcg tcc agc aac agt gcc cag ggc tct
        k   g   g   s   c   s   q   a   a   s   s   n   s   a   q   g   s

1072  gat gag tct ctc atc gct tgt aaa gcc tga
        d   e   s   l   i   a   c   k   a   -
```

HLA-DRA

```
   1  atg gcc ata agt gga gtc cct gtg cta gga ttt ttc atc ata gct gtg ctg
        m   a   i   s   g   v   p   v   l   g   f   f   i   i   a   v   l

  52  atg agc gct cag gaa tca tgg gct atc aaa gaa gaa cat gtg atc atc cag
        m   s   a   q   e   s   w   a   i   k   e   e   h   v   i   i   q

 103  gcc gag ttc tat ctg aat cct gac caa tca ggc gag ttt atg ttt gac ttt
        a   e   f   y   l   n   p   d   q   s   g   e   f   m   f   d   f

 154  gat ggt gat gag att ttc cat gtg gat atg gca aag aag gag acg gtc tgg
        d   g   d   e   i   f   h   v   d   m   a   k   k   e   t   v   w

 205  cgg ctt gaa gaa ttt gga cga ttt gcc agc ttt gag gct caa ggt gca ttg
        r   l   e   e   f   g   r   f   a   s   f   e   a   q   g   a   l

 256  gcc aac ata gct gtg gac aaa gcc aac ctg gaa atc atg aca aag cgc tcc
        a   n   i   a   v   d   k   a   n   l   e   i   m   t   k   r   s

 307  aac tat act ccg atc acc aat gta cct cca gag gta act gtg ctc acg aac
        n   y   t   p   i   t   n   v   p   p   e   v   t   v   l   t   n

 358  agc cct gtg gaa ctg aga gag ccc aac gtc ctc atc tgt ttc atc gac aag
        s   p   v   e   l   r   e   p   n   v   l   i   c   f   i   d   k

 409  ttc acc cca cca gtg gtc aat gtc acg tgg ctt cga aat gga aaa cct gtc
        f   t   p   p   v   v   n   v   t   w   l   r   n   g   k   p   v

 460  acc aca gga gtg tca gag aca gtc ttc ctg ccc agg gaa gac cac ctt ttc
        t   t   g   v   s   e   t   v   f   l   p   r   e   d   h   l   f

 511  cgc aag ttc cac tat ctc ccc ttc ctg ccc tca act gag gac gtt tac gac
        r   k   f   h   y   l   p   f   l   p   s   t   e   d   v   y   d

 562  tgc agg gtg gag cac tgg ggc ttg gat gag cct ctt ctc aag cac tgg gag
        c   r   v   e   h   w   g   l   d   e   p   l   l   k   h   w   e

 613  ttt gat gct cca agc cct ctc cca gag act aca gag aac gtg gtg tgt gcc
        f   d   a   p   s   p   l   p   e   t   t   e   n   v   v   c   a

 664  ctg ggc ctg act gtg ggt ctg gtg ggc atc att att ggg acc atc ttc atc
        l   g   l   t   v   g   l   v   g   i   i   i   g   t   i   f   i

 715  atc aag gga gtg cgc aaa agc aat gca gca gaa cgc agg ggg cct ctg taa
        i   k   g   v   r   k   s   n   a   a   e   r   r   g   p   l   -
```

HLA-DR1

```
   1  atg gtg tgt ctg aag ctc cct gga ggc tcc tgc atg aca gcg ctg aca gtg
        m   v   c   l   k   l   p   g   g   s   c   m   t   a   l   t   v

  52  aca ctg atg gtg ctg agc tcc cca ctg gct ttg gct ggg gac acc cga cca
        t   l   m   v   l   s   s   p   l   a   l   a   g   d   t   r   p
```

```
103  cgt ttc ttg tgg cag ctt aag ttt gaa tgt cat ttc ttc aat ggg acg gag
       r   f   l   w   q   l   k   f   e   c   h   f   f   n   g   t   e

154  cgg gtg cgg ttg ctg gaa aga tgc atc tat aac caa gag gag tcc gtg cgc
       r   v   r   l   l   e   r   c   i   y   n   q   e   e   s   v   r

205  ttc gac agc gac gtg ggg gag tac cgg gcg gtg acg gag ctg ggg cgg cct
       f   d   s   d   v   g   e   y   r   a   v   t   e   l   g   r   p

256  gat gcc gag tac tgg aac agc cag aag gac ctc ctg gag cag agg cgg gcc
       d   a   e   y   w   n   s   q   k   d   l   l   e   q   r   r   a

307  gcg gtg gac acc tac tgc aga cac aac tac ggg gtt ggt gag agc ttc aca
       a   v   d   t   y   c   r   h   n   y   g   v   g   e   s   f   t

358  gtg cag cgg cga gtt gag cct aag gtg act gtg tat cct tca aag acc cag
       v   q   r   r   v   e   p   k   v   t   v   y   p   s   k   t   q

409  ccc ctg cag cac cac aac ctc ctg gtc tgc tct gtg agt ggt ttc tat cca
       p   l   q   h   h   n   l   l   v   c   s   v   s   g   f   y   p

460  ggc agc att gaa gtc agg tgg ttc cgg aac ggc cag gaa gag aag gct ggg
       g   s   i   e   v   r   w   f   r   n   g   q   e   e   k   a   g

511  gtg gtg tcc aca ggc ctg atc cag aat gga gat tgg acc ttc cag acc ctg
       v   v   s   t   g   l   i   q   n   g   d   w   t   f   q   t   l

562  gtg atg ctg gaa aca gtt cct cgg agt gga gag gtt tac acc tgc caa gtg
       v   m   l   e   t   v   p   r   s   g   e   v   y   t   c   q   v

613  gag cac cca agt gtg acg agc cct ctc aca gtg gaa tgg aga gca cgg tct
       e   h   p   s   v   t   s   p   l   t   v   e   w   r   a   r   s

664  gaa tct gca cag agc aag atg ctg agt gga gtc ggg ggc ttc gtg ctg ggc
       e   s   a   q   s   k   m   l   s   g   v   g   g   f   v   l   g

715  ctg ctc ttc ctt ggg gcc ggg ctg ttc atc tac ttc agg aat cag aaa gga
       l   l   f   l   g   a   g   l   f   i   y   f   r   n   q   k   g

766  cac tct gga ctt cag cca aca gga ttc ctg agc tga
       h   s   g   l   q   p   t   g   f   l   s   -
```

HLA-DR2a

```
  1  atg gtg tgt ctg aag ctc cct gga ggt tcc tac atg gca aag ctg aca gtg
       m   v   c   l   k   l   p   g   g   s   y   m   a   k   l   t   v

 52  aca ctg atg gtg ctg agc tcc cca ctg gct ttg gct ggg gac acc cga cca
       t   l   m   v   l   s   s   p   l   a   l   a   g   d   t   r   p

103  cgt ttc ttg cag cag gat aag tat gag tgt cat ttc ttc aac ggg acg gag
       r   f   l   q   q   d   k   y   e   c   h   f   f   n   g   t   e

154  cgg gtg cgg ttc ctg cac aga gac atc tat aac caa gag gag gac ttg cgc
       r   v   r   f   l   h   r   d   i   y   n   q   e   e   d   l   r

205  ttc gac agc gac gtg ggg gag tac cgg gcg gtg acg gag ctg ggg cgg cct
       f   d   s   d   v   g   e   y   r   a   v   t   e   l   g   r   p
```

```
256  gac gct gag tac tgg aac agc cag aag gac ttc ctg gaa gac agg cgc gcc
       d   a   e   y   w   n   s   q   k   d   f   l   e   d   r   r   a

307  gcg gtg gac acc tac tgc aga cac aac tac ggg gtt ggt gag agc ttc aca
       a   v   d   t   y   c   r   h   n   y   g   v   g   e   s   f   t

358  gtg cag cgg cga gtt gag cct aag gtg act gtg tat cct gca agg acc cag
       v   q   r   r   v   e   p   k   v   t   v   y   p   a   r   t   q

409  acc ctg cag cac cac aac ctc ctg gtc tgc tct gtg aat ggt ttc tat cca
       t   l   q   h   h   n   l   l   v   c   s   v   n   g   f   y   p

460  ggc agc att gaa gtc agg tgg ttc cgg aac agc cag gaa gag aag gct ggg
       g   s   i   e   v   r   w   f   r   n   s   q   e   e   k   a   g

511  gtg gtg tcc aca ggc ctg att cag aat gga gac tgg acc ttc cag acc ctg
       v   v   s   t   g   l   i   q   n   g   d   w   t   f   q   t   l

562  gtg atg ctg gaa aca gtt cct cga agt gga gag gtt tac acc tgc caa gtg
       v   m   l   e   t   v   p   r   s   g   e   v   y   t   c   q   v

613  gag cac cca agc gtg acg agc cct ctc aca gtg gaa tgg aga gca cag tct
       e   h   p   s   v   t   s   p   l   t   v   e   w   r   a   q   s

664  gaa tct gca cag agc aag atg ctg agt gga gtc ggg ggc ttt gtg ctg ggc
       e   s   a   q   s   k   m   l   s   g   v   g   g   f   v   l   g

715  ctg ctc ttc ctt ggg gcc ggg cta ttc atc tac ttc aag aat cag aaa ggg
       l   l   f   l   g   a   g   l   f   i   y   f   k   n   q   k   g

766  cac tct gga ctt cac cca aca gga ctc gtg agc tga
       h   s   g   l   h   p   t   g   l   v   s   -
```

HLA-DR2b

```
  1  atg gtg tgt ctg aag ctc cct gga ggc tcc tgc atg aca gcg ctg aca gtg
       m   v   c   l   k   l   p   g   g   s   c   m   t   a   l   t   v

 52  aca ctg atg gtg ctg agc tcc cca ctg gct ttg tct ggg gac acc cga cca
       t   l   m   v   l   s   s   p   l   a   l   s   g   d   t   r   p

103  cgt ttc ctg tgg cag cct aag agg gag tgt cat ttc ttc aat ggg acg gag
       r   f   l   w   q   p   k   r   e   c   h   f   f   n   g   t   e

154  cgg gtg cgg ttc ctg gac aga tac ttc tat aac cag gag gag tcc gtg cgc
       r   v   r   f   l   d   r   y   f   y   n   q   e   e   s   v   r

205  ttc gac agc gac gtg ggg gag ttc cgg gcg gtg acg gag ctg ggg cgg cct
       f   d   s   d   v   g   e   f   r   a   v   t   e   l   g   r   p

256  gac gct gag tac tgg aac agc cag aag gac atc ctg gag cag gcg cgg gcc
       d   a   e   y   w   n   s   q   k   d   i   l   e   q   a   r   a

307  gcg gtg gac acc tac tgc aga cac aac tac ggg gtt gtg gag agc ttc aca
       a   v   d   t   y   c   r   h   n   y   g   v   v   e   s   f   t

358  gtg cag cgg cga gtc caa cct aag gtg act gta tat cct tca aag acc cag
       v   q   r   r   v   q   p   k   v   t   v   y   p   s   k   t   q
```

```
409  ccc ctg cag cac cac aac ctc ctg gtc tgc tct gtg agt ggt ttc tat cca
       p   l   q   h   h   n   l   l   v   c   s   v   s   g   f   y   p

460  ggc agc att gaa gtc agg tgg ttc ctg aac ggc cag gaa gag aag gct ggg
       g   s   i   e   v   r   w   f   l   n   g   q   e   e   k   a   g

511  atg gtg tcc aca ggc ctg atc cag aat gga gac tgg acc ttc cag acc ctg
       m   v   s   t   g   l   i   q   n   g   d   w   t   f   q   t   l

562  gtg atg ctg gaa aca gtt cct cga agt gga gag gtt tac acc tgc caa gtg
       v   m   l   e   t   v   p   r   s   g   e   v   y   t   c   q   v

613  gag cac cca agc gtg aca agc cct ctc aca gtg gaa tgg aga gca cgg tct
       e   h   p   s   v   t   s   p   l   t   v   e   w   r   a   r   s

664  gaa tct gca cag agc aag atg ctg agt gga gtc ggg ggc ttt gtg ctg ggc
       e   s   a   q   s   k   m   l   s   g   v   g   g   f   v   l   g

715  ctg ctc ttc ctt ggg gcc ggg ctg ttc atc tac ttc agg aat cag aaa gga
       l   l   f   l   g   a   g   l   f   i   y   f   r   n   q   k   g

766  cac tct gga ctt cag cca aca gga ttc ctg agc tga
       h   s   g   l   q   p   t   g   f   l   s   -
```

Bbc9 TRAV22S1

```
  1  atg aac tat tct cca ggc tta gta tct ctg ata ctc tta ctg ctt gga aga
       m   n   y   s   p   g   l   v   s   l   i   l   l   l   l   g   r

 52  acc cgt gga gat tca gtg acc cag atg gaa ggg cca gtg act ctc tca gaa
       t   r   g   d   s   v   t   q   m   e   g   p   v   t   l   s   e

103  gag gcc ttc ctg act ata aac tgc acg tac aca gcc aca gga tac cct tcc
       e   a   f   l   t   i   n   c   t   y   t   a   t   g   y   p   s

154  ctt ttc tgg tat gtc caa tat cct gga gaa ggt cta cag ctc ctc ctg aaa
       l   f   w   y   v   q   y   p   g   e   g   l   q   l   l   l   k

205  gcc acg aag gct gat gac aag gga agc aac aaa ggt ttt gaa gcc aca tac
       a   t   k   a   d   d   k   g   s   n   k   g   f   e   a   t   y

256  cgt aaa gaa acc act tct ttc cac ttg gag aaa ggc tca gtt caa gtg tca
       r   k   e   t   t   s   f   h   l   e   k   g   s   v   q   v   s

307  gac tca gcg gtg tac ttc tgt gct ttg ata ggc ttt ggg aat gtg ctg cat
       d   s   a   v   y   f   c   a   l   i   g   f   g   n   v   l   h

358  tgc ggg tcc ggc act caa gtg att gtt tta cca cat atc cag aac cct gac
       c   g   s   g   t   q   v   i   v   l   p   h   i   q   n   p   d

409  cct gcc gtg tac cag ctg aga gac tct aaa tcc agt gac aag tct gtc tgc
       p   a   v   y   q   l   r   d   s   k   s   s   d   k   s   v   c

460  cta ttc acc gat ttt gat tct caa aca aat gtg tca caa agt aag gat tct
       l   f   t   d   f   d   s   q   t   n   v   s   q   s   k   d   s

511  gat gtg tat atc aca gac aaa act gtg cta gac atg agg tct atg gac ttc
       d   v   y   i   t   d   k   t   v   l   d   m   r   s   m   d   f
```

```
562  aag agc aac agt gct gtg gcc tgg agc aac aaa tct gac ttt gca tgt gca
       k   s   n   s   a   v   a   w   s   n   k   s   d   f   a   c   a

613  aac gcc ttc aac aac agc att att cca gaa gac acc ttc ttc ccc agc cca
       n   a   f   n   n   s   i   i   p   e   d   t   f   f   p   s   p

664  gaa agt tcc tgt gat gtc aag ctg gtc gag aaa agc ttt gaa aca gat acg
       e   s   s   c   d   v   k   l   v   e   k   s   f   e   t   d   t

715  aac cta aac ttt caa aac ctg tca gtg att ggg ttc cga atc ctc ctc ctg
       n   l   n   f   q   n   l   s   v   i   g   f   r   i   l   l   l

766  aaa gtg gcc ggg ttt aat ctg ctc atg acg ctg cgg ctg tgg tcc agt tga
       k   v   a   g   f   n   l   l   m   t   l   r   l   w   s   s   -
```

Bbc9 TRBV9S1

```
  1  atg tgc agg ctc ctc tgc tgt gtg gtc ttc tgc ctc ctc caa gca ggt ccc
       m   c   r   l   l   c   c   v   v   f   c   l   l   q   a   g   p

 52  ttg gac aca gct gtt tcc cag act cca aaa tac ctg gtc aca cag atg gga
       l   d   t   a   v   s   q   t   p   k   y   l   v   t   q   m   g

103  aac gac aag tcc att aaa tgt gaa caa aat ctg ggc cat gat act atg tat
       n   d   k   s   i   k   c   e   q   n   l   g   h   d   t   m   y

154  tgg tat aaa cag gac tct aag aaa ttt ctg aag ata atg ttt agc tac aat
       w   y   k   q   d   s   k   k   f   l   k   i   m   f   s   y   n

205  aat aag gag ctc att ata aat gaa aca gtt cca aat cgc ttc tca cct aaa
       n   k   e   l   i   i   n   e   t   v   p   n   r   f   s   p   k

256  tct cca gac aaa gct cac tta aat ctt cac atc aat tcc ctg gag ctt ggt
       s   p   d   k   a   h   l   n   l   h   i   n   s   l   e   l   g

307  gac tct gct gtg tat ttc tgt gcc agc agc cag ggc aga tct agc aat cag
       d   s   a   v   y   f   c   a   s   s   q   g   r   s   s   n   q

358  ccc cag cat ttt ggt gat ggg act cga ctc tcc ata cta gag atc tcc cac
       p   q   h   f   g   d   g   t   r   l   s   i   l   e   i   s   h

409  acc caa aag gcc aca ctg gtg tgc ctg gcc aca ggc ttc ttc ccc gac cac
       t   q   k   a   t   l   v   c   l   a   t   g   f   f   p   d   h

460  gtg gag ctg agc tgg tgg gtg aat ggg aag gag gtg cac agt ggg gtc agc
       v   e   l   s   w   w   v   n   g   k   e   v   h   s   g   v   s

511  acg gac ccg cag ccc ctc aag gag cag ccc gcc ctc aat gac tcc aga tac
       t   d   p   q   p   l   k   e   q   p   a   l   n   d   s   r   y

562  tgc ctg agc agc cgc ctg agg gtc tcg gcc acc ttc tgg cag aac ccc cgc
       c   l   s   s   r   l   r   v   s   a   t   f   w   q   n   p   r

613  aac cac ttc cgc tgt caa gtc cag ttc tac ggg ctc tcg gag aat gac gag
       n   h   f   r   c   q   v   q   f   y   g   l   s   e   n   d   e

664  tgg acc cag gat agg gcc aaa ccc gtc acc cag atc gtc agc gcc gag gcc
       w   t   q   d   r   a   k   p   v   t   q   i   v   s   a   e   a

715  tgg ggt aga gca
       w   g   r   a
```

li-wt

```
          AscI
       ---+------
   1   cgg cgc gcc atg gat gac cag cgc gac ctt atc tcc aac aat gag caa ctg
                   m   d   d   q   r   d   l   i   s   n   n   e   q   l

  52   ccc atg ctg ggc cgt aga ccc ggc gct cct gag agc aag tgt agc aga ggg
         p   m   l   g   r   r   p   g   a   p   e   s   k   c   s   r   g

 103   gcc ctg tac acc ggc ttc agc atc ctc gtg aca ctg ctg ctg gcc gga cag
         a   l   y   t   g   f   s   i   l   v   t   l   l   l   a   g   q

 154   gct acc acc gcc tac ttt ctg tat cag cag cag ggc cgg ctg gac aag ctg
         a   t   t   a   y   f   l   y   q   q   q   g   r   l   d   k   l

 205   acc gtg acc agc cag aac ctg cag ctg gaa aac ctg cgg atg aag ctg ccc
         t   v   t   s   q   n   l   q   l   e   n   l   r   m   k   l   p

                     AgeI
                   --+-----
 256   aag ccc ccc aaa ccg gtg agc aag atg cgc atg gcc acc ccg ctg ctg atg
         k   p   p   k   p   v   s   k   m   r   m   a   t   p   l   l   m
                             >>.................CLIP...................>

                          KasI
                        -+-----
 307   cag gcg ctg ccc atg ggc gcc ctg cca cag gga cct atg cag aac gcc acc
         q   a   l   p   m   g   a   l   p   q   g   p   m   q   n   a   t
       >....CLIP....>>

 358   aaa tac ggc aac atg acc gag gac cac gtg atg cat ctg ctg cag aat gcc
         k   y   g   n   m   t   e   d   h   v   m   h   l   l   q   n   a

 409   gac ccc ctg aag gtg tac ccc cca ctg aag ggc agc ttt ccc gag aac ctg
         d   p   l   k   v   y   p   p   l   k   g   s   f   p   e   n   l

 460   cgg cac ctg aag aac acc atg gaa acc atc gac tgg aag gtg ttc gag agc
         r   h   l   k   n   t   m   e   t   i   d   w   k   v   f   e   s

 511   tgg atg cac cac tgg ctg ctg ttc gag atg agc cgg cac agc ctg gaa cag
         w   m   h   h   w   l   l   f   e   m   s   r   h   s   l   e   q

 562   aag cct acc gac gcc cct ccc aaa gag tcc ctg gaa ctg gaa gat ccc agc
         k   p   t   d   a   p   p   k   e   s   l   e   l   e   d   p   s

 613   agc ggc ctg ggc gtg acc aag cag gat ctg gga ccc gtg ccc atg tga
         s   g   l   g   v   t   k   q   d   l   g   p   v   p   m   -
```

delta-li

```
          AscI
       ---+------
   1   cgg cgc gcc atg agc tct ggt tcg tca aag tgt agc aga ggg gcc ctg tac
         r   r   a   m   s   s   g   s   s   k   c   s   r   g   a   l   y

  52   acc ggc ttc agc atc ctc gtg aca ctg ctg ctg gcc gga cag gct acc acc
```

```
      t   g   f   s   i   l   v   t   l   l   l   a   g   q   a   t   t

103  gcc tac ttt ctg tat cag cag cag ggc cgg ctg gac aag ctg acc gtg acc
       a   y   f   l   y   q   q   q   g   r   l   d   k   l   t   v   t

154  agc cag aac ctg cag ctg gaa aac ctg cgg atg aag ctg ccc aag ccc ccc
       s   q   n   l   q   l   e   n   l   r   m   k   l   p   k   p   p

         AgeI
       --+-----
205  aaa ccg gtg agc aag atg cgc atg gcc acc ccg ctg ctg atg cag gcg ctg
       k   p   v   s   k   m   r   m   a   t   p   l   l   m   q   a   l
                 >>.....................CLIP.........................>

256  ccc atg ggc gcc ctg cca cag gga cct atg cag aac gcc acc aaa tac ggc
       p   m   g   a   l   p   q   g   p   m   q   n   a   t   k   y   g
     >>>

307  aac atg acc gag gac cac gtg atg cat ctg ctg cag aat gcc gac ccc ctg
       n   m   t   e   d   h   v   m   h   l   l   q   n   a   d   p   l

358  aag gtg tac ccc cca ctg aag ggc agc ttt ccc gag aac ctg cgg cac ctg
       k   v   y   p   p   l   k   g   s   f   p   e   n   l   r   h   l

409  aag aac acc atg gaa acc atc gac tgg aag gtg ttc gag agc tgg atg cac
       k   n   t   m   e   t   i   d   w   k   v   f   e   s   w   m   h

460  cac tgg ctg ctg ttc gag atg agc cgg cac agc ctg gaa cag aag cct acc
       h   w   l   l   f   e   m   s   r   h   s   l   e   q   k   p   t

511  gac gcc cct ccc aaa gag tcc ctg gaa ctg gaa gat ccc agc agc ggc ctg
       d   a   p   p   k   e   s   l   e   l   e   d   p   s   s   g   l

562  ggc gtg acc aag cag gat ctg gga ccc gtg ccc atg tga
       g   v   t   k   q   d   l   g   p   v   p   m   -
```

5.3 Vector maps

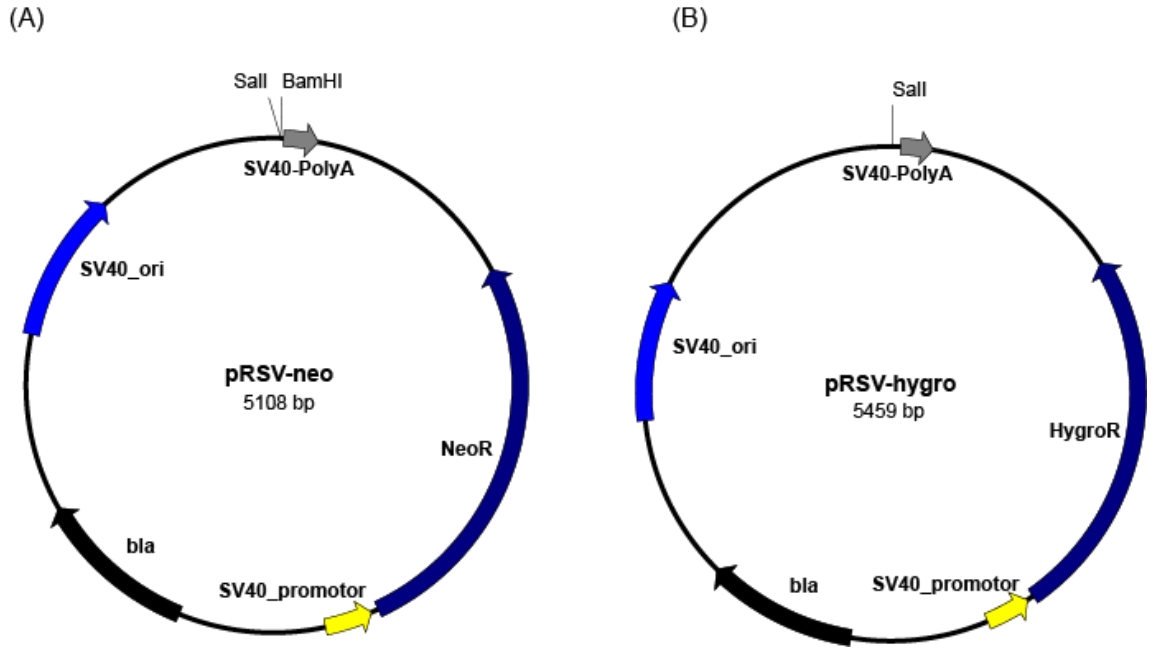

Figure 5-1 Vector map of pRSV-neo and pRSV-hygro for the expression of TCR β and TCRα. (A) TCR β chains are cloned into pRSV-neo. The Plasmid carries the gene of β-lactamase (bla), for ampicillin resistence, the origin of replication of Simian virus 40 (SV40_ori), SV40 polyadenylation site (SV40-PolyA), gene for neomycine resistance (NeoR) and SV40 promotor. The constant region of the β-chain was cloned into pRSV-neo, and can be complemented with BV-BN(D)N-BJ via SalI and AvaI. (B) TCR α chains are cloned into pRSV-hygro. It was constructed by replacing the neomycin resistance of pRSV-neo by the gene for hygromycine resistance. The constant region of the α-chain was cloned into the vector and can be complemented with the AV-AN-AJ via SalI and PvuII. The vector maps were constructed with Savvy Version 0.1.

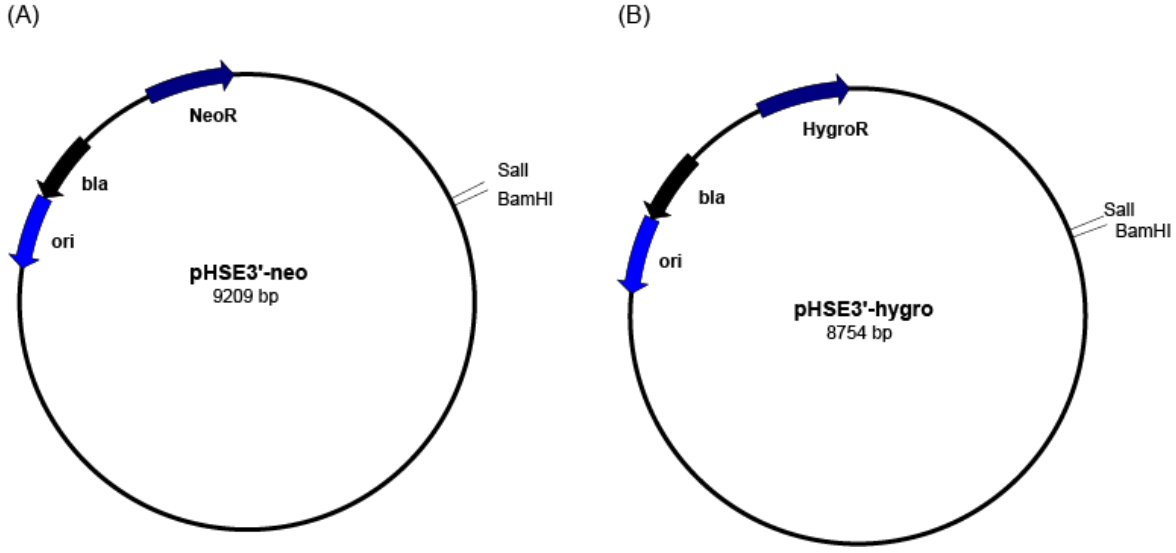

Figure 5-2 Vector maps of pHSE3'-neo and pHSE3'-hygro for the expression of MHC. MHC-I alleles as well as the β-chain of MHC-II alleles were cloned into pHSE3'-neo via the restriction sites SalI and BamHI. The vector carries gene for neomycine resistance, gene for β-lactamase (bla), for ampicillin resistance and an origin of replication (ori). (B) The α-chain of MHC-II was cloned into pHSE3'-hygro via the restriction sites SalI and BamHI. It is a derivate of pHSE3'-neo, however the neomycine resistance was substituted by hygromycine resistance. The vector maps were constructed with Savvy Version 0.1

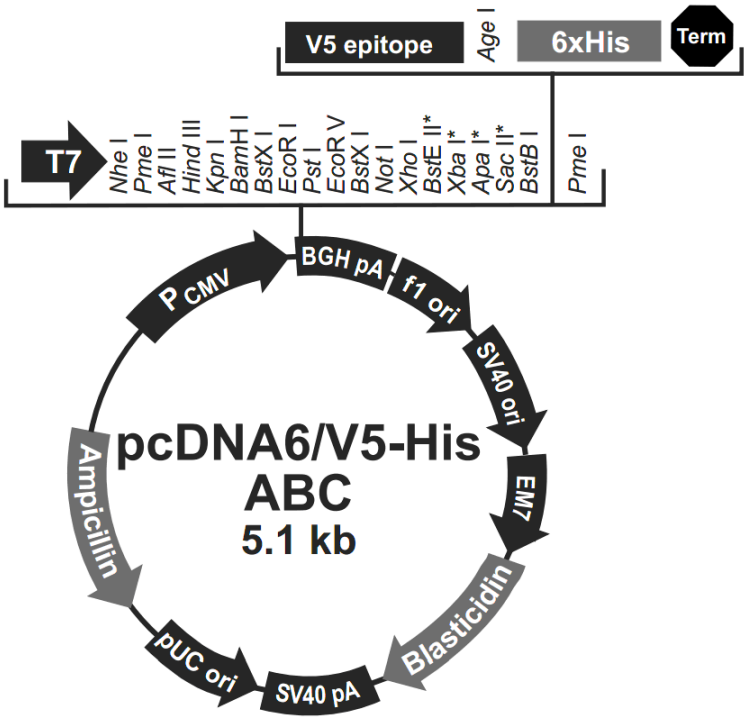

Figure 5-4 Vector map pcDNA6/V5-His ABC. Source: Invitrogen

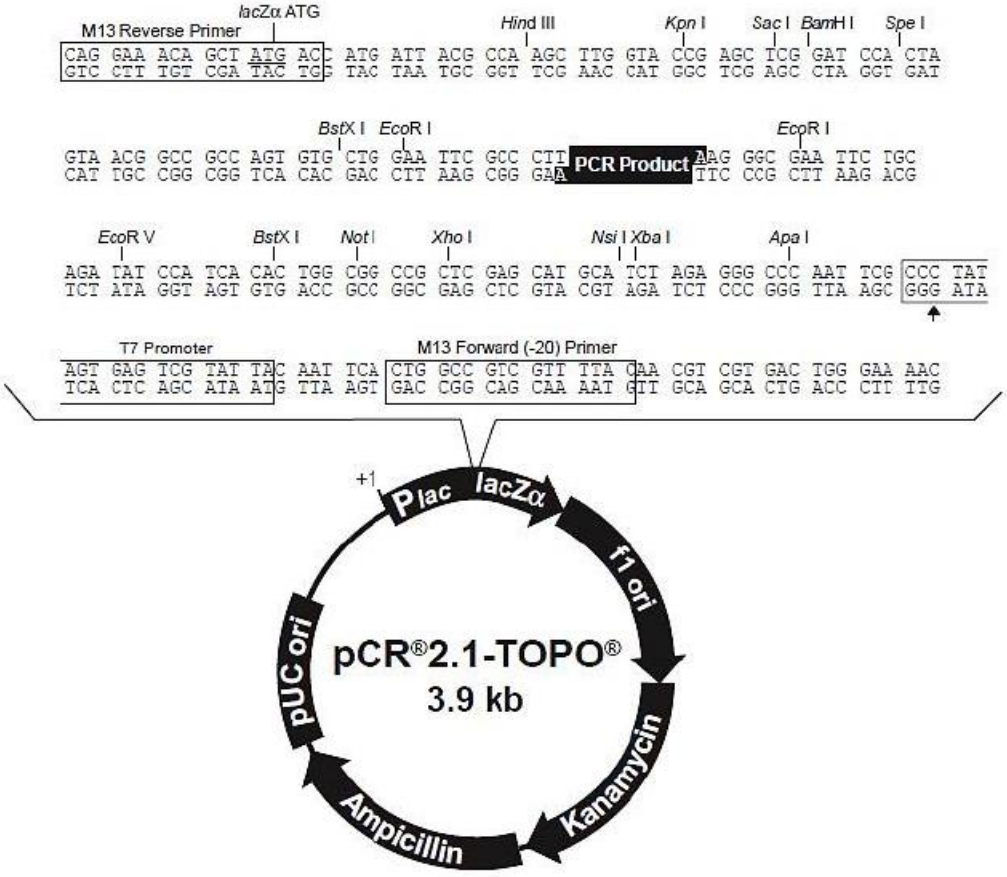

Figure 5-3 pCR®2.1-TOPO vector for TOPO-TA cloning. Source: Invitrogen.

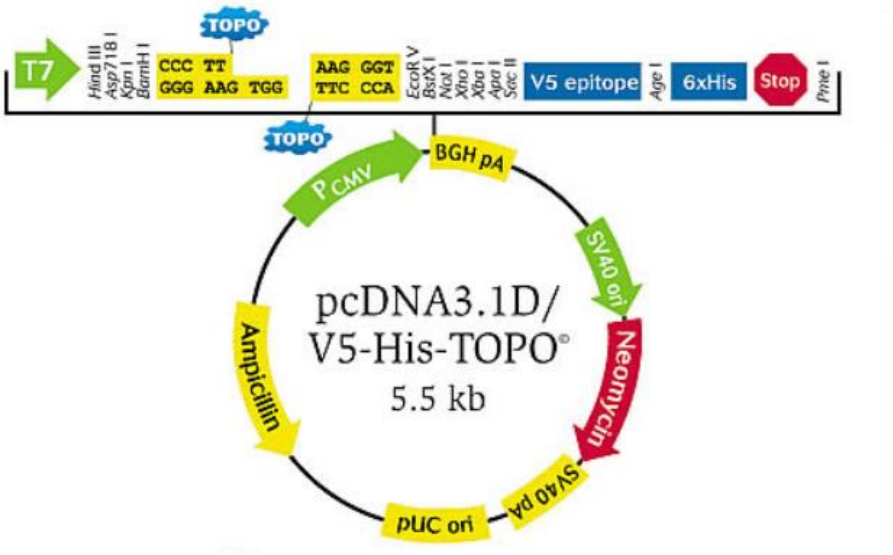

Figure 5-5 pcDNA3.1D/V5-His-TOPO for directional TOPO cloning. Source: Invitrogen

6 References

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Eidesstattliche Versicherung

Plappert, Aline

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema:

“Analysis of T Cell Receptors and their Antigens in $GABA_A$ Receptor Encephalitis”

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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