CHAPTER 3

RESULTS

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

3.1 Clonal dominance within MBP-specific CD8+ T cells in healthy subjects but not MS patients.

In this study, the myelin-specific CD4+ and CD8+ T-cell TCR repertoire in PBMC specimens from MS patients and healthy subjects were evaluated. As described in prior studies (53,38,51) we combined flow sorting and CFSE-labeled PBMC in order to obtain a high yield of antigen-specific T cells. In conjunction with a short term in vitro culture (7 days) and myelin antigen stimulation, we were successfully able to obtain myelin-specific CD4+ and CD8+ T cells (38,52). For molecular analysis of the TCR repertoire, we utilized a constant pair of primers for the anchor and TCRβ constant region in order to amplify the complete TCR in a given population of cells (52,53). This method allowed us to circumvent the use of multiple primer pairs and possible differences in amplification efficiencies, thus allowing us to more accurately assess the TCR distribution on a sorted T-cell population.

Individual TCR repertoire analysis of MBP-specific CD4+ and CD8+ T-cell responses from three healthy subjects and three treatment-naïve MS patients are depicted in Figure 3. The figure provides a visual depiction of the distribution of specific sequences within the total MBP-specific repertoire. Each bar represents a unique TCR sequence and the height of the bar depicts how often the sequence was represented within the total population. Overall, both untreated MS patients and

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healthy subjects exhibited a diverse clonotypic distribution within their MBP-specific T-cell responses. MBP-specific CD4+ T-cell responses showed at least one dominant clone (>10% of the total repertoire) in every subject. Overall, clonal dominance was similar between healthy subjects [2.2% (3 prominent sequences out of 134), Figures 3A, C, E] and MS patients [3.1% (4 out of 126), Figures 3G, I, K]. Interestingly, healthy subjects showed a slight trend toward a more focused MBP-specific response in the CD8+ compartment as revealed by 6.0% clonal dominance (7 out of 116, Figures 3B, D, F). In contrast, MBP-specific CD8+ T cells from MS patients were much more polytypic, similar to their CD4 responses, as revealed by a 2.1% clonal dominance (2 out of 93, Figures 3H, J, L).

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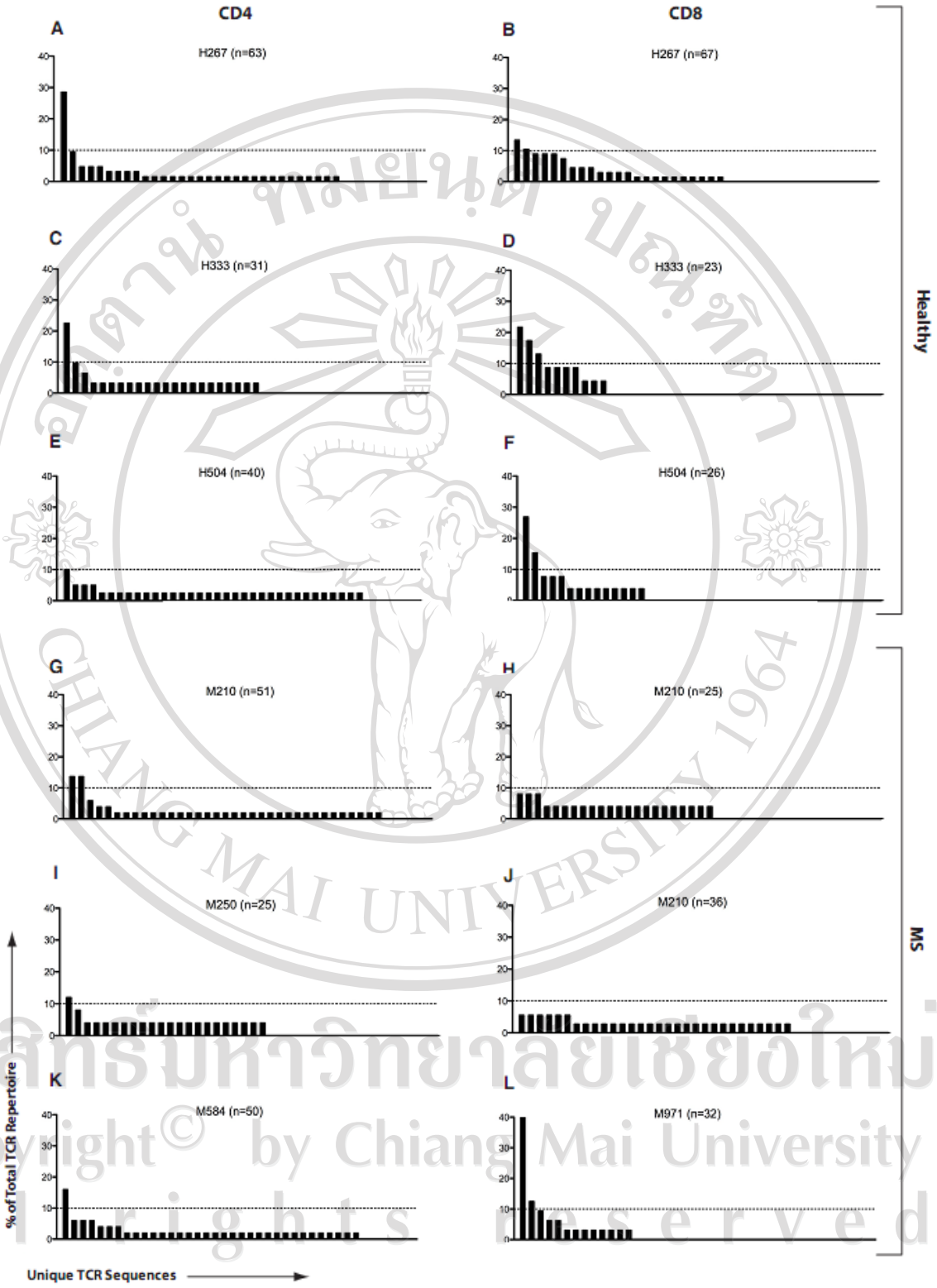

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Figure 3. TCR repertoire of MBP-specific T cells in MS and healthy subjects. PBMC from three healthy subjects (A-F) and three treatment-naïve RRMS patients (G-L) were used to obtain purified populations of MBP-specific CD4+ (left column) and CD8+ (right column) T cells using CFSE-based flow sorting. TCRβ repertoire was evaluated within each population. The total number of sequences (n) analyzed per population is denoted in the corresponding bar graph. Each bar along the X-axis represents a unique TCRβ sequence and the height of each bar represents its contribution to the total TCR repertoire (% of total). Dotted line is drawn at the 10% level, used as a cutoff for significant contribution by a single clone to the overall response.

3.2 Distinct TCRBV usage in MBP-reactive CD4+ T cells in MS

We next evaluated the specific TCR sequences of the antigen-specific clones. Tables 1-4 demonstrate the individual CDR3 sequences, showing clones that were represented at least two times within a total repertoire. Individual sequences of MBP-specific CD4+ and CD8+ TCR were distinct across different MS patients and healthy controls.

To gauge the overall variable region β chain usage in MS patients vs. healthy controls, we plotted the contribution of each TCRBV region within the MBP-specific CD4 and CD8 repertoires. Figure 4 demonstrates the TCRBV usage by T cells from three healthy subjects and three MS patients separately. The vertical bars represent the percentage of total TCRBV gene usage in a particular repertoire for that subject. Prominent TCRBV5 usage was observed in patients M210 and M584, similar to

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previous studies that have reported higher TCRBV5 family TCR usage in MS when compared to healthy subjects (57).

Figure 5 depicts these data in a cumulative manner so that MS patients and healthy subjects can be compared side by side. Interestingly, while there were clonal dominance differences in the MBP-specific CD8+ T cells (Figure 3), TCRBV usage differences were prominent in the MBP-specific CD4+ T-cell repertoires (Figure 5). Thus, MS patients showed a significantly higher usage of TCRBV4, TCRBV5, TCRBV7, TCRBV9 and TCRBV11 and significantly lower usage of TCRBV3 and TCRBV27 (Figure 5A). Cumulatively, TCRBV usage appeared to show similar distribution within the CD8+ T-cell repertoires of healthy subjects and MS patients (Figure 5B), with only marginal differences.

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Table 1. TCR repertoire of healthy MBP-specific CD4+ T cells.

Subject	BV locus	CDR3	BJ locus	#[a]	Total	Freq (%)
H333	2	CASRVGTGAQPQH	1-5	7	31	22.58
	2	CASGQGAGDQPQH	1-5	3	31	9.68
	6-5	CASSYSRRGRDTGPYNEQF	2-1	2	31	6.45
H267	2	CASISPGSYEQY	2-7	18	63	28.57
	29-1	CSVSSYNEQFF	2-1	6	63	9.52
	3-1	CASSQGGGVVTEAF	1-1	3	63	4.76
	10-2	CASSAAARGVGTDTQY	2-3	3	63	4.76
	9	CASSAPDSFYNSPLHF	1-6	3	63	4.76
	10-1	CASSLDGNNEQF	2-1	2	63	3.17
	19	CASKRGMSYNEQF	2-1	2	63	3.17
	29-1	CSVDVGGTDTQYF	2-3	2	63	3.17
	28	CASRESLDTQYF	2-3	2	63	3.17
H504	28	CASSLASIYEQY	2-7	4	40	10.00
	28	CASSLPARSYNEQF	2-1	2	40	5.00
	15	CATSREAGYYGYT	1-2	2	40	5.00
	7-2	CASSQGRLNTEAF	1-1	2	40	5.00

Data is restricted to those sequences that appeared at least twice within a sorted population.

[a] Indicates the number of instances this sequence was found within the total sequence population.

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Table 2. TCR repertoire of healthy MBP-specific CD8+ T cells.

Subject	BV locus	CDR3	BJ locus	#	Total	Freq (%)
H333	5-1	CASSLEGRPDHEQY	2-7	5	23	21.74
	2	CASSYSQGGTEAF	1-1	4	23	17.39
	11	CASSPYTDTQY	2-1	3	23	13.04
	6-5	CASSYADEQY	2-7	2	23	8.70
	2	CASKEVAGGRYTGELF	2-2	2	23	8.70
	7-8	CASSFRGDPFYGTY	1-2	2	23	8.70
	7-9	CASSLLDRGGGSTIY	1-3	2	23	8.70
H267	5-1	CASSLAGGYSPLH	1-6	9	67	13.43
	4-1	CASSRTTSSSYNEQF	2-1	7	67	10.45
	2	CASSDRGVGTGELF	2-2	6	67	8.96
	6-5	CASSYRTSQVINSPLH	1-6	6	67	8.96
	19	CASIKRTLPGASNTEAF	1-1	6	67	8.96
	7-4	CASSLFVSGDSPLH	1-6	5	67	7.46
	12-3	CASSPGLAGYEQF	2-1	3	67	4.48
	27	CASSSWDTDSPLH	1-6	3	67	4.48
	24	CASSLQGESGPLH	1-6	3	67	4.48
	28	CASSATGVIHNEQF	2-1	2	67	2.99
	6-2	CASSYGTQGQY	2-7	2	67	2.99
	20-1	CSARDLGDSNSPLH	1-6	2	67	2.99
	4-2	CASSGYRGGNQPQH	1-5	2	67	2.99
H504	27	CASSSGLIRF	2-1	7	26	26.92
	3-1	CASSRDSGFKDTQY	2-5	4	26	15.38
	5-5	CASSLETRGTNEQF	2-1	2	26	7.69
	2	CASRVGTGAQPQH	1-5	2	26	7.69
	5-1	CASSLAGSGYGYT	1-2	2	26	7.69

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Table 3. TCR repertoire of MS MBP-specific CD4+ T cells.

Subject	BV locus	CDR3	BJ locus	#	Total	Freq (%)
M210	27	CASSLGGGGTEQFGGG	1-1	7	51	13.73
	5-1	CASSPGSLSRETQY	2-5	7	51	13.73
	5-1	CASSLVLGGKNYGYT	1-2	3	51	5.88
	2	CASSEQNTQY	2-1	2	51	3.92
	6-6	CASSPGGRNEQF	2-1	2	51	3.92
M250	2	CASRVGTGAQPQH	1-5	3	25	12.00
	2	CASGQGAQDQPQH	1-5	2	25	8.00
M584	20-1	CSATVGAGTYEQY	2-7	8	50	16.00
	20-1	CSVSDRNNEQF	2-1	3	50	6.00
	11-3	CAPRTKIRANRAF	1-1	3	50	6.00
	5-1	CASSLAWGDTEAF	1-1	3	50	6.00
	5-1	CASSASRTGNTEAF	1-1	2	50	4.00
	5-7	CASSFYREAF	1-1	2	50	4.00
	7-9	CASSHTDRGRGNTIY	1-3	2	50	4.00

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Table 4. TCR repertoire of MS MBP-specific CD8+ T cells.

Subject	BV locus	CDR3	BJ locus	#	Total	Freq (%)
M210	6-5	CASSYDSATGELF	2-2	2	25	8.00
	18	CASSPPVIANYGYT	1-2	2	25	8.00
	20-1	CSASYGGFGYT	1-2	2	25	8.00
M250	2	CASSEFRG	1-3	2	36	5.56
	5-1	CASSHGLAEP	2-7	2	36	5.56
	6-2	CASRTHPGQL	2-7	2	36	5.56
	7-9	CASSLALTVM	1-1	2	36	5.56
	16	CASSSAFNSYNQF	2-1	2	36	5.56
	30	CAWGTGADYGYT	1-4	2	36	5.56
M971	7-2	CASSLEGISTDTQY	2-1	13	32	40.63
	5-1	CASSLAGQGVNTEAF	1-1	4	32	12.50
	6-2	CASSLTRTYEQY	2-7	3	32	9.38
	5-1	CASSTRTGSGTEAF	1-1	2	32	6.25
	27	CASSYLEIQGLKNIQY	2-4	2	32	6.25

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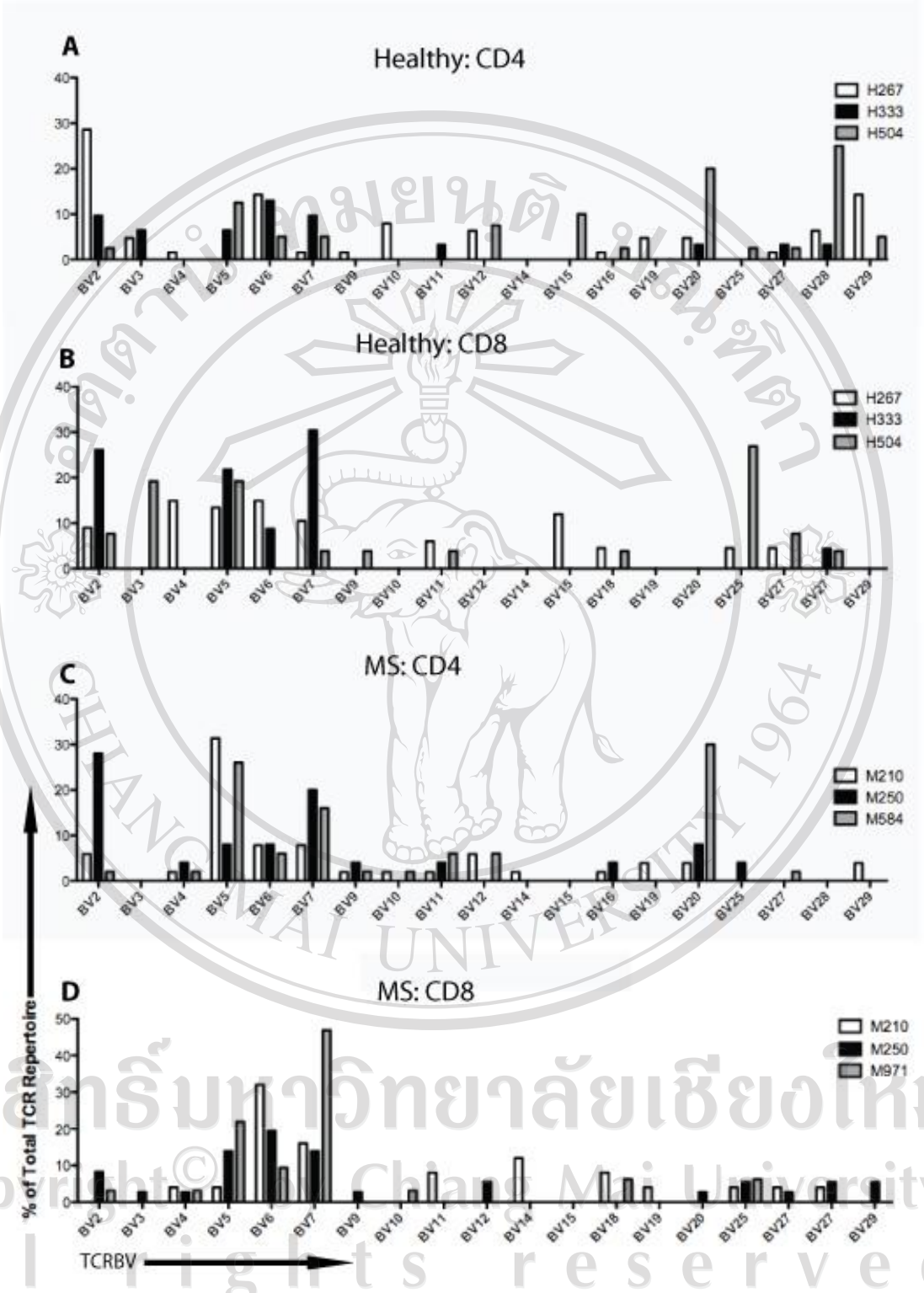

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Figure 4. TCRBV region gene usage in MBP-specific T cells. TCRBV gene usage by MBP-specific CD4+ and CD8+ T cells from three healthy subjects (A-B) and three MS patients (C-D) is depicted. The X-axis represents unique TCRBV genes while the Y-axis plots the contribution of each gene (percentage) within the antigen-specific repertoire.

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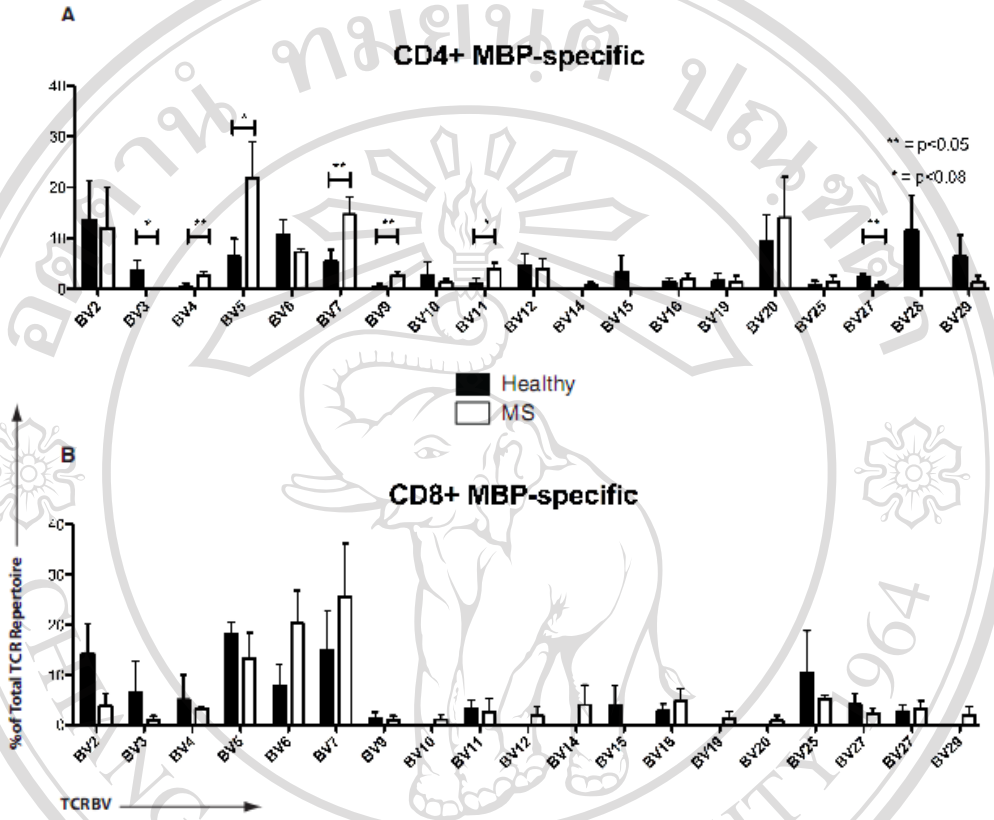

Figure 5. Cumulative TCRBV usage in MBP-specific T cells. These graphs represent cumulative data from Figure 3, depicting overall TCR usage among MBP-specific CD4+ (A) and CD8+ (B) T cells from healthy subjects vs. MS patients (*p<0.08; **p<0.05).

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3.3 Focused clonal composition of PLP-specific CD8+, unlike CD4+ T cells, in MS

In contrast to MBP-specific T-cell responses, PLP-specific CD4+ T cells exhibited a broadly polyclonal TCR repertoire (Figure 6 and Tables 5-8). The PLP-specific CD4+ and CD8+ TCR repertoire possessed no unique dominating sequence in healthy subjects. MS TCR analysis revealed only one dominating CD4 sequence (1.0%* or 1 out of 95, Figure 6D), whereas PLP-CD8+ TCR repertoire revealed a significantly more focused response as revealed by a 10.3%* clonal dominance (3 out of 29), *p=0.01 (Figures 6E, G). We had difficulty getting adequate evaluable PLP-specific CD8 responses from healthy subjects and thus, comparison between healthy subjects and MS patients was not possible.

TCRBV analysis of the PLP-specific T-cell repertoire revealed broad usage across MS patients and healthy controls (Figure 7), with no striking usage differences within the CD4 repertoires. Due to limited evaluation of the PLP-specific CD8 responses within the healthy subjects, we are unable to comment on their comparative attributes.

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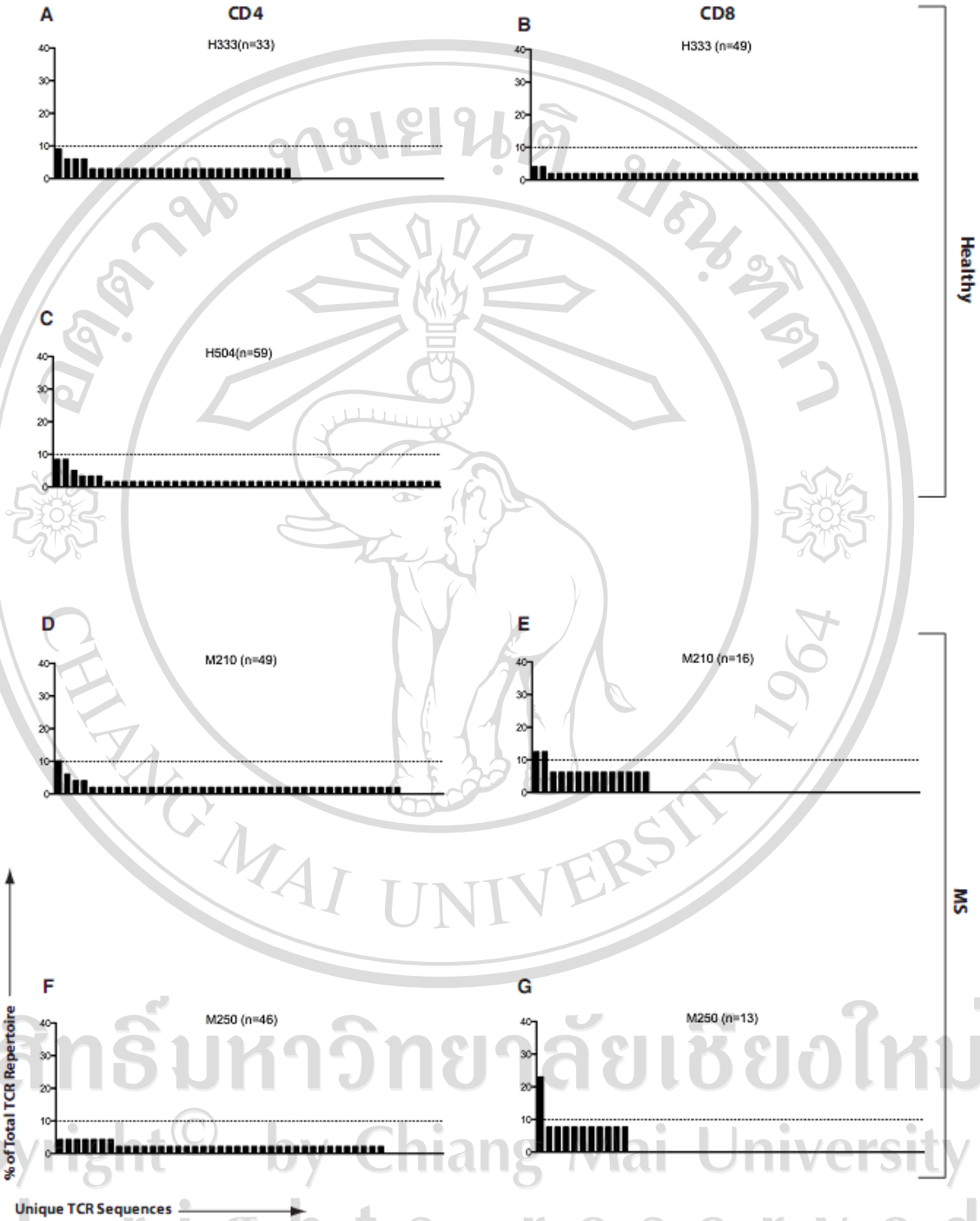

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Figure 6. TCR repertoire of PLP-specific T cells in MS and healthy subjects. The TCRβ repertoire was evaluated in sorted populations of PLP-specific CD4+ and CD8+ T cells from two healthy subjects and two MS patients. The figure is structured similar to Figure 3 (H504 did not have an evaluable CD8 response to PLP).

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Table 5. TCR repertoire of healthy PLP-specific CD4+ T cells.

Subject	BV locus	CDR3	BJ locus	#	Total	Freq (%)
H333	6-1	CASSADRGGYQY	2-1	2	49	4.08
	20	CSARPGLGGLSGNTIYF	1-3	2	49	4.08
H504	7-9	CASSLDRNYPSGNTIY	1-3	5	59	8.47
	28	CASSLPARSYNEQF	2-1	5	59	8.47
	3-1	CASSQELSFYNEQF	2-1	3	59	5.08
	20-1	CSARDPGTGNTGELF	2-2	2	59	3.39
	7-9	CASSPGTEDSWGY	2-5	2	59	3.39
	7-9	CASSPYSGLLGGELF	2-2	2	59	3.39

Table 6. TCR repertoire of healthy PLP-specific CD8+ T cells.

Subject	BV locus	CDR3	BJ locus	#	Total	Freq (%)
H333	12-3	CASRLTGNEQF	2-1	3	33	9.09
	5-5	CASSLGQGVLNEQF	2-1	2	33	6.06
	2	CASSVGGVDTEAF	1-1	2	33	6.06
	7-8	CASSRKGANVT	2-6	2	33	6.06

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Table 7. TCR repertoire of MS PLP-specific CD4+ T cells.

Subject	BV locus	CDR3	BJ locus	#	Total	Freq (%)
M210	27	CASSLGGGGTEAF	1-1	5	49	10.20
	5-1	CASSPGSLSRETQY	2-5	3	49	6.12
	18	CASSPGLAGGP	2-1	2	49	4.08
	5-1	CASSLGSPVGYEQY	2-7	2	49	4.08
M250	29	CSVEQANYGYT	1-2	2	46	4.35
	7-2	CASSFFLQGATEAF	1-2	2	46	4.35
	4-2	CASSQEGGQALQY	2-7	2	46	4.35
	6-5	CASSDRGFPRVTGELF	2-2	2	46	4.35
	18	CASSPEMSNQPQH	1-5	2	46	4.35
	11-2	CASSLVMNTEAF	1-1	2	46	4.35
	12-4	CASSLGQGGQTQY	2-5	2	46	4.35

Table 8. TCR repertoire of MS PLP-specific CD8+ T cells.

Subject	BV locus	CDR3	BJ locus	#	Total	Freq (%)
M210	2	CASVIRLPFNYGYT	1-2	2	16	12.50
	5-5	CASSQGFDDATEQY	2-7	2	16	12.50
M250	4-2	CASSHRLGAA	1-1	3	13	23.08

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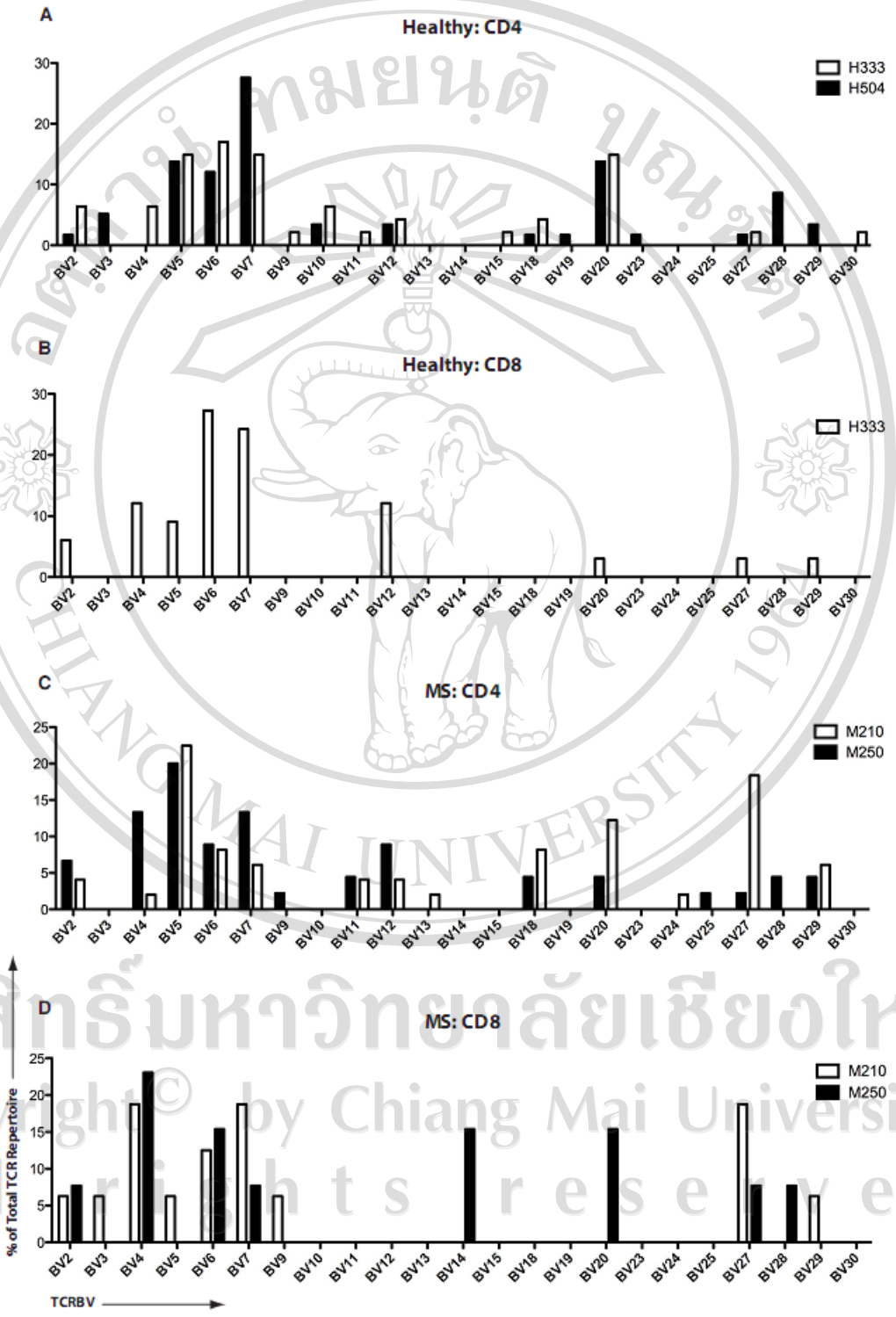

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Figure 7. TCRBV region gene usage in PLP-specific T cells. TCRBV gene usage by PLP-specific CD4+ and CD8+ T cells from two healthy subjects (A-B) and two MS patients (C-D) is depicted (Figure structured similar to Figure 3).

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3.4 Comparison of MBP- and PLP-specific TCR with published TCR sequences

Finally, we compared our set of CNS-specific TCR sequences to published sequences using open BLAST sequence analysis. A sequence was determined to be similar when at least four matching amino acids were located within the -NDN- hypervariable region, along with the same TCRBV and TCRJV usage.

Table 9 shows matches found against sequences derived from MS patients in our study. Interestingly, among all the published TCR sequences (not selected for any specific disease), the overwhelming majority of matches were found in sequences from other MS patients. Four positive matches were found among the published sequences from micro-dissected CNS-infiltrating CD8+ T cells taken from active lesions of MS patients (36). This prior study found oligoclonal expansion of CNS-infiltrating CD8+ T cells, suggesting an important function for these cells in the MS lesion. One match was found among a set of published TCR from a study of Chronic Encephalitis of Rasmussen (58). In this study, TCR sequences were analyzed among brain samples from seven patients, three MS patients, two viral encephalitis cases, four cases of medically refractory partial epilepsy and two cases with no detectable inflammatory neurologic condition. The only sequence found matching a myelin antigen-specific TCR was from one of the MS patients. The N-D-N sequence (GLAGG) also matched sequences published in other studies unrelated to MS including one of our own sequences from healthy subject H333, suggesting a more "public" CDR3 sequence (59). Of note, when TCR sequences from healthy subjects in our study were used to query the Genbank records, no matches were found that originated from any MS-related studies. Near matches were detected from other

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diseases such as chronic myeloid leukemia and non-Hodgkin lymphoma (data not shown), the significance of which is unclear.

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Table 9. Matching TCR clones with published TCR sequences.

Source clone	BV locus	CDR3	BJ locus
CD8+ MBP from M971 (case 1)	5-1	SALYLCASSLAGQGVNTEAFFGQ	1-1
CD8+ active MS lesion (Babbe et al., 2000)	5-1	SALYLCASSLAGQGYTGELFFGE	2-2
CD8+ active MS lesion (Babbe et al., 2000)	7-9	SAMYLCASSLSGQGDYGYTFGS	1-2
CD8+ MBP from M971 (case 2)	5-1	SALYLCASSTRTGSGTEAFFGQ	1-1
CD8+ active MS lesion (Babbe et al., 2000)	5-4	SALYLCASSSRTGSVSYEQYFGP	2-1
CD4+ MBP from M250	2	SAMYFCASRVGTGA---QPQHFGD	1-5
CD8+ active MS lesion (Babbe et al., 2000)	6-6	TSVYFCASRPGTGASNQPQHFGD	1-5
CD4+ PLP from M210	18	SAAYFCASSPGLAGGPSYNEQFFGP	2-1
Encephalitis study control MS patient (Li et al., 1997)	6-5	TSVYFCASSPGLAGGPNEQYFGP	2-1
CD4+ PLP from H333	27	TSLYFCASS--GLAGG-ADTQYFGP	2-3
Synovial fluid T cell in Arthritis patient (Striebich et al., 1998)	27	TSLYFCASSSPGLAGGTDTQYFGP	2-3
Infiltrating Lym. in calcified aortic stenosis (Wu et al., 2007)	27	TSLYFCASSPGLAGGPTDTQYFGP	2-3

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RESULTS

Project II: Phenotype of Primary T cells With Reduced Expression of CD147

3.5 CD147 is upregulated upon activation in primary T cells

The process began by establishing the relevance of the CD147 molecule with regard to primary T-cell activation. In Figure 8 the histograms show the CD3+ population from the culture activated with PHA compared to a resting culture. The activated culture is confirmed by the CFSE stain showing enhanced proliferation and the upregulation of CD25. CD147 is clearly upregulated upon T-cell activation as well.

3.6 Optimization of CD147 upregulation assay

In addition to stimulation with PHA several other methods were explored while trying to optimize the assay. Figure 9 shows the other activation methods that were used. Soluble anti-CD3 antibody OKT3 produced dependable but not robust upregulation of CD147 (Figure 9A). While activation of the culture twice did not produce satisfactory results because of the requisite drop in viability which was observed when adding the additional stress, it is worthy to note that an SEB activation alone produced moderate activation which was greatly enhanced by even a limited PHA re-stimulation of only one hour (Figure 9B). A significant improvement over soluble OKT3 was plate-bound OKT3 with the addition of anti-CD28 mAb. In Figure 9C, prominent upregulation of both CD25 and CD147 is shown in the T-cell population. The least amount of success was achieved with doses of PMA which did not produce dependable upregulation of CD147 in this study (Figure 9D). We settled on PHA stimulation as the method for activating our cultures because of the

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dependable upregulation of CD147 it achieved. It was also the most cost-effective method and the easiest to use.

In order to assess the minimal dosage required to achieve dependable upregulation of CD147 we titrated the dose of PHA from 0-5 μg/ml. Figure 10 shows the results of this assay. PHA at a concentration lower than 3 μg/ml produced less-than-optimal upregulation of CD147, however upregulation of CD25 was comparable to higher doses. At concentrations above 3 μg/ml CD147 expression did not improve, however CD25 expression increased in small increments with each higher concentration of 4-5 μg/ml. At the conclusion of these optimization experiments PHA at a concentration of 4 μg/ml was chosen for the standard activation method for all knockdown experiments.

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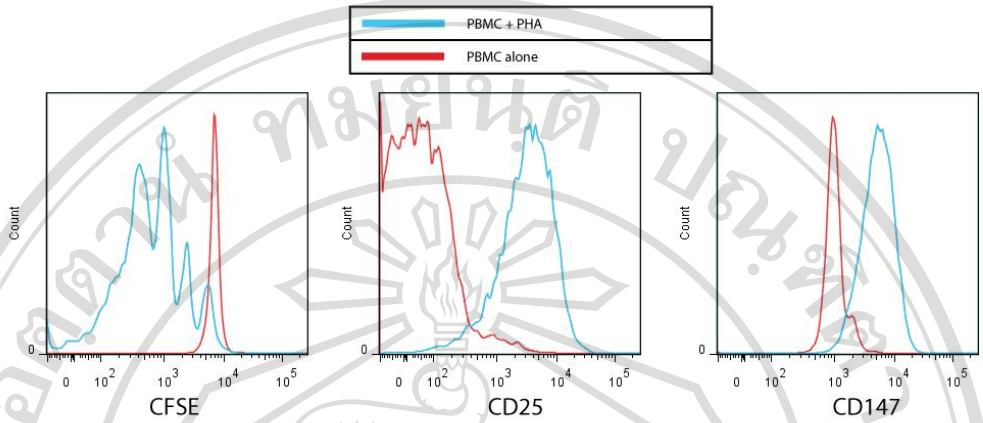

Figure 8. CD147 is upregulated upon activation**.** PBMC were cultured with PHA 4 µg/ml or media alone. Flow cytometry was performed on day 5. Stains for CFSE and CD25 show activated culture on gated lymphocyte population. CD147 is upregulated when compared to the non-activated culture.

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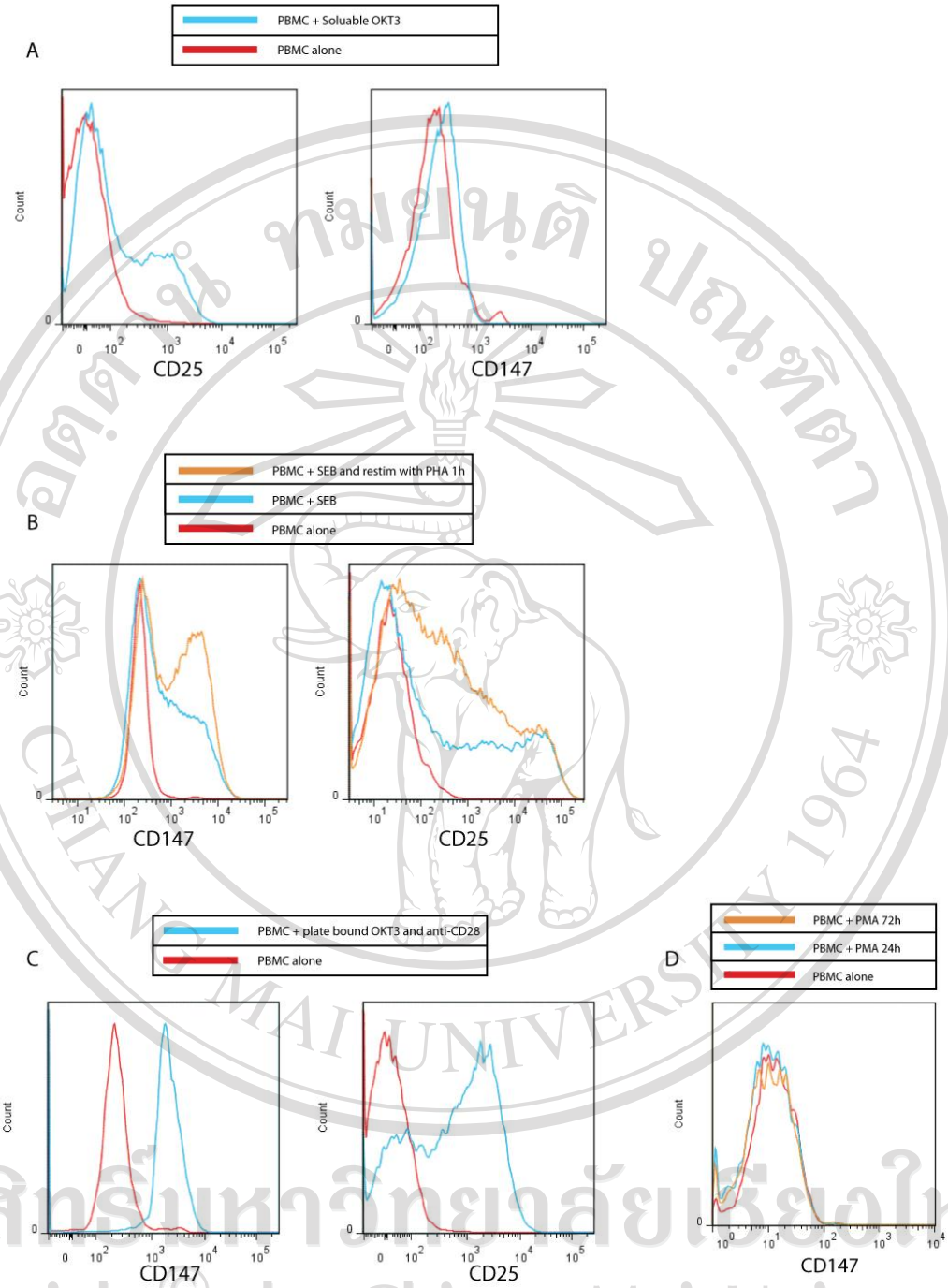

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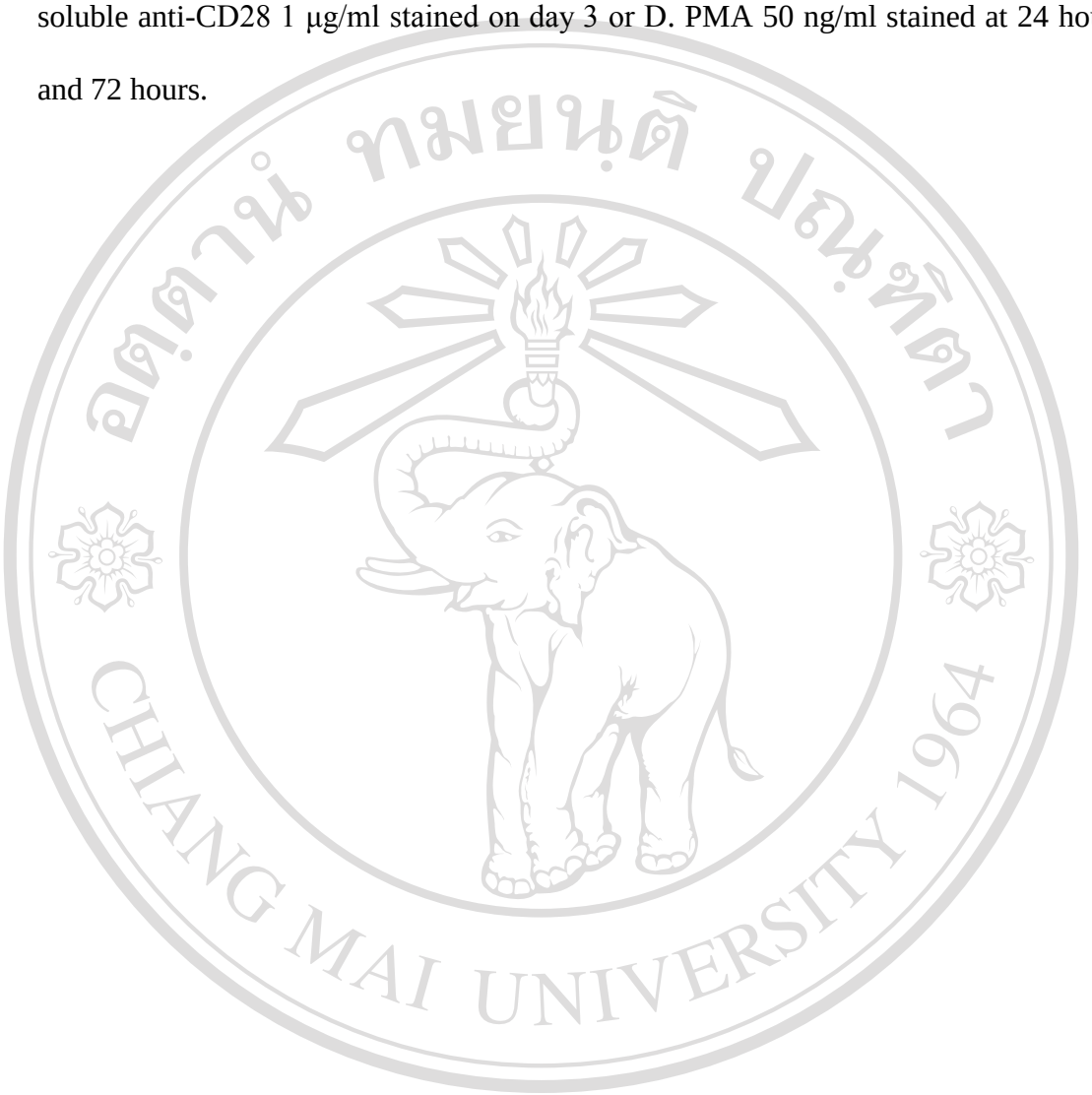
Figure 9. Alternate stimulation methods effect CD147 upregulation. PBMC were cultured with a variety of stimulating agents, including; A. soluble OKT3 1 µg/ml,

stained at 48 hours. B. SEB either alone or in conjunction with a secondary stimulation utilizing PHA 5 μg/ml for 1 hour on day 3. C. plate-bound OKT3 and soluble anti-CD28 1 μg/ml stained on day 3 or D. PMA 50 ng/ml stained at 24 hours and 72 hours.



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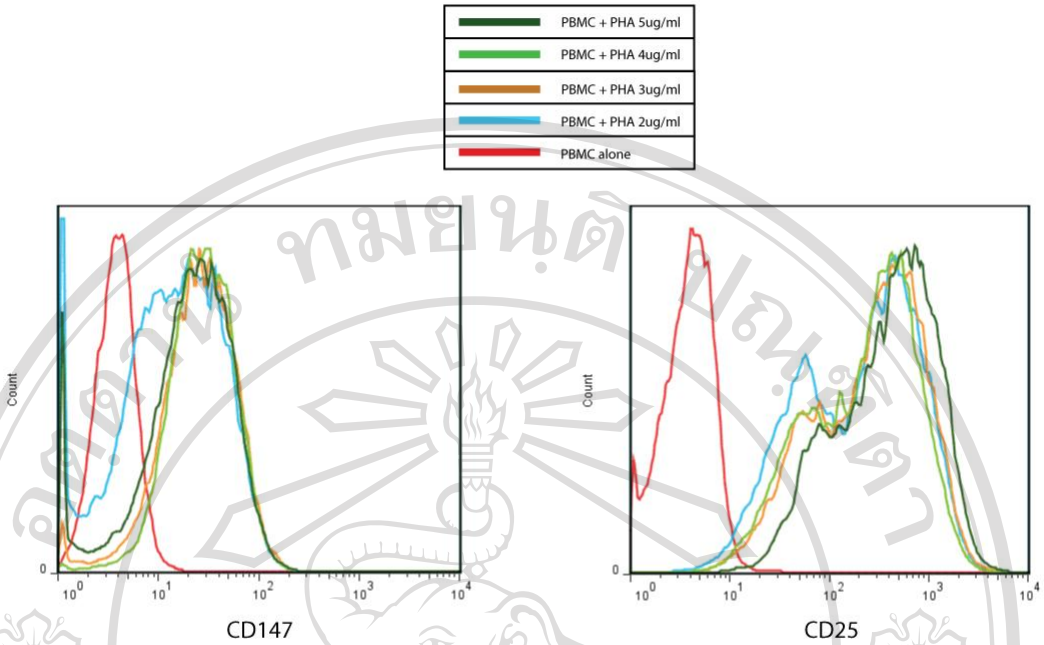

Figure 10. Titration of PHA dosage. PBMC were cultured with PHA in different concentrations or media alone. Flow Cytometry was performed 24 hours later. Stains for CD147 and CD25 show activated cultures on gated lymphocyte populations.

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3.7 CD147 expression on T-cell subsets

To my knowledge there has not been an evaluation of CD147 expression levels on subsets of T cells. Since a very basic assessment could be made by simply gating on CD4 and CD8 and measuring expression of CD147 on activated T-cell populations, we included these two markers in our standard panel of FLOW antibodies. Figure 11 shows the geo-mean fluorescence intensity of CD147 expression on CD4+ and CD8+ T-cell populations upon activation. The results indicate that CD147 upregulation could be observed in both CD4+ and CD8+ cells. The CD147 expression levels on both subsets of T cells were not statistically different.

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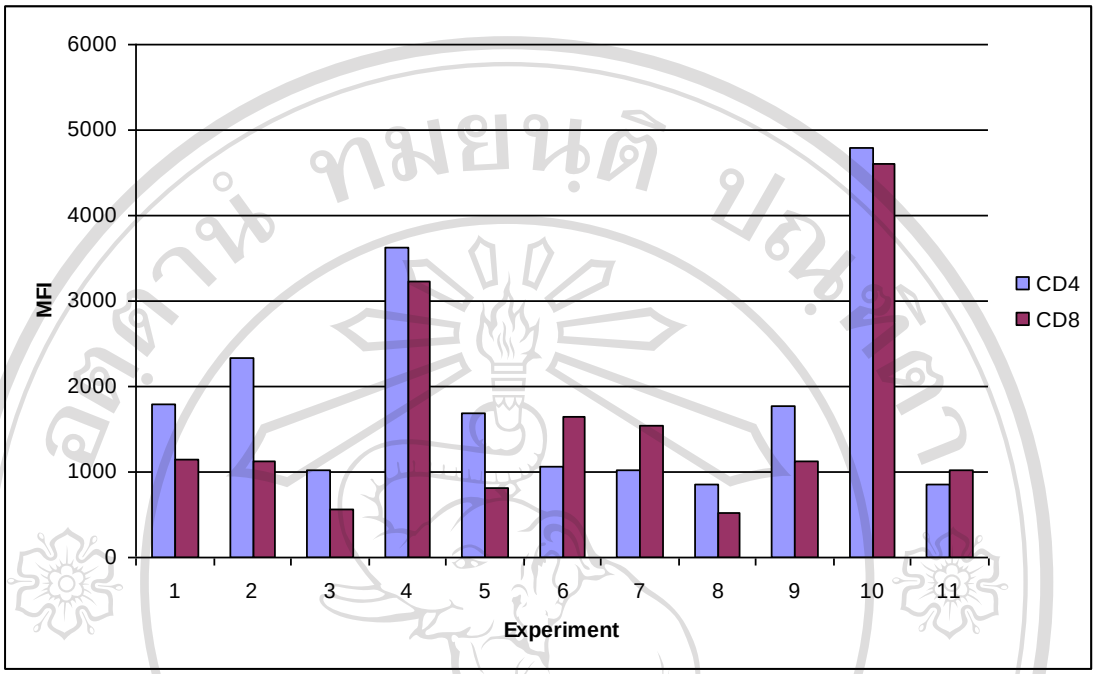

Figure 11. Upregulation of CD147 on CD4+ and CD8+ lymphocytes. PBMC were activated with PHA. CD147 expression on lymphocytes was determined by staining the activated cells with anti-CD147 mAb and anti-CD4 and anti-CD8 mAbs. CD4+ and CD8+ cells were gated and CD147 expression was analyzed by flow cytometry. Experiments 1-3 represent subject J, 4-5, subject B, and 6-11, subject H.

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3.8 Transfection

To ascertain transfection efficiency in different assay conditions a green fluorescent protein (GFP) expression plasmid was transfected into both primary T cells and Jurkat cells. The dot plots in Figure 12 show two individual samples of PBMC. GFP expression was measured 24 hours post-transfection. The dot plots on the left show GFP expression in the total lymphocyte gate and the plots on the right show GFP expression in the CD3+ population. Overall these control experiments went well and we was generally able to establish GFP expression in the range of 50-60% for the lymphocytes, with a slight reduction to 40-50% in the CD3+ population. Following the manufacture's protocol we tried using recommended programs for unactivated T cells and had success with both programs U-014 and V-024. Generally better results were obtained when using recently drawn blood as opposed to using the buffy coats supplied from the blood bank. Figure 13 shows a representative experiment with the freshly drawn blood sample shown on the left. Although transfection efficiency seems to be similar (62% vs. 52%) viability was always better when freshly drawn blood was used. Unsurprisingly when transfection efficiency in primary T cells was compared to that in Jurkat the cell line always had higher expression. Figure 14 shows the percent of GFP positive cells in the lymphocyte gate of both freshly drawn and bank blood when compared to the Jurkat T-cell lymphoma.

3.9 CD147 knockdown in Jurkat

Two pre-designed siRNAs targeting the Ig-like C2 coding region of the BSG gene, Ambion Silencer siRNA ID# 215973 and 10732, were selected for knockdown CD147. Initial experiments in the Jurkat cell line were only marginally successful. While the Jurkat cells offered high transfection efficiency and tolerated the

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transfection procedure well, achieving a substantial knockdown proved frustrating. Figure 15 shows Jurkat stained for CD147 and CD298. CD147 levels are constitutively very high on the Jurkat cells. Using each siRNA in a separate transfection reaction a marginal reduction was seen in CD147 expression at 24 hours however 72 hours post-transfection there was no difference between the knockdown and control groups. CD298 was stained in these cultures to show that the siRNA was indeed specific for CD147 as there was no knockdown of CD298 in all tested reactions.

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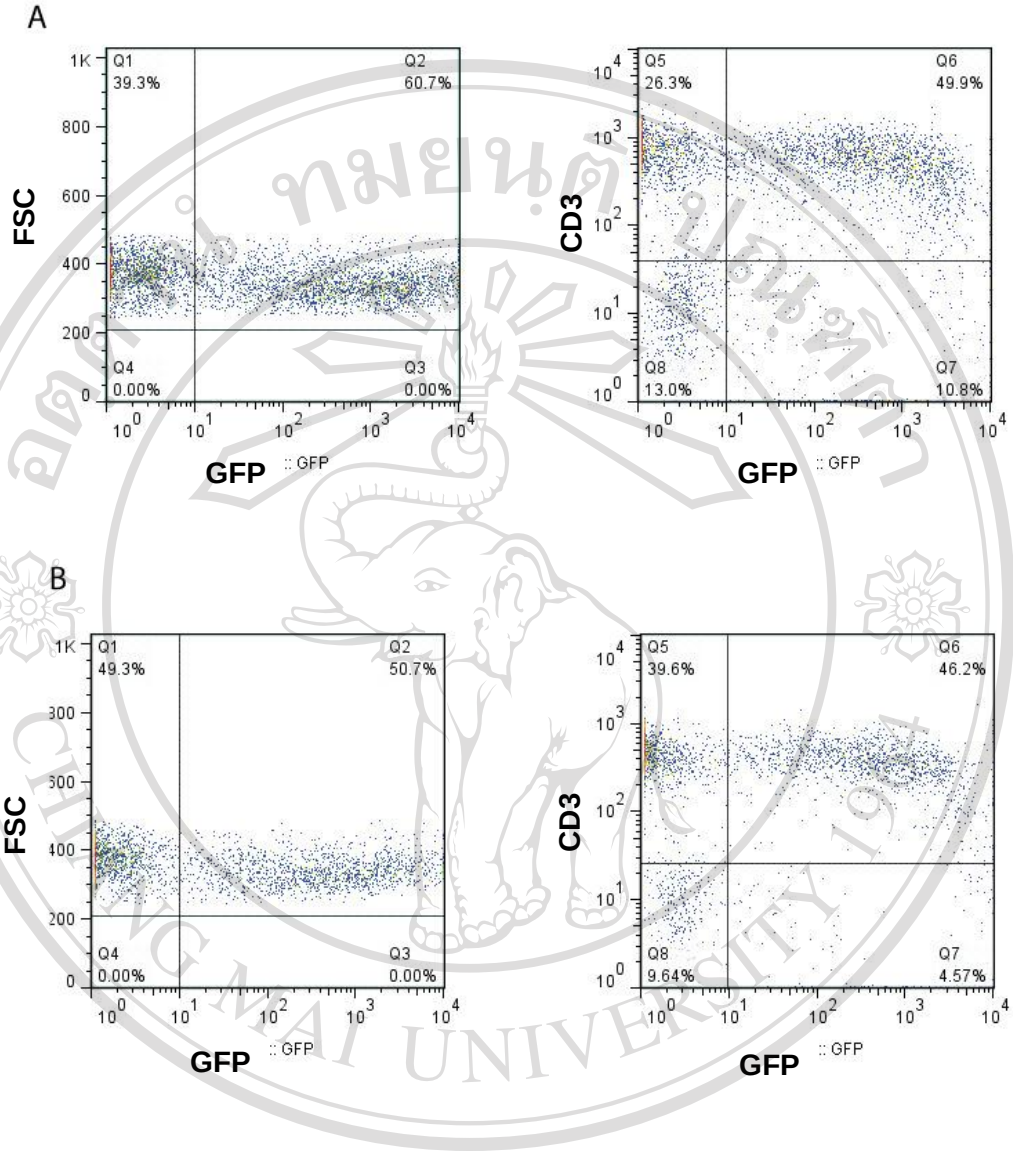

Figure 12. GFP expression in PBMC: lymphocyte gate vs. CD3+. Two independent samples of PBMC (subject A and B) were transfected with GFP-expressing plasmid in the Nucleofection protocol. After 24 hours GFP expression was checked by flow cytometry. Dot plots on the left show expression in total lymphocyte gate, while the plots on the right show GFP in CD3+ cells. The percentage of transfected CD3+ cells, while slightly lower than what was seen in the lymphocyte gate alone, was nearly 50% which is currently the gold standard for primary T-cell transfection .

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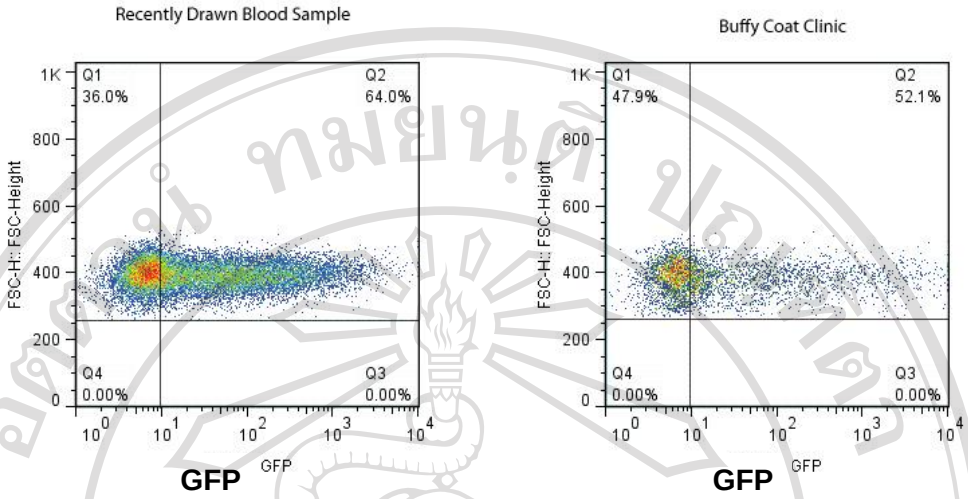

Figure 13. GFP expression in PBMC: recently drawn vs. 24 hours post draw. PBMC were transfected with GFP-expressing plasmid provided by Lonza. After 24 hours GFP expression was checked by flow cytometry. The dot plot on the left shows expression from a sample that was drawn and immediately ficol-hypaque separated. The plot on the right shows GFP expression in the buffy coat received from the blood bank which was drawn 24 hours previous to being processed and transfected.

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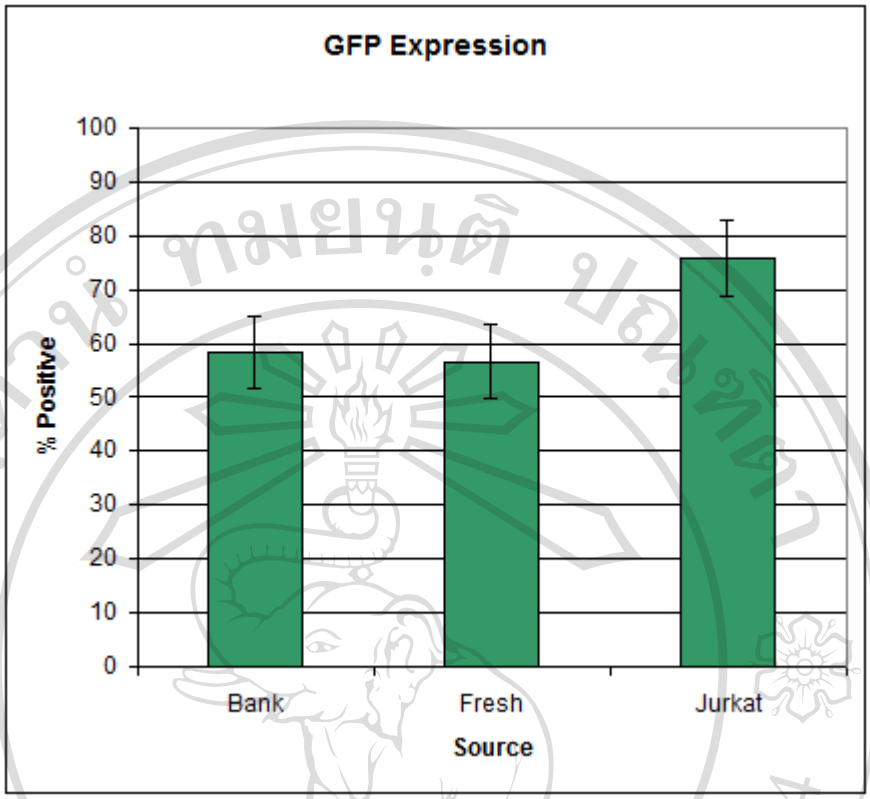

Figure 14. GFP expression in PBMC and Jurkat: source comparison. PBMC were transfected with GFP-expressing plasmid provided by Lonza. After 24 hours GFP expression was checked by flow cytometry. Percent of GFP positive cells; average of 3 experiments in each category.

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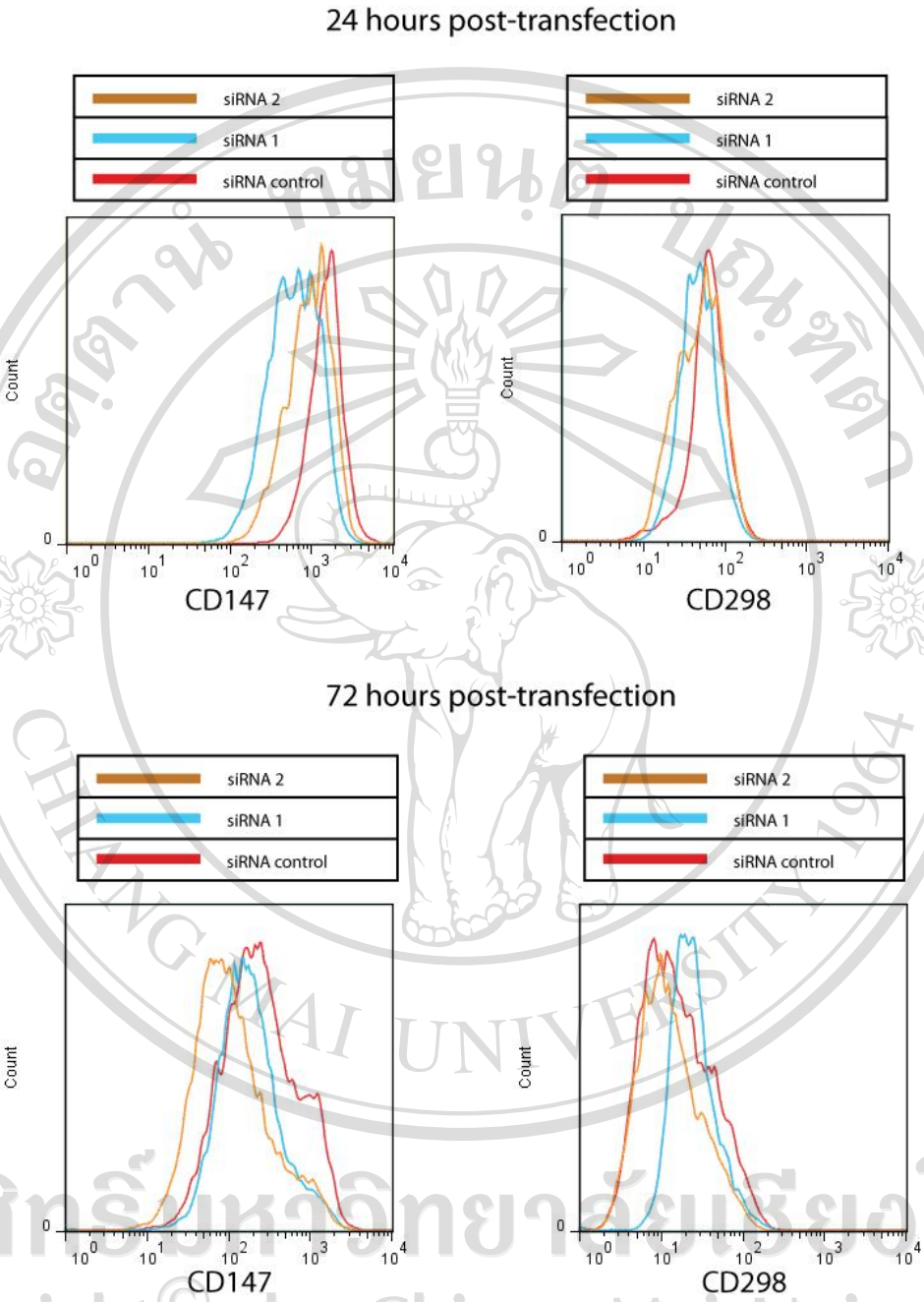

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Figure 15. CD147 knockdown in Jurkat T-cell lymphoma. Individual nucleofections using siRNA #10732 or #215973 at 100 nM. Cells were stained for CD147 or CD298 at 24 and 72 hours. CD147 knockdown was not very effective in Jurkat.

3.10 Knockdown in Primary T cells

Further attempts at knocking down CD147 expression in Jurkat were abandoned as the primary goal of my experiments was to lower expression in primary T cells. The histograms in Figure 16 show representative knockdowns in 3 subject samples 3 days after activation. The unactivated/untransfected culture is shown as a baseline, along with the activated/CD147 siRNA culture and the activated/non-specific siRNA culture. Successful transient knockdown was achieved by using both siRNAs in tandem at equal concentrations of 100 nM each. Four million cells were transfected in each reaction utilizing program U-014 for unactivated T cells. The cultures were activated 24 hours post transfection with PHA at a concentration of 4 µg/ml.

3.11 CD25 levels

The level of CD25 expression was measured by flow cytometry in each knockdown experiment. As can be seen in Figure 17, no meaningful correlation could be found between the total expression of CD25 and the percent reduction of CD147. While more often than not a reduction in CD147 produced a slight increase in CD25 expression, there were also cases where that trend was reversed.

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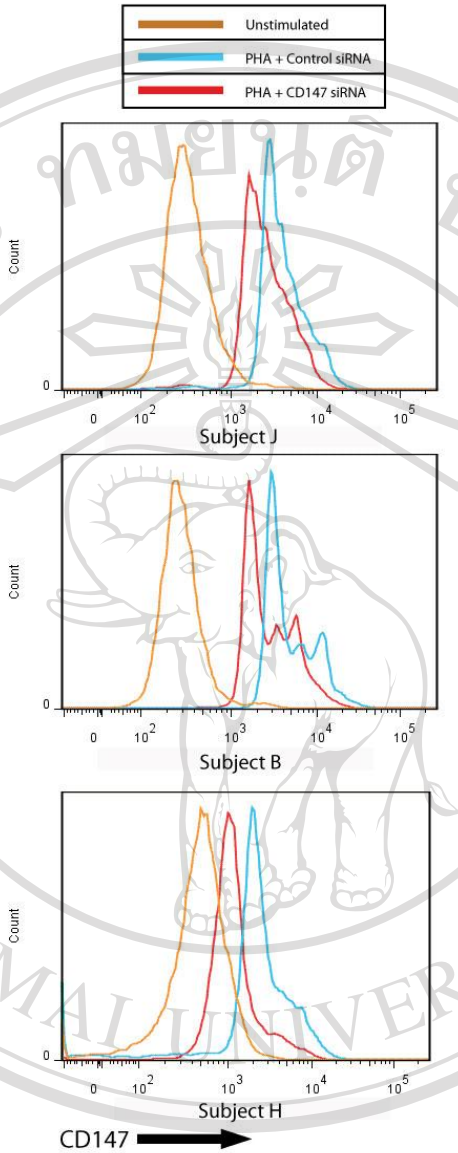

Figure 16. Successful knockdown of CD147 expression in primary T cells from three individual subjects. Histograms show three examples of CD147 knockdown. Unstimulated PBMC cultures at a concentration of $4x10^6$ per reaction (100µl) were transfected in the Nucleofector ® utilizing Program U-014. For silencing of CD147 mRNA targets Ambion Silencer siRNA ID# 215973 and 10732 were used - 200 nM siRNA 1 and 2 together (100nM each) OR 200nM control siRNA. Twenty-four hours post transfection cultures were stimulated with PHA 4 µg/ml. Harvested cells were

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stained and analyzed on a flow cytometer 3 days post-activation. Unstimulated, untransfected PBMC are shown for comparison

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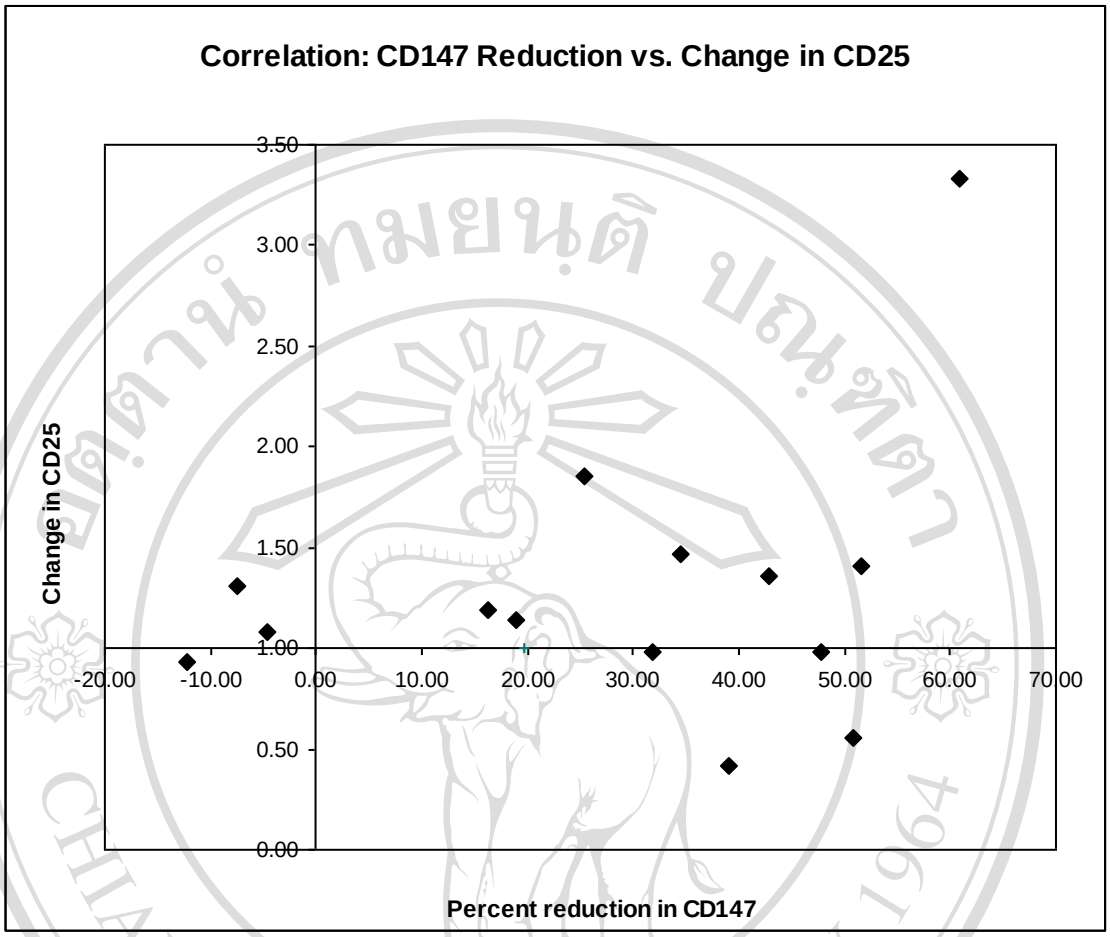

Figure 17. Correlation between reduction of CD147 expression and change in CD25 expression. Changes in surface expression of CD147 did not produce a trend in expression of CD25 in activated T lymphocytes. A value of 1.00 on the Y-axis denotes no difference between the knockdown and control culture. Data points greater than 1.00 show comparatively higher levels of CD25 expression in the knockdown culture, where data points below 1.00 show lower levels of CD25 expression compared to the control culture. These data represent 7 experiments with 2 timepoints each, except for one which had 3 timepoints. Four subjects were tested.

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3.12 Increased proliferation upon CD147 knockdown

The correlation between reduction of CD147 expression and increase in T-cell proliferation is shown in Figure 18. There is a clear correlation between the percent reduction of CD147 expression achieved and the percent increase in proliferation.

Negative proliferation/reduction corresponds to knockdown experiments that were unsuccessful, where there was no significant difference in CD147 expression or in fact the control culture's expression was lower than the knockdown cultures. The figure is a composite of replicate experiments conducted in 4 different subjects. In most cases measurements were taken on day 3 and day 5 post-activation. In one case measurements were also taken on day 7. When these cultures were analyzed the Lymphocyte gate was further isolated by including only the cells that fell into the category of CD4+/CD8- or CD8+/CD4-.

The results of experiments where knockdown was not achieved are shown in Figure 19. When surface levels of CD147 were the same, the result was an equal amount of CD25 and no change in proliferation. Two examples are shown here from different subjects.

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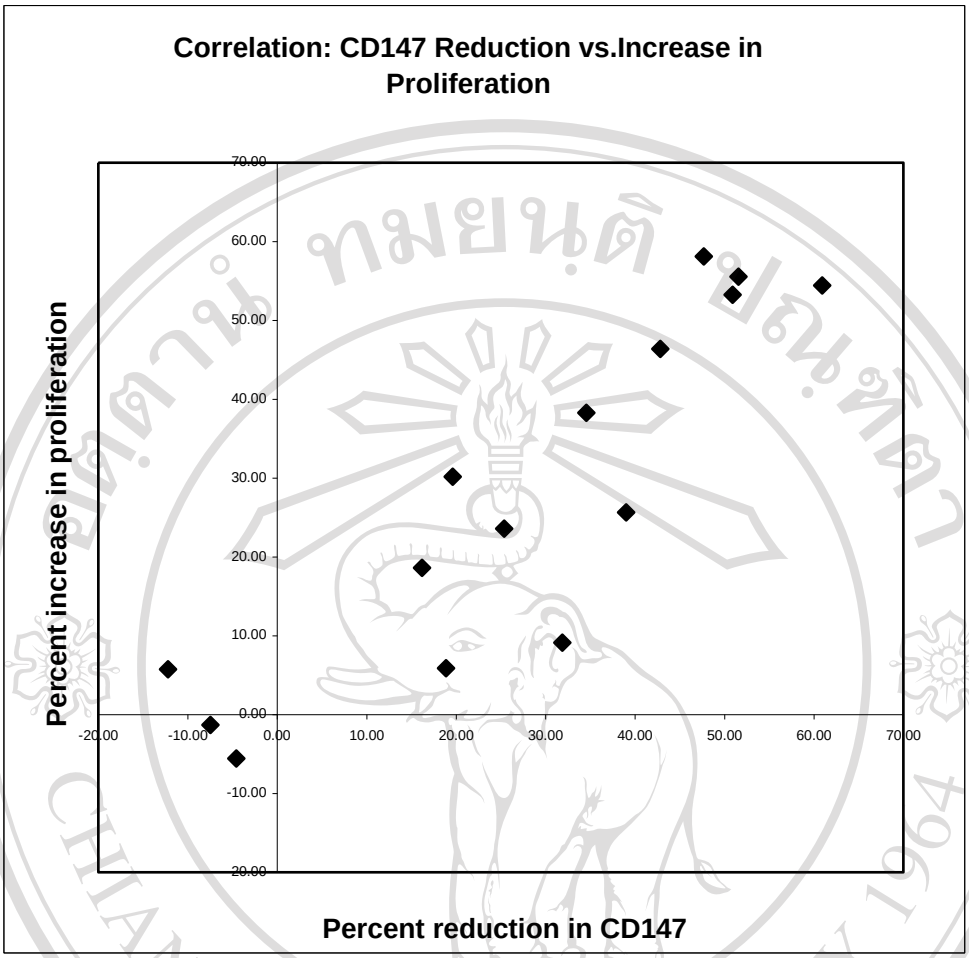

Figure 18. Correlation between reduction of CD147 expression and increase in proliferation in primary T cells. The reduction in CD147 expression correlates with a resulting increase in proliferation as measured by CFSE staining. These data represent 7 experiments with 2 timepoints each, except for one which had 3 timepoints. Four subjects were tested. Negative proliferation or reduction indicate experiments where the knockdown group showed higher expression/proliferation than the control.

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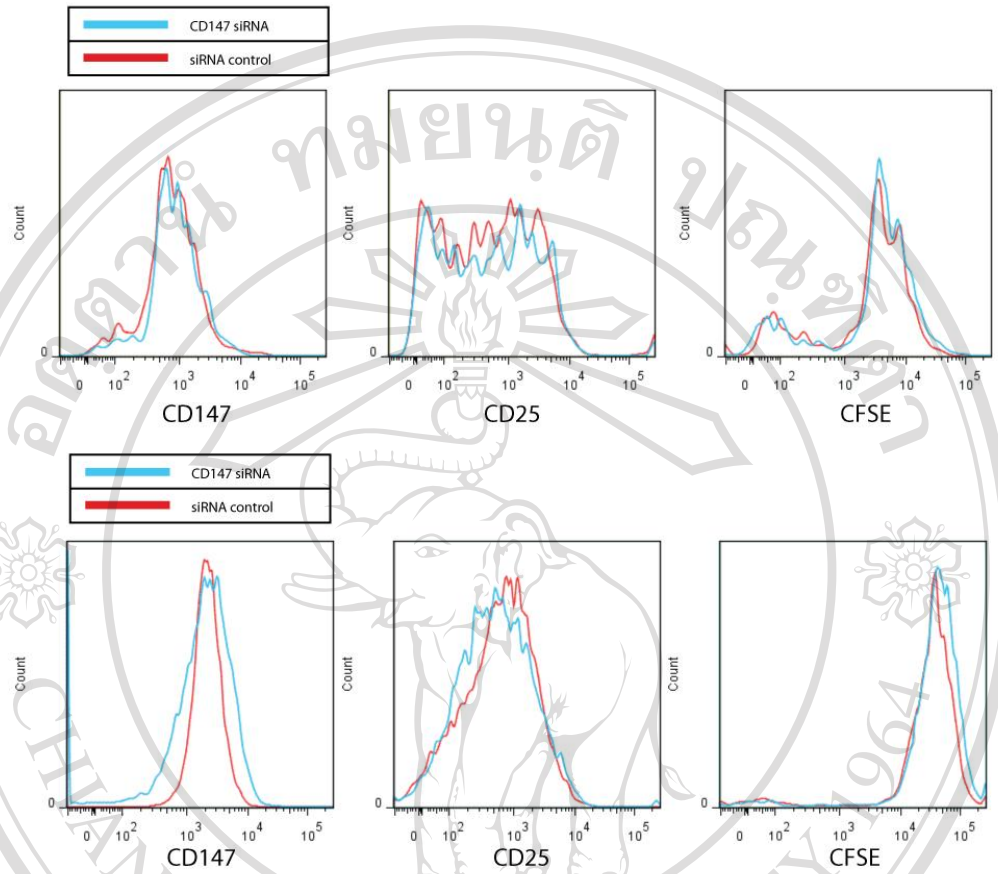

Figure 19. Experiments where CD147 knockdown was not achieved show comparable levels of proliferation and CD25 expression. Two individual experiments in two subjects show comparable levels of both proliferation and CD25 expression when CD147 was found to be expressed at the same level in a failed knockdown.

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3.13 Cytokine production of CD147 knockdown cells

Figures 20 and 21 show alterations in cytokine profiles of CD147 knockdown cells which were assayed by two different methods. In Figure 20, IFNγ, IL-10 and IL-4 were measured using an intracellular cytokine-staining protocol. Each bar graph represents an individual experiment and the amount of CD147 reduction is noted above the graph. IFNγ was increased in the two cultures which achieved significant knockdown, where a knockdown of 35% and 0% produced no significant change. The IL-10 and IL-4 measurements were divergent with an increase in one experiment and a large decrease in the other.

Figure 21 shows three of the experiments in which a cytometric bead array protocol was used to measure soluble cytokines in culture supernatants. Unfortunately no meaningful conclusions could be drawn from these measurements.

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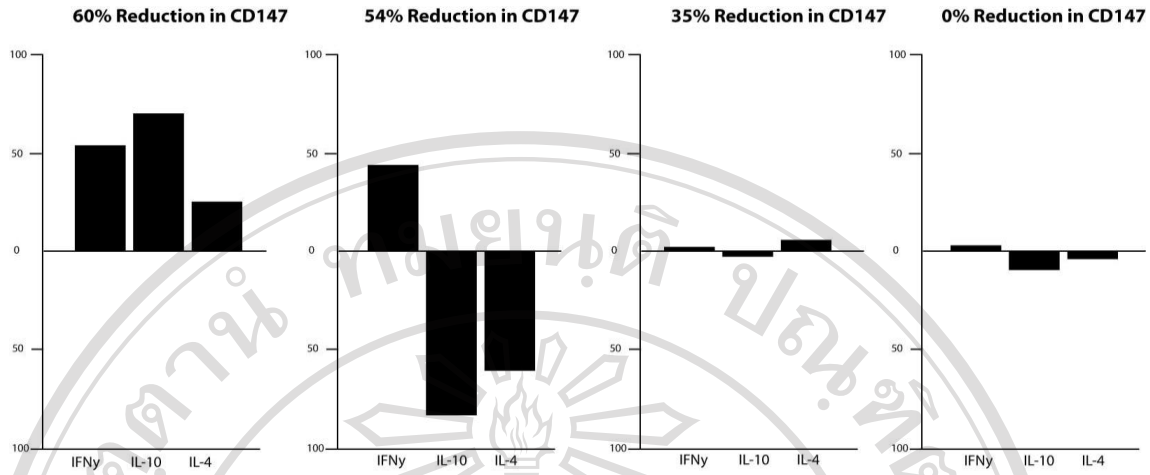

Figure 20. Intracellular cytokine staining in 4 samples. A significant reduction in CD147 levels in primary T cells produced substantial changes in intracellular cytokine levels. However, it is difficult to draw conclusions from this small sample due to the large variance in the observed IL-10 and IL-4 values. When the level of knockdown was reduced, the measured difference in cytokine levels was also reduced.

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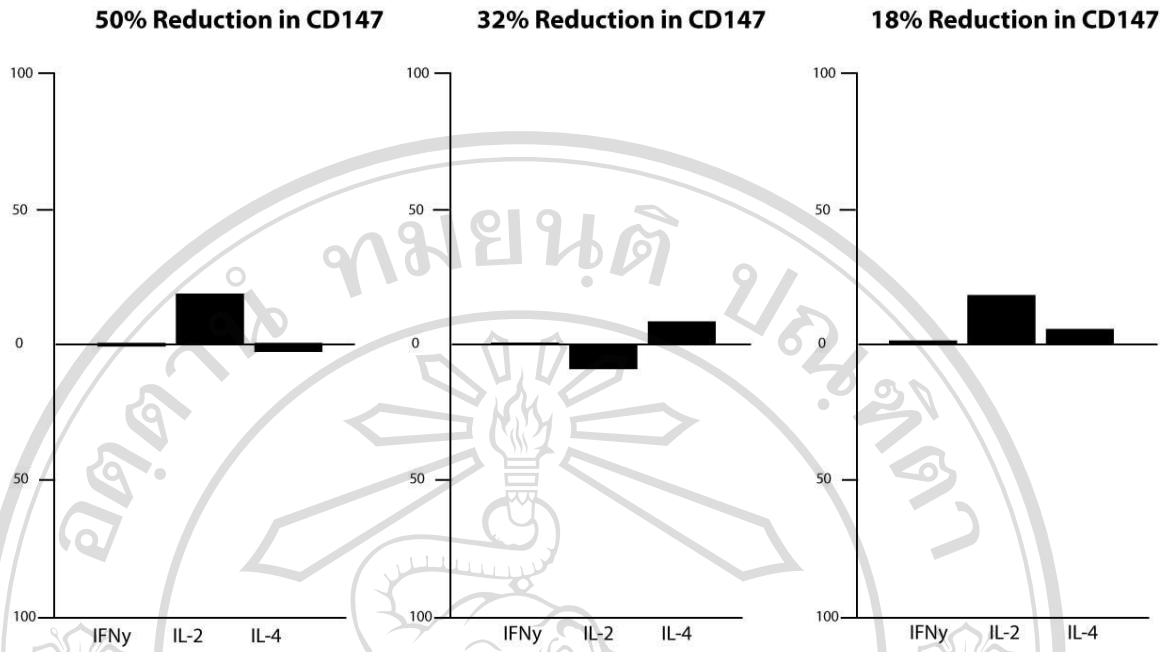

Figure 21. Cytometric Bead Array for soluble cytokines. Due to experimental difficulties no valuable information was gained from this experiment. Although some differences were observed between activated and unactivated cultures, the signal-to-noise ratio was too high to measure differences between knockdown and control cultures.

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