Comparison of surface molecule expression and interleukin-12 secretion between human monocytes-derived dendritic cells treated with cytokines according to maturation time

Dae Suk Kim

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Min Geol Lee

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Dae Suk Kim

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This certifies that the Master's Thesis

of Dae Suk Kim is approved

Thesis Supervisor : Min-Geol Lee

Thesis Committee Member: Chein-Soo Hong

Thesis Committee Member: In-Hong Choi

The Graduate School

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watching your charming face have given me huge boosts! Love you all!

Thank the Lord for all these things and what you have prepared! Ebenezer!!

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<ABSTRACT>

Comparison of surface molecule expression and interleukin-12 secretion between human monocytes-derived dendritic cells treated with cytokines according to maturation time

Dae Suk Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Min Geol Lee)

Dendritic cells (DCs) play an important role in the generation and regulation of immune responses. Immature DCs are very effective in processing native protein antigens for the MHC class II restricted pathway. Mature DCs are specialized to stimulate resting T cells to grow and differentiate. Interleukin-12 (IL-12) is a crucial factor in the moment of dendritic cell/T cell interaction in the lymphoid organ. IL-12 acts on T cells and NK cells by inducing proliferation, enhancing cytotoxicity, and promoting IFN-γ production. It is most important cytokine for induction of Th1 cells. Currently most studies on DCs rely on the in vitro development of DC-like cells from CD34+ progenitor cells of blood monocytes. In our unpublished previous report, proper production of IL-12, not fully expressed molecules representing mature DCs, was optimal criteria of DCs for DCs immunotherapy in murine system. Therefore the purpose of this study is to investigate whether there is discordance among the expressions of surface molecules, intracytoplasmic IL-12

expressions and IL-12 secretion in human monocytes-derived DCs (MoDCs). In this study the optimal day, which DCs become phenotypically most mature, was day 7 and intracytoplasmic IL-12p40 expressions were also highest on day 7. The amounts of IL-12 secreted by mature DCs in this study were so small that they were excluded in data analysis. In conclusion even though the amounts of IL-12 secretions were too small, the pattern of surface molecules expressions and intracytoplasmic IL-12 expressions coincided and both were highest on day 7 in human monocytes-derived DCs. So we think sufficiently matured day 7 DCs are proper candidate to be used in DC immunotherapies.

Key words: dendritic cell, interleukin-12

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I. INTRODUCTION

Dendritic cells (DCs) are highly specialized professional antigen-presenting cells (APCs) usually located at surveillance interfaces of the human body such as the skin or mucosa, and specialized to generate and regulate immune responses[1,2]. At different stages of their development they display a different functional repertoire. Immature DCs are very effective in processing native protein antigens for the MHC class II

restricted pathway[3]. Mature DCs are less able to capture new proteins for presentation but are much better at stimulating resting CD4+ and CD8+ T cells to grow and differentiate[4]. The interactions of MHC/peptide and TCR and of costimulatory molecules with their counterreceptors lead to the activation of T cells that, in turn, results in their proliferation and cytokine synthesis. An additional crucial factor in the moment of dendritic cell/T cell interaction in the lymphoid organ is the cytokine interleukin-12 (IL-12). IL-12 is a cytokine composed of two disulfide-linked subunits of 35kD (p35) and 40kD (p40), which are encoded by two separate genes. These associate to form the bioactive heterodimer of 70 kD (p70)[5]. IL-12 p70 acts on T cells and NK cells by inducing proliferation, enhancing cytotoxicity, and promoting IFN-γ production. It is the most important cytokine for induction of Th1 cells and plays a major role in resistance to bacterial, viral, and parasitic infections, as wells as to tumors[6]. Moreover this heterodimeric cytokine critically regulates the balance between Th1 and Th2 responses[7]. Dendritic cells have repeatedly been shown to produce IL-12 both in an unstimulated state[8] and, in much larger amounts, when stimulated by either bacteria or bacterial products[9,10], virus[11], or by ligation of their CD40 and/or MHC class II molecules[12-14].

Currently most experimental and clinical studies of dendritic cells rely on the in vitro development of DC-like cells from CD34+ progenitor cells or blood monocytes[15-17]. Commonly, monocytes are cultured for 5-7 days with GM-CSF and IL-4 to generate immature DCs that have to be activated for another 2-3 days with microbial, proinflammatory, or T-cell derived stimuli to obtain mature DCs with full T cell stimulatory capacity. The discovery that myeloid DCs can be easily generated from monocytes of CD34+ precursors allowed the procurement of these otherwise scarce cells (less than 0.2% of white blood cells) in considerable numbers[18]. This method has enabled the development of DC immunotherapy. Such DC immunotherapy has been tried in mice and humans, and positive results have been obtained from many tumors, especially from malignant melanoma, B cell lymphoma, colorectal cancer, and prostate cancer[19-22]. Despite some favorable results, DCs immunotherapies have yet to show widespread, long-term efficacy in the clinic. There are many issues of DCs immunotherapeutic challenges, including; source and ex vivo manipulation of DCs; antigen preparation and loading; and, route of administration. In addition, ways of measuring the immune response and clinical response need to be standardized and adopted using state of the art

procedures[23].

In our previously conducted mouse study[24], DCs treated with cytokine cocktail for 48 hours had more expression of surface molecules than those treated for 8 hours. However 8 hours treated DCs showed more IL-12 secretion compared to 48 hours treated DCs. Moreover 8 hours treated DCs showed better clinical results and strong immune responses than 48 hours treated DCs. Thus we concluded that proper production of IL-12, not fully expressed surface molecules representing mature DCs, were optimal criteria of DCs for DCs immunotherapy.

Therefore the purpose of this study is to investigate whether there is discordance among the expression of surface molecules, intracytoplasmic IL-12 expression and IL-12 secretion in human monocytes-derived DCs (MoDCs).

II. MATERIALS AND METHODS

1. Materials

Blood samples are obtained with informed consent from five healthy donors. 20 ml blood samples were collected from each patient for 3 consecutive days. Those who had received any systemic or topical treatment with immunosuppressive drugs for at least four weeks before collection of blood samples were excluded.

2. Culture of human dendritic cells

The culture media contains X-vivo 15 (Cambrex Bio Science, Walkersville, MD), 2 μM L-glutamine (Gibco), 100 IU/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco), and 10 % fetal bovine serum (Hyclone, Logan, UT). Monocytes from study participants are isolated from PBMCs via cell attachment method[25]. PBMCs are attached to six-well plates for 40 minutes. The supernatant and the floating cells are discarded, and the attached cells are used for culture. Monocytes are plated in six-well plates at a final concentration of 3×10^6 cells in 3 ml of culture medium. DCs are generated by culturing monocytes for five days

in medium supplemented with 500 U/ml GM-CSF, 1000 U/ml IL-4 at day 0, 2, 4. At days 2, 4 one-third of the medium is removed, and an equivalent volume of fresh medium supplements with the above mentioned cytokines. In each individual, medium was divided into halves on day 5, and each of separated media was treated with maturation cytokine cocktail(TNF-α: 10ng/ml, IL-1β: 10 ng/ml, IL-6: 0.8 ul/ml, $PGE_{2:}$ 1 ug/ml) on 5^{th} day. \

DCs culture was performed for 3 consecutive days with 24 hours intervals in each patient.

3. Flow cytometry

To identify the surface molecules expressed on DCs, day 7 DCs(D7) were collected from the DCs cultured at the first day and day 6 DCs(D6) from the second day, day 5 DCs(D5) from the third day. The surface molecules of D7, D6, D5 were analyzed by flow cytometry. In addition, day 8 DCs(D8) from the first day, day 7 DCs(D7) from the second day and day 6 DCs(D6) from the third day were collected, then these 3 groups of DCs were analyzed and compared by flow cytometry.

Cells were washed with 0.4% BSA/PBS and stained for 30 min at 4℃

with monoclonal antibodies conjugated to fluorochromes against CD1a(Pharmingen, San Diego, CA), CD14(Pharmingen), CD45(Pharmingen), CD80 (Pharmingen), CD83 (Pharmingen), CD86 (Pharmingen), HLA-ABC (Pharmingen), HLA-DR (Pharmingen). After 2 washes with 0.4% BSA/PBS, flow cytometric analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA).

4. Intracytoplasmic IL-12 examination

The method is just as same as that of preparation of surface molecule expression analysis by flow cytometry. Except before staining cells with IL-12p40 antibodies (Pharmingen), the cells were treated with BD cytofix/cytoperm solution (BD Biosciences, Pharmingen, San Diego, CA) for 20 minutes to make perforation on cell membrane and stabilize the cells.

5. Interleukin-12 ELISA

Cell-culture supernatants were collected from DC preparations on day 7 from the DCs cultured at the first day, day 6 from the second day, day 5 from the third day. Additionally, day 8 from the first day, day 7 from the

second day, day 6 from the third day were also collected. Each collected supernatants were frozen at -20℃. Supernatants were thawed and human IL-12p70 levels were determined using a commercial ELISA assay according to the manufacture's protocol.

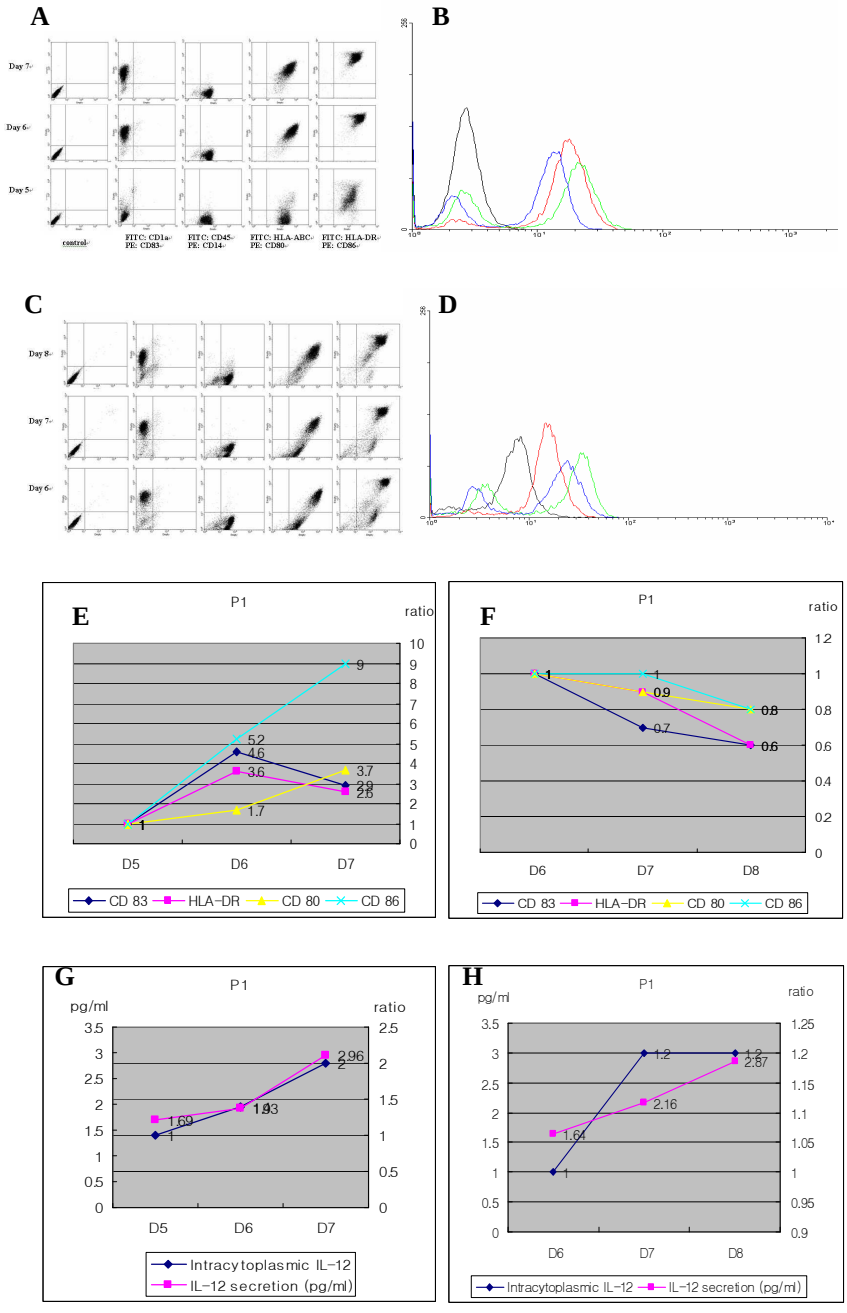
6. Data Analysis

Surface molecule expressions, intracytoplasmic IL-12p40 expression and IL-12p70 secretion quantities were compared between the DCs and supernatants collected at day 7 from DCs cultured at the first day, day 6 DCs from the second day and day 5 DCs from the third day. Moreover, day 8 DCs from the first day, day 7 DCs from the second day and day 6 DCs from the third day were also compared.

III. RESULTS

1. Surface molecules, intracytoplasmic IL-12p40, IL-12p70 secretion of first healthy donor (P1)

Comparing the surface molecules expressions of DCs in day 5~7 (Figure 1E), the expressions of CD83 and HLA-DR in day 5~7 were highest on day 6. It is well known that mature DCs, derived from the method that we used, usually mature at least for 48 hours after maturation stimuli supplement, so the decrease of CD83 and HLA-DR expressions from day 6 to day 7 are probably due to other factors. Experimental errors and individual factors, even though we used cells from healthy volunteer, might have caused the decreased expressions of CD83 and HLA-DR. The same phenomenon is also observed in DCs from day 6~8. The fact that mature DCs had failed to express more surface molecules on day 7 compared to day 6 prevents us from using the data obtained from first healthy donor.


A
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P1
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D5
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D7
CD 83
HLA-DR
CD 80
CD 86
F
P1
ratio
D6
D7
D8
CD 83
HLA-DR
CD 80
CD 86
G
P1
pg/ml
ratio
D5
D6
D7
Intracytoplasmic IL-12
IL-12 secretion (pg/ml)
H
P1
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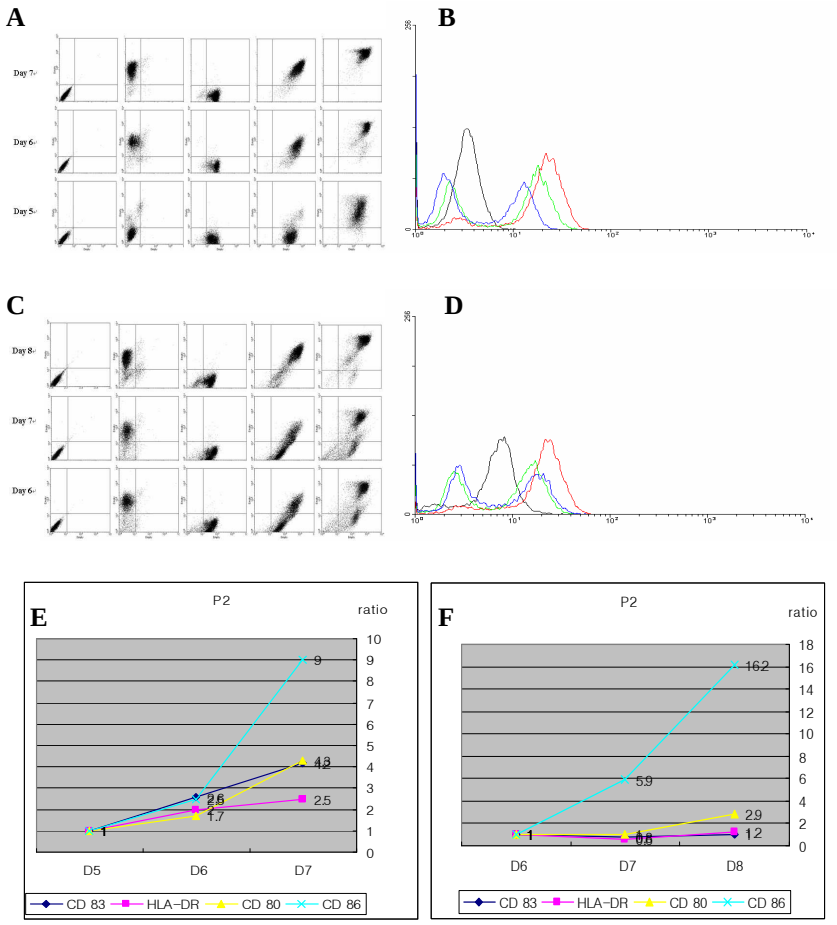
D6
D7
D8
Intracytoplasmic IL-12
IL-12 secretion (pg/ml)

Figure 1. The expressions of surface molecules (A) and intracytoplasmic IL-12p40 (B) of DCs from day 5, 6, 7 shown by flow cytometric analysis respectively (Red line: Day 7, Green: Day 6, Blue: Day 5). The expressions of surface molecules (C) and intracytoplasmic IL-12p40 (D) of DCs from day 6, 7, 8 shown by flow cytometric analysis respectively (Red line: Day 8, Green: Day 7, Blue: Day 6). The ratio of the surface molecules expressions of DCs from day 5, 6, 7 (E, day 5 value as 1). The ratio of the surface molecules expressions of DCs from day 6, 7, 8 (F, day 6 value as 1).The ratio of intracytoplasmic IL-12p40 expression and the amount of IL-12p70 secretion of DCs from day 5, 6, 7 (G, day 5 value as 1). The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 6, 7, 8 (H, day 6 value as 1).

2. Surface molecules, intracytoplasmic IL-12p40, IL-12p70 secretion of second healthy donor (P2)

Considering the expressions of surfaces molecules of DCs from day 5~7 (Figure 2E) and day 6~8 (Figure 2F), it is obvious that DCs became phenotypically most matured on day 8. If we put the results of intracytoplasmic IL-12 expressions on DCs in day 5~7 (Figure 2G) and day 6~8 (Figure 2H) together, it is apparent that intracytoplasmic IL-12

expression continued to increase till day 8. Therefore the phenotypically most matured DCs and DCs expressing most intracytoplasmic IL-12 were observed on the same day (Day 8). The amounts of IL-12 secreted from DCs on day 5~7 (Figure 2G) and day 6~8 (Figure 2H) were too small. Therefore comparing the differences between the amounts of IL-12 secretions was insignificant.



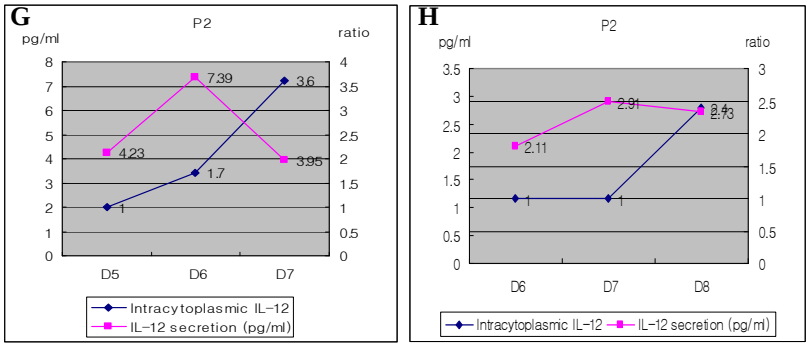
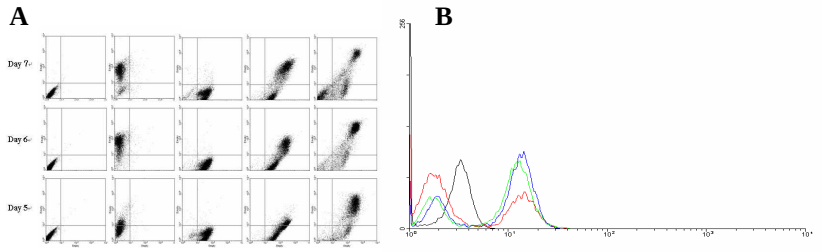


Figure 2. The expressions of surface molecules (A) and intracytoplasmic IL-12p40 (B) of DCs from day 5, 6, 7 shown by flow cytometric analysis respectively (Red line: Day 7, Green: Day 6, Blue: Day 5). The expressions of surface molecules (C) and intracytoplasmic IL-12p40 (D) of DCs from day 6, 7, 8 shown by flow cytometric analysis respectively (Red line: Day 8, Green: Day 7, Blue: Day 6). The ratio of the surface molecules expressions of DCs from day 5, 6, 7 of DCs (E, day 5 value as 1). The ratio of the surface molecules expressions of DCs from day 6, 7, 8 (F, day 6 value as 1).The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 5, 6, 7 (G, day 5 value as 1). The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 6, 7, 8 (H, day 6 value as 1).

3. Surface molecules, intracytoplasmic IL-12p40, IL-12p70 secretion of third healthy donor (P3)

The surface molecules expressions of DCs on day 5~7 (Figure 3E) were highest on day 6 except CD80. However there were only little differences between day 6 and day 7. All the surface molecules expressions of DCs on day 6~8 (Figure 3F) were highest on day 7. Putting the results of surface molecules expressions together, it is clear that phenotypically most matured DCs are achieved on day 7. Considering the intracytoplasmic IL-12 expressions of DCs in day 5~7 (Figure 3G) and day 6~8 (Figure 3H), it is obvious that intracytoplasmic IL-12 was most expressed on day 7. Therefore the phenotypically most matured DCs and DCs expressing most intracytoplasmic IL-12 were observed on the same day (Day 7). The amounts of IL-12 secreted from DCs in day 5~7 (Figure 3G) and day 6~8 (Figure 3H) were too small. Therefore comparing the differences between the amounts of IL-12 secretions was insignificant.



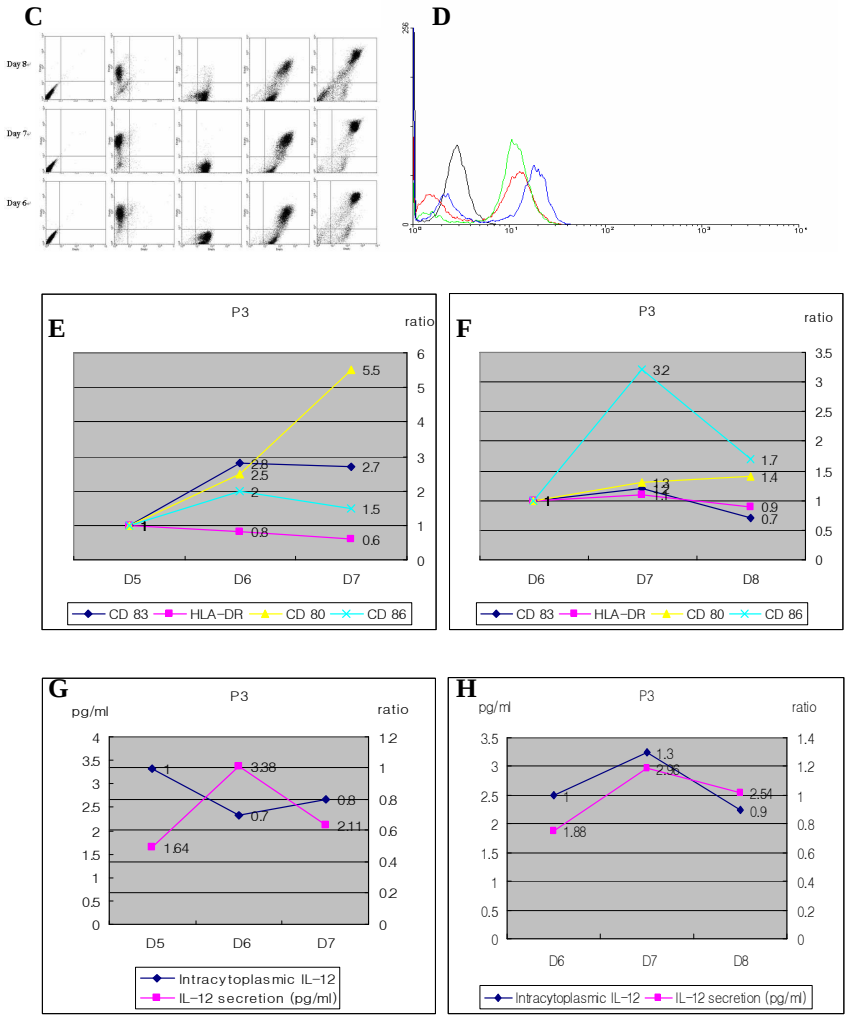


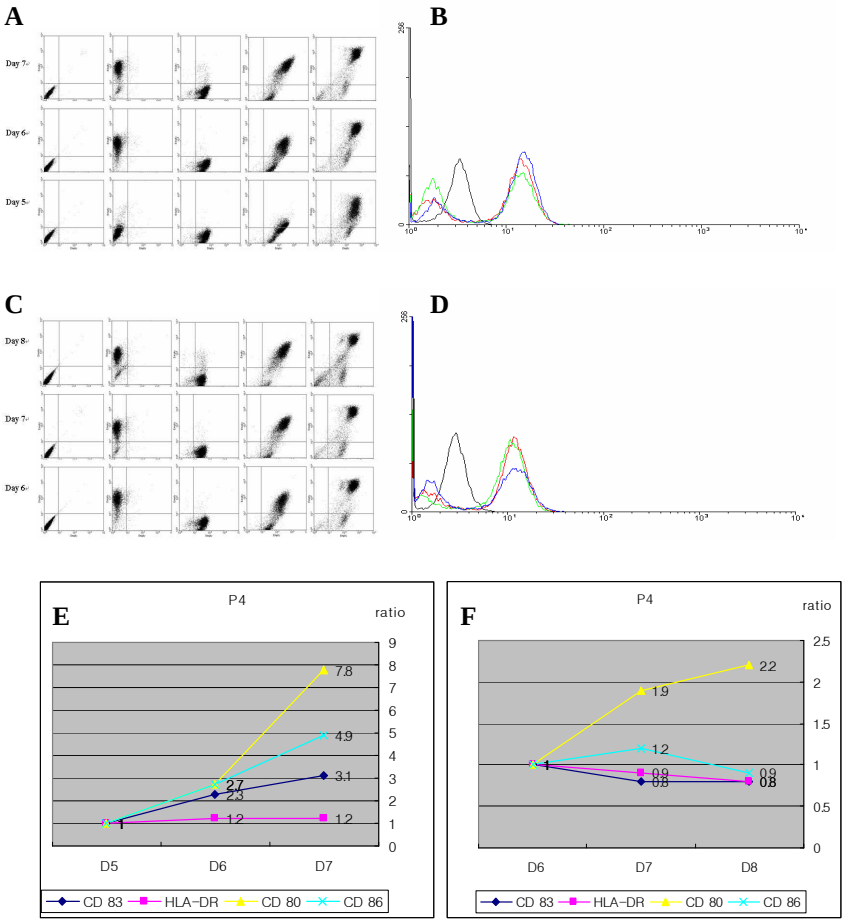
Figure 3. The expressions of surface molecules (A) and intracytoplasmic IL-12p40 (B) of DCs from day 5, 6, 7 shown by flow cytometric analysis respectively (Red line: Day 7, Green: Day 6, Blue: Day 5). The expressions of surface molecules (C) and intracytoplasmic IL-12p40 (D) of DCs from day 6, 7, 8 shown by flow cytometric analysis respectively (Red line: Day 8, Green: Day 7, Blue: Day 6). The ratio of the surface

molecules expressions of DCs from day 5, 6, 7 of DCs (E, day 5 value as 1). The ratio of the surface molecules expressions of DCs from day 6, 7, 8 (F, day 6 value as 1).The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 5, 6, 7 (G, day 5 value as 1). The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 6, 7, 8 (H, day 6 value as 1).

4. Surface molecules, intracytoplasmic IL-12p40, IL-12p70 secretion of fourth healthy donor (P4)

The surface molecules expressions of DCs on day 5~7 (Figure 4E) were highest on day 7. The surface molecules expressions of DCs on day 6~8 (Figure 4F) were all similar, but considering CD86 and CD80 expressions it seemed that mature DCs reached highest maturation status on day 7 and maintained till day 8. Considering the results of intracytoplasmic IL-12 expression from DCs in day 5~7 and day 6~8, intracytoplasmic IL-12 was most expressed on day 7 and 8. Therefore the phenotypically most matured DCs and DCs expressing most intracytoplasmic IL-12 were observed on the same day (Day 7~8). The amounts of IL-12 secreted from

DCs in day 5~7 (Figure 4G) and day 6~8 (Figure 4H) were too small. Therefore comparing the differences between the amounts of IL-12 secretions was insignificant.



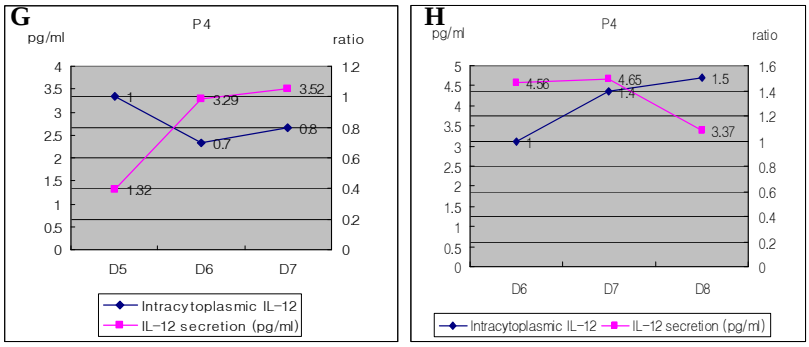
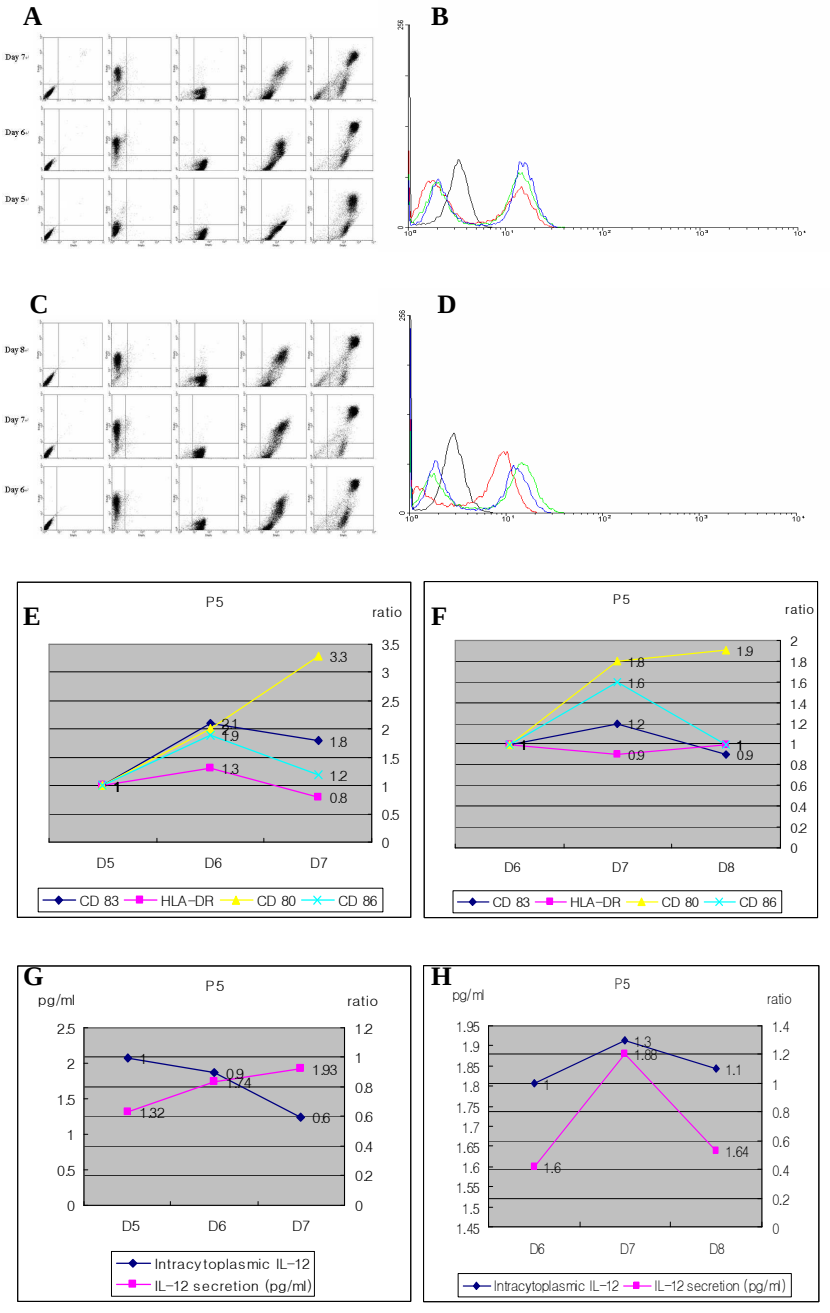


Figure 4. The expressions of surface molecules (A) and intracytoplasmic IL-12p40 (B) of DCs from day 5, 6, 7 shown by flow cytometric analysis respectively (Red line: Day 7, Green: Day 6, Blue: Day 5). The expressions of surface molecules (C) and intracytoplasmic IL-12p40 (D) of DCs from day 6, 7, 8 shown by flow cytometric analysis respectively (Red line: Day 8, Green: Day 7, Blue: Day 6). The ratio of the surface molecules expressions of DCs from day 5, 6, 7 of DCs (E, day 5 value as 1). The ratio of the surface molecules expressions of DCs from day 6, 7, 8 (F, day 6 value as 1).The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 5, 6, 7 (G, day 5 value as 1). The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 6, 7, 8 (H, day 6 value as 1).

5. Surface molecules, intracytoplasmic IL-12p40, IL-12p70 secretion of fifth healthy donor (P5)

The surface molecules expressions of DCs on day 5~7 were highest on day 6 and CD83, CD86, HLA-DR decreased on day 7 (Figure 5E). As mentioned earlier, it is well known that mature DCs, derived from the method that we used, usually mature at least for 48 hours after maturation stimuli supplement, so the decrease of surface molecules expressions from day 6 to day 7 are probably due to other factors and it is inappropriate to use these results. However the surface molecules expressions of DCs on day 6~8 were highest on day 7 (Figure 5F). The expression of intracytoplasmic IL-12 of DCs in day 5~7 was highest on day 5 and decreased following day 5 (Figure 5G) which is very odd results. The expression patterns of surface molecules of DCs on day 5~7 were incorrect so that we think the data from intracytoplasmic IL-12 expressions of DCs on day 5~7 were also inappropriate to utilize. The expression of intracytoplasmic IL-12 of DCs on day 6~8 was highest on day 7. Therefore the phenotypically most matured DCs and DCs expressing most intracytoplasmic IL-12 were observed on the same day (Day 7). The amounts of IL-12 secreted from DCs in day 5~7 (Figure 5G) and day 6~8 (Figure 5H) were too small. Therefore comparing the differences between the amounts of IL-12 secretions was insignificant.


A
B
C
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P5
ratio
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2.5
2
1.5
1
0.5
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3.3
2.1
1.9
1.8
1.3
1.2
1
0.8
D5
D6
D7
CD 83
HLA-DR
CD 80
CD 86
F
P5
ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.9
1.8
1.6
1.2
1
1
0.9
0.9
D6
D7
D8
CD 83
HLA-DR
CD 80
CD 86
G
pg/ml
P5
ratio
2.5
2
1.5
1
0.5
0
1.2
1
0.8
0.6
0.4
0.2
0
1
0.9
1.74
1.93
1.32
0.6
D5
D6
D7
Intracytoplasmic IL-12
IL-12 secretion (pg/ml)
H
pg/ml
P5
ratio
1.95
1.9
1.85
1.8
1.75
1.7
1.65
1.6
1.55
1.5
1.45
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.3
1.88
1.1
1
1.64
1.6
D6
D7
D8
Intracytoplasmic IL-12
IL-12 secretion (pg/ml)

Figure 5. The expressions of surface molecules (A) and intracytoplasmic IL-12p40 (B) of DCs from day 5, 6, 7 shown by flow cytometric analysis respectively (Red line: Day 7, Green: Day 6, Blue: Day 5). The expressions of surface molecules (C) and intracytoplasmic IL-12p40 (D) of DCs from day 6, 7, 8 shown by flow cytometric analysis respectively (Red line: Day 8, Green: Day 7, Blue: Day 6). The ratio of the surface molecules expressions of DCs from day 5, 6, 7 of DCs (E, day 5 value as 1). The ratio of the surface molecules expressions of DCs from day 6, 7, 8 (F, day 6 value as 1).The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 5, 6, 7 (G, day 5 value as 1). The ratio of intracytoplasmic IL-12p40 expressions and the amount of IL-12p70 secretions of DCs from day 6, 7, 8 (H, day 6 value as 1).

6. The mean fluorescent intensities of the surface molecules expressions and intracytoplasmic IL-12p40 and IL-12p70 secretions of DCs on day 5, 6, 7

		HLA-DR	CD 80	CD 86	CD 83	Intracytoplsamic IL-12	IL-12 secretion (pg/ml)
P1	Day 7	361.4	102.6	720.8	61.9	13.72	2.96
	Day 6	507.4	46.5	417.9	97.5	9.89	1.93
	Day 5	141.1	28.1	80.1	21.3	7.00	1.69
P2	Day 7	646.1	118.6	993.3	97.5	15.02	3.95
	Day 6	523.3	46.6	276.8	59.9	7.39	7.39
	Day 5	257.6	27.5	110.5	23.2	4.23	4.23
P3	Day 7	207.6	94.7	251.1	60.3	3.81	2.11
	Day 6	276.4	42.7	343.2	62.8	6.84	3.38
	Day 5	343.2	17.2	168.3	22.1	7.55	1.64
P4	Day 7	409.8	130.2	670.9	79.8	7.29	3.52
	Day 6	391.4	45.9	374.1	58.0	5.72	3.29
	Day 5	339.5	16.7	138.2	25.7	8.77	1.32
P5	Day 7	303.7	53.7	246.3	41.5	4.26	1.93
	Day 6	454.7	32.6	385.6	47.8	5.97	1.74
	Day 5	363.3	16.2	200.0	22.8	6.63	1.32

Table 1. Immunofluorecent intensities of surface molecules, intracytoplasmic IL-12p40 expressions by flow cytometry analysis and the amount of IL-12p70 secretion among day 7, 6, 5

7. The mean fluorescent intensities of the surface molecules expressions and intracytoplasmic IL-12p40 and IL-12p70 secretions of DCs on day 6, 7, 8

		HLA-DR	CD 80	CD 86	CD 83	Intracytoplsamic IL-12	IL-12 secretion (pg/ml)
P1	Day 8	402.6	116.5	584.0	65.2	13.37	2.87
	Day 7	595.0	117.8	752.7	81.3	13.56	2.16
	Day 6	700.0	137.7	732.8	109.5	10.88	1.64
P2	Day 8	573.5	147.3	830.2	77.3	17.37	2.73
	Day 7	285.4	49.9	303.2	61.4	7.34	2.91
	Day 6	492.8	51.2	51.2	74.5	7.12	2.11
P3	Day 8	250.0	97.7	400.2	47.9	5.72	2.54
	Day 7	299.0	95.6	751.7	84.2	8.62	2.96
	Day 6	275.6	72.2	237.6	71.0	6.71	1.88
P4	Day 8	300.0	131.9	466.9	68.5	7.14	3.37
	Day 7	332.9	112.4	630.4	70.0	7.05	4.65
	Day 6	370.3	59.1	539.2	85.8	4.88	4.56
P5	Day 8	415.7	66.5	421.6	44.0	4.72	1.64
	Day 7	398.9	65.4	644.6	60.1	5.80	1.88
	Day 6	425.8	35.6	412.1	51.1	4.45	1.60

Table 2. Immunofluorecent intensities of surface molecules, intracytoplasmic IL-12p40 expressions by flow cytometry analysis and the amount of IL-12p70 secretion among day 8, 7, 6

IV. DISCUSSIONS

Since DC-like cells were able to be developed in vitro from CD34+ progenitor cells or blood monocytes[15-17], numerous studies on DCs have been relied on this in vitro methods. Additionally, original methods have been variously modified in different studies to improve qualities and quantities of MoDCs. Immature DCs are usually obtained from monocytes following culture with GM-CSF and IL-4 for 5~7 days. DC maturation can then be induced using various inflammatory signals, such as tumor necrosis factor (TNF), interleukin-1 beta (IL-1β), or bacterial derivatives (e.g. lipopolysaccharide, lipoteichonic acid, or Ribomunyl), double-stranded RNA, interferons and/or prostaglandins[18]. After maturations signals are introduced, it takes 1 to 3 days for MoDCs to be fully maturated. Also, different culture medias are used in various protocols. Numerous protocols have been developed and utilized in many experiments. Currently, a cocktail described by Jonuleit and coworkers is used commonly in clinical studies for DC maturation[26]. However proper periods of time for culturing immature DCs with GM-CSF and IL4 are not definitely reported and also there is no recommendation for culture time

after treating DCs with maturation cocktail. Therefore we tried to confirm that 5 days, which is commonly applied time in many studies, is enough for culturing immature DCs and also compared the mature DC phenotype and IL-12 secretion according to their maturation time.

In this study, the DCs of P1 did not mature properly and they were inappropriate for data analysis. Besides the DCs of P1, the rest of the DCs (P2~P5) were all properly matured phenotypically. All of those four groups of mature DCs showed highest surface molecules expressions and most IL-12 secretion at the same time. In case of the mature DCs of P2, that time was day 8. However the mature DCs of P3, P4 and P5 showed highest surface molecules expressions and most IL-12 secretion on day 7. These results are quite different from our previously conducted experiment in mouse DCs[24]. In previous mouse study, the highest peak time was discordant between surface molecules expressions and IL-12 secretion. The highest expressions of surface molecules appeared later than the highest secretion of IL-12. And also in mouse study, IL-12 secretion reached the peak level on 8 hours after adding maturation cocktail, which was before the full maturation of DCs. However in current study, IL-12 expression reached its peak level when DCs were fully

maturated which was delayed compared to mouse study. The difference between the current study and mouse study might be due to fundamental species differences. However in recent review of the mouse and human DC populations by Liu YJ and coworker[127], they emphasized the basic similarities between the two systems. Most apparent discrepancies between two species have reflected the differences between culture-generated and freshly isolated DCs rather than fundamental species differences[127].

In human studies, there are conflicting reports about the timing of IL-12 and surface molecules expressing their highest levels. Romani and coworkers had demonstrated in human MoDCs that maturing DCs produce high levels of IL-12 and already mature DCs made less IL-12 than maturing DCs[28]. Lyerly and coworkers had reported that IL-12 expressions occurs specifically in mature DCs[29], which is just as same as our results. The difference between current study and Romani's study could be explained by several reasons. The first one is that the time period difference of priming culture between current study and the study done by Romani and coworkers. In our study 5-day priming culture in the presence of GM-CSF and IL-4 was done. Romani and coworkers had done initial 7-

day priming culture in the presence of GM-CSF and IL-4 then splitted immature cells in half. After that in the presence or absence of maturation stimuli, cells were cultured for 3 more days so finally they used 10-day old immature DCs and 10-day old mature DCs. We think the immature and mature DCs, that they have used, are too old and exhausted to compare IL-12 secretions and intracytoplasmic expressions. The second reason is that incomplete washing of priming culture media. We used X-vivo 15 with GM-CSF and IL-4 for priming culture. It is well known that IL-4 is potent enhancers of IL-12 production even though IL-4 is Th2 cytokine[30]. The effects of IL-4 on expression of the gene encoding p40 are bimodal: at early times during treatment (<24 hours), it inhibits p40 production, whereas at later times, it strongly enhances production[31]. Therefore if the washing of priming culture media was not enough, remaining IL-4 might have inhibited the production of IL-12 by early maturing DCs on day 6.

The opposite results of current study might be due to insufficient number of healthy donors. We could only evaluate the data from four healthy donors because of the exclusion of data from P1. We think further study will be necessary with more healthy donors to confirm our results.

One of the most unexpected data was the absolute amount of secreted IL-12p70. It only ranged between 1.32 and 7.39pg/ml. These are extremely minimum values to be used as proper data analysis. We tried to figure out the reasons and think that there can be several explanations for these results. Before mentioning the possible reasons, even though the amount of IL-12 secretions were insignificant to be used in data analysis the intracytoplasmic IL-12p40 expressions can be representative of bioactive IL-12 production. IL-12 is a unique heterodimeric cytokine composed of p35 and p40, and the heterodimeric structure was shown to be essential for its biological activity[32]. In cell lines, the expression of p40 transcripts was shown to correlate with the ability of the cells to produce IL-12, whereas p35 mRNA was reported to be ubiquitously expressed in almost all cell lines of either hematopoietic or nonhematopoietic origin[33]. These findings led to an assumption that p40 production is representative of bioactive IL-12 production[29,34].

The explanation is that experimental errors might have influenced the results. However standard concentration graph was properly obtained (1.5 pg/ml ~ 300 pg/ml) and ELISA technique was done by one of our most experienced co-worker. In addition, we had done other experiment with

exact same DC culture protocol just 1 month before this study (unpublised data) and detected IL-12 secretion was also negligible.

It is well known that IL-10 can be released by mature DCs[35]. IL-10 support Th2 response. We think IL-10 might have been secreted by maturing DCs which resulted in low quantity of IL-12 secretion by restraining Th1 response polarization. Actually there is previous study which proved IL-10 impairs IL-12 secretion in maturing DCs[36]. To check this possibility, we think the amount of IL-10 secretion as well as that of IL-12 secretion should have been measured.

Since Jonuleit and coworkers[26] used TNF-α, IL-1β, IL-6, PGE2 for maturation stimuli, this maturation cocktail has been used commonly in clinical studies for DC maturation. But there have been some reports about PGE2 being inhibitor of IL-12 secretion by mature DCs[35,36,38,39]. PGE2 is known to strongly synergizes with IL-1β and TNF-α in the induction of phenotypical and functional final maturation of DC[36]. PGE2 renders IL-1β and TNF-α effective at 100-fold lower concentrations[36]. Interestingly, however, in the absence of inflammatory cytokines PGE2 was completely ineffective implying that PGE2 is only a cofactor for IL-1β and TNF-α induced DC maturation[36]. These effects of PGE2 are

mediated by the elevation of intracellular cAMP levels. PGE2 also support induction of CCR7 expression and improve DC handling by reducing cell adherence[35]. In spite of PGE2 being stong inducer of phenotypical and functional DC maturation, DC matured in the additional presence of PGE2 show impaired IL-12 production and bias naïve T cell development toward the Th2 cells[36]. The IL-12 inhibitory potential of PGE2 is dose-dependent and with lower concentration of PGE2 as maturation cocktail, IL-12 secretion was increased[35]. Therefore we think that high concentration of PGE2 in our maturation cocktail might have inhibited the secretion of IL-12. So if we use lower concentration of PGE2, increased IL-12 secretion can be expected. We think further studies using various concentration of PGE2 as maturation cocktail will reveal better results.

Because the amount of IL-12p70 secretion was insignificant, it was frustrating that IL-12p70 secretion data were inappropriate to used in data analysis. However, there is a report that showed the expressions of intracytoplasmic IL-12p40 was associated with the amount of IL-12p70 secretion and even advocated intracellular IL-12p40 expression as a reliable marker of functionally mature DCs. Therefore even though IL-12p70 secretion data was excluded in our data analysis, we think

intracytoplasmic IL-12p40 expression can be representative.

V. CONCLUSION

In conclusion, DCs were most matured on day 7 and IL-12p40 expressions were also highest on day 7 in general. In contrast to our previous mouse study and other previous human study, IL-12p40 expressions were highest when DCs were phenotypically most matured. Discordance of our data and previous data might be explained by some reasons, but we think more standardized and extensive studies are necessary to resolve the controversy.

Decreased secretion of IL-12p70 might have been caused by experimental errors or mistake, but we think there are possibilities of other factors that caused the decrease of IL-12p70 secretion such as fundamental problems in protocol.

According to our data, day 7 DCs can be optimal candidate to be used in DCs immunotherapy. However, in vivo experiments and clinical trials are required for finding optimal mature DCs for DCs immunotherapy.

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< ABSTRACTS (IN KOREAN)>

싸이토카인에 의한 성숙 기간 차이에 따른 인간 단핵구 유래 수지상세포에서의 인터루킨-12의 표현 및 분비량 비교

<지도교수 이민걸>

연세대학교 대학원 의학과

김대석

수지상세포는 면역 반응의 유도 및 조절에 중요한 역할을 한다. 미성숙 수지상세포는 MHC class II 경로를 통한 단백질 항원 전달 기능을 매우 효율적으로 수행한다. 성숙 수지상세포는 T 세포의 성장 및 분화를 유발하는 역할을 한다. 인터루킨-12는 수지상세포와 T 세포의 상호작용에서 매우 중요한 역할을 하는 인자 중 하나이다. 인터루킨-12는 T 세포 및 NK 세포에 작용하여 세포의 성장과 독성을 유발 및 촉진하고, IFN-γ의 생성을 증가시킨다. 인터루킨-12는 Th1 세포를 유발하는데 있어서 가장 중요한 싸이토카인이다. 현재 수지상세포를 이용한 연구는 대부분 CD34 양성 기원세포나 혈액에서 추출한 단핵구를 수지상세포와 유사한 세포로 유도하는 기술을

기반으로 이루어진다.

쥐를 이용한 저자들의 실험에서 수지상세포의 표면항원 발현 정도가 아닌 적절한 인터루킨-12의 분비가 수지상세포 면역치료에 있어서 가장 적합한 기준이 된다는 것을 밝혀냈다. 따라서 이번 연구에서 저자들은 인간 단핵구 유래 수지상세포의 표면항원 발현과 세포내 인터루킨-12 발현, 인터루킨-12 분비 등이 일관된 양상을 나타내지 여부를 알아보고자 하였다.

연구 결과, 표면항원 발현상으로 수지상세포의 최고 성숙 시기는 7일째이었고, 마찬가지로 세포내 인터루킨-12 표현 정도도 7일째 최고로 높게 나타났다. 본 연구에서 인터루킨-12의 분비량은 그 양이 너무 적어서 의미를 부여하기 힘들기 때문에 자료 분석을 할 수 없었다. 결론적으로 비록 인터루킨-12의 분비량은 적었지만, 표면항원 발현과 세포내 인터루킨-12 발현은 모두 7일째 제일 높게 나타났다. 따라서 수지상세포 면역치료에 있어서 7일째의 수지상세포를 이용하는 것이 적절한 방법 중 하나라고 생각된다.

핵심되는 말: 수지상세포, 인터루킨-12