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The cationic lipid, diC14 amidine, extends the adjuvant properties of aluminum salts through a TLR-4- and caspase-1-independent mechanism

Arnaud Wilmar[a], Caroline Lonez[b], Marjorie Vermeersch[c], Mathieu Andrianne[a], David Pérez-Morga[c,d], Jean-Marie Ruysschaert[b], Michel Vandenbranden[b], Oberdan Leo[a], Stéphane T. Temmerman[a,*]

[a] Institute for Medical Immunology (IMI), Université libre de Bruxelles (ULB), CP305, Campus Gosselies, Rue Adrienne Bolland 8, 6041 Gosselies, Belgium
[b] Laboratory for Structure and Function of Biological Membranes, Centre for Structural Biology and Bioinformatics, Université Libre de Bruxelles, CP 206/2, Campus Plaine, Blvd. du Triomphe, 1050 Brussels, Belgium
[c] Laboratory of Molecular Parasitology, IBMM, Université Libre de Bruxelles, Campus Gosselies, Rue des Professeurs Jeener et Brachet 12, 6041 Gosselies, Belgium
[d] Center for Microscopy and Molecular Imaging (CMMI), Université Libre de Bruxelles, Campus Gosselies, Rue Adrienne Bolland 8, 6041 Gosselies, Belgium

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ABSTRACT

Adjuvant efficiency is critical for inducing a protective and long-lasting immune response against weak immunogenic antigens. Discovered more than 70 years ago, aluminum salts remain the most widely used adjuvant in human vaccine. Prone to induce a strong humoral response, alum fails to drive a cell-mediated immunity, which is essential to fight against intracellular pathogens. Adjuvant systems that contain more than one component may represent an excellent alternative for completing the lack of T cell immunity associated with the injection of alum-based vaccine. In this work, we demonstrated that the adjuvant effects of alum strongly benefited from combining with a cationic lipid, the diC14 amidine. Indeed, we measured a significant improvement of alum-driven IL-1β release when human macrophages were co-cultured with a mixed suspension of alum and the diC14 amidine. Morphological analysis suggested that diC14 amidine improved the alum uptake by phagocytes. Furthermore, the addition of diC14 amidine to alum efficiently enhanced antigen processing and cross-presentation by antigen presenting cells. The biological relevance of these *in vitro* data was assessed by measuring the *in vivo* development of a cytotoxic activity and the enhanced synthesis of antigen-specific immunoglobulins after immunization with alum combined to diC14 amidine. Mechanistically, we demonstrated that diC14 amidine supported the alum adjuvanticity independently of the TLR-4 and caspase-1 agonist activities of the cationic lipid. Based on our findings, we conclude that diC14 amidine works synergistically with alum to achieve higher immune protection after vaccination.

1. Introduction

Traditional vaccines based on live or attenuated pathogens typically elicit a protective immune response associated with a strong reactogenicity. In contrast, protein-based vaccines are well tolerated but display limited immunogenicity and require the addition of a material, termed adjuvant, to induce a protective and long-lasting immune response. Adjuvants usually target innate immunity, and more precisely antigen-presenting cells (APC), to efficiently induce adaptive and memory immune responses. Discovered at the beginning of last century, aluminum salts (alum) are still the most common used vaccine adjuvant and have been widely injected in human subjects, yet the underlying mechanism of action remains poorly understood [1]. Alum immunopotentiation may arise from a prolonged exposure of the immune system to slow released antigen, from particulate structure formation facilitating antigen phagocytosis, or from a higher number of immune sentinels recruited at the site of injection following local inflammation [2,3]. Whereas alum itself appears unable to trigger the production of common pro-inflammatory cytokines and the maturation of innate immune cells [4], the intracellular Nalp3 inflammasome complex has been recently implicated in the detection of alum crystals [5–7]. Nalp3 belongs to the Nod-like receptor (NLR) family of the pattern recognition receptors (PRR) and forms a complex with ASC and caspase-1 to catalyze the release of innate mediators, such as IL-1β and IL-18 [8]. Despite a large set of *in vitro* supporting evidences, the physiological relevance of the Nalp3 inflammasome in the alum adjuvanticity remains controversial [9,10].

Alum-formulated vaccines face a major restriction as they fail to protect against intracellular pathogens for which a cell-mediated immunity is required [11]. The induction of a cytotoxic T cell response against an exogenous-derived antigen relies on

* Corresponding author. Tel.: +32 2 650 95 87; fax: +32 2 650 95 63.
E-mail address: stephane.temmerman@ulb.ac.be (S.T. Temmerman).

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cross-priming by professional APCs. Dendritic cells (DC) engulf soluble molecules and further process and present the immunogenic epitopes onto their major histocompatibility complex (MHC) class I molecules to naive CD8+ T cells. While the mechanisms responsible for the cross-presentation remains to be fully elucidated, there is a growing interest for the identification of molecules that may promote cross-priming after vaccination. Adjuvant systems built on the combination of immunomodulatory molecules have given promising clinical results and are strongly considered for potentiating immune responses to various subunit antigens [12,13]. Recently licensed for human use, the adjuvant system AS04, consisting of a detoxified derivative of LPS (3-*O*-desacyl-4′-monophosphoryl lipid A – MPL) adsorbed onto aluminum salts, has been shown to augment and prolong the adaptive immune response to human papilloma virus antigens [14]. Despite increased Th1-associated cytokine production, the induction of a cytotoxic CD8+ T cell response after the addition of a TLR-4 ligand to alum lack supporting evidences [15]. Typically belonging to the family of vehicle adjuvant, cationic lipids emerge as a promising new adjuvant technology to efficiently deliver antigens to the host immune system [16]. Cationic particle-based vaccines are rapidly ingested by APCs and prone to activate a cell-mediated response [17]. Among cationic liposomes, diC14 amidine offers the unique ability to facilitate the entry of the antigen into the cell and stimulate the secretion of pro-inflammatory cytokines through a TLR-4 dependent mechanism [18]. However, the attempts to use this cationic agent as a vaccine adjuvant failed to substantially enhance the protection against a model antigen in mice (A. Legat personal communication and Fig. 6A). We thus ask whether we could combine diC14 amidine liposomes to alum in order to improve the strength and the scope of the antigen-specific immune response.

Our findings clearly demonstrate the benefit of adding diC14 amidine to alum for enhancing the production of pro-inflammatory cytokines, the antigen processing and the protein cross-presentation by APCs. Contrary to LPS, diC14 amidine and alum requires a pre-formulation step to maximize their effect on the immune functions. Hence, investigations into the underlying mechanism responsible for the diC14 amidine-mediated improvement of the alum-driven immune response ruled out the TLR-4 agonist activity of diC14 amidine. Finally, immunization protocols performed in various strain backgrounds definitively assessed the benefit to combine diC14 amidine and alum into an adjuvant system.

2. Materials and methods

2.1. Reagents and liposome preparation

A commercially available suspension of aluminum hydroxide and magnesium hydroxyde (Imject – Pierce, Rockford, US) displaying a good efficacy as an adjuvant and referred as alum in this manuscript, was used for cell stimulation and mouse immunization [19]. A single suspension of aluminum hydroxide ($Al(OH)_3$) obtained from GSK Biologicals, Rixensart, Belgium and licensed for use in humans, was considered in our experiments when indicated. The adjuvant system 04 (AS04) was also obtained from GSK Biologicals. Ultra-pure LPS from *Escherichia coli* 0111:B4 and Pam3CSK4 (Synthetic triacylated lipoprotein) were purchased from Invivogen (San Diego, CA). Anti-IL-1β, anti-caspase-1 antibodies were obtained from Cell Signaling (Danvers, MA). DQ-Ovalbumin and CFSE tracker dye were purchased from Invitrogen (Carlsbad, CA). Ovalbumin (grade VII) and SIINFEKL peptide of OVA (257–264 aa) were obtained from Sigma–Aldrich (St. Louis, MO) and Tebu-Bio (Le Perray en Yvelines, France), respectively. Inhibitors for phagocytosis (cytochalasin B) or caspase-1 (Z-YVAD-fmk) were purchased from Sigma–Aldrich and Bachem (Weil am Rhein, Germany), respectively. diC14 amidine liposomes (3-tetradecylamino-*tert*-butyl-*N*-tetradecylpropionamidine) were synthesized as described previously [20], prepared as in [18] and used at 5 μG/mL or stated otherwise.

2.2. Dynamic light scattering experiments and zeta-potential analysis

Measurements were performed on a Zetasizer–Nanoseries (Malvern), equipped with a 677 nm laser and dynamic light scattering (PCS) at 90 degree for particle sizing in a disposable sizing cuvette (for size and polydispersity index measurements) or a clear disposable zeta cell (for zeta potential measurements). For each experiments, 1.2 mg of alum (for alum alone or mixtures), or 120 μg diC14-amidine (for amidine alone or mass ratio amidine:alum 1:10), or 12 μg diC14-amidine (for mass ratio amidine:alum 1:100) were resuspended in 2 mL of Hepes buffer (10 mM Hepes, pH 7.2). Results were analyzed using the zetasizer software (Malvern). Numbers are means of at least 5 sets of 10 measurements ± standard deviation.

2.3. Electron microscopy

The cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, washed and let them set overnight at 4 °C onto glass coverslips coated with 0.1% poly-L-lysine. After washing, cells were post-fixed for 60 min with 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2. Thereafter, the samples were serially dehydrated in ethanol and acetone, critical point dried and mounted on SEM stubs. Observations were performed with an ESEM Quanta 200 FEG (Tecnai) microscope. Images were analyzed and processed by AnalySIS and Adobe Photoshop softwares.

2.4. Animals

Wild type females and males C57BL/6 (7 weeks old) were obtained from Harlan (AD Horst, The Netherlands). Genetically altered C57BL/6 mice for *TLR-4* and *caspase-1* genes have been previously described [21,22] and were kindly provided by F. Trottein (Institut Pasteur de Lille, France) and R. Beyaert (VIB, Ghent University, Belgium), respectively. Maintenance and care of mice complied with the guidelines of the Ethics Committee for the use of laboratory animals.

2.5. Generation and stimulation of human monocyte-derived macrophages

Monocyte-derived macrophages (MoM) were generated from buffy coat with approval of the ethic committee (protocol P2009/092). Briefly, mononucleated cells were isolated by gradient-density centrifugation. Monocytes were next purified using CD14 positive selection magnetic beads (Miltenyi, Bergisch Gladbach, Germany). *In vitro* differentiation into macrophage-like cells was performed by culturing the CD14 positive fraction in RPMI1640 (Lonza, Basel, Switzerland) complemented with 10% of fetal calf serum (FCS) and 800 U/mL of human granulocyte-macrophage colony stimulating factor (GM-CSF, Gentaur, Brussels, Belgium). After 6 days, adherent and non-adherent cells were harvested, washed and seeded in 24-well plates (Greener Bio-one, Wemmel, Belgium) for stimulation. diC14 amidine or LPS (100 nG/mL) were used to prime 1E6 MoM for 2 h before the addition of alum (50–500 μG/mL). When indicated, diC14 amidine or LPS were pre-mixed with alum for 30 min on rolling bars at room temperature before the addition to the cells. Supernatants were