

CHAPTER 1

INTRODUCTION

1.1 Statement of problems

1.1.1 T Lymphocytes

Of the different cells that comprise the adaptive immune system certainly no other cell receives more attention. T lymphocytes or T cells, the primary players in cell mediated immunity, have been studied in almost every imaginable context existing in the field of molecular biology. And yet, every month hundreds of new research papers are published divulging new and interesting ways to uncover the many remaining mysteries, mechanisms and associations of the linchpin of the immune system.

The two fundamental tenets of the adaptive immune response are under the control of the T cell: specificity and memory. The exquisite specificity of the T cell is provided by the virtually limitless possible recombinations of the T-cell receptor (TCR) alpha and beta chains, which make contact with the antigen/major histocompatibility complex (MHC) groove on the antigen presenting cell (APC).

When a T cell encounters its cognate antigen in the context of this MHC molecule, and adequate co-stimulation threshold events occur, the T cell is induced to activate and divide into an army of clones specific for whatever threat was encountered on the APC.

An event such as this; a self:non-self encounter, is paired with the other possibility of a self:self encounter in which the activation, division and finally attack by the T cell would constitute a pathological process, namely, autoimmunity. This

event is avoided by the deletion of potentially auto-reactive T cells in the thymus as they are developing. However all T cells which are potentially auto-reactive are not deleted and some remain to circulate in the periphery. A system of peripheral self-tolerance exists there to regulate the activation of these T cells by withholding co-stimulation for these self:self pairings and thusly rendering them anergic.

1.1.2 Multiple Sclerosis

An example of the failure of the tolerance machinery is multiple sclerosis (MS) which is accepted to be an autoimmune disease even though the precise etiology is as yet poorly understood. MS is a demyelinating disease of the central nervous system (CNS) in which the immune system turns on the host. A prevailing theory is that for some reason host T cells have recognized their cognate antigen in the myelin of the CNS and cross the blood-brain-barrier to assault the spinal cord and brain tissues.

1.1.3 T-cell receptor sequencing to study population dynamics

The foundation of using TCR sequence data to determine clonality in an immune response lies in the ability of this method to determine the outgrowth of specific clones of T cells by their TCR sequence. In this analysis TCR sequences that are over-represented in a heterogenous population of TCR represent a specific “responder” clone that is actively dividing in response to recognizing its cognate antigen. By combining the ability to identify individual clones with the ability to sort specific T-cell populations, a more concise picture of a T-cell response is revealed.

Why might this be valuable information? For starters, the overwhelming outgrowth of a single TCR would point to a dominant antigenic peptide. Since the etiological agent that initiates the onset of MS is unknown many plausible theories have grown around the existence of a “molecular mimic” that may match epitopes

found in the CNS. This unfortunate pairing with an HLA haplotype predisposed to MS vulnerability may be the autoimmune-initiating event. Second, it would be very interesting to find matching TCR sequences in different subjects, also supporting the dominant antigenic peptide. Third, the population dynamics of different T-cell subsets could be dissected: for instance, CD4+ vs. CD8+, dividing vs. non-dividing, and those responding to a particular peptide, or another.

1.1.4 Special methods

The idea of using TCR sequencing data in this way is not new. But, it has never been approached in this manner. The principle question at the outset of this thesis was this. Would it be possible to get the highest resolution image of total clonality, without having to rely on previous lower resolution methods? The new methods used included:

1. Short-term cultures
2. Proliferation and CD4+/CD8+ staining to segregate dividing and non-dividing cells using cell sorting
3. Pools of peptides spanning entire encephalitogenic proteins
4. Special modified reverse transcriptase-PCR that obviates the need for numerous cumbersome primer sets
5. Exhaustive search of a very large pool of TCR sequence data

1.1.5 Reducing expression of the CD147 surface molecule on T cells

The second major topic of this thesis takes on another question that required the application of special methods. In order to explore the role of CD147 in T-cell biology we asked whether a physiologically relevant knockdown of CD147 expression could be attained in primary T cells. The overwhelming majority of assays such as these are performed in immortalized cell lines such as the Jurkat T-cell Lymphoma which is a hearty cell that tolerates harsh transfection conditions, but suffers in that it does not present an accurate picture of how a non-cancerous cell behaves. With the same thought process that went into the TCR clonality study, we decided to put primary T cells into another short-term culture assay to investigate the effects of transient target-gene knockdown. This process would entail the application and optimization of relatively new technology for the transfection of primary T cells.

Recently Amaxa biological instruments company developed a proprietary electroporation system called the Nucleofector®. The Nucleofector technology is a novel transfection technology especially designed for the needs of primary cells and difficult-to-transfect cell lines. It is a non-viral method which is based on a unique combination of electrical parameters and cell-type specific solutions. Although Nucleofector technology is non-viral, it allows transfected DNA to directly enter the nucleus. In contrast, other commonly used transfection methods rely on cell division for the transfer of DNA into the nucleus. Thus, nucleofection provides the ability to transfect even non-dividing cells, such as neurons and resting blood cells. Over 150 published journal articles claim successful target gene knockdown in primary T cells using the Nucleofector.

1.1.6 CD147

CD147 is a novel and interesting target for knockdown in primary T cells as it has been implicated in a variety of physiological and pathological processes. It is upregulated upon T-cell activation, however little is known about its substrates or ligands and whether it directly effects critical cellular processes such as proliferation or upregulation of other molecules in primary T cells. Additionally, previous studies of CD147 knockdown in cancer cell lines show that measurable changes occur in the cell when this molecule's expression is reduced.

A recent study proposes that CD147 is a novel regulator of T-cell transmigration into the CNS in MS. As a regulator of matrix metalloproteinases CD147 is a potential player in the process which allows leukocytes to cross over into the CNS parenchyma. And in fact, increased CD147 levels were characteristic of brain samples from MS subjects, particularly in MS lesion-containing areas (68).

In order explore the role of CD147 in T-cell biology the aim of this study is to establish a physiologically relevant knockdown of CD147 expression in primary T cells. This will entail the application and optimization of relatively new technology for the transfection of primary T cells. After a suitable knockdown is achieved, basic comparisons can be made to a control group that will further elucidate the function of this pleiotropic molecule in the context of T-cell function.

1.2 Literature reviews

1.2.1 Clinical aspects of MS

Multiple sclerosis (MS) is an inflammatory, demyelinating disorder of the central nervous system (CNS) that affects more than two million people worldwide. The clinical presentation of MS may take a variety of different forms. First, relapsing remitting Multiple Sclerosis (RRMS), which is the most frequent, comprises 85-90% of new cases. This form of MS is characterized by attacks or flare-ups of neurological distress. The patient will then experience 100% recovery after an episode. This often progresses into secondary progressive MS (SPMS) where 100% recovery is not achieved and disability persists or worsens between attacks. A rare form of MS comprising a small percent of cases is primary progressive MS (PPMS). Here, the disease takes a steady, continuous decline into disability without the appearance of acute exacerbation or remission (1).

Multiple sclerosis primarily affects women (73% of cases) and primarily those from higher latitudes and of Northern European descent (2). MS is not directly inherited but genetics play an important role in who gets the disease. While the risk of developing MS in the general population is 1/750, the risk rises to 1/40 in anyone who has a close relative with the disease. (3).

Symptoms of MS can include a variety of neurological disorders resulting from damage to the myelin sheath or neuronal fibers of the CNS. Common symptoms include; fatigue, numbness, coordination and balance disorders, bladder and bowel dysfunction, vision problems, pain, dizziness and vertigo, emotional changes, and depression. Less common symptoms are; speech disorders, headache, hearing loss, seizures, tremors and respiratory problems (3). When making a diagnosis of MS some

common disorders to rule out are tumors of the CNS as well as inflammatory diseases such as vasculitis and systemic lupus erythematosus (SLE).

There is no single test for the diagnosis of MS. As there is no single symptom or physical finding that will confirm a case of MS, several strategies must be used to determine if a person meets the criteria for a diagnosis of MS. The criteria for a diagnosis of MS are as follows: 1. Find evidence of damage in at least two separate areas of the CNS. 2. Find evidence that the damage occurred at least one month apart. 3. Rule out all other diagnoses (4). The most common diagnostic test used to confirm MS is magnetic resonance imaging (MRI) which is used to find areas of damage in the CNS. Also a lumbar puncture can be performed to obtain cerebral spinal fluid to test for the presence of elevated immunoglobulins or myelin proteins such as myelin basic protein (MBP) and proteolipid protein (PLP).

The current treatment options for MS are limited to disease-modifying medications. There are 8 drugs approved by the United States Food and Drug Administration and all of these are used to treat relapsing forms of MS; currently no medication is approved for the treatment of primary progressive MS. Half of the medications in this group are analogues of human interferon- β (IFN- β). The method of action of IFN- β is not entirely understood however proposed activities of the molecule include; inhibition of proliferation of leukocytes, reduced antigen presentation, and modulation of cytokine production toward an anti-inflammatory phenotype (5).

Glatiramer acetate (GA; Copaxone®), a random copolymer of glutamic acid, lysine, alanine and tyrosine, emerged almost thirty years ago through experiments in an animal model of MS. Researchers were attempting to induce disease in mice with the core amino acids of myelin basic protein, but developed a mixture of peptides

with varying sequences that still maintained the intended ratios of each amino acid to the others. GA was found to inhibit experimental autoimmune encephalomyelitis (EAE) in rodents and primates instead of induce it (6,7,8,9). Subsequent to multiple clinical trials (10,11,12,13), GA is now an approved immunomodulatory therapy for RRMS.

The most recent drug approved for the treatment of MS is the first in a new class of selective adhesion-molecule inhibitors. Natalizumab® binds to the $\alpha 4$ subunit of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins and blocks binding to their endothelial receptors. Lymphocyte migration across the blood–brain barrier is thought to be an important early step in the formation of lesions (14). The interaction of $\alpha 4\beta 1$ integrin, a protein on the surface of lymphocytes, with vascular-cell adhesion molecule 1 (VCAM-1), which is expressed on the surface of vascular endothelial cells in brain and spinal cord blood vessels, mediates the adhesion and migration of lymphocytes in areas of inflammation (15,16,17,18). Furthermore, the interaction of $\alpha 4\beta 1$ integrin with ligands such as fibronectin (19) and osteopontin (20) modulate the survival, and activation of leukocytes that have gained access to the CNS. In phase-3 clinical trials Natalizumab® reduced the rate of clinical relapse at one year by 68% (21).

1.2.2 Research of MS

Although the etiology of MS is poorly understood, there is considerable evidence for an immune-mediated pathology, such as the presence of T-cell responses to myelin antigens (22) a strong genetic association with certain HLA Class II haplotypes such as HLA-DRB15 as well as polymorphisms in cytokine receptors such as the IL-7 receptor alpha chain (CD127)(23,24,25). Recently evidence was born out in a drug study when the immunosuppressive drug Mitoxantrone was approved as a treatment for RRMS and SPMS (26).

Evidence supporting the involvement of T cells is based upon a vast body of work in an animal model of MS, experimental autoimmune encephalomyelitis (EAE). This well-characterized model can be induced by immunization with myelin sheath proteins such as MBP (27), myelin oligodendrocyte glycoprotein (MOG) (28), or PLP (29,30) or conversely, adoptive transfer of neuroantigen-targeting T cells which can commute disease to new host (31).

Specifically, CD4⁺ Th1/Th17 T cells are believed to be the principle mediators of both EAE and MS, based on several observations, such as: infiltrating CD4⁺ T cells isolated from the CNS of mice with acute EAE producing IL-2 and IFN- γ (32), presence of Th1-inducing cytokines in inflammatory lesions and treatment of mice with Th1-inducing cytokines resulting in aggravation of autoimmune disease (33) and analysis of gene transcripts in chronic MS lesions revealed an increased level of IL-17 transcripts when compared to acute lesions or control tissues from healthy subjects (34).

Additionally, CD8⁺ T cells are also believed to play a role in this autoimmune disease as revealed by MS and EAE studies. For example, CD8⁺ and CD4⁺ T cells were found in demyelinating MS lesions (35). In another study microdissection was used to find CD8⁺ T cells predominating and exhibiting oligoclonal expansion within the lesions (36,37). It has also been previously shown that CD4⁺ and CD8⁺ T-cells responses to neuroantigens could be detected in MS as well as healthy individuals, with slightly higher CD8⁺ T-cell responses to myelin-associated oligodendrocytic basic protein (MOBP) in MS (38). Studies using transgenic and wildtype myelin-specific CD8⁺ T cells have revealed a potential pathogenic or regulatory role in certain models of EAE (39,40,41). In fact in a recent EAE study neuroantigen-specific CD8⁺ T cells were able to suppress the induction of EAE and to inhibit

ongoing EAE. These regulatory cells were able to kill myelin oligodendrocyte glycoprotein (MOG) loaded CD4⁺ T cells as well as CD4-depleted antigen presenting cells (42). In humans CD8⁺ T-cell-mediated suppression is similar between healthy subjects and clinically quiescent treatment-naïve MS patients, but is significantly deficient during acute exacerbation of MS and this suppression recovers post-relapse (43).

Earlier studies have evaluated mainly CNS-specific CD4⁺ T-cell repertoire from both the CNS as well as the periphery, using a combination of different PCR primers to amplify T-cell receptor (TCR) V β segments (37,44,45,46,47), an approach that could suffer from differing reaction efficiencies. Other studies have used antibodies to different V β segments (48), gaining a global overview of TCR usage. In addition, CDR3 spectratyping studies have been used to measure the Gaussian distribution of CDR3 lengths, with (49) or without (50) sequencing of interesting peaks.

1.2.3 The T-cell receptor

The T-cell receptor is a heterodimer consisting of two different polypeptide chains termed alpha (TCR α) and beta (TCR β) (66). The two chains which are linked by a disulfide bond are very similar in structure to the Fab fragment of an immunoglobulin molecule. Both the TCR α and TCR β chains make contact with the antigen binding cleft of the major histocompatibility complex (MHC) when antigen presentation occurs. The TCR α chain is comprised of variable (V α) and joining (J α) segments while the TCR β contains a diversity (D) segment in addition to V β and J β (Figure 1). The center of the TCR which makes contact with the bound antigen in the center of the MHC molecule is termed the complementarity determining region 3 (CDR3). This hypervariable region is composed of D and J segments whereas the

CDR1 and CDR2 loops which make contact with the less-variable parts of the MHC molecule are encoded by the germline V segments. In addition to the diversity offered by the germline gene segments, an enzyme, terminal deoxynucleotidyl transferase (TdT), contributes to the diversity of the TCR by adding non-templated or N-nucleotides which can be inserted into the space between the rearranged V, D and J segments. This allows for a great deal of variability in the final composition of the CDR3 region. Recently second-generation sequencing (which can generate millions of TCR β chain sequences) was used to find over 1 million individual TCRs from a single blood sample (56). This study also found that when comparing donors there was TCR sharing between individuals as high as 14.2% when comparing inferred amino acid sequences, and this high correlation rate was found when individuals shared HLA class I alleles.

1.2.4 TCR α and TCR β

The TCR α and TCR β chains are rearranged separately in sequence and several checkpoints exist in this early stage of development of the T cell while it resides in the thymus. The β -chain gene segments rearrange first. If no functional β chain can be produced from these rearrangements, the cell will not be able to produce a pre-T-cell receptor and will die unless it makes successful rearrangements at both the γ and δ loci. However there is a method by which the cell can be rescued due to the presence of two clusters of D β and J β genes upstream of two C β genes. Once the productive β -chain gene rearrangement is complete the β chain is expressed together with the invariant partner chain pT α and the CD3 molecules. This expression triggers events which halt any further β -chain rearrangement and thus ensure allelic exclusion at the β locus.

Once the β chain has been expressed rearrangement of the α chain can commence. Due to the high number of $V\alpha$ and $J\alpha$ gene segments spread over some 80 kb of DNA many successive rearrangements may take place thereby saving an initially non-productive α -gene rearrangement. This potential for many successive rearrangements at both alleles of the α -chain locus means that virtually all T cells will develop with functional α chains. Moreover, many T cells have in-frame rearrangements on both chromosomes which allows them to produce two productive α chains. And since expression of the TCR itself is not sufficient to shut off α -chain rearrangement, continued rearrangements occur even after the expression of the TCR at the cell surface. This process continues as the developing T cell is tested for self-peptide:self-MHC recognition in partnership with the same β chain. This process ceases only when positive selection occurs as a consequence of receptor engagement (66).

So, in a strict sense, allelic exclusion is imperfect for the α chain. Although receptor engagement ensures that only the one positively-selected receptor will be the functioning receptor, the T cell could very well express two α chains. This is why the focus of TCR sequencing clonality studies are restricted to the use of the β chain.

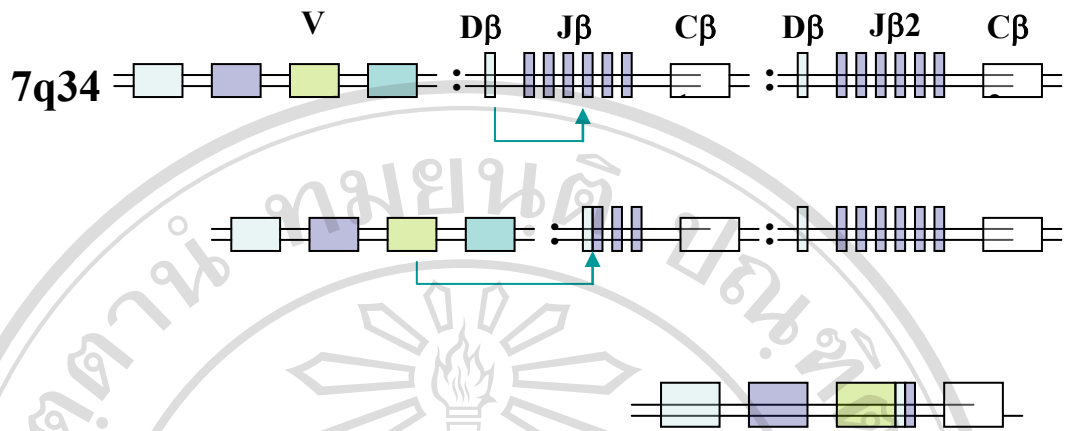


Figure 1. TCR β gene segment rearrangement.

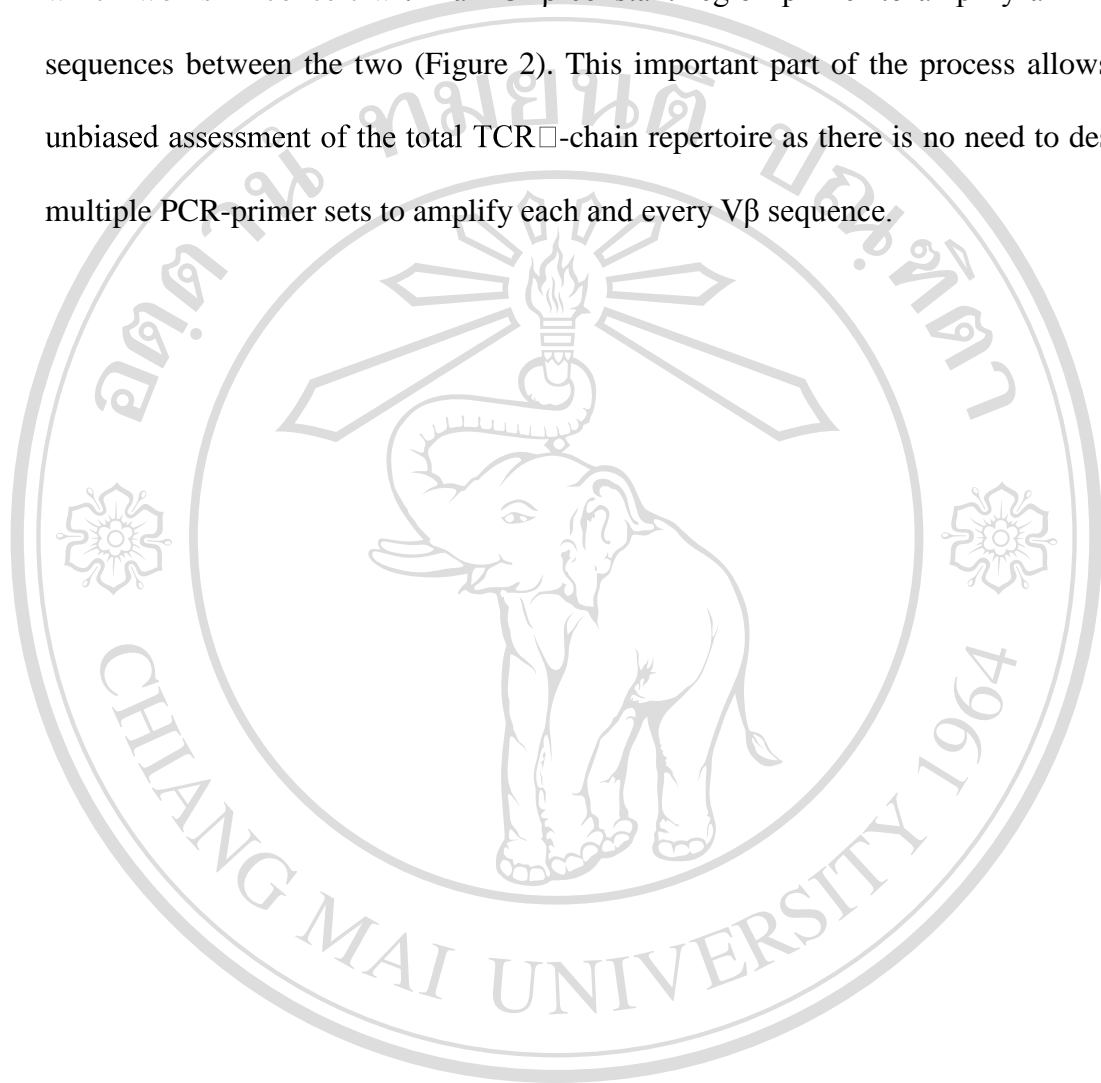
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1.2.5 SMART-RACE

Switching Mechanism At the 5' end of the RNA Transcript for Rapid Amplification of cDNA Ends (SMART-RACE)[™] is a PCR-based method for producing cDNA that overcomes the problems of truncated cDNA because the reverse-transcriptase (RT) cannot transcribe the entire mRNA sequence (121). SMART-PCR amplification of cDNA begins with conversion of mRNA to cDNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT), mutated in the RNase H domain, in the first strand synthesis reaction (Figure 2). This enzyme possesses RT activity, terminal deoxynucleotidyl transferase (TdT) activity, and DNA-dependent DNA polymerase activity. During first strand synthesis, an anchored oligo-dT primer (3' cDNA synthesis primer) anneals to the 5'-end of poly(A) tails. The 5'-end of this 3' cDNA synthesis primer contains a defined sequence that serves as a PCR primer target site in the subsequent amplification step. When the first strand has been extended to the end of an mRNA template, the TdT activity of the reverse transcriptase adds several un-templated deoxycytosine (d(C)) residues to the 3'-end of first strand cDNAs. The 5'cDNA synthesis primer contains three guanine residues at its 3' end that anneal to these un-templated d(C) "tails" and then serve as a template for the DNA-dependent DNA polymerase activity of the MMLV-RT. This Switching Mechanism allows for MMLV-RT-mediated 3'-extension of all first strand cDNA products to include a defined, contiguous sequence copied from the 5'cDNA synthesis primer. The terminal transferase and template switching activities occur during the first strand cDNA synthesis. Regions of sequence in the 5'cDNA synthesis primer and the 3'cDNA synthesis primer are identical, allowing for uniform amplification of all first strand cDNAs with a single PCR primer (120,122).

The TCR-sequencing protocol takes advantage of this reaction by using the defined, contiguous sequence in the second round of PCR as a universal primer site which works in concert with a TCR β -constant region primer to amplify all TCR β sequences between the two (Figure 2). This important part of the process allows an unbiased assessment of the total TCR α -chain repertoire as there is no need to design multiple PCR-primer sets to amplify each and every V β sequence.



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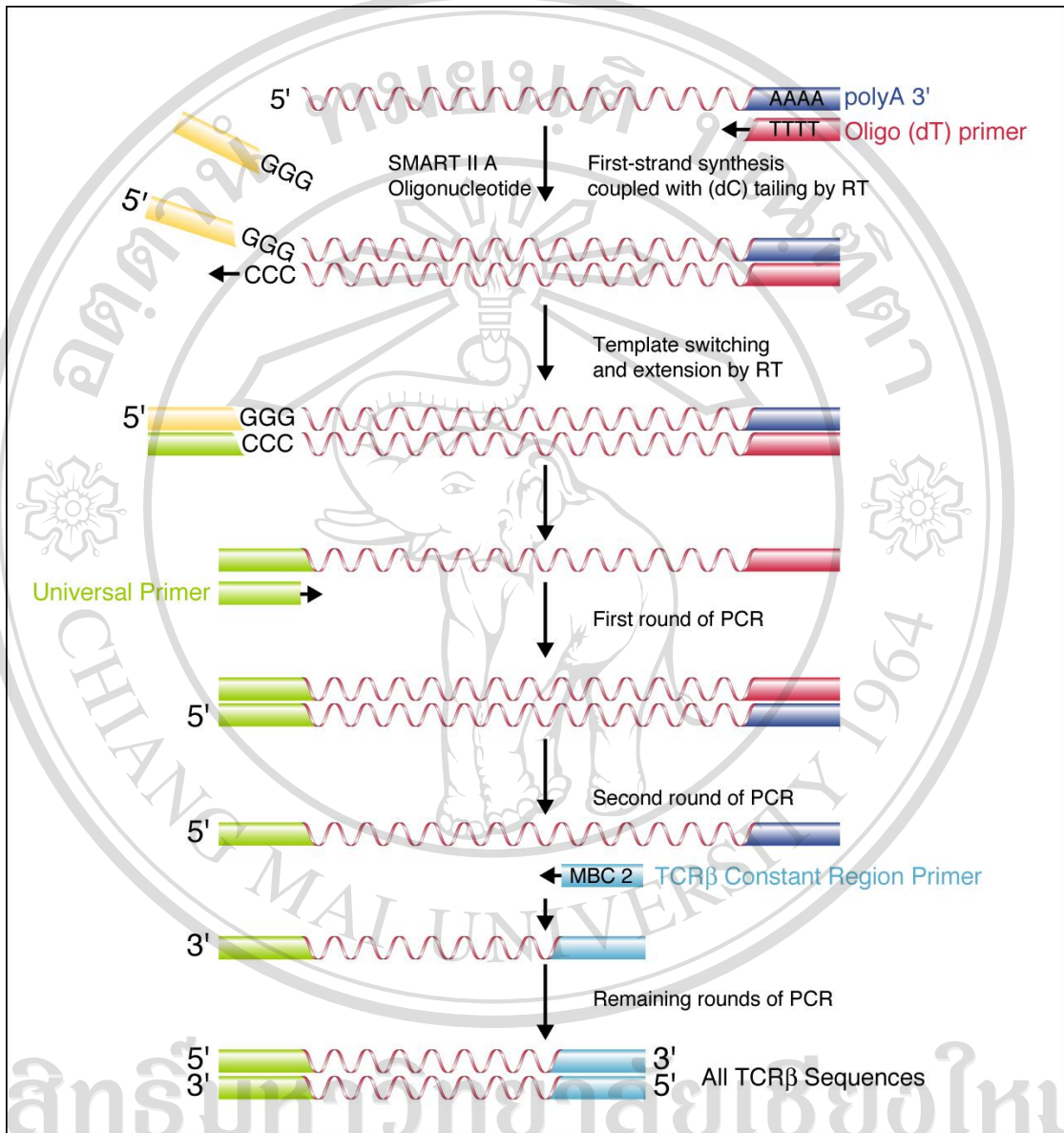


Figure 2. SMART-RACE approach to TCR analysis.

Figure Clontech

1.2.6 Background of the CD147 surface molecule

CD147 is a 35–60 kDa type 1 integral plasma membrane glycoprotein that belongs to the Immunoglobulin superfamily (69). In humans it is also known as M6 antigen (98), extracellular matrix metalloproteinase inducer (EMMPRIN) (71), and human basigin (BSG) (72). Homologues to CD147 exist in other species such as; the rat protein OX-47/CE9 (73), the chicken blood-brain barrier-related molecule neurothelin (74) and the mouse protein basigin/gp42 (72,75). The human CD147 molecule is 248 - 269 amino acids long and contains two immunoglobulin-like (Ig) domains. Recently a form has been characterized that contains another membrane-distal Ig-like domain (76). CD147 shows remarkable variation in size because of heterogeneous N-glycosylation occurring at three separate sites, two on the membrane proximal Ig domain and one on the membrane distal domain (77).

The transmembrane region of CD147 is 21- 24 residues long and is comprised of mostly hydrophobic amino acids save for one charged residue. This fact is interesting because a single charged residue is highly energetically unfavorable and is not normally found in proteins spanning the membrane only once. This structural feature suggests that CD147 forms a complex with other membrane proteins thereby shielding the charge in an energetically stable state (78). Similar to the leucine zipper motif, 3 leucines are repeated every seventh amino acid residue in the CD147 transmembrane domain and this domain is highly conserved between species, (96% between mouse and human or chicken) (73,79,80). CD147 is expressed on a variety of cell types, including: haematopoietic, epithelial, endothelial cells and leukocytes.

CD147 was first discovered as a factor that stimulates the production of the collagenase matrix metalloproteinase type 1, (MMP-1) by fibroblasts (81). Turnover

of extracellular matrix (ECM) is mediated by MMP's (a family of enzymes capable of degrading almost all ECM proteins). Subsequently CD147 was found to influence induction of other MMPs, including MMP-2 (82), MMP-3 (83), MMP-9 (84) and membrane type 1 (MT)1-MMP and (MT)2-MMP (85). The intracellular pathways by which CD147 induces MMPs are not yet clear (86).

1.2.7 CD147, MMPs and Cancer

The fact that CD147 stimulates fibroblasts to secrete these matrix-digesting enzymes has become very interesting when researched with regard to tumors. Peritumoral fibroblasts will secrete more MMP's in response to high levels of CD147 located on the surface of the tumor cells. The degradation of surrounding matrix is an important event in the metastatic evolution of tumors. CD147 has been found to be an important wide-range modulator of tumor-stroma cross-talk, based on the finding that it mediates MMP production and angiogenesis via stimulation of vascular endothelial growth factor (VEGF)(87) and CD 147 was found to be the most frequently upregulated mRNA and protein in micrometastatic cells from the bone marrow of cancer patients (88). Additionally CD147 expression correlates with tumor progression of gliomas (89), ovarian carcinoma (90) and melanoma (91). And in the case of breast carcinoma, positive CD147 expression correlated with decreased tumor-specific survival (92).

1.2.8 CD147-associated proteins

The presence of a single charged residue residing in the transmembrane domain of CD147 gives an indication that CD147 is a good candidate for protein-protein interaction. Studies with a CD2-CD147 chimera, as well as site directed mutagenesis studies have shown that the transmembrane domain is critical for protein-protein interactions within the plasma membrane (93). In fact, CD147 has

been shown to associate with itself. Yoshida et al. proved that CD147 forms homo-oligomers on the plasma membrane using their N-terminal Ig-like domains (94).

Monocarboxylic acid transporters (MCT) are responsible for exporting the byproducts of cellular metabolism (lactate) out of the cell. Several studies have shown an association between CD147 and MCT1, MCT3 and MCT4. In fact knockdown of CD147 expression via siRNA resulted in a reduction of MCT trafficking to the plasma membrane (95) and siRNA-mediated knockdown of MCT4 expression impaired the trafficking of CD147 to the cell surface resulting in CD147 accumulating in the endoplasmic reticulum (96). The CD147 molecule was found to co-precipitate with $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins and to co-localize with these integrins in areas of cell contact (97). Integrins are a superfamily of membrane proteins that mediate attachment and communication of a cell with its immediate external environment. Chief amongst their known functions is modification of cell structure via focal adhesion complexes responding to contact with the ECM.

It has also been demonstrated that some CD147 monoclonal antibodies (mAbs) induce homotypic aggregation of U937 cells (98). Induction of U937 cell aggregation is an LFA-1/ICAM-1-dependent pathway and involves the activation of protein kinases and reorganization of the cytoskeleton (98, 99)

1.2.9 CD147 and the cytoskeleton

Hepatocellular Carcinoma cells transfected with CD147siRNA showed significantly reduced expression of vinculin and focal adhesion kinase (FAK). Vinculin is membrane-cytoskeletal protein in focal adhesion plaques involved in linking integrins to the actin cytoskeleton. FAK is a tyrosine kinase recruited at focal adhesions/integrin receptor clusters and is important for cell migration and differentiation events associated with cell-to-cell and cell-to-ECM contacts, as well as

being a regulator of integrin-dependent MMP-2 and MMP-9 expression and release by T-cell lymphoma cells (101).

1.2.10 CD147 and cyclophilins

CD147 has been shown to associate with cyclophilins in both the intracellular and extracellular environment. Cyclophilins are a ubiquitously expressed family of proteins that have been shown to have chemotactic properties for many cells of the immune system including; neutrophils (102,103,104,105) eosinophils and monocytes (103,105) and T cells (105,106). In each of these studies the chemotactic effects are abrogated by the addition of anti-CD147 antibody or a factor which sequesters cyclophilins such as cyclosporine. Cyclophilins are also expressed inside the cell and have been shown to co-localize with CD147 inside the cell (107). Treatment with Cyclosporine, which binds cyclophilins, knockdown of cyclophilin 60, and altering a residue in the transmembrane region of CD147 all diminished transport of CD147 to the plasma membrane without reducing the total level of CD147 expression (108). Cyclosporine itself is a potent immunosuppressive drug that binds with cyclophilin A to form a complex that blocks dephosphorylation of nuclear factor of activated T cells (NF-AT) by calcineurin. NF-AT is therefore not able to translocate to the nucleus and act as a transcription factor.

1.2.11 CD147 and TGF- β

CD147 expression is linked to a critical aspect of tissue remodeling – fibroblast to myofibroblast differentiation. Myofibroblasts secrete MMPs and share many features with peritumoral stroma. In experiments conducted by Huet et al. knock down of CD147, or culture with anti-TGF- β inhibits this process, suggesting a relationship between the two (109).

1.2.12 CD147 and T cells

CD147 is expressed on T cells (123) and is upregulated upon activation of the T cell. This upregulation occurs whether the T cell is activated through its TCR or by a super-agonist such as PHA. Monoclonal antibodies specific for certain epitopes on CD147 strongly inhibit proliferation induced by anti-CD3 mAb (110,111). In a recent study using the Jurkat T Lymphoma cell line, one group reports decreased proliferation, decreased adhesion to fibronectin, cell cycle arrest, and reduced trans-endothelial migration when CD147 surface expression was knocked down with RNAi technology (112). However, contradictory to this report, a more detailed analysis by another group finds that indeed CD147 contributes to the negative regulation of T-cell responses as the knockdown of CD147 in Jurkat cells promotes higher levels of NF-AT stimulation and Pak1 phosphorylation upon T-cell activation (124). Also another study reports the mean fluorescence intensity (MFI) of CD147 flow staining, CD3+ T lymphocytes is higher in SLE patients than healthy controls. And in contrast to Healthy Donor cells, the T cells from the SLE patients showed a reduction in tyrosine phosphorylation levels when stimulated with; anti-CD3, anti-CD28 and anti-CD147 monoclonal antibodies (113). Additionally monoclonal antibodies to CD147 induced aggregation and adhesion of Jurkat T cells and capping of CD147 to cell contact zones (114).

Finally a study found that antibody targeted to CD147 induced a displacement of the GPI-anchored coreceptors CD48 and CD59 from microdomains in human T lymphocytes. This caused a disruption of the immunological synapse and reduced capping on T cells. This was accompanied by a selective inhibition of TCR-mediated T cell proliferation (115).

1.2.13 Principle of siRNA mediated knockdown

Experimental RNA interference involves the introduction of short RNA sequences, typically 21-23 bp duplexes with 3' dinucleotide overhangs into a target cell. When long double-stranded RNA is introduced into the cell an enzyme called Dicer recognizes the RNA duplex and cleaves it into shorter fragments. These fragments then associate with a ribonucleic protein complex called RISC (RNA induced silencing complex). When synthetically produced siRNA is used the sequences are already short enough to incorporate directly into the RISC complex. The RISC complex facilitates the unwinding of the double strand siRNA and then the binding to a target RNA sequence. Slicer, which is included in the RISC complex then cleaves the target RNA in the middle of the region of complementarity. This leads to the mRNA's destruction and ultimately the silencing of the gene from which the mRNA was transcribed.

Of the available methods for RNA interference, siRNA was chosen because it is the most expedient method for optimizing gene knockdown. Many parameters must be optimized to obtain meaningful data from a gene knockdown experiment. While much time and money was spent repeating experiments changing experimental conditions such as; stimulation agent, dosage and timing, cell source and concentration, siRNA concentration and finally staining profiles and timing, the usage of synthetic, validated siRNA sequences provided the shortest path to selecting the proper sequences that would give a physiologically relevant gene knockdown.

1.3 Objectives

This thesis is composed of two projects as follows:

Project I: Clonal distribution of neuroantigen-specific CD8⁺ and CD4⁺ T cells in multiple sclerosis

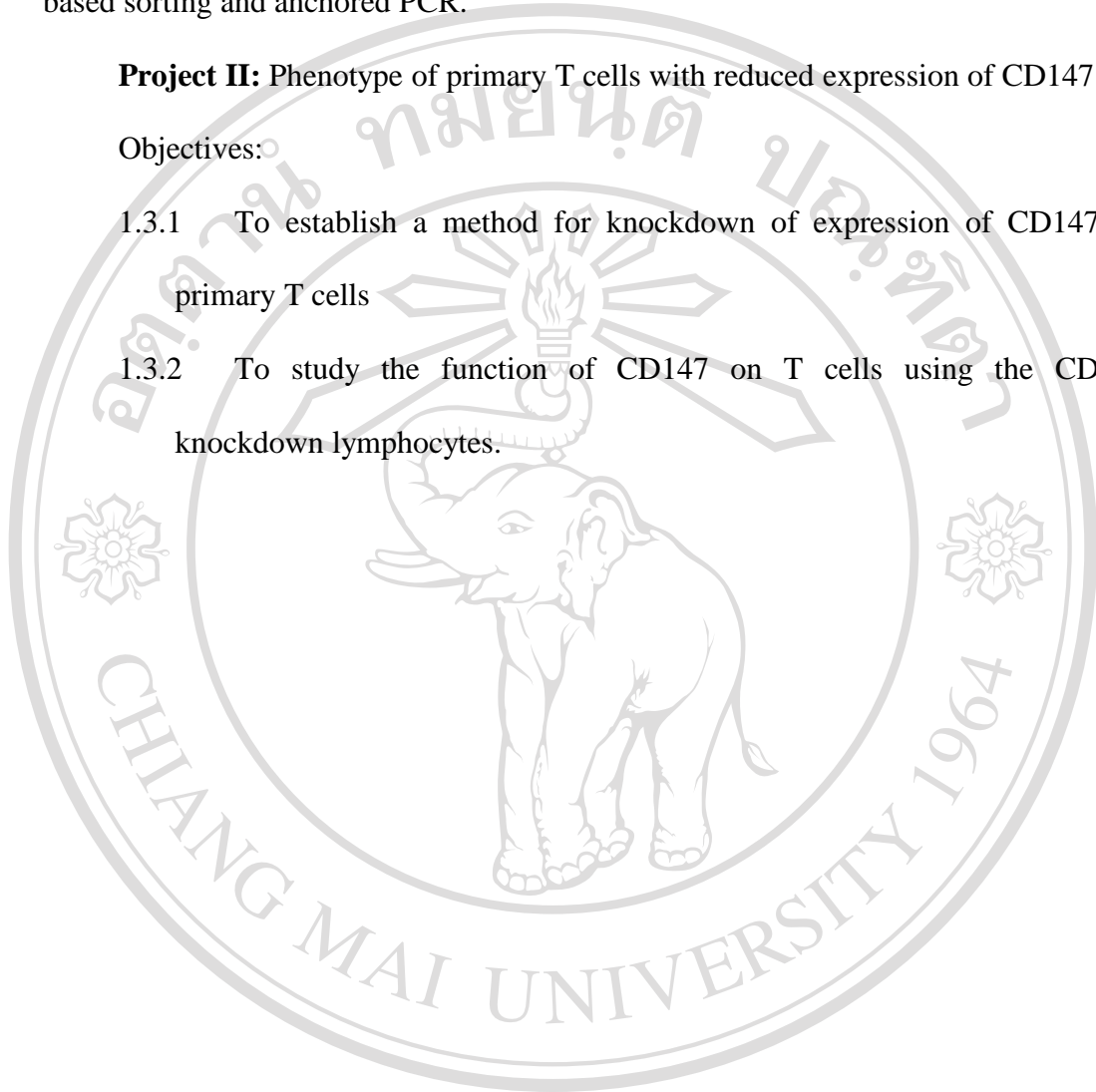
Objective:

To evaluate the clonal distribution of neuroantigen-specific T cells from RRMS patients and healthy donors using a novel combination of short-term culture, CFSE-based sorting and anchored PCR.

Project II: Phenotype of primary T cells with reduced expression of CD147

Objectives:

- 1.3.1 To establish a method for knockdown of expression of CD147 on primary T cells
- 1.3.2 To study the function of CD147 on T cells using the CD147 knockdown lymphocytes.



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