รายงานวิจัยฉบับสมบูรณ์

การศึกษาลักษณะและการทำงานของเดนไดรติกเซลล์ในผู้ป่วยเบาหวาน

โดย ดร.พญ. จารุภา สูงสถิตานนท์ และคณะ

พฤษภาคม 2554

สัญญาเลขที่ MRG5100810

รายงานวิจัยฉบับสมบูรณ์

การศึกษาลักษณะและการทำงานของเดนไดรติกเซลล์ในผู้ป่วยเบาหวาน

ผู้วิจัย	สังกัด
1. ดร.พญ. จารุภา สูงสถิตานนท์	มหาวิทยาลัยมหิดล
2. พญ. อภิรดี ศรีวิจิตรกมล	มหาวิทยาลัยมหิดล
3. นพ. วิศิษฏ์ ทองบุญเกิด	มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project Code : 5180010

Project Title : Phenotype and function of peripheral blood-derived dendritic cells in diabetic patients

Investigator : Jarupa Soongsathitanon, M.D., Ph.D.
Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University

E-mail Address : sijrt@mahidol.ac.th

Project Period : 15 May 2008 – 14 May 2010

Abstract:

Atherosclerosis and cardiovascular disease represent a leading cause of death in diabetes mellitus. Prolonged hyperglycemia gives rise to pathological lesion of blood vessel. Dendritic cells were evidenced to be present in atherosclerotic plaque and may be involved in the pathogenesis of atherosclerosis in diabetes. This study was designed to investigate the differences in phenotype and function of monocyte-derived dendritic cells in patients with different level of glycemic control and cells under exposure to hyperglycemic condition *in vitro*. Expression of marker of activation, CD80 and CD86, was compared between diabetic patients with good glycemic control, poor glycemic control, and healthy subjects. CD80 expression was significantly increased in patients with poor glycemic control in comparison to patients with good control and healthy subjects, while CD86 expression was higher in diabetic patients, with either poor or good glycemic control. Expression of CCR2, a receptor responsive to monocyte chemoattractant protein-1 that recruits inflammatory cells into atherosclerotic lesion, was also determined and showed a comparable level between diabetic patients and non-diabetic controls. *In vitro* exposure to high glucose and advanced glycation end products did not change the expression of CD80, CD86, and CCR2 in monocyte-derived dendritic cells. However, high glucose condition enhanced ubiquitin level in peripheral blood mononuclear cells which is associated with the inflammatory state in diabetes.

Keywords : diabetes mellitus, atherosclerosis, dendritic cell, hyperglycemia, advanced glycation end product

บทคัดย่อ

รหัสโครงการ : 5180010

ชื่อโครงการ : การศึกษาลักษณะและการทำงานของเดนไดรติกเซลล์ในผู้ป่วยเบาหวาน

ชื่อนักวิจัย : ดร.พญ. จารุภา สูงสถิตานนท์

ภาควิชาวิทยาภูมิคุ้มกัน คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

E-mail Address : sijrt@mahidol.ac.th

ระยะเวลาโครงการ : 15 พ.ค. 51 – 14 พ.ค. 53

บทคัดย่อ:

ภาวะหลอดเลือดแข็งและโรคระบบหัวใจและหลอดเลือด เป็นสาเหตุการตายอันดับต้นๆในผู้ป่วยโรคเบาหวาน ภาวะน้ำตาลในเลือดสูงนานๆทำให้เกิดรอยโรคที่หลอดเลือดได้ การศึกษาอื่นๆในภาวะหลอดเลือดแข็ง พบเดนไดรติกเซลล์จำนวนมากในรอยโรค และมีรายงานว่าเดนไดรติกเซลล์อาจเกี่ยวข้องกับกลไกการเกิดภาวะนี้ โครงการวิจัยนี้ทำขึ้นเพื่อศึกษาความแตกต่างระหว่าง phenotype และการทำงานของเดนไดรติกเซลล์ ระหว่างผู้ป่วยเบาหวานที่มีการควบคุมน้ำตาลได้ดีและไม่ดี และคนปกติ นอกจากนี้ยังศึกษาผลของภาวะน้ำตาลสูงต่อ phenotype และการทำงานของเดนไดรติกเซลล์ด้วย การแสดงออกโมเลกุลที่นำมาเปรียบเทียบกันคือ โมเลกุล CD80 และ CD86 ซึ่งเป็นตัวบ่งชี้ภาวะกระตุ้นในเดนไดรติกเซลล์ และ CCR2 ซึ่งเป็นตัวรับของไซโตคายน์ที่สำคัญในการดึงดูดเซลล์เข้าไปในผนังหลอดเลือดขณะเกิดภาวะหลอดเลือดแข็ง ผลการทดลองพบว่า CD80 สูงขึ้นในผู้ป่วยที่มีการควบคุมน้ำตาลไม่ดี ส่วน CD86 นั้นพบสูงขึ้นในผู้ป่วยเบาหวานทั้งที่ควบคุมน้ำตาลดีและไม่ดีเทียบกับคนปกติ แสดงให้เห็นถึงภาวะกระตุ้นของเดนไดรติกเซลล์ในผู้ป่วยเบาหวาน สำหรับโมเลกุล CCR2 ไม่มีความแตกต่างกันระหว่างผู้ป่วยและคนปกติ ในการทดลองซึ่งเดนไดรติกเซลล์ถูกเลี้ยงในภาวะน้ำตาลสูงหรือเติมสารโปรตีนซึ่งเป็น advanced glycation end products พบว่าระดับของโมเลกุลทั้ง CD80, CD86 และ CCR2 ไม่มีความแตกต่างกับกลุ่มที่เลี้ยงในน้ำตาลระดับปกติ เมื่อทำการเลี้ยงโมโนนิวเคลียร์เซลล์ในภาวะน้ำตาลสูงพบว่า เซลล์มีการแสดงออกของ ubiquitin เพิ่มขึ้นซึ่งโปรตีนนี้มีความสัมพันธ์กับภาวะอักเสบในเบาหวาน

คำหลัก : เบาหวาน, ภาวะหลอดเลือดแข็ง, เดนไดรติกเซลล์, น้ำตาลในเลือดสูง

หน้าสรุปโครงการ (Executive Summary)

Diabetes mellitus is a growing worldwide medical and public health problem. In addition to the disease itself, the co-morbidities which add the burden of medical care are long-term complications due to poor glycemic control. These complications include microvascular and macrovascular complications, such as vascular damage at kidney or retina, atherosclerosis, and cardiovascular diseases (CVD). Atherosclerosis and CVD represent a leading cause of death in diabetes. Prolonged high blood glucose level will give rise to pathological lesion of blood vessel. Mechanisms that are involved in the pathogenesis of atherosclerosis include various types of cells, such as endothelial cells, vascular smooth muscle cells, and inflammatory cells. Among these, dendritic cells have been evidenced to present in atherosclerotic plaque. By studying the behavior of dendritic cells in diabetes would provide knowledge of how dendritic cells may play role in the pathogenesis of atherosclerotic complication in diabetes and might lead to the development of new strategies to prevent and treat such complication.

Ten type 2 diabetic patients [5 patients with good control (HbA1C ≤ 7%) and 5 patients with poor control (HbA1C > 9%)] who were diagnosed according to the ADA criteria will be recruited from the Diabetes Clinic of Siriraj Hospital. Subjects included the one with age ranging from 35-65 years. Both men and women are included. Five non-diabetic control subjects will be healthy volunteers Subjects who have major organ diseases or take medications affecting immune function will be excluded.

To determine phenotype and function of peripheral blood-derived dendritic cells, blood will be collected from all subjects for mononuclear cell isolation. Monocyte-derived dendritic cells will be propagated in order to study phenotype and function. Phenotype of cells will be determined by detection of cell surface markers by flow cytometry. Function of cells will be evaluated by measuring cytokine production in culture media. Dendritic cell phenotype and function will be compared between well controlled diabetic, poorly controlled diabetic and normal subjects.

Peripheral blood mononuclear cells (PBMCs) will be isolated from peripheral blood of healthy subjects. Cells will be allowed to short termly incubate with high glucose or advanced glycation end products (AGEs), which is AGE-BSA. Then cells will be lysed and preceed to proteomic study.

วัตถุประสงค์

1. To determine differences in the phenotype and function of peripheral blood-derived dendritic cells of type 2 diabetic patients with good or poor glycemic control.
2. To determine the effect of exposure to high glucose and advanced glycation end products on proteome profiles of peripheral blood mononuclear cells.

วิธีทดลอง

Patients: Patients with type 2 diabetes, diagnosed according to the American Diabetes Association practice guidelines for the diagnosis of diabetes, are recruited from the Diabetes Clinic of Siriraj Hospital.

Patients will be divided into 2 groups; well controlled and poorly controlled, according to the level of glycosylated hemoglobin (HbA1C). Patients with HbA1C ≤ 7% are designated as well controlled. Patients with HbA1C > 9.0% are designated as poorly controlled.

Inclusion criteria

- Patients diagnosed as type 2 diabetes regardless of time of diagnosis
- Patients age between 35-65 years old
- Patients with HbA1C ≤ 7% and HbA1C > 9.0%
- Patients without other major organ diseases

Exclusion criteria

- Patients taking any medication known to affect immune function, i.e. immunosuppressive agents, anti-inflammatory drugs, cytokines, and some hormones.
- Patients receiving any blood products or immunoglobulins within 6 months prior to enrollment.
- Patients with impaired renal function, poorly controlled blood pressure, and abnormal lipid profile.
- Patients with a history of clinically significant heart disease, peripheral vascular disease, or pulmonary disease.

Controls:

Criteria for selection of normal controls

- Have no immunocompromised conditions

- Do not take any immunosuppressive agents or drugs that affect immune function.
- Women are not pregnant or lactating
- Are not in post-operative recovery period
- Do not have infection, inflammatory diseases, malignancy, or malnutrition

Subjects will be 35-65 years of age. Both men and women are included.
Number of subjects in each group is 5.

Methods:

1. **Blood collection.** At more than 2 hours after meal, thirty milliliters of peripheral venous blood of patients and control subjects were collected into a sterile tube containing EDTA.

2. **Isolation of peripheral blood mononuclear cell (PBMC).** PBMCs were separated from freshly collected whole venous blood by a Histopaque density gradient centrifugation. Blood was diluted 1:2 in PBS layered over Histopaque 1077 and centrifuged for 30 minutes at 2000 rpm at room temperature. The interface was recovered and washed 3 times in PBS.

3. **Propagation of monocyte-derived dendritic cell.** PBMCs wre seeded in culture plate at a density of $2.5x10^6$ cells/ml and were cultured for 6 days in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 ng/ml GM-CSF and 50 ng/ml IL-4 and incubated at 37°C in a humidified atmosphere with 5% CO_2.

4. **Phenotyping of dendritic cells by flow cytometry.** Cell surface molecules were identified by flow cytometry in FACSCalibur flow cytometer. For each analysis, $1x10^6$ cells were stained with the following monoclonal antibodies: Lineage cocktail 1 (CD3/CD14/CD16/CD19/CD20/CD56)-FITC, HLA-DR-PerCP, CD11c-PE, CD80-PE, CD86-PE and CCR2-APC. Mouse isotype-matched Ig is used as negative control. Data were collected for 50,000 cells/sample.

5. **Quantification of cytokine production.** The culture media were harvested for cytokine assay and stored at -70°C until used. The levels of multiple cytokines including IL-1β, IL-6, and TNF-α were measured employing Luminex assay according to the manufacturer's instructions.

6. ***In vitro* incubation of PBMCs.** PBMCs were seeded in T25 flask at a density of $5x10^6$ cells/ml. Cells were incubated for 24 hours in complete media containing 5 mM glucose (normal glucose), 25 mM glucose (high glucose), or 25 mM glucose plus 100

ug/mL AGE-BSA at 37□C in 5% CO_2 humidified atmosphere for 24 hours. After 24 hours of incubation, the adherent cells were detached by physical method using a pasture pipette to draw the culture medium up and down until all cells were detached. Thereafter, the cells were harvested by centrifugation at 1000 rpm for 5 min at 4□C and then washed with PBS 4 times to avoid contamination of proteins from bovine serum supplemented into the culture medium. Then the pellets were collected and preceded to protein extraction.

7. Two-dimensional gel electrophoresis and proteomic analysis. Cell proteins were extracted by 2D lysis buffer and total protein concentration was measured. Seven-cm-long, pH 3 to 10 non-linear strips were rehydrated for 18 h with rehydration buffer containing 150 µg of proteins. After 18 h of rehydration, the strips were submitted to isoelectric focusing (IEF). Then the strips were proceed to reduction in equilibration buffer I (containing dithiothreitol; DTT) and equilibration buffer II (containing iodoacetic acid; IAA) and then applied to the SDS-PAGE. SDS-PAGE was performed on 13% polyacrylamide gels and electrophoresis was carried out until the dye front reached the lower end of the gel. Protein spots were visualized by Deep purple staining. Deep purple stained gels were scanned with a Typhoon 9200 at 100 dpi resolution and the Tiff images generated were analyzed with Image Master 2D software (Amersham Bioscience). The individual gels were compared by the program. The different spots between each subject group were picked to identify.

8. Statistical analysis. Comparison between groups was done by using Student's *t* test. Multiple comparisons among groups were done by using ANOVA. P-value less than 0.05 was considered statistically significant. All data are reported as mean ± SEM.

ผลการทดลอง

1. Characterization of monocyte-derived dendritic cells

Dendritic cells were derived from peripheral blood monocytes of healthy subjects by separating peripheral blood mononuclear cells (PBMCs) from whole blood by Ficoll-Hypaque density gradient. PBMC were cultured in 6-well tissue culture plate and monocytes were selected by plastic adherence. Cells were incubated at 37°C for 3 hours to allow monocytes to adhere with bottom of the plate. Then the plates were wash to remove non-adherent cells, which are lymphocytes, and adherent cells were cultured in complete media supplemented with GM-CSF และ IL-4 to drive monocytes to become dendritic cells.

Phenotype of dendritic cells was characterized by determination of cell surface markers by immunofluorescence flow cytometry. Dendritic cells are cells that have negative lineage marker (CD3, CD14, CD16, CD19, CD20, CD56), positive HLA-DR and positive CD11c.

Cells were selected according to size and granularity (Figure 1) and then to surface markers. Cells with lineage-negative and HLA-DR-positive (Figure 2) were gated and determined for CD11c expression. Number of HLA-DR^{+}, CD11c^{+} cells were approximately 100% of gated cells (Figure 3) or 10-30% of total cells.

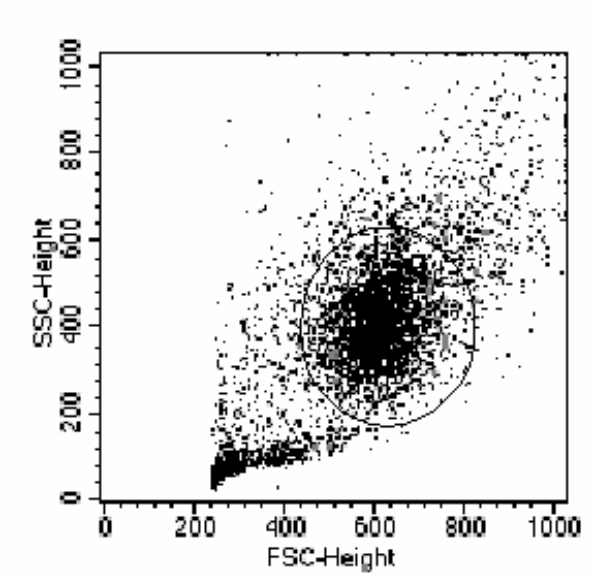

Figure 1. Cell selection from size and granularity

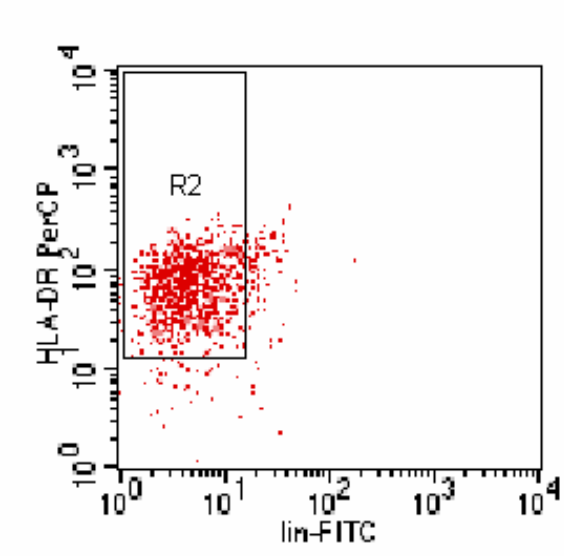

Figure 2. Lineage-negative, HLA-DR-positive cells

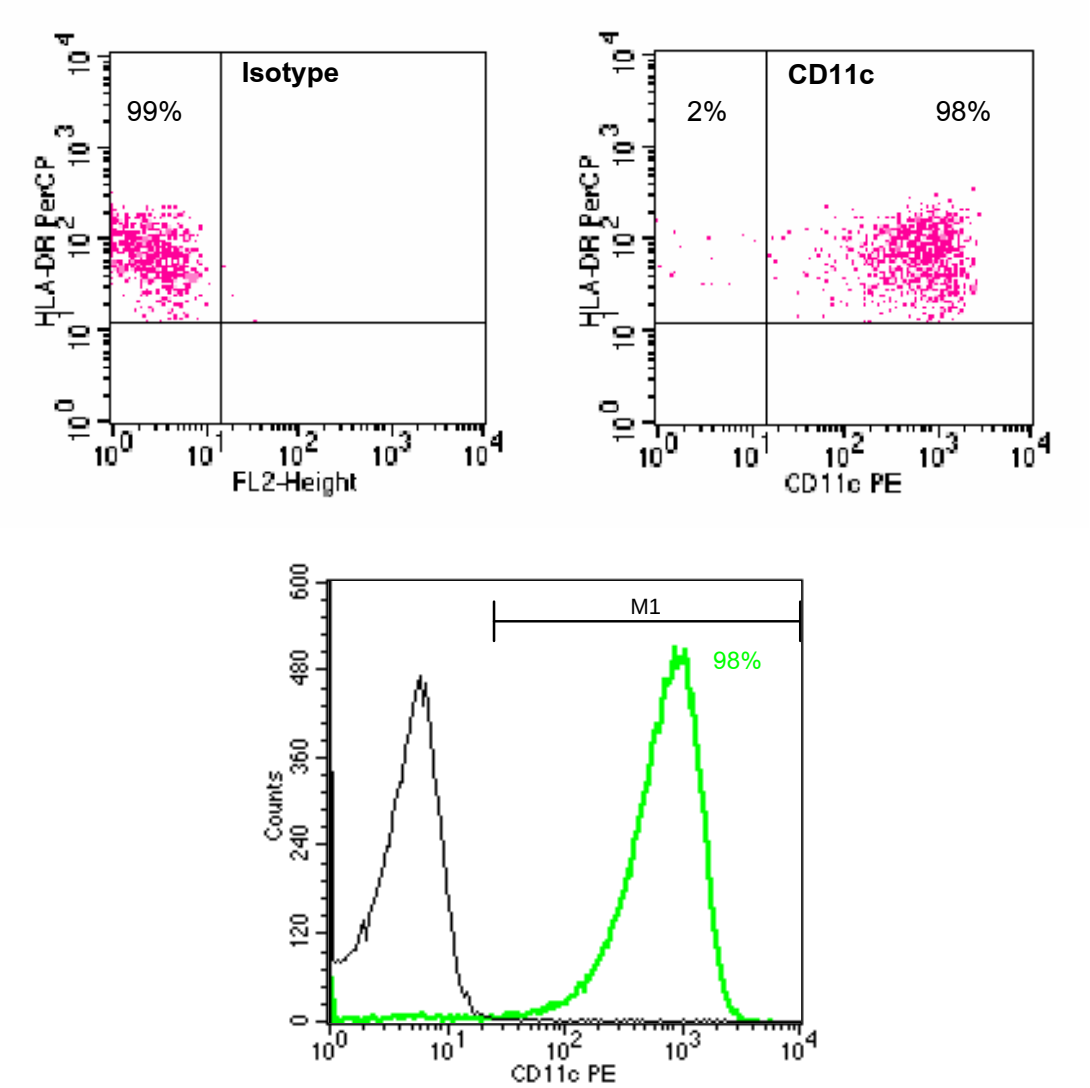

Figure 3. HLR-DR-positive, CD11c-positive cells

Activation markers, CD80 and CD86, were determined and showed that 78% of immature dendritic cells expressed CD80 while 74% of cells expressed CD86, ranging from low to high intensity (Figure 4). When monocyte-derived dendritic cells were stimulated with LPS, cells were more heterogeneous in size of cells and granularity. Upon LPS stimulation, CD80 expression increased from 87% to 99% and mean fluorescence intensity (MFI) was higher (Figure 5). CD86 also increased from 80% to 97% (Figure 6). For HLA-DR expression, percentage of cells expressed HLA-DR and MFI was higher (Figure 7).

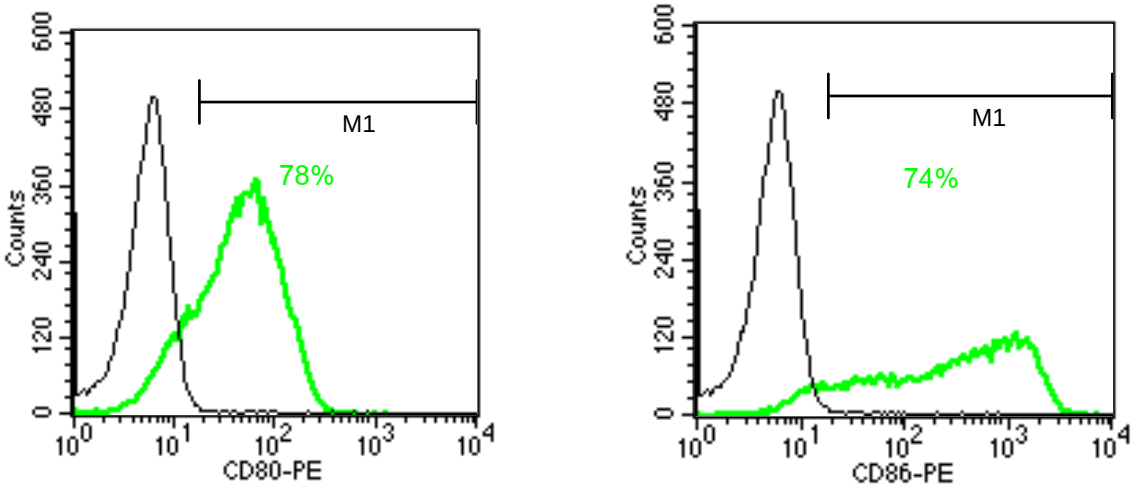

Figure 4. CD80 and CD86 expression on monocyte-derived dendritic cells

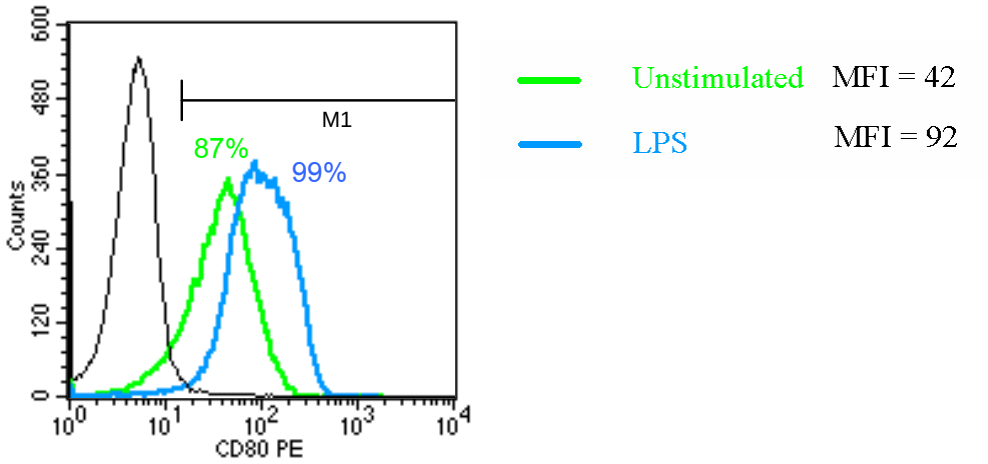

Figure 5. CD80 expression on LPS-stimulated monocyte-derived dendritic cells

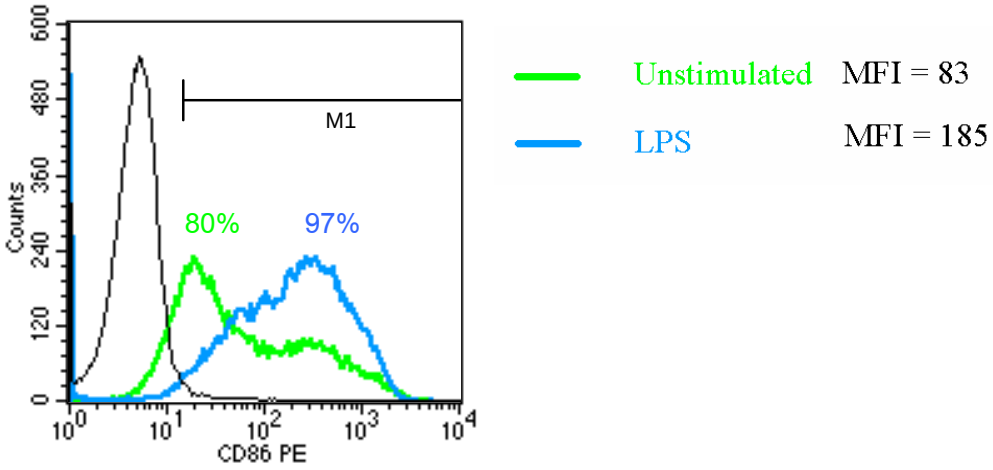

Figure 6. CD86 expression on LPS-stimulated monocyte-derived dendritic cells

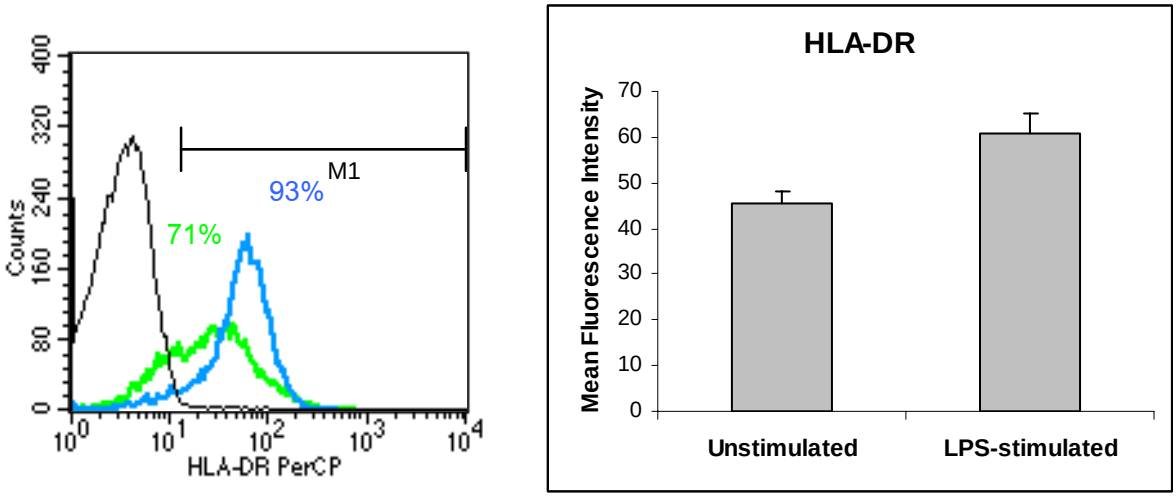

Figure 7. HLA-DR expression on monocyte-derived dendritic cells

CCR2 is a chemokine receptor that plays role in the recruitment of monocyte/dendritic cell to atherosclerotic lesion. This molecule has been shown to express at high level in monocytes of diabetic patients. CCR2 is also expressed on immature dendritic cell, therefore, in the conditions that prone to atherosclerosis formation, such as hyperglycemia, expression of CCR2 on dendritic cell may be affected. In monocyte-derived dendritic cells from healthy subjects, no CCR2 expression was detected on cells (Figure 8).

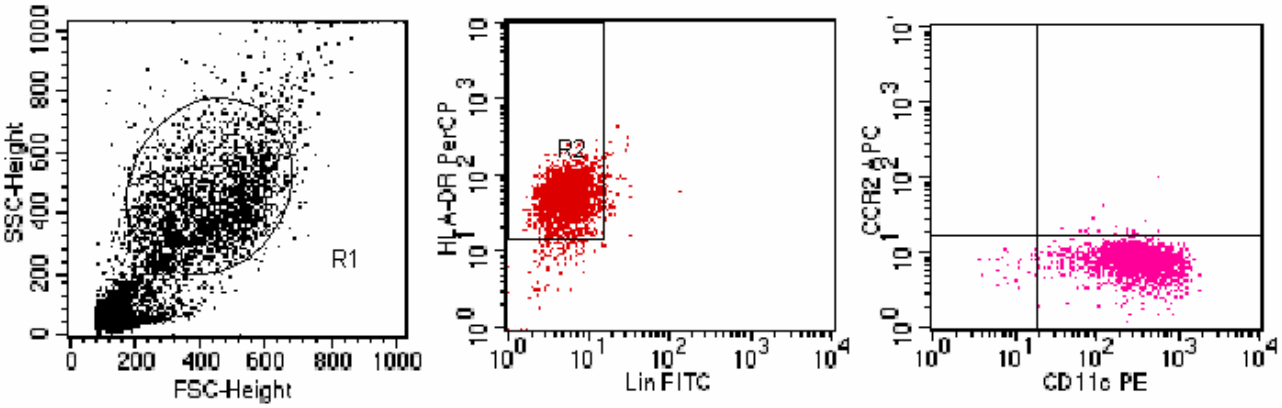

Figure 8. CCR2 expression on monocyte-derived dendritic cells

2. Comparison of phenotype and function of monocyte-derived dendritic cell between patients with good glycemic control and poor glycemic control

To compare phenotype and function of monocyte-derived dendritic cells between patients with good and poor glycemic control and non-diabetic subjects, cells

were isolated from patients and investigated for surface marker expression. Table 1 shows mean blood glucose level and HbA1C in each group.

Table 1. List of altered protein in PBMCs cultured in different conditions

	Non-diabetic	Good glycemic control	Poor glycemic control
Age	48	51	57
FBS (mg/dl)	88	123	188
HbA1C (%)	NA	7.0	10.4

NA = Not applicatble

Flow cytometry results showed that CD80 expression was significantly increased in poorly glycemic controlled patients in comparison to well glycemic controlled patients and healthy subjects (Figure 9; # = $p < 0.05$). CD86 expression was higher in diabetic patients, with either poor or good glycemic control, compared with non-diabetic subjects (Figure 10; ∗ = $p < 0.05$). CCR2 expression was at the same levels among three groups (Figure 11).

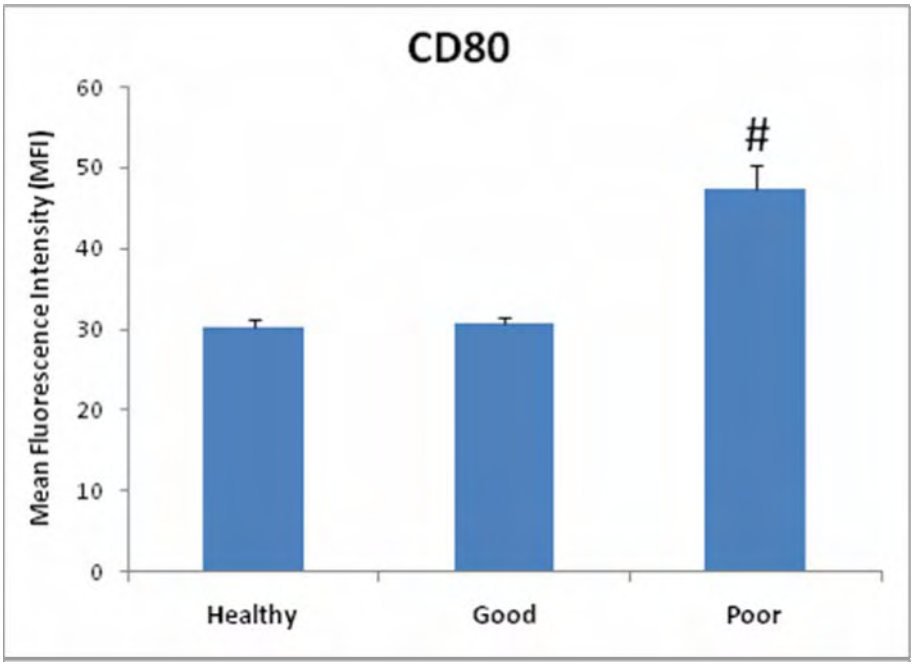

Figure 9. CD80 expression on dendritic cells of patients with good glycemic control, poor glycemic control and healthy subjects

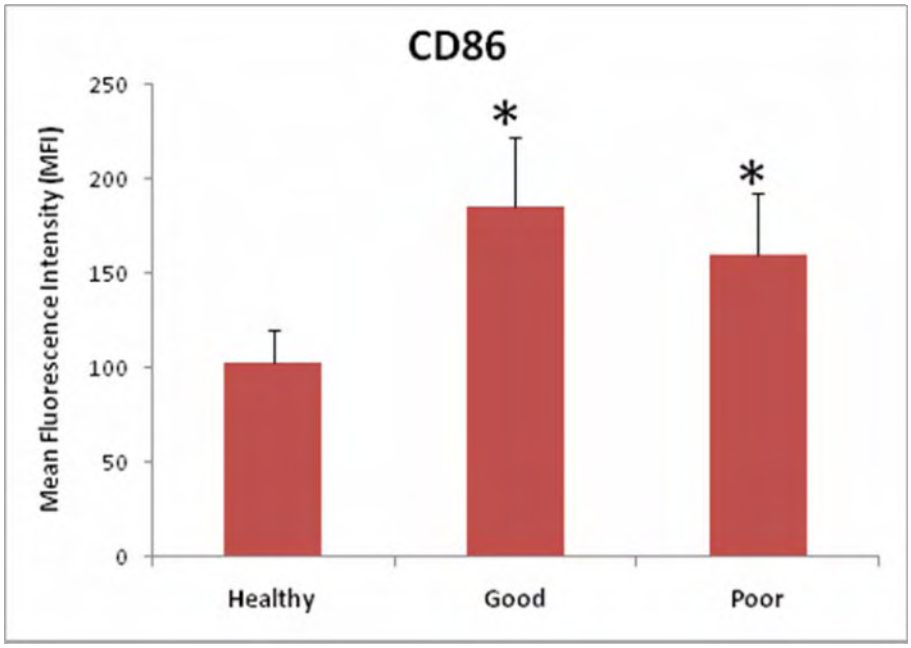

Figure 10. CD86 expression on dendritic cells of patients with good glycemic control, poor glycemic control and healthy subjects

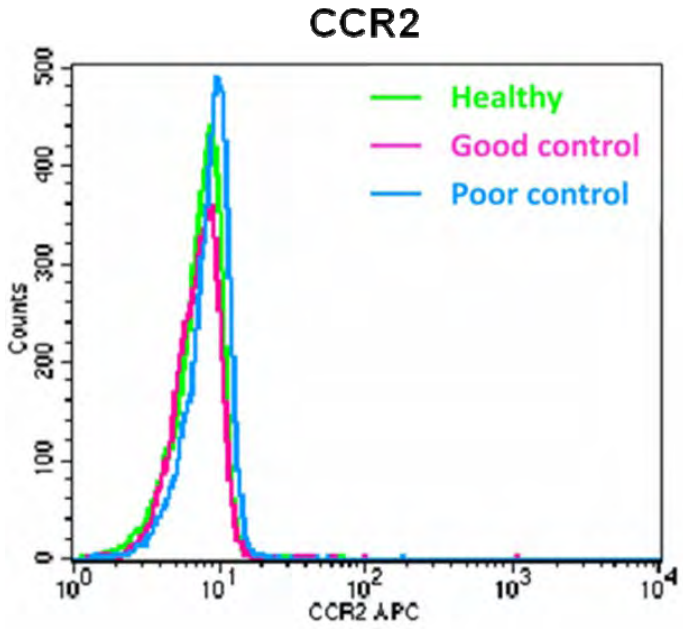

Figure 11. CCR2 expression on dendritic cells of patients with good glycemic control, poor glycemic control and healthy subjects

3. *In vitro* study of the effect of hyperglycemia on dendritic cells and peripheral blood mononuclear cells (PBMCs)

3.1 Effect of high glucose and AGEs on monocyte-derived dendritic cell

When effect of high glucose on dendritic cells was investigated, cells were incubated in media containing 25 mM glucose compared with 5 mM glucose (physiologic glucose level). CD80, CD86, and CCR2 expression on dendritic cells was comparable between the 2 groups (Figure 13 and 14). LPS was used as a positive control.

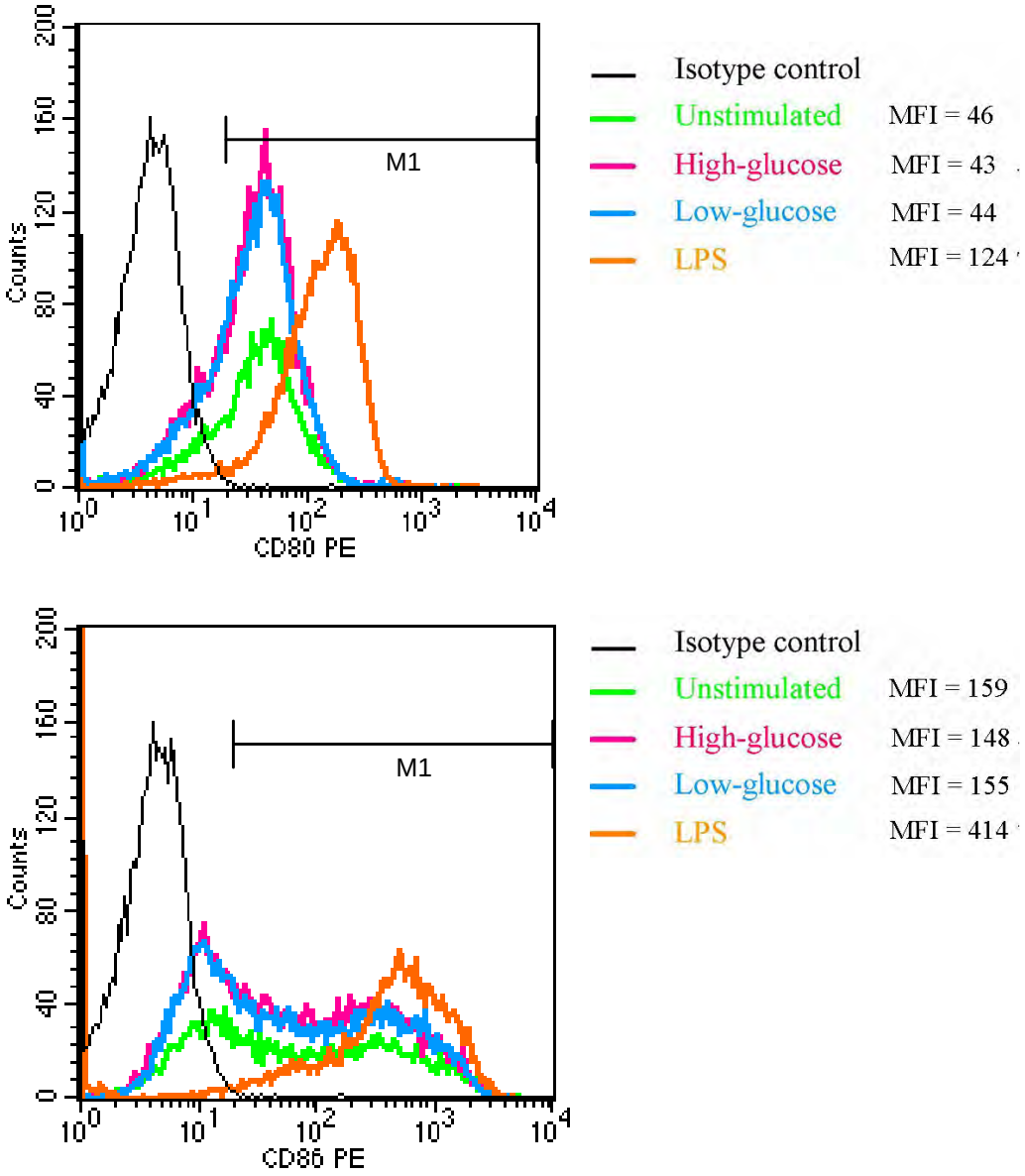

Figure 13. CD80 and CD86 expression of dendritic cells incubated with high glucose (25 mM) and normal glucose (5 mM)

As the signal of CCR2 expression was rather low, mean fluorescence intensity (MFI) was used to compare the level of expression between groups. There was no difference between normal glucose and high glucose exposure (Figure 14).

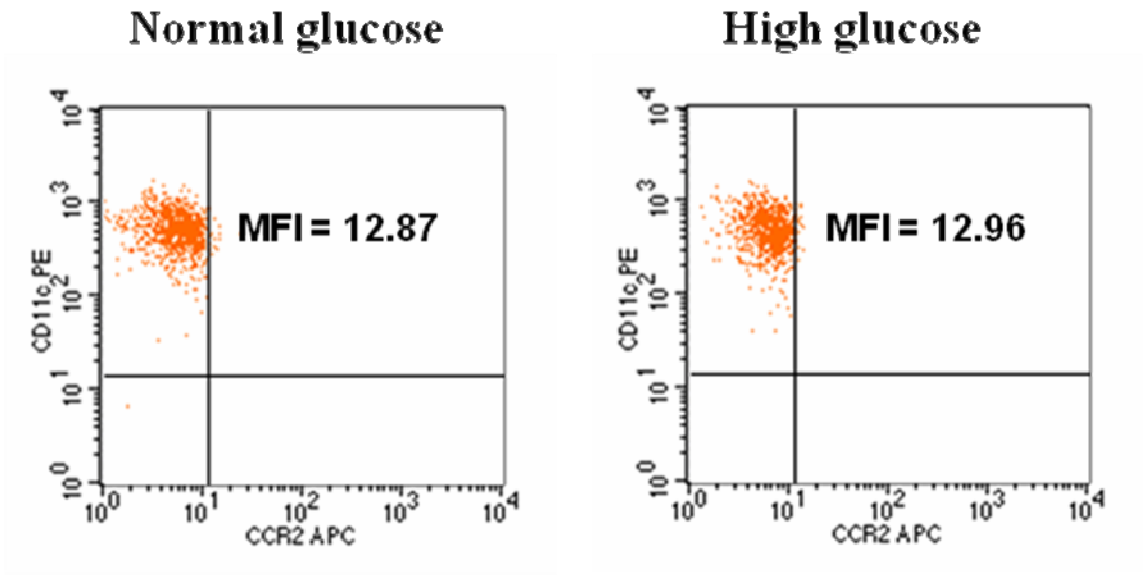

Figure 14. CCR2 expression of dendritic cells incubated with high glucose (25 mM) and normal glucose (5 mM)

Effect of AGEs, which is products of protein formed by prolonged hyperglycemia, on phenotype of dendritic cells was done by incubating cells with AGE-BSA for 24 hours. There was no change in the expression of CCR2 on cells exposed to short-term AGE-BSA (Figure 15.)

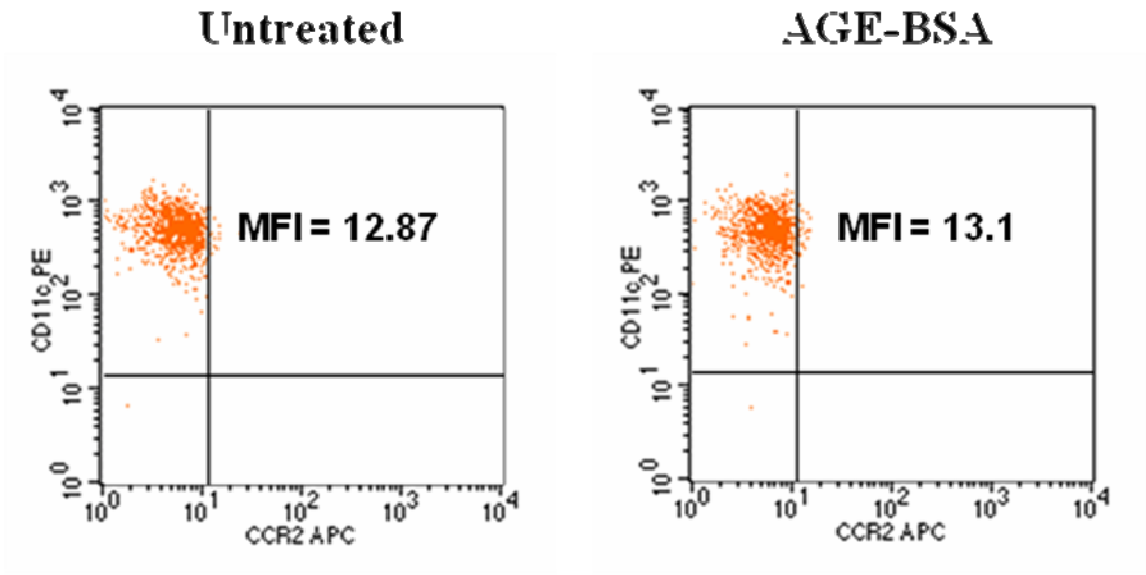

Figure 15. CCR2 expression of dendritic cells incubated with AGE-BSA

Cells incubated with AGE-BSA gave similar levels of CD80 and CD86 expression to cells incubated with BSA (Figure 16). LPS was used as a positive control.

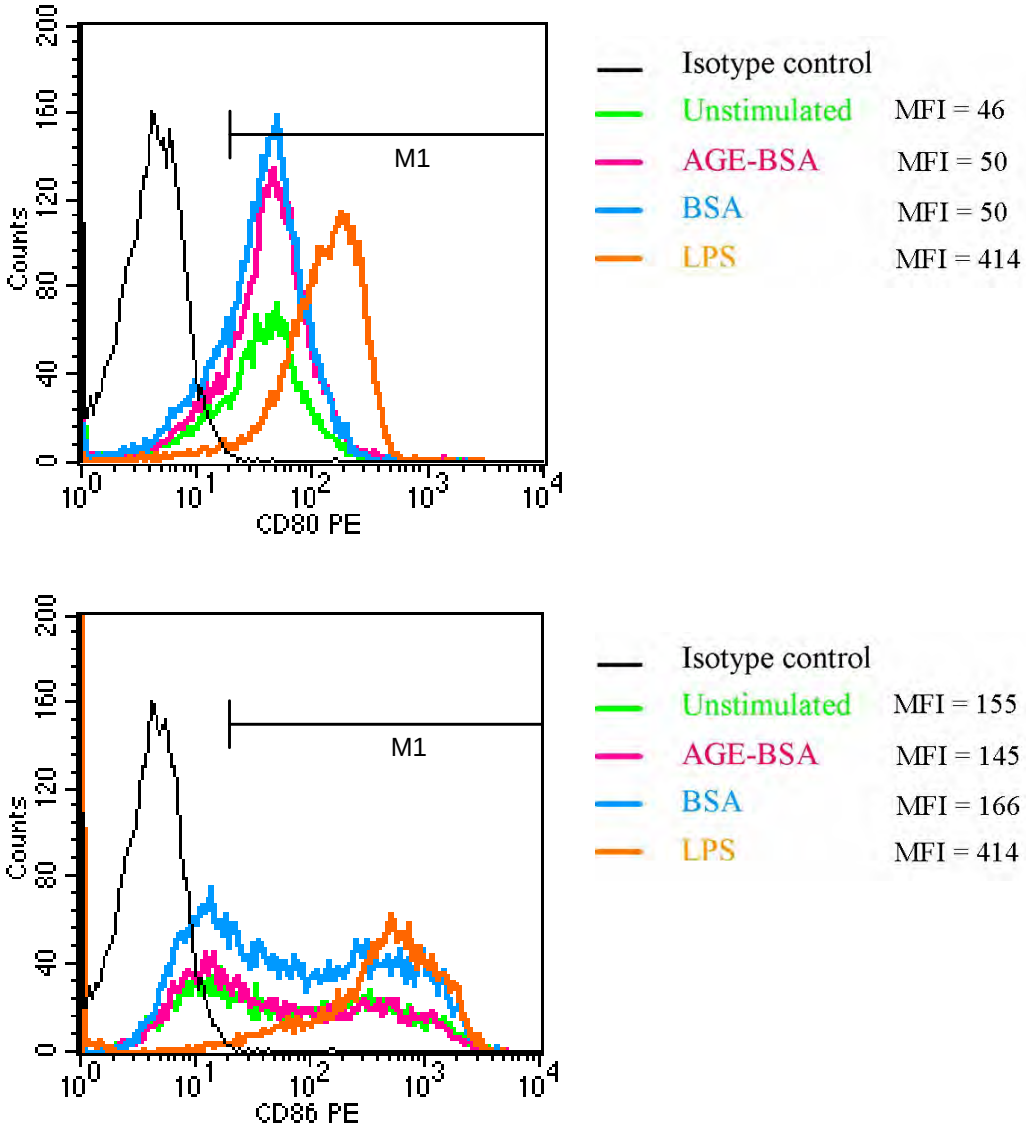

Figure 16. CD80 and CD86 expression of monocyte-derived dendritic cells incubated with AGE-BSA and BSA

3.2 Identification of differential proteomic expression in PBMCs cultured in different conditions

Since the yield and purity of monocyte-derived dendritic cells were poor when cells were harvested from the buffy coat of separated donated blood, PBMCs were used instead in the experiments subject to proteomic study. PBMCs were isolated from buffy coat and incubated in 3 different conditions at 37□C in 5% CO_2 humidified atmosphere for 24 hours. Then cells were harvested and cellular proteins were extracted for Two-

dimensional gel electrophoresis. Gels were stained with Deep purple staining to reveal protein spots.

NG = 5 mM glucose (normal glucose)

HG = 25 mM glucose (high glucose)

HG+AGE = 25 mM glucose plus 100 ug/mL AGE-BSA

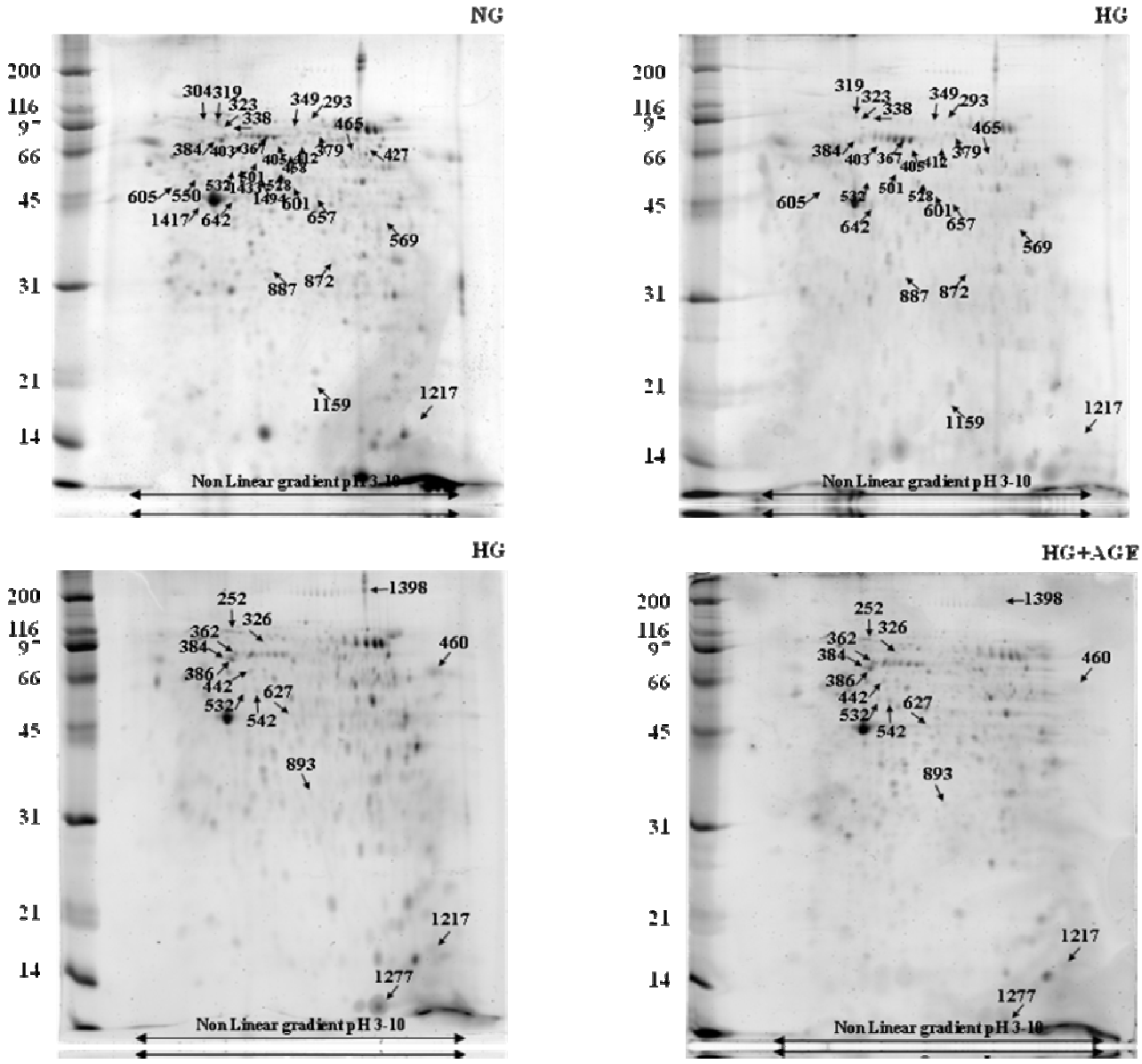

Figure 14. Protein spots on Deep purple stained gels from different conditions

Deep purple stained gels were scanned with a Typhoon 9200 at 100 dpi resolution and the Tiff images generated were analyzed with Image Master 2D software. The individual gels were compared by the program and list of altered protein was shown in Table 2. There were 31 spots different between normal glucose vs. high glucose, while 14 spots different were found between high glucose vs. high glucose plus AGE.

Table 2. List of altered protein in PBMCs cultured in different conditions

Spot no.	Protein name	NCBI ID	Identification scores (MS, MS/MS)	Ratio (Group2/ Group1)	*p* values
	NG vs HG				
293	L-arginine:glycine amidinotransferase	gi|47934917	69, NA	0.25	0.029
304	ATP/GTP binding protein-like 2	gi|149022584	70, NA	0.00	0.000
319	Adenylosuccinate lyase 1	gi|126338737	72, NA	0.27	0.021
323	Annexin A13	gi|149066347	67, NA	0.12	0.001
338	mCG145437	gi|148695953	76, NA	0.18	0.007
349	Calpain 14	gi|223468608	65, NA	0.11	0.005
367	KIAA1349 protein	gi|7243079	70, NA	2.04	0.029
379	WD repeat-containing protein 1 isoform 6	gi|114593199	90, 82	0.14	0.003
384	L-plastin isoform 1	gi|114651553	88, 159	0.57	0.043
403	T-box 3 (ulnar mammary syndrome), isoform CRA_b	gi|119618476	73, NA	0.12	0.007
405	Chain A, Crystal Structure Of The Human 70kda Heat Shock Protein 1a	gi|261825070	109, 97	0.17	0.006
412	Ubiquitin specific protease 28	gi|73955134	69, NA	0.10	0.001
427	Dynamin 1-like, isoform CRA_a	gi|149019701	63, NA	0.00	0.038
458	Serpin peptidase inhibitor, clade A, member 3 isoform 4	gi|114654581	77, NA	0.00	0.000
465	Immunoglobulin heavy chain variable region	gi|112699598	77, NA	0.50	0.024
501	Spindlin-like protein 2 (SPIN-2)	gi|27665034	73, NA	0.15	0.003
528	Immunoglobulin heavy chain variable region	gi|4234661	72, NA	0.27	0.026
532	rag1	gi|258546474	74, NA	0.49	0.037
550	Methyltransferase 10 domain containing	gi|149637021	64, NA	0.00	0.001
569	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	gi|149027822	74, NA	0.22	0.017
601	T-box 3, isoform CRA_a	gi|148687838	74, NA	0.62	0.046
605	ADP-ribosylation factor guanine nucleotide-exchange factor 2 (brefeldin A-inhibited), isoform CRA_a	gi|119596086	74, NA	0.15	0.017
642	Hypothetical protein	gi|109094285	67, NA	0.36	0.018
657	2-methylacyl-CoA racemase	gi|4995299	61, NA	0.25	0.008
872	PTK2B protein tyrosine kinase 2 beta isoform 3	gi|241982783	79, NA	0.17	0.001
887	NEDD4-binding protein 1 (N4BP1)	gi|109462432	72, NA	0.13	0.000
1159	Transmembrane protein 2 (Identities 100%, Gaps 0%)	gi|55957834	73, NA	0.12	0.006
1217	Ubiquitin-like protein 4	gi|74009181	60, NA	5.62	0.004
1417	Immunoglobulin heavy chain variable region	gi|37777992	70, NA	0.16	0.023
1433	Polyamine modulated factor 1 binding protein 1	gi|19705509	71, NA	0.00	0.003
1494	Sorbin and SH3 domain	gi|194041761	70, NA	0.00	0.004
	HG vs HG+AGE				
252	Odam protein	gi|74221723	76, NA	2.05	0.039
326	Glutathione synthetase (Identities 100%, Gaps 0%)	gi|4504169	67, NA	3.58	0.033
362	Chain A, 70kd Heat Shock Cognate Protein Atpase Domain, K71m Mutant	gi|157831588	61, 46	1.88	0.019
384	L-plastin isoform 1	gi|114651553	88, 159	2.58	0.004
386	L-plastin isoform 2	gi|114651554	99, 129	1.81	0.015
442	Glycine C-acetyltransferase (Identities 99%, Gaps 0%)	gi|109094131	73, NA	1.76	0.021
460	Chain C	gi|7766942	83, 189	0.21	0.002
532	rag1	gi|258546474	74, NA	3.29	0.038
542	Anti-MSP1 MAD20 block2 ScFv Ig heavy chain variable region	gi|12836983	64, NA	2.46	0.025
627	Pyruvate kinase	gi|2623945	74, NA	1.47	0.034
893	Transmembrane protein 2 (Identities 100%, Gaps 0%)	gi|55957834	85, NA	2.19	0.014
1217	Ubiquitin-like protein 4	gi|74009181	60, NA	0.24	0.010
1277	S100 calcium-binding protein A8	gi|21614544	61, 56	0.16	0.015
1398	Dysferlin, limb girdle muscular dystrophy 2B (autosomal recessive)	gi|197102454	77, NA	0.33	0.014

NCBI = National Center for Biotechnology Information

%Cov = %Sequence coverage [(number of the matched residues/total number of residues in the entire sequence) x 100%]

NA = Not applicable

Mass spectrometric analyses revealed that ubiquitin-like protein 4 was upregulated in high glucose condition compared with normal glucose, while the protein was downregulated in the presence of AGE. This was confirmed in 2D Western blot, showing upregulation of ubiquitin in high glucose and downregulation of ubiquitin in high glucose plus AGE (Figure 15).

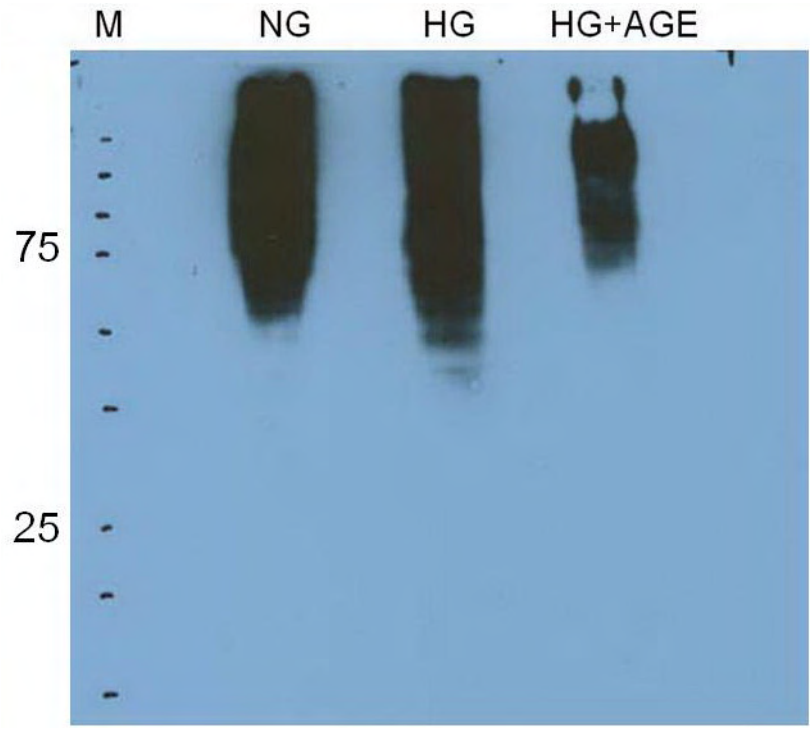

Figure 15. Western blots of ubiquitin protein in different conditions

สรุปและวิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต

1. Comparison of phenotype and function of monocyte-derived dendritic cell between patients with good glycemic control and poor glycemic control

CD80 and CD86 are markers of activation of dendritic cells. CD80 expression was significantly increased in patients with poor glycemic control in comparison to well controlled patients and healthy subjects, while CD86 expression was higher in diabetic patients, with either poor or good glycemic control. These results showed the activated state of dendritic cells in diabetic patients, especially the ones with poor glycemic control. This suggests that poorly controlled patients, which are predisposed to atherosclerosis formation, have activated dendritic cells and more inflammatory state which may lead to the pathogenesis of macrovascular complications.

CCR2 is a chemokine receptor for monocyte chemoattractant protein-1 (MCP-1) that plays role in the recruitment of monocyte/dendritic cell to atherosclerotic lesion. This molecule has been shown to express at high level in monocytes of diabetic patients. CCR2 is also expressed on immature dendritic cell, therefore, in the conditions that prone to atherosclerosis formation, such as hyperglycemia, expression of CCR2 on dendritic cell may be affected. In monocyte-derived dendritic cells, CCR2 expression was at the same levels among three groups. It is possible that the monoclonal antibody to CCR2 used in this study was not at optimal concentration and did not show the signal properly, as very low expression was found in healthy subjects (Figure 8). The use of other monocytic cell line, such as THP-1 cell, as a positive control may be useful to optimize the appropriate concentration of antibody. Moreover, interaction of other chemokine and chemokine receptor such as fractalkine and CX3CR1 has also been shown responsible for the recruitment of dendritic cells in vascular lesion.

2. Effect of high glucose and AGEs on markers of monocyte-derived dendritic cells *in vitro*

In vitro exposure of monocyte-derived dendritic cells to either high glucose condition or AGE compounds demonstrated a comparable expression of CD80, CD86, and CCR2 to normal glucose-treated or untreated cells. These results were obtained after 24 hours of incubation. Longer period of incubation with high glucose and AGEs may be required to see the differences, as in physiologic situation in diabetic patients cells were exposed to high glucose and AGEs for long time. However, when cells were added with high glucose or AGEs since day 3 of culture and allowed for incubation for 72 hours, there were still no differences in marker expression between groups. In the case that cells were incubated for longer than 1 week, the cells looked poor and died since we have found that primary monocyte-derived dendritic cells isolated from donated blood did not tolerate to longer period of culture.

3. Proteomic analysis

Differential expressions of cellular proteins of PBMCs under different conditions were investigated by proteomic analysis. In this experiment, cells were incubated with 5 mM glucose (normal glucose), 25 mM glucose (high glucose), or 25 mM glucose plus 100 ug/mL AGE-BSA for 24 hours. Deep purple staining reveals 31 spots different between normal glucose vs. high glucose and 14 spots different between high glucose vs. high glucose plus AGE.

Ubiquitin-like protein 4 was found upregulated in high glucose condition compared with normal glucose, while the protein was downregulated in the presence of AGE. This was confirmed in 2D Western blot, showing upregulation of ubiquitin in high glucose and downregulation of ubiquitin in high glucose plus AGE. This is consistent with other study showing that ubiquitin-proteasome system overactivity is associated with the inflammatory process of atherosclerosis in type 2 diabetic patients. The ubiquitin-proteasome activity was greater in atherosclerotic lesions of diabetic patients in comparison to lesions of non-diabetics, and the source of ubiquitin-proteasome system overactivity was macrophage. Therefore, in the proteomic analysis of PBMCs, we are able to show that ubiquitin was upregulated in high glucose condition. The explanation of the finding that ubiquitin was downregulated in the presence of AGEs in high glucose environment remains to be investigated.

Future plan:

1. Comparison of CX3CR1 expression on dendritic cells between diabetic patients with good glycemic control, poor glycemic control, and healthy subjects. CX3CR1 is a receptor for fractalkine which has been shown significant for dendritic cells to be recruited to atherosclerotic lesion. Diabetic patients with poor glycemic control, which are prone to diabetic vascular complications, may have higher level of CX3CR1. This would elucidate further the mechanism of how hyperglycemia affects dendritic cell in poor control diabetes which may develop atherosclerosis.
2. Expression of ubiquitin-proteasome system in dendritic cells under exposure to prolonged hyperglycemia. Ubiquitin-proteasome system has been shown related to oxidative stress and atherosclerotic lesion in diabetes. As the results showed that ubiquitin was upregulated in PBMCs in high glucose condition, it is interesting to know whether dendritic cells are the source of the change in ubiquitin-proteasome system.