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Spatiotemporal Regulation of Heat Shock Protein 90-Chaperoned Self-DNA and CpG-Oligodeoxynucleotide for Type I IFN Induction via Targeting to Static Early Endosome

Koichi Okuya,*† Yasuaki Tamura,* Keita Saito,*† Goro Kutomi,† Toshihiko Torigoe,* Koichi Hirata,† and Noriyuki Sato*

Recent studies have suggested that TLR9 signaling in early endosomes leads to IFN-α production by plasmacytoid dendritic cells (pDCs), whereas TLR9 signaling in late endosomes induces pDC maturation, IL-6, and TNF-α secretion. In this study, we show that human DNA as well as CpG-oligodeoxynucleotides (ODNs) in complex with heat shock protein 90 (Hsp90) stimulate pDCs to produce large quantities of IFN-α. The Hsp90–CpG-A complexes are targeted into the Rab5α, early endosomal Ag 1α-static early endosome postinternalization by DCs, suggesting that preferential sorting of Hsp90-chaperoned self-DNA/CpG-ODNs to the static endosome is required for signaling through TLR9 for IFN-α production. Interestingly, Hsp90-mediated preferential static early endosomal translocation of CpG-ODNs triggers robust IFN-α production from murine conventional DCs. Thus, extracellular Hsp90 converts inert self-DNA/CpG-ODNs into a potent trigger of IFN-α production via spatiotemporal regulation. The Journal of Immunology, 2010, 184: 7092–7099.

Heat shock proteins (HSPs) are molecular chaperones that control the folding and prevent the aggregation of proteins. It is well known that tumor-derived HSPs, such as Hsp70, Hsp90, and gp96, initiate efficient tumor-specific CTL responses and protective immunity (1–6). We have demonstrated that extracellular HSP–Ag peptide complexes are efficiently cross-presented via the endosome–recycling pathway (7, 8). In this HSP-mediated cross-presentation, the receptor-dependent endocytosed HSP–peptide complex is translocated to the early endosome, and thereafter, the Hsp90-chaperoned peptide is transferred onto recycling MHC class I molecules.

Bacterial and viral DNAs rich in CpG motifs or small synthetic oligodeoxynucleotides (ODNs) containing CpG motifs activate innate immune cells, such as dendritic cells (DCs), via TLR9 (9, 10). TLR9 is expressed within endolysosomal compartments in innate immune cells and recognizes distinct patterns of nucleic acids in the endolysosomal compartments (11–15). Upon TLR9 engagement, IFN-α induction depends on the MyD88–IFN regulatory factor (IRF)-7 signaling pathway (16, 17). DC subpopulations are characterized by expression of different surface markers and the ability to produce cytokines that modulates both innate resistance and the adaptive immune response (18). In the murine system, the plasmacytoid DCs (pDCs) are CD11c<sup>low</sup> B220<sup>high</sup> Ly6C<sup>high</sup> cells exhibiting plasmacytoid morphology and are able to produce a high level of IFN-α in response to several viruses or to CpG-ODN (19–21). Murine CD11c<sup>high</sup> conventional DCs (cDCs) have been further subdivided into subsets, such as CD8<sup>+</sup> DCs and CD8<sup>−</sup> DCs (18). In humans, CD11c<sup>−</sup> IL-3R<sup>high</sup> pDCs differ from CD11c<sup>+</sup> myeloid DCs (mDCs) in being uniquely able to produce a large amount of IFN-α in response to viral stimulation (22, 23) or to CpG-ODN (24). Previous studies revealed that CpG-A or various DNAs needed to be retained for long periods in the endosomes of pDCs for sufficient activation of TLR9 signaling (25, 26). Recently, CpG-ODNs have shown promising results as vaccine adjuvants for cancer immunotherapy due to their ability to induce potent Th1-type immune responses and anti-tumor responses (27–30).

Very recently, Lakadamyali et al. (31) have shown that early endosomes are comprised of two distinct populations called static early endosomes, which are slow maturing, and rapidly maturing dynamic early endosomes.

We have demonstrated that targeting of HSP–peptide complexes to the early endosomal Ag 1<sup>+</sup> Rab5<sup>+</sup>-static early endosome is crucial for cross-presentation (8). In this study, we show that Hsp90 can form complexes with CpG-ODNs in vitro and that these complexes act as potent inducers of IFN-α production not only by pDCs but also cDCs. Furthermore, we show that extracellular CpG-ODN–Hsp90 complexes accumulate in the static early endosome but not the dynamic early endosome when pulsed onto DCs. In the human system, Hsp90 can convert inert self-DNA into a potent trigger of IFN-α production by targeting static early endosomes via spatiotemporal regulation. Thus, extracellular Hsp90 can be an excellent immunomodulator for cancer immunotherapy via spatiotemporal regulation of chaperoned molecules.

Materials and Methods

Mice

C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Tbrα<sup>−/−</sup> mice were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). All mice were kept in a specific-pathogen-free environment.
mouse facility. Studies were performed by an approval of Animal Experiment Ethics Committee of Sapporo Medical University (Sapporo, Japan).

**Oligodeoxynucleotides**

Synthesized CpG ODNs were purchased from Sigma-Aldrich (St. Louis, MO) and Invivogen (San Diego, CA). The sequences of ODNs were: murine Cpg-A, 5'-ggtgcattgcgttgagggggg-3'; murine Cpg-B, 5'-ttatatagtttttgtgcggtcag-3'; and control Cpg ODN, 5'-ggtgcattgcgttgagggggg-3'. ODN2216 was Cpg ODN type A-human TLR9 ligand (5'-ggggctgatctgtgagggg-3') and purchased from Invivogen.

**Protein and Abs**

Purified human Hsp90 was purchased from Stressgen (Ann Arbor, MI). Organelles were detected by laser confocal microscopy with specific Abs against KDEL (Stressgen) for endoplasmic reticulum (ER), Rab5 (Santa Cruz Biotechnology, Santa Cruz, CA) and EEA1 (Abcam, Cambridge, MA) for early endosomes, and lysosome-associated membrane protein 1 (LAMP1) (Santa Cruz Biotechnology) for lysosomes. Each Ab was labeled with Alexa Fluor 488 or Alexa Fluor 594 ( Molecular Probes, Eugene, OR). Alexa Fluor 488 was used for labeling Hsp90.

**Preparation of DCs**

Murine cDCs and pDCs were isolated from bone marrow-derived DCs using magnetic beads and the MACS system (Miltenyi Biotec, Auburn, CA). Bone marrow cells were cultured for 5 d in a 5% CO2 environment at 37°C in complete RPMI 1640 medium with 10% FCS, 20 mg/ml GM-CSF (Endogen, Woburn, MA), and 50 µM 2-ME (Invitrogen, Carlsbad, CA). Murine pBc were purified using a PlasmaLytic Dendritic Cell Isolation Kit (Miltenyi Biotec) and cDCs were purified by negative selection with anti-murine pDC Ag 1 microbeads (Miltenyi Biotec), followed by positive selection with CD11c beads (Miltenyi Biotec). To confirm the expression of TLR9 and IRF7, pDCs and cDCs were lysed with 0.5% CHAPS containing protease inhibitor (Roche, Basel, Switzerland). Equal amounts of protein were separated by SDS-PAGE and analyzed by immunoblotting with anti-TLR9 (Invivogen), anti-IRF7 (Abcam), and β-actin Abs ( Sigma-Aldrich). For RT-PCR, mRNA was isolated from pDCs and mouse spleen cells using an RNaseasy mini kit (QIAGEN, Valencia, CA). Cells were pelleted at 4°C and resuspended in RLT lysis buffer (Qiagen). Total RNA was extracted following a column DNAse digestion using RNeasy mini columns and collected in RNase-free water. Oligo(dT)-primed reverse transcriptase of RNA into cDNA was performed with a Superscript III first-strand synthesis kit (Invitrogen), and 5% of the product was used for each RT-PCR sample using PCR buffer with hot-start Invitrogen Taq polymerase (Invitrogen). Primer pairs for TLR9 (forward 5'-gctttttgggcttcctgatgct-3' and reverse 5'-ttatatagtttttgtgcggtcag-3') and GAPDH (forward 5'-gactcgccgctaagctggtgg-3' and reverse 5'-tgaggtggatggaggtttgcc-3') were designed using Primer3. Human mDCs and pDCs were isolated from PBMCs of healthy donors with mDC and pDC isolation kits (Miltenyi Biotec) according to the manufacturer's instructions.

**Generation of CpG-ODN–Hsp90 complex in vitro**

To confirm whether CpG-ODN–Hsp90 complexes could be generated, 1.5 nmol CpG-ODN was end labeled with T4-poly nucleotide kinase and [γ-32P]ATP, followed by incubation with 0.5 µM purified Hsp90 (Stressgen) at 37°C for 30 min. These samples were then separated by native-PAGE, and the gels were analyzed with silver staining and exposed to a Fuji BAS-MS imaging plate (Fuji Medical Systems, Tokyo, Japan) for ~24 h. The images were scanned using a BAS-2000 Phosphor Imaging System(Fuji Medical Systems). To make 3 µM CpG-ODN–Hsp90 complex, 3 nmol CpG-ODN was mixed with 1 µM Hsp90, incubated for 30 min, and then diluted with 1 ml medium.

**Measurement of cytokine production**

DCs were plated at 5–10 x 10^5 cells/well in flat-bottomed, 96-well plates in 100 µl complete RPMI 1640 medium with 10% FCS and stimulated with various reagents for 24 h. Cpg-ODN or CpG-ODN-Hsp90 complex was added to medium at a final concentration of 3 µM. Supernatants were diluted and tested for various cytokines with mouse or human IFN-α (PBL InterferonSource, Picataway, NJ), TNF-α (Pierce, Rockford, IL), and IL-6 (R&D Systems, Minneapolis, MN) using a sandwich ELISA kit. Absorbance was determined at 450 nm.

**Flow cytometry**

For detection of cell surface-bound and intracellular Cpg-A that was transported by cDC and pDC, isolated mouse cDCs and pDCs were incubated with synthesized Cy5-labeled Cpg-A (Sigma-Aldrich) or a complex with Hsp90 at 37°C. After 30 min, flow cytometric analysis was performed on a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA).

**Confocal imaging**

Hsp90 was conjugated with Alexa Fluor 488 (Molecular Probes) according to the manufacturer’s instructions. To visualize the kinetics of exogenously loaded Hsp90, purified cDCs were seeded on glass cover slips for 12 h and were first incubated with Alexa Fluor 488-labeled Hsp90 (10 µg) at 4°C for 10 min and washed. The cells were then further incubated for 0–120 min, fixed with 4% paraformaldehyde for 5 min at room temperature, and visualized. For the detection of colocalization with exogenous Hsp90 and organelles, cDCs were incubated with Alexa Fluor 488-labeled Hsp90 at 37°C for 120 min and washed. The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then blocked with 10% goat serum for 40 min. Cells were stained with anti-KDEL for detecting ER, anti-LAMP1 for late endosomes and lysosomes, and anti-Rab5 and anti-EEA1 for early endosomes for 60 min, followed by Alexa Fluor 594-conjugated goat anti-rabbit IgG for 60 min, and mounted. To analyze the intracellular routing of Cpg-A, cDCs were seeded on glass coverslips for 12 h and stimulated with Cy5-labeled Cpg-A alone for 30 min. Then the cells were washed and incubated with medium without Cpg-A for 0, 60, and 120 min, fixed, permeabilized with 0.1% Triton X-100, and blocked with 10% goat serum for 40 min. Following this, they were stained with anti-EEA1 and anti-LAMP1 Abs followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. To analyze the intracellular routing of complexed Cpg-A with Hsp90, cDCs were seeded on glass cover slips for 12 h and stimulated with complexed Cy5-labeled Cpg-A with Hsp90 for 30 min. Then the cells were washed and incubated with medium without Cpg-A for 0, 60 and 120 min, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 10% goat serum for 40 min. Following this, they were stained with anti-Rab5, anti-EEA1, and anti-LAMP1 Abs followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. All samples were visualized using an LSM510 confocal microscope (Zeiss, Oberkochen, Germany), and images were captured and analyzed using the Zeiss LSM Image Browser (Zeiss). For evaluation of colocalization, a single z-plane of one cell was evaluated. For each protein and organelle combination, a total of 150 cells (50 cells each from three independent experiments) were analyzed.

**Mouse injections**

For analysis of in vivo cytokine production induced by stimulation with Cpg-A, C57BL/6 mice were anesthetized and challenged with normal saline (50 µl), Hsp90 (20 µg/mouse), and Cpg-A (50 µg/mouse), and Cpg-A (50 µg) complexed with Hsp90 (20 µg) by the i.p. route. After 12 h, blood was collected by cardiac puncture, and serum was prepared. Spleens were collected and crushed for isolation of spleen cells, and cDCs were enriched by negative selection using anti-murine pDC Ag 1 microbeads, followed by positive selection using anti-CD11c microbeads, and cultured for 24 h. Supernatants were then collected for IFN-α ELISA.

**Genomic DNA isolation**

Human genomic DNA was isolated from PBMCs of healthy donors using a DNeasy kit (Qiagen) according to the manufacturer’s instructions.

**Statistical analysis**

All experiments were independently performed three times in triplicate. Results were given as means ± SEM. Comparisons between two groups were performed using Student t test, whereas comparisons among multiple groups were done using ANOVA, with a value of p < 0.05 considered to be statistically significant.

**Results**

**Extracellularly loaded Hsp90 accumulates in static early endosomes within cDCs**

We first examined the intracellular trafficking of extracellular Hsp90 loaded onto cDCs. We labeled native Hsp90 protein with Alexa Fluor 488 and loaded it onto cDCs separated from mouse bone marrow-derived DCs using an anti-CD11c Ab at 4°C for 10 min and washed. The cells were then further incubated for 0–120 min, and we analyzed the kinetics of intracellular trafficking of Hsp90. Extracellular Hsp90 was gradually trafficked from the cell surface to the cytosol with the passage of time (Fig. 1A). To
investigate the intracellular localization of extracellular Hsp90, cDCs were incubated with the Alexa Fluor 488-labeled Hsp90 for 30 min and washed. Postincubation with medium without Hsp90 for 120 min, the cells were fixed and stained with organelle markers, such as KDEL for ER, LAMP1 for the late endosome and lysosome, and Rab5 and EEA1 for the early endosome. Extracellular Hsp90 accumulated in Rab5 and EEA1 positive-early endosomes but not in ER or late endosomes/lysosomes (Fig. 1 B).

These results indicated that extracellular Hsp90 was sorted into the static early endosomal pathway and retained for longer periods, but not the dynamic early endosomal pathway. These observations led us to investigate whether extracellular Hsp90 converted non–IFN-α stimulatory CpG-A ODN into a trigger of cDC activation to produce IFN-α.

In vitro generation of CpG-ODN–Hsp90 complex

As Hsp90 has been demonstrated to be a binder to CpG-ODNs (32), we first confirmed whether Hsp90 could be complexed with CpG-A and control CpG in vitro. We incubated 1.5 nmol end labeled CpG-ODN with 0.5 μM Hsp90 at 37°C for 30 min. Samples were subjected to native-PAGE and visualized by silver staining (Fig. 2A) and autoradiography (Fig. 2B). These results indicated that Hsp90 could bind CpG-ODNs. To make a 3 μM CpG-ODN–Hsp90 complex, 3 nmol CpG-A or CpG-B was mixed with 1 μM Hsp90, incubated for 30 min, and then diluted with 1 ml medium.

Hsp90 enhances IFN-α induction in response to CpG-A from pDC and cDC

Mouse pDCs, not cDCs, have previously been demonstrated to be the major cells secreting IFN-α following CpG-A stimulation. To elucidate the ability of Hsp90 to enhance secretion of IFN-α from the DC subset, freshly isolated pDCs were cultured for 24 h in the presence of 3 μM CpG-A or 3 μM CpG-A–Hsp90 complex, respectively. The CpG-A–Hsp90 complex enhanced the IFN-α production 2-fold more than CpG-A alone (Fig. 3A). Notably, a high amount of IFN-α production was observed in cDCs stimulated with the CpG-A–Hsp90 complex despite the lack of production of IFN-α.
when they were stimulated with CpG-A alone (Fig. 3B). Exogenous Hsp90 did not have any effect on IFN-α secretion by either pDCs or cDCs (Fig. 3A, 3B). Furthermore, the observed IFN-α production was completely inhibited poststimulation with the CpG-A–Hsp90 complex in both pDCs and cDCs derived from Tlr9−/− mice (Fig. 3A, 3B). Moreover, IFN-α production was potently inhibited by chloroquine, which blocks endosomal signaling (Fig. 3C, 3D). The degree of the inhibition of the IFN-α production by chloroquine was different between pDCs and cDCs, but this may have been due to the sensitivity to the chloroquine. These results suggested that Hsp90 might translocate the chaperoned CpG-ODNs into static early endosomes and efficiently activate the TLR9 signaling pathway for IFN-α production.

Both cDCs and pDCs uptake CpG-A to similar degrees

We compared the efficiency of binding and uptake of CpG-A or the CpG-A–Hsp90 complex by cDCs and pDCs to rule out the possibility that Hsp90 enhanced the uptake of CpG-A by cDCs and pDCs. Cy5-labeled CpG-A, either coupled with Hsp90 or alone, was added to cDC or pDC cultures, and the binding and uptake were analyzed. The percentage of cDCs that took-up CpG-A was 36.9% with CpG-A stimulation alone, and it was approximately the same, 39.5%, when it was coupled with Hsp90 (Fig. 4). The uptakes of CpG-A alone and the CpG-A–Hsp90 complex by pDCs were also similar. These results indicated that both cDCs and pDCs took up the labeled CpG-ODN at almost the same level and supported our hypothesis that Hsp90-mediated direction of CpG-ODN into the early endosome and efficiently activate the TLR9 signaling pathway for IFN-α production.

Mouse cDCs express TLR9 and IRF7

We examined whether TLR9 was expressed by murine cDCs. Mouse cDCs express TLR9 and IRF7 positive early endosomes of cDCs (Supplemental Fig. 1A). Laser confocal microscopic analysis revealed that TLR9 localized in the EEA1-positive early endosomes of cDCs (Supplemental Fig. 1C).

CpG-ODN–Hsp90 complex serves as a potent inducer for IFN-α in vivo

Next, we examined whether the CpG-A–Hsp90 complex had an in vivo effect similar to that observed in vitro. We administered PBS, Hsp90 alone, CpG-A alone, or the CpG-A–Hsp90 complex to C57BL/6 mice. After 12 h, we collected sera from the mice and measured the IFN-α. The production of IFN-α from mice injected with the CpG-A–Hsp90 complex was higher than that induced by CpG-A alone (Fig. 5A). Moreover, production of IFN-α by cDCs isolated from mice injected with the CpG-A–Hsp90 complex produced a high level of IFN-α. In contrast, cDCs from mice injected with CpG-A alone did not (Fig. 5B). These results indicated that Hsp90 could target chaperoned molecules to the early endosomal compartment within cDCs and induce robust INF-α secretion in vivo as well as in vitro.

Hsp90 retains CpG-ODN in static early endosomes

We assumed that in cDCs, as CpG-A was rapidly trafficked to late endosomes and lysosomes, CpG-A–mediated TLR9 signaling was not sufficient. We therefore investigated the intracellular routing of CpG-A after uptake of it in cDCs using laser confocal microscopy. Isolated cDCs from BMDCs were incubated with Cy5-labeled CpG-A for 30 min, and then the cells were washed and incubated with new medium without CpG-A for 0, 60, and 120 min. Following incubation, the cells were fixed and stained. Immediately poststimulation, most of the CpG-A localized within LAMP1+ late endosomes/lysosomes (Fig. 6A, Supplemental Fig. 2). In contrast, the frequency of colocalization with EEA1 was very low. After 120 min incubation, CpG-A was detected within late endosomes/lysosomes at high frequency and in a large area of the LAMP1+ organelle (Fig. 6A, Supplemental Fig. 2). Together with our observation that TLR9 localized in the EEA1-positive early endosomes of cDCs, these results indicated that CpG-A was rapidly trafficked to the LAMP1+ late endosome/lysosome pathway, and therefore could not activate the TLR9–MyD88–IRF7 signaling pathway. In contrast, Cy5-labeled CpG-A coupled with Hsp90 was pooled for at least 120 min during stimulation within Rab5+, EEA1−-static early endosomes, and lysosomal localization was poorly detected (Fig. 6B, Supplemental Fig. 3). Furthermore, when complexed with Hsp90, CpG-A appeared to form large aggregates that colocalized with early endosomes after 120 min (Fig. 6B). These results suggested that their ability to form aggregated structures was needed to induce IFN-α. Quantitative analysis of the colocalization between the Cy5-labeled CpG-A and EEA1 and LAMP1 revealed average colocalization incidences of 16.7% and 91.9%, respectively, immediately after

|FIGURE 4. Both pDCs and cDCs bind and uptake CpG-A or Hsp90–CpG-A complex to similar degrees. Flow cytometric analysis for binding and uptake of Cy5-labeled CpG-A alone (left panels) or in complex with Hsp90 (right panels) by cDCs (top panels) and pDCs (bottom panels) after 30 min stimulation. Data are representative of three independent experiments.|

|FIGURE 5. CpG-A–Hsp90 complex serves as a potent inducer for IFN-α in vivo. CpG-A (50 μg/mouse) or CpG-A–Hsp90 (50 μg/mouse) complex was administered i.p. to C57BL/6 mice 6–10 wk of age. A. After 12 h, mouse serum was obtained via cardiac puncture and levels of IFN-α were measured using ELISA. B. Mice were then euthanized for spleen removal. cDCs were isolated and cultured for 24 h, and the supernatant was measured for IFN-α production. Data are presented as means + SEM of triplicate wells. Data are representative of three independent experiments. *p < 0.01; paired Student t test. |
in early endosomes to produce IFN-α. When complexed with Hsp90, CpG-B induced robust IFN-α production from cDCs (Fig. 7A) and only low levels of TNF-α and IL-6 as compared with CpG-B alone (Fig. 7B, 7C). The CpG-B–Hsp90 complex also failed to induce cDC maturation (data not shown). Consistent with these results, the production of TNF-α and IL-6 from cDCs was inhibited when they were stimulated with the CpG-A–Hsp90 complex as compared with CpG-A alone (Supplemental Fig. 4).

**Extracellular Hsp90 enhances the IFN-α production by human pDCs**

In humans, pDCs and B cells express TLR9 (10, 33). We examined whether the CpG-A–Hsp90 complex also induced IFN-α from human DCs as observed in mouse DCs. The production of IFN-α when we stimulated PBMCs of healthy donors with the CpG-A–Hsp90 complex was enhanced when compared with CpG-A alone (p < 0.05) (Fig. 8A). We observed that PBMC-derived mDCs expressed little TLR9 and that CD19+ B cells expressed TLR9 at a low level (data not shown). Therefore, it was reasonable that there was little IFN-α production by these cells following stimulation with CpG-A or the CpG-A–Hsp90 complex (Fig. 8B, 8C). In contrast, as pDCs isolated from PBMCs have been shown to produce large amounts of IFN-α, we examined whether Hsp90 could affect the IFN-α production by human pDCs. The CpG-A–Hsp90 complex induced augmented production of IFN-α compared with CpG-A alone (Fig. 8D). We therefore concluded that Hsp90-mediated spatiotemporal targeting to static early endosomes of CpG-ODN boosted TLR9 activation and triggered efficient IFN-α induction in mouse pDCs, cDCs, and human pDCs.

**Hsp90 converts self-DNA into a potent trigger of IFN-α induction by human pDCs**

Finally, we determined whether the extracellular Hsp90 enabled human pDCs to sense self-DNA, leading to IFN-α production. Human genomic DNA, which was isolated from healthy volunteers, was unable to induce IFN-α from pDCs. However, we found that self-DNA complexed with Hsp90 could induce IFN-α production from pDCs (Fig. 9). Thus, Hsp90 converted inert self-DNA into an activator of pDCs.

**Discussion**

pDCs sense certain viral and microbial infections. In contrast to mDCs, pDCs uniquely express TLR7 and TLR9, intracellular receptors that recognize viral/microbial nucleic acids within endosomal compartments in humans. Together with the constitutive expression of IRF7, TLR7 and TLR9 permit pDCs to mount rapid and robust type I IFN responses to viral/microbial infections. However, pDCs normally do not respond to self-DNA, which may reflect the fact that viral/bacterial DNA sequences contain multiple CpG nucleotides that bind and activate TLR9, whereas mammalian self-DNA contains fewer such motifs, which are most likely masked by methylation. Recent evidence, however, suggests that self-DNA has the potential to trigger TLR9, but may fail to do so because it fails to access the TLR9-containing endolysosomal compartments. One of the mechanisms of this effect is attributed to the fact that DNase easily and rapidly breaks down the extracellular DNA, thereby hampering self-DNA localization into endocytic compartments. In contrast to the human system, murine cDCs, like pDCs, express TLR7 and TLR9.

Two classes of synthetic ODNs containing an unmethylated CpG motif have been classified: CpG-A ODN, which stimulates IFN-α production by pDCs, and CpG-B ODN, which does not. Instead, CpG-B ODN stimulates pDCs to produce IL-6 and TNF-α and induce DC maturation, such as the upregulation of CD80 and CD86. This effect is likely due to the presence of unmethylated CpG motifs in CpG-B ODNs, which are specifically recognized by pDCs. These results suggest that extracellular Hsp90 can enhance IFN-α production in human pDCs by converting self-DNA into a potent activator of these cells.

Recent studies have indicated that TLR9 signaling in late endosomes induces DC maturation and TNF-α and IL-6 secretion. We therefore tested whether Hsp90 could convert CpG-B, which was expected to stimulate TLR9 in late endosomes, into a ligand that triggered TLR9 signaling in DCs. Our results showed that Hsp90 converted CpG-B into a trigger of IFN-α production and TNF-α and IL-6 secretion. This effect was likely due to the ability of Hsp90 to induce the selective and continuous activation of late endosomal TLR9 via spatiotemporal targeting of CpG-ODNs.

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pDCs sense certain viral and microbial infections. In contrast to mDCs, pDCs uniquely express TLR7 and TLR9, intracellular receptors that recognize viral/microbial nucleic acids within endosomal compartments in humans. Together with the constitutive expression of IRF7, TLR7 and TLR9 permit pDCs to mount rapid and robust type I IFN responses to viral/microbial infections. However, pDCs normally do not respond to self-DNA, which may reflect the fact that viral/bacterial DNA sequences contain multiple CpG nucleotides that bind and activate TLR9, whereas mammalian self-DNA contains fewer such motifs, which are most likely masked by methylation. Recent evidence, however, suggests that self-DNA has the potential to trigger TLR9, but may fail to do so because it fails to access the TLR9-containing endolysosomal compartments. One of the mechanisms of this effect is attributed to the fact that DNase easily and rapidly breaks down the extracellular DNA, thereby hampering self-DNA localization into endocytic compartments. In contrast to the human system, murine cDCs, like pDCs, express TLR7 and TLR9.

Two classes of synthetic ODNs containing an unmethylated CpG motif have been classified: CpG-A ODN, which stimulates IFN-α production by pDCs, and CpG-B ODN, which does not. Instead, CpG-B ODN stimulates pDCs to produce IL-6 and TNF-α and induce DC maturation, such as the upregulation of CD80 and CD86. This effect is likely due to the presence of unmethylated CpG motifs in CpG-B ODNs, which are specifically recognized by pDCs. These results suggest that extracellular Hsp90 can enhance IFN-α production in human pDCs by converting self-DNA into a potent activator of these cells.
CD86 and the expression of MHC class II molecules. Recently, it has been demonstrated that the manner of CpG internalization and the retention time of CpG in endosomes differ between CpG-A and CpG-B, and the retention of the CpG/TLR9 complex in endosomes is the primary determinant of TLR signaling (17, 34). CpG-A ODNs are characterized by a poly G tail that forms large multimeric aggregates with a diameter \( \sim 50 \mu m \). In contrast, CpG-B ODNs are monomeric and do not form such higher order structures. In addition, multimeric CpG-A ODNs are retained for longer periods of time in the early endosomes, whereas CpG-B ODNs rapidly traffic through early endosomes into late endosomes or lysosomes of pDCs. The prolonged retention of multimeric CpG-A ODNs provides extended activation of the TLR9-MyD88-IRF7 signal-transducing complex, which leads to robust IFN-\( \alpha \) production. Therefore, we also examined the ability of Hsp90 to target and retain chaperoned CpG-DNA in static early endosomes of cDCs, resulting in type I IFN production. We found that Hsp90-chaperoned CpG-A was localized and retained within static early endosomes for longer periods in cDCs, thereby eliciting TLR9 signaling for IFN-\( \alpha \) production, but not inflammatory cytokines, such as IL-6 and TNF-\( \alpha \). In contrast, CpG-A alone moved into late endosomes and lysosomes within cDCs. Interestingly, not only CpG-A but also CpG-B could stimulate the TLR9 signaling within static early endosomes, resulting in the production of IFN-\( \alpha \). Thus, extracellular Hsp90 had the ability to direct associated molecules into static early endosomes. Moreover, as time passed, the CpG-A–Hsp90 complex formed large aggregates within early endosomes (Fig. 6B), again suggesting an important link between the physical size of TLR9 ligands and their stimulatory capacity. Thus, our data indicated that when DCs detected aggregated DNA structures in the early endosomes through TLR9, this was coupled with IRF7 activation and IFN-\( \alpha \) production. By contrast, when DCs sensed the linear DNA structures in the late endosomes or lysosomes through TLR9, this was coupled with NF-\( \kappa B \) activation, which led to IL-6 and TNF-\( \alpha \) production and DC maturation.

Why, however, are DNA–Hsp90 complexes selectively retained in early endosomes but not in late endosomes or lysosomes in DCs? We found that endocytosed CpG-A–Hsp90 complexes were selectively transferred into Rab5+, EEA-1+-static early endosomes. Very recently, Lakadamyali et al. (31) have shown that early endosomes are comprised of two distinct populations: a dynamic population that is highly mobile on microtubules and matures rapidly toward the late endosome and a static population that matures much more slowly. Cargos destined for degradation, including low-density lipoprotein, epidermal growth factor, and influenza virus, are internalized and targeted to the Rab5+, EEA1\(^+\) dynamic population of early endosomes, thereafter trafficking to Rab7\(^+\) late endosomes. In contrast, the recycling ligand

**FIGURE 7.** Hsp90 converts CpG-B to a trigger of IFN-\( \alpha \) production by cDCs. IFN-\( \alpha \) (A), TNF-\( \alpha \) (B), and IL-6 (C) production poststimulation with 3 \( \mu M \) CpG-B or in complex with Hsp90 by cDCs. Data are presented as means + SEM of triplicate wells. Data are representative of three independent experiments. *\( p < 0.005 \); **\( p < 0.01 \), paired Student t test.

**FIGURE 8.** CpG-A–Hsp90 complex enhances IFN-\( \alpha \) induction by human pDCs. IFN-\( \alpha \) production poststimulation with NS, Hsp90, 3 \( \mu M \) CpG-A alone or in complex with Hsp90 by human PBMC (A), CD19\(^+\) cells (B), mDCs (C), and pDCs (D). Data are presented as means + SEM of triplicate wells. Data are representative of three independent experiments. *\( p < 0.005 \); **\( p < 0.05 \), paired Student t test. NS, normal saline.

**FIGURE 9.** Hsp90 converts self-DNA into a potent trigger of IFN-\( \alpha \) induction by human pDCs. Human pDCs were stimulated with genomic DNA isolated from healthy volunteers (10 \( \mu g/ml \)) either alone or postcomplexing with Hsp90 (10 \( \mu g \)). Levels of IFN-\( \alpha \) were measured after overnight culture using ELISA. Error bars represent the SEM of triplicate wells. Data are representative of four independent experiments. *\( p < 0.005 \), paired Student t test. ND, not detected.
to stimulate TLR9 in late endosomes, into a ligand that triggers inflammation and autophagy. We therefore hypothesized that targeting Hsp90-mediated translocation of CpG-ODNs into static early endosomes and lysosomes but not at physiological pH (7.4), the CpG-A–Hsp90 complex was retained in the static early endosomes and lysosomes. Therefore, our observation that the CpG-B–Hsp90 complex made possible their retention in early endosomes and subsequent induction of higher levels of IFN-α and low levels of IL-6 and TNF-α as compared with CpG-B alone; the Hsp90 complex with CpG-B also failed to induce pDC maturation as revealed by the low surface expression of CD80 and CD86. These data suggested that the CpG-B–Hsp90 complex made possible their retention in early endosomes and presumably enabling the selective and sustained activation of early endosomal TLR9.

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Disclosures

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References


TARGETING HSP90–DNA COMPLEX TO STATIC ENDOSOME

Transferrin is delivered to Rab5−/−, EEA1−/− static early endosomes, followed by translocation to Rab11+ recycling endosomes. They also found that cargos trafficked into these static early endosomes were retained for longer periods and not internalized into late endosomes and lysosomes. Therefore, our observation that the CpG-A–Hsp90 complex was retained in the static early endosomes, leading to sustained activation of DCs and IFN-α production, was consistent with their findings. Moreover, as Rutz et al. (35) have demonstrated that TLR9–CpG ODN interaction occurs at the acidic pH (6.5–5.0) found in early endosomes and lysosomes but not at physiological pH (7.4), the Hsp90-mediated translocation of CpG-ODNs into static early endosomes (pH 6.5) might accelerate the TLR9 and CpG–ODN interaction. In contrast, CpG-A alone, which did not stimulate IFN-α production, targeted the EEA1− and LAMP1− dynamic early endosome–late endosome/lysosome pathway, leading to inflammatory cytokine responses as well as DC maturation through NF-κB–mediated signaling. These data suggested that Hsp90 shuttled the chaperoned DNA into the static early endosome, resulting in the formation of DNA aggregation under mildly acidic circumstances as well as preventing translocation to late endosomes/lysosomes. Therefore, we hypothesized that targeting of DNA to static early endosomes was critical for eliciting TLR9 signaling for IFN-α production by DCs. Furthermore, we examined whether Hsp90 could convert CpG-B, which is thought to stimulate TLR9 in late endosomes, into a ligand that triggers TLR9 in early endosomes. Indeed, when complexed with Hsp90, CpG-B induced pDCs to produce high levels of IFN-α of pDCs and only low levels of IL-6 and TNF-α as compared with CpG-B alone; the Hsp90 complexed with CpG-B also failed to induce pDC maturation as revealed by the low surface expression of CD80 and CD86. These data suggested that the CpG-B–Hsp90 complex made possible their retention in early endosomes and subsequent induction of higher levels of IFN-α and low levels of IL-6 and TNF-α and impaired pDC maturation.

Recently, it has been demonstrated that pDCs do sense and respond to self-DNA in human autoimmune diseases. In systemic lupus erythematosus (SLE), pDCs are activated to produce IFNs by circulating immune complexes consisting of autoantibodies and self-nucleic acids that stimulate endosomal TLR following FcγRII-mediated uptake. The aberrantly produced IFNs are major effectors in the pathogenesis of autoimmunity, mainly by inducing unabated maturation of peripheral mDCs that stimulate autoreactive T cells. Recent evidence implies that upon internalization of chromatin–IgG immune complexes via BCR or FcRIII of DCs, mammalian DNA displays robust immunostimulatory activities toward B cells or DCs (36, 37). Furthermore, the sera of patients with SLE containing immune complexes consisting of autologous DNA and anti-DNA Abs effectively activate pDCs to produce type I IFNs (38, 39). We have demonstrated that, upon Hsp90-mediated enforced endosomal translocation, both human self-DNA as well as CpG-ODN could activate DCs via TLR9 to produce type I IFN. Previous studies have demonstrated the presence of autoantibodies to the Hsp90 (40, 41) and enhanced expression of Hsp90 in PBMCs of patients with active SLE (42, 43), suggesting the role of Hsp90 in the pathogenesis. In addition, the Hsp90 has been shown to localize both in the cytoplasm and nucleus (44). Moreover, under stressful conditions, it has been shown that cytosolic Hsp90 translocates to the nucleus (45). This suggests that Hsp90 may bind self-DNA within the nucleus. When cells undergo necrosis, self-DNA associated with endogenous Hsp90 could be released into the extracellular space and might trigger IFN-α production by pDCs. Our findings support the idea that Hsp90, an endogenous danger signal found in the sera from patients with SLE, is the key mediator of pDC activation in SLE. Thus, Hsp90 may inactivate innate tolerance to self-DNA by forming a complex with self-DNA that is delivered to and retained within early endocytic compartments of pDCs to trigger TLR9 and induce IFN-α production. Thus, we determined a fundamental mechanism by which pDCs sense and respond to self-DNA coupled with Hsp90. Our data suggest that, through this pathway, pDCs drive autoimmunity in autoimmune diseases.

The release of host-derived (self) DNA into the extracellular environment is a common feature of both necrotic and apoptotic cell death (46). However, extracellular self-DNA usually does not lead to innate immune activation because it is rapidly degraded by DNase and fails to access endosomal compartments of DCs where TLR9 is located. The importance of this mechanism in preventing autoimmune responses is shown by the fact that mice deficient in DNase 1 develop an SLE-like syndrome. Several host factors that can convert self-DNA into a trigger of DC activation have been reported. Endogenous anti-microbial peptide LL37 (also known as CAMP), autoantibodies, and high mobility group box 1 protein have been demonstrated to do so. In this study, we found that extracellular self-DNA acquires the ability to trigger activation of TLR9 in human pDC by forming a complex with the host-derived endogenous Hsp90.

Together, our findings indicate that the ability of Hsp90 to convert self-DNA into a trigger of high levels of IFN-α production depends on its capacity to concentrate and retain DNA in static early endosomes, presumably enabling the selective and sustained activation of early endosomal TLR9.

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