Characterization of potentially autoaggressive brain infiltrating CD8+ T cells in multiple sclerosis patients

Dissertation

der Fakultät für Biologie der Ludwig-Maximilians-Universität München

zur Erlangung des Doktorgrades der Naturwissenschaften

vorgelegt von

Latika Bhonsle

aus Kolhapur, Indien

Dezember 2011

---

erstellt am Max-Planck-Institut für Neurobiologie, Abteilung Neuroimmunologie

Martinsried bei München

Erstgutachterin	Prof. Dr Elisabeth Weiss
Zweitgutachter	PD Dr Josef Mautner
Sondervotum	PD Dr Klaus Dornmair

Dissertation eingereicht am	21.12.2011
Mündliche Prüfung am	02.03.2012

Summary

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system. The disease is thought to be mediated by autoaggressive lymphocytes that attack the nervous myelin sheath, leading to the formation of characteristic lesions in the brain tissue. CD8+ T cells are thought to play a role in the immunopathogenesis of the disease because of the directed infiltration of an immune privileged structure followed by *in situ* clonal expansion. They are also the dominant brain infiltrating T cell population. However, the events that trigger this autoimmune attack, as well as the molecular target(s) of the autoaggressive T cells remain unknown. The current study is part of a long term strategy to identify the molecular target(s) of these autoaggressive CD8+ T cells. To achieve this, potentially autoaggressive T cells were isolated by laser microdissection from the brain tissue samples of MS patients. Their antigen specific T cell receptors (TCRs) were characterized, reconstructed *in vitro* in T hybridoma cells and applied in investigating antigen specificities.

The α- and β- chains of the MS brain derived TCRs were characterized using a multiplex PCR approach. The TCR chain sequences provide insights into their immune function in context of multiple sclerosis. Firstly, identical TCR β-chains were found to be coexpressed with more than one α-chain in three different instances. This suggests that antigen driven receptor development or the existence of dual TCR molecules may contribute to autoimmunity. Secondly, one TCR clone was detected in cells from three tissue blocks of one patient, indicating that the T cell infiltration is not anatomically restricted during the autoimmune attack. Thirdly, four out of nine T cell receptor molecules, that were characterized together with colleagues, were found to belong to an innate T cell population called the mucosal-associated invariant T (MAIT) cells. These cells have been previously detectected in MS patients and are thought to regulate anti-microbial immunity, possibly providing a link between microbial infection and autoimmunity. The MAIT T cell receptors were expressed in T hybridoma cell lines and used to screen candidate MS antigens and plasmid encoded peptide libraries. In addition, the MHC class I related molecule MR1 that restricts MAIT antigen recognition, was investigated for its anchor residues to shed light on the nature of the bound MAIT antigen. In the long run, these studies promise to contribute to the understanding of multiple sclerosis in particular and autoimmunity in general.

Acknowledgements

It is my pleasure to thank all the people who have made this thesis possible.

Dr Klaus Dornmair, my immediate supervisor at the Max Planck Institute of Neurobiology, who showed me how good research is done. I appreciate his logical thinking and the fact that he was always available for scientific discussions and hands on demonstrations. For giving me a challenging research topic to work on and helping me sharpen my scientific thinking and widen my technical skill set.

Prof Reinhard Hohlfeld for his motivating advice with regards to my research and development as a scientist. I am grateful that he gave me the opportunity to work at the interface of fundamental and clinical research at the Institute of Clinical Neuroimmunology.

Prof Hartmut Wekerle for initiating my move to Germany and inspiring me to work on clinically relevant research during my doctoral years. His valuable feedback during progress seminars, as well as scientific advice contributed greatly to my work and growth as a scientist.

Prof Elisabeth Weiss, my doctoral thesis supervisor at the faculty of Biology at the Ludwig-Maximilians-Universität München, for her intellectual support and guidance that have been invaluable to me. Her wide knowledge and motivating feedback during progress meetings, as well as advice on the formal procedures of thesis completion contributed greatly to this thesis.

Dr Josef Mautner and all **members of my thesis examination committee** for their contribution to the completion of this work.

Prof Martin Kerschensteiner and **Dr Dieter Jenne** for their guidance as members of my thesis advisory committee. I am appreciative of their contribution of ideas, opinions and time towards my research.

Dr Hans-Joerg Schaeffer, **Maxi Reif** and **Dr Ingrid Wolf** from the office of the International Max Planck Research School for Molecular and Cellular Life Sciences (IMPRS-LS) . I shall be grateful to the IMPRS-LS for support in resolving administrative matters, soft skill training and extracurricular activities. Additionally, I would also like to thank all my colleagues from the research school who made my time in Munich very memorable.

David, **Katherina**, **Wakiro, Anna** and **former colleagues** who contributed to the growing body of work on autoaggressive T cells and helped lay the foundation for this thesis. **Prof Jörg Prinz**, **Song Min** and **Gintare** for the productive collaborations that were an integral part of this research.

Joachim, **Ingrid**, **Reinhard** and **Martina** for their willingness to explain and demonstrate new techniques and providing a great work atmosphere. **Judy, Jana, Michael and Marija** for being my constant companions on this journey of a doctoral student. I would like to extend my heartfelt gratitude to all **members of the Dornmair laboratory** and the **Department of Neuroimmunology** for the productive work environment and the meaningful scientific and personal discussions.

My teachers **Anuradha Prasad**, **Milind Watve** and **Vani Brahmachari** who inspired me to follow the path of science and be true to my cause.

The gratitue I feel for my family and loved ones is immeasurable.

Rosi, **Hans**,**Verena** and **Manuel** , my extended family who have been a constant source of love and encouragement.

Julian, for his unconditional love and support. For happily sharing the highs, the lows and the lulls of doctoral life. But most of all for believing in me and being my source of inspiration and positive energy. I look forward to all the wonderful adventures that the future holds for us.

Jana my sister and best friend for her caring, love and good advice.

My parents **Ajit** and **Anjali** for raising me, teaching me, and supporting my dreams and decisions. They have been my role models for as long as I remember.

To them I dedicate this thesis.

Table of Contents

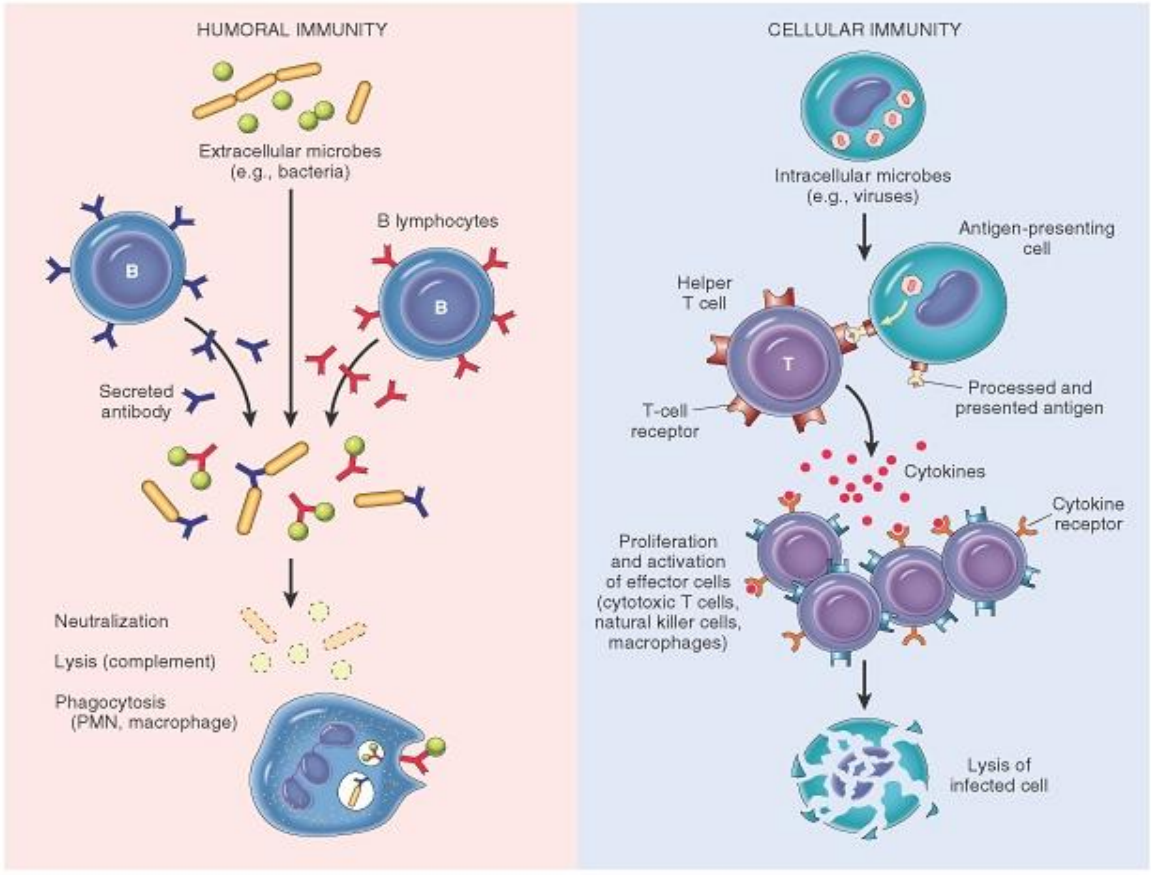
1 Introduction

1.1 The immune system

The human immune system is a combination of two fronts, the fast acting innate immune system and the versatile adaptive immune system, that protect an individual from threats such as infectious agents and cancer. When a pathogen infiltrates the first defenses of the body, the innate immune mechanism is brought into action. It brings together macrophages, natural killer T (NKT) cells, mast cells, granulocytes and the complement system to swiftly get rid of the pathogenic threat and stem subsequent infection. This system is rapid in action, but limited in the sense that it does not recognize all foreign microbes and substances, especially pathogens that have mutated and modified themselves over time. Based on its ability to continuously adjust to novel antigenic challenges, the second line of defense is known as the adaptive immune system. This system consists of two branches (**Figure 1-1)**, (a) humoral immunity mediated by B lymphocytes and antibodies and (b) cellular immunity mediated by CD4+ helper T lymphocytes and CD8+ effector T lymphocytes.

Humoral immunity prevents microbial infection by the production of antibodies that neutralize their antigens and facilitate the clearing of opsonized particles by phagocytocis. B cells recognize their antigen in the native form and also serve as professional antigen presenting cells (APCs). Immunological memory also guarantees that in case of another infection with an encountered pathogen, the immune response runs more efficiently. Cellular or cell mediated immunity is responsible for protection against microbes and tumor cells. CD4+ helper cells are capable of recognizing exogenous antigens (bacteria, parasites, and toxins) that are displayed on the major histocompatibility complex (MHC) class II molecules. Post activation, the cells may secrete interferon-gamma (Th1 response) that induces a cell mediated immune response against intracellular pathogens or interleukin-4 (Th2 response) that leads to a B cell mediated humoral immune response directed against extracellular pathogens and toxins. CD8+ effector T cells

recognize antigen in context of the MHC class I molecule. They possess cytotoxic activity and can induce the death of infected or dysfunctional cells. This T cell subgroup is dealt with in further detail in **Section 1-2**.



Copyright © 2002, Elsevier Science (USA). All rights reserved.

Fig. 1-1: The two branches of the adaptive immune system

(a) Humoral immunity: An infection with a toxin producing extracellular pathogen activates B cells that secrete antigen specific neutralizing (blue) and opsonizing or coating (red) antibodies. The invading pathogens are then killed by neutralization or complement system mediated lysis or phagocytosed by polymorphonuclear leucocytes (PMN) or macrophages. **(b)** Cellular immunity: The virus infected antigen presenting cell (APC) displays the processed antigen to the CD4+ helper T cell via its MHC class II molecule. Upon activation, the helper T cell secretes cytokines that induce the proliferation and activation of various effector cells including CD8+ effector T cells that mediate killing of the infected cell. (Castillo 2002)

1.2 Autoimmune disease

The lymphocytes of the adaptive immune system must be capable of distinguishing between the individual's healthy cells (termed as 'self') and the invading pathogen or tumor cells (termed as

'non-self'). During development, both B and T lymphocytes are inspected at various checkpoints to eliminate potentially autoreactive cells, i.e. lymphocytes that recognize a 'self' component (Wekerle 1992). However, if 'self' recognizing lymphocytes escape these control mechanisms and enter the periphery, they are capable of raising an autoimmune attack on the 'self components' of an individual's apparently healthy tissues. This attack may be systemic like in the case of systemic lupus erythematosus (SLE), where inflammation may occur in various organs like the heart, joints, skin, lungs, kidneys and liver. This condition is thought to be mediated by anti-DNA antibodies (Diamond et al. 1992). Alternately, the autoimmune attack may be localized in an organ specific manner like in the case of psoriasis. In this disease, autoreactive CD4+ and CD8+ T cells infiltrate the epidermis and cause a chronic inflammation of the skin (Prinz 2003). This also applies in the case of multiple sclerosis (MS), a chronic inflammation that is directed at the central nervous system (CNS) (**Section 1.5**). The effects of organ specific autoimmune attacks may extend beyond the target tissue and indirectly affect other organs and body systems. The causes for the loss of tolerance leading to the autoimmune reaction, and the underlying immune mechanisms are not well understood.

1.3 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS) characterized by multi-focal inflammatory demyelination, axonal loss, glial cell activation, and the infiltration of immune cells (Compston and Coles 2008). With respect to incidence, MS is the second most frequent neurological disease affecting young adults after epilepsy. The disease was first described by french neurologist Jean-Martin Charcot in the late 20th century. MS is believed to be an autoimmune disorder because of the characteristic infiltration of immune cells via a disrupted blood brain barrier into the CNS (Sospedra and Martin 2005).

1.3.1 Pathology

Sharply demarcated demyelinated plaques within CNS areas such as optic nerves, periventricular regions, brainstem, cerebellum and spinal cord are characteristic of chronic MS brain tissue (Lassmann et al. 2001). These lesions are infiltrated by oligoclonal T cells (CD4+, CD8+, and

γδ), occasional B cells, plasma cells and macrophages (Hauser et al. 1983; Traugott et al. 1983). The demyelination and axonal loss is not limited to the white matter (WM) and also affects the grey matter (GM) or cortex (Hohlfeld and Wekerle 2001).

1.3.2 Etiology

The etiology of MS is poorly understood but genetic and environmental factors are thought to play a role. Disease prevalence increases within family members of MS patients as suggested by the fact that the concordance rate in monozygotic twins is higher than that of dizygotic twins (Dyment et al. 2004). Genetic linkage studies have suggested that certain human leukocyte antigen (HLA) class II alleles such as the HLA-DRB1*1501 and -DQB1*0601 are associated with MS (Olerup and Hillert 1991) besides other genes like TCRβ, cytotoxic T-lymphocyte associated protein 4 and CD 45 (Dyment et al. 2004). Environmental triggers such as infectious agents and lifestyle are also believed to contribute to disease occurrence (Coo and Aronson 2004). The human herpesvirus, the Epstein-Barr virus (EBV) (Wandinger et al. 2000), is of particular interest in this regard. Of late, CD8+ effector T cells that are the dominant infiltrating T cell subpopulation (Woodroofe et al. 1986) have garnered interest for their contribution to MS.

1.4 CD8+ effector T cells

CD8+ effector T cells are responsible for mediating immune responses against invading pathogens and tumor cells. They recognize the 'non-self' or altered molecules, presented on the surface of these cells by MHC class I molecules, via antigen specific T cell receptors (TCRs) and eliminate the infected cells by the release of perforin and granzyme containing cytotoxic granules (Harty et al. 2000), or by expressing the Fas ligand (Medana et al. 2000).

1.4.1 TCR diversity generation

T cells are derived from haematopoietic stem cells in the bone marrow, and then develop into naïve T cells in the thymus. Every individual's T cell repertoire is capable of recognizing a broad

antigenic spectrum. To fulfill this requirement, T cells undergo 'somatic recombination', which is a random rearrangement of TCR gene segments resulting in highly variable TCRs. At the end of this process, each T cell clone expresses an exclusive TCR. The genetic loci that determine TCR diversity are presented in **Figure 1-2**.

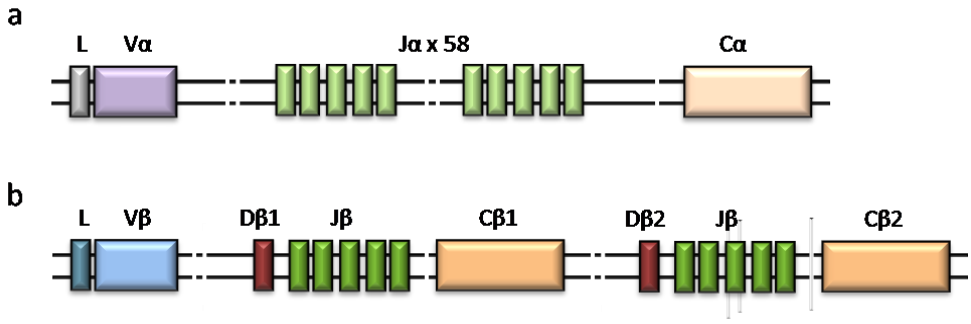


Fig. 1-2: Human TCRα and TCRβ genetic loci

(a) The human TCRα gene locus is composed of a combination of variable (V), joining (J) and constant (C) gene segments that are all derived from a genetic locus consisting of 70 to 80 Vα (or AV), 58 Jα or (AJ) and one Cα gene segment. **(b)** The TCRβ locus has a similar composition in addition to the diversity (D) segment between the V and J segments. The β-chain locus consists of 54 Vβ (or BV), 2 Dβ, 14Jβ (or BJ) and 2 Cβ segments. (Arden et al. 1995; Rowen et al. 1996) (Modified from Murphy et al. 2008)

The β-chain of the TCR is rearranged first by random combination of a D and J segment, followed by a V segment. During this combination, there occur insertions and deletion of palindromic (P) or non-germline (N) nucleotides that further add to the variability. The ultimate unison with the C segment occurs by post-transcriptional RNA splicing. The high variability of the TCR is accounted for in the three complementarity determining regions (CDR) CDR1, 2 and 3 (Jores et al. 1990). The CDR1 and CDR2 are coded mainly by the variable regions for both α- and β-chains. The centrally placed CDR3 region, termed as the N region in the α-chain and the NDN region in the β-chain, is the region containing the highest variability. It is this region that ultimately binds to the MHC-peptide molecule (**Section 1.4.3**) and is responsible for antigen recognition.

1.4.2 The αβ T cell receptor complex

The T cell receptor (TCR) is a heterodimer consisting of a α- and β-chain that are comprised of an antigen specific N-terminal variable region, and an immunoglobulin domain like C-terminal constant region. The C region is anchored in the cell membrane. The TCR is associated with a number of other molecules, forming the TCR complex (**Figure 1-3**).

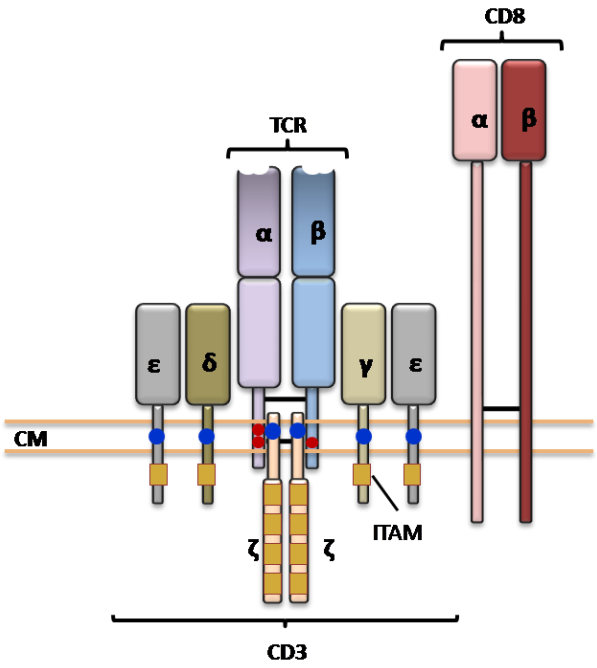


Fig. 1-3: The αβ TCR complex

The TCR complex consists of two variable chains α (purple) and β (blue) and the invariant dimers CD3γε, CDδε and ζζ. Basic residues within the membrane region (CM) are denoted as red dots and the acidic residues as blue dots. The immunoreceptor tyrosine-based activation motifs (ITAM) are denoted in orange. The CD8 co-receptor (red) may be composed of an αα homodimer or an αβ heterodimer, as depicted above. This molecule is responsible for stabilizing the TCR-MHC-peptide interaction and boosting the subsequent signal transduction cascade. Disulphide bonds that stabilize inter-chain interactions are shown in black. (Modified from Murphy et al. 2008)

An integral component of this complex is the CD3 glycoprotein complex that is responsible for downstream signaling post antigen recognition (Clevers et al. 1988). The TCR signaling cascade is initiated with the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) (Smith-Garvin et al. 2009) that recruit protein kinases to the activated TCR. This is followed by a flux of calcium ions and further signaling events that consequently activate a calcium ion-calmodulin (CaM)-dependent phosphatase called calcineurin. Calcineurin dephosphorylates proteins of the nuclear factor of activated cells (NFAT) family that facilitate their nuclear entry. Here the NFAT proteins collaborate with different co-activators to drive gene transcription (Katzav, 2004). NFAT is a transcription factor that regulates the expression of several genetic loci including interleukin-2 (IL-2). NFAT-dependent promoters can be used as molecular tools to

regulate reporter genes like β-galactosidase and detect TCR activation (Karttunen and Shastri 1991).

1.4.3 Antigen restriction by the MHC class I molecule

T lymphocytes recognize their specific antigens in context of the major histocompatibility complex (MHC) molecule, a phenomenon called MHC restriction. CD8+ T cells are restricted by the MHC class I molecule which is a membrane anchored structure consisting of an α-chain in association with β_2 microglobulin (**Figure 1-4**).

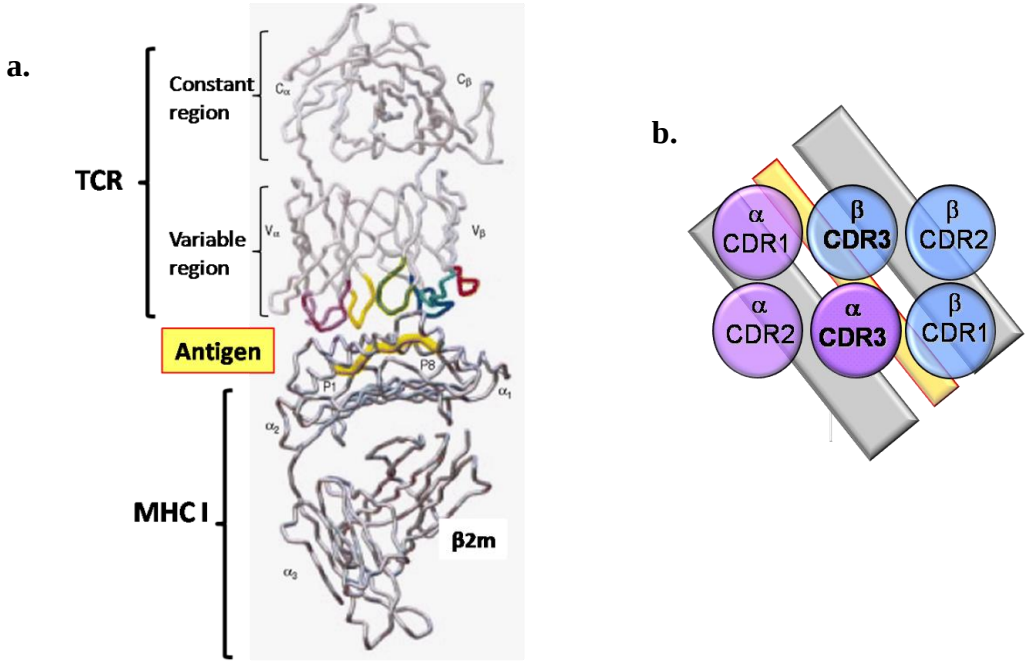


Fig. 1-4: Interaction of the αβ TCR with the MHC-peptide complex

(a) The crystal structure of the extracellular domains of an MHC class I molecule (MHC I) presenting antigen (yellow) to a TCR. The α1 and α2 domains of the MHC molecule contact the antigen, while the α3 domain is associated with β_2 microglobulin. The C-terminal of the α-chain spans the cellular membrane ending in a short cytoplasmic region (Rudolph et al. 2006). The bound octapeptide (P1 to P8) antigen interacts with the complementarity determining regions (CDR 1, 2 and 3) of the two TCR chains (depicted in color). These lie in the variable region (Vα, Vβ) of the TCR and are followed by a conserved constant region (Cα, Cβ). **(b)** A simplified representation of the contribution of the CDR regions of the TCR to antigen binding and recognition. The α-chain is depicted in purple while the β-chain is depicted in blue. The α1 and α2 domains of the MHC class I molecule are shown in grey, bound to the antigen (yellow). The CDR3 region of both TCR chains is mostly responsible for interacting with the MHC-peptide complex. (Modified from Garcia et al. 1996)

The MHC locus on chromosome 6 is a polygenic consortium of highly polymorphic genes. The gene locus codes for three MHC class I molecules that are HLA-A, HLA-B and HLA-C (where

HLA stands for human leukocyte antigen). Globally, there exist 767 HLA-A alleles, 1178 HLA-B alleles and 439 HLA-C alleles (from the IMGT-HLA database). These three loci are expressed in a co-dominant manner such that each cell can express up to six MHC class I molecules on its surface. MHC molecules are expressed on almost all nucleated cells. The cells that are capable of processing and presenting MHC bound antigen to the TCR complex are known as antigen presenting cells.

The presented peptides are sourced from cytoplasmic proteins that are processed by the proteasome. These peptides are trafficked to the endoplasmic reticulum (ER) by the antigen processing transporter (TAP). Here the peptides are loaded into the binding site of the respective MHC class I molecule aided by chaperones. The MHC-peptide complex now leaves the ER lumen and translocates via the Golgi apparatus to the cellular surface (Hansen and Bouvier 2009). In the absence of a binding partner, the MHC molecule is retained within the ER. Depending on the MHC molecule, all binding peptides usually carry anchor positions which are fixed amino acid residues that make close contact with the MHC molecule. For e.g. For HLA-A*0101, the nonapeptides mostly carry an asparagine residue at position 3 and a tyrosine residue at position 9.

1.4.4 Proposed role of T cells in MS

Both helper T cells and effector T cells have been associated with MS. CD4+ T cells were observed in lesions of MS and experimental autoimmune encephalomyelitis (EAE), the animal model of MS (Handel et al. 2011). Moreover linkage studies have indicated that some degree of genetic risk may be conferred by the HLA-DR and HLA-DQ molecules. Transgenic mice that expressed these MHC molecules were found to be susceptible to EAE (Kawamura et al. 2000) while transgenic mice that expressed these MHC molecules in combination with MS patient derived myelin basic protein (MBP) specific TCRs could develop spontaneous or induced EAE (Madsen et al. 1999).

Other studies have reported the preponderance of CD8+ effector T cells in MS lesions (Woodroofe et al. 1986) and genetic studies (Lincoln et al. 2005; Sawcer et al. 2011) suggest that an independent association exists between MHC class I alleles and MS (Fugger et al. 2009).

Moreover, CD8+ T cells are detected in numbers exceeding those of CD4+ T cells in acute and chronic lesions of MS (Babbe et al. 2000; Junker et al. 2007). Interestingly, the myelin specific CD8+ T cell response is seen to increase in MS patients (Zang et al. 2004) and myelin protein specific CD8+ T cells can induce EAE in animal models of the disease (Huseby et al. 2001; Sun et al. 2001). As depicted in **Figure 1-5**, it is believed that CD8+ T cells with autoimmune specificities can damage oligodendrocytes and neurons by direct cytotoxicity or cytokine secretion.

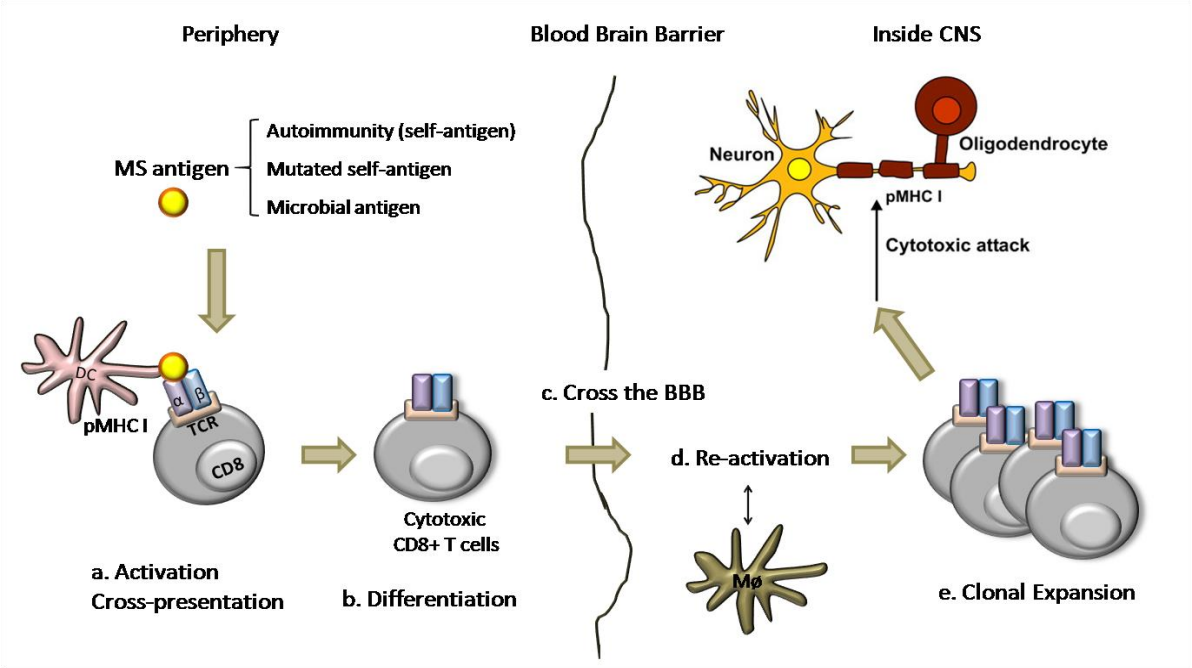


Fig. 1-5: The proposed role of CD8 + effector T cells in MS

The figure depicts how CD8+ T cells leave the periphery and enter the CNS to mediate an autoimmune attack in MS. Dendritic cells in the periphery present antigens to CD8+ T cells that in turn **(a)** get activated. These further **(b)** differentiate into different subsets such as cytotoxic effector T cells and regulatory T cells (not shown here). **(c)** When the integrity of the blood brain barrier (BBB) breaks down, the cytotoxic CD8+ T cells with 'self' antigen specificity cross the BBB. **(d)** Within the central nervous system (CNS) they encounter microglia or macrophages (MØ) that express MHC class I peptide complexes (pMHC I). By this interaction, the CD8+ cells get **(d)** reactivated and **(f)** expand clonally. On meeting their target antigen(s) that are presented on MHC class I expressing oligodendrocytes and neurons, they cause tissue damage ultimately leading to neurological deficits. (Modified from Friese and Fugger 2005)

1.5 Mucosal-associated invariant T (MAIT) cells

Human mucosal-associated T (MAIT) cells are innate CD8+ cells, characterized by the expression of an invariant TCR α-chain (AV7.2-AJ33 in humans) and homing to mucosal-associated lymphoid tissues (Le Bourhis et al. 2011). MAIT cells are detected in large numbers in blood (1-8% of all lymphocytes), liver (20-45% of all lymphocytes) and display an effector memory phenotype (Le Bourhis et al. 2011). After thymic development, the MAIT cells require the presence of B cell, the gut commensal flora and the evolutionary conserved MHC class I related molecule MR1 (also known as MHC-related molecule 1 or MHC-related protein 1) for peripheral expansion (Martin et al. 2009). The MR1 (**Section 1.5.1**) molecule is also responsible for restricting antigen presentation to the MAIT receptor (Huang et al. 2005). The role of the MAIT subset in the immune system is yet to be delineated, although it is associated with various diseases (**Section 1.5.2**) including MS. The antigen(s) recognized by the MAIT TCR (**Section 1.5.5**) remains to be characterized.

1.5.1 The antigen presenting MHC class I related molecule MR1

The MR1 protein is encoded on human chromosome 1 and is highly conserved among mammals (Riegert et al. 1998). It displays a high sequence identity to the MHC class I molecules in the putative ligand groove (α1 and α2 domains) (Lybarger et al. 2003). Similar to classical MHC molecules, MR1 stably associates with β_2 microglobulin and MR1 mRNA is ubiquitously expressed in humans and mice (Le Bourhis et al. 2011). Besides its indispensable role in MAIT cell development, MR1 is also believed to restrict antigen presentation to the MAIT TCR (Huang et al. 2005).

1.5.2 The association of MAIT cells in human diseases including MS

The MAIT subpopulation has been found to infiltrate diseased organs in various illnesses suggesting a much broader role in the immune response. This includes bacterial infection, kidney and brain tumors (Peterfalvi et al. 2008), as well as MS. The association between MAIT cells and MS was first described in a study (Illes et al. 2003) where the authors detected the MAIT

invariant α-chain AV7.2-AJ33 in autopsy central nervous system (CNS) lesions from MS patients as well as in a peripheral nervous system biopsy from a patient with chronic inflammatory demyelinating polyneuropathy. In a more recent study (Miyazaki et al. 2011) the same group reported a reduction in MAIT cell numbers in the peripheral blood of MS patients during remission and relapse. This number was seen to decrease in patients with active disease, and later increase in parallel with clinical recovery. The authors suggest that MAIT cells are capable of suppressing T_h1 responses *in vitro* and probably play a regulatory role in MS.

1.5.3 Hypotheses regarding the MAIT antigen

There exists conflicting evidence in support for the MAIT antigen being a lipid or a peptide. The activation of MAIT cells by α-mannosylceramide derivatives (Shimamura et al. 2007) could indicate that MAIT cells also respond to a lipid antigen. On the other hand, a delipidated antigen preparation of the *M. tuberculosis* cell wall was found to be highly antigenic for the MAIT TCR while proteolytic digestion of this preparation abolished this activity (Gold et al. 2010). In addition, molecular modeling of MR1 suggests that the antigen binding groove is most suited to binding a hydrophilic compound (Le Bourhis et al.2011). Furthermore, the mechanism of MR1 antigen presentation is thought to be independent of the known MHC class I and II pathways (Le Bourhis et al. 2011).

1.6 Characterizing the molecular target(s) of autoaggressive T cells

1.6.1 TCR characterization

Characterization of TCRs of unknown specificity, such as those with 'self' targets, is important for delineating the underlying protective and pathogenic mechanisms. Efforts in this direction have been hindered for various reasons such as the short survival of live T cells *in vitro* (Dooms and Abbas 2002), the need to distinguish relevant clones from an oligoclonal background *in vivo* (Moebius et al. 1990; Pantaleo et al. 1997; Goronzy et al. 1998; Hofbauer et al. 2003; Matsumoto et al. 2003) and the need for a method that can detect the entire repertoire (~10^{15}) of all possible αβ TCR heterodimers (Davis and Bjorkman, 1988). Early TCR repertoire studies in MS analyzed the germline TCR β-chain repertoire by restriction fragment length polymorphism (RFLP) (Biddison et al. 1989; Beall et al. 1989) or reverse transcription of whole RNA from frozen MS lesions (Wucherpfennig et al. 1992). However these studies were unable to study the contribution of single cells to the TCR repertoire.

More recently monoclonal antibodies that recognize limited TCR chains, RT-PCR amplification, spectratyping, random cloning and sequencing of β-chain transcripts have been used to characterize TCR chains. An experimental approach to investigate these T cells and eventually their molecular target(s) (Dornmair et al. 2003) was used in this study (**Figure 1-6**). A 'clone specific' approach that involves detecting the clone of interest with antibodies, followed by amplification of TCR genes using clone specific β-chain primers and unbiased α-chain primers was first applied in studying autoimmune T cells in muscle autoimmune disorders (Seitz et al. 2006). Similarly, an unbiased approach that involves unbiased primers covering all functional TCR α and β variable region genes was also applied. The two MS patients, a biopsy case (FE) (Skulina et al. 2004) and an autopsy case (MS-4) (Junker et al. 2007) were examined for matched α- and β-TCR chains of infiltrating CD8+ T cells. Several β-chain oligoclonal expansions were detected in these samples indicating an antigen driven immune reaction in the MS brain.

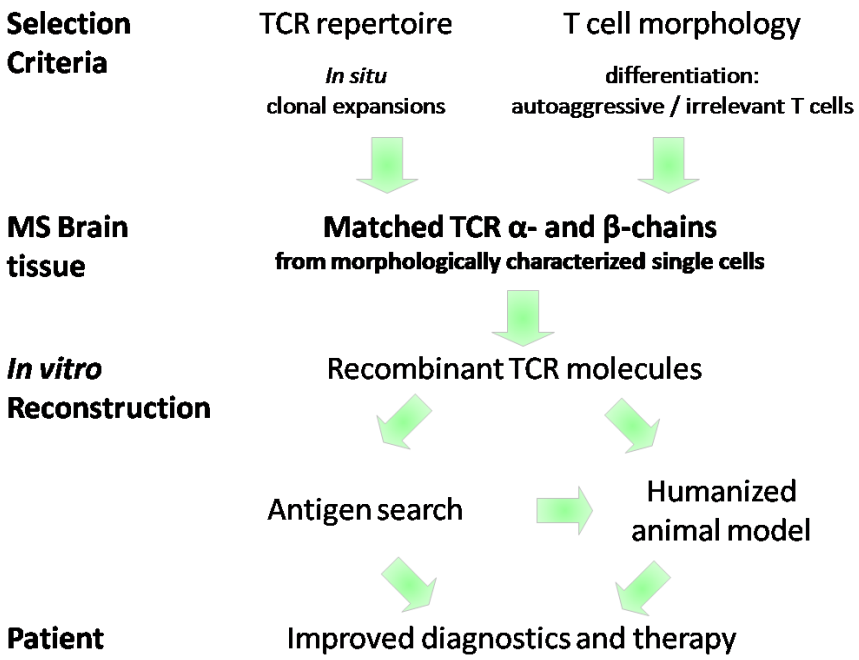


Fig. 1-6: Experimental approach to characterize autoaggressive T cells and their target(s)

CD8+ T cells are isolated from MS brain tissue on the basis of selection criteria that distinguish between autoaggressive and bystander cells. From these single cells, the matching α- and β-chains of the TCR may be characterized. These may be reconstructed as stably transfected recombinant TCR molecules in mouse cell lines that can be directly applied in antigen search. Alternatively, the recombinant TCR molecules may be reconstructed in an animal model of MS (EAE) to evaluate its contribution to the disease. Ultimately, all of these efforts would contribute to the understanding of the disease and could be relevant to the development of diagnostic tools and therapeutic options to combat MS. (Dornmair et al. 2003)

1.6.2 Antigen determination

T cell receptors of MS brain derived cells may be determined by different approaches. The first of these is the screening of candidate MS antigens (**Section 1.6.2.1**). Although this approach is focused and disease specific, it is restricted by the availability of patient matched antigen presenting cells. A second alternative would be the screening of patient derived full-length cDNA expression libraries as was done for the identification of melanoma associated tumor antigens (van der Bruggen et al. 2002) and other TCR antigens (Wong et al. 1999; Smith et al. 2001; Uenaka et al. 2003). Nevertheless, this method is also limited by the ability of the APC to correctly process the antigen. The need for accurate antigen processing may be overcome by the screening of peptide libraries. Hence, the third approach employs the screening of synthetic combinatorial peptide libraries in positional scanning format (PS-SCL) (**Section 1.7**). However

this method is successful only for polyspecific TCRs as a large number of peptides have to be recognized before a signal can be detected. The fourth approach involves the use of plasmid encoded combinatorial peptide libraries (Wang et al. 2005, Siewert et al. in press) wherein TCR mimotopes that activate the TCR are isolated and can reveal the naturally occurring ligand by peptide sequence database analysis (**Section 1.6.2.2**).

1.6.2.1 Screening of Candidate MS antigens

Various proteins are implicated in the immune pathogenesis of experimental autoimmune encephalitis (EAE), the animal model of MS. For example myelin oligodendrocyte protein (MOG) (Schluesener et al. 1987), myelin basic protein (MBP) (Eylar et al. 1970; Lennon et al. 1970) and neurofascin (Mathey et al. 2007; Hemmer et al. 2002). MS immunopathogenesis is also believed to have an association with viral infection, especially with the Epstein-Barr virus (EBV) (Sumaya et al. 1980; Bray et al. 1983; Lucas et al. 2011). Almost all MS patients are seropositive for EBV in contrast to only 90% of the healthy population. Moreover, EBV infected B cells were detected in the meninges of MS patients (Serafini et al. 2007). It is speculated that molecular mimicry might play a role in linking EBV to MS. The hypotheses is that after a microbial (EBV) infection, immune cells with specificities against the infectious agent might cross react with a similar epitope on healthy cells (of the CNS) leading to an autoimmune attack (Lucas et al. 2011). Candidate antigen screening is however restricted by the availability of the original HLA-matched antigen presenting cells. No other cell line would be capable of processing and presenting protein-derived peptides on MHC class I molecules (Ackerman and Cresswell 2004). To circumvent the need for patient matched APCs and correct antigen processing, one may screen unbiased peptide libraries (**Section 1.6.2.2**).

1.6.2.2 Unbiased plasmid encoded combinatorial peptide (PECP) libraries

Plasmid encoded combinatorial peptide (PECP) libraries code for peptides of fixed length that circumvent the need for intracellular protein processing. The PECP library contains mimotopes, or peptides that mimic the epitope recognized by the TCR. A recent method developed in our laboratory (Siewert et al., in press) describes the application of a PECP library in detecting single activated T cells in an APC-TCR hybridoma co-culture. The identification of different

mimotopes with converging motifs allows for the detection of the parent peptide antigen. The experimental approach is presented in **Figure 1-7**. The APCs (COS-7 cell line) that have been co-transfected with a plasmid encoded combinatorial peptide (PECP) library and MHC class I complementary DNA express the MHC-peptide complex on the cell surface. The TCR hybridoma coexpresses the TCR, CD8αβ and synthetic green fluorescent protein (sGFP) (Heim et al. 1995) under the regulation of NFAT. The sGFP protein is a modified version of GFP isolated from the jellyfish *Aequorea Victoria* where the serine residue at position 65 in the wild type is replaced with threonine. sGFP is observed to have a brighter fluorescence level than GFP (Heim et al. 1995). During co-culture, the TCR hybridoma cells that make contact with an APC presenting the correct antigen get activated, and turn bright green. The APC underlying the green TCR hybridoma is then isolated by a microcapillary pipette under a fluorescence microscope, and the plasmids coding for the antigenic peptide that it expresses can be isolated.

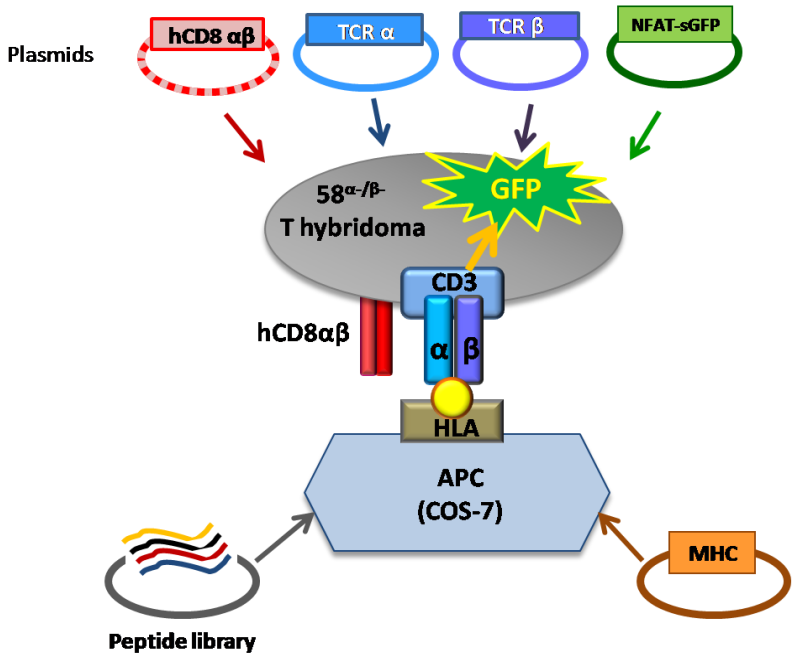


Fig. 1-7: Experimental strategy for antigen search using PECP libraries

First the TCR hybridoma cells (grey) are transfected with plasmids coding for TCR α-(blue) and β-(purple) chains (derived from T cell lines or clones), followed by the transfection of plasmids coding for the human CD8 α- and β-chains (pink, red) and sGFP (Heim et al. 1995) whose expression is regulated by a NFAT enhancer element (green). The antigen presenting cells (APCs) are COS-7 cells (blue). APCs are co-transfected with the suitable MHC molecule (brown) and a PECP library (peptide library). On presentation of the correct antigenic peptide (yellow), the TCR hybridoma expresses sGFP as a result of TCR activation, and is easily detected. (Based on Siewert et al., in press)

The application of PECP libraries in an unbiased screening of T cell antigens provides many advantages. First, it circumvents the need for intracellular APC specific protein processing. Second the antigen recognition mimics TCR recognition of a MHC-peptide complex. Third, low affinity TCR interactions can still be detected as the cells remain in close physical contact due to gravity. Most importantly, T cell activation can be detected at the level of single cells.

1.7 Anchor position determination

The anchor positions of a MHC molecule determine the peptides that it can efficiently bind to and present to the compatible TCR. The peptide ligands bound to the antigen presenting MHC molecules can be extracted and analyzed to reveal these anchor positions as done for endogenous ligands that are expressed by healthy cells (Falk et al. 1991; Rudensky et al. 1991), 'non self' ligands on infected cells (Rötzschke et al. 1990) or tumor cells (Schirle et al. 2000). The MHC molecules carrying bound peptides are purified from cellular lysates by immunoprecipitation, after which they undergo a mild acid treatment for peptide release. The released peptides can then be examined by Edman degradation (Edman 1950) or mass spectrometry. Edman degradation involves a sequential derivatization and removal of amino acids from the amino terminus, which are then identified by chromatography. Mass spectrometry can identify a peptide sample by determining the mass ratio of the ionized peptides. The detected ions are separated according to their mass-to-charge ratio, detected and processed into mass spectra. For MHC molecules where the anchor positions are not known, this would be a useful T cell independent approach.

1.8 Objectives

It has long been believed that CD8+ T cells play an important role in the immune mechanisms of the autoimmune disease MS. They are seen to infiltrate the brain in large numbers, and proliferate there giving rise to clonally expanded subpopulations. However, the processes that prompt this infiltration and the molecular target(s) of the autoaggressive T cells remain unknown.

In order to characterize potentially autoaggressive brain infiltrating CD8+ T cells from MS patients, this study had four objectives:

- The first objective was to identify the matching α- and β- TCR chains from brain infiltrating CD8+ effector T cells. This would include oligoclonal expansions as well as T cell clones that were activated *in situ* and provide a MS patient specific TCR repertoire from single, putatively autoaggressive T cells.

- The second objective was to reconstruct and recombinantly express these putatively autoagressive TCR molecules in mouse T hybridoma cell lines in order to employ them in cell based assays.

- The third objective was to search for the ligands of these TCR molecules using methods such as the screening of (i) candidate MS antigens, and (ii) unbiased plasmid encoded peptide libraries.

- The fourth objective was a TCR independent approach to characterize the anchor positions of antigen presenting molecules by screening a positional scanning combinatorial peptide library using recombinantly generated antigen presenting molecules.

2 Materials and Methods

2.1 Material

2.1.1 Chemicals and consumables

Chemicals were, unless mentioned otherwise, procured from the companies Merck (Darmstadt) and Sigma-Aldrich (Taufkirchen). Consumables such as pipette tips, reaction tubes and centrifuge tubes were obtained from Biozym (Hess, Oldendorf), Eppendorf (Hamburg) and Becton Dickinson (Heidelberg). Special tips and polypropylene tubes were ordered from the companies Biozym and Eppendorf. Cell culture dishes were from companies BD Falcon (Heidelberg), Corning (Wiesbaden) and Nunc (Langenselbold).

2.1.2 Oligonucleotides

All oligonucleotides were synthesized by the company Metabion (Martinsried) with the exception of the M13 primers (Invitrogen, Karlsruhe). All primers were obtained at a stock concentration of 100 µM. When primers had to be stored long prior to use, lyophilized primers were ordered and re-constituted in sterile buffer prior to use. The sequences and sources of all primers used in this study are listed in **Appendix I**.

2.1.3 Expression vectors

The expression vectors used are described in the relevant section. Vector maps are presented in **Appendix II**.

2.1.4 Antibodies

Specificity	Conjugated Fluorophore	Clone	Species/ Isotype	Concentration	Source
Mouse CD3e	-	145-2C11	Hamster/ IgG	0.5 mg/ml	eBioscience (Frankfurt)
Human CD8α	-	LT8	Mouse/ IgG1	1.0 mg/ml	AbD Serotec
Human CD8α	FITC	DK2F	Mouse/ IgG1k	2.0 mg/ml	DAKO (Glostrup, Denmark)
Human CD8β	-	2ST8.5H7	Mouse/ IgG2a	1.0 mg/ml	Beckman Coulter (Marseille, France)
Human CD134	FITC	ACT35	Mouse/ IgG1	50 µg/ml	BD Pharmingen
Human TCR Vβ 1	FITC	BL37.2	Rat/ IgG1	2.5 mg/ml	Beckman Coulter
Human β2 microglobulin	-	polyclonal	Mouse/ IgG	n.k	Abnova (Eching)
Human HLA-ABC	FITC/PE	W6/32	Mouse/ IgG2a	2.5 mg/ml	Beckman Coulter
c-myc	-	9E10.3	Mouse/ IgG1k	200 µg/ml	Millipore (USA)
6x His	-	HIS.H8	Mouse/ IgG2b	1.0 mg/ml	Abcam (Cambridge, UK)
Secondary antibodies					
Fluorescein	Alexa Fluor 488	polyclonal	Goat/ IgG	1 mg/ml	Molecular Probes (Leiden, The Netherlands)
Mouse	FITC	polyclonal	Goat/ F(ab')2	n.k	DAKO
Mouse	Peroxidase	polyclonal	Goat/ IgG + IgM (H+L)	n.k.	Dieter Jenne (MPI of Neurobiology)
Isotype Controls (IC)					
IC Mouse/ IgG1	-	X40	Mouse/ IgG1	50 µg/ml	Becton Dickinson
IC Mouse/ IgG2a	FITC	X39	Mouse/ IgG2a	50 µg/ml	Becton Dickinson

n.k: not known

MPI: Max Planck Institute

2.1.5 Patients and tissue samples

All specimens were characterized by genomic HLA typing at the Institute for Immunogenetics, Ludwig-Maximilians-Universität München (LMU). The HLA composition of the two patients analyzed in this study has been presented in **Table 2-1.**

Patient ID	HLA-				
	A*	B*	C*	DRB1*	DQB1*
FE	0101	0801	0701	0301	05
MS – 4	0201	0702	0702	04	0302
	0301	5101	1502	15	0602

Tab. 2-1: HLA composition of the MS patients

Column 1 displays the identification code (Patient ID) of the MS patients. This is followed by the HLA alleles determined by genomic HLA typing. Patient FE is seemingly homozygous for the haplotype as only one allele was detected for each of the loci analyzed. Patient MS-4 is heterozygous for all loci. (Skulina et al. 2004; Junker et al. 2007)

2.1.5.1 Biopsy case: Patient FE

2.1.5.1.1 Diagnosis

Patient FE, a male patient, was diagnosed at the age of 49 (1996) with a malignant glioma in the right temporooccipital lobe of the brain. The affected region was surgically removed and a detailed pathological examination resulted in the diagnosis of MS (Skulina et al. 2004). The tissue sample hails from a relatively early phase of the disease and data gathered might provide hints into the early events of pathogenesis. The patient is apparently homozygous for the MHC class I alleles (Skulina et al. 2004), meaning that the antigen must be researched in context of only three (instead of six) MHC class I molecules.

2.1.5.1.2 Samples

A part of the biopsy that was stored in liquid nitrogen was used in this analysis. In the course of this study, three tissue blocks (designated #9, #10 and #11) originating from the same lesion

were investigated. These blocks contained cortex or grey matter (GM), white matter (WM) and meninges.

2.1.5.1.3 TCR repertoire

Since biopsy acquisition in 1996, the TCR repertoire of this patient was studied in great detail in our laboratory (Skulina et al. 2004; Babbe et al. 2000). This is summarized in **Table 2-2.**

Single cell PCR (Babbe et al. 2000)			**CDR3-Spectratyping** (Skulina et al. 2004)					
Brain		**TCR BV CDR3 Sequence**	**Brain**	**CSF**	**PBL**			**PBL**
1996			**1996**	**2001**	**2001**			**2003***
Frequency of								
Par. CD8+ T cells	Per. CD8+ T cells				CD4	CD8	CD138	CD8
1/24	1/46	BV1-CAS-TPERDPS-NEQ-BJ2.1	+	-	-	-	-	-
1/24	**2/46**	**BV1-CASS-ISRKD-TQY-BJ2.3**	**+**	**(+)**	-	**+**	**+**	**+**
4/24	2/46	BV4-CS-VWEV-SGA-BJ2.6	+	+	-	+	-	-
2/24	-	BV13.1-CASS-LGA-DTQ-BJ2.3	+	-	-	-	-	-
2/24	1/46	BV13.2-CAS-RALVAT-YNE-BJ2.1	+	-	-	-	-	-
-	2/46	BV13.2-CASS-YP-GEL-BJ2.2	+	-	-	-	-	-
1/24	3/46	BV13.3-CASS-PGDRAQ-BJ2.1	+	+	-	**(+)**	-	-
1/24	1/46	BV14-CASS-PLWEGGIG-NTE-BJ1-1	+	+	-	+	-	-
3/24	-	BV22-CASS-EGAGEH-NEQ-BJ2.1	+	**(+)**	-	-	-	-

Tab. 2-2: Repertoire of clonally expanded β-chains in patient FE

+: readable sequence, (+): sequence of clonal expansion determined on an oligoclonal background, - : clone not identified by CDR3 spectratyping or direct sequencing, TCR: T cell receptor, BV: Variable region of the β-chain, BJ: Joining region of the β-chain, CSF: cerebrospinal fluid, PBL: peripheral blood lymphocytes, par.: parenchymal, per.: perivascular, CD4: helper T cell marker, CD8: effector T cell marker, CD138: cell proliferation and migration marker. (Babbe et al. 2000; Skulina et al. 2004). *Unpublished data from Klaus Dornmair and Joachim Malotka, MPI of Neurobiology.

The BV1-BJ2.3 expansion that was detected over a period of seven years in the patient could be expected to contribute to the disease. An analysis of the patient's peripheral blood lymphocytes yielded the sequence of a pairing AV7.2-AJ16 α-chain in six T cells (personal communication from Joachim Malotka and Klaus Dornmair, MPI of Neurobiology). In collaboration with David Laplaud and Katherina Siewert (MPI of Neurobiology), efforts were then made to confirm whether this TCR could also be detected in the brain tissue (**Section 3.1**).

2.1.5.2 Autopsy case: Patient MS-4

2.1.5.2.1 Diagnosis

The male patient was diagnosed at age 38 with primary progressive MS. The patient was affected with the disease for 4 years, and the cause of death was unknown. For this study, tissue blocks from the periventricular region (block #6: slowly expanding chronic lesion) and temporal white matter (block '9: inactive demyelinated lesion) were analyzed (Junker et al. 2007).

2.1.5.2.2 Samples

Tissue regions that included either visible lesions or normal appearing CNS tissue were dissected by the pathologist, snap frozen and stored at -80°C.

2.1.5.2.3 TCR repertoire

The analysis of the TCR repertoire in the four tissue blocks of patient MS-4 (Junker et al. 2007) revealed the presence of some clonal expansions, such as the BV1-BJ1.1 β-chain that was found in more than one tisuue block (blocks 6, 7 and 9) and may be termed as 'pervasive'. The repertoire of clonally expanded TCR β-chains is shown in **Table 2-3**. Such clonal expansions were equally interesting for further characterization of the pairing α-chain.

CDR3-Spectratyping					
Lesion number				NAWM	TCR BV CDR3 Sequence
5	6	7	9	12	
-	+	+	+	-	**BV1-CASS-VGDVRQM-NTEAFF-BJ1.1**
*	+	+	+	(+)	BV4-CS-VGTGGVGSAG-TNEKLF-BJ1.4
-	+	+	+	*	BV4-CS-ATGDRG-SNQPQHF-BJ1.5
+	+	+	+	+	BV4-CS-TQTGT-GANVLTF-BJ2.6
-	+	+	+	-	BV6-CASS-LGTGM-NTEAFF-BJ1.1
(+)	+	(+)	+	+	BV15-CATS-PGGQI-YGYTF-BJ1.2

Tab. 2-3: Repertoire of clonally expanded TCR β-chains in patient MS-4

TCR: T cell receptor, BV: Variable region of the β-chain, BJ: Joining region of the β-chain, NAWM: Normal appearing white matter, +: monoclonal expansion found at two or more anatomical site, (+): Oligo- or polyclonal expansion, *: NDN-sequence different from the pervasive clone, - : absence of PCR product in spectratyping reaction, Modified from Fig. 4, Junker et al. 2007

2.1.6 Laboratory Equipment

Autoclave	Varioklav Type 500 (liquids)	H+P Labortechnik (Oberschleißheim, Germany)
Autoclave	Webeco Type A5 (consumables)	Webeco (Selmsdorf, Germany)
Balance	L2200P	Sartorius (Göttingen, Germany)
Balance	2001 MP2	Sartorius
Centrifuge	5417 C	Eppendorf (Hamburg, Germany)
Centrifuge	5417 R	Eppendorf
Centrifuge	Heraeus® Megafuge 1.0R	Thermo Fisher Scientific (Schwerte, Germany)
Centrifuge	Sorvall RC-5C PLUS	Thermo Fisher Scientific
Cryotome	CM3050	Leica (Wetzlar, Germany)
DNA gel imager	Gel DocTM XR	Bio-Rad (München, Germany)
Incubator	Thermomixer 5436 (reaction tubes)	Eppendorf
Incubator	BBD 6220 (eukaryotic cell culture)	Heraeus (Hanau, Germany)
Incubator	Jouan EB 53 (bacterial cultures)	Thermo Fisher Scientific
Microinjector	CellTramVario	Eppendorf
Micromanipulator	LN25 Mini	Luigs und Neumann (Ratingen, Germany**)**
Microscope	AxioVert 200M	Zeiss (Munich, Germany)
Microscope	AxioVert 25	Zeiss
Microscope	Microbeam-Z	Zeiss
Microscope	Axio Observer	
Water preparation	Milli-Q Advantage	Millipore (Schwalbach, Germany)
ELISA-Reader	MR 4000	Dynatech (Rückersdorf, Germany)
Flow cytometer	FACS Calibur	Becton Dickinson (Heidelberg, Germany)
Flow cell sorter	FACS Sorter FACS Vantage SE	Becton Dickinson
Freezing box		Nalgene (Roskilde, Denmark)

Gel electrophoresis chambers		MPI of Neurobiology workshop
Gel electrophoresis power supply		GE Healthcare (Munich, Germany)
Electroporation device	Genepulser	Bio-Rad
Electroporation device	Micropulser	Bio-Rad
Electroporation device	Multiporator	Eppendorf
Cell counting chamber	Neubauer	Roth (Karlsruhe, Germany)
PCR cycler	GeneAmp PCR system 9600	Perkin Elmer (Rodgau, Germany)
pH-meter	pH521	WTW (Weilheim, Germany)
Pipettes		Gilson or Eppendorf
Photometer	Gene Quant II	Pharmacia Biotech
UV-Vis spectrophotometer	Nanodrop 1000	Thermo Fisher Scientific

Equipment or devices that were utilized exclusively for specific experiments are mentioned in the relevant section.

2.2 Microbiological methods

2.2.1 Preparation of electrocompetent *E. coli*

Electrocompetent *E. coli* are a prerequisite for the transformation of bacteria by electroporation. First, a pre-culture was prepared by inoculating 'MAX Efficiency® DH5αF'IQ^{TM,} *E. coli* in 10 ml LB-medium supplemented with 50 µg/ml Kanamycin and grown overnight at 37 °C in the shaker incubator. Then, 6 ml of this was further inoculated into a half liter volume of the same media and incubated until an optical density of 0.5 was measured. The growth curve was monitored at 600 nm. After this, the bacteria were concentrated to a 1 ml volume by a four course centrifugation (rotor SLA-1500: 4.000 rpm, 5 min, 4 °C) followed by resuspension of the sediment in sterile 10 % Glycerol pH 7.0 (Carl Roth, Karlsruhe) in a stepwise manner. Finally, the competent cells were distributed as 50 µl aliquots and stored at -80 °C. The transformation efficiency of each charge was tested by transforming 10 pg of pUC DNA per 50 µl cells as explained in **Section 2.2.2**. This is defined as the number of visible colonies per microgram of transformed DNA and was measured within a range of 10^8 und 10^9.

- MAX Efficiency® DH5αF'IQTM competent cells (Invitrogen)
- LB medium (Luria-Bertani)
  1 % (w/v) Bacto tryptone (Becton Dickinson), 0.5 % (w/v) yeast extract (Becton Dickinson), 0.5 % (w/v) NaCl, autoclaved

2.2.2 Bacterial transformation by electroporation

Electrocompetent DH5α-bacteria from **Section 2.2.1** were transformed with plasmid DNA via electroporation. During this process, the applied electric field leads to a short term permeabilisation of the bacterial cell membrane, allowing for the entry of plasmid DNA into the cell. In a typical experiment, 1.8 µl of the ligation reaction was added to 50 µl of the electrocompetent cells in a pre-cooled electroporation cuvette (Bio-Rad, 0.2 cm). Care was taken

to avoid the formation of air bubbles. After the electric shock using the 'Gene Pulser II' (machine settings: 2,5 kV, 25 µFD, 200 Ω) the cells were allowed to recover in 1 ml LB medium for 1 hour in the shaker incubator at 37°C. The bacteria were then plated on ampicillin (Amp) supplemented LB agar for the selection of positive clones, and grown overnight at 37°C. pUC DNA was used as a positive control, and the empty (lacking insert) linearized vector was used as a negative control.

The plasmid encoded peptide (PECP) libraries were maintained in electrocompetent ElectroMax DH10B™T1R *E. coli*. This electroporation procedure was optimized in the laboratory for best results. 50 µl of the bacteria were mixed with the purified ligation product (**Section 2.4.3.6**) and transferred, without bubble formation, to a pre-cooled cuvette (Bio-Rad, 0.1 cm). The electroporation was carried out at 2.5 kV as before. The bacteria were immediately re-suspended in 1 ml of pre-warmed SOC media and allowed to recover for 60 minutes at 37°C in the shaker incubator. Post recovery, the bacteria were expanded overnight in a 1000-fold greater volume, i.e. 50 ml for 50 µl, of LB agar (+Amp) media. This is of vital importance as the ElectroMax DH10B™T1R cells are provided at a very high density of 2.8 x 10^{11}/ml, as determined in the laboratory (by $OD_{600\ nm}$ measurements and plating), which demands a starting $OD_{600\ nm} \leq 0.2$ (OD = optical density) that would ensure that each individual clone was well represented in the final population. The number of individual clones present in the library were determined by plating aliquots (1/1000 and 1/500,000) of the transformation mixture on to LB agar (+Amp) plates (explained in **Section 2.2.4**). The empty linearized vector was used as a negative control.

- ElectroMax DH10B™T1R *E. coli* (Invitrogen)
- LB agar (+Amp)
  LB medium with 1.5 % (w/v) bacto agar (Becton Dickinson); autoclaved; (+ 100 µg/ml ampicillin)

2.2.3 Bacterial transformation by heat shock

The chemically competent 'One Shot® TOP10' *E. coli* strain was routinely used in TOPO-TA cloning. Once the bacterial aliquot was thawed on ice, 2 µl DNA was added and this mix was further incubated for 30 minutes on ice to allow the DNA to attach itself to the bacterial cell membrane. This was followed by a 30 second heat shock in a water bath that permeabilises the

cell membrane for a short duration and allows for cellular entry of DNA. Following 20 minute incubation on ice, the transformed bacteria were inoculated into pre-warmed S.O.C medium and grown in the shaker incubator at 37 °C for 1 hour. The pCR®2.1-TOPO vector renders the bacterial clones resistant to ampicillin. In addition, one may carry out a 'blue-white colony' selection by supplementing the LB (+Amp) agar plate with 40 µl X-Gal stock solution. The enzyme ß-galactosidase, coded by the lac-Z gene catabolizes the X-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopranoside) into galactose and indoxyl. Indoxyl gets oxidized to an indigo blue product that renders color to the cellular cytoplasm. The insertion of a PCR product into the pCR®2.1-TOPO vector interrupts the lacZ gene. As a result, the positive colonies are white in color.

- One Shot® TOP 10 *E. coli* (Invitrogen)
- pCR®2.1-TOPO
  3.1 kb, Kan^R, Amp^R, for vector map refer to **Appendix II**
- X-Gal stock solution (40 mg/ml):
  400 mg X-Gal in 10 ml dimethylformamide (DMF); sterile filtered and stored at -20 °C

2.2.4 Determination of bacterial cell numbers

Bacterial cell numbers were determined in parallel by $OD_{600\ nm}$ measurements and plating the bacteria on LB agar plates. The OD_{600nm} measurements were recorded on the UV-VISIBLE Recording spectrophotometer. A value of 1.0 corresponds to a bacterial population of $1x\ 10^9$ cells /ml and the apparatus is most accurate for values ranging from 0.1 to 0.5. Based on these observations, the bacteria were diluted to achieve desired concentrations. This was further verified by plating defined bacterial aliquots on LB-agar (+Amp) plates, growing overnight at 37°C and then counting the number of appearing clones.

2.2.5 Long term storage of bacterial cultures: glycerol stocks

Bacterial cultures were mixed homogenously with sterile glycerol in a 1:1 proportion, distributed in cryotubes and stored at -80°C.

2.3 Cell-culture based methods

2.3.1 Cultivation of cell lines

2.3.1.1 Cell line COS-7

The COS-7 cells were used as antigen presenting cells in antigen search experiments. They were stably transfected with human MR1 (pHSE3' expression vector) and transiently transfected with plasmid encoding peptide libraries (pcDNA6/-His A expression vector). COS-7 is an adherent, fibroblast-like, african green monkey kidney cell line that has been immortalized by transformation with the simian vacuolating virus 40 (SV40) that is able to produce the large T antigen. Consequently, when a vector such as the pcDNA plasmid bearing the SV40 promoter is introduced into the COS-7 cell, it can be replicated to a great extent. The cells were cultivated in RPMI complete media at 37°C under 5% CO_2 in different appropriate tissue culture vessels. During routine cell culture, the cells were detached from the vessel surface using trypsin preceded by a 1X phosphate buffered saline (PBS) wash.

- Cell line 'COS-7'
  ATCC number: CRL-1651 (Gluzman, 1981)
- pHSE3'-MR1
  10.2 kb, Amp^R, Neo^R, for vector map refer to **Appendix II**. Provided by Wakiro Sato, MPI of Neurobiology (Pircher et al. 1989)
- pcDNA6/V5-HisA (Invitrogen)
  5.1 kb, Amp^R, $Blasticidin^R$, for vector map refer to **Appendix II**
- RPMI complete medium
  RPMI 1640, 10 % FCS (Sigma, Deisenhofen, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 1X non-essential amino acids, (Invitrogen, Karlsruhe, Germany)
- 1X PBS
  150 mM NaCl, 8.4 mM Na_2HPO_4, 2 mM NaH_2PO_4
- Trypsin :
  Trypsin/EDTA 1X (Invitrogen)

2.3.1.2 Cell line $58^{-/-}$

$58^{-/-}$ is a TCR deficient, mouse T hybridoma cell line derived from the BW5147 thymoma cell line that lacks inherent functional TCR chains. Hence, it is a suitable recipient cell line for the expression of human TCRs. The cell line is cultivated in suspension in the RPMI complete medium. In addition to the two TCR chains cloned into pRSV vectors, this cell line was also used for the simultaneous expression of the human CD8 molecule (pLPC-hCD8α-IRES2-hCD8β expression vector) and sGFP under NFAT regulation (pcDNA-NFAT-sGFP expression vector). Each construct was selected for and maintained within the cells under antibiotic selection as summarized in **Table 2-4**.

Expression vector*	Antibiotic selection	Stock conc. (mg/ml)	Selection conc. (µg/ml)	Maintenance conc. (µg/ml)
TRAV pRSVhygro	Hygromycin (Invitrogen)	50	500-1000	300
TRBV pRSV5.neo	Geneticin (Invitrogen)	100	2000-3000	1500
pLPC-hCD8α-IRES2-hCD8β	Puromycin (Biomol)	1	2-4	1
pcDNA-NFAT-sGFP	Blasticidin (Invitrogen)	10	2-4	3

Tab. 2-4: Antibiotics used for selection in eukaryotic cell culture

The $58^{-/-}$ cells transfected with the expression vectors mentioned in the first column were cultured in a cocktail of four antibiotics to maintain the stable expression (**Section 2.3.3**). TRAV: TCR α-chain, TRBV: TCR β-chain, Stock conc.: the conc. at which the antibiotics are stored for long term; Selection conc.: antibiotic conc. at which clones are selected post transfection; Maintenance conc.: antibiotic conc. at which the selected clones are propagated for routine experiments; conc.: concentration. *Vector maps presented in **Appendix II**

- Cell line '$58^{-/-}$'
  Provided by Klaus Dornmair, MPI of Neurobiology, (Letourneur and Malissen, 1989)

- pRSVhygro
  5.9 kb, $Hygro^R$, Amp^R, for vector map refer to **Appendix II**, provided by Joachim Malotka, MPI of Neurobiology

- pRSV.neo
  6.1 kb, Neo^R, Amp^R, for vector map refer to **Appendix II**, provided by Joachim Malotka, MPI of Neurobiology

- pLPC-hCD8α-IRES2-hCD8β
  $Puro^R$, provided by Klaus Dornmair, MPI of Neurobiology

- pcDNA-NFAT-sGFP
  5.8 kb, Amp^R, $Blasticidin^R$, generated by Michael Ackman and Daniela Hackl, provided by Klaus Dornmair, MPI of Neurobiology

2.3.1.3 Cell line HEK293E

The HEK293E cell line was used for the expression of recombinant human MR1 and β2 microglobulin. It is a modified human embryonic kidney cell line that grows in suspension in serum free media. The cells are stably transfected with a plasmid coding for the nuclear EBV-antigen 1 (EBNA-1). EBNA-1 is required for the episomal replication of plasmids that carry the EBV specific replication of origin 'oriP'(Hennessey et al. 1983). The plasmid also carries a geneticin (G-418) resistance marker for maintenance of the plasmid within the cells. The pTT5-vector employed in the experiments carries not only the 'oriP', but also a promoter of the human cytomegalovirus (CMV). This genetic combination, in addition to the constitutive expression of the adenoviral protein E1a that transactivates the CMV promoter (Gorman et al. 1989), facilitates high expression rates. These factors make this cell line an ideal high protein expression system. All steps that involved cell line handling were carried out aseptically in the 'LaminAir®'cell culture hood.

- Cell line 'HEK293E Large Scale Transient Expression System'
- pTT5 (NRC Biotechnology Research Institute)
  4401 bp, Amp^R, for vector map refer to **Appendix II,** patented by Yves Durocher, NRC Biotechnology Research Institute, Ottawa, Ontario, CA, provided by Dieter Jenne (License holder), MPI of Neurobiology
- Cell culture medium
  GIBCO® FreeStyle™ 293 expression medium with 0.1 % (v/v) Pluronic® F-68 (Invitrogen) and 25 µg/ml G-418 sulfate (PAA laboratories, Cölbe, Germany)

2.3.2 Determination of cell count and viability

The viable cell count was determined by mixing the cell suspension and trypan blue solution in a 1:1 ratio and placing this mixture within a 'improved Neubauer' cell counting chamber. The perforated membranes of dead cells allow the trypan blue to enter these cells, rendering them blue while live cells exclude the dye. Live cells in the four large quadrants are counted under a compound microscope; the average of this value is multiplied by the dilution factor and then adjusted by the cell counting chamber constant 10^4. The resulting value describes the cells/ml in the suspension.

- Trypan blue solution (0.1 %)
  0.4 % Trypan blue solution 1:4 in 1X PBS pH 7.4; sterile filtered

2.3.3 Transfection of eukaryotic cells: stable protein expression

The $58^{-/-}$ cell lines were transfected for the stable expression of TCR molecules. Electroporation or Fugene HD reagent was used as a means of transfection. The plasmid DNA was linearized prior to transfection to facilitate chromosomal integration, and eventually, stable transfectants. The rare cutter restriction enzymes *Xmn*1 and *Nde*1 were used to linearize the TCR chains cloned into the pRSV vector. The linearized DNA was precipitated in ethanol at -80°C and then re-suspended in sterile water.

For electroporation, cells were harvested during the logarithmic growth phase and washed twice in non-supplemented RPMI. When using the GenePulser the cells were resuspended in non-supplemented RPMI, while in case of the multiporator the provided hypoosmolar buffer was used. To 0.8-8 x 10^6 cells, 16 µg of plasmid DNA (in 10 mM Tris-HCl, pH 8.5) was added and this suspension was pipetted into 0.4 cm electroporation cuvettes suited to the device. An electric pulse was applied at 280V, 960 µF for the GenePulser and 1200 V, 40 ms for the multiporator at room temperature. Following the pulse, the cells were immediately re-suspended in pre-warmed RPMI complete media, seeded to the desired density and grown under regular cell culture conditions.

Transfection by Fugene HD was done following the manufacturer's recommendations. To 2 µg of DNA in 100 µl of RPMI, 7 µl of Fugene HD was added. The transfection complex was mixed shortly, incubated for 15 minutes at room temperature and then 25 µl of it was added to one well of a 24-well plate that had been previously seeded with cells (100,000 cells / well). These parameters were followed for the up- and down scaling of similar experiments. Antibiotic selection was applied 2 days post transfection. The minimum amount of antibiotic used for this purpose was determined by varying antibiotic concentrations and plotting a 'killing curve'. These values are presented in **Table 2-4**. The stably transfected cell lines employed in this study are listed in **Table 2-5**.

Cell line	Transfected molecules	Application of cell line
Human TCRs transfected in the 58-/- T hybridoma cell line		
58-Fe-BV1-AV7.2-AJ16	TCRα-chain: AV7.2-AJ16 TCRβ-chain: BV1-BJ2.3 Human CD8αβ, NFAT-sGFP	TCR expression for antigen determination
58-Fe-BV1-AV7.2-AJ24.2	TCRα-chain:AV7.2-AJ24.2 TCRβ-chain: BV1-BJ2.3 Human CD8αβ, NFAT-sGFP	TCR expression for antigen determination
58-Fe-BV1-AV7.2-AJ33	TCRα-chain:AV7.2-AJ33 TCRβ-chain: BV1-BJ2.3 Human CD8αβ, NFAT-sGFP	TCR expression for antigen determination
58-Fe-BV1-AV7.2-AJ54	TCRα-chain:AV7.2-AJ54 TCRβ-chain: BV1-BJ2.3 Human CD8αβ, NFAT-sGFP	TCR expression for antigen determination
58-Fe-BV8.1-AV30.1	TCRα-chain: AV30.1-AJ18 TCRβ-chain:BV8.1-BJ1.1 Human CD8αβ	TCR expression for antigen determination
Human MR1 transfected in the COS-7 cell line*		
COS-7-hMR1*	Human MR1	Antigen presenting cell in antigen determination experiments

Tab. 2-5: Cell lines generated by stable transfection

TCR: T cell receptor, BV: TCR β-chain variable region, BJ: TCRβ-chain joining region; AV: TCR α-chain variable region, AJ: TCRα-chain joining region; hMR1: human MHC-related protein 1, *this cell line was provided by Wakiro Sato, MPI of Neurobiology

2.3.4 Transfection of eukaryotic cells: transient protein expression

The HEK293E cells were transfected for the transient expression (Durocher et al. 2002) of β2 microglobulin associated human MR1 (hMR1). The cells were diluted in pre-warmed media to a concentration of 10^6 cells / ml an hour prior to transfection. 1 µg plasmid DNA (precipitated in ethanol and re-suspended in sterile water) was transfected per ml of this cell suspension, i.e. $100x10^6$ cells were transfected with a combination of 50 µg of the hMR1-pTT5 expression vector and 50 µg of the β2 microglobulin-pTT5 expression vector (for vector maps see

Appendix II). The transfection reagent polyethylenimine (PEI) was used at a final concentration of 2 µg/ml (Boussif et al. 1995). The positively charged PEI binds to the negatively charged phosphate backbone of plasmid DNA, and this complex is then endocytosed into the cell (Godbey et al. 1999). First, 1 ml PEI was diluted in 4 ml OptiPro^{TM} SFM. At the same time, the plasmid DNA was diluted in OptiPro^{TM} SFM to a final volume of 5 ml. The PEI was then added to the DNA and this mixture was incubated for 30 minutes at room temperature (RT). Finally, this transfection complex was added in a drop wise manner to the cells. 0.5 % (w/v) Tryptone was added to the cells 24 hours after transfection. This protein hydrolysate is broken down to free amino acids by proteases and can be directly taken up by the cells for protein biosynthesis (Heidemann et al. 2000). In addition, it can also be removed easily from the cellular supernatant during protein purification (Pham et al. 2003). The supernatant was harvested 110 hours post transfection by centrifugation for 5 minutes, 1500 rpm at 4°C. Further protein purification steps are elaborated in **Section 2.5.2**. The plasmid encoded peptide libraries were transiently expressed in COS-7 cells using electroporation and FugeneHD as outlined in **Section 2.3.4.**

- PEI stock solution (1 mg/ml)
  500 mg 25 kDa linear PEI (Polysciences Inc. Warrington, Eppelheim) dissolved in H_2O at pH 2, adjusted to pH 7 with NaOH; diluted to 500 ml with H_2O, sterile filtered
- Tryptone stock solution (5 %)
  50 g Bacto TC lactalbumin (Becton Dickinson) in 1 L H_2O, sterile filtered

2.3.5 T hybridoma cell activation assay

2.3.5.1 T hybridoma activation by CD3 cross-linking

The CD3e antibody (clone 145-2C11) was used to determine the functionality of the recombinant TCR *in vitro*. TCR activation and subsequent downstream signaling (Call et al. 2002) leads to the nuclear transport and accumulation of nuclear factor of activated T cells (NFAT) that further results in interleukin 2 (IL-2) secretion. The activation status can be determined by assaying secreted IL-2 or by a NFAT driven GFP production that renders the activated cell fluorescent. The TCR antigen would display a similar effect. The CD3e antibody (1 µg/ml in PBS) or antigens to be tested were coated onto a 96 well cell culture plate by

incubation at 37°C for 3 hours. The plate was rinsed with 200 µl PBS and 40,000 hybridoma cells were added per well. After 16 hour incubation at 37°C, the samples were assayed for activation by IL-2 detection in cellular supernatant or fluorescence microscopy for the presence of GFP+ cells as explained in following **Section 2.3.5.2** and **Section 2.3.5.3**.

2.3.5.2 T hybridoma activation by antigenic stimulus

MS patient derived TCRs may be tested for their reactivity to brain tissue sections **(2.3.5.2.1**) and extracts (**2.3.5.2.2**). Similarly, candidate antigens that are observed to cause the MS equivalent (experimental autoimmune encephalitis or EAE) in animal models may be tested. These include autologous and Epstein-Barr virus (EBV) antigens (**2.3.5.2.3**), as well as myelin derived proteins (**2.3.5.2.4**).

The MAIT TCR derived from patient FE was tested as described above using antigen presenting cells isolated from a HLA-matched donor. Even though the antigen-restriction element of the MAIT TCR is thought to be MR1, APCs that specifically expressed MR1 were not used. This is because this series of experiments was performed at an early stage when it was not known that the AV7.2-AJ33 was the invariant MAIT α-chain.

2.3.5.2.1 Human brain tissue

To test whether the putatively autoaggressive TCRs recognized an antigen that was specific to the patient, or even to MS, the TCR expressing hybridoma cells were co-incubated with brain tissue and the resulting activation was measured. For this, tissue derived from the same MS patient, an unrelated MS patient and a meningitis patient was tested. 10 µm sections of each sample were placed on UV-illuminated glass cover slips that had been coated with poly-L-lysine (50-100 µl/slide). The slide was air dried for 10 minutes and then placed into a 3.5 cm culture dish containing RPMI complete medium to which 1.5 x 10^6 hybridoma cells were added. The set up was incubated for 16 hours at 37°C, and the number of activated cells was counted manually under a fluorescence microscope. In an additional experiment, a pan-MHC class I antibody, (clone W6/32) (1:50 dilution) was used to block MHC class I mediated antigen presentation and

eventual hybridoma activation. This was done to verify whether the activated cells were indeed a function of MHC-peptide complex recognition. The antibody was added to the culture media and incubated on wet ice for 30 minutes prior to the addition of hybridoma cells.

2.3.5.2.2 Human brain extracts

The activation of TCR hybridoma cells was tested in the context of grey matter (GM) and white matter (WM) homogenates as well as their respective glycoprotein fractions. These brain extracts were provided by Prof Meinl (MPI of Neurobiology). A fraction of peripheral blood lymphocytes containing dendritic cells and macrophages from a HLA-matched donor were used as antigen presenting cells (APCs). These were isolated from blood as follows. 40 ml of freshly drawn blood was diluted with 20 ml PBS and applied onto 15 ml of a 'PANCOLL human' separating agent solution. This mixture was centrifuged (500 g, 25 minutes, RT) for the separation of the PBMC fraction. The PBMCs were carefully removed, and the residual fraction containing the dendritic cells was washed with PBS. The cells were counted and seeded at a density of 100,000 cells per well on a 96-well plate. After an overnight incubation at 37°C, the immature dendritic cells remained adherent to the cell culture plates, and the supernatant was removed. The brain extracts were added and incubated for 6 hours, during which the APCs were given sufficient time to take up the extracts by endocytosis, process the antigens and present them appropriately. This was followed by a wash and addition of hybridoma cells. 16 hours after co-culture, the cellular supernatant was assayed for IL-2 as explained in **Section 2.3.5.3.1**.

- PANCOLL human separating agent solution
  PAN Biotech GmBh, Aidenbach, Germany

2.3.5.2.3 Autologous and Epstein-Barr Virus (EBV) antigens

To test whether the TCR hybridoma recognize 'self' antigens or antigens derived from the Epstein-Barr Virus, they were co-cultured with autologous EBV-transformed B cells of patient FE. 100,000 EBV transformed B cells were seeded per well of a 96-well plate in RPMI medium. The lytic cycle of replication was induced by transfecting the cells with the PZLF1 and BRLF1 plasmids (provided by Prof. Miller, Yale University). The T hybridoma cells were added 48

hours post transfection. 16 hours after co-culture, the cellular supernatant was assayed for IL-2 as outlined in **Section 2.3.5.3.1**.

2.3.5.2.4 Myelin derived antigens

The reactivity of the MS derived TCR to myelin proteins such as myelin oligodendrocyte protein (MOG) (Schluesener et al. 1987), myelin basic protein (MBP) (Eylar et al. 1970; Lennon et al. 1970) and neurofascin (Mathey et al. 2007) was examined. These proteins were provided by Judy Ng and Prof Meinl (MPI of Neurobiology). HLA-matched antigen presenting cells from a healthy donor were used as explained in **Section 2.3.5.2.2**. To these cells 5 mg of each protein was added and incubated for 6 hours. Then the T hybridoma cells were co-cultured for 16 hours, after which the cellular supernatant was assayed for secreted IL-2 as described in **Section 2.3.5.3.1**.

2.3.5.3 Readouts for T hybridoma activation assay

2.3.5.3.1 IL-2 ELISA

IL-2 is a leukocytotrophic signaling molecule that plays a role in the development of the immunologic memory of cytotoxic T cells. Physiologically, it is secreted during T cell response to antigenic challenges such as a microbial infection. This secreted IL-2 could be assayed in the cellular supernatants by the IL-2 enzyme linked immunosorbent assay (ELISA) kit. The manufacturer's instructions were followed while performing the assay. For each ELISA a standard curve with IL-2 was performed with concentrations ranging from 5-1,000 pg/ml. The detection limit of the kit was 5 pg/ml or 0.25 pg/ well.

- IL-2 ELISA kit
  Mouse IL-2 ELISA Ready-SET-Go, eBioscience
  50 µl of cellular supernatant was assayed for each sample

2.3.5.3.2 Fluorescence microscopy

On antigenic encounter, also simulated by CD3 cross-linking, the TCR expressing hybridoma cells express green fluorescent protein (GFP) that was best observed after 16 hours incubation at

37°C. GFP (238 amino acids, 26.9 KDa) displays green fluorescence when excited with blue light (emission at 498/516 nm). This fluorescence can be clearly detected on a fluorescence microscope, and even measured by flow cytometry (**Section 2.6.1**) by using a GFP filter. The details of the microscope set up are listed below.

- Microscope
  Axiovert 200M, Zeiss
- CCD camera
  CoolSNAP-HQ, Roper Scientific
- Fluorescence lamp
  HXP 120, Visitron
- Objectives
  5x, NA 0.15; ∞/0, Epiplan-NEOFLUAR
  10x, NA 0.45 Plan apochromat
  20x, NA 0.4; ∞/0-1.5 Achroplan, condenser Ph2
- Fluorescence filters
  Cy3 filter - excitation/emission: 545(25)/605(70) nm, Zeiss
  GFP filter - excitation/emission at 472(30)/520(35) nm, Semrock, BrightLine
- Automated scan system
  BioPresision2, Visitron
- Image acquisition and analysis software
  MetaMorph-Software, V7.7

2.3.6 Long term storage of eukaryotic cell lines

Eukaryotic cell lines were frozen at -80°C and then transferred to liquid nitrogen for long term storage. The cells were counted and then centrifuged for 6 minutes, 1200 rpm at 4°C. Dimethylsulfoxide (DMSO) used in the freezing media functions as a protective agent that hinders ice crystal formation and eventual cell destruction. The freezing media and cell number vary depending on the cell line being handled. This has been clarified in **Table 2-6** for the cell lines employed in this study. The cells were revived by thawing at 37°C in a water bath, followed by centrifugation for the removal of DMSO, and a wash with serum free media before re-suspension in the suitable growth media.

Cell line	Freezing media	Cells frozen /ml of freezing media
COS-7	Fetal calf serum with 10 % (v/v) DMSO (Sigma)	0.5 to 5 x 10^6
58 $^{-/-}$	FCS with 10 % (v/v) DMSO	1 to 5 x 10^6
HEK293E	GIBCO® FreeStyle™ 293 expression medium with 10 % (v/v) DMSO	1 x 10^7

Tab. 2-6: Specifications for freezing of eukaryotic cell lines

FCS is routinely used as a freezing medium, but has to be excluded in the case of HEK293E cells which are cultivated under serum free conditions.

2.4 Molecular biology based methods

2.4.1 RNA isolation

The RNA isolated from live TCR expressing hybridoma cells was used as a template to verify the expression of the transfected TCR α-chain as antibodies specific to the TCR were commercially unavailable. For this purpose, the TRIzol® reagent was used and the manufacturer's instructions were followed at each step. To 1 x 10^7 hybridoma cells, 0.75 ml of the reagent was added, and this led to a yield of 5 µg/µl total RNA. The reverse transcription (RT) reaction is outlined in **Section 2.4.3.1**.

- TRIzol® reagent
  Ambion, Austin, USA

2.4.2 DNA isolation

2.4.2.1 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* cultures using commercially available kits and the manufacturer's instructions were followed. The method is based on the alkaline lysis of the bacteria. Details are presented in **Table 2-7.**

Vol. of starting culture	Plasmid isolation kit	Vol. of isolated plasmids
1-3 ml	Spin miniprep kit	30 µl
250-500 ml	Plasmid maxi kit	200-300 µl

Tab. 2-7: Plasmid isolation kits
The bacteria were grown overnight in LB (+Amp). After precipitation, the plasmids were re-suspended in EB buffer (10 mM Tris, pH 7.5-8, 5, Qiagen). This volume has been mentioned in the last column.

2.4.2.2 Agarose gel electrophoresis

In the presence of an electric field, DNA migrates towards the anode due to its negatively charged phosphate groups. This property allows for the separation of DNA fragments on the

basis of size in a gel matrix. The agarose gel is prepared in TBE buffer with 0.5 µg/ml ethidium bromide (EtBr). The EtBr intercalates between the double helices of the DNA and being fluorescent under UV (λ = 254 to 366 nm) it is used to visualize the DNA. The size of the migrating DNA can be determined by direct comparison to DNA markers of varied sizes such as 50 bp, 100 bp and 1 kb The samples were mixed in loading buffer and then run at 180 V for gels of 250 ml and at 90 V for 45 ml gels.

- DNA loading buffer (6x)
  50 % (v/v) glycerol, 0.02 % (w/v) bromphenol blue (Serva, Heidelberg)
  0.02 % (w/v) xylenecyanol (Bio-Rad), 10 mM Tris, pH 7.5
- TBE buffer (10x):
  0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA (Bio-Rad), pH 8.0
- Ethidium bromide stock solution (1 mg/ml)
  1 % ethidium bromide solution (Carl Roth) 1:10 in H_2O
- DNA markers
  50 bp (Peqlab, Erlangen), 100 bp or 1 kb (New England BioLabs, Frankfurt)

2.4.2.3 DNA extraction from agarose gels

DNA fragments were visualized under a UV transilluminator and excised with a scalpel. They were extracted using commercially available kits in accordance with the manufacturer's instructions.

- Easypure® DNA Purification Kit(Biozym)
  10 µl elution volume
- QIAquick Gel Extraction Kit (Qiagen)
  30 µl elution volume

2.4.2.4 DNA precipitation

To increase the concentration of the sample, or exchange buffer, DNA samples were routinely precipitated in ethanol. Smaller samples (1 µg and below) were precipitated using the 'Pellet Paint® Co-precipitant' reagent in accordance with the manufacturer's instructions. This reagent allows for the visualization of the precipitated sample. Larger samples were precipitated using ethanol. At first the sample was mixed with 1/10 volume sodium acetate (3 M) and then with 2.5

volumes of 100 % ethanol. This was followed by 30 minute incubation at -80°C. The precipitated sample was centrifuged followed by the removal of the supernatant under sterile conditions. After air drying the sample for 5 minutes, it was re-constituted in 'ultra-pure' water. This sterile DNA was further used for transfection of eukaryotic cells.

- Pellet Paint® Co-precipitant' (Novagen)

2.4.2.5 Other methods of DNA sample preparation

PCR products or short DNA fragments were purified from the reaction mix by using the 'MinElute PCR Purification Kit'. Following the manufacturer's instructions, the samples were bound to a column, washed and eluted in 10 µl buffer. In addition, the 'QiaQuick Nucleotide Removal Kit' was used to get rid of residual nucleotides in the reaction mix. The sample was run on a column, and then eluted in 30 µl buffer.

2.4.2.6 Determination of DNA concentration

DNA has an absorption maximum at 260 nm, and can be measured photometrically at this wavelength. The photometer 'GeneQuantII' and 'NanoDrop 1000' were utilized for this purpose. When the DNA sample was not sufficiently pure, the amount could be estimated directly on the agarose gel by direct comparison to a known standard run in a parallel lane.

2.4.3 Enzymatic treatment of DNA

2.4.3.1 Reverse transcription (RT)

During the process of reverse transcription, single stranded RNA is used as a template to synthesize a complementary cDNA strand that can be further amplified by PCR (**Section 2.4.3.2**). The total RNA extracted from TCR hybridoma cells (**Section 2.4.3.1**) was reverse transcribed to cDNA for the verification of correct TCR expression. In addition, the african green monkey MHC related I (MR1) gene was reverse transcribed from COS-7 cells (Wakiro Sato, MPI of Neurobiology). The reaction was catalyzed by the SuperScript™III reverse transcriptase,

an engineered version of the moloney murine leukemia virus reverse transcriptase, and the manufacturer's instructions were followed. First, the 5 µg of RNA was mixed with 1µl oligo (dT) and 1 µl dNTP (10 mM) and incubated at 65°C for 5 minutes followed by incubation on ice for 1 minute. The reaction tube was centrifuged; 4 µl of the first strand buffer (5x), 1 µl DTT (0.1 M) and 1 µl enzyme were added. This reverse transcription reaction was carried out at 45 °C for 60 minutes. The cDNA synthesized was further amplified using Vα-chain specific primers for TCR and MR1 specific primers listed in **Appendix I**.

- SuperScript™III reverse transcriptase
  200 U/µl; 10,000 units (Invitrogen)

2.4.3.2 Polymerase Chain Reaction (PCR)

PCR is a method that allows for the amplification of a single or few copies of DNA to multiple copies by cycles of annealing, amplification and denaturation. Oligonucleotides flanking a specific DNA sequence (forward and reverse primers) are used by the Taq DNA polymerase as anchors to synthesize the sequence by the additions of nucleotides. The variations of the PCR that were used in this study are explained in detail below. In every case positive (known template) and negative (water instead of template) controls were included. The reactions were carried out in the 'GeneAmp PCR System 9600 Thermocycler' or in the 'T personal Thermocycler'. The results were analyzed by gel electrophoresis (**Section 2.4.2.2**).

2.4.3.2.1 Colony PCR

The colony PCR was used to determine the successful ligation of a DNA fragment into the desired vector. Single bacterial clones were picked with a pipette tip and transferred directly into the PCR mix. The primer pairs used for the amplification flank the site of insertion on the vector. These are listed in **Table 2-8.** The additions for the PCR reaction and the subsequent PCR program are listed below.

- PCR reaction (20 µl)
  1x PCR buffer (10X) (Roche), 200 µM dNTP (10 mM), 0.5 µM forward primer (100 µM) ,0.5 µM reverse primer (100 µM), 1 u Taq polymerase (5 u/µl) (Roche)

- PCR program

3 min	94 °C	Denaturation	
1 min	94 °C	Denaturation	} 25 cycles
1 min	53 °C	Hybridization	
1 min	72 °C	Elongation	
10 min	72 °C	Elongation	

Vector	Forward primer	Reverse primer
pCR®2.1-TOPO	M13 forward (-20)	M13 reverse
pTT5	pTT5-for seq	pTT5-rev seq

Tab. 2-8: Primer pairs employed in colony PCR

The listed primers were either provided by manufacturer or ordered from Metabion (desalted). Primer sequences are provided in **Appendix I**.

2.4.3.2.2 Single cell multiplex PCR for TCR chains

RT PCR was performed on single CD8+ T cells, isolated from MS patient brain tissue, for the characterization of putatively autoaggressive TCRs. In this technique the first template is RNA which is transcribed by the enzyme reverse transcriptase into its complementary DNA (cDNA) strand. Subsequent steps use this DNA as template for amplification as in a traditional PCR. The T cells to be analyzed were first isolated from the brain tissue via laser microdissection. Cells that were double positive for either T cell markers or only CD8+ were marked electronically using the PalmRobo software (Zeiss). After 1-propanol evaporation, the cells were cut and laser pressure catapulted into the mineral oil coated lids of single reaction 200 µl PCR tubes. The tubes were transferred to dry ice until addition of the PCR reagents.

Double positive (CD8+, TCRVβ+) cells were processed as described earlier (Seitz et al. 2006). The method employs clone specific, nested TCR β-chain primers and a pool of unbiased TCR α-chain primers. CD8 single positive were processed using an unbiased protocol (developed in collaboration with Jörg Prinz, LMU). These two approaches are depicted in **Figure 2-1**. The PCR mix was pipetted into the cap of the PCR tube, and the tube was centrifuged at 14,000 rpm for 3 minutes at 4°C. A touch-down PCR, beginning with a high annealing temperature that

gradually reduces in each subsequent step, was applied to minimize unspecific primer binding and amplification. Between reactions, samples were stored on ice to maintain sample integrity. In every experiment, a negative control was included where water was added instead of the template. After PCR 3 and 4, the products were analyzed by gel electrophoresis (**Section 2.4.2.2**) before proceeding to the next step, and eventually sequenced. The TCR chains were named according to Arden et al. 1995. The reagent additions as well as PCR programs for each approach are mentioned in full detail below. To avoid contaminations due to carryovers, the TCR specific primers were aliquoted in sterile reaction tubes, stored at -20°C and thawed fresh for each experiment. All primer sequences are listed in **Appendix I**.

Clone specific PCR

- RT reaction (20 µl)
  QIAGEN OneStep RT-PCR enzyme Mix (Sensiscript and Omniscript reverse transcriptases and HotStarTaq DNA polymerase) (1 µl), 5x QIAGEN OneStep RT-PCR buffer (2µl), dNTP mix (0.5 µl, 10 mM), RT primers (0.625 µm each), RNase-free water

  PCR program: 30 min, 50°C

- PCR 1 (5 µl)
  Template: product from RT PCR (20 µl), QIAGEN OneStep RT-PCR enzyme mix (0.2 µl), 5x QIAGEN OneStep RT-PCR buffer (1 µl), dNTP mix (0.2 µl, 8 mM), Cα-rev-out primer (0.15 µl, 0.6 µm), Vα-i-for-out primer pool (0.45 µl , 0.062 µm each), BV-JBx-for-out (0.6 µM , 0.15 µl), BV-JBx-rev-out (0.6 µM , 0.15 µl) , RNase-free water

  PCR program:

15 min	95 °C		
1 min	94 °C	Denaturation	
1 min	61 °C	Hybridization	4 cycles
1 min	72 °C	Elongation	
1 min	94 °C	Denaturation	
1 min	58 °C	Hybridization	4 cycles
1 min	72 °C	Elongation	
1 min	94 °C	Denaturation	
1 min	56 °C	Hybridization	4 cycles
1 min	72 °C	Elongation	
1 min	94 °C	Denaturation	
1 min	53 °C	Hybridization	30 cycles
1 min	72 °C	Elongation	
10 min	72 °C	Elongation	

- PCR 2 (20 µl)
  Template: product from PCR 1 (1 µl), Taq DNA Polymerase (5 U/µl, Roche Diagnostics) (1.5 U), 10x PCR buffer (Roche Diagnostics) (2 µl), dNTP Mix (0.2 µl, 10 mM), BV-JBx-for-nest (10 µM, 0.1 µl), BV-JBx-rev-rest (10 µM, 0.1 µl), RNase-free water

  PCR program:

2 min	94 °C		
1 min	94 °C	Denaturation	40 cycles
1 min	53 °C	Hybridization	
1 min	72 °C	Elongation	

- PCR 3
  Additions and PCR program same as for PCR 3. Primers: BV-JBx-for-in (10 µM, 0.1 µl), BV-JBx-rev-in (10 µM, 0.1 µl)

- PCR 4
  Template: product from PCR 1 (1 µl), Taq DNA polymerase (1 U), 10x PCR buffer (2 µl), dNTP Mix (0.4 µl, 4 mM), Vα-j-for-in primer pool (2 µl, 0.5 µM each), Cα-rev-in (2 µl, 0.5 µM), RNase-free water

  PCR program:

2 min	94 °C		
30 sec	94 °C	Denaturation	4 cycles
1 min	61 °C	Hybridization	
1 min	68 °C	Elongation	
30 sec	94 °C	Denaturation	4 cycles
1 min	58 °C	Hybridization	
1 min	68 °C	Elongation	
30 sec	94 °C	Denaturation	4 cycles
1 min	56 °C	Hybridization	
1 min	68 °C	Elongation	
30 sec	94 °C	Denaturation	40 cycles
1 min	53 °C	Hybridization	
1 min	68 °C	Elongation	
10 min	68 °C	Elongation	

- PCR 5
  The template from PCR 1 is re-amplified by clone-specific primers from the positive pool. Additions of reagents and PCR program in keeping with PCR4.

Unbiased PCR

- RT Reaction (12.5 µl)
  QIAGEN OneStep RT-PCR enzyme mix (0.5 µl), 5x QIAGEN OneStep RT-PCR buffer (1.3 µl), dNTP Mix (0.75 µl, 10 mM each), Cα/Cβ RT primers: Cα-out and Cβ-out, (10 µM each), RNase-free water
  PCR program: 30 min 50°C

- PCR 1 (12.5 µl)
  QIAGEN OneStep RT-PCR enzyme mix (0.5 µl), 5x QIAGEN OneStep RT-PCR buffer (2.5 µl), dNTP mix (0.5 µl, 10 mM each), Vα and Vβ primer pools (0.6 µl, 3 µM each), RNase-free water

PCR program:

15 min	95 °C		
30 sec	94 °C	Denaturation	
1.5 min	60 °C	Hybridization	10 cycles
1 min	68 °C	Elongation	
30 sec	94 °C	Denaturation	
1 min	53 °C	Hybridization	30 cycles
1 min	68 °C	Elongation	
15 min	68 °C	Elongation	

- PCR 2 (20 µl)
  Taq DNA polymerase (0.10 µl), 10x PCR buffer (2 µl), dNTP mix (0.4 µl, 10 mM each), Vp1-UP to Vp-9-UP primers (0.2 µl, 11.1 µM each), RNase-free water

  PCR program:

5 min	94 °C	Denaturation	
2.5 min	53 °C	Hybridization	10 cycles
15 min	68 °C	Elongation	

- PCR 3 (20 µl)
  Taq DNA polymerase (0.20 µl), 10x PCR buffer (2 µl), dNTP Mix (0.4µl, 10 mM each), UP and Cβ-in primer (2 µl, 2.5 µM each), RNase-free water

  PCR program:

2 min	94 °C		
30 sec	94 °C	Denaturation	
1 min	58 °C	Hybridization	50 cycles
1 min	68 °C	Elongation	
15 min	68 °C	Elongation	

- PCR 4 and 5 (same as for the clone specific PCR)

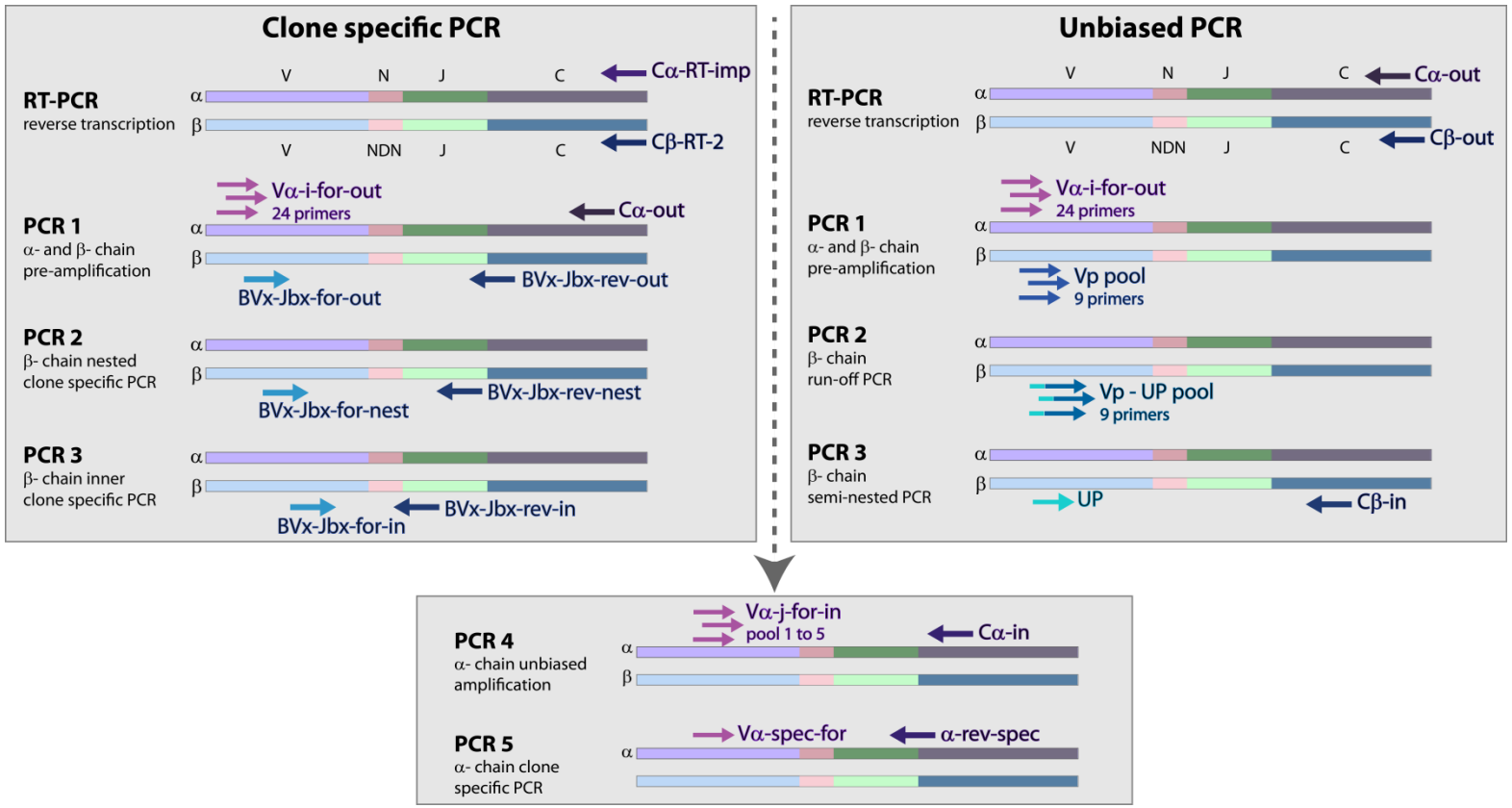


Fig. 2-1 Relative positions of primers in the clone specific and unbiased PCR approaches for amplification of TCR α- and β- chains

The TCR chains are shown with the variable (V), hypervariable (NDN), joining (J) and constant (C) regions (not drawn to scale). Both PCR approaches consist of 5 different PCR reactions. In the clone specific approach, β-chain specific primers were designed based on CDR3 spectratyping results while the unbiased approach uses universal primer pools. After reverse transcription, the cDNA is used for the pre-amplification of the α- and β- chains. This PCR product serves as a template for further β- chain amplification. The last two nested PCR reactions (4 and 5) for the α-chain are common in both approaches. The outer primers bear the suffix '-out', followed by the suffix '-nest' and '-in' for nested primers. The suffix 'for' indicates that the primer anneals in the 5' to 3' direction, and the opposite is true for the suffix 'rev'. The primers 'Cα-RT-imp, Cα-out, Cα-in' corresponds to the constant region of the TCR α-chain (order: outer to inner). The Vα-i-for-out pool consists of 24 primers that lie in the variable region of the α-chain and the nested Vα-j-for-in pool comprises 36 primers divided into 5 different pools. The Vα-spec-for and α-rev-spec primers are designed to specifically amplify the CDR3 region of a particular clone. Similarly, the Cβ-RT-2, Cβ-out and Cβ-in primers correspond to the TCR β-chain constant region. In the clone specific PCR, 'BVx-Jbx' indicates the clonal expansion of interest, for e.g. BV1-BJ2.3 in Patient FE. The Vp pool consists of 9 primers that lie in the β-chain variable region. The Vp-UP pool is nested to the Vp primers, and adds a 22 nucleotide sequence designated as 'UP' (denoted in light blue) to the amplified product from the previous PCR reaction. This addition makes it possible to further amplify all amplicons in a nested reaction using a universal UP primer that is complementary to the added region. Modified in part from Seitz et al. 2006.

2.4.3.2.3 Single cell PCR for amplification of library plasmids

Single APCs were isolated with the aid of a microcapillary and immediately placed into a PCR tube containing ammonia solution for DNAse inactivation. In some cases, the PCR tubes were additionally incubated at 80°C for 5 minutes to inactivate the DNAse. After this, the PCR tubes were placed under a sterile hood for up to 60 minutes to allow for complete evaporation of ammonia. Following the first PCR reaction, a second nested PCR was carried out using the 1:100 dilution of the first PCR product as a template. Both PCR products were run on a gel (**Section 2.4.2.2**) for estimation of DNA concentration.

- First (outer) PCR reaction mix (50 -100 µl)
  1X PCR buffer (10X) (Roche), 0.2 mM dNTP (10 mM) (Qiagen), 0.5 µM pcDNA-for-1 primer, 0.5 µM pcDNA-rev-1 primer, 0.05 U/µl Taq polymerase (Roche)

  PCR program:

3 min	94 °C		
1 min	95 °C	Denaturation	40 cycles
1 min	53 °C	Hybridization	
1 min	72 °C	Elongation	
10 min	72 °C	Elongation	

- Second (nested) PCR reaction mix (50 µl) for cloning by restriction digestion
  Additions same as for first PCR except primers, 0.5 µM pcDNA-2nd-for primer, 0.5 µM pcDNA-rev-10 primer

- Second (nested) PCR reaction mix (50 µl) for directional TOPO cloning
  1X iProof HF Master mix (iProof™ High-Fidelity PCR Kit, Bio-Rad), 0.5 µM pcDNA-for-2-TOPO or pcDNA-2nd-for primer, 0.5 µM pcDNA-rev-3 or pcDNA-rev-2 primer

  PCR program:

3 min	98 °C		
20 sec	98 °C	Denaturation	40 cycles
20 sec	56 °C	Hybridization	
30 sec	72 °C	Elongation	
10 min	72 °C	Elongation	

2.4.3.3 TOPO TA cloning

'TOPO-TA Cloning®' (Invitrogen) is based on the fact that the Taq polymerase enzyme adds a desoxyadenosine to the 3'end of the PCR product due to its transferase activity. The linearized pCR®2.1-TOPO®-vector carries complementary 3'-desoxythymidine overhangs. The PCR product was ligated into the vector via 5 minute incubation at RT by following the manufacturer's instructions. The ligated product was then transformed into chemically competent 'One Shot® TOP10' *E. coli* bacteria as explained in **Section 2.2.3**.

- pCR®2.1-TOPO® vector (Invitrogen)
  3.9 kb, Amp^R, KanR, for vector map refer to **Appendix II**

2.4.3.4 Directional TOPO TA cloning

Directional TOPO TA cloning was performed for the cloning of library plasmids that were isolated from antigen presenting hMR1-COS-7 cells (**Section 2.4.3.4)**. The peptide coding inserts on the library plasmid were amplified (**Section 2.4.3.9.2)** and the resulting blunt ended PCR products carried 5'CACC ends. This facilitated the ligation into the topoisomerase activated pcDNATM3.1D/V5-His-TOPO vector that carries a complementary sequence. All steps were carried out according to the manufacturer's instructions. The only exception was the use of 0.5 µl vectors, instead of the recommended 1µl, and the extension of the incubation at 16°C to overnight. The resulting ligated plasmids were precipitated in ethanol (**Section 2.4.2.4**) and further used to transform the ElectroMax DH10B bacteria (**Section 2.2.2)**.

- pcDNATM3.1D/V5-His-TOPO vector (Invitrogen)
  5.5 kb, Amp^R, Neo^R, for vector map refer to **Appendix II**

2.4.3.5 Restriction digestion of DNA

Restriction enzymes are endonucleases that are capable of recognizing specific sequences in double stranded DNA and then cutting the phosphodiester bonds at a related position. This property can be exploited to create DNA fragments with complimentary overhangs that can eventually be ligated. One unit of a restriction enzyme is defined as that quantity which can digest a fixed amount of substrate DNA, for e.g. 1 µg of λ DNA (48.5 kb), within one hour under optimal conditions of buffer and temperature. The amount of enzyme units required per reaction

is determined by calculating for the size, amount and number of restriction sites present in the sample DNA sequence. All restriction enzymes used in this study were purchased from New England Biolabs (Frankfurt, Germany) and used in accordance with the manufacturer's instructions. The reactions were set up in volumes between 20 to 200 µl for 1 to 16 hours at 37°C. Double digests could be performed when the two restriction sites were separated by more than 100 bp and compatible buffers were available. In case of sequential digests in compatible buffers, the first enzyme was heat inactivated prior to addition of second enzyme. For buffer change, the restriction product was first ethanol precipitated (**Section 2.4.2.4**) and the reconstituted as required. The rare cutting *Asc*I and *Not*I were employed sequentially with an intermediate ethanol precipitation step for the generation of plasmid encoded combinatorial peptide libraries (**Section 2.4.3.8**) and the recovery of antigen coding plasmids from APCs (**Section 2.4.3.9**). For the latter, the 9 and 10 bp flanking fragments were removed by the application of the 'Qiaquick nucleotide removal kit' (Qiagen). The vector maps are presented in **Appendix II**.

2.4.3.6 Ligation of DNA

The T4 DNA Ligase enzyme (Invitrogen) catalyses the formation of a phosphodiester linkage between the free 3' hydroxyl group of one DNA fragment and the free 5' phosphate group of the second fragment. The ATP required for this reaction is provided in the ligase buffer (5X, Invitrogen) and used at a final concentration of 1 mM. To prevent the self-ligation of the vector, the DNA fragment to be inserted is added at a four times higher molar concentration than the vector. Self-ligation may be tested by setting up a negative control in parallel that lacks the insert. The ligation reaction is incubated overnight at 16°C. The incubation time for the ligation of library plasmids to the pcDNArc-spacer backbone (**Section 2.4.3.8.2**) was extended to ≥ 48hrs to ensure high yields. The ligated products were precipitated in ethanol (**Section 2.4.2.4**) and stored at -80°C before transformation into *E. coli* cells (**Section 2.2.2 and 2.2.3**).

- Ligation mix (20 µl reaction)
  1 u T4 DNA ligase, 100 ng vector, 4x molar concentration of insert, in 1x T4 ligase buffer

2.4.3.7 Cloning of TCRs

The TCR chains detected in the patient tissue were cloned into the pRSV vector for generating transgenic TCR cell lines for antigen studies. Based on the single cell PCR product, part of the- and β- chains were chemically synthesized (**Section 2.4.3.11**). These fragments started with a 5' *SalI* restriction site, covered the leader, variable, N (D) N-J and joining region and ended in a unique site in the conserved constant region. The pBRDel vector was used as a subcloning vector for the β-chain. The V-NDN-J region was cloned using the *Sal*I and *Bgl*III sites. The constant region lies between the *BglIII* and *BamHI* sites. Hence the entire TCR β-chain could be cut out via a *SalI-BamHI* restriction. The TCR chains were incorporated into the expression plasmids $pRSV_{hygro}$ (α-chain) and $pRSV_{neo}$ (β-chain) between the *SalI* and *BamHI* restriction sites. These were then co-transfected via electroporation (**Section 2.3.3**) into the T hybridoma cell line $58^{\alpha-/\beta-}$ for stable TCR expression. The vector maps are presented in **Appendix II**.

2.4.3.8 Cloning of human MR1 and β2 microglobulin

Human MR1 and β2 microglobulin were cloned into pTT5 vectors with leader sequences from Fab fragments (that had been successfully expressed in the HEK293E cell line). The pTT5 vectors were obtained from Birgit Obermeier, MPI of Neurobiology. In the MR1-pTT5 vector, the MR1 coding region followed by a myc tag, polyhistidine ($His_{(6)}$) tag, BirA (for biotin tagged purification) was inserted into the vector after the leader sequence of the heavy chain via a *Sac*I and *BamH*I restriction. In the β2 microglobulin vector, the β2 microglobulin coding region was inserted after the leader sequence of the light chain via *BssII* and *BamHI* restriction sites. These were then co-transfected into the HEK293E cell line for protein expression. The leader sequences of the heavy and light chains were used as it was known that the signal peptidase efficiently cleaves these sequences (Klaus Dornmair and Birgit Obermeier, MPI of Neurobiology, personal communication). The vector maps are presented in **Appendix II**.

2.4.3.9 Generation of plasmid encoded combinatorial peptide (PECP) libraries

The PECP libraries were a pre-requisite for the unbiased determination of peptide antigens of TCRs. They were constructed by ligating peptide coding library inserts into the pcDNArc-spacer expression plasmid.

2.4.3.9.1 Preparation of peptide coding library inserts

In the course of this study the unbiased N library was used to screen for MR1 antigens. This library was provided by Katherina Siewert, MPI of Neurobiology. In addition, two libraries with anchor residues matched to patient FE HLA class I molecules A*0101 and B*0801 were synthesized. For this purpose, high-performance liquid chromatography (HPLC) purified single stranded oligonucleotides were custom designed and procured from Metabion. A fill-in reaction was then carried out to generate a complementary strand yielding a double stranded product. This was followed by a denaturation step of 100°C for 5 minutes. The reaction mix was gradually cooled to RT over the period of 60 minutes and to it an equal volume of the PCR mix was added. The following reaction was carried out according to the cycling conditions mentioned below to amplify the double stranded products. These products were sequentially digested with *Asc*I and NotI and purified as explained in **Section 2.4.3.5**.

- Strand fill-in reaction mix (100 µl)
  1X PCR buffer (10X) (Roche), 20 µM oligonucleotide (N27-all-lib-*ASc*-for / N27-A1-39-lib-*Asc*-for / N27-B8-359-lib-*Asc*-for), 20 µM N27-*Not*-rev primer (Metabion) (Primer sequences in **Appendix I**)

- PCR mix (100 µl)
  1X PCR buffer 200 µM dNTP (10 mM) (Qiagen), 5 U Taq polymerase (Roche)
  PCR program: 60°C 5 min, 63°C 4 min, 65°C 60 min

2.4.3.9.2 The 'pcDNArc-spacer' backbone for ligation of the library inserts

The 'pcDNArc-spacer', where 'rc' stands for 'rare cutter' was used as the vector backbone for the PECP libraries. This expression plasmid was provided by Katherina Siewert, MPI of Neurobiology. Prior to ligation of the library inserts, the backbone was digested with *Asc*I/*Not*I (**Section 2.4.3.5**), and purified with agarose gel electrophoresis (**Section 2.4.2.2**) and the 'QIAquick gel extraction kit' (Qiagen). After determination of DNA concentration (**Section**

2.4.2.6), the backbone was dephosphorylated with the 'Rapid DNA Dephos and Ligation' kit (Roche) according to the manufacturer's instructions (**Section 2.4.3.10**) before ligation (**Section 2.4.3.6**) with the library inserts.

- pcDNArc-spacer
  7.6 kb, Amp^R, Neo^R
  Provided by Katherina Siewert, MPI of Neurobiology

2.4.3.10 Recovery of antigen coding plasmids from antigen presenting cells

The live antigen presenting cells were isolated by micromanipulation (**Section 2.4.3.9.1**) and their contained antigen coding plasmids were amplified by a nested PCR reaction (**Section 2.4.3.2.3**). These amplified inserts were cloned into expression plasmids (**Section 2.4.3.9.2**) and expanded in *E. coli* before repeating the transfection into COS-7 cells to evaluate enrichment of the activation signal.

2.4.3.10.1 Isolation of positive APCs by micromanipulation

The TCR hybridoma cells were co-cultured for 16 hours with APCs expressing the PECP libraries. The co-culture was screened under a fluorescence microscope for activated, GFP+ TCR hybridoma cells. The APCs of interest were those that were in stable contact with these, activated TCR hybridoma cells. These were isolated by the use of custom designed glass capillaries (custom-tips, **Ø** 15-30 µm, beveled edge, Eppendorf) attached to a microinjector (Cell Tram Vario) that allowed one to dislodge the cell from the cell culture dish with minimal amounts of cell culture medium (1-3 µl). This entire method is depicted in **Figure 2-2** using the MAIT TCR as an example. The isolation was closely followed on the computer monitor. The isolated cell was directly transferred into a PCR tube and the antigen coding plasmids were amplified as outlined in **Section 2.4.3.2.3.**

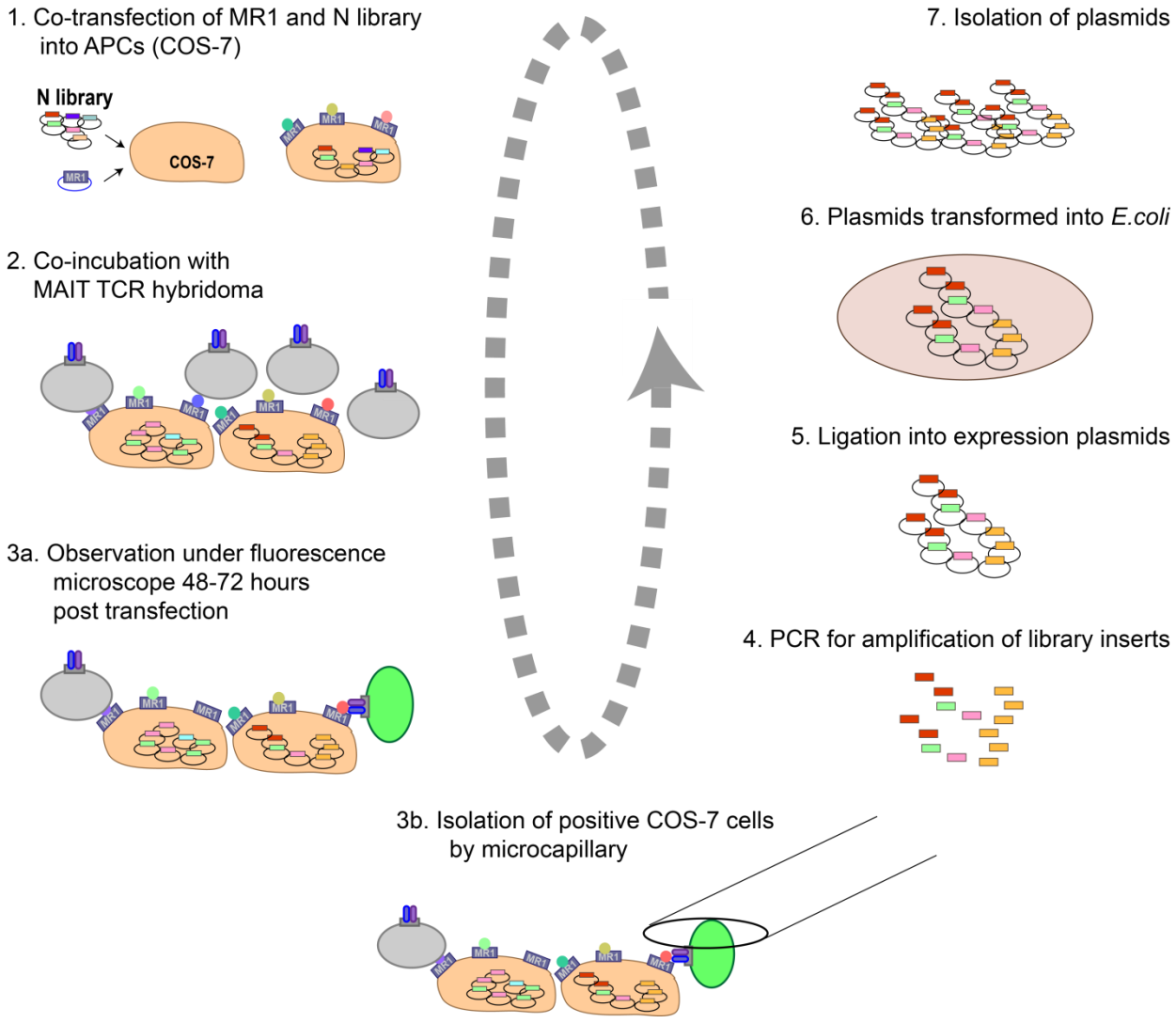


Fig. 2-2: Recovery of antigen coding plasmids from APCs

(1) The N library was transfected into MR1 expressing COS-7 cells (8 µg library plasmids/4 x 10^6 cells). **(2)** 32-60 hours post transfection the MAIT TCR hybridoma cells (1.6 x 10^6 cells/ 3ml culture medium) were added and incubated for 16 hours. **(3a)** The co-culture was monitored under a fluorescence microscope for the detection of activated, GFP+ hybridoma cells that were in contact with the underlying APC (that contained the antigen that had caused activation). **(3b)** The underlying APC was isolated and **(4)** the intracellular antigen encoding plasmids were amplified. **(5)** The recovered plasmids were cloned into the expression plasmid and **(6)** expanded in bacteria. **(7)** The plasmids were further enriched by another round of APC transfection followed by the aforementioned steps.

2.4.3.10.2 Cloning of PCR products into expression plasmid

The PCR products were inserted into the pcDNArc-spacer backbone by restriction digestion and ligation (**Section 2.4.3.6**) or directional TOPO TA cloning (**Section 2.4.3.4**). The PCR products were purified on a column (**Section 2.4.2.5**) and then ligated to the pcDNA backbone (**Section**

2.4.3.6). The plasmids were then precipitated in ethanol and transformed into electrocompetent ElectroMax DH10B™T1R *E. coli* (Invitrogen) by electroporation (**Section 2.2.2**). To ensure that all clones were represented in the pool, the bacterial were inoculated in higher volumes of LB media, such as 500-750 ml prior to DNA isolation (**Section 2.4.2.1**). The isolated plasmids were transfected into COS-7-MR1 cells as outlined in **Section 2.3.4.** These were tested for T hybridoma activation, and the obtained signal was compared to that from the first round to see if an increase had been caused due to enrichment.

2.4.3.11 Dephosphorylation of DNA

Dephosphorylation of digested vector DNA prior to ligation reduces the background caused due to vector self-ligation. The alkaline phosphatase enzyme catalyzes the removal of 5' phosphate groups from nucleic acids and prevents self-ligation of vector via phosphodiester linkage. The pcDNArc-spacer backbone (**Section 2.4.3.8.2**) of the peptide encoding library was dephosphorylated prior to insertion of library encoding fragment. The reaction was carried out as recommended by the manufacturer.

- Rapid DNA Dephos and ligation kit (Roche)

2.4.3.12 Gene synthesis and DNA sequencing

The TCR α- and β- fragments (variable region to beginning of constant region) were synthesized at Geneart (Regensburg, Germany).The samples were sequenced at the service centers of the MPI of Biochemistry and the Biocenter of the LMU.

2.4.4 Analysis of DNA sequences

The obtained sequences were analyzed with the software 'Chromas' (Version 1.45, Griffith University, Australia). The detailed analysis of sequences from TCRs was carried out with the NCBI Blast software (Version 2.2.25), and the IMGT database provided at 'http: //www.imgt.org/'. The sequences were aligned using the clustwal software (Version W2) (Thompson et al. 1994). Nucleotide sequences could be translated into the amino acid sequence using the 'Translate Tool' provided at 'http: //www.expasy.ch/tools/dna.html' (Swiss Institute of Bioinformatics).

2.5 Protein analysis

2.5.1 Determination of protein concentration

The concentration of proteins in solution can be determined by measuring the absorbance at 280 nm. The aromatic ring groups present in amino acids such as tryptophan, and to a lesser extent tyrosine and phenylalanine, are responsible for this effect. An absorption peak at 190-230 nm may be attributed to peptide bonds. All measurements were carried out at the spectrophotometer 'NanoDrop 100'.

2.5.2 Protein purification

2.5.2.1 Protein G affinity chromatography

Protein G affinity chromatography using Dynabeads® was applied for the purification of myc tagged hMR1 using a myc tag recognizing antibody (clone 9E10.3). The expression of hMR1 in HEK293E cells is explained in **Section 2.3.4**. To 100 ml of cell supernatant (containing approximately 25 µg MR1), 80 µg of commercially available human β2 microglobulin and the myc tag recognizing antibody (clone 9E10.3) were added. After incubation for 60 minutes at RT, 100 µl of Protein G Dynabeads® were added. The recombinant protein G, coupled to the magnetic bead matrix, binds to the Fc region of an IgG antibody hence purifying it from the solution. The magnetic beads can be easily collected by applying a Dynal® magnet to the exterior wall of the reaction tube. Before use, the beads were washed thrice in the wash buffer to get rid of residual ethanol. Post incubation, the beads were washed to get rid of all unbound protein. The supernatant was then removed and the beads were re-suspended in the chemically synthesized positional scanning nonapeptide library to screen peptides in order to determine the anchor positions of human MR1 (Section 2.5.4).

- Dynabeads® Protein G, Invitrogen

  Dynabeads® wash buffer: 1 M sodium acetate, 150 mM NaCl, pH 5.0

- Human β2 microglobulin, Sigma

2.5.2.2 Ni-NTA-Affinity chromatography

The recombinant protein expressed by the HEK293E cells carries a $His_{(6)}$ peptide tag that allows it to bind with high affinity to the Ni-NTA matrix as a function of the aromatic imidazole group. The stationary phase is a composed of nitriloaceticacid (NTA) and Ni^{2+} ions coupled onto an agarose matrix. The bound proteins are later eluted in excess of imidazole that in turn displaces the $His_{(6)}$ peptide from the matrix. The entire purification procedure was carried out in columns. A 5 ml bed volume of the Ni-NTA-agarose was washed with 10 column volumes water for the removal of residual ethanol. The matrix was then equilibrated with 10 mM imidazole in 1X PBS (pH 8.0). The cellular supernatant was cleared of cell debris by a centrifugation step (Rotor SLA-1500, 12,500 rpm, 30 min, 4 °C) followed by sterile filtration (0.2 µm filter) to get rid of residual micro debris. The pH of the supernatant was adjusted to pH 7.5 using 1 M Tris (pH 8.6) and then equilibrated to 1X PBS with a final concentration of 10 mM imidazole. This low imidazole concentration is thought to reduce unspecific binding of the protein to the matrix. Furthermore, a protease inhibitor phenylmethanesulfonylfluoride (PMSF) was added to a final concentration of 100 µM. 200 ml supernatant was loaded onto the column at a flow rate of 1.5 ml per minute. Post binding, the column was washed with 20 ml PBS/20 mM imidazole, pH8.0 for the removal of the nonspecifically bound protein fraction. The elution was carried out with 20 ml PBS/200 mM imidazole, pH8.0 at the FPLC apparatus (**Section 2.5.2.3**). All buffers were sterile filtered (0.2 µm filter) and degassed prior to use.

- Ni-NTA agarose, Qiagen
- 1.5 x 10 cm Luer-lock columns , flow through, Sigma
- 0.2 µm filter, Milipore

2.5.2.3 Fast protein liquid chromatography (FPLC)

The proteins, purified on the Ni-NTA matrix (**Section 2.5.2.2**), were eluted using an FPLC system from Amersham Pharmacia Biotech. This elution can be followed by monitoring the absorbance value of the component aromatic amino acids at $\lambda = 277$ nm on the in-built UV detector (Uvicord SD). The FPLC apparatus is composed of two pumps, one of which is filled with 1X PBS while the second with PBS/200 mM imidazole. The in-built control system (LCC-

501 Plus) is used to determine the proportion to which the two buffers are mixed before running through the column. The washing step at PBS/20 mM imidazole was carried out until all nonspecifically bound proteins had been eluted. The imidazole concentration was then raised to 200 mM, and as the absorption value rose, the eluate was collected. This was followed by dialysis (**Section 2.5.2.4**) against PBS for the removal of imidazole and concentration determination. The purified proteins were stored at 4°C in PBS, 0.07 % sodium azide and 100 µM PMSF.

2.5.2.4 Dialysis

Dialysis is a technique employed to get rid of certain buffer components form the sample, or for the complete buffer exchange. The technique is based on the creation of a concentration gradient across a semi-permeable membrane, whereby small molecules are able to cross the membrane whereas larger molecules are retained. Depending on the size of the said molecules, dialysis membranes of different molecular weight cut offs (MWCO) can be used. The ‚D-tube ™ Dialyzer Mini'- tube was used for volumes up to 200 µl while for larger volumes up to 3 ml, the 'D-Tube™ Dialyzer Maxi' tube were used (Novagen, Darmstadt). Both of these have a MWCO between 12 to 14 kDa. The dialysis for the reduction in imidazole was carried out against PBS in the cold room. The buffer was constantly stirred during this time.

2.5.3 Gel electrophoresis

Gel electrophoresis is based on the migration of charged molecules in the presence of an electric field. Smaller molecules are able to migrate faster than larger ones, in a porous gel matrix, and are hence separated from each other. In contrast to DNA, where the phosphate groups are negatively charged, proteins exhibit a wide spectrum of net charge and this property is exploited during this charge based separation procedure.

2.5.3.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins may be separated on the basis of their size by concealing their inherent charges. This is achieved by mixing the protein sample with an excess of the anionic detergent sodium dodecyl

sulphate (SDS). The SDS also leads to the unfolding of secondary structure. Hence the separation is truly size based and independent of both charge and protein folding. Depending on the complexity of the protein mixture to be separated, SDS-PAGE Novex® Tris-Glycine mini gels (8 cm x 8 cm x 1 mm, Invitrogen) were used. The 'Wide Range SigmaMarker™' was taken as the molecular weight standard. Prior to loading, the samples were mixed either with the reducing or non-reducing loading buffers. The reducing buffer contains β-mercaptoethanol which breaks the proteins down to their sub units by the breakage of disulfide bonds. The complete reduction and denaturation of the sample was achieved a 95°C. Electrophoresis was carried out in the 'MightySmall' apparatus (Hoefer) with the SDS running buffer with a constant voltage of 130 V for 90 minutes.

- SDS running buffer (10X)
  1 % (w/v) SDS, 0.24 M Tris, 1.92 M glycine
- Loading buffer ,non- reducing (5X)
  0.2 M Tris-HCl pH 6.8, 7.5 % (w/v) SDS, 20 % (v/v) glycerol, 0.02 % (w/v) bromphenol blue
- Loading buffer ,reducing (3X)
  0.15 M Tris-HCl pH 6.8, 15 % (w/v) SDS, 45 % (v/v) glycerol, 0.01 % (w/v) bromphenol blue, 6% (v/v) ß-mercaptoethanol

2.5.3.2 Staining of protein gels

2.5.3.2.1 Coomasie Brilliant Blue staining

The Coomassie® Brilliant Blue dye can be used when the sample contains at least 500 ng of protein. This dye does not interfere with downstream mass spectrometry analysis. The Novex® mini gels were incubated for 30 minutes with the dye on a swinging surface. The acetic acid in the solution precipitates the proteins, thereby fixing them to the gel. Nonspecific background staining was reduced by washing the stained gel in the destaining solution.

- Coomassie® dye solution
  0.1 % (w/v) Coomassie® Brilliant-Blue R-250, 40 % methanol, 10 % acetic acid
- Destaining solution
  50 % methanol, 7 % acetic acid

2.5.3.2.2 Silver staining (Heukeshoven protocol)

Silver staining is more sensitive in comparison to Coomassie® Brilliant blue dye and can detect protein levels of 5 ng or less. Following the Heukeshoven protocol , the proteins were first fixed on the gel for 30 minutes and then sensitized for 60 minutes. The cross linking effect of glutaraldehyde does not allow later analysis by mass spectroscopy. After washing three times with water, the gels were stained for 45 minutes during which Ag^+ ions bind to the proteins. The gel development is carried out in an alkaline solution that reduces the ions to metallic silver. The protein bands are stained brown to black depending on protein concentration. Once the desired stain was achieved, the reaction was stopped.

- H-fixation solution
  40 % ethanol, 10 % acetic acid
- H-sensitization solution
  0.2 % (w/v) sodium thiosulfate, 0.5 M sodium acetate 0.5 % (v/v) glutaraldehyde, 30 % ethanol
- H-staining solution
  0.1 % (w/v) silver nitrate, 0.02 % (v/v) formaldehyde
- H-development solution
  0.01 % (v/v) formaldehyde, 2.5 % (w/v) sodium carbonate
- H-stop solution
  1.5 % (w/v) EDTA

2.5.3.2.3 Silver staining (Shevchenko protocol)

The silver staining protocol from Shevchenko, based on (Rabilloud et al. 1988), does not include cross-linking by glutaraldehyde, hence making the sample compatible with mass spectroscopy analysis. The detection limit is approximately 5 ng. The protein gel was fixed for 20 minutes and then rinsed in 50% methanol for 10 minutes. Following a 1 minute sensitization it was washed three times in water. During the 20 minute staining that followed, the nonspecifically bound silver ions were trapped in soluble complexes due to the sodium thiosulfate pre-treatment and these were removed by a two time water wash. During development the solution was refreshed when a yellow discoloration was observed. The reaction was stopped by lowering the pH of the solution.

- S-fixation solution
  50 % methanol, 5 % acetic acid

- S-sensitization solution
  0.02 % (w/v) sodium thiosulfate
- S-staining solution
  0.1 % (w/v) silver nitrate; pre-cooled
- S-development solution
  0.04 % (v/v) formaldehyde, 2 % (w/v) sodium carbonate
- S-stop solution
  5 % acetic acid

2.5.3.3 Electro-blotting

During electro-blotting, an electric field is employed to transfer proteins separated on the polyacrylamide gel onto a membrane surface. For this purpose, a ‘semi-dry-system’ apparatus was used. Three Whatman-filter papers (GE Healthcare) were soaked in the anode buffer and placed onto the lower anode plate. A polyvinylidene fluoride (PVDF) membrane was shortly activated in methanol, washed in water and dipped in the anode buffer, before placing onto the filter papers. The polyacrylamide gel was placed on top of the membrane and further covered by three Whatman filter papers that had been soaked in the cathode buffer. The blot ‘sandwich’ was completed by the placing of the upper cathode plate. The transfer was carried out at 35 mA for 3 hours.

- Anode buffer
  50 mM boric acid, 20 % ethanol, pH 9.0
- Cathode buffer
  50 mM boric acid, 5 % ethanol, pH 9.0

2.5.4 Peptide screening for anchor position determination

2.5.4.1 Synthetic combinatorial peptide library in positional scanning format (PS-SCL)

Synthetic combinatorial peptide libraries in positional scanning format (PS-SCL) are composed of peptide pools. Within a given pool, each peptide contains fixed amino acids at one position and randomized amino acids at all other positions (Gundlach et al. 1996; Sospedra et al. 2003; Nino-Vasquez et al. 2004). For instance, a PS-SCL nonapeptide library would contain 180 peptide pools for the 20 amino acids that can be arranged in 9 positions. Such libraries have so far been applied for TCR based antigen searches (Rubio-Godoy et al. 2002). In this study, recombinant MHC molecules (MR1) were incubated with a PS-SCL in order to isolate their nonapeptide binding partners. The experimental design for determining the anchor positions of a MHC molecule (using HLA-A*0101 as an example) by this approach is shown in **Figure 2-3**.

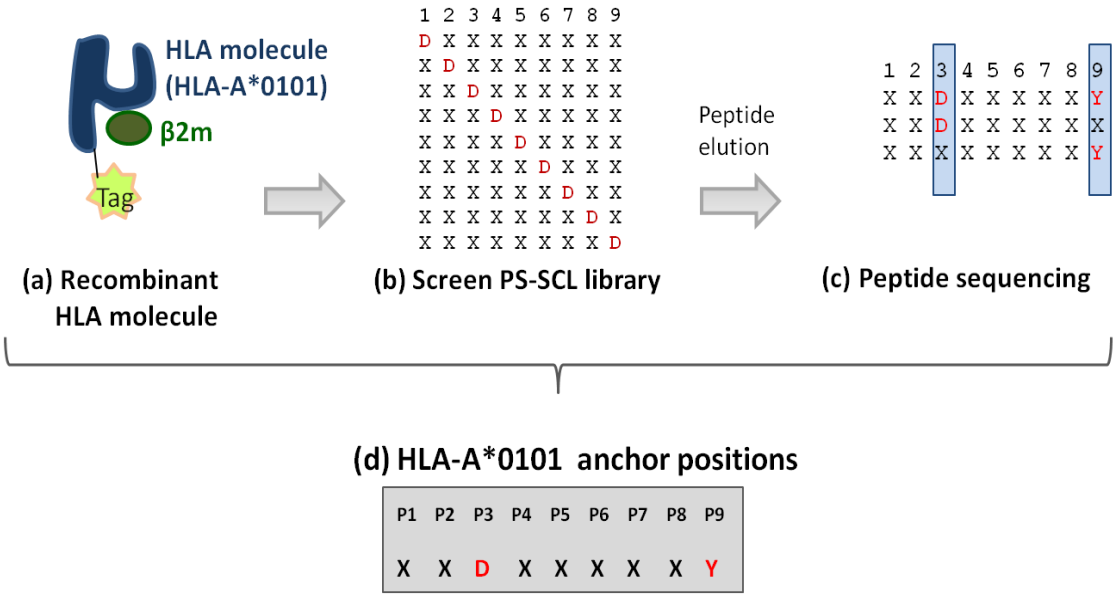


Fig. 2-3: Experimental design for anchor position determination of a HLA molecule (HLA-A*0101)

(a) Recombinant β2 microglobulin associated human HLA-A*0101 is produced and purified via a terminal tag such as His $_{(6)}$. **(b)** The HLA molecule may now be used to screen a synthetic combinatorial peptide library in positional scanning format (PS-SCL). This is shown as a nonapeptide library where a given amino acid (here aspartic acid) is fixed from position 1 to 9 while every other position is randomized (denoted by 'X'). **(c)** The peptides that bind to the HLA molecule can be eluted by acid treatment, and sequenced. The sequences shown here are examples for a hypothetical, successful experiment where the anchor positions of the MHC molecule HLA-A*0101 were revealed. **(d)** The sequence of the eluted peptides will reflect the anchor positions of the MR1 molecule. For HLA-A*0101 these are aspartic acid (D) at position 3 and tyrosine (Y) at position 9. The amino acids in **(b)**, **(c)** and **(d)** have been denoted according to standard one letter nomenclature.

The recombinant human MR1 (approximately 25 µg) purified via the myc tag and protein G affinity chromatography and was immobilized on magnetic beads (**Section 2.5.2.1**). The MR1 immobilized on magnetic beads was added to 450 µl of a chemically synthesized nonapeptide PS-SCL (gift from Clemencia Pinilla, Torrey Pines Institute for Molecular Studies). Individual peptides have a concentration of about $1x\ 10^{-15}$ M in this library. 25 µg of commercially available β2 microglobulin was also added to the mixture. This MR1-peptide- β2 microglobulin mixture was incubated for 48 hours to allow sufficient time for binding of the peptides to MR1 after which the peptides were eluted by acid treatment (**Section 2.5.4.2**).

2.5.4.2 Elution of bound peptides

After the incubation, with the PS-SCL nonapeptide library, the MR1 (and binding partners) were separated by magnetic purification and washed three times with 1X PBS to remove unbound peptides and once shortly with water to remove residual salt. 250 µl of 0.2 % trifluoroacetic acid was added for elution of bound peptides from MR1. The eluate was diluted to 0.1 % trifluoroacetic acid and sequenced by Edman degradation (Reinhard Mentele, MPI of Biochemistry) and mass spectrometry (Ignasi Forné, LMU).

2.6 Immunological methods

2.6.1 Flow cytometry

Flow cytometry enables multiparametric cellular analysis such as the detection of multiple cellular markers, both intracellular and those expressed on the surface. The cells to be analyzed are suspended in solution and stained for markers of interest by using fluorophore tagged antibodies. A beam of (laser) light is applied on a hydrodynamically focused single cell suspension. Cells passing through this beam scatter the light, as well as emit light from the excited fluorophores. This scatter and emission is analyzed by detectors positioned in front of and perpendicular to the beam. Information based on the forward scatter (FSC) and side scatter (SSC) produced by the cell, correlating to cellular volume and inherent granularity respectively, allows one to characterize the cells.

Flow cytometry was employed for analyzing the surface expression of transfected molecules as well as intracellular sGFP expression. A FACS Calibur (Becton Dickinson) with excitation lasers for 488 nm and 633 nm was used $1x10^4$ - $1x10^6$ cells were taken per well of a 96 well conical bottomed plated, washed twice with 200 µl FACs buffer, re-suspended in 50 µl of primary antibody solution and incubated on ice, in the dark for 30 minutes. The cells were then re-washed and suspended either in a secondary antibody solution or in the dead cell counterstain solution. The antibodies used for staining are tabulated in **Section 2.1.2**. 10,000 cells were acquired per sample and analyzed with the software Cell Quest (Becton Dickinson, V3.3) or FlowJo (Tree Star, V7.6).

- FACS buffer
  10 % FCS in 1X PBS
- Antibody solution
  Primary-1:50 diluted in FACS buffer,
  Secondary-1:200 diluted in FACS buffer

- Dead cell counter stain solution
  TOPRO-3 (Invitrogen), stock solution: 1 mM in DMSO, final conc. 1:4000

2.6.1.1 Fluorescence-activated cell sorting (FACs)

Fluorescence-activated cell sorting was used to sort human peripheral blood lymphocytes (PBLs) directly into PCR tubes (Wolfgang Klinkert, MPI of Neurobiology) as a positive control to validate the unbiased β-chain primers. Details of the PCR protocol are presented in **Section 2.4.3.2.2**. FACS allows for the separation of single cells from a heterogeneous suspension based on their light scattering and fluorescence characteristics. The cell suspension is hydrodynamically focused so that droplets, each containing single cell, are formed. The droplets are charged and then analyzed for fluorescence, after which they are electrostatically deflected based on their charge. The charge returns to neutral when the droplet has broken off from the stream.

15 ml of human blood was collected in EDTA and diluted 1:1 in PBS. 15 ml of Lymphoprep was added to this mixture and then centrifuged for 20 minutes at 500 g. The cellular phase was collected in a separate tube, washed with PBS and centrifuged at 500 g for 15 minutes. The cells were incubated on ice for 30 minutes with FITC labeled CD3 antibody that was diluted 1:50 in PBS containing 2 % fetal calf serum (FCS). The cells were washed twice with PBS and re-suspended in PBS containing 1:4000 TOPRO 3 dye (for identification of dead cells). Prior to cell sorting, the cell suspension was passed through a cell strainer to ensure a homogenous single cell suspension. CD3+ and TOPRO 3- cells (live T cells) were gated and single cells were sorted directly into pre-cooled PCR tubes containing 5.5 µl of the RT PCR Buffer. The tubes were immediately stored at -80°C until the RT reaction.

- Cell strainer
  BD Falcon tube with cell strainer cap
- RT PCR buffer
  QIAGEN OneStep RT-PCR kit

2.6.2 Staining of brain tissue

2.6.2.1Staining for laser microdissection

Brain tissue sections of patient FE and MS-4 were stained with antibodies specific for CD8 (effector T cell marker) and CD134 (T cell activation marker) to identify T cells that had infiltrated the MS lesion. Polyethylene tretraphtalate (PET) membrane slides (P.A.L.M Microlaser) were baked at 180°C for 4 hours, followed by UV irradiation and coating with poly-L-lysine hydrobromide. 10 µm thick cryostat sections from frozen tissue specimens were mounted onto these slides and stored at – 80°C. All aqueous staining solutions were treated with RNase inhibitor and DEPC for ribonuclease (RNase) inactivation.

Prior to staining, the slides were thawed to ambient temperature, fixed briefly in 100% acetone and then rehydrated in PBS for 10 seconds. Blocking was performed for 3 minutes in PBS containing 2 % BSA. Next, the sections were co-incubated for 5 minutes with 1:100 diluted Cy3 labeled anti-CD8 beta chain antibody (clone 2ST8.547) and 1:20 diluted FITC labeled anti CD 134 antibody (clone ACT35) or 1:25 diluted FITC labeled anti BV1 antibody (clone BL37.2) . The sections were then rinsed with 1ml of PBS and incubated for 3 minutes with a 1:00 diluted Alexa-488 labeled anti-FITC antibody (polyclonal) in order to enhance the fluorescence signal. All incubation steps were carried out in a humidified staining chamber. After a second PBS rinse, the sections were covered with isopropanol to retard RNase activity as well as specimen drying, and immediately analyzed under the P.A.L.M Microbeam-Z microscope. Laser microdissection and the RT PCR that followed are elaborated in **Section 2.4.3.2.2**.

The entire staining and cell isolation was carried out within a room meant specifically for this purpose. All surfaces within the room are cleaned with alcohol followed by an RNase removal agent (RNaseZAP™, Sigma) and the room is illuminated with UV light for 3 hours before and after use. To prevent RNase transfer from body surface, the user is dressed in lab coat, gloves, face mask, head and shoe covers.

- Poly-L-lysine hydrobromide (Sigma)
- Protector RNase inhibitor, 1U/ml, (Roche)

- Bovine serum albumin (BSA), (Sigma)
- Cy3-mAb labeling Kit, (GE Healthcare)

2.6.2.2 Staining for localization of T cells in white and grey matter

Sequential 10 µm sections from frozen tissue (block # 9, 10, 11) patient FE were stained with luxol fast blue (LFB) for differentiation of white and grey matter, and with CD8 for detection of effector T cells to see visualize the infiltration in each region. The LFB stained sections were additionally stained with a haematoxylin nuclear stain to visualize all cells. The staining reagents were a gift from Edgar Meinl, MPI of Neurobiology.

2.6.2.2.1 Luxol fast blue and haematoxylin staining

The luxol fast blue (LFB) stain, also known as the modified Klüver's stain is used to stain the myelin sheath in white matter blue, hence differentiating it from the grey matter. LFB is an alcohol soluble sulfonated copper phthalocyanine dye that works via an acid-base reaction wherein the myelin lipoprotein base is replaced with the base of the dye. The haematoxylin nuclear staining involves the application of haemalum, a complex of aluminium ions and oxidized haematoxylin. This occurs due to the binding of the dye-metal complex to arginine-rich, basic nucleoproteins such as histones. Prior to staining, the section was first placed at room temperature for 15 minutes followed by fixing in 4 % paraformaldehyde for 30 minutes at 4°C. Following a 4X wash in PBS, the sections were placed in 0.1 % Luxol fast blue solution overnight at 55°C. The section was then washed in 96 % ethanol, followed by deionised water, and differentiated in 0.1 % Lithium carbonate solution and 70 % ethanol. The reaction was stopped by washing with deionized water. The LFB stained section was then dipped for 5 seconds in the haematoxylin solution followed by a water wash to remove excess dye. The stained slides were dehydrated by dipping in a series of increasing ethanol concentration (70% - 90 % - 96 %), followed by 100 % isopropanol and xylene. A drop of the mounting medium Eukitt was then applied before placing a cover glass. The slides may now be observed under the microscope.

- Paraformaldehyde (Sigma)
- Luxol fast blue solution (Sigma)

- Haematoxylin solution (Merck)
- Mounting medium 'Eukitt' (Gröppel)

2.6.2.2.2 CD8 staining

The brain infiltrating CD8 T cells were detected using the Cy3 labeled anti-CD8 beta chain antibody mentioned in **Section 2.6.2.1**. Incubation times were longer as RNA did not have to be isolated from the tissue. After fixing the sections as done in Section **2.6.2.2.1**, blocking was performed for 30 minutes in PBS containing 2 % BSA. The section was then incubated with the antibody for one hour at room temperature. Both blocking and antibody incubation was carried out in a humidified chamber. After antibody incubation, the sections were washed 3 times in PBS. A drop of fluorescence mounting medium (Dako) was placed on the section and covered with cover glass before analysis at the fluorescence microscope.

2.6.3 Western Blot

The recombinant hMR1 and β2 microglobulin, purified from HEK293E cells, were detected using western blot. First, the proteins that had been differentiated according to molecular weight on a gradient gel (4-20%) (**Section 2.5.3**) were immobilized onto a PVDF membrane. Next, all unspecific binding sites on the membrane were blocked by incubation in the blocking buffer for 2 hours. The membrane was then incubated overnight at 4°C with the primary detection antibody (anti c-myc for myc tagged MR1 and anti-β2 microglobulin) diluted in the wash buffer. This was followed by a 45 minute 5 step washing procedure for the removal of unbound antibody. Then the secondary antibody diluted in wash buffer was added for 1 hour and followed by the 45 minute wash. The enhanced chemiluminescence (ECL-system) of detection was used. The secondary antibody is coupled to the enzyme horse radish peroxidase (HRP) that oxidizes the luminol present in the ECL-A solution in the presence of hydrogen peroxide (H_2O_2), thereby driving it into an excited state. It decays to the ground state by emitting light. This emission darkens the corresponding part of the X-ray film, marking the location of the protein of interest. The ECL-B solution contains p-hydrocoumarin acid for enhancement of this light signal. For

each membrane, a mixture of 10 ml ECL-A solution, 3.1 µl of 30 % H_2O_2 and 100 µl ECL-B solutions was used. The membrane was incubated in this mix for 1 minute and then immediately placed into a film cassette and overlaid with an X-ray film which was developed in the dark room.

- Wash buffer (PBS-T)
  PBS with 0.05 % (v/v) tween-20
- Blocking buffer
  5% (w/v) skimmed milk, blotting grade (Bio-Rad) in PBS-T
- Primary antibody (1:1000)
  For MR1: anti-c-myc, clone 9E10.3 (Millipore)
  For β2 microglobulin: polyclonal (Abnova)
- Secondary antibody (1:2500)
  Goat anti-mouse IgG + IgM (H+L), peroxidase conjugated
  From Dieter Jenne, MPI of Neurobiology
- ECL-A solution
  0.25 % (w/v) luminol in 1M Tris/HCl
- ECL-B solution
  0.11 % (w/v) para-hydroxycoumarin acid in DMSO
- X-ray film
  Amersham Hyperfilm ECL, (GE Healthcare)

3 Results

3.1 Identification of TCR α- and β-chains from single T cells infiltrating the MS brain

Multiple sclerosis (MS) is an autoimmune demyelinating condition characterized by axonal loss, glial cell activation and the infiltration of immune cells into the CNS. CD8+ T cells are the predominant lesion infiltrating subset in MS and represent the effector population that can direct the 'autoimmune attack' on the nervous tissue (Friese and Fugger 2009). To date, the molecular target of the T cell receptor (TCR) of these autoaggressive T cells remains unknown. The identity of the autoaggressive TCR, therefore, would provide important information about the antigen(s) that it recognizes. To shed some light on the identity of the MS antigen(s), the putatively autoaggressive T cells, that had infiltrated the brain tissue in MS patients, were characterized. In order to differentiate between autoaggressive and bystander cells, certain criteria were followed while isolating cells from MS tissue cryosections. First, the cellular appearance had to be in keeping with T cell morphology (diameter: 7 -21 µm). Second, the cell of interest should express cytotoxic T cell surface makers such as CD8 besides activation markers such as CD134. Clonally expanded T cell populations could be identified by CDR3 spectratyping and such cells were analyzed (i) when TCR-specific antibodies were available, and (ii) with clone-specific primers. When TCR-specific antibodies were not available, the CD8+ T cells were additionally stained for an activation marker (such as CD134). Cells fulfilling the above criteria were isolated from the frozen tissue sections by laser microdissection (LMD) as depicted in **Figure 3-1**. To characterize their TCRs, a clone specific PCR approach (Seitz et al. 2006) (**Section 3.1.1)** and an unbiased PCR approach developed for live T cells (**Section 3.1.2**) was applied for the first time to frozen brain tissue originating from two MS patients (patient FE, biopsy; patient MS-4, autopsy).

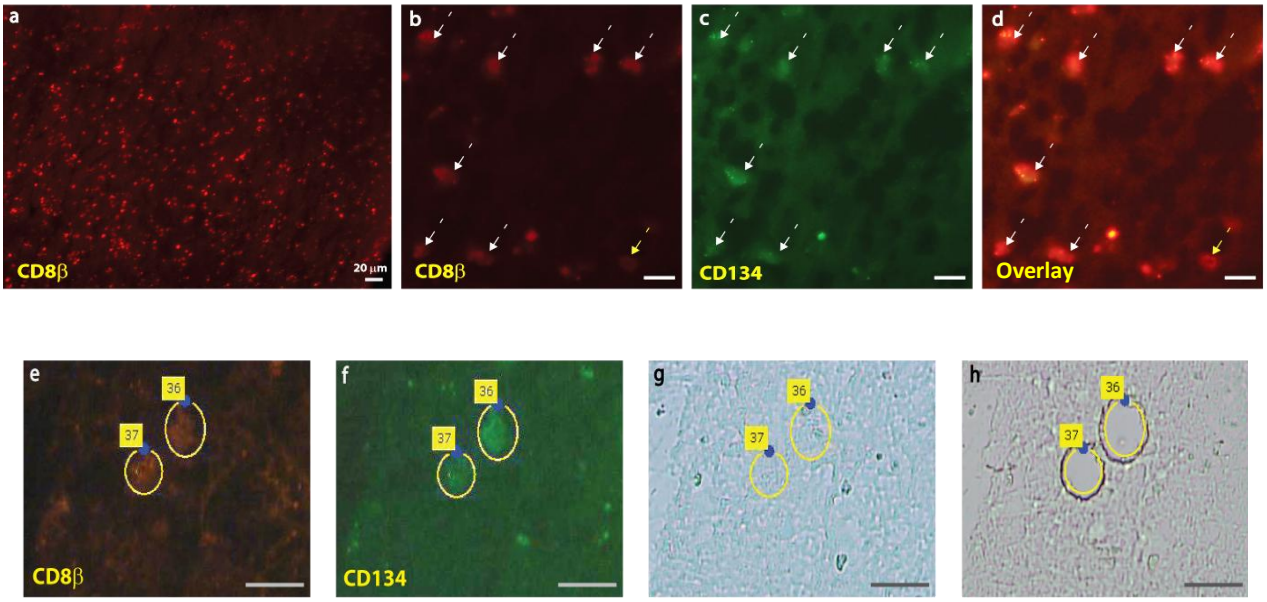


Fig. 3-1: CD8+ T cells infiltrating MS brain and their isolation by laser microdissection

Cryosections from patient FE brain sample stained for effector T cell marker CD8 (red) and T cell activation marker CD134 (green) **(a)** CD8+ T cells infiltrating brain tissue, **(b-d)** immunolocalization of activated effector T cells that are double positive (white arrows) for **(b)** CD8+ and **(c)** CD134+ as well as non activated CD8+ T cells (yellow arrow), **(e-h)** representative micrographs of the cryosections on membrane-covered polyethylene tretraphtalate (PET) slides used for laser microdisscetion (LMD). Activated single T cells are co-stained for CD8 **(e)** and a TCR chain or T cell activation marker CD134 **(f)**. They are then dissected and catapulted out of the tissue directly into the cap of a PCR tube for subsequent amplification of the expressed TCR. Panels **(g)** and **(h)** represent the bright-light image of the tissue before **(g)** and after **(h)** LMD. The numbers in the yellow field refers to apparatus parameters **(a-d)**. The image quality is affected as the tissue is not covered with a cover slip during cell isolation. The scale bars correspond to 20 µm.

3.1.1 Application of the clone specific PCR approach

It was possible to delineate the clonally expanded TCR populations within the different tissue blocks of the two MS patients FE (Skulina et al. 2004) and MS-4 (Junker et al. 2007) by CDR3 spectratyping. Clonal expansions belonging to TCRs (i) that had persisted in the patient for a long time and (ii) for which antibodies were commercially available were analyzed with this approach. The methodology was developed in the context of muscle infiltrating T cells (Seitz et al. 2006) and applied for the first time to MS patient derived frozen brain tissue.

3.1.1.1 TCR α- and β-chains derived from Patient FE

The BV1-BJ2.3 expansion that had persisted in the patient for seven years (1996-2003) was studied (Skulina et al. 2004; Babbe et al. 2000). An earlier analysis of peripheral blood cells of the patient had revealed the coexpression of an AV7.2-AJ16 α-chain (Malotka and Dornmair, unpublished). During this study, the expression of this α-chain in the brain was investigated in collaboration with Katherina Siewert and David Laplaud. Vβ1+ CD8+ single T cells were isolated from the brain and a TCR specific RT PCR was carried out. The peptide sequences of the recovered TCR chains are presented in **Table 3-1**.

a. β-chain sequence

Variable region	Peptide sequence Variable-CDR3-Joining	Joining region	Frequency	Detected in
1	CASS-ISGRKD-TQYFGPG	2.3	36	Blood, Brain (Skulina et al. 2004) (Babbe et al. 2000)

b. α-chain sequence

Variable region	Peptide sequence Variable-CDR3-Joining	Joining region	Frequency (Total: 15)	Detected In
7.2	CAV-RDNLE-LLFARG	16	6	Blood (#)
7.2	CAV-RDQA-TDSWGKLQFGAG	24.2	1	Brain
7.2	CAV-RDS-NYQLIWGAG	33	7	Brain
7.2	CAV-QEP-SGSLTFGEG	58	1	Brain

Tab. 3-1: TCR sequences derived from patient FE using the clone specific approach

(a) The BV1-BJ2.3 β-chain that persisted in patient FE for 7 years (Skulina et al. 2004; Babbe et al. 2000). **(b)** The four AV7.2 α-chains that are coexpressed with this β-chain. This work was done in collaboration with colleagues at the MPI of Neurobiology. (#) Klaus Dornmair and Joachim Malotka detected the AV7.2-AJ16 α-chain in blood. The following three α-chain sequences were found together with David Laplaud and Katherina Siewert. The variable regions of the TCRs are named in accordance with the Arden nomenclature (Arden et al., 1995). The CDR3 region is represented as flanked by the terminal amino acid (AA) residues of the variable region up till the underlined 'FGXG' consensus sequence of the joining region. These are separated by dashes. 'Frequency' relates to the number of cells where a particular TCR sequence was detected.

Of the 490 cells isolated, 36 carried the β-chain of interest and these were further analyzed using a pool of unbiased α-chain primers. The α-chain sequences could be recovered from 15 cells.

Within these 15 cells, some sequences appeared more than once, and these added to a total of 4 TCR pairs. Within these TCR pairs, the four different α-chains were coexpressed with the identical β-chain (Vβ1-Jβ2.3). Further analysis revealed that these α-chains were homologous at the peptide level and belonged to the mucosal- associated invariant T cell (MAIT) subset, with the AV7.2-AJ33 expansion being the classical MAITα-chain. These observations are discussed in **Section 4.2**.

3.1.1.2 TCR α- and β-chains derived from MS-4

All experiments carried out on material from Patient MS-4 were done in collaboration with Gintare Kemezyte (MPI of Neurobiology). The BV1-BJ1.1 expansion that had been earlier detected in both tissue blocks numbered 6 and 9 was studied (Junker et al. 2007). Of the 375 cells analyzed with clone specific primers, the sequence was recovered from one cell. This is presented in **Table 3-2**. The pairing alpha chain sequence could not be recovered.

Variable region	Peptide sequence Variable-CDR3-Joining	Joining region	Frequency (Total: 1)
1	CASS-VGDVRQM-NTEAFFGQG	1.1	1

Tab. 3-2: TCRβ-chain sequence derived from Patient MS-4 using the clone specific approach

The variable regions of the TCRs are named in accordance with the Arden nomenclature (Arden et al. 1995). The CDR3 region is represented as flanked by the terminal amino acid (AA) residues of the variable region up till the underlined 'FGXG' consensus sequence of the joining region. These are separated by dashes. 'Frequency' relates to the number of cells positive for the TCR sequence. This work was done in collaboration with Gintare Kemezyte, MPI of Neurobiology.

3.1.2 Application of the unbiased PCR approach

The clone specific PCR approach, although successful, allows one to only study TCRs for which antibodies were commercially available. To overcome this limitation, an unbiased β-chain primer pool was developed in collaboration with Song-Min Kim and Jörg Prinz (LMU) that, in combination with the pre-established universal α-chain primer pool (Seitz et al. 2006), allows for the detection of all TCR chains in a manner that is independent of CDR3 spectratyping. With this

approach all lesion infiltrating CD8+ T cells, including those that were also positive for the CD134 activation marker, could be analyzed. The protocol was developed for analyzing live T cells isolated directly from skin tissue. The RNA derived from live cells would be of better quality and of larger quantity, than that of T cells isolated from frozen tissue. Hence, it was essential to modify the protocol to suit the needs of this study. This was first tested on live TCR expressing cell lines (**Section 3.1.2.1**), followed by human peripheral blood lymphocytes (**Section 3.1.2.2**) and then ultimately applied to frozen brain tissue (**Section 3.1.2.3**).

3.1.2.1 Validation of protocol on human TCR transfected cell lines

The universal β-chain primer pool was first tested on live, recombinant human TCR expressing mouse hybridoma cell lines (**Table 3-3**). Since these cell lines express the TCR under the influence of a strong CMV promoter, it would be the appropriate gold standard for checking the accuracy of the protocol. The $58^{-/-}$ cell line that does not express human TCRs ($58^{-/-}$ (untransfected)) and a $58^{-/-}$ cell line expressing the γ/δ TCR ($58^{-/-\ \gamma/\delta}$) were used as negative controls (**Table 3-3**).

TCR hybridoma cell line	Result	Yield
$58^{-/-}$ BV1	correct sequence	6/16
$58^{-/-}$ BV9	correct	15/16
$58^{-/-}$ BV17	correct	2/16
$58^{-/-}$ (untransfected)	no product	13/16
	BV1 (contamination)	3/16
$58^{-/-\ \gamma/\delta}$	no product	12/16
	BV17 (contamination)	4/16

Tab. 3-3: TCR sequences recovered from TCR expressing hybridoma cell lines

The universal β chain-primer pool was tested on live, human TCR expressing, mouse hybridoma cell lines ($58^{-/-}$). The cell line has been designated with the TCR β-chain that it expresses. For each cell line, 16 single cells were tested. The obtained PCR products were sequenced to ascertain correct identity. The 'yield' relates to positive sequences for a particular cell line. A fraction of the negative controls gave a PCR product (contamination) resulting from a carryover from the test samples. The TCRs are named according to Arden nomenclature.

For the three test samples (BV1, BV9, BV17), correct sequences were recovered. However, PCR products were not observed for all processed samples. This might indicate that within the primer pool, some primers are more efficient than others. β-chain TCR sequences were recovered from some negative control samples as well. Since the experiments were not carried out in a clean room, the carryover of samples may have occurred. However, these results also point to the sensitivity of the technique, which is an essential prerequisite to single cell experiments. The 47.91 % yield indicates that the technique was successful in amplifying human TCRs from strongly expressing live cells.

3.1.2.2 Validation of protocol on human peripheral blood lymphocytes

Next, the protocol was tested on FACs sorted single T cells that were isolated from the peripheral blood lymphocytes of a healthy donor. The T cells were frozen at -80°C immediately after isolation to replicate actual experimental conditions. The RT-PCR was carried out directly on the thawed cells. Of the 40 single cells analyzed, PCR products were recovered from 20 cells with a yield of 50%. On further analysis, four α-chains were recovered. The results are presented in **Table 3-4**.

a. β-chain sequences

		CDR3 Sequence				Frequency
β-chain	**Vβ**		**N(D)N**	**Jβ**		**(Total: 20)**
1	1	CASS	VGG	LQFFGPG	2.1	1
2	2.1	CSA	SLCQQLSP	NEQFFGPG	2.1	1
3	3	CAS	RPAPKGWGDTES	EAFFGQG	1.1	1
4	3	CAS	THGRGD	TEAFFGQG	1.1	1
5	3	CASS	DRT	EKLFFGSG	1.4	1
6	5.2	CASS	ISDG	TQYFGPG	2.3	1
7	6.2	CASS	SLPSP	TYTFGSG	1.2	1
8	6.4	CASSL	VPY	TDTQYFGPG	2.3	1
9	7.1	CASS	HAGTGNP	NEKLFFGSG	1.4	1
10	7.2	CAS	RVPT	LTFGAG	2.6	1
11	8.1	CAS	PLAWR	EQFFGPG	2.1	1

12	9	CASS	HGTRLLARR	NYGYTFGSG	1.2	1
13	12.4	CAISE	STLSKP	FFGQG	1.1	1
14	13.5	CASS	GRQ	NEQFFGPG	2.1	1
15	19.1	CAS	FLIQYYN	QFFGPG	2.1	1
16	20	CAW	PRSLCLR	FFGSG	1.4	1
17	21.3	CASSL	DAPL	EAFFGQG	1.1	1
18	25.1	CASS	EQGV	TIYFGEG	1.3	1
19	27.1	CTS	GTQ	LFFGEG	2.2	1
20	32.1	CGS	QQSLDQG	QFFGPG	2.1	1

b. α-chain sequences

Coexpression with β-chain	CDR3 Sequence					Frequency
	Vα		N(D)N	Jβ		(Total: 4)
6	2.1	CAV	VNYT	FKTIFGAG	9	1
15	19.1	CAV	CQSS	SGSRLTFGEG	58	1
18	1.3	CAV	CDLLT	NVLHCGSG	35	1
19	20.1	CLVG	GPRPI	SARQLTFGSG	22	1

Tab. 3-4: TCR sequences from human peripheral blood lymphocytes (unbiased PCR approach)

(a) β-chain sequences from 20 single peripheral blood lymphocytes (PBLs). These are arranged according to the increasing order of the variable region number, followed by the TCR chain peptide sequence. **(b)** Matching α-chain sequences from 4 PBLs. These are arranged according to the β-chain sequence that was coexpressed in the same cell. A total of 4 TCR pairs were recovered by applying the unbiased PCR to 40 single PBLs. The sequence begins at the conserved terminal cysteine residue of the variable region and ends in the underlined 'FGXG' consensus sequence of the joining region. Frequency corresponds to the number of times a particular sequence was recovered. Vβ: variable region of β-chain (Arden nomenclature), Jβ: joining region of β-chain; N (D) N: hypervariable region; Vα: variable region of α-chain, Jα: joining region of α-chain

3.1.2.3Unbiased identification of TCR chains from frozen brain tissue

Once the protocol had been validated, it was applied to T cells isolated from frozen tissue. As the sample tissue had been in -80°C storage for a prolonged time period, and was repeatedly thawn and frozen for making tissue sections, the quality of the RNA, and hence the efficiency of the RT-PCR would be expected to be greatly reduced. To make up for this, a larger sample size was considered, and all reactions were carried out in quick short steps. In certain PCR steps, the reaction volume was modified. Ultimately, TCR sequences were successfully recovered from frozen brain tissue by the application of the unbiased PCR approach.

3.1.2.3.1 Patient FE

Three tissue blocks (#9, #10, and #11), originating from the same lesion of the patient, were analyzed during this study. Tissue infiltrating T cells were stained for the marker of cytotoxic cells (CD8) as well as the T cell activation marker (CD134). Of the 643 laser-microdissected single CD8+ T cells isolated, TCR β-chain sequences were amplified from 68 samples. Some β-chains were recovered more than once, and 13 distinct chains were detected in total. On further analysis of these samples, 5 different α-chains were detected from 13 cells. Interestingly, in two cases, the identical β-chain paired with 2 different α-chains. Both of these β-chains belonged to dominant expansions that were detected in 46 and 8 cells. Previously, we have reported a similar observation in the context of inflammatory muscle diseases (Seitz et al. 2006). The fifth TCR pair was found in just one cell, but was known to be expanded from previous studies (Babbe et al. 2000). These results are presented in **Table 3-5**.

a. β-chain sequences

	CDR3 Sequence					Frequency in different tissue blocks			
β-chain	**Vβ**	**N(D)N**		**Jβ**		**# 9**	**# 10**	**# 11**	**Total**
1	6.2	CASS	PYPH	TEAFFGQG	1.1	0	0	1	1
2	6.2	CASS	SRDRG	GYTFGSG	1.2	0	0	1	1
3	6.2	CASSL	RPN	GELFFGEG	2.2	1	0	0	1
4	6.2	CASS	PTSL	TDTQYFGPG	2.3	1	0	0	1
5	6.3	CASSL	AFTGES	EQYFGPG	2.7	1	0	0	1
6[#†]	6.5	CASSL	APN	GELFFGEG	2.2	5	3	0	8
7	8.1	CAS	THRGHG	NTEAFFGQG	1.1	1	0	0	1
8[#†]	8.1	CAS	TQGWGD	TEAFFGQG	1.1	23	13	10	46
9	13.1	CASS	TSPGGARG	GNTIYFGEG	1.3	1	0	0	1
10[¤†]	13.1	CASS	LGA	DTQYFGPG	2.3	1	2	0	3
11[¤]	13.2	CAS	RALVAT	YNEQFFGPG	2.1	0	0	1	1
12[†]	17.1	CASS	SRS	SYEQYFGPG	2.7	1	1	0	2
13	21.3	CASS	LAY	GELFFGEG	2.2	1	0	0	1

b. α-chain sequences

	CDR3 Sequence	Frequency in

Coexpression with β-chain						different tissue blocks			
	Vα		N(D)N	Jα		1	2	3	Total
6a†	20.1	CLVGD	SRKG	DDKIIFGKG	30	1	1		2
6b†	20.1	CLVG	AT	GNTGKLIFGQG	37	1	1		2
8a	1.5	CAV	SA	TDKLIFGTG	34	1			1
8b†	30.1	CAV	PF	DRGSTLGRLYFGRG	18	5	1	1	7
11	21.1	CAAS	G	GSNYKLTFGKG	53			1	1

Tab. 3-5: TCR sequences recovered from patient FE using the unbiased PCR approach

A total of 5 TCR pairs were recovered from 68 single cells. All the β-chains and α-chains are arranged according to the increasing order of the variable region number. This is followed by the TCR chain peptide sequence beginning with the conserved terminal cysteine residue of the variable region and ending in the 'FGXG' consensus sequence of the joining region. The term 'frequency' corresponds to the number of times a particular sequence was recovered, and this has been distributed amongst the three blocks analyzed in the study. **(a)** β-chain sequences from 13 cells. **(b)** α-chain sequences that were found to be coexpressed with a particular β-chain. The designation of 'a' or 'b' following the TCR chain number corresponds to the two different alpha chains that pair with the identical beta chain. Vβ: variable region of β-chain (Arden nomenclature), Jβ: joining region of β-chain, N (D) N: hypervariable region Vα: variable region of α-chain, Jα: joining region of α-chain; †: detected in more than one tissue block; #: β-chain found in coexpression with two different Vα rearrangements; ¤: also detected in earlier studies (Babbe et al. 2000)

A study in our laboratory (Junker et al. 2007) demonstrated that T cell clonal expansions are found in different physical regions of the MS brain. Having found the β-chains of T cell clones 6, 8, 10, 12 in more than one tissue block from patient FE, we could confirm that these clonal expansions were not localized in one part of the brain, but were present as 'pervasive clones' in other parts as well.

3.1.2.3.1.1 Effector T cell distribution in white and grey matter

The demyelinating inflammation in MS has been mainly thought to be a white matter (WM) disorder of the brain and the involvement of grey matter (GM) in the disease was noted early, but came to the forefront only recently (Geurts and Barkhof, 2008; Geurts et al. 2009). By staining sequential sections for infiltrating CD8+ effector cells, and luxol fast blue (white matter/ grey matter differentiation), we were able to visualize the infiltration in these two regions. Representative images for block # 9 and # 11 from patient FE are depicted in **Figure 3-2**.

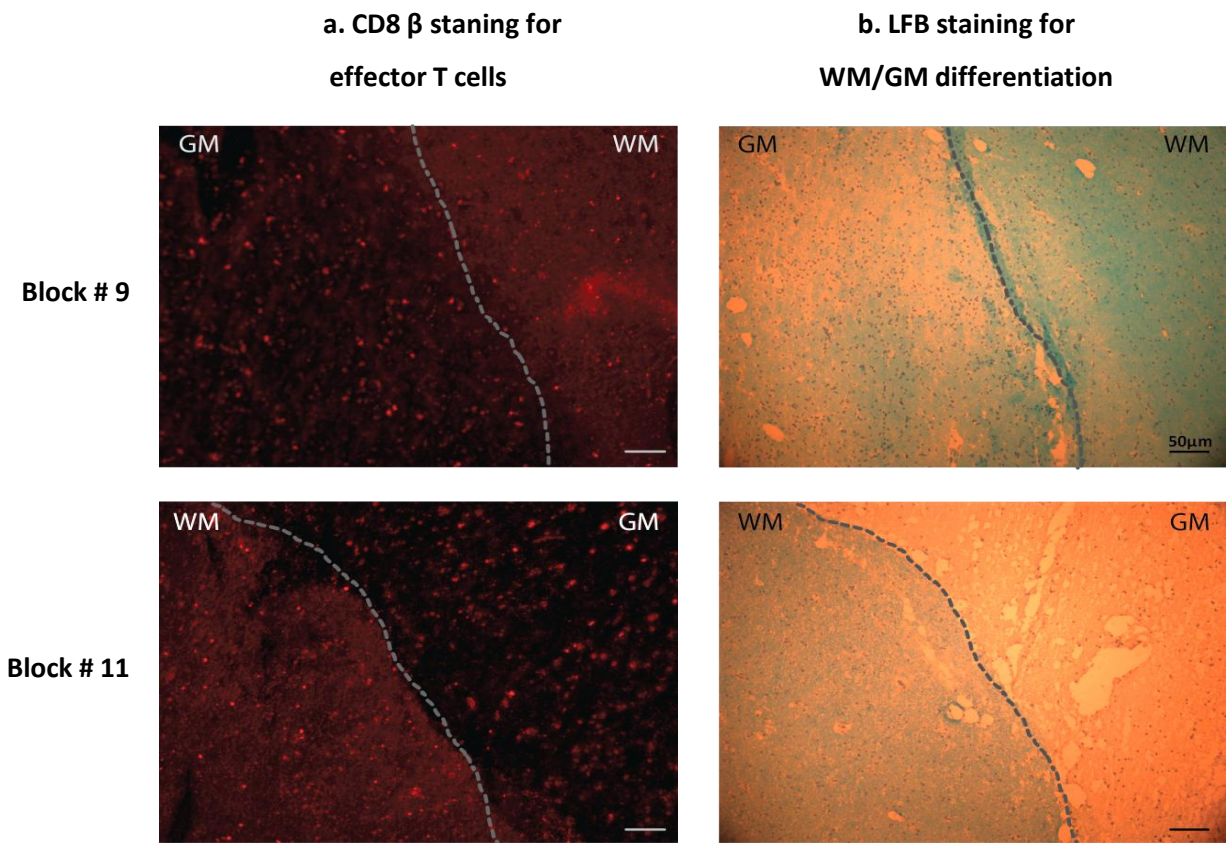


Fig. 3-2: CD8+ T cell infiltration in grey matter (GM) and white matter (WM) of patient FE

Sequential cryosections from block #9 and #11 of patient FE brain sample stained for **(a)** infiltrating CD8+ effector T cells (red) using a Cy3 labeled CD8β antibody. The cells are seen in both white matter (WM) and grey matter (GM). **(b)** The WM/GM differentiation by luxol fast blue (LFB) that stains the myelin in the white matter blue. The boundaries between WM and GM are depicted in all images as dotted grey lines. The sections were additionally stained with the haematoxylin nuclear stain that stains the nuclei of all cells purple. The staining reagents were a gift from Edgar Meinl, MPI of Neurobiology. The scale bars correspond to 50 µm

CD8+ T cells were observed to infiltrate white matter, grey matter and the meninges (data not shown here). The T cells isolated from patient FE (**Section 3.1.2.3.1**) were isolated bonafide from both grey and white matter. Since it was not technically possible to perform a stain for effector T cells and differentitate between white and grey matter on the sections used for laser microdissection, it is not possible to determine the exact localization of these cells.

3.1.2.3.2 Patient MS-4

All experiments carried out on material from patient MS-4 were done in collaboration with Gintare Kemezyte (MPI of Neurobiology, Martinsried). The unbiased PCR primer approach was applied to a total of 969 single CD8+ cells and from these 6 β-chain sequences could be recovered. Of these 6, one TCR chain was found twice. The low yield may be due to the mediocre RNA quality of the frozen sample as discussed in **Section 4.1.4**. These sequences are presented in **Table 3-6**.

β-chain sequences

		CDR3 Sequence				Frequency
β-chain		**Vβ**	**N(D)N**	**Jβ**		**(Total: 6)**
1	6.2	CQQ	RTEGGRRG	DTIYFGEG	1.3	1
2	6.3	CASS	LIGSV	TDTQYFGPG	2.3	2
3	6.5	CASS	FTQF	NQPQHFGDG	1.5	1
4	6.5	CASS	RIGG	SYNEQFFGPG	2.1	1
5	8.1	CASS	RDRG	IQYFGAG	2.4	1

Tab. 3-6: TCR sequences derived from patient MS-4 using the unbiased PCR approach

A total of 5 β-chain re-arrangements were recovered from 969 single cells. These are arranged according to the increasing order of the variable region number. This is followed by the TCR chain peptide sequence beginning with the conserved terminal cysteine residue of the variable region and ending in the underlined 'FGXG' consensus sequence of the joining region. Frequency corresponds to the number of times a particular sequence was recovered. Vβ: variable region of β-chain (Arden nomenclature), Jβ: joining region of β-chain; N (D) N: hypervariable region

3.2 *In vitro* expression of recombinant TCRs

The four MAIT TCRs (BV1-BJ2.3 β-chain coexpressed with four α-chains AV7.2-AJ16, AV7.2-AJ33, AV7.2-AJ24.2 and AV7.2-AJ58) and BV8.1-AV30.1 TCR (BV8.1-BJ1.1 coexpressed with AV30.1-AJ18) derived from patient FE were reconstructed during the course of this study. The variable and NDN region of the BV1-BJ2.3 and AV7.2-AJ33 were cloned from patient FE cDNA by David Laplaud, MPI of Neurobiology. For the other five chains, the recombinant TCR was put together by ligating a chemically synthesized 'leader-variable-joining region' fragment into a pRSV vector containing the relevant TCR constant region. The $58^{-/-}$ mouse hybridoma cell line was chosen as an expression system as it lacks the inherent expression of functional TCR chains. The $58^{-/-}$ cells were first co-transfected with the expression vectors for the two chains of the TCR heterodimer (**Section 3.2.1**). Following antibody selection, positive clones were subjected to a CD8 super-transfection (**Section 3.2.2**) and an ultimate NFAT-GFP transfection (**Section 3.2.3**). This scheme is illustrated in the **Figure 3-3**.

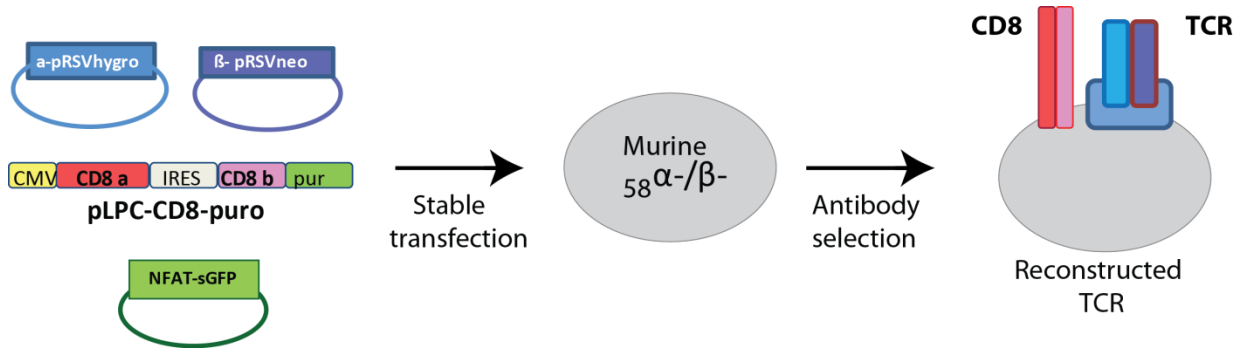


Fig. 3-3: Pictorial representation of the expression of recombinant TCR molecules

The α- (hygromycin selection) and β- (geniticin selection) TCR chains cloned into pRSV vectors were first co-transfected into the murine $58^{-/-}$ cell line, followed by super transfection with CD8 heterodimer expression plasmid (puromycin selection) and a final NFAT-sGFP expression plasmid (blasticidin selection) transfection. After each transfection, suitable clones were chosen by antibody selection pressure. By cultivating the cell line with a cocktail of four antibodies, it was possible to maintain stable transfectants in culture. The vector maps can be found in **Appendix II**.

3.2.1 Expression of TCR

The four MAIT TCRs and the BV8.1-AV30.1 TCR were successfully expressed in the $58^{-/-}$ cell line. Stable expression of the TCR chain was confirmed by flow cytometry using a murine CD3 and human TCR β-chain specific antibody (**Figure 3-4**). Given that α-chain specific antibodies

were not commercially available it was feasible to ascertain mRNA level expression by a RT-PCR (data not shown here).

3.2.2 Expression of human CD8

The four MAIT TCR expressing cells were now transfected with the construct coding for the human CD8 co-receptor. Stable expression of both CD8 chains (α and β) was verified by flow cytometry as shown in **Figure 3-4**.

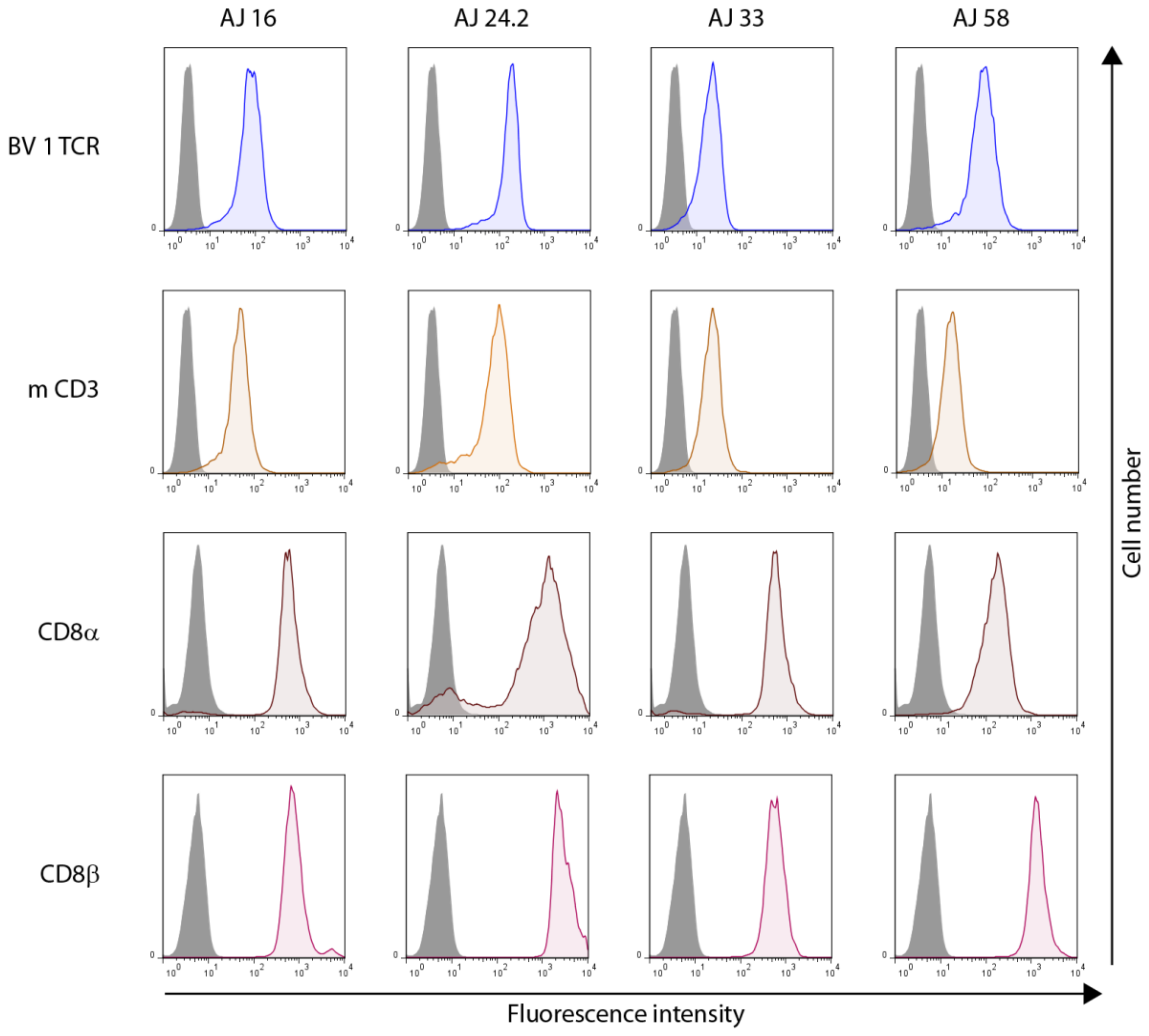


Fig. 3-4: Stable expression of human TCR and CD8 αβ co-receptor in the 58$^{-/-}$ cell line

The MAIT TCR expressing cells (denoted as AJ 16, AJ 24.2, AJ 33 and AJ 58 according to the J region of the α-chain. All four TCRs share the same BV1-BJ2.3 β-chain.) were super-transfected with CD8 expression plasmids. Stable surface expression of the human BV1 TCR chain (blue), the inherent murine CD3 (mCD3) (orange) molecule, and the two chains of the CD8 molecule (α: maroon, β: magenta) was confirmed by flow cytometry (plot of fluorescence intensity vs. cell number). The grey shaded curve delineates the isotype controls for each antibody.

Next, the functionality of the expressed MAIT TCR complex was tested. This was done by assaying for IL-2 secretion in the cellular supernatant in response to CD3 cross-linking of the TCR by a murine CD3 antibody (clone 145-2C11). Clones that demonstrated stable and high expression were selected and cultivated further for experiments. These results are presented in **Figure 3-5**.

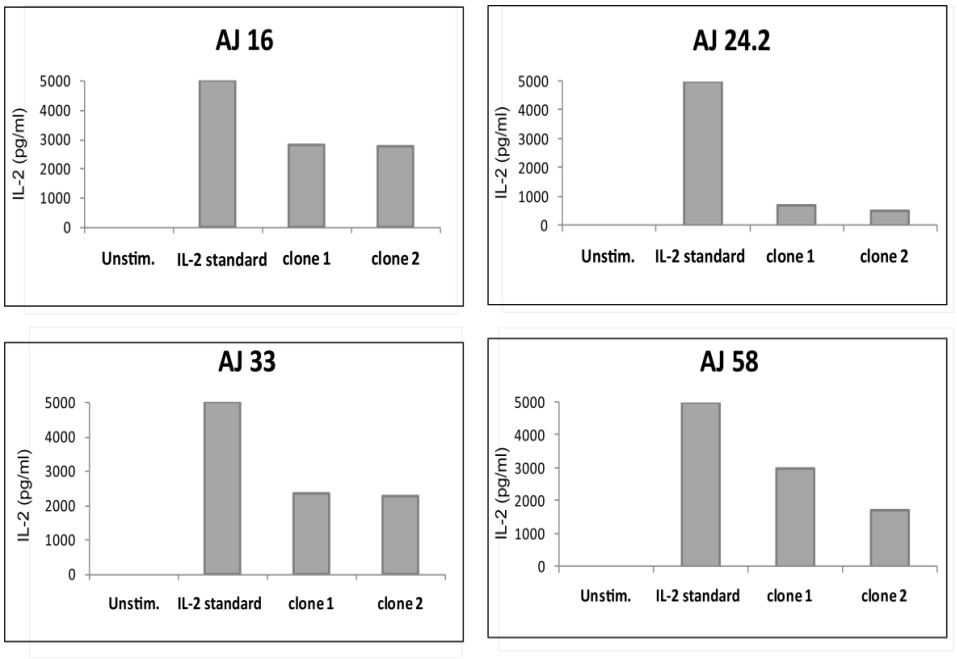


Fig. 3-5: IL-2 secretion in response to CD3 cross-linking as assayed by an ELISA

A murine CD3 specific antibody was used to elicit IL-2 secretion as a means to check for correct functioning of the transfected TCR for the four MAIT TCR hybridomas (denoted as AJ 16, AJ 24.2, AJ 33 and AJ 58 according to the J region of the α-chain. All four TCRs share the same BV1-BJ2.3 β-chain). The best expressing clones of each cell line (designated as clone 1 and clone 2) were used for further study. Unstimulated (Unstim.) cells served as negative controls while an IL-2 standard served as the positive control. For cell line AJ 16, and AJ 58, the reaction time was 1.6 minutes, for AJ 33 it was 3 minutes and for AJ 24.2 it was 6 minutes.

3.2.3 Expression of NFAT-GFP

The final transfection in this series was that of an NFAT-GFP construct which would render the cells GFP positive upon antigen recognition and subsequent TCR activation. This effect could be simulated *in vitro* by using a murine CD3 antibody (clone 145-2C11). The resultant GFP expression construct could be easily detected by flow cytometry and fluorescence microscopy (**Figure 3-6**). The GFP expression was never observed in 100% of the population, and the

individual cells expressed GFP to varied degrees. Similar observations were made for a NFAT driven lacZ construct (Sanderson and Shastri, 1994) used to report T cell activation.

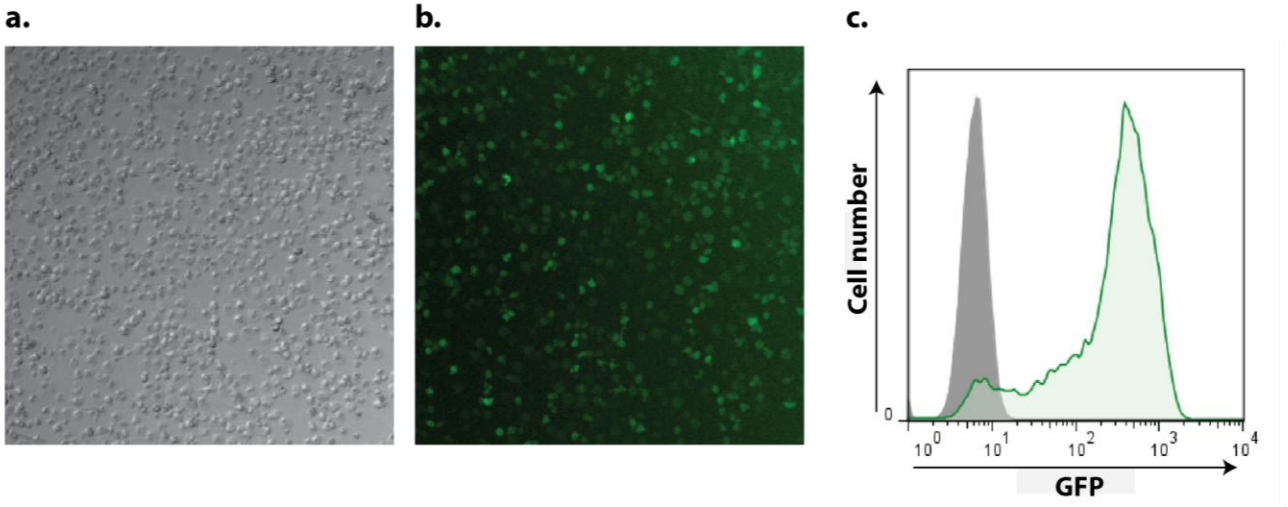


Fig. 3-6: GFP expression upon TCR activation by CD3 cross-linking *in vitro*

Crosslinking of the CD3 chains of the TCR complex leads to the same downstream signaling cascade that follows TCR activation. This is translated into an NFAT driven GFP expression in the activated MAIT hybridoma cells as detected by **(a, b)** Fluorescence microscopy images and **(c)** Flow cytometry histogram. Almost 85% of the cells are GFP + in the above example as can be discerned by a direct comparison between the bright light **(a)** and fluorescence channel **(b)** images. In the flow cytometry image **(c)**, the grey curve corresponds to the unstimulated negative control while the green curve depicts the stimulated, GFP + cells. This data corresponds to the AJ33 MAIT TCR hybridoma cell line**.**

3.3 MAIT TCR antigen search: Testing of candidate MS antigens

The classical MAIT (BV1-BJ2.3 and AV7.2-AJ33) TCR was found to be expressed most frequently in the lesion infiltrating CD8+ T cells of patient FE. Also, the possibility that the other three related TCRs (identical BV1-BJ2.3 β-chain co-expressed with AV7.2-AJ16, AV7.2-AJ24.3 and AV7.2-AJ58) might recognize a similar antigen, qualified this TCR as an interesting subject of further study. All antigen search experiments that follow were directed towards this TCR.

The initial antigen search experiments (**Section 3.3.1 to 3.3.4**) were carried it out at a stage when it was not known that the AV7.2-AJ33 α-chain belonged to the MAIT TCR. These experiments were designed to test whether the antigen recognized by the TCR (BV1-BJ2.3/ AV7.2-AJ33) was indeed 'MS specific'. Consequently, molecules put forward as 'candidate MS antigens' were first tested using patient HLA matched antigen presenting cells (instead of strictly MR1 expressing APCs). This included co-incubation of the TCR hybridoma with human brain tissue (**Section 3.3.1**), white and grey matter brain extracts (**Section 3.3.2**), autologous and EBV derived antigens (**Section 3.3.3**) and myelin derived antigens (**Section 3.3.4**). The experiments in Sections 3.3.2 to 3.3.4 were carried out in collaboration with Katherina Siewert (MPI of Neurobiology).

3.3.1 Human brain tissue

The objective of this experiment was to ascertain whether the antigenic target of the TCR was a 'brain specific' or even 'patient specific' entity. The hybridoma cells were co-incubated with 10 µm thick tissue sections of brain tissue originating from the patient (FE), and as controls; an unrelated MS patient and a meningitis patient. 12-16 hours later, the samples were analyzed under a fluorescence microscope and the GFP + activated cells in the vicinity of the tissue were counted (cells/mm^2). The experiment was repeated with the application of a pan-MHC class I antibody (clone W6/32) in an attempt to block antigen presentation, as at this stage it was assumed that the TCR was restricted by a classical MHC class I molecule. A drop in activation would be expected if the antigenic signal was in fact brain or patient specific. The results are

depicted in **Figure 3-7**. In general, a higher activation was observed in response to the patient tissue > unrelated MS patient > meningitis patient. This effect could not be eliminated completely by blocking with the pan-MHC class I antibody. At face value, this observation may be attributed to the expression of an antigen that is specific to the MS patient, but also found in other brain samples. This experiment would need to be repeated with an antibody capable of blocking MR1 in order to obtain significant results.

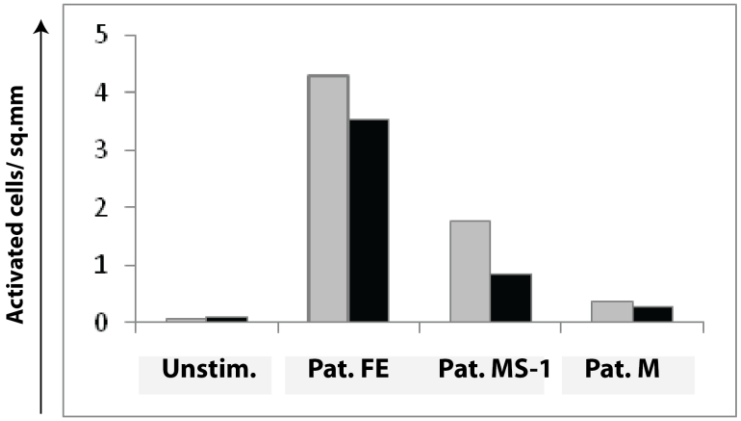


Fig. 3-7: TCR activation in response to brain tissue

The number of GFP+ cells observed per mm^2 (sq.mm) of the scanned area was plotted for each tissue sample tested in the presence (black) or absence (grey) of the pan-MHC class I blocking antibody (clone W6/32). Unstimulated cells (Unstim.) were used as a negative control. Tissue test samples were sourced from the MS patient in whom the TCR was detected (Pat. FE); an unrelated MS patient (Pat. MS-1) and a meningitis patient (Pat. M)

3.3.2 Human brain extracts

Next the TCR was investigated in the context of brain derived antigens that were present in human brain white matter (WM) and grey matter (GM) homogenates. In addition, the glycoprotein fractions were also tested. The extracts were added to a (HLA matched) fraction of peripheral blood cells that contained dendritic cells and macrophages and were capable of endocytosing external protein, processing it and presenting it subsequently via the HLA molecules on the surface. The hybridoma cells were incubated with a murine CD3 antibody (clone 145-2C11) to mimic TCR activation in the positive control and no antigen was added in the negative control. The TCR activation was measured by estimating the IL-2 secreted in the supernatant by the hybridoma cells. The outcome of the experiment is presented in **Figure 3-8**. No activation was observed in response to the human brain extracts of the white and grey matter fractions. Via the positive control it was ascertained that the TCR was functionally active. Absence of activation may be due to low amounts or inadequate processing of the antigen.

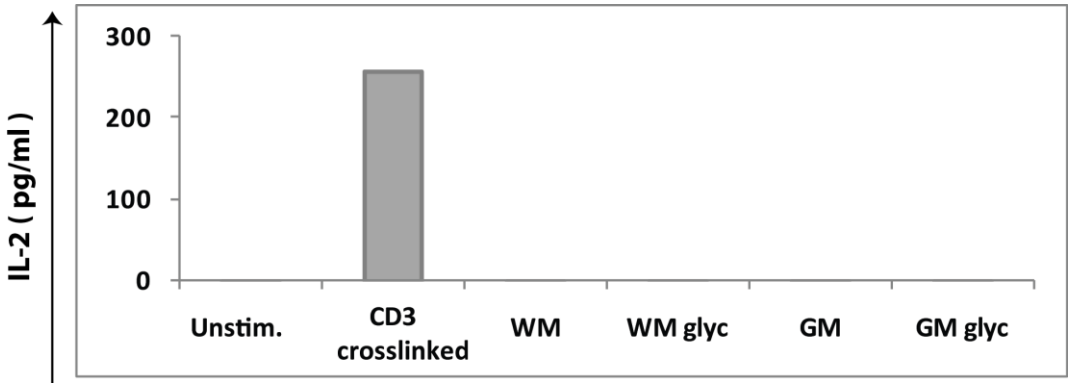


Fig. 3-8: TCR response to human brain extracts

The BV1-BJ2.3/AV7.2-AJ33 TCR hybridoma was co-cultured with a (patient FE HLA matched) fraction of peripheral blood cells containing dendritic cells and macrophages that presented antigens derived from brain homogenates. TCR activation was measured as a function of secreted IL-2. No significant response was elicited from the tested fractions. CD3 cross-linked (antibody clone 145-2C11) TCR hybridoma cells served as positive control and gave a measurable response. Unstimulated cells (Unstim.) were used as the negative control. WM: white matter; GM: grey matter; glyc: glycoprotein fraction. The brain extracts were provided by E. Meinl (MPI of Neurobiology)

3.3.3 Autologous antigens and EBV derived antigens

The examination of autologous or 'self' antigens is an integral part of studying autoimmune conditions. In addition, antigens of microbial origin that may mimic the autologous antigen or be inherently self-reactive have also been implicated in autoimmunity and MS immune pathogenesis (Hafler 1999; Van Noort et al. 2000). More specifically, the Epstein-Barr virus (EBV) has been linked to MS incidence (Serafini et al. 2007) making it an interesting candidate.

With the intention of testing these two hypotheses, the BV1-BJ2.3/AV7.2-AJ33 TCR hybridoma cells were co-cultured along with patient FE derived autologous B cells that had been transformed with EBV. These B cells carry all patient specific HLA molecules, and are hence capable of presenting EBV antigens of the latent phase in addition to autologous antigens. In a parallel experiment, the B cells were transfected with 'lytic phase inducing' plasmids BZLF1 and BRLF1 (gift of Prof. Miller, Yale University) prior to co-culture, so that they were capable of presenting lytic phase antigens as well. CD3 stimulation (antibody clone 145-2C11) of the cells was used as a positive control while absence of antigen served as the negative control. IL-2 secreted in the cellular supernatant was a measure of activation. The results are summarized in

the following **Figure 3-9**. The hybridoma cells were not activated to a level that was clearly distinguishable from that of the negative control. Such low levels of IL-2 production cannot indicate antigen recognition based TCR activation.

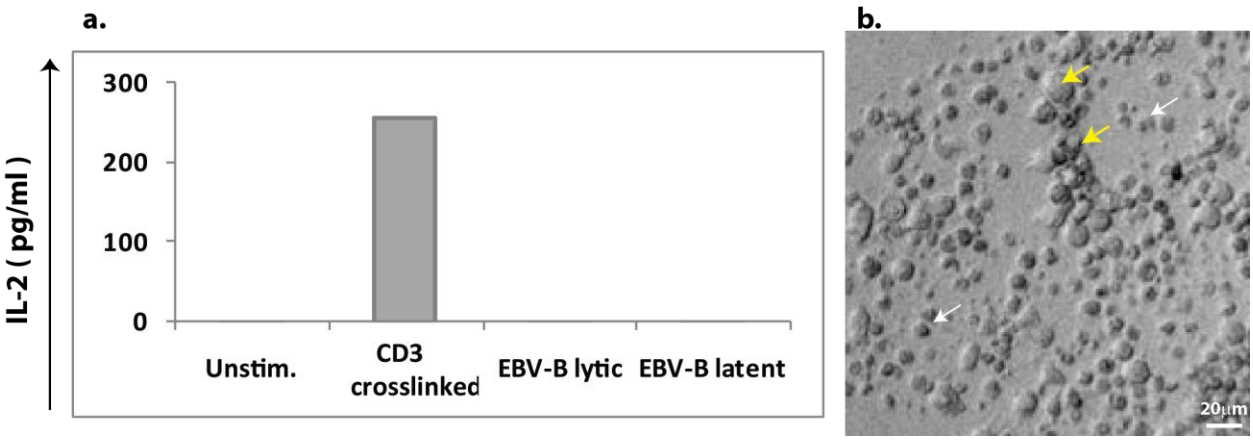


Fig. 3-9: TCR response to autologous and EBV derived antigens

The BV1-BJ2.3/AV7.2-AJ33 TCR hybridoma cells were co-cultured with EBV transformed autologous B cells of patient FE. **(a)** No IL-2 could be detected in the supernatant from the co-cultures. CD3 crosslinked cells (antibody clone 145-2C11) served as a positive control and showed a measurable response. Unstimulated cells (Unstim.) served as negative control. EBV-B lytic: EBV transformed B cells of patient FE that had been induced to enter the lytic phase of viral life cycle; EBV-B latent: EBV transformed B cells of patient FE that carried the virus in latency. **(b)** The co-culture of the MAIT TCR hybridoma (white arrows) that are seen as rounded, isolated cells and EBV transformed autologous B cells of patient FE (yellow arrows) that are larger in size, and clumped together. The scale bar corresponds to 20 µm.

3.3.4 Myelin derived MS candidate antigens

Myelin derived antigens such as myelin oligodendrocyte protein (MOG), myelin basic protein (MBP) and neurofascin are believed to be the antigenic targets in MS based on a number of studies in animal models of the disease. These candidates were also tested by presentation via a (HLA matched) fraction of peripheral blood cells that contained dendritic cells and macrophages that were co-cultured with the BV1-BJ2.3/AV7.2-AJ33 TCR bearing hybridoma cells. TCR activation was measured based on amounts of secreted IL-2. The outcome of this study is depicted in **Figure 3-10**. No significant IL-2 secretion could be detected in response to myelin derived MS candidate antigens. This indicates non-recognition of these antigens by the TCR.

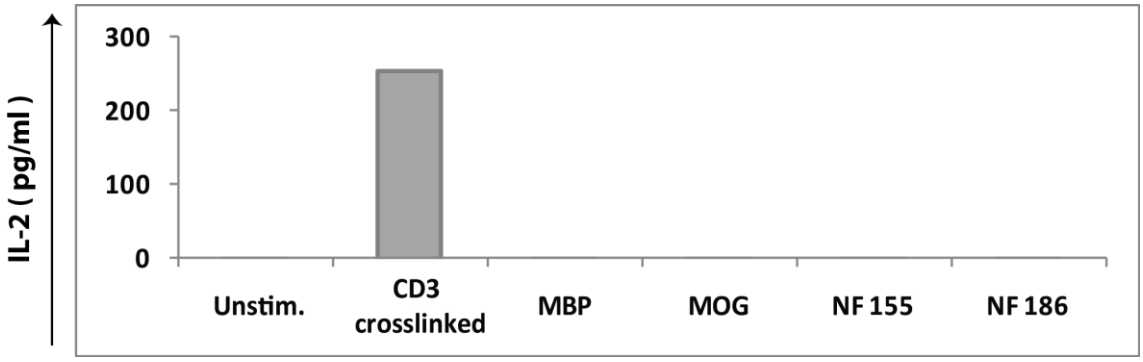


Fig. 3-10: MAIT TCR response to myelin derived MS candidate antigens

BV1-BJ2.3/AV7.2-AJ33 TCR hybridoma cells were co-cultured with a (patient FE HLA matched) fraction of peripheral blood cells containing dendritic cells and macrophages that presented the candidate antigens to the TCR. TCR activation was measured as a function of secreted IL-2. No significant response was elicited from the tested molecules. CD3 crosslinked cells (antibody clone 145-2C11) served as a positive control, validating the functionality of the TCR. Unstimulated cells (Unstim.) served as the negative control. MBP: myelin basic protein, MOG: myelin oligodendrocyte protein, NF: neurofascin (isoforms 155 and 186)

3.4 MAIT antigen unbiased search: screening of PECP libraries

The failure to detect the antigen of the BV1BJ2.3-AV7.2-AJ33 TCR by screening of 'ubiquitously expressed' and 'candidate MS' antigens made it necessary to apply an unbiased method of antigen search. At this stage, it became known that the BV1-BJ2.3-AV7.2-AJ33 TCR might be a MAIT TCR. The $58^{-/-}$ cell line expressing the entire TCR complex (MAIT TCR, CD8, and NFAT-GFP) is from now on referred to as the MAIT hybridoma. Since not much is known about the antigen specificities of this TCR, the need for an unbiased method of antigen search was great. Such a method would not only have to allow the screening of all possible peptide antigens, but also ensure proper antigen processing and presentation to the TCR. In addition, the method would have to be sensitive enough to detect even the lowest levels of activation and have a scheme in place that would allow for the isolation of the antigen. Keeping all of the above in mind, the combination of plasmid encoded combinatorial peptide (PECP) libraries combined with the NFAT-GFP TCR activation readout was applied for antigen search (Siewert et al., in press).

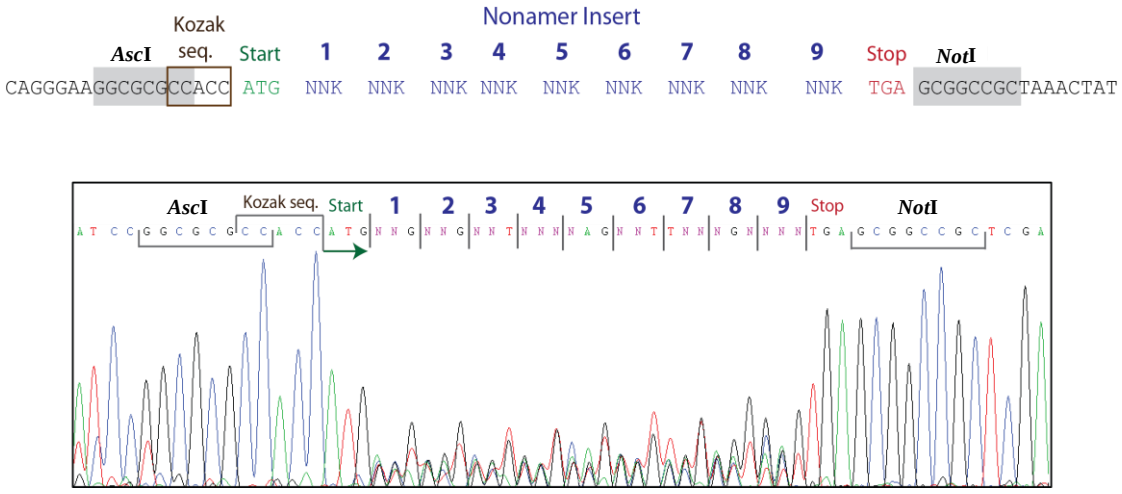
3.4.1 Library synthesis (A0101, B0801)

MHC class I molecules are capable of presenting peptides that are 8-10 amino acids in length. In addition, these peptides contain conserved amino acid residues or 'anchor positions' that are specific to the MHC class I molecule that binds them. For example peptides presented by MHC class I molecule A*0101 are anchored at position 3 and 9 by an aspartic acid and tyrosine residue (**Figure 3-11**). Keeping the above in mind, the plasmid encoded combinatorial libraries (PECP) were designed to code for nonamers (9 amino acid length) that carried the specific anchor molecules for preferential binding to a specific MHC class I molecule. Opposed to a randomized library, an anchored library is composed of an increased number of peptides capable of being presented by the HLA molecule. This in turn adds greatly to the potential repertoire of antigens that a TCR may recognize and hence bind to. The PECP libraries were all designed in a similar manner, and several copies were generated. HLA matched PECP libraries take conserved amino acid anchors into consideration and code for antigenic peptides that would best bind to a given HLA molecule.

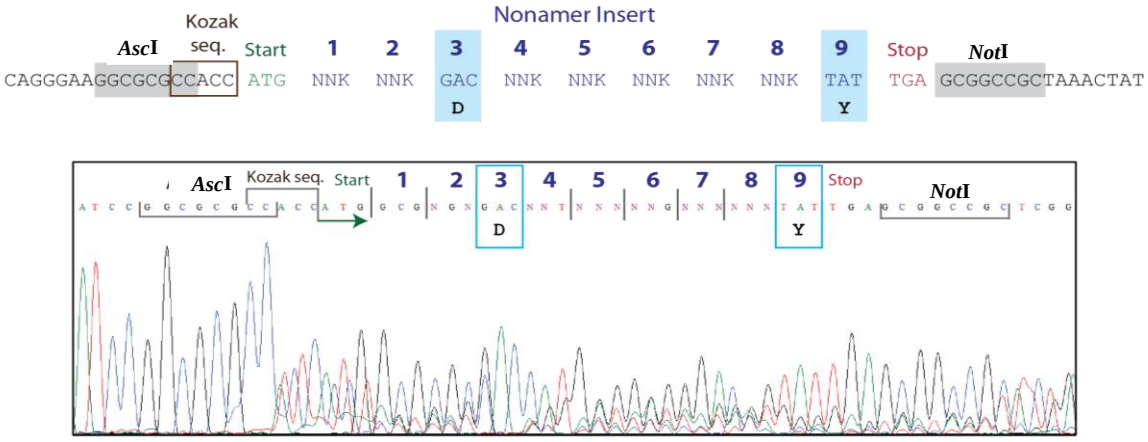
The patient FE is homozygous for HLA-A*0101, -B*0801 and -Cw*0701 (Skulina et al. 2004). PECP libraries were generated for the HLA molecules whose anchor positions are known and well defined, that is the HLA-A*0101 and HLA-B*0801. A randomized unbiased PECP library called the N library (provided by Katherina Siewert) was used to investigate other HLA molecules that are not as well defined such as HLA-Cw*0701 or the MHC class I related protein (MR1). At this stage, it was known that the BV1-BJ2.3/ AV7.2-AJ33 TCR might be a MAIT TCR that was restricted by MR1.

The nonamer coding region of the library was 27 nucleotides long. It is flanked upstream by a Kozak sequence and start codon and downstream by a stop codon to ensure correct initiation and termination of the peptides during translation. This region was further flanked by two 'rare cutting' restriction enzyme recognition sites of 8 nucleotides each (upstream: *Not*I, downstream: *Asc*I). The presence of these sites ensures that the frequency of randomly occurring restriction sites remains low (more than 16 fold less than a 6 nucleotide recognition site restriction enzyme), hence facilitating accurate cloning of fragments. Randomized nucleotide sequences often code for stop codons, and this can inadvertently lead to the generation of truncated and non-functional peptides. To lower the incidence of such randomized stop codons, guanine and thymidine residues were exclusively introduced at position three of every codon. This ensured that the total number of codons was reduced by half while the stop codons were reduced from three to one. Hence, for a randomized library, the number of non-functional truncated peptides was decreased from 35 % to 25 %. The sequence details of the libraries used in this study are summarized below in F**igure 3-11**.

a. Randomized nonapeptide library/ N library (N27-K)



b. HLA-A *0101 matched nonapeptide library (A1-39-K)



c. HLA-B *0801 matched nonapeptide library (B8-359-K)

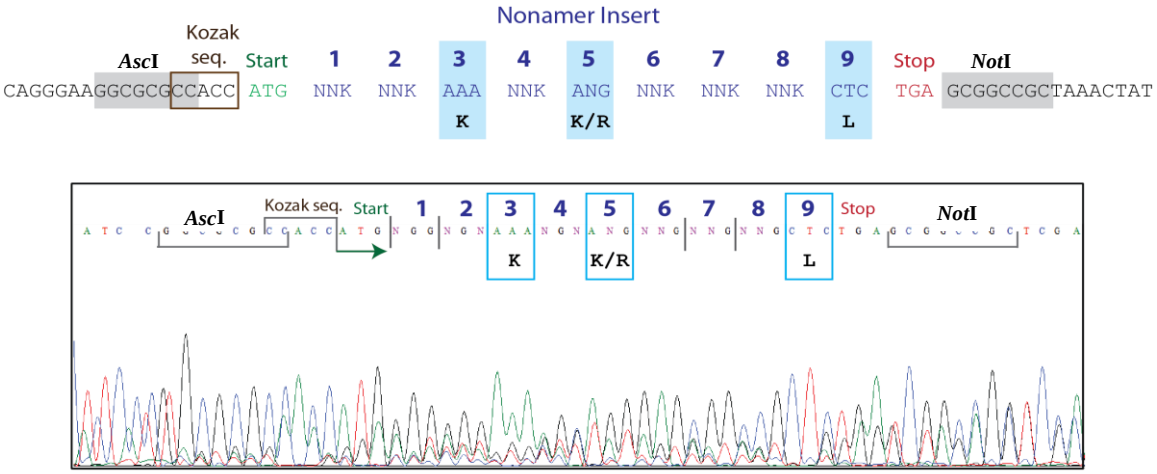


Fig. 3-11: PECP libraries generated for antigen search

Pictorial representation and electropherograms of the peptide coding region (blue) of the PECP libraries generated for antigen search. The nonamer coding amino acid residues are flanked on either side by rare cutter restriction sites of *Asc*I and *NotI* (grey). They begin with a guiding kozak sequence, the start codon (green) and finish in the stop codon (red). The remainder sequence of the expression plasmid has not been presented in this figure. For all the three libraries, the upper panel depicts a schematic of library design followed by the electropherogram of the actual library. In the cDNA sequences depicted in blue, 'N' represents the presence of any of the four (adenine, cytosine, thymidine and guanine) residues while a 'K' residue corresponds to a guanine or thymidine residue. **(a)** N27-K library encoding randomized nonapeptides (provided by Katherina Siewert, MPI of Neurobiology). This library was applied in searching for the MAIT antigen as the MR1 anchor residues are unknown. **(b)** A1-39-K library containing nonapeptides anchored at positions 3 and 9 by aspartic acid (D) and tyrosine (Y) residue. **(c)** B8-359-K library containing nonapeptides anchored at positions 3, 5 and 9 by a lysine (K), lysine or arginine (R) and leucine (L) residue respectively. The anchor positions are depicted in blue numerals.

3.4.2 TCR Activation by the unbiased PECP (N) library

The MAIT antigen is unknown to this date. Some studies strongly argue in the favor of it being a peptide (Huang et al. 2005; Miley et al. 2003) but there are other reports that support it being a lipid (Shimamura et al. 2007). To test whether the antigen was a peptide, the activation of the MAIT TCR in response to a plasmid encoded combinatorial library (PECP) was examined. MAIT TCR hybridoma cells were co-cultured with antigen presenting cells (COS-7) that had been transfected with the expression vectors for the PECP N-library and one HLA molecule. The different MHC class I molecules (HLA-A*0101, -B*0801, -Cw*0701) of the patient FE and MR1 were tested. Untransfected COS-7 cells were used as negative control.

Activated GFP + hybridoma cells were observed in all experiments, including the control. This indicated a 'background' level activation of the MAIT TCR hybridoma cells. As a first step, the experiment was repeated on a larger scale and activation in response to each HLA molecule was quantified (**Section 3.5**). As MR1 is highly conserved amongst mammals, the same molecule from COS-7 (African green monkey cell line) that was used as an antigen presenting cell might be capable of presenting antigen to and activating the MAIT TCR. This presumably contributed to a 'background activation' of the MAIT TCR **(Section 3.6 and 3.7)** and posed a challenge in narrowing down the antigenic signal. In order to figure out the source of the background activation, it was necessary to quantify the observed signal and identify its source.

3.5 Investigation of HLA restriction of the MAIT TCR

To confirm that the BV1-BJ2.3/AV7.2-AJ33 TCR isolated from the MS lesion of patient FE was indeed a MAIT TCR, it was necessary to study TCR activation in context of all patient MHC class I molecules and MR1. The antigen presenting COS-7 cells were transfected with the expression vectors for the randomized nonapeptide library (N library) in combination with the four different MHC molecules (A, B, C and MR1). As controls, COS-7 cells transfected only with MR1 or the library and untransfected cells were analyzed. At 48 and 72 hours after transfection, the MAIT hybridoma cells were co-cultured with the APCs. After 16 hours of co-incubation, the GFP +, activated hybridoma cells were manually counted under the fluorescence microscope by two independent observers.

Activated cells were observed in all samples including the controls, as represented in the following **Figure 3-12**. No exclusive bias for a particular MHC molecule was apparent, and the background activation was evident by the presence of GFP + cells in the negative controls (transfection of empty expression plasmid, and no transfection). However, the MAIT TCR hybridoma activation in response to the N library presented by MR1 was higher than in all other samples. This effect was maintained at samples checked at 48 and 72 hours after transfection. The activation was diminished post 72 hours in comparison to that at 48 hours. The experiments were carried out four times. In the first experiment, despite high background activation, the highest number of GFP + activated cells was observed in the samples where MR1 and the N library were co-transfected.

This indicated that MR1 might be involved in antigen restriction to the MAIT TCR, but was not conclusive because the second follow up experiment showed a random distribution of activation and a high background. In order to reduce this background, the MAIT TCR hybridoma cells were extensively re-cloned to select clones with minimum background **(Figure 3-13)**. During the third and fourth experiment (**Figure 3-12**), the MR1-N library sample consistently contained more activated cells than the other HLA class I molecules. These observations indicate that MR1 might indeed be the appropriate antigen presenting partner for the MAIT TCR. However, the 'background activation', that seems to be an inherent property of the MAIT TCR hybridoma, must be taken into account during antigen search experiments. Only the activation that was above this background level could be deemed as 'real' or coming from the real MR1 antigen.

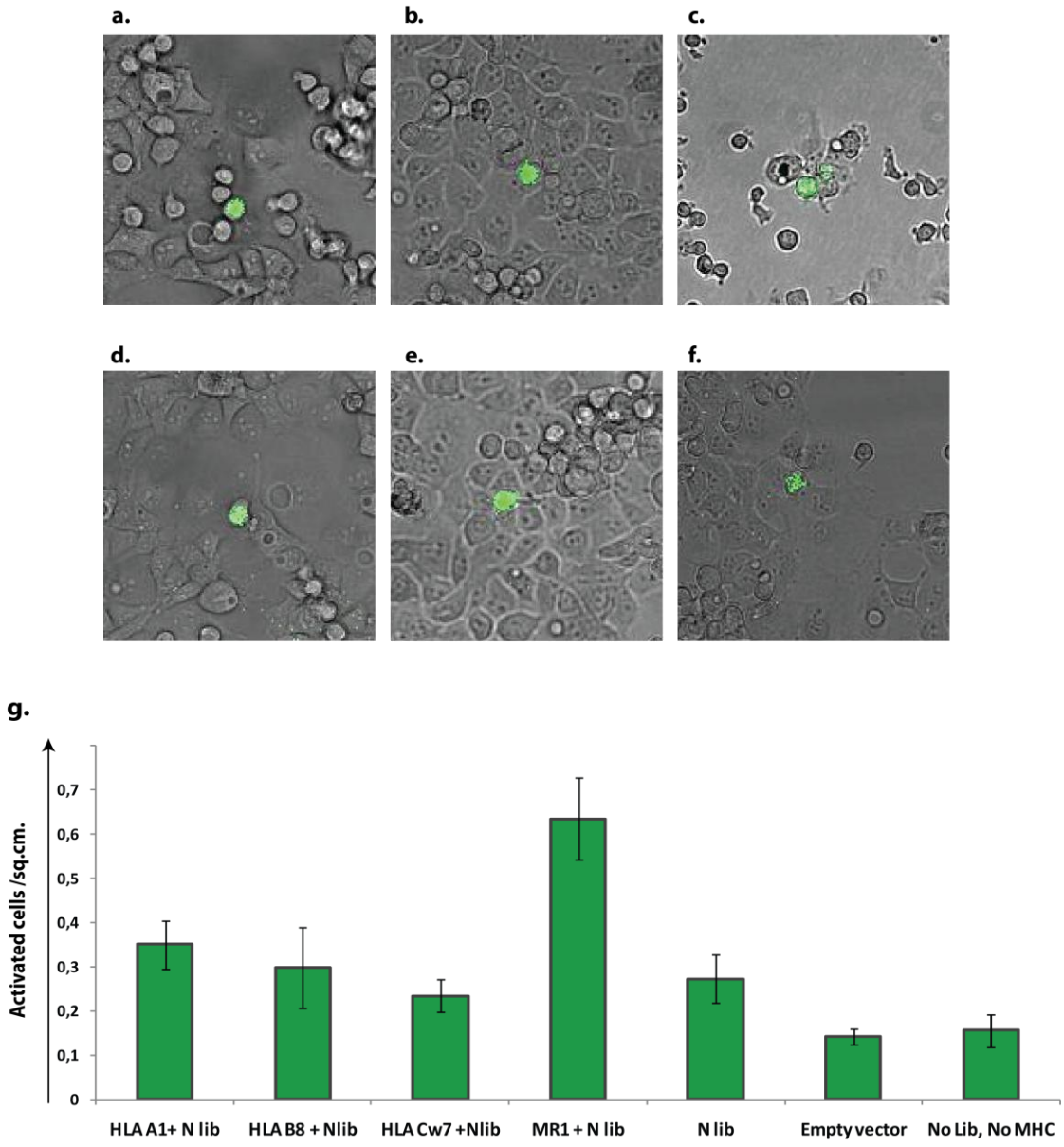


Fig. 3-12: MAIT TCR activation by N library presented by different MHC molecules

(a-f) Activated MAIT TCR hybridoma cells observed in co-culture with COS-7 transfected with **(a)** N library and HLA-A*0101 ,**(b)** N library and HLB-A*0801, **(c)** N library and HLA-Cw*0701, **(d)** N library and MR1, **(e)** empty expression plasmid and **(f)** untransfected COS-7 cells. **(g)** Plot of activated (GFP +) MAIT hybridoma cells counted per unit area (cm^2) in response to the N library presented by different MHC molecules. The MAIT TCR hybridoma cells were co-cultured for up to 16 hours with COS-7 (APC) cells transfected transiently with the different HLA molecules. This experiment was repeated four times and the co-culture was initiated 48 and 72 hours post transfection. The GFP +, activated MAIT TCR hybridoma cells per unit area (cm^2) was recorded for each HLA molecule to be tested. The three MHC class I alleles of the patient FE (only three, as the patient is homozygous) and the MHC Related molecule-1 (MR1) were tested. As negative controls, the empty pCDNA expression plasmid and untransfected COS-7 (APCs) were used. HLA: human leukocyte antigen, A1: A*0101, B8: B*0801, Cw7: Cw*0701

3.6 Background activation of the MAIT TCR

The background activation observed might be due to an endogenous MAIT antigen sourced from the COS-7 cells or the cell culture media, the capability of COS-7 MR1 to present the MAIT antigen to human TCRs (**Section 3.7**) or even a clone with a TCR signaling defect. This situation might be circumvented by the selection of a more 'stable' clone by re-cloning (**Section 3.6.1**).

3.6.1 Reduction of background activation by re-cloning

Re-cloning of the MAIT TCR hybridoma cells was done to select a 'stable' clone that met two criteria. First, the background activation had to be as minimal as possible. In case the clone contained GFP + cells in the resting phase that were detectable by fluorescence microscopy, the clone was discarded to avoid picking of false positives. Second, in the presence of antigenic stimuli, the cells should respond with a high signal, that can be clearly measured by flow cytometry or observed with fluorescent microscopy. This could be simulated by TCR activation following CD3 cross-linking. It is important to point out that CD3 cross-linking does not furnish a 100 % response in $58^{-/-}$ TCR hybridoma cells. At no stage did the entire population respond completely, irrespective of the cell numbers and antibody concentration applied. This seems to be an inherent property of the cell line, and has to be accepted. The reduction in unspecific background in a resting, unstimulated phase and the increase in activation upon TCR activation due to cross-linking are depicted in **Figure 3-13**.

In the three clones presented in the figure, clone 3 shows the optimal parameters, that is high activation upon CD3 cross-linking (up to 87% of the population is activated and GFP +). These cells were also clearly visualized via fluorescence microscopy (data not depicted here). Most importantly, clone 3 shows a very low background (0.14%), and the cells that are in the lower right quadrant (background) are not bright enough to be detected under the fluorescence microscope as false positives. This is not true for clone 2, which includes bright GFP+ cells in the resting phase, that were detected under the fluorescence microscope, and these could contribute to false positives during an experiment.

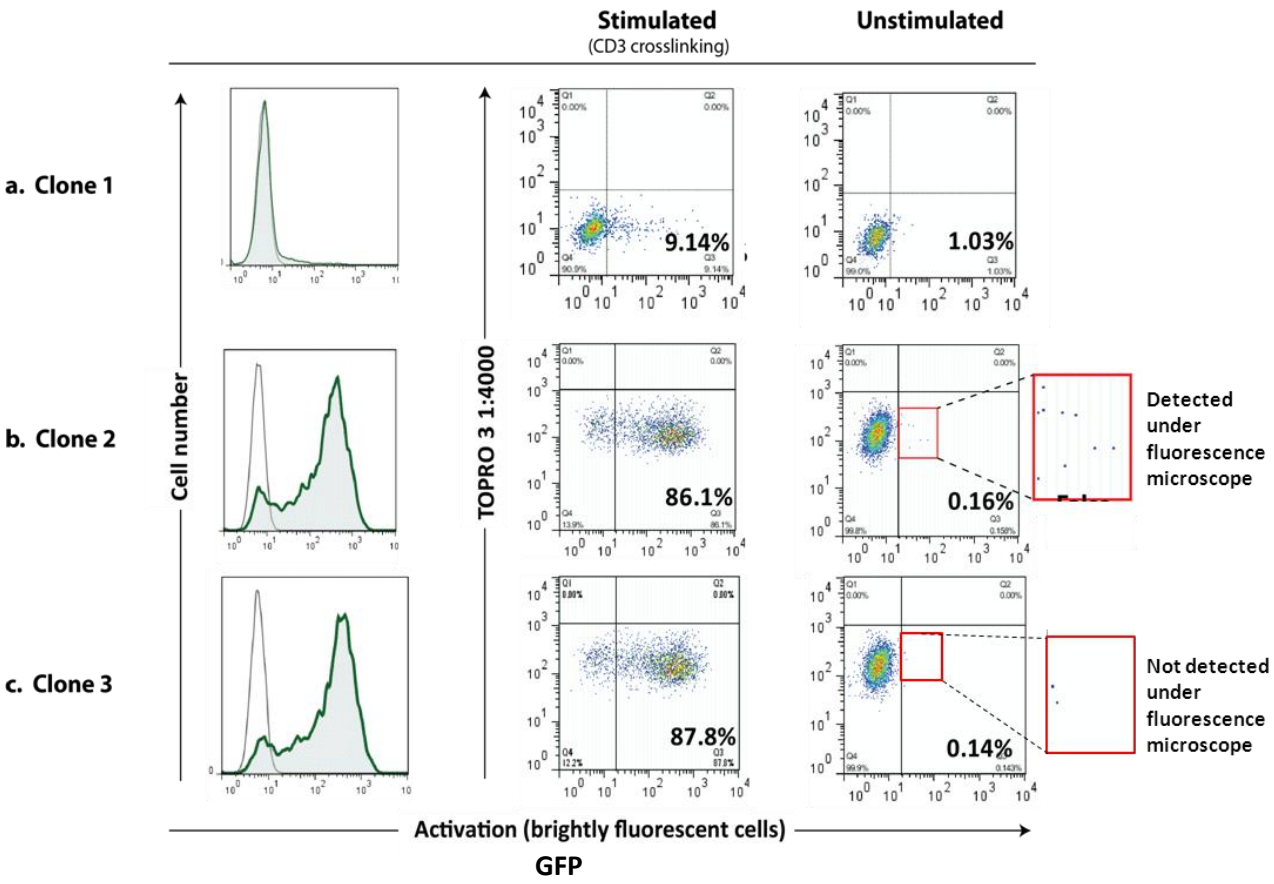


Fig. 3-13: Re-cloning reduces the background due to unspecific activation

Flow cytometry histograms and scatter plots depicting the activation response (GFP expression) of three different MAIT TCR hybridoma clones following CD3 cross-linking (stimulation). This was done to simulate antigen recognition, in order to select a clone that responds specifically to antigen stimuli and has low background activation. The first column depicts histograms that show the stimulated sample in green and the unstimulated cells (negative control) in grey. This is followed by scatter plots divided into 4 quadrants based on the negative control. The lower right quadrant contains the fraction of the cell population that was GFP +. The cells were stained with the TOPRO 3 dye and only the live cells were gated and further analyzed.

(a) Clone 1, depicted in the topmost panel, does not respond well to stimulation and contains cells that express GFP even in the absence of stimulation (background).

(b) Clone 2, depicted in the middle panel, does show a strong response upon stimulation (up to 86% of the cells are GFP +) but also contains GFP + cells in the unstimulated fraction that were sufficiently bright to be detected as false positives under the fluorescence microscope. These are highlighted in the inset in a red box.

(c) Clone 3, in the lowest panel, is an ideal case which shows low background and high activation only upon stimulation. The GFP + cells in the lower right quadrant of the unstimulated fraction (red box) were not visible under the fluorescence microscope. Hence, this clone may be used for further experiments.

3.7 Investigation of cross presentation between monkey MR1 (COS-7) and human MAIT TCR

MR1 is known to be conserved in mammals (Riegert et al. 1998), and hence it is quite likely that the African green monkey (COS-7) derived MR1 may be capable of antigen presentation to the human MAIT TCR. To examine this hypothesis, the COS-7 MR1 polypeptide sequence (provided by Wakiro Sato, MPI of Neurobiology) was compared with that of human MR1. The differences within human and African green monkey MR1 are depicted on a crystal structure of a MHC class I molecule (HLA-A*0201) in **Figure 3-14**.

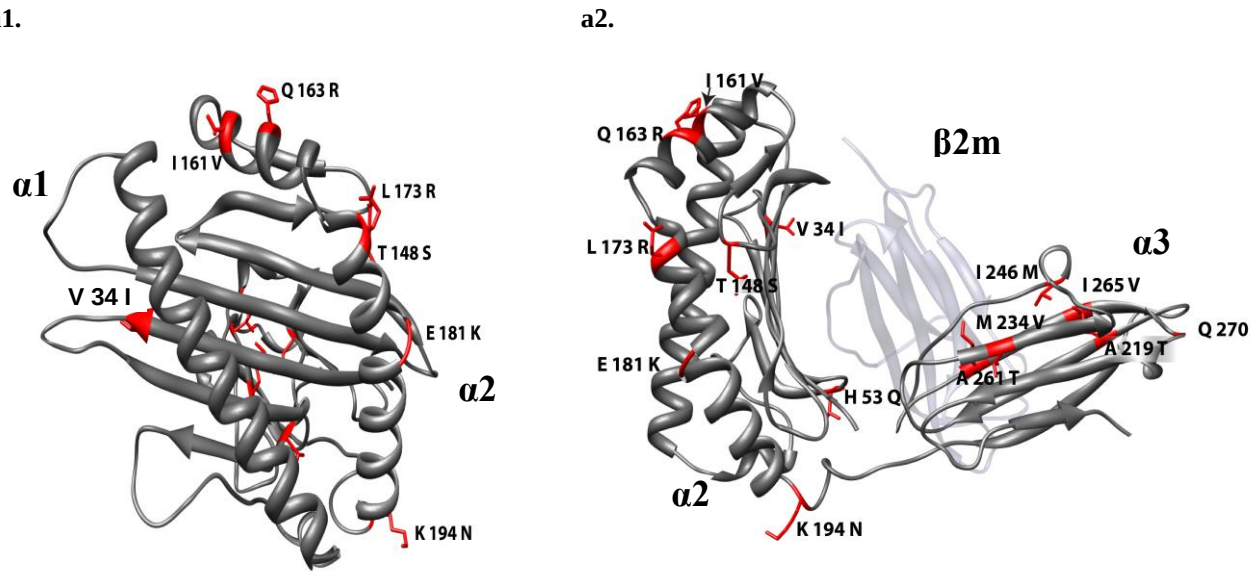


Fig. 3-14: Comparison of human and monkey (COS-7) MR1 molecules

(a) The crystal structure (dark grey) of a MHC class I molecule (HLA-A*0201) (Orth et al. 2003) used as a template to compare amino acid exchanges between human and monkey MR1. These exchanges have been highlighted in red. The orientation of their side chains is also visible. All amino acid residues are named according to the standard one letter code. **(a1)** A top view of the MHC class I molecule that shows the α1/α2 domains that bind the peptide ligand and interact with the T cell receptor. **(a2)** The molecule viewed after turning it 90°C to the left. This view shows all the three (α1, α2, α3) domains of the MHC class I molecule in addition to the β2 microglobulin (light grey). The monkey MR1 molecule differs from the human MR1 molecule at 16 amino acid residues within the α1, α2 and α3 domain. 8 of these exchanges lie within the ligand binding α1/α2 domains and 8 lie within the α3 domain. These 16 exchanges do not include any of the amino acid residues that are implicated to be important for interaction with the ligand and the T cell receptor (Huang et al. 2005). The structure was downloaded from the protein database (DOI:10.2210/pdb1qew/pdb) and molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081).

There are 16 amino acid exchanges between the human and monkey MR1 molecule, where 8 lie within the α1 and α2 ligand binding domains and 8 in the α3 domain. The residues at position 161, 163 and 173 may lie in close proximity to the interacting TCR while all other residues are oriented away from the interaction surface. The exchange at position 161 (I161V) is conservative. At position 163, a polar glutamine (Q) residue is exchanged for a charged arginine (R) residue. The exchange at position 173 (L173R) is from a large hydrophobic aliphatic amino acid to a positively charged one. Further E181K (change from negative to positively charged residue) is present on the side of the model, but in the human MR1, it may be at the top of the helix. However, none of these exchanges are at positions that are thought to be responsible for interaction with the ligand or T cell receptor (Huang et al 2005). This suggests that despite the differences, the African green monkey MR1 may bind to its ligand in a manner similar to that of human MR1.

The sequence similarity between the African green monkey and human MR1 is as high as 94%, and is second only to the relatedness between human and chimpanzee MR1 (99%). Within the ligand binding region of MR1, 3 residues are deemed most important (Huang et al. 2005) for ligand positioning and discrimination. All these residues are perfectly conserved in the human and African green monkey MR1. This indicates that the MR1 from the African green monkey might very well be capable of presenting the MAIT antigen to and activating the human MAIT TCR. In view of the background activation, MAIT TCR activation arising from an endogenous antigen presented by the monkey MR1 cannot be prevented. This background has to be accounted for in all experiments, and only activation above this level may be deemed as ‘real’ or coming from an actual antigenic stimulus.

3.8 TCR activation by antigen pools isolated from single APCs

Once it was observed that the MAIT TCR activation was higher than the background in the presence of the N library and human MR1, the MAIT antigen was searched for using MR1 presented N library. MR1 expressing COS-7 cells were transfected with the N library and co-cultured with the MAIT TCR hybridoma. APCs underlying GFP + MAIT hybridoma cells were isolated. APCs were only isolated when the hybridoma was well attached to the APC surface to avoid picking false positives. Due to the presence of the background, it was necessary to screen a large number of cells in order to locate the antigenic signal. Some representative examples of APC isolation by microcapillary during antigen search experiments are presented in **Figure 3-15**.

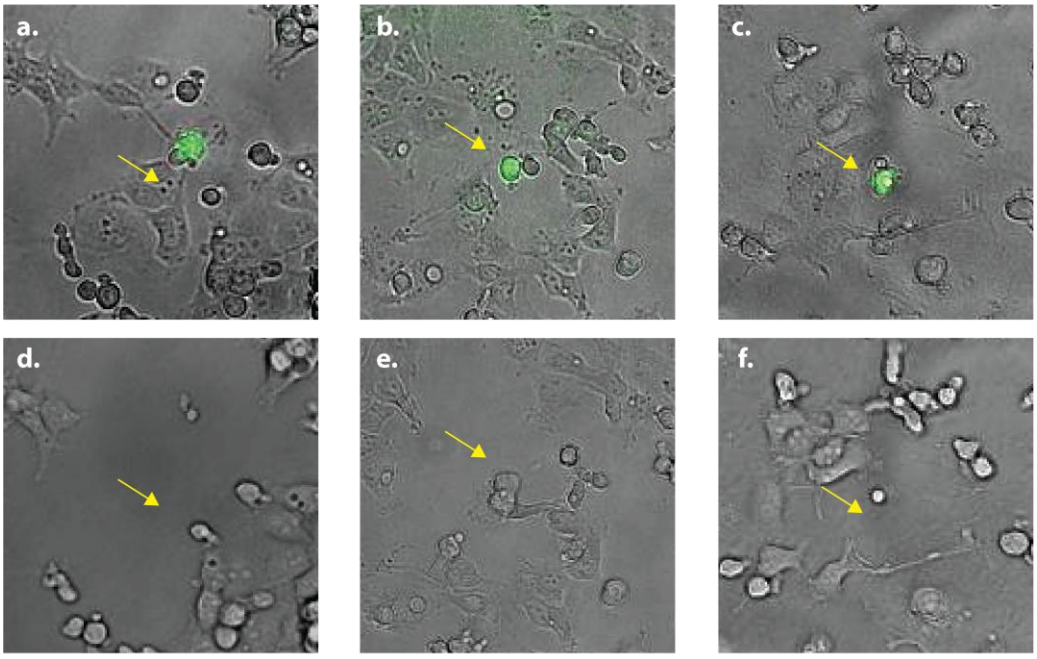


Fig. 3-15: Isolation of APCs (COS-7 cells) from co-culture with MAIT TCR hybridoma cells

Images acquired by fluorescence microscope at 10x magnification. MAIT TCR hybridoma cells were co-cultured with COS-7 cells that stably expressed human MR1 and transiently expressed the randomized PECP nonapeptide N library. 16 hours after co-culture, activated hybridoma cells were detected under a fluorescence microscope and the underlying COS-7 cell was isolated. **(a-c)** Antigen presenting COS-7 cell in direct contact with an activated (GFP +) MAIT TCR hybridoma cell (depicted with yellow arrow) from three different experiments. **(d-f)** Image after the COS-7 cell has been isolated from the cell culture dish by a microcapillary (microcapillary not in image).

To this end, 267 single cells were isolated and the library plasmids within them were amplified in separate single cell PCRs to create smaller plasmid pools, henceforth called sub-pools, from the mother library. These sub-pools were re-cloned into the pcDNA backbone either via directional TOPO cloning or conventional restriction digestion followed by ligation. The sub-pools were then co-transfected with MR1 into the COS-7 antigen presenting cells for a second round of screening. To enhance the signal, cells were re-picked and these smaller sub-pools were then screened as mentioned above. However, in subsequent rounds of screening, the activation signal could not be enhanced or enriched especially because in some cases it was difficult to differentiate between 'background' and 'real' TCR activation. Hence, this method could not be applied for identifying the antigen of this particular TCR.

3.9 Investigation of the MR1 anchor residues

3.9.1 Expression of recombinant human MR1 and β2 microglobulin

The application of an unbiased plasmid encoded peptide library for the identification of the MAIT TCR may have been unsuccessful due to the fact that the binding anchors of MR1 are not known. A MR1 anchored library would contain only those antigens that would bind MR1, and hence increase the chances of the correct mimotope causing activation. To fulfill this need, a pragmatic approach was devised that would provide the first insights into MR1 anchor positions. This could be achieved by analysis of endogenous MR1 ligands (whose sequences would represent the MR1 anchor positions if they did exist). If MR1 follows a pathway similar to that of MHC class I molecules, it would only translocate from the endoplasmic reticulum to the surface when bound to an endogenous peptide. Recombinant human MR1 (associated with β2 microglobulin) may be used for screening chemically synthesized peptide libraries. Elution of the bound peptides, and subsequent sequencing would shed light on the MR1 anchor positions.

To this end, human MR1 and ß2 microglobulin were cloned into pTT5 vectors (vector maps presented in **Appendix II**) for expression in HEK293E cells. The pTT5 vector was previously used in our laboratory for the expression of monovalent Fab fragments (Birgit Obermeier, MPI of Neurobiology). The leader sequences of the Fab fragments were combined with the MR1 and β2 microglobulin sequence (Heavy chain leader => MR1, light chain leader => β2 microglobulin) as it was known that these leader sequences facilitated the secretion of the Fab fragments into the cell supernatant (personal communication from Klaus Dornmair and Birgit Obermeier, MPI of Neurobiology). In the MR1 construct, the MR1 coding region was followed by a myc tag and polyhistidine ($His_{(6)}$) tag to allow for purification and detection of the protein. These experiments were done in collaboration with Wakiro Sato (MPI of Neurobiology).

MR1 was secreted in the supernatant in low quantities of approximately 7-10 µg from $3x10^8$ cells. As reported by others, the majority of the MR1 protein was retained within the cells (Miley et al. 2003, Le Bourhis et al. 2011). It is believed that in the absence of a binding partner, the

MR1 is unable to leave the endoplasmic reticulum, and is retained there. β2 microglobulin was secreted and detected both in the supernatant and cell lysate.

MR1 and ß2 microglobulin were detected in both supernatant and cell lysate by western blot. The cellular supernatant and lysate were separated by SDS-PAGE (4-20% gradient gel) and then transferred to a PVDF membrane. The MR1 was detected at 38 kDa via the myc tag (antibody clone 9E10.3, raised in mouse) followed by a secondary HRP-coupled anti mouse antibody that was detected by chemiluminescence. The β2 microglobulin at 12 kDa was detected via a polyclonal antibody against human β2 microglobulin followed by the secondary HRP-coupled anti mouse antibody that was detected by chemiluminescence. (**Figure 3-17**).

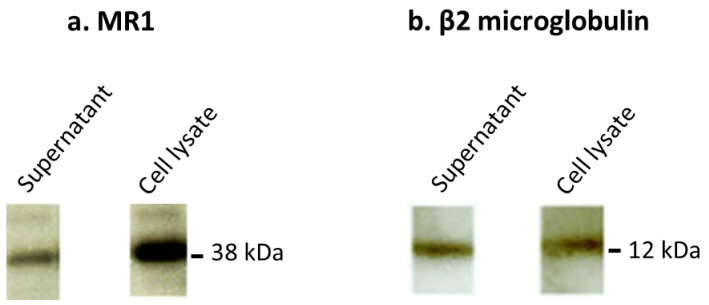


Fig 3-16: Western blot of the recombinantly expressed human MR1 and β2 microglobulin

20 µl of the undiluted supernatant from 3x 10^8 cells and 20 µl of cellular lysate from 5x 10^4 cells were run under reducing conditions on a gradient gel (4-20%), separated on the basis of molecular weight and immobilized onto a PVDF membrane. **(a)** MR1 (38 kDa) was detected via the C terminal myc tag followed by a secondary HRP-coupled anti-mouse antibody. This was detected by chemiluminescence. Exposure time: 15 seconds **(b)** The β2 microglobulin (12 kDa) was detected using a polyclonal antibody preparation that recognizes human β2 microglobulin followed by a secondary HRP-coupled anti-mouse antibody. This was detected by chemiluminescence.. Exposure time: 5 seconds

3.9.2 Analysis of MR1 bound peptides

In order to learn more about the anchor positions of the human MR1 molecule, a nonamer positional scanning synthetic combinatorial peptide library was screened with recombinant MR1. The bound peptides were eluted by treatment with 0.2% trifluoroacetic acid and analyzed by Edman degradation (Reinhard Mentele, MPI of Biochemistry) and mass spectrometry (Forne

Ignasi, LMU). The reverse phase HPLC run of the phenylthiohydantoin-amino acids analyzed by Edman degradation sequencing is presented in **Appendix IV**. There was no discernible signal detected above the background. No pattern, that could be suggestive of fixed anchor positions, was observed in the elution profile. When analysed by mass spectrometry, 38 nonapeptides were sequenced. The nonapeptides analyzed by mass spectrometry do not reveal highly conserved residues, albeit a pattern that is presented as a frequency plot in **Figure 3-18**. MR1 seems to bind to peptides that begin and end with a positively charged amino acid with a hydrophobic stretch in between. The peptide sequences are listed in **Appendix V**.

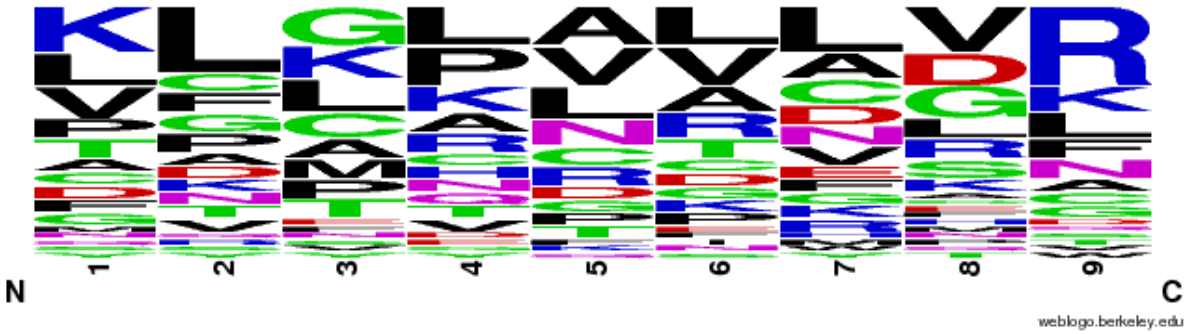


Fig. 3-17: Frequency plot of nonapeptide sequences

The nonapeptide sequences obtained from mass spectrometry analysis were analyzed with the WebLogo software (Crooks et al. 2004). The amino acids are represented in the standard one letter code. The plot consists of a stack of letters, and each position corresponds to one stack. The height of each letter corresponds to the relative frequency of each amino acid at that particular position. For the nonapeptides pulled down by MR1, there is no stringent sequence conservation of a residue at any one particular position but we may discern an overall pattern starting with a positively charged or large aliphatic residue followed by a second large hydrophobic residue at position 2. This is followed by a relatively hydrophobic patch extending from positions 4 to 7. Position 8 may comprise of a hydrophobic or charged residue and the peptide ends in a positively charged residue like arginine (R). Color code: KRH (positive charge): blue, DE (negative charge): red, NQ (polar): pink and AVLIPWFM (large, hydrophobic): black. N: amino terminal, C: carboxyl terminal, #1-9: amino acid positions

4 Discussion

Multiple Sclerosis (MS) is an autoimmune condition mediated in part by autoaggressive T cells that infiltrate the central nervous system in large numbers, and bring about the hallmark inflammatory demyelination that leads to plaque formation. The immunopathogenic components of this phenomenon, in particular the mechanism of T cell activation and the identity of target antigens is not known. Methods to characterize the specificity of T cell mediated immune responses would greatly benefit the understanding of autoimmune disorders in general. To cover this gap of knowledge, a two pronged PCR approach that could amplify αβ TCR re-arrangements either selectively or in an unbiased manner was applied.

Over thousand single cells were analyzed, and a total of nine T cell receptors (TCRs) were identified in twenty eight cells of patient FE **(Section 4.1)**. Of these, four were found in a collaborative effort and belong to the mucosal-associated invariant T cell (MAIT) subset (**Section 4.2)**. The remaining five are apparently CD8+ effector T cells. The MAIT TCRs and one effector TCR were recombinantly expressed in a mouse cell line and the dominant MAIT clonal expansion (BV1-BJ2.3/ AV7.2-AJ33) was focused on for antigen characterization. Efforts in this direction included the screening of candidate antigens (**Section 4.3**), investigating the antigen restriction of the MAIT TCR (**Section 4.4**), screening of an unbiased plasmid encoded peptide library (**Section 4.5**) and investigating the anchor positions of the MHC class I related molecule MR1 (**Section 4.6**).

4.1 Characterization of TCR chains from MS brain

This study is first of its kind in applying a multiplex RT PCR approach for the detection of matching TCR α- and β- chains from clonally expanded CD8+ T cells in the MS brain (**Section 4.1.1**). All of the detected TCR pairs were expanded in the brain of the patient, and are expected

to contribute to the course of the disease. Clonal expansion is a hallmark of activated cells that have multiplied locally upon antigenic stimulus, meaning that these T cells recognize a local antigen. In three instances identical β-chains were found in combination with more than one α-chain, giving rise to similar but distinct TCR heterodimer molecules (**Section 4.1.2**). Furthermore, some clonal expansions were found in different tissue blocks and were not restricted to one anatomical site within the MS brain (**Section 4.1.3**). The technical aspects of the method will be discussed in **Sections 4.1.4**.

4.1.1 Significance of the technique for studying T cells and their targets

T cells play an important role in protective immune responses against threats like microbial infections and tumors as well as pathogenic responses in autoimmune diseases like multiple sclerosis. The approach used in this study for the characterization of T cell receptors may be applied in any of the above situations to delineate the immune mechanisms of T cell responses. Unlike earlier efforts that adopted indirect approaches such as fragment length analyses, random cloning and sequencing of TCR cDNA to characterize TCRs (Biddison et al. 1989; Beall et al. 1989; Wucherpfenning et al. 1992), the current PCR based technique allows for the direct characterization of paired αβ TCR chains from single T cells. TCR analysis at the level of the single cell would provide a better picture of a complex T-cell population such as oligoclonal expansions that are observed in pathogenic, protective and autoimmune responses. Moreover, it allows for the study of a small number of cells that may be important because of their activation markers, their location in the tissue or the subgroup that they belong to, like MAIT cells (Le Bourhis et al. 2011).

The study of the resulting infiltrate, be it pathogen-, tumor- or autoimmunity driven is often complicated by the presence of bystander cells (Moebius et al. 1990; Hofbauer et al. 2003; Matsumoto et al. 2003). The current technique enables the study of specific T cell clones by the application of CD8 co-receptor and/or TCR specific antibodies coupled with single cell isolation by laser microdissection. The use of unbiased primer pools for both α- and β-chain rearrangements affords further independence from the limited repertoire of commercially available TCR antibodies. Ultimately, the cDNA of TCR chains recovered from patient samples

may be used to reconstruct the receptor in an experimental system such as a TCR hybridoma cell line (Seitz et al. 2006) or animal model (Madsen et al. 1999) to characterize their antigenic specificity. In the current study, more than half of the characterized TCRs were reconstructed, and one was applied in antigen studies. This would eventually lead to functional and disease progression studies in response to particular antigenic stimuli and improve our understanding of immune reactions of unknown specificity such as those in MS.

4.1.2 Identical TCR β-chains pair with different α-chains

In three instances, the same β-chain (BV1-BJ2.3, BV6.5-BJ2.2 and BV8.1-BJ1.1) was found to be coexpressed with more than one α-chain in patient FE. The BV1-BJ2.3 TCR β-chain persisted in the patient for seven years and during this time was detected in blood, cerebrospinal fluid (CSF) and brain tissue (Babbe et al. 2000, Skulina et al. 2004). It was coexpressed with four α-chains that are identical or related to the invariant α-chain of the MAIT sub-population. The significance of detecting MAIT cells in the MS brain has been addressed in **Section 4.2**. All four TCR pairs share the identical β-chain and the CDR1, CDR2 regions of the α-chains. The CDR3 region, that is important for antigen binding and recognition, is very similar and the joining regions are not identical. On aligning these sequences, as depicted in **Figure 4-1**, one notices that in three out of four TCRs, the CDR3 region begins with arginine, a positively charged amino acid. This is followed by a negative residue (aspartic acid).

In two other cases, the dominant BV6.5-BJ2.2 and BV8.1-BJ1.1 clonal expansions were found to be coexpressed with two different functional α-chains (**Figure 4-1**). In case of the BV6.5-BJ2.2 β-chain, the two α-chains share the identical variable region. Such relatedness might point to the recognition of identical antigenic epitopes, or even antigen driven TCR development. Considering the fact that MS pathogenesis has a T cell mediated component, such oligoclonally expanded T cells, especially those isolated from the site of the autoimmune attack, would be of high significance in understanding the disease.

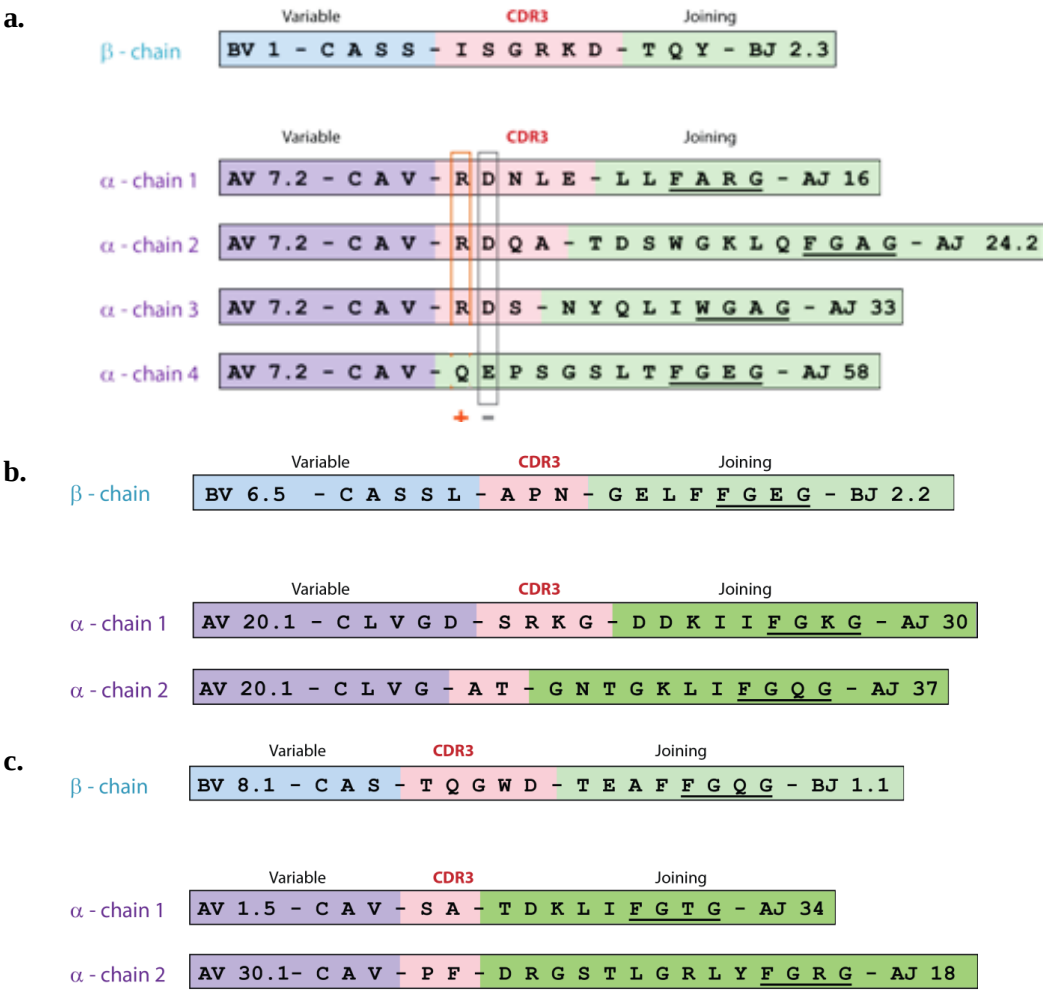


Fig. 4-1: Three instances of TCRs where an identical β-chain pairs with more than one α-chain

The peptide sequences depict the terminal variable region (β-chain: blue, α-chain: purple), followed by the CDR3 region (red) that plays a pivotal role in antigen binding and recognition, and the joining region (green). TCRs were named in accordance with the Arden nomenclature. The underlined residues represent the 'FGXG' consensus sequence of the joining region. **(a)** All four MAIT TCRs share the identical BV1-BJ2.3 β-chain (blue), and are homologous. This work was done in collaboration with Klaus Dornmair, Joachim Malotka, David Laplaud and Katherina Siewert. **(b)** The two α-chains that are coexpressed with the BV6.5-AV2.2 chain carry the identical variable region and joining regions of comparable length. **(c)** The BV8.1-BJ1.1 chain is coexpressed with two α-chains where the CDR3 regions are similar in length unlike the joining regions.

Similar results have been reported in earlier studies. A study in a mouse tumor model reported the pairing of several distinct albeit structurally related α-chains with the same expanded β-chain in infiltrating T cells (Hamrouni et al. 2003). Further evidence comes from the study during which the technique used in this study was developed. In the study of inflammatory muscle disease, the authors described a case in which three α-chains paired with the same β-chain (Seitz

et al. 2006). Taken together, these data suggest that the same antigen may recruit related or 'sister' αβ TCR heterodimers and influence TCR development. This situation may occur when the antigen-driven T cell expansion selects the two different clones with an identical β-chain rearrangement, but distinct α-chain rearrangements.

Alternately, the same T cell clone may also express two distinct α-chains simultaneously, but inadequate mRNA preservation may allow the detection of only one α-chain from the cell. The existence of such 'dual TCRs' was shown for α-chains (Padovan et al. 1993; Heath et al. 1995) in humans and mice. The expression of one specific TCR is usually guaranteed by the defined order of TCRα and TCRβ gene locus rearrangements during T cell development. First, one TCRβ chain locus is rearranged followed by the expression of the TCRβ chain in combination with the constant pre-TCR α-chain on the cell surface (Saint-Ruf et al. 1994). This triggers cell proliferation and halts further β-chain rearrangement. After this, the TCRα locus is rearranged. However, T cells expressing more than one TCR can be formed if incomplete allelic exclusion takes place. This happens relatively often for the TCR α-chain, and it was proposed that up to 30% of human T cells can contain two rearranged TCRα gene loci and can express two different TCRs (Padovan et al. 1993). It was suggested that these naturally occurring dual TCR cells can contribute to the high proportion of alloreactive T cells (Morris and Allen 2009). In view of these results it is clear that a 'dual' TCR with different specificities could play a relevant role in an autoimmune disease like MS.

4.1.3 Incidence of identical clonal expansions in different anatomical sites

Some of the clonally expanded T cells were not limited to one tissue block and may be thought of as 'pervasive' as they are not restricted to one anatomical site within the brain of patient FE. The three tissue blocks are different parts of the same lesion that was surgically removed from the patient's brain. The presence of 'pervasive clones' confirmed the observations of an earlier study (Junker et al. 2007) where MS brain infiltrating T cells were detected in different anatomical regions of the brain. This widespread presence may be due to an antigen that is ubiquitously expressed in brain tissue but not equally available due to differential processing of local APCs. This in turn would prompt the responsible T cell clone to widen its antigen search

and not restrict itself to a particular region. Moreover, CD8+ T cells were observed to infiltrate both white matter (WM) and grey matter (GM) in the tissue of patient FE. It would be interesting to make a comparative study of the TCR repertoire of CD8+ T cells infiltrating the WM and GM as discussed in **Section 4.7.1**.

4.1.4 Technical challenges of TCR characterization

The characterization of nine TCRs from the MS brain demonstrates the successful application of this approach. However, the TCR yield is restricted due to some technical challenges. The first challenge arises due to compromised mRNA quality that is a result of inadequate preservation in frozen tissues. Even though degradation of RNA during staining and cell isolation procedures is minimized by a short staining protocol facilitated by the use of directly labeled antibodies and RNAse inhibitors, the yield of TCR sequences is low. This difference is evident from the better RNA integrity from RNA extracted from freshly isolated live cells, and higher yields when the same technique is applied (**Section 3.1.2.2**). Data representing the difference in RNA quality between the frozen tissue from patient FE in comparison to that from live peripheral blood lymphocytes is represented in **Appendix III**. This loss of RNA quality might be a result of non-optimal tissue isolation, wherein the tissue sample was not immediately placed under refrigeration. The mRNA quality is further compromised during cryostorage and frequent freeze-thaw cycles during section preparation. To resolve this problem, one would have to ensure that the time between tissue isolation and cryostorage is reduced to the minimum. Ideally, the tissue should be placed directly on dry ice post isolation and then moved to 80°C. Moreover, freeze thaw cycles should be minimized and fresh cryosections should be used whenever possible. The tissue may also be immediately fixed in a RNA preserving fixative (Vincek et al. 2003) that preserves histomorphology.

The second challenge is the fact that the tissue is cut into 10 µm sections for optimal isolation by laser microdissection. Since T cells have a diameter ranging from 7-20 µm, there is a possibility that the cell is cut away or partially damaged during sample preparation. This could allow the mRNA to get washed out of the cytoplasm during the staining procedures, lowering the efficiency of the PCR. Since increasing the thickness of the tissue section would impair cell

isolation, this is not an option. One may consider minimizing the number of intermittent washes or crosslinking the RNA to the cell membrane, in order to reduce RNA loss.

Thirdly, this method requires the simultaneous use of multiple primers in one PCR reaction. Unspecific interactions between different primer oligonucleotides may make them unavailable for template amplification. In order to sufficiently cover the entire T cell receptor repertoire, a pool of primers must be applied. One may think of adding a pre-amplification step that amplifies the cDNA in a sufficient quantity such that this may be further amplified in nested reactions with multiple pools containing fewer primers.

4.2 Characterization of MAIT cells in the MS brain

Four TCRs characterized from patient FE belong to the mucosal-associated invariant T (MAIT) cell subset. MAIT cells are a T cell subset that has a limited germ-line encoded TCR repertoire (Porcelli et al. 1993; Tilloy et al. 1999) characterized by an invariant TCR α-chain. They are named based on their preferential residence in the gut mucosa. MAIT cells are $CD4^-/CD8^-$ or $CD8^{\alpha/\alpha}$, constitute up to 4 % of all blood T cells in a healthy individual and are believed to function at the interface of the innate and adaptive immune system (Porcelli et al. 1993). Antigen presentation is restricted by the MHC related molecule I (MR1). To date, the exact function of MAIT cells in diseased and healthy individuals, as well as the identity of the MAIT TCR ligand is unclear.

4.2.1 The significance of MAIT sister TCR pairs found in the MS patient

The four MAIT sister pairs found in MS patient FE are interesting for various reasons. First, all TCRs share the identical β-chain. Second, this β-chain is part of a clonal expansion that persisted in the patient for seven years, and was detected in both blood and brain tissue. Third, one of the α-chains is the classical invariant (AV7.2-AJ33) MAIT chain. This chain was detected seven times from MS brain infiltrating CD8+ T cells. Fourth, all four α-chains demonstrate a sequence homology within the 'antigen recognizing' CDR3 regions (**Fig. 4-1**). This observation may point to the fact that all the 4 MAIT TCRs recognize a common antigenic epitope and that the α-chains evolved under its influence. These results also present a first time report of MAIT sister pairing with the BV1 β-chain.

4.2.2 Proposed role and significance of MAIT cells in autoimmunity and MS

Interesting evidence comes from a report (Berer et al. 2011) where the contribution of commensal microflora to autoimmune demyelination was demonstrated in an animal model of MS. The commensal gut microbiota was shown to be capable of triggering a relapsing-remitting course of the disease in the absence of pathogenic agents, and also played a role in B cell

recruitment and activation. In light of the fact that MAIT cells preferentially reside in the gut mucosa and recognize microbial antigens (Gold et al. 2010; Le Bourhis et al. 2010), their detection in the MS brain might indicate their involvement in microbiota mediated autoimmunity. Another study (Miyazaki et al. 2011) showed that MAIT cell numbers in peripheral blood of MS patients were reduced in comparison to healthy controls. This number was influenced by course of disease being lower during active disease and increasing with clinical recovery. The MAIT cells also suppressed the IFN-γ production of T cells *in vitro* indicating that they might play a role in regulating pathogenic T_h1 cells in MS.

The invariant MAIT α-chains were all found to be coexpressed with a β-chain that persisted in the patient in detectable numbers for a prolonged period of time. Up to seven years after the disease was first diagnosed, this clone was detected in peripheral blood, as well as in brain biopsy tissue. This indicates that the MAIT cells did contribute not only to the early stages of MS in this patient, but also played a part in the stages that followed. In the light of these observations, it is interesting to note that MAIT cells home to the gut mucosa where they naturally come in close contact with the commensal microflora. This interaction could follow on the lines of molecular mimicry, and prime effector T cells to recognize a 'self' antigen, while priming MAIT cells to regulate this autoimmune T cell population. The ability of MR1 to present a self-ligand has been shown in the context of rare MAIT cell hybridomas that were activated by uninfected, MR1-overexpressing cells (Treiner et al. 2003; Huang et al. 2009). In the course of an autoimmune attack, the MAIT cells would leave the periphery, get enriched at the site of autoimmunity, and regulate the pathogenicity of the CD8+ T effector cells.

4.3 Testing the recognition of candidate MS antigens by the MAIT TCR

4.3.1 Human brain tissue

To evaluate the reactivity of the MAIT TCR to a brain antigen, the TCR expressing hybridomas were co-cultured with freshly sectioned tissue. In general, a much higher activation was observed in response to the patient tissue in comparison to the unrelated MS patient and the meningitis patient. This indicated that the MAIT TCR might recognize an antigen that was more readily processed and presented in the brain of patient FE rather than the two control patients. Since the second MS patient was not analyzed for MAIT TCR infiltration, it would be difficult to speculate whether this was indeed the case. In addition, the TCR activation was reduced but not completely eliminated upon application of a pan MHC class I antibody. This observation could have two reasons. On one hand, the incomplete blockage of activation may be simply due to insufficient amounts of blocking antibody in the milieu. On the other hand, MAIT antigen presentation may indeed be independent of the class I MHC molecule and is hence not affected by HLA blocking. In such a case one has to assume that the antibody binds an epitope that is (a) not shared between MHC class I and MR1 or (b) shared between the two molecules, but not important for antigen binding and/ or MAIT TCR activation.

4.3.2 Human brain extracts, myelin antigens, autologous and EBV antigens

Since the MAIT TCR was detected a clonal expansion within the MS brain, human brain extracts of WM and GM as well as myelin antigens, that have been suggested to play a role in MS pathogenesis, were presented via a fraction of peripheral blood cells containing dendritic cells and macrophages to the MAIT TCR hybridomas. No significant activation of the MAIT TCR was observed. It is quite unlikely that the MAIT antigen is not expressed in the CNS, as studies by us and others (Illes et al. 2003) have reported clonal expansions of MAIT cells within the MS brain. These results may be affected by the antigen processing capabilities of the APCs used in this experiment, as the peripheral blood fraction was not treated with growth factors for the induction of mature dendritic cells. The crude cell preparation used in the experiment may have

been dominated by cells that were not efficient antigen presenters, and compromised the antigen presentation to the TCR. The significance of correct antigen processing and accessibility is addressed in the following **Section 4.3.3**. In another experiment, the MAIT TCR hybridomas were also co-cultured with the Epstein-Barr virus (EBV) transformed autologous B cells, but no significant activation was observed. It is also probable that cellular interaction was impeded during co-culture, as the EBV-B cells grow in clumps, and fail to form a uniform layer in culture. This may have disrupted efficient antigen presentation to the MAIT TCR. However, this non-recognition of viral antigens supports the observations of a study that showed that the MAIT cells do not recognize cells infected with viruses (Le Bourhis et al. 2010). This may be extended to our findings, where the MAIT TCR did not respond to the EBV antigens.

4.3.3 Antigen processing and accessibility as limiting factors

The experiments in which candidate brain and MS antigens were tested might be hindered by the lack of suitable APCs that are efficient in antigen processing and presentation. There are many studies supporting the notion that different cell types possess variable protein processing capabilities. The source of antigenic peptides might be the well characterized proteasome, or the immunoproteasome (Hoppes et al. 2010) that has altered substrate specificity, or even an organ specific proteasome such as the one described in the Thymus (Murata et al. 2007). Alternately, the peptides may be produced in a proteasome independent fashion by tissue-specific enzymes (Parmentier 2010). One cannot accurately predict the mechanisms that govern the generation of a TCR antigen (Morel et al. 2000; Chapiro et al. 2006) and hence it becomes increasingly important to closely replicate the *in vivo* environment during antigen search experiments. This becomes difficult in a situation when the antigen presenting partner of a TCR of unknown specificity is also unknown, and unavailable for *in vitro* cell culture. Keeping the above evidence in mind, we decided to adopt an antigen search strategy that would be unbiased, and overcome the challenges of antigen processing by coding for nonamer peptides that would cover virtually all antigenic epitopes. The related findings are discussed in **Section 4.5**.

4.4 Investigation of HLA restriction of the MAIT TCR

The MS derived MAIT TCR, carrying the classical invariant MAIT α-chain (AV7.2-AJ33) was investigated to determine its HLA restriction. The HLA molecules tested were the patient matched MHC class I molecules and the MR1 molecule. Antigen presenting cells that coexpressed each MHC molecule of interest, as well as a source of antigenic peptides were co-cultured with the MAIT TCR hybridoma and subsequently analyzed for MAIT TCR activation. Surprisingly, activation was not restricted to any one test molecule, but the highest level of activation was seen in the presence of the MR1 molecule. This observation indicates that the MR1 molecule is possibly best suited to present antigen to the MS derived MAIT TCR and may in fact be responsible for restricting the antigen presentation to this particular TCR.

4.5 Unbiased search for the antigen of the MAIT TCR

4.5.1 Background activation of the MAIT TCR hybridoma

'Background activation' or activation in the absence of antigenic stimuli was often observed for the MAIT hybridoma. The cells were re-cloned extensively to reduce this 'background' but it could not be completely eliminated. This background may be due to the presence of an endogenous antigen (**Section 4.1.1.1**) that is capable of activating the MAIT TCR. Additionally, it can also arise if the African green monkey MR1 molecule (from COS-7 cells) is capable of cross-presentation to the human MAIT TCR (**Section 4.1.1.2**).

4.5.1.1 Is the MAIT TCR activated by an endogenous antigen?

Evidence from literature suggests that MR1 is capable of binding endogenous antigens. It is known that MAIT cell ontogeny is independent of B cells, but requires MR1 expression on hematopoietic cells in the thymus suggesting that these cells present an endogenous ligand to the developing MAIT cells (Chua et al.2011). Furthermore, a recent study (Abos et al. 2011) detected MR1 on the surface of specific MR1 expressing cell lines without the addition of exogenous antigen(s). It has been suggested that MR1 might need to assume the 'folded' or

ligand bound conformation to reach the cell surface. In order to do this independent of an exogenous ligand, the MR1 molecule would have to bind to an endogenous partner or a culture medium component. These observations suggest that the MR1 cell surface expression occurs in the presence of a ligand. The recognition of an endogenous component could contribute to background activation, as was observed for the MS derived MAIT TCR expressed in hybridoma cells. This background was observed to fluctuate during cell culture, and could be minimized by re-cloning and selecting for 'low background' clones. However, if the background activation is indeed due to an endogenous antigen, then selecting 'low background' clones would result in cell lines that are less responsive to the endogenous stimuli. This could explain why an enrichment of antigenic signal was not observed even after repeated re-analysis of isolated antigen plasmids during the screening of plasmid encoded peptide libraries.

4.5.1.2 Does the monkey MR1 cross present antigen to the human MAIT TCR?

MR1 is known to be very well conserved across mammalian species. Human and murine MR1 are highly homologous at the level of protein sequence within the α1 (89.7% similarity) and α2 (89.1% similarity) domains (Treiner et al. 2005). Cross-species activation between human and mouse MAIT cells (Le Bourhis et al. 2010), was observed in MR1 over-expressing cell lines. In light of the above observations, it is quite likely that the African green monkey MR1 (from the COS-7 cell line) can similarly cross activate human MAIT cells. The amino acid residues that have been suggested to be potentially important for antigen binding and recognition (Huang et al. 2005) are identical and conserved between human and monkey MR1. The differences between the African green monkey (COS-7) and human MR1 molecule are not included in these 'important' residues. Hence the COS-7 MR1 is likely to bind to its ligand in a manner that is similar to that of human MR1. If it is indeed capable of cross-presentation to the human MR1, presentation of an endogenous ligand would additionally contribute to the observed background activation. This 'background activation' of the MAIT hybridoma cannot be eliminated completely by recloning as it is an inherent part of the system.

4.6 Investigation of the MR1 anchor positions

The first step in creating a human MR1 matched PECP library would be the characterization of its anchor positions. A pragmatic approach was followed that would provide preliminary insights into the anchor positions of the MR1 molecule. This would be achieved by analysis of endogenous MR1 ligands (whose sequences would represent the MR1 anchor positions if they did exist) or to screen chemically synthesized peptide libraries with recombinant human MR1, and then study the bound peptides.

To this end, recombinant human MR1 (hMR1) was expressed in association with β2 microglobulin and used to screen chemically synthesized peptide libraries. hMR1 was secreted in the cell supernatant, but a greater part of the hMR1 was detected within the cell. It is possible that this 'non-secreted' form was sequestered within the endoplasmic reticulum (ER) due to the non-availability of a binding partner that would stabilize the folded MR1 conformer. A similar observation is made in the case of classical MHC molecules and MR1. In the absence of a ligand, MR1 is unable to exit the ER and accumulates there (Miley et al. 2003; Le Bourhis et al. 2011). To circumvent this, the recombinant hMR1 may be coexpressed with a peptide encoding combinatorial plasmid library such as the randomized N library. The PECP library may serve as a ligand source, and facilitate MR1's exit from the ER.

Although the amount of secreted MR1 was low, it was sufficient for screening of chemically synthesized nonamers belonging to a combinatorial positional scanning peptide library. The peptides that bound to MR1 were eluted and analyzed via Edman degradation and mass spectrometry. No signal could be detected for the peptides analyzed by Edman degradation and this may be due to low sensitivity of the method. Mass spectrometry proved to be more sensitive, and the sequences of the eluted peptides indicate that there is no strict sequence conservation at any one position within the nonapeptide, albeit a pattern that begins and ends with a charged amino acid residue with a stretch of hydrophobic residues in between. Using this pattern as a guide, efforts in this direction may screen related peptides to test MAIT TCR reactivity in the future.

4.7 Further experiments

4.7.1 Studying the TCR repertoire of CD8+ T cells that infiltrate WM and GM

The demyelination in MS has long been thought to be focused in the WM and only recently have the demyelinating plaques in the GM come to the fore (Geurts et al. 2009). In this study, CD8+ T cells were observed to infiltrate both the white matter (WM) and grey matter (GM) in the brain tissue of patient FE. Firstly it would be interesting to quantify this infiltration to see if the infiltrates in both regions are comparable in number and density. Secondly, a detailed study of the TCR repertoire in both regions may be carried out to understand the antigen specificities of these T cells. It would be interesting to see whether an overlap in TCR sequences exists between the CD8+ effector populations infiltrating GM and WM, or whether these are mutually exclusive. The WM and GM may first be macrodissected and analyzed for oligoclonal expansions by spectratyping. The dominant expansions detected by spectratyping may then be looked for in single cells isolated by laser microdissection using the clone specific or unbiased multiplex PCR approach.

4.7.2 Delineating the role of MAIT cells in MS pathogenesis

In light of a study linking gut flora to MS pathogenesis (Berer et al. 2011), the contribution of MAIT cells in MS seems significant and not just a mere coincidence. To delineate this one may examine MAIT cells in MS patients or animal models with different courses of the disease. Studies that evaluate the presence of MAIT cells in the blood prior to, during and after a relapse may be helpful in revealing patterns. Besides, brain tissue could also be stained for the MAIT invariant α-chain to get an overall picture of the numbers and density of infiltrating MAIT cells. This may then be compared to the numbers of effector T cells infiltrating the same region to know which subpopulation is dominant locally. A ‘humanized’ MAIT TCR animal model would be useful in understanding the role and location of MAIT cells at different time points in the disease. A spontaneous EAE animal model in mice suggests that the human MAIT TCR may play a protective role during the disease (personal communication, Wakiro Sato, MPI of

Neurobiology). This is in keeping with studies that suggest that the MAIT subpopulation plays a regulatory role in MS (Miyazaki et al., 2011).

4.7.3 Identifying the anchor positions of the MR1 molecule

The use of plasmid encoded combinatorial peptide libraries for antigen identification could thus far not narrow down onto the MAIT antigen, although it did provide evidence supporting the peptidic nature and MR1 restriction of the TCR derived from patient FE. This approach involved the screening of an unbiased nonamer library, and excluded peptides of other lengths. Some studies suggest that the MR1 antigen processing and presentation is distinct from that of classical MHC molecules (Huang et al. 2008; Gapin et al. 2009). If this is indeed the case, it is possible that the MAIT antigen may not be a nonamer. It is possible that nonapeptides are capable of a weak association that allows for activation, but not a strong antigenic signal that can be isolated. Hence screening of randomized PECP libraries of varied lengths would be an interesting follow up experiment.

Furthermore, the PECP library that was used so far could not be matched to the antigen presenting molecule since the MR1 anchor positions are not known. The experiments to identify the anchor positions of MR1, using a chemically synthesized positional scanning combinatorial peptide library, provide a preliminary pattern of nonapeptides that bind to MR1. This may be used as a guide for future experiments. The experiment may first be scaled in order to screen more peptides (of variable lengths) in order to increase the signal. A confirmation of the peptide pattern should be followed by the generation of plasmid encoded peptides that follow this pattern. By testing whether they successfully activate the MAIT TCR detected in patient FE, one would be able to delineate the (a) anchor positions of MR1 and (b) show that the MAIT TCR indeed recognizes a peptide.

It would be interesting to see if all the four MAIT T cell receptor pairs from patient FE recognize the same or similar peptide(s). This is speculated as the TCR sequences are related and indicate antigen driven development. More information on the anchor positions of the MR1 molecule could be gained by analyzing peptide libraries of varied lengths. Additionally, efforts

can also be made in eluting the endogenous ligand of MR1. Although this entity would not be expected to have a pathogenic potential, it would conform to the MR1 anchor positions. The chemical synthesis of mimotopes and database mining would be helpful in further revealing its pathogenic association. To cater to a situation where the MR1 ligand is not a peptide, one may for instance analyze MR1 binding to lipid ligands.

4.7.4 Characterizing the antigen of the (non-MAIT) effector T cells

Of the nine T cell receptors characterized in this study, five apparently belong to the cytotoxic T cell subset. Reconstruction in mouse hybridoma cell lines would allow for their application in antigen search studies. In order to find the antigens that they recognize and respond to, one would also have to determine their HLA restriction as a first step. This knowledge will allow for the development and application of HLA anchor specific PECP libraries and antigen presenting cell lines that are stably transfected with the responsible HLA molecule, both of which are essential for antigen search experiments. The screening of these libraries would provide mimotopes that would in turn shed light upon the antigen(s) that these T cells recognize. Besides being useful in *in vitro* studies, these TCRs may be studied in *in vivo* in 'humanized' mouse models of the disease.

List of figures and tables

Abbreviations

Quantities are denoted in accordance to the international SI system of units while the amino acids have been named according to the standard code.

A	Adenosine	FCM	Flow cytometry
Amp	Ampicillin	FCS	Fetal calf serum
APC	Antigen presenting cell	FITC	Fluoresceinisothiocyanate (fluorophore)
ATP	Adenosine triphosphate	FPLC	Fast protein liquid chromatography
β2m	Beta 2 microglobulin	G	Guanine
bp	Base pairs	HEK293E	Human embryonic kidney 293 EBNA-1
C	Constant (region of TCR chains)	HLA	Human leukocyte antigen
C	Cytosine	hMR1	Human MR1
CD	Cluster of differentation	HRP	Horseradish peroxidase
cDNA	Complementary DNA	IL-2	Interleukin-2
CDR	Complementarity determining region	ITAM	Immunoreceptor tyrosine-based activation motif
CMV	Cytomegalovirus	J	Joining (region of TCR chains)
CNS	Central nervous system	K	K-nucleotide, stands for random insertion of guanine or thymine nucleotides
C-terminal	Carboxy terminal	kb	Kilo base pairs/ kilobases
Cy3	Cytochrome 3 (fluorophore)	LB	Luria-Bertani culture medium
D	Diversity (region of TCR α- or β-chains)	LC	Liquid chromatography
DEPC	Diethylpyrocarbonate	mAb	Monoclonal Antibody
DMSO	Dimethylsulfoxid	MAIT	Mucosal-associated invariant T cells
DNA	Deoxyribonucleic acid	MBP	Myelin basic protein
dNTP	Desoxy-nucleoside-triphosphate	MHC	Major histocompatibility complex
E. coli	*Escherichia coli*	MOG	Myelin oligodendrocyte glycoprotein
EAE	Experimental Autoimmune Encephalomyelitis	MPI	Max Planck Institute
EBNA	Epstein-Barr virus nuclear antigen	MS	Multiple sclerosis
EBV	Epstein-Barr virus	MWCO	Molecular weight cut-off
ECL	Enhanced chemiluminescence	N	N- or non-templated nucleotide, stands for random insertion of any nucleotide
EDTA	Ethylenediamine tetraacetate	n.k	Not known
ELISA	Enzyme linked immunosorbent assay	OD	Optical density
ER	Endoplasmatic reticulum	Ori	Origin of replication

PAGE	Polyacrylamide- gel electrophoresis	RT	Room temperature
PBS	Phosphate buffered saline	SDS	Sodium dodecyl sulfate
PCR	Polymerase chain reaction	sGFP	Synthetic green fluorescent protein (S65T) (Heim et al. 1995)
PFA	Paraformaldehyde	SLE	Systemic lupus erythematous
pMHC	Peptide-MHC complex	T	Thymine
PS-SCL	Positional scanning-synthetic combinatorial library	TAP	Transporter associated with antigen processing
PVDF	Polyvinylidene fluoride	Taq	Polymerase of *Thermophilus aquaticus*
NCBI	National Center for Biotechnology Information	TBE	Tris/borate/EDTA (buffer)
NF	Neurofascin	TCR	T cell receptor
NFAT	Nuclear factor of activated T cells	Tris	Tris (hydroxymethyl)-amino methane
NK- T cells	Natural killer T cells	u	Unit
NTA	Nitriloacetate	U	Uracil
N-terminal	Amino terminal	UV	Ultraviolet light
RE	Restriction enzyme	V	Variable (region of TCR chains)
RNA	Ribonucleic acid	v/v	volume per volume
RNAse	Ribonuclease	w/v	weight per volume
RPM	Rounds per minute	X-Gal	5-Bromo-4-chloro-3-indoxyl-β-D-galactopyranoside
RPMI	„Roswell Park Memorial Institute“ (cell culture medium)		

Appendix

I. Primer sequences

All oligonucleotide primers used in this study are listed below. The primer label and its nucleotide sequence are followed by its intended site of hybridization. With regards to primers used for nested PCRs, the designation 'out' refers to primers used in the first reaction and 'in' refers to primers used in the following second reaction. The designation 'for' indicates that the primer would amplify its target in the 5' to 3' direction, while 'rev' refers to a primer that achieves the opposite. Wobble nucleotides that were included in the sequences follow the standard IUPAC nomenclature: R (A or G), Y (C or T), K (G or T), S (G or C), W (A or T), B (G or T or C). All TCR primers are named according to the Arden nomenclature.

- **Reverse Transcription (RT)(Section 2.4.3)**

Label	Sequence	Hybridization site
hMR1-for	5'-ATGGGGGAACTGATGGCGTT	Human MR1 signal peptide
hMR1-rev	5'-TCATCGATCTGGTGTTGGAAG	Human MR1 transmembrane region
Vα7.2-lead	5'-ACGCGTCGACATGTGGGGAGTTTTCCTTC	Leader-Region Vα7.2 (TCR)
Cα-rev-out	5'-GCAGACAGACTTGTCACTGG	C-Region α-chain (TCR)

- **Colony PCR (Section 2.4.3.2.1)**

Label	Sequence	Hybridization site
M13 Forward (-20)	5'-GTAAAACGACGGCGTC	pCR®2.1-TOPO-Vector
M13 Reverse	5'-GAGGAAACAGCTATGAC	pCR®2.1-TOPO-Vector
pTT5-for seq	5'-CTTTCTCTCCACAGGTGTC	pTT5-Vector
pTT5-rev seq	5'-CCTTCCGAGTGAGAGACAC	pTT5-Vector

- **Single cell multiplex PCR for TCR chains (Section 2.4.3.2.2)**

Clone specific PCR

- RT PCR

Label	Sequence	Hybridization site
C α RT imp	5'-GCCACAGCACTGTTGC	C-region α-chain (TCR)
C β-RT-2	5'-GAAGAAGCCTGTGGCC	C-region β-chain (TCR)

- PCR 1

Label	Sequence	Hybridization site
Vα-1^{14}-for-out	5′-AGSAGCCTCACTGGAGTTG	Variable region α-chain (TCR)
Vα-1^{235}-for-out	5′-CTGAGGTGCAACTACTCATC	Variable region α-chain (TCR)
Vα-2-for-out	5′-CARTGTTCCAGAGGGAGCC	Variable region α-chain (TCR)
Vα-3,25-for-out	5′-GAARATGYCWCCATGAACTGC	Variable region α-chain (TCR)
Vα-4,20-for-out	5′-WTGCTAAGACCACCCAGCC	Variable region α-chain (TCR)
Vα-5-for-out	5′-AGATAGAACAGAATTCCGAGG	Variable region α-chain (TCR)
Vα-6,14-for-out	5′-RYTGCACATATGACACCAGTG	Variable region α-chain (TCR)
Vα-7-for-out	5′-CACGTACCAGACATCTGGG	Variable region α-chain (TCR)
Vα-8,21-for-out	5′-CCTGAGYGTCCAGGARGG	Variable region α-chain (TCR)
Vα-9-for-out	5′-GTGCAACTATTCCTATTCTGG	Variable region α-chain (TCR)
Vα-10,24-for-out	5′-ASTGGAGCAGAGYCCTCAG	Variable region α-chain (TCR)
Vα-11-for-out	5′-TCTTCAGAGGGAGCTGTGG	Variable region α-chain (TCR)
Vα-12-for-out	5′-GGTGGAGAAGGAGGATGTG	Variable region α-chain (TCR)
Vα-13,19,26-for-out	5′-SAASTGGAGCAGAGTCCTC	Variable region α-chain (TCR)
Vα-15-for-out	5′-CCTGAGTGTCCGAGAGGG	Variable region α-chain (TCR)
Vα-16-for-out	5′-ATGCACCTATTCAGTCTCTGG	Variable region α-chain (TCR)
Vα-17-for-out	5′-TGATAGTCCAGAAAGGAGGG	Variable region α-chain (TCR)
Vα-18-for-out	5′-GTCACTGCATGTTCAGGAGG	Variable region α-chain (TCR)
Vα-22,31-for-out	5′-CCCTWCCCTTTTCTGGTATG	Variable region α-chain (TCR)
Vα-23,30-for-out	5′-GGCARGAYCCTGGGAAAGG	Variable region α-chain (TCR)
Vα-27-for-out	5′-CTGTTCCTGAGCATGCAGG	Variable region α-chain (TCR)
Vα-28-for-out	5′-AGACAAGGTGGTACAAAGCC	Variable region α-chain (TCR)
Vα-29-for-out	5′-CAACCAGTGCAGAGTCCTC	Variable region α-chain (TCR)
Vα-32-for-out	5′-GCATGTACAAGAAGGAGAGG	Variable region α-chain (TCR)
BV-1-for-i-out	5′-TGTACTGGTACCAACAGAGC	Vβ 1, β-chain (TCR)
Fe-BV1-BJ2.3-2	5′-CCTGGGCCAAAATACTGCG	Jβ 2.3, β-chain (TCR)

- PCR 2

Label	Sequence	Hybridization site
BV-1-for-i-nest	5′-GGAGAAGAGAGAGCAAAAGG	Vβ 1, β-chain (TCR)
Fe-BV1-BJ2.3-1	5′-ATACTGCGTATCCTTCCTCC	NDN region, Patient FE specific

- PCR 3

Label	Sequence	Hybridization site
BV1-for-inner	5′-ATTCTCCGCACAACAGTTCC	Vβ 1, β-chain (TCR)
Fe-BV1-nDn-in-impr	5′-TCCTTCCTCCCGCTAATGC	NDN region, Patient FE specific

- PCR 4

Label	Sequence	Hybridization site
Vα-4/1-for-in	5′-ACAGAAGACAGAAAGTCCAGC	Variable region α-chain (TCR)
Vα-4/2-for-in	5′-GTCCAGTACCTTGATCCTGC	Variable region α-chain (TCR)
Vα-6-for-in	5′-GCAAAATGCAACAGAAGGTCG	Variable region α-chain (TCR)

Vα-8/1-for-in	5′-CAGTGCCTCAAACTACTTCC	Variable region α-chain (TCR)
Vα-8/2-for-in	5′-GCCTCAGACTACTTCATTTGG	Variable region α-chain (TCR)
Vα-14-for-in	5′-ACAGAATGCAACGGAGAATCG	Variable region α-chain (TCR)
Vα-24-for-in	5′-CCTTCAGCAACTTAAGGTGG	Variable region α-chain (TCR)
Vα-28-for-in	5′-TCTCTGGTTGTCCACGAGG	Variable region α-chain (TCR)
Vα-2/1-for-in	5′-TGGAAGGTTTACAGCACAGC	Variable region α-chain (TCR)
Vα-2/2-for-in	5′-TGGAAGGTTTACAGCACAGG	Variable region α-chain (TCR)
Vα-5-for-in	5′-CAGCATACTTACAGTGGTACC	Variable region α-chain (TCR)
Vα-10-for-in	5′-TCACTGTGTACTGCAACTCC	Variable region α-chain (TCR)
Vα-12-for-in	5′-TACAAGCAACCACCAAGTGG	Variable region α-chain (TCR)
Vα-22-for-in	5′-AGGCTGATGACAAGGGAAGC	Variable region α-chain (TCR)
Vα-31-for-in	5′-GTGGAATACCCCAGCAAACC	Variable region α-chain (TCR)
Vα-7-for-in	5′-CTCCAGATGAAAGACTCTGC	Variable region α-chain (TCR)
Vα-13-for-in	5′-TTAAGCGCCACGACTGTCG	Variable region α-chain (TCR)
Vα-17-for-in	5′-CTGTGCTTATGAGAACACTGC	Variable region α-chain (TCR)
Vα-18-for-in	5′-CCTTACACTGGTACAGATGG	Variable region α-chain (TCR)
Vα-21-for-in	5′-TGCTGAAGGTCCTACATTCC	Variable region α-chain (TCR)
Vα-23-for-in	5′-GTGGAAGACTTAATGCCTCG	Variable region α-chain (TCR)
Vα-32-for-in	5′-TCACCACGTACTGCAATTCC	Variable region α-chain (TCR)
Vα-3-for-in	5′-TTCAGGTAGAGGCCTTGTCC	Variable region α-chain (TCR)
Vα-11-for-in	5′-AGGGACGATACAACATGACC	Variable region α-chain (TCR)
Vα-15-for-in	5′-CCTCCACCTACTTATACTGG	Variable region α-chain (TCR)
Vα-19-for-in	5′-CCTGCACATCACAGCCTCC	Variable region α-chain (TCR)
Vα-25-for-in	5′-AGACTGACTGCTCAGTTTGG	Variable region α-chain (TCR)
Vα-26-for-in	5′-CCTGCATATCACAGCCTCC	Variable region α-chain (TCR)
Vα-29-for-in	5′-ACTGCAGTTCCTCCAAGGC	Variable region α-chain (TCR)
Vα-1/235-for-in	5′-AAGGCATCAACGGTTTTGAGG	Variable region α-chain (TCR)
Vα-1/14-for-in	5′-CTGAGGAAACCCTCTGTGC	Variable region α-chain (TCR)
Vα-9-for-in	5′-ATCTTTCCACCTGAAGAAACC	Variable region α-chain (TCR)
Vα-16-for-in	5′-TCCTTCCACCTGAAGAAACC	Variable region α-chain (TCR)
Vα-20-for-in	5′-ACGTGGTACCAACAGTTTCC	Variable region α-chain (TCR)
Vα-27-for-in	5′-ACTTCAGACAGACTGTATTGG	Variable region α-chain (TCR)
Vα-30-for-in	5′-CTCTTCACCCTGTATTCAGC	Variable region α-chain (TCR)
Cα-rev-out	5′-GCAGACAGACTTGTCACTGG	Variable region α-chain (TCR)

- PCR 5

Label	**Sequence**	**Hybridization site**
Valpha 7i.2	5′-CACGTACCAGACATCTGGG	Vα 7.2, α-chain (TCR)
Valpha 7 Fe-2	5′-**TTCTGGTACCAGCAACATGC**	Vα 7.2, α-chain (TCR)
Valpha 7 Fe-3	5′-GATGGTTTGGAGGAGAAAGG	Vα 7.2, α-chain (TCR)
Fe 7.2 J-C rev cl	5′-GTTCTGGATATTAAGATCCACC	Patient FE specific, joining-constant region
Fe-AV7.2J16-rev-1	5′-GATCCACCTTTAACATGGTYC	Patient FE specific, Jα 16 region
Fe-AV7.2J16-rev-2	5′-TTTAACATGGTYCCCCTTGC	Patient FE specific, Jα 16 region
Fe-AV7.2J16-rev-3	5′-TTGCAAAGAGCAGCTCGAGG	Patient FE specific, Jα 16 region
Cα-in-impr	**5′-AGTCTCTCAGCTGGTACACG**	C-Region α-chain (TCR)

Unbiased PCR

- RT PCR

Label	Sequence	Hybridization site
Cα out	5′-GCAGACAGACTTGTCACTGG	C-region α-chain (TCR)
Cβ out	5′-TGGTCGGGGAAGAAGCCTGTG	C-region β-chain (TCR)

- PCR 1

Label	Sequence	Hybridization site
VP 1	5′-TSYTTTGTCTCCTGGGAGCA	β chain leader 1, 5, 16, 17, 23
VP 2	5′-CCTGAAGTCGCCCAGACTCC	Vβ 22, 25
VP 3	5′-GTCATSCAGAACCCAAGAYACC	Vβ 18, 24
VP 4	5′-GGWTATCTGTMAGMGTGGAACCTC	Vβ 2, 4
VP 5	5′-ATGTACTGGTATCGACAAGAYC	Vβ 3, 11, 12, 13, 14, 15
VP 6	5′-CACTGTGGAAGGAACATCAAACC	Vβ 20
VP 7	5′-TCTCCACTCTSAAGATCCAGC	Vβ 6, 8, 21
VP 8	5′-CAGRATGTARATYTCAGGTGTGATCC	Vβ 6
VP 9	5′-CCAGACWCCAARAYACCTGGTCA	Vβ 7, 9

- PCR 2

Label	Sequence	Hybridization site
VP 1-UP	5′-ACAGCACGACTTCCAAGACTCACYTTTGTCTCCTGGGAGCA	UP anchor-VP 1
VP 2-UP	5′-ACAGCACGACTTCCAAGACTCACCTGATGTCGCCCAGACTCC	UP anchor-VP 2
VP 3-UP	5′-ACAGCACGACTTCCAAGACTCAGTCATSCAGAACCCAAGAYACC	UP anchor-VP 3
VP 4-UP	5′-ACAGCACGACTTCCAAGACTCAGGWTATCTGTMAGMGTGGAACCTC	UP anchor-VP 4
VP 5-UP	5′-ACAGCACGACTTCCAAGACTCAATGTACTGGTATCGACAAGAYC	UP anchor-VP 5
VP 6-UP	5′-ACAGCACGACTTCCAAGACTCACACTGTGGAAGGAACATCAAACC	UP anchor-VP 6
VP 7-UP	5′-ACAGCACGACTTCCAAGACTCATCTCCACTCTSAAGATCCAGC	UP anchor-VP 7
VP 8-UP	5′-ACAGCACGACTTCCAAGACTCACAGRATGTARATYTCAGGTGTGATCC	UP anchor-VP 8
VP 9-UP	5′-ACAGCACGACTTCCAAGACTCATCAGACWCCAARAYACCTGGTCA	UP anchor-VP 9

- PCR 3

Label	Sequence	Hybridization site
UP	5′-ACAGCACGACTTCCAAGACTCA	UP anchor
Cβ in	5′-TCTGATGGCTCAAACACAGC	C-Region β-chain (TCR)

- PCR 4

Same as for the clone specific PCR

- PCR 5

Label	Sequence	Hybridization site
Va 1.5 Lead for	5′-ATGCTCCTGCTGCTCGTCC	Vα 1.5 leader, α-chain (TCR)
AJ 34-out	5′-GACTTGTAATCTGGTCCCAG	Jα 34 joining, α-chain (TCR)
Va 30.1 Lead for	5′-ATGGAGAAAATGTTGGAGTGTG	Vα 30.1 leader, α-chain (TCR)
AJ-18-out	5′-GACAGTCAACTGAGTTCCTC	Jα 18 joining, α-chain (TCR)
AV-20.1-Lead for	5′-CGTCGACATGAGGCAAGTG	Vα 20.1 leader, α-chain (TCR)
Ca-start-1	5′-GCAGGGTCAGGGTTCTGG	C-Region α-chain (TCR)

- **Single cell PCR for amplification of library plasmids (Section 2.4.3.2.3)**

Label	Sequence	Hybridization site
pcDNA-for-1	5′-CACTGCTTACTGGCTTATCG	pcDNA6/V5-HisA-lib
pcDNA-for-2-TOPO	5′-CACCCGACTCACTATAGGGAGACC	pcDNA6/V5-HisA-lib
pcDNA-2nd-for	5′-TCCGGCGCGCCACCATG	pcDNA6/V5-HisA-lib
pcDNA-rev-1	5′-ACTAGAAGGCACAGTCGAGG	pcDNA6/V5-HisA-lib
pcDNA-rev-2	5′-CTGATCAGCGGGTTTAAACTC	pcDNA6/V5-HisA-lib
pcDNA-rev-3	5′-TGGTGATGGTGATGATGACC	pcDNA6/V5-HisA-lib
pcDNA-rev-10	5′-CTAGACTCGAGCGGCCGC	pcDNA6/V5-HisA-lib

- **Linker oligonucleotides for construction the PECP libraries (Section 2.4.3.8)**

The coding sequences are underlined. Nucleotides contributing to restriction sites are boxed. The Kozak sequences are highlighted in blue. Nucleotides coding for start and stop signals are shown in red.

Label	Sequence
	*Asc*I-Kozak- Start-Coding sequence-Stop – *Not*I
N27-all-lib-*ASc*-for	5′-CAGGGAAGGCGCGCCACCATG**NNKATCNNKNNKNNKGTGNNKNNKCTATGA**GCGGCCGCTAAACTAT
N27-A1-39-lib-*Asc*-for	5′-CAGGGAAGGCGCGCCACCATG**NNKNNKGACNNKNNKNNKNNKNNKTAT**TGAGCGGCCGCTAAACTAT
N27-B8-359-lib-*Asc*-for	5′-CAGGGAAGGCGCGCCACCATG**NNKNNKAAANNKARGNNKNNKNNKCTN**TGAGCGGCCGCTAAACTAT
N27-*Not*I-rev	5′-TAGTTTAGCGGCCGCTCA

II. Vector maps

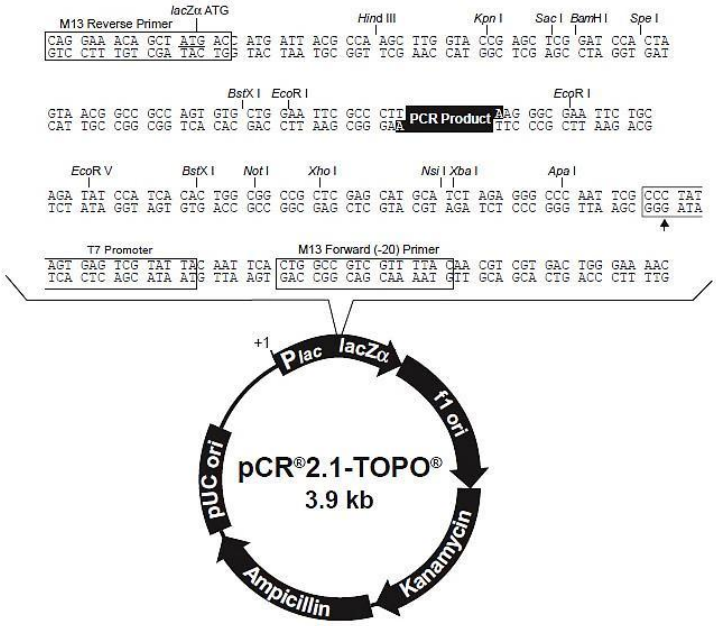


Fig. II-1: pCR®2.1-TOPO vector

P*lac* = *lac*Z-Gen promoter, *lacZ*α = gene coding for β-galactosidase (α-fragment), f1 ori = origin of replication of the f1 phage, kanamycin- and ampicillin-resistance gene, pUC ori = bacterial (*E. coli*) origin of replication; Source: handbook'TOPO-TA Cloning®' (Invitrogen)

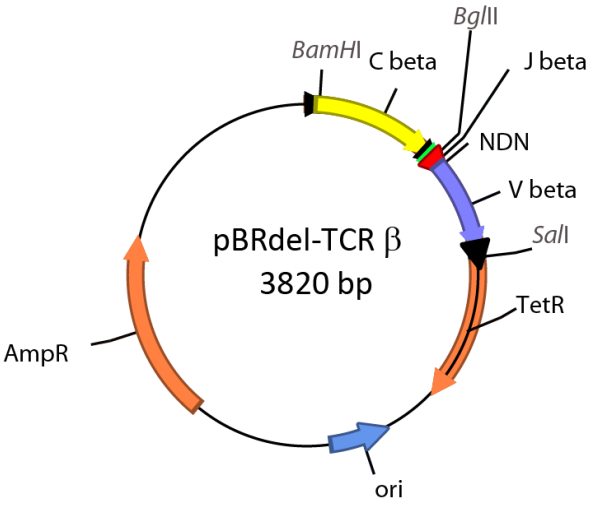


Fig. II-2: pBRdel for subcloning the TCR β-chain

The pBRdel vector serves as an intermediate subcloning vector prior to cloning the entire TCR β-chain into the pRSV5neo vector. The standard pBRDel vector (3403 bp) contains an origin of replication (ori), ampicillin resistance (Amp^r) and tetramycin (Tet^r) resistance genes. The subcloning vector contains a complete TCR β-chain sequence lying between the *Sal*I and *BamH*I recognition sites. Since the constant region sequence is the same for all β-chains, the upstream V-NDN-J-DNA sequence can be exchanged with a different one via the flanking *Sal*I and *Bgl*II recognition sites. The complete TCR sequence may now be cut out via a *Sal*I-*BamH*I restriction digestion and cloned into the pRSV5neo vector (Fig. II-4). Plasmid map constructed in: 'ApE- A plasmid editor' freeware program (Version 1.1.7) by M.Wayne Davis. Source: Joachim Malotka, MPI of Neurobiology

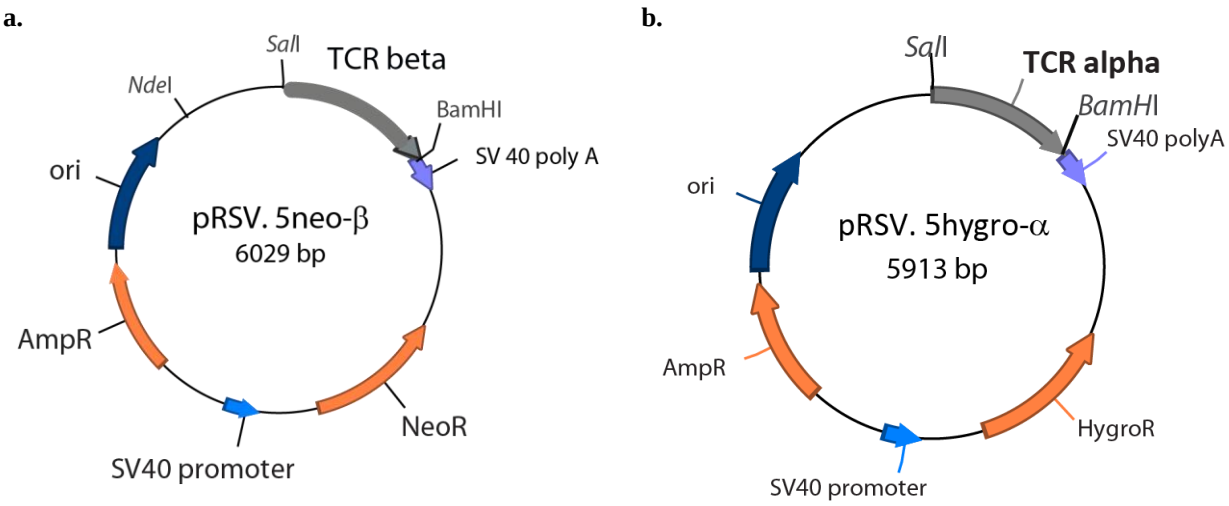


Fig. II-3: pRSV5neo vector for cloning of the TCRα- and β-chains

(a) The pRSV.5 neo vector with the TCR β-chain (BV8.1-BJ1.1) insert. The vector carries genetic elements such as the long terminal repeats of the Rous sarcoma virus (RSV LTR), polyA stretch from simian virus 40 (SV40-polyA), neomycin resistance gene (NeoR), origin of replication of the simian virus 40 (SV40-ori) , ampicillin resistance gene (AmpR), origin of replication (ori). The recognition sites of all relevant restriction enzymes are indicated.

(b) The prSV.5 hygro vector with the TCR α-chain (AV30.1-AJ18) insert. The pRSVhygro vector is a derivative of the prSV.5 neo vector wherein the neomycin resistance gene is replaced with a hygromycin resistance gene (HygroR) via a *Sma*I and *Hind*III restriction site. The pRSV.5neo and prSVhygro vector was provided by Joachim Malotka, MPI of Neurobiology. Plasmid map constructed in: 'ApE- A plasmid editor' freeware program (Version 1.1.7) by M.Wayne Davis.

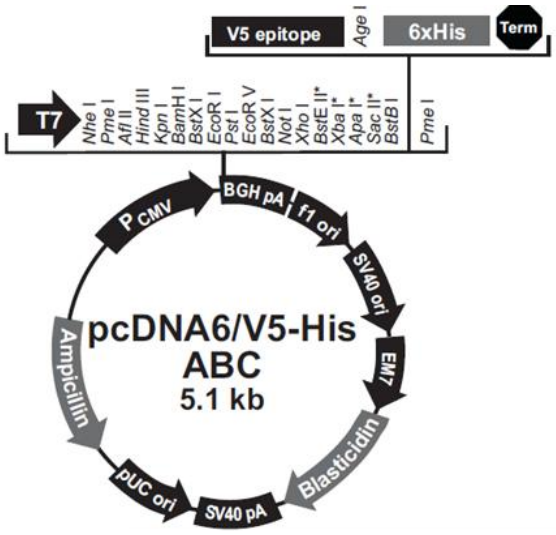


Fig. II-4: pcDNA6/V5-HisA vector
P_{CMV} = CMV promoter, T7 = T7 promoter, 6xHis = polyhistidine tag, BGH pa = BGH polyadenylation sequence, f1ori= origin of replication f 1 phage, SV40 ori= SV40 promoter and origin or replication, EM7= EM7 promoter, resistance genes for blasticidin and ampicillin, SV 40= SV40 polyaednylation sequence, pUC ori= pUC origin. Source: Invitrogen.

pcDNA6/V5-His-A-PECP lib (5145 bp): Antigenic peptide coding region

CTTGGTACCGAGCTCGGATCCGGCGCGCCACCATGNNNNNNNNNNNNNNNNNNNNNNNNNNNNTGAGCGGCCGCTCGAGTC
TAGAGGGCCCTTCGAAGGTAAGCCTATCCCTAACCCTCTCCTCGGTCTCGATTCTACGCGTACC

Fig. II-5: The antigen peptide coding region in pcDNA6/V5-HisA-PECP library
Open reading frame of the antigen peptide coding region of the plasmid encoded combinatorial peptide (PECP) library, Grey: Flanking sequences of the pcDNA6/V5-His A vector, The recognition sites of restriction enzymes *BamH*I (blue) and *Not*I (red) are highlighted in boxes.

pcDNA6/V5-HisA-sGFP (5870bp):GFP-coding region

GTCCAGTGTGGTGGAATTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGC
TGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG
AAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCACATATGGCGTGCAGTGCTT
CAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCA
TCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG
CTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTTCAACAGCCACAACGTATA
CATCATGGCTGACAAGCAGAAGAACGGAATCAAGGCCAACTTCAAGACCCGCCACAACATCGAGGACGGCGGCGTGCAGC
TGGCCGACCACTACCAGCAGAACACCCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAA
TCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGATTACACA
TGGCATGGATGAACTATACAAGGGATCCCATCACCATCACCATCACTAAGCTCTCGAGTCTAGAGGGCCCTT

Fig. II-6: Nucleotide sequence of the s-GFP coding region in pcDNA6/V5-HisA-sGFP
Open reading frame of the s-GFP gene, Grey: Flanking sequences of the pcDNA6/V5-His A vector, The recognition sites of restriction enzymes *EcoR*I (blue) and *Xho*I (red) are highlighted in boxes.

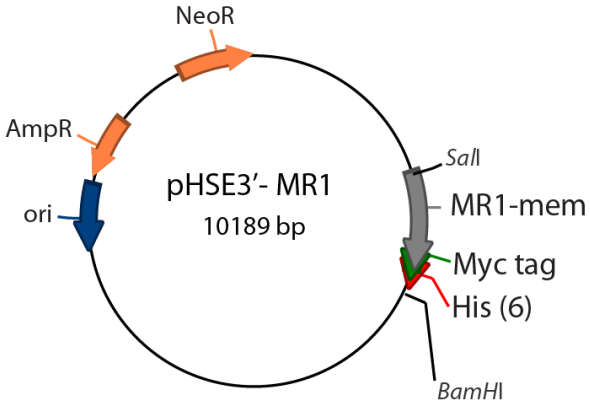


Fig. II-7: pHSE3'- MR1
The human MR1 gene was cloned into the pHSE3' vector (Pircher et al. 1989) by Wakiro Sato, MPI of Neurobiology. It contains the extracellular membrane bound (MR1-mem) region carrying a myc tag and His(6) tag for subsequent purification and/or detection by western blot. The pHSE3' vector carries genetic elements such as the origin of replication (ori), and resistance genes for ampicillin (AmpR) and Neomycin (NeoR). The recognition sites of the enzymes relevant for cloning of the MR1 gene are also depicted. Sequence source: Wakiro Sato. Plasmid map constructed in: 'ApE- A plasmid editor' freeware program (Version 1.1.7) by M.Wayne Davis.

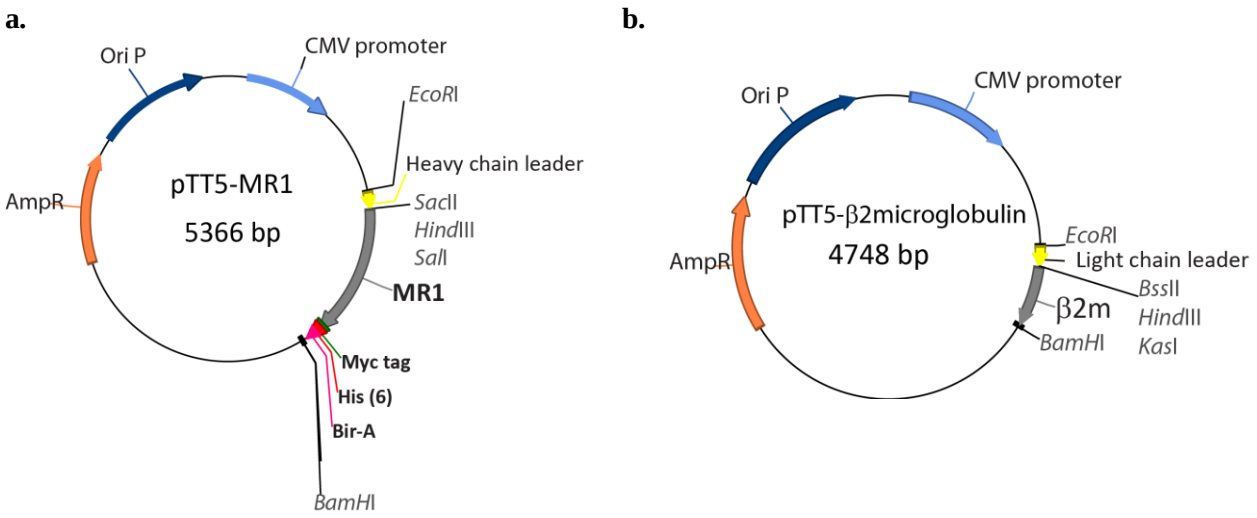


Fig. II-8: pTT5-MR1 and pTT5-β2 microglobulin
Human MR1 and β2 microglobulin were cloned into the pTT5 vector. The pTT5 vector contains an EBV specific origin of replication (oriP) as well as a promoter of the cytomegalovirus (CMV) and an ampicillin resistance gene (AmpR). All the relevant recognition sites of the restriction enzymes are depicted. **(a)** pTT5-MR1 carries the leader sequence (yellow) of the Fab heavy chain, followed by the MR1 gene (MR1), and a myc tag, a polyhistidine tag (His (6)), and a C terminal Bir-A site. **(b)** pTT5-β2m carries the leader sequence of the Fab light chain, followed by the β2 microglobulin coding region. Plasmid source: Birgit Obermeier, MPI of Neurobiology. Plasmid map constructed in: 'ApE- A plasmid editor' freeware program (Version 1.1.7) by M.Wayne Davis.

III. Electropherograms depicting RNA integrity

The electropherograms depict RNA integrity on a plot of fluorescence (FU) Vs., Svedberg units (s). A comparison of the RNA integrity of RNA isolated from peripheral blood lymphocytes (Fig. III-1) in comparison to that isolated from frozen tissue of patient FE (Fig. III-2, III-3 and III-4) shows that the amount of degraded RNA in frozen tissue sections exceeds that in live cells. This loss in RNA integrity in the tissue samples is possibly due to delayed cryopreservation post isolation and repeated freeze thaw cycles. This low RNA quality translates into reduced yields of cDNA during reverse transcription. Data provided by Joachim Malotka and Anna Backes, MPI of Neurobiology.

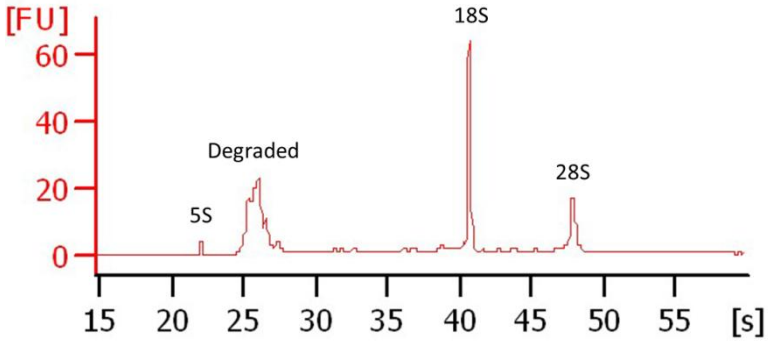


Fig. III-1: Electropherogram of RNA from peripheral blood lymphocytes

The peaks depict different regions of the sample such as the 5S, degraded RNA, 18S and 28S subunits. Even though this RNA is sourced from live cells, in the absence of RNAse inhibitors, some of the RNA is degraded during sample processing. However, the RNA integrity of this sample is sufficient for cDNA synthesis.

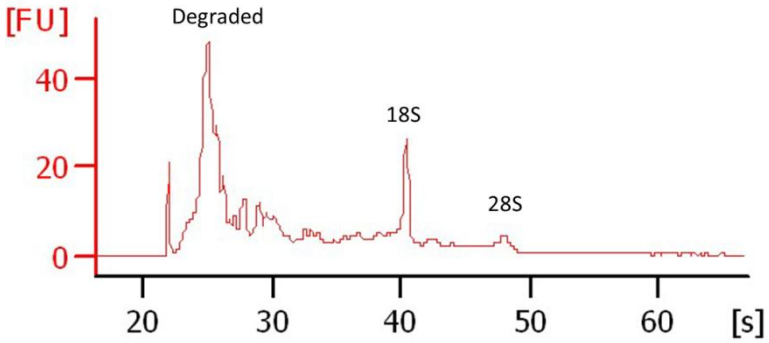


Fig. III-2: Electropherogram of RNA isolated from block # 9

The peaks depict the degraded RNA, 18S and 28S subunits. The amount of degraded RNA is much higher in comparison to the intact 18S and 28S subunits. The RNA integrity of this sample is compromised and hardly sufficient for cDNA synthesis.

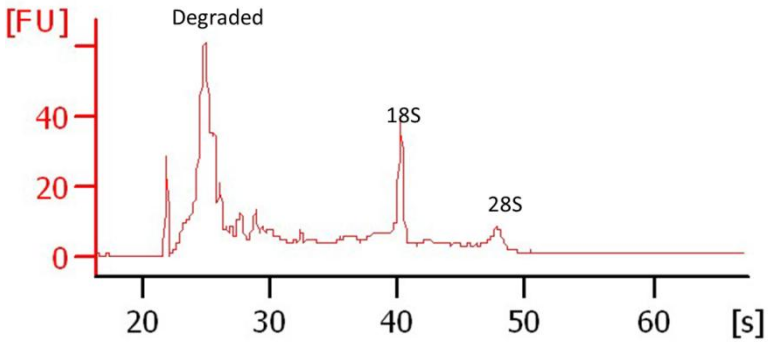


Fig. III-3: Electropherogram of RNA isolated from block # 10

The peaks depict the degraded RNA, 18S and 28S subunits. The amount of degraded RNA is much higher in comparison to the intact 18S and 28S subunits. The RNA integrity of this sample is compromised and hardly sufficient for cDNA synthesis.

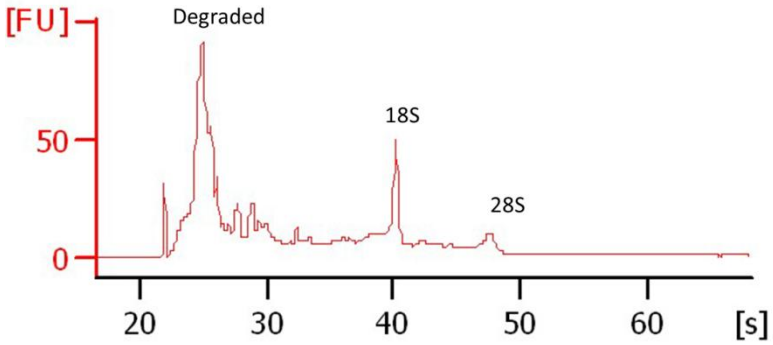


Fig. III-4: Electropherogram of RNA isolated from block # 11

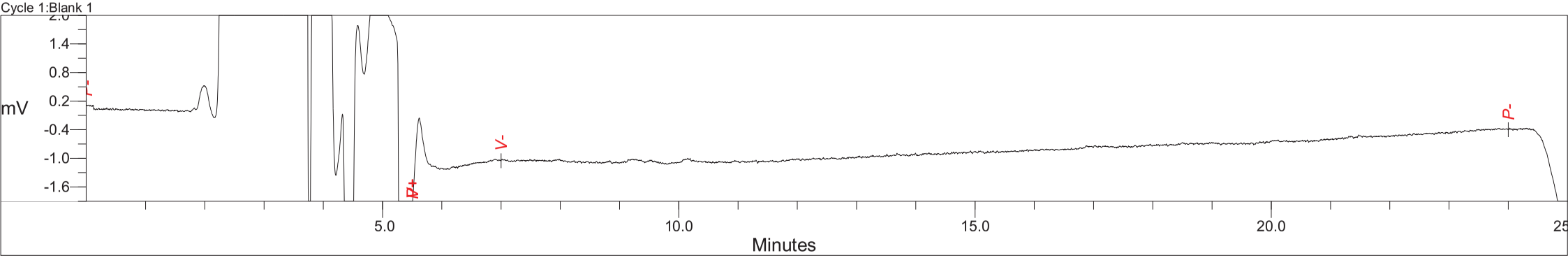
The peaks depict the degraded RNA, 18S and 28S subunits. The amount of degraded RNA is much higher in comparison to the intact 18S and 28S subunits. The RNA integrity of this sample is compromised and hardly sufficient for cDNA synthesis.

IV. Edman degradation data

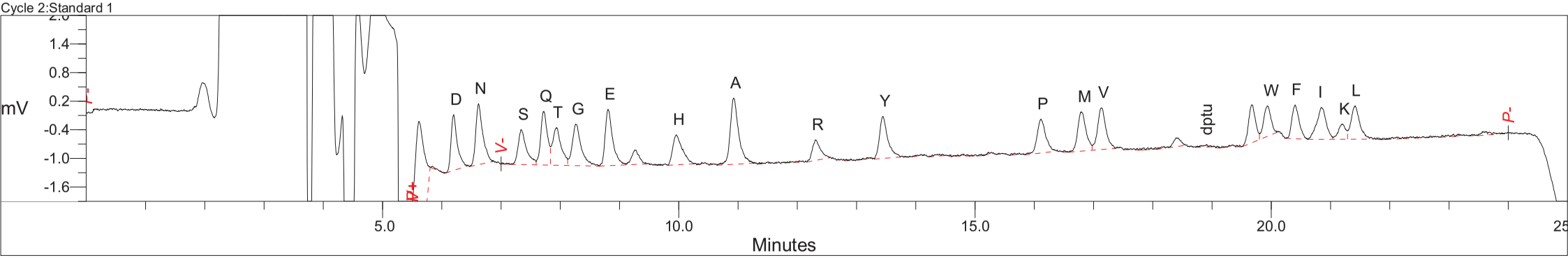
Detection of phenylthiohydantoin-amino acids via reverse phase HPLC

Edman degradation was used to sequence the nonapeptides (from the synthetic combinatorial peptide libraries in positional scanning format) that were eluted from recombinant human MR1. However, the signal from the eluate was too low to be detected. Even after 13 cycles, there was no clear signal from the sample that could be distinguished from the background. The amino acid residues are denoted as per the standard one alphabet code. Y axis: Absorbance unity, X axis: Retention time. Data provided by Reinhard Mentele, MPI of Biochemistry.

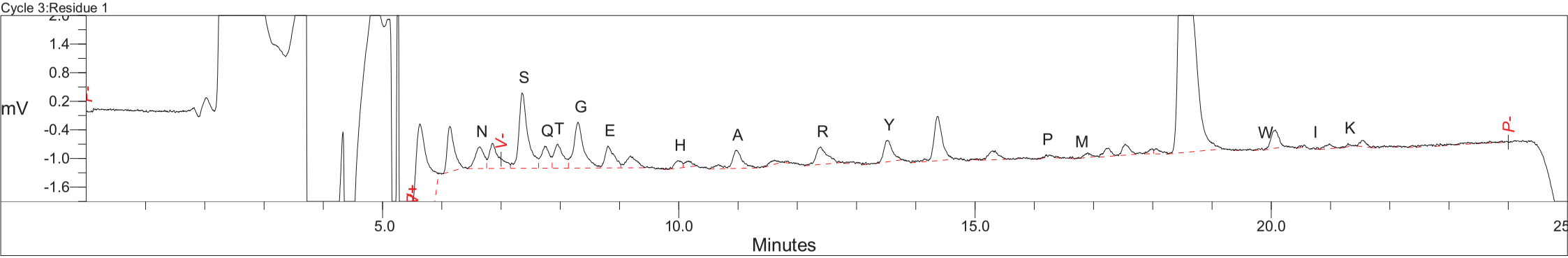
Cycle 1:Blank 1



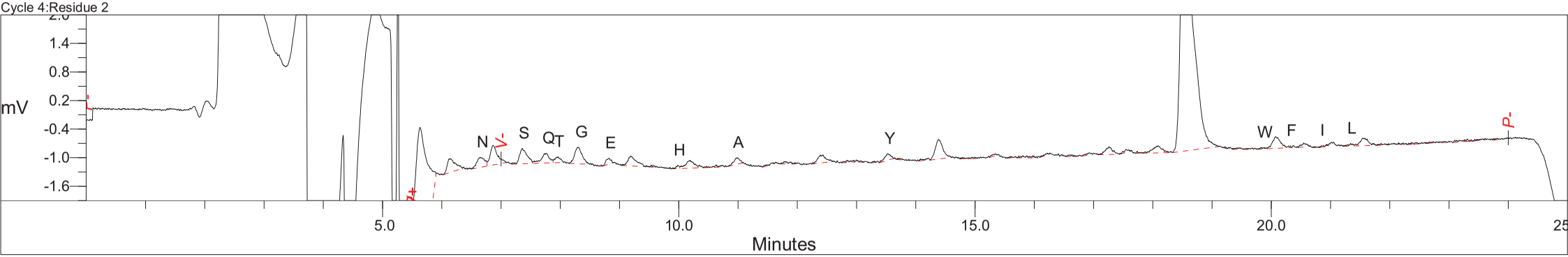
Cycle 2:Standard 1



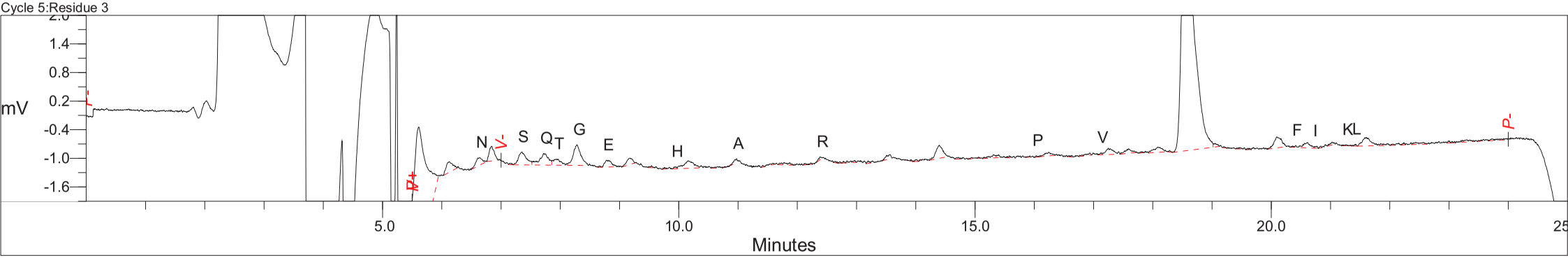
Cycle 3:Residue 1



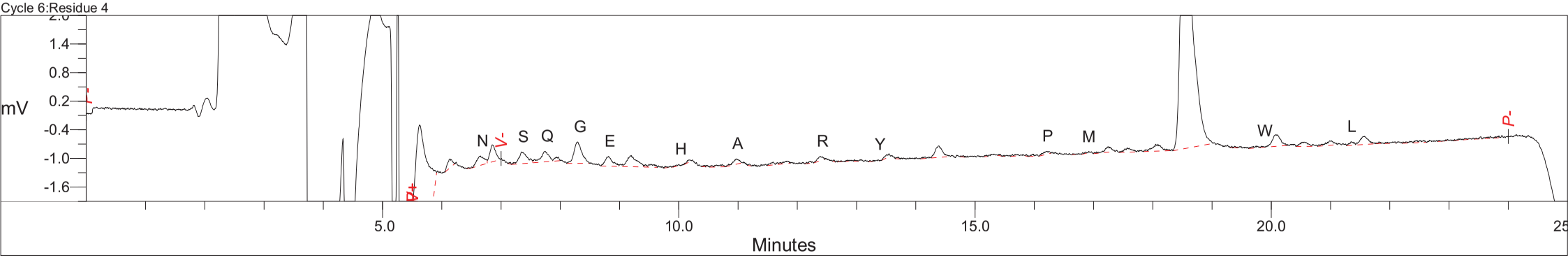
Cycle 4:Residue 2



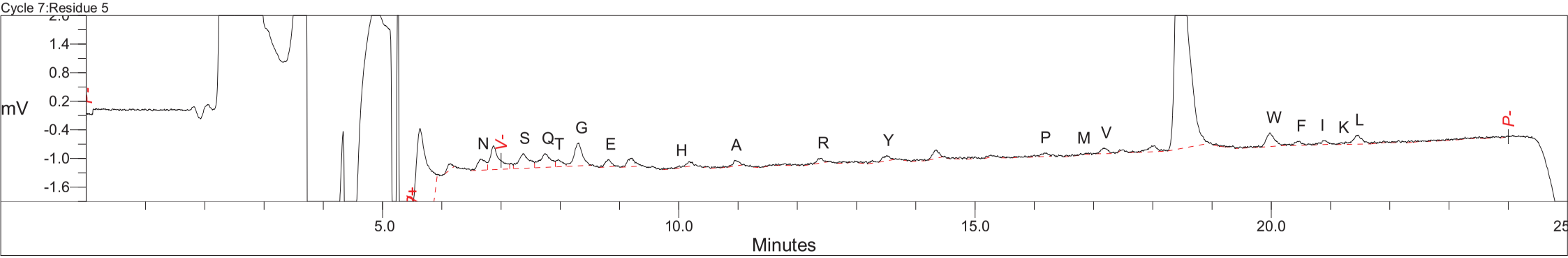
Cycle 5:Residue 3



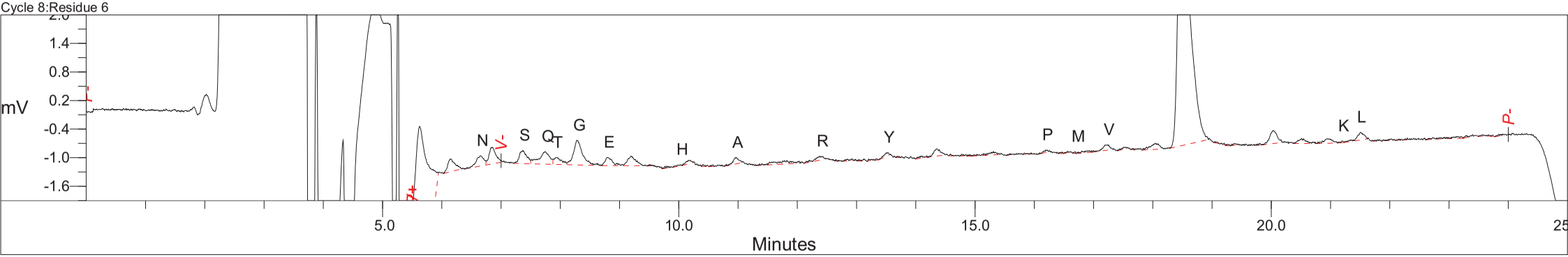
Cycle 6:Residue 4



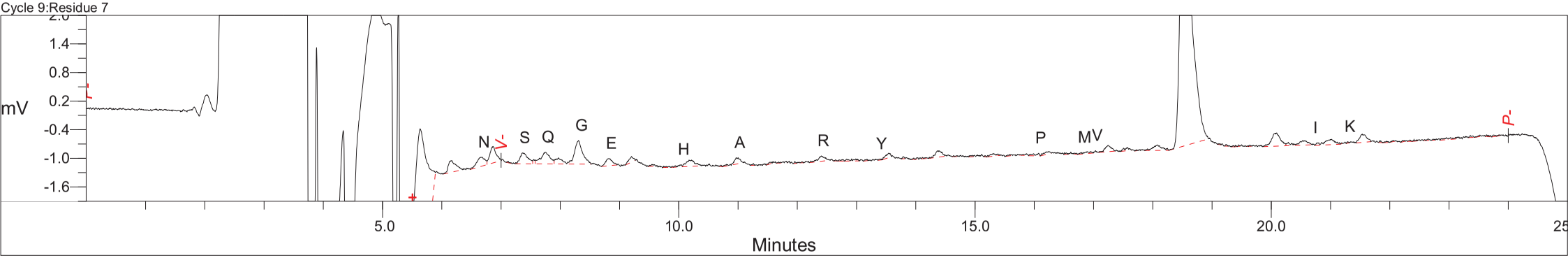
Cycle 7:Residue 5



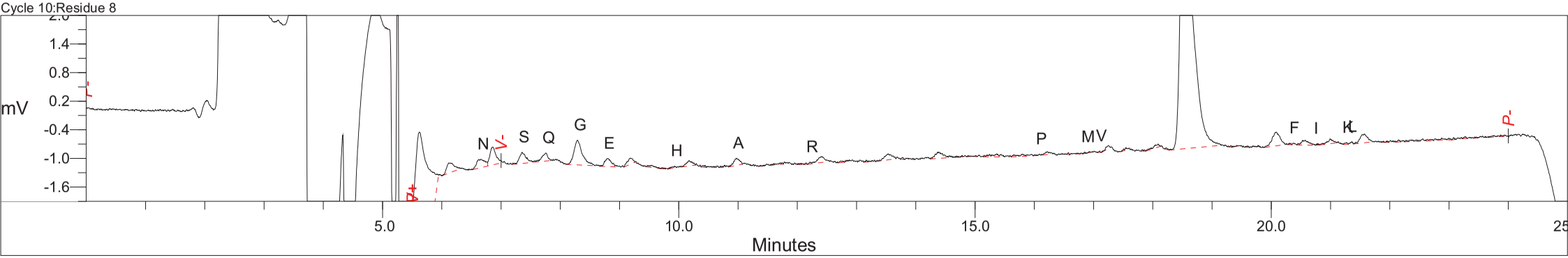
Cycle 8:Residue 6



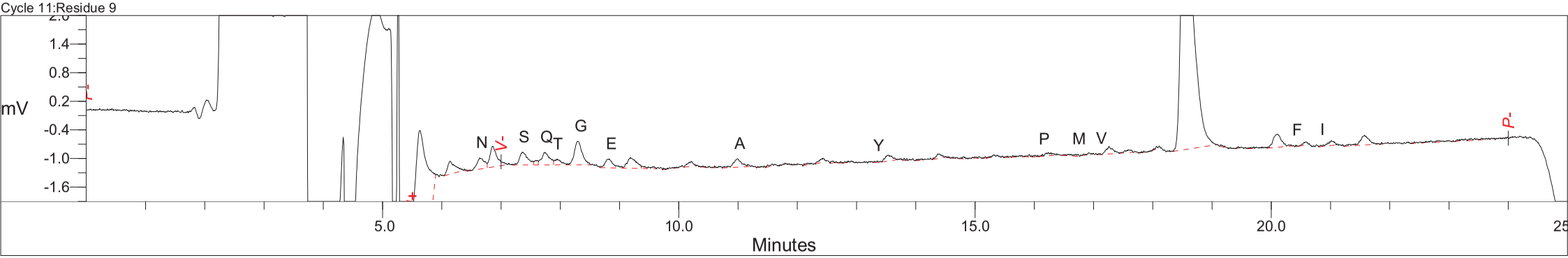
Cycle 9:Residue 7



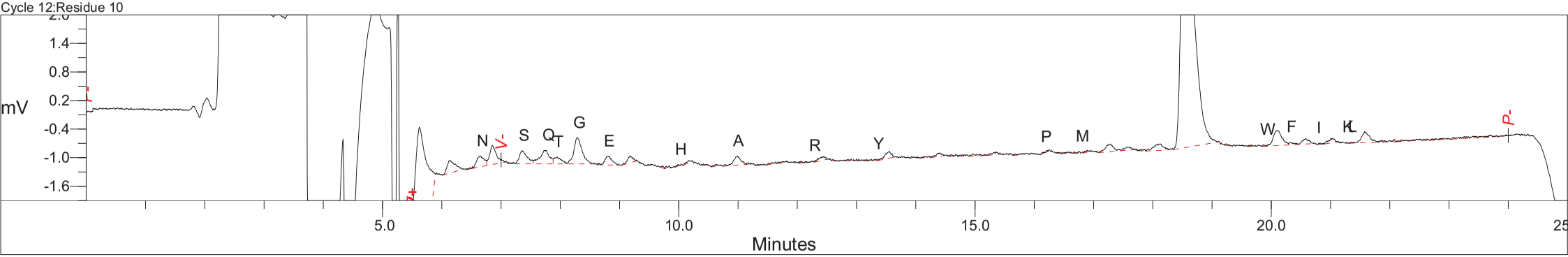
Cycle 10:Residue 8



Cycle 11:Residue 9

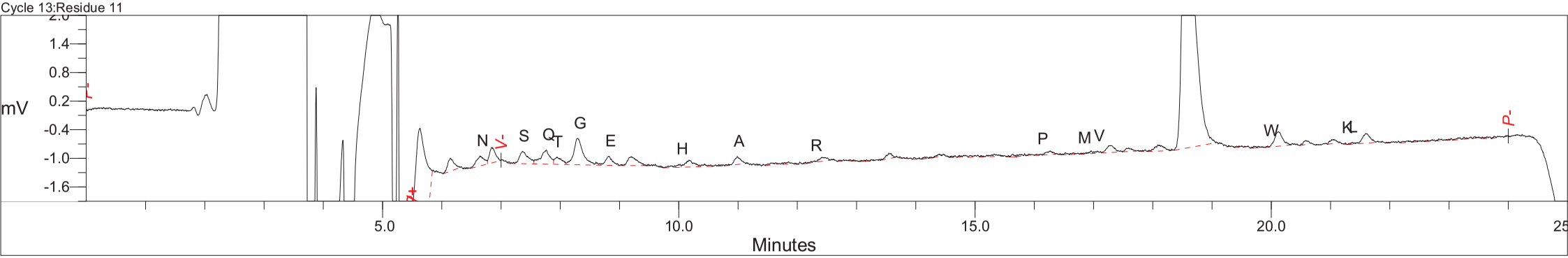


Cycle 12:Residue 10



No lab name
No address

Cycle 13:Residue 11



V. Sequence of peptides examined by mass spectrometry

Data provided by Dr Forne Ignasi, Ludwig-Maximilians-Universität München

No.	P1	P2	P3	P4	P5	P6	P7	P8	P9
1	A	Q	Y	E	D	I	A	N	R
2	A	T	M	Q	N	L	N	D	R
3	C	C	C	D	C	C	C	M	D
4	C	L	G	P	A	T	K	S	A
5	D	C	C	C	C	C	M	D	C
6	D	G	P	P	K	T	C	S	S
7	F	G	E	R	A	K	L	V	L
8	F	G	T	L	L	T	K	K	R
9	G	D	M	K	N	L	N	D	R
10	G	Y	S	R	P	G	A	G	G
11	K	K	A	L	L	P	F	V	F
12	K	L	G	K	F	L	G	K	W
13	K	L	L	L	V	P	D	G	N
14	K	N	K	T	T	F	L	R	R
15	K	P	K	K	R	G	F	T	T
16	K	P	T	R	A	L	V	H	L
17	K	S	K	A	G	N	L	A	R
18	L	K	P	P	A	V	L	R	R
19	L	L	G	L	L	L	G	S	A

No.	P1	P2	P3	P4	P5	P6	P7	P8	P9
20	L	L	G	P	R	A	A	F	V
21	L	L	P	N	R	V	R	Q	R
22	L	V	L	P	N	R	C	G	R
23	M	D	C	C	C	D	C	C	C
24	N	F	C	V	A	V	L	V	L
25	P	A	A	L	L	V	V	G	G
26	P	P	K	T	V	A	H	L	K
27	P	R	G	K	V	R	V	G	N
28	Q	F	A	G	D	D	A	P	R
29	S	T	L	V	L	V	L	V	F
30	T	A	M	Q	N	L	N	D	R
31	T	L	G	A	Q	E	D	D	K
32	T	L	T	L	V	K	L	V	N
33	V	C	N	Y	V	S	W	L	K
34	V	F	F	H	G	A	R	R	K
35	V	L	L	H	A	R	E	V	L
36	V	L	L	P	T	A	E	E	R
37	V	V	K	A	V	R	D	V	F
38	Y	N	V	N	P	V	Y	L	Q

The above table presents the 38 nonapeptide sequences (from the synthetic combinatorial peptide libraries in positional scanning format) that were eluted from recombinant human MR1. These were sequenced by electrospray mass spectrometry. The first column contains an arbitrary reference number, while the following columns denote the 9 positions (P1 to P9) of each peptide. Sequence alignment analysis of the peptides did not reveal a stringent position bias, albeit a pattern that could be suggestive of fixed anchor positions of the human MR1 molecule.

Ehrenwörtliche Erklärung

gemäß der Promotionsordnung für Biologie der LMU München

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die erlaubten Quellen und Hilfsmittel verwendet habe. Sämtliche Experimente wurden von mir selbst durchgeführt, sofern nicht explizit auf Dritte verwiesen wird.

Ich habe weder anderweitig versucht, eine Dissertation oder Teile einer Dissertation einzureichen, noch eine Doktorprüfung durchzuführen.

München, den 21.12.2011 Latika Bhonsle

Curriculum Vitae

Name	Latika Bhonsle
Date of birth	23.10.1985
Place of birth	Agra, India
Nationality	Indian

Educational history

Since 09/2008	Doctoral candidate in the research group of Dr. Klaus Dornmair, P.D at the Max Planck Institute of Neurobiology, Martinsried and the Institute for Clinical Neuroimmunology of the Ludwig-Maximilians-Universität München, Germany
07/2006 – 05/2008	MSc in Biomedical Sciences at the University of Delhi, India
Master Thesis	An analysis of host molecular mimicry and the effect of insertion element sequences on the *Mycobacterium tuberculosis* H37Rv genome 73.42%, first class with distinction
06/2007 – 07/2007	Internship at the Advanced Centre for Treatment, Research and Education in Cancer, Mumbai, India
Project	Characterization of human regulatory T cells
05/2003 – 04/2006	BSc in Microbiology at the University of Delhi, India
Bachelor Thesis	Establishing a relation between nutrient medium concentrations and oligophilic actinomycete morphology 79.95% , first rank, first class with distinction
07/2003 – 03/2003	Senior School Certificate Examination at the Kendriya Vidyalaya, Pune, India 79.20%, first class with distinction

Academic achievements and awards

Since 09/2008	Member of the International Max Planck Research School for Molecular and Cellular Life Sciences
07/2006 – 05/2008	'Catch them young' scholarship by the Government of India for academic excellence in the MSc Biomedical Sciences course
04/2006	First rank in the BSc Microbiology course
042002	Distinctive performance in the all India Science Olympiad for high school students

Data presentation at scientific conferences

07/2011	Poster presentation: FASEB 'Autoimmunity' Summer Conference, Vermont, United States of America
11/2010	Oral presentation: Seminar for the young scientists of the SFB 571 'autoimmune Reactions : From Manifestations to Therapy'
10/2010	Poster presentation: 10^{th} International Congress of Neuroimmunology, Sitges, Spain

References

Alvarez-Lafuente,R., Garcia-Montojo,M., Heras,V.D.L., Dominguez-Mozo,M.I., Bartolome,M., Benito-Martin,M.S., and Arroyo,R. (2008). Herpesviruses and human endogenous retroviral sequences in the cerebrospinal fluid of multiple sclerosis patients. MS 14, 595-601.

Abós B, Gómez Del Moral M, Gozalbo-López B, López-Relaño J, Viana V, Martínez-Naves E.(2011). Human MR1 expression on the cell surface is acid sensitive, proteasome independent and increases after culturing at 26°C; Biochem Biophys Res Commun.;411(3),632-6.

Ackerman,A.L. and Cresswell,P. (2004). Cellular mechanisms governing cross-presentation of exogenous antigens. Nat. Immunol. 5, 678-684.

Alvarez-Lafuente,R., Las Heras,V., Bartolomé,M., Garcia-Montojo,M., and Arroyo,R. (2006). Human herpesvirus 6 and multiple sclerosis: A one-year follow-up study. Brain Pathol. 16, 20-27.

Arden B, Clark SP, Kabelitz D, Mak TW. (1995). Human T-cell receptor variable gene segment families.Immunogenetics; 42, 455-500.

Ascherio,A. and Munger,K.L. (2007). Environmental risk factors for multiple sclerosis. Part I: The role of infection. Ann. Neurol. 61, 288-299.

Babbe,H., Roers,A., Waisman,A., Lassmann,H., Goebels,N., Hohlfeld,R., Friese,M., Schroder,R., Deckert,M., Schmidt,S., Ravid,R., and Rajewsky,K. (2000). Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J. Exp. Med. 192, 393-404.

Beall SS, Concannon P, Charmley P, McFarland HF, Gatti RA, Hood LE, McFarlin DE, Biddison WE.(1989). The germline repertoire of T cell receptor beta-chain genes in patients with chronic progressive multiple sclerosis.J Neuroimmunol.21(1),59-66.

Berer K, Mues M, Koutrolos M, Rasbi ZA, Boziki M, Johner C, Wekerle H, Krishnamoorthy G.(2011). Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination; Nature; 479(7374),538-41.

Biddison WE, Beall SS, Concannon P, Charmley P, Gatti RA, Hood LE, McFarland HF, McFarlin DE.(1989). The germline repertoire of T-cell receptor beta-chain genes in patients with multiple sclerosis.Res Immunol.;140(2),212-5.

Bolton, J.S. (1900). The Exact Histological Localisation of the Visual Area of the Human Cerebral Cortex. Proc. R. Soc. Lond. 67, 216-218.

Boussif ,O., Lezoualc'h,F., Zanta,M.A., Mergny,M.D., Scherman,D., Demeneix,B., and Behr,J.-P. (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo, Polyethylenimine. Proceedings of the National Academy of Sciences of the United States of America 92, 7297-7301.

Bray PF, Bloomer LC, Salmon VC, Bagley MH, Larsen PD. (1983) Epstein-Barr virus infection and antibody synthesis in patients with multiple sclerosis. Arch Neurol. 40(7), 406-8.

Bray,P.F., Luka,J., Culp,K.W., and Schlight,J.P. (1992). Antibodies against Epstein-Barr nuclear antigen (EBNA) in multiple sclerosis CSF, and two pentapeptide sequence identities between EBNA and myelin basic protein. Neurol. 42, 1798-1804.

Brennan,R.M., Burrows,J.M., Bell,M.J., Bromham,L., Csurhes,P.A., Lenarczyk,A., Sverndal,J., Klintenstedt,J., Pender,M.P., and Burrows,S.R. (2010). Strains of Epstein-Barr virus infecting multiple sclerosis patients. MS 16, 643-651.

Buljevac,D., Van Doornum,G.J.J., Groen,J., Osterhaus,A.D.M.E., Hop,W., Van Doorn,P.A., van der Meché,F.G.A., and Hintzen,G. (2005). Epstein-Barr virus and disease activity in multiple sclerosis. J. Neurol. Neurosurg. Psych. 76, 1377-1381.

Call,M.E., Pyrdol,J., Wiedmann,M., and Wucherpfennig,K.W. (2002). The organizing principle in the formation of the T cell receptor-CD3 complex. Cell 111, 967-979.

Castillo R., (2002) Immunopathology, Elsevier Science

Cepok,S., Zhou,D., Srivastava,S., Nessler,S., Stei,S., Büssow,K., Sommer,N., and Hemmer,B. (2005). Identification of Epstein-Barr virus proteins as putative targets of the immune response in multiple sclerosis. J. Clin. Invest. 115, 1352-1360.

Chapiro,J., Claverol,S., Piette,F., Ma,W., Stroobant,V., Guillaume,B., Gairin,J.E., Morel,S., Burlet-Schiltz,O., Monsarrat,B., Boon,T., and Van den Eynde,B.J. (2006). Destructive cleavage of antigenic peptides either by the immunoproteasome or by the standard proteasome results in differential antigen presentation. J. Immunol. 176, 1053-1061.

Chua WJ, Kim S, Myers N, Huang S, Yu L, Fremont DH, Diamond MS, Hansen TH.(2011) Endogenous MHC-related protein 1 is transiently expressed on the plasma membrane in a conformation that activates mucosal-associated invariant T cells. J Immunol;186(8),4744-50

Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. (1988a). p. 629-62.

Clevers HC, Dunlap S, Wileman TE, Terhorst C. (1988b). Human CD3-epsilon gene contains three miniexons and is transcribed from a non-TATA promoter. Proc Natl Acad Sci U S A, 85(21),8156-60.

Compston,A. and Coles,A. (2008). Multiple sclerosis. Lancet 372, 1502-1517.

Davis, M.M. and Bjorkman, P.J. (1988). T-cell antigen receptor genes and T-cell recognition.Nature 334, 395-402.

Coo, H. and Aronson, K.J. (2004). A systematic review of several potential nongenetic risk factors for multiple sclerosis. *Neuroepidemiology*, **23**, 1-12.

Crooks GE, Hon G, Chandonia JM, Brenner SE (2004). WebLogo: A sequence logo generator, Genome Research, 14,1188-1190.

Diamond B, Katz JB, Paul E, Aranow C, Lustgarten D, Scharff MD. (1992) The role of somatic mutation in the pathogenic anti-DNA response.Annu Rev Immunol; 10, 731-757.

Dooms, H. and Abbas, A.K.(2002). Life and death in effector T cells.Nat Immunol 3, 797-8.

Dornmair K, Goebels N, Weltzien HU, Wekerle H, Hohlfeld R. T-cell-mediated autoimmunity - Novel techniques to characterize autoreactive T-cell receptors. Am J Path. 2003; 163, 1215-1226.

Durocher,Y., Perret,S., and Kamen,A. (2002). High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Res. 30.

Dyment DA, Ebers GC, Sadovnick AD. Genetics of multiple sclerosis. (2004). Lancet Neurology; 3, 104-110.

Edman P, (1950) Method for determination of the amino acid dequence in peptides; Acta Chem Scand.; 4,283-93.

Eylar,E.H., Caccam,J., Jackson,J.J., Westall,F.C., and Robertson,R.B. (1970). Experimental allergic encephalomyelitis: Synthesis of disease inducing site of the basic protein. Science 168, 1220-1223.

Falk K, Rötzschke O, Stevanović S, Jung G, Rammensee HG.(1991). Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecule; Nature. 351(6324),290-6.

Friese, M.A. and Fugger, L. (2005). Autoreactive CD8+ T cells in multiple sclerosis: a new target for therapy? Brain, 128, 1747-1763.

Friese, M.A. and Fugger,L. (2009). Pathogenic CD8+ T cells in multiple sclerosis. Ann. Neurol. 66, 132-141.

Fugger,L., Friese,M.A., and Bell,J.I. (2009). From genes to function: the next challenge to understanding multiple sclerosis. Nature Rev. Immunol. 9, 408-417.

Garcia KC, Scott CA, Brunmark A. /1996). CD8 enhances formation of stable T-cell receptor/MHC classI complexes. Nature; 384, 577-581.

Geurts, J.J.G. and Barkhof, F. (2008). Grey matter pathology in multiple sclerosis. Lancet Neurology 7, 841-851.

Geurts,J.J.G., Stys,P.K., Minagar,A., Amor,S., and Zivadinov,R. (2009). Gray matter pathology in (chronic) MS: Modern views on an early observation. J. Neurol. Sci. 282, 12-20.

Gluzman,Y. (1981). SV40-transformed simian cells support the replication of early SV40 mutants. Cell 23, 175-182.

Godbey,W.T., Wu,K.K., and Mikos,A.G. (1999). Poly (ethylenimine) and its role in gene delivery. Journal of Controlled Release 60, 160.

Gold,M.C., Cerri,S., Smyk-Pearson,S., Cansler,M.E., Vogt,T.M., Delepine,J., Winata,E., Swarbrick,G.M., Chua,W.-J., Yu,Y.Y.L., Lantz,O., Cook,M.S., Null,M.D., Jacoby,D.B., Harriff,M.J., Lewinsohn,D.A., Hansen,T.H., and Lewinsohn,D.M. (2010). Human Mucosal Associated Invariant T cells detect bacterially infected cells. PLoS Biology 8, e1000407.

Gorman,C.M., Gies ,D., McCray ,G., and Huang ,M. (1989). The human cytomegalovirus major immediate promotor can be trans-activated by adenovirus early proteins. Virology 171, 377-385.
Goronzy, J.J., Zettl, A. and Weyand, C.M. (1998) T cell receptor repertoire in rheumatoid arthritis.Int Rev Immunol 17, 339-63.

Gundlach,B.R., Wiesmuller,K.H., Junt,T., Kienle,S., Jung,G., and Walden,P. (1996). Determination of T cell epitopes with random peptide libraries. J. Immunol. Methods *192*, 149-155.

Hafler,D.A. (1999). The distinction blurs between an autoimmune versus microbial hypothesis in multiple sclerosis. J. Clin. Invest. 105, 527-529.

Hamrouni,A., Aublin,A., Guillaume,P., and Maryanski,J.L. (2003). T cell receptor gene rearrangement lineage analysis reveals clues for the origin of highly restricted antigen-specific repertoires. J. Exp. Med. 197, 601-614.

Handel AE, Lincoln MR, Ramagopalan SV(2011).Of mice and men: experimental autoimmune encephalitis and multiple sclerosis.Eur J Clin Invest. 41(11), 1254-8.

Hansen TH, Bouvier M. (2009). MHC classI antigen presentation: learning from viral evasion strategies. Nat Rev Immunol. 9(7),503-13.

Harty JT, Tvinnereim AR, White DW (2000). CD8+ T cell effector mechanisms in resistance to infection. ARI; 18, 275-308.

Hauser SL, Bhan AK, Gilles FH (1983). Immunohistological staining of human brain with monoclonal antibodies that identify lymphocytes, monocytes and the Ia antigen. J Neuroimmunol; 5, 197-205.

Heath WR, Carbone FR, Bertolino P, Kelly J, Cose S, Miller JF.(1995) Expression of two T cell receptor alpha chains on the surface of normal murine T cells;Eur J Immunol ;25(6),1617-23.

Heim, R., Cubitt, A. B. & Tsien, R. Y. (1995). Improved green Fluorescence.Nature 373, 663-664.

Heidemann,R., Zhang,C., Qi,H., Rule,J.L., Rozales,C., Park,S., Chuppa,S., Ray,M., Michaels,J., Konstantinov,K., and Naveh,D. (2000). The use of peptones as medium additives for the production of a recombinant therapeutic in high density perfusion cultures of mammalian cells. Cytotechnology 32, 157-167.

Hemmer B, Archelos JJ, Hartung H-P (2002). New concepts in the immunopathogenesis of multiple sclerosis. NRN; 3, 291-301.

Hemmer,B., Gran,B., Zhao,Y.D., Marques,A., Pascal,J., Tzou,A., Kondo,T., Cortese,I., Bielekova,B., Straus,S.E., McFarland,H.F., Houghten,R., Simon,R., Pinilla,C., and Martin,R. (1999). Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease. Nature Med. *5*, 1375-1382.

Hennessey,K., Heller,M., Santen,v.V., and Kieff,E. (1983). Simple repeat array in Epstein-Barr virus DNA encodes part of the Epstein-Barr nuclear antigen. Science 220, 1396-1398.

Hofbauer, M., Wiesener, S., Babbe, H., Roers, A., Wekerle, H., Dornmair, K., Hohlfeld, R. and Goebels, N. (2003) Clonal tracking of autoaggressive T cells in polymyositis by combining laser

microdissection, single-cell PCR, and CDR3-spectratype analysis. Proc Natl Acad Sci U S A 100, 4090-5.

Höllsberg,P., Hansen,H.J., and Haahr,S. (2003). Altered CD8+ T cell responses to selected Epstein-Barr virus immunodominant epitopes in patients with multiple sclerosis.Clin. Exp. Immunol. 132, 137-143.

Hoppes,R., Ekkebus,R., Schumacher,T.N., and Ovaa,H. (2010). Technologies for MHC classI immunoproteomics. J. Proteomics. 73, 1945-1953.

Huang S, Gilfillan S, Cella M, Miley MJ, Lantz O, Lybarger L, Fremont DH, Hansen TH., (2005). Evidence for MR1 antigen presentation to mucosal-associated invariant T cells.,J Biol Chem. 3; 280(22),21183-93.

Huang S, Gilfillan S, Kim S, Thompson B, Wang X, Sant AJ, Fremont DH, Lantz O, Hansen TH.(2008). MR1 uses an endocytic pathway to activate mucosal-associated invariant T cells.J Exp Med.205(5):1201-11

Huang S, Martin E, Kim S, Yu L, Soudais C, Fremont DH, Lantz O, Hansen TH (2009). MR1 antigen presentation to mucosal-associated invariant T cells was highly conserved in evolution; PNAS;106(20),8290-5.

Huseby, E.S., Liggitt, D., Brabb, T., Schnabel, B., Ohlen, C. and Goverman, J. (2001). A pathogenic role for myelin-specific CD8 (+) T cells in a model for multiple sclerosis. J Exp Med, 194, 669-676.

Illés Z, Shimamura M, Newcombe J, Oka N, Yamamura T (2004). Accumulation of Valpha7.2-Jalpha33 invariant T cells in human autoimmune inflammatory lesions in the nervous system. Int Immunol., 16(2),223-30.

Illes,Z., Shimamura,M., Newcombe,J., Nobuyuki,O., and Yamamura,T. (2003). Accumulation of Va7.2±Ja33 invariant T cells in human autoimmune in¯ammatory lesions in the nervous system. International Immunology 16, 223-230.

Inoko, H., David, C.S. and Tabira, T. (2000). Hla-DR2-restricted responses to proteolipid protein 95-116 peptide cause autoimmune encephalitis in transgenic mice. J Clin Invest, 105, 977-984.

Jores R, Alzari PM, Meo T. (1990). Resolution of hypervariable regions in T-cell receptor beta chains by a modified Wu-Kabat index of amino acid diversity.PNAS 87, 9138-42.

Junker,A., Ivanidze,J., Malotka,J., Eiglmeier,I., Lassmann,H., Wekerle,H., Meinl,E., Hohlfeld,R., and Dornmair,K. (2007). Multiple sclerosis: T-cell receptor expression in distinct brain regions. Brain 130, 2789-2799.

Karttunen J, Shastri N. (1991). Measurements of ligand induced activation in single viable T cells using lacZ reporter gene. Proc Natl Acad Sci USA; 88, 3972-3976.

Katzav S. (2004). Vav1: an oncogene that regulates specific transcriptional activation of T cells. Blood; 107, 2443-51.

Kawamura, K., Yamamura, T., Yokoyama, K., Chui, D.H., Fukui, Y., Sasazuki, T.,Inoko, H., David, C.S. and Tabira, T. (2000). Hla-DR2-restricted responses to proteolipid protein 95-116 peptide cause autoimmune encephalitis in transgenic mice. J Clin Invest, 105, 977-984.

Lassmann,H., Niedobitek,G., Aloisi,F., Middeldorp,J.M., and The NeuroproMiSe EBV Working Group (2011). Epstein-Barr virus in the multiple sclerosis brain: a controversial issue-report on a focused workshop held in the Centre for Brain Research of the Medical University of Vienna, Austria. Brain 134, 2772-2786.

Le Bourhis L, Guerri L, Dusseaux M, Martin E, Soudais C, Lantz O. (2011). Mucosal-associated invariant T cells: unconventional development and function.Trends Immunol, 32(5),212-8.

Le Bourhis,L., Martin,E., Péguillet,I., Guihot,A., Froux,N., Coré,M., Lévy,E., Dusseaux,M., Meyssonnier,V., Premel,V., Ngo,C., Riteau,B., Duban,L., Robert,D., Rottman,M., Soudais,C., and Lantz,O. (2010). Antimicrobial activity of mucosal-associated invariant T cells .Nature Immunol. 11, 701-708.

Lennon VA, Wilks AV, Carnegie PR. (1970) Immunologic properties of the main encephalitogenic peptide from the basic protein of human myelin.J Immunol.105(5),1223-30.

Letourneur,F. and Malissen,B. (1989). Derivation of a T cell hybridoma variant deprived of functional T cell receptor a and b chain transcripts reveals a nonfunctional a-mRNA of BW5147 origin. Eur. J. Immunol. 19, 2269-2274.

Lincoln,M.R., Montpetit,A., Cader,M.Z., Saarela,J., Dyment,D.A., Tiislar,M., Ferretti,V., Tienari,P.J., Sadovnick,A.D., Peltonen,L., Ebers,G.C., and Hudson,T.J. (2005). A predominant role for the HLA class II region in the association of the MHC region with multiple sclerosis. Nature Genet. 37, 1108-1112.

Lucas RM, Hughes AM, Lay ML, Ponsonby AL, Dwyer DE, Taylor BV, Pender MP. (2011). Epstein-Barr virus and multiple sclerosis.J Neurol Neurosurg Psychiatry; 82(10),1142-8.

Madsen, L.S., Andersson, E.C., Jansson, L., krogsgaard, M., Andersen, C.B.,Engberg, J., Strominger, J.L., Svejgaard, A., Hjorth, J.P., Holmdahl, R., Wucherpfennig, K.W. and Fugger, L. (1999) A humanized model for multiple sclerosis using HLA-DR2 and a human T-cell receptor. Nat Genet, 23, 343-347.

Marsh SG, Albert ED, Bodmer WF, Bontrop RE, Dupont B, Erlich HA, Geraghty DE, Hansen JA, Hurley CK, Mach B, Mayr WR, Parham P, Petersdorf EW, Sasazuki T, Schreuder GM, Strominger JL, Svejgaard A, Terasaki PI, Trowsdale J.(2005).Nomenclature for Factors of the HLA System, 2004. Hum Immunol. 66(5),571-636.

Martin E, Treiner E, Duban L, Guerri L, Laude H, Toly C, Premel V, Devys A, Moura IC, Tilloy F, Cherif S, Vera G, Latour S, Soudais C, Lantz O. (2009). Stepwise development of MAIT cells in mouse and human. PLoS Biol. 7(3),e54.

Mathey,E.K., Derfuss,T., Storch,M.K., Williams,K.R., Hales,K., Woolley,D.R., Al-Hayani,A., Davies,S.N., Rasband,M.N., Olsson,T., Moldenhauer,A., Velhin,S., Hohlfeld,R., Meinl,E., and Linington,C. (2007). Neurofascin as a novel target for autoantibody-mediated axonal injury. J Exp Med.204, 2363-2372.

Matsumoto, Y., Yoon, W.K., Jee, Y., Fujihara, K., Misu, T., Sato, S., Nakashima, I. and Itoyama, Y. (2003). Complementarity-determining region 3 spectratyping analysis of the TCR repertoire in multiple sclerosis. J Immunol 170, 4846-53.

Medana IM, Gallimore A, Oxenius A, Martinic MMA, Wekerle H, Neumann H. (2000) MHC classI-restricted killing of neurons by virus specific CD8+ T lymphocytes is effected through the Fas/FasL, but not the perforin pathway. Eur J Imm ; 30, 3623-3633.

Miley, M.J., Truscott,S.M., Yu,Y.Y.L., Gilfillan,S., Fremont,D.H., Hansen,T.H., and Lybarger,L. (2003). Biochemical Features of the MHC-Related Protein 1 Consistent with an immunological Function. J Immunol 170, 6090-6098.

Miyazaki Y, Miyake S, Chiba A, Lantz O, Yamamura T. (2011). Mucosal-associated invariant T cells regulate Th1 response in multiple sclerosis., Int Immunol;23(9),529-35.

Moebius, U., Manns, M., Hess, G., Kober, G., Meyer zum Buschenfelde, K.H. and Meuer, S.C. (1990). T cell receptor gene rearrangements of T lymphocytes infiltrating the liver in chronic active hepatitis B and primary biliary cirrhosis (PBC): oligoclonality of PBC-derived T cell clones. Eur J Immunol 20, 889-96.

Morel,S., Lévy,F., Burlet-Schiltz,O., Brasseur,F., Probst-Kepper,M., Peitrequin,F., Monsarrat,B., Van Velthoven,R., Cerottini,J.-C., Boon,T., Gairin,J.E., and Van den Eynde,B.J. (2000). Processing of some antigens by the standard proteasome but not the immunoproteasome results in poor presentation by dendritic cells. Immunity 12, 107-117.

Morris GP, Allen PM; (2009) Cutting edge: Highly alloreactive dual TCR T cells play a dominant role in graft-versus-host disease; J Immunol; 182(11),6639-43.

Murata,S., Sasaki,K., Kishimoto,T., Niwa,S., Hayashi,H., Takahama,Y., and Tanaka,K. (2007). Regulation of CD8+ T cell development by thymus-specific proteasomes. Science 316, 1349-1353.

Nino-Vasquez,J.J., Allicotti,G., Borras,E., Wilson,D.B., Valmori,D., Simon,R., Martin,R., and Pinilla,C. (2004). A powerful combination: the use of positional scanning libraries and biometrical analysis to identify cross-reactive T cell epitopes. Mol. Immunol. *40*, 1063-1074.

Padovan E, Casorati G, Dellabona P, Meyer S, Brockhaus M, Lanzavecchia A.(1993) Expression of two T cell receptor alpha chains: dual receptor T cells; Science ;262(5132),422-4.

Pantaleo, G., Demarest, J.F., Schacker, T., Vaccarezza, M., Cohen, O.J., Daucher, M., Graziosi, C., Schnittman, S.S., Quinn, T.C., Shaw, G.M., Perrin, L., Tambussi, G., Lazzarin, A., Sekaly, R.P., Soudeyns, H., Corey, L. and Fauci, A.S. (1997). The qualitative nature of the primary immune response to HIV infection is a prognosticator of disease progression independent of the initial level of plasma viremia. Proc Natl Acad Sci U S A 94, 254-8.

Parmentier,N., Stroobant,V., Colau,D., De Driesbach,P., Morel,S., Chapiro,J., Van Endert,P., and Van den Eynde,B.J. (2010). Production of an antigenic peptide by insulin-degrading enzyme. Nature Immunol. 11(5),449-54.

Peterfalvi A, Gomori E, Magyarlaki T, Pal J, Banati M, Javorhazy A, Szekeres-Bartho J, Szereday L, Illes Z, (2008). Invariant Valpha7.2-Jalpha33 TCR is expressed in human kidney and brain tumors indicating infiltration by mucosal-associated invariant T (MAIT) cells. Int Immunol. 20(12),1517-25.

Petersdorf EW, Sasazuki T, Strominger JL, Svejgaard A, Terasaki PI, Tiercy JM, Trowsdale J.(2010) Nomenclature for factors of the HLA system. Tissue Antigens. 75(4),291-455.

Pham,P.L., Perret,S., Doan,H.C., Cass,B., St-Laurent,G., Kamen,A., and Durocher,Y. (2003). Large-Scale transient transfection of serum-free suspension-growing HEK293 EBNA1 cells: peptone additives improve cell growth and transfection efficiency. Biotech. & Bioeng. 84, 332-342.

Pinilla,C., Appel,J.R., and Houghten,R.A. (1994). Investigation of antigen-antibody interactions using a soluble, non-support-bound synthetic decapeptide library composed of four trillion (4 x 10(12) sequences. Biochem. J. 301 (Pt 3), 847-853.

Pircher H, Mak TW, Lang R, Ballhausen W, Rüedi E, Hengartner H, Zinkernagel RM, Bürki K. (1989). T cell tolerance to Mlsa encoded antigens in T cell receptor V beta 8.1 chain transgenic mice. EMBO J. 8(3):719-27.

Porcelli,S., Yockey,C.E., Brenner,M.B., and Balk,S.P. (1993). Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- T cells demonstrates preferential use of several V genes and an invariant TCR chain. J. Exp. Med. 178, 1-16.

Prinz JC. (2003).The role of T cells in psoriasis. J Eur Acad Derm Ven. 7(3), 257-70.

Rabilloud,T., Carpentier,G., and Tarroux,P. (1988). Improvement and simplification of low-background silver-staining of proteins by using sodium dithionite. Electrophoresis 9, 288-291.

Riegert P, Wanner V, Bahram S., (1998). Genomics, isoforms, expression, and phylogeny of the MHC classI-related MR1 gene. J Immunol. 161(8),4066-77.

Rötzschke O, Falk K, Deres K, Schild H, Norda M, Metzger J, Jung G, Rammensee HG. (1990). Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells; Nature 348(6298),252-4.

Rowen L, Koop BF, Hood L. (1996). The complete 685-kilobase DNA sequence of the human b T cell receptor locus. Science 272, 1755-1762.

Rubio-Godoy,V., Pinilla,C., Dutoit,V., Borras,E., Simon,R., Zhao,Y., Cerottini,J.C., Romero,P., Houghten,R., and Valmori,D. (2002). Toward synthetic combinatorial peptide libraries in positional scanning format (PS-SCL)-based identification of CD8+ Tumor-reactive T-Cell Ligands: a comparative analysis of PS-SCL recognition by a single tumor-reactive CD8+ cytolytic T-lymphocyte clone. Cancer Res. *62*, 2058-2063.

Rudensky A, Preston-Hurlburt P, Hong SC, Barlow A, Janeway CA Jr.(1991) Sequence analysis of peptides bound to MHC classII molecules.Nature,353(6345),622-7.

Saint-Ruf C, Ungewiss K, Groettrup M, Bruno L, Fehling HJ, von Boehmer H.(1994) Analysis and expression of a cloned pre-T cell receptor gene; Science;266(5188),1208-12.

Salvetti,M., Giovannoni,G., and Aloisi,F. (2009). Epstein-Barr virus and multiple sclerosis.Curr.Opin. Neurol. 22, 201-206.

Sanderson,S. and Shastri,N. (1994). LacZ inducible, antigen/MHC-specific T cell hybrids. Int. Immunol. 6, 369-376.

Sawcer,S. et. al (2011). Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. Nature 476, 214-219.

Schirle M, Keilholz W, Weber B, Gouttefangeas C, Dumrese T, Becker HD, Stevanović S, Rammensee HG.(2000). Identification of tumor-associated MHC classI ligands by a novel T cell-independent approach; Eur J Immunol.;30(8),2216-25.

Schluesener,H.J., Sobel,R.A., Linington,C., and Weiner,H.L. (1987). A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. J. Immunol. 139, 4016-4021.

Seitz,S., Schneider,C.K., Malotka,J., Nong,X., Engel,A.G., Wekerle,H., Hohlfeld,R., and Dornmair,K. (2006). Reconstitution of paired T cell receptor a- and b-chains from microdissected single cells of human inflammatory tissues. Proc. Natl. Acad. Sci. USA 103, 12057-12062.

Serafini,B., Rosicarelli,B., Franciotta,D., Magliozzi,R., Reynolds,R., Cinque,P., Andreoni,L., Trivedi,P., Salvetti,M., Faggioni,A., and Aloisi,F. (2007). Dysregulated Epstein-Barr virus infection in the multiple sclerosis brain.J. Exp. Med. 204, 2899-2912.

Serafini,B., Severa,M., Columba-Cabezas,S., Rosicarelli,B., Veroni,C., Chiappetta,G., Magliozzi,R., Reynolds,R., Coccia,E.M., and Aloisi,F. (2010). Epstein-Barr virus latent infection and BAFF expression in B cells in the multiple sclerosis brain: Implications for viral persistence and intrathecal B-cell activation. J. Neuropathol. Exp. Neurol. 69, 677-693.

Shimamura,M., Huang,Y.Y., Okamoto,N., Suzuki,N., Yasuoka,J., Morita,K., Nishiyama,A., Amano,Y., and Mishina,T. (2007). Modulation of Va19 NKT cell immune responses by a-mannosyl ceramide derivatives consisting of a series of modified sphingosines. Eur. J. Immunol. 37, 1836-1844.

Siewert,K., Malotka,J., Kawakami, N., Wekerle, H., Hohlfeld, R., and Dornmair, K. (2011) Unbiased identification of target antigens of CD8+ T cells with combinatorial libraries coding for short peptides, (Nature Medicine, in press)

Skulina,C., Schmidt,S., Dornmair,K., Babbe,H., Roers,A., Rajewsky,K., Wekerle,H., Hohlfeld,R., and Goebels,N. (2004). Multiple sclerosis: Brain-infiltrating CD8+ T cells persist as clonal expansions in the cerebrospinal fluid and blood. Proc. Natl. Acad. Sci. USA 101, 2428-243.

Smith,E.S., Mandokhot,A., Evans,E.E., Mueller,L., Borrello,M.A., Sahasrabudhe,D.M., and Zauderer,M. (2001a). Lethality-based selection of recombinant genes in mammalian cells: Application to identifying tumor antigens. Nature Med. 7, 967-972.

Smith-Garvin JE, Koretzky GA, and Jordan MS. (2009). T cell activation. Annu Rev Immunol. 27,591-619.

Sospedra M, Martin R. (2005). Antigen-specific therapies in multiple sclerosis. Int Rev Immunol 24, 393-413.

Sospedra,M., Pinilla,C., and Martin,R. (2003). Use of combinatorial peptide libraries for T-cell epitope mapping. Methods 29, 236-247.

Sumaya CV, Myers LW, Ellison GW. (1980). Epstein-Barr virus antibodies in multiple sclerosis. Arch Neurol. 37(2), 94-6.

Sun, D., Whitaker, J.N., Huang, Z., Liu, D., Coleclough, C., Wekerle, H. and Raine, C.S. (2001). Myelin antigen-specific CD8+ T cells are encephalitogenic and produce severe disease in C57BL/6 mice. J Immunol, 166, 7579-7587.

Thompson J.D., Higgins D.G., Gibson T.J. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." Nucleic Acids Res. 22, 4673-4680.

Tilloy,F., Treiner,E., Park,S.-H., Garcia,C., Lemonnier,F.A., De la Salle,H., Bendelac,A., Bonneville,M., and Lantz,O. (1999). An invariant T cell receptor chain defines a novel TAP-independent, major histocompatibility complex class Ib restricted T cell subpopulation in mammals. J. Exp. Med. 189, 1907-1921.

Treiner,E., Duban,L., Bahram,S., Radosavljevic,M., Wanner,V., Tilloy,F., Affaticati,P., Gilfillan,S., and Lantz,O. (2003). Selection of evolutionarily conserved mucosal-associated invariant T cells by MR1. Nature 422, 164-169.

Uenaka,A., Hirano,Y., Hata,H., Win,S., Aji,T., Tanaka,M., Ono,T., Skipper,J.C., Shimizu,K., and Nakayama,E. (2003). Cryptic CTL epitope on a murine sarcoma Meth A generated by exon extension as a novel mechanism. J. Immunol. 170, 4862-4868.

van der Bruggen,P., Traversari,C., Chomez,P., Lurquin,C., De,P.E., Van den Eynde,B., Knuth,A., and Boon,T. (1991). A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254, 1643-1647.

Van Noort,J.M., Bajramovic,J.J., Plomp,A.C., and Van Stipdonk,M.J.B. (2000). Mistaken self, a novel model that links microbial infections with myelin-directed autoimmunity in multiple sclerosis. J. Neuroimmunol. 105, 46-57.

Vincek V, Nassiri M, Nadji M, Morales AR.(2003) A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples; Lab Invest;83(10),1427-35.

Wandinger, K., Jabs, W., Siekhaus, A., Bubel, S., Trillenberg, P., Wagner, H., Wessel, K., Kirchner, H. and Hennig, H. (2000) Association between clinicaldisease activity and Epstein-Barr virus reactivation in MS. *Neurology*, **55**, 178-184.

Wang,Y., Rubtsov,A., Heiser,R., White,J., Crawford,F., Marrack,P., and Kappler,J.W. (2005a). Using a baculovirus display library to identify MHC classI mimotopes. Proc. Natl. Acad. Sci. U. S. A 102, 2476-2481.

Wekerle H. (1992) Myelin specific, autoaggressive T cell clones in the normal immune repertoire: their nature and their regulation;Int Rev Immunol. 9(3),231-41.

Wong,F.S., Karttunen,J., Dumont,C., Wen,L., Visintin,I., Pilip,I.M., Shastri,N., Pamer,E.G., and Janeway,C.A. (1999). Identification of an MHC classI-restricted autoantigen in type I diabetes by screening an organ-specific cDNA library. Nature Med. 5, 1026-1031.

Woodroofe MN, Bellamy AS, Feldmann M, Davison AN, Cuzner ML. (1986). Immunocytochemical characterisation of the immune reaction in the central nervous system in multiple sclerosis.Possible role for microglia in lesion growth. J Neurol Sci. 74(2-3), 135-52.

Wucherpfennig KW, Newcombe J, Li H, Keddy C, Cuzner ML, Hafler DA.(1992). T cell receptor V alpha-V beta repertoire and cytokine gene expression in active multiple sclerosis lesions. J Exp Med.175(4), 993-1002.

Zang, Y.C., Li, S., Rivera, V.M., Hong, J., Robinson, R.R., Breitbach, W.T., Killian, J.and Zhang, J.Z. (2004). Increased CD8+ cytotoxic T cell responses to myelin basic protein in multiple sclerosis. J Immunol, 172, 5120-5127.