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CpG-A stimulates Hsp72 secretion from plasmacytoid dendritic cells, facilitating cross-presentation

Tsutomu Tanaka[a,b], Toshimitsu Kajiwara[a], Goro Kutomi[c], Takehiro Kurotaki[c], Keita Saito[c], Takayuki Kanaseki[a], Tomohide Tsukahara[a], Yoshihiko Hirohashi[a], Toshihiko Torigoe[a], Koichi Hirata[c], Yoshiharu Okamoto[d], Noriyuki Sato[a], Yasuaki Tamura[a,e,*]

[a] Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan
[b] United Graduate School of Veterinary Sciences, Yamaguchi University, Yamaguchi, Japan
[c] Department of Surgery, Sapporo Medical University School of Medicine, Sapporo, Japan
[d] Joint Department of Veterinary Medicine, Tottori University, Tottori, Japan
[e] Department of Molecular Therapeutics, Center for Food & Medical Innovation, Hokkaido University, Sapporo, Japan

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ABSTRACT

Plasmacytoid dendritic cells (pDCs) are the main producers of IFN-α in response to unmethylated DNA molecules, including cytosine guanine dinucleotide (CpG)-DNA *in vivo*. pDCs specifically express toll-like receptor (TLR) 9 and are therefore able to recognize the unmethylated DNAs. It has recently been shown that not only conventional DCs (cDCs) but also pDCs efficiently cross-present exogenous antigens after TLR9 activation. However, the precise molecular mechanism has remained unclear. Here, we show that pDCs secreted heat shock protein 72 (Hsp72) in response to CpG-A administration in a TLR9-dependent manner. Extracellular Hsp72 bound to an Hsp90-peptide complex and enhanced binding of Hsp90-peptide complex to pDC, resulting in efficient cross-presentation. Our experiments therefore suggest a mechanism for orchestration of immune responses by stimulation of pDCs with CpG-A.

1. Introduction

Cross-presentation is a crucial mechanism in tumor and microbial immunity because it allows internalized exogenous antigens (Ags) to be delivered into the MHC class I pathway. This pathway is important for the development of $CD8^+$ T-cell responses against tumors and microbes. Although cross-presentation is considered to be a unique property of $CD8^+$ conventional DCs [1], Mouries et al. have recently shown that splenic plasmacytoid dendritic cells (pDCs) efficiently capture exogenous Ags *in vivo* but are not able to cross-present these Ags in a steady state [2]. However, *in vivo* and *in vitro* stimulation by TLR7 or TLR9 or viruses licenses pDCs to cross-present soluble or particulate Ags by a mechanism dependent on transporter associated with antigen processing. These findings suggest that pDCs may play a crucial role in the induction of adaptive immune responses against pathogens that do not infect tissues of hematopoietic origin. Considering the host defense against viral infection, it is beneficial that stimulation by TLR ligands or viruses activates not only the innate immune response, but also the adaptive immune response including $CD8^+$ T-cell responses *via* cross-presentation by pDCs [3]. Orchestration of innate immunity and adaptive immunity is required for host defense against viral infection. However, the mechanism by which the orchestration of innate immunity and adaptive immunity occurs remains unclear.

Heat shock proteins (HSPs) are molecular chaperones that control the folding of proteins and prevent protein aggregation. They are capable of interacting with a broad range of peptides within the cell, resulting in HSP-peptide complexes that elicit $CD8^+$ T-cell responses by cross-presentation. We have shown that Hsp90-cancer antigen peptide complexes were efficiently cross-presented by conventional dendritic cells (cDCs) [4,5]. Immunization with the Hsp90-peptide complex could elicit powerful cytotoxic T lymphocytes (CTLs). Moreover, the role of extracellular HSPs in the stimulation of innate immunity has drawn much attention in recent

Abbreviations: DC, dendritic cell; CpG, cytosine guanine dinucleotide; TLR, toll-like receptor; Hsp72, heat shock protein 72; Hsp90, heat shock protein 90; Ag, antigen; mAb, monoclonal antibody.

* Corresponding author at: Department of Molecular Therapeutics, Center for Food & Medical Innovation, Hokkaido University, Sapporo, Japan. Fax: +81 11 706 9463.
E-mail address: ytamura@gmail.com (Y. Tamura).

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years [6,7]. Here, we show that TLR9 stimulation by CpG-A induced the secretion of Hsp72 in a TLR9-dependent manner, leading to increased cross-presentation of exogenous Hsp90-peptide complex by pDCs. Moreover, immunization with the Hsp90-peptide complex in combination with CpG-A was more effective for CTL induction than immunization with Hsp90-peptide complex alone. We found that extracellular Hsp72 bound to the Hsp90-peptide complex enhanced the binding of the complex to pDC, increasing cross-presentation by pDCs. These findings suggest that CpG-A-mediated stimulation makes an Hsp90-based cancer vaccine more immunogenic.

2. Materials and methods

2.1. Mice

C57BL/6 mice were obtained from The Jackson Laboratory. *Tlr9*$^{-/-}$ mice were kindly provided by Dr. S. Akira (Osaka University, Japan). HLA-A*2402/K^b transgenic mice were purchased from SLC Japan (Shizuoka, Japan). All mice were kept in a specific pathogen-free mouse facility. Studies were performed with the approval of the Animal Experiment Ethics Committee of Sapporo Medical University.

2.2. Oligodeoxynucleotides and TLR ligands

Synthesized CpG oligodeoxynucleotides (ODNs) were purchased from InvivoGen (San Diego, CA, USA). The sequences of ODNs were: murine CpG-A, 5′-ggTGCATCGATGCAgggggG-3′. ODN2216 was CpG ODN type A human TLR9 ligand (5′-ggGGGACGATCGTCgggggg-3′) and ODN2006 was CpG type B. TLR2 ligand PamCys-Ser-(Lys)$_4$, TLR7 ligand imiquimod (R837), and lipopolysaccharide (LPS) were purchased from InvivoGen. Human IFN-αA was purchased from PBL Biomedical Laboratories (Piscataway, NJ, USA).

2.3. Proteins and antibodies

Purified human Hsp90 and recombinant human Hsp72 were purchased from StressGen (Ann Arbor, MI, USA). Monoclonal antibodies (mAbs) anti-H-2K^b (clone AF6-88.5) and anti-I-A^b (clone AF6-120.1) were purchased from BD Pharmingen (San Jose, CA, USA).

2.4. Cells

The B3Z cell line (kindly provided by Dr. N. Shastri, University of California, Berkeley, CA, USA) is a CD8$^+$ T cell hybridoma cell line specific for the OVA$_{257–264}$ epitope (SL8) in the context of H-2K^b. RMA-S-A*2402 cells were RMA-S cells transfected with the gene encoding for HLA-A*2402.

2.5. Generation of Hsp90-peptide complex in vitro

The following peptides were used (the precise MHC I binding epitopes are underlined): survivin-2B$_{80–88}$ peptide (AYACNTSTL), survivin-2B$_{75–93}$ precursor peptide (GPGTV<u>AYACNTSTL</u>GGRGG), SL8 (SIINFEKL), and SL8C precursor peptide (<u>SIINFEKL</u>TEWTS). *In vitro* reconstitution was carried out as previously described [4]. Briefly, Hsp90 was mixed with a 50:1 peptide to a protein molar ratio in 0.7 M NaCl containing sodium-phosphate buffer, heated at 45 °C for 10 min, and then incubated at room temperature for 30 min.

2.6. Preparation of pDCs

Murine pDCs were purified from spleen cells using a Plasmacytoid Dendritic Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Human pDCs were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors with the plasmacytoid DC isolation kit II (Miltenyi Biotec), according to the manufacturer's instructions. Human cDCs were isolated from PBMCs using a Myeloid Dendritic Cell isolation kit (Miltenyi Biotec). The healthy donors gave informed consent for the use of blood samples in our research.

2.7. Measurement of Hsp72, Hsp90, Hsp60, and cytokine production

Human or murine DCs were plated at 5×10^4 cells/well in 96-well flat-bottomed plates in 100 μl of complete RPMI 1640 medium with 10% FCS and stimulated with various reagents for 24 h. Either 3 μM CpG-A or CpG-B, 1 or 10 μg LPS, or 100 U/ml human IFN-αA was added to the wells. After stimulation for 24 h, the supernatant fractions were diluted and tested for human or murine Hsp72 (StressGen), human Hsp90 (StressGen), human Hsp60 (Enzo Life Sciences, Farmingdale, NY, USA) or murine TNF-α (Pierce, Rockford, IL, USA) using a sandwich ELISA kit. Absorbance was measured at 450 nm.

2.8. Vaccination with SL8C and Hsp90–SL8C complex

C57BL/6 mice were injected subcutaneously on days 0 and 7 with Hsp90 (50 μg)-SL8C (50 μg) or SL8C (50 μg) with or without CpG-A (100 μg /mouse). Seven days after the second immunization, splenocytes from immunized mice were cultured in the presence of SL8 peptide (1 μg/ml) at 5×10^6 cells/ml for 5 days. On day 5, cells were harvested for a standard 4-h ^{51}chromium release assay.

2.9. In vitro cross-presentation assay

Splenic pDCs (1×10^4) from C57BL/6 mice were pulsed with the SL8C peptide (10 μg/ml) or Hsp90 (10 μg/ml)-SL8C peptide (10 μg/ml) complex generated *in vitro*, and SL8 peptide-specific B3Z cells (1×10^5) were added to each well and cultured overnight. The absorbance at 595 nm of the cleavage product of chlorophenol red β-pyranoside was used to quantify β-galactosidase activity.

2.10. Laser confocal microscopic analysis

Hsp90 was conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions. Isolated pDCs were incubated with BSA (10 μg/ml) or Hsp72 (10 μg/ml) for 30 min at 4 °C and then with Alexa 488-labeled Hsp90-peptide complex for 1 h at 4 °C. After incubation, the cells were fixed with cold acetone and visualized using an LCM510 confocal microscope (Zeiss, Oberkochen, Germany).

2.11. Immunoprecipitation

To examine whether Hsp90–SL8C complex bound to Hsp72 *in vitro*, the two proteins were mixed at a 1:1 molar ratio and incubated for 30 min at 37 °C. The complex was pretreated with 30 μl of protein G beads and immunoprecipitated using a mouse anti-Hsp90 mAb, a mouse anti-Hsp72 mAb, or a mouse control IgG. After resolving 10% SDS-PAGE, western blot analysis was performed using mouse anti-Hsp90 mAb or mouse anti-Hsp72 mAb.