Minireview

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

Rimma Libanova,1* Pablo D. Becker1,2 and Carlos A. Guzmán1
1Department of Vaccinology and Applied Microbiology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany.
2Peter Gorer Department of Immunobiology, King’s College London, London SE1 9RT, UK.

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.

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 vaccines.
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 specific 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-dispersable 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 (Manichal 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).
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 *Glycanococcus bacter 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; 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).

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

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., 2003).
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 argued 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 awakened 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. haemagglutinin 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 toxoin, 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 pri-mates 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 virome-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 experi-mental 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-xB 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, cyclic di-nucleotides are not signaling 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 p38 and RSK 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.
any longer. However, subunit vaccines are poorly immunogenic, raising the need to address 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.

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 uncharactarized 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.

Fig. 2. Putative intracellular cascades activated by cyclic di-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.
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.

Acknowledgements

The work was in part supported by the European Commission under the project ‘PANFLUVC’ (Contract No. 044115) and ‘TRANSVAC’ (Contract No. 228403), the German Research Foundation by the Excellence Cluster ‘From Regenerative Biology to reconstructive Therapy’ (EXC 62/1), the Helmholtz Association through the Indo-German Science Centre for Infectious Diseases (IG-SCID TwinPro-03), and the German Federal Ministry of Education and Research through the project ‘GERONTO’ (03I 5890 A).

References

Caccamo, N., Guggino, G., Joosten, S.A., Gelsomino, G., Di Carlo, P., Titone, L., et al. (2010) Multifunctional CD4(+) T cells correlate with active Mycobacterium tuberculosis infection. Eur J Immunol 40: 2211–2220.

Chen, W., Patel, G.B., Yan, H., and Zhang, J. (2010) Recent advances in the development of novel mucosal adjuvants and antigen delivery systems. Hum Vaccin 6: 706–714.

Chin, K.H., Lee, Y.C., Tu, Z.L., Chen, C.H., Tseng, Y.H., Yang, J.M., et al. (2010) The CAMP receptor-like protein CLP is a novel c-di-GMP receptor linking cell-cell signaling to virulence gene expression in Xanthomonas campestris. J Mol Biol 396: 646–662.

Coffman, R.L., Sher, A., and Seder, R.A. (2010) Vaccine adjuvants: putting innate immunity to work. Immunity 33: 492–503.

Crimmins, G.T., Herskovits, A.A., Rehder, K., Sivick, K.E., Lauer, P., Dubensky, T.W., Jr, et al. (2008) Listeria monocytogenes multidrug resistance transporters activate a cytosolic surveillance pathway of innate immunity. Proc Natl Acad Sci USA 105: 10191–10196.

De Gregorio, E., Tritto, E., and Rappuoli, R. (2008) Alum adjuvanticity: unravelling a century old mystery. Eur J Immunol 38: 2068–2071.

Dey, A.K., and Srivastava, I.K. (2011) Novel adjuvants and delivery systems for enhancing immune responses induced by immunogens. Expert Rev Vaccines 10: 227–251.

Dostert, C., Guarda, G., Romero, J.F., Menu, P., Gross, O., Tardivel, A., et al. (2009) Malarial hemozoin is a Nalp3 inflammasome activating danger signal. PLoS ONE 4: e6510.

Ebensen, T., and Guzman, C.A. (2009) Immune modulators with defined molecular targets: cornerstone to optimize rational vaccine design. Adv Exp Med Biol 655: 171–188.

Ebensen, T., Schulze, K., Riese, P., Link, C., Morr, M., and Guzman, C.A. (2007a) The bacterial second messenger cyclic dGMP exhibits potent adjuvant properties. Vaccine 25: 1464–1469.

Ebensen, T., Schulze, K., Riese, P., Morr, M., and Guzman, C.A. (2007b) The bacterial second messenger cdGMP exhibits promising activity as a mucosal adjuvant. Clin Vaccine Immunol 14: 952–958.

Ebensen, T., Libanova, R., Schulze, K., Yevsa, T., Morr, M., and Guzman, C.A. (2011) Bis-(3′,5′)-cyclic dimeric adenosine monophosphate: strong Th1/Th2/Th17 promoting mucosal adjuvant. Vaccine 29: 5210–5220.

Eisenbarth, S.C., Colegio, O.R., O’Connor, W., Sutterwala, F.S., and Flavell, R.A. (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 453: 1122–1126.

Flach, T.L., Ng, G., Hari, A., Desrosiers, M.D., Zhang, P., Ward, S.M., et al. (2011) Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nat Med 17: 479–487.

Franchi, L., and Nunez, G. (2008) The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. Eur J Immunol 38: 2085–2089.

Garcon, N., and Van Mechelen, M. (2011) Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems. Expert Rev Vaccines 10: 471–486.

Garcon, N., Chomez, P., and Van Mechelen, M. (2007) GlaxoSmithKline adjuvant systems in vaccines: concepts, achievements and perspectives. Expert Rev Vaccines 6: 723–739.

Gomelsky, M. (2010) cAMP, c-di-GMP, c-di-AMP and now e6510. PLoS ONE 5: e174.

Hengge, R. (2009) Principles of c-di-GMP signalling in bacteria. Nat Rev Microbiol 7: 263–273.

Higgins, S.C., and Mills, K.H. (2010) TLR, NLR Agonists, and other immune modulators as infectious disease vaccine adjuvants. Curr Infect Dis Rep 12: 4–12.

Hu, D.L., Narita, K., Hyodo, M., Hayakawa, Y., Nakane, A., and Karaiolos, D.K. (2009) c-di-GMP as a vaccine adjuvant enhances protection against systemic methicillin-resistant Staphylococcus aureus (MRSA) infection. Vaccine 27: 4867–4873.

Ishii, K.J., Kawagoe, T., Koyama, S., Matsui, K., Kumar, H., Kawai, T., et al. (2008) TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines. Nature 451: 725–729.
Kuroda, E., Ishii, K.J., Uematsu, S., Ohata, K., Coban, C., Kufer, T.A., and Sansonetti, P.J. (2011) NLR functions beyond Microbial Biotechnology © 2011 Society for Applied Microbiology and Blackwell Publishing Ltd, © 2011 The Authors

Ishikawa, H., and Barber, G.N. (2011) The STING pathway and regulation of innate immune signaling in response to DNA pathogens. Cell Mol Life Sci 68: 1157–1165.

Ishikawa, H., Ma, Z., and Barber, G.N. (2009) STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. Nature 461: 788–792.

Janeway, C.A., Jr (1989) Approaching the asymptote? Evolution and revolution in immunity. Cold Spring Harb Symp Quant Biol 54 (Pt 1): 1–13.

Karaolis, D.K., Rashid, M.H., Chythanya, R., Luo, W., Hyodo, M., and Hayakawa, Y. (2005a) c-di-GMP (3′,5′-cyclic diguanylic acid) inhibits Staphylococcus aureus cell-cell interactions and biofilm formation. Antimicrob Agents Chemother 49: 1029–1038.

Karaolis, D.K., Cheng, K., Lipsky, M., Elnabawi, A., Catalano, J., Hyodo, M., et al (2005b) 3′,5′-Cyclic diguanylic acid (c-di-GMP) inhibits basal and growth factor-stimulated human colon cancer cell proliferation. Biochem Biophys Res Commun 329: 40–45.

Karaolis, D.K., Means, T.K., Yang, D., Takahashi, M., Yoshimura, T., Muraile, E., et al (2007a) Bacterial c-di-GMP is an immunostimulatory molecule. J Immunol 178: 2171–2181.

Karaolis, D.K., Newstead, M.W., Zeng, X., Hyodo, M., Hayakawa, Y., Bhan, U., et al. (2007b) Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. Infect Immun 75: 4942–4950.

Kawai, T., and Akira, S. (2011) Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity 34: 637–650.

Kool, M., Pettrilli, V., De Smedt, T., Rolaz, A., Hammad, H., van Nimwegen, M., et al. (2008) Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome. J Immunol 181: 3755–3759.

Koyama, S., Ishii, K.J., Kumar, H., Tanimoto, T., Coban, C., Uematsu, S., et al. (2007) Differential role of TLR- and RLR-signaling in the immune responses to influenza A virus infection and vaccination. J Immunol 179: 4711–4720.

Kufer, T.A., and Sansonetti, P.J. (2011) NLR functions beyond pathogen recognition. Nat Immunol 12: 121–128.

Kuroda, E., Ishii, K.J., Uematsu, S., Ohata, K., Coban, C., Akira, S., et al. (2011) Silica crystals and aluminum salts regulate the production of prostaglandin in macrophages via NALP3 inflammasome-independent mechanisms. Immunity 34: 514–526.

Libanova, R., Ebensen, T., Schulze, K., Bruhn, D., Norder, M., Yevsa, T., et al. (2010) The member of the cyclic di-nucleotide family bis-(3′, 5′)-cyclic dimeric iminosine monophosphate exerts potent activity as mucosal adjuvant. Vaccine 28: 2249–2258.

Ma, Q., Yang, Z., Pu, M., Peti, W., and Wood, T.K. (2010) Engineering a novel c-di-GMP-binding protein for biofilm dispersal. Environ Microbiol 13: 631–642.

McWhirter, S.M., Barbalat, R., Monroe, K.M., Fontana, M.F., Hyodo, M., Joncker, N.T., et al. (2009) A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP. J Exp Med 206: 1899–1911.

Madhun, A.S., Haasheim, L.R., Nostbakken, J.K., Ebensen, T., Chichester, J., Yusibov, V., et al. (2011) Intranasal c-di-GMP-adjuvanted plant-derived H5 influenza vaccine induces multifunctional Th1 CD4(+) cells and strong mucosal and systemic antibody responses in mice. Vaccine 29: 4973–4982.

Makedonas, G., and Betts, M.R. (2006) Polymicrobial analysis of human t cell responses: importance in vaccine immunogenicity and natural infection. Springer Semin Immunopathol 28: 209–219.

Marichal, T., Ohata, K., Bedoret, D., Mesnil, C., Sabatel, C., Kobiyama, K., et al. (2011) DNA released from dying host cells mediates aluminum adjuvant activity. Nat Med 17: 996–1002.

Mbou, M.L., De Gregorio, E., and Ulmer, J.B. (2011) Alum’s adjuvant action: grease is the word. Nat Med 17: 415–416.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature 388: 394–397.

Miyaji, E.N., Carvalho, E., Oliveira, M.L., Raw, I., and Ho, P.L. (2011) Trends in adjuvant development for vaccines: DAMPs and PAMPs as potential new adjuvants. Braz J Med Biol Res 44: 500–513.

Niebuhr, M., Muhradtf, P.F., Wittmann, M., Kapp, A., and Werfel, T. (2008) Intracutaneous injection of the macrophage-activating lipopeptide-2 (MALP-2) which accelerates wound healing in mice – a phase I trial in 12 patients. Exp Dermatol 17: 1052–1056.

Ogunniyi, A.D., Paton, J.C., Kirby, A.C., McCullers, J.A., Cook, J., Hyodo, M., et al. (2008) c-di-GMP is an effective immunomodulator and vaccine adjuvant against pneumococcal infection. Vaccine 26: 4676–4685.

O’Riordan, M., Yi, C.H., Gonzales, R., Lee, K.D., and Portnoy, D.A. (2002) Intracellular recognition of bacteria by a macrophage cytosolic surveillance pathway. Proc Natl Acad Sci USA 99: 13861–13866.

Poltorak, A., He, X., Smirnova, I., Liu, M.Y., Van Huffel, C., Du, X., et al. (1998) Defective LPS signaling in C3H/HeJ and C3H/HeN mice: mutations in Tlr4 gene. Science 282: 2085–2088.

Rappuoli, R. (2000) Reverse vaccinology. Curr Opin Microbiol 3: 445–450.

Rappuoli, R., and Covacci, A. (2003) Reverse vaccinology and genomics. Science 302: 602.

Ribeiro, C.M., and Schijns, V.E. (2010) Immunology of vaccine adjuvants. Methods Mol Biol 626: 1–14.

Romling, U. (2008) Great times for small molecules: c-di-AMP, a second messenger candidate in Bacteria and Archaea. Sci Signal 1: pe39.

Romling, U. (2009) Cyclic Di-GMP (c-Di-GMP) goes into host cells – c-Di-GMP signaling in the obligate intracellular pathogen Anaplasma phagocytophilum. J Bacteriol 191: 683–686.

Romling, U., and Simm, R. (2009) Prevailing concepts of c-di-GMP signaling. Contrib Microbiol 16: 161–181.

Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., et al. (1987) Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. Nature 325: 279–281.

Rynkiewicz, D., Rathkopf, M., Sim, I., Waytes, A.T., Hopkins, R.J., Giri, L., et al. (2011) Marked enhancement of the immune response to BioThrax(R) (Anthrax Vaccine...
Adsorbed) by the TLR9 agonist CPG 7909 in healthy volunteers. Vaccine 7: 724–735.

Sauer, J.D., Sotelo-Troha, K., von Moltke, J., Monroe, K.M., Rae, C.S., Brubaker, S.W., et al. (2010) The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides. Infect Immun 79: 688–694.

Sauer, J.D., Sotelo-Troha, K., von Moltke, J., Monroe, K.M., Rae, C.S., Brubaker, S.W., et al. (2010) The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides. Infect Immun 79: 688–694.

Trinchieri, G. (2010) Type I interferon: friend or foe? J Exp Med 207: 2053–2063.

Vivanco, I., and Sawyers, C.L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489–501.

Witte, G., Hartung, S., Buttnér, K., and Hopfner, K.P. (2008) Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. Mol Cell 30: 167–178.

Woodward, J.J., Iavarone, A.T., and Portnoy, D.A. (2010) c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response. Science 328: 1703–1705.

Yan, H., KuoLee, R., Tram, K., Qiu, H., Zhang, J., Patel, G.B., et al. (2009) 3′,5′-Cyclic diguanylic acid elicits mucosal immunity against bacterial infection. Biochem Biophys Res Commun 387: 581–584.

Zaru, R., Ronkina, N., Gaestel, M., Arthur, J.S., and Watts, C. (2007) The MAPK-activated kinase Rsk controls an acute Toll-like receptor signaling response in dendritic cells and is activated through two distinct pathways. Nat Immunol 8: 1227–1235.

Zhang, Z., Kim, S., Gaffney, B.L., and Jones, R.A. (2006) Polymorphism of the signaling molecule c-di-GMP. J Am Chem Soc 128: 7015–7024.