# A Signaling Polypeptide Derived from an Innate Immune Adaptor Molecule Can Be Harnessed as a New Class of Vaccine Adjuvant[1]

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**Modulation of intracellular signaling using cell-permeable polypeptides is a promising technology for future clinical applications. To develop a novel approach to activate innate immune signaling by synthetic polypeptides, we characterized several different polypeptides derived from the caspase recruitment domain (CARD) of IFN-$\beta$ promoter stimulator 1, each of which localizes to a different subcellular compartment. Of particular interest was, N′-CARD, which consisted of the nuclear localization signal of histone H2B and the IFN-$\beta$ promoter stimulator 1CARD and which localized to the nucleus. This polypeptide led to a strong production of type I IFNs and molecular and genetic analyses showed that nuclear DNA helicase II is critically involved in this response. N′-CARD polypeptide fused to a protein transduction domain (N′-CARD-PTD) readily transmigrated from the outside to the inside of the cell and triggered innate immune signaling. Administration of N′-CARD-PTD polypeptide elicited production of type I IFNs, maturation of bone marrow-derived dendritic cells, and promotion of vaccine immunogenicity by enhancing Ag-specific Th1-type immune responses, thereby protecting mice from lethal influenza infection and from outgrowth of transplanted tumors in vivo. Thus, our results indicate that the N′-CARD-PTD polypeptide belongs to a new class of vaccine adjuvant that directly triggers intracellular signal transduction by a distinct mechanism from those engaged by conventional vaccine adjuvants, such as TLR ligands.** *The Journal of Immunology,* **2009, 182: 1593–1601.**

Accumulating evidence from basic research and from clinical studies clearly indicates that type I IFNs are key to the elimination of viral infection (1, 2), suppression of tumor progression (3, 4), and to vaccine immunogenicity (5). Type I IFNs, such as IFN-$\alpha$ and -$\beta$, are produced from a wide variety of cell types upon viral infection or in response to foreign nucleic acids, such as DNA and RNA (6–8). Recent research has dissected and elucidated the molecular basis of the ability of the immune system to sense a variety of nucleic acids as pathogen-associated molecular patterns (9) or to sense the presence of aberrant self-DNA under dangerous situations (10, 11). RIG-I-like helicases (RLHs)[4] mediate innate immune signaling in human cells induced by immunostimulatory RNAs, such as 5′-triphosphate RNA or dsRNA, or right-handed B-form DNA (B-DNA) (12–14). RLHs trigger cellular signaling through adaptor molecules, such as IFN-$\beta$ promoter stimulator 1 (IPS-1, also known as MAVS/VISA/Cardif), TNFR-associating factor (TRAF) 3, and TRAF family member-associated NF-$\kappa$B activator (TANK), thereby coordinating the activation of I$\kappa$B kinase (IKK) family members, such as NF-$\kappa$B essential modulator, IKK-$\alpha$, IKK-$\beta$, TANK-binding kinase 1 (TBK1), and inducible IKK (IKKi). Once activated by such cytoplasmic kinases, NF-$\kappa$B, IFN regulatory factor 3 (IRF3), and IRF7 transmigrate into the nucleus and act as master regulators of type I IFN-related gene promoters (15).

These signaling molecules contain distinct domains, and thereby associate with specific target molecules and modulate downstream signal transmission. IPS-1 plays a central role in this signaling pathway and its caspase recruitment domain (CARD) forms the death domain fold, which is structurally similar to domains of Fas-associated via death domain and caspase family members (16). The CARD of IPS-1 is essential for signal transmission through homotypic interactions with the CARDs of upstream RLHs (9). Mitochondrial sorting of IPS-1 is also crucial for its canonical

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[4] Abbreviations used in this paper: RLH, RIG-I-like helicase; B-DNA, B-form DNA; IPS-1, IFN-$\beta$ promoter stimulator 1; TRAF, TNFR-associating factor; TANK, TRAF family member-associated NF-$\kappa$B activator; TBK1, TANK binding kinase 1; IKK, I$\kappa$B kinase; IKKi, inducible IKK; IRF3, IFN regulatory factor 3; CARD, caspase recruitment domain; N′-CARD, fusion of the $NH_2$-terminal nuclear localization signal of histone H2B to the IPS-1 CARD; PTD, protein transduction domain; TMD, transmembrane domain; NLS, nuclear localization signal; NDH, nuclear DNA helicase II; ODN, oligodeoxynucleotide; flu vax, influenza split-product vaccine; DC, dendritic cell; FL, full length; BM-DC, bone marrow-derived dendritic cell.

signaling because human hepatitis C virus NS3/4A protease inactivates IPS-1 by cleaving a region adjacent to the transmembrane domain (TMD), which is required for IPS-1 localization to the mitochondrial outer membrane (17).

To develop a novel approach to modulate innate immune signaling by synthetic polypeptides, we generated several different IPS-1 CARD-fusion polypeptides, each of which localizes to a different subcellular compartment. Of interest, the nuclear localization of a fusion polypeptide between the nuclear localization signal (NLS) of histone H2B and the IPS-1 CARD (hereafter referred to as N′-CARD) activated a distinct signaling pathway initiated from the nucleus and led to a strong production of type I IFN. Molecular and genetic analyses showed that nuclear DNA helicase II (NDH) is critically involved in this signaling pathway. Fusion of N′-CARD to the protein transduction domain (PTD), originally derived from the HIV Tat protein (18), facilitated transduction of N′-CARD from outside to inside the cell without loss of its original intracellular function. Finally, we demonstrate that the N′-CARD-PTD polypeptide acts as a novel vaccine adjuvant by directly triggering innate intracellular immune signaling to augment vaccine immunogenicity. Such a mechanism is distinct from TLR-mediated signaling, which is engaged in innate immune activation by conventional vaccine adjuvants, such as monophosphoryl-lipid A (an LPS derivative) and CpG oligodeoxynucleotide (ODN).

## Materials and Methods

### *Cells and reagents*

HEK293, HeLa, RAW264.7, and TC-1 cells were purchased from American Type Culture Collection and maintained in DMEM supplemented with 10% FCS and 50 μg/ml penicillin/streptomycin. Sf9 cells were maintained in Sf900 II SFM (Invitrogen). LPS was purchased from Sigma-Aldrich. CpG ODN, 5′-ATC GAC TCT CGA GCG TTC TC-3′, was synthesized by Gene Design. Mouse GM-CSF and Flt3L were purchased from PeproTech. Influenza split-product vaccine (flu vax) was prepared at The Research Foundation for Microbial Diseases of Osaka University (Kanon-ji city, Kagawa, Japan) from the purified influenza virus A/New Caledonia/20/99 strain (H1N1) by sequential treatment with ether and formalin, according to the method of Davenport et al. (19, 20).

### *Expression plasmids*

The IPS-1 expression plasmid was described previously (21). The IPS-1 CARD, aa 1–100 of the IPS-1 ORF, was PCR-amplified. Fusion cDNAs were generated by ligating aa 1–100 and 514–540 of IPS-1 ORF (CARD-TMD), aa 1–37 of histone H2B ORF and aa 1–100 of IPS-1 ORF (N′-CARD), N′-CARD and aa 514–540 of hIPS-1 ORF (N′-CARD-TMD), and were amplified by PCR. These fragments were introduced in-frame into pFLAG CMV5b (Sigma-Aldrich) or pGEX6P-2 (GE Healthcare). GST-N′-CARD was further fused to the PTD (Tyr-Ala-Arg-Ala-Ala-Ala-Arg-Gln-Ala-Arg-Ala) and introduced into pFastBac HT-B (Invitrogen). TBK1, IKKi, NDH, and chloride channel 1A (CC1A) cDNAs were amplified by PCR using a human spleen cDNA library (Takara). These fragments were introduced in-frame into pFLAG-CMV4 (Sigma-Aldrich), pCIneo-HA, pCAGGS-Flag-m1SECFP, pCAG-His Venus, or pcDNA3-RFP. The N′-CARD T54A expression plasmid was generated by site-directed mutagenesis, as described previously (22). The sequences of the PCR products were confirmed using an ABI PRISM Genetic Analyzer (PE Applied Biosystems).

### *Luciferase assay*

The luciferase assay was conducted as described previously (23).

### *Confocal microscopy*

HeLa cells were transfected with CARD-YFP, CARD-TMD-YFP, N′-CARD-YFP, N′-CARD-TMD-YFP, IPS-1-YFP, YFP-IKKi, YFP-TBK1, and/or mRFP-NDH and incubated for 48 h. In some cases, the cells were treated with Hoechst 33258 (Invitrogen) and/or MitoTracker reagent (Invitrogen) at 37°C for 15 min. Alternatively, HeLa cells were treated with CARD or N′-CARD-PTD for 30 min. Cells were treated with Hoechst 33258 for 15 min before fixation and incubation with mouse anti-FLAG M2-Cy3. After washing with PBS containing 1% BSA, the cells were examined under an FV 500 confocal microscope (Olympus).

### *Immunoprecipitation and immunoblotting*

Immunoprecipitation and immunoblotting was performed as described previously (24) using anti-FLAG M2 (Sigma-Aldrich), anti-FLAG M2-HRP (Sigma-Aldrich), anti-HA (Covance), anti-HA-HRP (Roche Diagnostics), anti-ubiquitin-HRP (Santa Cruz Biotechnology), anti-NDH (provided by J. D. Parvin, Brigham and Women's Hospital, Boston, MA), anti-p-JNK, anti-p-p38, anti-p-ERK, and anti-β-actin (Cell Signaling Technology).

### *RNA interference*

An siRNA targeting NDH mRNA (stealth RNAi) was chemically synthesized by Invitrogen (Carlsbad, CA): sense, 5′-AUU GCU UGC AAA UCA UGA UCC UGU U-3′; antisense, 5′-AAC AGG AUC AUG AUU UGC AAG CAA U-3′. HEK293 cells ($6 \times 10^5$) were transfected with 120 pmol of control or NDH siRNA using Lipofectamine RNAi MAX reagent (Invitrogen) according to the manufacturer's protocol.

### *Purification of recombinant polypeptides*

DH10Bac competent cells (Invitrogen) were transformed with pFastBac HT-B-GST or with GST-N′-CARD-PTD to generate recombinant Bacmids. Sf9 cells were transfected with Bacmid-encoding GST or GST-N′-CARD-PTD to generate recombinant seed baculoviruses. Seventy-two hours after infection, the Sf9 cells were washed once with PBS and suspended in sonication buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 mM DTT) containing 10% Triton X-100. After sonication, cell lysates were centrifuged at 15,000 rpm, at 4°C for 30 min. The supernatants were collected and dialyzed with sonication buffer. Recombinant polypeptides were purified using GSTrap (GE Healthcare) according to the manufacturer's protocol. In brief, after the column was equilibrated with 2 ml sonication buffer, the cell lysate was applied and the column then washed three times with 10 ml PBST (PBS containing 0.5% Triton X-100) and with PBS once. Recombinant polypeptide (GST or GST-N′-CARD-PTD) was eluted with sonication buffer containing 10 mM reduced glutathione and then dialyzed with PBS. Recombinant proteins (1 μg) used in all the experiments contained <20 fg endotoxins (*Limulus* J Single Test, Wako).

### *ELISA and RT-PCR*

Bone marrow-derived dendritic cells (DCs) were generated by 5 days of culture with GM-CSF (20 ng/ml) (GM-DCs) or Flt3L (100 ng/ml) (FL-DCs). GM-DCs or FL-DCs were treated with or without 1, 3, or 10 μg/ml N′-CARD-PTD or 1 μM of CpG ODN for 24 h and the supernatants were subjected to ELISA for mouse IFN-α, IFN-β (PBL Biomedical Laboratories), or IL-12 p40 (Invitrogen). RAW264.7 cells were treated with 1 μg/ml LPS or 10 μg/ml N′-CARD-PTD for 3, 6, 12, 18, 24, and 48 h. The levels of mRNA for TNF-α, IL-6, IFN-α, IFN-β, IP-10, and β-actin were examined by RT-PCR as described previously (5, 22).

### *Immunization*

Eight-week-old female BALB/c mice were administered s.c. with N′-CARD-PTD (5 μg), CpG ODN (5 μg), or flu vax (0.7 μg) alone, flu vax (0.7 μg) plus N′-CARD-PTD (5 μg), or flu vax (0.7 μg) plus CpG ODN (5 μg) at 0 and 10 days. Blood was drawn at 20 days and serum Ab titer was measured by ELISA as described previously (25). Alternatively, 8-wk-old female C57BL/6 mice were immunized with E7 peptide (E7, Arg-Ala-His-Tyr-Asn-Ile-Val-Thr-Phe, 3 μg), E7 plus N′-CARD-PTD (5 μg), or E7 plus CpG ODN (5 μg) at 0 and 2 wk. Splenocytes were harvested 2 wk after final immunization. The cells were incubated with 1 μg/ml E7 or NP peptide (Ala-Ser-Asn-Glu-Asn-Met-Glu-Thr-Met) for 18 h at 37°C. Total RNA was isolated and real-time PCR was performed as described previously (22).

### *Influenza challenge*

Ten days after final immunization, mice were challenged intranasally with $2 \times 10^4$ pfu (8 $LD_{50}$) of influenza virus A/PR/8/34 (25). The body weights and mortality of the challenged mice were monitored for the next 14 days.

### *Tumor transplantation*

Eight week-old C57BL/6 mice were administered subcutaneously with TC-1 ($1 \times 10^5$ cells/mouse), a mouse lung carcinoma expressing E7 Ag (25). Mice were immunized with control NP peptide (3 μg), E7 (3 μg), N′-CARD-PTD (5 μg), or E7 (3 μg) plus N′-CARD-PTD (5 μg) at 3, 4, 5, 6, and 7 day after TC-1 inoculation. The sizes of local tumor mass were monitored for the next 20 days.

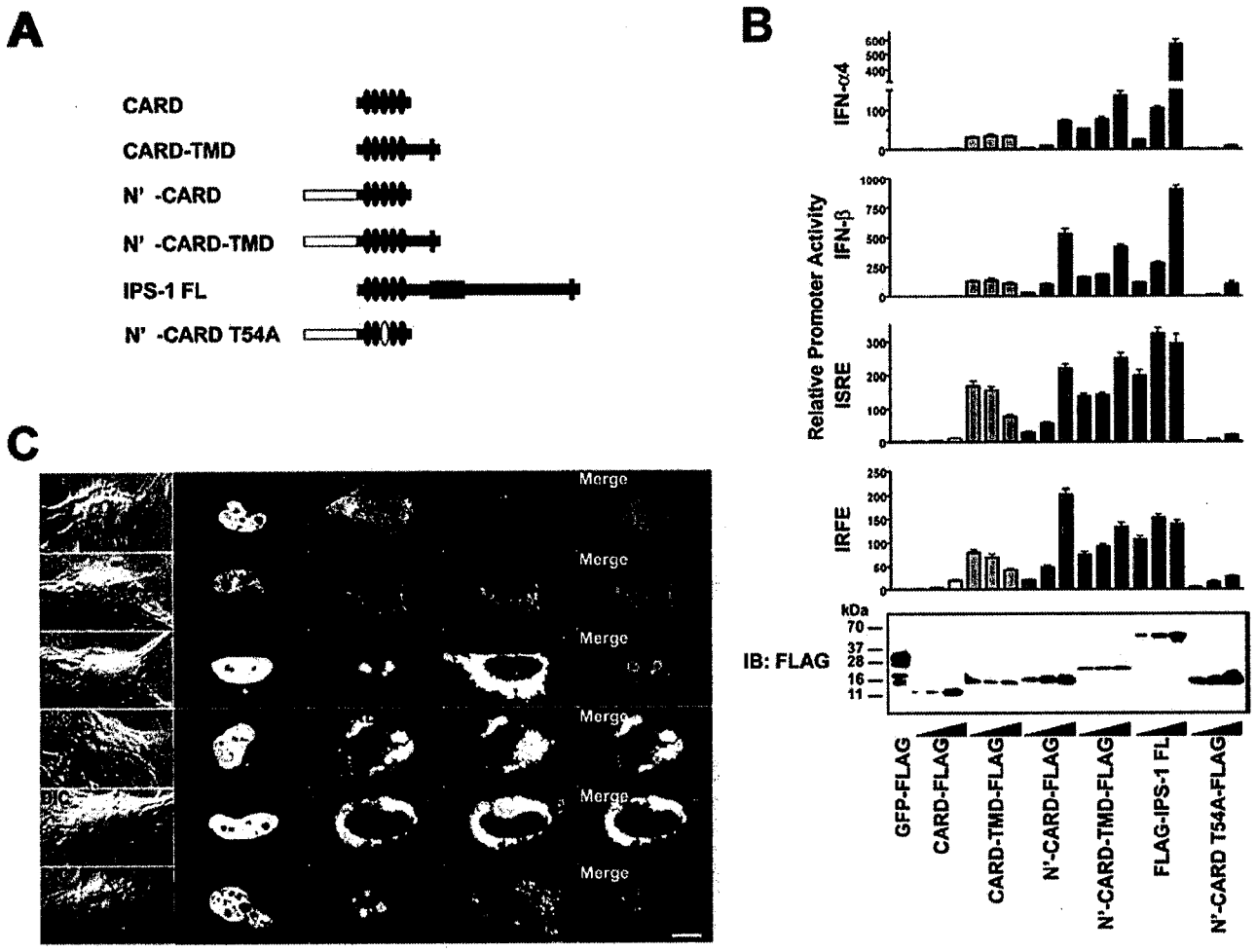


**FIGURE 1.** Synthetic IPS-1 CARD fusion molecules induce activation of type I IFN-related promoters. *A*, Schematic diagram of synthetic fusion molecules consisting of domains derived from IPS-1 and histone H2B. *B*, HEK293 cells were transfected with the expression plasmids, GFP-FLAG, CARD-FLAG, CARD-TMD-FLAG, N′-CARD-FLAG, N′-CARD-TMD-FLAG, FLAG-IPS-1 FL, and N′-CARD T54A-FLAG in the presence of TK-RL plus a reporter plasmid expressing firefly luciferase under the control of either the IFN-$\alpha$4 promoter (*top panel*), the IFN-$\beta$ promoter (*second panel from the top*), the ISRE-dependent promoter (*third panel from the top*), or the IRFE-dependent promoter (*fourth panel from the top*). Data represent means $\pm$ SD of the relative luciferase activity of six samples. Cell lysates were also subjected to immunoblot analysis to examine levels of target polypeptide expression (*bottom panel*). *C*, HeLa cells were transfected with the expression plasmids, YFP-CARD, YFP-CARD-TMD, YFP-N′-CARD, YFP-N′-CARD-TMD, YFP-IPS-1 FL, and YFP-N′-CARD T54A. Genomic DNA or mitochondria were stained with Hoechst 33258 or Mitotracker reagent, respectively, and then analyzed under a confocal microscope. The data represent one of three independent experiments with similar results. Scale bar, 10 $\mu$m.

*Statistical analysis*

The Student's *t* test or the Mantel-Cox log rank test was used for statistical analysis.

## Results

*The nuclear redistribution of IPS-1 CARD elicits type I IFN promoter activation*

To elucidate the mechanisms underlying IPS-1 CARD-mediated signaling, plasmids encoding either the IPS-1 CARD alone or the IPS CARD fused to the IPS-1 TMD or to the NLS of histone H2B were generated and their abilities to induce type I IFN-related promoter activation were characterized (Fig. 1*A*). Although the CARD alone had minimal activity in eliciting such promoter activation, fusion of the TMD to the CARD (CARD-TMD) resulted in a significant activation, suggesting that the TMD facilitates CARD-mediated signaling, consistent with previous data (Fig. 1*B*; Ref. 26). Of interest, fusion of the $NH_2$-terminal NLS of histone H2B to the IPS-1 CARD (N′-CARD) conferred strong promoter activation, suggesting that nuclear localization of N′-CARD triggers signal activation. Indeed, N′-CARD induced phosphorylation of IRF3 at a comparable level to full length IPS-1 (FL) (Supplemental Fig. 1).[5] The mutant polypeptide N′-CARD T54A, in which the third $\alpha$-helical structure of the CARD was disrupted (22), induced significantly lower levels of promoter activation, suggesting that the conformation of the IPS-1 CARD is also critical for its activity. Although N′-CARD fused to the IPS-1 TMD (N′-CARD-TMD) induced significant levels of promoter activation, the levels were comparable to those induced by N′-CARD or CARD-TMD, suggesting that the effects of CARD distribution mediated by the NLS and the IPS-1 TMD are redundant.

*N′-CARD localizes to the nucleus and signals through NDH*

To elucidate the signaling mechanisms triggered by N′-CARD and CARD-TMD, we examined the subcellular localizations of these fusion molecules. Confocal microscopy analysis showed that CARD-TMD fused to YFP (YFP-CARD-TMD) was present in

[5] The online version of this article contains supplemental information.

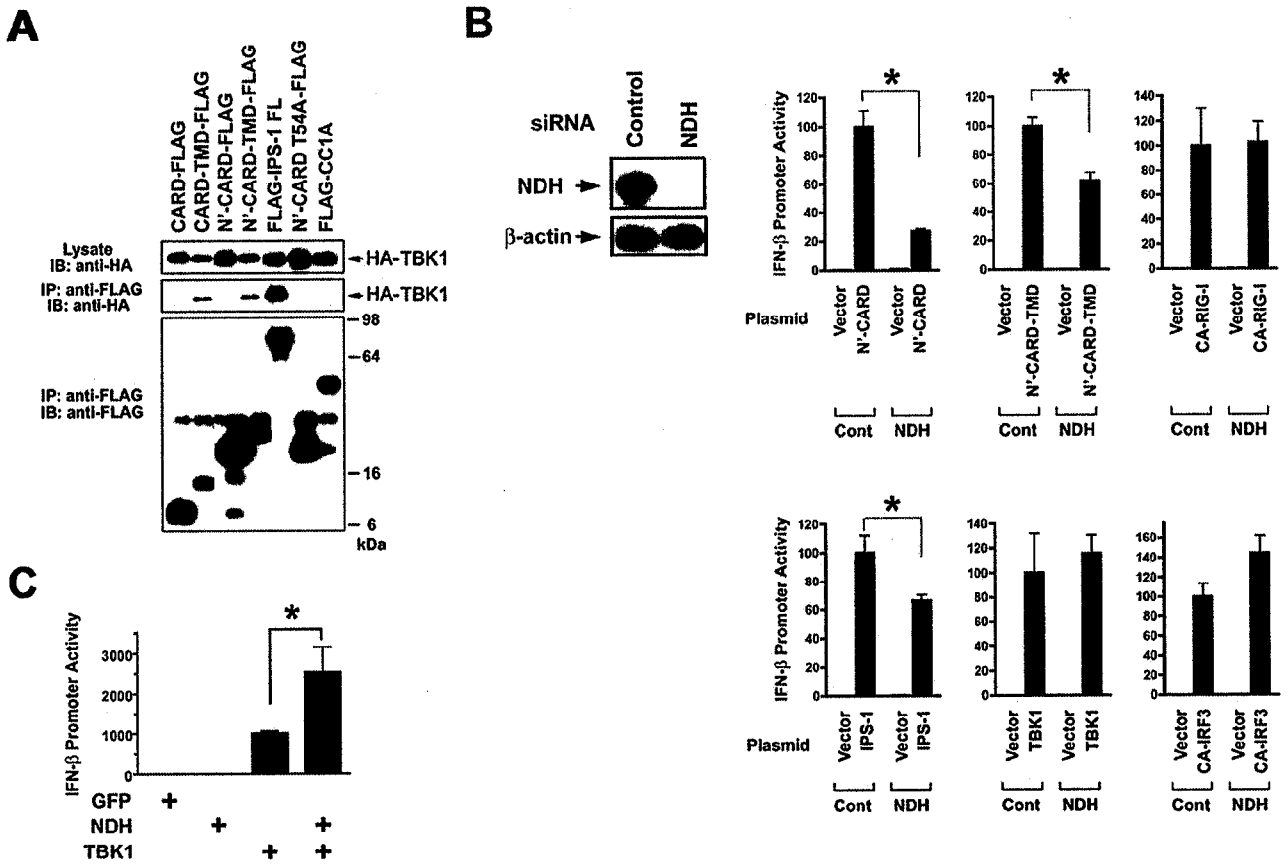


**FIGURE 2.** Role of NDH in N′-CARD-mediated signaling. *A*, Cell lysates from HEK293 cells transfected with the expression plasmids for HA-TBK1 plus CARD-FLAG, CARD-TMD-FLAG, N′-CARD-FLAG, N′-CARD-TMD-FLAG, FLAG-IPS-1 FL, N′-CARD T54A-FLAG, or FLAG-CC1A were prepared and immunoprecipitated with anti-FLAG Ab. The immune complexes were analyzed by immunoblotting using anti-HA or anti-FLAG Ab. *B*, After HEK293 cells were transfected with control or NDH siRNA, the levels of NDH protein were examined by immunoblotting. The cells were further transfected with the expression plasmid for N′-CARD, N′-CARD-TMD, CA-RIG-I, IPS-1, TBK1, and CA-IRF3 in the presence of TK-RL plus a reporter plasmid expressing firefly luciferase under the control of the IFN-β promoter. *C*, HEK293 cells were transfected with the expression plasmid(s) for GFP, NDH, and/or TBK1 in the presence of TK-RL plus a reporter plasmid expressing firefly luciferase under the control of the IFN-β promoter. *B* and *C*, Forty eight hours after transfection, luciferase assay was performed. Data represent means ± SD of the relative luciferase activity of eight samples. *, $p < 0.05$.

mitochondria, with a localization pattern similar to that of IPS-1 FL (YFP-IPS-1 FL), while N′-CARD fused to YFP (YFP-N′-CARD) was mostly present in the nuclear interchromosomal space (Fig. 1*C*). Because CARD alone (YFP-CARD) was present diffusely within the cell and both YFP-N′-CARD and YFP-N′-CARD T54A localized to the nucleus, it was suggested that the NLS directed the nuclear distribution of the IPS-1 CARD (Fig. 1*C*). These results implied that N′-CARD triggers cellular signaling pathways that originate in the nucleus and that are distinct from those triggered by CARD-TMD or IPS-1 FL, which originate from mitochondria.

TBK1, and its closely related IKK family member IKKi, are kinases acting downstream of IPS-1 and are required for a type I IFN production (21, 26, 27). We next examined the molecular interactions between each CARD-fusion molecule and TBK1 by immunoprecipitation analysis. As a control, TBK1 was coprecipitated with IPS-1 FL (Fig. 2*A*). A significant amount of TBK1 was also detected after precipitation with CARD-TMD or N′-CARD-TMD, but not after precipitation with CARD, N′-CARD, or N′-CARD T54A, suggesting that the TMD supports the association of the CARD with TBK1 (Fig. 2*A*).

To examine the signaling mechanisms triggered by N′-CARD, we tried to identify cellular molecules that associate with N′-CARD using a tandem-affinity purification system and TOF-MS analysis (data not shown). Among the N′-CARD interacting molecules identified, we were particularly interested in nuclear DNA helicase II (NDH, also known as RNA helicase A), a 1270 amino acid protein containing two copies of a dsRNA binding domain, a DEIH (Asp-Glu-Ile-His) helicase core, and an RGG (Arg-Gly-Gly) box nucleic acid-binding domain.

To examine the functional role of NDH in the signaling pathway leading to type I IFN production, NDH mRNA was ablated by RNA interference. Endogenous NDH protein was specifically decreased by NDH siRNA but not by control siRNA treatment (Fig. 2*B*). Knockdown of NDH resulted in a suppression of N′-CARD-induced IFN-β promoter activation by 73%. The level of promoter activation induced by IPS-1 or N′-CARD-TMD was also partially suppressed in NDH-knockdown cells by 33 and 38%, respectively. The levels were comparable when a constitutively active form of RIG-I (RIG-I 2CARDs), TBK1, or a constitutively active form of IRF3 (IRF3CA) was examined (Fig. 2*B*). Although over-expression of NDH had no effect, and over-expression of TBK-1 had a minimal effect on IFN-β promoter activation, over-expression of NDH plus TBK1 synergistically activated the IFN-β promoter, suggesting that NDH had the ability to up-regulate TBK1 activity (Fig. 2*C*). These results, taken together, suggest that NDH is involved in the events downstream of N′-CARD, and partially in

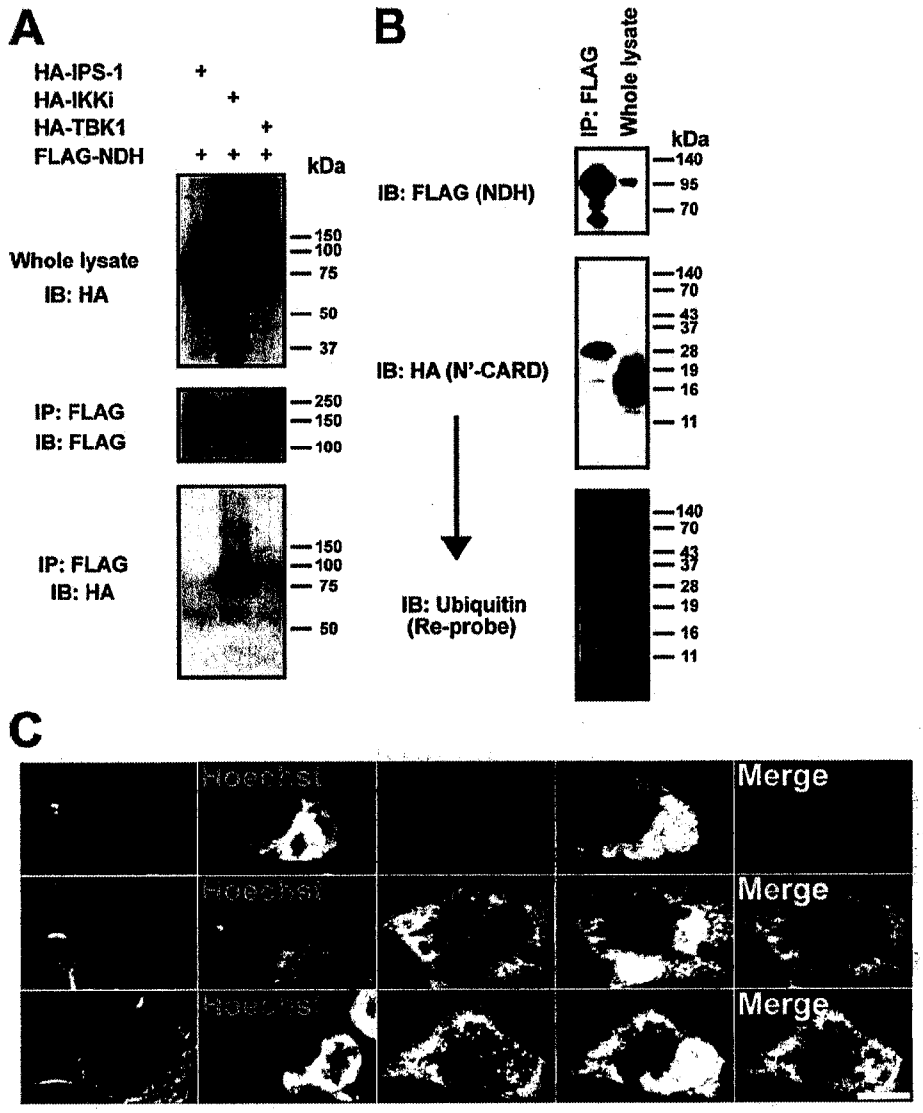


**FIGURE 3.** NDH associates with N′-CARD, TBK1, and IKKi. *A* and *B*, The lysates of HEK293 cells transfected with the expression plasmids for FLAG-NDH plus HA-IPS-1, HA-IKKi, HA-TBK1 (*A*) or N′-CARD-HA (*B*) were prepared and immunoprecipitated with anti-FLAG Ab. The immunoblots were probed with anti-HA or anti-FLAG Ab (*A* and *B*) or sequentially probed with anti-HA and anti-ubiquitin Ab (*B*). *C*, HeLa cells were transfected with an expression plasmid for mRFP-NDH alone or with those for mRFP-NDH and YFP-IKKi or YFP-TBK1. After staining with Hoechst 33258, the cells were examined under a confocal microscope. Data represent one of three independent experiments with similar results. Scale bar, 10 $\mu$m.

those downstream of IPS-1, and that it plays a role in signaling upstream of TBK1.

To confirm the physical interactions among NDH, IKKi, TBK1, and N′-CARD, immunoprecipitation analysis was performed. A strong interaction was detected between NDH and IKKi or TBK1, while there was no apparent association of NDH with IPS-1 in this assay (Fig. 3*A*). By contrast, NDH was confirmed to interact with N′-CARD. Of interest, the mobility of N′-CARD coprecipitated with NDH was retarded in SDS-PAGE (~25 kDa) when compared with that in whole cell lysate (~18 kDa) (Fig. 3*B*). The retarded N′-CARD was detected by anti-ubiquitin Ab, suggesting that mono-ubiquitinated N′-CARD, directly or indirectly, has the ability to associate with NDH (Fig. 3*B*). We also examined the subcellular localization of NDH, IKKi, and TBK1 by confocal microscopy analysis (Fig. 3*C*). Both YFP-IKKi and YFP-TBK1 were mostly present in the cytoplasm, while mRFP-NDH was diffusely present within the cell. Most NDH present within the cytoplasm colocalized with IKKi or TBK1 (Fig. 3*C*).

### *Recombinant N′-CARD polypeptide fused to the protein transduction domain (N′-CARD-PTD) induces type I IFN production and exerts innate immune responses in vitro*

To examine the potent ability of N′-CARD in modulating innate immune responses, we generated a recombinant N′-CARD polypeptide fused to the PTD, which enables transduction of extracellular protein into intracellular compartments. When the N′-CARD-PTD polypeptide was added to the culture medium of HeLa cells, it entered the nucleus within 30 min (Fig. 4*A*). By contrast, when the same amount of CARD polypeptide was added, only a minimal level of the polypeptide was observed inside the cell (Fig. 4*A*). The addition of the N′-CARD-PTD polypeptide alone induced significant levels of IFN-$\beta$ promoter activation in HEK293 cells, suggesting that N′-CARD-PTD has the ability to transmigrate into the cell and trigger NDH-mediated cellular signaling to elicit type I IFN production (Supplemental Fig. 2).

We next examined whether administration of the N′-CARD-PTD polypeptide activates immune cells in vitro. As shown in Fig. 4*B*, N′-CARD-PTD induced production of a proinflammatory cytokine (TNF-$\alpha$), type I IFNs (IFN-$\alpha$ and -$\beta$), and an IFN-stimulated gene product (IP-10) in a mouse macrophage cell line, RAW264.7. The expression of IFN-$\alpha$ and -$\beta$ mRNAs was detected within 18 h; the expression of IFN-$\alpha$ mRNA continued for more than 48 h after N′-CARD-PTD treatment. By contrast, LPS, an activator of TLR4-mediated innate immune responses, induced IFN-$\beta$ mRNA within 3 h, but this induction lasted for less than 18 h. The overall level of IFN-$\alpha$ mRNA production was higher in cells stimulated with N′-CARD-PTD compared with those stimulated with LPS, while that of IFN-$\beta$ was lower in those stimulated

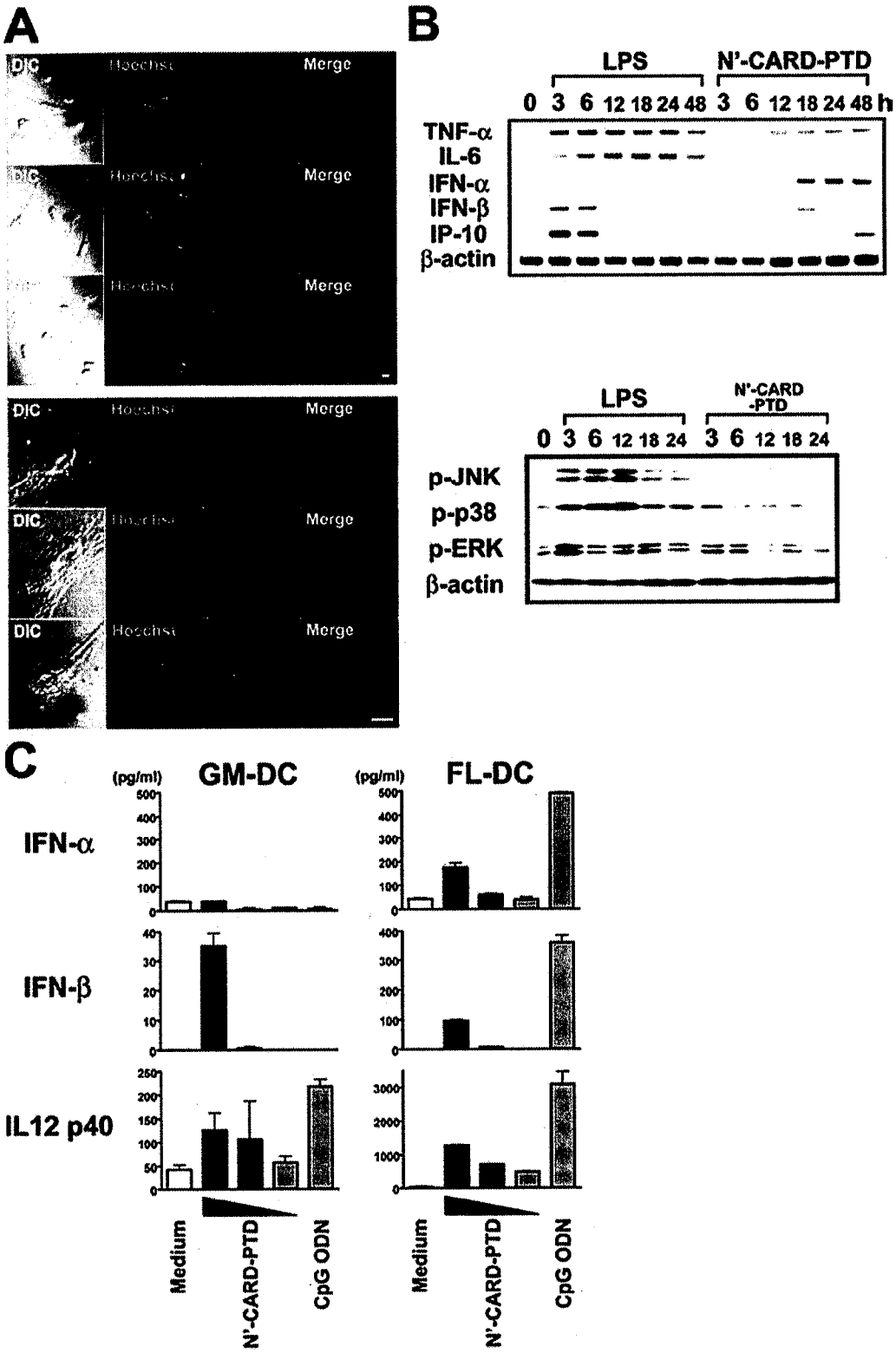


**FIGURE 4.** The N′-CARD-PTD polypeptide induces type I IFN production and DC maturation. *A*, Recombinant CARD or N′-CARD-PTD polypeptide was administered into the culture medium of HeLa cells. Thirty minutes after addition, the cells were permeabilized, stained with anti-FLAG M2-Cy3 and Hoechst 33258, and subjected to confocal microscopy analysis. *Upper panel,* Lower magnification. *Lower panel,* Higher magnification. Scale bar, 10 μm. *B*, RAW264.7 cells were treated with 1 μg/ml LPS or 10 μg/ml N′-CARD-PTD for 3, 6, 12, 18, 24, and 48 h. The levels of mRNA expression for TNF-α, IL-6, IFN-α, IFN-β, IP-10 and β-actin were examined by RT-PCR (*upper panel*). The levels of phosphorylated JNK, p38, or ERK were examined by immunoblotting (*lower panel*). *C*, GM-DCs or FL-DCs were treated with or without 1, 3, or 10 μg/ml N′-CARD-PTD or 1 μM of CpG ODN for 24 h and the supernatants were subjected to ELISA for mouse IFN-α, IFN-β, or IL-12 p40. Data represent one of two or three independent experiments with similar results.

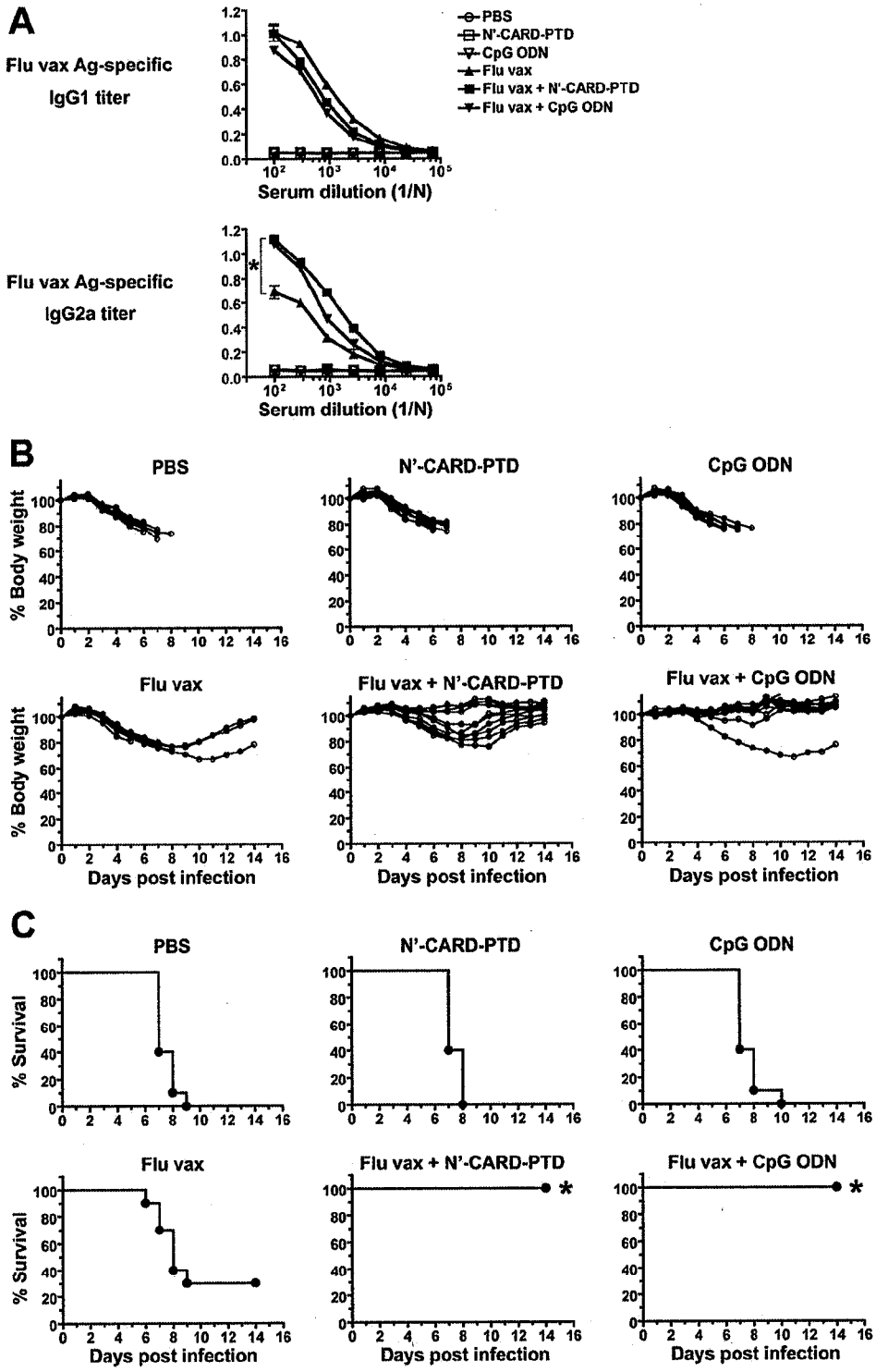


**FIGURE 5.** Coadministration of N′-CARD-PTD enhances Ag-specific IgG2a production and superior protection against lethal influenza infection. *A–C*, Eight-week-old female BALB/c mice ($n = 10$) were immunized s.c. with flu vax (0.7 $\mu$g), N′-CARD-PTD (5 $\mu$g), CpG ODN (5 $\mu$g), flu vax (0.7 $\mu$g) plus N′-CARD-PTD (5 $\mu$g), or flu vax (0.7 $\mu$g) plus CpG ODN (5 $\mu$g) at 0 and 10 days. *A*, Anti-flu vax Ab titer was examined 10 days after the final immunization. *B* and *C*, Ten days after the final immunization, mice were challenged with 8 $LD_{50}$ doses of influenza A/P/R8 (H1N1). The body-weight changes (*B*) and the mortality (*C*) were monitored for the next 14 days. Data represent one of two independent experiments with similar results. *, $p < 0.05$.

with N′-CARD-PTD compared with those stimulated LPS (Fig. 4*B*). These results suggest that N′-CARD-PTD activates a distinct innate immune signaling pathway(s) from those engaged by LPS. In fact, LPS induced phosphorylation of MAPK such as JNK, p38, and ERK within 3 h, while N′-CARD-PTD had little effects on activation of these kinases except for ERK at 3 and 6 h (Fig. 4*B*). We also examined whether N′-CARD-PTD activates bone marrow-derived dendritic cells (BM-DCs). As a control, CpG ODN activated BM-DCs induced in vitro by Flt3L (FL-DCs) but not BM-DCs induced in vitro by GM-CSF (GM-DCs) to produce type I IFNs. N′-CARD-PTD, by contrast, activated both GM-DCs and FL-DCs to produce type I IFNs (Fig. 4*C*). We also observed that N′-CARD-PTD weakly but significantly up-regulated cell surface expression of MHC class I, class II, CD40, and CD86 on both GM-DCs and FL-DCs (data not shown). Up-regulation of such cell surface molecules was dependent on type I IFN production but independent on myeloid differentiation factor 88 (MyD88) nor Toll-IL-1R domain-containing adaptor-inducing IFN-$\beta$ (TRIF) (data not shown).

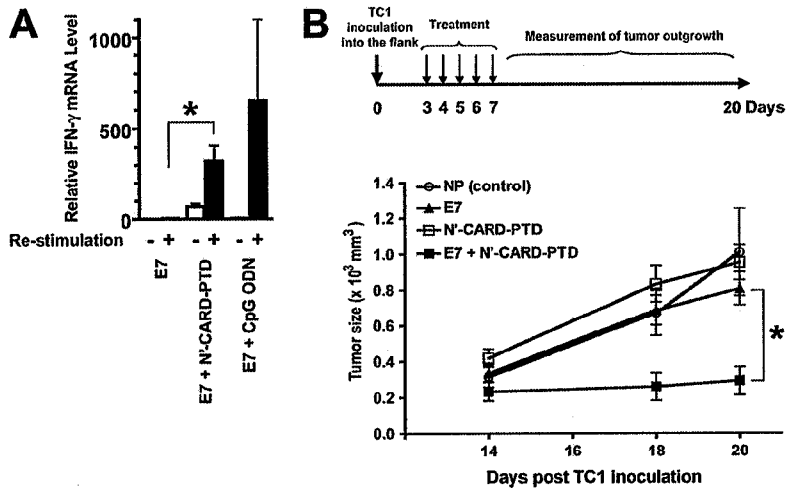


**FIGURE 6.** Coadministration of N′-CARD-PTD plus tumor-associated Ag E7 confers superior protection against tumor outgrowth. *A*, Eight-week-old female BALB/c mice ($n = 5$) were immunized subcutaneously with E7 peptide (3 $\mu$g), E7 peptide (3 $\mu$g) plus N′-CARD-PTD (5 $\mu$g), or E7 peptide (3 $\mu$g) plus CpG ODN (5 $\mu$g) at 2 and 4 wk. Splenocytes were prepared from each individual mouse and restimulated in vitro with control NP (−) or E7 peptide (±). The relative expression levels of IFN-$\gamma$ mRNA were measured by real-time PCR and normalized to 18S rRNA levels. *B*, Eight-week-old C57BL/6 mice ($n = 10$) were inoculated subcutaneously with $1 \times 10^5$ TC-1 cells/mouse at 0 days and then immunized with control NP peptide (3 $\mu$g), E7 (3 $\mu$g), N′-CARD-PTD (5 $\mu$g), or E7 (3 $\mu$g) plus N′-CARD-PTD (5 $\mu$g) at 3, 4, 5, 6, and 7 days. Tumor sizes were measured at 14, 18, and 20 days. Data represent one of two independent experiments with similar results. *, $p < 0.01$.

*N′-CARD-PTD augments Ag-specific acquired immune responses to protect against influenza virus infection and tumor outgrowth in vivo*

To examine the in vivo effects of N′-CARD-PTD on innate and acquired immune responses, we used a mouse model of influenza virus infection and of tumor transplantation. Influenza split-product vaccine (flu vax) was used to evaluate the adjuvanticity of N′-CARD-PTD. Flu vax was prepared at The Research Foundation for Microbial Diseases of Osaka University from the purified influenza virus A/New Caledonia/20/99 strain treated sequentially with ether and formalin. As shown in Fig. 5*A*, s.c. administration of flu vax plus N′-CARD-PTD or CpG ODN induced significant levels of specific IgG1 production that were comparable to that of flu vax alone. Administration of flu vax plus N′-CARD-PTD or CpG ODN, by contrast, resulted in significantly higher levels of specific IgG2a production compared with that of flu vax alone, suggesting that N′-CARD-PTD and CpG ODN have the ability to modulate Th1-deviated immune responses. In accordance with such adjuvant effects, immunization with flu vax plus N′-CARD-PTD conferred superior protection against a lethal influenza challenge relative to that with flu vax alone (Fig. 5, *B* and *C*).

We next examined whether N′-CARD-PTD has an ability to enhance Ag-specific cellular immune responses. Immunization with MHC class I-restricted HPV E7 peptide (E7) alone induced minimal levels of E7-specific IFN-$\gamma$ production from splenocytes (Fig. 6*A*). Treatment with E7 plus N′-CARD-PTD or CpG ODN induced higher levels of E7-specific IFN-$\gamma$ production, suggesting that N′-CARD-PTD has an adjuvant effect on cell-mediated immune responses (Fig. 6*A*). Thus, mice were s.c. transplanted with TC-1 cells expressing E7 as a model tumor Ag, and then immunized with E7 in the presence or absence of N′-CARD-PTD, as shown in Fig. 6*B*. The outgrowth of TC-1 tumors in mice treated with either N′-CARD-PTD or E7 alone was comparable to that in mice treated with control NP peptide. In accordance with E7-specific IFN-$\gamma$ production from splenocytes, the sizes of established tumors in mice treated with E7 plus N′-CARD-PTD were significantly smaller compared with those in mice treated with E7 alone or with N′-CARD-PTD alone (Fig. 6*B*). These in vivo results, taken together, indicate that N′-CARD-PTD, an activator of NDH-mediated innate immune responses, acts as a vaccine adjuvant, thereby enhancing protective immune responses against pathogens or tumors.

## Discussion

This study provides the first evidence that the N′-CARD-PTD polypeptide directly enters the nucleus and triggers the innate immune signaling pathway leading to type I IFN production through NDH. NDH is a member of the DEXH (Asp-Glu-X-His) family of helicases and is highly conserved in higher eukaryotes, from *Drosophila* to mammals. Previous studies have shown that NDH interacts with molecules of the transcription machinery, such as the RNA polymerase II complex (28), cAMP-response element-binding protein (28), and NF-$\kappa$B p65 (29), thereby regulating the transcription of responsive genes. NDH also acts together with the RNA editing enzyme to coordinate the editing and splicing of numerous cellular and viral RNAs (30, 31). Knockout of the *Ndh* gene led to early embryonic lethality (<E10.5) due to a high frequency of apoptosis in embryonic ectodermal cells during gastrulation (32). In addition to these properties of gene regulation and cellular homeostasis, our results suggest that NDH has a distinct property of mediating innate immune signaling upstream of TBK1. Recently, it was shown that a DEAD (Asp-Glu-Ala-Asp) box helicase, DDX3X, is a kinase substrate of TBK1 and acts as a critical component of TBK1-dependent innate immune signaling, particularly in the type I IFN production pathway (33).

MAPK activation plays a significant role in LPS- or CpG DNA-mediated signaling (Fig. 4*C* and Ref. 34), however, the signaling pathway induced by N′-CARD-PTD may not involve MAPK. This suggested that, unlike TLR-mediated signaling pathways, activation of MAPK is not crucial for N′-CARD-PTD-mediated type I IFN production. Rather, the action of N′-CARD-PTD resembles the signal activation induced by IFN stimulatory DNA, which was originally reported by Stetson et al. as having a similar action to B-DNA, which is critical in the control of DNA vaccine immunogenicity (5, 35). Because MAPK activation is associated with deleterious effects, ranging from hyperinflammation to cancer (36), the lack of such kinase activation would be an advantage for the repeated clinical application of N′-CARD-PTD. Further study will be needed to elucidate the molecular basis of the NDH-mediated signaling pathway and to determine the value of N′-CARD-PTD for clinical use.

Many TLR ligands and related compounds have been tested as vaccine adjuvants and as anti-allergy and anti-cancer drugs in humans (37). Among these, some clinical trials of TLR9-targeting molecules, including CpG ODN and its conjugated products, have recently been abandoned due to unexpectedly weaker responses in

humans relative to those observed in mice. This result was attributable to a lower frequency of TLR9 expression in human immune cells; expression was found in only a portion of B cells and plasmacytoid DCs that combined made up just 1% of the total immune cell population (38). In contrast, immunostimulatory RNA or B-DNA activates innate immune responses through cytosolic receptors but only when they are introduced into intracellular compartments, i.e., they have almost no effects when they are present outside the cell. Taking such observations into account, N′-CARD-PTD may have the advantage of self-transmigration into the nucleus and of triggering innate immune signaling in the absence of TLRs but in the presence of NDH and TBK1, which are ubiquitously expressed in a wide-variety of cell types.

In conclusion, this study showed concrete evidence that the activation of a distinct NDH-mediated signaling pathway up-regulates innate immune responses and that N′-CARD-PTD is a candidate vaccine adjuvant in future vaccine development. These findings may also provide insights that will be helpful in the design of immunomodulatory agents, such as using constitutively active signaling molecules of the innate immune responses.

## Disclosures

The authors have no financial conflict of interest.

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