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International Reviews of Immunology, 32:209–220, 2013

ISSN: 0883-0185 print / 1563-5244 online
DOI: 10.3109/08830185.2013.773326

informa
healthcare

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

Accepted 1 February 2013.
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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

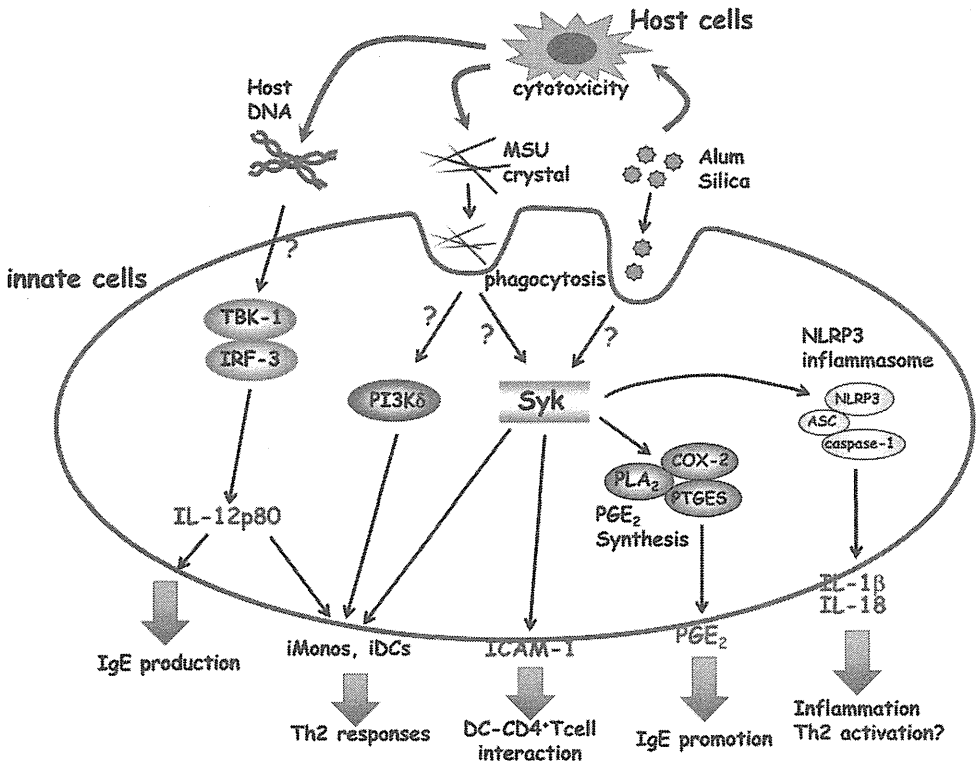

FIGURE 1. Proposed mechanisms of particulate adjuvants (alum, MSU, silica) in innate immunity. Alum induces cell death, and the damaged host cells, such as macrophages and neutrophils, release genomic DNA and uric acid as DAMPs. The recognition mechanisms of genomic DNA are still unclear, but the TBK-1-IRF3 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

C.C. and K.J.I are supported by a Health and Labour Sciences Research Grant "Adjuvant Database Project" of the Japanese Ministry of Health, Labour and Welfare. E.K. was supported in part by the Regional Innovation Strategy Support Program and the Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant number 24591145).

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

VIP	vasoreactive intestinal polypeptide
PACAP	pituitary adenylate cyclase-activating polypeptide
COX	cyclooxygenase
PTGES	PGE synthase
IRF3	interferon regulatory factor 3
TBK1	TANK-binding kinase 1
TNF	tumor necrosis factor
NF-κB	nuclear factor kappa-B
STAT	signal transducers and activator of transcription
MPL	monophosphoryl lipid A

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The Chemotherapeutic Agent DMXAA as a Unique IRF3-Dependent Type-2 Vaccine Adjuvant

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Abstract

5,6-Dimethylxanthenone-4-acetic acid (DMXAA), a potent type I interferon (IFN) inducer, was evaluated as a chemotherapeutic agent in mouse cancer models and proved to be well tolerated in human cancer clinical trials. Despite its multiple biological functions, DMXAA has not been fully characterized for the potential application as a vaccine adjuvant. In this report, we show that DMXAA does act as an adjuvant due to its unique property as a soluble innate immune activator. Using OVA as a model antigen, DMXAA was demonstrated to improve on the antigen specific immune responses and induce a preferential Th2 (Type-2) response. The adjuvant effect was directly dependent on the IRF3-mediated production of type-I-interferon, but not IL-33. DMXAA could also enhance the immunogenicity of influenza split vaccine which led to significant increase in protective responses against live influenza virus challenge in mice compared to split vaccine alone. We propose that DMXAA can be used as an adjuvant that targets a specific innate immune signaling pathway via IRF3 for potential applications including vaccines against influenza which requires a high safety profile.

Citation: Tang CK, Aoshi T, Jounai N, Ito J, Ohata K, et al. (2013) The Chemotherapeutic Agent DMXAA as a Unique IRF3-Dependent Type-2 Vaccine Adjuvant. PLoS ONE 8(3): e60038. doi:10.1371/journal.pone.0060038

Editor: Eng Eong Ooi, Duke-National University of Singapore Graduate Medical School, Singapore

Received December 28, 2012; **Accepted** February 21, 2013; **Published** March 21, 2013

Funding: This work was supported by a Health and Labour Sciences Research Grant 'Adjuvant database Project' of the Japanese Ministry of Health, Labour and Welfare. CKT was funded by A*STAR, Singapore. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

DMXAA was developed as a vascular disruptive agent for use in cancer therapy. Several clinical trials, including a recently completed phase III clinical trial for non-small cell lung carcinoma, have shown that DMXAA is safe and well-tolerated in humans [1]. It is a cell-permeable small molecule which reduces tumor load by inducing apoptosis in tumor vascular endothelium thereby reducing blood flow to solid tumor [2]. Further investigations into the properties of DMXAA have revealed that it is a strongly immunogenic molecule. The anti-neoplastic property of DMXAA is largely attributed to its induction of TNFα which can be detected in the serum and tumor micro-environment within hours of administration [3]. It can activate several inflammatory cell signaling pathways, including extracellular signal-regulated kinases 1 and 2, c-Jun N-terminal kinases, and cytosolic nucleotide-binding oligomerization domain 1 and 2-like receptors [4,5]. In addition, DMXAA is a strong inducer of reactive oxygen species (ROS) [6]. The most striking immunogenic feature of DMXAA is its induction of immediate and predominant type-I-IFN [7]. DMXAA resembles viral infections and double stranded DNA (dsDNA) in the inflammatory signaling events it triggers to induce type-I-IFN production [8]. It utilizes the TBK1-IRF3 signaling pathway without the involvement of Toll-like receptors (TLRs) or RNA helicases for its mechanism of type-I-IFN induction. For the cell signaling events that are upstream of TBK1 phosphorylation, DMXAA was shown to initiate the translocation of the E3 ubiquitin ligase tripartite motif 56 (TRIM56) from the cytoplasm into intracellular punctate structures where the Stimulator of Interferon Genes (STING) was simultaneously recruited [9]. STING is an adaptor molecule that is vital to the induction of type-I-IFN during viral infection [10] and stimulation with cytosolic dsDNA [11] and the bacterial second messenger product, cyclic diguanylate (c-di-GMP) [12]. DMXAA was recently demonstrated to require STING for the production of IFN-β [13]. Due to its ability to induce strong type-I-IFN, DMXAA was found to be an effective antiviral agent against influenza [14,15].

In addition to the induction of pro-inflammatory cytokines, DMXAA can induce the direct activation of antigen presenting cells (APCs) such as macrophages and dendritic cells (DCs). *In-vivo* administration of DMXAA induced maturation of DCs in draining lymph node of tumor bearing mice within 24 h. This was shortly followed by the increase of tumor antigen specific CD8 T cells and their migration to tumor sites due to chemokines such as CCL2 and CXCL10 that were released by the activated APCs [16]. Based on these immunogenic properties of DMXAA, we hypothesize that DMXAA could function as an adjuvant. In this report, we demonstrate in mouse models that DMXAA could indeed promote the adaptive immune response in immunization studies against influenza virus and be a potential adjuvant candidate.

Materials and Methods

Mice and immunizations

Ifnar$^{-/-}$ and *Irf3*$^{-/-}$ mice were of C57BL/6 background and *IL-33*$^{-/-}$ mice were of BALB/c background. The development of these animals was described elsewhere [17–19]. Wild-type (WT) controls were purchased from CLEA, Japan. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Research Institute for Microbial Diseases and Immunology Frontier Research Center of Osaka University, who specifically approved this study. All animal experiments were performed to ameliorate suffering according to the guideline of ASUDC of RIMD and IFREC of Osaka university. Endotoxin-free chicken egg Ovalbumin (OVA) (Seikagaku Biobusiness) was mixed with various adjuvants, including DMXAA (Sigma-Aldrich), aluminum hydroxide suspension (Sigma Aldrich) and K-type CpG ODN 2006 (InvivoGen), in PBS prior to immunization. DMXAA was dissolved in 5% $NaHCO_3$ and was ensured endotoxin-free by analysis with LAL testing (Lonza). In all immunization experiments, mice were injected intradermally at the base of tail on days 0 and 14 and were bled on day 21.

Generation and *in-vitro* stimulation of bone marrow derived dendritic cells

In-vitro grown DCs were prepared by incubating red blood cells-lysed bone marrow cells from WT and various knockout mice with 20 ng/ml of GM-CSF (Peprotech, NJ, USA) for 5 days as previously described in [20]. On day 5, DCs were stimulated with DMXAA, lipopolysaccharides (LPS) (Sigma Aldrich, MO, USA), and Lipofectamine 2000 (Invitrogen, NY, USA) complexed c-di-GMP (Biolog, Bremen, Germany) for 6 h before the supernatant were collected and cytokines measured. The level of DC maturation induced by the various stimuli were determined by using flow cytometry to detect CD86 expression on $CD11c^+$ cells and presented as histogram plots.

Cytokine ELISA

TNFα was measured using the R&D DuoSet® ELISA Development Systems (R&D Systems). IFNβ was measured by ELISA, using rat monoclonal [7F–D3] antibody to Interferon beta (ab24324, Abcam) and rabbit polyclonal antibody to Interferon β (#AB2215, Millipore) and finally with sheep antibody to rabbit IgG (H&L-HRP; ab97095, Abcam). Standard curves were generated using recombinant mouse IFNβ (12400-1, Interferon Source PBL). Results reported in the figures are averages of three samples with errors displayed as standard deviations. Antibody responses to OVA and SV were determined by ELISA where plates were coated with OVA protein and SV respectively. The OVA and SV specific antibodies were detected using goat anti-mouse IgG, IgG1, IgG2a or IgG2c-HRP (Southern Biotech). The relative antibody titers were determined directly from the standard curve generated from positive serum by solving the regression line equation. All ELISAs were developed with the KPL TMB Microwell Peroxidase Substrate System (KPL).

Influenza virus infection and vaccination

Mice were immunized intradermally, at the base of the tail, on days 0 and 14, with 100 μg DMXAA and 0.75 μg of New Caledonia/20/1999 (H1N1), prepared as described [21]. On day 21, the immunized mice were anesthetized with ketamine before they were intranasally infected with 1×10^5 pfu of A/Puerto Rico/8/34 (PR) (H1N1) virus. All efforts were made to reduce suffering to the animal. Challenged mice were monitored daily for their body weight loss and any signs of sickness. Mice that were in a moribund condition or had loss more than 25% of body weight were considered to have reached an experimental endpoint and were humanely euthanized by cervical dislocation.

Statistical analysis

All data were reported as means ± standard deviation. Students t-test was used to compare significant differences between two groups, whereas one-way analysis of variance with Bonferroni's post-test was used to compare differences among three or more groups. Log-rank (Mantel-Cox) tests was used to analyze significant difference between survival curves.

Results

DMXAA has adjuvant properties and induces preferential type-2 response

To determine if the immunogenic property of DMXAA could adjuvant vaccines, we utilized the OVA model antigen system where C57BL/6 mice were immunized with OVA mixed with DMXAA. We found that DMXAA could significantly augment specific immune responses against OVA, as indicated by the increase in serum anti-OVA total IgG (tIgG) titers compared to OVA alone immunized group (Figure 1A). The adjuvant effect was dependent on the dose of DMXAA. In addition, it was observed to have noticeable but insignificant adjuvant effect at a low dose of 10 μg. The immune response induced by the combination of DMXAA and OVA was long-lasting and could be detected as late as 150 days after the final immunization (Figure 1B). To evaluate its efficacy, we compared DMXAA with the established adjuvants, Alum and CpG DNA, which induce predominantly T_H2 and T_H1 immune responses, respectively. Mice immunized with OVA plus DMXAA (100 μg) generated comparable anti-OVA tIgG titers as Alum (665 μg) and CpG DNA (25 μg) adjuvanted groups (Figure 1C). DMXAA resembled Alum in generating predominantly T_H2 type responses as indicated by the induction of higher IgG1 than IgG2c titers (Figure 1D and 1E). In contrast, CpG DNA induced higher IgG2c and lower IgG1 levels. We have also analyzed OVA specific T cell responses by stimulating splenocytes of immunized mice with whole OVA protein or its CD4 and CD8 epitopes followed by measuring IFN-γ secretion. No T-cell responses could be detected in OVA plus Alum or DMXAA groups, whereas splenocytes from the OVA plus CpG group responded with high IFN-γ secretion in the presence CD8 peptide and whole OVA protein (Figure 1F). *In-vivo* depletion of CD4 T cells prior to immunization with OVA and DMXAA completely abrogated the production of OVA-specific antibodies (Figure 1G), suggesting that the generation of adaptive immune responses by DMXAA was CD4 T cell-dependent. These results indicate that DMXAA possesses immuno-stimulatory properties that can function effectively as an adjuvant for vaccines.

Adjuvant effect of DMXAA is dependent on the type-I-IFN response induced by IRF3 signaling

DMXAA has been shown to activate the TBK1-IRF-3 signaling pathway to induce strong IFNβ response from mouse embryonic fibroblasts (MEFs), macrophages and dendritic cells [7]. A recent study also reported that DMXAA could induce IL-33 up-regulation through IRF3 dependent mechanism [22]. IL-33 promotes humoral immunity by triggering the release of T_H2 cytokines such as IL-4, IL-5 and IL-13 from polarised naive T cells [23]. Therefore we would like to determine if the adjuvant effect of DMXAA requires IRF3-dependent type-I-IFN secretion and

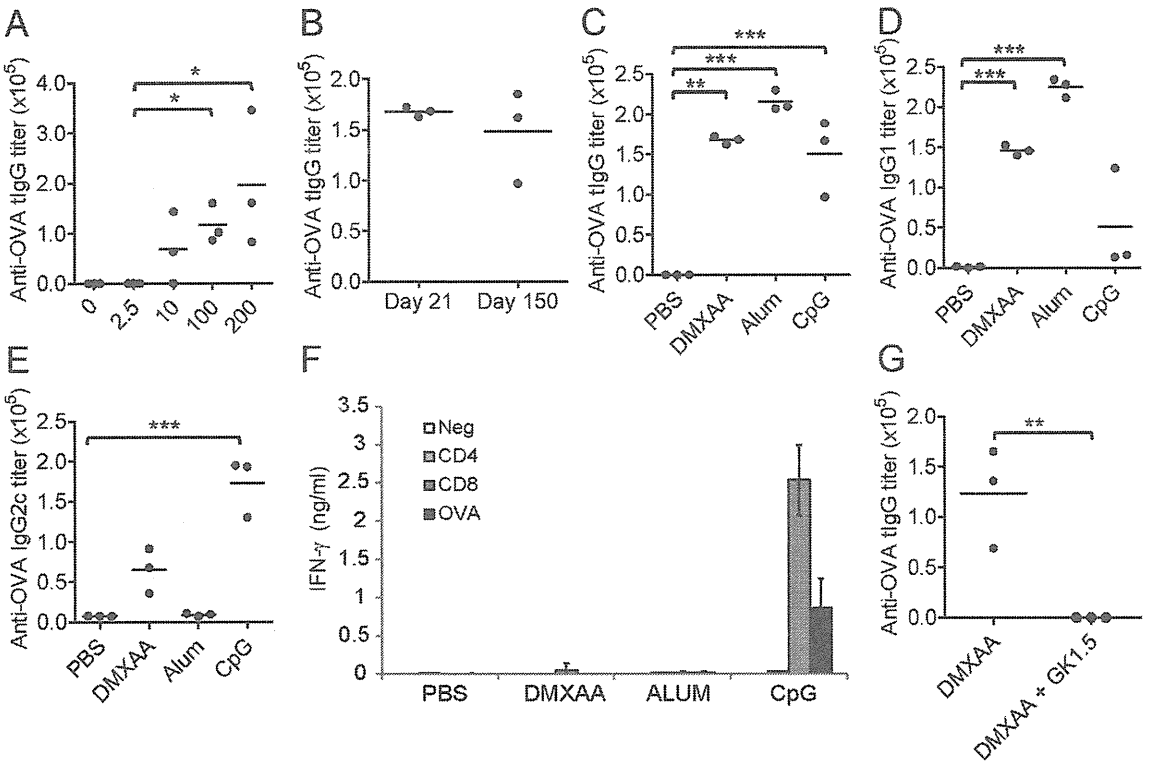

Figure 1. DMXAA acts as a potent adjuvant. (A) Anti-OVA tIgG titers of C57BL/6 mice immunized with 100 μg OVA plus the indicated doses of DMXAA (μg). (B) Anti-OVA tIgG titers of C57BL/6 mice 21 days and 150 days after immunization with 100 μg OVA and 100 μg DMXAA. (C–E) C57BL/6 mice were immunized twice i.d. with 100 μg OVA plus DMXAA (100 μg), Alum (665 μg) or CpG DNA (25 μg) and the induction of (C) tIgG, (D) IgG1 and (E) IgG2c antibody responses against OVA were assessed. (F) IFN-γ secretion from splenocytes of immunized mice that were stimulated for 48 h with CD4 and CD8 OVA peptides and whole OVA protein. (G) Anti-OVA tIgG titers of C57BL/6 mice injected i.v. with 200 μg anti-CD4 (GK1.5) antibodies prior to immunization with 100 μg OVA and 100 μg DMXAA. Results presented are representatives of three separate experiments. *<0.05, ** $P<0.01$, *** $P<0.001$ by Students t-test when comparing between two groups and one-way ANOVA with Bonferroni's post-test when comparing three or more groups.
doi:10.1371/journal.pone.0060038.g001

could the induction of preferential T_H2 type response be due to its up-regulation of IL-33.

To address this, OVA immunization studies were performed on mice lacking IRF3 (*Irf3*$^{-/-}$), IFNαβ receptor (*Ifnar*$^{-/-}$) and IL-33 (*Il-33*$^{-/-}$). As observed in Figure 2A, the anti-OVA tIgG (Figure 2A) titers from *Irf3*$^{-/-}$ and *Ifnar*$^{-/-}$ mice were significantly inhibited compared to WT C57BL/6 mice. This indicates that the adjuvant effect of DMXAA was strongly dependent on IRF3 mediated transcription and responses mediated by type-I-IFN. In contrast to *Irf3*$^{-/-}$ and *Ifnar*$^{-/-}$ mice, *Il-33*$^{-/-}$ mice showed comparable levels of tIgG antibody response as WT BALB/c immunized mice (Figure 2B). Moreover, the preference for the induction of IgG1 (Figure 2C) over IgG2a (Figure 2D) subtype as observed in WT BALB/c mice remained the same in *Il-33*$^{-/-}$ mice. To further support the dependence on IRF3 mediated type-I-IFN for DMXAA adjuvant effect, bone marrow derived DCs from *Irf3*$^{-/-}$, *Ifnar*$^{-/-}$and WT mice were stimulated with DMXAA (Figure 2E–G). Cyclic diguanylate (c-di-GMP) is an IRF3-dependent type-I-IFN inducer and was included as a control. As observed in figure 2E, *Irf3*$^{-/-}$ DCs were unable to induce IFNβ response whilst *Ifnar*$^{-/-}$ DC responded with levels comparable to WT DCs. Therefore indicating that the lack of DMXAA adjuvant effect observed in *Ifnar*$^{-/-}$ mice was not due to the inability to induce type-I-IFN but rather it was the inability to respond to it. Although *Irf3*$^{-/-}$ and *Ifnar*$^{-/-}$ mice did not respond to the adjuvant effect of DMXAA, it was found to be capable of inducing IL-6 (Figure 2F) and TNFα (Figure 2G) response from *Irf3*$^{-/-}$ and *Ifnar*$^{-/-}$ DCs. In addition, the DC maturation effect of DMXAA was still present in *Irf3*$^{-/-}$ and *Ifnar*$^{-/-}$ DCs in the same order of magnitude as WT DCs (Figure 2H). These data suggest that other stimulatory pathways of DMXAA remained intact in *Irf3*$^{-/-}$ mice but they did not play a role in the adjuvant effects of DMXAA. Collectively, we demonstrate that the adjuvant effect of DMXAA is directly dependent on IRF3 mediated type-I-IFN induction and that the reported IL-33 up-regulation by DMXAA is not involved in raising immunogenicity of the vaccine or the skewing towards Th2 type response.

DMXAA is a potent adjuvant for influenza split virus vaccine and enhances protection against influenza challenge

In our previous report, we have demonstrated that in contrast to influenza whole virus vaccine (WV), split vaccine (SV) was unable to induce type-I-IFN production from plasmacytoid DCs [21]. This was due to the lack of RNA content in the SV preparation required to trigger TLR7 activation. As a result, SV immunizations were less protective against lethal influenza challenge as compared to WV immunizations. Hence, we would like to

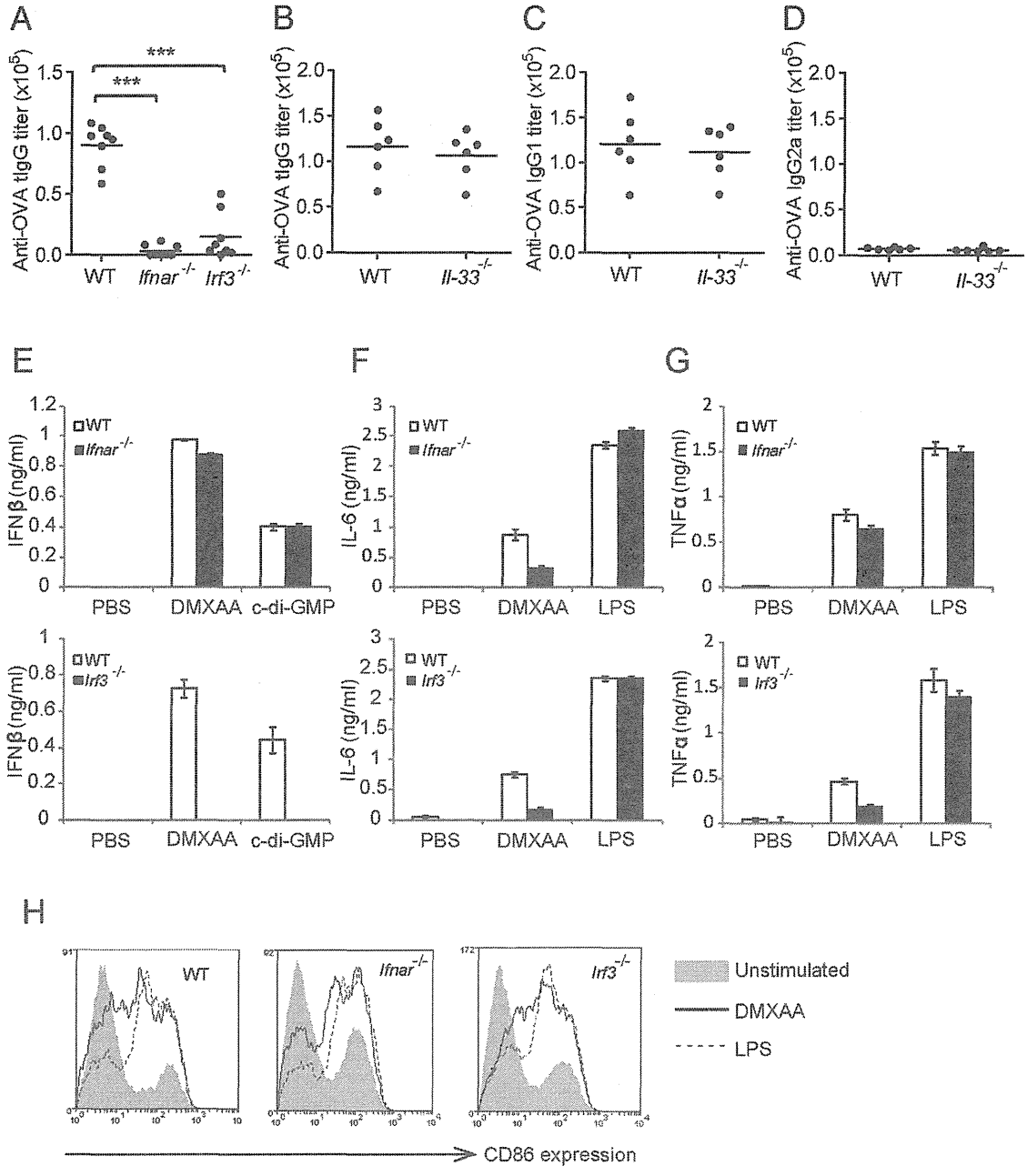

Figure 2. Adjuvant effects of DMXAA require type-I-IFN responses induced by IRF3 activation. Anti-OVA (A) tIgG antibody responses of WT C57BL/6, *Ifnar*$^{-/-}$ and *Irf3*$^{-/-}$ mice immunized twice i.d. with 10 μg OVA plus 100 μg DMXAA. (B) tIgG, (C) IgG1 and (D) IgG2a antibody titers against OVA in WT BALB/c and *Il-33*$^{-/-}$ mice immunized twice i.d. with 10 μg OVA plus 100 μg DMXAA. Results presented are pooled titers from two separate experiments. *In-vitro* cultured DCs derived from WT, *Ifnar*$^{-/-}$ and *Irf3*$^{-/-}$ mice were stimulated with DMXAA (2.5 μg/ml), LPS (1 μg/ml) or lipofectamine complexed c-di-GMP (10 μg/ml) for 6 h before the supernatant were collected and analysed for (E) IFNβ, (F) IL-6 and (G) TNFα secretion and CD11c^{+} cells were analysed for CD86 expression (H). Results presented are average of triplicate conditions ± SD and are representative of three separate experiments. *** $P<0.001$ one-way ANOVA with Bonferroni's post-test.
doi:10.1371/journal.pone.0060038.g002

determine if the type-I-IFN dependent adjuvant effect of DMXAA could adjuvant SV and immunized mice from live flu challenges. C57BL/6 WT mice were immunized intradermally with SV prepared from New Caledonia/20/1999 (H1N1) and mixed with DMXAA. We found that SV plus DMXAA induced higher tIgG antibody responses than SV alone immunizations (Figure 3A). Similar to OVA immunization studies, the adjuvant effect of DMXAA induced higher IgG1 than IgG2c titers to SV (Figure 3B

and 3C). Next, we challenged the immunized mice with a high dose of A/Puerto Rico/8/34 (PR) (H1N1). As seen in Figure 3D, naive mice were quick to succumb to the selected dose of live influenza challenge whilst SV alone immunized mice were offered low level protection (Figure 3A). Although SV alone immunizations had low antibody titers, it was found to be mildly immunogenic and capable of inducing detectable CXCL10 and Anti-HA BALF IgA production [21] that may account for the low level of protective response observed. In contrast, mice immunized with SV plus DMXAA had significantly higher survival rates than naive mice and mice immunized with SV alone (Figure 3D). 11 out of 12 mice that were immunized with SV + DMXAA survived the lethal challenge compared to the 3 out of 12 SV alone group. It was also observable from the rate of weight-loss that SV plus DMXAA immunized mice had a lesser degree of disease-induced morbidity and were able to recover from the infection at a faster rate than control groups (Figure 3E). To exclude the possible role of DMXAA-induced innate immune responses in the protection against lethal challenge, the survival rate of mice injected with DMXAA alone without SV was determined and found to be similar as naïve mice (Figure 3D). Therefore the protective response observed in SV + DMXAA immunized group was due to the adaptive response generated from the immunization and not the innate immune response triggered by DMXAA. These results demonstrate that DMXAA is an efficacious adjuvant for SV vaccine.

Discussion

A large cohort Phase III clinical trial of DMXAA on patients with non-small cell lung carcinoma was recently halted due to inefficacy although it was shown to be well tolerated [1]. As opposed to an earlier successful Phase II clinical trial [24], the Phase III trial showed no overall survival between DMXAA and placebo treated groups. The researchers conducting the clinical trial reasoned that a smaller sample size in the phase II trial overestimated the efficacy of DMXAA. The future of DMXAA as a vascular disruptive agent for cancer therapy is therefore uncertain. In this report, we have demonstrated that the immunogenic properties of DMXAA could be harnessed to adjuvant vaccines with its acceptable safety profile. A local low-dose of DMXAA was capable of adjuvanting vaccines with efficacy that was comparable to the well-studied adjuvants, Alum and CpG. The adjuvant activity was observed using amounts as low as 10 μg per mouse, which was a smaller dose than the 30 mg/kg required for the vascular disruptive effect [25]. When extrapolated to human use, the lower dose required for the adjuvant activity serves to promote DMXAA as a candidate for vaccine adjuvant.

Despite the activation of several distinct inflammatory signaling pathways, we narrowed the immune activity responsible for the adjuvant effect of DMXAA to the IRF3 mediated activation of type-I-IFN. This is surprising as DMXAA induced biased T_H2 response while type-I-IFN is commonly associated with the

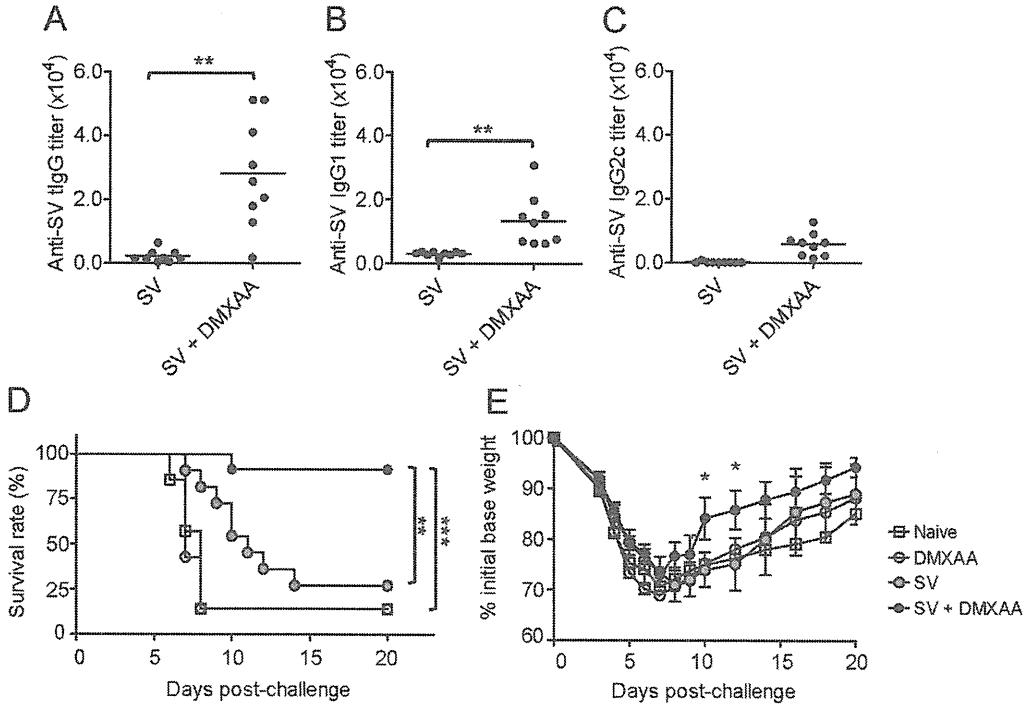

Figure 3. Adjuvant effect of DMXAA can improve potency of influenza SV vaccine and protect mice from a lethal challenge. C57BL/6 mice were immunized twice i.d. with 0.75 μg SV and 100 μg DMXAA, and their sera were assessed for anti-SV (A) tIgG, (B) IgG1 and (C) IgG2c antibodies. ** $P<0.01$ by Students t-test. Results presented are pooled from two separate experiments. (D) C57BL/6 mice that had received two i.d. injections of PBS (open square), 100 μg DMXAA alone (open circle), 0.75 μg SV alone (grey-filled circle) or SV plus DMXAA (black filled circle) were challenged with a lethal dose of A/Puerto Rico/8/34 (PR) (H1N1) 7 days after the final immunization (n = 6 mice per experimental group). Results presented are pooled from two separate experiments. Survival rates were recorded daily and statistical analyses were performed using the log-rank (Mantel-Cox) test where ** and *** denotes $p<0.01$ and $p<0.001$ respectively (E) The rate of weight-loss by the challenged mice were monitored and presented as an average percentage of the initial base weight ± standard error. * denotes $p<0.05$ vs SV alone by Student's T-test.
doi:10.1371/journal.pone.0060038.g003

generation of T_H1 response *in-vivo* [26]. The recent study reporting that DMXAA could induce IL-33 up-regulation through IRF3 dependent mechanism made us question if this could be the reason for the unusual observation [22]. However, immunization studies performed on *Il-33*$^{-/-}$ mice confirmed that IL-33 was not involved in the adjuvant effect of DMXAA or its skewing towards T_H2 response. We have recently reported that Alum mediates enhancement of T_H2 response through the DNA sensing pathway triggered by the release of dsDNA from dying host cells [18]. However, we found that DMXAA did not induce significant increase in free dsDNA in the peritoneal lavage of mice when injected intraperitoneally as opposed to Alum (data not shown). Therefore the mechanism through which DMXAA induced preferential T_H2 type responses remains elusive and requires further investigation. It is possible that the production of IL-6 by DMXAA to be involved as it has been known to inhibit T_H1 polarization by activating NFAT, c-maf and SOCS-1 [27,28] and induce the humoral immunity promoting cytokine, IL-21.

The revealing of DMXAA adjuvant property suggests that it could adjuvant tumor associated antigens and activate the adaptive immune system against cancer cells as part of its anti-tumor response. So far, there are no reports on DMXAA raising humoral immunity against tumor cells with its T_H2 enhancing capability. However, there is evidence which suggests that DMXAA could act as a cancer vaccine adjuvant. For example, it was demonstrated that the administration of DMXAA in tumor bearing mice could increase the number of circulating specific CD8 T-cells [16]. It was also shown to have a positive influence in a separate study which investigated if the anti-cancer property of systemic high-dose DMXAA could work in combination with the adaptive immune response generated by DNA vaccine to protect mice against tumor challenges [29].

In summary, results from this report have shown that DMXAA is capable of functioning as an adjuvant with a defined mechanism that acts specifically on the IRF3 dependent induction of type-I-IFN. DMXAA has already been investigated for applications in antiviral [15] and anti-bacterial [30] therapies and here we demonstrate that it is capable of adjuvanting vaccines as well.

Acknowledgments

We thank Yukiko Fujita and Aki Konishi for their excellent technical assistance for animal husbandry and genotyping.

Author Contributions

Conceived and designed the experiments: CKT CC KJI. Performed the experiments: CKT JI NJ. Analyzed the data: CKT CC KJI. Contributed reagents/materials/analysis tools: TA KO KK BHD EK KM SA. Wrote the paper: CKT CC KJI.

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Cell Type-Specific Subcellular Localization of Phospho-TBK1 in Response to Cytoplasmic Viral DNA

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Abstract

Cytoplasmic viral RNA and DNA are recognized by RIG-I-like receptors and DNA sensors that include DAI, IFI16, DDX41, and cGAS. The RNA and DNA sensors evoke innate immune responses through the IPS-1 and STING adaptors. IPS-1 and STING activate TBK1 kinase. TBK1 is phosphorylated in its activation loop, leading to IRF3/7 activation and Type I interferon (IFN) production. IPS-1 and STING localize to the mitochondria and endoplasmic reticulum, respectively, whereas it is unclear where phosphorylated TBK1 is localized in response to cytoplasmic viral DNA. Here, we investigated phospho-TBK1 (p-TBK1) subcellular localization using a p-TBK1-specific antibody. Stimulation with vertebrate DNA by transfection increased p-TBK1 levels. Interestingly, stimulation-induced p-TBK1 exhibited mitochondrial localization in HeLa and HepG2 cells and colocalized with mitochondrial IPS-1 and MFN-1. Hepatitis B virus DNA stimulation or herpes simplex virus type-1 infection also induced p-TBK1 mitochondrial localization in HeLa cells, indicating that cytoplasmic viral DNA induces p-TBK1 mitochondrial localization in HeLa cells. In contrast, p-TBK1 did not show mitochondrial localization in RAW264.7, L929, or T-23 cells, and most of p-TBK1 colocalized with STING in response to cytoplasmic DNA in those mammalian cells, indicating cell type-specific localization of p-TBK1 in response to cytoplasmic viral DNA. A previous knockout study showed that mouse IPS-1 was dispensable for Type I IFN production in response to cytoplasmic DNA. However, we found that knockdown of *IPS-1* markedly reduced p-TBK1 levels in HeLa cells. Taken together, our data elucidated the cell type-specific subcellular localization of p-TBK1 and a cell type-specific role of IPS-1 in TBK1 activation in response to cytoplasmic viral DNA.

Citation: Suzuki T, Oshiumi H, Miyashita M, Aly HH, Matsumoto M, et al. (2013) Cell Type-Specific Subcellular Localization of Phospho-TBK1 in Response to Cytoplasmic Viral DNA. PLoS ONE 8(12): e83639. doi:10.1371/journal.pone.0083639

Editor: Haitao Guo, Drexel University College of Medicine, United States of America

Received August 12, 2013; **Accepted** November 5, 2013; **Published** December 9, 2013

Funding: This work was supported in part by grants-in-aid from the Ministry of Education, Science, and Culture of Japan, and the Ministry of Health Labor and Welfare of Japan, the Kato Memorial Bioscience Foundation, Yasuda Cancer Foundation, and the Ono Foundation. Financial supports by a MEXT Gran-in-Project "the Carcinogenic Spiral", the Natinal Cancer Center Research and Development Fund (23-A-44), and the Japan Initiative for Global Research Network on Infectious Disease (J-GRID) are gratefully acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interest exist.

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Introduction

RIG-I-like receptors (RLRs) are cytoplasmic viral RNA sensors that play an essential role in Type I interferon (IFN) expression in response to RNA virus infection [1]. RLRs recognize cytoplasmic double-stranded RNA (dsRNA) and the dsRNA analog polyI:C [1]. A recent study reported that RLRs localize on antiviral stress granules in response to cytoplasmic polyI:C or viral infection [2]. IPS-1 (also called MAVS, Cardif, and VISA) is a solo adaptor of RLRs and localizes on the outer-membrane of mitochondria and peroxisomes [3–7]. A recent study reported that a part of IPS-1 localizes on mitochondria-associated membranes (MAMs), which is a distinct membrane compartment that links the endoplasmic reticulum (ER) to the mitochondria [8]. RIG-I is then recruited to MAMs to bind IPS-1 [8]. There are several regulatory proteins on mitochondria such as MFN-1 and MFN-2 [9,10]. Association of RLRs with IPS-1 induces the formation of IPS-1 prion-like aggregates, leading to TBK1 activation [11] and consequent Type I IFN production [12,13]. Toll-like receptor 3 (TLR3) also recognizes viral dsRNA and polyI:C; however, TLR3 localizes to early endosomes or the cell surface and requires the adaptor TICAM-1 to induce Type I IFN expression [14–16].

Cytoplasmic DNA sensors, such as DAI, IFI16, DDX41, cGAS, and Mre11, recognizes DNA viruses [17–19]. These DNA sensors recognize not only viral DNA but also cytoplasmic