Nonagonistic Dectin-1 ligand transforms CpG into a multitask nanoparticulate TLR9 agonist

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Edited by Rafi Ahmed, Emory University, Atlanta, GA, and approved January 16, 2014 (received for review October 12, 2013)

CpG DNA, a ligand for Toll-like receptor 9 (TLR9), has been one of the most promising immunotherapeutic agents. Although there are several types of potent humanized CpG oligodeoxynucleotide (ODN), developing "all-in-one" CpG ODNs activating both B cells and plasmacytoid dendritic cells forming a stable nanoparticle without aggregation has not been successful. In this study, we generated a novel nanoparticulate K CpG ODN (K3) wrapped by the nonagonistic Dectin-1 ligand schizophyllan (SPG), K3-SPG. In sharp contrast to K3 alone, K3-SPG stimulates human peripheral blood mononuclear cells to produce a large amount of both type I and type II IFN, targeting the same endosome where IFN-inducing D CpG ODN resides without losing its K-type activity. K3-SPG thus became a potent adjuvant for induction of both humoral and cellular immune responses, particularly CTL induction, to coadministered protein antigens without conjugation. Such potent adjuvant activity of K3-SPG is attributed to its nature of being a nanoparticle rather than targeting Dectin-1 by SPG, accumulating and activating antigen-bearing macrophages and dendritic cells in the draining lymph node. K3-SPG acting as an influenza vaccine adjuvant was demonstrated in vivo in both murine and nonhuman primate models. Taken together, K3-SPG may be useful for immunotherapeutic applications that require type I and type II IFN as well as CTL induction.

innate immunity | two-photon microscopy | MARCO | Siglec-1 | β-glucan

CpG oligodeoxynucleotide (CpG ODN) is a short (~20 bases), single-stranded synthetic DNA fragment containing the immunostimulatory CpG motif, a potent agonist for Toll-like receptor 9 (TLR9), which activates dendritic cells (DCs) and B cells to produce type I interferons (IFNs) and inflammatory cytokines (1, 2) and acts as an adjuvant toward both Th1-type humoral and cellular immune responses, including cytotoxic T-lymphocyte (CTL) responses (3, 4). Therefore, CpG ODN has been postulated as a possible immunotherapeutic agent against infectious diseases, cancer, asthma, and pollinosis (2, 5).

There are at least four types of CpG ODN, each of which has a different backbone, sequence, and immunostimulatory properties (6). D-type (also called A) CpG ODNs typically comprise one palindromic CpG motif with a phosphodiester (PO) backbone and phosphorothioate (PS) poly(G) tail, and activates plasmacytoid DCs (pDCs) to produce a large amount of IFN-α but fails to induce pDC maturation and B-cell activation (7, 8). The three other types of ODN consist of a PS backbone. K-type (also called B) CpG ODN contains nonpalindromic multiple CpG motifs, and strongly activates B cells to produce IL-6 and pDCs to maturation but barely produces IFN-α (8, 9). Recently, C and P CpG ODNs have been developed; these contain one and two palindromic CpG sequences, respectively, both of which can activate B cells like K-type and pDC like D-type, although C CpG ODN induces weaker IFN-α production compared with P CpG ODN (10–12).

D and P CpG ODNs have been shown to form higher-order structures, Hoogsteen base pairing to form parallel quadruplex structures called G tetrads, and Watson–Crick base pairing between *cis*- and *trans*-palindromic portions, respectively, that are required for robust IFN-α production by pDCs (12–14). Although such higher-order structures appear necessary for localization to early endosomes and signaling via TLR9, they suffer from product polymorphisms, aggregation, and precipitation, thereby hampering their clinical application (15). Therefore, only K and C CpG ODNs are generally available as immunotherapeutic agents and vaccine adjuvants for human use (16, 17). Although K CpG ODN enhances the immunogenicity of vaccines targeting infectious diseases and cancers in human clinical trials (6, 17), chemical or physical conjugation between antigen and K CpG ODN is necessary for optimal adjuvant effects. These results indicate that these four (K, D, P, and C) types of CpG ODN have advantages and disadvantages; however, the

Significance

CpG oligodeoxynucleotide (ODN), a Toll-like receptor 9 ligand, is a promising immunotherapeutic agent; however, developing an IFN-inducing CpG ODN forming a stable nanoparticle without aggregation has been unsuccessful. Here we generated a nanoparticulate CpG ODN (K3) wrapped by the nonagonistic Dectin-1 ligand schizophyllan (SPG), K3-SPG. K3-SPG stimulates human peripheral blood mononuclear cells to produce large amounts of both type I and II IFN. K3-SPG thus became a potent adjuvant, especially for cytotoxic T-lymphocyte (CTL) induction to coadministered protein antigens without conjugation, which is attributable to its nanoparticulate nature rather than to targeting Dectin-1. Protective potency of K3-SPG as an influenza vaccine adjuvant was demonstrated in both murine and nonhuman primate models. K3-SPG may be used as an IFN inducer as well as a CTL inducer for immunotherapeutic applications.

Author contributions: K.K., T.A., C.C., and K.J.I. designed research; K.K., T.A., H.N., M.H., and Y.K. performed research; T.A., H.N., E.K., M.H., K.T., S.M., K.S., Y.K., Y.Y., S.S., Y.I., and S.A. contributed new reagents/analytic tools; K.K., T.A., H.N., E.K., S.K., C.C., and K.J.I. analyzed data; and K.K., T.A., E.K., and K.J.I. wrote the paper.

Conflict of interest statement: K.S. holds a patent related to schizophyllan forming a complex with nucleic acids. K.K., T.A., and K.J.I. have filed a patent application related to the content of this manuscript.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1319268111/-/DCSupplemental.

development of an "all-in-one" CpG ODN activating both B cells and pDCs that forms a stable nanoparticle without aggregation has yet to be accomplished. A better strategy, targeting CpG ODN toward antigen-presenting cells (APCs), is desired to improve immunostimulatory specificity and immunotherapeutic efficacy of CpG ODNs.

Schizophyllan (SPG), a soluble β-glucan derived from *Schizophyllum commune*, is a drug that has been approved in Japan as an enhancer of radiotherapy in cervical carcinoma patients for the last three decades (18). It has been shown to form a complex with polydeoxyadenylic acid (dA) as a triple-helical structure (19). Although we previously demonstrated that mouse and humanized CpG ODN with PO poly(dA) at the 5′ end complexed with SPG enhanced cytokine production and acted as an influenza vaccine adjuvant (20, 21), it has been difficult to achieve high yields of the CpG–SPG complex toward its more efficient and cost-effective preclinical as well as clinical development. Recently, when the PS backbone of the dA sequence was linked to CpG ODN, the efficacy of complex formation was elevated by nearly 100% (22). However, a thorough investigation has yet to be conducted to identify the best humanized CpG sequence and optimization of factors to gain all-in-one activities of the four types of CpG ODN.

To do this, we sought to optimize a humanized CpG–SPG complex as a vaccine adjuvant and immunostimulatory agent in humans (in vitro), mice (in vitro and in vivo), and nonhuman primates (in vivo). In this study, we identified a novel K CpG ODN (K3) and SPG complex, namely K3-SPG. It forms a higher-order nanoparticle that can be completely solubilized. We found that this all-in-one K3-SPG displayed a more potent activity than, and different characteristics from, any other type of CpG ODN and previous CpG–SPG complexes.

Results

A Rod-Shaped Nano-Sized Particle of K3-SPG Gains Dual Characteristics of K- and D-Type CpG ODNs. To make a complex between CpG ODNs and schizophyllan (SPG), CpG ODNs need additional sequences of phosphorothioate backbone of 40-mer polydeoxyadenylic acid (dA_{40}) at the 5′ or 3′ end (20, 22). Fig. 1*A* shows methods of CpG ODN and SPG complexation through denaturing–renaturing procedures. In this study, we selected K3 as a K-type CpG ODN. At first, we examined the immunostimulatory impacts of the 5′ and 3′ ends of CpG ODN. 5′-K3-dA_{40}-3′, but not 5′-dA_{40}-K3-3′, complexed with SPG-activated human peripheral blood mononuclear cells (PBMCs) to produce a robust amount of IFN-α (Fig. 1*B* and Fig. S1). K3, K3-dA_{40}, or dA_{40}-K3, which are able to activate human PBMCs to produce other cytokines such as IL-6, failed to produce IFN-α (Fig. 1*B* and Fig. S1). These results indicate that the 5′-CpG sequence (K3-SPG) is more desirable than the 3′-CpG sequence as a novel TLR9 agonist. Although some CpG ODN-induced cytokine production is known to have a dose-dependent correlation, K3-SPG–induced IFN-α production is not. Given that previous reports showed that IFN-α pro-duction by K CpG ODN stimulation has a bell-shaped dose–response correlation (7), altogether these results suggest that K3-SPG still has the character of K CpG ODN.

Qualification and quantitation of K3-SPG were conducted by scanning electron microscopy (SEM) and dynamic light scattering (DLS). K3-SPG had a rod-like structure, consistent with that seen in a previous report (23) (Fig. 1*C*). It appeared to be a soluble monomeric nanoparticle with an average diameter of 30 nm, comparable to SPG itself and smaller than D CpG ODN (D35) (14, 24) (Fig. 1*D*). Given that K3-SPG forms a nanoparticle, we compared the immunostimulatory activities of K3-SPG with D, C, and P CpG ODNs. PBMCs stimulated with K3-SPG produced larger amounts of IFN-α and IFN-γ but at far lower concentrations than those induced by D35 (Fig. 1*E*) and P and C CpG ODNs (Fig. S2). These results suggest that K3-SPG gains the characteristic of D CpG ODN without losing that of the K type, because these IFNs are known to be D type-specific cytokines (7, 8, 25). To understand the dual functions of K and D

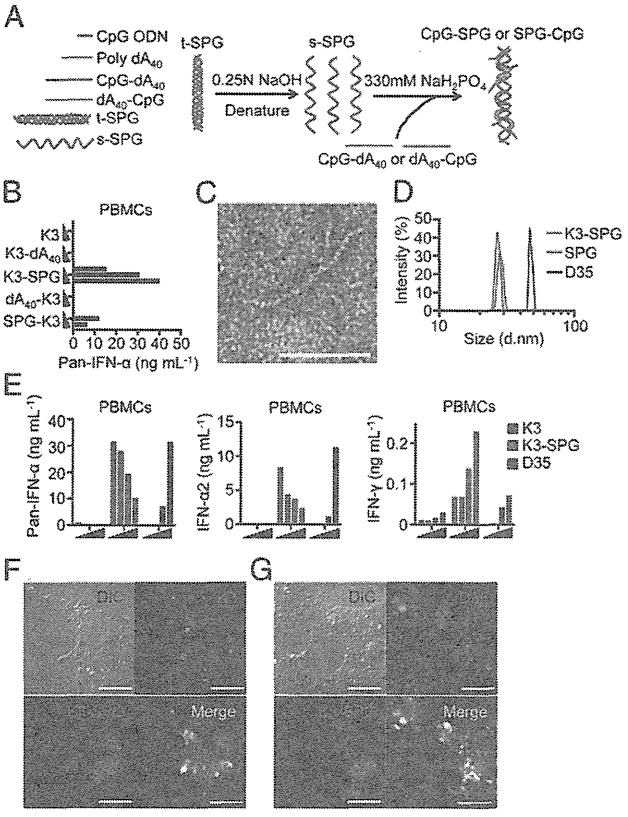

Fig. 1. K (B) CpG ODN and SPG complex forms nanoparticles and gains D (A) CpG ODN characteristics. (*A*) Methods of CpG ODN and SPG complexation. tSPG, triple-stranded SPG; sSPG, single-stranded SPG. (*B*) Production of IFN-α by human PBMCs stimulated with K3, K3-dA_{40}, K3-SPG, dA_{40}-K3, or SPG-K3 (adjusted for K3 ODN concentration at 0.1, 0.3, or 1 μM) for 24 h was measured by ELISA. (*C*) K3-SPG processed for SEM. (Scale bar, 50 μm.) (*D*) Size of K3-SPG, SPG, and D35 was analyzed by DLS. (*E*) Production of type I and II IFNs by PBMCs stimulated with K3, K3-SPG, or D35 for 24 h was measured by ELISA. (*F* and *G*) Mouse BMDMs were stimulated with Alexa 488-K3 (*F*) or Alexa 488-D35 (*G*) and Alexa 647-K3-SPG at 1 μM for 3 h. The cells were incubated with Hoechst 33258, fixed, and analyzed by fluorescence microscopy. DIC, differential interference contrast. (Scale bars, 10 μm.) Data represent one of three independent experiments with similar results.

CpG ODNs, we analyzed the intracellular localization of K3-SPG in bone marrow-derived macrophages (BMDMs). K3-SPG was colocalized with not only the endosomes containing K CpG ODN but also those containing D CpG ODN (Fig. 1 *F* and *G*) such as C CpG ODN (26), suggesting that K3-SPG may transduce endosome-mediated innate immune signaling pathways by K and D CpG ODNs. These results strongly suggest that K3-SPG forms a nano-sized higher-order and completely solubilized particle and found that this all-in-one K3-SPG displayed a more potent activity than, and different characteristic from, any other CpG ODNs and previously known CpG–SPG complex.

K3-SPG Is a Prominent Vaccine Adjuvant That Induces Potent CTL Responses to Protein Antigen Without Conjugation. We compared the adjuvant effects of K3, K3-dA_{40}, and K3-SPG in a murine immunization model. When wild-type mice were immunized with LPS-free chicken ovalbumin protein (OVA) alone or OVA with each K3-derived adjuvant, K3-SPG induced significantly higher humoral immune responses (Fig. 2*A*) and stronger T-cell responses than that induced by K3 (Fig. 2*B*). Of note, tetramer assays revealed a significantly greater number of OVA-specific CD8 T cells (Fig. 2*C*). We also observed very strong in vivo CTL activity against

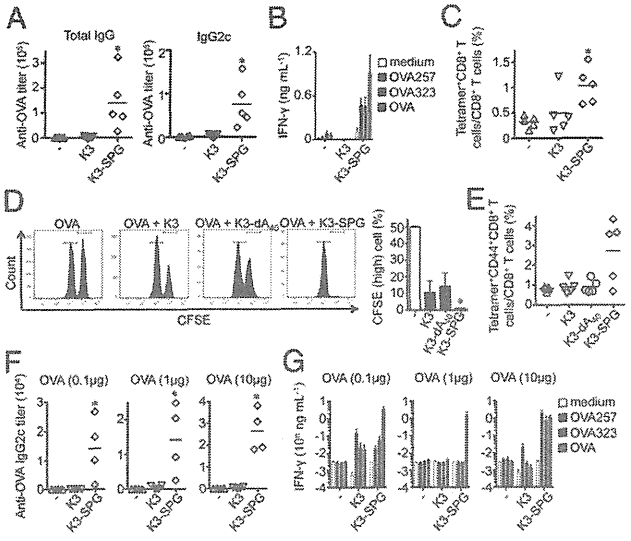

Fig. 2. K3-SPG acts as a potent vaccine adjuvant by simple mixture with antigen. Adjuvant activities of K3-SPG were analyzed. C57BL/6J mice (n = 4 or 5) were immunized s.c. with OVA protein antigen and various adjuvants. OVA-specific serum IgG (*A*), IFN-γ (*B*), and $OVA_{257-264}$-specific tetramer (*C*) were monitored (d17) after immunization (d0 and d10) with OVA (100 μg) with or without K3 (10 μg) or K3-SPG (10 μg). (*D*) In vivo CTL assay 7 d after priming with OVA and various adjuvants as indicated. (*E*) Immunization with $OVA_{257-264}$ peptide (10 μg) with or without adjuvant as indicated. (*F* and *G*) Dose-sparing study; OVA-specific serum IgG and IFN-γ were monitored after immunization as in *A* and *B*. $^*P < 0.05$ (Mann–Whitney *U* test). Data represent one of two or three independent experiments with similar results.

coadministered protein antigens lacking any covalent conjugation (Fig. 2*D*). This strong CTL induction by K3-SPG was reproduced by peptide vaccination (Fig. 2*E*) and was dose-dependent (Fig. S3). The antigen-sparing ability of K3-SPG was so potent that comparable antibody and CD4 T-cell responses were achieved using one-hundredth the amount of OVA antigen (Fig. 2 *F* and *G*). These results clearly indicate that K3-SPG is a more prominent adjuvant than K3 alone.

SPG Is a Soluble Dectin-1 Ligand but Is Not a Dectin-1 Agonist. We examined the role of Dectin-1 in cellular uptake of, and following activation by, SPG and K3-SPG, as Dectin-1 has been shown to be a receptor for β-glucans such as Zymosan (27). Using flow cytometry, we found that HEK293 cells expressing Dectin-1 but not Dectin-2 or a control (vector) increased the uptake of SPG or K3-SPG in vitro regardless of ODN presence (Fig. 3 *A* and *B*). It has recently been reported that the soluble form of β-glucan does not activate Dectin-1 signaling (28). Additionally, Dectin-1 signaling inhibits TLR9-mediated cytokine production through suppressor of cytokine signaling 1 induction (29). Therefore, we examined the agonistic activity of SPG. When splenocytes were stimulated with Zymosan-Depleted but not SPG, dose- and Dectin-1–dependent TNF-α and other cytokine production was observed, whereas cytokine production by Zymosan and Curdlan was Dectin-1–independent (Fig. 3*C* and Fig. S4). Zymosan-Depleted inhibited CpG ODN-induced IFN-α, with this inhibition relieved by Dectin-1 deficiency (Fig. 3*D*). In contrast, SPG did not inhibit CpG ODN-induced IFN-α production (Fig. 3*E*). These results indicate that SPG is a ligand but not an agonist of Dectin-1; therefore, SPG does not interfere with TLR9-mediated IFN-α production.

Adjuvant Effects of K3-SPG Are Dependent on TLR9 and Partially Dependent on Dectin-1. Because K3-SPG is a complex of CpG ODN and β-glucan, we examined the role of TLR9 (1) and Dectin-1 (30) using receptor knockout mice. When splenocytes and Flt3 ligand-induced bone marrow-derived DCs (FL-DCs) from *Tlr9*- and *Dectin-1*–deficient mice were stimulated with K3-SPG, cytokine production was completely dependent on TLR9 but not Dectin-1, excluding IL-12 p40 production (Fig. 4 *A–D*). K3-SPG–induced IL-12 p40 production showed two peaks, where the first peak of its production, but not the second peak at a higher dose, was dependent on Dectin-1 (Fig. 4*D*). This result may imply that Dectin-1 expression is involved in IL-12 p40 production at a lower dose of K3-SPG in vitro. Consistent with in vitro results, immunization of *Tlr9*-deficient mice with K3-SPG plus OVA resulted in diminished humoral and T-cell responses (Fig. 4 *E–G*). *Dectin-1*–deficient mice showed comparable immune responses with wild-type mice when the mice were immunized with OVA plus 10 μg of K3-SPG (Fig. S5). When *Dectin-1*–deficient mice were immunized with OVA plus 1 μg of K3-SPG, mice exhibited a reduced CD8 T-cell response according to the tetramer assays (Fig. 4*J*), with no significant changes in antibody and cytokine production from T cells (Fig. 4 *H* and *I*). These results suggest that the adjuvant effect of K3-SPG is dependent on TLR9 signaling. Although SPG and K3-SPG do not stimulate Dectin-1 signaling, the effect of K3-SPG is still partially dependent on Dectin-1 in vivo.

MARCO⁺, but Not Siglec-1⁺, Macrophages in Draining Lymph Nodes Dominantly Capture K3-SPG with Antigen. Given that K3-SPG provides potent adjuvant effects in vivo through immunization with a simple antigen mixture, we hypothesized that cells that capture both antigen and K3-SPG should play a critical role in mediating adjuvant effects. To examine in vivo distribution of fluorescence-labeled OVA and K3-SPG, we used fluorescence microscopy and two-photon microscopy. After an injection at the

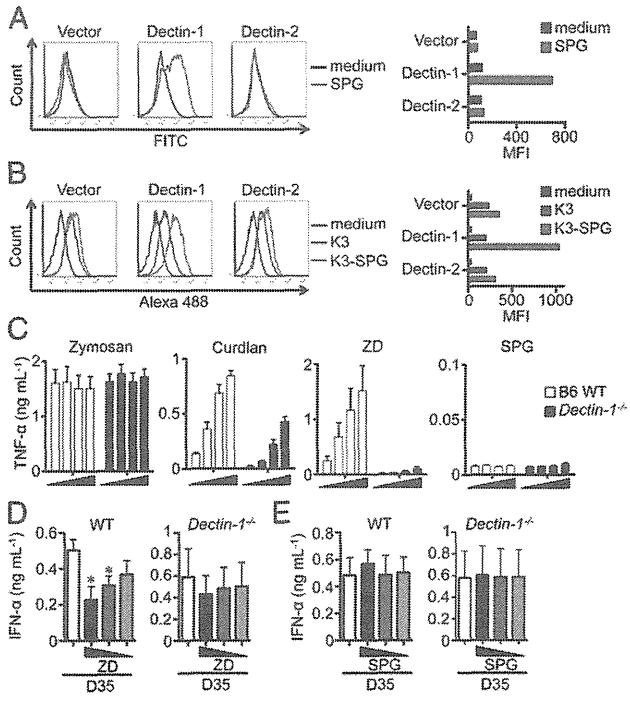

Fig. 3. SPG is a nonagonistic Dectin-1 ligand, but does not interfere with TLR9-mediated IFN-α production. (*A* and *B*) HEK293 cells transiently expressing Dectin-1 or Dectin-2 were treated with SPG-FITC (*A*), Alexa 488-K3, or Alexa 488-K3-SPG (*B*) for 60 min, and then their cellular uptake was monitored by flow cytometry [*Left*, histogram; *Right*, mean fluorescent intensity (MFI)]. Splenocytes from C57BL/6J and *Dectin-1*$^{-/-}$ mice (n = 3) were stimulated with Zymosan, Curdlan, Zymosan-Depleted (ZD), or SPG (3.7–100 μg/mL) (*C*), with D35 (1 μM), or with or without ZD (11.1–100 μg/mL) (*D*) or SPG (*E*) for 24 h and supernatant cytokines were monitored by ELISA. $^*P < 0.05$ (*t* test). Data represent one of three independent experiments with similar results.

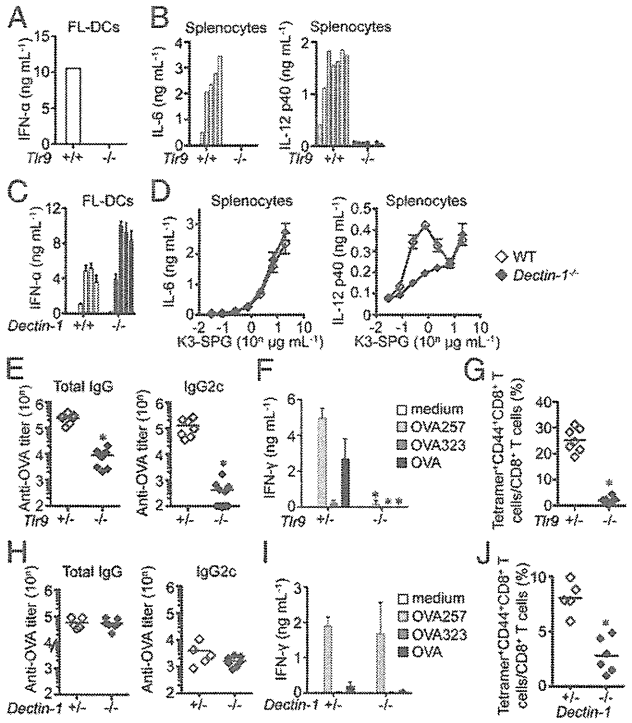

Fig. 4. Adjuvant effects of K3-SPG were completely dependent on TLR9 and partially on Dectin-1. FL-DCs (*A* and *C*) or splenocytes (*B* and *D*) from C57BL/6J, *Tlr9*$^{-/-}$, or *Dectin-1*$^{-/-}$ mice were stimulated with K3-SPG [20 μg/mL (*A*), 0.014–10 μg/mL (*B*), or 0.014–10 μg/mL (*C* and *D*)] for 24 h, and their cytokine production was monitored by ELISA. *Tlr9*$^{+/-}$ (n = 7) or *Tlr9*$^{-/-}$ mice (n = 10) (*E–G*) and *Dectin-1*$^{+/-}$ (n = 5) or *Dectin-1*$^{-/-}$ mice (n = 6) (*H–J*) were immunized s.c. with OVA (100 μg) and K3-SPG [10 μg (*E–G*) or 1 μg (*H–J*)] at days 0 and 10. Seven days after the last immunization, OVA-specific serum IgG (*E* and *H*), IFN-γ (*F* and *I*), and $OVA_{257-264}$-specific tetramer (*G* and *J*) were monitored. *P < 0.05 (Mann–Whitney *U* test). Data represent one of two or three independent experiments with similar results.

base of the tail, both antigen and adjuvant reached the surface of draining inguinal lymph nodes (iLNs) within 1 h (Fig. 5 *A*, *B*, and *D*). After 24 h, some K3-SPG had moved to the CD3e$^+$ T-cell area and colocalized with DQ-OVA (Fig. S6*A*). Those cells that contained both K3-SPG and DQ-OVA in the T-cell area of the iLNs were CD11c$^+$ DCs (Fig. S6*B*).

Of interest, the majority of fluorescence signals remained on the surface of the iLNs (Fig. 5*A*), prompting us to focus on two types of macrophages known to be distributed on the LN surface, Siglec-1$^+$ (also called CD169 or MOMA-1) macrophages (also known as subcapsular sinus macrophages) and MARCO$^+$ macrophages (31). Histological analysis using conventional fluorescence microscopy did not suitably reveal the entire iLN surface; moreover, these macrophages were difficult to isolate for flow cytometric analysis (32, 33). Hence, we used two-photon microscopy imaging analysis to clarify the distribution of antigen and K3-SPG ex vivo. After the injection of anti-MARCO and –Siglec-1 antibodies, specific macrophages were visualized (Movie S1). When the iLN surface was monitored by two-photon microscopy at 1 h postinjection, OVA and K3-SPG were colocalized with MARCO$^+$ but not Siglec-1$^+$ macrophages (Fig. 5 *B* and *D*, Fig. S7 *A–D*, and Movies 2–5). Previous reports suggest that the immune complex and inactivated influenza virus are captured by Siglec-1$^+$ macrophages to induce humoral immune responses (34, 35). The distribution pattern perfectly matched that for MARCO$^+$ macrophages in the iLNs and did not colocalize with Siglec-1$^+$ macrophages, as confirmed by Volocity's colocalization analysis (Perkin Elmer) (Fig. 5 *B–E*). In contrast, K3 was more diffusely distributed between MARCO$^+$ and Siglec-1$^+$ areas compared with K3-SPG (Fig. 5 *D* and *E*, Fig. S7 *C–E*, and Movies 6 and 7). Additionally, both *Tlr9*- and *Dectin-1*–deficient mice showed comparable localization of K3-SPG (Fig. S7 *F* and *G*).

To determine the contribution of these macrophages toward the adjuvant effects of K3-SPG, we examined different recovery kinetics of macrophages and DCs following an injection of clodronate liposomes into the base of the tail. After the injection, the macrophages were completely depleted by day 2. These cells did not recover for at least 1 wk, whereas DCs were mostly recovered by day 7, as previously reported (36). When both macrophages and DCs were depleted, immune responses were significantly suppressed [Fig. 5*F*, Clo (−d2)]. When only macrophages, but not DCs, were depleted, the immune responses were comparable to those in untreated mice [Fig. 5*F*, Clo (−d7)]. This would suggest that although both OVA and K3-SPG were mainly captured by

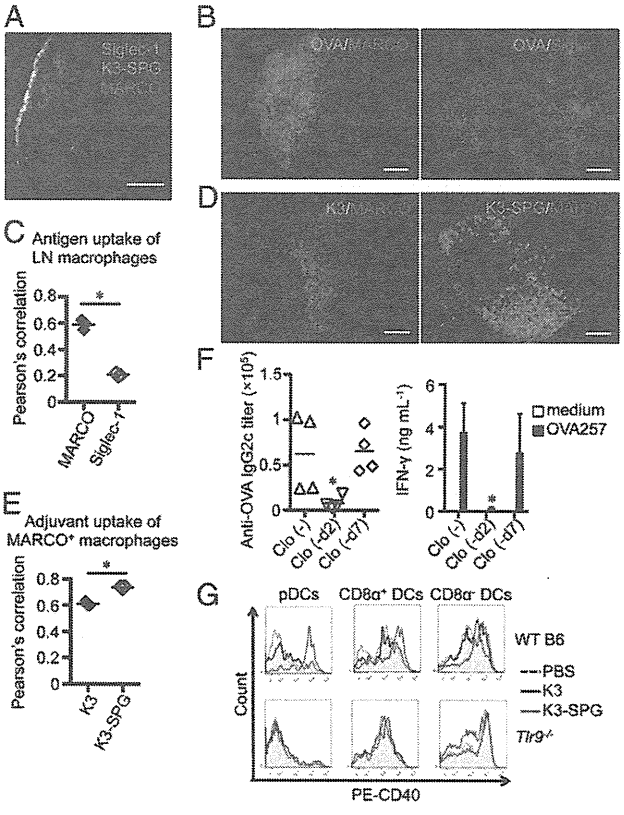

Fig. 5. Role of lymph node macrophages and dendritic cells in uptake and adjuvant effects of K3-SPG. (*A*) Immunohistochemistry of mouse inguinal LNs after Alexa 488-K3-SPG injection. One hour after injection, the LNs were collected and stained with anti–MARCO-phycoerythrin (PE) and anti–Siglec-1-APC antibodies. (*B–E*) Two-photon microscopic analysis of LNs. DQ-OVA, Alexa 488-K3, or Alexa 488-K3-SPG was injected as indicated, and anti–MARCO-PE or anti–Siglec-1-PE antibodies were administered. The LNs were collected 1 h later and analyzed by two-photon microscopy. (*C* and *E*) Colocalization of antigen or adjuvant with the stained macrophages was analyzed by Pearson's correlation. (*F*) Clodronate liposomes were injected into C57BL/6J mice either 2 or 7 d before immunization (n = 4). Mice were administered OVA (100 μg) plus K3-SPG (10 μg) at day 0. Eight days after immunization, OVA-specific serum IgG and IFN-γ were monitored. (*G*) C57BL/6J and *Tlr9*$^{-/-}$ mice were administered s.c. with K3 (10 μg) or K3-SPG (10 μg). At 24 h postadministration, the LNs were collected and the prepared cells were stained and analyzed by flow cytometry. (Scale bars, 100 μm.) *P < 0.05 (*t* test or Mann–Whitney *U* test). Data represent one of two or three independent experiments with similar results.

MARCO$^+$ macrophages in the LNs after injection, the macrophages were dispensable to inducing adaptive immune responses. In other words, the adjuvant effect of K3-SPG was largely dependent on the DC population.

K3-SPG Targets and Strongly Activates the Antigen-Bearing DC Population in Vivo. Our findings suggest that although a large portion of nanoparticulate K3-SPG was taken up by MARCO$^+$ macrophages in iLNs after injection, the adjuvant effects appear to be controlled by DCs. We focused on antigen and adjuvant uptake by the DC population in iLNs. At 24 h postinjection, the uptake of antigen and adjuvants by the DC population was analyzed by flow cytometry. The frequency of CpG-positives in three DC subsets (pDCs, CD8α$^+$ DCs, and CD8α$^-$ DCs) was significantly increased after K3-SPG injection than with K3 (Fig. S8*A*). In contrast, the frequency of OVA-positive DCs was comparable after K3 and K3-SPG injections (Fig. S8*B*). When we focused on both antigen- and adjuvant-positive DCs, there was a substantial increase for K3-SPG over K3 (Fig. S9). Both pDCs and CD8α$^+$ DCs in iLNs were strongly activated by K3-SPG but not by K3 24 h postinjection, and this was completely dependent on TLR9 (Fig. 5*G*). Our results indicate that pDCs and CD8α$^+$ DCs preferentially capture nanoparticulate K3-SPG rather than nonparticulate K3 for maturation and to exert adjuvant effects.

K3-SPG Is a Potent Adjuvant for Influenza Vaccine in Murine and Nonhuman Primate Models. Finally, we sought the adjuvant effect of K3-SPG by using more clinically relevant influenza vaccination models in both mice and nonhuman primates. When mice were immunized with ether-treated hemagglutinin antigen-enriched virion-free split vaccine (SV) plus the indicated adjuvant, K3-SPG demonstrated superior adjuvant effects to K3 when antibody responses (Fig. S10*A*) and T-cell responses (Fig. S10*B*) were compared. More importantly, SV plus K3-SPG immunization resulted in a 100-fold greater antibody response, even compared with vaccination using a whole (virion) inactivated vaccine (WIV) (0.2 μg per mouse) (Fig. 6*A*), which contains viral RNA as a built-in adjuvant (21). Interestingly, SV (0.1 μg per mouse) plus K3-SPG strongly induced both CD8 and CD4 T-cell responses (Fig. 6*B*). Mice immunized with SV and K3-SPG exhibited less body weight loss than WIV-immunized mice (Fig. 6*C*). Strikingly, K3-SPG conferred 100% protection against lethal PR8 virus challenge at the dose of which only 10% of WIV-vaccinated mice survived (Fig. 6*D*). These results strongly support the notion that K3-SPG works as a potent adjuvant for protein or protein-based vaccines in a murine model, prompting us to extend this finding to a nonhuman primate model using the cynomolgus monkey (*Macaca fascicularis*). Each group of three cynomolgus monkeys was immunized with SV plus K3 or K3-SPG at days 0 and 14. Serum antibody titers were then monitored for 8 wk. The SV plus K3-SPG induced significantly higher antibody titer at 2 wk postimmunization, and titer levels remained high for at least another 6 wk (Fig. 6*E*). Although antibody titers were reduced at 110 wk after immunization, the K3-SPG group had higher antibody titers than the K3 group (Fig. 6*E*). When PBMCs were stimulated with SV and WIV, IFN-γ was detected from the SV plus K3-SPG–immunized group (Fig. 6*F*). Taken together, these results suggest that K3-SPG is a prominent vaccine adjuvant in a nonhuman primate model.

Discussion

The medical need for novel, potent, and safe adjuvants is ever-increasing these days as (*i*) recombinant vaccine antigens such proteins and peptides are short on natural adjuvants, unlike attenuated or inactivated whole microbial antigens, (*ii*) conventional aluminum salts and oil adjuvants are limited or preferred for enhancing humoral immune responses, and (*iii*) new adjuvants that can induce cellular immune responses, including CTLs, are needed, for example for cancer vaccines. The last two decades have resulted in tremendous progress with respect to adjuvant research and development. A hallmark of the new generation of adjuvants is that nucleic acids have been rediscovered to be immunologically active in stimulating specific innate immune receptors of the host, in particular TLRs. CpG DNA, a ligand for TLR9, is one of the most promising immunotherapeutic agents that has been identified.

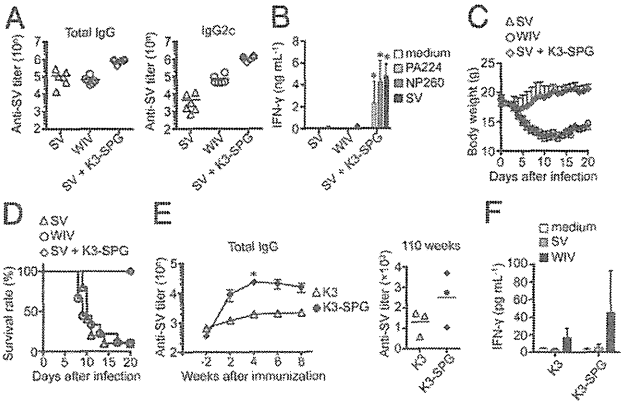

Fig. 6. K3-SPG acts as an influenza vaccine adjuvant in mice and nonhuman primates. (*A–D*) C57BL/6J mice (n = 6 or 10) were immunized with SV (0.1 μg), whole inactivated vaccine (WIV) (0.2 μg), or SV (0.1 μg) plus K3-SPG (10 μg) at days 0 and 14. Seven days after the final immunization, SV-specific serum IgG titers (*A*) and IFN-γ (*B*) [specific to SV antigen, $PA_{224-233}$ (PA224) (10 μg/mL) or $NP_{260-283}$ (NP260)] were monitored. (*C* and *D*) Fourteen days after the final immunization, mice were challenged with a 10-LD_{50} dose of influenza virus A/PR/8 (H1N1). Changes in body weights (*C*) and mortality (*D*) were monitored for the next 20 d. (*E* and *F*) Cynomolgus monkeys (n = 3) were immunized with SV (5 μg) plus K3 (5 nmol) or SV plus K3-SPG (5 nmol) at days 0 and 14. (*E*) Serum samples were collected at −2, 2, 4, 6, 8, and 110 wk. Antigen-specific serum antibody titers were measured by ELISA. (*F*) PBMCs were prepared from individual cynomolgus monkey blood at 4 wk after the first immunization and restimulated in vitro with medium, SV (10 μg), or WIV for 24 h. Mouse IFN-γ in the supernatants was determined by ELISA. *P < 0.05 (t test or Mann–Whitney U test).

Although there are several types of potent humanized CpG ODN—K (also called B), D (A), C, and P types—the development of an all-in-one CpG ODN activating both B cells and pDCs to form a stable nanoparticle without aggregation has been less than successful. In this study, we generated a novel K CpG ODN that we designated K3-SPG. Although it had been reported that there are molecular interactions between single-stranded nucleic acids and β-glucan (37) and that murine and humanized CpG ODNs can be wrapped by SPG to increase their original TLR9-agonistic activities (20), our report demonstrates that a rod-shaped nano-sized K3-SPG particle exhibits dual characteristics of K and D CpG ODNs (Fig. 1). K3-SPG is distinct from other previously reported K CpG ODNs, including K3. In turn, K3-SPG becomes a D CpG ODN, stimulating human PBMCs to produce large amounts of both type I and type II IFN, targeting the same endosome where the IFN-inducing D type resides without losing its K-type activity (Fig. 1 *F* and *G*). Another surprising finding is that this K3-SPG forms a rod-like single nanomolecule (Fig. 1 *C* and *D*). This is advantageous over previously demonstrated D or P types, whose ends form higher-order structures that may hamper further development as prodrugs, including good manufacturing practice assignment.

Another prominent feature of this K3-SPG is its potency as an adjuvant for induction of both humoral and cellular immune responses, especially CTL induction, to coadministered protein antigens without conjugation. Such potent adjuvant activity of K3-SPG is attributable to its nanoparticulate nature (Figs. 1 *C* and *D* and 2) rather than targeting Dectin-1 by SPG (Figs. 3 and 4). Initially, we hypothesized that K3-SPG becomes such a potent adjuvant because it targets Dectin-1, because SPG is a β-1,3-glucan, and seems to be a clear Dectin-1 ligand (Fig. 3*A*). Our other results, however, led us to conclude that the role of Dectin-1

in vivo with respect to the adjuvant activity of K3-SPG was minimal (Fig. 4). More importantly, the in vivo activity of K3-SPG was completely dependent upon TLR9 (Fig. 4 *E–G*). SPG is a soluble Dectin-1 ligand but not a Dectin-1 agonist, and thus does not interfere with TLR9-mediated DC activation (Fig. 3 *D* and *E*). The adjuvant activity of K3-SPG is mostly independent of Dectin-1, except at very low doses during the immunization protocol (Fig. 4*J*). Instead, some other receptors such as C-type lectins, Siglecs, and scavenger receptors may play roles in delivering SPG into macrophages and/or DCs, accumulating and activating antigen-bearing macrophages and DCs in draining lymph nodes (Fig. 5). In this regard, we also found that $MARCO^+$, but not Siglec-1^+, macrophages in draining lymph nodes are dominant in capturing K3-SPG, and coadministered antigen (LPS-free OVA protein), and that K3-SPG targets the antigen-bearing DC population in vivo. Although the depletion of macrophages did not ameliorate adjuvant effects, large amounts of antigen and K3-SPG are taken up by the same $MARCO^+$ macrophages, and the two-photon microscopic data suggest that they are activated as they become much bigger than nonstimulated macrophages. Whether this massive accumulation of antigen and adjuvant in $MARCO^+$ macrophages contributes to the following DC activation and adaptive T- and B-cell activation is yet to be elucidated in future work.

The protective potency of K3-SPG as an influenza vaccine adjuvant was demonstrated in vivo in both murine and nonhuman primate models. In the murine model, intradermal immunization with a very low dose of seasonal influenza split vaccine mixed with K3-SPG in solution provoked robust IgG responses and offered better protection than a low but physiological dose of whole inactivated virion vaccination against the heterologous challenge of lethal virus (Fig. 6 *C* and *D*). These data provide better protective potency than our previous results, where we used approximately 10 times higher doses of influenza antigens (21), because many factors for K3-SPG have been improved for its potency: K3–SPG complexation efficiency and optimization of the order between K3 and poly(dA_{40}) (Fig. 1); the immunization route is different as well. The data above prompted us to develop K3-SPG as a potent adjuvant for influenza split vaccine, especially for those urgently needing improvement: seasonal influenza vaccination for the elderly, immunodeficient patients (transplant recipients), and pandemic influenza vaccination.

Taken together, these data suggest that K3-SPG can be used as a potent adjuvant for protein vaccines such as influenza split vaccines, and may be useful for immunotherapeutic applications that require type I and type II IFN as well as CTL induction.

Materials and Methods

All animal studies using mice and monkeys were conducted in accordance with the Institutional Animal Care and Use Committee at the National Institute of Biomedical Innovation. All of the ODNs used in this manuscript were synthesized by GeneDesign. Other details are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. This study was supported by a Health and Labour Sciences Research Grant and the Japan Science and Technology Agency Core Research for Evolutionary Science and Technology Program.

1. Hemmi H, et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* 408(6813):740–745.
2. Krieg AM (2006) Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov* 5(6):471–484.
3. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* 186(10):1623–1631.
4. Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL (1998) CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc Natl Acad Sci USA* 95(26):15553–15558.
5. Klinman DM (2004) Immunotherapeutic uses of CpG oligodeoxynucleotides. *Nat Rev Immunol* 4(4):249–258.
6. Vollmer J, Krieg AM (2009) Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv Drug Deliv Rev* 61(3):195–204.
7. Krug A, et al. (2001) Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. *Eur J Immunol* 31(7): 2154–2163.
8. Verthelyi D, Ishii KJ, Gursel M, Takeshita F, Klinman DM (2001) Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. *J Immunol* 166(4):2372–2377.
9. Hartmann G, Krieg AM (2000) Mechanism and function of a newly identified CpG DNA motif in human primary B cells. *J Immunol* 164(2):944–953.
10. Hartmann G, et al. (2003) Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. *Eur J Immunol* 33(6):1633–1641.
11. Marshall JD, et al. (2003) Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J Leukoc Biol* 73(6):781–792.
12. Samulowitz U, et al. (2010) A novel class of immune-stimulatory CpG oligodeoxynucleotides unifies high potency in type I interferon induction with preferred structural properties. *Oligonucleotides* 20(2):93–101.
13. Kerkmann M, et al. (2005) Spontaneous formation of nucleic acid-based nanoparticles is responsible for high interferon-alpha induction by CpG-A in plasmacytoid dendritic cells. *J Biol Chem* 280(9):8086–8093.
14. Klein DC, Latz E, Espevik T, Stokke BT (2010) Higher order structure of short immunostimulatory oligonucleotides studied by atomic force microscopy. *Ultramicroscopy* 110(6):689–693.
15. Puig M, et al. (2006) Use of thermolytic protective groups to prevent G-tetrad formation in CpG ODN type D: Structural studies and immunomodulatory activity in primates. *Nucleic Acids Res* 34(22):6488–6495.
16. McHutchison JG, et al. (2007) Phase 1B, randomized, double-blind, dose-escalation trial of CPG 10101 in patients with chronic hepatitis C virus. *Hepatology* 46(5): 1341–1349.
17. Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM (2011) CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines* 10(4):499–511.
18. Okamura K, et al. (1986) Clinical evaluation of schizophyllan combined with irradiation in patients with cervical cancer. A randomized controlled study. *Cancer* 58(4): 865–872.
19. Sakurai K, Mizu M, Shinkai S (2001) Polysaccharide—polynucleotide complexes. 2. Complementary polynucleotide mimic behavior of the natural polysaccharide schizophyllan in the macromolecular complex with single-stranded RNA and DNA. *Biomacromolecules* 2(3):641–650.
20. Shimada N, et al. (2007) A polysaccharide carrier to effectively deliver native phosphodiester CpG DNA to antigen-presenting cells. *Bioconjug Chem* 18(4):1280–1286.
21. Koyama S, et al. (2010) Plasmacytoid dendritic cells delineate immunogenicity of influenza vaccine subtypes. *Sci Transl Med* 2(25):25ra24.
22. Minari J, et al. (2011) Enhanced cytokine secretion from primary macrophages due to Dectin-1 mediated uptake of CpG DNA/β-1,3-glucan complex. *Bioconjug Chem* 22(1):9–15.
23. Bae AH, et al. (2004) Rod-like architecture and helicity of the poly(C)/schizophyllan complex observed by AFM and SEM. *Carbohydr Res* 339(2):251–258.
24. Costa LT, et al. (2004) Structural studies of oligonucleotides containing G-quadruplex motifs using AFM. *Biochem Biophys Res Commun* 313(4):1065–1072.
25. Gürsel M, Verthelyi D, Gürsel I, Ishii KJ, Klinman DM (2002) Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotide. *J Leukoc Biol* 71(5):813–820.
26. Guiducci C, et al. (2006) Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation. *J Exp Med* 203(8):1999–2008.
27. Herre J, et al. (2004) Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* 104(13):4038–4045.
28. Goodridge HS, et al. (2011) Activation of the innate immune receptor Dectin-1 upon formation of a 'phagocytic synapse.' *Nature* 472(7344):471–475.
29. Eberle ME, Dalpke AH (2012) Dectin-1 stimulation induces suppressor of cytokine signaling 1, thereby modulating TLR signaling and T cell responses. *J Immunol* 188(11):5644–5654.
30. Saijo S, et al. (2007) Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. *Nat Immunol* 8(1):39–46.
31. Martinez-Pomares L, Gordon S (2012) $CD169^+$ macrophages at the crossroads of antigen presentation. *Trends Immunol* 33(2):66–70.
32. Aoshi T, et al. (2009) The cellular niche of *Listeria monocytogenes* infection changes rapidly in the spleen. *Eur J Immunol* 39(2):417–425.
33. Gray EE, Cyster JG (2012) Lymph node macrophages. *J Innate Immun* 4(5-6):424–436.
34. Suzuki K, Grigorova I, Phan TG, Kelly LM, Cyster JG (2009) Visualizing B cell capture of cognate antigen from follicular dendritic cells. *J Exp Med* 206(7):1485–1493.
35. Gonzalez SF, et al. (2010) Capture of influenza by medullary dendritic cells via SIGN-R1 is essential for humoral immunity in draining lymph nodes. *Nat Immunol* 11(5): 427–434.
36. Aoshi T, et al. (2008) Bacterial entry to the splenic white pulp initiates antigen presentation to $CD8^+$ T cells. *Immunity* 29(3):476–486.
37. Sakurai K, Shinkai S (2000) Phase separation in the mixture of schizophyllan and poly (ethylene oxide) in aqueous solution driven by a specific interaction between the glucose side chain and poly(ethylene oxide). *Carbohydr Res* 324(2):136–140.

Vaccines **2013**, *1*, 278-292; doi:10.3390/vaccines1030278

OPEN ACCESS

vaccines

ISSN 2076-393X

www.mdpi.com/journal/vaccines

Review

Innate Immune Signaling by, Genetic Adjuvants for, DNA Vaccination

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Received: 13 June 2013; in revised form: 6 July 2013 / Accepted: 9 July 2013 / Published: 18 July 2013

Abstract: DNA vaccines can induce both humoral and cellular immune responses. Although some DNA vaccines are already licensed for infectious diseases in animals, they are not licensed for human use because the risk and benefit of DNA vaccines is still controversial. Indeed, in humans, the immunogenicity of DNA vaccines is lower than that of other traditional vaccines. To develop the use of DNA vaccines in the clinic, various approaches are in progress to enhance or improve the immunogenicity of DNA vaccines. Recent studies have shown that immunogenicity of DNA vaccines are regulated by innate immune responses via plasmid DNA recognition through the STING-TBK1 signaling cascade. Similarly, molecules that act as dsDNA sensors that activate innate immune responses through STING-TBK1 have been identified and used as genetic adjuvants to enhance DNA vaccine immunogenicity in mouse models. However, the mechanisms that induce innate immune responses by DNA vaccines are still unclear. In this review, we will discuss innate immune signaling upon DNA vaccination and genetic adjuvants of innate immune signaling molecules.

Keywords: DNA vaccine; innate immune responses; adjuvant; DNA sensor

1. Introduction of DNA Vaccines

Almost two decades ago, it was reported that plasmid DNA could induce adaptive immune responses against plasmid-encoded antigens [1], indicating it could be used in novel therapeutic applications as a human vaccine for the prevention of various pathogen infections [2], autoimmunity [3], allergy [4], neurological disorders [5], and cancer [6]. In the veterinary field, some DNA vaccines are already licensed for West Nile virus in horse, infectious hematopoietic necrosis virus in salmon, and melanoma in dogs [7]. For human use, DNA vaccines have not been licensed, however, many candidate DNA vaccines are being studied in ongoing clinical trials. The clinical benefits of DNA vaccine are low cost, vaccine stability, high productivity, and easy modification of antigen in comparison with traditional protein vaccines. Conversely, it was reported that the immunogenicity of DNA vaccines was quite low according in clinical trials. Indeed, the immunogenicity of DNA vaccines tended to be weaker than other types of vaccines using live virus, virus vectors, or traditional protein plus adjuvant vaccines. Therefore, the immunogenicity of DNA vaccines was improved by changing promoters, codon usage of antigen sequences, the insertion of genetic adjuvants such as cytokines and innate immune activation molecules, strategies to prime and boost vaccination, and the route of administration [8].

Furthermore, elucidation of the molecular mechanisms of DNA vaccines is also important for developing DNA vaccines for human use. TANK-binding kinase 1 (TBK1), and stimulator of interferon genes (STING), was identified as an essential molecule for the induction of adaptive immune responses by DNA vaccination. In addition, double-stranded DNA (dsDNA) is a critical ligand of the STING-TBK1 signaling cascade [9]. These results indicate that dsDNA-induced innate immune signaling lead to induction of DNA-encoded antigen specific adaptive immune responses, like an adjuvant. However, DNA sensing machinery is still controversial. In this review, we will discuss innate immune signaling of DNA vaccines and genetic adjuvants of innate immune signaling molecules.

In 1990, Wolf *et al.* showed that the intramuscular administration of naked DNA led to the induction of DNA-encoded reporter genes in muscle cells [10]. Subsequently, Ulmer *et al.* demonstrated that the intramuscular administration of plasmid DNA encoding influenza viral protein induced encoded antigen-specific cytotoxic T lymphocyte (CTL) responses, which protected against lethal influenza virus infection [1]. These findings were the first evidence that naked DNA administration alone could induce adaptive immune responses against antigens expressed from plasmid DNA, and suggested that DNA vaccine strategies might be useful for clinical use. Indeed, many researchers evaluated novel DNA vaccines using experimental infectious diseases models [11]. The properties of DNA vaccines represent greater stability, low cost, high productivity, and possibility to improve immunogenicity. In 1998, the first human clinical trial of DNA vaccines against human immunodeficiency virus was reported [12]. This study evaluated the safety and efficacy of DNA vaccines. Importantly, one of the safety concerns for DNA vaccines was the integration of plasmid DNA into the host genome [13]. If integration occurs following DNA vaccination, the integrated-DNA may cause oncogene activation, tumor suppressor gene inactivation, or chromosomal instability. Fortunately, experimental data showed the rate of

plasmid DNA integration was lower than the natural rate of mutation in mammalian genomes [14]. Another safety concern is development of anti-DNA antibodies, associated with autoimmune disorders [15]. Anti-dsDNA antibody was increased in mouse after DNA vaccination [16]. In the clinical trials, anti-DNA antibody did not increase in any study subject [17]. However, the improvement of DNA vaccines to enhance immunogenicity may increase the risk of integration and development of anti-DNA antibody. Therefore, evaluation of safety concerns is essential before clinical trials are initiated. Subsequently, research groups have developed novel DNA vaccines against cancer, influenza virus, human papillomavirus, hepatitis, and malaria. However, the early clinical trials showed disappointing results.

1.1. Mode of Action

Although DNA vaccines can induce both humoral and cellular immune responses against plasmid-encoded antigens, the mode of action of DNA vaccines is still unclear. However, when DNA plasmids are administered to muscle, skin, subcutaneous, or the nasal cavity, it is believed that the DNA plasmid enters cells, translocates to the nucleus, and antigen is expressed by the host cellular machinery. In most cases, myocytes and antigen presenting cells (APCs), such as dendritic cells (DCs) or macrophages, appear to capture plasmid DNA. Subsequently, antigen protein is degraded and presented by major histocompatibility complex (MHC)-I in immune cells. Additionally, expressed-antigens can be secreted from cells by active secretion of the protein or released due to apoptosis of the transfected cell. Secreted antigen proteins are taken up, degraded, and presented by APCs on MHC-I and MHC-II molecules. Finally, APCs recruited to the draining lymph nodes activate naïve B cells, CD4+ and CD8+ T cells. In many cases, secreted antigen proteins could induce both IgG1 and IgG2a/c antibody, and cytosolic protein antigens could induce IgG2a/c antibody.

1.2. Methods of DNA Vaccination

Intramuscular electroporation (imEPT) is one method of DNA vaccine administration, which overcomes limitations such as low transfection efficacy and insufficient recruitment of APCs to the injection site, by inducing transient enhancement of cell membrane permeability. Consequently, the increased uptake of DNA into the host cell and induction of low level of inflammation can enhance the influx of APCs to the injection site [18]. This method induces potent immune responses including CTL responses, and is therefore a convenient method for analyzing the intracellular signaling cascade of DNA vaccines. Indeed, for most cases, the contribution of innate immune activation by DNA vaccination is evaluated using imEPT in mouse models. Gene gun [19], needle-free systems [20], and mucosal delivery [21] are studied as other methods for DNA vaccination; however, these methods have not been examined to elucidate the innate immune signaling of DNA vaccination. It is important whether these vaccination methods activate same innate immune signaling cascade.

2. Innate Immunity and DNA Vaccines

2.1. Immunostimulatory Properties of Double-Stranded DNA

At present, it is known that nucleic acids such as DNA and RNA induce innate immune responses such as type I interferon (IFN) and inflammatory cytokine production. Interestingly, the innate immune

activation of DNA is affected by DNA structure and conformation. In 1963, it was reported that rat liver derived-DNA or RNA stimulation could produce type I IFN from chick cells [22]. In 1984, Bacillus Calmette-Guérin-derived DNA was shown to have strong anti-tumor activity [23]. These findings were the first evidence that both host and bacterial DNA induced innate and adaptive immune responses. Subsequently, bacteria-derived unmethylated CpG DNA and synthetic CpG oligonucleotide (ODN) were shown to be direct stimulators of B cells [24]. Additionally, Toll-like receptor 9 (TLR9) was identified as a receptor for CpG motif DNA that activated innate immune responses in immune cells, such as DCs, B cells, and macrophages [25]. Meanwhile, host DNA-induced innate immune activation was forgotten and ignored. In 1999, the independent effects of unmethylated CpG motifs or specific DNA sequences were shown as at least 25 base pairs of synthetic double-stranded (ds), but not single-stranded (ss) DNA up-regulated the expression of genes related to immune responses [26]. Later, the B-form conformation of dsDNA was shown to be more effective at inducing innate immune responses than the Z-form of dsDNA [27]. Stimulation with synthetic B-form dsDNA, poly (dA-dT) poly (dA-dT), resulted in the induction of type I IFN and IFN-inducible chemokines, whereas stimulation with synthetic Z-form dsDNA, brominated poly (dG-dC) poly (dG-dC) only induced CXCL10 release.

Studies then focused on adaptive immune responses and demonstrated genomic DNA derived from dead cells induced the maturation of APCs and cellular immune responses, especially CTL responses [28]. In addition, traditional aluminum adjuvant induced cell death and host-derived DNA release, which induced antigen specific IgE production [29]. These results indicate that the immunostimulatory effect of self-DNA could cause the induction of innate immune responses and side-effects in the host. Adverse effects of aberrant DNA have been shown in relation to the function of DNase, an enzyme that digests DNA. DNase II-deficient mice failed to digest DNA from engulfed nuclei of erythroblasts in hepatic macrophages and resulted in the robust production of type I IFN and inflammatory cytokines, which caused severe anemia and rheumatoid arthritis (RA)-like symptoms in a TLR9-independent manner [30,31]. DNase I and DNase III knockout mice developed systemic lupus erythematosis-like symptoms and inflammatory myocarditis, respectively [32–34]. The functional mutations of DNase I and DNase III in humans were also shown to cause several autoimmune disorders, such as systemic lupus erythematosis [33,35], Aicardi-Goutieres syndrome [36], familial chilblain lupus [37], or retinal vasculopathy with cerebral leukodystrophy [38]. Thus, DNA-induced immune responses are not only involved in the prevention of microbial infection but also of autoimmune responses. These findings indicate that normal cells are equipped with innate sensing machineries to remove aberrant genomic DNA fragments.

2.2. Cellular Signaling of DNA Vaccines

In general, DNA vaccines derived from bacterial plasmids contain unmethylated CpG motifs recognized by TLR9, which induce innate immune responses [25]. Therefore, many researchers have attempted to clarify whether TLR9-induced innate immune responses are required for immunogenicity of DNA vaccines. Unexpectedly, some reports suggested that TLR9 was not essential for the induction of immune responses of DNA vaccines *in vivo*, although plasmid-induced cytokine production from immune cells was completely dependent on TLR9 *in vitro* [39,40]. Importantly, dsDNA, including plasmid DNA, could activate both immune cells and non-immune cells such as fibroblasts or

keratinocytes. Therefore, TLR9-independent DNA sensing machinery might also be involved in the immunogenicity of DNA vaccines [39,40].

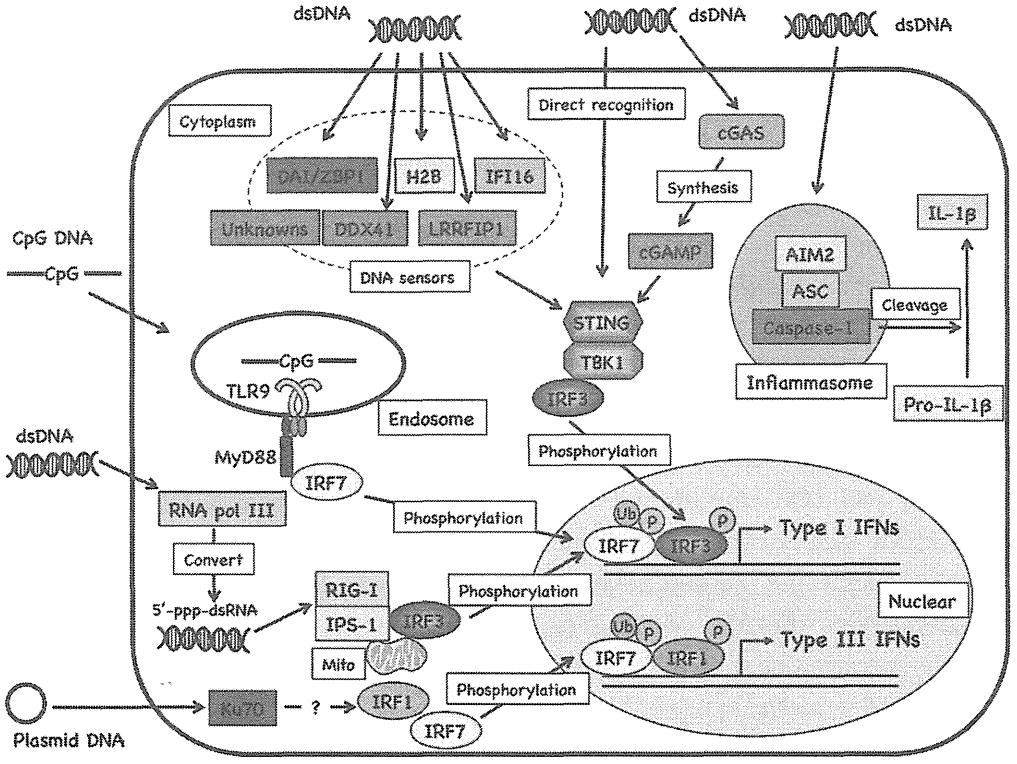
TBK1 is noncanonical IκB kinase that directly phosphorylates interferon regulatory factor 3 (IRF3) to produce type I IFN by TLR-dependent and -independent pathways [27,41]. Thus, TBK1 is important for the activation of innate immune responses upon pathogen infection, tumor development, or autoimmune disease. TBK1-deficient mouse embryonic fibroblasts (MEFs) do not induce cytokine production when stimulated with B-form DNA [27]. Interestingly, TBK1-deficient mice were not able to induce either humoral or cellular immune responses upon DNA vaccination [42]. In addition, type I IFN receptor-deficient mice also showed abolished induction of adaptive immune responses. These results strongly suggest that TBK1-dependent but TLR9-independent mechanisms for the type I IFN signaling cascade are critical for the induction of adaptive immune responses following DNA vaccination. Another important molecule is STING (also known as MITA, ERIS, and MYPS) [43–46] that was firstly reported to be associated with MHC-II-mediated cell death [37]. Subsequently, STING was shown to function as an adaptor molecule that activates innate immune signaling upon cytosolic dsDNA recognition [43]. STING-deficient MEFs did not activate dsDNA-mediated innate immune signaling. Furthermore, STING deficient mice could not induce humoral and cellular immune responses by DNA vaccination [47]. Surprisingly, a recent study showed that STING directly binds to dsDNA to induce innate immune activation [48]. However, it is still unclear whether STING directly binds to plasmid DNA and contributes to DNA vaccine immunogenicity. Other innate immune signaling molecules have been evaluated for their involvement in DNA vaccine immunogenicity and demonstrated that IRF3 is only involved in cellular immune responses but not humoral immune responses [49]. Although STING and TBK1 studies were examined by imEPT to evaluate their contribution to the immunogenicity of the DNA vaccine, IRF3 research has not used the electroporation method. Studies indicate that dsDNA-mediated, but not TLR9-dependent, innate immune signaling regulates the immunogenicity of DNA vaccines [42,47]. Interestingly, our preliminary data showed that other transcription factors are involved in the immunogenicity of DNA vaccines, which are dependent on antigen properties [50].

2.3. Cytosolic Sensors for DNA Fragments and Their Metabolites

To date, several cellular molecules are reported as DNA sensors that recognize aberrant cytosolic DNA (Figure 1). These sensors are involved in the elimination of invasive pathogens, and induce innate immune signaling. In most cases, recognition of cytosolic DNA by these sensors results in the induction of innate immune responses through the STING-TBK1 signaling cascade [27,43], suggesting that the detection of dsDNA structure of plasmid DNA by cytosolic DNA sensing machinery contributes to the enhanced adaptive immune responses against DNA vaccine-encoded antigens.

Z-DNA binding protein 1/DNA-dependent activator of IFN-regulatory factors (ZBP1/DAI) was reported as the first cytosolic dsDNA sensor [51]. Overexpression of ZBP1/DAI increased type I IFN gene expression by dsDNA stimulation such as bacterial and mammalian DNA. Knockdown of ZBP1/DAI resulted in decreased IFN-β production by dsDNA and DNA virus infection but not synthetic dsRNA and RNA virus infection. In addition, ZBP1/DAI directly interacted with B-form DNA in the cytoplasm. Of interest, however, ZBP1/DAI deficient MEFs responded normally to dsDNA, and ZBP1/DAI deficient mice showed normal adaptive immune responses against DNA-encoded antigen [42].

Figure 1. Cytosolic DNA sensing machinery.



Retinoic acid-inducible gene I (RIG-I), and melanoma differentiation-associated gene 5 (MDA5) were identified as cytosolic RNA sensors and activated innate immune responses to protect RNA virus infection [52]. These receptor-mediated signaling pathways are completely regulated by adaptor molecule IFN-β promoter stimulator 1 (IPS-1) (also known as MAVS, VISA, and Cardif) [53–56]. Although RIG-I acts as a cytosolic RNA receptor, it was shown to be involved in the indirect recognition of cytosolic dsDNA. Knockdown of RIG-I resulted in reduced type I IFN production by both dsDNA and dsRNA stimulation in a human hepatocellular carcinoma cell line, HuH-7. Subsequently, it was shown that RNA polymerase III transcribed 5'-triphosphate RNA from poly(dA·dT)·poly(dT·dA) or pathogen genome DNAs as a template, and facilitated the RIG-I-mediated type I IFN production cascade. Intracellular bacteria-induced type I IFN production was abrogated by inhibitors of specific RNA polymerase III, resulting in the promotion of bacterial growth [57]. Although RIG-I-mediated innate immune signaling is completely regulated by IPS-1, IPS-1-deficient mice had normal adaptive immune responses against plasmid DNA vaccinations [42]. In addition, at least in human cells, knockdown of IPS-1 resulted in decreased type I IFN production after dsDNA stimulation [27]. The involvement of RIG-I-IPS-1 signaling in human DNA vaccination is still controversial.

Double stranded DNA induces both innate immune responses and cell death. It was reported that electroporated DNA could induce cell death in murine macrophages [58]. Absence in melanoma 2 (AIM2) was identified as a cytosolic DNA sensor that activated the inflammasome to produce IL-1β and dsDNA-induced cell death. On recognition of cytosolic dsDNA, AIM2 interacts with inflammasome-related molecules to induce pyroptosis, a type of programmed cell death characterized by the activation of caspase-1 and IL-1β production. Deficiency of AIM2 resulted in enhanced susceptibility to bacteria

and DNA virus [59,60]. Collectively, electroporation of plasmid DNA might cause aberrant DNA to induce inflammasome activation or cytokine production via AIM2.

Histone H2B is a component of chromatin. Recently, we demonstrated that histone H2B recognized dsDNA in the cytosol to induce innate immune responses through IPS-1 and COOH-terminal importin 9-related adaptor organizing histone H2B and IPS-1 (CIAO). In addition, histone H2B sensed host-derived dsDNA after cell damage by electroporation [61]. Taken together, histone H2B might contribute to the recognition of administered plasmid DNA and electroporated-derived DNA to induce adaptive immune responses against DNA vaccines. In addition, interferon gamma inducible protein 16 (IFI16) [62], high mobility group box protein 1 (HMGB1) [63], Ku70 [64], leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) [65], and DDX41 [66] were also identified as cytosolic DNA sensors.

Nucleotide second messenger, cyclic-di-GMP, is synthesized by bacteria from two GTP precursors and induced innate immune activation through the STING-TBK1 signaling cascade [67]. Recently, it was reported that after DNA transfection or DNA virus infection cyclic GMP-AMP (cGAMP) was produced by cGAMP synthase (cGAS), a member of the nucleotidyltransferase family. This endogenous nucleotide second messenger induced innate immune responses. Indeed, cGAS binds to DNA in the cytoplasm and catalyzes cGAMP synthesis to act as a cytosolic dsDNA sensor [68]. Furthermore, cGAMP directly interacted with STING to activate IRF3, and knockdown of cGAS suppressed IFN-β production by dsDNA transfection or DNA virus infection. It will be interesting to examine whether DNA vaccination induces cGAMP using plasmid DNA as a template to induce adaptive immune responses.

Studies of DNA sensors were performed using different cell types, synthetic DNAs, bacteria, and viruses. However, only limited type of knockout mice have been used for DNA vaccines, although DNA-mediated innate immune signaling is related to the immunogenicity of DNA vaccines. To elucidate which DNA sensors contribute to the immunogenicity of DNA vaccines, the data by using various DNA sensor gene-deficient mice should be accumulated.

3. Genetic Adjuvant

Innate Immune Activation Molecules

In general, the immunogenicity of DNA vaccines is lower than for traditional protein vaccines or live vaccines, although DNA vaccines contain a "built-in" adjuvant, the CpG motif. Indeed, addition of several CpG motifs into plasmid DNA resulted in improved immunogenicity of DNA vaccines [69]. Additionally, human specific CpG motifs containing DNA vaccines induced the maturation of human monocytes [70] suggesting that improvements to plasmid DNA for innate immune signaling activation are important for the enhancement of immunogenicity and induction of optimal immune responses.

Recently, TLR adaptor molecules, such as myeloid differentiation primary response gene (MyD88) and Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β (TRIF) was inserted into plasmid DNA as a genetic adjuvant and enhanced humoral immune responses against plasmid-encoded antigen (Table 1). In contrast, TRIF genetic adjuvant potently enhanced cellular immune responses. Indeed, TRIF genetic adjuvant elicited protection against lethal influenza virus infection and tumor progression [71]. These studies suggest that TLR agonists may act as DNA vaccine adjuvants.

Flagellin is a TLR5 agonist that activates innate immune responses. Dermal injection of plasmids encoding flagellin, and influenza A virus nucleoprotein enhanced both humoral and cellular immune responses. Interestingly, the flagellin vaccine adjuvant induced antigen-specific IgA production and enhanced protective immunity to lethal influenza A virus infection [72]. These results demonstrate that expression of DNA-encoded TLR agonists can improve the immunogenicity of DNA vaccines.

In addition, IRF1, 3, and 7 were also evaluated as genetic adjuvants for influenza virus DNA vaccines. IRF1 genetic adjuvant strongly enhanced humoral immune responses. In contrast, IRF3 genetic adjuvant induced stronger cellular immune responses. Interestingly, IRF7 genetic adjuvant enhanced both humoral and cellular immune responses [73]. These results suggest that IRF genetic adjuvants can improve both humoral and/or cellular immune responses. In addition, constitutive active forms of IRF3 and IRF7 were evaluated as DNA vaccine adjuvants and elicited both humoral and cellular immune responses to protect against vaccinia virus infection [74]. Furthermore, DNA binding domain-lacked IRF1 (ΔIRF1) was superior to full length IRF1 on HIV TAT DNA vaccines, as ΔIRF1 genetic adjuvant enhanced cellular immune responses [75].

Recently, we showed that TBK1 acts as a genetic DNA vaccine adjuvant. *Plasmodium falciparum* serine repeat antigen 36 (SERA36)-encoded DNA vaccine administration with TBK1 genetic adjuvant enhanced at least humoral immune responses but not detect any cellular immune responses in this immunization [76]. These results suggest that TBK1 genetic adjuvant improves the immunogenicity of DNA vaccines, at least in anti-malarial immunogenicity.

It was reported that ZBP1/DAI interacted with receptor-interacting protein kinase 3 to mediate virus-induced necrosis [77], and electroporated DAI-encoded plasmid DNA facilitated the transcription of type I IFN and proinflammatory cytokines *in vivo*. In addition, DAI genetic adjuvant enhanced CTL responses by type I IFN and NF-κB-dependent but IRF3-independent mechanisms. Co-administration of DAI-encoded plasmid with melanoma-associated antigen tyrosinase-related protein-2 (TRP2) DNA vaccine resulted in enhanced tumor rejection and protection against B16 melanoma challenge [78]. However, whether the improvement of DNA vaccine immunogenicity involves DAI-mediated cell death is still unclear. These results suggest that at least DAI genetic adjuvant can improve the immunogenicity of DNA vaccines.

HMGB1 was also evaluated as a genetic adjuvant for DNA vaccines. Co-immunization with HMGB1 expressing plasmid with HIV-1 Gag and Env expressing DNA vaccines resulted in enhanced humoral and cellular immune responses [79]. In addition, HMGB1 genetic adjuvant also enhanced the immunogenicity of influenza DNA vaccines [80]. Furthermore, chicken (chMDA5) acted as a genetic adjuvant for avian H5N1 influenza virus DNA vaccine. MDA 5 is a RIG-I like receptor that recognizes cytosolic RNAs to induce innate immune responses. In chickens, MDA5 seems to recognize avian influenza virus infection, because chickens lack RIG-I. chMDA5 genetic adjuvant enhanced humoral immune responses and protected against a lethal H5N1 infection [81].

Table 1. Adjuvant effects of innate immune signaling molecules.

Genetic Adjuvant	DNA vaccine-induced immune responses			Vaccine model	Reference
	Ab* responses	CD4+ T cells	CD8+ T cells		
MyD88	↑↑	Not tested	↑	Tumor, Influenza	[71]
TRIF	↑	Not tested	↑↑	Tumor, Influenza	[71]
IRF1	↑↑	↑	↑	Influenza	[73]
ΔIRF1	→	↑	↑↑	HIV-1	[75]
IRF3	↑	↑↑	↑↑	Influenza	[73,74]
IRF7	↑	↑	↑	Influenza	[73,74]
Flagellin	↑	↑	↑↑	Influenza	[72]
TBK1	↑	→	→	Malaria	[76]
HMGB1	↑	↑	↑	HIV, Influenza	[79,80]
DAI/ZBP1	Not tested	Not tested	↑	Tumor	[78]
chMDA5	↑	Not tested	Not tested	Avian Influenza	[81]

*Ab, antibody.

4. Conclusions

About 15 years have passed since the first human clinical trial for DNA vaccines. At present, DNA vaccines are not yet approved for human use. However, many researchers have attempted to improve plasmid DNA, using codon optimization, proper antigen selection, localization changes and addition of antigen signal sequences, appropriate delivery systems and routes, cytokines, and costimulatory molecules as adjuvants, innate immune signaling molecules as adjuvants, targeting for vaccine delivery systems and presentation, and prime boost strategies, amongst others. Indeed, some approaches have succeeded in improving the immunogenicity of DNA vaccines. However, it is important to elucidate the modes of action, such as the cellular and intracellular mechanisms of DNA vaccines. Currently, only dsDNA-mediated STING/TBK1 signaling cascade has been shown to mediate the induction of adaptive immune responses by DNA vaccination. Therefore, it is important to understand how to recognize and induce innate and adaptive immune responses to develop novel, safe, and effective DNA vaccines.

References

1. Ulmer, J.B.; Donnelly, J.J.; Parker, S.E.; Rhodes, G.H.; Felgner, P.L.; Dwarki, V.J.; Gromkowski, S.H.; Deck, R.R.; DeWitt, C.M.; Friedman, A.; *et al.* Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* **1993**, *259*, 1745–1749.
2. Ingolotti, M.; Kawalekar, O.; Shedlock, D.J.; Muthumani, K.; Weiner, D.B. DNA vaccines for targeting bacterial infections. *Expert Rev. Vaccines* **2010**, *9*, 747–763.
3. Silva, C.L.; Bonato, V.L.; dos Santos-Junior, R.R.; Zarate-Blades, C.R.; Sartori, A. Recent advances in DNA vaccines for autoimmune diseases. *Expert Rev. Vaccines* **2009**, *8*, 239–252.
4. Spiegelberg, H.L.; Takabayashi, K.; Beck, L.; Raz, E. DNA-based vaccines for allergic disease. *Expert Rev. Vaccines* **2002**, *1*, 169–177.
5. Shimamura, M.; Sato, N.; Morishita, R. Experimental and clinical application of plasmid DNA in the field of central nervous diseases. *Curr. Gene Ther.* **2011**, *11*, 491–500.

6. Alam, S.; McNeel, D.G. DNA vaccines for the treatment of prostate cancer. *Expert Rev. Vaccines* **2010**, *9*, 731–745.
7. Redding, L.; Weiner, D.B. DNA vaccines in veterinary use. *Expert Rev. Vaccines* **2009**, *8*, 1251–1276.
8. Saade, F.; Petrovsky, N. Technologies for enhanced efficacy of DNA vaccines. *Expert Rev. Vaccines* **2012**, *11*, 189–209.
9. Desmet, C.J.; Ishii, K.J. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. *Nat. Rev.* **2012**, *12*, 479–491.
10. Wolff, J.A.; Malone, R.W.; Williams, P.; Chong, W.; Acsadi, G.; Jani, A.; Felgner, P.L. Direct gene transfer into mouse muscle *in vivo*. *Science* **1990**, *247*, 1465–1468.
11. Laddy, D.J.; Weiner, D.B. From plasmids to protection: A review of DNA vaccines against infectious diseases. *Int. Rev. Immunol.* **2006**, *25*, 99–123.
12. MacGregor, R.R.; Boyer, J.D.; Ugen, K.E.; Lacy, K.E.; Gluckman, S.J.; Bagarazzi, M.L.; Chattergoon, M.A.; Baine, Y.; Higgins, T.J.; Ciccarelli, R.B.; *et al.* First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: Safety and host response. *J. Infect. Dis.* **1998**, *178*, 92–100.
13. Wang, Z.; Troilo, P.J.; Wang, X.; Griffiths, T.G.; Pacchione, S.J.; Barnum, A.B.; Harper, L.B.; Pauley, C.J.; Niu, Z.; Denisova, L.; *et al.* Detection of integration of plasmid DNA into host genomic DNA following intramuscular injection and electroporation. *Gene Ther.* **2004**, *11*, 711–721.
14. Faurez, F.; Dory, D.; le Moigne, V.; Gravier, R.; Jestin, A. Biosafety of DNA vaccines: New generation of DNA vectors and current knowledge on the fate of plasmids after injection. *Vaccine* **2010**, *28*, 3888–3895.
15. Rekvig, O.P.; Nossent, J.C. Anti-double-stranded DNA antibodies, nucleosomes, and systemic lupus erythematosus: a time for new paradigms? *Arthritis Rheum.* **2003**, *48*, 300–312.
16. MacColl, G.; Bunn, C.; Goldspink, G.; Bouloux, P.; Gorecki, D.C. Intramuscular plasmid DNA injection can accelerate autoimmune responses. *Gene Ther.* **2001**, *8*, 1354–1356.
17. Tavel, J.A.; Martin, J.E.; Kelly, G.G.; Enama, M.E.; Shen, J.M.; Gomez, P.L.; Andrews, C.A.; Koup, R.A.; Bailer, R.T.; Stein, J.A.; *et al.* Safety and immunogenicity of a Gag-Pol candidate HIV-1 DNA vaccine administered by a needle-free device in HIV-1-seronegative subjects. *J. Acquir. Immune Defic. Syndr.* **2007**, *44*, 601–605.
18. Van Drunen Littel-van den Hurk, S.; Hannaman, D. Electroporation for DNA immunization: Clinical application. *Expert Rev. Vaccines* **2010**, *9*, 503–517.
19. Haynes, J.R.; McCabe, D.E.; Swain, W.F.; Widera, G.; Fuller, J.T. Particle-mediated nucleic acid immunization. *J. Biotechnol.* **1996**, *44*, 37–42.
20. Rao, S.S.; Gomez, P.; Mascola, J.R.; Dang, V.; Krivulka, G.R.; Yu, F.; Lord, C.I.; Shen, L.; Bailer, R.; Nabel, G.J.; *et al.* Comparative evaluation of three different intramuscular delivery methods for DNA immunization in a nonhuman primate animal model. *Vaccine* **2006**, *24*, 367–373.
21. Torrieri-Dramard, L.; Lambrecht, B.; Ferreira, H.L.; van den Berg, T.; Klatzmann, D.; Bellier, B. Intranasal DNA vaccination induces potent mucosal and systemic immune responses and cross-protective immunity against influenza viruses. *Mol. Ther.* **2011**, *19*, 602–611.
22. Isaacs, A.; Cox, R.A.; Rotem, Z. Foreign nucleic acids as the stimulus to make interferon. *Lancet* **1963**, *2*, 113–116.

23. Tokunaga, T.; Yamamoto, H.; Shimada, S.; Abe, H.; Fukuda, T.; Fujisawa, Y.; Furutani, Y.; Yano, O.; Kataoka, T.; Sudo, T.; *et al.* Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* **1984**, *72*, 955–962.
24. Krieg, A.M.; Yi, A.K.; Matson, S.; Waldschmidt, T.J.; Bishop, G.A.; Teasdale, R.; Koretzky, G.A.; Klinman, D.M. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature* **1995**, *374*, 546–549.
25. Hemmi, H.; Takeuchi, O.; Kawai, T.; Kaisho, T.; Sato, S.; Sanjo, H.; Matsumoto, M.; Hoshino, K.; Wagner, H.; Takeda, K.; Akira, S. A Toll-like receptor recognizes bacterial DNA. *Nature* **2000**, *408*, 740–745.
26. Suzuki, K.; Mori, A.; Ishii, K.J.; Saito, J.; Singer, D.S.; Klinman, D.M.; Krause, P.R.; Kohn, L.D. Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. *Proc. Natl. Acad.Sci. USA* **1999**, *96*, 2285–2290.
27. Ishii, K.J.; Coban, C.; Kato, H.; Takahashi, K.; Torii, Y.; Takeshita, F.; Ludwig, H.; Sutter, G.; Suzuki, K.; Hemmi, H.; *et al.* A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat. Immunol.* **2006**, *7*, 40–48.
28. Ishii, K.J.; Suzuki, K.; Coban, C.; Takeshita, F.; Itoh, Y.; Matoba, H.; Kohn, L.D.; Klinman, D.M. Genomic DNA released by dying cells induces the maturation of APCs. *J. Immunol.* **2001**, *167*, 2602–2607.
29. Marichal, T.; Ohata, K.; Bedoret, D.; Mesnil, C.; Sabatel, C.; Kobiyama, K.; Lekeux, P.; Coban, C.; Akira, S.; Ishii, K.J.; *et al.* DNA released from dying host cells mediates aluminum adjuvant activity. *Nat. Med.* **2011**, *17*, 996–1002.
30. Yoshida, H.; Okabe, Y.; Kawane, K.; Fukuyama, H.; Nagata, S. Lethal anemia caused by interferon-beta produced in mouse embryos carrying undigested DNA. *Nat. Immunol.* **2005**, *6*, 49–56.
31. Kawane, K.; Ohtani, M.; Miwa, K.; Kizawa, T.; Kanbara, Y.; Yoshioka, Y.; Yoshikawa, H.; Nagata, S. Chronic polyarthritis caused by mammalian DNA that escapes from degradation in macrophages. *Nature* **2006**, *443*, 998–1002.
32. Napirei, M.; Karsunky, H.; Zevnik, B.; Stephan, H.; Mannherz, H.G.; Moroy, T. Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat. Genet.* **2000**, *25*, 177–181.
33. Yasutomo, K.; Horiuchi, T.; Kagami, S.; Tsukamoto, H.; Hashimura, C.; Urushihara, M.; Kuroda, Y. Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat. Genet.* **2001**, *28*, 313–314.
34. Morita, M.; Stamp, G.; Robins, P.; Dulic, A.; Rosewell, I.; Hrivnak, G.; Daly, G.; Lindahl, T.; Barnes, D.E. Gene-targeted mice lacking the Trex1 (DNase III) 3'→5' DNA exonuclease develop inflammatory myocarditis. *Mol. Cell. Biol.* **2004**, *24*, 6719–6727.
35. Lee-Kirsch, M.A.; Gong, M.; Chowdhury, D.; Senenko, L.; Engel, K.; Lee, Y.A.; de Silva, U.; Bailey, S.L.; Witte, T.; Vyse, T.J.; *et al.* Mutations in the gene encoding the 3'–5' DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat. Genet.* **2007**, *39*, 1065–1067.
36. Crow, Y.J.; Hayward, B.E.; Parmar, R.; Robins, P.; Leitch, A.; Ali, M.; Black, D.N.; van Bokhoven, H.; Brunner, H.G.; Hamel, B.C.; *et al.* Mutations in the gene encoding the 3'–5' DNA exonuclease TREX1 cause Aicardi-Goutieres syndrome at the AGS1 locus. *Nat. Genet.* **2006**, *38*, 917–920.

37. Lee-Kirsch, M.A.; Chowdhury, D.; Harvey, S.; Gong, M.; Senenko, L.; Engel, K.; Pfeiffer, C.; Hollis, T.; Gahr, M.; Perrino, F.W.; *et al.* A mutation in TREX1 that impairs susceptibility to granzyme A-mediated cell death underlies familial chilblain lupus. *J. Mol. Med.* **2007**, *85*, 531–537.
38. Richards, A.; van den Maagdenberg, A.M.; Jen, J.C.; Kavanagh, D.; Bertram, P.; Spitzer, D.; Liszewski, M.K.; Barilla-Labarca, M.L.; Terwindt, G.M.; Kasai, Y.; *et al.* *C*-terminal truncations in human 3'–5' DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. *Nat. Genet.* **2007**, *39*, 1068–1070.
39. Spies, B.; Hochrein, H.; Vabulas, M.; Huster, K.; Busch, D.H.; Schmitz, F.; Heit, A.; Wagner, H. Vaccination with plasmid DNA activates dendritic cells via Toll-like receptor 9 (TLR9) but functions in TLR9-deficient mice. *J. Immunol.* **2003**, *171*, 5908–5912.
40. Babiuk, S.; Mookherjee, N.; Pontarollo, R.; Griebel, P.; van Drunen Littel-van den Hurk, S.; Hecker, R.; Babiuk, L. $TLR9^{-/-}$ and $TLR9^{+/+}$ mice display similar immune responses to a DNA vaccine. *Immunology* **2004**, *113*, 114–120.
41. Sato, S.; Sugiyama, M.; Yamamoto, M.; Watanabe, Y.; Kawai, T.; Takeda, K.; Akira, S. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J. Immunol.* **2003**, *171*, 4304–4310.
42. Ishii, K.J.; Kawagoe, T.; Koyama, S.; Matsui, K.; Kumar, H.; Kawai, T.; Uematsu, S.; Takeuchi, O.; Takeshita, F.; Coban, C.; Akira, S. TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. *Nature* **2008**, *451*, 725–729.
43. Ishikawa, H.; Barber, G.N. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature* **2008**, *455*, 674–678.
44. Jin, L.; Waterman, P.M.; Jonscher, K.R.; Short, C.M.; Reisdorph, N.A.; Cambier, J.C. MPYS, a novel membrane tetraspanner, is associated with major histocompatibility complex class II and mediates transduction of apoptotic signals. *Mol. Cell. Biol.* **2008**, *28*, 5014–5026.
45. Zhong, B.; Yang, Y.; Li, S.; Wang, Y.Y.; Li, Y.; Diao, F.; Lei, C.; He, X.; Zhang, L.; Tien, P.; *et al.* The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* **2008**, *29*, 538–550.
46. Sun, W.; Li, Y.; Chen, L.; Chen, H.; You, F.; Zhou, X.; Zhou, Y.; Zhai, Z.; Chen, D.; Jiang, Z. ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 8653–8658.
47. Ishikawa, H.; Ma, Z.; Barber, G.N. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* **2009**, *461*, 788–792.
48. Abe, T.; Harashima, A.; Xia, T.; Konno, H.; Konno, K.; Morales, A.; Ahn, J.; Gutman, D.; Barber, G.N. STING recognition of cytoplasmic DNA instigates cellular defense. *Mol. Cell* **2013**, *50*, 5–15.
49. Shirota, H.; Petrenko, L.; Hattori, T.; Klinman, D.M. Contribution of IRF-3 mediated IFNbeta production to DNA vaccine dependent cellular immune responses. *Vaccine* **2009**, *27*, 2144–2149.
50. Tozuka, M.; Kobiyama, K.; Jounai, N.; Takeshita, F.; Koyama, S.; Coban, C.; Ishii, K.J. Laboratory of Adjuvant Innovation, National Institute of Biomedical Innovation, Ibaraki, Japan. Personal communication, 2013.

51. Takaoka, A.; Wang, Z.; Choi, M.K.; Yanai, H.; Negishi, H.; Ban, T.; Lu, Y.; Miyagishi, M.; Kodama, T.; Honda, K.; *et al.* DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **2007**, *448*, 501–505.
52. Yoneyama, M.; Kikuchi, M.; Matsumoto, K.; Imaizumi, T.; Miyagishi, M.; Taira, K.; Foy, E.; Loo, Y.M.; Gale, M., Jr.; Akira, S.; *et al.* Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J. Immunol.* **2005**, *175*, 2851–2858.
53. Kawai, T.; Takahashi, K.; Sato, S.; Coban, C.; Kumar, H.; Kato, H.; Ishii, K.J.; Takeuchi, O.; Akira, S. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* **2005**, *6*, 981–988.
54. Meylan, E.; Curran, J.; Hofmann, K.; Moradpour, D.; Binder, M.; Bartenschlager, R.; Tschopp, J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* **2005**, *437*, 1167–1172.
55. Seth, R.B.; Sun, L.; Ea, C.K.; Chen, Z.J. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* **2005**, *122*, 669–682.
56. Xu, L.G.; Wang, Y.Y.; Han, K.J.; Li, L.Y.; Zhai, Z.; Shu, H.B. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol. Cell* **2005**, *19*, 727–740.
57. Chiu, Y.H.; Macmillan, J.B.; Chen, Z.J. RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. *Cell* **2009**, *138*, 576–591.
58. Stacey, K.J.; Ross, I.L.; Hume, D.A. Electroporation and DNA-dependent cell death in murine macrophages. *Immunol. Cell Biol.* **1993**, *71*, 75–85.
59. Fernandes-Alnemri, T.; Yu, J.W.; Juliana, C.; Solorzano, L.; Kang, S.; Wu, J.; Datta, P.; McCormick, M.; Huang, L.; McDermott, E.; *et al.* The AIM2 inflammasome is critical for innate immunity to *Francisella tularensis*. *Nat. Immunol* **2010**, *11*, 385–393.
60. Rathinam, V.A.; Jiang, Z.; Waggoner, S.N.; Sharma, S.; Cole, L.E.; Waggoner, L.; Vanaja, S.K.; Monks, B.G.; Ganesan, S.; Latz, E.; *et al.* The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat. Immunol.* **2010**, *11*, 395–402.
61. Kobiyama, K.; Takeshita, F.; Jounai, N.; Sakaue-Sawano, A.; Miyawaki, A.; Ishii, K.J.; Kawai, T.; Sasaki, S.; Hirano, H.; Ishii, N.; *et al.* Extrachromosomal histone H2B mediates innate antiviral immune responses induced by intracellular double-stranded DNA. *J. Virol.* **2010**, *84*, 822–832.
62. Unterholzner, L.; Keating, S.E.; Baran, M.; Horan, K.A.; Jensen, S.B.; Sharma, S.; Sirois, C.M.; Jin, T.; Latz, E.; Xiao, T.S.; *et al.* IFI16 is an innate immune sensor for intracellular DNA. *Nat. Immunol.* **2010**, *11*, 997–1004.
63. Yanai, H.; Ban, T.; Wang, Z.; Choi, M.K.; Kawamura, T.; Negishi, H.; Nakasato, M.; Lu, Y.; Hangai, S.; Koshiba, R.; *et al.* HMGB proteins function as universal sentinels for nucleic-acid-mediated innate immune responses. *Nature* **2009**, *462*, 99–103.
64. Zhang, X.; Brann, T.W.; Zhou, M.; Yang, J.; Oguariri, R.M.; Lidie, K.B.; Imamichi, H.; Huang, D.W.; Lempicki, R.A.; Baseler, M.W.; *et al.* Cutting edge: Ku70 is a novel cytosolic DNA sensor that induces type III rather than type I IFN. *J. Immunol.* **2011**, *186*, 4541–4545.
65. Yang, P.; An, H.; Liu, X.; Wen, M.; Zheng, Y.; Rui, Y.; Cao, X. The cytosolic nucleic acid sensor LRRFIP1 mediates the production of type I interferon via a beta-catenin-dependent pathway. *Nat. Immunol.* **2010**, *11*, 487–494.

66. Zhang, Z.; Yuan, B.; Bao, M.; Lu, N.; Kim, T.; Liu, Y.J. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat. Immunol.* **2011**, *12*, 959–965.
67. McWhirter, S.M.; Barbalat, R.; Monroe, K.M.; Fontana, M.F.; Hyodo, M.; Joncker, N.T.; Ishii, K.J.; Akira, S.; Colonna, M.; Chen, Z.J.; *et al.* A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. *J. Exp. Med.* **2009**, *206*, 1899–1911.
68. Sun, L.; Wu, J.; Du, F.; Chen, X.; Chen, Z.J. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science* **2013**, *339*, 786–791.
69. Kojima, Y.; Xin, K.Q.; Ooki, T.; Hamajima, K.; Oikawa, T.; Shinoda, K.; Ozaki, T.; Hoshino, Y.; Jounai, N.; Nakazawa, M.; *et al.* Adjuvant effect of multi-CpG motifs on an HIV-1 DNA vaccine. *Vaccine* **2002**, *20*, 2857–2865.
70. Coban, C.; Ishii, K.J.; Gursel, M.; Klinman, D.M.; Kumar, N. Effect of plasmid backbone modification by different human CpG motifs on the immunogenicity of DNA vaccine vectors. *J. Leukoc. Biol.* **2005**, *78*, 647–655.
71. Takeshita, F.; Tanaka, T.; Matsuda, T.; Tozuka, M.; Kobiyama, K.; Saha, S.; Matsui, K.; Ishii, K.J.; Coban, C.; Akira, S.; *et al.* Toll-like receptor adaptor molecules enhance DNA-raised adaptive immune responses against influenza and tumors through activation of innate immunity. *J. Virol.* **2006**, *80*, 6218–6224.
72. Applequist, S.E.; Rollman, E.; Wareing, M.D.; Liden, M.; Rozell, B.; Hinkula, J.; Ljunggren, H.G. Activation of innate immunity, inflammation, and potentiation of DNA vaccination through mammalian expression of the TLR5 agonist flagellin. *J. Immunol.* **2005**, *175*, 3882–3891.
73. Sasaki, S.; Amara, R.R.; Yeow, W.S.; Pitha, P.M.; Robinson, H.L. Regulation of DNA-raised immune responses by cotransfected interferon regulatory factors. *J. Virol.* **2002**, *76*, 6652–6659.
74. Bramson, J.L.; Dayball, K.; Hall, J.R.; Millar, J.B.; Miller, M.; Wan, Y.H.; Lin, R.; Hiscott, J. Super-activated interferon-regulatory factors can enhance plasmid immunization. *Vaccine* **2003**, *21*, 1363–1370.
75. Castaldello, A.; Sgarbanti, M.; Marsili, G.; Brocca-Cofano, E.; Remoli, A.L.; Caputo, A.; Battistini, A. Interferon regulatory factor-1 acts as a powerful adjuvant in tat DNA based vaccination. *J. Cell. Physiol.* **2010**, *224*, 702–709.
76. Coban, C.; Kobiyama, K.; Aoshi, T.; Takeshita, F.; Horii, T.; Akira, S.; Ishii, K.J. Novel strategies to improve DNA vaccine immunogenicity. *Curr. Gene Ther.* **2011**, *11*, 479–484.
77. Upton, J.W.; Kaiser, W.J.; Mocarski, E.S. DAI/ZBP1/DLM-1 complexes with RIP3 to mediate virus-induced programmed necrosis that is targeted by murine cytomegalovirus vIRA. *Cell Host Microbe* **2012**, *11*, 290–297.
78. Lladser, A.; Mougiakakos, D.; Tufvesson, H.; Ligtenberg, M.A.; Quest, A.F.; Kiessling, R.; Ljungberg, K. DAI (DLM-1/ZBP1) as a genetic adjuvant for DNA vaccines that promotes effective antitumor CTL immunity. *Mol. Ther.* **2011**, *19*, 594–601.
79. Muthumani, G.; Laddy, D.J.; Sundaram, S.G.; Fagone, P.; Shedlock, D.J.; Kannan, S.; Wu, L.; Chung, C.W.; Lankaraman, K.M.; Burns, J.; *et al.* Co-immunization with an optimized plasmid-encoded immune stimulatory interleukin, high-mobility group box 1 protein, results in enhanced interferon-gamma secretion by antigen-specific CD8 T cells. *Immunology* **2009**, *128*, e612–e620.