Vaccine 29 (2011) 2400–2410

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Dendritic cells stimulated with outer membrane protein A (OmpA) of *Salmonella typhimurium* generate effective anti-tumor immunity

Min Ja Jang[a,1], Jee-Eun Kim[b,1], Yoon Hee Chung[a], Won Bok Lee[a], Yong Kyoo Shin[c], Jun Sik Lee[d], Daejin Kim[a,*], Yeong-Min Park[e,f,g,**]

[a] Department of Anatomy, Chung-Ang University College of Medicine, Dongjak-Gu, Seoul, Republic of Korea
[b] Research Institute of Women's Health, Sookmyung Women's University, Seoul, Republic of Korea
[c] Department of Pharmacology, Chung-Ang University College of Medicine, Seoul, Republic of Korea
[d] Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA
[e] Department of Microbiology and Immunology, Pusan National University, College of Medicine, Yangsan, Republic of Korea
[f] National Research Laboratory of Dendritic Cells Differentiation and Regulation, Pusan National University, College of Medicine, Yangsan, Republic of Korea
[g] Medical Research Institute, Pusan National University, College of Medicine, Yangsan, Republic of Korea

ARTICLE INFO

Article history:
Received 22 May 2010
Received in revised form
30 December 2010
Accepted 14 January 2011
Available online 26 January 2011

Keywords:
Dendritic cells
Outer membrane protein A
Anti-tumor immunity

ABSTRACT

Gram-negative bacterial outer membrane proteins (Omps) have an important role in pathogenesis and signal reception. We previously reported that *Acinetobacter* OmpA (*Ab*OmpA) induced maturation of bone marrow-derived dendritic cells (BMDCs) and that *Ab*OmpA-primed DCs produced IL-12 which generated Th1 $CD4^+$ T-cells. We analyzed the effects of *Salmonella typhimurium* OmpA (OmpA-Sal) on dendritic cell (DC) maturation in the present study, and determined that tumor antigen-pulsed DCs stimulated with OmpA-Sal induced anti-tumor responses in a mouse model. OmpA-Sal activated BMDCs by augmenting expression of MHC class II and of the co-stimulatory molecules CD80 and CD86. RT-PCR revealed that IL-12(p40) gene expression is highly augmented in OmpA-Sal-stimulated BMDCs. DNA (CRT/E7) vaccination combined with OmpA-Sal stimulation generated more antigen-specific $CD8^+$ T-cells in the present study. Certain antigen-pulsed BMDCs stimulated with OmpA-Sal induced strong PADRE-specific $CD4^+$ and E7-specific $CD8^+$ T-cell responses. In addition, BMDCs stimulated with OmpA-Sal (OmpA-Sal-BMDCs) and pulsed with both E7 and PADRE peptide generated greater numbers of E7-specific $CD8^+$ effector and memory T-cells than those pulsed with E7 peptide alone. E7- and PADRE-expressing OmpA-Sal-BMDC vaccines resulted in significant long-term protective anti-tumor effects in vaccinated mice. Our data suggested that E7- and PADRE-expressing BMDCs that were matured in the presence of OmpA-Sal might enhance anti-tumor immunity and support the therapeutic use of OmpA-Sal in DC-based immunotherapy.

1. Introduction

Naive $CD4^+$ or $CD8^+$ T cells encounter dendritic cells (DCs) during priming of adaptive immune responses. DCs are professional antigen-presenting cells (APCs) that are closely involved in naïve T-cell activation into cytotoxic T-cells (CTLs) and contribute to the production of cytokines and the generation of immune responses against infections or tumors [1,2]. Several studies have identified the chemical nature of adjuvants for the promotion of immune responses to antigens. Toll-like receptors (TLRs) and the complement system elicit specific signaling cascades and can result in enhancement of T- and B-cell responses [3,4]. Therefore, improvements in DC vaccines are necessary before they can be applied as useful modalities in conventional cancer treatment.

Purified and synthetic components of microbial extracts have potent adjuvant effects on the immune system [5–7]. These include variable lipids and glycolipids, such as mycolic acid, lipoarabinomannan (LAM), LPS, and microbial polynucleotides (including bacterial DNA with unmethylated CpG sequences). Innate cells recognize highly conserved microbial structures designated as pathogen-associated molecular patterns (PAMPs). PAMP recognition by innate cells initiates adaptive immune responses [8–10] and results in the production of pro-inflammatory mediators and the up-regulation of stimulatory molecule expression in APCs [11]. Immature DCs in the periphery differentiate into mature DCs through interactions between molecular patterns of microbial components and pattern recognition receptors, including Toll-like receptors (TLRs) [12].

* Corresponding author at: Department of Anatomy, Chung-Ang University, College of Medicine, Dongjak-Gu, Seoul, Republic of Korea. Tel.: +82 2 740 5648; fax: +82 2 815 4814.
** Corresponding author at: Department of Microbiology and Immunology, College of Medicine, Pusan National University, Yangsan, Republic of Korea. Tel.: +82 51 510 8091; fax: +82 55 382 8090.
E-mail addresses: kimdj@cau.ac.kr (D. Kim), immunpym@pusan.ac.kr (Y.-M. Park).
[1] These authors contributed equally.

0264-410X/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2011.01.036

Outer membrane proteins (Omp) are released during the growth and lysis of Gram-negative bacteria, and are associated with bacterial pathogenesis [13]. The Gram-negative bacterial cell wall protein OmpA has been recently identified as an initiator of adaptive immune responses. OmpA is considered as a new PAMP since it is conserved among *Euterobacteriaceae* and is essential for bacterial survival and virulence [14,15]. *Klebsiella pneumoniae* OmpA binds to a wide range of immune effector cells [16]. In addition, *K. pneumoniae* OmpA (*Kp*OmpA) activates both macrophages and dendritic cells [17]. We previously reported that the OmpA of *Acinetobacter baumannii* (*Ab*OmpA) induces DC maturation and stimulates interferon-γ production (rather than IL-4 production) from T-cells in mixed lymphocyte reactions [18]. We also found out that OmpA-Sal induced the expression of surface markers and Th1-polarizing cytokine production in dendritic cells was mediated by the TLR4 signaling pathway in a TLR4 Knock-out system [19]. These findings suggested that OmpA-Sal has potential as an anti-tumor vaccine adjuvant, particularly in DC-based immunotherapy.

Salmonella-associated Omps can induce immune responses and elicit T-cell responses, both of which may contribute to protect against bacterial infections in mouse models [20–23]. In the present study, we evaluated the adjuvant effects of OmpA derived from *Salmonella typhimurium* (OmpA-Sal). OmpA-Sal is not only a protein required for the formation of hydrophobic porins in the outer membranes in *Salmonella* but also a molecule inducing the maturation of murine bone marrow derived dendritic cells (BMDCs). We also analyzed antigen-specific $CD4^+$ and $CD8^+$ T-cell immune responses with BMDCs stimulated by OmpA-Sal and translated into *in vivo* tumor treatment and prevention experiments with tumor cell lines. Taken together, our data suggested that OmpA-Sal could serve as a component of DC-based immunotherapy

2. Materials and methods

2.1. Antibodies(Abs), peptides, cell line and mice

The HPV-16E7 (RAHYNIVTF) and PADRE (AKFVAAWTLKAAA) peptides were synthesized by Macromolecular Resources (Denver, CO, USA) at a purity of ≥90%. Antibodies (Abs) against CD4 (PE-conjugated, clone L3T4), CD8 (PE-conjugated, clone Ly-1), IFN-γ (FITC-conjugated, clone XMG1.2), CD11c (PE-conjugated), CD80 (PE-conjugated), CD86 (PE-conjugated, clone), I-A^b (PE-conjugated, clone AF6-120.1), and H-$2D^b$ (PE-conjugated, clone AF6-88.5) were purchased from BD Pharmingen. The HPV-16 E7-expressing murine tumor model TC-1 (kindly provided by Dr. T.-C. Wu, Johns Hopkins University, Baltimore, MD, USA) has been previously described [24]. All cells were maintained in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Female C57BL/6 mice were acquired from the Chung-Ang laboratory animal service (Seoul, Korea). All animal procedures were performed according to approved protocols and were in accordance with recommendations for the proper use and care of laboratory animals at our institution.

2.2. Preparation of OmpA of Salmonella typhimurium (OmpA-Sal)

The full-length OmpA-Sal gene (X02006.1) was amplified by PCR, and a chromosomal preparation of X02006.1 was used as a PCR substrate. The upstream primer 5′-GCGGATCCCACGA AGCCGGAGAA-3′ was designed to carry the EcoRI restriction site. The downstream primer 5′-GCAAGCTTAGAAACGATAGCC-3′ carried the HindIII restriction site. PCR products digested with EcoRI and HindIII were ligated into the pMAL™ expression vector (*New England Biolabs Inc.*). *E. coli* BL21 (DE3)/pMAL™ harboring a OmpA-Sal gene was grown in Luria–Bertani (LB) medium at 37 °C. Recombinant proteins were over-expressed by a bacteria protein expression system with Xgal (80 μg/ml) and IPTG (0.1 mM) at 25 °C for 4 h. Bacterial cells were sonicated, and supernatants with soluble OmpA-Sal were collected by centrifugation [25]. OmpA-Sal was incubated with endotoxin removal resin overnight to remove LPS and was concentrated by Centricon (2000 MW cut-off; Millipore). Endotoxin was assayed under endotoxin-free experimental conditions using the Limulus Amebocyte Lysate (LAL) pyrogen kit (Biowhittaker, Walkersville, MD). The quantity of OmpA endotoxin was ≤0.01 ng/mg. In addition, OmpA-Sal purity was determined 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) in boiling water for 5 min.

2.3. Plasmid DNA construction, preparation and DNA vaccination

The generation of pcDNA3-CRT/E7 has been described previously [26]. Plasmid constructs were confirmed by DNA sequencing. Amplification and purification of DNA were previously described [27]. DNA constructs were kindly provided by Drs. T.-C. Wu and Chien-Fu Hung from Johns Hopkins University, Baltimore, MD, USA. Intramuscular (i.m.) DNA vaccination was performed with 100 μg of pcDNA3-CRT/E7 DNA per mouse; mice received booster vaccines 1 week later.

2.4. Generation of bone marrow derived DCs and phenotypic analysis

DCs were generated from murine bone marrow cells with previously described modifications. Briefly, bone marrow was flushed from the tibiae and femurs of C57BL/6 mice and was depleted of red blood cells with ammonium chloride. Cells were plated in six-well culture plates (1×10^6 cells/well) in RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids, 50 μM β-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% FBS, and 10 ng/ml recombinant mouse GM-CSF at 37 °C and 5% CO_2. DCs were stimulated with 800 ng/ml of LPS or OmpA-Sal on culture day 6. DCs were harvested, washed, and re-suspended with FACS buffer (PBS containing 5% BSA) on culture day 7. Cells were stained with CD11c, CD80, CD86, I-A^b, and H-$2D^b$ for 30 min at 4 °C. Stained cells were analyzed with flow cytometry.

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

The mouse bone marrow derived DCs were treated with or without LPS(μg/ml) and omp-sal(μg/ml) for 24 h, total RNA was extracted using Trizol (Invitrogen Life Technologies, Inc., Carlsbad, CA), and cDNA was synthesized using AMV RT and oligo(dT_{15}) as a primer (Intron Biotechnology, Seongnam, Korea). Then cDNA was amplified using the following primers: IL-12p40 (sense, 5′-CACCTGCCCAACTGCCGAGG-3′; and antisense, 5′-TAG CTCCCTG-GCTCTGCGGG -3′)/IL-4 (sense, 5′-TCAACCCCCAGCTAGTTGTCAT-3′; and antisense, 5′-TTGCATGATGCTCTTTAGGC-3′)/IL-10 (sense, 5′-GCAGGACTTTAAGG GTTACT-3′; and antisense, 5′-TTCATGGCCTTGTAGACACC-3′)/GAPDH (sense, 5′-GTGGAGTCTACTGGCGTCTT-3′; and antisense, 5′-GCCTGCTTCACCACCTTCTT-3′). Cycling conditions for IL-12p40 were 30 s at 95 °C, 60 s at 60 °C, and 1 min at 72 °C for 35 cycles; conditions for IL-4 were 30 s at 95 °C, 60 s at 56 °C and 1 min at 72 °C for 35 cycles; conditions for IL-10 were 30 s at 95 °C, 60 s at 49 °C and 1 min at 72 °C for 35 cycles; and conditions for GAPDH