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. • IL-4 is required for IgE production, but not for Th2 cell differentiation.	37), 38) 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

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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Role of extrachromosomal histone H2B on recognition of DNA viruses and cell damage

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Histones are essential components of chromatin structure, and histone modification plays an important role in various cellular functions including transcription, gene silencing, and immunity. Histones also play distinct roles in extrachromosomal settings. Extrachromosomal histone H2B acts as a cytosolic sensor to detect double-stranded DNA (dsDNA) fragments derived from infectious agents or damaged cells to activate innate and acquired immune responses in various cell types. It also physically interacts with interferon (IFN)-β promoter stimulator 1 (IPS-1), an essential adaptor molecule that activates innate immunity, through COOH-terminal importin 9-related adaptor organizing histone H2B and IPS-1 (CIAO), resulting in a distinct signaling complex that induces dsDNA-induced type I IFN production. Such a molecular platform acts as a cellular sensor to recognize aberrant dsDNA in cases of viral infection and cell damage. This mechanism may also play roles in autoimmunity, transplantation rejection, gene-mediated vaccines, and other therapeutic applications.

Keywords: DNA sensor, extrachromosomal histone, virus infection, DNA damage, epigenetic modifications

INTRODUCTION

Epigenetic modifications of histones, the primary protein component of chromatin, contribute to diverse homeostatic cellular activities such as transcriptional regulation, chromosome condensation (mitosis), apoptosis, and DNA repair (Bradbury, 1992; Koshland and Strunnikov, 1996; Rogakou et al., 2000; Fernandez-Capetillo et al., 2004). Histones are divided into two groups based on their principal functions. Histones H2A, H2B, H3, and H4 are known as the core histones. Two of each core histone form the histone octamer, which genomic DNA wraps around to form a nucleosome (Luger et al., 1997). Histone H1, the linker histone, binds and rearranges the DNA between nucleosome units (linker DNA) to assist chromatin compaction. Interestingly, histones are present in cytosol (Kobiyama et al., 2010) as well as in the nucleus, mitochondria (Konishi et al., 2003), and cell surface (Radic et al., 2004), particularly during viral infections, apoptosis, and cell damage. Histone H2B transits in and out of the nucleosome more rapidly than other core histones, such as H3 and H4. Thus, about 3% of total H2B is exchanged within 6 min ($t_{1/2}$), ~40% within 130 min, and ~50% by 8.5 h (Kimura, 2005). Histones have microbicidal activity in neutrophil extracellular traps (NETs), which are composed of DNA, elastase, and histones. Treatment of NETs with histone neutralizing antibodies resulted in reduced bactericidal activity against species such as *Shigella flexneri* and *Staphylococus aureus* (Brinkmann et al., 2004). Thus, these "extrachromosomal" histones play important roles in physiological conditions, including innate and adaptive immune responses. We recently reported that extrachromosomal histone H2B is involved in the recognition of cytosolic double-stranded DNA (dsDNA) generated by DNA viruses (non-self) and genomic DNA from damaged cells (self) (Kobiyama et al., 2010; Kawashima et al., 2011a).

DNA-MEDIATED IMMUNE RESPONSE

In 1963, Alick Isaacs found that nucleic acids, both DNA and RNA, strongly induce innate immune responses, such as type I interferon (IFN) production (Isaacs et al., 1963; Rotem et al., 1963). Although this finding generated a great deal of excitement in the field of immunology at that time, it was forgotten or largely ignored until it was shown that unmethylated CpG DNA stimulates immune cells to produce cytokines (Tokunaga et al., 1984; Krieg et al., 1995). As a result, most immunologists presumed that unmethylated CpG DNA was the essential element within self and non-self DNA that activated innate immunity. Toll-like receptor 9 (TLR9) was subsequently identified as a cellular receptor for unmethylated CpG DNA in the activation of innate immune responses in immune cells, such as dendritic cells (DCs), B cells, and macrophages (Hemmi et al., 2000, 2003). In the meantime, dsDNA independent of unmethylated CpG motifs or any other specific sequence was shown to up-regulate the expression of genes related to the immune response (Suzuki et al., 1999). In particular, genomic dsDNA released by injured cells induces maturation of antigen presenting cells and adaptive immune responses (Ishii et al., 2001).

Furthermore, TLR9-dependent and -independent IFN-α production is induced in response to herpes simplex virus-1 (HSV-1) infection (Hochrein et al., 2004). It was later confirmed that the right-handed helical structure (B-form) of DNA is the component responsible for induction of robust type I IFNs in both immune and non-immune cells through TLR9-independent recognition and signaling cascades (Ishii et al., 2006; Stetson and Medzhitov, 2006).

The harmful effects of aberrant DNA have been shown in relation to the function of enzymes that digest DNA (DNases). Thus, hepatic macrophages in DNase II-deficient mice failed to digest DNA from engulfed nuclei of erythroblasts and exhibited robust production of type I IFN, which resulted in severe anemia and development of rheumatoid arthritis (RA)-like symptoms in a TLR9-independent manner (Yoshida et al., 2005; Kawane et al., 2006). DNase I and DNase III knockout mice showed systemic lupus erythematosus (SLE)-like symptoms and inflammatory myocarditis, respectively (Napirei et al., 2000; Yasutomo et al., 2001; Morita et al., 2004). The functional mutations of DNase I and DNase III in humans have also been associated with several autoimmune disorders, such as SLE (Yasutomo et al., 2001; Lee-Kirsch et al., 2007b), Aicardi–Goutieres syndrome (Crow et al., 2006), familial chilblain lupus (Lee-Kirsch et al., 2007a), and retinal vasculopathy with cerebral leukodystrophy (Richards et al., 2007). Thus, DNA-induced immune responses are involved in the prevention of both microbial infection and autoimmune responses. These findings also suggest that normal cells are equipped with innate machinery that senses and removes aberrant genomic DNA fragments before they produce pathological effects.

CYTOSOLIC SENSORS FOR DNA FRAGMENTS AND THEIR METABOLITES

Several proteins have been identified as DNA sensors that recognize aberrant cytosolic DNA fragments and their metabolites. These sensors are involved in the elimination of invasive pathogens and the induction of inflammation. In most cases, recognition of cytosolic DNA by these sensors results in induction of innate immune responses through several key proteins such as stimulator of interferon genes (STING) and TANK-binding kinase 1 (TBK1) (Ishii et al., 2006; Ishikawa and Barber, 2008). STING and TBK1 are also essential factors in the immunogenicity of plasmid DNA vaccines (Ishii et al., 2008; Ishikawa et al., 2009). The underlying mechanisms for the immunological advantages of DNA vaccines have not been fully elucidated. However, it has been suggested that the detection of the double-stranded structure of plasmid DNA by cytosolic DNA sensors contributes to an enhanced adaptive immune response to the vaccine antigen.

Z-DNA binding protein 1/DNA-dependent activator of IFN-regulatory factors (ZBP-1/DAI) was the first reported cytosolic DNA sensor (Takaoka et al., 2007). ZBP-1/DAI contains two Z-DNA binding domains and a D3 domain, all of which are essential for its activation. Overexpression of ZBP-1/DAI enhanced dsDNA-mediated gene expression and knockdown of ZBP-1/DAI impaired IFN-β production by HSV-1 infection, but not Newcastle disease virus (NDV) infection, in a mouse fibroblast cell line (Takaoka et al., 2007). However, fibroblasts from ZBP-1/DAI deficient mice normally responded to dsDNA, and the mice also showed normal immunogenicity to plasmid DNA vaccinations (Ishii et al., 2008).

In 1993, it was reported that electroporated DNA induces cell death in murine macrophages (Stacey et al., 1993). Recently, absence in melanoma 2 (AIM2) was identified as a cytosolic DNA sensor for activation of the inflammasome, a large multimolecular complex that regulates activation of the enzyme caspase-1, to induce IL-1β production and DNA-induced cell death. AIM2 is a member of the hematopoietic IFN-inducible nuclear protein with a 200-amino-acid repeat (HIN-200) family, which contains a pyrin domain and a DNA-binding HIN-200 domain. AIM2 recognizes cytosolic DNA and interacts with inflammasome-related molecules to induce pyroptosis, a type of programed cell death characterized by activation of caspase-1 and IL-1β production upon inflammatory antimicrobial responses. Deficiency of AIM2 results in an enhancement of susceptibility to bacteria and DNA viruses (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009).

Interferon gamma inducible protein 16 (IFI16) is a member of the pyrin and HIN domain-containing (PYHIN) protein family that contains a pyrin domain and two DNA-binding HIN domains. IFI16 directly binds viral DNA in the cytosol and induces IFN-β production through STING (Unterholzner et al., 2010). Small interfering RNA (siRNA) for IFI16 inhibited DNA-induced but not RNA-induced IFN-β production. Knockdown of p204, a mouse ortholog of IFI16, impaired activation of transcription factors and gene inductions upon DNA virus infection.

Although retinoic acid-inducible gene I (RIG-I) was initially identified as a cytosolic RNA receptor, it is also involved in the recognition of cytosolic dsDNA. Thus, knockdown of RIG-I in human hepatocellular carcinoma cell line, HuH-7, attenuated dsDNA-induced type I IFN production. Subsequently, it was shown that poly(dA·dT)·poly(dT·dA) and DNA virus-derived DNAs were converted into 5′-triphosphate RNA by RNA polymerase III to induce RIG-I-mediated type I IFN production. This IFN production induced by intracellular bacteria was abolished by a specific inhibitor of RNA polymerase III, which in turn resulted in a promotion of bacterial growth (Chiu et al., 2009).

High mobility group box protein 1 (HMGB1), initially identified as a non-histone DNA-binding and chromatin-associated protein, is involved in DNA organization and transcriptional regulation (Goodwin et al., 1973; Bustin, 1999). Although most of HMGB1 is localized to the nucleus, HMGB1 acts as an "alarmin" to promote inflammation upon its release from the nucleus during necrosis (Scaffidi et al., 2002). In addition, extracellular HMGB1 is involved in the pathogenesis of autoimmune diseases, as evidenced by the presence of anti-HMGB1 autoantibodies in sera from RA and drug-induced SLE patients (Wittemann et al., 1990; Ayer et al., 1994). The HMGBs (HMGB1, HMGB2, and HMGB3) also bind immunogenic nucleic acids, e.g., virus-derived RNAs and genomic DNAs, and activate innate immune signaling through receptor for advanced glycation and end products (RAGE). In fact, knockdown of HMGBs resulted in a reduction of innate immune responses against immunogenic nucleic acids (Yanai et al., 2009).

In human cells, various types of DNA reportedly induce type III IFNs, especially IFN-λ1 (or interleukin29; IL29). Ku70, whose original functions were reported as DNA repair, V(D)J recombination and telomerase maintenance, was identified as a cytosolic DNA sensor that is responsible for the induction of IFN-λ1 (Zhang et al., 2011a). Knockdown of Ku70 suppressed IFN-λ1 activation in human cells. Whereas other known DNA sensors are involved in type I IFN production, Ku70 is unique in the production of type III IFN upon dsDNA stimulation.

Leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) was initially identified as an RNA-binding protein, but it was eventually recognized as a receptor for both exogenous DNA and RNA (Yang et al., 2010). LRRFIP contains a DNA-binding domain, and is responsible for the production of IFN-β through interaction with β-catenin and recruitment of acetyltransferase p300 in cases of vesicular stomatitis virus (VSV) and *Listeria monocytogenes* infection.

RNA and DNA helicases are members of the DEADbox family, the name of which was derived from one of the conserved amino-acid sequences in the proteins. Members of the DExD/H-box (where x can be any amino acid) helicase superfamily, such as DHX9 and DHX36, were identified as cytosolic CpG DNA sensors for the induction of type I IFN production in plasmacytoid DCs (Kim et al., 2010). Another helicase, DDX41, a member of the DEXDc family, was identified as an intracellular dsDNA sensor that is responsible for type I IFN production in myeloid DCs (Zhang et al., 2011b). After stimulation with dsDNA, DDX41 interacts with STING in the microsome, mitochondria, and mitochondria-associated endoplasmic reticulum membrane fractions. DDX41 also recognizes bacterial second messenger cyclic di-GMP and cyclic di-AMP, and activates type I IFN production by interacting with STING, leading to TBK1-IRF3 activation (Parvatiyar et al., 2012).

DNA transfection or DNA virus infection leads to a production of cyclic GMP-AMP (cGAMP) via the function of cGAMP synthase, cGAS, which belongs to the nucleotidyltransferase family, and an endogenous second messenger to induce innate immune responses. cGAS binds to DNA in the cytoplasm and catalyzes cGAMP synthesis to function as a cytosolic dsDNA sensor that induces type I IFNs (Sun et al., 2013). It was also shown that cGAMP directly interacts with STING to activate IRF3, and knockdown of cGAS results in the suppression of IFN-β production induced by dsDNA transfection or DNA virus infection (Sun et al., 2013).

These studies were performed using different types of cells, synthetic DNAs, bacteria, and viruses as shown in **Table 1**. Therefore, it should be noted that multiple recognition machineries for sensing cytosolic DNA and DNA metabolites might differ among species and/or cell types.

EXTRACHROMOSOMAL HISTONE H2B IS INVOLVED IN DNA SENSING

To identify molecules responsible for cytosolic dsDNA-mediated type I IFN production, we screened a cDNA expression library using HEK293T cells stably transfected with a luciferase gene cassette under an IFN-β promoter. Among >960,000 independent clones examined, a single clone encompassing the histone H2B ORF exhibited a striking enhancement of dsDNA-induced IFN-β promoter activation (Kobiyama et al., 2010). In a separate set of experiments, cellular proteins that bind dsDNA were purified from rat thyroid cell line FRTL-5, cells previously proven to respond well to dsDNA (Suzuki et al., 1999). Protein extracts were passed through ssDNA sepharose and absorbed onto dsDNA sepharose columns before electrospray ionization (ESI)-MS/MS mass spectrometry analysis. Among the molecules identified, histone H2B showed a significantly high MASCOT (probability) score (Kawashima et al., 2011a). Thus, two independent approaches implied that extrachromosomal H2B functionally mediates IFN-β promoter activation in human kidney cells following dsDNA stimulation and physically associates with dsDNA in rat thyroid cells.

Type I IFN production induced by dsDNA was significantly suppressed in HEK293 cells treated with H2B siRNA, but not by those treated with siRNAs for other core histones. Although most histone H2B localizes in the nucleus, it appears to sense DNA in the cytoplasm by interacting with IFN-β promoter stimulator 1 (IPS-1) (Kobiyama et al., 2010), an essential adaptor molecule for signal activation triggered by cytoplasmic dsRNA and single stranded 5′-triphosphate RNA (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). Human, but not mouse, IPS-1 was involved in the dsDNA-mediated signal transduction (Kumar et al., 2006; Ishii et al., 2008). Therefore, histone H2B interacts with IPS-1 in the cytoplasm following dsDNA stimulation only in human cells.

Yeast two-hybrid screening identified KIAA1192 as a molecule that interacts directly with histone H2B; therefore, it was renamed CIAO (C-terminal importin 9-related adaptor organizing histone H2B and IPS-1) based on its novel role. While high similarities of amino acid sequences were detected between human and mouse H2B (>70.1%) and between human and mouse CIAO (99.2%), the amino acid sequence of IPS-1 was largely different between human and mouse (30.3%). The observed interaction of CIAO and IPS-1 only in human molecules is a possible reflection of this difference in IPS-1 sequence (Kobiyama et al., 2010). These results strongly suggest that there is species-specific involvement of IPS-1 in dsDNA-mediated signaling.

We further examined the role of histone H2B on cell-autonomous antiviral responses. Knockdown of histone H2B suppressed IFN-β production and STAT1 phosphorylation when DNA viruses, in this case modified vaccinia virus Ankara (MVA), were infected (Kobiyama et al., 2010). Multiplication of adenovirus type 5 was significantly enhanced in the H2B knockdown cells, while multiplication of RNA viruses, such as encephalomyocarditis virus (EMCV), was not affected by the presence or absence of histone H2B (**Figure 1A**). Multiplication of other DNA viruses, such as human papilloma viruses (HPV11 and HPV16) and adenovirus serotype 5, was significantly enhanced in cells to which histone H2B siRNA was transfected. These results suggested that extrachromosomal histone H2B is involved in the sensing of DNA viruses and mediates cell-autonomous antiviral immune responses in human cells. The human immunodeficiency virus (HIV) is a lentivirus, a class of retrovirus, which has two copies of positive single stranded RNA that codes viral genes. Upon infection in target cells, the viral RNA genome is reverse transcribed into dsDNA in the peri-integration complex (PIC). When

Table 1 | Cytosolic DNA sensors.

DNA sensor	Localization	Pathogens	Nucleic acid ligand	Reference
ZBP-1/DAI	Cytoplasm	HSV	Poly(dA:dT), ISD	Takaoka et al. (2007)
AIM2	Cytoplasm	VV, MCMV, *L. monocytogenes*, *F. tularensis*	Calf thymus DNA, poly(dA:dT)	Burckstummer et al. (2009), Fernandes-Alnemri et al. (2009), Hornung et al. (2009), Roberts et al. (2009)
IFI16	Cytoplasm	VV, HSV-1	Poly(dA:dT)	Unterholzner et al. (2010)
RNA pol III/RIG-I	Cytoplasm	*L. pneumophila*, AdV, HSV-1, EBV	Poly(dA:dT)	Chiu et al. (2009)
HMGB1	Nucleus, extracellular	VSV, HSV-1	dsDNA, dsRNA, ssDNA, ssRNA	Yanai et al. (2009)
Ku70	Cytoplasm	HIV?	Plasmid DNA	Zhang et al. (2011a)
LRRFIP1	Cytoplasm	*L. monocytogenes*, VSV	Poly(dA:dT)	Yang et al. (2010)
DDX41	Cytoplasm	*L. monocytogenes*, AdV, HSV-1, VV	Poly(dA:dT), c-d-GMP, c-d-AMP	Zhang et al. (2011b), Parvatiyar et al. (2012)
cGAS	Cytoplasm	HSV-1	cGAMP	Sun et al. (2013)
Histone H2B	Nucleus, cytoplasm	HPV, AdV, HIV	Poly(dA:dT), genomic DNA	Kobiyama et al. (2010), Kawashima et al. (2011a)

HSV, herpes simplex virus; VV, vaccinia virus; MCMV, mouse cytomegalovirus; AdV, adenovirus; EBV, Epstein–Barr virus; VSV, vesicular stomatitis virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; dA:dT, poly(dA-dT)·poly(dT-dA); ISD, immunostimulatory DNA.

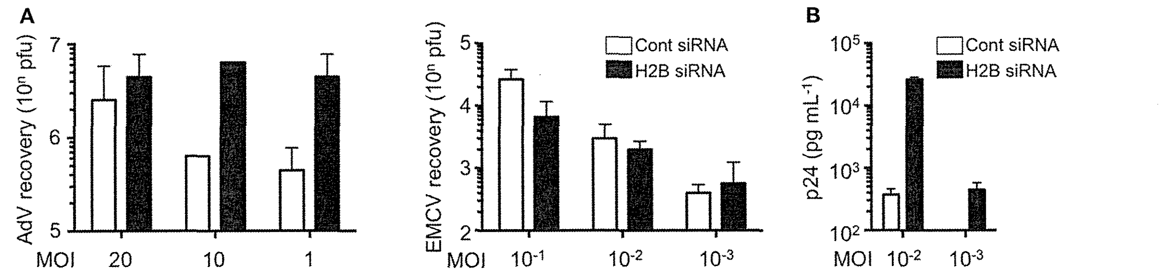

FIGURE 1 | Histone H2B is a key factor for the suppression of viral replication. (A) HEK293 and HeLa cells were pretreated with control siRNA (Cont siRNA) or histone H2B siRNA (H2B siRNA). The cells were infected with AdV type 5 or EMCV. Twenty-four hours after infection, viral multiplication was determined by a plaque assay. **(B)** Magic 5 cells were pretreated with Cont siRNA or H2B siRNA. The cells were infected with HIV-1 IIIB for 3 h. Seventy-four hours after infection, viral multiplication was determined by HIV-1 p24 ELISA using culture supernatant.

histone H2B was knocked-down in CCR5-expressing HeLa/CD4$^+$ cell clone 1–10 (Magic 5) cells, HIV-1 replication was significantly enhanced (**Figure 1B**). These data clearly indicate that histone H2B discriminates between foreign DNA and RNA upon viral infection to evoke IPS-1-mediated signaling through association with a novel adaptor protein, CIAO. It has also been suggested that human IPS-1 has evolutionarily gained the potential to transmit dsDNA-initiated, histone H2B-mediated signaling to combat human viruses that produce DNA intermediates within the cell. Whether histone H2B has a role in infection in mice, probably by interacting with molecules other than IPS-1, is currently unknown.

We next examined the involvement of genomic DNA-mediated immune responses in light of a possible role in the triggering of autoimmune disorders. When FRTL-5 thyroid cells were exposed to progressively higher levels of electric pulsing, in the absence of pathogens or immune cells, genomic DNA was released to the cytoplasm, which was associated with activation of the expression of certain genes, such as those encoding type I IFN and chemokines. More importantly, the expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules was also induced in thyroid cells (Suzuki et al., 1999; Kawashima et al., 2011a), suggesting that the autoimmune target cell itself might present autoantigens upon cell damage (Kawashima et al., 2011b). It has been assumed that autoimmune thyroid diseases, such as Graves's disease and Hashimoto's thyroiditis, develop by a combination of genetic susceptibility and environmental factors. The data suggested that thyroid cell injury results in the release of genomic DNA fragments into the cytosol, which are recognized by extrachromosomal histone H2B to activate genes involved in both innate and acquired immune responses. Such responses may relate to the development of thyroiditis that in turn may increase the chance to present self-antigens to immune cells and initiate autoimmune reactions. Thus, our findings suggest that extrachromosomal histone H2B acts as a cytosolic DNA sensor for both self and non-self DNA, and that this recognition mechanism may be involved in preventing microbial infections and triggering of autoimmune disorders.

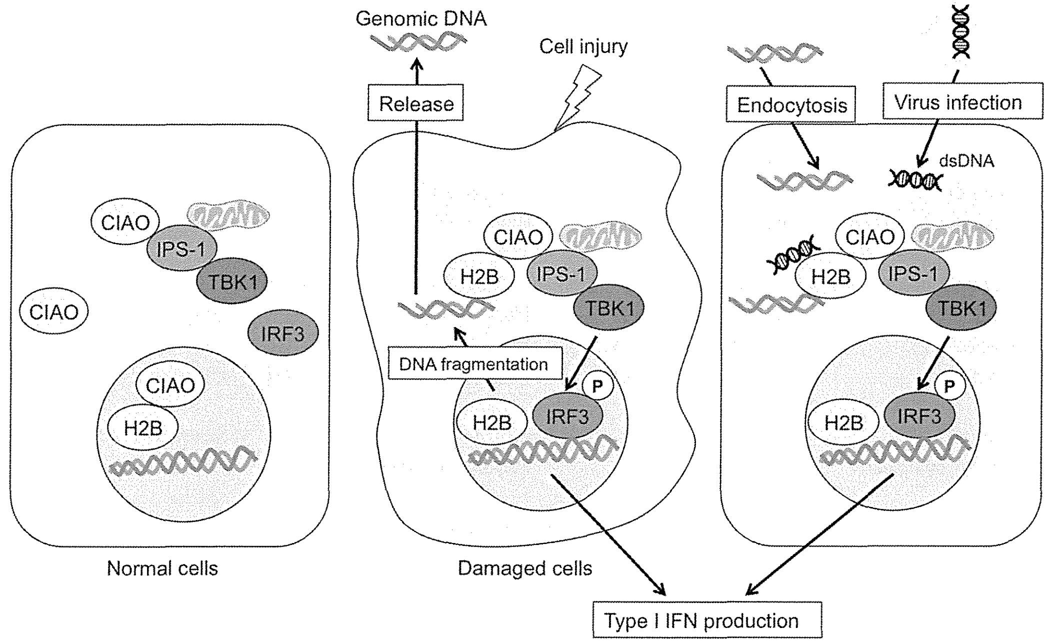

FIGURE 2 | Mode of extrachromosomal histone H2B-mediated innate immune responses. Under normal conditions, histone H2B primarily localizes to the nucleus. In cases of cell damage or viral infection, histone H2B recognizes aberrant self- or non-self-derived dsDNA and forms an H2B-CIAO-IPS-1 interaction complex in the cytoplasm, which in turn activates TBK1 and induces IRF3 phosphorylation to produce type I IFN.

EPIGENETIC MODIFICATION AND VIRUS INFECTION

Epigenetic modifications, including histone modifications and chromatin remodeling, regulate cellular processes that require access to genomic DNA. DNA viruses utilize the chromatin-mediated regulation of gene transcription and DNA replication of the host cell (Liang et al., 2009). In the case of herpes viruses, chromatin modulation is a regulatory factor of viral latency and reactivation cycles. Infection of cells with herpes virus results in the deposition of nucleosomes bearing repressive K9 methylation of histone H3 (H3-K9) on the viral genome. Inhibition of lysine-specific demethylase (LSD1) results in an accumulation of repressive chromatin and blockage of viral gene expression (Liang et al., 2009). In the case of HIV-1, histone H3-K9 methyltransferase G9a is responsible for chromatin-mediated HIV-1 transcriptional latency through methylation of H3 (Imai et al., 2010). In addition, K9 methylation of histone H3 is involved in repression of the human cytomegalovirus gene (Ioudinkova et al., 2006). Thus, since viruses utilize the host gene regulation system for their replication, its modification blocks initial gene expression of a DNA virus, including adenovirus (Liang et al., 2013).

Histone H2B can also be modified by acetylation (Schiltz et al., 1999), GlcNAcylation (Fujiki et al., 2011), phosphorylation (Fernandez-Capetillo et al., 2004), sumoylation (Nathan et al., 2006), and ubiquitination (Zhu et al., 2005), but not by citrullination and methylation. Thus, histone H2B acetylation (K12 and K15) is involved in transcriptional activation (Schiltz et al., 1999; Kawasaki et al., 2000), and phosphorylation of histone H2B (S14) is an epigenetic marker of apoptotic cells (Cheung et al., 2003). Deacetylation of K15 is essential for H2B S14 phosphorylation, and inhibition of deacetylation suppresses internucleosomal DNA degradation (Ajiro et al., 2010). Histone H2B is phosphorylated by irradiation, which accumulates in irradiation-induced foci (Fernandez-Capetillo et al., 2004). Ubiquitination of histone H2B is involved in DNA breaks (Wu et al., 2009). Since our findings suggest that histone H2B was involved in the recognition of both virus- and host-derived DNA, modification of histone H2B may also affect immune responses.

CONCLUDING REMARKS

It was long believed that the sole function of histones is to wrap genomic DNA for nucleosome assemblage. However, recent studies suggest a potential role for histones in other physiological functions in extrachromosomal settings. Histone H2A.X is phosphorylated in response to dsDNA breaks and recruited to the site of the break (Redon et al., 2002). Histone H3.3 accumulates in condensed chromatin where gene transcription is activated (Janicki et al., 2004). Also, histone H1.2 is a cytochrome *c*-releasing factor that appears in the cytoplasm after exposure to X-ray-irradiation (Konishi et al., 2003). More striking evidence is that extracellular histones have a cytotoxic ability and act as major mediators of death in cases of sepsis (Xu et al., 2009). In addition, human histone H2A and H2B have microbicidal activity, and are involved in killing promastigotes of *Leishmania amazonensis (L. amazonensis)*, *L. major*, *L. braziliensis*, and *L. mexicana*. Exposure to histones markedly decreased the infectivity of promastigotes in murine macrophages *in vitro* (Wang et al., 2011). These data strongly suggest that extrachromosomal and extracellular histones work as an alarmin to maintain cellular homeostasis by changing their modifications and subcellular localizations. Thus, extrachromosomal histone H2B acts as a sensor for dsDNA aberrantly present within the cell, alerting cells to dangerous situations, such as infection, apoptosis, DNA breaks, and cell injury (**Figure 2**). This mechanism may also play an important role in autoimmunity, transplantation rejection, gene-mediated vaccines, and other therapeutic applications.

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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.

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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

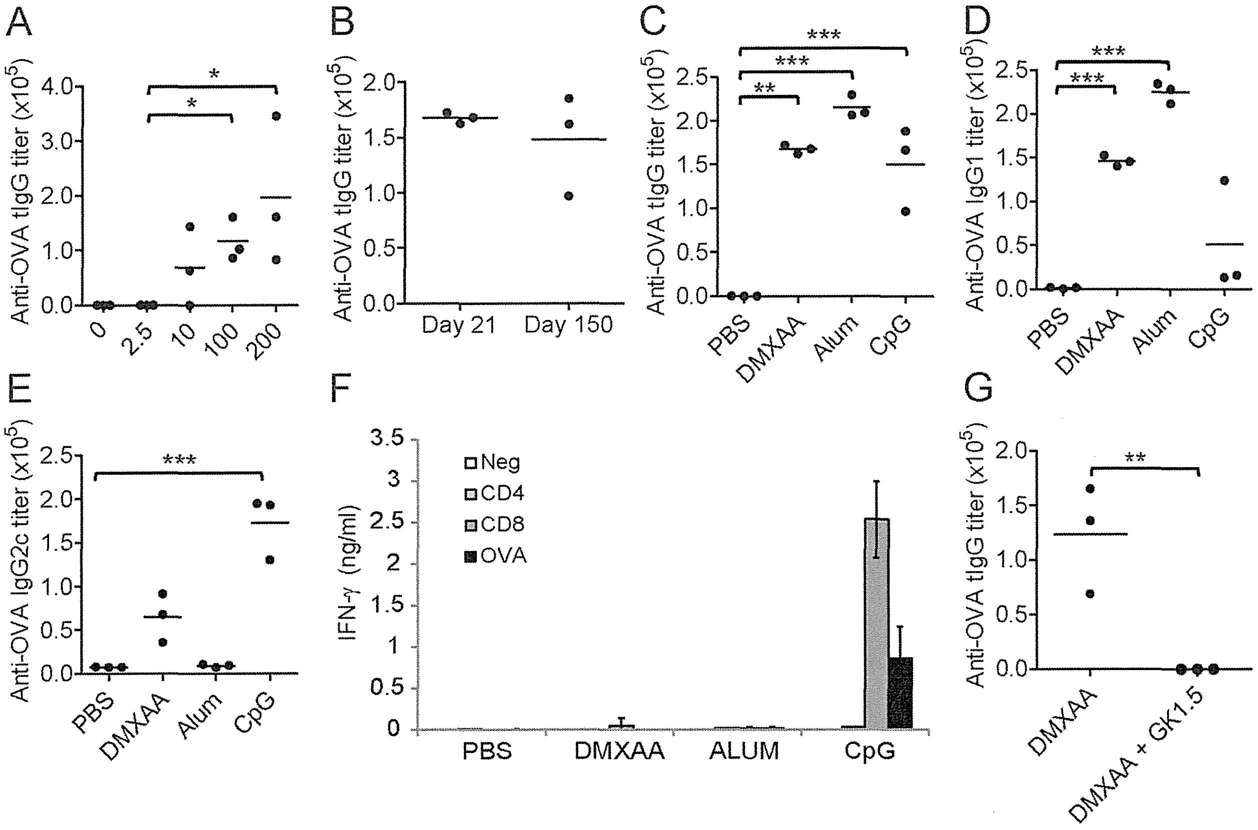

Figure 1. DMXAA acts as a potent adjuvant. (A) Anti-OVA tIgG titers of C57BL/6 mice immunized with 100 µg OVA plus the indicated doses of DMXAA (µg). (B) Anti-OVA tIgG titers of C57BL/6 mice 21 days and 150 days after immunization with 100 µg OVA and 100 µg DMXAA. (C–E) C57BL/6 mice were immunized twice 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.
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could the induction of preferential T$_H$2 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

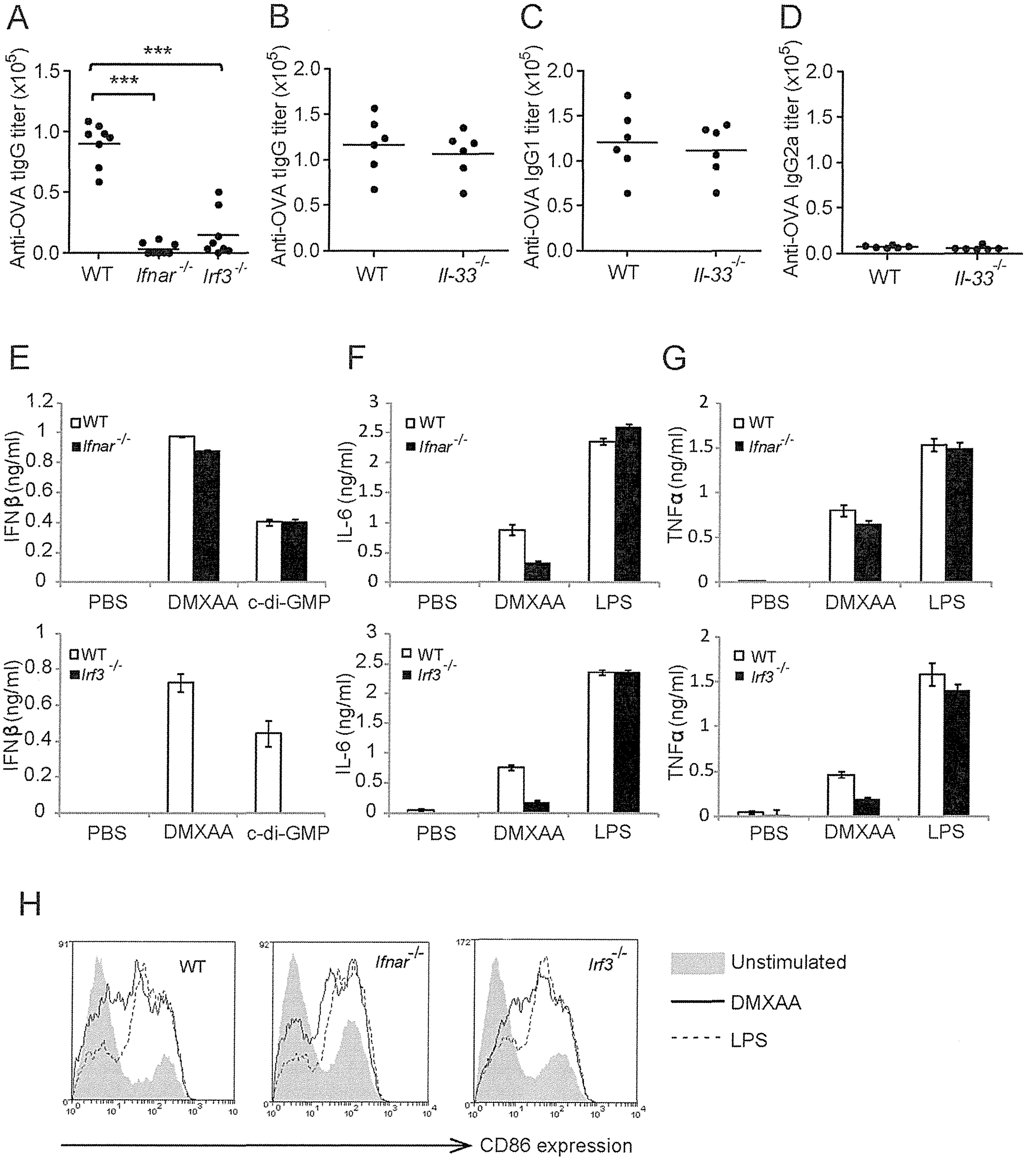

Figure 2. Adjuvant effects of DMXAA require type-I-IFN responses induced by IRF3 activation. Anti-OVA (A) tIgG antibody responses of WT C57BL/6, *Ifnar*$^{-/-}$ and *Irf3*$^{-/-}$ mice immunized twice i.d. with 10 µg OVA plus 100 µg DMXAA. (B) tIgG, (C) IgG1 and (D) IgG2a antibody titers against OVA 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.
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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 $T_{H}2$ response while type-I-IFN is commonly associated with the

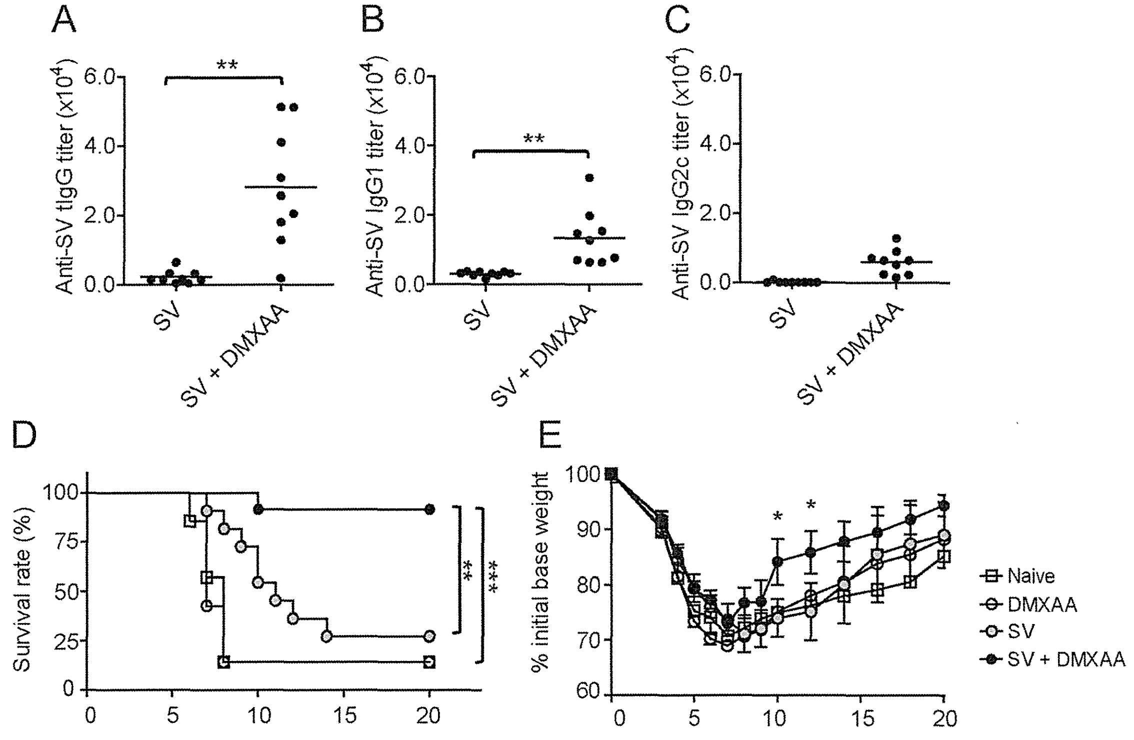

Figure 3. Adjuvant effect of DMXAA can improve potency of influenza SV vaccine and protect mice from a lethal challenge. C57BL/6 mice were immunized twice i.d. with 0.75 µg SV and 100 µg DMXAA, and their sera were assessed for anti-SV (A) tIgG, (B) IgG1 and (C) IgG2c antibodies. ** *P*<0.01 by Students 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.
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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.

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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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