The carbomer-lecithin adjuvant Adjuplex™ has potent immune activating properties and elicits protective adaptive immunity against influenza challenge in mice

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Abstract

The continuing discovery and development of adjuvants for vaccine formulation is important to safely increase potency and/or reduce antigen dose of existing vaccines, and tailor the adaptive immune response to newly-developed vaccines. Adjuplex™ is a novel adjuvant platform based on purified lecithin and carbomer homopolymer. Here we analyzed the adjuvant activity of Adjuplex™ in mice for the soluble hemagglutinin (HA) glycoprotein of influenza A. Titration of Adjuplex™ revealed an optimal dose of 1% for immunogenicity, eliciting high titers of HA-specific HA, but inducing no significant weight loss. At this dose, Adjuplex™ completely protected mice from an otherwise lethal influenza challenge, and was at least as effective as adjuvants MPL and alum in preventing disease. Adjuplex™ elicited a balanced Th1/Th2-type immune response with accompanying cytokines, and triggered antigen-specific CD8+ T cell proliferation. Use of the peritoneal inflammation model revealed that Adjuplex™ recruited dendritic cells (DCs), monocytes and neutrophils in the context of innate cytokine and chemokine secretion. Adjuplex™ neither triggered classical maturation of DCs nor activated a pathogen recognition receptor (PRR)-expressing NFκB reporter cell line, suggesting a mechanism of action different from that reported for classical pathogen-associated molecular pattern (PAMP)-activated innate immunity. Taken together, these data reveal Adjuplex™ as a potent and well-tolerated adjuvant with application for subunit vaccines.
Introduction

Vaccines based upon recombinant, purified or inactivated microorganism-derived antigens generally have sub-optimal immunogenicity in the absence of an adjuvant. The field of vaccine adjuvant discovery has gained rapid momentum following the discovery of innate immune pathogen-recognition receptors (PRRs) including Toll-Like-, Nod-Like- and Rig-Like Receptors (TLRs, NLRs and RLRs respectively) that activate and condition innate and adaptive immunity (1). Despite this, the absolute requirement to demonstrate adjuvant safety along with other considerations has limited licensing of vaccines containing novel adjuvants. New vaccines, particularly those aimed at driving robust adaptive immune responses in situations where these are limited such as influenza vaccination in the aged or HIV-1 antibody-based vaccines, may require more potent and/or tailored adjuvants. Adjuvant discovery therefore remains an essential area of vaccine research (1).

Adjuplex™ is a biodegradable matrix of carbomer homopolymer (also known as carbopol™) and submicron sized liposomes (nanoliposomes) derived from purified soy lecithin. Carbomers, a species of cross-linked polyacrylic acid with long and broad use in biomedicine (2), have been evaluated as experimental adjuvants in veterinary vaccines against swine parvovirus (3) and circovirus type 2 (4), Staphylococcus aureus in sheep (5) and equine influenza virus (6). These reports demonstrate that biodegradable carbomers such as carbopol™ are not harmful to mammals and stimulate a more robust immune response than antigen alone. Indeed, carbomer is a component of a licensed veterinary vaccine in pigs (Suvaxyn™, Wyeth). Despite its veterinary use, there is little information available in the
published literature relating to the type and magnitude of innate or adaptive immune response induced by carbomers compared to other well-characterized adjuvants. Lecithin is a biocompatible naturally occurring surfactant derived from the lipid matrix of biological membranes (7). The term lecithin is loosely applied to various fractions of lipids commonly sourced from plant seeds, such as soybeans, or egg yolk. Natural lecithin contains a complex mixture of phosphatides as well as triglycerides, fatty acids and carbohydrates whereas refined, de-oiled lecithin contains exclusively phosphatides or simply phosphatidylcholine, the major component of the phosphatide fraction (7). Lecithin's properties as emulsifier, stabilizer, antioxidant and dispersing agent, and its propensity to form multi-lamellar vesicles (liposomes), are utilized throughout the pharmaceutical industry, notably for drug and vaccine delivery (7-9). Lecithin is a principal component of the widely used commercial veterinary adjuvant, Amphigen® (10).

Our own recent work has highlighted the potent adjuvanting activity of carbomers, either used alone with subunit antigens (11) or co-formulated with the proprietary oil-in-water formulation MF59 (12, 13), and suggested that they compare favourably with other adjuvants in terms of tolerability and potency. The co-formulation of carbomer and lecithin that comprises Adjuplex™ has also been demonstrated as a potent, yet well-tolerated adjuvant suitable for use with a variety of antigens and immunization regimens. In a range of animal species from mice to non-human primates, Adjuplex™ induced antibodies to various HIV-1 (14, 15) and malaria (16) antigens, and small molecule addictive drug analogs (cocaine, nicotine) (17-22). Notably, in a non-human primate study of HIV-1 envelope glycoprotein
trimer antigen comparing DNA prime/protein boost regimens, in which all 107 animals received protein antigen adjuvanted with Adjuplex™, neutralizing 108 antibody breadth and titers exceeded levels achieved by previous vaccine 109 regimens in primates (15).

Despite these studies, little is known of the immunological profile of 111 Adjuplex™, or its ability to elicit protective antibodies against a common 112 human pathogen. For these reasons we assessed the adjuvant activity of 113 Adjuplex™ in the context of soluble influenza HA antigen, the target of 114 neutralizing antibodies and a potential component of a human vaccine against 116 this virus. We find that Adjuplex™ is well tolerated in mice and elicits a strong 117 and balanced adaptive immune response driving potent antibody production 118 that is protective against influenza challenge. Based on these results, we 119 propose that further exploration and development of this adjuvant is 120 warranted.

Methods

Antigens and Adjuvants

Influenza hemagglutinin (HA) bromelain-released and purified from the H1N1 124 PR8 isolate (23) was obtained from Dr. J. Skehel, NIMR, UK. The following 125 adjuvants were used: Adjuplex™ was supplied by Advanced BioAdjuvants 126 LLC. as a 100% solution used at the doses shown; Aluminium salts 127 (alhydrogel, Brenntag; referred to as “alum”, stock 2%) used at 100 µg per 128 dose; Monophosphoryl Lipid A (MPLA-SM VacciGrade, from hereon termed 129 MPL, Invivogen Inc.) used at 10 µg per dose. All adjuvants were mixed with
HA diluted in endotoxin-free PBS at room temperature approximately 30 mins prior to administration.

Animals, Immunizations and viral challenge

C57BL/6 mice were purchased from Harlan Inc. and were immunized in groups as described in figure legends, between 6-8 weeks of age. Antigen-adjuvant formulations were prepared under sterile conditions in endotoxin-free PBS in a total volume of 100 µL, and were administered subcutaneously in prime or prime and boost regimens as described in the text. Blood samples were taken via tail bleed at the time points described in the text. In some experiments mice were sacrificed and spleens were taken, disaggregated and cultured in vitro as described below. For protection experiments, animals were intranasally challenged with 16 HAU (corresponding to $1.17 \times 10^5$ plaque forming units, PFU) of mouse-adapted PR8 H1N1 on day 15 after immunization. Animals were monitored for weight loss and euthanized when a humane endpoint was reached, defined based on clinical scoring or weight loss. All animal experiments were performed under the appropriate national licenses in accordance with the UK Animals (Scientific Procedures) Act 1986 and were authorized by the UK Home Office and the Oxford local institutional ethical review board.

ELISA

Serum samples were allowed to clot for 30 mins at room temperature (RT), cleared by centrifugation and stored at $-20^\circ$C until analyzed. Antigen-coated and blocked ELISA plates were incubated with serial dilutions of samples.
Bound antibodies were detected with the appropriate secondary reagents (anti-mouse IgG-HRP (STAR120P, Serotec); anti-mouse IgG1-HRP and IgG2c-HRP (Genetex, gtx77297) and the ELISA developed with TMB substrate (Thermo Fisher Scientific) using previously described methods (24).

Hemagglutination Inhibition (HI) assay

Sera were pretreated for 2 h at 4°C with 0.5% turkey red blood cells (TRBC, bioTRADING Benelux B.V., the Netherlands) in PBS. TRBC were removed and the sera were incubated overnight at 37°C in 10% CO$_2$ with 1/25 dilution of receptor-destroying enzyme (Sigma-Aldrich). The sera were serially 2-fold diluted from 1/8 in duplicates, and were incubated for 1 h at RT with 8 HAU influenza virus A/PuertoRico/8/1934 in 50µL. After incubation, 1% TRBC were added and incubated for 1 h at RT. The HAI titer was determined as the reciprocal of the final dilution without agglutination and reported as log$_2$-transformed values.

T cell assays

For assay of HA-specific responses, splenocytes were cultured in the presence or absence of 20 µg/mL HA for 72 h and $^3$H thymidine incorporation measured overnight using a scintillation counter (Beckman-Coulter). Alternatively, proliferation was determined using a BrdU labeling kit (eBioscience, CA, USA), and fluorescent staining for CD3 and CD4 or CD8 (BD Biosciences, NJ, USA), acquired on a CyAn™ ADP analyzer (Beckman)
Coulter, USA) and analyzed using FlowJo analysis software (Tree Star, Ashland, USA). Supernatants were cleared by centrifugation and analyzed by multiplex cytokine assay.

**Cell recruitment assays**

Adjuplex™ in endotoxin-free PBS, or PBS alone, was administered (100 µL) intraperitoneally. Mice were euthanized 24 h later and small volume (2 mL) and large volume (5 mL) peritoneal lavages sequentially performed using ice-cold PBS-EDTA. Supernatants from small volume lavages were used in cytokine/chemokine analyses, and cells from both lavages were combined for flow cytometric analysis.

**Flow cytometry and antibodies**

The following antibodies were used for flow cytometry: CD11b (M1/70), CD19 (ID3), CD3 (145-2C11), Ly6C (AL-21), Ly6G (1A8), F4/80 (Cl:A3-1), CD11c (HL3), MHC-II (2G9). Peritoneal leukocytes were stained for flow cytometry and absolute numbers of monocytes (CD11b+Ly6C++Ly6G−F4/80int), macrophages (CD11b+F4/80hiLy6G−Ly6C−), neutrophils (CD11b+Ly6GhiLy6C+F4/80), and dendritic cells (CD11b−/intCD11chiF4/80−Ly6C−MHC-IIhi) were determined.

**Multiplex cytokine assays**

Supernatants were separated from either peritoneal lavage or cultured cells via centrifugation and stored at -80°C until use. Cytokine concentrations of undiluted cell culture supernatants or peritoneal lavage were determined via
Bio-plex® cytokine array (Bio-Rad Laboratories). The panel tested was TNFα, IL-12 p40 and p70, IL-1β, IL-4, IL-6, RANTES, and GM-CSF. Only those assayed as positive compared to control are shown in Figure 6.

**DC maturation and reporter cell line assays**

Immature BMDC were derived as described in (24). Cells were pulsed with Adjuplex™ at the concentrations shown for 24, washed, blocked in BD mouse FC block (BD biosciences) and stained for surface markers with anti-mouse MHC class II (Biolegend), CD80 (Serotec), CD86 (BD biosciences), CD11c (APC), CD40 (Biolegend), and OX40L (Biolegend), and subsequently acquired and analyzed using a FACSCalibur flow cytometer (BD biosciences) and FlowJo analysis software (Tree Star). Thp1-Blue™ cells (InvivoGen) expressing TLR1/2, TLR2, TKLR2/6, TLR4, TLR5, TLR8, NOD1 and NOD2 were stimulated for with Adjuplex™ or PRR ligands (InvivoGen) for 24h and supernatants tested for secreted embryonic alkaline phosphatase (SEAP) expressed following NFkB or AP1 activation, using QUANTI-Blue™ substrate (InvivoGen).

**Statistical analysis**

Antibody titer data were log₁₀-transformed and then tested for normality (Kolmogorov-Smirnov test). If data showed a normal distribution within each compared group a one-way ANOVA was used to assess for statistical significance defined as $p<0.05$. If the data were not normally distributed, they were analyzed using a Kruskal-Wallis test with the same significance limit.
Direct comparisons between individual groups were tested for significance using the appropriate post-tests as indicated.

Results

Dose optimization of Adjuplex™

To define the optimal combination of tolerability and potency, Adjuplex™ was titrated from stock over three doses (1, 5 and 10%) using two doses of antigen (0.54 and 1.6 µg HA). The HA used was soluble bromelain-cleaved purified from whole influenza virus A lysates (23). Mice were immunized subcutaneously on day 0 and day 14 and antigen-specific IgG endpoint titers determined by ELISA. IgG titers rose more rapidly for HA adjuvanted in Adjuplex™ than HA alone, and reached maximal values of ~10⁶ for adjuvanted HA that were higher than those for non-adjuvanted HA (Fig. 1a). Area under the curve (AUC) analysis revealed no significant differences in overall IgG titer between the 1, 5 and 10% Adjuplex™ groups, but no obvious difference between the two antigen doses (Fig. 1b). A similar pattern of results was obtained using a hemagglutination inhibition (HI) assay (Fig. 1c). We therefore chose the 0.54 µg antigen dose for further study. Weight loss as an indication of adjuvant tolerability was measured over 72 h after immunization. The two highest does of Adjuplex™ showed clear transient decreases in weight compared to the lowest dose and HA alone (Fig. 1d). Quantification of these data by AUC analysis for both doses of antigen pooled, confirmed no significant difference between antigen alone and antigen with 1% Adjuplex™, whereas both 5 and 10% Adjuplex™ triggered significant weight loss (Fig. 1e). No signs of local toxicity or intolerance, assessed by swelling or
scratching caused by irritation, were noted at any adjuvant dose (results not shown). When the immunogenicity to toxicity (determined by weight loss) ratio was calculated, 1% Adjuplex™ was significantly better than HA alone, and showed a trend towards a more beneficial outcome than 5 or 10% Adjuplex™ (Fig. 1f). We therefore chose 1% Adjuplex™ for further study.

Comparative adjuvanticity of Adjuplex™, alum and MPL

We evaluated the comparative adjuvanticity and safety of Adjuplex™ with two well-characterized adjuvants: alum, the most widely used licensed vaccine adjuvant and Monophosphoryl Lipid A (MPL), a TLR-4 ligand-based licensed adjuvant used in hepatitis B and HPV vaccines (1). Alum and MPL were used at doses similar to those previously published to be safe and immunogenic in mice (25-27). Mice were immunized on day 0 and day 18 of the study and total antigen-specific IgG responses assayed post-prime at day 11 and post-boost at day 34. Adjuplex™ and MPL induced faster kinetics of IgG production compared to HA alone and HA in alum (Fig. 2a). When quantified by AUC analysis, Adjuplex™ induced significantly higher responses than HA alone or in alum, but was indistinguishable from MPL (Fig. 2b). HI titers were determined post-prime and post-boost and reflected the ELISA binding values (Fig. 2c). Weight loss was measured over 48 h from prime, and quantification revealed no significant differences between any of the groups (Fig. 2d, e). When the immunogenicity:weight loss index was calculated, Adjuplex™ and MPL showed a strong increased trend compared to alum (Fig. 2f). Thus Adjuplex™ is equivalently well tolerated and potent in IgG induction as MPL.
Antigen-specific B and T cell responses

To investigate T cell responses we first carried out an IgG isotype analysis as this informs the type of T helper cell bias imposed upon the B cells producing antibody. HA formulated in Adjuplex™ gave significantly higher IgG1 titers than HA alone, equivalent titers to HA in alum, but significantly lower titers than HA in MPL (Fig. 3a). IgG2c titers were highest in the Adjuplex™ and MPL groups and significantly higher than HA alone or HA in alum (Fig. 3b).

The IgG1 to IgG2c ratio confirmed a significantly greater Th2-type balance for HA alone or adjuvanted with alum or MPL compared to Adjuplex™, that gave a balanced IgG1:IgG2c response (Fig. 3c). In vitro HA restimulation of splenocytes followed by $^{3}$H thymidine incorporation showed a strong proliferative response for HA in Adjuplex™ that was significantly greater than that obtained with HA alone or HA in MPL (Fig. 3d). To define whether the antigen-specific proliferative response triggered by Adjuplex™ was shared by both CD4$^+$ and CD8$^+$ T cells, mice were primed and boosted and splenocytes restimulated in vitro as before, then CD4$^+$ and CD8$^+$ T cell subsets were analyzed for activation by BrdU labeling. Figure 3e shows that CD4$^+$ T cells were equivalently activated by exposure to HA in Adjuplex™ or alum, but that these responses were not significantly greater than that elicited by antigen alone. By contrast Adjuplex™ elicited a robust CD8$^+$ T cell response that was significantly greater than that achieved with HA alone, whereas the response to HA in alum was not significantly greater than antigen alone (Fig. 3f). To further characterize antigen-specific T cell responses we analyzed cytokines released from the restimulated splenocyte culture. Adjuplex™-adjuvanted HA stimulated release of several Th1-associated cytokines (TNFα, IFNγ, IL-
12p70, GMCSF and IL-2) to levels significantly higher than HA alone or MPLA-adjuvanted HA, and levels of IL-12p70 and GMCSF that were higher than HA with alum (Fig. 4). By contrast IL-17 levels were not significantly different between groups. The Th-2 associated cytokine IL-4 was significantly greater in the Adjuplex™ group than all the other groups, and the Adjuplex™ group elicited greater greater IL-5 and IL-10 release than HA alone or in MPL (Fig. 4h-j). When these data are taken together, and consistent with the literature, alum is a Th2-biasing adjuvant (28, 29). By contrast MPL induces relatively modest levels of Th1 and Th2-type cytokines with IFN-γ being the only cytokine that is highly induced compared to HA alone, and Adjuplex™ elicits a robust and balanced Th1/Th2 response, as previously described for carbopol™ (11).

Protection from influenza virus challenge

To assess whether adjuvanting HA might lead to increased protection from an otherwise lethal intranasal inoculation of mouse-adapted influenza virus, mice were subcutaneously inoculated with a single dose of 0.54 µg HA alone, Adjuplex™ alone, or the same dose of HA formulated in MPL or alum. Mice were challenged 15 days later with 16 HAU, corresponding to 1.17 x 10⁵ PFU, of PR8 H1N1 influenza A virus, and weight loss measured over the subsequent 10 days. As shown in Figure 5a, mice administered Adjuplex™ or HA alone lost weight dramatically over the first 5 days and all reached the humane endpoint by day 5. By contrast, 3/5 alum-adjuvanted mice and all MPL and Adjuplex™-adjuvanted mice survived with minimal or no weight loss (Fig. 5b). AUC analysis of the weight loss data revealed that all three
adjuvants protected mice, with a trend towards Adjuplex™ eliciting greatest protection (Fig. 5c). Measurement of HA-specific IgG elicited in each group at challenge showed robust responses in all adjuvanted groups (Fig. 5d) and the pattern of HI titers was similar to that of the ELISA binding antibodies (Fig. 5e). Titers of antigen-specific IgG and HI both correlated strongly with protection from disease (Fig. 5f, g).

Immune cell recruitment and cytokine production after peritoneal administration

The administration of adjuvants into the peritoneal cavity, whilst not representing a route relevant to human vaccination, allows facile analysis of local immune cell infiltration and exit and cytokine production. This route has been used for analysis of alum-elicited immune cell recruitment and cytokine responses (29, 30), and so is relevant to the current study. Mice were administered 100 µg alum or 5% Adjuplex™ in 200 µL and 24 h later peritoneal lavage was obtained to capture innate immune cell infiltration and local cytokine release. Cells were labeled with a panel of antibodies and individual populations were gated and quantified (Fig. 6a). Data summarized in Figure 6b show that alum and Adjuplex™ recruited equivalent numbers of monocytes, whereas Adjuplex™ had a trend towards decreased neutrophil and increased dendritic cell (DC) counts compared to alum. Cytokine responses were analyzed at the 24 h timepoint, and those positive are shown in Figure 6c. Adjuplex™ induced greater IL-12 p40 and RANTES responses than alum, whereas alum induced greater IL-6 and MCP-1 responses. In sum, these data imply that Adjuplex™ induces an early burst of cytokines and
chemokines, and attracts inflammatory monocytes and DCs to the site of adjuvant administration. The presence of DCs and monocytes is of particular interest as the former are professional antigen presenting cells that would be expected to trap antigen and migrate with it to the draining lymph nodes for presentation to T cells (30), and the latter may differentiate into antigen presenting dendritic cells (31, 32) in the inflammatory cytokine milieu induced by Adjuplex™.

**Adjuplex™ does not trigger classical DC maturation**

Adjuvants that bind PRRs trigger NFκB activation pathways leading to maturation of immature DCs and upregulation of costimulatory surface molecules that allows efficient antigen-specific priming of T cells. We therefore tested whether Adjuplex™ might represent a PRR ligand by pulsing DCs with two concentrations of Adjuplex™ and reading out surface HLA and costimulatory marker expression. The highest dose (5%) of Adjuplex™ generated a high fluorescent background in the BMDC and so could not be used for the analysis. By contrast with LPS that upregulated MHC class II, CD80, CD86, CD40, and OX40L expression, there was no significant increase of any surface markers in the presence of Adjuplex™ ([Fig. 7a](#)). To confirm that Adjuplex™ was unable to trigger PRRs directly, we pulsed an NFκB myeloid reporter cell line, Thp1-blue, expressing many functional PRRs including TLR1/2, TLR2, TKLR2/6, TLR4, TLR5, TLR8, NOD1 and NOD2, with a range of Adjuplex™ concentrations and read out NFκB activation. Whereas known ligands LPS (TLR4), LTA-SA (TLR2), FSL-1 (TLR2/6) and MDP (NOD2) all activated NFκB, none of the 3 concentrations of Adjuplex™
induced any significant activation (Fig. 7b). Taken together with the lack of canonical DC maturation, we therefore conclude that the major adjuvant activity of Adjuplex™ is unlikely to depend upon direct DC activation via PRRs.

Discussion

Adjuplex™ compared favourably in terms of tolerability and potency with both licensed adjuvants tested, alum and MPL, inducing antigen-specific IgG titers of equal (MPL) or greater (alum) magnitude without imposing detectable weight loss. In line with this, Adjuplex™ was at least as effective as the other adjuvants in protecting mice from an otherwise lethal influenza virus challenge, and highly significantly superior to antigen alone. By contrast with currently used alum-based adjuvanted vaccine formulations, carbomer-based formulations are non-toxic in vitro and exhibit excellent biocompatibility and biodegradability (33, 34).

Adjuplex™ imposed a more balanced Th response than the other adjuvants as defined by the IgG1/IgG2a ratio, the increased levels of Th1 cytokines including IL-12 and GM-CSF, and the antigen-induced proliferation of CD8+ T cells. This is unsurprising with respect to alum which has well-characterized Th2-biasing properties (35). The TLR4 agonist MPL has been demonstrated to act primarily by triggering TRIF-mediated signaling (36) and thus induces, by contrast with LPS, a non-proinflammatory innate immune response, which may be suboptimal for the effective induction of memory CD8+ T cell activity (37). In addition to the potent induction of Th1-related cytokines, Adjuplex™
also induced robust Th2-type cytokines, most notable of which was IL-4, a cytokine favoring B cell activation and differentiation into plasma cells.

Adjuplex™ is a co-formulation between carbomer and lecithin. It seems likely that the major immune activating activity will come from the carbomer component, while the lecithin, configured as nanoliposomes, may function primarily to enhance bioavailability of the antigen and facilitate delivery to antigen presenting cells. Because of their biocompatibility, biodegradability, and smaller size than conventional liposomes, nanoliposomes are thought to enhance the performance of bioactive agents by improving their solubility, bioavailability, and stability (38). Moreover, the type of adaptive immune activating properties of Adjuplex™ we report here are coordinate with the balanced Th response and the robust elicitation of cytokines we observed previously for carbopol™ (11). The lecithin nanoliposomes may carry intrinsic immune stimulating activity (39), but may impart additional favorable properties to the carbopol formulation, such as eliciting enhanced antibody titers and avidity as observed for co-formulations of carbomer and the oil-in-water emulsion MF59 (12, 13). Indeed, in oral immunization studies in mice, formulation of BSA with lecithin had no adjuvanting effect on the induction of anti-BSA antibodies and carbomer was only moderately effective, whereas the combination of lecithin with carbomer increased IgG antibody titers five-fold over carbomer alone (40).

Unlike MPL, which has a clearly defined mode of action by triggering TLR4 on antigen presenting cells leading to their maturation and enhanced priming of T
cells, the mechanism of Adjuplex™ action remains to be defined. We have clearly demonstrated that Adjuplex™ does not trigger canonical DC activation, and therefore it must act by an alternative mechanism. Since we assume that at least part of Adjuplex™ adjuvant activity is derived from the carbopol component, further study of Adjuplex™ and carbomer mode of action is warranted.

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**Figure legends**

**Figure 1. Optimization of Adjuplex™ and antigen dose.** a) Mice (C57Bl/6, 3 per group) were immunized on days 0 and 14 with HA in Adjuplex and blood taken on days -1, 14 and 28. b) Total antigen-specific IgG production was quantified by AUC analysis. c) Hemagglutination inhibition (HI) titers were measured and expressed as Log2 values. d) Weight was measured over 48 h and plotted as % change from starting weight (100%). e) Weight loss was quantified by AUC. f) The immunogenicity : toxicity ratio was determined by
Figure 2. Comparison of relative activity of Adjuplex™ with other commonly used adjuvants. a) Mice (C57Bl/6, 5 per group) were immunized on days 0 and 18 with HA alone, Adjuplex™ alone or HA in Adjuplex™, alum or MPL, blood taken on days -1, 10 and 36, and HA-specific IgG titers determined. b) HA-specific IgG titers were quantified by AUC analysis. c) Hemagglutination inhibition (HI) titers were measured and expressed as Log2 values. d) Weight was measured over 48 h and plotted as % change from starting weight (100%). e) Weight loss was quantified by AUC analysis. f) Toxicity relative to adjuvanticity for each condition was quantified by determining the ratio of weight loss to IgG titer. Data in b – e represent mean + SEM; *p<0.05, **p<0.01; n.s. = not significant.

Figure 3. B and T cell activity induced by immunization. a-d) Mice (C57Bl/6, 5 per group) were immunized for the experiment in Figure 2, and HA-specific IgG1 (a) and IgG2c (b) endpoint titers were determined. c) The ratio of IgG1:IgG2c was calculated. d) Splenocytes were harvested on day 38 post-prime and HA-specific responses assayed by ³H thymidine incorporation. e, f) Splenocytes from mice immunized with Adjuplex™ alone, HA alone or in Adjuplex™ or alum were HA-pulsed in vitro and then fixed and labeled for incorporated nuclear BrdU, and surface CD3 and either CD4 or CD8, and analyzed by flow cytometry. *p<0.05; **p<0.01; ***p<0.001; n.s. = not significant.
Figure 4. Cytokines induced by immunization. Supernatants from HA-restimulated splenocytes from mice (C57Bl/6, 5 per group) immunized for the experiment in Figure 2 were analyzed by multiplex array for cytokines. a-d) Th1 cytokines; e-g) other cytokines and ratio of IL-17:IL-12. h-j) Th2 cytokines. *p<0.05; **p<0.01; ***p<0.001; n.s. = not significant.

Figure 5. Protection from influenza challenge. a) Mice (C57Bl/6, 5 per group) were immunized on day 0 with Adjuplex™ or 0.5 µg HA alone or HA formulated in Adjuplex™, MPL or alum, and mice were challenged on day 15 after immunization with 16 HAU corresponding to 1.17 x 10^5 PFU of influenza A H1N1 PR8. Weight loss was measured over 10 days and expressed as % change from starting weight set at 100%. b) Kaplan-Meier survival curves for each group: mice losing 25% or greater weight were humanely sacrificed. c) Weight change over the period of measurement was calculated using AUC analysis. d) Blood was taken on the day of challenge and HA-specific serum IgG endpoint titers determined. e) Hemagglutination inhibition (HI) titers were determined and expressed as Log2 values. f) Linear regression analysis of correlation between weight loss and antigen-specific IgG titer. g) Linear regression analysis of correlation between weight loss and HI titer. Data expressed in a - e represent mean ± SEM; * p<0.05; ** p<0.01; ***p<0.001; ****p<0.000.

Figure 6. Immune cell recruitment and local cytokine production induced by Adjuplex™. Intraperitoneal administration of PBS, Adjuplex™ or alum into
n = 2-3 mice was followed 24 h later by peritoneal lavage and analysis of cell subsets by flow cytometry for DC, monocytes and neutrophils (a) and results summarized for each subset in (b). c) Innate immune cytokines and chemokines in peritoneal exudate were analyzed by multiplex bead array.

Figure 7. Lack of innate immune cell and receptor triggering by Adjuplex™. a) Bone marrow derived dendritic cells (BMDC) were incubated with the concentrations of Adjuplex™ or the PRR ligands indicated, and surface marker expression analyzed by flow cytometry and expressed as percent positively stained cells compared to isotype controls. b) THP1-blue reporter cells were pulsed with the compounds shown for 24 h and supernatants assayed for secreted embryonic alkaline phosphatase.

References


