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Minireview

Cyclic di-nucleotides: new era for small molecules as adjuvants

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Summary

The implementation of vaccination as an empiric strategy to protect against infectious diseases was introduced even before the advent of hygiene and antimicrobials in the medical practice. Nevertheless, it was not until a few decades ago that we really started understanding the underlying mechanisms of protection triggered by vaccination. Vaccines were initially based on attenuated or inactivated organisms. Subunit vaccines were then introduced as more refined formulations, exhibiting improved safety profiles. However, purified antigens tend to be poorly immunogenic and often require the use of adjuvants to achieve adequate stimulation of the immune system. Vaccination strategies, such as mucosal administration, also require potent adjuvants to improve performance. In the 1990s, immunologists found that pathogens could be sensed as 'danger signals' by receptors recognizing conserved motifs. Although our knowledge is still limited, tremendous advances were made in the understanding of host defence mechanisms regulated by these evolutionary conserved receptors, and the molecular structures which are recognized by them. This opened a new era in adjuvant development. Some of the latest players arrived to this field are the cyclic di-nucleotides, which are ubiquitous prokaryotic intracellular signalling molecules. This review is focused on their potential for the development of vaccines and immunotherapies.

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Introduction

The primary goal of vaccination is the generation of a strong and long-lasting immune response able to protect the host against infectious diseases. Live attenuated vaccines, such as some paediatric vaccines (e.g. measles, mumps, rubella, oral polio and BCG), stimulate the immune system via a transient infection caused by replicating live organisms without causing clinical illness. However, they are able to cause diseases in immune compromised individuals. Thus, a safer vaccination approach is based on the use of inactivated organisms (e.g. inactivated vaccines against polio or whooping cough) or non-replicating subunits (e.g. subunit vaccines against polio and hepatitis B virus). Although purified antigens by general rule are weak immunogens, the problem can be solved by incorporating adjuvants into the vaccine formulation. Adjuvants are components able to stimulate the immune system, which potentiate/modulate antigen-specific immune responses without exhibiting antigenicity on their own (Ebensen and Guzman, 2009; Coffman *et al*., 2010; Ribeiro and Schijns, 2010). Thus, adjuvants are critical not only for obtaining strong immune responses, but they also enable to (i) reduce the antigen dose, (ii) accelerate the process for establishing protective immunity, (iii) stimulate long term memory, and (iv) modulate the quality of the response according to the specific clinical needs. However, for optimal modulation of immune responses to vaccination, the availability of a toolbox of adjuvants exhibiting different effector functions is a prerequisite.

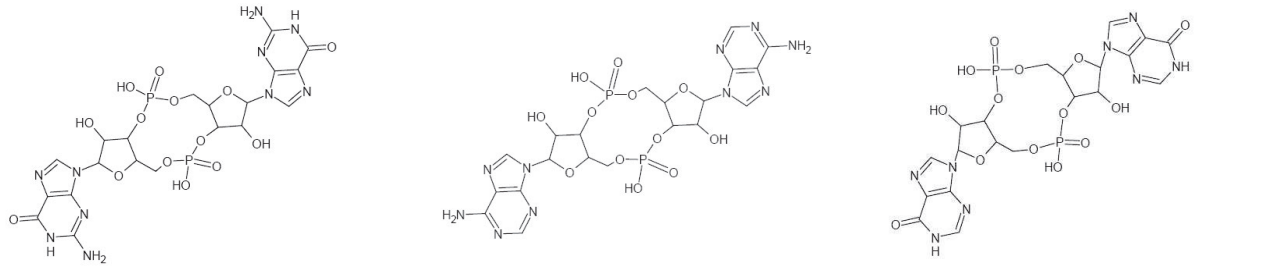
Aluminum salts (alum) were the first adjuvants licensed for human vaccines in the 1920s (De Gregorio *et al*., 2008). However, it was not until recently that we begin to understand the molecular events leading to their adjuvanticity (Eisenbarth *et al*., 2008; Kuroda, *et al*., 2011; Marichal *et al*., 2011; Mbow *et al*., 2011). Once the purely empirical process for vaccine developing became no longer accepted by the medical community, regulatory authorities and the society, the development of subunit vaccines faced a new problem, namely the lack of knowledge on the mechanisms of adjuvanticity for the candidate adjuvants, which are necessary for developing new

vaccine formulations. Hence, it became clear that new adjuvants are needed, particularly those which are amenable for the implementation of mucosal vaccination strategies. This approach leads to the induction of responses not only at systemic level, but also at the pathogen portal of entry. In this quest, the better understanding of host–pathogen interactions, in particular the mechanisms of immune recognition, immune scape and immune clearance, turned out to be crucial for the development of new adjuvants (Dey and Srivastava, 2011).

Since microbial pathogens are evolutionarily different from their hosts, they possess a large number of structures which are not present in the host and can be recognized as foreign. However, due to high mutation and division rates, pathogens have the potential to very rapidly adapt themselves to the immune system of their hosts. In 1997, Charles Janeway published the discovery of the Toll-like receptor-4 (TLR-4), the first human homologue of the *Drosophila* Toll. TLR-4 is a receptor sensing lipopolysaccharide (LPS), which is able to activate the immune system (Medzhitov *et al*., 1997; Poltorak *et al*., 1998). He also hypothesized that the innate immune system, the first and most ancient unspecific line of defence, has evolved a simple and efficient strategy to identify threats (Janeway, 1989). The immune system is armed with a very limited pool of sensors, which are able to recognize conserved and non-dispensable microbial molecules. These 'pathogen-signatures', termed pathogen-associated molecular patterns (PAMPs), are recognized by the innate immune system, making it aware of the nature of the threat. Once the first line of defence is mounted, the innate immune system instructs the adaptive immune system in the kind of response needed to effectively clear the pathogen and avoid re-infection. This theory gained popularity and still holds true, although some changes were introduced. Matzinger proposed in 2004 that the immune system is not sensing pathogens but damage-associated molecular patterns (DAMPs) induced by pathogens or other kind of processes, such as tumours (Seong and Matzinger, 2004; Miyaji *et al*., 2011). These signals are specifically recognized by receptors term pattern-recognition receptors (PRRs), a group of conserved molecules including intra- and extracellular TLRs, cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid inducible gene-1 (RIG-I)-like receptors (RLRs) expressed in different types of immune cells, such as dendritic cells (DCs) (Steinman and Hemmi, 2006; Koyama *et al*., 2007; Kufer and Sansonetti, 2011). Following PAMPs and DAMPs mediated triggering of the innate immune system via PRRs, the release of pro-inflammatory cytokines leads to the stimulation of the adaptive immune system (Miyaji *et al*., 2011).

The same signal transduction pathways activated during pathogen recognition, which lead to the promotion of an adaptive immune response able to clear infection can be subverted to promote responses to vaccination. In this context, we have assisted in recent years to the development of candidate adjuvants based on TLR agonists, which have proven their usefulness in preclinical and/or clinical studies (Higgins and Mills, 2010). New PRR agonists have shown to be effective in clinical trials, such as the TLR-2/6 and TLR-9 agonists MALP-2 and oligodeoxynucleotides containing CpG motifs (Niebuhr *et al*., 2008; Rynkiewicz *et al*., 2011). A non-toxic derivative of the TLR-4 agonist LPS, the 3-O-desacyl-4′-monophosphoryl lipid A (MPL), has been incorporated in combination with other compounds (e.g. alum) in formulations of licensed or candidate vaccines (Garcon *et al*., 2007; Garcon and Van Mechelen 2011). Recently, it was also suggested that alum, which was used for almost 80 years as adjuvant in human vaccines, activates the NACHT, LRR and PYD domains-containing protein 3 (NALP3; also known as NLRP3) inflammasome, which in turn leads to the release of caspase-1-dependent cytokines (Eisenbarth *et al*., 2008; Kool *et al*., 2008). Although the actual mechanism by which NALP3 activation leads to caspase-1 cleavage remains unknown (Dostert *et al*., 2009). On the other hand, other reports do not fully support a role for the inflammasome as alum primary target (Franchi and Nunez, 2008; Kuroda *et al*., 2011). A recent study suggests that the interaction of alum with the membrane lipid structures of DCs is essential for activity (Flach *et al*., 2011). This mechanism was proposed as a new paradigm for interactions between crystalline structures and the immune system. Finally, experimental evidence has been provided demonstrating that alum causes cell death, thereby promoting the release host cell DNA that acts as a potent endogenous immunostimulatory signal, which mediates in turn alum adjuvant activity (Marichal *et al*., 2011). These studies point out to the complexity of the underlying mechanisms of adjuvanticity. However, an in-depth description of agonists of TLR and other PRRs exceed the scope of this review (for a comprehensive review on the subject see Kawai and Akira, 2011).

In the following sections we will focus on recent findings that lead to the identification of nucleotides bis-(3′,5′)-cyclic dimeric guanosine monophosphate (c-di-GMP), bis-(3′,5′)-cyclic dimeric adenosine monophosphate (c-di-AMP) and bis-(3′,5′)-cyclic dimeric inosine monophosphate (c-di-IMP) (Fig. 1) and their potential as adjuvants. These small molecules serve as PAMPs, which are targets for immune recognition and central for the survival/virulence of pathogens; however, the mechanisms used by the host to sense them still remain poorly characterized (Libanova *et al*., 2010; Woodward *et al*., 2010; Chen *et al*., 2010; Ebensen *et al*., 2011).



bis-(3',5')-cyclic dimeric guanosine monophosphate
c-di-GMP

bis-(3',5')-cyclic dimeric adenosine monophosphate
c-di-AMP

bis-(3',5')-cyclic dimeric inosine monophosphate
c-di-IMP

Fig. 1. Chemical structure of c-di-GMP, c-di-AMP and c-di-IMP.

Origin and chemical properties of the cyclic di-nucleotides

Naturally occurring cyclic di-nucleotides are small second messenger molecules of bacterial origin with a regulatory role in several processes. The c-di-GMP was first identified as a cellulose synthase activator in *Glycanocetobacter xylinus* (Fig. 1) (Ross *et al*., 1987). Further studies demonstrated that it is essential for the regulation of biofilm formation, motility, virulence gene expression and other critical aspects of bacterial physiology in a number of pathogens (Tischler and Camilli, 2004; Zhang *et al*., 2006; Romling, 2009; Romling and Simm, 2009; Chin *et al*., 2010; Gomelsky, 2010; Ma *et al*., 2010). High levels of c-di-GMP enhance biofilm formation and repress virulence factor expression and motility, whereas low levels of c-di-GMP repress biofilm formation and induce virulence factor expression and motility (Tischler and Camilli, 2005; Chin *et al*., 2010). Several recent reports have described another compound of the cyclic di-nucleotide family, c-di-AMP (Fig. 1). This compound was initially described as a second messenger signalling for DNA integrity in *Bacillus subtilis* during sporulation (Romling, 2008). From the chemical point of view, these second messengers are small cycles of RNA, containing two bases, such as guanine or adenine, linked to ribose and phosphate. Previous reports showed many similarities in the *in vivo* biosynthesis of c-di-GMP and c-di-AMP (Romling, 2008; 2009; Witte *et al*., 2008). Both seem to be controlled by di-guanylate cyclases (DGCs) and di-adenosine cyclases (DACs) respectively (Romling, 2008; 2009; Hengge, 2009). Di-guanylate cyclases can convert two molecules of guanosine-triphosphate (GTP) to c-di-GMP (690.4 Da) and DACs two molecules of adenosine-triphosphate (ATP) into c-di-AMP (658.4 Da) (Ross *et al*., 1987; Romling, 2008; Witte *et al*., 2008). The c-di-GMP is highly soluble in water and physiological saline. It is stable when boiled in acidic (pH = 3) or alkaline (pH = 10) conditions for 1 h (Karaolis *et al*., 2005a). The c-di-GMP is also stable at temperatures ranging from −78°C to 20°C (Karaolis *et al*., 2005a) for at least a month. In addition to its stability, toxicity studies revealed that c-di-GMP is non-toxic on normal rat kidney cells in the range of 2 to 400 μM and also non-lethal in CD1d mice after 24 h (dose: 50 μl of 200 μM c-di-GMP) (Karaolis *et al*., 2005b).

Immunomodulatory properties of the cyclic di-nucleotides

Karaolis *et al*. showed that exogenous c-di-GMP reduces *in vitro* cell–cell interactions and biofilm formation of *Staphylococcus aureus* (Karaolis *et al*., 2005a) and also demonstrated that treatment with c-di-GMP attenuates *S. aureus* infection *in vivo*, reducing the number of recovered bacterial cells in a mouse infection model (Karaolis *et al*., 2007a). Unexpectedly, they found that c-di-GMP has no apparent inhibitory or bactericidal effect on *S. aureus in vitro*, thereby suggesting that the observed reduction in colonization by biofilm-forming *S. aureus* strains was due to a biological effect on the host immune system (Karaolis *et al*., 2005a). In fact, we showed that *in vitro*, c-di-GMP and to a greater extent c-di-AMP stimulate the production of nitric oxide by pre-activated murine macrophages at a concentration of 200 and 1.6 ng ml^{-1} respectively (Ebensen *et al*., 2011). Similar results were obtained for *Klebsiella pneumoniae* infection models (Karaolis *et al*., 2007b).

Since microbes often mediate their pathogenesis by directly entering the cytosol, the host immune system possesses several distinct pathways to control it. One of these pathways is known as the cytosolic surveillance pathway (CSP), which leads after activation to the induction of type I interferons (IFNs) O'Riordan *et al*., 2002; Crimmins *et al*., 2008). The group of Portnoy identified c-di-AMP as a critical activator of the CSP during *Listeria monocytogenes* infections (Woodward *et al*., 2010). However, the direct advantage resulting from c-di-AMP expression for bacteria still remains unclear, although Portnoy hypothesized that c-di-AMP might be involved in extracellular signalling by *L. monocytogenes* (Woodward

et al., 2010). Also c-di-GMP is thought to interact with the CSP, being able to trigger type I IFNs (McWhirter *et al*., 2009). Since they seem to be crucial for bacterial survival, it can be assumed that these molecules could serve as danger signals recognized by the host immune system. It can be argue that other structurally related albeit chemically distinct compounds could act as danger signals able to evoke an immune response in the host. An example of this is the c-di-IMP (Fig. 1), which was synthesized out of the parental compound c-di-AMP through adenosine-deaminase. This novel compound demonstrated strong *in vivo* as well as *in vitro* adjuvant properties (Libanova *et al*., 2010). Consequently, cyclic di-nucleotides seem to be PAMPs sensed by the immune system.

The above-mentioned properties awaked a growing interest in their evaluation as potential adjuvants. Several studies demonstrated the potent immunostimulatory and immunomodulatory properties of c-di-GMP, c-di-AMP and c-di-IMP, as well as their high potential as vaccine adjuvants (Ebensen *et al*., 2007a,b; 2011; Karaolis *et al*., 2007a; Libanova *et al*., 2010; Madhun *et al*., 2011). All three cyclic di-nucleotides are able to efficiently promote activation and maturation of murine DCs *in vitro*, as it was demonstrated by the increased expression of MHC class II, co-stimulatory (CD80/CD86), activation (CD40) and adhesion (CD54) markers, and by their improved capacity to process and present antigens to T cells (Karaolis *et al*., 2007a; Libanova *et al*., 2010; Ebensen *et al*., 2011). In addition, human immature DCs stimulated with c-di-AMP strongly increase the expression of activation (HLA-DR, CD80/CD86) and maturation (CD83) markers. In contrast, human DC treatment with c-di-GMP resulted in a less prominent activation (Karaolis *et al*., 2007a; Ebensen *et al*., 2011). Interestingly, c-di-AMP seems to be a very efficient activator of macrophages, whereas c-di-IMP and c-di-GMP exhibit a more modest activity (Libanova *et al*., 2010; Ebensen *et al*., 2011).

In vivo studies performed by different groups on mice immunized by systemic or mucosal route with model (e.g. β-galactosidase, ovalbumin), as well as disease-relevant antigens (e.g. haemaglutinin from the H5N1 flu virus, pneumococcal antigens, *S. aureus* antigens) using c-di-nucleotides as adjuvants demonstrated their capacity to promote strong antigen-specific humoral and cellular immune responses (Karaolis *et al*., 2005b; Hu *et al*., 2009; Yan *et al*., 2009; Libanova *et al*., 2010; Ebensen *et al*., 2011; Madhun *et al*., 2011). Administration of c-di-nucleotides by mucosal route also induced strong secretory IgA responses detected both locally, as well as at distant mucosal territories (Ebensen *et al*., 2007b; 2011; Libanova *et al*., 2010). Side-by-side comparison with golden standards (e.g. B subunit of cholera toxin, TLR-2 agonists, alum) suggested that c-di-nucleotides exert equal or even stronger adjuvant properties than many standard compounds (Ogunniyi *et al*., 2008; Hu *et al*., 2009; Libanova *et al*., 2010; Ebensen *et al*., 2011).

Co-administration of cyclic di-nucleotides with model-antigens resulted in the stimulation of balanced T helper (Th1/Th2/Th17) immune responses (Karaolis *et al*., 2007a; Libanova *et al*., 2010; Ebensen *et al*., 2011). Thus, in contrast to other candidate adjuvant, which by and large promote Th1 or Th2 polarized responses, the c-di-nucleotides are characterized by broader response breadth, which in turn make them suitable for a wider range of applications. This is particular attractive in the context of applications where in addition to antibody responses a strong Th1 response is needed, since most adjuvants promoting a Th1 response are not equally efficient in terms of promoting antibody production. In fact, c-di-nucleotides showed a strong capacity to promote the stimulation of cytotoxic responses, as determined by measuring cytotoxic T lymphocytes *in vivo* (Ebensen *et al*., 2007b; 2011; Libanova *et al*., 2010).

Recent studies performed in mouse, non-human primates and patients showed a strong correlation between the frequency of polyfunctional T cells and protection against disease (Makedonas and Betts, 2006; Seder *et al*., 2008; Caccamo *et al*., 2010), although the underlying mechanisms behind the observed protection are still unknown. Interestingly, we have shown that the use of c-di-nucleotides as mucosal adjuvants for virosome-based vaccine formulations resulted in the stimulation of polyfunctional T cells against flu antigens (Madhun *et al*., 2011). Additionally, this vaccine formulation promoted the induction of $CD4^+$ cells with high cross-reactivity against different H5N1 strains (Madhun *et al*., 2011).

Challenge studies on different mouse infection models (e.g. *S. aureus, Streptococcus pneumoniae, K. pneumoniae*) also demonstrated that both intranasal and parenteral pre-treatment of mice with c-di-GMP alone or co-administrated with bacterial antigens protect against invasive bacterial infections (Karaolis *et al*., 2007a,b; Ogunniyi *et al*., 2008). Although these studies suggest that c-di-nucleotides exhibit a considerable potential as immune therapeutics, their true value remain to be elucidated.

Putative mechanism of action of the cyclic di-nucleotides

During the last years, it became clear that adjuvants cannot only potentiate immune responses, but can also modulate them. Thus, the understanding their mechanism of action is critical to make optimal use of them, as well as to foresee potential safety issues. Therefore, it is important to identify cellular receptors and elucidate downstream signalling cascades. Although there is an increasing body of experimental evidence dissecting the signalling events triggered

by c-di-nucleotides in prokaryotes (Gomelsky, 2010; Ma *et al*., 2010; Zhang *et al*., 2006; Romling, 2008; 2009; Romling and Simm, 2009), much less is known about their effector functions on immune cells. Initial experiments performed using TLR-expressing cell lines suggested that immune activation by c-di-GMP does not involve TLRs 1–9 or NODs 1 and 2 (Karaolis *et al*., 2007a). This was further confirmed by both *in vitro* studies showing the independency of TLR and RLR signalling by using MyD88/TRIF [myeloid differentiation primary response gene 88/toll-interleukin-1 receptor (TIR)-domain-containing adapter-inducing interferon-β and MAVS (mitochondrial antiviral signalling)] deficient macrophages (McWhirter *et al*., 2009), and *in vivo* immunization studies using MyD88 and TIRAP (TIR-domain containing adaptor protein) knockout mice (Ebensen *et al*., unpublished data). On the other hand, the potential contribution of the NOD-like receptor pathway was ruled out by the intact responsiveness of macrophages from mice deficient for *Rip2–/–*, *Nalp3–/–* and *Nod1/2–/–* to c-di-GMP (McWhirter *et al*., 2009). Additional work suggested that c-di-GMP can be acting, at least in part, by a novel mechanism leading to transcriptional activation of type I IFN via CSP (McWhirter *et al*., 2009). Interestingly, studies on *L. monocytogenes* secreted c-di-AMP also showed TLR and RLR independency and postulated a comparable induction of host CSP (Woodward *et al*., 2010). The CSP was initially described as an innate host surveillance mechanism, which specifically distinguishes bacteria in the cytosol from bacteria in the vacuole, leading to the upregulation of *ifnb* (O'Riordan *et al*., 2002; Crimmins *et al*., 2008; Stetson and Medzhitov, 2006).

Interestingly, there are *in vitro* studies showing the induction of similar transcriptional profiles in cells stimulated by cyclic di-nucleotides and DNA. Both are able to trigger type I IFNs and co-regulated genes via induction of Tank-binding kinase 1 (TBK1) and its substrate the IRF-3, as well as nuclear factor NF-κB and MAP kinases (Ishii *et al*., 2008; McWhirter *et al*., 2009; Woodward *et al*., 2010). On the other hand, *in vivo* studies showed that c-di-GMP activates both IRF-3 and IRF-7 (McWhirter *et al*., 2009). However, cylic di-nucleotides are not signalling through the cytosolic DNA sensor DAI (DNA-dependent activator of IRFs) (McWhirter *et al*., 2009; Trinchieri, 2010), as it is the case for DNA (Fig. 2).

Thus, great importance should be attached to the MAPK signalling pathways, which play important roles in many cellular processes, including growth, differentiation, apoptosis and the stimulation of immune responses. It has been reported by McWhirter *et al*. that c-di-GMP is able to activate all three MAPK pathways in mouse bone marrow macrophages, the extra cellular signal-regulated kinase (ERK), p38 kinase, and Jun N-terminal kinase (JNK) (McWhirter *et al*., 2009). Interestingly, activation of the p38 kinase is also required for the induction of gene expression by the CSP. Our studies also showed that stimulation with c-di-AMP of the murine macrophage-like cell line J774A.1 results in the activation of p38, ERK, JNK and RSK1/2 kinases (Libanova *et al*., unpublished data). This is in line with the observation that ERK and p38 are responsible for the activation of RSK (Zaru *et al*., 2007). This could in turn close the circle, since protein kinases are involved in type I IFN signalling, and deliver anti-apoptotic and pro-survival signals (Vivanco and Sawyers, 2002). Recent studies showed that an ER-localized stimulator of interferon genes (STING) is required for the type I IFN response to c-di-GMP (both *in vitro* and *in vivo*) and c-di-AMP (only tested *in vitro*) (Sauer *et al*., 2010; Ishikawa and Barber, 2011). Interestingly, former studies showed that the STING protein is critical for regulating the production of IFN in response to cytoplasmic DNA virus (Ishikawa *et al*., 2009) (Fig. 2).

Although cyclic di-nucleotides have similarities in some general features of their molecular structure, there are clear chemical and biological differences that make each of them a unique compound. Beyond their being distinct chemical entities, c-di-AMP is able to promote efficient activation of macrophages *in vitro*, a capacity not observed with c-di-GMP and to a less with c-di-IMP. Moreover, c-di-AMP is showing more potency in terms of its capacity to activate murine and, particularly, human DCs. Although the *in vivo* studies show the stimulation of balanced Th1/Th2/Th17 immune responses after the administration of the c-di-nucleotides by intranasal route, there are also some differences in the potency of these small molecules, suggesting that c-di-AMP exerts the most potent adjuvant activity. Furthermore, some preliminary studies on type I IFN responses induced *in vivo* suggested that c-di-GMP triggers earlier responses than c-di-AMP. This is further supported by the distinct transcriptional profiles observed on activated macrophages *in vitro* (Libanova *et al*., unpublished data). The balance between differences in structure and effector functions needs further elucidation in order to exploit these compounds for fine-tuning responses to vaccine antigens.

Conclusion

In recent years we have assisted to the advent of new approaches and technologies, which have in turn facilitated the identification of candidate antigens for developing novel subunit vaccines. For example, the promising genomic-based approach known as 'reverse vaccinology' has been exploited to identify antigens, which have been translated into the clinical development pipeline (Rappuoli, 2000; Rappuoli and Covacci, 2003; De Gregorio *et al*., 2008; He *et al*., 2010; Sette and Rappuoli, 2010). Thus, in the post-genomic era the identification of suitable candidate antigens does not represent a major bottleneck

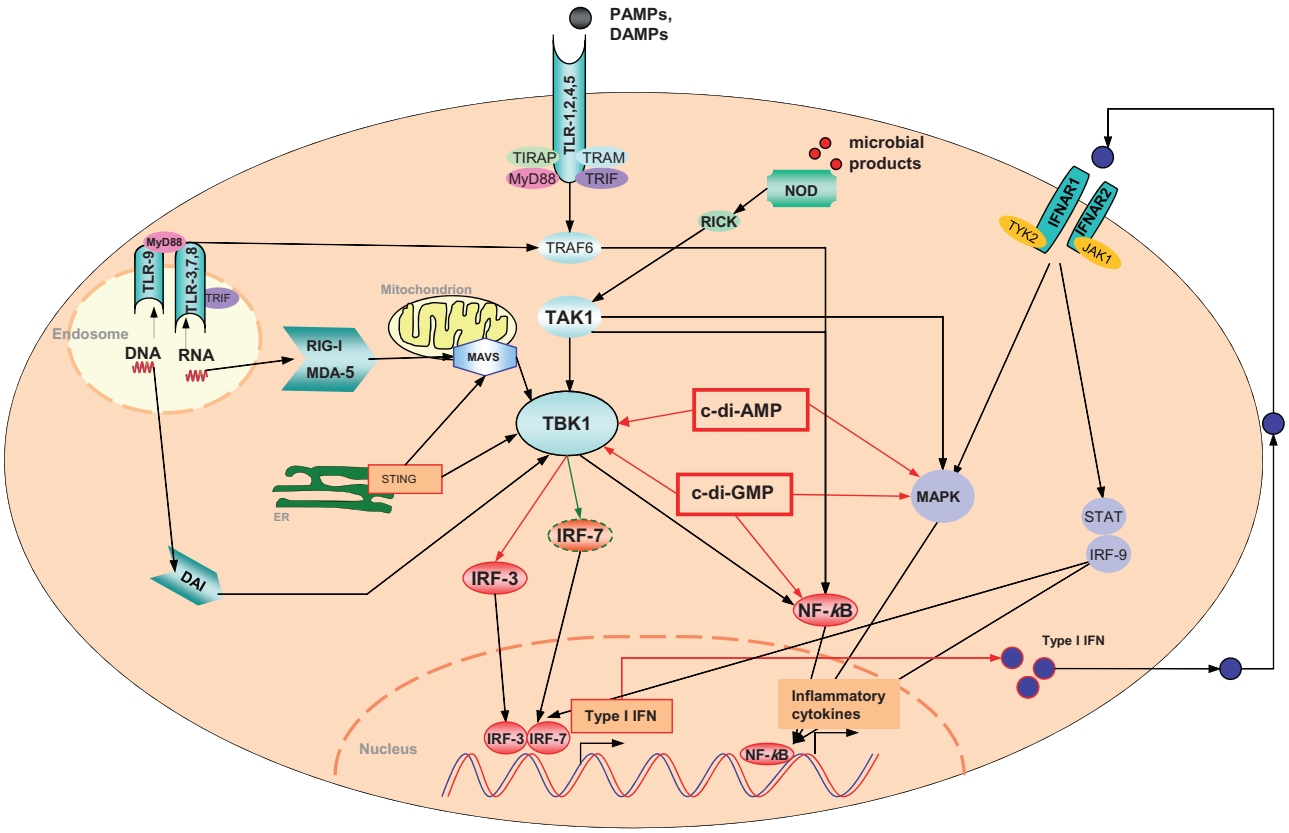


Fig. 2. Putative intracellular cascades activated by cyclic di-nucleotides.
In this schematic representation, TLRs are separated in two major groups, those associated to the membrane and those located in the endosomal compartment. For the sake of clarity, in this scheme there is no discrimination between the different TLR at either the membrane or endosomal compartment (for details in TLRs pathways see review by Kawai and Akira, 2011). The membrane-bound TLRs (TLR-1, TLR-2/1, TLR-2/6, TLR-4 and TLR-5) detect PAMPs and DAMPs on the cell surface and bind to specific TIR domain containing adapters, such as TRIF, MyD88, TIRAP and TRAM. Other TLRs, such as TLR-3, TLR-7 and TLR-8, are localized in intracellular vesicles and recognize RNA, whereas the intracellular TLR-9 recognizes DNA. The important players downstream in these signalling cascades are TRAF6, TAK1 and TBK1, which in turn phosphorylates IRF-3 and IRF-7, leading to their homo-dimerization and translocation into the nucleus, where they drive transcription of IFNs. This signalling cascades result in the activation of NF-κB and MAPK, which in turn are leading to the production of pro-inflammatory cytokines and type I IFN.
On the other hand, TLR-independent recognition of PAMPs is mediated by the intracellular receptors NLRs (NODs) and RLRs (RIG-I, MDA-5) present in the cytosol, which after activation trigger a subset of responses, which are similar to those promoted by TLRs. Exogenous or viral dsRNA is recognized by the RNA helicase RIG-I (or MDA-5), and signals through the mitochondrial antiviral signalling adaptor MAVS (also known as IPS-1), which activates TBK1, thereby leading to phosphorylation of IRF-3, NF-κB release, translocation of the transcriptional regulators and gene induction. DNA is sensed by DAI, leading to activation of the same TBK1/IRF pathway as RIG-I/MDA-5. The ER-localized STING protein was shown to be critical for regulating the production of IFN in response to cytoplasmic DNA virus.
In vitro and *in vivo* studies suggest that c-di-GMP and c-di-AMP are sensed through a cytosolic pathway leading to type I IFN induction. The induction of type I IFNs by c-di-nucleotides is dependent on TBK1/IRF-3 signalling, although it is independent of known cytosolic receptors. The adaptor molecule STING also seems to be required for the type I IFN responses induced by c-di-nucleotides. Preliminary studies suggest that the adjuvanticity of c-di-GMP relies on activation by IRF-3/IRF-7. However, it is still unclear if c-di-nucleotides need to reach the cytosol to exert their activity or they are acting via up-to-now uncharacterized surface receptors. It is also unknown to which extend the induction of type I IFN is sufficient to explain the complex and pleiotropic adjuvant properties of these molecules. Additional information is also needed, to assert the molecular processes responsible for the observed differences between the biological activities of different c-di-nucleotides. Red lines: putative c-di-nucleotide driven pathways for which strong experimental evidence exists. Green lines: Presumptive pathway for which preliminary data is available. Black lines: non c-di-nucleotide driven pathways.

any longer. However, subunit vaccines are poorly immunogenic, raising the need for adjuvants able to promote the elicitation of responses of appropriate quality and strength. Thus, it is becoming obvious in the field of vaccinology that for vaccine development, the availability of an optimal adjuvant is as important as the selection of the antigen. This need is becoming more pressing when issues such as antigen sparing in the context of pandemic threats, immune modulation for therapeutic vaccines, or vaccines tailored for specific population groups (e.g. newborns, elderly) are considered.

A single adjuvant would not be able to address all these complex and intrinsically different needs. Furthermore, factors such as pre-existing immunity against the adjuvant

might render a particular candidate useless. Recent vaccine formulations brought into the market have also proven that there is a huge potential associated to the combination of different adjuvants (e.g. AS04 and AS01). Therefore, the availability of an adjuvant toolbox with a broad palette of compounds able to address different specific clinical needs would represent a clear asset. In this context, synthetic cyclic di-nucleotides are non-immunogenic and very well-defined chemical entities, which are able to stimulate a broad spectrum of effector cells and clearance mechanisms, exerting strong immune modulatory activities when administered by either systemic or mucosal route. Up to now, preclinical studies suggest that they would also exhibit an adequate safety profile; however, their true potential in terms of safety and efficacy for human vaccine development remains to be proven in the field.

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