Vaccine 27 (2009) 4662–4671

Contents lists available at ScienceDirect

Vaccine

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

A novel vaccine adjuvant comprised of a synthetic innate defence regulator peptide and CpG oligonucleotide links innate and adaptive immunity

Jason Kindrachuk[a], Håvard Jenssen[a], Melissa Elliott[a], Rebecca Townsend[b], Anastasia Nijnik[a], Song F. Lee[b], Volker Gerdts[c], Lorne A. Babiuk[d], Scott A. Halperin[b], Robert E.W. Hancock[a,*]

[a] *Centre for Microbial Diseases and Immunity Research, University of British Columbia, 2259 Lower Mall Research Station, Vancouver, BC, V6T 1Z3, Canada*
[b] *Canadian Center for Vaccinology, Dalhousie University, IWK Health Centre, 5850/5980 University Avenue, Halifax, NS, B3K 6R8, Canada*
[c] *Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, SK, S7N 5E3, Canada*
[d] *University of Alberta, 3-7 University Hall, Edmonton, AB, T6G 2J9, Canada*

ARTICLE INFO

Article history:
Received 30 January 2009
Received in revised form 12 May 2009
Accepted 21 May 2009
Available online 17 June 2009

This manuscript is dedicated to the memory of Aaron W. Wyatt who passed away suddenly on December 24th, 2008. Aaron was not only a superb colleague but also a good friend.

Keywords:
Adjuvant
Innate defence regulator peptide
Host defense peptide
CpG DNA
Oligodeoxynucleotides
Toll-like receptors
Formulation

ABSTRACT

There has been an increased demand for the development of novel vaccine adjuvants that lead to enhanced induction of protection from infectious challenges and development of immunological memory. A novel vaccine adjuvant was developed comprising a complex containing CpG oligonucleotide and the synthetic cationic innate defence regulator peptide HH2 that has enhanced immune modulating activities. The complex of HH2 and the CpG oligonucleotide 10101 was a potent inducer of cytokine/chemokine expression *ex vivo*, retained activity following extended storage, had low associated cytotoxicity, and upregulated surface marker expression in dendritic cells, a critical activity for a vaccine adjuvant. Immunization of mice with a coformulation of the HH2–CpG complex and pertussis toxoid significantly enhanced the induction of toxoid-specific antibody titres when compared to toxoid alone, inducing high titres of IgG1 and IgG2a, typical of a balanced Th1/Th2 response, and also led to high IgA titres.

This study demonstrates the potential application of the HH2–CpG complex as a vaccine adjuvant.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

To decrease the adverse events associated with whole cell bacterial vaccines, there has been a trend towards the development of better defined vaccines containing a limited number of antigens and highly purified components [1]. Although this has diminished vaccine-associated adverse events, this trend has also resulted in vaccines with reduced ability to promote protective immune responses. Thus, alternative strategies have focused on the design and development of vaccine adjuvants in an effort to enhance immunogenicity.

Adjuvants have an important role in current vaccine development as they modulate immune responses, through immune cell recruitment, the direct or indirect activation of immune cells to produce cytokines and chemokines, and/or a poorly explained depot effect (localization of antigen in an appropriate tissue) [1]. Alum is the most widely used adjuvant for a wide-range of antigens although it demonstrates limited adjuvanticity reflecting its modest ability to activate immune cells through Toll-like receptors (TLR). However there are significant concerns regarding its potential to cause certain pathologies including granulomas, increased IgE production, and allergenicity [2–4]. Thus there is great interest in adjuvants that are safe, stable, have low associated costs of manufacturing, and promote an appropriate cellular and/or antibody immune response [5].

The identification of pattern recognition receptors, and in particular the TLR family, has provided a cellular target for adjuvant design since activation of these receptors stimulates innate immunity, which is recognized to be critical for the induction of antigen-specific adaptive immune responses. Many adjuvants signal through such pathways leading to induction of cytokine and chemokine expression [1]. Thus it seemed reasonable to develop adjuvants that activate multiple pathways in an effort to enhance antigen-specific immune responses. Previous studies

* Corresponding author. Tel.: +1 604 822 0172.
E-mail address: bob@cmdr.ubc.ca (R.E.W. Hancock).

0264-410X/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2009.05.094

demonstrated partial synergy between CpG oligonucleotides (ODN; a TLR9 agonist) and the human host defence peptide LL-37 in inducing certain cytokines [6]. Therefore we sought here to combine synthetic innate defence regulator peptides (IDRs), with enhanced immunomodulatory activities relative to LL-37, with CpG oligonucleotides, to provide a new adjuvant formulation for antigen presentation.

IDRs are synthetic mimics of cationic host defence peptides (HDPs) that are ubiquitous in nature; HDPs represent important components of the immune systems of all complex life forms ranging from insects to humans. Traditionally these peptides are characterized by their limited size (∼12–50 residues in length), cationic charge (due to high proportions of arginine and lysine residues), and amphipathic nature (≥30% hydrophobic amino acid residues), and adopt ordered structures upon interaction with hydrophobic environments such as cell membrane bilayers. Recently, there has been an increased appreciation that many HDPs are able to modulate immunity, while some also possess direct antimicrobial activity. The immunomodulatory activities of these peptides include the up-regulation of chemokines and cytokines and their receptors, direct (or indirect) recruitment of leukocytes to sites of infection, stimulation of histamine release from mast cells, angiogenesis, dendritic cell (DC) maturation and wound healing [7–10]. HDPs also enhance adaptive immune responses through activities such as chemoattraction of immature DCs and lymphocytes [11–14] and the induction of DC maturation [15]. Indeed HDPs such as the human neutrophil peptides (HNP) 1–3 and LL-37 are able to act as adjuvants, promoting adaptive immunity [16,17]; however, these peptides are quite long and/or complex and are thus limited by difficulties in synthesis and associated cost of manufacturing. Thus, we have undertaken to create short, novel synthetic mimics, IDRs, with optimized immunomodulatory activities. Recently, we demonstrated that the 13-aa synthetic peptide IDR-1 was able to confer protection to bacterial challenge through modulation of innate immunity [18], indicating that it may be possible to produce a short peptide that modulates adaptive responses through activation of innate immunity.

Bacterial DNA is a potent activator of innate immune responses in mammals [19]. The immunostimulatory activity of microbial DNA is due to the presence of unmethylated CpG dinucleotide sequences frequently found in microbial DNA but largely absent from host cell genetic material. Single-stranded CpG ODNs have garnered much interest as novel immunomodulators due to their relatively low toxicity, chemical stability, and low cost of production [19]. The immunomodulatory properties of CpGs have resulted in a number of potential medical applications including: (1) priming the innate immune response, (2) as anti-allergens, (3) for the treatment of a variety of malignancies, and (4) as adjuvants for improving vaccination efficiency, especially in individuals with poor immune responses [19]. Indeed these molecules have been demonstrated to enhance human, murine, and porcine neonatal immune responses [20–22], although the use of CpGs in adjuvant formulations was previously demonstrated to skew vaccine-induced immune responses towards a Th1-bias [1]. In the context of a vaccine adjuvant, a balanced Th1/Th2 response is arguably the most desirable response since the modulation of Th1 and Th2 contributions influences the balance between protection and immunopathology [23]. The Th1 response results in the initiation of inflammatory immune responses, primarily addressing intracellular infections, whereas the Th2 response evokes antibody responses to extracellular pathogens, and most allergies [24]. However, over-activation of the Th1 pathway can result in the generation of organ-specific autoimmune disease [23] whereas Th2 overactivation is associated with allergy and a predisposition to systemic autoimmune disease [25].

Here we present a novel vaccine adjuvant comprising a mixture of CpG ODN and a synthetic 12-amino acid IDR, HH2 (VQLRIRVAVIRA-NH_2), which had been optimized for immunomodulatory activities. The *in vitro*, *ex vivo* and *in vivo* performance of this complex suggests the potential of this complex as a new vaccine adjuvant.

2. Materials and methods

2.1. Materials

Phosphorothioate-stabilized CpG ODN 10101 (TCGTCGTT-TTCGCGCGCGCCG) was kindly provided by Merial (Deluth, GA, USA). Peptides HH2 (VQLRIRVAVIRA-NH_2), HH18 (IWVIWRR-NH_2) and 1047 (IIRRWWV-NH_2) were synthesized using solid phase Fmoc chemistry, purified to a purity >90% using reverse phase HPLC and analyzed by mass spectrometry, at either the Brain Research Center (University of British Columbia, Vancouver, Canada) or GenScript (Piscataway, NJ, USA). The genetically detoxified form of pertussis toxoid was generously donated by Novartis Vaccines (Siena, Italy).

2.2. Formulation of HH2–CpG complexes and agarose electrophoretic mobility shift assays (AEMSA)

Binding assays were performed by incubating CpG ODN 10101 with peptide HH2 in a final volume of 50 μL of 10 mM Tris–HCl, 270 mM Sorbitol (pH 7.4). Reactions were incubated at 37 °C for 10 min, stopped by the addition of 10 μL of agarose gel loading solution (30% glycerol), and immediately subjected to electrophoresis through a 0.8% agarose gel at 95 V for 1 h. DNA was visualized by ethidium bromide staining.

2.3. Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) measurements were performed with a Voyager DE-STR mass spectrometer (Applied Biosystems) equipped with a pulsed nitrogen laser (20 Hz, 337 nm) with the kind permission of Dr Suzanne Perry (Michael Smith Laboratory Proteomics Core Facility, UBC). All samples were acquired in linear mode and negative ion spectra were acquired. The accelerating voltage was −20 kV with a delay time of 400 ns. 200 laser shots were obtained from across the sample surface and then averaged. For these measurements, 1 μL of sample was mixed on a stainless steel sample plate with the same volume of 6-aza-2-thiothymine (ATT) [10 mg/mL in 1:1 (v/v) solution of acetonitrile and 20 mM ammonium citrate] matrix solution. The preparation was allowed to dry at room temperature and subjected to mass spectrometry. Close external calibration with peptide standards was performed.

2.4. Human PBMC isolation and stimulation with HH2–CpG formulations

Venous blood from healthy volunteers was collected in Vacutainer® collection tubes containing sodium heparin as an anti-coagulant (BD Biosciences, NJ, USA) in accordance with University of British Columbia ethical approval and guidelines. Blood was diluted with an equal volume of complete RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate (all from Invitrogen Life Technologies, Carlsbad, CA, USA) and separated by centrifugation over a Ficoll-Paque Plus (GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA) density gradient. The buffy coat was collected, washed twice in RPMI 1640 complete medium, and peripheral blood mononuclear cell (PBMC) numbers determined by trypan blue exclusion. PBMC

(5×10^5) were seeded into 24-well tissue culture dishes (Falcon; BD Biosciences) at 1×10^6 cells/mL and rested for 1 h at 37 °C in 5% CO_2. The cells were then stimulated with the HH2–CpG formulations for 24 h. All experiments were repeated on at least three separate occasions.

2.5. Chemokine/cytokine induction

Following exposure of cells to peptides for 24 h at 37 °C in 5% CO_2, the tissue culture supernatants were collected by centrifugation at $16,000 \times g$ at 4 °C for 5 min to obtain cell-free samples. Supernatants were aliquoted and then stored at −20 °C. MCP-1 and TNF-α secretion into the tissue culture supernatants was detected by sandwich enzyme-linked immunosorbent assay (ELISA) (Invitrogen Life Technologies, Carlsbad, CA, USA). All assays were performed in triplicate in three separate experiments. The concentration of the chemokines in the culture medium was quantified by establishing a standard curve with serial dilutions of recombinant human MCP-1 and TNF-α.

2.6. Cytotoxicity assay

Fresh human venous blood was collected as described above and PBMCs separated from the blood cells over a Ficoll-Paque Plus gradient. The toxic effect of HH2–CpG complexes on human red blood cells was assayed by measuring haemoglobin release due to cell lysis. Red blood cells were washed three times in sterile 0.85% NaCl (saline) and centrifuged at 1500 rpm for 10 min. Concentrated red blood cells were diluted 3-fold in saline, and 100 μL of this cell suspension was mixed in standard non-coated 96-well polypropylene microtiter plates with 50 μL of HH2–CpG complexes at the concentrations indicated and incubated on a rocking table at 37 °C under 5% CO_2 pressure for 24 h. Triton X-100 (1%) was used as a positive control demonstrating 100% cell lysis, and sterile saline was considered as a negative control. Haemoglobin release was monitored at 414 and 546 nm using an ELISA plate reader. To further test toxicity, PBMC (2×10^5) were seeded into 96-well plates (Sarstedt, Newton, NC, USA) and incubated at 37 °C in 5% CO_2 overnight. The release of cytosolic lactate dehydrogenase (LDH) release assay was then assessed [7] after 24 h of incubation with the HH2–CpG complexes. All experiments were done in triplicate.

2.7. Flow cytometry

Flow cytometry data was collected on FACSCalibur™ and analyzed using CellQuest^Pro software (Becton Dickinson). For analysis of cytokine production, the cells were treated with appropriate stimuli for 18 h, with the Golgi-STOP™ inhibitor (BD Biosciences) added 2 h after the beginning of the stimulation. The cells were fixed and permeabilized using the Cytofix/Cytoperm Plus™ kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol, and stained with the anti-human IFNα2-specific antibody conjugated to fluorescein isothiocyanate (FITC) (clone 225.C, eBioscience). For the analysis of cell surface marker expression, the cells were re-suspended in Hanks balanced salt solution pH 7.4, supplemented with 2% FCS (Invitrogen Life Technologies, Carlsbad, CA, USA), and containing 0.2% (w/v) sodium azide and 20 mM HEPES, pH 7.4. The staining was carried out at 4 °C for 20 min, and the antibodies used were allophycocyanin-conjugated anti-CD123 (clone 6H6), AF647-conjugated anti-human CD11c and CD14 (clones 3.9 and M5E2), PerCP-conjugated anti-human HLA-DR (clone L243), or phycoerythrin-conjugated anti-human CD80 and CD86 (clones 2D10 and IT2.2; all antibodies from BioLegend, USA). Lineage marker antibodies were either FITC or phycoerythrin conjugated anti-human CD3, CD14, CD16, and CD20, purchased from ImmunoTools, Germany as part of the IT-Box 341 set.

2.8. Immunization of mice

Cohorts of 5–6-week-old female BALB/c mice (Charles River Laboratories, St. Constant, QC) were immunized intranasally with various vaccine formulations on days 1 and 14. To examine the synergistic effects of the adjuvant components, mice were immunized with either 0.5 μg pertussis toxoid alone (Novartis Vaccines, Siena, Italy), or in combination with 10 μg CpG ODN 10101, 50 μg HH2 or the two adjuvants formulated together. Mice were sedated with ketamine and xylazine and 13 μL/nostril of vaccine formulation was delivered using a pipette. Saliva was collected on day 20 following stimulation with 0.05 mg/mL carbamylcholine chloride (Sigma–Aldrich Chemical Co., Oakville, ON) and mice were asphyxiated on day 21 with CO_2. Sera, vaginal washes and bronchoalveolar lavages (BAL) were collected for determination of antibody titres.

2.9. Analysis of antibody titres

Antigen-specific IgG and IgA from sera and secretory IgA from saliva, vaginal washes and broncheoalveolar lavage (BAL) were determined by end-point ELISA using methods previously described [26]. Briefly, twofold diluted samples were assayed on polystyrene microtitre plates coated with 100 ng pertussis toxoid/well. Specific total IgG antibodies were detected by an alkaline-phosphatase-conjugated goat anti-mouse IgG (1:8000; Sigma–Aldrich). IgG sub-types were detected with goat anti-mouse IgG1 and goat anti-mouse IgG2a (1:8000; Southern Biotechnology Assoc., Inc., Birmingham, AL) followed by an alkaline-phosphatase-conjugated rabbit anti-goat IgG (1:20,000; Sigma–Aldrich). Pertussis toxoid specific IgA was detected with biotinylated goat anti-mouse IgA (α-chain specific, 1:20,000; Sigma–Aldrich) followed by an avidin–alkaline-phosphatase conjugate (1:10,000; Sigma–Aldrich). The end-point antibody titre was defined as the reciprocal of the dilution that gave an A_{405} value 0.05 greater then the pooled pre-immune samples.

2.10. Statistical analyses

Statistical comparisons were performed with Prism 4.0 Software (GraphPad Inc.), using the two-tailed Student's *t*-test for comparisons of two data sets, and analysis of variance (ANOVA) for multiple comparisons.

3. Results

3.1. Formulation of the HH2–CpG complex

Two libraries of short cationic synthetic IDR peptides were developed that demonstrated enhanced immunomodulatory activities *ex vivo* and *in vivo* as compared to a model parent peptide (Jenssen et al.; manuscript in preparation). From these libraries, peptide HH2 was chosen as a model peptide for use in the development of a novel vaccine adjuvant, based on its superior ability, compared to IDR-1, to induce chemokines in human PBMC. HH2 was combined with the CpG ODN 10101, which induces IFN-α secretion from DCs and stimulates B-cells [27–29]. It was hypothesized that the formulation of an enhanced immunomodulatory IDR peptide and a CpG ODN would lead to a complex that could synergistically enhance the immunogenic response to a particular antigen.

Various ratios of the peptide HH2 and the CpG ODN 10101 were formulated and visualized by AEMSA and staining of the

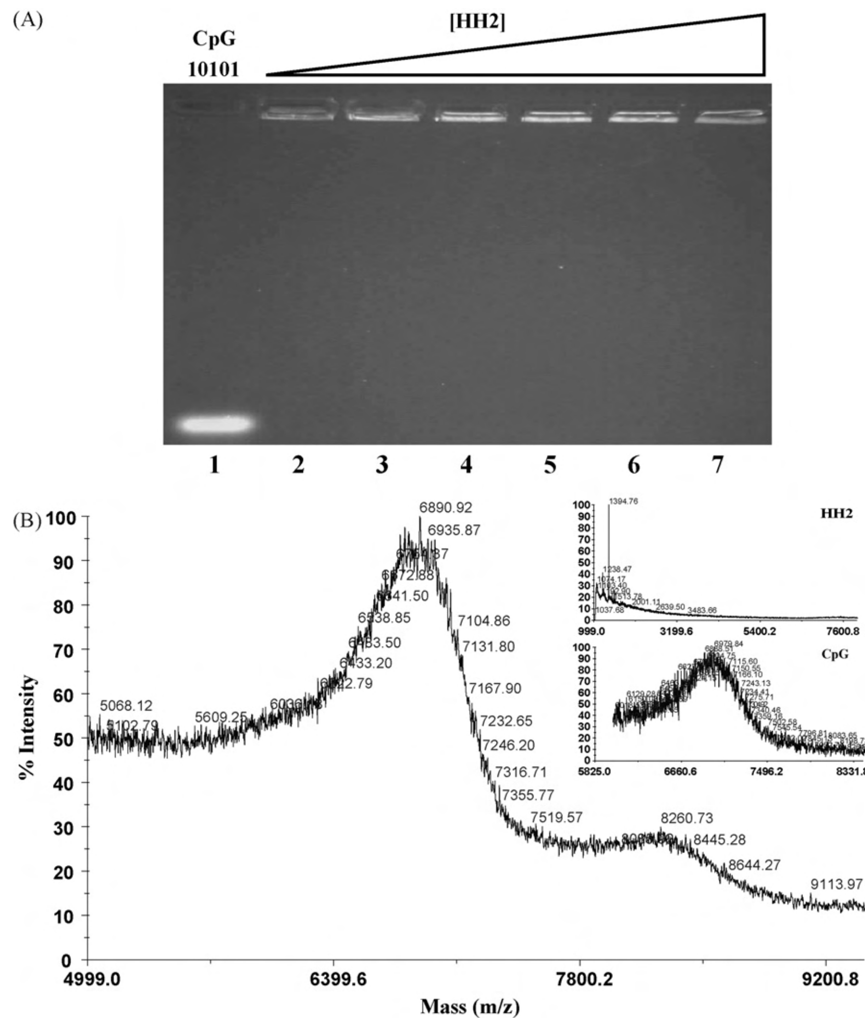

Fig. 1. Complexing of HH2 with CpG resulting in the formation of a novel nucleopeptide complex. HH2 and CpG were pre-complexed at various component concentrations in 10 mM Tris–HCl, 270 mM sorbitol buffer, pH 7.5. (A) AEMSA of HH2–CpG complexes. Lane 1: CpG (5 μg); lane 2: CpG (5 μg) + HH2 (1.25 μg); lane 3: CpG (5 μg) + HH2 (2.5 μg); lane 4: CpG (5 μg) + HH2 (5 μg); lane 5: CpG (5 μg) + HH2 (10 μg); lane 6: CpG (5 μg) + HH2 (20 μg); lane 7: CpG (5 μg) + HH2 (40 μg). (B) MALDI-TOF analysis of the HH2–CpG complex. Two μg of HH2 was complexed with 1 μg of CpG and analyzed by MALDI-TOF mass spectrometry. HH2 alone (mass 1394.76 Da) and CpG alone (mass ~6900 Da) are displayed in the inset. The formulated HH2–CpG sample demonstrated two broad peaks, one at corresponding to CpG at a mass of ~6900 Da, and a second at ~8300 Da corresponding to the combined masses of peptide and the CpG.

CpG to monitor for complex formation (Fig. 1). As anticipated, the co-incubation of HH2 and CpG ODN 10101 resulted in the formation of a slow migrating aggregate when compared to the mobility of the CpG alone; however, as AEMSA EMSA does not resolve complexes solely by molecular weight an estimate of the size of the HH2–CpG complex could not be made. Complex formation was independent of pH and high salt concentrations, and titration of $MgCl_2$ or $CaCl_2$ (0–100 mM) into preformed HH2–CpG complexes (2:1; wt/wt) resulted in no discernable dissociation of these complexes (data not shown). A representative sample of the 2:1 (wt/wt) ratio formulation of HH2:CpG was selected for MALDI-TOF mass spectrometry to directly confirm complex formation between the peptide and CpG. HH2 alone had a distinct peak at 1394.76 Da corresponding to the correct molecular weight of the peptide whereas CpG 10101 alone resolved with a broad peak with an apparent mass of ~6900 Da (Fig. 1B). The formulated HH2–CpG sample demonstrated two broad peaks, one at corresponding to CpG at a mass of ~6900 Da, and a second at ~8300 Da, corresponding to the combined masses of peptide HH2 and the CpG ODN (Fig. 1B). Thus, it was confirmed that incubation of CpG ODN 10101 and HH2 resulted in the formation of an HH2–CpG complex.

3.2. Synergistic induction of chemokine in PBMCs by the HH2–CpG complex

Preliminary studies demonstrated that peptide HH2, as well as CpG [30], is a potent inducer of monocyte chemoattractant protein (MCP)-1, a chemokine that is chemotactic for monocytes/macrophages, T cells, NK cells, and neutrophils. Therefore MCP-1 was chosen for analyzing chemokine induction by the HH2–CpG complex. HH2 and CpG were pre-complexed with a range of CpG:HH2 ratios prior to PBMC stimulation. For these chemokine screens four CpG concentrations were chosen that best represented those currently utilized in other *in vivo* studies and these were held constant across a range of seven ratios of CpG:HH2 ranging from 8:1 (wt/wt) CpG:HH2 to 1:8 (wt/wt) CpG:HH2 (Fig. 1). Thus, e.g., the 2:1 (wt/wt) ratio of HH2:CpG was tested by combining the four different concentrations of CpG (1.25, 2.5, 5, and 10 μg/ml) with HH2 concentrations of 2.5, 5, 10 and 20 μg/ml, respectively. Human PBMCs were stimulated with the various HH2–CpG complexes immediately following complex formation and monitored for induction of MCP-1 by ELISA. The 1:2, 1:4 and 1:8 (wt/wt) ratios of CpG:HH2 led to potent induction of chemokine expression *ex vivo* (Fig. 2A). These results were particularly striking for those CpG

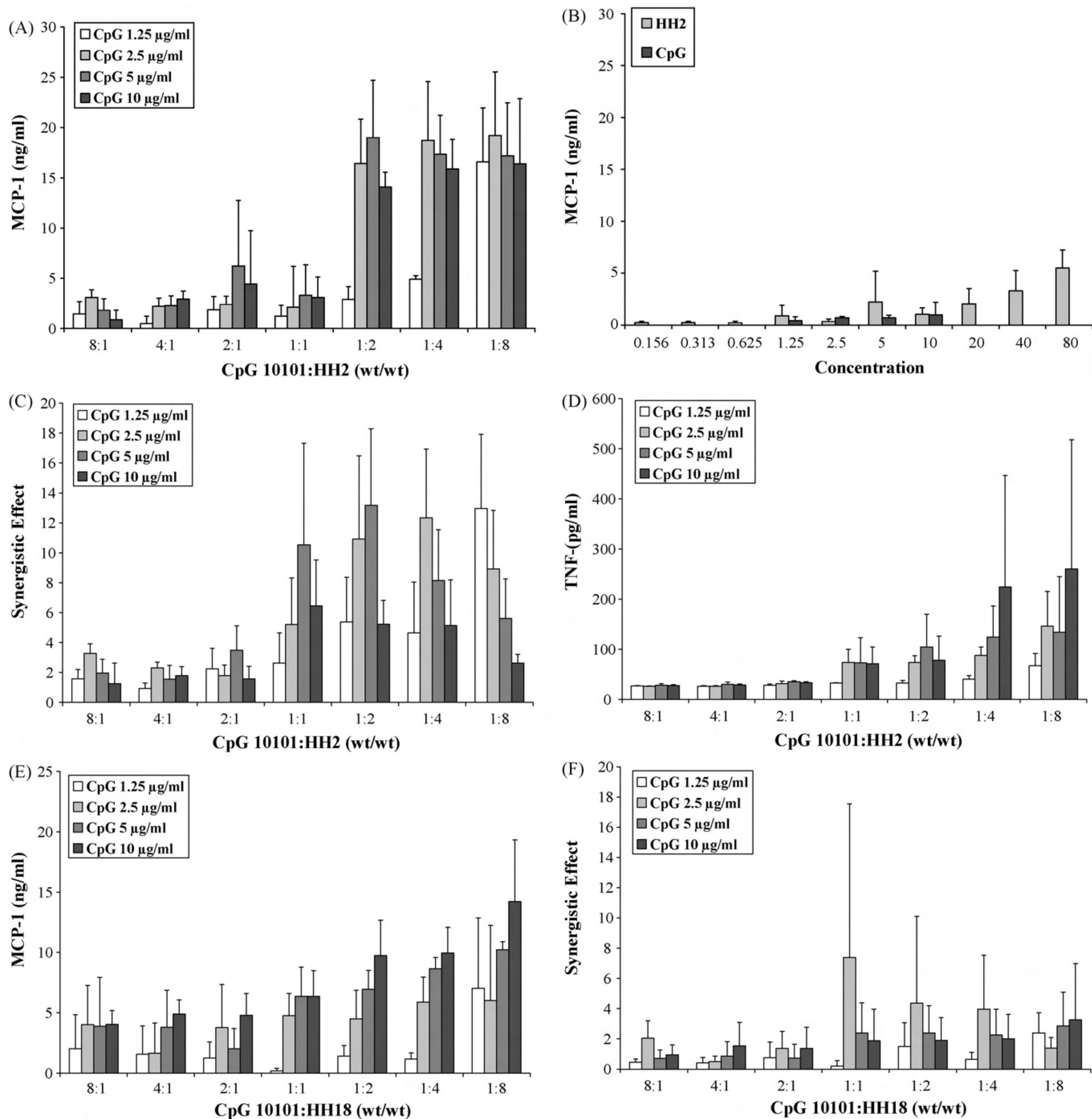

Fig. 2. Complexes of HH2 and CpG resulting in synergistic chemokine induction at defined HH2:CpG ratios. Human PBMCs were stimulated with a range HH2–CpG complexes, or the components alone, for 18 h. Following stimulation, supernatants were collected and monitored for MCP-1 release by ELISA. (A) Total MCP-1 release induced by HH2–CpG complexes. (B) Induction of MCP-1 release by either HH2 or CpG alone at the concentrations used to form complexes. (C) Calculation of synergy for MCP-1 release by complex formation between HH2 and CpG. Synergy was calculated as the total MCP-1 release by the HH2–CpG complex divided by the summed MCP-1 release caused by HH2 alone and CpG alone, at the concentrations found in the complex. (D) Induction of TNF-α by the various HH2–CpG complexes described in (A). (E) Total MCP-1 release induced by HH18-CpG complexes. (F) Calculation of synergy for MCP-1 release induced by the HH18-CpG complex. All data are representative of at least 3 independent experiments.

concentrations >1.25 μg/ml. As the enhanced induction of MCP-1 for a particular HH2–CpG complex ratio might simply have been due to the additive effects of the individual components on MCP-1 induction, the synergistic MCP-1 induction (synergistic effect) was calculated for each ratio screened. Synergistic effect was calculated as the total release of MCP-1 by the HH2–CpG complex divided by the additive release of MCP-1 induction by the individual complex components. A cut-off level of ≥2 was chosen to indicate synergy (Fig. 2C). In particular the 2:1 (10 μg/ml/5 μg/ml) HH2–CpG complex was a strong inducer of MCP-1 (18,999 ± 3879 pg/ml) and showed a synergistic effect of 13 ± 5 (Fig. 2C). Induction of the pro-inflammatory cytokine TNF-α was also examined for all of the HH2–CpG formulations and only modest increases of the cytokine could be demonstrated (Fig. 2D), in contrast to the very potent MCP-1 induction. As a control, peptide HH18 (IWVIWRR-NH2), a peptide created in the same library as HH2 but having weaker MCP-1 induction activity, resulted in considerably diminished total MCP-1 induction and synergistic induction values when complexed to CpG ODN 10101, as compared to complexed HH2 (Fig. 2E and F). It should be noted that complex formation between CpG ODN 10101 and peptide 1047 (IIRRWWV-NH2), an IDR with minimal chemokine induction activity, or 2-dioleoyl-3-trimethylammonium-propane

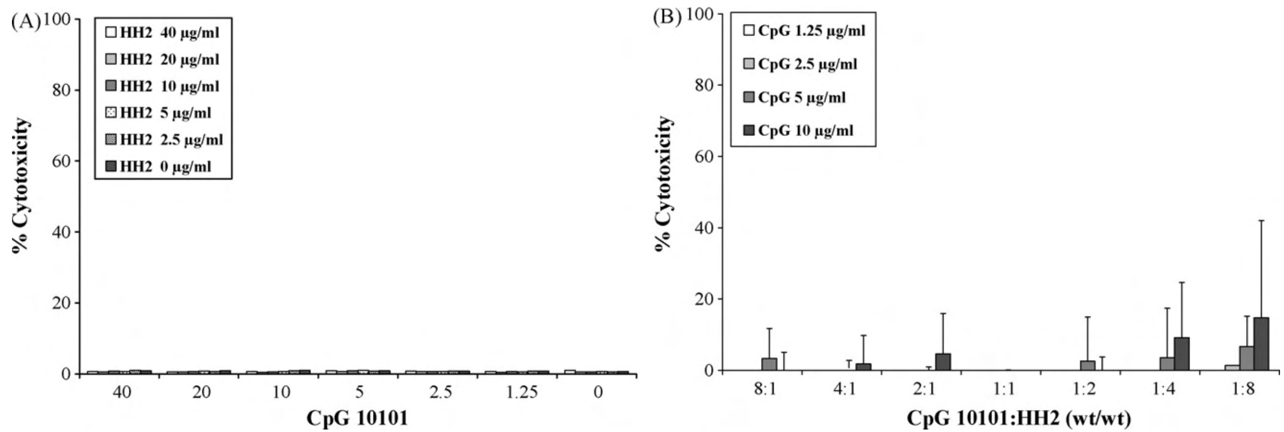

Fig. 3. Minimal cytotoxicity of the HH2–CpG complex. Human red blood cells or PBMCs were stimulated with a range of HH2–CpG complexes, or the components alone, for 18 h. Following stimulation, supernatants were collected and monitored for the release of haemoglobin or LDH. (A) Total haemoglobin release from red blood cells induced by the HH2–CpG complexes. (B) LDH release from PBMCs following stimulation with the HH2–CpG complexes. All data are representative of at least 3 independent experiments.

(DOTAP), a cationic transfection reagent, led synergy values of <1 for MCP-1 induction; even though complex formation could still be demonstrated by AEMSA (data not shown).

As vaccine adjuvants must demonstrate safety as well as potency, possible cytotoxicities associated with the HH2–CpG complexes were investigated by monitoring the release of LDH from PBMCs and haemoglobin from red blood cells. Previous investigations with cationic peptides had determined that some peptides have strong membrane lytic properties [31]. In contrast the tested HH2–CpG complexes resulted in minimal or no release of haemoglobin or LDH from RBCs and PBMCs, respectively (Fig. 3A and B).

The 2:1 (wt/wt) HH2–CpG complex was used to investigate the retention of MCP-1 induction activity following prolonged storage. To examine storage stability, HH2 and CpG were complexed and either left in solution or lyophilized to powder and incubated at room temperature over a 28 day period (Fig. 4A). AEMSA confirmed that the HH2–CpG complexes were preserved over extended incubation as either a solution or powder with no evidence of unbound CpG over the period monitored. To verify the retention of chemokine induction activity following extended storage, PBMCs were stimulated with the HH2–CpG complexes after 1, 3, 5, 7, 14, 21 and 28 day incubations and MCP-1 induction measured by ELISA. It was demonstrated that the immunomodulatory activity of the complexes was fully retained and in fact increased following these extended incubations (Fig. 4B).

3.3. HH2–CpG complex induction of IFNα production in pDCs and up-regulation of surface marker expression on monocytes and conventional DCs

Cationic peptides are able to enhance immune responses as assessed by induction of chemokine/cytokine expression and up-regulation of co-stimulatory markers in monocytes and DCs [15,32,33]. For example, the human cathelicidin LL-37 promotes DC activation and the induction of Th1 polarized adaptive immune responses [15] while LL-37 and human neutrophil peptides (HNP) 1–3 are known to have adjuvant properties [16,17]. Based on these observations and the recent demonstration that LL-37 coupled with self-DNA strongly augments pDC responses [34] it was postulated that HH2 in complex with CpG ODN, may also augment pDC activation.

Human PBMCs were stimulated with either the formulated HH2–CpG complex or the individual components HH2 (20 μg/ml) or CpG ODN 10101 (10 μg/ml) for 18 h. Normal endocytic uptake of the individual components or HH2–CpG complex was allowed to proceed for 2 h with subsequent addition of the monensin-based vesicular transport inhibitor Golgi-STOP to inhibit protein secretion and induce cytokine accumulation inside the cells. Samples were analyzed for IFN-α production by flow cytometry, gating on the plasmacytoid DCs as positive for MHCII and CD123 and neg-

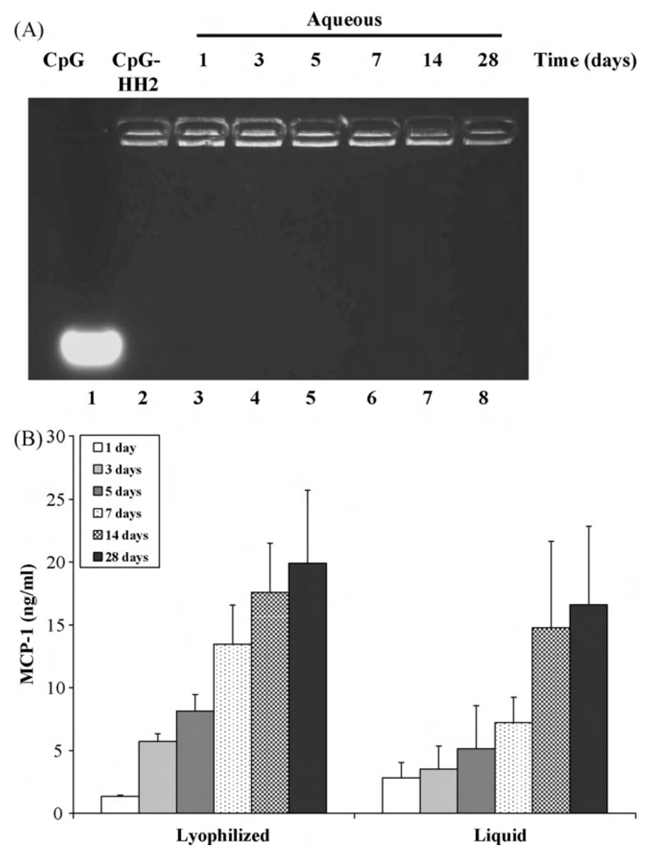

Fig. 4. Stability and activity of the HH2–CpG adjuvant complex following prolonged storage. Following formation of a 2:1 (wt/wt) complex of CpG (20 μg/ml) and HH2 (40 μg/ml), HH2–CpG complex was stored and monitored for stability and activity. (A) HH2–CpG remained complexed following prolonged incubation in 10 mM Tris/270 mM sorbitol, pH 7.5. (B) Chemokine induction by the HH2–CpG complex was retained following incubation as a lyophilized powder or aqueous suspension stored at room temperature.

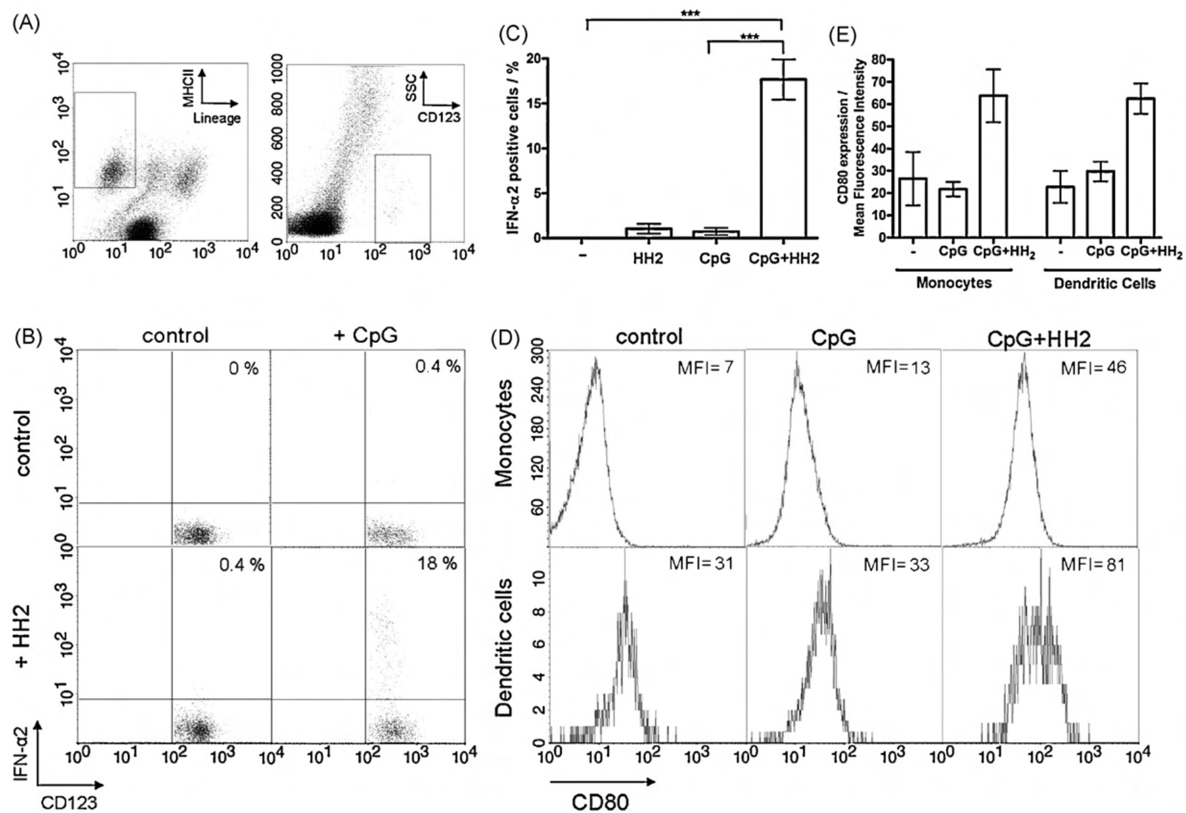

Fig. 5. Induction, by the HH2–CpG complex, of IFNα production in plasmacytoid dendritic cells and activation of monocytes and conventional dendritic cells. Human PBMC were stimulated with HH2 (20 μg/ml), CpG (10 μg/ml), or the formulation of the two components, for 18 h. GolgiSTOP™ inhibitor was used in the analysis of cytokine production, as described in Section 2. (A) Plasmacytoid dendritic cells gated as CD123^{+ve} HLA-DR^{+ve} Lineage Marker^{-ve} cells. (B and C) Percentage of IFNα2^{+ve} cells within the plasmacytoid dendritic cell gate. (D and E) CD80 expression on monocytes (gated as CD14^{+}) and conventional dendritic cells (gated as CD11c^{+ve} HLA-DR^{+ve} Lineage Marker^{-ve}), mean fluorescence intensities (MFI) of the cells are shown and compared. All data were representative of at least 3 independent experiments; statistical analysis employed a one-way ANOVA test, *$p < 0.05$, ***$p < 0.001$.

ative for lineage markers CD3, CD14, CD16, and CD20, according to standard protocols; the pDC population constituted on average 0.2% of the total PBMC (Fig. 5A). The percentage of IFN-α containing cells within the pDC gate was compared between the samples and a strong synergistic induction of IFN-α was observed in response to the HH2–CpG complex (Fig. 5B and C). As IFN-α is known to strongly augment antigen-presenting-cell activation, stimulate adaptive immune responses, and promote Th1 polarization, the IFN-α induction by the HH2–CpG complex could have contributed to adjuvant activity.

The ability of the HH2–CpG formulation to activate other classes of antigen-presenting-cells in blood was also analyzed. The induction of the co-stimulatory receptor CD80 was measured on monocytes and conventional DCs (Fig. 5D), gated as CD14^{+} and lineage-marker^{-ve} MHCII^{+ve} CD11c^{+ve} cells, respectively. As with the induction of IFN-α, an overall synergistic induction of CD80 was seen for both monocytes and cDCs in response to the HH2–CpG complex (Fig. 5E). It remains possible that the effects of the complex on monocytes and DCs may be an indirect result of the activation of other cell types, such as the IFN-α producing pDCs, since human monocytes and conventional DCs are considered to be devoid of functional TLR9 [19].

3.4. In vivo induction of antigen-specific immune responses by formulation of the HH2–CpG complex with pertussis toxoid

Detoxified pertussis toxin (PTd) was used to assess the ability of the HH2–CpG complex to enhance mucosal immune responses in mice. PTd, a component of all commercial pertussis subunit vaccines, was formulated with or without the various adjuvants and the PTd-specific humoral response monitored following intranasal immunization with two doses on days 1 and 14. For the immunizations a 5:1 (wt/wt) ratio of HH2:CpG was selected as this ratio fell within the range of our most active HH2–CpG complexes identified from the initial MCP-1 screens (Fig. 2A). Sera from BALB/c mice immunized with PTd alone, or in combination with either CpG or HH2, or co-formulated with the HH2–CpG complex, were analyzed by ELISA for the presence of total specific IgG as well as the different IgG subtypes; IgG1 as an indicator of a Th2 response and IgG2b as an indicator of a Th1 response. PTd alone failed induce a strong immune response, while the addition of CpG led to virtually no improvement over the antigen alone for any of the IgG sub-types (titres less than 10^1; Fig. 6). The combination of HH2 with PTd led to a strong increase in antibody titres but this was strongly biased towards an IgG1 (Th2) response with absolutely no IgG2a observed. In contrast the co-formulation of PTd with the HH2–CpG adjuvant complex resulted in higher titres (>10^4) of total IgG, as well as both the IgG1 and IgG2a subclasses indicating a balance between a Th1 and Th2 type responses following co-formulation with the HH2–CpG complex.

The intranasal administration of PTd with the HH2–CpG adjuvant complex also induced significant increases in PTd-specific serum IgA antibody titres as well as secretory IgA in the brocheoalveolar lavage while PTd alone or co-formulated separately with either CpG or HH2 led to no increase in IgA titres. There was no significant change in antibody titres of vaginal or saliva IgA in response to either PTd alone or in combination with any adjuvant utilized here.

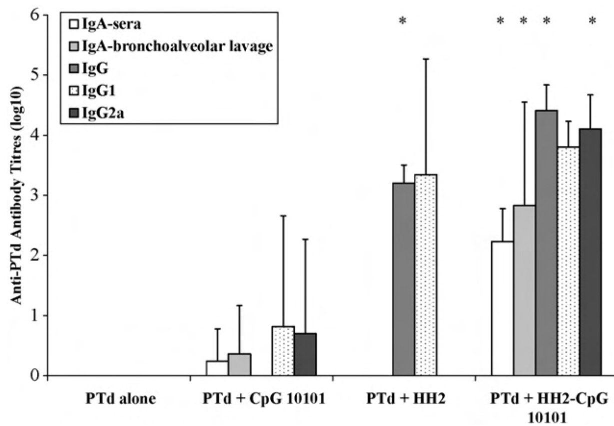

Fig. 6. Induction of humoral type 1/2 responses in mice by immunization with HH2–CpG co-formulated with pertussis toxoid (PTd). BALB/c mice (5/group) were immunized intranasally with the indicated vaccine formulations on days 1 and 14. For determination of pertussis toxoid-specific antibody titres, sera from each experimental group were pooled on day 21 and analyzed using specific ELISA as described in Section 2. NB for the CpG + PTd samples there were marginal levels of IgG1 and IgG2a that were not detected when total IgG was assessed.

4. Discussion

With the ongoing development of novel and improved vaccines, there has been an increasing interest in developing improved adjuvants that can enhance immune responses to an antigen while minimizing both vaccine- and adjuvant-related adverse events. Effective adjuvant approaches should bridge the innate and adaptive immune responses in the host. New vaccine adjuvant development to date has focused on the production of novel adjuvants that are stable, biodegradable, have low associated costs of manufacturing, and result in the induction of appropriate cellular or antibody immune responses; however, this has proven difficult due to a high incidence of injection-site and systemic toxicities [5]. Here we report on the development of a novel vaccine adjuvant comprising an immune modulating IDR peptide HH2 and a single-stranded CpG ODN 10101. HH2 was selected from a library of synthetic IDRs as having enhanced immune modulating activities and was able to confer protection to bacterial challenges in animal models (manuscript in preparation). It was postulated here that the addition of a peptide optimized for immunomodulatory activity along with single stranded CpG ODN, which might skew vaccine-induced immune responses towards a Th1-type response, would create a potent vaccine adjuvant platform. Importantly, the use of an optimized chemokine inducing IDR would likely enhance cell recruitment to the site of immunization and thus improve immunogenicity.

CpG oligonucleotides have been studied extensively for their immunomodulatory activities that include induction of TLR9 pathways leading to modest up-regulation of pro-inflammatory cytokines, up-regulation of co-stimulatory molecule expression, and anti-apoptotic activity. CpGs induce Th1-type responses in vitro [35] and in vivo in experimental vaccines for hepatitis B [36–39], anthrax [40], and HIV [38]. Interestingly, CpGs have shown even greater adjuvanticity when co-administered with other adjuvants [41–43]. On the other hand, IDRs have well-documented immunomodulatory activities such as chemokine activity and chemokine induction [32,33], wound healing [44], activation or inhibition of apoptosis [45], and anti-inflammatory activities [46]; importantly, these peptides also enhance and modulate adaptive immune responses. For example, the chemoattraction of immature DCs, lymphocytes, monocytes, and macrophages has been demonstrated for HNP 1–3 and human β-defensins (HBD) 1-2 [11–13] and co-culture of immature DCs with LL-37 induces DC maturation [15]. Co-formulation of HNP 1-3 with ovalbumin (OVA) or keyhole limpet hemocyanin (KLH) of B-cell lymphoma idiotype Ag resulted in enhanced OVA-specific immune responses and resistance to subsequent tumour challenge, respectively [16,17]. Recently, it was reported that IC31, a vaccine adjuvant comprised of the synthetic peptide $KLKL_5KLK$ with a single-stranded ODN comprised of IC repeats (ODN1a), induced strong antigen-specific cellular and humoral immune responses [47].

The work described here demonstrated that an adjuvant complex comprised of the peptide HH2 and CpG oligonucleotide induced strong chemokine release in PBMC, dependent on the specific ratio of HH2 to CpG. Furthermore, the most active HH2–CpG complex ratios had no discernable cytotoxicity and minimal potential for inducing the pro-inflammatory cytokine TNFα. Using these *ex vivo* investigations of HH2–CpG as a guide, an HH2–CpG complex ratio (5:1, wt/wt) that demonstrated both strong synergistic chemokine induction was utilized to demonstrate that intranasal immunization of BALB/c mice with this complex co-formulated with PTd resulted in the induction of strong and balanced humoral responses.

Initial assessment of HH2–CpG vaccine adjuvant formulations relied on the demonstration that HH2 and CpG interact to form a stable complex and subsequent studies were aimed at identifying the optimal concentrations of HH2 peptide and CpG for induction of chemokines following complex formation. The HH2–CpG complex likely results from the strong electrostatic interaction between the cationic peptide and anionic nucleic acid, as demonstrated previously for nucleic acids and polycationic amino acids or peptides [47,48]. Previous studies with a simple peptide and a polyIC–ODN indicated an immediate formation of peptide–ODN complexes leading to enhanced immune modulating properties as compared to the components alone; however, no systematic investigation was performed concerning the optimal ratios of components or their concentrations [47,49]. The work described here demonstrates that the immune modulating activities associated with HH2 as well as the overall ratio of HH2 to CpG in the complex have a significant impact on the chemokine inducing activities of the adjuvant complexes. In contrast complexes of CpG 10101 with the peptide 1047, a relatively inert cationic IDR in terms of immune modulating activities, or between the cationic transfection reagent DOTAP and CpG 10101 did not result in the synergistic induction of MCP-1, demonstrating that the immune modulating activity of the complex was not simply due to non-specific complex formation between the CpG and a cationic molecule. Similarly HH18, a moderately immunomodulatory peptide when complexed with CpG complex led to much weaker chemokine induction activities and calculated synergy values as compared to HH2.

Cell stimulation experiments also verified that specific ratios of CpG:HH2 are required for optimal chemokine induction activity; CpG:HH2 ratios outside the ranges of 1:2–1:8 resulted in decreased induction of chemokine. Using these *ex vivo* chemokine induction screens as a guide also it was demonstrated that these optimized HH2–CpG complexes induced synergistic activation of DCs, a process required for activation of naïve T-cells and for the consequent induction of adaptive immune responses. These results were consistent with actual adjuvant studies employing PTd co-formulated with an optimized HH2–CpG adjuvant complex, which resulted in the induction of a potent mixed Th1/Th2 immune response in mice, confirming that the HH2–CpG adjuvant formulation increased a balanced immune response to this antigen.

Important to all candidate vaccine adjuvant platforms was the demonstration that the adjuvant did not induce systemic toxicity. Highlighting this was the demonstration that the HH2–CpG adjuvant complex did not induce cytotoxic responses or LDH release from human cells. Similarly immunization with the HH2–CpG complex resulted in no obvious lesions at the injection site nor adverse

symptoms upon injection, suggesting that the adjuvanticity associated with the complex is not an artefact of HH2–CpG mediated toxicity. It is possible that the lack of toxicity of the HH2–CpG complex, as well as the storage stability of the complex, might be correlated strong electrostatic interaction between the two components. Upon extended storage of the HH2–CpG complex in a liquid medium, or as a lyophilized powder, an increasing ability to stimulate MCP-1 induction was observed. This may reflect additional complex formation over extended periods of time leading to formation of more stable structures between peptide and ODN molecules.

In addition to assisting in recruitment and activation of immune cells, the vaccine adjuvant comprised of the HH2–CpG complex likely might also enhance cellular delivery of the accompanying antigen to antigen-presenting cells, and improve CpG uptake it has been demonstrated for complexes of the cationic peptide LL-37 with CpG ODN [34]. The precise mechanism utilized by the HH2–CpG complex for cell entry, and the signalling pathways involved in initiation of the immune response, remain to be investigated.

Acknowledgements

We gratefully acknowledge financial support from the Bill and Melinda Gates Foundation and Canadian Institutes for Health Research through the Grand Challenges in Global Health Research Initiative. REWH is a recipient of a Canada Research Chair and SAH is a recipient of a CIHR/Wyeth Pharmaceuticals Chair in Clinical Vaccine Research. J.K. was supported by a fellowship from the Canadian Cystic Fibrosis Foundation.

References

[1] Garlapati S, Facci M, Polewicz M, Strom S, Babiuk LA, Mutwiri G, et al. Strategies to link innate and adaptive immunity when designing vaccine adjuvants. Vet Immunol Immunopathol 2009;128:184–91.
[2] Schirmbeck R, Melber K, Mertens T, Reimann J. Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implication for the pathogenesis of HBV-induced hepatitis. J Virol 1994;68:1418–25.
[3] Traquina P, Morandi M, Contorni M, Van Nest G. MF59 adjuvant enhances the antibody response to recombinant hepatitis B surface antigen vaccine in primates. J Infect Dis 1996;174:1168–75.
[4] Brewer JM, Conacher M, Satoskar A, Bluethmann H, Alexander J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production. Eur J Immunol 1996;26:2062–6.
[5] Aguilar JC, Rodriguez EG. Vaccine adjuvants revisited. Vaccine 2007;25:3752–62.
[6] Mookherjee N, Brown KL, Bowdish DM, Doria S, Falsafi R, Hokamp K, et al. Modulation of the TLR-mediated inflammatory response by the endogenous human host defense peptide LL-37. J Immunol 2006;176:2455–64.
[7] Bowdish DM, Davidson DJ, Scott MG, Hancock REW. Immunomodulatory activities of small host defense peptides. Antimicrob Agents Chemother 2005;49:1727–32.
[8] Heilborn JD, Nilsson MF, Kratz G, Weber G, Sorensen O, Borregaard N, et al. The cathelicidin anti-microbial peptide LL-37 is involved in re-epithelialization of human skin wounds and is lacking in chronic ulcer epithelium. J Invest Dermatol 2003;120:379–89.
[9] Rehaume LM, Hancock REW. Neutrophil-derived defensins as modulators of innate immune function. Crit Rev Immunol 2008;28:185–200.
[10] Mookherjee N, Hancock REW. Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. Cell Mol Life Sci 2007;64:922–33.
[11] Biragyn A, Surenhu M, Yang D, Ruffini PA, Haines BA, Klyushnenkova E, et al. Mediators of innate immunity that target immature, but not mature, dendritic cells induce antitumor immunity when genetically fused with nonimmunogenic tumor antigens. J Immunol 2001;167:6644–53.
[12] Soruri A, Grigat J, Forssmann U, Riggert J, Zwirner J. beta-Defensins chemoattract macrophages and mast cells but not lymphocytes and dendritic cells: CCR6 is not involved. Eur J Immunol 2007;37:2474–86.
[13] Territo MC, Ganz T, Selsted ME, Lehrer R. Monocyte-chemotactic activity of defensins from human neutrophils. J Clin Invest 1989;84:2017–20.
[14] Yang D, Chertov O, Oppenheim JJ. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defenses and adaptive immunity. Cell Mol Life Sci 2001;58:978–89.
[15] Davidson DJ, Currie AJ, Reid GS, Bowdish DM, MacDonald KL, Ma RC, et al. The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. J Immunol 2004;172:1146–56.
[16] Lillard Jr JW, Boyaka PN, Chertov O, Oppenheim JJ, McGhee JR. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. Proc Natl Acad Sci USA 1999;96:651–6.
[17] Tani K, Murphy WJ, Chertov O, Salcedo R, Koh CY, Utsunomiya I, et al. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. Int Immunol 2000;12:691–700.
[18] Scott MG, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, Thompson A, et al. An anti-infective peptide that selectively modulates the innate immune response. Nat Biotechnol 2007;25:465–72.
[19] Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov 2006;5:471–84.
[20] Angelone DF, Wessels MR, Coughlin M, Suter EE, Valentini P, Kalish LA, et al. Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF-alpha production in vitro and in vivo. Pediatr Res 2006;60:205–9.
[21] Butler JE, Francis DH, Freeling J, Weber P, Krieg AM. Antibody repertoire development in fetal and neonatal piglets. IX. Three pathogen-associated molecular patterns act synergistically to allow germfree piglets to respond to type 2 thymus-independent and thymus-dependent antigens. J Immunol 2005;175:6772–85.
[22] Linghua Z, Yong G, Xingshan T, Fengzhen Z. Co-administration of porcine-specific CpG oligodeoxynucleotide enhances the immune responses to pseudorabies attenuated virus vaccine in newborn piglets in vivo. Dev Comp Immunol 2006;30:589–96.
[23] Singh VK, Mehrotra S, Agarwal SS. The paradigm of Th1 and Th2 cytokines: its relevance to autoimmunity and allergy. Immunol Res 1999;20:147–61.
[24] Muller B, Gimsa U, Mitchison NA, Radbruch A, Sieper J, Yin Z. Modulating the Th1/Th2 balance in inflammatory arthritis. Springer Semin Immunopathol 1998;20:181–96.
[25] Kidd P. Th1/Th2 balance: the hypothesis, its limitations, and implications for health and disease. Altern Med Rev 2003;8:223–46.
[26] Chan KG, Mayer M, Davis EM, Halperin SA, Lin TJ, Lee SF. Role of D-alanylation of *Streptococcus gordonii* lipoteichoic acid in innate and adaptive immunity. Infect Immun 2007;75:3033–42.
[27] Vollmer J, Weeratna R, Payette P, Jurk M, Schetter C, Laucht M, et al. Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities. Eur J Immunol 2004;34:251–62.
[28] Hartmann G, Battiany J, Poeck H, Wagner M, Kerkmann M, Lubenow N, et al. Rational design of new CpG oligonucleotides that combine B cell activation with high IFN-alpha induction in plasmacytoid dendritic cells. Eur J Immunol 2003;33:1633–41.
[29] Marshall JD, Fearon K, Abbate C, Subramanian S, Yee P, Gregorio J, et al. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. J Leukoc Biol 2003;73:781–92.
[30] Lee JG, Lee SH, Park DW, Lee SH, Yoon HS, Chin BR, et al. Toll-like receptor 9-stimulated monocyte chemoattractant protein-1 is mediated via JNK-cytosolic phospholipase A2-ROS signaling. Cell Signal 2008;20:105–11.
[31] Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 2003;55:27–55.
[32] Van Wetering S, Mannesse-Lazeroms SP, Van Sterkenburg MA, Daha MR, Dijkman JH, Hiemstra PS. Effect of defensins on interleukin-8 synthesis in airway epithelial cells. Am J Physiol 1997;272:L888–96.
[33] Sakamoto N, Mukae H, Fujii T, Ishii H, Yoshioka S, Kakugawa T, et al. Differential effects of alpha- and beta-defensin on cytokine production by cultured human bronchial epithelial cells. Am J Physiol Lung Cell Mol Physiol 2005;288:L508–13.
[34] Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 2007;449:564–9.
[35] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002;20:709–60.
[36] Halperin SA, Van Nest G, Smith B, Abtahi S, Whiley H, Eiden JJ. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine 2003;21:2461–7.
[37] Halperin SA, Dobson S, McNeil S, Langley JM, Smith B, McCall-Sani R, et al. Comparison of the safety and immunogenicity of hepatitis B virus surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide and a licensed hepatitis B vaccine in healthy young adults. Vaccine 2006;24:20–6.
[38] Cooper CL, Davis HL, Angel JB, Morris ML, Elfer SM, Seguin I, et al. CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. Aids 2005;19:1473–9.
[39] Cooper CL, Davis HL, Morris ML, Efler SM, Adhami MA, Krieg AM, et al. CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind phase I/II study. J Clin Immunol 2004;24:693–701.
[40] Cooper CL, Davis HL, Morris ML, Efler SM, Krieg AM, Li Y, et al. Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. Vaccine 2004;22:3136–43.

[41] Datta SK, Cho HJ, Takabayashi K, Horner AA, Raz E. Antigen-immunostimulatory oligonucleotide conjugates: mechanisms and applications. Immunol Rev 2004;199:217–26.
[42] Tafaghodi M, Jaafari MR, Sajadi Tabassi SA. Nasal immunization studies using liposomes loaded with tetanus toxoid and CpG-ODN. Eur J Pharm Biopharm 2006;64:138–45.
[43] Turanek J, Ledvina M, Kasna A, Vacek A, Hribalova V, Krejci J, et al. Liposomal preparations of muramyl glycopeptides as immunomodulators and adjuvants. Vaccine 2006;24(Suppl. 2). S2-90-1.
[44] Steinstraesser L, Koehler T, Jacobsen F, Daigeler A, Goertz O, Langer S, et al. Host defense peptides in wound healing. Mol Med 2008;14:528–37.
[45] Barlow PG, Li Y, Wilkinson TS, Bowdish DM, Lau YE, Cosseau C, et al. The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. J Leukoc Biol 2006;80:509–20.
[46] Scott MG, Davidson DJ, Gold MR, Bowdish D, Hancock RE. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. J Immunol 2002;169:3883–91.
[47] Schellack C, Prinz K, Egyed A, Fritz JH, Wittmann B, Ginzler M, et al. IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses. Vaccine 2006;24:5461–72.
[48] Lingnau K, Egyed A, Schellack C, Mattner F, Buschle M, Schmidt W. Poly-L-arginine synergizes with oligodeoxynucleotides containing CpG-motifs (CpG-ODN) for enhanced and prolonged immune responses and prevents the CpG-ODN-induced systemic release of pro-inflammatory cytokines. Vaccine 2002;20:3498–508.
[49] Kritsch CE, Berger A, Heinrich-Cseh C, Bugajska-Schretter A, Zauner W. Separation and quantification of a novel two-component vaccine adjuvant. J Chromatogr B Analyt Technol Biomed Life Sci 2005;822:263–70.