CHAPTER III

CHARACTERIZATION OF ACTIVATED CD4$^+$T CELLS PRIMED BY SEB PULSED NAÏVE B CELLS IN COMPARISON OF OTHER APC CONTROL

3.1 QUESTIONS

3.1.1 Do activated CD4$^+$T cells primed by SEB pulsed human peripheral naïve B cells constitute with unique characteristics?

3.1.2 Can activated CD4$^+$T cells primed by SEB pulsed human peripheral naïve B cells suppress allo-CD4$^+$T cell proliferation?

3.2 HYPOTHESIS

Activated CD4$^+$T cells primed by SEB pulsed human peripheral naïve B cells illustrate unique cell characteristics, including their induced regulatory function.

3.3 OBJECTIVES

3.3.1 To define phenotypes of activated CD4$^+$T cells primed by SEB pulsed human peripheral naïve B cells in comparison with other APC controls

3.3.2 To imply the role of human naïve B cell as APC of both conventional Ag and T cell superantigen

3.3.3 To compare human peripheral naïve B cell's SEB presentation with that of mouse splenic naïve B cell's Ag presentation

3.4 MATERIALS AND METHODS

3.4.1 ANTIBODIES AND REAGENTS

Anti-human CD40 (5C3), CD45RO (UCHL1), CD19 (4g7), CD3 (ucht1), CD25 (m-a251), CD45RA (hi100), HLA-II (tu39), CD4 (RFT-4G), CD11c (b-ly6), CD62L (SK11), FAS (DX2), TNF-α (MAb11), IFN-γ (4SB3), IL-2 (5344.1111), IL-4 (8D4-8) and IL-10 (JES3-19F1) antibodies were purchased from BD Pharmingen (San Diego, CA). IL-4 and granulocyte macrophage colony-stimulating factor (GM-CSF) were obtained from R&D Systems (Minneapolis, MN). Carboxyfluorescein succinimidyl ester (CFSE) was from

Molecular Probes (Merelbeke, Belgium). CpG ODN 2006 was from IMGENEX (San Diego, CA). Polyinosinic:polycytidylic acid (Poly I:C) was from Sigma-Aldrich (St. Louis, MO, USA). Propidium iodide (PI) and PKH-26 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Staphylococcal enterotoxin B, from *Staphylococcus aureus* (SEB) was purchased from Sigma-Aldrich (Singapore). RosetteSep® human B lymphocyte enrichment cocktail and RosetteSep® Human $CD4^+$T cell enrichment cocktail were purchased from StemCell Technologies Inc. (Vancouber DC, CA). Dynabeads® CD25 was purchased from Invitrogen Dynal (AS, Oslo, Norway). Naïve B cell isolation kit II, anti-PE microbeads, anti CD14 microbeads were purchased from Miltenyi Biotec (Auburn, USA).

3.4.2 HUMAN BLOOD SAMPLE

Blood samples were collected from healthy donors at National Blood Centre Thai Red Cross Society into heparin vacutainers. All samples were collected after the donors had signed a written informed consent in accordance with the institutional reviewed board-approved protocols of the Faculty of Medicine, Chulalongkorn University. Blood samples were collected and used for preparations of various cell types: 10 ml for naïve B cells, 5-10 ml for monocyte-derived dendritic cells (MoDCs), and 5 ml for naïve $CD4^+$T cells.

3.4.3 ISOLATION OF PERIPHERAL NAÏVE B CELLS

Total B cells were enriched directly from blood sample using RosetteSep® human B lymphocyte enrichment cocktail (StemCell, DC, CA). The enriched B cells were subsequently treated for naïve B cells ($CD19^+CD27^-$cells) isolation using Human naïve B cell isolation kit. In brief, 20-minutes incubation of donor blood sample with the RosetteSep cocktail (50 µl cocktail/1 ml blood) was performed to allow non-B cells (CD2, CD3, CD16, CD36, CD56, CD66b and CD235a positive cells) to crosslink with red blood cells (RBCs). Enriched human B cells were separated from crosslinked cells by density-gradient sedimentation on Ficoll-Hypaque Isoprep Robbins Scientific, Sunnyvale, CA) at 1200 g for 20 minutes at 23°C. Enriched total B cells were then

incubated with a cocktail of biotinylated antibodiesspecific to non-naïve B cells and anti-biotin microbeads for 15 and 20 minutes. In this study, the amount of antibodies as well as beads was estimated based on approximation of 1×10^6 PBMCs/1 ml blood sample. Labeled total B cells were washed with cold MAC buffer and then flushed through the magnetic column. The naïve B cell fraction (flow-through fraction) was collected, and determined for B cell purity (%$CD19^+$cells), naïve B cell purity (%$CD19^+CD27^-$cells) and contaminated memory B cell (%$CD19^+CD27^+$cells). For 4 samples, 5 ml of blood was used to estimate the number of naïve B cells/ml blood after their separation using gradient centrifugation on Ficoll-Hypaque Isoprep (Robbins Scientific) at 1500 rpm, 23°C for 30 minutes. The acquired data was included for yield calculation. Enriched naïve B cells were maintained in RPMI 1640 with L-glutamine (Invitrogen GIBCO, Grand Island, NY) supplemented with 10% Fetal bovine serum (FBS) (Cambrex Bio Science Walkersville, Walkersville, MD). The cells were cultured in 96-well U-bottom plate (5×10^4 cells/well) for 18 hours prior to subsequent experiment.

3.4.4 PREPARATION OF MONOCYTE-DERIVED DENDRITIC CELLS (MODCS)

To prepare monocyte-derived dendritic cells (MoDCs), collected PBMCs were subsequently isolated for $CD14^+$cells using CD14 MicroBeads (Miltenyi, Auburn, USA) according to the manufacturer protocol. Enriched $CD14^+$cells ($1\text{-}2\times10^6$ cells/well) were cultured in the presence of 3 μg/ml IL-4 and GM-CSF in RPMI supplemented with 10%FBS for 6 days. Purity of $CD11c^+CD209^+CD3^-$ MoDCs was >90%.

3.4.5 PREPARATION OF CPG OLIGODEOXYNUCLEOTIDE ACTIVATED B CELLS AND POLYINOSINE-POLYCYTIDYLIC ACID (POLY I:C) STIMULATED MONOCYTE-DERIVED DENDRITIC CELLS (MODCS)

Isolated naïve B cells were cultured in presence of 5 μg/ml ODN 2006 (InvivoGen, San Diego, USA), a class B CpG oligodeoxynucleotides (or CpG ODN) for 18 hours. To obtain poly I:C stimulated monocyte derived dendritic cell (MoDC), MoDCs were differentiated as indicated previously with addition of 5 μg/ml poly I:C (Sigma-Aldrich) on the last 2 days of culture. Cell viability, surface CD40, CD69, CD80, CD83,

CD86 and HLA class II were determined with all acquired cells immediately after preparation.

3.4.6 ISOLATION AND CULTURE OF PERIPHERAL NAÏVE $CD4^+$T CELLS

Naïve $CD4^+$T cells ($CD4^+CD25^-CD45RA^+CD45RO^-$cells) were isolated from blood sample using RosetteSep® Human $CD4^+$T cell enrichment cocktail, Dynabeads® CD25 (Invitrogen, Oslo, Norway) and anti-PE microbeads (Miltenyi, Auburn, USA). Briefly, human $CD4^+$T cells were enriched directly from blood sample using RosetteSep® Human $CD4^+$T cell enrichment cocktail by process similar to that of B cell enrichment. The enriched total $CD4^+$T cells were then depleted for $CD25^+$cells by Dynabeads® CD25 according to the manufacturer protocol to collect $CD4^+CD25^-$T cells. This fraction was subsequently incubated with anti-CD45RO-PE monoclonal antibody and anti-PE microbeads for 25 and 30 minutes. Bead labeled-total $CD4^+CD25^-$T cells were flushed through the magnetic column (MS). The flow-through fraction considered as naïve T cells was maintained in 10%FBS RPMI at 37°C, 5% CO2 in a humidified incubator lesser than 18 hours prior to co-culturing or further process.

3.4.7 STAPHYLOCOCCAL ENTEROTOXIN B (SEB) PULSATION AND ACTIVATION OF NAÏVE $CD4^+$T CELL

Naïve B cells, CpG activated B cells, MoDCs Poly I:C stimulated MoDCs, and allo naïve $CD4^+$T cells were pulsed with 0.5 μg SEB per 1-1.5x10^5 cells for an hour at 37°C with 5% CO_2. Immediately after pulsation, autologous naïve $CD4^+$T cells were co-cultured with SEB pulsed-antigen presenting cells (Naïve B cells, CpG activated B cells, MoDCs Poly I:C stimulated MoDCs) in a 96-well U-bottom plate at the ratio of 1:1 for 68 hours to acquire total 8-10x10^4 cells. Increases of cell size (FSC) and activated T cells ($CD4^+CD25^+$cells) were then determined in each co-culture.

3.4.8 ISOLATION AND STIMULATION OF NATURAL REGULATORY T CELL

$CD4^+CD25^+CD127^{lo}$T cells were regarded as representatives of natural regulatory T cells in this by study. The cells were isolated by applying $CD4^+CD25^+CD127^{lo}$T cell

isolation kit (Miltenyi Biotec, Auburn, USA) with enriched $CD4^+$T cells. In brief, total $CD4^+$T cells were enriched directly from blood sample using RosetteSep® Human $CD4^+$T cell enrichment cocktail as previously described. Enriched total T cells were then incubated with a cocktail of biotinylated antibodiesspecific to non- $CD4^+CD25^+CD127^{lo}$T cells and anti-biotin microbeads for 15 and 20 minutes (Miltenyi Biotec, Auburn, USA). In this study, the amount of antibodies as well as beads was estimated based on approximation of 1×10^6 PBMCs/1 ml blood sample. Labeled total $CD4^+$T cells were washed with cold MAC buffer and then flushed through the magnetic column. The $CD4^+CD25^+CD127^{lo}$T cell fraction (flow-through fraction) was collected, and determined for cell purity (97%). Acquired $CD4^+CD25^+CD127^{lo}$T cells were cultured in the presence of 1 μg/ml anti-human CD3 and CD28 (BD Biosciences) for 86 hours to enhance their suppressive function prior to usage.

3.4.9 SUPPRESSIVE FUNCTION TEST ON ALLO $CD4^+$T CELL'S PROLIFERATION

Target allo-$CD4^+CD25^-$T cells were isolated and labeled with CFSE as described in experiment 1. To purify total $CD4^+$T cells from 68-hour co-culture between naïve $CD4^+$T and SEB pulsed naïve/CpG activated B cells, Dynabeads® CD19 pan B cells (Invitrogen Dynal AS, Oslo, Norway) for B cell depletion were applied. Briefly, the co-cultured cells were incubated with anti-CD19 macrobeads on ice for 30 minutes in isolation buffer, and then put in magnetic stand for 2 minutes. The supernatant containing total $CD4^+$T cells was collected and determined for purity (>97%). Acquired total $CD4^+$T cells were determined for their property to suppress allo-$CD4^+$T cell proliferation. Briefly, 5×10^4 $CFSE^+CD4^+CD25^-$T cells were co-cultured with the cells of interest at the indicated ratios. The culture was performed in the presence of 1 μg/ml anti-human CD3 and CD28 antibodies (BD Biosciences) or allo-MoDCs (1 MoDC : 3 $CFSE^+$target cells) with 0.1 μg/ml SEB (Sigma-Aldrich) in U-shape-96-well plate for 5 days. Live proliferating target cells were achieved and selected by PI exclusion. Suppression of cell proliferation was determined by calculating the decrease of proliferating $CFSE^+CD4^+$T cells co-cultured with the cells of interest in comparison with resting naïve $CD4^+$T cells.

3.4.10 FLOW CYTOMETRY

Surface molecules' fluorescence staining was previously described in the first experiment. For intracellular molecules' fluorescence staining, 0.5-2x10^4 cells of interest were separated into each well of V-shape 96-well plate. Surface molecules' fluorescence staining was performed prior to intracellular staining (previously described). Cells of interest were fixed and permeabilized with working Fix/Perm reagent (eBioscience, CA, USA) (100 µl/well) prepared according to manufacturer's protocol in the dark for 45 minutes at 4°C. The cells were washed twice with 1XPerm buffer and centrifuged at 2200 rpm for 5 minutes at 4°C prior to discarding the supernatant. The cells were incubated with intracellular antibodies' cocktail in the dark for 45 minutes at 4°C, and then washed/spun once more prior to fixation in 1% formaldehyde PBS. The stained cells were then applied onto a flow cytometer (FACSCaliber, BD). To The data were acquired and analyzed using CellQuestPro (Becton Dickinson, San Jose, USA) and Summit program (Dako, CO, USA), accordingly.

3.4.11 STATISTICAL ANALYSIS

Differences among experiment groups were detected by One-way analysis of Variance (1-way ANOVA). To further detect significant differences among experimental groups, Bonferroni post hoc test was performed. Significant difference between two experiment groups was detected by Unpaired t-test. All statistic analyzes were performed using Graph Pad Prism 3.0.

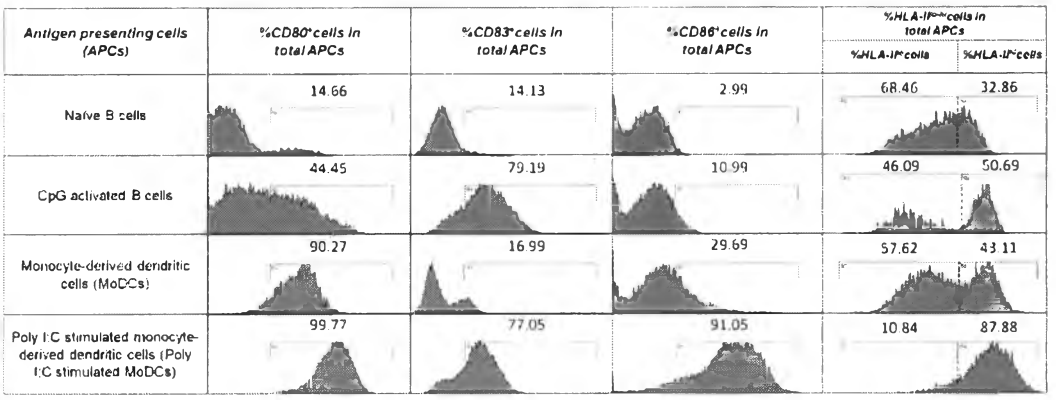
3.5 RESULTS

3.5.1 NAÏVE B CELLS EXPRESSED LOWEST CD80 AND CD86 WHEN COMPARED WITH OTHER ANTIGEN PRESENTING CELL CONTROLS

The expression levels of HLA class II (HLA-II), CD80, CD83 and CD86 on naïve B cells, CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs were determined (Figure 11). Naïve B cells expressed lowest CD80 and CD86 when compared with other antigen presenting cell (APC) types ($p<0.001$ to $p<0.05$) (Figure 11A and 11B). CpG activated B cells and Poly I:C stimulated MoDCs significantly expressed CD83 when

compared with resting naïve B cells and MoDCs, respectively (p<0.001). However, only Poly I:C stimulated MoDCs illustrated a decrease of HLA-II^{lo}cells (p<0.01) while increasing HLA-II^{hi} cells (p<0.05) when compared with MoDC (Figure 11A and 11B).

11A



11B

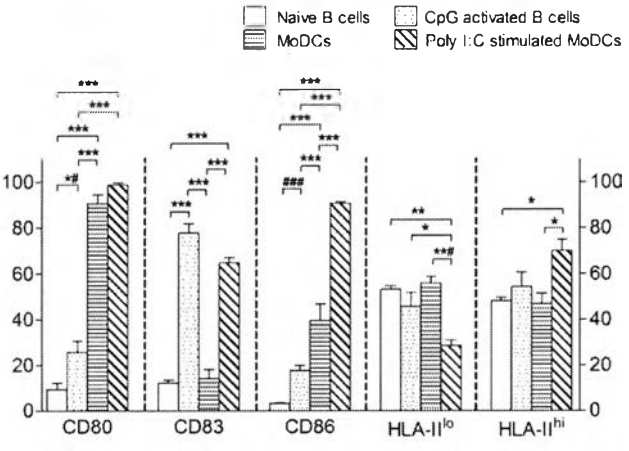


Figure 11. B7 co-stimulatory molecule and HLA class II expressions on naïve B cells, CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs. (A) Expressions of CD80, CD83, CD86 and HLA class II of resting naïve B cells CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs acquired from representative samples. (B) Comparison of positive cell number of CD80, CD83, CD86, low (HLA-II^{lo}) and high (HLA-II^{hi}) expression among naïve B cells (n=6), CpG activated B cells (n=6), MoDCs (n=5) and Poly I:C stimulated MoDCs (n=3). *Asterisk within the graph denoted significantly different values acquired from one-way ANOVA with subsequent Turkey's Multiple comparison test. * indicated p<0.05, ** indicated p<0.01, and *** indicated p<0.001.

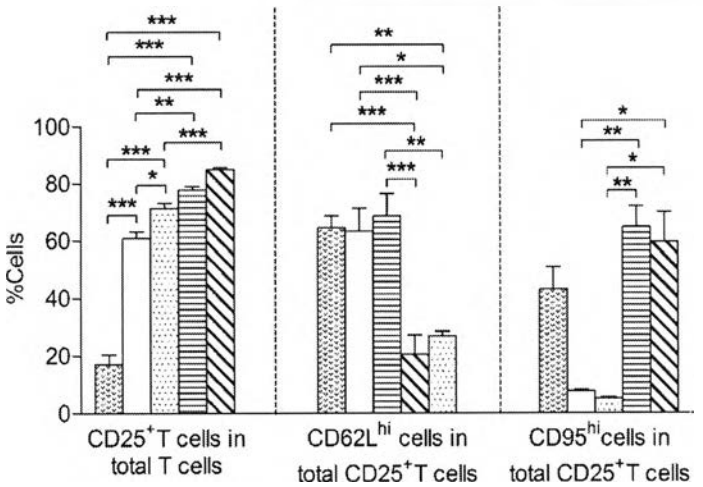
[#]Number sign within the graph denoted significantly different values acquired from paired t-test. [#] indicated $p<0.05$ and [###] indicated $p<0.001$.

3.5.2 NAÏVE AND CPG ACTIVATED B CELLS PRIMED CD4$^+$T CELL ACTIVATION TO ACQUIRE CD25(+)CD62L(hi)CD95(lo) PHENOTYPES DURING STAPHYLOCCOCUS ENTEROTOXIN B (SEB) PRESENTATION

Activated T cell phenotypes were analyzed by flow cytometry in naïve CD4$^+$T cells co-cultured for 68 h with SEB-pulsed naïve B cells, SEB-pulsed CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs. Naïve CD4$^+$T cell cultured in absence of APCs (resting naïve T cells) and SEB pulsed naïve CD4$^+$T cells were regarded as controls (Figure 12). Significant T cell activation was detected in all cultures with the presences of SEB-pulsed APCs when compared with resting and the direct SEB-pulsed naïve T cell controls. These were indicated by increases of both cell size and CD25 expression (Figure 12A and 12B) ($p<0.001$). Lowest CD25$^+$T cell numbers were presented when the naïve T cells were SEB-presented by naïve B cells in comparison with those by other APCs ($p<0.001$ to 0.05) (Figure 12B). Interestingly, most of activated T cells (CD25$^+$T cells) of SEB-presented by naïve and CpG activated B cells still retained high CD62L ($p<0.001$ to <0.05), but CD95 expressions ($p<0.01$ to <0.05) when compared with those of SEB-presented by MoDCs and Poly I:C stimulated MoDCs (Figure 12A and 12B).

APCs	SEB pulsation	Naïve CD4⁺T cells	Cell size (FSC-H)	%CD25⁺cells in total CD4⁺T cells	%CD62L^hi cells in total CD4⁺CD25⁺T cells		%CD95^hi cells in CD4⁺CD25⁺T cells	
					CD62L^lo	CD62L^hi	CD95^lo	CD95^hi
-	-	+	80	0.35	NA		NA	
-	+	+	90	12.77	55.32		27.39	
Naïve B cells	+	+	138	73.19	79.50		10.37	
CpG activated B cells	+	+	148	76.72	69.87		6.23	
MoDCs	+	+	159	78.12	22.99		61.47	
Poly I:C stimulated MoDCs	+	+	165	84.63	4.00		51.04	

12A



12B

Symbols	APCs	SEB pulsation	Naïve CD4⁺T cells
	-	+	+
	Naïve B cells	+	+
	CpG activated B cells	+	+
	MoDCs	+	+
	Poly I:C stimulated MoDCs	+	+

Figure 12. Determination of T cell activation and phenotypes after 68 hours of culture. (A) Cell size (FSC-H), %CD25$^+$ cells in total CD4$^+$T cells's number of resting naïve CD4$^+$T cells, SEB pulsed naïve CD4$^+$T cells, naïve CD4$^+$T cells co-cultured with SEB pulsed naïve B cells and naïve CD4$^+$T cells co-cultured with SEB pulsed MoDCs prepared from representative samples. Comparisons of (B) FSC-H and (D) CD25$^+$T cell's number among all experiment groups (n=5). The resting T cells were not included

in the figure due to their extremely limited number of activated T cell. *Asterisk within the graph denoted significantly different values acquired from one-way ANOVA with subsequent Turkey's Multiple comparison test. * indicated $p<0.05$, ** indicated $p<0.01$, and *** indicated $p<0.001$.

3.5.3 NAÏVE B CELLS POORLY PRIMED NAÏVE CD4$^+$T CELL ACT IVATION TO PRODUCE TH1 AND TH2 ASSOCIATED CYTOKINES WHEN COMPARED WITH THOSE OF OTHER ANTIGEN PRESENTING CELLS

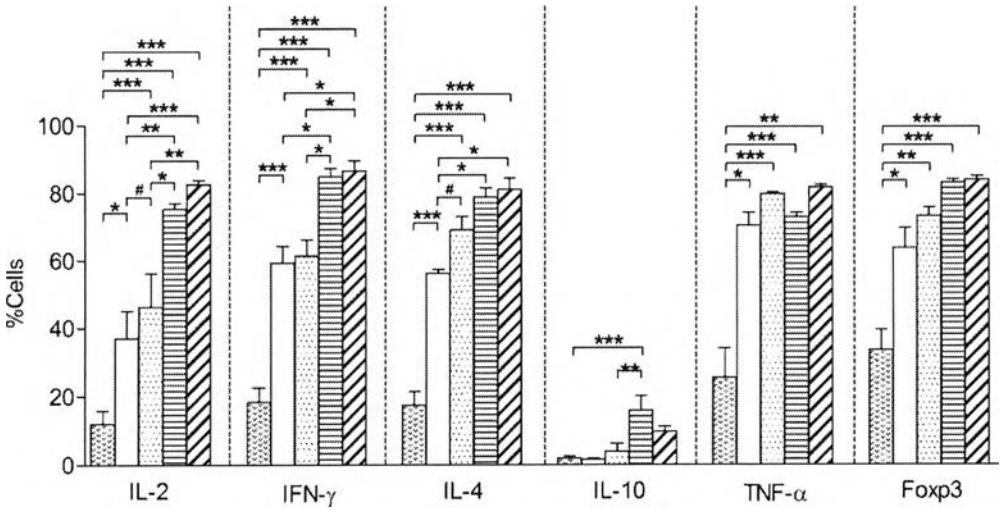
Cytokine producing T cells in naïve CD4$^+$T cells co-cultured for 68 h with SEB-pulsed naïve B cells, CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs were determined. Naïve CD4$^+$T cell cultured in absence of APCs (resting naïve T cells) and SEB pulsed naïve CD4$^+$T cells were regarded as controls (Figure 13). In the presence of SEB pulsed APCs, significant IL-2, IFN-γ, IL-4, TNF-α cytokine productions and Foxp3 upregulation of activated CD4$^+$T cells were detected in all cultures when compared with those of resting and the direct SEB-pulsed naïve T cell controls (Figure 13A-13C) ($p<0.001$). Lowest degrees of IL-2, IFN-γ and IL-4 productions were presented when the naïve T cells were SEB-presented by naïve B cells in comparison with those induced by other APCs ($p<0.001$ to 0.05) (Figure 13B-13C). Interestingly, only MoDCs seemed to successfully induce IL-10 production of T cells after SEB presentation (Figure 13C) ($p<0.001$).

APCs	SEB pulsation	Naïve CD4⁺T cells	%IL-2⁺cells in total CD4⁺T cells	%IFN-γ⁺cells in total CD4⁺T cells	%TNF-α⁺cells in total CD4⁺T cells
-	-	+	0.72	3.05	1.76
-	+	+	11.11	34.35	12.57
Naïve B cells	+	+	14.34	57.53	57.17
CpG activated B cells	+	+	14.26	65.54	74.47
MoDCs	+	+	86.13	87.32	74.45
Poly I:C stimulated MoDCs	+	+	92.26	97.33	74.88

13A

APCs	SEB pulsation	Naïve CD4⁺T cells	%IL-4⁺cells in total CD4⁺T cells	%IL-10⁺cells in total CD4⁺T cells	%Foxp3⁺cells in total CD4⁺T cells
-	-	+	0.91	1.26	1.04
-	+	+	8.61	2.08	19.46
Naïve B cells	+	+	71.26	1.84	51.39
CpG activated B cells	+	+	73.75	1.70	78.13
MoDCs	+	+	72.84	14.67	80.51
Poly I:C stimulated MoDCs	+	+	75.72	9.39	81.47

13B



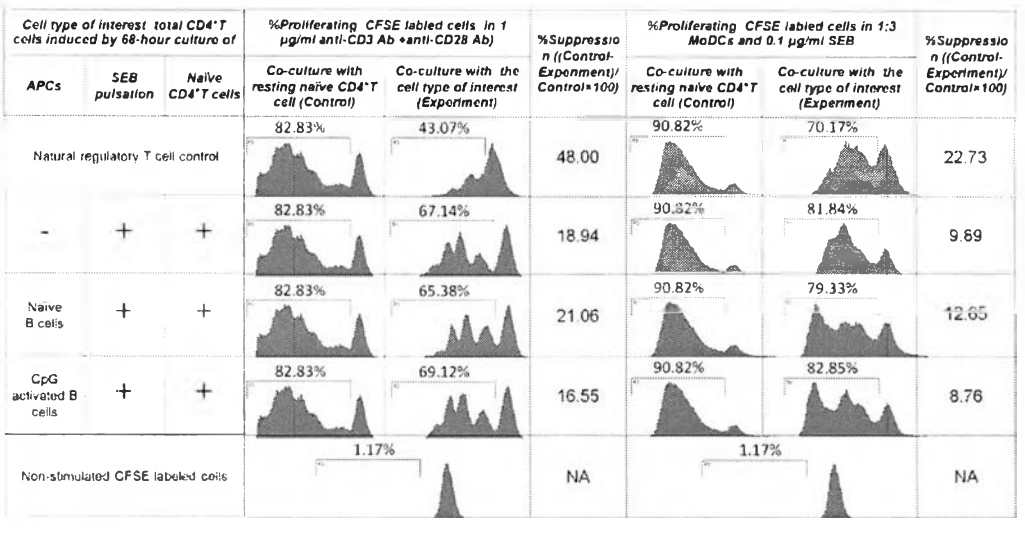
Symbols	APCs	SEB pulsation	Naïve CD4⁺T cells
	-	+	+
	Naïve B cells	+	+
	CpG activated B cells	+	+
	MoDCs	+	+
	Poly I:C stimulated MoDCs	+	+

13C

Figure 13. Cytokine production profile and Foxp3 expression of activated T cells primed by various SEB pulsed APCs. (A) IL-2, IFN-γ, TNF-α producing cells and (B) IL-4, IL-10, Foxp3 expressing cells in total $CD4^+$T cells acquired from resting naïve $CD4^+$T cells, SEB pulsed naïve $CD4^+$T cells, naïve $CD4^+$T cells co-cultured with SEB pulsed naïve B cells and naïve $CD4^+$T cells co-cultured with SEB pulsed MoDCs prepared from representative samples. (C) Comparisons of cytokine producing and Foxp3 expressing cell numbers among all experiment groups. Resting T cells were not included in the figure due to their extremely limited number of cytokine producing T cells presented. *Asterisk within the graph denoted significantly different values acquired from one-way ANOVA with subsequent Turkey's Multiple comparison test. * indicated $p<0.05$, ** indicated $p<0.01$, and *** indicated $p<0.001$. #Number sign within the graph denoted significantly different values acquired from paired t-test. # indicated $p<0.05$.

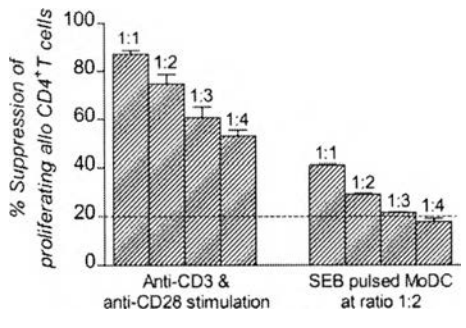
3.5.4 ACTIVATED $CD4^+$T CELLS PRIMED BY BOTH SEB-PRESENTED NAÏVE B CELLS AND CpG ACTIVATED B CELLS ILLUSTRATED NO SIGNIFICANT SUPPRESSIVE FUNCTION ON ALLO-$CD4^+$T CELL PROLIFERATION.

Mouse splenic naïve B cell's oval albumin (OVA) presentation was previously shown to prime naïve $CD4^+$T cells to acquire both *in vitro* and *in vivo* immunosuppressive function (Reichardt et al., 2007b). To determine whether SEB-presented naïve B cells was able to constitute such a function to their primed T cells in this study, total $CD4^+$T cells in populations acquired from naïve $CD4^+$T cells co-cultured for 68 h with SEB-pulsed naïve B cells were isolated and tested for their suppression on allo-$CD4^+$T cell proliferation. Direct SEB-pulsed naïve T cells were regarded as the control cells. The suppressive function test was first determined on stimulated $CD4^+CD25^+CD127^{lo}$T cells which were regarded as natural regulatory T cell control (Figure 14A). A decrease of %suppression correlated with decreasing co-cultured cell's ratio was presented indicating the suppressive function of acquired $CD4^+CD25^+CD127^{lo}$T cells (Brusko et al., 2007). The suppression's potency of $CD4^+CD25^+CD127^{lo}$T cells in SEB pulsed MoDC system was lower than that of anti-CD3 & anti-CD28 stimulation system at all co-cultured ratio (Figure 14B). The significant %suppression used in this study was at least 20%(Brusko et al., 2007). After optimization, the systems were subsequently applied for suppressive functions of total $CD4^+$T cells primed both by SEB-presented naïve B cells and CpG activated B cells. The 1:3 ratio (T cells of interest : Target cells) were applied, since higher co-culture ratio resulted in >20% suppression of direct SEB-pulsed naïve T cell control possibly due to competition for nutrients and cytokine of these pre-stimulated cells (Valencic et al., 2007). At 1:3 ration, both total $CD4^+$T cells primed by SEB-presented naïve B cells and -CpG activated B cells illustrate no significant suppressive function when compared with direct SEB-pulsed naïve T cell control (Figure 14A and 14C).

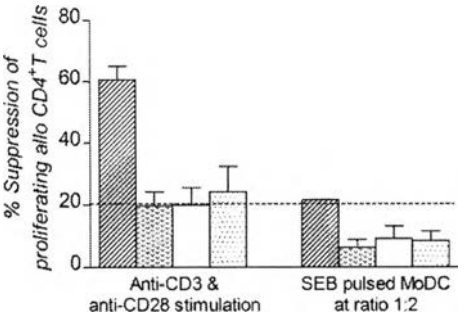


Cell type of interest total CD4⁺T cells induced by 68-hour culture of			%Proliferating CFSE labled cells in 1 µg/ml anti-CD3 Ab +anti-CD28 Ab)		%Suppression ((Control-Experiment)/Control×100)	%Proliferating CFSE labled cells in 1:3 MoDCs and 0.1 µg/ml SEB		%Suppression ((Control-Experiment)/Control×100)
APCs	SEB pulsation	Naïve CD4⁺T cells	Co-culture with resting naïve CD4⁺T cell (Control)	Co-culture with the cell type of interest (Experiment)		Co-culture with resting naïve CD4⁺T cell (Control)	Co-culture with the cell type of interest (Experiment)	
Natural regulatory T cell control			82.83%	43.07%	48.00	90.82%	70.17%	22.73
-	+	+	82.83%	67.14%	18.94	90.82%	81.84%	9.89
Naïve B cells	+	+	82.83%	65.38%	21.06	90.82%	79.33%	12.65
CpG activated B cells	+	+	82.83%	69.12%	16.55	90.82%	82.85%	8.76
Non-stimulated CFSE labeled cells			1.17%		NA	1.17%		NA

12A



12B



12C

Symbols	APCs	SEB pulsation	Naïve CD4⁺T cells
▨	-	+	+
□	Naïve B cells	+	+
▤	CpG activated B cells	+	+
▨	Natural regulatory T cells		

Figure 14. Allo-CD4$^+$T cell proliferation suppression's determination of total CD4$^+$T cells primed by SEB presented B cells. (A) Demonstration of %Suppression calculation of representative samples (B) %Suppression of proliferating allo-CD4$^+$T cells generated from stimulated CD4$^+$CD25$^+$CD127^{lo}T cells co-culturing with target CFSE labeled CD4$^+$CD25$^-$T cells at 1:1, 1:2, 1:3 and 1:4 ratios (C) %Suppression of proliferating allo-CD4$^+$T cells generated from directly SEB pulsed naïve CD4$^+$T cells, naïve CD4$^+$T cells co-cultured with SEB pulsed naïve B cells, and SEB pulsed CpG activated B cells at 1:3 ratio.

3.6 DISCUSSION

To determine the SEB priming effect of naïve B cell on $CD4^+$T cell activation, naïve $CD4^+$T cells co-cultured with SEB pulsed naïve B cells for 68 hours were determined for phenotypes, cytokine production and cell suppressive function in comparison with those primed by other APCs (CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs). The result has shown that SEB-presented naïve B cell primes naïve $CD4^+$T cell activation to acquire CD25(+)CD62L(hi)CD95(lo) phenotypes (Figure 12) with limited T_H1 and T_H2 associated cytokine productions (Figure 13). However, the acquired cells demonstrated no significant suppressive function (Figure 14). This suggested us that human regulatory T cell induction by naïve B cell should require crucial factors that were not presented in the SEB presentation system investigated in this study.

In addition to naïve B cells, other three APC types were included in this study as the controls; CpG activated B cells, MoDCs and Poly I:C stimulated MoDCs. These cells respectively represented TLR activated B cell, immature DC and mature DC control (Avalos et al., 2009; Sato and Fujita, 2007; Wagner et al., 2004). With Toll-like receptor ligands, both CpG activated B cells and Poly I:C stimulated MoDCs were thus comparably stimulated naïve CD4 T cells. B7 peripheral protein family, CD80, CD83 and CD86 expressions on each APC type were determined. As previously described, CD80 and CD86 are important co-stimulatory molecules of antigen presentation to T cells upregulated during B cell activation and DC maturation (Reichardt et al., 2007a). On the contrary, CD83 has no significant co-stimulatory function but is recognized as a mature dendritic cell marker (Breloer et al., 2007) and TLR activated B cell marker (Breloer et al., 2007; Prazma et al., 2007). Successful induction of CpG activated B cell and Poly I:C stimulated MoDCs were implied by their CD83 and CD86 upregulations (Figure 11). However, CD80, CD86 and HLA-II expressions on CpG activated B cells were still not comparable to those of MoDC supporting the knownledge that TLR stimulation in absence of CD40L signal from $CD4^+$T cell is not sufficient to activate B cell effectively (Wagner et al., 2004). Due to their lowest CD80 and CD86 expressions, naïve B cells

should be the least immunogenic APC in this experiment (Reichardt et al., 2007a; Watt et al., 2007).

Significant T cell activation was presented in all 68-hour co-cultures between naïve $CD4^+$T cells and SEB-pulsed APCs (Figure 12). By determining $CD25^+$T cell number (Figure 12B), naïve B cells ineffectively primed naïve $CD4^+$T cell activation when compared with other APCs (Figure 12B). Variations in CD62L and CD95 expressions on activated T cells primed by different APCs were presented in this study. CD62L or L-selectin is an adhesion molecule functioning as lymphocyte's homing receptor to 2^{nd} lymphoid compartment highly expressed on naïve and central memory T cells (Nicholson, 2002). During early T cell's response to both conventional antigen and SEB, CD62L downregulation is needed for releasing the cells from recirculation among lymph nodes to effector site (Berg et al., 1989; Cauley et al., 2000; Rajagopalan et al., 2006). CD95 upregulation is also another common sign during T cell activation (Arens et al., 2005; Slifka and Whitton, 2000) and regarded as an important apoptosis mediator of activated T cells (Slifka and Whitton, 2000). While SEB presentation by DCs downregulated CD62L but upregulated CD95 expressions on activated T cells, SEB presentation by B cells resulted in high CD62L detainment and poor CD95 upregulation (Figure 12A and 12B). Interestingly, these phenotypes were shared with activated $CD4^+$T cells primed by naïve B cells previously reported in transgenic mouse studies (Croft et al., 1997; Reichardt et al., 2007b).

After activation in a particular milieu, naïve $CD4^+$T cells can differentiate into T helper (T_H) cells (T_H1, T_H2 or T_H17), inducible regulatory T cells, or anergic T cells after several cell generations (Zhu et al.). As described in introduction, this differentiation is already determined since early state of T cell response (Ivars, 2007; Nakata et al., 1995; Vella et al., 1995). As early as 68-hour of culture, limited IL-2, IFN-γ and IL-4 productions of T cells induced by SEB-presenting naïve B cells were presented (Figure 13). Limited production of these cytokines from early activated T cells is generally used to predict their later anergic T cell's generation (Fathman and Lineberry, 2007; Reichardt et al., 2007a). According to this notice, the acquired result thus implied later anergic T cell

differentiation by naïve B cell's SEB presentation. This notice thus supported tolerance inducing role of naïve B cells during bacterial superantigen response (Ashour and Seif, 2007; Raimondi et al., 2006a; Stark Aroeira et al., 1997).

Since a study in transgenic mouse also demonstrated that naïve B cell antigen presentation was able t o induce regulatory T cell differentiation directly from naïve $CD4^+$T cell (Reichardt et al., 2007b), we thus determined the suppressive function of activated $CD4^+$T cells primed by SEB presented naïve B cells (Reichardt et al., 2007b). Activated $CD4^+$T cells primed by SEB presented naïve B cells show no significant suppressive function (Figure 14). The data thus suggested that regulatory T cell's differentiation could not be induced by the SEB presentation system used in this study.

When compared the culture system of the current study with that previously reported in transgenic mouse (Reichardt et al., 2007b), several variations in factors that could affect regulatory T cell differentiation were presented. One of these factors is the nature of immune response against SEB, itself. Though SEB was reported to successfully induce regulatory T cell differentitation, most of them were actually achieved *in vivo* or ex *vivo* within 1-2 week culture period (reviewed in Ivars, 2007). The lack of both *in vivo* complexity and adequate culture time may partially contribute to unsuccessful regulatory T cell induction. In addition, some intrinsic differences between peptide antigen and SEB may also affect consequent T cell differentiation. While peptide pulsation weakly triggered APCs, SEB pulsation resulted in strong signaling and even stimulate TNFα/IL-1β production of the cells (Kissner et al., ; Mooney et al., 1990) which thus would consequently affect regulatory T cell differentiation (Collins and Oldham, 1995; Curtsinger and Mescher, ; Del Prete et al., 1994; Freedman et al., 1988; Lens et al., 1996; Nambu and Nakae). However, these modulatory effects need further investigation in such SEB pulsation system.

Source of naïve B cell may also affect their priming properties. The naïve B cell isolation of this study relied on depletion of non-naïve B cells, and regarded CD27 as the most important marker. Though peripheral $CD27^-$B cells are universal human naïve B cell representatives (Agematsu et al., 1997; Crotty et al., 2004; Klein et al., 1998), recent

detailed characterization has revealed heterogeneity of this population. Transitional B cells and intermediate B cells of which shared several phenotypes have been identified among peripheral $CD27^{-}$B cell population (Bofill et al., 1985; Campana et al., 1985; Lee et al., 2009; Palanichamy et al., 2009; van Zelm et al., 2007). This suggested us the presence of these cell types among isolated naïve B cells used in this study. On the contrary, mouse naïve B cells were commonly referred to resting splenic B cells isolated from naïve mice (Croft et al., 1997; Evans et al., 2000; Reichardt et al., 2007b). Though contaminationed memory B cells is extremely limited (<1%), considerable proportions of transitional B cells, type-1 B cells and marginal zone B cells (10-30%) are common in resting splenic B cells (Pape et al., ; Pers et al., 2002; Robert C. Hsueh, 2002; van Zelm et al., 2007). Presences of both type-1 and marginal zone B cells can affect the differentiation of naïve $CD4^{+}$T cells, since the cells are able to to enhance regulatory T cell differentiation, at least by their IL-10 production (Bouaziz et al., 2008; Lund and Randall, ; Mauri and Ehrenstein, 2008; Mizoguchi and Bhan, 2006; Reichardt et al., 2007b; Yanaba et al., 2008). In addition, possible intrinsic differences between peripheral and splenic naïve B cells should also be considered. Splenic microenvironment normally provides numerous differentiation factors which are absence from peripheral blood (Pillai and Cariappa, 2009; Thomas et al., 2006). The differences in modulatory factors should thus directly affect splenic naïve B cell's immunoproperty including its APC function. However, the knowledge about possible variations in characteristics among naïve B cells of different sources is still very limited in human.

The current experiment demonstrated that *in vitro* SEB presentation by naïve B cells cannot induce regulatory T cell differentiation directly from naïve $CD4^{+}$T cells. However, possible tolerance induction is implied by ineffectiveness of naïve B cells to prime effector phenotypes on T cells. Several factors can contribute to the different outcome of T cell differentiation in this study when compared to that performed in transgenic mouse model. Since these factors were known to affect various outcomes of T cell differentiation, these issues needed further investigation to make us deeply understand the mechanisms of naïve B cell priming role in both human and mouse

species. Among these possible contributors, we are particularly interested in possible variation in intrinsic immunoproperties among naïve B cells isolated from various organ sites. Due to the lack of biological knowledge and ethical limitation for such a study, we thus tried to approach this issue by applying bioinformatic metaanalysis in the next experiment.