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Immunostimulatory Activity of Dendritic Cells Pulsed with Carbonic Anhydrase IX and *Acinetobacter baumannii* Outer Membrane Protein A for Renal Cell Carcinoma

Bo Ra Kim[1,2†], Eun Kyoung Yang[1†], Sun Hee Kim[1], Dong Chan Moon[2], Hwa Jung Kim[3], Je Chul Lee[2*], and Duk Yoon Kim[4*]

[1]Department of Physiology and [2]Department of Microbiology, Kyungpook National University School of Medicine, Daegu 700-422, Republic of Korea
[3]Department of Microbiology, Chungnam National University, College of Medicine, Daejeon 301-747, Republic of Korea
[4]Department of Urology, Catholic University of Daegu, School of Medicine, Daegu 705-718, Republic of Korea

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Dendritic cell (DC)-based immunotherapy is a potent therapeutic modality for treating renal cell carcinoma (RCC), but development of antigens specific for tumor-targeting and anti-tumor immunity is of great interest for clinical trials. The present study investigated the ability of DCs pulsed with a combination of carbonic anhydrase IX (CA9) as an RCC-specific biomarker and *Acinetobacter baumannii* outer membrane protein A (AbOmpA) as an immunoadjuvant to induce anti-tumor immunity against murine renal cell carcinoma (RENCA) in a murine model. Murine bone-marrow-derived DCs pulsed with a combination of RENCA lysates and AbOmpA were tested for their capacity to induce DC maturation and T cell responses *in vitro*. A combination of RENCA lysates and AbOmpA up-regulated the surface expression of co-stimulatory molecules, CD80 and CD86, and the antigen presenting molecules, major histocompatibility (MHC) class I and class II, in DCs. A combination of RENCA lysates and AbOmpA also induced interleukin-12 (IL-12) production in DCs. Next, the immunostimulatory activity of DCs pulsed with a combination of CA9 and AbOmpA was determined. A combination of CA9 and AbOmpA up-regulated the surface expression of co-stimulatory molecules and antigen presenting molecules in DCs. DCs pulsed with a combination of CA9 and AbOmpA effectively secreted IL-12 but not IL-10. These cells interacted with T cells and formed clusters. DCs pulsed with CA9 and AbOmpA elicited the secretion of interferon-γ and IL-2 in T cells. In conclusion, a combination of CA9 and AbOmpA enhanced the immunostimulatory activity of DCs, which may effectively induce anti-tumor immunity against human RCC.

***Keywords*:** renal cell carcinoma, dendritic cells, outer membrane protein A, carbonic anhydrase IX

Renal malignancies account for 3-4% of all newly diagnosed cancers and result in over 13,000 deaths each year (Jemal *et al*., 2010). Among urologic malignancies, renal cell carcinoma (RCC) is particularly difficult to treat because one third of the patients diagnosed with RCC present with metastases and the advanced RCC responds poorly to conventional treatments such as chemotherapy or radiotherapy (Skinner *et al*., 1971; Leibovich *et al*., 2003). As an alternative approach to RCC treatment, immunotherapy, including cytokine therapy using interferon-α (IFN-α) or interleukin-2 (IL-2), has been introduced and produces response rates of 15-20% (Bukowski, 2000; Ko and Atkins, 2005). In view of the non-specific character of cytokine therapy, dendritic cell (DC)-based immunotherapy elicits more specific immune responses against tumors and has been shown to have therapeutic potential for treating RCC (Bleumer *et al*., 2007; Lim *et al*., 2007; Driessens *et al*., 2009). DCs are the most powerful cells for tumor immunotherapy (Steinman and Banchereau, 2007; Driessens *et al*., 2009). However, several limitations such as selection of tumor-associated antigen (TAA), efficient antigen loading, and full activation of DCs for eliciting anti-tumor immunity remain to be overcome for developing DC-based immunotherapy against RCC.

Carbonic anhydrase IX (CA9) is an RCC-specific biomarker and is a potential target for RCC-specific immunotherapy (Uemura *et al*., 1999; Grabmaier *et al*., 2000). CA9 is present in more than 80% of primary and metastatic RCC, but normal tissues, including kidney, do not express this marker. A CA9-specific immune response shows anti-tumor activity against RCC *in vitro* (Vissers *et al*., 1999, 2002), but patients with advanced or metastatic RCC have a poor prognosis, suggesting that an immune response against CA9 is not sufficient to generate an anti-tumor response *in vivo*. To enhance the immunostimulatory potential of DC vaccines against RCC, a combination of autologous tumor cell lysates or TAA with cytokines such as IFN-γ, tumor necrosis factor (TNF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) has been introduced (Hernández *et al*., 2003; Inoue *et al*., 2008; Bauer *et al*., 2009). However, DC vaccines activated by CA9 combined with cytokines have produced inconsistent results and there is a need for a potent immunostimulator for DC-based immunotherapy against RCC. Pathogen-associated molecular patterns, such as lipopolysaccharides, peptidoglycans, unmethylated cytosine-phosphorothionateguanine-rich oligo-

† These authors contributed equally to this work.
* For correspondence. (J.C. Lee) E-mail: leejc@knu.ac.kr; Tel: +82-53-420-4844; Fax: +82-53-427-5664 (D.Y. Kim) E-mail: dykim@cu.ac.kr ; Tel: +82-53-650-4663; Fax: +82-53-623-4660

deoxynucleotides (CpG-ODN), and membrane proteins, are able to activate non-specific immune responses (Tsan and Baochong, 2007). Among these, CpG-ODN has been widely used to enhance immune responses as an immunoadjuvant and to potentiate anti-tumor activity of DCs against murine RCC (RENCA) (Chagnon *et al*., 2005). Additionally, a major outer membrane protein of *Acinetobacter baumannii*, outer membrane proteins A (AbOmpA), was found to induce DC maturation and drive the Th1 immune response (Lee *et al*., 2007). We have recently shown that DC vaccine pulsed with autologous tumor cell lysates combined with AbOmpA induces strong anti-tumor activity against murine melanoma (Lee *et al*., 2008). Moreover, AbOmpA alone greatly improved the therapeutic efficacy of the DC vaccine, suggesting that AbOmpA is a potent immunoadjuvant for anti-tumor immunotherapy.

RENCA is very useful for studying RCC-targeted DC vaccines (Hillman *et al*., 1994; Chagnon *et al*., 2001) because transplanted RENCA produces solid tumors in mice that metastasize to distant organ. This progressive disease process is similar to what is observed in RCC patients. In this study, we investigated the ability of DCs pulsed with CA9 combined with AbOmpA to induce anti-tumor immunity in a RENCA model.

Materials and Methods

Animals and tumor cells

Balb/c male mice (6-8 weeks old) were purchased from the Bio Korea (Gyunggi-do, Korea) and housed in a specific pathogen-free animal facility for at least one week before use. All procedures involving animals were approved by the Animal Care Committee of Kyungpook National University. A RENCA cell line, RCC syngeneic to the Balb/c mouse (Wiltrout *et al*., 1995), was kindly provided by Dr. Sung Joon Hong (Yonsei University College of Medicine, Seoul, Korea). RENCA cells were cultured and maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 1,000 U/ml penicillin, 50 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS). Cells were maintained at 37°C in 5% CO_2.

Reagents and antibodies

Recombinant mouse IL-4 and GM-CSF were purchased from R&D Systems (USA). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies were used to detect the expression of CD4 (L3T4), CD8 (Ly-2), CD11c (HL3), CD80 (16-10A1), CD86 (GL1), major histocompatibility complex (MHC) class I (H-2K^b), and MHC class II (I-A^b) (BD pharmingen, USA). An isotype-matched control monoclonal antibody, biotinylated anti-CD11c (N418) (BD pharmingen), was used for flow cytometry. CpG-ODN (5′-TCCATGA CGTTCCTGACGTT-3′) was synthesized by Bioneer (Daejeon, Korea).

Preparation of recombinant proteins and cell lysates

The recombinant AbOmpA was produced as previously described (Lee *et al*., 2007, 2008). cDNA from the *CA9* gene was purchased from the Korean Research Institute of Bioscience and Biotechnology (Daejeon, Korea). The full length of *CA9* gene was amplified by polymerase chain reaction (PCR) using the primers 5′-TGTCTAGACCAT GGCTCCCCTGTGCCC-3′ and 5′-CGCAATTGGGCTCCAGTCTC GGCTAC-3′. The PCR products of *AbOmpA* and *CA9* genes were cloned into the pET28a expression vector. *Escherichia coli* BL21 (DE3)/pET28a harboring the *AbOmpA* and *CA9* genes was grown in Luria-Bertani medium at 37°C and both recombinant proteins were overexpressed by treatment with 1 mM of isopropyl-1-thio-β-D-galactopyranoside. Recombinant proteins were purified by using a nickel-column (Sigma-Aldrich, USA) and endotoxins were removed by polymyxin B-coated beads (Sigma-Aldrich). Tumor cell lysates were prepared by four cycles of a freezing and thawing of the RENCA cells. Cellular debris was removed by centrifugation and the soluble fraction was passed through a 0.2 μm membrane filter. The protein concentration was determined using a modified BCA assay (Thermo Scientific, USA). The recombinant proteins and tumor cell lysates were stored at -80°C.

Generation of DCs and antigen pulsing

DCs were generated from murine bone marrow-derived cells as previously described with some modifications (Winzler *et al*., 1997; Lee *et al*., 2007). Bone marrow-derived cells were obtained from the tibia and femur of Balb/c mice and then depleted of erythrocytes. Cells were cultured in OptiMEM (Invitrogen, USA) supplemented with 10% FBS, 20 ng/ml recombinant mouse GM-CSF, and 20 ng/ml IL-4 at 37°C in 5% CO_2. On day 3 of culturing, floating cells were removed and fresh medium containing GM-CSF and IL-4 was added. On day 6, non-adherent cells and loosely adherent proliferating DC aggregates were harvested. To obtain a high purity of DC populations, DCs were labeled with a bead-conjugated anti-CD11c monoclonal antibody (Miltenyi Biotec, Germany), followed by positive selection through paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions. DCs (10^6 cells/ml) were pulsed with either CpG-ODN (4 μg/ml), RENCA lysates (10 μg/ml), AbOmpA (200 ng/ml), CA9 (200 ng/ml) or a combination of CA9 (200 ng/ml) and AbOmpA (200 ng/ml) at 37°C for 24 h.

Preparation of primary lymphocytes

Balb/c mice were euthanized by CO_2 inhalation to prepare splenic lymphocytes. Spleens were mechanically disrupted and passed through

Table 1. Phenotypic maturation of DCs pulsed with different antigens[a]

Antigens	Mean fluorescence intensity (MFI)[b]			
	CD80	CD86	MHC class I	MHC class II
Control[c]	54	356	152	768
RENCA lysates	86	383	141	771
CpG-ODN	84	505	196	805
AbOmpA	139	728	275	1,120
RENCA lysates and AbOmpA	164	776	239	1,198

[a] Cells were stained with antibodies against each antigen and analyzed by flow cytometry with isotype controls
[b] The data are representative of three independent experiments that gave similar results
[c] The control consisted of immature DCs incubated with RPMI media alone

a sterile nylon mesh filter (BD Biosciences, USA). The resulting cells were centrifuged at 400 g for 5 min and erythrocytes were removed. The cells were suspended and incubated in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES (pH 7.4), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol.

Flow cytometric analysis

Single cell suspensions of DCs or lymphocytes were stained with specific monoclonal antibodies. Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4°C and stained with appropriate fluorescence-conjugated antibodies for 30 min at 4°C. The stained cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, USA). The intracellular expression IL-12 p40/p70 (C15.6) and IL-10 (JESS-16E3) (BD pharmingen) was also measured by flow cytometry.

ELISA

The culture supernatants from DCs treated with antigens or T cells co-cultured with DCs were collected and stored at -80°C for ELISA. The levels of secreted IL-2, IL-4, IL-5, IL-10, IL-12p40, TNF-α, and IFN-γ were assessed according to the manufacturer's instructions (BD Pharmingen). The concentrations of cytokines were calculated using standard curves generated from the recombinant cytokines.

Cluster formation and cytokine production by mixed lymphocyte reaction

Responder T cells, which participate in syngeneic T cell reactions, were isolated from spleen of Balb/c mice by using a MACS column (Miltenyi Biotec). Staining with FITC-conjugated anti-CD3 antibody revealed that they consisted mainly of $CD3^+$ cells (>95%). Stimulator DCs (1×10^4 cells) were pulsed with CpG-ODN, RENCA lysates, AbOmpA, CA9 or a combination of CA9 and AbOmpA for 24 h and then washed thoroughly. DCs pulsed with antigens were co-cultured with T cells (1×10^5 cells) in 96-well U-bottom plates for 48 or 72 h, and the cluster formation was observed by phase contrast microscope (Nikon, Japan). In this same set of experiment, cytokine production was measured in culture supernatants.

Statistical analysis

Statistical analyses were performed using SPSS (SPSS Inc., USA). Differences were compared using a one-way ANOVA. *P*-values <0.05 were considered to be statistically significant.

Results and Discussion

AbOmpA potentiates maturation of DCs pulsed with RENCA lysates

The ability of DCs to activate T cells largely depends on their maturation. When immature DCs develop into mature DCs, the expression of costimulatory molecules and antigen presenting molecules is up-regulated on their surface (Winzler *et al*., 1997). We examined DC maturation induced by RENCA lysates. DCs were pulsed with RENCA lysates for 24 h and the expression of cell surface molecules involved in T cell activation was analyzed by flow cytometry. DCs pulsed with RENCA lysates slightly up-regulated the expression of B7 family of immune-regulatory ligands (CD80 and CD86) compared to immature DCs, but antigen presenting molecules MHC class I and class II were not up-regulated (Table 1). These findings suggest that RENCA lysates alone are not sufficient for DC maturation, but immunoadjuvants are essential for full maturation of DCs.

AbOmpA is a potent immunoadjuvant for DC-based immunotherapy against murine melanoma (Lee *et al*., 2008). CpG-ODN is also known to be a potent immunoadjuvant for

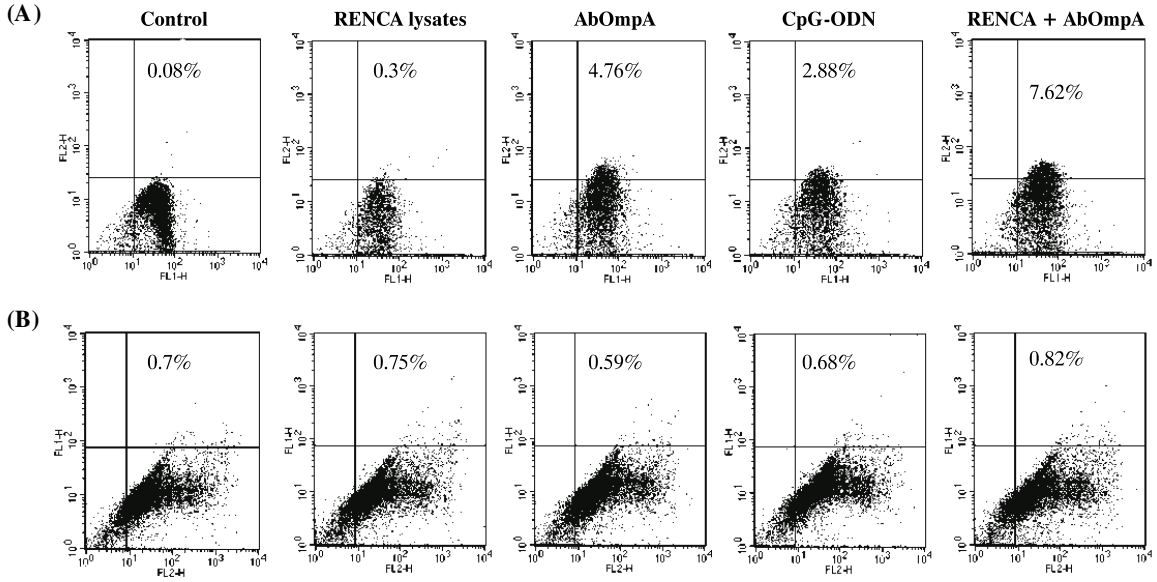

Fig. 1. Cytokine production in DCs pulsed with various antigens. Mature DCs were generated by stimulating immature DCs with RENCA lysates, AbOmpA, CpG-ODN, and a combination of RENCA lysates and AbOmpA for 24 h. After 24 h of culture, the production of IL-12 (A) and IL-10 (B) was measured by flow cytometry. The data are representative of two independent experiments that showed a similar tendency.

Table 2. Expression of cell surface markers on DCs treated with CA9, AbOmpA, and a combination of CA9 and AbOmpA[a]

Antigens	Mean fluorescence intensity (MFI)[b]			
	CD80	CD86	MHC class I	MHC class II
Control[c]	20,029	11,238	74,720	65,119
Lipopolysaccharides	35,709	17,209	94,862	83,769
AbOmpA	20,939	12,120	90,241	86,258
CA9	26,123	18,487	83,417	80,135
CA9 and AbOmpA	26,652	18,960	92,680	81,952

[a] Cells were stained with antibodies against the indicated antigens and analyzed by flow cytometry
[b] The data are representative of more than three independent experiments that gave similar results
[c] The control consisted of immature DCs incubated with RPMI media alone

DC vaccine against a murine RCC (Chagnon *et al*., 2005). We therefore compared the maturation of DCs pulsed with AbOmpA and CpG-ODN. Although DCs pulsed with both immunoadjuvants up-regulated the expression of surface markers such as CD80, CD86, and MHC class I and class II molecules compared to immature DCs, the expression of surface markers in DCs pulsed with AbOmpA was higher than that of DCs pulsed with CpG-ODN (Table 1). This result suggests that AbOmpA is a potent immunoadjuvant that can be used for maximizing the activation of DCs.

To determine whether AbOmpA potentiated DC maturation by RENCA lysates, DCs were pulsed with RENCA lysates combined with AbOmpA. The expression of CD80, CD86, and MHC class I and class II was up-regulated in DCs pulsed with a combination of RENCA lysates and AbOmpA compared to DCs pulsed with RENCA lysates alone (Table 1). This result suggests that AbOmpA promotes the maturation of DCs pulsed with RENCA lysates.

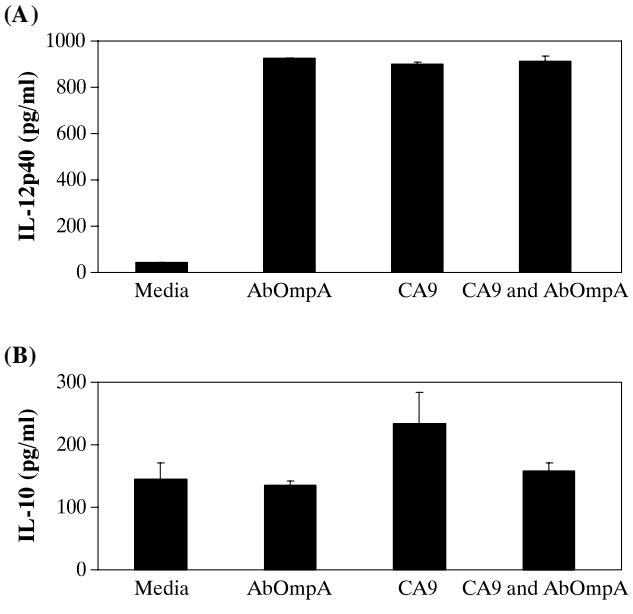

Fig. 2. Cytokine production in DCs pulsed with various antigens. Mature DCs were generated by stimulating immature DCs with AbOmpA, CA9, and a combination of AbOmpA and CA9 for 24 h. The culture supernatant was collected and the production of IL-12p40 (A) and IL-10 (B) was measured by ELISA.

AbOmpA enhances immunostimulatory activity of DCs pulsed with RENCA lysates

Early signals produced by DCs determine the nature and magnitude of an immune response (Chagnon *et al*., 2005). IL-12 and other cytokines produced by DCs induced both non-specific innate immune responses and specific cytotoxic T cell responses against tumors. These specific and non-specific immune responses are partly due to Th1 cells secreting cytokines such as IFN-γ (Hilkens *et al*., 1997). A potent driving factor for the development of Th1 cells is IL-12 from antigen-presenting cells. To determine whether DCs pulsed with a combination of RENCA lysates and rAbOmpA secreted IL-12, IL-12-secreting DCs were analyzed by flow cytometry. The cell populations positive for IL-12p40p70 were increased among DCs pulsed with RENCA lysates, CpG-ODN, and AbOmpA, but the expression of IL-12p40p70 was the highest in DCs pulsed with a combination of RENCA lysates and AbOmpA (Fig. 1A). 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 in DCs pulsed with RENCA lysates, AbOmpA, CpG-ODN, and a combination of RENCA lysates and AbOmpA was similar with that of untreated control DCs (Fig. 1B). These results suggest that AbOmpA enhances immunostimulatory activity of DCs pulsed with RENCA lysates.

CA9 is a potential target for human RCC-specific immunotherapy

After observing the immunostimulatory activity of DCs pulsed with a combination of RENCA lysates and AbOmpA, we searched for a potential target for human RCC-specific immunotherapy. CA9 has been identified as a RCC-specific TAA with potential prognostic and therapeutic value (Mukouyama *et al*., 2004; Bleumer *et al*., 2007; Zhou *et al*., 2010). CA9 shed from RCC plays a role in stimulating an adaptive immune response (Wang *et al*., 2008). His-tagged CA9 proteins were generated and used to determine DC maturation. Recombinant CA9 proteins up-regulated the expression of CD80, CD86, and MHC class I and MHC class II in DCs (Table 2). The expression of these surface markers was higher in DCs pulsed with a combination of CA9 and AbOmpA than that in DCs pulsed with CA9 or AbOmpA alone. Next, we examined the secretion of IL-12 and IL-10 from DCs pulsed with CA9, AbOmpA, and a combination of CA9 and AbOmpA. The secretion of IL-12p40 was increased in DC pulsed with two different antigens alone or in combination compared to untreated immature DCs (Fig. 2A). Secretion of IL-10 was

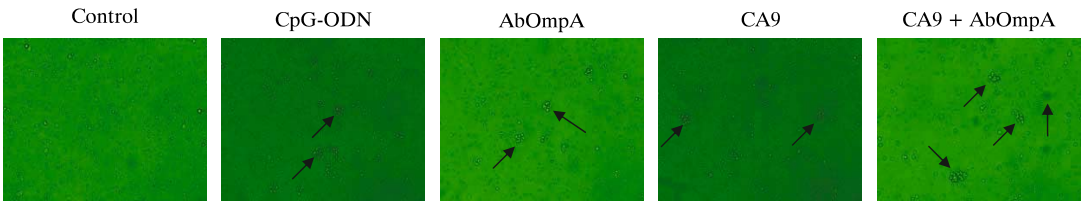

Fig. 3. Cluster formation of DCs with T cells. DCs were incubated for 24 h in medium alone, or with AbOmpA, CpG-ODN, CA9, and a combination of CA9 and AbOmpA. DCs were washed and co-cultured with syngeneic T cells. Cluster formation was assessed by inverted phase contrast microscope after 48 h. Magnification, 200×. Arrows indicate cell clusters.

not increased in DCs pulsed with AbOmpA or a combination of CA9 and AbOmpA, but CA9 alone slightly induced IL-10 secretion in DCs (Fig. 2B). These results suggest that a combination of CA9 and AbOmpA enhances DC immunostimulatory activity.

The increased expression of co-stimulatory molecules in DCs enhances the presentation of antigen to T cells. To determine whether DCs pulsed with antigens interacted with T cells, antigen-treated DCs and splenic T cells were mixed and cluster formation was observed. Phase contrast microscopy showed the most prominent cluster formation among DCs pulsed with CA9 and AbOmpA (Fig. 3). These results suggest that DCs pulsed with CA9 and AbOmpA favorably present antigen to T cells, which may induce a specific T cell response against human RCC.

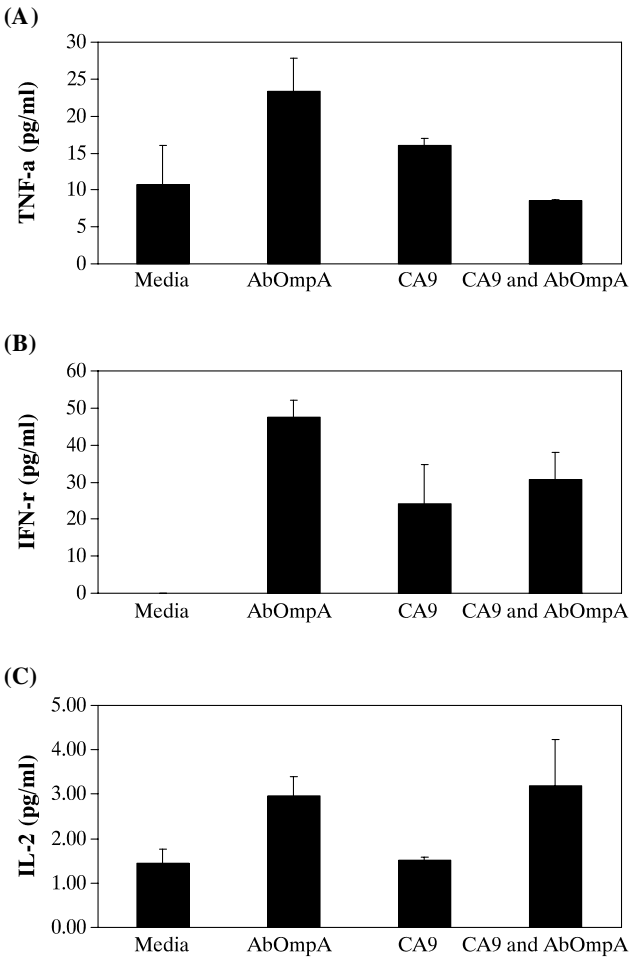

Fig. 4. Cytokine production in T cells stimulated by DCs pulsed with various antigens. DCs were pulsed with AbOmpA, CA9, and a combination of AbOmpA and CA9 for 24 h. Splenic T cells were co-cultured with the antigen-pulsed DCs at a ration of 10:1 (T cells: DCs) for 72 h. The culture supernatant was collected and production of TNF-α (A), IFN-γ (B), and IL-2 (C) was measured by ELISA.

A combination of CA9 and AbOmpA induces strong T cell response against tumors

IL-2 is an important growth and activation factor for cytotoxic T lymphocytes (CTLs), and typical Th1 cells produce a large amount of IFN-γ (Hilkens *et al*., 1997). To evaluate the cytokine production of T cells stimulated by DCs pulsed with various antigens, primary splenic T cells were co-cultured with antigen-pulsed DCs at 1:10 DCs/T cell ratio for 72 h. TNF-α was secreted in T cells co-cultured with DCs pulsed with AbOmpA, but not in T cells co-cultured with DC pulsed with CA9 alone or a combination of CA9 and AbOmpA (Fig. 4A). IFN-γ production increased in T cells co-cultured with DCs pulsed with AbOmpA, CA9, and a combination of both antigens, but was the highest in T cells co-cultured with DCs pulsed with AbOmpA alone (Fig. 4B). IL-2 production increased in T cells co-cultured with DCs pulsed with AbOmpA alone and a combination of CA9 and AbOmpA, but not in T cells co-cultured with DCs pulsed with CA9 alone (Fig. 4C). IL-4 and IL-5 secretion was not increased in T cells co-cultured with DCs pulsed with any of the antigens tested (data not shown). These results suggest that T cells activated by DCs pulsed with a combination of CA9 and AbOmpA differentiate into Th1 cells secreting IFN-γ and CTLs secreting IL-2, which can induce therapeutic anti-tumor immunity.

DC-based vaccines are a promising alternative approach for RCC treatment, and many studies have evaluated the efficacy of DC vaccines against RCC in animal models or clinical trials (Chagnon *et al*., 2005; Bleumer *et al*., 2007; Driessens *et al*., 2009). In this report, we showed that a combination of an RCC-specific TAA, CA9, and an immunoadjuvant, AbOmpA, elicited strong anti-tumor immunity. We postulate that CA9 targets tumors and induces specific anti-tumor immunity, and that AbOmpA potentiates the immunostimulatory activity of DCs. Moreover, AbOmpA can activate DCs in a way that overcomes the immunosuppressive effects of RENCA tumors. Since RENCA down-regulates the immune response (Chagnon *et al*., 2005), fully-activated DCs are essential for

inducing anti-tumor activity. AbOmpA activates DCs and polarizes Th1 immunity via Toll-like receptor 2 and subsequent mitogen-activated protein kinases and nuclear factor-κB pathways (Lee *et al*., 2007). In conclusion, our results highlight the potent immunoadjuvant properties of AbOmpA against RCC, which was previously observed in murine melanoma. Further studies are necessary to determine whether DCs pulsed with a combination of CA9 and AbOmpA can generate anti-tumor immunity *in vivo*.

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