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

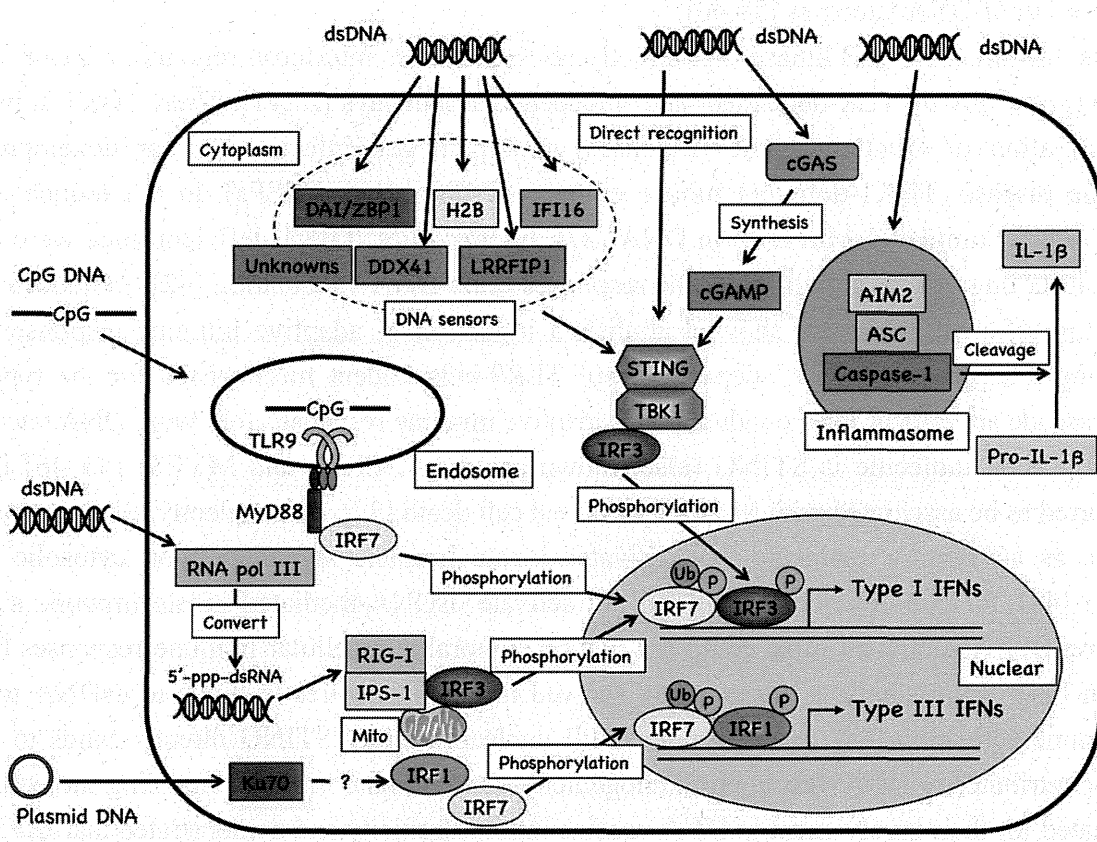
TBK1 is noncanonical IκB kinase that directly phosphorylates interferon regulatory factor 3 (IRF3) to produce type I IFN by TLR-dependent and -independent pathways [27,41]. Thus, TBK1 is important for the activation of innate immune responses upon pathogen infection, tumor development, or autoimmune 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.

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REVIEW ARTICLE

Particulate Adjuvant and Innate Immunity: Past Achievements, Present Findings, and Future Prospects

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Particulates and crystals stimulate the immune system to induce inflammatory responses. Several nanometer- to micrometer-sized particulates, such as particle matter 2.5 (PM2.5), diesel particles, and sand dust, induce pulmonary inflammation and allergic asthma. Conversely, nanometer- to micrometer-sized crystal, sphere, and hydrogel forms of aluminum salts (referred to as "alum") have been used as vaccine adjuvants to enhance antibody responses in animals and humans. Although most of these particulates induce type-2 immune responses *in vivo*, the molecular and immunological mechanisms of action as a vaccine adjuvant are poorly understood. In this review, recent advances in particulate adjuvant research from the standpoint of innate immune responses are discussed.

Keywords adjuvant, alum, innate immunity, particulates, vaccine

INTRODUCTION: ADJUVANT AND INNATE IMMUNITY

Immune responses are categorized into two types: innate and adaptive. Innate immunity is mediated by macrophages and dendritic cells (DCs), which engulf and kill microbes. In contrast, adaptive immunity involves antigen-specific responses mediated by T cells, B cells, and memory cells. It had long been believed that the innate immune response functions as a temporal defense system against infection until the adaptive immune response can be elicited. However, recent studies have demonstrated that innate immunity is essential for the effective induction of adaptive immunity [1–3].

Vaccination mimics natural infection and induces pathogen-specific adaptive immunity effectively. Typically, vaccines contain two main components: antigens and adjuvants. An adjuvant is a substance that enhances antigen-specific (adaptive) immune responses when used in combination with a specific antigen. An adjuvant is thought to be an activator of innate immunity. In general, innate immune cells recognize pathogen-derived factors [e.g. pathogen-associated molecular patterns

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(PAMPs)], through pattern recognition receptors (PRRs) and induce inflammatory responses. There are four classes of PRRs: Toll-like receptors (TLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and C-type lectin receptors (CLRs) [4–7]. These receptors "sense" pathogen-derived factors and transduce activating signals into cells, triggering adaptive immunity against pathogens. Therefore, the ligands for PRRs, such as PAMPs and damage-associated molecular patterns (DAMPs), exhibit potent adjuvant properties that elicit adaptive immunity, and PRRs are considered to be receptors for adjuvants [1, 8].

However, the molecular and immunological mechanisms of many adjuvants used clinically (or those under development) have yet to be fully elucidated. For example, oil emulsions (e.g. Freund's adjuvant and MF-59) and saponin-based adjuvants (e.g., QS-21 and ISCOM) exhibit strong adjuvant activities and could be promising candidates as adjuvants for new human vaccines [9, 10], yet no specific PRR(s) is identified.

An increasing number of particulates and nanoparticles have been reported to exhibit adjuvant activity. A well-known and widely used particulate adjuvant is aluminum salts, which is referred to as "alum" [11–13]. The mechanisms of induction of adaptive immunity by alum or a particulate adjuvant are also unclear, even though alum has been used as a human vaccine adjuvant for more than 80 years. The induction of adaptive immunity requires innate immunity. Hence, it has been proposed that particulates can activate innate cells, and that this action is accompanied by the induction of cytokines, chemokines, and other factors.

PARTICULATES AND THE ADJUVANT EFFECT

Several particulates are known to exhibit adjuvant effects in immune responses. Alum selectively stimulates humoral immune responses, especially type-2 helper (Th2) immune responses, which are characterized by the production of interleukin (IL)-4 and IL-5 and the induction of immunoglobulin (Ig) E and IgG1 [11–13]. (In the case of mice, IgG1 is categorized into Th2-dependent antibody, but the IgG isotype for human Th2 responses has not been clarified fully.) Similar to alum, crystalline silica (which causes a type of pulmonary fibrosis referred to as "silicosis") induces Th2 responses and antigen-specific IgE and IgG1 [14]. It has been reported that synthesized particles, such as poly(lactic-co-glycolic acid) (PGLA), polystyrene particles, nickel oxide nanoparticles, and carbon nanotubes, induce humoral immunity, especially antigen-specific production of IgG1 and IgE [15–19]. Several particulate pollutants, such as diesel exhaust particles, have been shown to induce Th2 responses after intratracheal instillation and are thought to be the source of allergic diseases [20, 21]. In addition to artificial particulates, several crystals generated in the body induce inflammatory responses and possess adjuvant activity. Monosodium urate (MSU) crystals are formed if the concentration of uric acid released from damaged cells reaches saturation. MSU crystals act as DAMPs, and are the causative agent of gout. MSU crystals also act as Th2 adjuvants [22–26]. The biocrystalline substance hemozoin is a hemin detoxification byproduct of malaria parasites. Hemozoin exhibits a potent adjuvant effect and induces humoral immune responses [27]. Chitin particles, which are biopolymers of N-acetyl-D-glucosamine found in fungi, helminthes, and insects, induce the accumulation of IL-4-producing eosinophils and basophils, and are associated with allergy [28]. In contrast to PAMPs such as lipopolysaccharide (LPS) and CpG oligodeoxynucleotides, almost all particulates preferentially elicit Th2 responses and the induction of IgE. Therefore, it has been hypothesized that the specific signals evoked by particulates in innate cells are involved in triggering adaptive (Th2) responses.

PARTICLE SIZE AND IMMUNE RESPONSES

Particle size is thought to affect particulate-induced immune responses. Sharp et al. investigated the relationship between particle size and DC activation. They showed that the polystyrene particles measuring between 430 nm and 1 μm activated DCs efficiently to produce IL-1β [15]. Hornung et al. demonstrated that the optimal size of silica crystals engulfed by macrophages was approximately 1 μm [29]. Coban et al. investigated the adjuvanticity of hemozoin of different sizes. They reported that hemozoin particles measuring between 50 nm and 200 nm exhibited a stronger adjuvant effect compared with larger (2–20 μm) and smaller (<50 nm) particles [27, 30]. These results suggest that particles measuring between 200 nm and 1 μm are the optimal size for phagocytosis and the stimulation of immune responses.

DEPOT EFFECT

Antigen persistence and prolonged release, an effect referred to as the "depot effect" (first proposed by Glenny et al. in 1926), is believed to be responsible for the adjuvanticity of alum [31]. Harrison verified this hypothesis in 1935 by transferring the alum nodules from one guinea pig into a second guinea pig [32]. However, the depot effect has been questioned in several reports. Holt demonstrated that the antibody responses were normal if alum nodules were excised 2 weeks after immunization [33]. In particular, a recent report by Hutchison et al. demonstrated that the removal of the injection site 2 hours after the administration of antigen/alum had no effect on antigen-specific antibody and T-cell responses [34]. These studies suggest that the antigen depot does not play an important part in alum adjuvanticity, and that alum exhibits additional effects that account for its adjuvant properties.

TH2 CYTOKINES AND IL-4-PRODUCING CELLS

Alum preferentially induces Th2 responses (which are characterized by the production of IgG1 and IgE) and IL-4 is a crucial factor for the induction of such Th2 responses. Alum and several other particulates induce the recruitment of IL-4-producing myeloid cells. Jordan et al. reported that alum induces IL-4-producing Gr-1$^+$ cells, and that these cells and IL-4 are required for the expansion of antigen-specific B cells *in vivo* [35]. Furthermore, Wang et al. demonstrated that alum-elicited Gr-1$^+$ cells are IL-4-producing eosinophils [36]. As stated above, chitin-induced IL-4-producing cells were eosinophils and basophils. Moreover, eosinophil recruitment is dependent on the leukotriene B_4 produced by macrophages [28]. However, it has been reported that the antigen-specific antibody responses are normal in several eosinophil-deficient mice (IL-5-deficient, GATA1Δ, and Phil mice) compared with wild-type (WT) control mice after immunization with ovalbumin (OVA)-alum [37]. In addition, Ohnmacht el al. demonstrated that antigen-specific IgG1 and IgE responses were comparable in WT and basophil-deficient mice immunized with OVA and alum [38]. These studies suggest that IL-4-producing myeloid cells such as eosinophils and basophils do not participate in alum adjuvanticity or Th2 responses. Recently, it has been reported that CD1d-deficient [both type-I and -II natural killer T (NKT) cell-deficient]-mice, but not Jα18-deficient (only type-I NKT cell-deficient)-mice exhibited reduced levels of antigen-specific IgG1 [39]. Type-II NKT cells appear to be required for alum-induced antigen-specific IgG1 responses in the regulation of IL-4-producing T cells.

There are several reports on IL-4 signaling and alum adjuvanticity [40, 41]. Brewer et al. reported on the involvement of IL-4 in the immunization of alum using IL-4-, IL-4Rα-, and STAT6-deficient mice. These strains of mice did not induce the production of IgE and exhibited reduced levels of IgG1. However, T cells from IL-4Rα- and STAT6-deficient mice produced normal or higher amounts of IL-4 and IL-5 in response to

a specific antigen. These results indicate that IL-4- and IL-13-mediated signaling is required for Th2-associated antibody production but is dispensable for alum-induced Th2 responses.

Recently, several reports focused on the importance of thymic stromal lymphopoietin (TSLP) on Th2 activation, and Al-Shami et al. demonstrated that TSLP receptor-deficient mice displayed reduced Th2 responses after immunization with OVA and alum [42]. However, allergen (without adjuvant)-induced Th2 responses were also reduced in TSLP receptor-deficient or anti-TSLP antibody-treated mice [43, 44]. These results indicate that TSLP receptor-deficient mice are Th1 prone, and that reduced Th2 responses are not specific to immunization with alum.

PARTICULATES AND MYD88 SIGNALING

All TLR ligands are thought to be potent immune adjuvants through the activation of the adaptor molecules MyD88 and TRIF. Schnare et al. demonstrated that MyD88-deficient mice produced normal levels of OVA-specific IgG and IgE, but that elevated levels of total IgE were produced after immunization with OVA in alum [45]. The excessive amounts of total IgE appeared to be caused by the increased production of IL-13 in MyD88-deficient T cells. Gavin et al. also reported alum adjuvanticity in mice deficient in MyD88 and TRIF, which lack TLR signaling. The antibody responses in these double-knockout (KO) mice were comparable with those in WT mice immunized with trinitrophenol (TNP)-hemocyanin in alum [46]. These results suggest that TLR signaling does not account for the action of alum and indicate that TLRs may acts as negative regulators of IgE production. However, Da Silva et al. demonstrated that MyD88 pathway was required for alum-induced Th2 responses in asthma models [47]. The reason for these discrepant results is unclear. There might be differences in the alum (Imject alum, aluminum hydroxide, aluminum phosphate, or aluminum potassium sulfate) and OVA (endotoxin-free or not) used.

Conversely, hemozoin crystals seem to act as MyD88-dependent adjuvants in natural and synthetic forms [27, 30]. The mechanism(s) underlying this dissimilarity between alum and hemozoin particulates remains to be investigated.

NLRP3 INFLAMMASOME

In 2008, several reports focused on the discovery that particulate adjuvants activate the NLRP3 inflammasome [29, 48]. The inflammasome is a PRR, and there are four classes of inflammasome: NLRP1, NLRP3, NLRC4, and AIM2 [5]. The NLRP3 inflammasome is one of the best characterized inflammasomes and is activated by particulates and crystals [15, 29, 48–53]. NLRP3 forms a multiprotein complex with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1. The NLRP3 inflammasome promotes the secretion of inflammatory cytokines such as IL-1β and IL-18 as active mature forms cleaved by activated caspase-1. In addition to activation by PAMPs, several reports have demonstrated that particulates such as silica and alum stimulate macrophages and DCs to produce IL-1β and IL-18 through activation of the inflammasome, and that alum-induced antigen-specific IgG1 responses are significantly reduced in NLRP3-, ASC-, and caspase-1-deficient mice [48, 54]. Similar to alum, most particulate adjuvants are considered to have an adjuvant effect via inflammasome activation because silica, asbestos, PLGA, and MSU act as activators of the NLRP3 inflammasome. However, other reports have shown that the NLRP3 inflammasome is not required for antibody production in response to vaccination using a particulate adjuvant, including alum [27, 37, 55, 56]. These contradictory reports on the role of the NLRP3 inflammasome may be because of different experimental conditions. Several studies used Imject alum [48, 54, 55], whereas other studies used aluminum hydroxide [56]. Differences in genetic background of the animal used, such

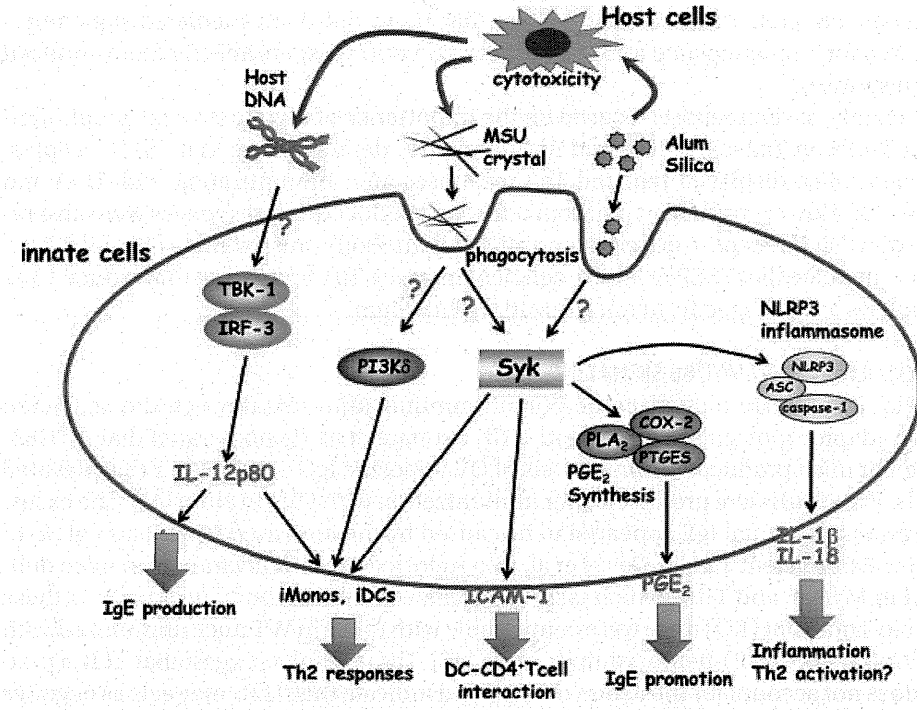

FIGURE 1. Proposed mechanisms of particulate adjuvants (alum, MSU, silica) in innate immunity. Alum induces cell death, and the damaged host cells, such as macrophages and neutrophils, release genomic DNA and uric acid as DAMPs. The recognition mechanisms of genomic DNA are still unclear, but the TBK-1-IRF3 axis plays an important part in IgE production and iMono/iDC migration via the IL-12p80 production. The released uric acid forms MSU crystals, which are recognized by lipid sorting on DCs. The engulfed MSU crystals trigger the activation of Syk and PI3Kδ, and induce inflammatory cells or a strong interaction between DCs and $CD4^+$ T cells. However, the released uric acid has not been shown to form crystals at the site of alum injection. Alum and silica stimulate macrophages and DCs to produce NLRP3 inflammasome-dependent IL-1β and IL-18. These cytokines contribute (at least in part) to acute inflammation and Th2 activation. Macrophages and DCs also induce PGE_2 in response to alum and silica via Syk activation. PGE_2 is involved in IgE production. iMonos: inflammatory monocytes; iDCs: inflammatory DCs.

as C57BL/6 [48, 54, 55] and mixed C57BL/6–129 [56], might contribute to the contrasting results. The involvement of inflammasome-dependent cytokines in alum adjuvanticity is an important issue. It has been demonstrated that IL-18 plays an important part in alum-mediated Th2 responses [57]. However, IL-1 and IL-18 signaling triggers MyD88-dependent signaling, and MyD88 signaling is dispensable for alum adjuvanticity (as described above). The NLRP3 inflammasome may participate in adjuvant activity through IL-1β- and IL-18-independent mechanisms, but the role of the NLRP3 inflammasome in the induction of adjuvant activity remains unclear (Figure 1).

MSU AS A DAMP

Uric acid is a purine catabolite that is released from dying or stressed cells. Uric acid forms MSU crystals if the concentration of uric acid is saturated. Shi et al. demonstrated that uric acid and MSU crystals act as DAMPs and stimulate DCs to induce the maturation and activation of cells [58]. Interestingly, similar to alum, MSU crystals are known to activate Th2 responses preferentially [22–26]. Kool et al. demonstrated that uric acid is released in the peritoneal cavity after the injection of alum, and that antigen-specific T-cell responses were prevented after uricase treatment [22]. Alum is

known to induce cell death, and uric acid and MSU crystals induced by alum cytotoxicity appear to contribute to alum adjuvanticity (whether uric acid forms crystal *in vivo* in alum-injected sites is of considerable interest). In addition, this study demonstrated that uric acid-primed inflammatory monocytes and DCs have an important role in the activation of antigen-specific T cells [22]. However, this study reported that MyD88 signaling was required for this mechanism, which is a controversial proposal. Similar to alum and silica, MSU crystals have been reported to activate the NLRP3 inflammasome [50], and this finding is suspected to be linked to the adjuvant activity through the activation of the NLRP3 inflammasome. However, it has been reported that IL-1β, MyD88, and the NLRP3 inflammasome are dispensable for uric acid-dependent adjuvant activity, and that spleen tyrosine kinase (Syk) and PI3-kinase δ in inflammatory monocytes and DCs are required for Th2 activation by uric acid (Figure 1) [25]. Syk is a nonreceptor tyrosine kinase and a key mediator of immunoreceptor signaling in immune cells. It has been demonstrated that Syk is involved in particulate-mediated innate cell activation [17, 51, 59]. The relationship between uric acid-induced Th2 activation and Syk is interesting. Although Syk is known to be activated by immunoreceptor tyrosine-based activation motif (ITAM)-bearing receptors [60], the underlying mechanisms of Syk activation by particulates is unclear.

Recently, several studies demonstrated the unique recognition mechanisms of particulates. Ng et al. analyzed the recognition of MSU crystals by DCs using atomic force microscopy. MSU crystals were shown to interact with DCs via receptor-independent mechanisms by directly engaging cell surface lipids (mainly cholesterol) [61]. The aggregation of lipid rafts triggers the recruitment and activation of Syk, and ultimately, Syk activates PI3-kinase, phagocytosis, and cytokine secretion (Figure 1) [61]. Flach et al. reported that alum also binds to the surface of DCs, leading to lipid sorting that is similar to MSU crystal-mediated activation of Syk and PI3-kinase. However, the uptake of alum is not required, and activated DCs interact with $DC4^+$ T cells via binding with intracellur adhesion molecule (ICAM)-1 and leukocyte function-associated antigen (LFA)-1 (Figure 1) [62]. Syk appears to be a key molecule for the activation of DCs via lipid sorting, but the mechanisms of Syk activation by MSU or alum are unclear. In general, Syk is known to be activated by Src family kinases such as Hck, Fgr, and Lyn, and ITAM-containing FcRγ and DNAX-activating protein of 12 kDa (DAP12). However, DCs double-deficient in ITAM-containing FcRγ and DAP12 or triple-deficient in Src family kinases ($Hck^{-/-}$, $Fgr^{-/-}$, and $Lyn^{-/-}$) retain their function after activation by MSU crystals [61].

LIPID MEDIATOR

Recently, we found that Th2-inducing particulate adjuvants have another unique mechanism for the activation of innate immune cells: alum and silica particulates stimulate macrophages to produce prostaglandins (PGs) in a similar way to the secretion of IL-1β and IL-18 via NLRP3 activation [17]. In addition to proinflammatory cytokines, lipid mediators such as PGs are involved in the induction of inflammatory responses. The well-characterized proinflammatory lipid mediator PGE_2 is a metabolite of arachidonic acid that is produced by various types of cells, including antigen-presenting cells [63]. Studies have shown that PGE_2 suppresses Th1 responses by elevating intracellular concentrations of cyclic adenosine monophosphate (cAMP) in DCs and macrophages, thereby inhibiting the production of IL-12 and interferon [64–66]. In addition, PGE_2 enhances IL-23 production by DCs and favors Th17 polarization [67, 68]. More recently, PGE_2 has been shown to facilitate the differentiation of Th1 cells in the presence of IL-12 and high doses of the co-stimulatory CD28 antibody via the activation of the PI3-kinase pathway [68]. Thus, PGE_2 exhibits various functions in the regulation of immune responses.

Silica and alum stimulate macrophages and DCs to produce IL-1β, IL-18, and PGE_2. The PGE_2 production induced by silica and alum has been shown to be independent of the NLRP3 inflammasome because inflammasome-deficient ($NLRP3^{-/-}$, $ASC^{-/-}$, caspase-$1^{-/-}$) macrophages produced normal levels of PGE_2 in response to silica and alum compared with WT counterparts. Treatment with a Syk inhibitor or the knockdown of Syk using small interfering RNA (siRNA) molecules markedly suppressed the production of PGE_2 after stimulation with silica and alum, demonstrating that Syk regulates particulate-induced PGE_2 production. In this case, the mechanisms of Syk activation by alum and silica are unclear. However, several reports (including those involving studies on MSU crystals) have demonstrated that particulates stimulate innate immune cells via Syk activation. Therefore, Syk may be a key molecule for particulate-induced immune responses (Figure 1). PGE_2 synthesis is regulated by cyclooxygenase (COX) and PGE synthase (PTGES), and COX-2 and PTGES (also known as mPGES-1) in particular have been reported to regulate stimulation-dependent PGE_2 production in macrophages [69]. PTGES-deficient macrophages do not produce detectable amounts of PGE_2 after stimulation with silica or alum. In addition, PTGES-deficient mice display reduced amounts of antigen-specific IgE after immunization with alum and silica. In contrast, the levels of antigen-specific IgG are normal in PTGES-deficient mice compared with WT mice. These results indicate that particulate-induced PGE_2 is involved in IgE production *in vivo* (Figure 1) [17]. Several reports have demonstrated that PGE_2 facilitates IgE production by the accumulation of increased levels of intracellular cAMP [70, 71]. Interestingly, neuropeptides such as vasoreactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) preferentially activate Th2 responses and increase intracellular cAMP levels in a receptor-dependent manner [72, 73]. In addition, the soluble extract of birch pollen consists of a lipid that is thought to be the causative agent of allergic asthma and to resemble the structure and function of PGE_2. This lipid induces Th2 responses and intracellular cAMP accumulation in DCs [74, 75]. Agents for cAMP elevation may act as Th2 adjuvants.

Many particulates that exhibit adjuvant activity, such as MSU crystals, PLGA, chitin particles, nickel oxide, amorphous silica, and carbon nanotubes, stimulate macrophages to produce inflammasome-dependent IL-1β and inflammasome-independent PGE_2. In addition, we have found that, similar to the release of uric acid, increased amounts of PGE_2 are released from damaged cells, suggesting that PGE_2 also works as a DAMP (Kuroda et al., unpublished data). These findings suggest that PGE_2 is a useful marker for the screening of particulate (Th2) adjuvants.

RELEASE OF NUCLEIC ACIDS FROM HOST CELLS

Activation of innate immunity by DAMPs appears to be a critical mechanism for adjuvant activity. Recently, it was reported that the DNA released from host cells mediates the adjuvant activity of alum [76]. In this study, alum induced the local accumulation of host DNA at the injection site during alum-induced cell death, and interestingly, treatment with DNase I decreased the antigen-specific antibody responses in mice immunized with OVA in alum. Purified genomic DNA mixed with OVA induced OVA-specific IgG1 and IgE responses as efficiently as the alum adjuvant. These results indicate that the alum-induced release of host DNA triggers initial innate immune responses. These responses are not dependent on TLRs, RLRs, or inflammasomes, and the mechanisms by which the host DNA triggers the immune response are unclear. However, interferon regulatory factor 3 (IRF3) and TANK-binding kinase 1 (TBK1) are required for the adjuvant activity of alum (Figure 1). It has been reported that antigen-specific IgE responses, but not IgG1 responses, are significantly reduced in IRF3-deficient and TBK1/tumor necrosis factor (TNF)-double-deficient mice.

TABLE 1. Summary of the effect of particulate (alum) adjuvant on immune system

	Proposed mechanisms	Adjuvant activity	References
Depot effect	Antigen persistence and prolonged release	• Depot effect is not required.	33), 34)
IL-4-producing cells and IL-4 signals	Th2 and IgE induction	• Eosinophils and basophils are not required.	37), 38)
		• IL-4 is required for IgE production, but not for Th2 cell differentiation.	40), 41)
MyD88 pathway and TLRs	Innate cell activation	• MyD88 and TLRs are not required.	42), 43)
NLRP3 Inflammasome	Activation of NLRP3 inflammasome and IL-1β, IL-18 release	• The involvement of inflammasome in adjuvant activity remain unclear.	27), 37), 44) 50)~52)
Uric acid (MSU crystal)	Released from damaged cells by alum Work as DAMPs	• Th2 induction by inflammatory monocytes and DCs via Syk and PI3 kinase activation.	25), 57)
Lipid mediator	Induced from macrophage and DCs by alum or silica	• PGE_2 is induced by Syk activation and promote IgE production.	17)
Nucleic acid (DNA)	Released from damaged cells by alum Work as DAMPs	• Th2 and IgE induction by inflammatory monocytes and DCs through the secretion of IL-12p80 and activation of TBK1-IRF3.	75)

Inflammatory DCs (derived from inflammatory monocytes) were identified as the cells responsible for the induction of Th2 responses. In addition, a reduced number of inflammatory DCs in the draining lymph nodes were observed in IRF3-deficient mice immunized with OVA in alum, and the transfer of WT inflammatory monocytes to IRF3-deficient mice increased Th2 cytokine and IgE production. This study also demonstrated that IL-12p80 (a p40 homodimer) is required for alum-induced migration of inflammatory monocytes, and that treatment with anti-IL-12p80 antibody partially attenuated the IgE responses in alum-treated WT mice (Figure 1). IL-12p80 is known to induce DC migration and to activate nuclear factor kappa-B (NF-κB) and p38 MAP kinase but not signal transducers and activator of transcription (STAT) proteins [77, 78]. Identifying the IL-12p80-producing cells involved in responses to alum or host DNA would be interesting.

IgG1 and IgE responses are uncoupled, i.e. the TBK1-IRF3 axis is required only for the IgE responses. It is believed that the Th2-related antibodies IgE and IgG1 are regulated by identical mechanisms. As described above, PGE_2 is only involved in IgE production, not IgG1 production. Although the mechanisms of the regulation of IgE and IgG1 production and the relationship between IRF3 and PGE_2 remain unclear, the investigation of these mechanisms may help to improve the adjuvants currently in use.

FUTURE PROSPECTS AND CONCLUSION

A summary of the effects of particulate adjuvants is shown in Table 1. Particulate adjuvants (including alum) induce adaptive immunity. The development and modulation of adaptive immunity is regulated by innate immunity. However, the basis for the adjuvanticity of particulates and the mechanisms by which particulates activate

innate immunity are not fully understood. Alum has been used as a safe vaccine adjuvant in humans, but the limitations of alum include local reactions and the augmentation of IgE antibody responses [11, 79]. These limitations reflect the need for continuing research, and these limitations may be overcome by elucidation of the mechanisms of the effect of particulate adjuvants on immune responses.

Alum in combination with another adjuvant, an AS04, a combination of alum with monophosphoryl lipid A (MPL), has been licensed. In addition, a combination with potent Th1 stimulator such as IL-12 and CpG oligodeoxynucleotides shows a great promise, with improvement in alum-induced Th2 responses [11, 12, 80]. Other adjuvant combinations might be explored further. Thus, advances in adjuvant research could open new possibilities for the treatment of not only infectious diseases but also allergic inflammation and cancer.

Declaration of Interest

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ABBREVIATIONS

DC	dendritic cell
PAMP	pathogen-associated molecular pattern
PRR	pattern-recognition receptor
TLR	Toll-like receptor
NLR	Nod-like receptor
RLR	RIG-I-like receptor
CLR	C-type lectin receptor
DAMP	damage-associated molecular pattern
Th2	type-2 helper
IL	interleukin
Ig	immunoglobulin
PGLA	poly(lactic-co-glycolic acid)
MSU	monosodium urate
LPS	lipopolysaccharide
WT	wild-type
OVA	ovalbumin
NKT	natural killer T
TSLP	thymic stromal lymphopoietin
KO	knockout
TNP	trinitrophenol
ASC	apoptosis-associated speck-like protein containing a caspase recruitment domain
Syk	spleen tyrosine kinase
ITAM	immunoreceptor tyrosine-based activation motif
ICAM	intracellular adhesion molecule
LFA	leukocyte function-associated antigen
DAP	DNAX-activating protein of 12 kDa
PG	prostaglandin
cAMP	cyclic adenosine monophosphate
siRNA	small interfering RNA