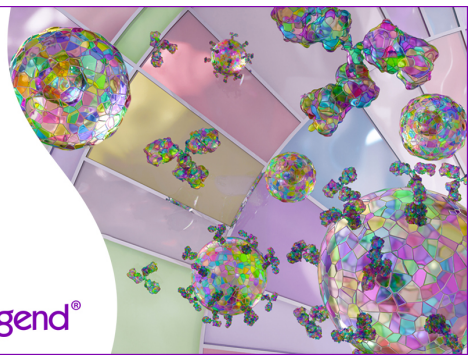

The Journal of Immunology
RESEARCH ARTICLE | JUNE 15 2017

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*J Immunol* (2017) 198 (12): 4707–4715.
https://doi.org/10.4049/jimmunol.1600731

# CD63-Mediated Antigen Delivery into Extracellular Vesicles via DNA Vaccination Results in Robust CD8$^+$ T Cell Responses

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**DNA vaccines are attractive immunogens for priming humoral and cellular immune responses to the encoded Ag. However, their ability to induce Ag-specific CD8$^+$ T cell responses requires improvement. Among the strategies for improving DNA vaccine immunogenicity are booster vaccinations, alternate vaccine formulations, electroporation, and genetic adjuvants, but few, such as extracellular vesicles (EVs), target natural Ag delivery systems. By focusing on CD63, a tetraspanin protein expressed on various cellular membranes, including EVs, we examined whether a DNA vaccine encoding an Ag fused to CD63 delivered into EVs would improve vaccine immunogenicity. In vitro transfection with plasmid DNA encoding an OVA Ag fused to CD63 (pCD63-OVA) produced OVA-carrying EVs. Immunizations with the purified OVA-carrying EVs primed naive mice to induce OVA-specific CD4$^+$ and CD8$^+$ T cells, whereas immunization with EVs purified from cells transfected with control plasmids encoding OVA protein alone or a calnexin-OVA fusion protein delivered into the endoplasmic reticulum failed to do so. Vaccinating mice with pCD63-OVA induced potent Ag-specific T cell responses, particularly those from CD8$^+$ T cells. CD63 delivery into EVs led to better CD8$^+$ T cell responses than calnexin delivery into the endoplasmic reticulum. When we used a mouse tumor implantation model to evaluate pCD63-OVA as a therapeutic vaccine, the EV-delivered DNA vaccination significantly inhibited tumor growth compared with the control DNA vaccinations. These results indicate that EV Ag delivery via DNA vaccination offers a new strategy for eliciting strong CD8$^+$ T cell responses to the encoded Ag, making it a potentially useful cancer vaccine.** ***The Journal of Immunology*****, 2017, 198: 4707–4715.**

Deoxyribonucleic acid vaccination may become a novel preventive and/or therapeutic method for combating human diseases, applicable not only to infectious diseases, but also to noncommunicable diseases (1, 2). The clinical benefits of DNA vaccines are their low cost, stability, and high productivity, as well as the ease with which their Ag sequence(s) can be modified to work against highly mutated pathogens. In the veterinary field, DNA vaccines are already licensed for West Nile virus for horses, infectious hematopoietic necrosis virus for salmon, and melanoma for dogs (3). Although the results of early clinical trials in humans showed that DNA vaccines are safe and well-tolerated, their immunogenicities were much lower than expected based on the animal experiment results. To overcome this issue, several different approaches have been evaluated for improving the immunogenicity of these vaccines, such as altering the promoter sequence or altering the codon usage in the Ag-encoding sequence, inserting genetic adjuvants, adding booster vaccinations, mixing the vaccines with external adjuvants, or administering the vaccines through novel routes or devices (4–6).

Extracellular vesicles (EVs) such as exosomes, apoptotic bodies, and microvesicles are membrane-enclosed vesicles released by cells by various mechanisms, including budding off the plasma membrane (microvesicles), or formation of intraluminal vesicles in compartments of the endosomal pathway, followed by fusion of these compartments with the plasma membrane (7). They exist in various bodily fluids, such as plasma, breast milk, semen, saliva, and urine. Interestingly, DNA, RNA, microRNA, proteins, and lipids are all contained inside EVs, but the precise roles played by EVs are still not fully understood (8). One study showed that these small vesicles mediate cell-to-cell communication and are closely linked with immunomodulation (9). APCs that express both MHC class I and II, as well as costimulatory molecules that stimulate CD4$^+$ and CD8$^+$ T cells, secrete EVs (10, 11). Additionally, EVs from mature dendritic cells (DCs) can activate immature DCs to increase their Ag-presenting ability (12). Ag-loaded EVs can also be used as vaccines for cancer therapy (13). These findings suggest that use of EVs or Ag-loaded EVs could present a novel strategy worth pursuing for vaccine formulations.

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Received for publication April 29, 2016. Accepted for publication April 11, 2017.

This work was supported by grants from the Ministry of Health, Labour and Welfare, the Ministry of Education, Culture, Sports, Science and Technology of the Japanese government, the Research on Development of New Drugs, the Emerging/Re-emerging Infectious Diseases Project of Japan, and the Research Program on HIV/AIDS from the Japan Agency for Medical Research and Development.

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The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; EGFP, enhanced GFP; ER, endoplasmic reticulum; EV, extracellular vesicle; i.d., intradermal; imEPT, i.m. electroporation; NP, nucleoprotein; pCal-OVA, pOVA fused to calnexin; pCD63-OVA, pOVA fused to CD63; pOVA, plasmid DNA encoding an OVA Ag.

In previous studies, the role played by EVs in the induction of immune responses (14, 15) and the EV-targeting strategy via the C1C2 domain of MFG-E8/lactadherin have both been described (16–18). Because the Ag-specific immunogenicity of a vaccine can be enhanced by Ag targeting to EVs, we consider that the strategy to target the C1C2 domain is an elegant one. However, MFG-E8, which is secreted mainly by activated macrophages in the spleen and lymph nodes, binds to apoptotic cells (19, 20). Therefore, we propose a new vaccine strategy based on the use of a surface marker protein (CD63) on EVs as a ubiquitously expressed protein, but not the C1C2 domain of MFG-E8. CD63 is a tetraspanin protein expressed on various cellular membranes, including EVs.

In this study, we investigated whether a DNA vaccine encoding an Ag fused to CD63 could be delivered into EVs to improve vaccine immunogenicity. We constructed an OVA Ag fused to a CD63 expression plasmid to deliver the Ag into the EVs. We also sought to identify the types of immune responses that were induced or enhanced by delivering the DNA vaccine into the EVs via a CD63-fused Ag and also evaluated the potential of the EV-based approach as an anticancer vaccine.

## Materials and Methods

### *DNA constructs*

The full-length CD63 (aa 1–238), CD9 (aa 1–226), CD81 (aa 1–236), and calnexin (aa 1–591) cDNAs were PCR amplified from a mouse lung cDNA library from C57BL/6J as a template. The cDNA fragments were verified by DNA sequencing and then introduced into pCI mammalian expression vectors (Promega Corporation, Madison, WI), pCIneo-FLAG (Promega), pEGFP-N1 (Clontech, Palo Alto, CA), pEGFP-C1 (Clontech), and pmCherry-N1 (Clontech), as described previously (21). The full-length OVA (aa 1–386)-encoding cDNA was PCR amplified from plasmid DNA encoding an OVA Ag (pOVA) (22). The OVA cDNA fragment was verified by DNA sequencing, introduced into pCIneo-FLAG, and named pOVA-FLAG. The full-length OVA protein was fused to CD63, CD9, CD81, or calnexin in the N-terminal position. As a linker, a glycine hexamer (6× glycine) was inserted between OVA and the fused gene. The fused genes were verified by DNA sequencing and then introduced into the pCI vectors, pCIneo-FLAG vectors, or pEGFP-N1 vectors, and named pCD63-FLAG (pCD63), pOVA–6×Gly-CD63 (pOVA-CD63), pCD63-6×Gly-OVA-FLAG (pOVA fused to CD63 [pCD63-OVA]), pCD9-6×Gly-OVA-FLAG (pCD9-OVA), pCD81-6×Gly-OVA-FLAG (pCD81-OVA), pCalnexin-6×Gly-OVA-FLAG (pOVA fused to calnexin [pCal-OVA]), or pCD63-6×Gly-OVA-enhanced GFP (EGFP; pCD63-OVA-EGFP). All the plasmids were transformed into *Escherichia coli* DH5α cells and, after expansion, purified using Qiagen Plasmid Endo-free Maxiprep kits (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

### *Cells*

293T, HeLa, and EL-4-OVA (E.G-7) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). FreeStyle 293-F cells (293F cells) were purchased from Life Technologies (Carlsbad, CA). The cells were cultured at 37˚C under 5% $CO_2$ in DMEM (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS and 50 μg/ml penicillin-streptomycin (Nacalai Tesque). The 293F cells were cultured at 37˚C under 8% $CO_2$ in Free Style 293 Expression medium, which is free of animal serum-derived exosomes (Life Technologies). The E.G-7 cells were cultured at 37˚C under 5% $CO_2$ in RPMI 1640 supplemented with 10% FCS, 50 μg/ml penicillin-streptomycin, 0.05 mM 2-ME, 1 mM sodium pyruvate, 10 mM HEPES, and 1× nonessential amino acids (Life Technologies).

### *Isolating EVs from the cell culture medium*

Serum (EV)-free 293F cells were transfected with various DNA constructs for 48–72 h. The culture supernatants were then collected and centrifuged at 2000 × *g* for 30 min. EVs were isolated from the cell culture medium using a Total Exosome Isolation kit (Life Technologies) following the manufacturer's protocol. In brief, 1.5 ml of the Total Exosome Isolation reagent was added to 3 ml of cell culture medium and then incubated at 4˚C overnight, followed by centrifugation at 10,000 × *g* for 1 h at 4˚C. The EV pellets were resuspended in 100 μl of PBS. The EVs collected were characterized qualitatively and quantitatively by electron microscopy, Western blotting, and flow cytometric assays, as described later.

### *Cell transfection and Western blotting*

Cell transfections were performed as described previously (21). Transient transfections were conducted with Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Batches of 293F cells ($1 \times 10^6$/ml) were individually transfected with each expression plasmid. The transfected cells were harvested 48 h after transfection. The culture medium containing the transfected cells was centrifuged at 2000 × *g* for 30 min. The cells were lysed with radioimmunoprecipitation assay buffer (Nacalai Tesque) and incubated for 15 min on ice, followed by centrifugation at 20,000 × *g* for 15 min at 4˚C. The supernatants and isolated EV samples were diluted in 3× SDS buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol, 0.01% bromophenol blue) and heated for 5 min at 95˚C. Immunoblotting analyses were performed as described previously (21) using an anti-CD63 Ab (R5G2; MEDICAL & BIOLOGICAL LABORATORIES, Nagoya, Japan), an anti-calnexin Ab (ab22595; Abcam, Cambridge, U.K.), or an anti-FLAG M2-Peroxidase (HRP) Ab (A8592; Sigma-Aldrich, St. Louis, MO).

### *Immunogold electron microscopy and fluorescence microscopy*

Immunogold electron microscopy assays were performed as described previously (23). Isolated EVs were immunolabeled with a mouse anti-CD63 Ab as the primary Ab and gold-labeled anti-mouse IgG as the secondary Ab (10 nm Gold) (Abcam). Fluorescence analysis was performed with a fluorescence deconvolution microscope (BZ-9000; Keyence, Osaka, Japan).

### *Flow cytometry analyses*

293F cells ($1 \times 10^6$/ml) were transfected with pCD63-OVA-EGFP. The culture medium containing the transfected cells was collected at 48 h posttransfection and centrifuged. The resulting supernatants were characterized by Influx (BD Biosciences, San Jose, CA) (24). Beads (100 and 200 nm; Polysciences, Warrington, PA) were used to determine the appropriate gating for the EV fraction (Supplemental Fig. 1).

### *Animals and immunizations*

Six-week-old female C57BL/6J mice were purchased from CLEA Japan, and OT-I and OT-II mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were immunized by i.m. electroporation (imEPT) with 50 μg of plasmid DNA encoding the control (OVA or CD63) or fusion proteins (CD63-OVA, CD9-OVA, CD81-OVA, or Cal-OVA) in nuclease-free saline at 50 μl/muscle. The injection site was the same as that described previously (25).

For the EV immunizations, the mice received two tail-based immunizations with 600 μg of the EV proteins (40 ng of OVA) or with the OVA protein (40 ng) alone (Hyglos, Bernried, Germany) in PBS (total volume: 100 μl per mouse) on day 7 after the first immunization. The purified EVs used to immunize the mice were isolated from pOVA-, pCD63-OVA–, or pCal-OVA–transfected cells. The concentrations of the purified EV proteins and the cell culture supernatants were tested using an *RC DC* Protein Assay (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. One week after the final vaccination, the mice were sacrificed and their Ag-specific immune responses were measured.

All of the animal experiments were conducted in accordance with the institutional guidelines for the National Institutes of Biomedical Innovation, Health and Nutrition animal facility (Osaka, Japan).

### *Evaluation of cellular immune responses*

To evaluate cellular immune responses, we prepared splenocytes ($2 \times 10^6$ per well) and then incubated them in complete RPMI 1640 medium containing 20 μg/ml OVA peptides [$OVA_{257–264}$, an H-$2K^b$-restricted OVA class I epitope, or $OVA_{323–339}$, an I-A (d)-restricted OVA class II epitope] or OVA Ag (Calbiochem, La Jolla, CA). Cellular immune responses were measured as described previously (26).

### *ELISA*

Batches of 293T cells ($1 \times 10^6$/ml) were individually transfected with each expression plasmid. The cells were incubated for 48 h after transfection, and the OVA expression levels in the cells and cell culture supernatants were measured by ELISA (Institute of Tokyo Environmental Allergy, Tokyo, Japan) according to the manufacturer's protocol. The OVA-specific serum Ab titers were measured as described previously (26).

### *Tetramer assays*

The tetramer assays were performed as previously described (27). In brief, splenocytes were stained with PE-labeled H-$2K^b$ OVA tetramer

(SIINFEKL) (MEDICAL & BIOLOGICAL LABORATORIES) for 20 min at room temperature. Next, the cells were stained with FITC-labeled anti-CD8α (KT15; BioLegend, San Diego, CA), allophycocyanin-labeled anti–TCRβ-chain (H57-597; BioLegend), Brilliant Violet 421–labeled anti-CD62L (MEL-14; BioLegend), and allophycocyanin/Cy7-labeled anti-CD44 (IM7; BioLegend) Abs in PBS. The number of OVA tetramer$^+$ CD44$^+$ CD8a$^+$ TCRb$^+$ cells was determined by flow cytometry.

### *In vivo CTL assay*

The in vivo CTL assay was performed as described previously (27). In brief, 6-wk-old C57BL/6J mice were vaccinated with the DNA vaccines. On day 21, splenocytes from naive C57BL/6J mice were labeled with either 2 or 0.2 μM CFSE (Life Technologies) for 10 min at 37°C. The CFSE-labeled cells were subjected to peptide pulsing by incubating them with 10 μg/ml of the $OVA_{257–264}$ peptide for 90 min at 37°C. Next, the cells were washed, and equal numbers of them from each treatment group were transferred intravascularly to the immunized mice. Splenocytes were isolated 24 h after the transfer, and the CFSE-labeled cells were analyzed by flow cytometry.

### *Tumor challenge*

Tumor inoculation and tumor volume measurements were performed in the experimental mice as described previously (28). The mice were s.c. injected with $1 \times 10^6$ E.G-7 cells in 100 μl of PBS. When the average tumor volumes reached 1 $cm^3$, the mice were given primary immunizations of pOVA, pCD63-OVA, pCal-OVA, or the pCIneo-FLAG negative control. For the tumor prevention experiments, the mice were immunized with the earlier-named plasmid DNA vaccines and 10 d later, they were injected s.c. with E.G-7 cells ($1 \times 10^6$ cells in 100 μl of PBS).

### *Statistical analysis*

The statistical significances of the differences between the various groups were determined using Mann–Whitney *U* tests.

## Results

### *CD63-fused Ags entrapped in EVs act as vaccines*

Previous studies have suggested that vaccination with Ag-loaded EVs induces Th1 responses and CTL activation (14, 29). To determine whether CD63-fused Ag expression in the cell leads to more efficient Ag secretion in the EVs than the simple expression of the Ag alone, we constructed a plasmid DNA encoding a fusion protein of CD63 and OVA (pCD63-OVA). We aimed to deliver the encoded OVA Ag into the EVs via CD63 because CD63 is expressed on EV membranes and is already used as a common EV marker (30). We also prepared a control plasmid DNA that encodes a pCal-OVA to deliver the encoded Ag to the endoplasmic reticulum (ER) (31–33).

Serum (exogenous EV)-free 293F cells were transfected with each of the plasmid DNAs described earlier, and the EVs in the supernatant were collected and purified from the cell culture medium using exosome isolation reagents. We then analyzed the encoded fusion protein expression levels in the cell lysates, supernatants, and EV fractions using Western blotting (Fig. 1A). The EV fractions, but not the supernatants, from all the groups contained another EV marker, CD81, and the cellular fractions contained only calnexin, thus confirming the purity of each fraction (Fig. 1A). Under these conditions, the OVA protein was detected in the supernatant after pOVA transfection (Fig. 1A, 1B). In contrast, neither CD63 nor the CD63-OVA fusion protein was detected in the supernatants, but they were both detected in the cell lysates and EV fractions (Fig. 1A, 1B). Delivery of the pCal-OVA via the ER was detected in the cell lysate, but not in the EV fraction (Fig. 1A, 1B). Next, we examined whether the CD63-OVA protein localized in or on the EVs. By using EGFP- or FLAG-fusion protein-encoding plasmids, we analyzed CD63 expression by flow cytometry as well as by electron microscopy. The results of both analyses suggest that the CD63-OVA fusion protein is in fact delivered into the EVs (Fig. 1C, Supplemental Fig. 1A).

Using fluorescence microscopy, we also examined whether fusing another protein to the $NH_2$- or COOH-terminal of CD63 altered its localization. To investigate this, we constructed a plasmid with EGFP fused to the N terminus of CD63 or mCherry fused to the COOH terminus. HeLa cells were cotransfected with these plasmid DNAs. As a result, the signals from the EGFP fused to the N terminus of CD63 and mCherry fused to the COOH terminus proteins colocalized, and a merged signal was seen (Supplemental Fig. 1B).

We next examined whether the purified EVs from the serum-free cell culture supernatants of cells transiently transfected with the CD63-OVA protein acted as a vaccine and induced sufficient OVA-specific immune responses. We collected the EVs from the cell culture supernatants after transfecting the cells with pOVA, pCD63-OVA, or pCalnexin-OVA, and the C57/BL6 naive mice were intradermally immunized with these purified EVs at the base of their tails. One week after the primary immunizations, we evaluated the Ag-specific humoral and cellular immune responses in the mice. The CD63-OVA–containing EV immunization induced higher Ag-specific Ab responses than any of the other immunizations we tested (Fig. 1D). More interestingly, immunization with the CD63-OVA–containing EVs induced Ag-specific IFN-γ–producing CD8$^+$ T cells, whereas the other immunizations we tested failed to do so (Fig. 1E, 1F, Supplemental Fig. 1C). These results strongly suggest that CD63-OVA from the EV fraction can induce a stronger CD8$^+$ T cell response than OVA alone or calnexin-OVA from the same fraction.

### *Ag delivery into EVs improves DNA vaccine immunogenicity*

Based on the earlier results, we hypothesized that DNA vaccines incorporating Ag delivery into EVs would have enhanced immunogenicities. To examine whether the pCD63-OVA DNA vaccine had enhanced immunogenicity compared with the other control DNA vaccines, we immunized mice with plasmid DNAs twice by imEPT to induce efficient transfection in vivo. One week after the booster immunization, the pCD63-OVA–immunized mice had significantly lower titers of anti-OVA IgG1 in their serum samples than the pOVA-immunized mice did, although there was no difference in the anti-OVA total IgG and IgG2c titers between these two groups (Fig. 2A). When we compared the anti-OVA IgG2c/IgG1 ratios between pCD63-OVA and pOVA, we observed higher IgG2c/IgG1 values in the pCD63-OVA–immunized mice compared with those of the pOVA group (Fig. 2A). Next, we checked the frequency of $OVA_{257–264}$–specific tetramer$^+$ CD44$^+$ CD8$^+$ T cells in the immunized mice. We observed higher frequencies of $OVA_{257–264}$–specific tetramer$^+$ CD44$^+$ CD8$^+$ T cells in the pCD63-OVA immunized mice than in the pOVA-immunized mice (Fig. 2B). We also stimulated the splenocytes with either class I ($OVA_{257–264}$) or class II ($OVA_{323–333}$) OVA peptide ex vivo, and the resulting cytokine production from these splenocytes was measured by ELISA. We detected significantly higher levels of IFN-γ production after pCD63-OVA vaccination compared with pOVA vaccination for each peptide stimulation test (Fig. 2C).

We also examined the efficacy of the DNA vaccine by fusing OVA to the $NH_2$- or COOH-terminal of CD63 (Supplemental Fig. 2). The number of OVA-specific tetramer$^+$ CD8$^+$ T cells increased after pOVA-CD63 and pCD63-OVA vaccination compared with the pOVA vaccination, and there was no difference in terms of the number of these cells after the pOVA-CD63 and pCD63-OVA vaccinations. These results suggest that the immunogenicity of the DNA vaccine-expressing CD63 fused to OVA at the $NH_2$ or COOH terminus is equivalent.

In addition to the CD63-based fusion protein, we also created plasmid DNAs encoding CD9-OVA (pCD9-OVA) or CD81-OVA (pCD81-OVA) fusion proteins, which also delivered the encoded Ag into the EVs (34, 35). We checked that the CD9-OVA protein

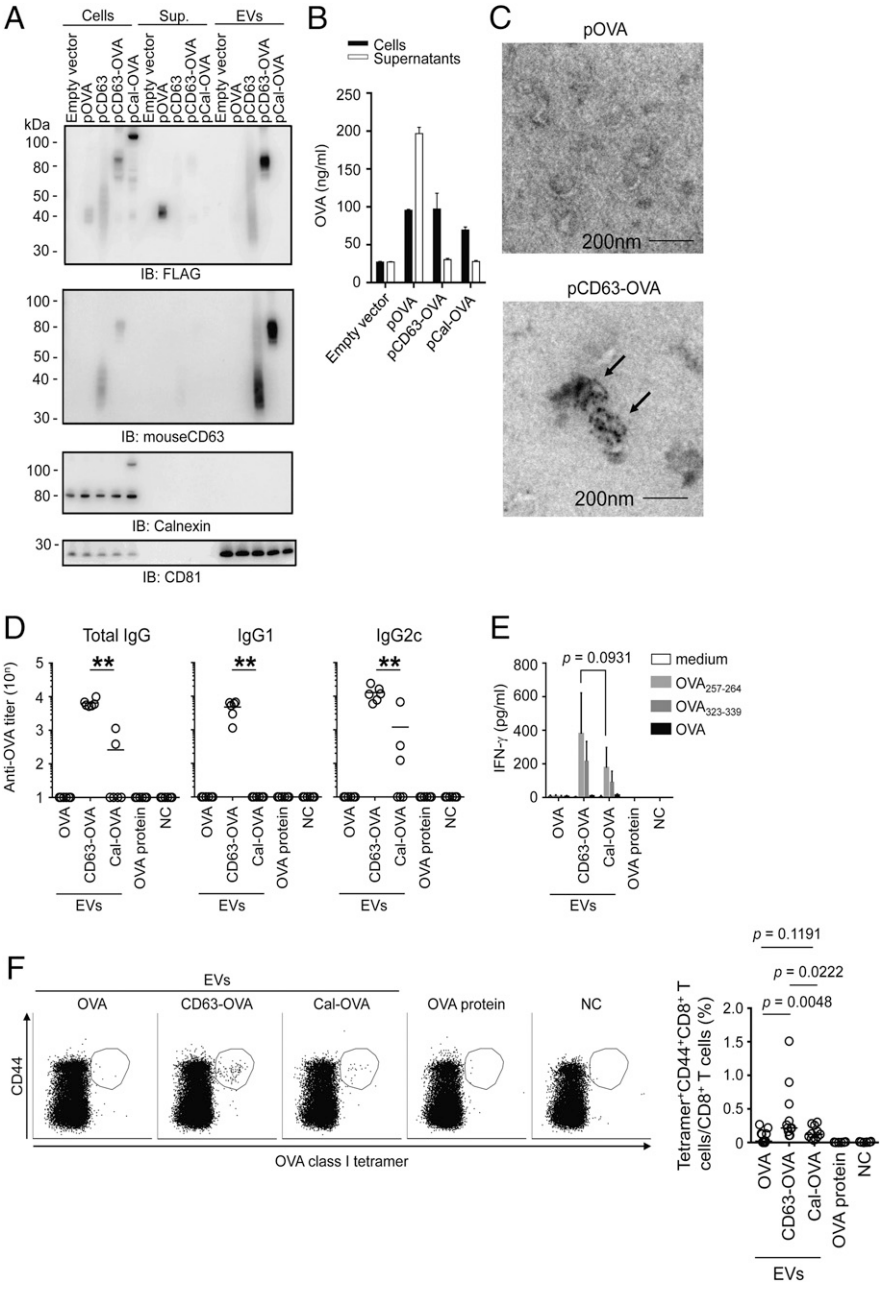


**FIGURE 1.** Assessment of Ag-expressing EVs as CTL-inducing immunogens. (**A** and **B**) 293F and 293T cells were transiently transfected with the plasmids indicated. At 48 h posttransfection, the EV fraction was purified from culture supernatants. The cells were then subjected to immunoblotting (IB) analyses (A) or used in ELISAs to analyze their OVA protein concentrations. (**C**) EV detection by transmission electron microscopy. Empty vector: EVs purified from 293F cells transfected with an empty vector were used as a negative control. pCD63-OVA: EVs purified from pCD63-OVA–transfected 293F cells. Arrows indicate specific anti-mouse CD63 staining. (**D**–**F**) C57BL/6J mice ($n = 6$) were intradermally immunized with the purified EVs isolated from pOVA-, pCD63-OVA–, or pCal-OVA–transfected cells or OVA proteins. On day 14 after priming, sera from the immunized mice were subjected to ELISA for OVA-specific IgG titers, OVA-specific serum IgG1 titers, and OVA-specific serum IgG2c titers (D), whereas splenocytes from the mice were subjected to cytokine ELISA to measure IFN-γ levels in response to OVA protein, $OVA_{257–264}$, $OVA_{323–339}$, or medium (E). In addition, splenocytes were also subjected to FACS analysis to evaluate the percentage of $CD8^+$ T cells recognized by the $OVA_{257–264}$–specific tetramer (F). Data are representative of two independent experiments; error bars denote the SD. $^{**}p < 0.005$ (Mann–Whitney *U* test).

and the CD81-OVA protein were both expressed in the EVs (Fig. 2D, 2E). DNA vaccination with pCD9-OVA or pCD81-OVA resulted in higher anti-OVA IgG2c/G1 ratios than vaccination with the pOVA control. Furthermore, Ag-specific $CD8^+$ T cell responses were also enhanced at similar levels as those seen after pCD63-OVA vaccination (Fig. 2F–H).

These results indicate that Ag delivery into the EVs during DNA vaccination improves the immunogenicity of the vaccine, especially the $CD8^+$ T cell responses against it.

### *Ag delivery to EVs is essential for improving DNA vaccine immunogenicity*

We next investigated whether the strategy of delivering the encoded Ag into the EVs would offer any advantages over alternative strategies for enhancing T cell activation by the DNA vaccine, such as, for example, Ag delivery to other cellular membranes such as the ER. To test this, we compared the magnitude and type of immunogenicity generated by the two plasmid DNAs that encode pCD63-OVA or pCal-OVA, one of which (pCD63-OVA) is delivered into the EVs, or the other of which (pCal-OVA) is delivered across the ER. Mice that received two DNA vaccinations via imEPT with pCD63-OVA or pCal-OVA displayed similar anti-OVA Ab titers and anti-OVA IgG2c/G1 ratios (Fig. 3A). However, mice vaccinated with pCal-OVA had a higher frequency of $OVA_{257–264}$–specific tetramer$^+$ $CD44^+$ $CD8^+$ T cells than mice vaccinated with pCD63-OVA (Fig. 3B). In addition, splenocytes from the mice immunized with pCD63-OVA produced significantly higher amounts of IFN-γ upon stimulation than those of mice immunized with pCal-OVA (Fig. 3C). These results indicate that Ag delivery into EV membranes, but not to other membranes, is sufficient to improve the immunogenicity of the DNA vaccine.

### *CD63 does not act as a genetic adjuvant for the coadministered DNA vaccine*

Because the pCD63-OVA vaccine induced potent $CD8^+$ T cell responses (Figs. 2, 3), we examined whether the CD63-expressing plasmid DNA would act as a genetic adjuvant for the OVA-expressing DNA vaccine. To evaluate the potential adjuvant

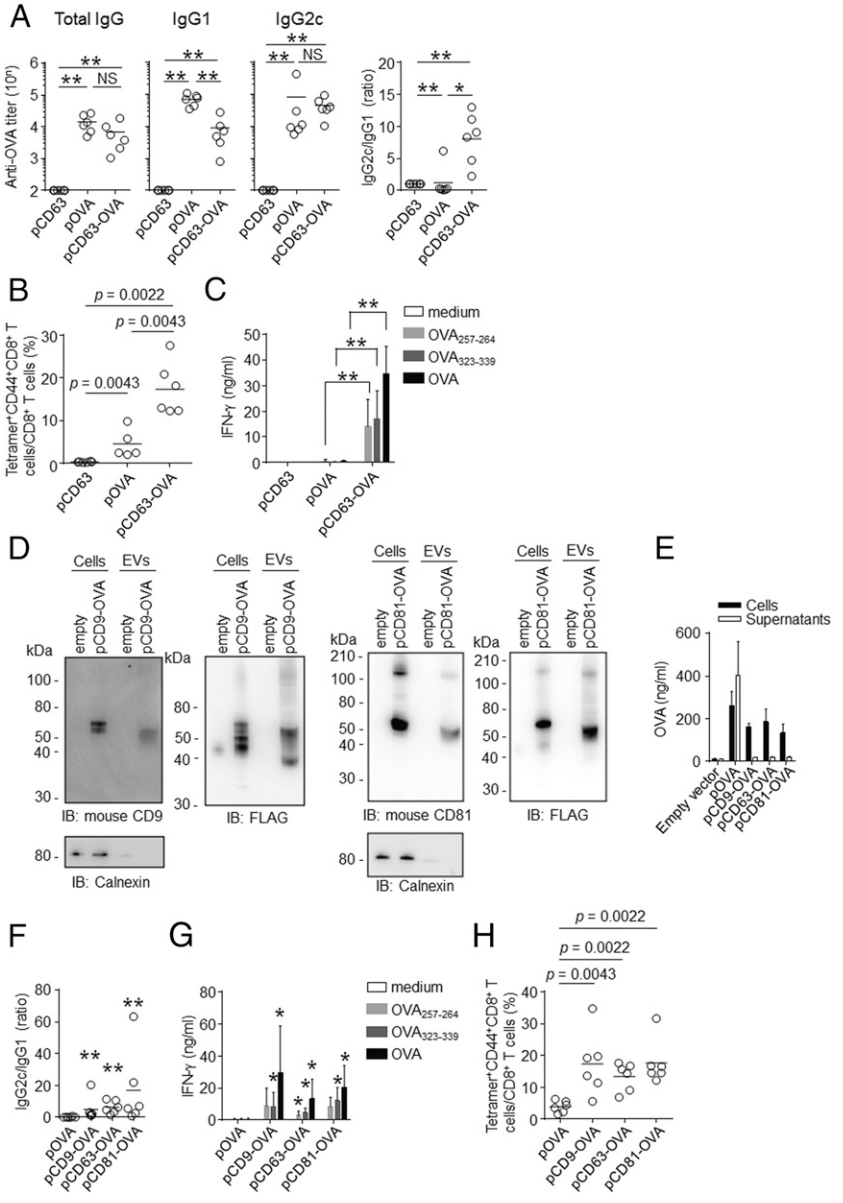


**FIGURE 2.** Effect of Ag delivery into EVs on the immunogenicities of the DNA vaccines. (**A**–**C**) C57BL/6J mice ($n = 6$) were immunized via imEPT with pOVA, pCD63, or pCD63-OVA (50 μg/mouse) at days 0 and 14. Sera from the mice were subjected to ELISA to obtain the OVA-specific IgG titers, the OVA-specific serum IgG1 titers, and the OVA-specific serum IgG2c titers (A). The ratios of the OVA-specific serum IgG2c to OVA-specific serum IgG1 were calculated by dividing the OVA-specific serum IgG2c titers by the OVA-specific IgG titers (A). The percentage of $CD8^+$ T cells recognized by the $OVA_{257–264}$–specific tetramer (B) and the IFN-γ levels produced from the splenocytes by OVA, $OVA_{257–264}$, $OVA_{323–339}$, or medium (C) were monitored on day 21 postimmunization with these plasmids. (**D** and **E**) The 293F and 293T cells were transiently transfected with the plasmids indicated. At 48 h posttransfection, the EVs were purified from the cell supernatants. The cells and EVs were subjected to immunoblotting (IB) analyses (D). The OVA protein concentrations were analyzed by ELISA (E). (**F**–**H**) C57BL/6J mice ($n = 6$) were immunized via imEPT with pOVA, pCD9-OVA, pCD63-OVA, or CD81-OVA (50 μg/mouse) at days 0 and 14. The ratios of OVA-specific serum IgG2c to OVA-specific serum IgG1 (F), levels of IFN-γ produced from splenocytes in response to OVA, $OVA_{257–264}$, $OVA_{323–339}$, or medium (G), and the percentage of $CD8^+$ T cells recognized by the $OVA_{257–264}$–specific tetramer (H) were monitored on day 21 postimmunization with these plasmids. Data are representative of two or three independent experiments; error bars denote the SD. $^*p < 0.05$, $^{**}p < 0.005$ (Mann–Whitney $U$ test).

effects of CD63, we immunized mice with pOVA, pCD63, a mixture of pOVA and pCD63 (pOVA + pCD63), or pCD63-OVA. One week after the second immunization, the pCD63-OVA–immunized mice had significantly lower anti-OVA IgG1 titers in their serum samples than those from the pOVA + pCD63–immunized mice, but there was no difference in the total anti-OVA IgG, IgG2c, and IgG2c/IgG1 ratios between these two groups (Fig. 4A). In addition, no enhancement of the OVA-specific $CD4^+$ and $CD8^+$ T cell responses in the mice that received pCD63 and pOVA together was observed compared with the mice that received pOVA alone; however, mice vaccinated with pCD63-OVA showed a significant increase in their OVA-specific $CD4^+$ and $CD8^+$ T cell responses (Fig. 4B, 4C). These results indicate that CD63 itself does not function as a genetic adjuvant unless it is conjugated to the OVA Ag as a fusion protein. Therefore, delivering Ags in or on EVs can improve the immunogenicity of DNA vaccines.

*Ag delivery into EVs is useful for tumor vaccines*

Finally, we examined whether the pCD63-OVA vaccine has potential as a cancer vaccine and/or as an immunotherapy. We performed in vivo CTL cytotoxicity assays to evaluate the level of functional $CD8^+$ T cell activity because CTL responses are essential for cancer vaccine efficacy. Immunizing mice with pCD63-OVA significantly enhanced the OVA-specific $CD8^+$ T cell–mediated functional cytotoxicity in them compared with that induced by immunization with pOVA alone (Fig. 5A). To examine whether pCD63-OVA is able to suppress tumor growth, we used an explanted syngeneic tumor model with OVA-expressing mouse lymphoma cells (E.G-7 cells). Before inoculation with the E.G-7 tumor cells, the mice were immunized with pCD63-OVA or the other control plasmid DNA vaccines. After tumor inoculation, the tumor growth was significantly suppressed in the pCD63-OVA–immunized mice compared with that in the mice immunized with the control plasmid DNA (Fig. 5B).

To further evaluate the potential of pCD63-OVA to act as a therapeutic vaccine, mice were immunized with DNA vaccines 10 d after tumor inoculation in the same E.G-7 tumor model. The group immunized with pCD63-OVA showed significantly less tumor growth than the group immunized with the empty vector (Fig. 5C). Although there was no statistically significant difference between pOVA and pCD63-OVA vaccination in terms of tumor suppression ($p = 0.1641$), a clear trend of better tumor growth control by the

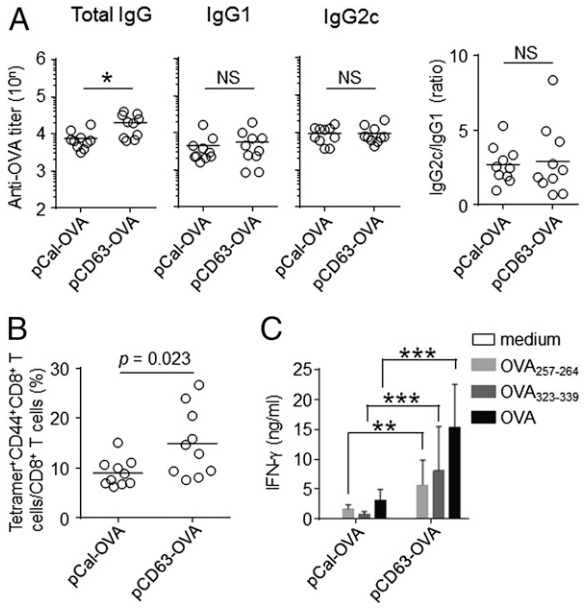


**FIGURE 3.** Ag delivery by EVs is essential for improving DNA vaccine immunogenicity. C57BL/6J mice ($n$ = 10) were immunized via imEPT with pCal-OVA or pCD63-OVA (50 μg/mouse) at days 0 and 14. Sera from the mice were subjected to ELISA to obtain the OVA-specific IgG titers, the OVA-specific serum IgG1 titers, and the OVA-specific serum IgG2c titers (**A**). The ratios of the OVA-specific serum IgG2c to OVA-specific serum IgG1were calculated by dividing the OVA-specific serum IgG2c titers by the OVA-specific IgG titers (A). The percentage of $CD8^+$ T cells recognized by the $OVA_{257–264}$–specific tetramer (**B**) and the IFN-γ levels produced from the splenocytes by OVA, $OVA_{257–264}$, $OVA_{323–339}$, or medium (**C**) were monitored on day 21 postimmunization with these plasmids. Data are representative of two or three independent experiments; error bars denote the SD. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$ (Mann–Whitney $U$ test).

pCD63-OVA–vaccinated group was apparent compared with the pOVA-vaccinated group. The number of OVA-specific $CD8^+$ T cells increased after pCD63-OVA vaccination when compared with the pOVA or pCal-OVA vaccination groups (Fig. 5D). These results indicate that Ag delivery into EVs during DNA immunization is a potential new strategy for eliciting Ag-specific $CD8^+$ T cell responses. This strategy may also be applicable to cancer vaccines.

## Discussion

Both humoral and cellular immune responses are inducible by DNA vaccination in animal models of disease. However, with humans, many individuals experience weaker than expected immunological responses to DNA vaccines. In this study, the effectiveness of the pCD63-OVA DNA vaccination implies that the immunogenicity of DNA vaccines can be improved by Ag delivery into EVs. Our results suggest that: 1) pCD63-OVA vaccination successfully delivers the encoded OVA Ag into the secreted EVs; 2) pCD63-OVA vaccination induces potent type 1 immune responses in vivo, such as Ag-specific and functional cytotoxic $CD8^+$ T cell responses; and 3) pCD63-OVA vaccination suppresses the growth of syngeneic explanted E.G-7 cell–derived tumors, both prophylactically and therapeutically.

After DNA vaccination, stroma cells at the injection site and DCs are directly transfected with the plasmid DNA construct (36). Then, the transfected cells transcribe and translate the encoded Ag, and it is subsequently presented to T cells directly by DCs or indirectly via stromal cells. This results in the induction of not only $CD4^+$ Th1 cells that are specific to the encoded Ag, but also of $CD8^+$ T cells via the indirect mechanism of cross-presentation (37). Concurrently, the plasmid DNA is sensed by intracellular DNA sensors and acts as an endogenous adjuvant for DNA vaccine-induced humoral and cellular immune responses (38, 39) to the encoded Ag(s). Although both the cellular immunological mechanisms of DNA vaccination and the immunological function of the EVs have been extensively studied recently (40, 41), the role of EVs in the DNA vaccine-induced immune response has not been fully explored. In previous studies, the role played by EVs in the induction of immune responses has been described (14–18). Although we did not directly compare our strategy with that of using the C1C2 domain for DNA vaccination, our data clearly indicate that delivery of the Ag into EVs is a useful strategy as evidenced by the

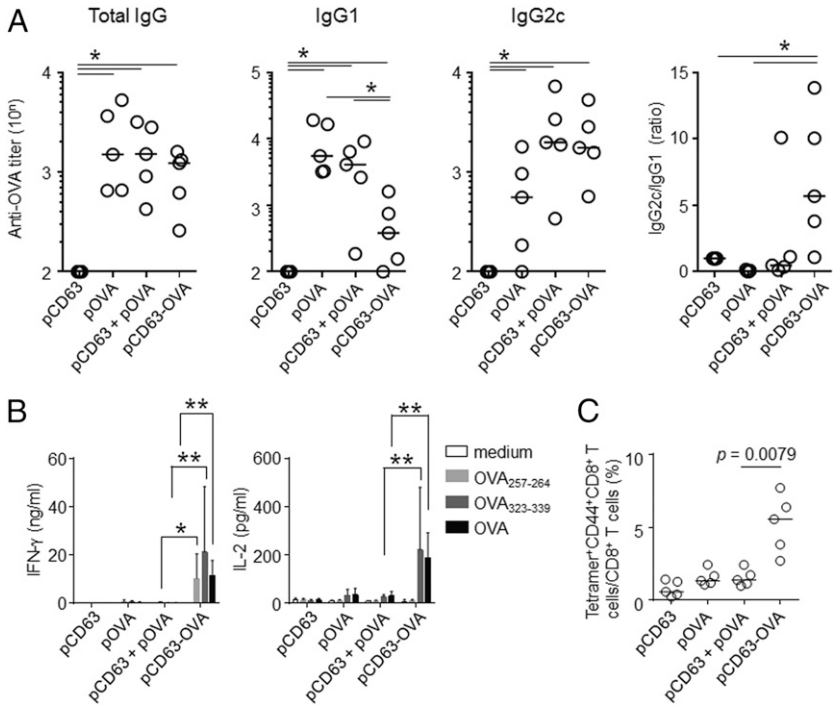


**FIGURE 4.** Assessment of the CD63-expressing plasmid as a genetic adjuvant for the coadministered DNA vaccine. C57BL/6J mice ($n$ = 5) were immunized via imEPT with pOVA, pCD63, pOVA + pCD63, or pCD63-OVA (50 μg/mouse) at days 0 and 14. Sera from the mice were subjected to ELISA to obtain the OVA-specific IgG titers, the OVA-specific serum IgG1 titers, and the OVA-specific serum IgG2c titers (**A**). The ratios of the OVA-specific serum IgG2c to OVA-specific serum IgG1 were calculated by dividing the OVA-specific serum IgG2c titers by the OVA-specific IgG titers (A). The IFN-γ or IL-2 levels produced from the splenocytes by OVA, $OVA_{257–264}$, $OVA_{323–339}$, or medium (**B**), and the percentage of $CD8^+$ T cells recognized by the $OVA_{257–264}$–specific tetramer (**C**) were monitored on day 21 postimmunization with the plasmids. Data are representative of two independent experiments; error bars denote the SD. $^*p < 0.05$, $^{**}p < 0.005$ (Mann–Whitney $U$ test).

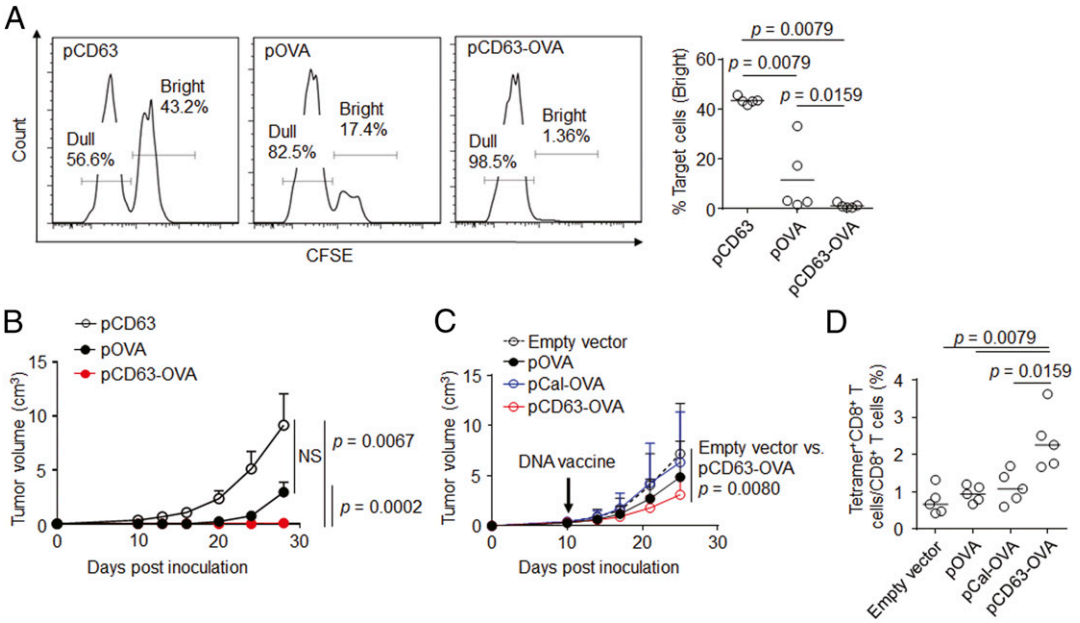


**FIGURE 5.** Effect of Ag delivery into EVs on the efficacies of the prophylactic and therapeutic tumor vaccines. (**A**) The in vivo CTL assay was performed on day 7 after the last vaccination with pOVA ($n = 5$), pCD63-OVA ($n = 5$), or pCD63 ($n = 5$). Data are representative of two independent experiments; error bars denote the SD. (**B**) C57BL/6J mice ($n = 10$) were immunized via imEPT with pOVA, pCD63, or pCD63-OVA (50 μg/mouse) at days 0 and 14. On day 7 after the last immunization, the mice were inoculated with $1 \times 10^6$ E.G-7 cells. Tumor growth was monitored over the following 28 d. Data are representative of two independent experiments; error bars denote the SEM. (**C**) On day 10 before vaccination, the mice were inoculated with $1 \times 10^6$ E.G-7 cells. Ten mice per group were immunized with 50 μg of pOVA, pCD63-OVA, pCal-OVA, or an empty vector. Tumor growth was monitored for the following 25 d. Data are representative of three independent experiments; error bars denote the SEM. (**D**) The percentages of $CD8^+$ T cells recognized by the $OVA_{257–264}$–specific tetramer were evaluated at day 25 after inoculation with E.G.-7 cells. Data are representative of two independent experiments.

EV injections and DNA vaccination experiments conducted in this study. Therefore, our data clearly demonstrate, for the first time to our knowledge, that plasmid DNA encoding an Ag protein fused to CD63, CD9, or CD81 enhances vaccine immunogenicity.

The most notable feature of the plasmid DNA encoding the CD63-OVA fusion protein Ag was its ability to induce potent $CD8^+$ T cell responses. This enhanced $CD8^+$ T cell response to the encoded Ag can be attributed to delivery of the encoded Ag via CD63 to the EVs. However, further studies are needed to confirm whether EV-mediated cross-presentation of the encoded Ag to $CD8^+$ T cells exists and whether this is essential for the DNA vaccine-induced $CD8^+$ T cell responses. We analyzed whether the effect of the CD63-OVA fusion protein on in vivo $CD8^+$ T cell priming required actual EV secretion. Using OT-I mouse splenocytes, we showed that OVA-specific IFN-γ was produced by stimulation with the purified CD63-OVA EVs derived from 293F, unlike the OVA EVs (Supplemental Fig. 3A). Also, as shown in Fig. 4, the importance of Ag delivery into the EVs was confirmed by the higher levels of Ag-specific cytokines and CTL in the mice immunized with the DNA vaccine expressing CD63 fused to OVA than those of the DNA vaccine expressing OVA alone. Therefore, we surmise that the EVs loaded with OVA Ags are actually secreted at the immunization sites with the DNA vaccine expressing CD63-OVA, thereby efficiently inducing $CD8^+$ T cell priming in vivo. It is also conceivable that CD63 and the other tetraspanin molecules we tested were not delivered into the EVs exclusively; rather, they possibly directed the encoded Ags to other cellular membranes as well. Clearly, more evidence is needed to clarify the precise mechanism of the cross-presentation required for DNA vaccines to induce $CD8^+$ T cell responses.

We measured the concentration of OVA proteins in the purified EVs by ELISA. As a result, the concentration of OVA protein in the CD63-OVA EVs or Cal-OVA EVs was lower than that of the OVA EVs (data not shown). However, as shown in Fig. 1D–F, OVA-specific immune responses in the mice increased after CD63-OVA EV immunization, suggesting that the drug delivery system of the Ag into the EVs contributes to higher immunogenicity. We consider that the secreted Cal-OVA protein itself might be contaminated in the EV fractions after kit purification, and that OVA-specific immune responses might be induced by contaminants in the Cal-OVA protein preparations. Because the EV fraction was isolated by a density gradient, it is likely that it contained vesicles with sizes similar to the EVs, such as small cellular debris, OVA protein aggregates, and possibly small amounts of Cal-OVA protein as well.

We used 293F cells to produce EVs because we have consistently been able to obtain high transfection efficacies with them and deliver protein expression in them, and these cells are also commonly used to produce EVs with which to immunize mice (42–45). However, an experiment should be conducted to confirm that there are no differences between the EVs produced by a human cell line and those produced by a murine cell line. Hence we used a murine cell line (NIH3T3) to produce CD63-OVA EVs, and we used them to examine whether OVA-specific immune responses were induced. When we used the same amount of OVA in the EVs (20 ng for each preparation) to simulate the splenocytes from OT-I mice in vitro, both CD63-OVA EVs, but not OVA EVs, derived from 293F (Supplemental Fig. 3A) and NIH3T3 (Supplemental Fig. 3B, 3C) cells induced OVA-specific IFN-γ production. Therefore, we conclude that using EVs produced by a human cell line or a murine cell line in this study did not influence OVA-specific IFN-γ production.

According to previous reports, naive OVA protein has an atypical secretion sequence and vaccination with pOVA induces anti-OVA IgG1 compared with anti-OVA IgG2a (46). Furthermore, immunization with the cell-associated forms of pOVA (not secreted OVA, where the secretion sequence is deleted) induced anti-OVA IgG2a, but not IgG1 (46). For the above reasons, we conclude that

the pCD63-OVA immunizations induced anti-OVA IgG2c, but not anti-OVA IgG1, and polarized the Th1 responses linked with more efficient CTL induction and antitumor activity.

High concentrations of EVs administered by i.v. injection are unsuitable for mice, because such injections cause rapid asphyxiation when doses exceeding 400 μg are used (47). However, for the in vivo EV injection experiments, we performed intradermal (i.d.) injections, and the mice did not show any signs of inflammation at the infection site (data not shown). Furthermore, the safety and utility of i.d. injection, as a vaccine, compared with i.v. injection has been highlighted by the World Health Organization (48). Therefore, we consider that i.d. injections containing 600 μg of EVs for mice might be acceptable for vaccine development, although further analyses would be required to exclude any safety concerns for the EV-based vaccines.

In our tumor model, we used E.G-7 tumor cells, which express OVA protein as a tumor Ag. Previous reports have suggested that the growth of tumor cells expressing OVA decreased after concurrent immunization with OVA protein and adjuvant, or with a DNA vaccine composed of OVA-expressing plasmid DNA (49, 50). Although other reports have already indicated that EVs can mediate a $CD4^+$ and $CD8^+$ T cell–dependent antitumor effect (29), our results confirm their findings and also demonstrate for the first time, as far as we are aware, that the growth of E.G-7 tumor cells can be further suppressed by DNA vaccination with pCD63-OVA, which delivers the encoded Ag into the EVs. Recently, tumor-specific mutated Ags, neoantigens, have been reported to contribute to T cell therapy in cancer immunotherapy (51–53). Neoantigens differ individually from each other, but if the nucleotide sequence of a neoantigen was inserted into plasmid DNA, neoantigen-specific immune responses would be induced. Therefore, we expect that neoantigen-specific CTL responses might be strongly induced by immunization of the CD63 plasmid DNA fused to a neoantigen.

The number of OVA-specific $tetramer^+$ $CD8^+$ T cells in the mice implanted with tumors increased significantly after DNA vaccination with pCD63-OVA (Fig. 5D), indicating that it may be possible to break the tolerant state of tumor Ag-specific $CD8^+$ T cells by vaccination with DNA encoding the tumor Ag fusion protein and EV delivery molecules, such as CD63, CD9, or CD81. It should be noted, however, that these tetraspanin EV marker proteins are also expressed in other cellular membranes. Therefore, their potential contribution to the enhanced immunogenicity of other cellular membranes cannot be excluded. This lack of EV specificity also raises safety concerns for clinical application because tetraspanin-containing fusion proteins may cause off-target effects.

DNA vaccines have been approved and used in veterinary applications (54), and, in humans, many DNA vaccines for infectious diseases or cancer therapy are currently in clinical trials (55–58). These vaccines and vaccine candidates use various strategies to improve the low immunogenicity of DNA vaccines. Our results suggest that the strategy of delivering the encoded Ag into EVs through fusion between the Ag and the EV marker, such as CD63, may offer another viable method for improving DNA vaccine immunogenicity. In addition to the OVA strategy, we also examined whether robust CTLs were induced after vaccination with CD63 fused to influenza virus nucleoprotein (NP) as another Ag. This experiment showed that the number of NP-specific CTLs, as determined by NP-specific tetramer staining using DNA-vaccinated mice splenocytes, increased after CD63-NP DNA vaccination compared with the NP DNA vaccinations (Supplemental Fig. 4). Notably, this strategy seems especially well suited at enhancing $CD8^+$ T cell responses to the encoded Ag. Our findings provide insights into ways with which to improve DNA vaccine immunogenicity and efficacy. They also further our biological and immunological understanding of how the DNA-encoded Ags in DNA vaccines are delivered and processed by the immune system to provide Ag-specific immune responses.

## Acknowledgments

We thank Dr. Cevayir Coban, Dr. Jun Kunisawa, and the other members of their and K.J.I.'s laboratories for valuable comments and assistance. We also thank Akiko Okabe and Mariko Nakamura for excellent technical assistance.

## Disclosures

The authors have no financial conflicts of interest.

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