CHAPTER 2

MATERIALS AND METHODS

2.1 Relapsing remitting multiple sclerosis patients and healthy subjects

MS patients and healthy controls (HC) were recruited at the UT Southwestern Medical Center and peripheral blood mononuclear cells (PBMC) were obtained by performing leukepheresis under approved IRB protocol. At the time of donation, MS patients had not received steroid treatment in the preceding three months and had never received interferon-β, glatiramer acetate or any disease-modifying immunomodulatory therapy. All MS patients were clinically defined as RRMS and were in remission at the time of leukapheresis. The age (in years) and gender distribution was as follows – MS patients: M584-41/F; M971-47/F; M210-47/F; M250-48/F and healthy controls: H267-48/F; H548-32/M; H333-53/F; H504-36/F. Ficoll-Paque Plus (Amersham Biosciences) separation was used in order to obtain PBMC, which were immediately cryopreserved on the day of collection (51).

2.2 Myelin antigens

Because of the diverse specificity of MS immune responses, PBMC were stimulated *in vitro* using pools of serial 15-mer peptides (overlapping by 10) spanning two entire putative MS antigens (human sequences); myelin basic protein (MBP) and proteolipid protein (PLP). No single pool contained more than 45 peptides, so for the larger peptide PLP, 2 pools were created containing a total of 54 peptides. And MBP was used in a single pool containing 43 peptides. The final concentration of each individual peptide was 2 mg/10^6 cells in all experiments described. Peptides were

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dissolved in dimethyl sulfoxide (DMSO), such that cultures contained less than 1 μl DMSO/ml of media as described before (38).

2.3 CFSE-based flow sorting

MBP and PLP-specific CD4+ and CD8+ T cells were sorted from CFSE-stained PBMC cultures, as described (38, 51). Briefly, PBMC were first suspended at 1×10^6/ml in phosphate-buffered saline (PBS) and incubated at 37°C for 7 mins. with 0.25 μM CFSE. Following addition of serum and two PBS washes, cells were resuspended at 2×10^6/ml in H5 media (RPMI 1640 supplemented with glutamine, 5% human AB serum, penicillin and streptomycin) and cultured in 15-30 ml of media in T25 or T75 flasks (BD Biosciences) with MBP or PLP peptide pools at 10 μg/ml (per 15-mer peptide). On day 7, cells were washed and stained with fluorescently tagged anti-CD4 and anti-CD8 antibodies and sorted by electronic gating into CFSE low (antigen responding) and CFSE high (non-responding), CD4+ and CD8+ T-cell populations using a BD FACSVantage SE sorter. On populations with adequate yields (>200,000 cells), a "post-sort" run was performed revealing >95% purity. Sorted cells were collected in 1.5 ml Sarstedt tubes, pelleted and frozen at –80°C in RNAlater (Ambion, Austin, TX) for subsequent molecular analyses. This technique acquires a highly enriched population of antigen-specific, HLA-restricted CD4+ and CD8+ T-cells (38).

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2.4 Evaluation of TCR repertoire

A detailed evaluation of the clonal repertoire was performed on each sorted antigen-specific T-cell population using an anchored PCR approach (52, 53) thus allowing for the characterization of endogenous levels of TCR Vβ usage. Total RNA was isolated (RNAEasy kit, Qiagen, Valencia, CA) and split evenly to make two

pools of cDNA. First a normal cDNA is made (Ready-to-go T-primed first strand kit, GE Healthcare) and then this product is used to check the RNA extraction by performing a β-actin and GAP-DH PCR. The remaining portion is used for anchored RT-PCR using a modified version of the Switching Mechanism at 5"end of RNA Transcript procedure (SMART Race cDNA Amplification Kit, BD Clonetech). A TCRβ constant region 3' primer for the PCR was used to obtain TCRβ PCR products from the 5' end to the start of the TCRβ constant region.

After the a purification step the PCR product was ligated into the pGEMT Easy vector (Promega, Madison, WI) and used to transform *Escherichia coli* (Max Efficiency DH5α, Invitrogen). White colonies were selected, amplified by PCR with M13 primers, and sequenced using the ABI BigDye Terminator V3.1 Cycle Sequencing Kit and sequenced on an ABI 3300 sequencer (ABI, Foster City, CA). Sequences were translated and then defined using the nomenclature from the International ImMunoGeneTics information website (55).

2.5 Data analysis

T-cell clonality was assessed by evaluating unique TCR sequences represented in the populations [representation of a single clone at >10% was considered significant, as described previously (53)]. Prism 5.0c students' t- test was used to compare the overall distribution of TCR clones between the different groups.

2.6 Subjects for CD147 study

Healthy normal subjects were recruited at the Biomedical Technology Research Center, Faculty of Associated Medical Sciences, Chiang Mai University and University of Texas Southwestern Medical Center Campus. All subjects were between the ages of 25 and 39.

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2.7 Cell cultures

Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-hypaque density gradient separation. The isolated PBMC fraction was suspended at $2x10^6$/ml in complete RPMI (RPMI 1640 supplemented with glutamine, 10% fetal calf serum, penicillin and streptomycin) or in H5 media (RPMI 1640 supplemented with glutamine, 5% human AB serum, penicillin and streptomycin). Jurkat T-cell lymphoma cultures were maintained in Dulbecco's Modified Eagle Medium supplemented 10% fetal calf serum in a CO_2 incubator at 37^oC.

2.8 Activation methods

Cultures were stimulated with several stimuli, including soluble anti-CD3 mAb clone OKT3 at 1µg/ml, plate-bound anti-CD3 mAb clone OKT3 and soluble anti-CD28 mAb, solution of 10 µg/ml OKT3 mAb and incubated for 3 hours, washed and then anti-CD28 mAb was added, SEB at 1µg/ml, PMA at 50ng/ml, or PHA at 3-5 µg/ml.

2.9 siRNA for knockdown CD147

Two pre-designed siRNAs targeting the coding region of the BSG (CD147) gene (Ambion Silencer siRNA ID# 215973 and 10732) were used in this study. The sequences are as follows: #10732 – 5’>3’ GGUUCUUCGUGAGUUCCUCtt , #215973 – 5’>3’ GCUACACAUUGAGAACCUGtt. Both sequences target the C2 immunoglobulin domain of the CD147 molecule.

Silencer Select negative control siRNA #1 (cat #4390843) was used as a control siRNA targeting no known human genes.

2.10 Transfection

Each Nucleofection reaction contains 3-5 $x10^6$ cells, 2 µg pmaxGFP vector or 30-300 nM siRNA (3-30 pmol/sample), 100 µl Human T-cell Nucleofector solution

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that has been supplemented with the included supplement solution. Six or 12-well plates were prepared by filling with 1.5-3.0 ml of culture media which were placed in a cell-culture incubator to equilibrate to 37°C. Cells were then soft-spun at 90xg for 10 minutes at room temperature and the supernatant was aspirated off until no residual media remained. The pellet was then resuspended in 100 µl room temperature Nucleofector solution per sample, or PBS in the case of control samples. Samples were not maintained in the Nucleofector solution for longer than 20 minutes total. The suspension was then combined with siRNA, Pmax GFP, or PBS alone. The cell suspension was then transferred to a Nucleofector cuvette, covered, tapped and placed in the machine. The samples were then transfected using protocols U-014 or V-024 for unactivated T cells or X001 for the Jurkat cell line. Once the transfection was complete 500 µl of the pre-equilibrated culture media was added to the cuvette and then redrawn with the sample to add to the culture plate.

2.11 Staining timing

Knowing that siRNA-mediated gene knockdown is transient, the selection of time-points was crucial to view the gene silencing as it runs its course. The Nucleofection protocol suggests a check of GFP 24 hours after the transfection. After the transfection was verified by GFP expression, the staining of CD147 can begin as soon as 36 hours after transfection. After the initial check, staining commenced along an ordered time line that stretched one week. For optimization, checks were made at 24 and/or 48 hour intervals until the best possible protocol was selected. While getting data on days 3, 5, and 7 was my goal, it was simply not possible due to limited cell numbers. The fact that the optimized number of cells per reaction was $4x10^6$ made it almost impossible to have enough cells alive by day 7 and still have a manageable transfection experiment that was also cost-effective.

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2.12 Activation timing

Two possible timing strategies are available for the gene-silencing protocol; activate the culture and then transfect, or transfect and then activate. Each method has its benefits and drawbacks. In this study, both were tested.

Traditional thought on transfection would lead one to believe that activation and then transfection is the best strategy. With chemical based transfection methods the cells need to be dividing for the oligomer to successfully enter the nucleus. The general outlook on the topic is that a dividing cell is more apt to uptake an oligomer. The drawback is that transfection is very traumatic for the primary cell. The substantial shock of superantigen activation followed by electroporation can and will cause a significant amount of apoptosis. Also, this sequence mandates a wash step after activation as the transfected cells need to be washed to remove as much of the transfection reagents as possible. This washes out your soluble activation agent and may necessitate a re-activation, which puts even more stress on the cells. The Nucleofector literature claims that it's proprietary process will deliver the transfected oligomer into the nucleus whether the cell is activated or resting.

The other approach is to transfect the unstimulated cell culture and allow it to rest for 6-24 hours before putting any additional stress on the cells. This is followed by a wash and then soluble or bound antigen can be used to stimulate the cells. The benefit of this method is that it is less traumatic for the cells. Also the siRNA has time to traffic to the nucleus before the cellular machinery activates in response to stimulation. If turnover of the molecule of interest is 12-24 hours, and the cell has already upregulated expression following a stimulation, there will be that much more of the molecule expressed that needs to be reduced to observe significant

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physiological changes. The drawback of this method is the possibility that the resting cells will not take up enough of the oligomer to establish a significant knockdown.

After trying both methods I finally decided on transfection and then activation. The result of activating the cells before transfection was reduced viability and generally unusable results.

2.13 Flow Cytometry

Cultures were washed 2 times in FACS buffer (PBS containing 1% BSA and 0.1% Sodium azide) by centrifugation at 1400 RPM for 10 minutes at 4° C. After the supernatant was decanted cell pellets were disturbed and fluorochrome-conjugated antibodies were added to tubes. Tubes were then incubated for 30 minutes at 4° C in the dark. After incubation the wash procedure was repeated and finally the samples were resuspended in 1% paraformaldehyde (PFA) to fix the stain until they could be run on the flow cytometer.

2.14 CFSE-based proliferation assay

This methodology detects dividing cells by using the green fluorescent dye 5-(and 6)-carboxyfluorescien diacetate succinimidyl ester (CFSE) (116). Dividing cells are detected by sequential halving of their fluorescence and can be immunophenotyped with cell surface markers. Thus, PBMC were first suspended at $1x10^6$/ml in PBS and incubated at 37°C for 7 minutes with 0.25 µM CFSE. Following addition of serum and two PBS washes, cells were suspended at $2x10^6$/ml in human media (H5) (RPMI 1640 supplemented with glutamine, 5% human AB serum, penicillin, and streptomycin).

2.15 Intracellular cytokine staining

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Cytokine flow cytometry assays for the detection of antigen-specific IFN-γ, IL-10 and IL-4 responses were performed as described previously (51, 117). PBMC cultures in 5% human media (H5) (RPMI 1640 supplemented with glutamine, 5% human AB serum, penicillin and streptomycin) were cultured for five hours in the presence of Brefeldin A (BFA - Sigma-Aldrich, St Louis, MO) in 5 ml polypropylene FACS tubes. At the end of the culture period they were washed with FACS buffer (PBS containing 1% BSA and 0.1% Sodium azide) for 10 minutes at 1400 RPM. The cells were re-suspended in volume of 100 μl and stained with fluorescently tagged antibodies for 30 minutes. The surface stain was washed off with FACS buffer and followed by permeabilization for 10 minutes with permeabilization buffer (30% Tween 20 in FACS Lyse- Becton Dickinson, (BD) Franklin Lakes, NJ). After two washes, intracellular staining was performed with and incubation time of 30 minutes at 4° C in the dark. Cells were washed again and fixed in 1% Paraformaldehyde solution.

2.16 Cytometric Bead Array

Cytometric bead array (CBA) employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes from a single sample (Becton Dickinson, (BD) Franklin Lakes, NJ). The CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes with a particle-based immunoassay. The specific capture beads are mixed with the phycoerythrin (PE) conjugated detection antibodies and then incubated with recombinant protein standards or test samples to form sandwich completes. The samples and standards are then run on a flow cytometer.

As per the manufacturers protocol the procedure is as follows. Lyophilized standards must first be reconstituted with assay diluent and then set up in a serial

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dilution in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. Following this the 50 µl of mixed capture beads were added to the empty assay tubes and the human cytokine standards were added to the control tubes. The unknown samples were then added to the appropriate assay tubes and 50 µl of PE detection reagent was added to every assay tube. The tubes were then incubated for 3 hours at room temperature in the dark. The samples were then washed once with 300 µl of wash buffer and after the supernatant was aspirated they were resuspended in another 300 µl of wash buffer. Samples were then acquired on a BD LSRII flow cytometer and analyzed using FCAP array software (BD).

2.17 Flow cytometric data acquisition

Flow cytometric data was acquired on a 4-Laser, 17-color custom BD LSRII using FACSDiva software. At least 25,000 events were acquired in most experiments. Higher numbers of collected events would have been preferable but this is one of the limitations of the transfection reactions. Generally this amount still allowed to visualize, gate and display populations clearly. Linear uncompensated data was then transferred as FCS 3.0 files and analyzed after compensation and transformation using FlowJo version 6.4.1 (TreeStar, San Carlos, CA), as recommended (118, 119). FCS 3.0 as opposed to FCS 2.0 is a newer method of storing data where even the data in the negative axis are stored along with the relevant uncompensated data files. This allows recompensation and transformation by the analyzing program over and above the compensation of the data acquiring machine. Recompensation and transformation at the time of analysis is essential to avoid poly color flow cytometry (PFC) associated artifacts.

2.18 Flow cytometric data display – bi-exponential and transformation

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Previously used flow cytometric dot plots suffered from a significant draw back in display in that it was not possible to visualize cell populations lying below the axis. Even thought it is not possible for a cell to express negative amounts of a marker, the flow cytometric graph can show such an artifact. This artifact arises due to the inherent errors and limitations of flow cytometers. The error in calculating the position of a cell can be either above or below the axis. Bi-exponential display is a newer method of display available in recent versions of FlowJo and FACS DIVA where the X and Y axes start in the negative range below zero. This display allows one to visualize even cells lying below the axis. This display allows us to accurately compensate since the whole population and its mean can be visualized. This method of display was used in most of my graphs.

Transformation is another technique used to overcome data display errors that arise to problems inherent in flow cytometry. It is known in flow cytometry that data spread occurs in certain channels and dot plots as one goes away from the zero on the log display scale. This is because there is a greater spread of data at higher values compared to lower values again due to inherent limitations of the data collected by a flow cytometer. This data spread can result in cell populations being buried below the axis. This can lead to errors in drawing the negative/control gates during analysis besides resulting in very few cells on the actual display plot. Transformation can compress these populations for better visualization by compressing the display axis. Both bi-exponential display and transformation do not change the actual proportion of cells in any gate or any other parameter of data; they just result in a more aesthetic display.

We have also used color coded contour plots ("Pseudo color plots") to display my data in all of my flow cytometric plots. Color contour plots are a combination of

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black and white dot plots and contour plots. They are used to overcome the limitations in dot plots and contour plots. In dot plots, the density of cell populations cannot be visualized so one cell on the plot could potentially represent any number of cells. The limitation of contour plots is that outlying populations cannot be visualized so small populations like cytokine secreting populations can be missed. Pseudo color plots overcome these limitations by displaying both information in a single plot.

2.19 Multi-color flow cytometry

Flow cytometry using more that 6 colors constitutes polycolor flow cytometry. It is different from flow cytometry using less than 6 colors in that it is more complicated and presents new problems and challenges. More controls were needed to ensure results obtained are not artifactual. 5-8 color phenotyping panels were set up using different combinations of CD4-PECY5, IL-4-PECY7, CD8-Pacific Blue/, CD147-PE (e bioscience), CD25-APC-CY7, IFNγ-Alexa 700, IL-10-APC, and CFSE in the FITC channel (all antibodies from BD Biosciences unless indicated otherwise). Same concentrations of antibodies were used to ensure comparability in different experiments.

In order to arrive at the appropriate staining combinations and panels, the following general guidelines were adhered to. The number of antigens and conjugates used were kept to the minimum necessary to answer the question posed to avoid the complications of PFC. The lasers and the fluorochromes detected by the machine are first taken in consideration. Then the conjugates available for all the desired antigens are also noted down. The next problem is to how to select various antigens for different conjugates. As a rule of thumb, high density common antigens like CD3, CD4, CD8 etc were used in rare colors like pacific blue, Alexa 700 etc. Important antigens in my experiment like CD25, CD4 and CD8 were measured off of different

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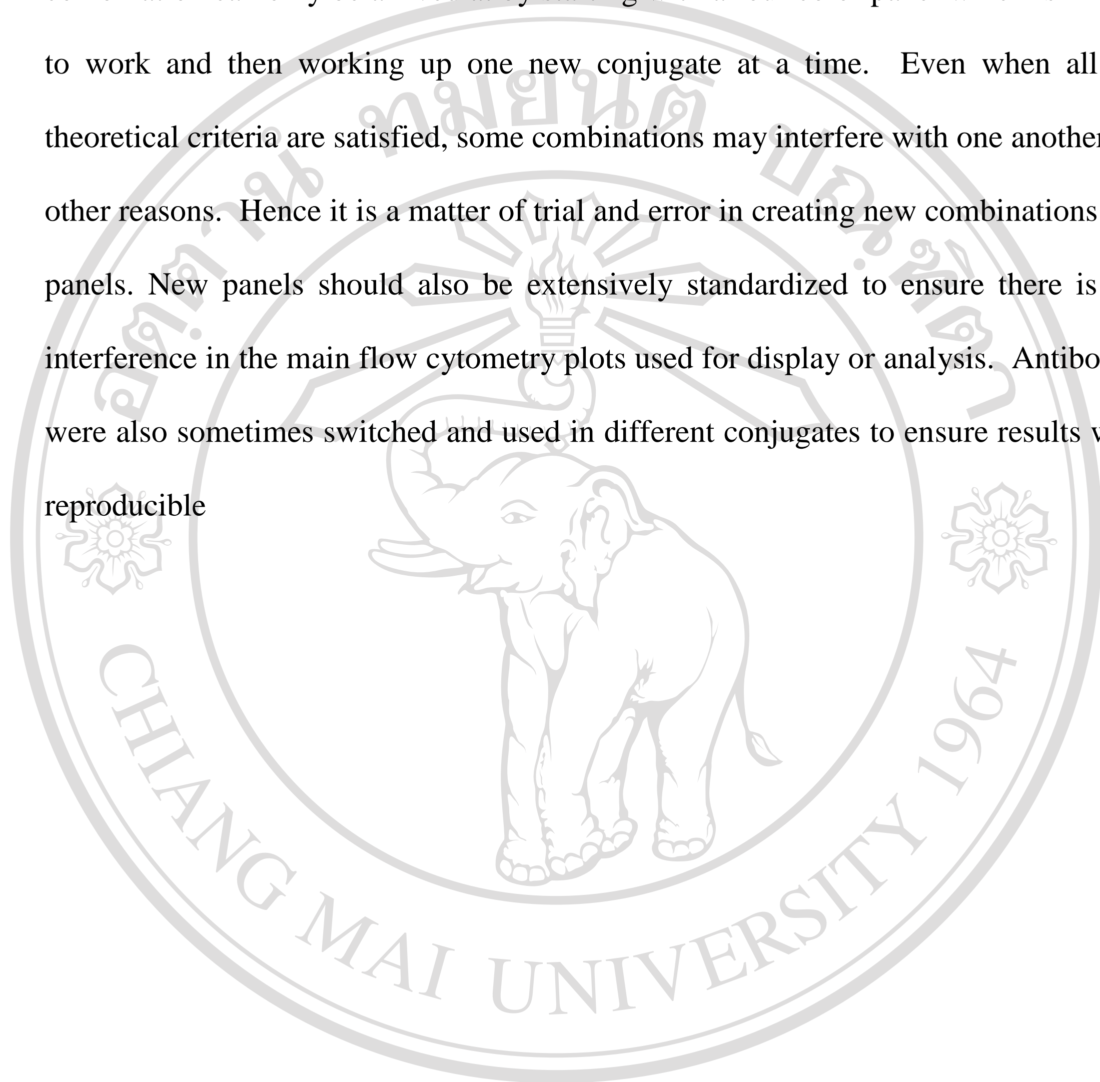
lasers to minimize interference. In the case of CD147 there was no choice as that marker is currently available in limited supply, namely FITC or PE. The correct combination can only be arrived at by starting with a four color panel which is known to work and then working up one new conjugate at a time. Even when all the theoretical criteria are satisfied, some combinations may interfere with one another for other reasons. Hence it is a matter of trial and error in creating new combinations and panels. New panels should also be extensively standardized to ensure there is not interference in the main flow cytometry plots used for display or analysis. Antibodies were also sometimes switched and used in different conjugates to ensure results were reproducible .



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