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Outer membrane protein A of *Acinetobacter baumannii* induces differentiation of CD4$^+$ T cells toward a Th1 polarizing phenotype through the activation of dendritic cells

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ABSTRACT

Acinetobacter baumannii is an increasing hospital-acquired pathogen that causes a various type of infections, but little is known about the protective immune response to this microorganism. Outer membrane protein A of *A. baumannii* (AbOmpA) is a major porin protein and plays an important role in pathogenesis. We analyzed interaction between AbOmpA and dendritic cells (DCs) to characterize the role of this protein in promoting innate and adaptive immune responses. AbOmpA functionally activates bone marrow-derived DCs by augmenting expression of the surface markers, CD40, CD54, B7 family (CD80 and CD86) and major histocompatibility complex class I and II. AbOmpA induces production of Th1-promoting interleukin-12 from DCs and augments the syngeneic and allogeneic immunostimulatory capacity of DCs. AbOmpA stimulates production of interferon-γ from T cells in mixed lymphocyte reactions, which suggesting Th1-polarizing capacity. CD4$^+$ T cells stimulated by AbOmpA-stimulated DCs show a Th1-polarizing cytokine profile. The expression of surface markers on DCs is mediated by both mitogen-activated protein kinases and NF-κB pathways. Our findings suggest that AbOmpA induces maturation of DCs and drives Th1 polarization, which are important properties for determining the nature of immune response against *A. baumannii*.

1. Introduction

Acinetobacter baumannii (*A. baumannii*) have emerged as important nosocomial pathogen that give rise to various infections, including bacteremia, meningitis, pneumonia, skin and wound infections and urinary tract infections [1–3]. *A. baumannii* is of great concern in clinical setting because of multiple drug resistance and high mortality of infected

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patients. Emergence of pan-drug resistant *A. baumannii* has been reported a few years ago [4–6] and innovative therapeutic approaches such as development of new drugs and vaccines are necessary to treat infectious diseases caused by pan-drug resistant *A. baumannii*. It is likely that the most effective intervention for pan-drug resistant *A. baumannii* will be therapeutic vaccine. However, a lack of understanding of protective immunity against *A. baumannii* caused little progress in the development of an efficacious vaccine.

Dendritic cells (DCs) are antigen-presenting cells (APCs) that play key roles in the regulation of immune responses to a variety of antigens [7,8]. DCs orchestrate both the quality and the magnitude of the immune response, but the ability of DCs to activate naïve T cells depends on their maturation. Mature DCs have a high surface expression of major histocompatibility complex (MHC) and costimulatory molecules. Immature DCs are mainly distributed in tissues interfacing with the external environment where they capture and process antigens with high efficiency [9–11]. In addition, DCs respond to microbes by producing proinflammatory cytokines that activate innate lymphocytes ($CD8^+$ and natural killer T cells) to directly kill infected cells [12,13]. Thus, DCs provide a link between innate and adaptive immune systems [14].

Outer membrane proteins are released during the growth and lysis of Gram (−) bacteria and their roles are increasingly recognized in pathogenesis of bacteria [15]. In addition, major outer proteins have potent immunostimulatory effects. Meningococcal outer membranes induce maturation of human monocyte-derived DCs (BMDCs) and augment the allo-stimulatory properties of these cells, which induce proliferation of $CD4^+$ T cells [16]. More interestingly, OmpA from *Klebsiella pneumonia* specifically binds to immature DCs and is endocytosed via a receptor-dependent mechanism. OmpA from *K. pneumonia* (KpOmpA) induces DCs to produce interleukin 12 (IL-12) and to mature, and KpOmpA-mediated signaling is occurred via tole-like receptor 2 (TLR2) [17]. AbOmpA of *A. baumannii* (AbOmpA, previously called Omp38) is a major porin protein in the outer membrane and is partly responsible for apoptosis of eukaryotic cells [18]. However, immune responses against AbOmpA remain unclear. The understanding the cellular responses of DCs against AbOmpA is likely to be important for the development of effective *A. baumannii* vaccines. In the current study, we investigated the ability of AbOmpA to interact with murine DCs. The data imply that AbOmpA induces maturation of DCs and initiates adaptive immune response through Th1 polarization.

2. Materials and methods

2.1. Animals

Male 6–8-week-old BALB/c (H-$2K^d$ and I-A^d) and C57BL/6 (H-$2K^b$ and I-A^b) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). C.Cg-Tg(DO11.10, H-2^d) mice carrying the MHC class II-restricted rearranged T cell and C57BL/6-Tg(TcraTcrb, H-2^b, OT-1) mice containing transgenic inserts for mouse Tcra-V2 and Tcrb-V5 genes recognize $OVA_{257–264}$ were purchased from the Jackson Laboratory (Bar Harbor, ME). They were housed in a specific pathogen-free environment within our animal facility for at least 1 week before use.

2.2. Reagents and antibodies (Abs)

Recombinant mouse GM-CSF and IL-4 were purchased from R&D Systems. Dextran-FITC (molecular mass, 40,000) and LPS from *Escherichia coli* 055:B5 were obtained from Sigma–Aldrich. An endotoxin filter (END-X) and an endotoxin removal resin (END-X B15) were acquired from Associates of Cape Cod. Cytokine ELISA kits for murine IL-4, and IFN-γ were purchased from BD Pharmingen. FITC- or PE-conjugated mAbs used to detect the expression of CD11c (HL3), CD80 (16-10A1), CD86 (GL1), CD40 (1C10), CD54 (YN1/1.7.4), IA^b β-chain (AF-120.1), $H2K^b$ (AF6-88.5), CD4 (L3T4), or the intracellular expression of IL-12 p40/p70 (C15.6) and IL-10 (JESS-16E3) by flow cytometry, as well as isotype-matched control mAbs, biotinylated anti-CD11c (N418) mAb, were purchased from BD Pharmingen. To detect protein levels, anti-phospho-ERK, anti-ERK, anti-phospho-p38, anti-p38, anti-IκB, anti-phospho-JNK, anti-JNK and anti-p65 Ab were purchased from Santa Cruz Biotechnology. To confirm the effects of ERK JNK and p38 MAPK activation and p65 on the expression of surface molecules in DCs, inhibitors PD98059, SP600125 and SB203580 were purchased from Calbiochem.

2.3. Preparation of AbOmpA

Full-length AbOmpA gene (1317 bp, GenBank Accession number AY485227) was amplified by PCR. A chromosomal preparation of *A. baumannii* ATCC 19606^T was used as a PCR substrate. The upstream primer 5′-ACAGGATCCATGAAATT-GAGTCGTATT-3′ was designed to carry *Bam*HI restriction site and the downstream primer 5′-ACAAGCTTTTATT-GAGCTGCTGCA-3′ carried the *Hin*dIII restriction site. PCR products digested with *Bam*HI and *Hin*dIII were ligated into the pET28a expression vector (Novagen). *E. coli* BL21 (DE3)/pET28a harboring a *ompA* gene was grown in Luria–Bertani (LB) medium at 37 °C and recombinant proteins were overexpressed with 1 mM IPTG at 25 °C for 4 h. After sonication of bacterial cells, the pellet containing the inclusion bodies was discarded and the supernatant containing the soluble form of AbOmpA was collected by centrifugation. The protein samples were loaded on a 5 ml HiTrap™ FF column (Amersham Biosciences) equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl and 5 mM imidazole). His-tagged OmpA was eluted by elution buffer (20 mM sodium phosphate, 500 mM NaCl and 500 mM imidazole). The samples were massively dialyzed against elution buffer without imidazole and then dialyzed with phosphate-buffered saline (PBS, pH 7.4) to reduce salt concentrations. rAbOmpA was incubated with endotoxin removal resin for overnight to remove LPS and concentrated by Centricon (2,000 MW cut-off; Millipore). rAbOmpA was analyzed for purity by SDS–PAGE (10% T, Ready Gel J, Bio-Rad) after denaturation with sample buffer (60 mM Tris–HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue) at 100 °C for 5 min. After electrophoresis, the gels were stained with 0.1% (w/v) Coomassie blue R-250 and destained as described previously [19]. Also, endotoxin was assayed under endotoxin-free

experimental conditions using a Limulus Amebocytes Lysate (LAL) pyrogen kit (Biowhittaker, Walkersville, MD). The experiments were conducted according to the manufacturer's protocol. The quantity of endotoxin in the OmpA was $\leq$0.01 ng/mg.

2.4. Generation of DCs

DCs were generated from murine bone marrow cells with modifications as previously described [20]. Briefly, bone marrow was flushed from the tibiae and femurs of C57BL/6 and depleted of red cells with ammonium chloride. The cells were plated in six-well culture plates (10^6 cells/ml) in OptiMEM (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.4), 20 ng/ml recombinant mouse GM-CSF and IL-4 at 37 °C in an atmosphere of 5% CO_2. On day 3 of the culture, floating cells were gently removed and fresh medium was added. On day 6–7 of the culture, non-adherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation or, in some experiments, replated in 60-mm dishes (10^6 cells/ml). On day 7, 80% or more of the non-adherent cells expressed CD11c. In certain experiments, to obtain highly purified populations for subsequent analyses, DCs were labeled with bead-conjugated anti-CD11c mAb (Miltenyi Biotec) followed by positive selection through paramagnetic columns (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the selected cell fraction was >90%.

2.5. Stimulation of DCs by AbOmpA

AbOmpA was dissolved in culture media and was added into cultures of isolated DCs in six-well plates (10^6 cells/ml) to be 200 ng/ml finally. For the analysis of apoptosis, DC were stimulated with LPS or left without any stimuli, and apoptosis was analyzed over time by staining of phosphatidylserine translocation with FITC-annexin V in combination with PI kit (BD Pharmingen) according to the manufacturer's instructions.

2.6. Flow cytometric analysis

On day 7, BMDCs were harvested, washed with PBS and resuspended in fluorescence activated cell sorter (FACS) washing buffer (2% fetal bovine serum and 0.1% sodium azide in PBS). Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4 °C and stained with appropriate Ab fluorescence-conjugated Ab described in *Reagents and antibodies* for 30 min at 4 °C. The stained cells were analysed using a FACSCalibur flow cytometer (Becton Dickinson).

2.7. Quantitation of antigen uptake

Endocytosis was quantitated as following. In brief, 2×10^5 cells were equilibrated at 37 °C or 4 °C for 45 min and then pulsed with fluorescein-conjugated dextran (Sigma–Aldrich) at a concentration of 1 mg/ml. Cold staining buffer was added to stop the reaction. The cells were washed three times and stained with PE-conjugated anti-CD11c Abs and then analyzed by FACSCalibur. The medium used in the culture, to stimulate DCs with AbOmpA, was supplemented with GM-CSF, because the ability of DCs to capture antigens is lost if DCs are cultured without GM-CSF [21].

2.8. Cytokines assay

Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4 °C and then stained with FITC-conjugated $CD11c^+$ antibody for 30 min at 4 °C. Cells stained with the appropriate isotype-matched immunoglobulin were used as negative controls. The cells were fixed and permeated with the Cytofix/Cytoperm kit (PharMingen) according to manufacturer's instructions. Intracellular IL-12p40/p70, IL-10 and IFN-γ were stained with fluorescein R-phycoerythrin (PE)-conjugated antibodies (PharMingen) in a permeation buffer. The cells were analyzed by flow cytometry. Furthermore, murine IL-4 and IFN-γ from DCs were measured using an ELISA kit (PharMingen) according to manufacturer's instructions.

2.9. T cell proliferation by syngeneic MLR

Syngeneic MLR assay was chosen because, although it is not specific for a given antigen, it provides adequate information on the overall antigen-presenting function of DCs. Responder $CD8^+$ T cells recognizing $OVA_{257–264}$ were isolated from spleen of C57BL/6-Tg(TcraTcrb, H-2^b, OT-1) mice via a MACS column (Miltenyi Biotec, Gladbach, Germany). Staining with fluorescein PE-labeled anti-CD8 monoclonal Ab (BD PharMingen) revealed that they consisted mainly of $CD8^+$ T cells (>95%). The lymphocyte population (95% of $CD8^+$ cells) was then washed twice in PBS and labeled with CFSE, as previously described [22]. The cells were resuspended in 1.5 μM CFSE in PBS. After being shaken for 5 min at room temperature, the cells were washed once in pure FBS and twice in PBS with 10% FBS. Stimulator DCs (1×10^4) derived from C57BL/6 mice were exposed to AbOmpA (200 ng/ml) or LPS (200 ng/ml) for 24 h and washed thoroughly, and were co-cultured with 1×10^5 CFSE-labeled T cells in 96-well U-bottom plates for another 4 days. A negative control ($CD8^+$ T cells in media alone), a specific antigen control (1 μM $OVA_{257–264}$) and a positive control ($CD8^+$ T cells in 200 ng/ml LPS) were created for each experiment. After 4 days, the cells were harvested and washed in PBS. CFSE dilution was assessed by flow cytometry.

2.10. Evaluation of cluster formation and cytokine production by allogeneic MLR

Responder T cells (1×10^5), which participate in allogeneic T cell reactions, were isolated from spleen of BALB/c mice in a MACS column (Miltenyi Biotec). Staining with FITC-conjugated anti-CD3 Abs (BD Biosciences PharMingen) revealed that they consisted mainly of $CD3^+$ cells (>95%). Stimulator DCs (1×10^4) derived from C57BL/6 mice were exposed to AbOmpA (200 ng/ml) or LPS (200 ng/ml) for 24 h and washed thoroughly, and were co-cultured with T cells in 96-well U-bottom plates for another 24, 48 and 72 h. And then, cluster formation was assessed. In this same set of experiment, we measured the cytokine production (IFN-γ and IL-4) in culture supernatants at 48 h after culture initiation.

2.11. Evaluation of CD4+T cell polarization by DCs

To find out whether AbOmpA-stimulated DCs treated T cells and induced Th1 cell polarization, we used OVA as antigen because antigen presentation can be readily monitored using $CD4^+$ OVA-specific TCR transgenic T cells. $CD4^+$ splenic T cells (2×10^6/ml) from DO11.10 mice were co-cultured with (2×10^5/ml) BALB/c DCs in 12-well plates for 24 h in the presence or absence of 200 ng/ml AbOmpA. All groups of experiment except negative control were added 2 μM $OVA_{323–339}$ peptide. The profiles of cytokines, eg., IFN-γ (Th1) and IL-4 (Th2) were analyzed by flow cytometry. Supernatants from these cultures were collected after 2 days and analyzed IFN-γ and IL-4 by ELISA.

2.12. Nuclear and cytoplasmic extracts and Western blot for ERK, p38 kinase JNK and NF-κB analysis

The cells were exposed to LPS (200 ng/ml) or 200 ng/ml AbOmpA. After incubation for 30, 60 and 90 min at 37 °C, cells were washed twice with cold PBS and lysed with modified RIPA buffer (1.0% NP-40, 1.0% sodium deoxycholate, 150 nM NaCl, 10 mM Tris–HCl [pH 7.5], 5.0 mM sodium pyrophosphate, 1.0 mM $NaVO_4$, 5.0 mM NaF, 10 mM leupeptin and 0.1 mM phenylmethylsulfonyl fluoride) for 15 min at 4 °C. Lysates were cleared by centrifuging at 14,000 × *g* for 20 min at 4 °C. The protein content of cell lysates was determined using the Micro BCA assay kit (Pierce). Equivalent amounts of proteins were separated by SDS–10% PAGE and analyzed by Western blotting using an antiphospho-ERK1/2, anti-phospho-JNK or anti-phospho-p38 MAPK mAb for 2 h, as described by the manufacturer of the antibodies. Following washing three times with PBS, membranes were incubated with secondary HRP-conjugated anti-mouse IgG for 1 h. After washing, the blots were developed using the ECL system (Amersham) by following manufacturer's instructions. Nuclear extracts of DCs were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). NF-κB p65 subunits in the nuclear extracts were determined by Western blot analysis with anti-NF-κB p65 subunit Ab.

2.13. TLR analysis

DCs cultured in RPMI1640 media containing 10% FBS at 37 °C, 5% CO_2. The cells were pretreated with 5 μg/ml of TLR2 and TLR4 blockers (Santa Cruz, CA, USA), respectively, for 2 h, and followed by washing twice with complete media pre-warmed to 37 °C and then performed TLR blocking experiments. AbOmpA and LPS were used at a final concentration of 200 ng/ml, respectively.

2.14. Statistics

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean ± SEM. ANOVA was used to compare experimental groups to control values. While comparisons between multiple groups were done using Tukey's Multiple Comparison test. Statistical significance was determined as *P* value less than 0.05.

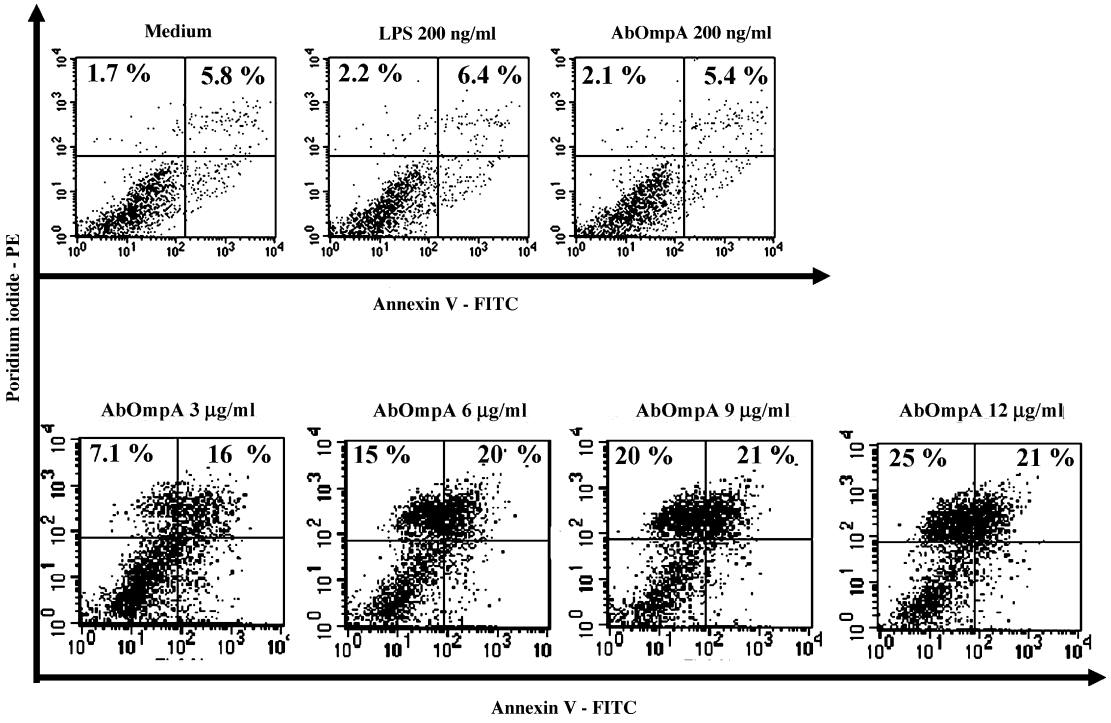

Fig. 1 – OmpA was determined to be cytotoxic to DCs at various concentrations. DCs were generated as described in Section 2. On day 6, the cells were cultured at standard conditions for another 24 h in the presence of 100 ng to 12 μg/ml AbOmpA and harvested. The cells were firstly gated on $CD11c^+$ cells, and analyzed by two-color flow cytometry using annexin v/PI staining kit. This result is a representative of three experiments that gave similar results.

3. Results

3.1. Purification and characterization of AbOmpA

We purified AbOmpA from *Escherichia coli* and assessed its cytotoxicity on DCs, since the purified AbOmpA form *A. baumannii* ATCC19606^T induced apoptosis of eukaryotic cells [18]. BMDCs were treated with various concentrations of AbOmpA for 24 h. Relatively low concentration (≤200 ng/ml) of AbOmpA appeared to be non-cytotoxic effects in DCs, while high concentrations (≥3 μg/ml) of AbOmpA induces cytotoxicity, as evidenced by annexin-V/propidium iodide (PI) staining of CD11c$^+$ cells (Fig. 1). Therefore, it was adjusted to 200 ng/ml of AbOmpA to stimulate DCs in the following studies.

3.2. AbOmpA induces maturation of BMDCs

To determine the effects of AbOmpA on the maturation of sentinel DCs into effector DCs, BMDCs were cultured with GM-CSF and IL-4 for 6 days under standard conditions, followed by another 1 day in the presence of 100 and 200 ng/ml of AbOmpA. Lipopolysaccharides (LPS) were used as a positive control. The resulting populations of DCs were analyzed by flow cytometry for expression of cell surface molecules involved in T-helper cell activation. AbOmpA-treated DCs showed increased expression of surface markers CD80, CD86, CD40, CD54, MHC class I and MHC class II (Fig. 2). The expression of CD80, CD86, CD54 and MHC class II in AbOmpA-treated DCs was higher than that of LPS-treated DCs. In contrast, the untreated DCs retained an immature

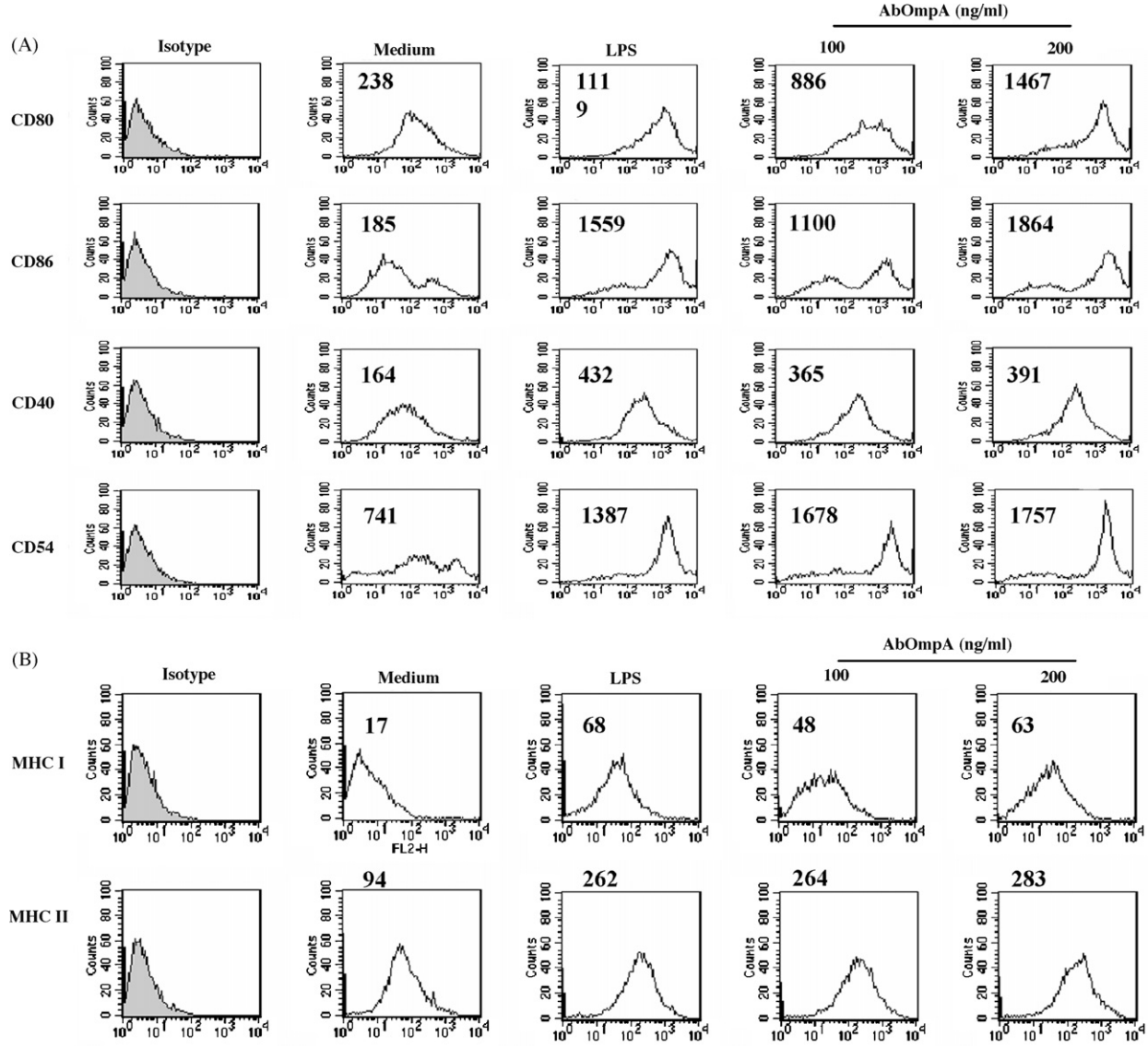

Fig. 2 – Comparison of DCs phenotypes cultured with OmpA and LPS. DCs were generated as described in Section 2. On day 6, the cells were cultured at standard conditions for another 24 h in the presence of 100 and 200 ng/ml OmpA or 200 ng/ml LPS and harvested, and analyzed by two-color flow cytometry. The cells were firstly gated on CD11c$^+$. Medium represents the untreated control and LPS represents the positive control. (A) OmpA induced the expression of costimulatory molecules CD80, CD86, CD40, and CD54 on DCs. (B) OmpA induced the expression of MHC class I and MHC class II molecules on DCs. The data are representative of three experiments that gave similar results.

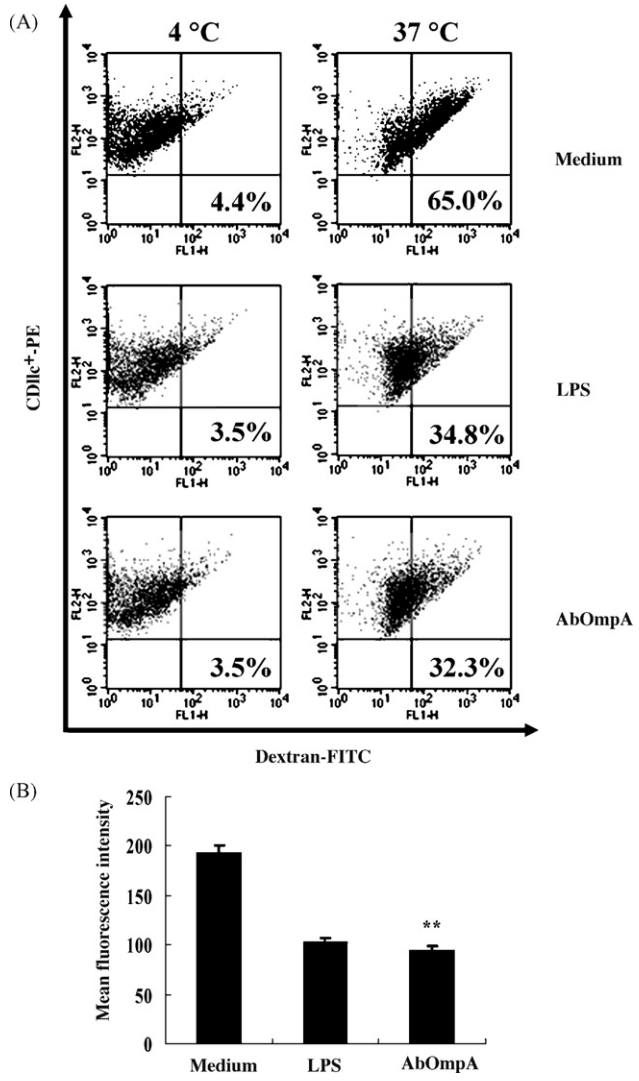

Fig. 3 – Measurements of endocytic capacity of OmpA-treated DCs. FITC-dextran uptake was analyzed by CD11c$^+$-PE/ FITC-dextran-positive cells using flow cytometry. DCs (1×10^5 cells) were treated with OmpA (200 ng/ml) or LPS (200 ng/ml) in the presence of FITC-dextran for 24 h. The cells were then washed twice in cold HBSS and stained with PE-conjugated anti-CD11c$^+$ antibody. Endocytic activity of the control was determined after incubation at 4 °C. The numbers represent the percentage of FITC-dextran-/CD11c$^+$-PE double positive cells (A) and mean fluorescence intensity (MFI) (B). The results are representative of three independent experiments that gave similar tendency.

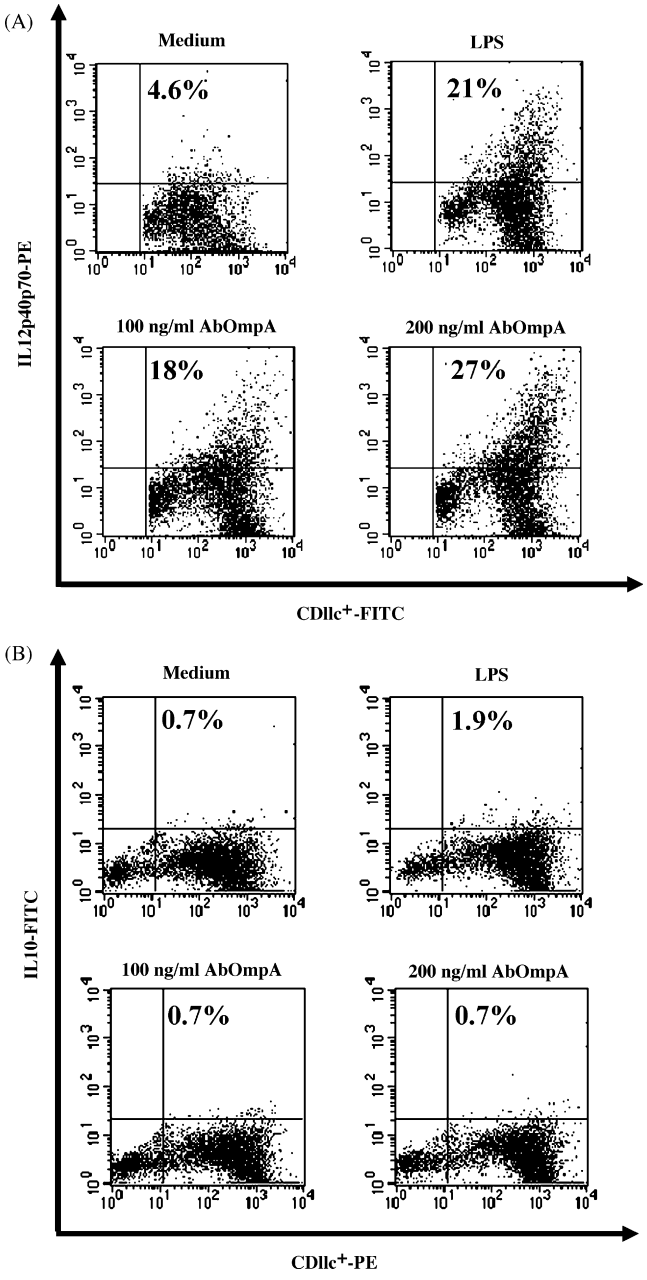

Fig. 4 – Cytokine production by OmpA-treated DCs. DCs (5×10^5/ml) were generated by stimulating immature DCs with 100 and 200 ng/ml OmpA or 200 ng/ml LPS for 24 h. After 24 h, the production of IL-12 (A) and IL-10 (B) was measured by flow cytometry. The data are representative of three independent experiments that gave similar tendency.

phenotype. Immature DCs are efficient in capturing and endocytosis of antigens. To determine whether AbOmpA modulates DCs in ability of endocytosis of antigens, the endocytic activity of DCs was measured. The uptake of dextran in AbOmpA- and LPS-treated DCs was significantly decreased compared with the untreated immature DCs (Fig. 3). All of these changes indicate that AbOmpA induces maturation of DCs.

3.3. *AbOmpA increases the number of IL-12 secreting DCs, but not IL-10 secreting DCs*

CD4$^+$ T cells may differentiate into different subsets of effector cells that perform distinct effector functions. The potent factors driving the development of Th1 and Th2 cells are IL-12 from antigen-presenting cells and IL-4 from T cells, respectively. To determine whether AbOmpA induces development

Table 1 – Confirmation of the roles of ERK, JNK, and p38 of MAPK and NF-κB p65 in AbOmpA-induced expression of surface molecules on DCs

Surface Ag	Mean fluorescence intensity					
	Medium	AbOmpA	U0126	SB203580	SP600125	PD98059
CD80	120 ± 5	1510 ± 35*	160 ± 10**	152 ± 7	140 ± 12	135 ± 2**
CD86	532 ± 12	1412 ± 42*	120 ± 9	85 ± 4**	81 ± 7**	86 ± 1
MHC class I	54 ± 8	115 ± 7*	102 ± 6**	80 ± 3	81 ± 2	105 ± 5**
MHC class II	170 ± 6	371 ± 12	291 ± 1	230 ± 5**	281 ± 15**	221 ± 17

Immature DCs were pretreated with a specific MEK 1 inhibitor (SB203580), JNK inhibitor (SP600125), p38 MAPK inhibitor (PD98059), or U0126 (NF-κB p65 inhibitor) for 30 min before OmpA stimulation, and then stimulated with 200 ng/ml OmpA for 24 h. Cells were firstly gated on $CD11c^+$ and analyzed the expression of CD80, CD86, MHC class I and MHC class II by flow cytometry. Similar results were observed in three independent experiments.

* The statistical significance between samples with and without specific inhibitor is indicated (*$p < 0.01$ vs. unstimulated DC (medium).

** The statistical significance between samples with and without specific inhibitor is indicated (**$p < 0.01$ vs. inhibitor-stimulated DC).

of Th1 subsets, IL-12 secreting DCs were analyzed by flow cytometry. The cell populations of IL-12p40p70-positivity were increased in both AbOmpA- and LPS-treated DCs compared with untreated DCs. The expression of IL-12p40p70 was more augmented in AbOmpA-treated DCs (27%) than that of LPS-treated DCs (21%) (Fig. 4A). IL-10 is a pleiotropic cytokine known to have inhibitory effects on the accessory functions of DCs and appears to play a role in preventing Th1 or Th2 responses. The expression of IL-10 was just detectable similar with that of negative control (media) (Fig. 4B).

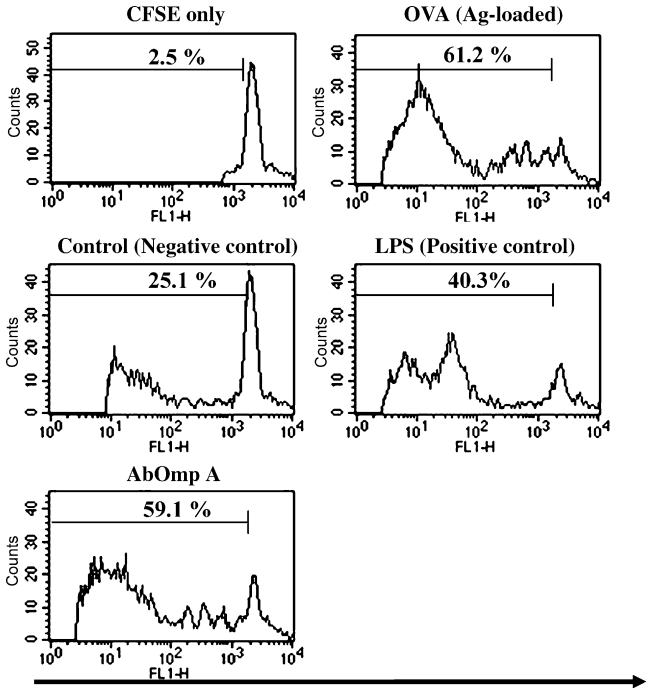

Fig. 5 – Syngeneic T cell stimulatory capacity of DCs differentiated with OmpA or LPS. Naïve T cells were co-cultured with DCs as described in Section 2. T cell proliferation was analyzed by flow cytometry and presented as percentage of dividing cells. A negative control ($CD8^+$ T cells in media alone), a specific Ag control (1 μM $OVA_{257–264}$) and a positive control ($CD8^+$ T cells in 200 ng/ml LPS) were created for each experiment. Similar results were obtained in three separate experiments.

3.4. *AbOmpA enhances immunostimulatory activity of DCs*

The increased expression of costimulatory molecules in DCs favorably involves in the presentation of antigen to T cells. We determined whether AbOmpA-treated DCs stimulate the proliferation of T cells in a syngeneic mixed lymphocytes reaction (MLR). This revealed that OmpA-treated DCs derived from C57BL/6 mice stimulated the proliferation of T cells derived from C57BL/6-Tg (OT-1) mice (Fig. 5). The stimulatory effects of AbOmpA were similar with ovalbumin-specific T cell stimulation, but higher than those of LPS. Successful induction of an adaptive immune response is determined by the context in which antigen is presented, its dose and the duration of its presentation [23]. In these respects, cluster formation is important for efficient induction of an adaptive immune response by interactions between T cells and DCs. To determine the interaction between DCs and T cells, LPS- or AbOmpA-treated DCs from C57BL/6 mice and T cells from BALB/c mice were mixed. Phase contrast microscopy showed the prominent cluster formation in AbOmpA- and LPS-treated DCs (Fig. 6A). These results suggest that AbOmpA enhances immunostimulatory activity of DCs to stimulate T cells.

Furthermore, we evaluated the cytokine production of primary allogeneic T cell responses stimulated by OmpA-treated DCs and LPS-treated DCs. OmpA-treated DCs co-cultured with T cells at 1:10 DCs/T cell ratio secreted large amounts of IFN-γ, but little IL-4. The production of IFN-γ by T cells co-cultured with OmpA-treated DCs was similar with that of LPS-treated DCs (Fig. 6B). These results suggests that T cells co-cultured with OmpA-treated DCs turned into typical Th1 cells, which produced large quantities of IFN-γ and released small amounts of IL-4. These responses were next confirmed using Ag (OVA)-specific T cell stimulation experiments.

3.5. *AbOmpA-primed DCs polarize $CD4^+$ T cells towards Th1 type response*

It is the unique ability of DCs to induce a primary immune response through activation and polarization of naïve T cells. We evaluated the cytokine production of primary allogeneic T cell responses stimulated by AbOmpA-treated DCs. T cells co-cultured with OmpA-treated DCs secreted large quantities of interferon-γ (IFN-γ), but the secretion of IL-4 was not affected

by AbOmpA-treated DCs (Fig. 7A). These results suggest that $CD4^+$ T cells co-cultured with AbOmpA-treated DCs polarize Th1 cells. Next, we determined the ability of OmpA-activated DCs to induce a Th1 phenotype in naïve $CD4^+$ T cells. $CD4^+$ splenic T cells from C.Cg-Tg(DO11.10) mice were co-cultured with AbOmpA-treated DCs derived from BALB/c mice in the presence 2 μM of $OVA_{323–339}$ peptide. The number of IFN-γ secreting naïve $CD4^+$ cells and subsequent secretion of IFN-γ were increasing by AbOmpA, while the number of IL-4 secreting $CD4^+$ cells and secretion of IL-4 were not affected (Fig. 7B). These results suggest that AbOmpA directs $CD4^+$ T cell differentiation towards a Th1 response.

3.6. *AbOmpA activates mitogen-induced protein kinases (MAPKs) and nuclear translocation of the NF-κB in DCs*

The activation of MAPKs, including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and p38 MAPK, and NF-κB is important events in DC maturation [7]. Recent studies suggest that three MAPK signaling pathways differentially regulate all aspects of phenotypic maturation, cytokine production and functional maturation of DCs [24]. In order to characterize the effects of AbOmpA on the MAPKs and NF-κB signaling pathways, DCs were treated with 200 ng/ml of AbOmpA. The activation of p38 MAPK, ERK and JNK was measured as phosphorylation of the proteins and the NF-κB pathway was determined as nuclear translocation of NF- κB p65 subunit by Western blot. AbOmpA induced phosphorylation of JNK, ERK1/2 and p38 MAPK in DCs (Fig. 8A). In addition, nuclear translocation of p65 subunit was observed in AbOmpA-treated DCs (Fig. 8B). To confirm whether the high expression of costimulatory molecules on DCs by AbOmpA was mediated by MAPKs-dependent pathway, immature DCs were pretreated with the specific inhibitors before stimulation of AbOmpA. Pretreatment of DCs with PD98059 for MEK 1, SP600125 for JNK, SB203580 for p38 MAPK and U0126 for ERK

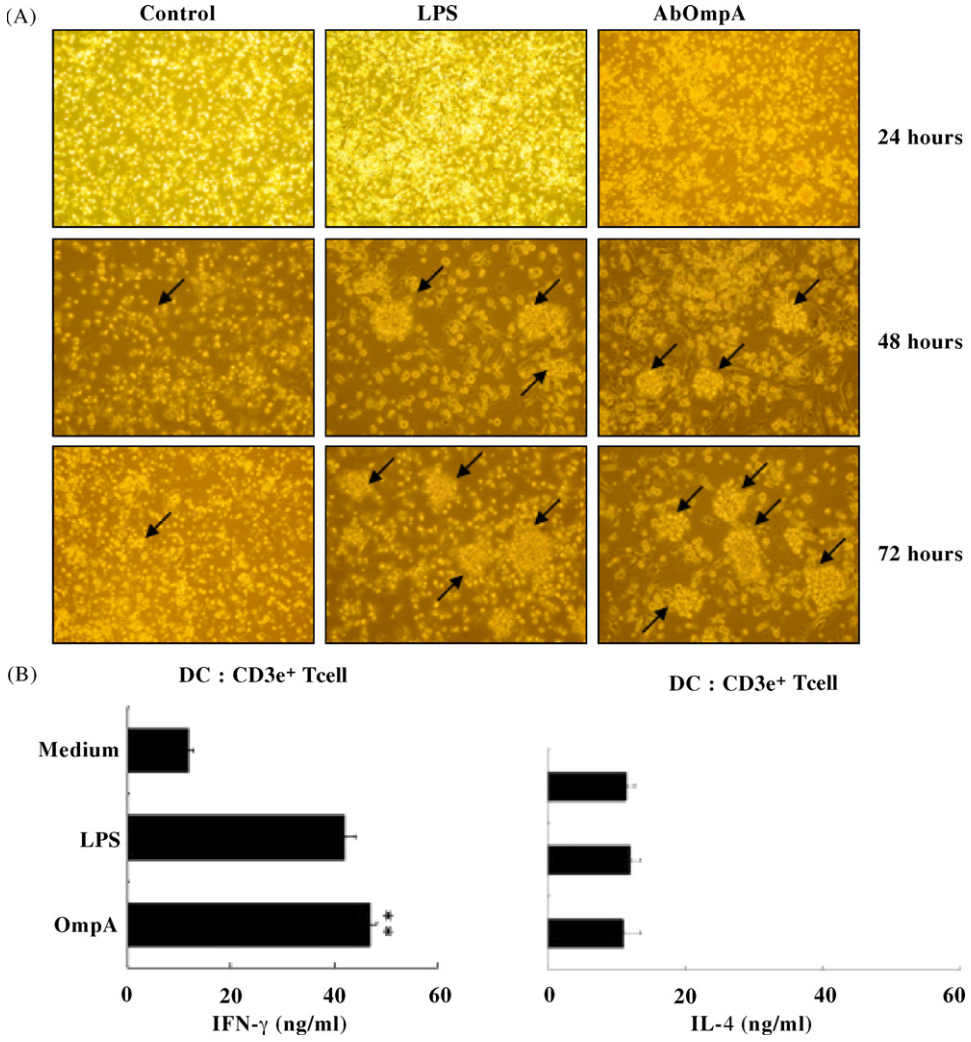

Fig. 6 – Evaluation of cluster formation and cytokine production profile. DCs were incubated for 24 h in medium alone, 200 ng/ml OmpA or 200 ng/ml LPS. DCs were washed and co-cultured with allogeneic T cells as described in Section 2. Cluster formation was assessed after 24, 48, and 72 h, respectively. A negative control (media alone) and a positive control (T cells in 200 ng/ml LPS) were created for each experiment. One representative experiment of three, showing a representative field in a culture well photographed using an inverted phase contrast microscope. In this same set of experiment, we measured the cytokine production (IFN-γ and IL-4) in culture supernatants at 48 h after culture initiation by ELISA.

remarkably inhibited AbOmpA-induced expression of CD80, CD86, HMC I and MHC class II (Fig. 9, Table 1). These results suggest that AbOmpA induces the expression of cell surface molecules involved in T-helper cell activation through both MAPKs and NF-κB pathways.

3.7. *TLR2 is involved in AbOmpA-mediated signaling*

It has been reported that KpOmpA correlates between TLR2 expression and the ability to respond to OmpA [17]. To elucidate this observation, we analysed IL-12 production with various sets of experimental group. The cells were incubated at 37 °C for 2 h with TLR blocking peptides, and then stimulated with LPS or AbOmpA after 24 h. After 24 h, intracellular IL-12 levels were measured. AbOmpA enhances the IL-12 synthesis. Enhancing effect of AbOmpA on IL-12 production was inhibited by TLR2 blocking peptide pretreatment, but it had no effect on untreated- and TLR4-pretreated DCs (Fig. 10). These results indicates that AbOmpA activates dendritic cells through TLR2.

4. Discussion

We have shown that AbOmpA, a major outer membrane protein of *A. baumannii*, is a highly immunogenic protein that

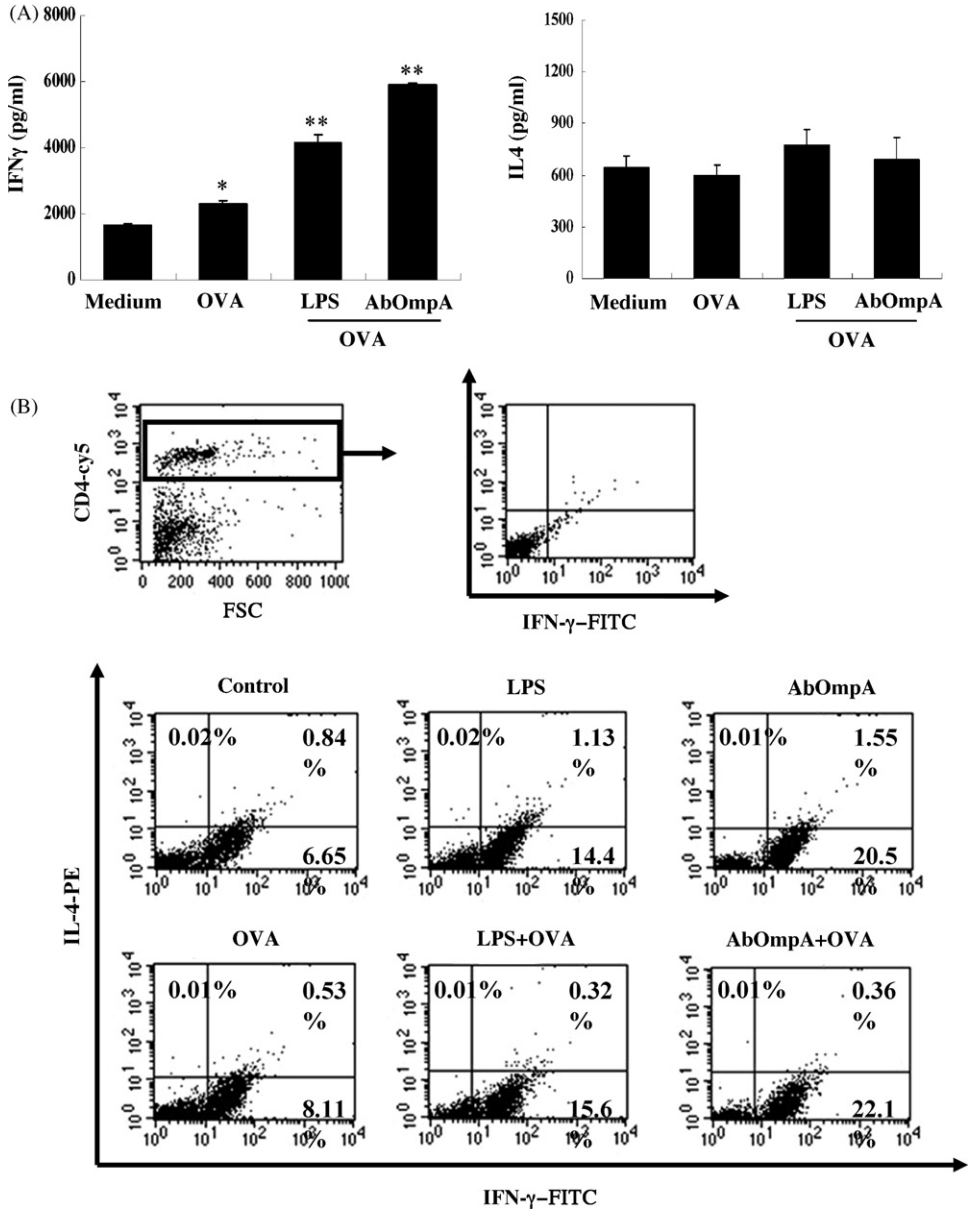

Fig. 7 – OmpA-treated DCs induced the differentiation of T cells to a Th1 response at 1:10 DCs/T cell ratio. To analyze cytokine production, T cells were co-cultured AbOmpA- or LPS-treated DCs for 48 h. After 48 h of expansion, IFN-γ and IL-4 were measured by ELISA in culture supernatants (A). Intracellular cytokine (IFN-γ and IL-4) concentrations were measured by flow cytometry (B). Data are the means ± S.D. of three independent experiments. (P < .001 vs. T cells treated with immature DC) (A). Medium represents the chemically-untreated control group. A representative data from three independent experiment is shown (B).**

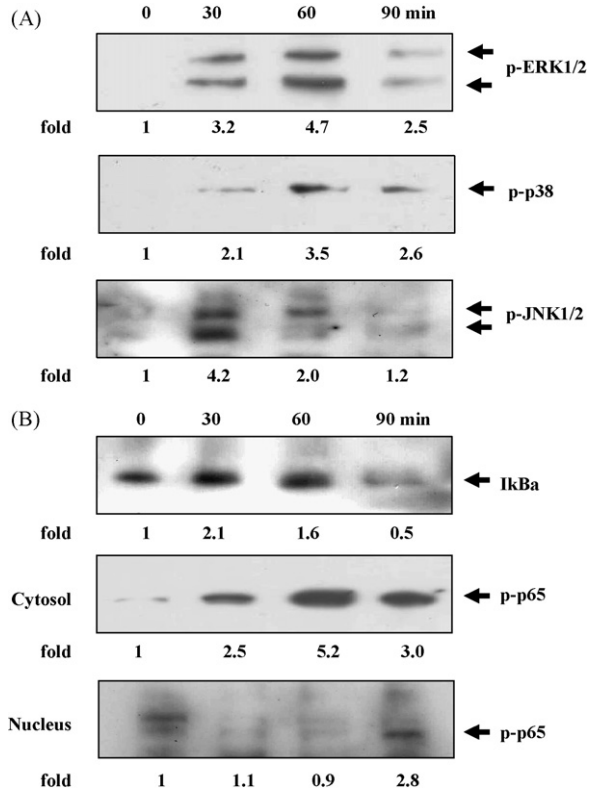

Fig. 8 – OmpA enhanced MAPK activity and NF-κB translocation in DCs. DCs were treated with 200 ng/ml OmpA for 30, 60 or 90 min. (A) For MAPKs analysis, cell lysates were prepared and blotted with anti-phospho-ERK1/2 (p-p44/42), anti-ERK1/2 (p44/42), anti-phospho-JNK1/2, anti-JNK1/2, anti-phospho-p38 (p-p38), and anti-p38 (p38), anti-phospho-IκBα, and anti-phospho-p65 Abs. (B) For OmpA-induced nuclear translocation of the NF-κB p65 subunit analysis, nuclear extracts were blotted with anti-p65 Ab. The bound antibodies were visualized using biotinylated goat anti-rabbit IgG. The activation levels were quantitated by measuring band intensities and shown as fold increase relative to background. The results shown represent three independent experiments.

induces $CD4^+$ Th1 response via DCs. AbOmpA induces maturation and activation DC, as demonstrated by high expression of costimulatory molecules on cell surfaces and the reduced antigen uptake. Functionally, AbOmpA-primed DCs have enhanced primary T cell stimulatory activity in an allogeneic MLR and drive Th1 polarization in a syngeneic ovalbumin (OVA)-specific $CD4^+$ T cells.

DCs are the most potent antigen presenting cells that initiate and amplify immune responses. Maturation of DCs by pathogen-associated molecular patterns (PAMPs) enables DCs to convey pathogen-associated signals to the adaptive immune system. We have shown in the current study that a relatively low concentration (200 ng/ml) of AbOmpA induces high expression of CD80, CD86, CD40, CD54, MHC class I and MHC class II in murine DCs. Moreover, AbOmpA-treated DCs produced high level of IL-12, but not IL-10. These results suggest that AbOmpA directly induces maturation and activation of DCs and consequently DCs convey AbOmpA to the adaptive immune system. However, high concentrations of AbOmpA ($\geq$3 μg/ml) induce death of DCs. Accordingly, the appropriate concentration of AbOmpA is critical for maturation and activation of DCs. We previously demonstrated that AbOmpA (high doses) was a cytotoxic molecule that induced apoptosis of epithelial cells and macrophages [18]. Apoptosis of epithelial cells may disrupt the mucosal lining and allows for the access of bacteria or bacterial products to deep tissues under mucosal epithelial cells. This situation may triggers the action mechanisms of DCs, such as death or activation relying on the OmpA dose, residing in the tissues under the disrupted epithelial cell lining by apoptosis. In this regard, the outcome of the infection with *A. baumannii* may depend on the induction of apoptosis in epithelial cells and consequently, OmpA-produced by *A. baumannii* triggered the activation or apoptosis in DCs that acts as sentinels by discarding foreign antigens and patogens in the surrounding tissue. Maturation of DCs has been also observed following treatment with OmpA of *K. pneumoniae* [17], chlamydial major outer membrane protein [25] and PorA of *Neisseria meningitidis* [26]. However, AbOmpA is a potent immunostimulator than other membrane proteins from Gram (−) bacteria, because 100 ng/ml of AbOmpA can induce maturation of DCs.

Successful induction of an adaptive immune response is determined by the context in which antigen is presented, its dose, and the duration of its presentation [23].

Pathogen-derived signals induce tissue-resident DCs to mature and migrate to local lymph nodes where they present antigen to naïve T cells. This professional competence for antigen presentation correlates with high expression of MHC, costimulatory (CD80 and CD86) and adhesion molecules (ICAM-1 and -3) [14]. Initial adhesion of T cells to DCs is antigen independent, allowing scanning of specific antigen–MHC complexes by TCR [11]. In the case of antigen recognition, an intracellular signaling cascade ensues in the T cell, which in turn promotes further maturation of DCs and eventually results in clonal proliferation of T cells and their differentiation into effector cells [27]. In these respects, cluster formation is very important for efficient induction of an adaptive immune respons.e by interactions between T cells and DCs. Here, we showed that OmpA remarkably stimulates the T cells/DCs cluster formation. On the basis of our result with production of IL-12 in AbOmpA-treated DCs, AbOmpA remarkably stimulates cluster formation of allogeneic T cells and DCs and, moreover, allogeneic T cells produced IFN-γ, which is a signature cytokine of Th1 cells. In the current study, we showed that AbOmpA favors Th1 polarization through the enhancement of IL-12 production by DCs. These properties may contribute to explain the distinct immunogenicity of this class of protein. However, PorA of *N. meningitidis* directs differentiation of $CD4^+$ T cells towards a Th2 type response [26]. Recombinant chlamydial major outer membrane protein antigen (rMOMP) induces $CD4^+$ Th1 immunity *in vivo*, but DCs pulsed *ex vivo* with rMOMP and adoptively transferred to naïve mice generate Th2 immune response [25]. These findings suggest that the nature and source of outer membrane protein antigens influence the Th1-Th2 balance of the immune response.

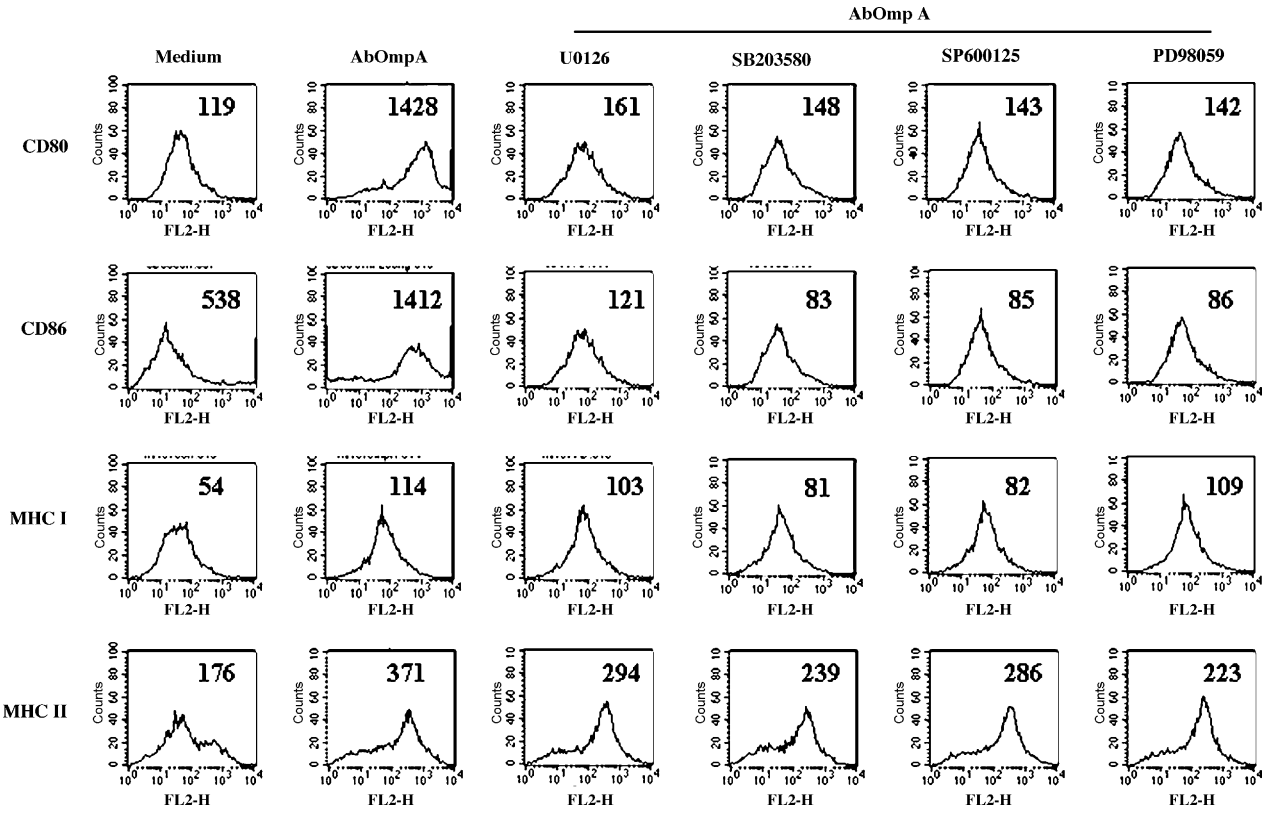

Fig. 9 – Confirmation of the roles of ERK, JNK, and p38 of MAPK and NF-κB p65 in AbOmpA-induced expression of surface molecules on DCs. To confirm the roles of ERK, JNK,and p38 of MAPK and NF-κB p65 in OmpA-induced expression of surface molecules on DCs, immature DCs were pretreated with a specific MEK 1 inhibitor (SB203580), JNK inhibitor (SP600125), p38 MAPK inhibitor (PD98059), or U0126 (NF-κB p65 inhibitor) for 30 min before OmpA stimulation, and then stimulated with 200 ng/ml OmpA for 24 h. Cells were firstly gated on $CD11c^+$ and analyzed the expression of CD80, CD86, MHC class I and MHC class II by flow cytometry. Similar results were observed in three independent experiments.

The recognition of PAMPs by Toll-like receptors (TLRs) leads to the activation of several MAPK pathways [28,29]. The activation of a MAPK pathway subsequently induces gene expression by activating several transcription factors, including NF-κB and AP-1 [30]. NF-κB activation is essential for the expression of a variety of cytokines in the proinflammatory cytokines [31]. Our results provided the evidence for AbOmpA stimulation of DCs resulting in the activation of MAPKs and NF-κB. Pretreatment with MAPKs inhibitors remarkably inhibited AbOmpA-induced expression of CD80, CD86 and MHC class II. The results indicate that both signaling pathways are involved in activation of DCs by AbOmpA. JNK and p38 MAPK signaling pathways coordinate to positively regulate the phenotypic maturation of human BMDCs, but ERK signaling pathway appears to negatively regulate the phenotypic maturation of human BMDCs to some degree [24]. However, our data showed that all MAPKs inhibitors inhibited expression of surface molecules in AbOmpA-treated DCs. Further studies are remained to clear this contradiction and TLR signaling by AbOmpA.

In summary, AbOmpA is a potent immunomodulator and contributes to the development of the initiation of a specific immune response, e.g., Th1 polarization. AbOmpA, other than LPS, may play roles in determining the nature of the immune

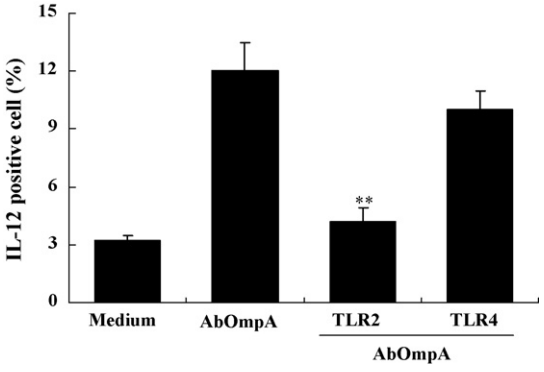

Fig. 10 – . AbOmpA induces intracellular IL-12 levels through TLR2. AbOmpA stimulates TLR2 (5 μg/ml) and TLR4 (5 μg/ml) blocking peptide with or without in dendritic cells. After TLR blocker treated for 2 h, the cells were treated with LPS or AbOmpA for 24 h. The IL-12 porduction levels were measured by flow cytometry. Mean ± S.D. of data from three independent experiments were shown. $^*p < 0.05$.

response against *A. baumannii*. Further understanding of the mechanisms by which AbOmpA modulates DCs function may lead to development not only of effective *A. baumannii* vaccines, but also of effective immunotherapy for cancer or other infectious diseases.

Acknowledgments

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