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The novel adjuvant combination of CpG ODN, indolicidin and polyphosphazene induces potent antibody- and cell-mediated immune responses in mice

J. Kovacs-Nolan[a], L. Latimer[a], A. Landi[a], H. Jenssen[b], R.E.W. Hancock[b], L.A. Babiuk[c], S. van Drunen Littel-van den Hurk[a,*]

[a] Vaccine and Infectious Disease Organization, University of Saskatchewan, Saskatoon, SK, S7N 5E3, Canada
[b] Centre for Microbial Diseases and Immunity Research, University of British Columbia, Vancouver, BC, V6T 1Z4, Canada
[c] University of Alberta, 3-7 University Hall, Edmonton, AB, T6G 2J9, Canada

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ABSTRACT

The need to enhance the immunogenicity of purified subunit antigens and modulate resulting immune responses has prompted the development of new adjuvants. Here, the ability of CpG oligodeoxynucleotides (ODN), a bovine host defence peptide indolicidin, and polyphosphazene to synergistically combine and enhance innate and adaptive immune responses was examined in mice. *In vitro*, the adjuvant combination of CpG ODN, indolicidin and polyphosphazene (CpG/indol/PP) enhanced the secretion of TNF-α, IL-12p40, and IL-6 by bone marrow-derived DCs (BMDCs) when compared to the individual components. When co-formulated with ovalbumin (OVA), CpG/indol/PP formed antigen-adjuvant complexes, and enhanced antibody and cell-mediated responses in mice, via both MHC I and II pathways, promoting a more balanced antibody-mediated and type 1-biased cell-mediated immune response. Furthermore, substitution of the proline residues of indolicidin with arginine increased the synergistic adjuvant effect of the peptide, and induced significantly higher IgG1 and IgG2a titers and IFN-γ secretion, as well as increased uptake by antigen presenting cells. These results clearly demonstrate that the use of a combination of CpG ODN, indolicidin, and polyphosphazene as adjuvant can significantly enhance an antigen-specific immune response.

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1. Introduction

The mammalian defense system against pathogens involves early recognition by the innate immune system followed by a transition to an antigen-specific adaptive immune response [1]. In previous vaccines live attenuated or inactivated pathogens were used for immunization. Recent vaccine development, however, has focused on the use of pathogen-derived protein/subunit or peptide antigens, which are often weakly immunogenic and rely heavily on stimulation of innate immune mechanisms through the use of adjuvants to ensure a vigorous adaptive response and consequent vaccine efficacy [2–4].

The choice of vaccine adjuvant is crucial, as they can selectively bias the immune response towards type 1 responses, characterized by the enhanced secretion of IFN-γ, TNF-α, and IL-12, and the induction of IgG2a and cytotoxic T lymphocytes (CTLs), or towards a type 2 response and the secretion of IL-4 and IL-5, the generation of IgG1 and IgE antibodies, and weak or no CTLs [5,6]. Generally speaking it is considered that type 1 responses are favourable for countering intracellular pathogens while type 2 responses are more targeted towards extracellular pathogens, although optimal protection might require balanced type 1/type 2 responses. For example, aluminium adjuvants are widely used, but are limited by their inability to induce cell-mediated responses, and their tendency to skew the immune response towards a type 2 response [6–8]. Furthermore, the choice of adjuvant can influence the number of injections required to achieve protection, can allow immunization by distinct routes (e.g. mucosal vaccines), and elicit optimal immune responses in individuals in whom conventional vaccination strategies may not be optimally effective, including infants and the elderly [2,9]. Therefore, the identification of novel adjuvants and/or adjuvant combinations that can effectively enhance and modulate the antigen-specific immune response is of prime importance in successful vaccine development for both human and veterinary applications.

Adjuvants may augment immune responses by one or more mechanisms, including direct or indirect immunomodulation, formation of an antigen-adjuvant depot, chemoattraction of appropriate immune cells to the site of antigen administration, and/or targeting or delivery to antigen presenting cells (APCs). For example

* Corresponding author. Tel.: +1 306 966 1559; fax: +1 306 966 7478.
E-mail address: sylvia.vandenhurk@usask.ca (S. van Drunen Littel-van den Hurk).

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molecules that modulate the host's innate immune response are able to promote the transition to adaptive immunity and enhance immunogenicity. The vertebrate innate immune system has developed various strategies to detect pathogens based on the recognition of certain pathogen signature molecules, including Toll-like receptors (TLRs) [6]. Oligonucleotides containing unmethylated CpG dinucleotides, characteristic of bacterial DNA, are detected by TLR9 and serve as a 'danger signal' for the innate immune system, triggering a protective immune response. Similarly, synthetic CpG ODNs can stimulate B cells, monocytes, macrophages and dendritic cells (DCs), inducing the secretion of pro-inflammatory (IL-1, IL-6, IL-18 and TNF-α) and type 1 cytokines (IL-12, IFN-γ), resulting in the generation of a type 1-biased immune response [6,10,11]. CpG ODNs enhance immune responses when co-administered with numerous viral and bacterial antigens (reviewed in [6]), and have been shown to overcome the type 2 bias associated with conventional adjuvants such as alum or oil-based adjuvants [12,13].

Host defense peptides (HDPs) are immune-modulating compounds endogenous to the host's immune system. Natural gene-encoded HDPs are typically 12–50 amino acids in length, have a net positive charge, and contain a substantial number of hydrophobic residues [14–17]. In mammals, HDPs are largely represented by defensins and cathelicidins [18]. They are constitutively present in the mucosa and bodily fluids at low levels released following exposure to pathogens and/or cell death and are able to both recruit and selectively activate APCs in a non-TLR-dependent manner, thereby serving as early warning signals to activate innate and adaptive immune responses [16]. The single known human cathelicidin peptide, LL-37, possesses a number of immune-modulating functions, including being chemotactic for human neutrophils, monocytes, T cells, and mast cells [19,20], inhibiting LPS-induced cytokine production [21], and upregulating genes involved in the innate immune response, such as IL-8, MCP-1, differentiation factors, and anti-inflammatory cytokines [21]. Mouse cathelin-related antimicrobial peptide (CRAMP), the orthologue of LL-37, was also shown to be chemotactic *in vitro* for human monocytes, neutrophils, and macrophages, as well as *in vivo* in mice, where it induced leukocyte activation and chemotaxis, and promoted antigen-specific immune responses when co-administered with the antigen ovalbumin (OVA) [18]. These results suggest that cathelicidin peptides are promising novel immunotherapeutic agents; however, the relatively large size of LL-37 and CRAMP, the cost of synthesis, and their protease lability [22] may limit their applications as vaccine adjuvants. Indolicidin, a 13-amino acid peptide isolated from the cytoplasmic granules of bovine neutrophils [23], is one of the smallest of the naturally occurring linear host defense peptides [24]. It possesses anti-endotoxin properties and induces expression of the chemokine IL-8 in human bronchial epithelial cells [22], and may therefore be an attractive candidate for vaccine applications.

Adjuvants may also enhance immune responses by aiding in antigen display and delivery. Polyphosphazenes are ion cross-linkable water soluble polymers, containing a long chain backbone of alternating nitrogen and phosphorous atoms, with two side groups attached to each phosphorous atom [25,26]. The use of polyphosphazene hydrogels and microparticles has previously been described for the delivery of antigens, immunoadjuvants, and nucleic acids [25,26]. More recently, studies have shown that polyphosphazenes, such as poly[di(carboxylatophenoxy)phosphazene] (PCPP), form water-soluble, non-covalent complexes with protein, and can enhance immune responses when simply mixed with antigens in solution [27,28]. While the mechanisms by which they exert their adjuvant activity are not fully understood, it has been suggested that polyphosphazenes may contribute to the immune response via multivalent antigen presentation and targeting of the antigen to the cell surfaces [27]. PCPP has demonstrated adjuvant activity with a number of bacterial and viral antigens [28–31] and has been found to be capable of inducing long lasting immune responses, even after a single immunization [29]. Furthermore, PCPP has also shown promise as an adjuvant for mucosal immunization [32].

Table 1
Sequences of indolcidin and substituted indolicidin derivative peptides.

Name	Sequence	Net charge
Indolicidin	ILPWKWPWWPWRR	+3
IN41	– – K– – – – – – – – –	+4
IN42	– – – – – K– – – – – – –	+4
IN44	– – K– – – – – – K – – –	+5
IN50	– – R– – – R – –R – – –	+6

Horizontal lines indicate same amino acid as parent peptide.

When used in combination, immunomodulating compounds may contribute to enhance the resulting immune response, acting as a more potent adjuvant than the individual components alone, and recently a novel adjuvant combination consisting of a cationic antimicrobial peptide and synthetic ODN, termed IC31, has been described [1]. In the present study, we investigated the immunostimulatory effect of the combination of CpG ODN 1826 and the cationic peptide indolicidin, alone and when used in combination with polyphosphazene, as well as the effect of substituted indolicidin derivatives to further enhance both cell-mediated and antibody-mediated immune responses.

2. Materials and methods

2.1. Adjuvants

Phosphorothioate-stabilized CpG ODN 1826 (TCCATGACGTTCC TGACGTT) was provided by Merial (Duluth, GA). Indolicidin (ILPWKWPWWPWRR-NH_2) was chemically synthesized in-house on a Pioneer solid-phase peptide synthesizer (PerSeptive Biosystems, Foster City, CA) using 9-fluorenylmethoxy carbonyl (Fmoc) chemistry. Indolicidin derivative peptides (Table 1) were synthesized by Fmoc chemistry at the University of British Columbia Brain Research Centre. The polyphosphazene polymer, a 90% substituted poly(di-p-dicarboxylatophenoxy)-phosphazene (PCPP)/10% hydroxylate (90:10 PCPP/OH), was synthesized as described previously [33] and was provided by Dr. John Klaehn, Idaho National Laboratory (Idaho Falls, ID).

2.2. Electrophoretic mobility shift assay (EMSA)

The formation of CpG ODN-indolicidin complexes was visualized by electrophoretic mobility shift assay (EMSA). A constant amount of CpG ODN 1826 was combined with increasing concentrations of indolicidin, and incubated for 30 min at room temperature. Samples were run on 1% agarose containing 0.5 μg/mL ethidium bromide in TAE running buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3). Samples were loaded in 5% (v/v) glycerol and run for 15 min at 100 V.

2.3. Immunization of mice

To evaluate the effect of the combination of CpG ODN and indolicidin as an adjuvant, OVA (Sigma–Aldrich, St. Louis, MO) was co-formulated with CpG ODN-peptide complexes as described by Schellack et al. [1]. Briefly, CpG ODN 1826 (10 μg) was mixed with 10 or 100 nmol of indolicidin in 10 mM Tris–HCl buffer, pH 7.6, containing 0.27 M sorbitol (Sigma–Aldrich), and then combined with 50 μg OVA in a final volume of 100 μL. Six- to eight-week-old female C57BL/6 mice ($n = 5$) (Charles River Laboratories, Inc., Montreal, Quebec) were immunized twice subcutaneously at a 3 week interval with OVA co-formulated with CpG ODN-indolicidin,

or each of the individual components. Serum was collected 2 weeks following each immunization (days 14 and 35), and OVA-specific antibody titers were measured by enzyme-linked immunosorbent assay (ELISA).

To evaluate the adjuvant activity of CpG ODN, indolicidin and polyphosphazene 6- to 8-week-old female C57BL/6 mice ($n = 8$) (Charles River Laboratories, Inc.) were immunized twice subcutaneously at a 3 week interval with 100 μL of vaccine containing 10 μg OVA (Sigma-Aldrich), either alone or co-formulated with CpG ODN 1826 (CpG), indolicidin (indol), and/or polyphosphazene (PP). CpG ODN, indolicidin and polyphosphazene were given at doses of 10 μg, 10 nmol (∼15 μg), and 50 μg, respectively. The doses of CpG ODN 1826 and polyphosphazene used correspond to those previously reported by Davis et al. [34] and Mutwiri et al. [35], respectively. Animals in the placebo group were injected with buffer only. Two weeks following the second immunizations, mice were euthanized and spleens were collected for analysis of cell-mediated immune responses by enzyme-linked immunospot (ELISPOT) assay, and OVA-specific antibody titers were measured by ELISA.

To examine the immune-enhancing effects of substituted indolicidin peptide derivatives, C57BL/6 ($n = 6$) mice were immunized twice subcutaneously, at a 3 week interval, with 100 μL of vaccine containing 10 μg OVA combined with 10 nmol of peptide, alone or in combination with 10 μg of CpG ODN 1826. Animals in the placebo group were injected with buffer only. Two weeks following the second immunizations, mice were euthanized and spleens were collected for analysis of cell-mediated immune responses by ELISPOT assay, and OVA-specific antibody titers were measured by ELISA.

All protocols were carried out in accordance with the Canadian Council of Animal Care Guide to the Care and Use of Experimental Animals, and approved by the University Committee on Animal Care and Supply (UCACS).

2.4. Enzyme-linked immunosorbent assay (ELISA)

To measure OVA-specific antibody responses, 96-well microtiter plates (IMMULON® 2; Thermo Electron Corp., Milford, MA) were coated overnight at 4 °C with 1 μg/well OVA in sodium carbonate buffer, pH 9.6. Plates were then washed and incubated overnight at 4 °C with four-fold serially diluted sera, with a starting dilution of 1:40. Bound antibodies were detected using alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:5000, and visualized with *p*-nitrophenyl phosphate (PNPP) (Sigma–Aldrich). OVA-specific mouse IgG1 and IgG2a titers were measured using biotinylated goat anti-mouse IgG1 and IgG2a antibodies (Caltag Laboratories, Burlingame, CA), diluted 1:5000, followed by streptavidin-AP (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1:20,000. ELISA titers were expressed as the reciprocal of the highest dilution resulting in a value of two standard deviations above the negative control serum.

2.5. IFN-γ and IL-5 ELISPOT assay

To measure the relative numbers of IFN-γ and IL-5 secreting cells 96-well nitrocellulose plates (MULTISCREEN®-HA; Millipore Corp.) were coated overnight, at 4 °C, with 0.2 μg/well of mouse IFN-γ- or IL-5-specific monoclonal antibodies (BD Biosciences, San Jose, CA). Plates were washed with phosphate-buffered saline (PBS) (Invitrogen Corp., Grand Island, NY) and blocked with 1% (w/v) bovine serum albumin (BSA) (Sigma–Aldrich) in PBS. Splenocytes were isolated as described previously [36], and resuspended to a final concentration of 10^7 cells/mL in AIM V® medium (Invitrogen Corp.) containing 0.1 mM non-essential amino acids (Invitrogen Corp.), 1 mM sodium pyruvate (Invitrogen Corp.), 10 mM HEPES (Invitrogen Corp.), and 50 μM 2-mercaptoethanol (Sigma–Aldrich). Splenocytes were added to triplicate wells at a concentration of 10^6 cells/well, and cultured in the presence of endotoxin-free OVA (Profos AG, Regensburg, Germany) or the H-2K^b MHC class I-restricted epitope $OVA_{257-264}$ (SIINFEKL) or the I-A^b MHC class II-restricted epitope $OVA_{265-280}$ (TEWTSSNVMEERKIKV) (GenScript Corporation, Piscataway, NJ), at 10 μg/mL, or medium, for 18–20 h. Plates were washed and incubated with biotinylated rat anti-mouse IFN-γ or IL-5 (BD Biosciences) at 2 μg/mL. Bound antibodies were detected using streptavidin-AP (Jackson) diluted 1:1000, and visualized using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitroblue tetrazolium chloride (NBT) substrate tablets (Sigma–Aldrich). Plates were washed with water and air-dried. Stained spots were counted, and the numbers of IFN-γ- and IL-5-secreting cells were expressed as the difference between the number of spots in OVA-, $OVA_{257-264}$-, or $OVA_{265-280}$-stimulated wells and control (medium) wells per 10^6 cells.

2.6. Native polyacrylamide gel electrophoresis (PAGE)

To characterize the physical nature of the experimental vaccine formulations, they were analyzed by native polyacrylamide gel electrophoresis (PAGE). Samples were loaded in 5% (v/v) glycerol, 0.02% (w/v) bromophenol blue (Sigma–Aldrich), and resolved on a 10% polyacrylamide gel, containing no sodium dodecyl sulphate (SDS). Gels were stained with 0.5% (w/v) Coomassie Brilliant Blue R-250 (Sigma–Aldrich). To visualize the simultaneous migration of CpG ODN and OVA, formulations were prepared using CpG ODN and antigen labelled with infrared dye. IRDYE® 800-labelled CpG ODN 1826 was synthesized by Li-Cor Biosciences (Lincoln, NE) and OVA was labelled with IRDYE 680 using the IRDYE 680 Microscale Protein Labeling Kit (Li-Cor Biosciences, Lincoln, NE) according to manufacturer's instructions. Samples were loaded in 5% (v/v) glycerol, and resolved in a 10% polyacrylamide gel containing no SDS. Gels were analyzed using an ODYSSEY® Infrared Imaging System (Li-Cor Biosciences).

2.7. Cytokine secretion by bone marrow-derived dendritic cells (BMDCs)

Cytokine secretion by bone marrow-derived dendritic cells (BMDCs) in response to CpG ODN, indolicidin and polyphosphazene was used to assess the immunostimulatory effects of the adjuvants. BMDCs were prepared as previously described [37], with modifications. Briefly, femurs and tibiae from naïve C57BL/6 mice were flushed with PBS, and erythrocytes were lysed with Tris-ammonium chloride buffer (17 mM Tris, 144 mM NH_4Cl, pH 7.2). Cells were cultured in 6-well plates (Corning Incorporated, Corning, NY) in RPMI 1640 medium (Invitrogen Corp.) supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 50 μg/mL gentamicin, 50 μM 2-mercaptoethanol, and 20 ng/mL murine GM-CSF (PeproTech Inc., Rocky Hill, NJ). On day 3 of culture, medium was removed and replaced with fresh culture medium. On day 5, cells were harvested and used for cytokine secretion and antigen uptake assays.

BMDCs (1×10^6 cells/mL) were cultured in 24-well culture plates (Corning Incorporated) in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 50 μg/mL gentamicin, and 50 μM 2-mercaptoethanol. CpG ODN 1826, indolicidin, and/or polyphosphazene were added in duplicate to the cells at final concentrations of 1 μg/mL, 1 nmol/mL and 5 μg/mL, respectively. Buffer alone was added as a negative control. After 24 h, culture supernatants were collected, and IL-6, IL-12p70, and TNF-α concentrations were measured using

QUANTIKINE® mouse IL-6, IL-12p70 and TNF-α ELISA kits (Research and Diagnostic Systems, Minneapolis, MN), according to the manufacturer's instructions. To measure MCP-1 secretion in response to the indolicidin derivatives, cells were cultured with 10 nmol/mL peptide, alone or in combination with 10 μg/mL CpG ODN 1826, and MCP-1 concentrations were measured using a BioSource™ MCP-1 ELISA Kit (MEDICORP Inc., Montreal, Quebec), according to the manufacturer's instructions.

2.8. Effect of CpG ODN and cationic peptide on antigen uptake by BMDCs

FITC-labelled OVA (FITC-OVA) (Molecular Probes Inc., Eugene, OR) was used to evaluate the effect of CpG ODN and peptide on antigen uptake by DCs. BMDCs (2×10^6 cells/mL) were incubated with 5 μg FITC-OVA alone or in the presence of 5 μg CpG ODN 1826 and/or 5 nmol peptide for 30 min at 4 °C or 37 °C. BMDCs were washed thoroughly with ice cold PBS, and fluorescence was measured by flow cytometry (FACSCALIBUR™, Becton Dickinson, San Jose, CA). Results are expressed as mean fluorescence intensity (MFI) relative to cells alone.

2.9. Statistical analysis

Statistical analysis was carried out using GRAPHPAD™ PRISM version 5.00 for WINDOWS® (GraphPad Software, San Diego, CA). Differences between groups were determined using a Mann-Whitney *U*-test. Results were considered significant if $P < 0.05$.

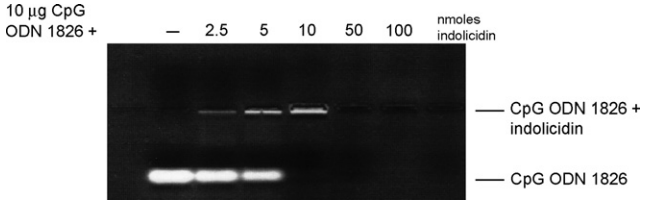

Fig. 1. Electrophoretic mobility shift assay of CpG ODN and indolicidin complexes. CpG ODN 1826 (10 μg) was combined with increasing amounts of indolicidin, and CpG ODN-indolicidin complexes were visualized by agarose gel electrophoresis in the presence of ethidium bromide.

3. Results

3.1. Formation of CpG ODN-indolicidin complexes and effect on anti-OVA antibody response

To examine interactions between CpG ODN and the cationic peptide indolicidin, a constant amount of CpG ODN 1826 (10 μg) was combined with increasing concentrations of indolicidin and analyzed by EMSA (Fig. 1). When ≤5 nmol of indolicidin was used, free ODN was visible, along with a larger, slow-migrating ODN-peptide complex. At ≥10 nmol of peptide, no free ODN was visible, but instead was present as a slow- or non-migrating ODN-peptide complex with indolicidin.

To examine the effect of the combination of CpG ODN and indolicidin *in vivo*, mice were immunized with OVA co-administered with CpG ODN, indolicidin (10 or 100 nmol), or CpG ODN combined with 10 or 100 nmol indolicidin (corresponding to molar ratios of

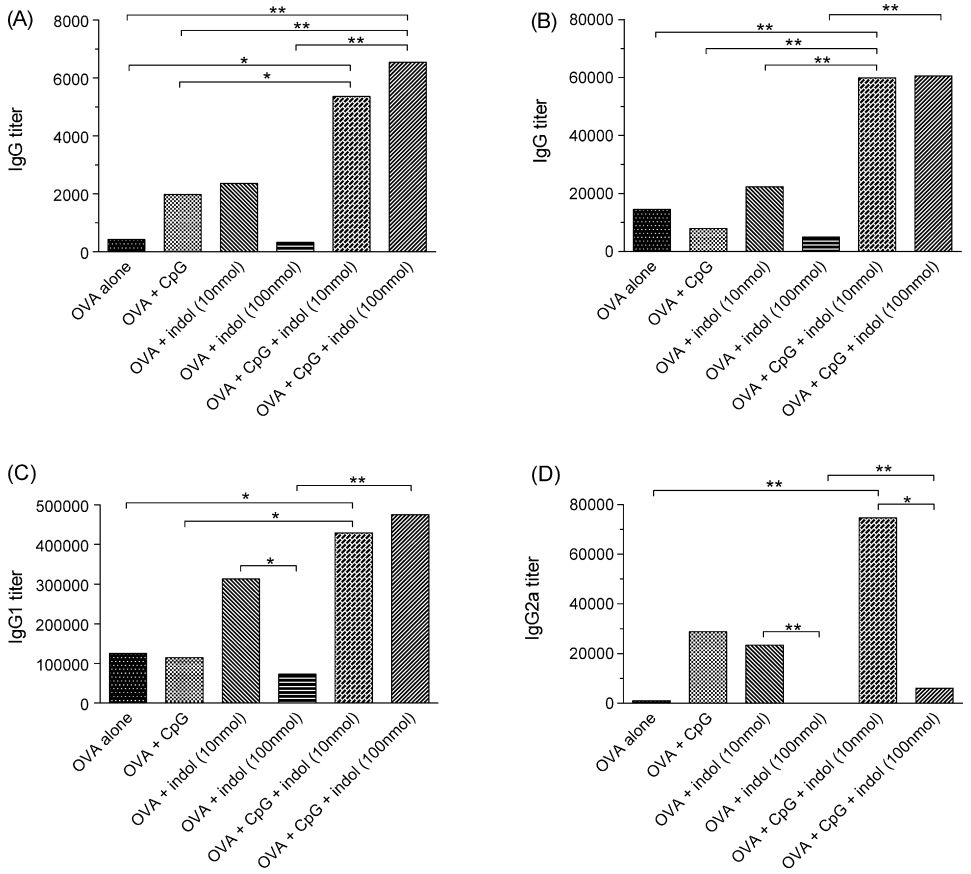

Fig. 2. OVA-specific antibody responses in mice immunized with OVA adjuvanted with CpG ODN 1826 and indolicidin. C57BL/6 mice ($n = 5$) were immunized twice subcutaneously with 50 μg OVA formulated with 10 μg CpG ODN, indolicidin, or 10 μg CpG ODN+indolicidin, at the indicated doses. Serum anti-OVA IgG titers were measured 14 days following primary immunization (A) and following secondary immunization (B). OVA-specific mouse IgG1 (C) and IgG2a (D) titers were measured 14 days following secondary immunization. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. Bars represent the median values of each group. $^*P < 0.05$; $^{**}P < 0.01$.

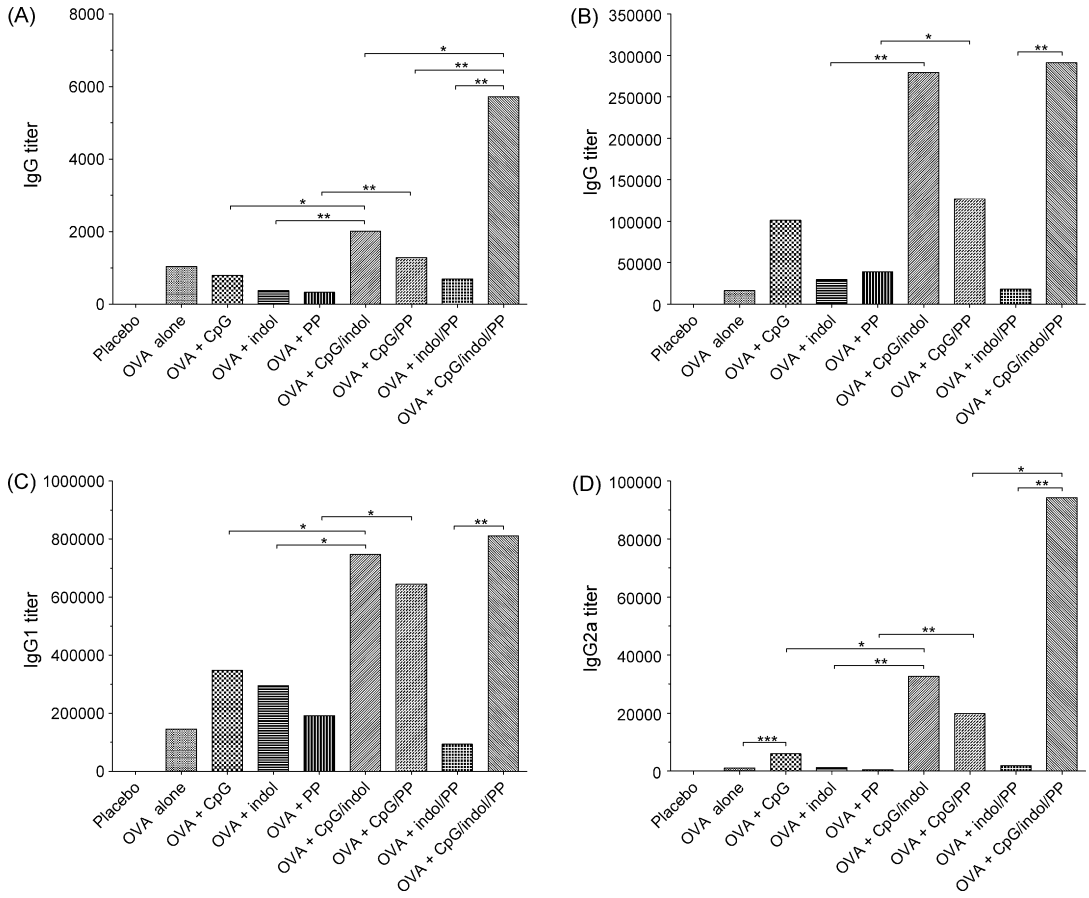

Fig. 3. OVA-specific antibody responses in mice immunized with OVA adjuvanted with CpG ODN 1826, indolicidin, and polyphosphazene. C57BL/6 mice ($n = 8$) were immunized twice subcutaneously with 10 μg OVA formulated with 10 μg CpG ODN, 10 nmol indolicidin, and/or 50 μg polyphosphazene. Serum anti-OVA IgG titers were measured 14 days following primary immunization (A) and following secondary immunization (B). OVA-specific mouse IgG1 (C) and IgG2a (D) titers were measured following secondary immunizations. Four of the mice in the OVA + indol/PP group died and were excluded from analyses. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. Bars represent the median values of each group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

ODN to peptide of 1:6.7 and 1:67, respectively). Peptide concentrations were chosen based on the absence of free CpG ODN as seen in Fig. 1, and a high and low dose were selected to determine if a dose-dependent effect of peptide concentration on adjuvanticity could be observed. A similar trend in IgG titers was seen after both one (Fig. 2A) and two (Fig. 2B) immunizations. While neither indolicidin or CpG ODN by themselves significantly increased antibody titers, after two immunizations the anti-OVA IgG titers induced by CpG ODN combined with 10 nmol of indolicidin were significantly higher than those induced by immunization with OVA alone, OVA+CpG, or OVA+indol (10 nmol) ($P < 0.01$) and nearly double that of the combined titres in the presence of CpG or indolicidin alone. There was no significant difference in the IgG titers of groups immunized with CpG ODN combined with 10 or 100 nmol of indolicidin. While both the high and low doses of indolicidin, when combined with CpG ODN, significantly increased serum subclass IgG1 titers when compared to OVA alone (Fig. 2C), only the lower dose (10 nmol) of indolicidin significantly increased IgG2a titers (Fig. 2D), reflecting the performance of indolicidin alone which was immunostimulatory for both IgG1 and IgG2a production at 10 nmol, but not at 100 nmol. These results suggested that indolicidin at a dose of 10 nmol, when complexed with CpG ODN, could effectively enhance the antigen-specific antibody response, and when used at low doses, resulted in a more robust and balanced immune response. Therefore the lower dose (10 nmol) of indolicidin was chosen for further study.

3.2. Co-formulation of OVA with CpG ODN, indolicidin, and polyphosphazene

To examine the adjuvant activities of CpG ODN, indolicidin and polyphosphazene, mice were immunized with 10 μg of OVA, which was previously determined to be a sub-optimal dose (data not shown), either alone or co-formulated with CpG ODN (CpG), indolicidin (indol), and/or polyphosphazene (PP), at doses of 10 μg, 10 nmol, and 50 μg, respectively. Negative control mice were injected with buffer alone (placebo).

Serum IgG titers were measured by ELISA after one immunization (Fig. 3A), and again 2 weeks later following the second immunization (Fig. 3B). After one immunization, mice immunized with OVA + CpG/indol/PP developed significantly higher IgG titers when compared to those of all other groups or even when compared to the summed titers of the three adjuvants alone. As observed in the previous trial, the combination of OVA with CpG ODN complexed with indolicidin induced significantly higher serum IgG titers than OVA with either CpG ODN or indolicidin alone, following one ($P < 0.05$ and $P < 0.01$) and two ($P < 0.001$) immunizations. After two immunizations, IgG titers induced by OVA formulated with either CpG/indol ($P < 0.001$) or CpG/PP ($P < 0.05$ and $P < 0.001$) were significantly higher than those induced by the individual adjuvants, however no significant differences were observed between the titers in these two groups and those induced by the combination of all three adjuvants, CpG/indol/PP.

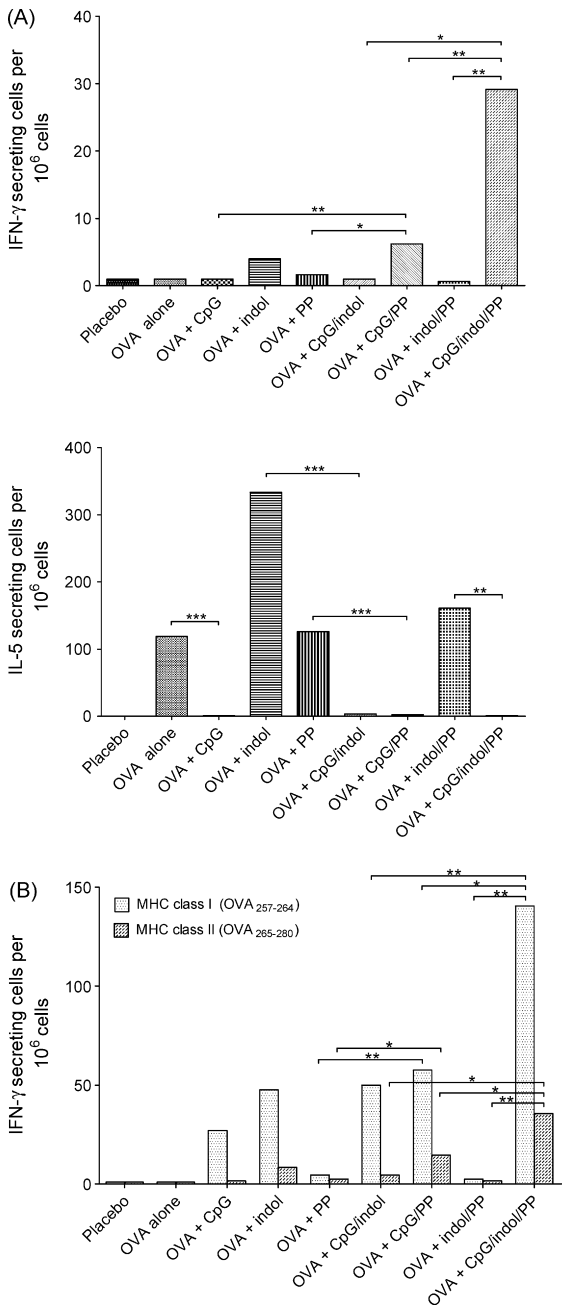

Fig. 4. OVA-specific cell-mediated immune responses in mice ($n = 8$) immunized with OVA co-formulated with CpG ODN, indolicidin and/or polyphosphazene were measured 14 days following secondary immunization. Splenocytes were re-stimulated with 10 μg/mL of OVA (A) or the OVA-derived peptides $OVA_{257-264}$ and $OVA_{265-280}$ (B) and numbers of cytokine-secreting cells were measured by ELISPOT assay. Four of the mice in the OVA + indol/PP group died and were excluded from analyses. Results are expressed as the difference between the number of cytokine-secreting cells in OVA/peptide-stimulated wells and medium-control wells per 10^6 cells. Bars represent the median values of each group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Further analysis of the anti-OVA antibody response induced by each of the formulations was carried out by measuring serum IgG1 and IgG2a-related antibody titers following two immunizations. A trend similar to that observed for the IgG titers was observed for IgG1, with higher IgG1 titers observed in mice immunized with OVA + CpG/indol, CpG/PP, and CpG/indol/PP (Fig. 3C). However, significantly higher IgG2a-associated anti-OVA antibody levels were observed in groups immunized with OVA + CpG/indol and CpG/indol/PP, with the triple adjuvant combination leading to approximately three-fold higher amounts of IgG2a when compared to the combination of CpG and indol (Fig. 3D), suggesting that the combination of CpG and indolicidin may support the induction of a more balanced immune response.

To further characterize the immune response induced by each of the formulations, IFN-γ production by OVA-exposed splenocytes from immunized mice was examined by re-stimulating splenocytes with OVA as well as the epitopes $OVA_{257-264}$ and $OVA_{265-280}$. Following re-stimulation with OVA, significantly higher numbers (>5-fold) of IFN-γ-secreting cells were observed in mice immunized with antigen co-formulated with CpG/indol/PP when compared to IFN-γ secretion by splenocytes from all other vaccine groups, including those formulated with the two adjuvant combinations ($P < 0.01$) (Fig. 4A). All formulations that did not contain CpG ODN resulted in significantly higher frequencies of IL-5-secreting cells when compared to OVA formulations incorporating CpG ODN, with indolicidin alone being superior as an adjuvant for this response relative to the other compounds (Fig. 4A). Moreover, immunization with OVA co-formulated with CpG/indol/PP significantly increased IFN-γ secretion in response to both of the OVA-derived antigenic peptides $OVA_{257-264}$ and $OVA_{265-280}$ (Fig. 4B) indicating that co-formulation of OVA with CpG/indol/PP induced a potent type 1 cell-mediated immune response to OVA, which was both MHC class I- and class II-restricted. Higher numbers of IFN-γ-secreting cells were induced against $OVA_{257-264}$, suggesting that co-formulation with CpG/indol/PP also enhanced antigen-specific CD8+ T cell responses. These results suggest that while the combination of CpG ODN and indolicidin was capable of enhancing anti-OVA antibody titers, it did not as effectively enhance cellular immune responses. Co-formulation of OVA with CpG ODN, indolicidin, and polyphosphazene, however, led to significant increases in both antibody- and cell-mediated immune responses, and resulted in a type 1-biased cell-mediated immune response and mixed type 1/type 2 antibody-mediated response.

3.3. Physical characterization of vaccine formulations

To examine the physical nature of the vaccine formulations, they were analyzed by native PAGE, which revealed the presence of a slower migrating complex when both indolicidin and polyphosphazene were included in the vaccine formulation (Fig. 5A). CpG ODN was not required for complex formation, as complexes were apparent both in the presence and absence of CpG ODN. Further analysis of this complex using infrared dye-labelled CpG ODN and OVA, in order to simultaneously visualize the migration of both CpG ODN (green) and OVA (red) within the gel, was carried out (Fig. 5B). Consistent with the previous EMSA observations, CpG ODN migration was retarded in the formulation containing CpG ODN and indolicidin. However, in the formulation containing all three adjuvants (CpG/indol/PP), CpG ODN appeared to co-migrate with OVA as part of a slower migrating complex (seen here as a yellow band), suggesting that the addition of polyphosphazene may induce the formation of antigen-adjuvant complexes.

3.4. DC activation by CpG ODN, indolicidin, and polyphosphazene

The immunostimulatory effects of CpG ODN, indolicidin and polyphosphazene on BMDCs were investigated by analyzing cytokine secretion in response to the adjuvant formulations. BMDCs were cultured in the presence of CpG ODN 1826, indolicidin and/or polyphosphazene, and concentrations of IL-12p70, TNF-α, and IL-6 were measured by ELISA. As would be expected, the DCs were activated by CpG ODN to produce IL-12p70, TNF-α, and IL-6 (Fig. 6). Indolicidin and polyphosphazene, on the other hand, induced little or no IL-12p70, TNF-α, and IL-6. However, the

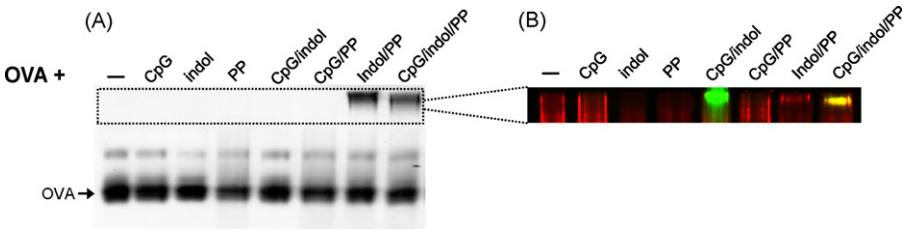

Fig. 5. Native PAGE analysis of vaccine formulations. Vaccines containing OVA formulated with CpG ODN, indolicidin and/or polyphosphazene were prepared and resolved on a 10% polyacrylamide gel and stained with Coomassie Brilliant Blue (A) or prepared using IRDYE® 800-labelled CpG ODN 1826 and IRDYE 680-labelled OVA and visualized using an ODYSSEY® Infrared Imaging System (B).

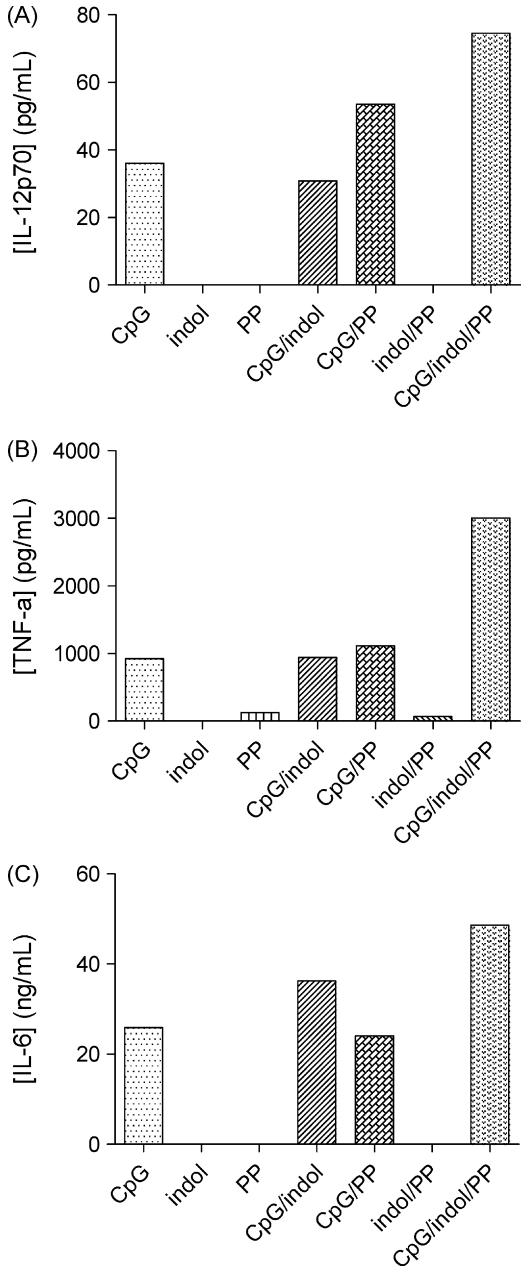

Fig. 6. *In vitro* cytokine secretion by DCs stimulated with CpG ODN 1826, indolicidin and polyphosphazene. Day 5 BMDCs from C57BL/6 mice were cultured in the presence of CpG ODN 1826, indolicidin, and/or polyphosphazene at final concentrations of 1 μg/mL, 1 nmol/mL and 5 μg/mL, respectively, for 24 h. Levels of IL-12p70, TNF-α, and IL-6 were measured by ELISA. Buffer alone was added to BMDCs as a negative control, and was subtracted from treatments as background cytokine levels. Data shown are representative values of three experiments.

combination of CpG/indol/PP tended to enhance production of all three cytokines.

3.5. *Effect of substituted indolicidin derivatives on innate and antigen-specific immune responses*

To further examine the role of the cationic peptide component when combined with CpG ODN, peptide derivatives based on the sequence of indolicidin were designed and selected based on previous *in vitro* chemokine induction, where they were shown to induce MCP-1 secretion in human PBMCs (data not shown). Likewise, here the indolicidin derivative peptides induced MCP-1 secretion in BMDCs, which was significantly increased ($P < 0.05$) when combined with CpG ODN (Fig. 7).

These peptides were then examined for their ability to enhance the OVA-specific immune response compared to the parent sequence of indolicidin, when combined with CpG ODN *in vivo*. As observed with indolicidin, all peptides increased anti-OVA serum IgG levels when combined with CpG ODN (data not shown). Furthermore, while a similar trend was observed in the IgG1 (Fig. 8A) and IgG2a (Fig. 8B) titers induced by all of the peptides, antibody titers induced by OVA + IN50/CpG were substantially higher than those of all other peptides. Examination of the cell-mediated immune responses by *in vitro* re-stimulation of splenocytes with OVA revealed that immunization with IN50/CpG resulted in a significant increase in the number of IFN-γ-secreting cells ($P < 0.01$) (Fig. 8C). A reduction in the number of IL-5-secreting splenocytes was observed in all groups immunized with formulations containing CpG ODN, and did not differ significantly from each other (Fig. 8D). These results suggest that the IN50 peptide, when co-administered with CpG ODN, may be a more potent enhancer of both antibody-mediated and cell-mediated immunity than indolicidin.

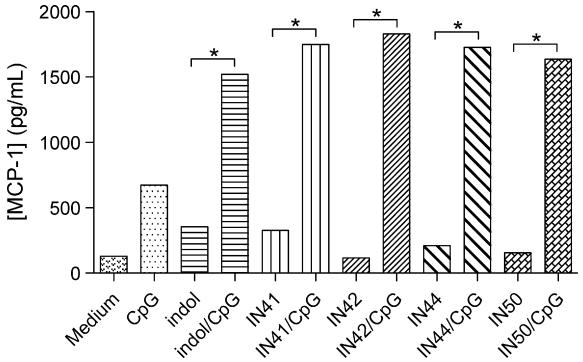

Fig. 7. MCP-1 secretion induced by indolicidin and indolicidin derivatives. Day 5 BMDCs from C57BL/6 mice were cultured in the presence of indolicidin and indolicidin derivative peptides combined with CpG ODN 1826, at final concentrations of 10 nmol/mL and 10 μg/mL, respectively, for 24 h. MCP-1 concentrations were measured by ELISA. Bars represent median values obtained from three experiments. $^{*}P < 0.05$.

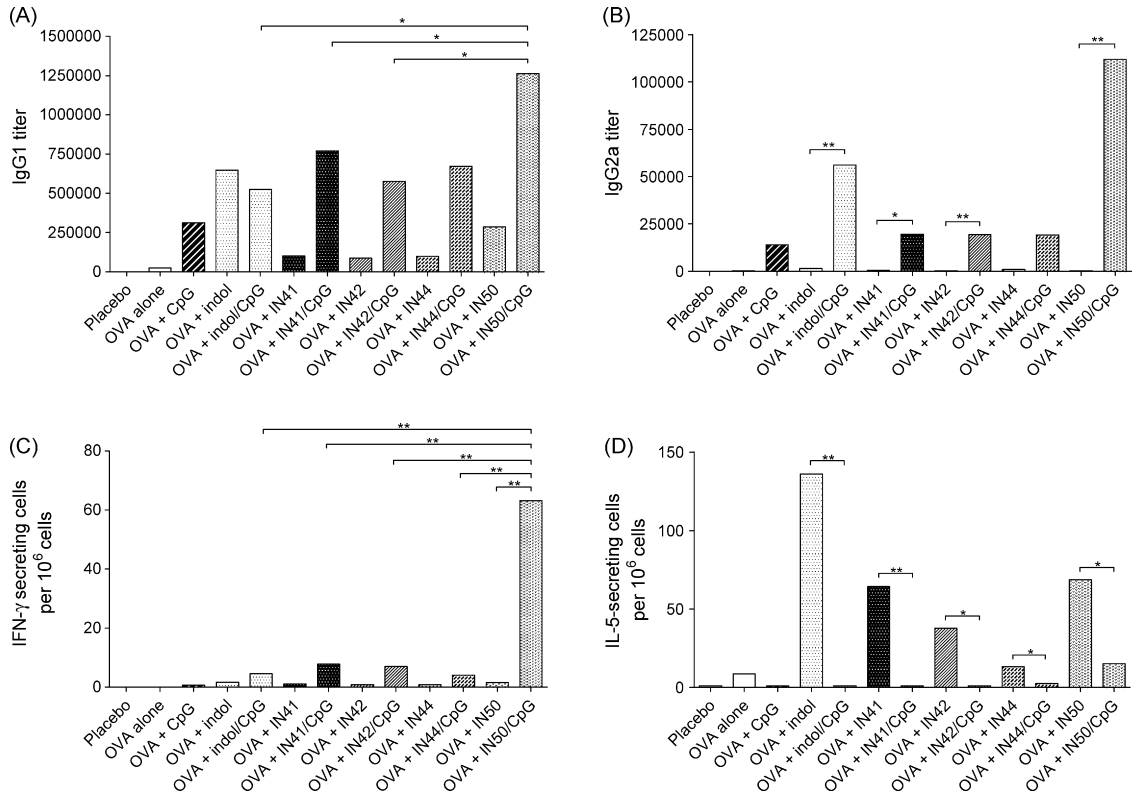

Fig. 8. OVA-specific antibody and cell-mediated immune responses in mice immunized with OVA adjuvanted with CpG ODN 1826 and substituted indolicidin derivative peptides. C57BL/6 mice ($n = 6$) were immunized twice subcutaneously with 10 μg OVA formulated with 10 nmol peptide alone or combined with 10 μg CpG ODN. Fourteen days following secondary immunizations, OVA-specific mouse IgG1 (A) and IgG2a (B) titers were determined, and numbers of IFN-γ- (C) and IL-5- (D) secreting cells were measured by ELISPOT assay. ELISA titers are expressed as the reciprocal of the highest dilution resulting in a reading of two standard deviations above the negative control. ELISPOT results are expressed as the difference between the number of cytokine-secreting cells in OVA-stimulated wells and medium-control wells per 10^6 cells. Bars represent the median values of each group. $^*P < 0.05$; $^{**}P < 0.01$.

3.6. Effect of indolicidin and IN50 on OVA uptake by DCs

To elucidate the mechanism by which the IN50 derivative peptide enhanced antigen-specific immune responses *in vivo*, the effect of antigen uptake *in vitro* by DCs was examined. FITC-labelled OVA was combined with peptide and/or CpG ODN, and MFI of the cells was determined as a measure of OVA uptake. While neither CpG ODN or peptide alone influenced antigen uptake, CpG ODN and IN50 resulted in a 3-fold increase in OVA uptake as measured by MFI of the FITC labelled OVA, when compared to labelled OVA alone, suggesting that the adjuvant combination of CpG ODN and IN50 may enhance immune responses in part by facilitating antigen uptake by APCs (Fig. 9)

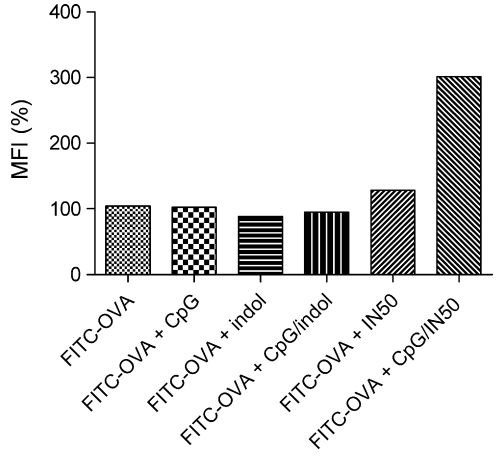

Fig. 9. Antigen uptake by DCs in the presence of CpG ODN and peptide. Day 5 BMDCs were incubated with FITC-labelled OVA alone or in the presence of 5 μg CpG ODN 1826 and/or 5 nmol of indolicidin or IN50 peptide for 30 min. Uptake of FITC-OVA was measured by FACS, and results are reported as mean fluorescence intensity (MFI) relative to untreated cells. Data shown are representative of three experiments.

4. Discussion

The discovery of key mechanisms of innate immunity has allowed the identification of specific targets for the induction of adaptive immunity [38], and has guided the discovery of novel adjuvant compounds. Previous reports have demonstrated that the combination of cationic peptide and synthetic ODN can induce type 1 antigen-specific immune responses [1]. Here, using the model antigen OVA, we have shown that the novel adjuvant combination of CpG ODN, indolicidin and polyphosphazene can enhance antigen-specific antibody and cell-mediated immune responses when compared to the peptide indolicidin and CpG ODN alone. Moreover, we have demonstrated that the indolicidin peptide can be engineered to further enhance its adjuvant activity when co-formulated with CpG ODN.

Prolonging the bioavailability and duration of action of immunomodulatory compounds, such as CpG ODN, may improve their therapeutic potential [39], and previous studies have shown that CpG ODN can form stable complexes with polycationic amino acids and peptides via electrostatic interactions [1,40,41]. These complexes led to the formation of an antigen-adjuvant depot at the site of injection and enhanced antigen-specific immune responses [41]. Here, we found that indolicidin and CpG ODN 1826 indeed formed complexes when mixed, and *in vivo* the combination significantly enhanced the antibody-mediated immune response, and producing a more balanced immune response when compared

to either of the adjuvants on their own. Ensuring that antigen and adjuvant components remain associated results in the generation of a homogeneous antigen-presenting and activated cell population [42]. To this end, a number of studies have shown that immune responses could be improved by chemically coupling CpG ODNs directly to the antigen [43] or incorporating them into liposome vesicles [39] or protein-loaded microparticles [44], suggesting that optimal immune stimulation occurs when antigen and immunomodulator are presented to the immune system in close proximity [10]. The ability of polyphosphazenes to non-covalently interact with proteins and nucleic acids suggests that they may similarly be capable of binding and presenting antigen and adjuvant components simultaneously. While the combination of CpG/indol enhanced the antigen-specific antibody-mediated immune response here, co-formulation of the two adjuvants with polyphosphazene was required for the induction of a more robust cell-mediated immune response, and increased numbers of IFN-γ-secreting spleen cells were observed when re-stimulated with the OVA protein, as well as both OVA-derived MHC class I- and II-restricted peptides.

Polyphosphazenes have been shown to form soluble protein-polymer complexes, and may stabilize and protect antigen [27], therefore inclusion of polyphosphazene in the vaccine formulation may have promoted the formation of complexes containing both antigen and adjuvant. Indeed, analysis by native PAGE revealed large molecular weight complexes in vaccine formulations that contained polyphosphazene and the cationic indolicidin peptide, consistent with the observation that polyphosphazenes may associate and form particles in the presence of multivalent cations [25]. Co-migration of CpG ODN and OVA, however, was only observed in the vaccine formulation consisting of OVA and CpG/indol/PP, suggesting that the three component adjuvant combination did in fact form complexes which may have facilitated the delivery of both antigen and adjuvants to APCs.

DCs are potent APCs, which are essential for the regulation of both innate and adaptive immune responses. In response to antigenic stimuli, immature DCs undergo activation and maturation, during which they release cytokines and chemokines, and migrate from peripheral tissues to the lymph nodes where they present antigen to naïve T cells [45]. Sparwasser et al. [46] previously demonstrated that CpG ODN could activate BMDCs to produce high levels of IL-12 and the pro-inflammatory cytokines IL-6 and TNF-α. These cytokines are important in the development of immune responses, as IL-12 and IFN-γ promote type 1 cytokine production and play important roles in the elimination of human pathogens, while IL-6 can facilitate the differentiation of T and B lymphocytes and stimulates antibody production [47–50]. The trend in the secretion of IL-12p70 and TNF-α by BMDCs in response to CpG/indol/PP correlates with our *in vivo* observations, most notably the increased secretion of IL-12p70, which has been shown to be instrumental in directing the development of T helper (Th) 1 cells that produce high amounts of IFN-γ [51], and suggests that CpG ODN, indolicidin and polyphosphazene can act in combination to enhance cytokine production by BMDCs.

The facilitation of antigen transport, uptake and presentation by APCs is important for the effectiveness of vaccines, and can be achieved by repeated or prolonged accessibility of antigen at the injection site and/or increased loading of APCs with antigen [41]. Chemokines, such as MCP-1, regulate trafficking of DCs and other lymphocytes into lymphoid organs [52], and the significant increase in MCP-1 induction in the presence of CpG ODN and indolicidin and its derivative peptides may, in part, explain the enhanced antibody production observed *in vivo*, but does not explain the enhancement in the cell-mediated immune response displayed in mice immunized with OVA + CpG/IN50. The efficient uptake of antigen by APCs is crucial for the induction of an effective immune response [2], and Fritz et al. [41] have shown that cationic peptides can enhance the delivery of antigen to APCs. Here, we observed that the combination of CpG/IN50 markedly enhanced the uptake of OVA by BMDCs. Recent evidence has shown that the human cathelicidin peptide, LL-37, could electrostatically bind DNA, promoting uptake by dendritic cells and resulting in prolonged exposure to TLR9 and activation of innate immune responses [53]. While indolicidin has a characteristic extended conformation, secondary structure prediction (NNPREDICT, available online at http://alexander.compbio.ucsf.edu/~nomi/nnpredict.html, D. Kneller, 1991) indicated that IN50 contained α-helical conformation, analogous to that of the well characterized human cathelicidin LL-37 [54]. Furthermore, studies using truncated LL-37 peptides have suggested that increasing the relative content of positively charged amino acids in the peptide may enhance their innate immune-modulating capacity, including an increased ability to suppress LPS-induced cytokine production by CD14+ cells [55] and increased chemotactic ability [56]. The increased net positive charge contributed by the substitution of proline residues with arginine in IN50 may further facilitate peptide-ODN interactions as well as enhance cellular uptake, as arginine-rich peptides have been found to efficiently translocate across biological membranes and enhance protein uptake [57,58]. These results would suggest that the IN50 peptide may be a promising new candidate for immunotherapeutic applications; however, additional studies will be required to further elucidate the mechanisms of immune enhancement by this novel peptide.

We have demonstrated here that, while the combination of CpG and indolicidin (CpG/indol) enhanced OVA-specific antibody titers when compared to either of these adjuvants alone, only CpG/indol/PP enhanced cytokine production by DCs *in vitro* and enhanced antigen-specific antibody-mediated and cell-mediated immune responses *in vivo*. Furthermore, we have shown that a substituted indolicidin derivative peptide may act as an even more potent immune enhancer, and when combined with CpG ODN, shows promise as novel immune-enhancing compounds to mediate and enhance antigen-specific immune responses.

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