Vaccine 27 (2009) 4388–4401

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Review

ISCOMs and ISCOMATRIX™

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ARTICLE INFO

Article history:
Received 15 August 2008
Received in revised form 22 February 2009
Accepted 9 May 2009
Available online 28 May 2009

Keywords:
ISCOMs
ISCOMATRIX™
Adjuvant
Preparation
Immune responses
Protection
Mechanism

ABSTRACT

Immunostimulatory complexes (ISCOMs) are particulate antigen delivery systems composed of antigen, cholesterol, phospholipid and saponin, while ISCOMATRIX™ is a particulate adjuvant comprising cholesterol, phospholipid and saponin but without antigen. The combination of an antigen with ISCOMATRIX™ is called an ISCOMATRIX™ vaccine. ISCOMs and ISCOMATRIX™ combine the advantages of a particulate carrier system with the presence of an in-built adjuvant (Quil A) and consequently have been found to be more immunogenic, while removing its haemolytic activity of the saponin, producing less toxicity. ISCOMs and ISCOMATRIX™ vaccines have now been shown to induce strong antigen-specific cellular or humoral immune responses to a broad range of antigens of viral, bacterial, parasite origin or tumor in a number of animal species including non-human primates and humans. These vaccines produced by well controlled and reproducible processes have also been evaluated in human clinical trials. In this review, we summarize the recent progress of ISCOMs and ISCOMATRIX™, including preparation technology as well as their application in humans and veterinary vaccine designs with particular emphasis on the current understanding of the properties and features of ISCOMs and ISCOMATRIX™ vaccines to induce immune responses. The mechanisms of adjuvanticity are also discussed in the light of recent findings.

Contents

1. Introduction

There is a growing interest in the use of colloidal particles as antigen delivery systems [1]. Liposomes, for example, allow for the encapsulation of antigenic proteins and peptides in a multimeric particulate form. However, due to the lack of sufficient

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doi:10.1016/j.vaccine.2009.05.032

inherent immunogenicity, they usually require the use of additional adjuvants if they are to be effective in stimulating an immune response [2]. Immunostimulatory complexes (ISCOMs) are particulate antigen delivery systems composed of antigen, cholesterol, phospholipid and saponin [3]. A description of ISCOMs can be found in a report dating back to 1973, but at that time their potential as subunit vaccine delivery systems was not recognised. The initial use of ISCOMs came from Morein by demonstrating that formulating micelles of the saponin Quil A extracted from the bark of *Quillaja saponaria* with viruses resulted in an effective particulate vaccine [4], which may act as an antigen delivery system with powerful immunostimulating activity. ISCOMATRIX™ is a particulate adjuvant comprising cholesterol, phospholipid and saponin but without antigen. It has essentially the same structure of ISCOMs [5]. Antigens can be formulated with the ISCOMATRIX™ to produce ISCOMATRIX™ vaccines that can provide the similar antigen presentation and immunomodulatory properties as the ISCOMs but with much broader application as they are not limited to hydrophobic membrane proteins [6].

ISCOMs and ISCOMATRIX™ combine the advantages of a particulate carrier system with the presence of an in-built adjuvant (Quil A) and consequently have been found to be more immunogenic than other colloidal systems such as liposomes and protein micelles [7]. Critically, formulation of ISCOMs and ISCOMATRIX™ retained the adjuvant activity of the Quil A, while increasing its stability, reducing its haemolytic activity, and producing less toxicity. They also required substantially less antigen and adjuvant to induce immunity in the host than vaccination with simple mixtures of free antigen and saponins [8]. Many studies have demonstrated the ability of ISCOMs and ISCOMATRIX™ vaccines to induce strong antigen-specific cellular or humoral immune responses to a wide range of antigens in a number of animal model [9,10]. As such, ISCOMs and ISCOMATRIX™ vaccines have been approved for veterinary use and are currently undergoing clinical trials for human use [8,11]. Both vaccines have been shown to be safe and effective in numerous animal and clinical trials, including anti-cancer, anti-bacterial, anti-viral, and anti-parasite vaccines [10]. Here we summarise recent progress of the ISCOMs and ISCOMATRIX™, including preparation technology as well as their application in humans and veterinary vaccine designs with particular emphasis on the current understanding of the ability of ISCOMs and ISCOMATRIX™ vaccines to induce immune responses and the mechanisms underlying this property.

2. Preparation of ISCOMs and ISCOMATRIX™ vaccines

2.1. Components of ISCOMs and ISCOMATRIX™

ISCOMs and ISCOMATRIX™ are the versatile and flexible systems with various phospholipids and saponin components, and possess the same particulate structure. The saponin and cholesterol molecules interact to form a subunit, ring-like micelle which, in the presence of a phospholipid, creating a cage-like structure, approximately 40 nm in diameter. The most commonly used saponin in ISCOMs and ISCOMATRIX™ is Quil A or its purified compounds. Quil A is a semi-purified preparation of *Quillaia* saponin that is composed of a heterogenous mixture of probably more than 100 closely related saponins. Quil A is suitable for veterinary applications but is considered unsatisfactory for human applications [12]. However, further characterisation of Quil A has identified several saponin fractions, which have adjuvant activity and retain the capacity to form ISCOMs and ISCOMATRIX™ [13]. Three purified saponin fractions from Quil A, referred to as QH-A, QH-B and QH-C, have been characterised with regard to their toxicity, haemolytic activity, ISCOMs forming ability and adjuvant properties [14]. The fraction QH-B, although having high adjuvant activity, was found to be too toxic for clinical applications. A combination of seven parts of QH-A and three parts of QH-C was referred to as ISCOPREP®703 or QH-703. Hu et al. [15] reported that ISCOMs vaccines against human respiratory syncytial virus (RSV) formulated with the QH-703 induced a significantly greater antigen-specific immune response than either QH-A or QH-C alone. Most recently, Pham et al. [16] investigated which of six fractions of Quil A were able to form ISCOMs by the method of ether injection. The injection of ether solutions of lipids into aqueous solutions of QS-17, QS-18 or QS-21 all resulted in homogeneous ISCOMATRIX™. The combination of lipids and QS-7 produced lamellae and liposomes as the prominent structures and a minor amount of ISCOMATRIX™. The remaining two hydrophilic, low molecular weight fractions of Quil A did not produce ISCOMs, instead liposomes and helical structures predominated in the samples. In addition, Bomford et al. [17] examined the adjuvant activity and formation of ISCOMs by a series of saponins differing in the structures of their aglycones and sugar chains. The only two saponins apart from *Quillaia* that were adjuvant-active were *Gypsophila* and *Saponaria* saponins, which resemble *Quillaia* saponins in that they contain saponins with branched sugar chains attached to positions 3 and 28 of the aglycone. *Saponaria* saponins formed irregular ISCOMs-like structures, and *Gypsophila* saponins produced a sheet of joined pore-like structures.

Some researchers have also attempted to alter the properties of ISCOMs vaccines by varying the phospholipid component. Lee et al. [18] compared the structure and adjuvanticity of ISCOMs vaccines formulated with either glycoglycerolipids from marine algae or with egg phosphatidylcholine. However, no difference was found in the antigen-specific immune responses raised against the membrane pore-forming protein from the human pathogen *Yersinia pseudotuberculosis* (YP-MPFP), following immunization with either type of ISCOMs vaccine. Another research concerning modification of ISCOMATRIX™ by the replacement of the phospholipid for the glycolipid (monogalactosyl-diacylglycerol) from sea macrophytes, and saponin Quil A for triterpene glycoside of cucumarioside from *Cucumaria japonica* showed that this newly designed vaccine termed TI-complexes exhibited considerably lower toxicity than ISCOMs. Under conditions of experimental immunization of mice by YP-MPFP, TI-complexes with antigen provided stronger humoral immune response to antigen than with classical ISCOMs, liposomes and Freund's adjuvant, thus showing the prospect of the use of TI-complexes as a new type of adjuvant carriers for antigens [19].

An intriguing alternative approach to the classical ISCOMs vaccine incorporates both an additional adjuvant plus an antigen presenting cell targeting moiety [20]. Cholera toxin (CT) is the most potent mucosal adjuvant known. As an intact holotoxin, however, CT is unsuitable for use in humans due to its potent induction of diarrhoea and demonstrated accumulation in the olfactory nerve. A novel adjuvant CTA1-DD was formed by the fusion of the highly active A1 subunit of CT to protein "D", a synthetic analogue of protein-A from *Staphylococcus aureus* which binds to B-lymphocytes. This fusion protein is thought to enhance antigen presentation by B-lymphocytes via a process which is dependent upon both the ADP-ribosylating activity of the CTA1 subunit, plus the B-cell binding of the D component [20]. As CT and saponin adjuvants appear to have distinct mechanisms of immune activation, Mowat et al. [21] combined CTA1-DD and ISCOMs to create a rationally designed adjuvant vector CTA1-DD/ISCOM. The vector was highly immunogenic by the intranasal as well as the oral route even with nanogram doses of Ag, inducing Ag-specific serum Abs, CD4 T cell priming, and IFN-γ production. Immunisation of mice with CTA1-DD/ISCOMs via a range of mucosal and systemic routes suggested that the combined vaccine is very effective at inducing both cellular and humoral immunity to antigens incorporated into the

vaccine particle [20]. Helgeby et al. [22] further extended the potential of CTA1-DD/ISCOMs as an effective mucosal vaccine delivery vehicle by incorporating CTA1-DD and the influenza virus PR8 Ag into the same ISCOMs. This combined vector was a highly effective enhancer of a broad range of immune responses, including specific serum Abs and balanced Th1 and Th2 $CD4^+$ T cell priming as well as a strong mucosal IgA response. Unlike unmodified ISCOMs, Ag incorporated into the combined vector could be presented by B cells *in vitro* and *in vivo* as well as by dendritic cells; it also accumulated in B cell follicles of draining lymph nodes when given subcutaneously and stimulated much enhanced germinal center reactions. Strikingly, the enhanced adjuvant activity of the combined vector was absent in B cell-deficient mice, supporting the idea that B cells are important for the adjuvant effects of the combined CTA1-DD/ISCOM vector [22].

Similarly, others have used the biotin–streptavidin system to tag antigens onto the ISCOMATRIX™. One such method was based on expression of a biotinylated fusion protein that was associated to Ni^{2+}-coated matrix via a His_6-tagged streptavidin fusion protein. This method utilizes the binding of the hexahistidyl (His_6)-tag to bivalent metal ions, often used for affinity purification of recombinant proteins. ISCOMATRIX™ containing a chelating lipid was prepared and then incubated first with Cu^{2+} or Ni^{2+} ions and then with the His_6-tagged protein [23]. The second method was based on the strong binding between biotin and streptavidin. The second approach was to express the immunogen with streptavidin as a fusion partner, which would bind to biotinylated matrix. Wikman et al. [24] used NcSRS2 and the malaria peptide M5 as model antigens to prepare ISCOMs using the above both approaches. When NcSRS2 ISCOMs produced according to the first approach were used to immunise mice, antibodies reacting with native *N. caninum* antigen were produced, indicating that the recombinant protein was correctly folded. Furthermore, immunisation resulted in partial protection against clinical disease and reduced the amounts of *N. caninum* DNA in the brain of immunised mice after challenge infection [25]. Pinitkiatisakul et al. [26] further evaluated and compared the immunogenicity of NcSRS2 ISCOMs prepared according to three different methods based on biotin–streptavidin binding and/or Ni^{2+}–His_6-tag interaction. While all these ISCOMs preparations induced *N. caninum* specific antibodies at similar levels, His_6-SA-SRS2′ coupled to biotinylated matrix generated the strongest cellular responses measured as *in vitro* proliferation and production of IFN-γ and IL-4 after antigen stimulation of spleen cells. However, the relationship between the levels of these cytokines as well as between IgG1 and IgG2a titres in serum induced by the three ISCOMs preparations were similar, indicating that the balance between Th1 and Th2 responses did not differ. After challenge infection, mice immunised with His_6-SA-SRS2′ coupled to biotinylated matrix had significantly lower amounts of parasite DNA in their brains compared to the other immunised groups.

2.2. Methods of ISCOMs and ISCOMATRIX™ formulation

ISCOMs and ISCOMATRIX™ can be prepared by various methods, which essentially differ in the pre-dispersion of the lipid components and the use of additional solubilisers [27]. The different methods produce colloidal dispersions which differ in homogeneity, occurrence of particle species and time to reach equilibration. To date, five different methods have been described in the literature: dialysis, centrifugation, lipid-film hydration, ethanol injection and ether injection. The colloidal structures depends not only on the preparation method, but also on the ratios of the saponin, cholesterol and phospholipid components (Figs. 1–4). Consequently, there appears to be an ideal, method-specific ratio of the three components to maximise formation of "typical" ISCOMs and ISCOMATRIX™ in the sense of the cage-like structures.

Two methods have traditionally been used for the preparation of ISCOMs, one based on centrifugation and the other on dialysis. The centrifugation method originally described for the preparation of ISCOMs was based on ultra-centrifugation and relies on the presence of lipids and antigen which are co-extracted from whole organisms [28]. In this method, the protein, cholesterol and phospholipids are first solubilised using a surfactant such as Triton X-100 in the absence of Quil A. This micellar solution is then centrifuged through a sucrose gradient containing Quil A. During the centrifugation process, ISCOMs are formed and can be collected as a band in the sucrose gradient. Finally, sucrose is removed by a dialysis step. The method is difficult and expensive to scale up due to requiring four steps and the limited quantities of ISCOMs that can be prepared in each run [29]. The dialysis method is more commonly used than the centrifugation method. In this method, the lipid components and Quil A are first solubilised in a surfactant system such as octylglucoside or MEGA 10, which is subsequently removed by dialysis. Compared to the lipid-film hydration and the ethanol injection methods, dialysis was reported to result in the most homogenous formulations with a very narrow particle size distribution [30]. However, it is a batch process and a time consuming procedure requiring at least 3 days dialysis against a suitable buffer. The major limitations of centrifugation and dialysis method are firstly the tedious or time-consuming nature of the procedure and secondly the possibility of residual surfactant remaining in the preparation [31]. The residual surfactants may have an influence on formation and prevalence of the colloidal structures. Furthermore, the presence of a surfactant in the final formulation may not be desirable for use in humans.

Several attempts have been made to develop alternative, quicker and simpler methods of production, including the use of an aqueous saponin solution to hydrate the vaccines [32]. The lipid-film hydration method is a much simpler procedure for the preparation of ISCOMs [33]. This method is based on the classical Bangham method used for the preparation of liposomes and involves the hydration of dried phospholipid/cholesterol films using an aqueous solution of Quil A. The main advantages of this approach are the simplicity and the absence of additional surfactants or solvents. A disadvantage is that more heterogeneous samples are obtained when compared to dialysis and ethanol injection. Moreover, the samples require extended equilibration times. Demana et al. [34,35] refined this methodology by introducing a freeze-drying step in order to promote intimate mixing of Quil A, cholesterol and phospholipid. The incorporation of a freeze-drying step following hydration of the lipid-film improves the quality of the end product when redispersed in an aqueous medium. The freeze-drying process also offers the opportunity to isolate lipid powder mixtures which spontaneously form different colloidal structures upon hydration [34]. Demana et al. [36] prepared solid Quil A–cholesterol–phospholipid formulations by physical mixing or by freeze-drying of aqueous dispersions of these components in ratios that allow spontaneous formation of ISCOMs and other colloidal structures upon hydration. Pellets prepared by compression of freeze-dried powders released PE–FITC–OVA slower than those prepared from physically mixed powders, and the antigen was released in the form of ISCOMs from pellets prepared by compression of freeze-dried powders. To investigate the application of hydration of freeze-dried lipid monophase matrices as a novel technique to produce ISCOMs encapsulating lipopeptides, Liang et al. [37] measured the size, polydispersity and morphology of the resulting colloidal particles. The homogeneity of ISCOMs preparations produced by this method was found to be influenced by the amount of matrix-forming material as well as the ratio of phospholipid: Quil A: cholesterol used for ISCOMs preparation. Further, it was observed that more homogeneous ISCOMs dispersions were produced in method hydrating the lipid matrix with an aqueous buffer containing Quil A compared to the method

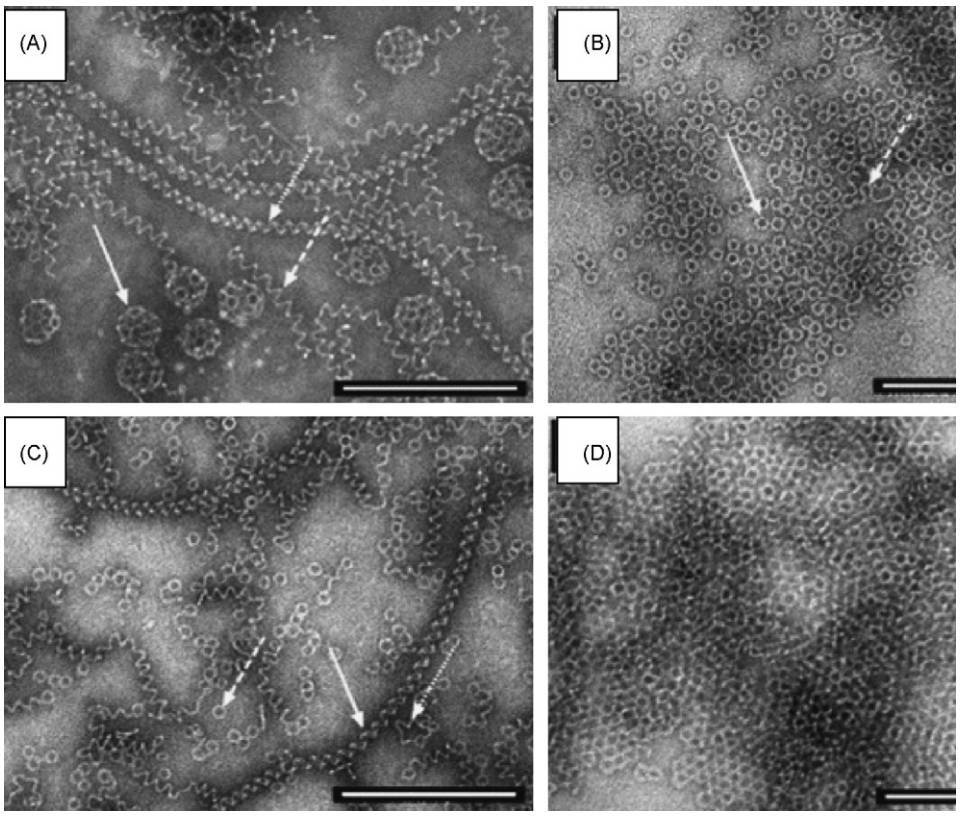

Fig. 1. Transmission electron microscopy (TEM) micrographs of colloidal particles prepared by dialysis method. (A) Typical (cage-like) ISCOM matrices (solid arrow) as well as helices (dashed arrow) and double helices (dotted arrow) at a weight ratio of Quil-A: cholesterol (CHOL): phosphatidylcholine (PC) (4:1:1), (B) ring-like micelles (solid arrow) and worm-like micelles (dashed arrow) at a weight ratio of Quil-A: CHOL (4:1), (C) double helices (solid arrow), ring-like micelles (dashed arrow) and worm-like micelles (dotted arrow) at a composition of Quil-A: CHOL (4:1), (D) Lamellae structures at a composition of Quil-A: CHOL (2:1). Bar = 200 nm [27].

hydrating a lipid matrix incorporating Quil A with an aqueous buffer.

The preparation methods based on ethanol and ether injection have been published as a surfactant-free protocol to form ISCOMATRIX™. Ethanol injection method is adapted from the ethanol injection technique described for the preparation of unilamellar liposomes [30]. Ethanolic solutions of cholesterol and phospholipid are injected into aqueous solutions of Quil A. This technique results in the formation of large numbers of cage-like particles within 2 h. The method is simple, rapid, and efficient and offers the possibilities for large scale commercial production [30]. Ether injection method is again based on the analogous method for the preparation of liposomes and is a variation of the ethanol injection method [38]. In this method, phosphatidylcholine and cholesterol were dissolved in ether, which was injected into an aqueous solution of Quil A, maintained at 55 °C. The use of ether has the advantages that higher quantities of lipids can be dissolved and that removal of the organic solvent is achieved by gentle heating.

2.3. Methods for antigen association

Association of the antigen with the ISCOMATRIX™ is thought to be required for optimal induction of cellular responses induced by ISCOMATRIX™ vaccines [39]. Historically, this association has been achieved by the use of ISCOMs vaccines where the antigen is incorporated into the ISCOMs structure via a hydrophobic region. This method was limited in both the amount and type of antigen that could be incorporated [7,9]. Recent effort has been applied to develop techniques for promoting the association of antigens with the ISCOMATRIX™ and several approaches have proven successful.

ISCOMs and ISCOMATRIX™ have glucuronic acid on their surface, which renders the surface of these nanostructures negative. The cationic ISCOMATRIX™ particles have been utilised to attract anionic proteins. Lendemans et al. [40] substituted normal cholesterol with a cationic derivative, 3β-[*N*-(N^1,N^1-dimethylamineoethane)-carbamoyl]-cholesterol, to produce positively charged ISCOMs particles. Lendemans et al. [41] also substituted the phospholipid component in classical ISCOMs with the cationic lipid dioleoyl-trimethyl-ammonium-propane (DOTAP). DOTAP can be incorporated with Quil-A and cholesterol to form cage-like particles, it appears to be unsuitable to prepare cationic equivalents of ISCOMs. In a different approach, the addition of a lipid tail to the protein can provide a hydrophobic domain allowing attachment to the ISCOMATRIX™ core structure. For example, the palmitification (addition of a terminal palmityl moiety) of hydrophilic ovalbumin was used to produce pOVA ISCOMs, which were shown to be functional, inducing protection in mouse tumor models [39]. Similarly, fusion proteins have been produced with the addition of hydrophobic peptide tags from either designed sequences, or viral or bacterial transmembrane regions, which also allowed hydrophilic proteins to be incorporated into ISCOMs vaccines [42].

Another interesting association technique utilizes chelation, the combination of a metal ion with a chemical compound to form a ringed structure. The antigen requires the addition of a poly-histidine tag to either the N- or C-terminal, a modification frequently utilised in recombinant protein purification. Chelating ISCOMATRIX™ comprises the same components as the standard adjuvant, with the addition of 1,2-dipalmityl-*rac*-glycerol-3,8-(3,6-dioxy)octyl-1-amino-*N*,*N*-diacetic acid (DPIDA) [43]. When the poly-histidine tagged protein is mixed with the chelating ISCOMATRIX™ in the presence of copper ions, the protein becomes chelated to the adjuvant. These chelated ISCOMATRIX™ vaccines have been shown to induce strong CTL responses in mouse mod-

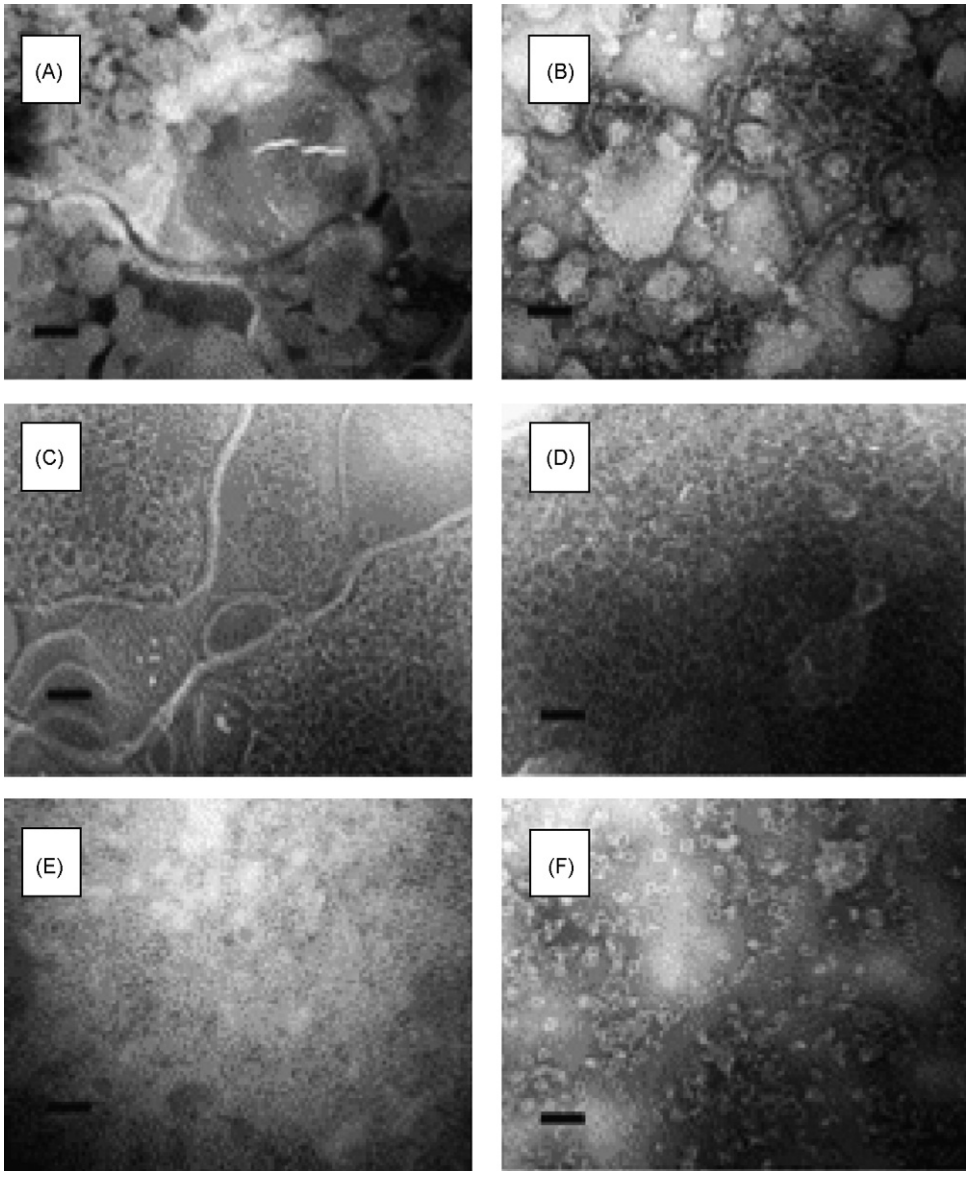

Fig. 2. TEM micrographs of colloidal particles formed when a dried lipid-film (2 mg CHOL, 12 mg phospholipid) was hydrated with various concentrations of Quil A solutions. (A) 0 mg/ml Quil A, (B) 2 mg/ml Quil A, (C) 3 mg/ml Quil A, (D) 4 mg/ml Quil A, (E) 6 mg/ml Quil A, (F) 8 mg/ml Quil A. Bar represents 100 nm [33].

els [43]. While this approach requires the production of a modified adjuvant, once produced it can be utilised to generate a wide range of associated vaccines, using any poly-histidine tagged recombinant protein.

3. Immunity and protective effects of ISCOMs and ISCOMATRIX™ vaccine

3.1. Vaccination against pathogens

ISCOMs and ISCOMATRIX™ vaccines containing antigens from a great variety of pathogens have been tested for the immunogenicity and protection in laboratory animals and human, as summarized in Table 1. The used antigens are most from the viruses including influenza, hepatitis virus, herpes virus, immunodeficiency virus, measles virus, mmetapneumovirus, newcastle disease virus, respiratory syncytial virus, rinderpest virus and rotavirus. Antigens from bacteria and parasites (*Anaplasma marginale*, *Mycoplasma mycoides*, *Corynebacterium diphtheriae*, *Helicobacter pylori*, *Moraxella bovis*, *Mycobacterium tuberculosis*, *Streptococcus pyogenes, Chlamydia trachomatis*, *Neospora caninum*, *Toxoplasma gondii*) have also been shown to evoke protective responses when prepared into ISCOMs and ISCOMATRIX™ vaccines.

3.2. Vaccination against cancer

Due to their ability to rapidly generate antigen-specific CTL, T helper cells and antibodies, ISCOMs and ISCOMATRIX™ vaccines have great potential as cancer immunotherapeutics. Human papilloma virus type 16 (HPV16) E6 and E7 oncoproteins are associated with cervical cancer development and progression, and may facilitate the host to eliminate the infected dysplastic epithelial cells that are precursors of cervical cancer. When co-administered with ISCOMATRIX™ adjuvant, E6E7 proteins consistently induced E7 specific CTL, IgG, IgG1 and IgG2a antibody, delayed-type hypersensitivity (DTH) responses, and *in vivo* tumor protection in mice [113]. In phase I study of HPV16-specific immunotherapy with E6E7 protein and ISCOMATRIX™ adjuvant in women with cervical

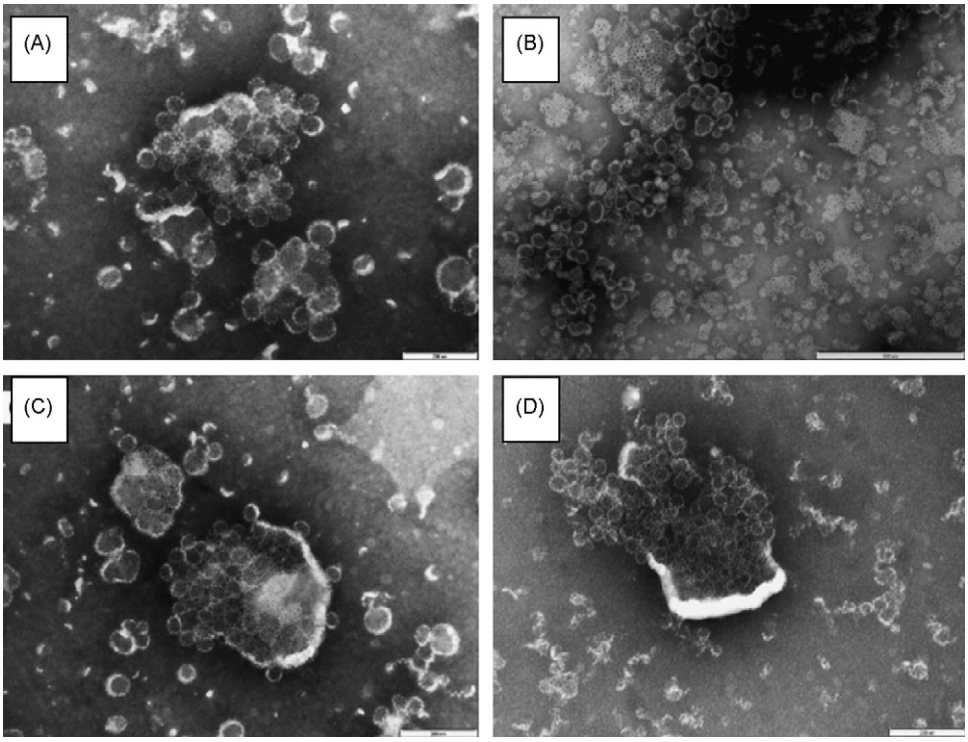

Fig. 3. TEM micrograph of colloidal particles prepared by hydration of freeze-dried matrix. (A) Sample prepared from PC/CHOL matrix with 200 mg sucrose and hydrating with a Quil A buffer using a ratio of PC:Quil A:CHOL (8:8:4), (B) sample prepared from PC/CHOL matrix with 300 mg sucrose and hydrating with a Quil A buffer using a lipid ratio of PC:Quil A:CHOL (8:8:4), (C) sample prepared from PC/CHOL matrix with 200 mg sucrose and hydrating with a Quil A buffer using a lipid ratio of PC:Quil A:CHOL (10:6:4)10:6:4, (D) sample was prepared from PC/Quil A/CHOL matrix with 200 mg sucrose and hydrating with buffer using a lipid ratio of PC:Quil A:CHOL (8:8:4) [35].

intraepithelial neoplasia ($n = 31$), it was reported that the specific antibody, DTH, *in vitro* cytokine release, and CD8 T-cell responses to E6 and E7 proteins were each significantly greater in the immunised subjects than in placebo recipients, while the frequency of loss of HPV16 was not statistically different between the vaccinated and placebo groups [114].

NY-ESO-1 (ESO) is one of the most immunogenic tumor antigens expressed by many different tumor types and belongs to the family of "cancer-testis" antigens. Maraskovsky et al. [115] studied the preclinical immunogenicity and efficacy of ESO protein formulated with ISCOMATRIX™ adjuvant (ESO–IMX). *In vitro*, the ESO–IMX was readily taken up by human monocyte-derived dendritic cells, and on maturation, and epitopes of ESO protein were presented on MHC class II molecules to ESO-specific $CD4^+$ T cells. ESO–IMX also induced strong ESO-specific IFN-γ and IgG2a responses in C57BL/6 mice, and ESO-specific $CD8^+$ CTLs in HLA-A2 transgenic mice. ESO–IMX further reduced incidence of tumors in C57BL/6 mice against challenge with a B16 melanoma cell line expressing ESO. These data illustrated that ESO–IMX represented a potent therapeutic anticancer vaccine. ESO–IMX was also evaluated for the safety and immunogenicity in a placebo-controlled clinical trial [116,117]. ESO–IMX induced high-titer antibody responses, strong DTH reactions, and circulating $CD8^+$ and $CD4^+$ T cells specific for a broad range of ESO epitopes. Among 42 patients, with a median followup of 748 days, 16 have relapsed: five of seven placebo patients, nine of 16 who received protein alone and two of 19 who received ESO–IMX. Thus, the patients in cohorts with higher immune response scores appeared to have longer the relapse free survivals than those from cohorts with low scores. The phase II trial of ESO–IMX was subsequently undertaken to assess objective clinical responses safety and immunogenicity in 27 patients with advanced ESO positive melanoma [118]. However, no objective responses (antibody titers, DTH reaction and clinical responses) were observed. The vaccine-induced immunity appeared to be attenuated in the presence of advanced metastatic disease.

Chen et al. [119] used autologous dendritic cells (DCs) pulsed with ESO–IMX in combination with overlapping synthetic peptides to identify the immunodominant T cells in ten patients vaccinated with ESO–IMX. Two novel $CD4^+$ T cell epitopes were identified and characterized. T cells specific to these epitopes not only recognized autologous dendritic cells loaded with ESO but also NY-ESO-1-expressing tumor cell lines treated with IFN-γ. One of the two epitopes identified was greater than the previously identified immunodominant HLA-DP4-restricted epitopes and correlated with ESO-specific $CD8^+$ T cell induction after vaccination. This T-cell response was vaccinated in most patients who expressed HLA-DR2. The study on immunodominant $CD4^+$ T cells and their determinants should help us to improve vaccine design. Schnurr et al. [120] reported that the type of human DC, the mode of activation, and the strategy for delivery of antigen are 3 critical factors for efficient stimulation of tumor-specific $CD8^+$ and $CD4^+$ T cells. Only $CD1c^+$ blood DCs and monocyte-derived DCs were capable of presenting epitopes of ESO on both MHC class I (cross-presentation) and MHC II, whereas plasmacytoid DCs were limited to MHC II presentation. Cross-presentation was inefficient for ESO alone, but highly efficient for ESO–ISCOMs and for ESO–IMX. The mode of antigen delivery was found to be a determining factor for cytosolic proteolysis by DCs. ISCOMs targeted a slow, proteasome-dependent cross-presentation pathway, whereas ISCOMATRIX™ targeted a fast, proteasome-independent pathway. Both cross-presentation pathways resulted in a long-lived, T-cell stimulatory capacity, which was maintained for several days longer than for DCs pulsed with peptide. This may provide DCs with ample opportunities for sensitizing tumor-specific T cells against a broad array of tumor antigen epitopes in lymph nodes. A pilot trial of ESO–IMX pulsed onto peripheral blood dendritic cells (PBDC) was also performed

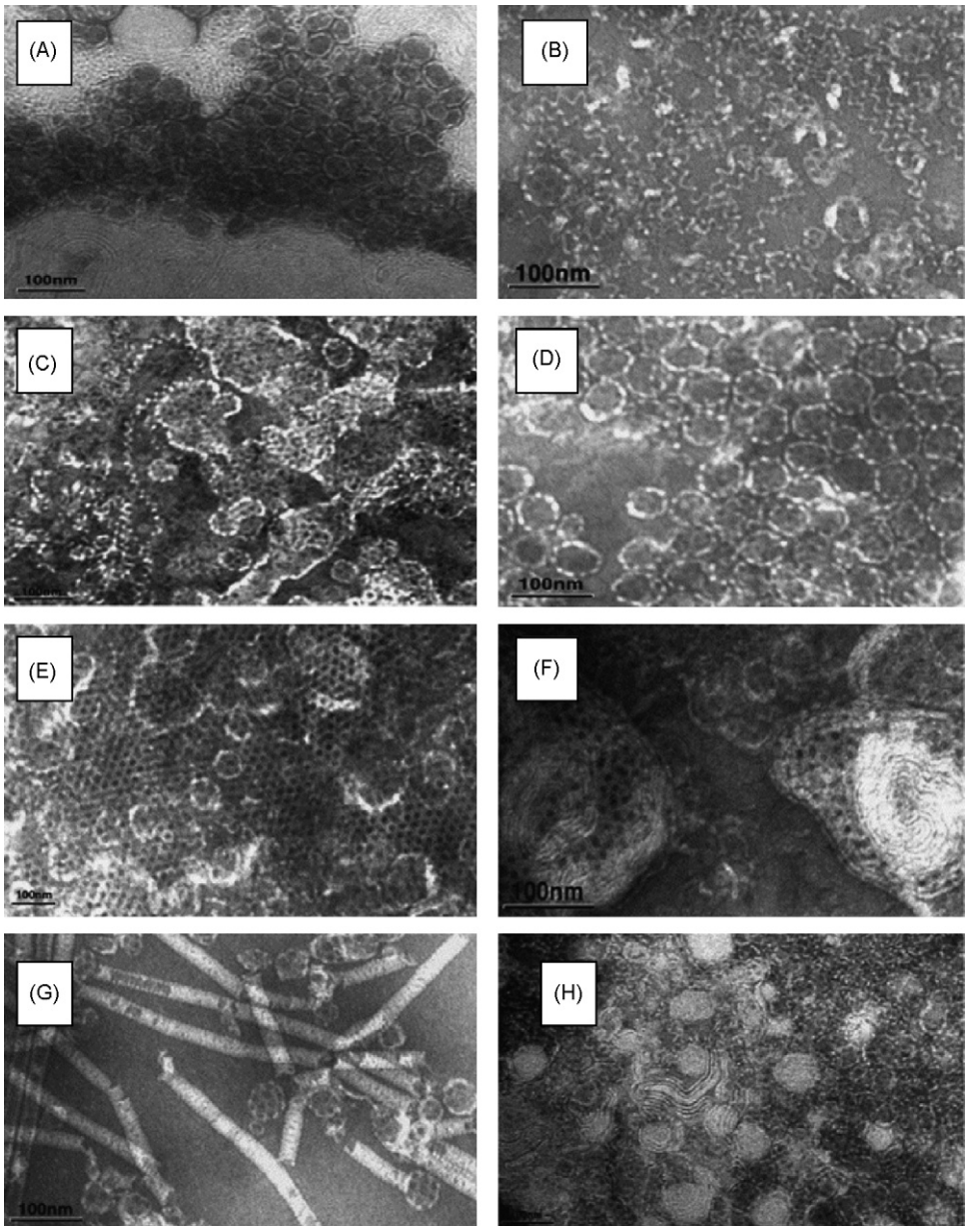

Fig. 4. TEM micrograph of colloidal particles prepared by ether injection method. (A) ISCOMATRIX™ together with liposomes, (B) worm-like micelles together with few ISCOMATRIX™, (C) lamellae and helical structures together with few ISCOMATRIX™, (D) ISCOMATRIX™, (E) lamellae structures together with ISCOMATRIX™, (F) liposomes and lamellae structures together with few ISCOMATRIX™, (G) spiral structures together with ISCOMATRIX™, (H) lipidic particles together with liposomes and ISCOMs [38].

to evaluate toxicity and the induction of ESO-specific immune responses in patients [121].The results indicated that pulsing with ESO–IMX leads to activation of PBDC, and the treatment of patients with ESO–IMX pulsed PBDC is safe.

4. Mechanism of ISCOMs and ISCOMATRIX™ vaccines

ISCOMs and ISCOMATRIX™ combine antigen presentation by both MHC class I and class II pathways, and the powerful immunomodulatory capability of the saponin [6]. ISCOMs and ISCOMATRIX™ vaccines have been shown to become highly immunogenic *in vivo*, and to induce antibody and cellular immune responses. The properties of ISCOMs and ISCOMATRIX™ included their *in vivo* distribution, interactions with antigen presenting cells (APC), stimulation of T helper (Th) cell subsets and activation of CTL [9,10]. This broad immune response is dependent on the induction of multiple innate and adaptive immune mediators, cellular processes and the interplay between these elements [122].

4.1. Antigen presentation

Lymphocyte recruitment from blood into the lymph node is thought to be initiated by the presence of antigen. The studies using a sheep afferent lymph node cannulation model have shown that ISCOMATRIX™, in the absence of antigen, can induce pro-inflammatory cytokines (IL-1, IL-6, IL-8 and IFN-γ) production, node shut down and lymphocyte recruitment [123]. The production of inflammatory mediators by immune effector cells is now recognized as being critical for the induction of primary immune responses. Smith et al. [124] investigated the ability of ISCOMs to recruit and activate innate immune responses as measured in peritoneal exudate cells. The intraperitoneal injection of ISCOMs

Table 1
Immune responses and protection in animals immunized with ISCOMs and ISCOMATRIX™ vaccines against infectious diseases.

Antigen	Animal model	Antibody	Cellular immune responses	Protection	Ref.
H1N1 influenza–ISCOMs (subvirion)	Mouse	Serum HAI titres	HA specific CTL	Protection against H1N1 and H2N2 (dose dependent)	[44]
H1N1 influenza–ISCOMs (disrupted)	Mouse	Serum IgG and IgA; Lung, nasal and faecal IgA	Virus specific CTL	100% against homologous challenge	[45]
H1N1 influenza–IMX (HA)	Mouse, sheep	Serum HAI titres, IgA and mucosal IgA			[46]
H1N1 influenza–IMX (disrupted)	Merino ewe	Serum HAI titres, IgA and mucosal IgA			[47]
Trivalent equine influenza–ISCOMs	Pony	Serum EIV-specific IgGa and IgGb and nasal IgA	EIV-specific IFN-γ	Clinical signs and virus excretion	[48–50]
Trivalent, subunit influenza–ISCOMs	Mice	Serum HI antibody			[51]
H3N2 influenza–ISCOMs (disrupted)	Mouse			94–100% protection dependent on antigen dose	[52]
H1N1 influenza–ISCOMs	Human		Virus-specific CTL		[53]
H1N1 influenza–ISCOMs (detergent-split)	Mice	Serum IgG1 and IgG2a	IFN-γ and IL-5; HA-specific CTL	67–100% cross-protection dependent on antigen dose	[54]
Influenza-ISCOMs	Macaque			100% protection against homologous challenge	[55]
H5N1 influenza–ISCOMs (surface gp)	Rooster			Protection against homologous challenge	[56]
H7N7 influenza–ISCOMs (HA and NA)	Rooster			Protection against homologous challenge	[57]
Trivalent flu vaccine–ISCOMs	Human	Serum HAI titres	T cell proliferation; H1-, HA-specific CTL		[58]
H3N2 influenza–ISCOMs (disrupted)	*Cynomolgus macaques*	Serum HAI antibody		Protection against homologous, not protect against heterologous challenge	[59]
HCV core protein–IMX	Rhesus macaque	Serum core specific antibody	IFN-γ, IL-2, IL-5 and IL-10; core protein-specific CTL		[60]
HCV proteins–IMX	Mice	Envelope glycoprotein specific antibodies	$CD4^+$ T helper responses but no $CD8^+$ T-cell responses		[61]
HBsAg–ISCOMs	Mice		spleen lymphoyte transformation and IL-2		[62]
HSV-2–ISCOMs (gp)	Mouse	Serum IgG, IgG1, IgG2a and neutralising antibody	IL-2 and IFN-γ; lymphoproliferation	80% and 56% survival from HSV-2 and HSV-1	[63]
PhHV-1–ISCOMs (gB and/orgD)	Cat or seal	Serum specific neutralizing antibody	Proliferative responses in vaccinated seals	Reduce viral titre in cats	[64,65]
EHV-1–IMX (gD)	Horse	Serum neutralizing and gD-specific IgGa and IgGb			[66]
HSV-1–ISCOMs (gGp)	Guinea pig			Reduce viral titre	[67]
HSV-1–ISCOMs (gp)	Mouse			93% protection from viral latency in CNS	[68]
HIV-1–ISCOMs (gp120)	*Macaca mulatta* macaques	HIV-1 gp120 specific virus neutralizing antibodies	IL-2 and IFN-γ; no cytotoxicity but T-cell proliferation; I		[69]
HIV-I–ISCOMs (env and gag peptides)	Mouse	Serum specific IgG2a and IgG2b	High T-cell stimulation index		[70]
HIV-1–IMX (gp120)	Guinea pig	Neutralizing antibody (100-fold)			[71]
HIV and SIV–ISCOMs (Th and CTL epitopes)	Rhesus macaques	Neutralizing antibody	CTLs	Reduce viral loads	[72]
HIV PR8-Flu–ISCOMs I	Mice, rhesus macaques	gp120-specific IgA in mice, but not in rhesus macaques			[73]
FIV–ISCOMs (accessory proteins Rev and OrfA)	Cats			No protection against FIV	[74]
MV–ISCOMs (H or F)	Mouse monkey	HI and neutralizing antibody, and F-specific antibody	Measles virus-specific T cells in mice	Full protection from acute encephalopathy	[75]
MV–ISCOMs (Inactivated virus)	Cotton rat			100% protection	[76]
MV–ISCOMs and MV–IMX	Macaques	Neutralizing antibody	MV-specific IFN γ-producing cells		[77]
HMPV–IMX (F)	Syrian golden hamsters	Neutralizing antibody		Homologous or heterologous protection; reduce viral titre	[78]
HMPV–IMX (F)	cynomolgus macaques	F-specific Ig antibody and neutralizing antibody	Specific lymphoproliferation	Reduce virus shedding	[79]
NDV–ISCOMs (HN and F)	Chicken	Serum HI and neutralizing antibody		>80%protection	[80]
RSV–ISCOMs (F and G)	Mice	RSV-specific IgG and IgA			[81]
RSV–ISCOMs (F and G)	Mice	Serum IgG, IgG2a, IgG1 and neutralizing antibody and IgA inbronchoalveolar lavage	IFN-γ, reduced Th2-cytokine expression	Reduce lung RSV titers	[82]

Table 1 (*Continued*)

Antigen	Animal model	Antibody	Cellular immune responses	Protection	Ref.
RSV–ISCOMs (F and G)	Mice	RSV-specific IgG and IgG2a	Th1 type of profile; IFN-γ; specific CTL		[15,83]
BRSV–ISCOMs (F and G)	Guinea pig	Serum VN antibody			[84]
BRSV–ISCOMs (F and G)	Calves	bRSV-specific nasal IgG, serum IgG_1 and IgG_2 titers		Clinical and virological protection	[85]
RPV–ISCOMs (H)	Cattle	Neutralizing antibody		100% protection	[86]
Rotavirus VLP–IMX	Gnotobiotic pigs			No protection	[87]
Rotavirus VP6 protein–IMX	Gnotobiotic lambs	Serum specific neutralizing and IgG antibody	Increased $CD8^+$ T cells in jejunal PP	Reduced period of viral excretion	[88]
Rotavirus VLP–IMX	Gnotobiotic pigs			70% protection	[89]
Rotavirus VLP–IMX	Gnotobiotic pig, gnotobiotic calve	serum VN, IgA, IgG and intestinal IgA antibody		Protection against viral shedding and diarrhea dependent on dose and maternal antibody	[90–94]
Rotavirus VLP–IMX	Gnotobiotic pig	IgM, IgA and IgG-secreting cells in intestine		75% and 100% protection against diarrhea and viral shedding	[95]
EBV–ISCOMs (gp340)	Cottontop tamarin	gp340 specific serum antibody	EBV gp340-specific CTL		[96]
AM-MSPs–ISCOMs and AM-MSPs–IMX	Mice, calf	IgG, IgG1, and IgG2a im mice			[97]
MmmSC–ISCOMs	Mice, cattle	Serum specific antibody in mice	Lymphoproliferation in mice	Reduce mortality, but higher gross pathological and histopathological scor in cattle	[98,99]
DT–ISCOMs	Mice and guinea pigs	Serum IgG, IgG1, IgG2a, IgA, neutralising antibody	IFN-γ and IL-5; DTH reaction	Protection against DT	[100]
HP–ISCOMs and HP–IMX (HpaA and catalase)	Mice	Catalase-specific IgA and IgG antibody		Reduce bacterial colonisation	[101]
MB–IMX (cytotoxin)	Bred beef calf	Serum neutralizing and cytotoxin-specific IgG		Reduce cumulative proportion of calves with ulcerated eyes	[102,103]
TB–ISCOMs (38-kDa lipoglycoprotein)	Mice	Serum specific IgG (IgG1 and IgG2a)	Specific CTL		[104]
Ag85B-ESAT-6 CTA1-DD–ISCOMs	Mice	IgA	IFN-γ		[105]
Sfb1–SAMA4	Mice	Sfb1-specific IgG		No protection	[106]
Chlamydia trachomatis –ISCOMs (MOMP)	Mice	IgA in lung	IFN-γ, DTH	Partial protection	[107–109]
NcSRS2–ISCOMs	Mice		IFN-γ, IL-4 and IL-5	Reduce the proliferation of *N. caninum* in the brains	[23,24,110]
TGRP–ISCOMs	Pigs	IgG		Partial protection against clinic signs	[111,112]

AM-MSPs, surface proteins of *Anaplasma marginale*; BRSV, bovine respiratory syncytial virus; CNS, central nervous system; DT, palmitified diphtheria toxoid; EHV-1, equine herpesvirus 1; EIV, equine influenza virus; F, fusion protein; FHV, feline herpesvirus; G, attachment protein; gB, glycoproteins B; gD, glycoproteins; H, haemagglutinin protein; HA, haemagglutinin; HAI, haemagglutinin inhibition; HBsAg: a recombinant hepatitis B vaccine; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HMPV, Human metapneumovirus; HN, haemagglutinin-neuraminidase; HP, *Helicobacter pylori*; HRV, human rotavirus; HSV, herpes simplex virus; IMX, ISCOMATRIX™; MB, *Moraxella bovis*; MmmSC, *Mycoplasma mycoides* subsp. *mycoides* small colony; MOMP, major outer membrane protein; MT, *Mycobacterium tuberculosis*; MV, measles virus; NA, neuraminidase; NDV, Newcastle disease virus; NcSRS2, *Neospora caninum* surface antigen; PhHV-1, *Phocid herpesvirus* type-1; OMP, outer membrane protein; PP, peyer's patches; RPV, rinderpest virus Rinderpest virus; RSV, Respiratory syncytial virus; SAMA4, a liposome/ISCOM combination adjuvant; Sfb1, fibronectin binding protein 1 of *Streptococcus pyogenes*; SIV, simian immunodeficiency virus; TB, tuberculosis; TGRP, *Toxoplasma gondii* rhoptry proteins; VLP, VP2/6-rotavirus-like particles; VP, viral protein.

induced intense local inflammation, with early recruitment of neutrophils and mast cells followed by macrophages, dendritic cells, and lymphocytes. Many of the recruited cells had phenotypic evidence of activation and secreted a number of inflammatory mediators, including nitric oxide, reactive oxygen intermediates, IL-1, IL-6, IL-12, and IFN-γ. Of these factors further only IL-12 appeared to be essential for the immunogenicity of ISCOMs, as IL-6- and inducible nitric oxide synthase knockout (KO) mice developed normal immune responses to OVA in ISCOMs, whereas these responses were markedly reduced in IL-12 KO mice. The recruitment of peritoneal exudate cells following an injection of ISCOMs was impaired in IL-12 KO mice, indicating a role for IL-12 in establishing the proinflammatory cascade. Thus, ISCOMs prime Ag-specific immune responses at least in part by activating IL-12-dependent aspects of the innate immune system. In addition, ISCOMs initiate mucosal as well as systemic immune responses in an IL-12 dependent manner but independently of IL-4 [125]. Together these observations suggest that ISCOMs vaccines activate innate immune responses, with IL-12 playing a key role [6].

ISCOMs and ISCOMATRIX™ vaccines have also been shown to induce upregulation of MHC class II and I expression on APCs, and to be superior to the other colloidal structures in terms of the activation of DC, which is required for the generation of antigen-specific immunity [126]. Because of their particulate nature, ISCOMs and ISCOMATRIX™ are directly targeted to and more efficiently taken up by APC, such as DCs and macrophages via endocytosis [127]. Further, the included *Quillaja* saponins exhibit adjuvant effects, providing the necessary signals to stimulate cells of the innate immune system (such as DCs) and consequently initiate antigen-specific acquired immunity. It was demonstrated that the adjuvancy of *Quillaja* saponins was dependent on an aldehyde functional group present within the individual molecules and aliphatic side-chains [128]. Saponin-mediated targeting of DEC-205 (a macrophage mannose receptor family of c-type lectin endocytic receptors) on the surface of DCs might account for higher uptake and more efficient presentation of antigens to T cells [129,130]. Antigen processing can occur in the endosome for both MHC class II and class I presentation [131], possibly by the recently described cross-presentation pathway [132–134]. The ability of the ISCOMs and ISCOMATRIX™ vaccines to induce these broad immune responses is due to the combination of antigen presentation by both MHC class I and class II pathways [6]. DC is now recognized as the most potent at priming

both $CD4^+$ and $CD8^+$ T cells *in vivo* and activation of DC enhances their ability to present antigen to $CD4^+$ T cells. ISCOMs recruit a number of accessory cells *in vivo*, including DC, but very little are known of which APC presents the ISCOMs-associated antigen and of how ISCOMs are processed by defined APC subsets. In particular, it is not known how ISCOMs can prime $CD8^+$ T-cell responses so efficiently *in vivo*, an unusual, but important, property for a vaccine adjuvant. In addition, unlike $CD4^+$ T-cell responses, where the role of DC maturation is well characterized, there is little information on the effects of DC maturation on class I-restricted presentation to $CD8^+$ T cells, particularly when exogenous antigens have been used. To test the hypothesis that presentation by DC underlies the priming of $CD8^+$ T cells by ISCOMs, Robson et al. [135] investigated the role of DC in the priming of antigen-specific $CD8^+$ T cells *in vitro* by ISCOMs containing ovalbumin. The results show that bone marrow-derived DC are extremely effective at presenting ISCOMs-associated antigen to resting $CD8^+$ T cells *in vitro* and this is greatly enhanced when ISCOMs-pulsed resting DC are induced to differentiate with lipopolysaccharide. The priming of $CD8^+$ T cells is independent of cognate $CD4^+$ T-cell help and the intracellular processing involves elements of both the class I and II pathways. Interestingly, the presentation by resting DC is dependent on TAP, but independent of CD40–CD40 ligand (CD40L), whereas the maturation effect reveals a CD40L-dependent, TAP-independent pathway. These findings show that ISCOMs can be presented very efficiently to $CD8^+$ T cells by DC and also suggest the existence of a novel pathway of MHC class I-restricted antigen processing which may be an important target for vaccine adjuvants. Schnurr et al. [120] reported that antigen cross-presentation was inefficient for ESO alone, but highly efficient for ESO–ISCOMs and for ESO–ISCOMATRIX™, and that ISCOMs targeted a slow, proteasome-dependent cross-presentation pathway, whereas ISCOMATRIX™ targeted a fast, proteasome-independent pathway.

4.2. Cytokine induction

Regulatory Th cells are essential for the development of antibody and CTL responses to foreign antigens. With respect to their cytokine production after activation, murine Th cells can be divided into at least two functionally distinct sub-populations. Th1 cells produce the cytokines IL-2 and IFN-γ and mediate certain antibody-independent immune responses as well as promoting certain antibody responses. Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and are considered to provide help essential for antibody production. The importance of the Th cell subsets for generation of protective immunity has been demonstrated in several experimental models and human diseases and suggests that vaccination against certain infectious diseases may be dependent on efficient means to induce T-cell responses with desired properties. The use of adjuvants with distinct immunomodulatory properties represents one approach to achieve this aim.

The ability of saponin based formulations to induce strong Th cell responses is well established. Studies in sheep demonstrated that high levels of IFN-γ was present in the lymph following injection of Quil A in the presence or absence of antigen; replacing the Quil A with $Al(OH)_3$ did not induce a similar response. A number of studies have examined the development of Th1-like and Th2-like T cell responses after injection of saponin containing vaccines, particularly ISCOMs and QS-21. Activation of T cells by ISCOMs was first described by Fossum et al. [136], who reported that immunization with influenza-ISCOMs induced spleen cells which proliferated and secreted IL-2 after antigen stimulation *in vitro*. These observations were extended in a study [137] which showed that the production of IL-2 and IFN-γ by spleen cells primed with ISCOMs was dependent on $CD4^+$ T cells. The ability of ISCOMs to induce T cells producing IL-2 and IFN-γ has since been demonstrated for a number of antigens and it is now well established that ISCOMs strongly promote the development of Th1 type T cell responses. Additional support for this observation is that ISCOMs elicit high levels of antibodies of the IgG2a subclass. A shift to production of antibodies of this subclass is dependent on IFN-γ, whereas IL-4 is important for the generation of high levels of IgG1 [138]. The relative production of these IgG subclasses in mice can therefore be used as a surrogate marker for the generation of immune responses of a Th1 or Th2 type.

Clearly, cytokine induction is an important component of ISCOMs and ISCOMATRIX™ vaccine. Upregulation of pro-inflammatory IL-1 was the first cytokine response observed to be related to the adjuvant activity of ISCOMs vaccines [139]. Since then many other cytokines have been shown to be up-regulated in response to ISCOMs and ISCOMATRIX™ vaccines, these include IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IFN-γ [9,140]. Mohamedi et al. [63] reported that ISCOMs favored the capacity to enhance a Th1 type of immune response. In his study, HSV-2 antigen was prepared following its formulation into ISCOMs in a murine model. The results showed that higher IgG2a and neutralising antibody levels, IL-2 and IFN-γ levels and lymphoproliferative responses were noted in mice immunized with the HSV-2 ISCOMs vaccine preparation. However, there were no differences between any of the HSV-2 vaccine formulations in terms of IL-4 induction in spleen cell cultures, indicating Th1 bias in this vaccine design. Similar report conducted by Rinaldo and Torpey [141] revealed that high levels of the cytokines IL-2 and IFN-γ, indicative of a bias towards Th1 immune responses, have been correlated with protection against HSV. ISCOMs were also reported to induce a concomitant Th2 response, resulting in a so-called balanced Th1/Th2 response [125]. The broad range of cytokines is consistent with the mixed Th1/Th2 responses observed with ISCOMs vaccines [122].

The generation of Th2 responses after ISCOMs immunization is less clear cut and appears to vary with the antigen used, the choice of cytokines analysed and the type of cytokine assay. The production of IL-4 by T cells primed with ISCOMs has been reported to be low or undetectable when determined as the cytokine concentration in cell culture supernatants. However, IL-4 may be rapidly consumed [142,143] and therefore, the low levels of IL-4 in culture supernates may not therefore reflect the true responses *in vivo*. In support of this, immunization with ISCOMs containing an antigen (PSA-2) from the parasite *Leishmania major* induced high numbers of T cells producing IL-4 as detected in an ELISPOT assay but only trace amounts of IL-4 were detected in parallel cell culture supernates. Moreover, vaccination of C3H/He mice with PSA-2-ISCOMs did not protect them against *L. major* infection despite the activation of high numbers of T cells secreting IFN-γ. As protection against *L. major* depends on the generation of Th1-like T cells producing IFN-γ and susceptibility correlates with the presence of IL-4, these findings suggest that the activation of Th2-like T cells by ISCOMs vaccination was sufficient to abrogate the protective Th1 effects. In addition, the induction of IL-4 by OVA in ISCOMs has been reported to be comparable to that of OVA in $Al(OH)_3$, an adjuvant with extremely high capacity to induce Th2 responses. The strong ability to increase IgG1 responses to antigens provides further support for the involvement of IL-4 in immune responses to ISCOMs. ISCOMs have also been reported to induce production of IL-5 and IL-10. Immunization with OVA–ISCOMs or PSA-2–ISCOMs generated T cells producing significant amounts of IL-5 [144]. The effects of ISCOMs on IL-10 production are unclear as both an increase and decrease in production of IL-10 have been reported. *Quillaia* saponins may down-regulate the production of IL-10 in a dose-dependent way [145]. It can be concluded that ISCOMs, in most cases, function as a potentiator of a Th1 type immune response but are also able to induce a concomitant Th2 response.

4.3. CTL induction

The induction of CTL responses generally requires that antigens are processed in the cell cytosol to generate peptides which are presented at the cell surface in the context of MHC class I molecules. Exogenous antigens must therefore be able to enter the cytosol to give rise to peptides which can be presented to MHC class I restricted CTL. Adjuvants can be useful for CTL induction by facilitating this process. One-way to achieve this is for the adjuvant to interact with the cell membranes so that antigen together with the adjuvant is deposited into the cytosol. The adjuvant can also induce the production of Th1-like cytokines which are necessary for the development of these cellular immune responses.

ISCOMs have been demonstrated to induce $CD8^+$ MHC class I restricted CTL to a number of antigens after immunization by several different routes of administration. This was first illustrated with recombinant HIV-1 gp160-ISCOMs and influenza-ISCOMs. The adjuvant potential of ISCOMs and ISCOMATRIX™ vaccine can be achieved by optimal CTL induction [39]. And the capacity to deliver antigen to the cytosol paves the way for a MHC class I-restricted antigen presentation resulting in a strong CTL response [146]. The most likely explanation for this is that ISCOMs and ISCOMATRIX™, because of their particulate nature, are targeted to and more efficiently taken up by cells of the immune system such as APCs followed by processed and presented to $CD8^+$ T-cells [115]. Le et al. [147] conformed the delivery of polytope vaccines in the form of either synthetic polypeptides or recombinant polytope proteins by ISCOMs and showed that induction of multiple protective CTL responses by these polytope-ISCOMs formulations were comparable to viral vector or DNA based delivery modalities as assessed by IFN-γ ELISAPOT, chromium release and viral challenge assays. A possible mechanism of the CTL effect elicited by the ISCOMs can be explained by the apoptotic and necrotic effects induced by saponin in EL4 mouse lymphoma cells [148].

The mechanism by which ISCOMs and ISCOMATRIX™ induce CTL responses is likely that these adjuvants associate with antigen and facilitate entry into the cell cytoplasm. Due to their surface-active properties, it is possible that the *Quillaia* saponins play a role in this process by intercalating with cholesterol in the cell membrane to form pores, which have been observed in electron micrographs, through which the saponin and antigen could pass into the cytoplasm. Supporting this mechanism is the finding that ISCOMs containing the measles virus F-protein have been reported to sensitize target cells *in vitro* for lysis by $CD8^+$ MHC class I-restricted CTL clones [149]. When a cell line which had lost the ability to generate peptides presented by MHC class I molecules was used as the APC, no lysis was detected, demonstrating that processing in the cytosol of measles F protein contained in the ISCOMs was necessary. Thus, the ISCOMs might incorporate into cell or endosomal membranes, thereby exposing the incorporated antigen to cytosolic proteases [150].

4.4. Apoptosis

The mechanism for adjuvant activities of saponin was also investigated by the apoptotic and necrotic effects induced by saponin in EL4 mouse lymphoma cells, which were expected to be a possible mechanism of CTL effect elicited by the ISCOM [16]. Since optimal cross-presentation of an antigen required an additional step of DC maturation induced by necrosis [150], cross-presentation of antigens to $CD8^+$ T cells was found to take place after phagocytosis of apoptotic cells by immature DCs, which provide antigenic signals for MHC class I presentation. Analysis of EL4 cells by flow cytometry after Annexin V/propidium iodide staining demonstrated that saponin induced both apoptosis and necrosis, after which immature DCs were shown to phagocytose both the antigen–saponin complexes and the saponin-induced dead cells, indicating that saponin induced both apoptosis and necrosis in EL4 cells and these events are critical for antigen processing and presentation [148].

The depot effect, whereby, antigen is trapped at the site of administration, in order to attract APCs is considered to be an important function of adjuvants. However, unlike aluminium- and oil-based adjuvants, ISCOMATRIX™ based vaccines are cleared rapidly from the site of injection to the draining lymph nodes, although there is some evidence of dose site effects such as cellular infiltration [122]. To date there is little evidence to suggest that ISCOMs and ISCOMATRIX™ binds to specific receptors and unlike other activators of innate immune responses such as CpGs, LPS and DNA they do not appear to activate Toll-Like Receptors (TLRs). It, therefore, remains unclear how ISCOMs and ISCOMATRIX™ induce cellular activation and up-regulate cytokine expression [122].

5. Conclusion

ISCOMs and ISCOMATRIX™ vaccines have now been tested with numerous antigens both in humans and in veterinary vaccine designs, and been shown to be highly immunogenic including antibody-mediated immunity, cell-mediated immunity as well as innate immune responses. The major features of ISCOMs and ISCOMATRIX™ vaccines for humoral responses include the magnitude, speed and longevity of the antibody response, as well as the capacity for antigen dose reduction, making them suitable for vaccine designs that require a rapid response and for antigen that is limited or expensive to manufacture. The major features of ISCOMs and ISCOMATRIX™ vaccines for cellular immune responses are the ability to induce strong and long lasting $CD4^+$ and $CD8^+$ T-cell responses or/and long lived CTL responses. The ability of ISCOMs and ISCOMATRIX™ to induce CTL in primates and humans makes them ideal for use in vaccines directed against chronic infectious diseases as well as for therapeutic cancer vaccines. Additionally, ISCOMs and ISCOMATRIX™ also demonstrates significant potential as a mucosal adjuvant, particularly for intranasal administration.

Novel data from animal or human models have provided insight into the mechanisms underlying the adjuvant functions of ISCOMs and ISCOMATRIX. These include the activation of IL-12-dependent aspects of the innate immune system, or inducing the abilities of antigen presentation by both MHC class I and class II pathways and relative cytokines. These events most likely create an optimal environment for the maturation of APC such as DC, thus enhancing their ability to present antigens and provide costimulatory signals that will facilitate the subsequent amplification of the antigen-specific immune response.

In all cases, the studies have shown a good safety and tolerability profile in humans and animals and as well as induction of both humoral and cellular immune responses. Although there are currently registered ISCOMs vaccines for veterinary applications, the properties and features of the ISCOMs and ISCOMATRIX™ vaccines need further clinical investigation for novel human vaccines and further cellular or humoral immune responses should also be required to demonstrate efficacy in humans in the future use.

Acknowledgements

This work was supported by a Grant-in-Aid from the National Natural Science Foundation of China (No. 30871888), the Zhejiang Provincial Natural Science Foundation of China (No. M303749), the National Key Project of Scientific and Technical Supporting Programs Funded by Ministry of Science & Technology of China (2008BADB4B06-2) and the Administration of Traditional Chinese Medicine of Zhejiang Province (No. A2006Z017).

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