Receptors that recognize uric acid have been reported and Liu-Bryan et al. revealed that TLR2, TLR4, and their adaptor molecule MyD88 are important for uric acid-mediated inflammation (Liu-Bryan et al., 2005). In contrast, the uric acid-mediated activation of DCs was shown to be TLR4-independent, suggesting the possible existence of other receptors that recognize uric acid in addition to TLR2 and TLR4 (Shi et al., 2003). To solve this question, Martinon et al. demonstrated that uric acid could be sensed by another receptor, NOD-like receptor family, pyrin domain-containing 3 (NLRP3), and induced to produce interleukin (IL)-1β through caspase-1 activation (Martinon et al., 2006). NLRP3 is a member of the NLR family, and a component of the inflammasome, a platform that induces IL-1β and IL-18 production. NLRP3 senses various types of pathogen infections or irritants such as *Candida albicans*, *Legionella pneumophila*, *Listeria monocytogenes*, *Malaria hemozoin*, alum, silica, and asbestos as well as uric acid (Kanneganti et al., 2006; Martinon et al., 2006; Dostert et al., 2008, 2009; Eisenbarth et al., 2008; Gross et al., 2009). Collectively, these results revealed that NLRP3 is a promiscuous receptor that senses PAMPs and DAMPs and can induce inflammatory responses.

ADENOSINE TRIPHOSPHATE (ATP)

ATP is an essential purine base required for almost all physical responses such as glucose metabolism, muscle contraction, biosynthesis, and molecular transfer. However, extracellular ATP from injured cells or non-apoptotic cells also serves as a danger signal through the activation of NLRP3 and caspase-1 (Communi et al., 2000). Previous detailed research has shown the importance of other ion channel molecules, namely, P2X7 and pannexin-1, in inducing extracellular ATP-mediated caspase-1 activation following IL-1β maturation (Ferrari et al., 2006; Kanneganti et al., 2007). The formation of the NLRP3 inflammasome requires an adaptor molecule, apoptosis-associated speck-like protein containing a carboxy-terminal caspase recruitment domain (ASC). ASC-deficient mice cannot activate caspase-1 and thus do not produce mature IL-1β following exposure to large amounts of ATP, suggesting that ATP-mediated IL-1β production is dependent on the NLRP3 inflammasome (Mariathasan et al., 2004). However, although extracellular ATP has been suggested to act as a DAMP molecule, there is no correlation between high amounts of extracellular ATP acting as DAMPs *in vitro* and physiological conditions *in vivo*. Eckle et al. suggested that most extracellular ATP might be immediately hydrolyzed by ectonucleotidases (Eckle et al., 2007). Taken together, investigation into the roles of extracellular ATP in inducing pathological and immune responses *in vivo* may provide important clues regarding the mechanism underlying inflammation induction by DAMP molecule recognition or in the development of inflammatory diseases.

NUCLEIC ACID-RELATED DAMPs

UNMETHYLATED CpG MOTIF AND GENOMIC DNA

As described above, uric acid and ATP are products of purine metabolism. Nucleic acids such as adenine or guanine are also purine metabolites. Nucleic acids exist in all organisms including pathogens, and function as a store of genetic information for protein translation and synthesis. Bacterial genomic DNA can be recognized as a PAMP, as it contains unmethylated CpG motifs whose frequency is higher in genomic DNA derived from pathogens compared with that of vertebrates. The earliest research related to bacterial genomic DNA as PAMPs was reported more than hundred years ago. Bruns et al. investigated heat-killed gram-negative or gram-positive bacteria as an immunotherapeutic agent termed Coley's toxin, for cancer (Swain, 1895). Although LPS is a major factor in mediating anti-tumor effects, other factors may be connected with its physiological function, as gram-positive bacteria do not express LPS. A hundred years on from the discovery of Coley's toxin, several studies have shown that bacterial DNA can activate natural killer (NK) cells or B cells, suggesting that the bacterial genomic DNA in Coley's toxin could contribute to its anti-tumor activity by stimulating NK cells (Shimada et al., 1986; Messina et al., 1991). Krieg et al. further revealed that bacterial genomic DNA contains unmethylated CpG motifs that can stimulate B cells and NK cells, and induce inflammatory cytokine production. Interestingly, methylated bacterial DNA failed to stimulate immune cells, indicating that unmethylated CpG motifs may act as PAMP molecules (Krieg et al., 1995; Klinman et al., 1996). However, whether genomic DNA containing methylated CpG motifs is incapable of innate immune activation remains controversial. In 1962, Glasgow et al. reported that ultraviolet-inactivated vaccinia virus, a DNA virus, resulted in IFN production in mouse cells (Glasgow and Habel, 1962). In addition, Suzuki et al. showed that viral DNA, vertebrate DNA and bacterial DNA induced the upregulation of major histocompatibility complex (MHC) class I expression and the type I IFN-related activation of transcription factors such as STAT3 in rat thyroid cells, suggesting that genomic DNA also activates innate immune signaling in a CpG-motif-independent manner (Suzuki et al., 1999). Interestingly, the structure of DNA strongly affects DNA-mediated innate immune activation. Double-stranded, right-handed B-form DNA, but not the left-handed Z-form DNA, strongly induced type I IFN production. Genomic DNA has a high content of B-form DNA, indicating that it may also function as a PAMP or DAMP (Ishii et al., 2006). Mitochondrial DNA has been also reported to function as a DAMP molecule. Zhang et al. reported that cellular injury caused the release of mitochondrial DNA, and induced systemic inflammatory responses via p38 MAPK activation in a TLR9-dependent manner. In addition, trauma patients had higher amounts of mitochondrial DNA than did healthy volunteers, suggesting that mitochondrial DNA could be considered a marker of inflammatory disease (Zhang et al., 2010). When the clearance of mitochondrial DNA by autophagy was inhibited, IL-1β production was augmented via the NLRP3 inflammasome to activate caspase-1, indicating that the amount of mitochondrial DNA DAMP activity is regulated by autophagy to suppress erroneous activation of innate immunity (Nakahira et al., 2011). Indeed, it was revealed that autophagy negatively regulates RNA-mediated type I IFN production, possibly to maintain cellular homeostasis (Jounai et al., 2007).

CORRELATION BETWEEN AUTOIMMUNE DISEASE AND DNA DAMPs

Both DNA and RNA can function as PAMPs and DAMPs, and are closely connected with inflammatory responses and the development of inflammatory disease. Direct evidence for DNA acting as a DAMP was shown using DNase-deficient mice. DNase I is present in extracellular compartments such as the sera and urine, and functions to degrade single-stranded DNA (ssDNA), double-stranded DNA (dsDNA), or chromatin, which are released from damaged or necrotic cells. Napirei et al. constructed DNase I-deficient mice, and reported that they presented with the classical symptoms of systemic lupus erythematosus (SLE) and glomerulonephritis (Napirei et al., 2000). In addition, DNase II deficient mice showed a similar phenotype to DNase I knockout mice. DNase II in the lysosomes of macrophages degrades DNA from apoptotic cells or nuclear genome DNA from liver erythroblasts. Interestingly, DNase II-deficient mice presented with lethal anemia owing to high levels of type I IFN production, caused by the accumulation of non-degraded genomic DNA in liver macrophages (Yoshida et al., 2005). In support of this, *DNase II* and *IFNRa/b* double knockout mice showed a non-lethal phenotype, but developed rheumatoid arthritis-like symptoms (Kawane et al., 2006), which could be attenuated by anti-TNF-α antibody treatment. This suggested that the accumulation of genomic DNA in macrophages induced inflammatory cytokines, including type I IFNs and TNF-α, and the synergistic action of these inflammatory cytokines resulted in lethal systemic inflammation (Kawane et al., 2006). Furthermore, studies on DNase III, also known as TREX1, also revealed that DNA could function as a DAMP. TREX1 is the major 3′ → 5′ DNA exonuclease for DNA editing in DNA replication or DNA repair. Morita et al. showed that *trex1*-deficient mice had a reduced survival rate owing to high susceptibility to inflammatory myocarditis, although null mice showed no spontaneous mutations or tumor development (Morita et al., 2004). To explain why *trex1*-deficient mice develop inflammatory myocarditis, Crow et al. demonstrated that the mutation in the *trex1* gene that abolished TREX1 enzyme activity was responsible for the development of Aicardi-Goutieres syndrome (AGS), a severe neurological brain disease with high levels of IFN-α in cerebrospinal fluid or serum, suggesting that TREX1 is a suppressor of DNA DAMP-mediated inflammatory responses (Crow et al., 2006). Furthermore, it was previously shown that the abolishment of interferon regulatory factor 3 (IRF3) or IFN-α receptor 1 ameliorated the AGS symptoms in *trex1*-deficient mice (Stetson et al., 2008). Collectively, these findings suggest that the dysregulation of self-DNA results in severe inflammatory responses such as high levels of type I IFNs leading to autoinflammatory disease.

NUCLEIC ACID SENSORS

Host cells are equipped with numerous types of receptors to recognize nucleic acids as PAMPs or DAMPs. These receptors function to protect the host from pathogen infection, but may also cause autoimmune disorders by inducing the constitutive activation of inflammatory responses (**Figure 1**). In this section, we introduce the well-characterized nucleic acid sensors.

TLRs

A large body of research exists demonstrating the TLR-mediated sensing of nucleic acids. TLR3 preferentially senses double-stranded RNA (dsRNA) species, which can originate from some viruses, and TLR3 is associated with induction of innate immunity in response to infection with West Nile virus, respiratory syncytial virus, and encephalomyocarditis virus (Wang et al., 2004; Groskreutz et al., 2006; Hardarson et al., 2007) (**Figure 2**). In addition, artificial dsRNA, poly (I:C), has been well-characterized as a ligand for TLR3. Although pathogen-related dsRNAs act as PAMPs, Kariko et al. reported that host messenger RNA could be sensed by TLR3 to induce inflammatory responses (Kariko et al., 2004). RNA released from necrotic cells can also elicit type I IFN production, suggesting that host RNA might function as a DAMP upon cellular injury (Kariko et al., 2004).

TLR7 and TLR8 recognize single-stranded RNA (ssRNA), and induce anti-viral innate immune responses against influenza virus or vesicular stomatitis virus (Lund et al., 2004) (**Figure 2**). Regardless of their common ligands, the cellular and tissue distribution of TLR7 expression is in contrast to that of TLR8. Human TLR7 is highly expressed in plasmacytoid DCs that preferentially induce type I IFN production, and is expressed at lower levels in myeloid cells. Conversely, the level of TLR8 expression is higher in monocytes and in monocyte-derived DCs than in plasmacytoid DCs (Hornung et al., 2002). Furthermore, mouse TLR8 did not respond to ssRNA, but human TLR8 did, suggesting that TLR8 might be inactivated in mice, although several papers have also linked mouse TLR8 with neuronal apoptosis and autoimmunity (Heil et al., 2004; Gorden et al., 2006; Ma et al., 2006).

In addition to the recognition of PAMPs, Vollmer et al. revealed that promiscuous recognition through TLR7 or TLR8 causes the development of SLE with high levels of type I IFNs and TNF-α production (Vollmer et al., 2005). Because the sera from SLE patients contains high levels of autoantibodies against self-antigens, such as small nuclear ribonucleoprotein particles (snRNPs) including ssRNA, TLR7, or TLR8 could recognize the immunocomplex of snRNPs with autoantibodies thorough Fc receptor-mediated internalization (Vollmer et al., 2005). Interestingly, TLR7 appears to be a specific sensor for the induction of type I IFN production from plasmacytoid DCs, whereas TLR8 is specific for TNF-α production from monocytes in SLE patients, suggesting that plasmacytoid DCs and monocytes collaborate to develop inflammatory responses in SLE via distinct sensors.

TLR9 senses ssDNA containing unmethylated CpG motifs. Previous studies have revealed that TLR9 recognizes genomic DNA from pathogens such as murine cytomegalovirus and Herpes simplex virus type 1 or type 2 as PAMPs (Hemmi et al., 2000; Lund et al., 2003; Krug et al., 2004a,b) (**Figure 2**). With regard to the development of autoinflammatory disease, TLR9 has been also been reported to recognize self-antigens complexed with autoantibodies. Leadbetter et al. revealed that autoreactive B cells were activated by a chromatin-autoantibody complex in a TLR9- and MyD88-dependent manner (Leadbetter et al., 2002). In addition, self-DNA-containing immune complexes, which are a well-characterized marker for SLE, were recognized by TLR9 through FcγRIIA-mediated internalization in plasmacytoid DCs

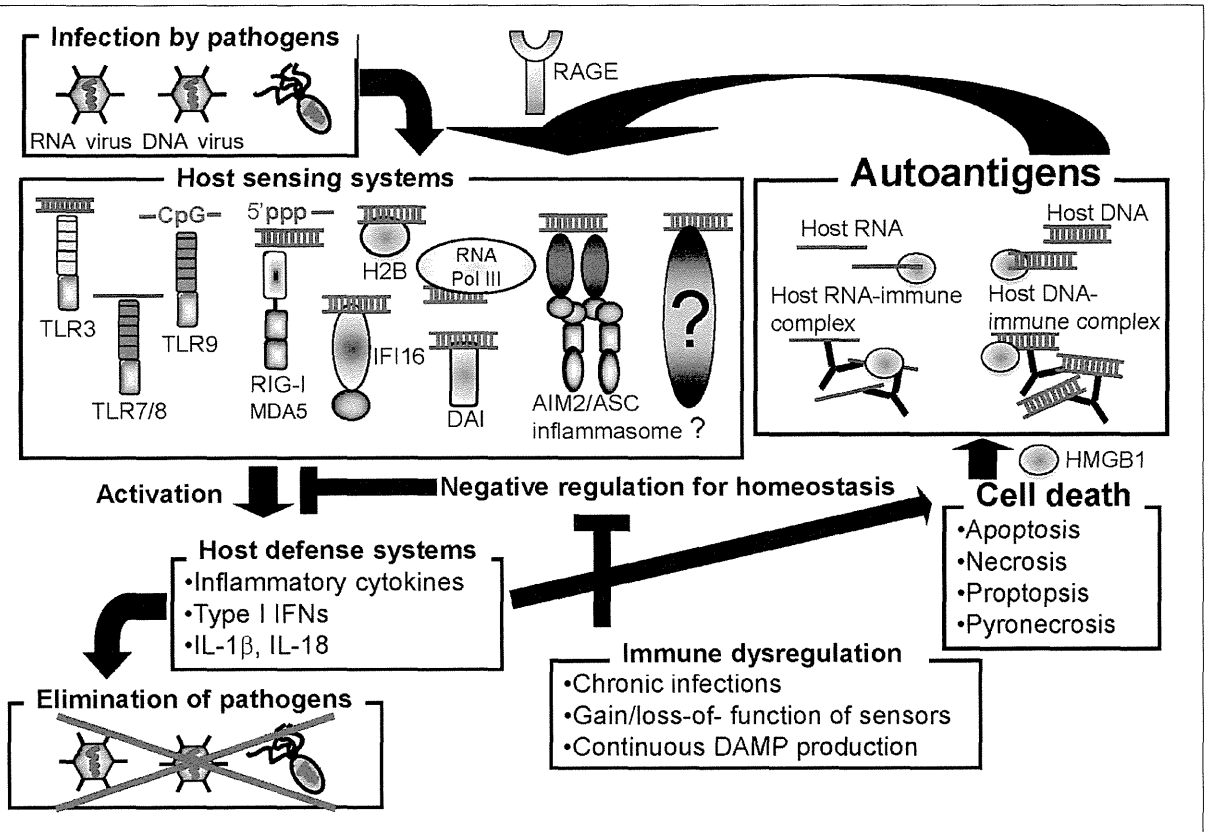

FIGURE 1 | Autoimmune disorders may be induced by promiscuous sensing of nucleic acids.

(Means et al., 2005). Thus, immune complexes containing self-DNA may signal as DAMPs through TLR9, although extracellular receptors such as FcγRIIA may be required for the delivery of autoimmune complexes to the TLR9-localizing compartment.

As described previously, the subcellular localization of TLRs is important for the recognition of DNA, because TLR3, 7, 8 and 9 localize to the endosomal compartment. Previous studies identified three adaptor molecules, Unc93B1, PRAT4A, and gp96, which are important for the trafficking of TLRs to sites for sensing their ligands. Unc93B1 functions to control the trafficking of TLRs 3, 7, and 9 from the endoplasmic reticulum (ER) to the endosome. PRAT4A is localized in the ER and acts as a regulator of the subcellular distribution of most TLRs except for TLR3. Gp96 is a member of the heat shock protein (HSP) 90 family, and resides in the ER where it controls the maturation of TLRs 2, 4, 5, 7, and 9 (Saitoh and Miyake, 2009). Because TLR7 and TLR9 are regulated by the same molecular machinery, the crosstalk between TLR7 and TLR9 may affect the sensing of auto-nucleic acids and the development of autoinflammatory disease. Christensen et al. showed that a deficiency of TLR9 results in malignant symptoms in a mouse model of lupus, despite the levels of antibody production specific for DNA and chromatin being down-regulated (Christensen et al., 2005). In contrast, TLR7-deficient mice developed attenuated lupus symptoms (Christensen et al., 2006). In addition, a recent study revealed that TLR9 suppressed the progression of autoinflammatory disease by antagonizing TLR7, suggesting that TLR9 counteracts TLR7 upon the recognition of self-immunocomplexes containing ssRNA or ssDNA (Nickerson et al., 2010). To support the interaction between TLR7 and TLR9 upon the development of autoimmune disease, Fukui et al. generated Unc93B1$^{D34A/D34A}$ knock-in mice to show that TLR9 competes with TLR7 for binding to Unc93B1 in the healthy state, while TLR7 is constitutively activated upon autoinflammatory responses because TLR9 has a lower affinity for the Unc93B1-like Unc93B1$^{D34A/D34A}$ mutant (Fukui et al., 2011).

RIG-I-LIKE RECEPTORS (RLRs)

Although TLRs can sense both non-self and self nucleic acids, fibroblasts, and endothelial cells that do not express TLRs also produce type I IFNs in response to infection with pathogens, indicating the existence of other receptors that sense nucleic acids. Yoneyama et al. determined that a cytoplasmic DExD/H box RNA helicase, RIG-I, senses infection by RNA viruses as well as artificial dsRNA, and induces innate antiviral immune responses mediated by type I IFNs (Yoneyama et al., 2004) (**Figure 2**). In addition to RIG-I, melanoma differentiation factor-5 (MDA5)

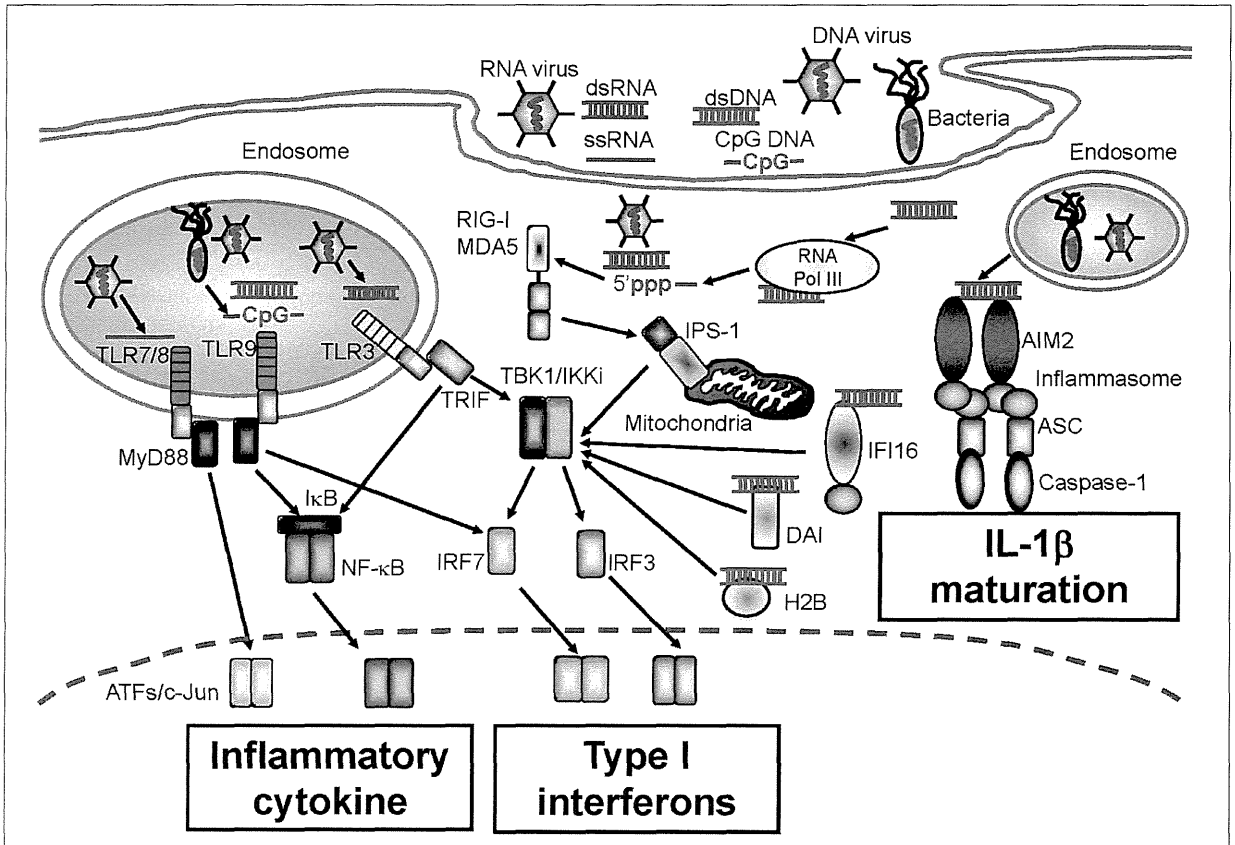

FIGURE 2 | Intracellular sensors for nucleic acids.

and laboratory of genetics and physiology-2 (LGP2) were also identified; these receptors were classified as RLRs because their protein structures were similar to that of RIG-I (Yoneyama et al., 2005). To induce an anti-pathogen immune response, a CARD domain in RIG-I and MDA5 transmits down-stream signals through homophilic interactions with the CARD adaptor molecule, IFN-β promoter stimulator-1 (IPS-1, also known as MAVS, Cardif, or VISA) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005). The function of LGP2 is controversial. Some *in vitro* studies showed that LGP2 negatively regulates RIG-I- or MDA5-mediated innate immune responses by competing for binding with their RNA ligands (Yoneyama et al., 2005; Bamming and Horvath, 2009). However, *in vivo* studies using *lgp2*-deficient mice revealed that LGP2 is a cofactor of RLR-mediated innate immune signaling (Venkataraman et al., 2007; Satoh et al., 2010).

RLRs sense pathogen-derived RNA species as PAMPs to induce type I IFN production, while MDA5 has been detected as an autoantigen in clinically amyopathic dermatomyositis patients (Sato et al., 2009; Nakashima et al., 2010). Although it is not clear how extracellular MDA5 is produced, the accumulation of immunocomplexes containing MDA5 is a marker for the frequency of rapidly progressive interstitial lung disease (Sato et al., 2009; Nakashima et al., 2010). Accompanying these observations, loss of function single nucleotide polymorphisms have been found in RIG-I and IPS-1 that are closely related to the development of autoimmune disease (Pothlichet et al., 2011), suggesting that inhibition of RLR signaling may be important in the progression of autoimmune disease. However, as described earlier, excessive production of inflammatory cytokines including type I IFNs appears to result in autoinflammatory disease. In contrast, the dysfunction of RLRs induces poor type I IFN production, but leads to autoimmune disease (Nakashima et al., 2010; Pothlichet et al., 2011). One possibility to explain this phenomenon is that non-functional RLRs result in an increased susceptibility against various types of virus infections, and the subsequent virus-mediated cell death may cause the release of DAMPs and signaling through DAMP receptors. Support this possibility, the loss of MDA5 function increased the susceptibility of beta cells to viral infection with picornavirus or encephalomyocarditis virus-D, and resulted in type 1 diabetes, whose types of diabetes are often caused by virus infection or autoimmunity (Colli et al., 2010; McCartney et al., 2011). Further analyses are required to elucidate the cross-talk between RLR signaling and the development of autoimmune disease.

ABSENT IN MELANOMA 2 (AIM2)-LIKE RECEPTORS (ALRs)

Although various NLR family members that can induce the activation of caspase-1 and maturation of IL-1β, IL-18, and IL-33 in response to a wide range of PAMP and DAMP molecules have been identified, no sensor of intracellular dsDNA for IL-1β maturation has been identified. However, four research groups concurrently reported a role for the novel intracellular DNA sensor, AIM2, in the activation of caspase-1 following IL-1β production (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). AIM2 belongs to a family of hematopoietic interferon-inducible nuclear proteins with a 200-amino acid repeat (HIN-200), known as the p200 or PYHIN family. Currently, four HIN-200 family molecules have been identified in humans, and six in mice. HIN-200 family molecules share similar structural features, including a pyrin domain at the NH_2 terminus, and a HIN-200 domain at the COOH terminus. Similar to the role of NLRP3 in IL-1β production, AIM2 causes oligomerization of the inflammasome upon DNA binding. The AIM2 inflammasome recruits ASC, an essential adaptor molecule, and induces NLRP3 inflammasome formation through homophilic interactions between the pyrin domain in AIM2 and that in ASC (**Figure 2**). The importance of the AIM2 inflammasome upon PAMP recognition has been confirmed by infection experiments using *aim2*-deficient macrophages infected with *Francisella tularensis*, *L. monocytogenes*, vaccinia virus, herpes simplex virus-1 and mouse cytomegalovirus (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010).

A second ALR, interferon-inducible protein 16 (IFI16) in humans (a homologue of p204 in mice), has been also investigated as an intracellular dsDNA sensor. However, while AIM2 induces IL-1β production in response to intracellular dsDNA binding, IFI16 is a sensor for type I IFN production upon recognition of intracellular dsDNA (Unterholzner et al., 2010). Although IFI16 also contains a pyrin domain, the pyrin in IFI16 is quite distinct from that in AIM2 as it has a lower affinity for ASC. Consistent with these different features of pyrin, IFI16-mediated type I IFN production upon intracellular dsDNA stimulation was not affected by ASC deficiency, suggesting that the two HIN-200 family molecules regulate both IL-1β and type I IFN production upon the recognition of intracellular dsDNA (Unterholzner et al., 2010). Although AIM2-mediated signaling appears to be distinct from IFI16-mediated type I IFN production, recent research has revealed that IFI16 negatively regulates the AIM2-mediated activation of caspase-1 (Veeranki et al., 2011). As increased inflammatory cytokine production is closely related to the development of autoinflammatory disease, the regulation between AIM2-mediated innate immune signaling and IFI16 might be deregulated in patients with autoimmune disease.

Roberts et al. identified p202 and AIM2 as cytosolic DNA binding proteins in mice. p202 is another ALR molecule without a pyrin domain, indicating an inability to bind ASC for inflammasome formation (Roberts et al., 2009). p202 appears to be a negative regulator for AIM2-mediated signaling, as the reduction of p202 results in higher AIM2-mediated activation of caspase-1 in response to intracellular DNA. However, elevated levels of p202 have been reported to induce SLE-like symptoms in mice (Rozzo et al., 2001). Interestingly, p202 levels are varied among mouse species, while AIM2 is expressed at the same level, indicating that p202 expression is tightly correlated to SLE development. Furthermore, Ravichandran et al. revealed that ablation of the *aim2* gene leads to higher expression of p202 and type I IFNs in mice, and *aim2*-deficient mice are prone to SLE (Panchanathan et al., 2010). Taken together, these findings suggest that mouse p202 might be homologous to human IFI16. In support of this, expression levels of IFI16 and anti-IFI16 autoantibodies were dramatically increased in SLE patients, indicating that IFI16 has similar features to p202 (Mondini et al., 2006).

A recent article described a correlation between psoriasis symptoms and AIM2 activation. Psoriasis is a chronic autoinflammatory disease caused by increased IL-1β production leading to Th17 cell maturation (Ghoreschi et al., 2010). Dombrowski et al. observed increased levels of cytosolic DNA fragments in skin lesions from psoriatic patients, which could be sensed by AIM2 (Dombrowski et al., 2011). Interestingly, those DNA fragments, which might be released from skin lesions in psoriatic patients, were internalized through binding to the antimicrobial peptide LL-37 (Dombrowski et al., 2011). Previous studies have shown that the complex of self-DNA with LL-37 can activate plasmacytoid DCs to produce type I IFNs, and complex-mediated type I IFN production is closely related with skin lesion development in psoriasis (Nestle et al., 2005; Lande et al., 2007). AIM2 is an interferon-inducible gene, suggesting that LL-37 complexes with self-DNA activate plasmacytoid DCs to produce type I IFNs, and that the subsequent upregulation of AIM2 leads to IL-1β production, and finally, psoriatic skin lesions occur because of the increased levels of type I IFN production as well as IL-1β production.

HIGH MOBILITY GROUP BOX 1 (HMGB1)

HMGB1 has been reported to be a major DAMP molecule. Goodwin et al. first identified HMGB1 from calf thymus chromatin as a non-histone DNA-binding protein (Goodwin et al., 1973). However, Wang et al. showed that a mouse macrophage cell line released HMGB1 in response to LPS stimulation. In addition, LPS-treated mice developed increased serum levels of HMGB1, similar to human patients with sepsis, suggesting that HMGB1 is a DAMP molecule in regard to sepsis symptoms (Wang et al., 1999). Accumulating evidence suggests that cellular injury results in the release of HMGB1 leading to inflammation (Abraham et al., 2000; Scaffidi et al., 2002). Consistent with these observations, numerous studies have showed a correlation between HMGB1 and autoimmune/inflammatory diseases such as atherosclerosis, diabetes, SLE, rheumatoid arthritis and Sjögren syndrome (Taniguchi et al., 2003; Porto et al., 2006; Urbonaviciute et al., 2008; Devaraj et al., 2009).

As described previously, higher serum levels of immunocomplexes of self-DNA with autoantibodies is a hallmark of SLE. Previous research has shown that HMGB1 is also contained in immunocomplexes and can elicit inflammatory cytokine production, suggesting that HMGB1 may be a carrier of DNA DAMPs (Tian et al., 2007; Urbonaviciute et al., 2008). Furthermore, HMGB1 appears to promiscuously bind numerous molecules such as LPS, IFN-γ, IL-1β, and CXCL12 to induce synergistic

physiological responses (Sha et al., 2008; Youn et al., 2008; Campana et al., 2009). Moreover, HMGB1 can sense pathogen-derived nucleic acids, which induce type I IFN production (Yanai et al., 2009). Collectively, HMGB1 might be a promiscuous carrier that enhances innate immune responses against PAMPs and DAMPs.

The receptors for HMGB1 have been investigated, but are still controversial. A well-studied receptor for HMGB1 is the receptor for advanced glycation end products (RAGE). Similar to HMGB1, RAGE is a promiscuous receptor that can bind to various ligands including DNA, RNA, SAA protein, HSPs and prion protein, suggesting that RAGE may sense a variety of DAMP molecules in an HMGB1-dependent or -independent manner (Sims et al., 2010). Experiments with *rage*-deficient mice revealed that HMGB1-mediated DNA sensing requires RAGE for internalization of DNA complexes to produce type I IFNs via TLR9 (Tian et al., 2007). Interestingly, RAGE could associate with TLR9 upon recognition of the A type of CpG-HMGB1 complex, indicating a possible function for RAGE as a bridge molecule between the extracellular HMGB1-DNA complex and the TLR9 compartment (Tian et al., 2007). In contrast to this observation, nucleosomes could sense HMGB1 complexes independently of RAGE. Instead of RAGE, TLR2 appears to be important for the recognition of HMGB1-nucleosome complexes, suggesting that the sensing machinery of the HMGB1-nucleosome complex might be distinct from that of the HMGB1-DNA complex, as the HMGB1-nucleosome complex could not elicit production of type I IFNs even though TNF-α or IL-10 were induced (Urbonaviciute et al., 2008). Furthermore, recent research identified a novel ligand for RAGE, complement C3a, that binds human stimulatory CpG DNA to induce type I IFNs in an HMGB1-independent manner. This suggests that RAGE-mediated DNA sensing may involve numerous ligands (Ruan et al., 2010). Although there are many varieties of HMGB1- or RAGE-mediated DNA recognition, both molecules are strongly associated with the induction of inflammation and the development of chronic inflammatory disease.

DNA-DEPENDENT ACTIVATOR OF IFN-REGULATORY FACTORS (DAI)

DAI has been identified as a molecule that recognizes intracellular DNA. Previous studies have revealed that DAI senses Z-type DNA; however, it may also bind to B-type DNA and induce type I IFN production through associations with TBK1 and IRF3 (Takaoka et al., 2007). Interestingly, DAI-deficient mice responded normally to cytosolic dsDNA stimulation, suggesting that DAI may function as one of a number of DNA sensors in a cell type-specific fashion (Ishii et al., 2008). Currently, the function of DAI is controversial, although the genetic adjuvanticity of DAI has been shown to induce strong cytotoxic T cell responses (Lladser et al., 2011). Although the ability of DAI to recognize DNA DAMPs has not been determined yet, DAI might be a link between the development of autoimmune disease and host DNA immune complexes.

HISTONES

Histone H2B (H2B) is a component of chromatin, and Kobiyama et al. identified that H2B also functions to sense intracellular dsDNA. Previous reports showed that histones act as DAMPs, and that excessive intracellular dsDNA induces type I IFNs through H2B (Kobiyama et al., 2010). In confirmation of this, H1 or H2 are released from the nucleus after DNA damage, and are translocated to mitochondria following the induction of apoptosis. In addition, H1, H2A, and H2B may act as antimicrobial proteins in certain animals, suggesting that H2B is an intracellular dsDNA sensor that recognizes dsDNA PAMPs and DAMPs (Kawashima et al., 2011). Histones may be related to autoimmune diseases as anti-histone antibodies were detected in patients with such diseases. Further analyses are required to clarify the relationship between histones and autoimmune disease.

Ku70

Ku70 functions in DNA repair, V(D)J recombination and in retaining the telomere. Zhang et al. showed that various DNA species-induced the production of type III interferon, IFN-λ1, and identified Ku70 as a novel DNA sensor by pull-down assay from the nucleus compartment (Zhang et al., 2011a). While other DNA sensors are important for the production of type I IFNs, Ku70 appears to be important for type III IFN production through IRF1 and IRF7. Furthermore, Ku70-mediated type III IFN production is restricted when the length of intracellular DNA stimuli is greater than 500 base pairs.

RNA POLYMERASE III

As described above, RIG-I senses intracellular RNA species, but may also recognize intracellular dsDNA. siRNA treatment of a human hepatoma cell line, Huh7, suppressed dsDNA-mediated type I IFN production. Subsequently, Chiu et al. showed that RIG-I senses the transcribed RNA byproducts of DNA templates that are generated by RNA polymerase III (as is the case for poly(dA·dT)·poly(dT·dA) and EBV genomic DNA) and induces production of type I IFNs (Chiu et al., 2009). An inhibitor of RNA polymerase III suppressed DNA-mediated type I IFN production, suggesting that RNA polymerase III is a distinct DNA sensor. However, RNA polymerase III-mediated dsDNA sensing is restricted to sequences of DNA stimuli containing less dA·dT than dG·dC.

DHX9 AND DHX36

Although the DExD/H box RNA helicase family contains RIG-I and MDA5, which function as RNA sensors, recent reports have revealed a similar RNA helicase family of molecules (DExDc family) that contain DHX9 and DHX36, which function as ssDNA sensors in plasmacytoid DCs (Kim et al., 2010). Interestingly, while DHX36 senses CpG-A, DHX9 senses CpG-B in a MyD88-dependent manner. This may suggest that ssDNA PAMPs or DAMPs are recognized by either DHX9 or DHX36, but recent research has shown that DHX9 collaborates with IPS-1 to recognize dsRNA in myeloid DCs, indicating the promiscuous sensing of DHX9 (Zhang et al., 2011b).

LEUCINE-RICH REPEAT FLIGHTLESS-INTERACTING PROTEIN 1 (Lrrfip1)

Some sensor molecules such as TLRs or NLRs share common molecular patterns, such as leucine rich repeats (LRRs), which are important for ligand recognition or protein–protein interactions.

An LRR-containing molecule, Lrrfip1, has been reported to sense intracellular DNA or RNA (Yang et al., 2010). Interestingly, whereas other DNA sensors often regulate type I IFN-related transcription factors such as IRF3/7 or caspase-1 to induce maturation of IL-1β, Lrrfip1 stimulates β-catenin and CBP/p300 to enhance *ifnb1* transcription, indicating a novel pathway involving β-catenin for type I IFN production upon cytosolic DNA sensing. Because Wnt/β-catenin signaling is also linked to tumor development, further analyses may identify the machinery involved in the regulation of type I IFN signaling by Lrrfip1 under tumor development.

STING (STIMULATOR OF INTERFERON GENES PROTEIN)

The major function of MHC class II is antigen presentation, while monoclonal antibodies against MHC class II can cause cell activation or apoptotic cell death. Jin et al. identified a novel tetraspanin family molecule, MPYS, associated with MHC-II-mediated cell death (Jin et al., 2008). Three research groups performing cDNA library screening to identify molecules associated with activation of the type I IFN promoter identified the same molecule, STING (also known as MITA, and ERIS). STING is a novel adaptor molecule that activates innate immune signaling mediated by intracellular nucleic acid stimuli (Ishikawa and Barber, 2008; Zhong et al., 2008; Sun et al., 2009). Surprisingly, the Barber research group further revealed that STING is essential for the induction of type I IFN production following sensing of cytosolic dsDNA, using STING-deficient mice. Based on their imaging analysis, STING appears to localize to the ER during the steady state, but translocates to the Golgi apparatus upon intracellular dsDNA stimulation to activate down-stream molecules such as TBK1. This suggests that STING is an essential adaptor molecule for cytosolic dsDNA-mediated type I IFN production in mice.

Cyclic-di-GMP and c-di-AMP are small molecules that function as second messengers and are important for cell survival, differentiation, colonization, and biofilm formation. Recent research has revealed that the cytosolic delivery of c-di-GMP or c-di-AMP-induced type I interferon (IFN) production from bone marrow macrophages, suggesting that c-di-GMP and c-di-AMP are bacterial PAMP molecules (McWhirter et al., 2009; Woodward et al., 2010). As type I IFN production by c-di-GMP or c-di-AMP requires their internalization, live invasive bacteria possibly produce these second messenger molecules after internalization into cells.

Recent reports have revealed that STING is a direct sensor of bacterial second messenger molecules, such as c-di-GMP or c-di-AMP (Burdette et al., 2011; Jin et al., 2011). This indicates the novel possibility that cytosolic dsDNA stimulation might produce c-di-GMP/c-di-AMP or related molecules that can be sensed by STING and induce type I IFN production.

ADJUVANTICITY THROUGH DNA DAMPs

Although DNA DAMPs are closely associated with the development of autoimmune disease, DNA DAMPs also contribute to the activation of acquired immune responses following vaccination with alum adjuvant. Previous studies have shown that genomic DNA from dying cells induces the maturation of antigen-presenting cells as well as antigen-specific antibody and cytotoxic T cell responses. This suggests that self-DNA DAMPs can activate innate immune responses that induce acquired immunoresponses. Recently, Marichal et al. demonstrated that the adjuvanticity of alum was dependent on self-DNA released from cells at the alum inoculation site (Marichal et al., 2011). NLRP3 appears to be a key sensor in the induction of alum-mediated innate immunity, although its function is only partially dependent upon alum adjuvanticity. Intraperitoneal inoculation of mice with alum induced the recruitment of neutrophils, and the resulting alum deposits contained high amounts of genomic DNA. Because treatment with DNase I attenuated alum adjuvanticity, the alum-mediated release of genomic DNA may account for its potent adjuvanticity. In addition, the alum-mediated induction of antibody production is dependent on TBK1 and IRF3 as demonstrated using knockout mice, suggesting that alum-mediated genomic DNA induces high adjuvanticity of alum via the TBK1/IRF3 pathway, while alum-mediated uric acid production is less related to alum adjuvanticity via NLRP3 (Marichal et al., 2011). Furthermore, self-DNAs from alum inoculation can activate inflammatory monocytes, and homodimers of IL-12p40 are more important than type I IFN production upon alum adjuvanticity. Taken together, these findings suggest that self-DNA DAMPs are important for pathogen elimination, the development of autoimmune disease and the adjuvanticity of alum. Further analyses are required to elucidate which types of cells produce self-genomic DNA after adjuvant inoculation, and which sensors recognize extracellular genomic DNAs.

In addition to alum adjuvant, there are many licensed adjuvants such as MF59®, AS03®, and AS04®. Both MF59® and AS03® are emulsions of oil/water containing squalene. Although both adjuvants elicit antibody responses as well as cell-mediated immune responses specific for antigens, their mode of action has not been identified. Information on the receptors for and signaling induced by these adjuvants is needed, because unfortunate side effects can be expected more easily.

CONCLUDING REMARKS

Many sorts of nucleic acid species exist in the environment. These species affect all organisms such as the evolution of organisms, the inflammatory response, and the advent of drug-resistant microorganisms. To prevent pathogen infection, mammalian cells have equipped themselves with many sorts of sensors to recognize exogenous nucleic acid species as PAMPs, while those sensors are also stimulated by endogenous nucleic acids species as DAMPs. Dysfunction of the machineries sensing both PAMPs and DAMPs is strongly associated with chronic inflammatory disease or autoimmunity. In addition, both PAMPs and DAMPs underlie the action of vaccines, because most modern vaccines contain adjuvants, which are composed of both PAMP- and DAMP-associated molecules. Therefore, the machinery responsible for sensing nucleic acids species should be further elucidated to help us understand machinery of chronic infection, autoimmune development, identifying the side effects of vaccines, and developing safe vaccine adjuvants.

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Review

Alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) induced IgG1 and IgG4 antibody responses in young children

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ABSTRACT

IgG subclass antibody responses are not fully understood. Alum-adjuvanted H5N1whole virion inactivated vaccine (WIV), a genetically reassortant vaccine seed strain originating from H5N1/A/Vietnam/1194/2004 and PR-8, induced significantly stronger antibody responses in neutralizing antibodies in children. In this report, IgG subclass antibody responses were investigated, and most serum samples were positive for IgG1 antibody before immunization. A significant response (more than 4-fold increase) of IgG1 antibody was observed in 67/193 (34.7%) and that of gG4 antibodies in 42/193(21.8%). Children <4 years of age showed a significant increase in IgG subclass antibodies but those ≥4 years showed lower responses. Alum- adjuvanted H5N1WIV induced an efficient immune response in young children especially <4 years.

Contents

1. Introduction

The 20th century saw three pandemics of influenza. The most devastating pandemic dated back to 1918, known as Spanish flu, and killed an estimated 40–50 million people, caused by H1N1 influenza virus transmitted through some animals not directly from an avian influenza virus [1]. Asian influenza A/H2N2 caused the second pandemic in 1957, and Hong Kong influenza A/H3N2 the third in 1968. After the 1968 pandemic, small local outbreaks were reported. Caused by H5N1, H7N7, or H9N2, they were considered to be from poultry. There was a regional outbreak of H5N1 in Hong Kong in 1997, and six of 18 patients died, causing a pandemic threat [2]. H5N1 is considered to be a target for pandemic vaccine, and WHO addressed sharing viruses and sequence information for a future pandemic vaccine development [3–5], and the development of an effective and safe vaccine is expected to mitigate the threat of a pandemic.

In Japan, alum-adjuvanted H5N1 whole virion inactivated vaccine (WIV) (alum concentration: 300 μg/ml) was developed using a genetically engineered reassortant, the NIBRG-14 strain, originating from H5N1/A/Vietnam/1194/2004. In a clinical phase II/III trial in healthy adults, alum-adjuvanted WIV (HA protein: 15 μg) led to favorable immunogenicity (>70% sero-conversion rate in NT antibodies) without causing any serious systemic illnesses [6]. However, when it was administered to young infants and children at a reduced dose, 7.5 or 3 μg, a high body temperature (≥38.0 °C) was observed in >60% of recipients <7 years of age, but, unexpectedly, NT antibody titers were higher than those observed in the clinical trial in adults.

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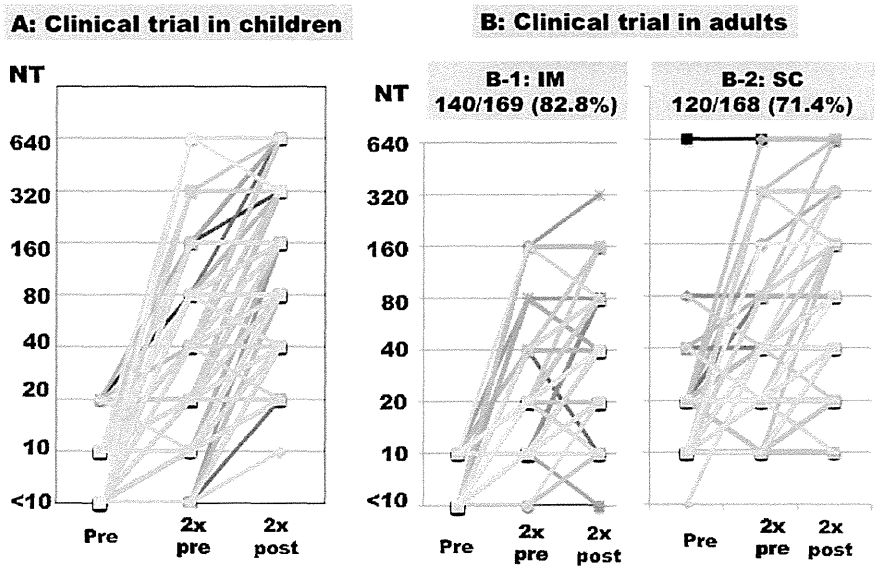

Fig. 1. Development of NT antibodies in clinical trials. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). The development of NT antibodies is shown in a clinical trial in children (A), in adults for intramuscular immunization (B-1), and for subcutaneous immunization (B-2). NT antibody titters are shown in *Y*-axis.

Functionally different IgG subclass antibody responses have been extensively investigated in mouse models, but human IgG subclass antibodies are not always functionally similar to the mouse. In mice, Th1 responses correlate with IgG2a, IgG2b and IgG3, regulated by the production of type I interferon (IFN), but in humans, IgG responses have not been strictly identified [7,8]. IgG1 is most abundant more than 50% of total IgG and IgG4, least abundant [9]. Human IgG1 reflected not only Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depends on IL-4 and IL-13, which are considered part of a Th2 response [10,11]. Primary antibody responses require T-cell help through functionally different Th1 and Th2 cytokines secreted by antigen-presenting macrophages or dendritic cells [12,13]. Therefore, the analysis of IgG subclass antibody responses after vaccination provides supportive evidence of CD4-positive T cell functions for modulating acquired immunity. In this report, IgG subclass responses were investigated in children immunized with alum-adjuvanted H5N1 WIV.

2. Summary of alum-adjuvanted vaccine trials in adults and children

An alum-adjuvanted H5N1 WIV clinical study was conducted, involving 337 subjects aged 20–59 years. Two doses H5N1 vaccine were administered with an approximately 4 weeks interval. Serum samples were obtained just before the first dose, and just before and one month after the second dose. H5N1 vaccines induced poor immunogenicity when assayed by HI tests. The NT assay was carried out by micro-neutralization methods using homologous vaccine strain [14]. NT antibody responses against H5N1 in adults and children are shown in Fig. 1. In adult study, 337 subjects were enrolled and divided into two groups: 169 of intramuscular inoculation and 168 of subcutaneous inoculation. The results of NT response are shown in Fig. 1B. Sero-conversion was observed in 260/337 subjects (77%), demonstrating four-fold or higher responses after the second dose immunization: 140/169 (82.8%) in the intramuscular immunization group (Fig. 1B-1) and 120/168 (71.4%) in the subcutaneous immunization group (Fig. 1B-2). The incidence of a febrile reaction ≥37.5 °C was reported at 3% in an adult vaccination study [15]. Using the same vaccine, a clinical trial was performed involving 374 subjects aged 6 months to 19 years. The results of NT antibodies are shown in Fig. 1A. All recipients became sero-converted in NT antibodies, but, unexpectedly, a high incidence of a febrile reaction ≥38.0 °C was demonstrated in recipients aged less than 7 years. The incidence of a febrile reaction (≥38.0 °C) after vaccination declined with age: 5/5 (100%) in subjects less than one year, 52/92 (57%) in those 1–3 years, 48/90 (53%) in those 4–6 years, 39/134 (29%) in those 7–12 years, and 3/53 (6%) in those 13–19 years. Higher NT antibody titers seemed to be found in those with a higher body temperature after vaccination [15].

3. IgG subclass antibodies against H5N1

A quantitative enzyme immunoassay (EIA) was performed to detect IgG subclass antibodies against the H5N1 vaccine virus in 193 cases where informed consent was re-obtained. H5N1 WIV antigen was adjusted to 333 ng/ml in PBS (−) and wells of a 96-well plate were coated with 33 ng. Serial dilutions of serum samples were incubated, starting at 1:200 for IgG1, IgG2, and IgG4, and 1:20 for IgG3. HRP-conjugated monoclonal antibody against each human IgG1, G2, G3, and G4 was added and stained with o-Phenylenediamine enzyme substrate. The EIA titer was expressed as the reciprocal dilution of 100×2^n that gave two-fold OD in the negative control wells by linear regression assay. The results of IgG1 antibody responses in 193 children are shown in Fig. 2A, where informed consent was re-obtained for EIA assay. Many subjects possessed high levels of IgG1 antibodies ($\geq 100 \times 2^4$) before vaccination and did not demonstrate a significant immune response after vaccination. A significant IgG1 antibody response was observed in 67 (34.7%). The IgG1 antibody response was examined in 20 randomly chosen adults and high levels of IgG1 titer were noted just before immunization without any significant increase after the vaccination (Fig. 2B).

A significant increase in IgG2 antibodies was observed in 12 subjects (6.2%) and that in IgG3 antibodies in four (2.1%). The IgG4

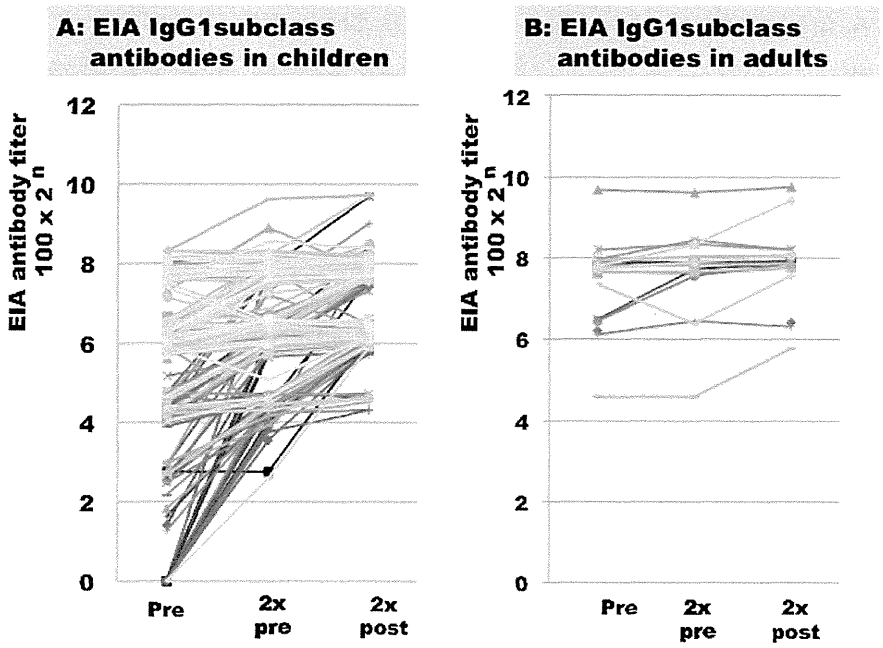

Fig. 2. IgG1 antibody responses in children and adults. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA antibody titers are shown in *Y*-axis for 100×2^n. EIA IgG1 subclass response in children is shown in panel A and that in adults in panel B.

response is shown in Fig. 3A. In 134 recipients, IgG4 antibody was negative before vaccination without a significant response after two dose vaccinations and 42 recipients (21.8%) showed positive responses. Competition assay was performed and the results of three sera are shown in Fig. 3B. Serial dilutions of antigen were mixed with serum samples and binding activity was examined. All serum samples became negative after competition and thus the IgG subclass assay was specific against H5N1 antigens.

4. IgG1 responses in different age groups

193 children were classified into four groups: aged <4 years (47 subjects), 4–6 years (42 subjects), 7–12 years (72 subjects), and ≥13 years (32 subjects). IgG1 EIA antibody responses are shown in Fig. 4 and Table 1. Among 47 subjects aged <4 years, 22 showed positive for IgG1 antibody prior to vaccination and 15 showed positive response after vaccination. Among the 25 infants in which no IgG1

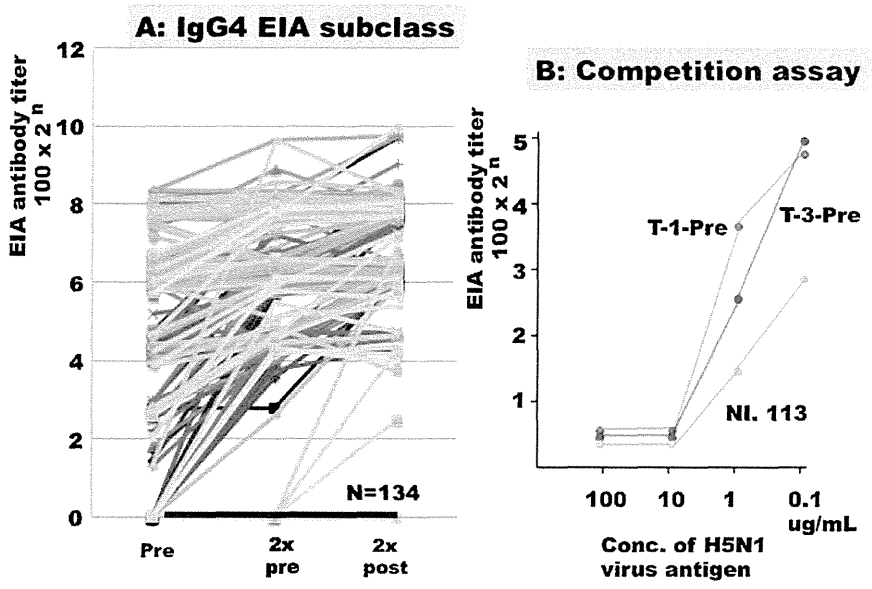

Fig. 3. IgG4 responses in children and the results of competition assay. Serum samples were obtained before the first dose (Pre) and before (2× Pre) and four weeks after the second immunization (2× post). EIA IgG4 antibody titers are shown in *Y*-axis for 100×2^n in panel A. Among 193 subjects, 134 had no EIA responses in IgG4. The results of competition assay are shown in panel B. Serial 10-fold dilutions of H5N1 WIV antigen were mixed with serum samples. The EIA activity was examined.

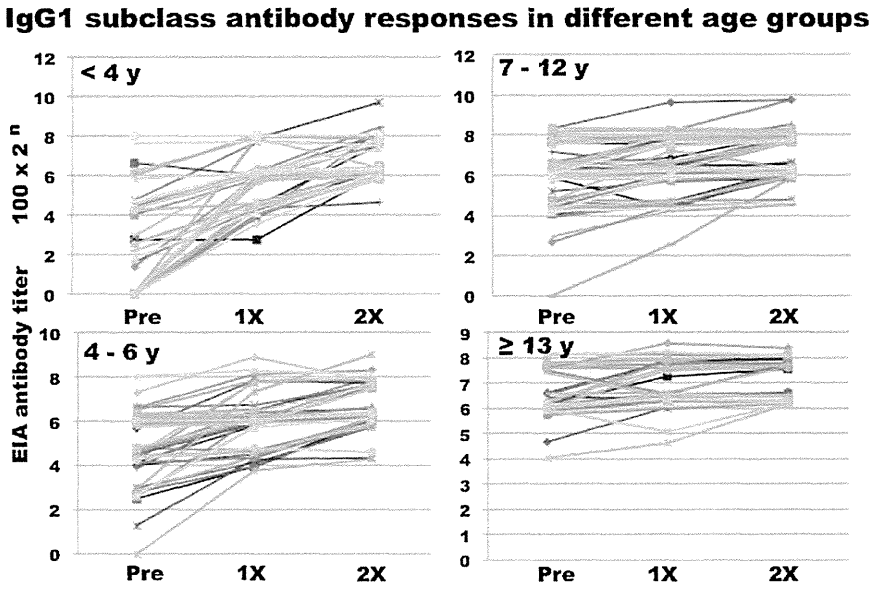

Fig. 4. Different IgG1 responses in different age groups: ≤4 years, 4–6 years, 7–12 years, and ≥13 years. Serum samples were obtained before the first dose (Pre) and before (1X) and four weeks after the second immunization (2X). IgG1 antibody titers are shown in *Y*-axis for 100×2^n.

EIA antibodies were detected before immunization, 17 showed positive after the first dose and the remaining 8 after the second dose. Of 42 subjects aged 4–6 years, 41 showed positive for IgG1 antibody before vaccination and 18 showed a significant response. One was negative pre-vaccination and sero- converted after the first dose. Of the 72 subjects aged 7–12 years, 71 were positive before vaccination and 7 showed a significant response. Among the 32 subjects aged ≥13 years, all tested positive before vaccination and only one showed a significant response. Most subject aged ≤4 years sero-converted, demonstrating four-fold or higher responses after the second immunization. Whereas, in subjects ≥7 years, IgG1 EIA antibody was detected before vaccination with a lower sero-conversion rate, similar to that observed in children ≥13 years and adults.

5. Discussion

Recent investigation on innate immunity has suggested that the development of acquired immunity against a specific antigen is modulated by the production of cytokines through functionally different Th1 and Th2 antigen-specific CD4-positive T lymphocytes [13]. The innate immune system consists of Toll-like receptors (TLRs), retinoic acid inducible gene-based (RIG)-like receptors, and nucleotide oligomerization domain (NOD)-like receptors (NLRs), known as inflammasome [16–18]. Investigation on cytokine production is essential for the better understanding of immune responses. In Japan, alum-adjuvanted H5N1 WIV has been licensed for adults but not for children. In a clinical trial of alum-adjuvanted H5N1 WIV in a pediatric group, a higher incidence of febrile reactions (≥38.0 °C) was observed with sufficient immune responses after vaccination. Cytokine productions were investigated in PBMCs obtained from non-vaccinated donors to know the reason behind these phenomena. H5N1WIV induced the higher levels of IFN-α, IL-6, IL-17, TNF-α, and MCP-1 than the control culture. With alum-adjuvanted H5 WIV, enhanced production of IL-1ß was demonstrated and IL-6 and TNF-α were produced similar to the levels obtained with H5N1 WIV [15]. WIV has genomic RNA that is recognized by TLR-7, inducing the production of IFN-α, which was essential for the antibody response in mice [19]. Inflammasome consists of NLRP3 and IL-1ß, IL-6, TNF-α, and IL-18 are induced in response to alum adjuvants through NLRP3 or other mechanisms. Alum-adjuvanted H5N1 WIV generated high titers of NT antibodies in young children, and, in this report, IgG subclass antibodies were investigated after immunization with alum adjuvanted H5N1 WIV.

IgG1 antibodies against H5N1 WIV antigens were detected in children ≥4 years of age and adult recipients before vaccination. The H5N1 influenza virus is not spread from human to human and has no history of large-scale outbreaks. The H5N1 WIV was a reassortant strain, whose envelop proteins, HA and NA were from H5N1/Vietnam/1194/2004 and remaining inner protein genes were from H1N1/PR8. Therefore, most subjects had IgG1 antibodies before vaccination. Approximately half of the recipients <4 years

Table 1
IgG1 EIA antibody responses in different age groups.

IgG1	<4 years (n=47)	4–6 years (n=42)	7–12 years (n=72)	≥13 years (n=32)
+++	22 (15)[a]	41 (18)[a]	71 (7)[a]	32 (1)[a]
– ++	17	1	1	0
– – +	8	0	0	0
– – –	0	0	0	0

+++: IgG1 antibody was positive before vaccination, one month after immunization of the first and second dose.
– ++: IgG1 antibody was negative before vaccination, and became positive after the first dose.
– – +: IgG1 antibody was negative before vaccination, and became positive after the second dose.
– – – IgG1 antibody was negative before vaccination, without antibody response after vaccination.
[a] Number of recipients with a significant responses.

had no detectable IgG1 antibodies before vaccination with a significant immune response, who did not experience H1N1 and H3N2 infections.

As for influenza infections, Garcon et al. [20] first reported high levels of IgG1 antibodies with lower amounts of IgG2 and IgG3 after immunization with different vaccine formulations; cold-adapted live recombinant, trivalent inactivated, and purified HA-conjugated vaccines to diphtheria toxoids. Hocart et al. [21] compared the subclass responses in natural infection with H3N2, and IgG1 levels in natural infection showed an 18-fold increase after infection and the other IgG subclasses, a 5- to 8-fold increase. The levels of IgG1 and IgG3 increased after immunization with live cold-adapted vaccines, and inactivated vaccines produced IgG1, IgG2, and IgG3 subclasses. IgG subclass responses were different from the vaccine formulations and also the increased levels of IgG1 differed with the serological status before vaccination. Stepanova et al. [22] reported different responses according to vaccine formulation and age. IgG1 and IgG4 responses were observed only in young adults immunized with the live influenza vaccine, the inactivated vaccine generating IgG1 and IgG3 in young adults, and IgG1 alone in the elderly. Human IgG1 reflected not only a Th1 cytokine response but also Th2 cytokine activation. IgG4 subclass switch depends on IL-4 and IL-13, which are considered part of a Th2 response [10,11].

In this report, a significant IgG1 and IgG4 antibody responses were observed after immunization with alum-adjuvanted H5 WIV especially in young infants <4 years. It provided efficient immune response in young naïve infants. Considering the previous report that alum-adjuvanted vaccine induced inflammatory cytokines, including IFN-α, IL-1ß, IL-6, and TNF-α, they would modulate the expression of co-stimulatory molecules recognized by naïve CD4 helper T cells. Therefore, the IgG4 antibody response seems to be T cell-dependent, induced by innate immune impacts of WIV with alum adjuvant. But, it caused high incidence of febrile reactions, and efficient influenza vaccine formulation for priming in young infants is expected with low incidence of febrile reactions.

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Review

Adjuvants in influenza vaccines

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ABSTRACT

The effectiveness of influenza vaccines is still controversial, and the role of adjuvants in such vaccines is briefly reviewed in this paper. Inactivated whole virus vaccines may include components that function as adjuvants, meaning that additive adjuvants are often not required. MF59 and AS03 showed higher adjuvanticity than aluminum salts in several clinical studies. Recent research has suggested that immune cell recruitment is the main mechanism underlying adjuvant actions in general, and that aluminum salts induce this recruitment via inflammation at the injected site. The aspect of how oil-based adjuvants, such as MF59 and AS03, recruit immune cells remains to be clarified.

Contents

1. Introduction

Influenza vaccines have been proven to induce high immunity in various trials. However, the coverage of seasonal influenza vaccine remains around half in Europe, America, and Asia [1], that may partially because its social usefulness is not yet fully shared in the population.

Vaccine effectiveness consists of vaccine immunogenicity, safety, and cost, and these aspects should be reviewed for assessment of influenza vaccines. In particular, vaccine adjuvants, vaccine administration routes, and/or immunization schedules may be the keys to improve vaccine efficacy and safety.

An adjuvant is used to enhance vaccine immunogenicity per se. The adjuvant effect, or adjuvanticity, would be measured by the ratio of immunogenicity (increase in geometric mean of antibody titer, percent responders, or seroconversion rate) of vaccine-with-adjuvant to vaccine-without-adjuvant in either non-clinical or clinical conditions. Recent clinical studies have suggested that AS03 or MF59 shows good adjuvanticity in influenza vaccines, but these adjuvants also increase local and systemic adverse reactions, although they are not severe.

Recently developed alternative vaccination routes such as nasal, skin patch or oral route vaccines often show better efficiency than classical administration. Several nasal vaccines (influenza [3], measles [4]), microneedle skin patch vaccines [5,6], oral vaccines (rotavirus vaccine [7]) are well studied.

Boosting immunization is promising for improving protection. Even when the priming is not sufficiently immunogenic, sequential immunization has been shown to provide enough protection.

In this review, adjuvants for influenza vaccines are briefly overviewed and the current knowledge of their functions based on molecular biology is reviewed.

2. Clinical experiences of influenza vaccines: effects of adjuvants

The World Health Organization's list of influenza vaccine developments [8] includes several studies analyzing the immunogenicity and safety profiles of adjuvanted vaccines versus non-adjuvanted vaccines (Table 1). Aluminum salts, the most world-wide and historically used adjuvants, were mostly used in the listed studies, followed by MF59® from Novartis and AS03 from GlaxoSmithKline.

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Table 1
Profiles of reviewed clinical studies that compared vaccines with and without adjuvants (numbers indicate references).

Vaccine type		Adjuvant			
		Aluminum	AS03	MF59	Others[a]
Pandemic	Whole virion	9, 21, 22	Nil	Nil	Nil
	Subunit/split	12–15	10, 11, 22	16–19	20, 24
	Recombinant	23	Nil	Nil	Nil
Seasonal	Subunit	Nil	Nil	25–27	Nil

[a] One study used Matrix M™ [20] and the other used Inulin [24].

Immunogenicity was reviewed by the increase in geometric mean of the antibody titer (GMT), vaccinee ratio of seropositivity, and ratio of seroconversion. The antibody titer was measured by either hemagglutinin inhibition assays or microneutralizing assays. The safety profile was reviewed as the frequency of vaccine-related adverse reactions, comprising local reactions of pain, induration, erythema, etc., and systemic reactions of fever, malaise, headache, etc. Since the trial designs differed, especially in doses, schedules, subject backgrounds, and details of the definitions of immunogenicity, inter-trial comparisons were not reasonable, but the authors gained the impression that adjuvanted vaccines caused more frequent adverse reactions, regardless of the adjuvant used. The severity of the adverse events was slight or moderate, and no serious adverse events were reported, indicating that these influenza vaccines adjuvanted with aluminum salts, MF59 or AS03 are tolerable.

Seven studies on aluminum adjuvanted vaccines included various types of whole virion vaccines [9,21,22], subunit/split vaccines [12–15] and recombinant vaccines [23]. They satisfied the European Medical Agency's criteria for assessment of influenza vaccine [28,29], no matter which type of vaccine were used. For example in the two doses whole-virus H5N1 vaccine study, GMT increase on 21days after the second administration was between 2.7 and 5.2 when Aluminum adjuvant was added, and was between 3.2 and 5.9 without adjuvant [9].

On the other hand, compared with studies on vaccine with other adjuvants (AS03 [10,11,22], MF59 [16–19,25–27] and others [20,24]) the trends for the adjuvant effects on the vaccine immunogenicity differed among the adjuvants, in that aluminum showed lower adjuvanticity than MF59, AS03, or other adjuvants, irrespective of the dose of aluminum (300–1000 μg/dose) or the form of aluminum (hydroxide or phosphate). One study with two doses split vaccine (7.5 μg HA per dose) adjuvanted with MF59 showed 406.9 of GMT on 21days after the second administration, while non-adjuvanted vaccine showed 156.6 [19]. Higher adjuvanticity of MF59 than aluminum salts has also been shown in a trial on hepatitis B virus vaccines [30], etc.

The protective efficacy of influenza vaccines is mostly assessed by the clinical occurrence of confirmed influenza or influenza-like illness. Direct comparisons between MF59 adjuvanted and non-adjuvanted trivalent subunit influenza vaccines showed that adjuvanted vaccines exhibited higher effectiveness in both young children in Canada [27] and elderly people in Italy [31]. In the former study where influenza illness was confirmed by means of real-time polymerase-chain-reaction in nasopharyngeal aspirates or swabs, the effectiveness of the adjuvanted vaccine was shown by decreased influenza occurrence by 75%; 13 cases among 1937 adjuvanted vaccine group presented influenza illness whereas 50 cases of 1772 non-adjuvanted vaccine group showed influenza illness [27]. In the latter study in elderly people, the protective efficacy of the adjuvanted vaccine appeared to be less, since the odds ratio for developing influenza-like illness with the non-adjuvanted vaccine (versus adjuvanted vaccine) was 1.52, while the odds ratio for non-vaccinated people (versus vaccinated) was 2.16 [31].

From these experiences, it can be said that adjuvants in subunit influenza vaccines enhance the immunogenicity except for aluminum salts, but their adjuvanticity may need more improvement to prevent clinical influenza illness sufficiently.

3. Whole virion vaccines: vaccines with "unintended adjuvant"?

While subunit/split vaccines contain virus surface proteins as the vaccine antigens, whole virion vaccines are made of whole influenza virus particles that have been inactivated, typically by formaldehyde treatment. Therefore, these vaccines are composed of not only surface proteins, such as neuraminidase and hemagglutinin (for type A and type B, as the most commonly used vaccine antigens) or hemagglutinin esterase (for type C), but also matrix proteins and genomic RNA.

A review of three whole virion vaccines suggested that they were effective even though they were without aluminum adjuvants, and one of them was more effective than the aluminum-adjuvanted whole virion vaccine [9]. Superior immunogenicity of a whole virion influenza vaccine has been demonstrated in several Toll-like receptor (TLR) 7-knockout mouse experiments, which suggested it was dependent on TLR7 signaling [32,33]. Sialo-suger chains of host bind to influenza viruses but TLR7 specifically recognizes RNA of pathogens. These studies suggest that remaining RNA of influenza virus in the whole virion vaccine might unintentionally function as an adjuvant through TLR7 signaling. It is an interesting concept that a whole virion vaccine product might contain a "built-in adjuvant" when we call aluminum salts, MF59, or AS03 are artificially added as adjuvants. However, its generalization to other single-stranded RNA virus vaccines is controversial, since TLR7 and TLR8 polymorphisms did not affect the measles vaccine antibody response [34] and a transcriptional analysis of human blood cells found similar results for a vaccine against yellow fever and poly ICLC, the specific ligand of TLR3 [35].

4. Mechanisms of influenza vaccine adjuvants

The differences in the mechanisms of aluminum and other adjuvants are not yet fully understood, but they are commonly known to induce mild inflammation with immune cell recruitment at the injection site and not to induce Th1 cellular immunity.

Aluminum salts are generally thought to catch antigens and keep them at the local injection site for periods of days to weeks, such that the antigen is slowly presented and processed by the immune system. This "depot effect" was shown historically in diphtheria toxin experiments, in which immunity was impaired when the injection site was removed, while animals with transplantation of the injection site showed transferred immunity in parallel [2]. In addition, inflammation and cell damage caused by aluminum salts were recently shown to be a critical step in their Th2-biased adjuvanticity.

MF59 is still known to be effective when it is administered in advance of a vaccine antigen. However, when MF59 is administered

at 24 h after an antigen, it is not sufficiently immunogenic. These observations show MF59 does not act via a "depot effect", but instead is supposed to condition the immune system to respond effectively. At 2 days after injection, MF59 is found in lymph node mature macrophages and the gene profile of the "adjuvant core response genes" found in microarray analyses of the injected muscle of mice suggests that the mechanism of action of MF59 involves strong recruitment of antigen-presenting cells to the injection site as early as 12 h after injection [36].

A recent comparison study between aluminum salts and MF59 in mice [37] has suggested that the degree of cell recruitment may represent the current description of adjuvanticity. Specifically, in the first 24 h, MF59 recruited significantly more neutrophils, monocytes, eosinophils, macrophages, and dendritic cells than aluminum salts.

MF59 is composed of 0.5% Tween-80 as a water-soluble surfactant, 0.5% Span85 as an oil-soluble surfactant, 4.3% squalene oil, and water. It is an oil-in-water preparation and its emulsion droplet size is approximately 130 nm. Experience with nanoparticle adjuvants suggests that the particle size may be a key factor for adjuvanticity, since microspheres with diameters of <10 nm activate antigen-presenting cells, while those with diameters of 30–100 nm act via a "depot effect". A study comparing the sizes of silica particles showed that 30-nm-diameter particles induced the most inflammation and toxicity compared with 70-nm- or 300-nm-diameter particles [38]. If this situation is universal, the cell recruitment by MF59 may not depend on its size, but on its components. A recent study [39] compared several kinds of oil for particle size, emulsion stability, and adjuvanticity in a malaria vaccine candidate and an influenza vaccine, and found that the physical/chemical characters were similar among squalene, sesame oil, grape seed oil, and soybean oil, and that squalene oil showed the highest adjuvanticity in both vaccines.

5. Concluding remarks

Adjuvanticity of MF59 and AS03 has been shown in various studies, but their mechanisms of action still remain unclear. Regardless of how MF59 and AS03 act as vaccine adjuvants, there appears to be more to do to achieve social agreement on the importance of influenza vaccines. Vaccines that are "safer and more immunogenic" and "for the high-risk population" are the goals for vaccine development.

Conflicts of interest

None declared.

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