Supporting Information

For

Enzymatic Stability and Immunoregulatory Efficacy of a Synthetic Indolicidin Analog with

Regular Enantiomeric Sequence

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Supporting Information Contents:

1. Background and Terminology

1.1. Vaccine adjuvants

A vaccine typically contains an agent dubbed antigen that resembles a disease-causing microorganism (or pathogen) against which adaptive immune responses are elicited. Vaccine adjuvants are conferred on the substance who helps to elicit a robust and effective antigen-specific immune response. They are added to many vaccines to increase their immunogenicity and efficacy:[1-3] they can make existing vaccines more effective; they can help make previously impossible vaccines a reality; they can shrink vaccine dosage as antigen-sparing strategies; they can improve the efficacy of vaccines in specific human populations; they can overcome antigenic competition; or they can alternate immunization routes. Broadly, they can be separated into two classes based on their principal mechanisms of action: immunomodulatory adjuvants and delivery systems. In contrast to the former which are thought to trigger the immune systems, the latter are generally particulate and mainly function as a depot to ensure the immunoavailability of antigen.[1]

1.2. Host defense peptides (HDPs)

HDPs, also termed antimicrobial peptides (AMPs), are essential components of the defense systems to protect the host from invading pathogens.[2,3] Typically, the HDPs function by disrupting the integrity of the cytoplasmic membrane of microorganisms.[2] To date, two HDP analogs, omiganan (derived from bovine indolicidin) and pexiganan (derived from frog magainin), have demonstrated efficacy in clinical trials in preventing microbial infections.[2] In addition to the antimicrobial activity, recent advances have revealed that HDPs are immunomodulatory against antigen-presenting cells of the innate immune system.[3] For example, β-defensins have been reported to mediate the recruitment of tissue dendritic cells (DCs) and T cells through interaction with CCR6 to the site of microbial invasion.[4] Furthermore, a submember of β-defensins, β-defensin 2, acts through TLR-4 to induce up-regulated expression of costimulatory molecules (CD24a, CD40), and DC maturation.[5] Human cathelicidin (often referred to as LL-37) is chemotactic for neutrophils, monocytes, and T cells but not for DCs.[6] These events, in turn, suggest that HDP may act on the cells of the innate immune system and may play important roles in immune surveillance against pathogens, self antigens, and tumor antigens.

2. Safety aspects and B-cell Immunity

2.1. *In vivo* biocompatibility summary

To investigate whether the high stability of LD-indolicidin can be harmful to the host, the tissue or organ integrities of the immunized mice was histopathologically examined, in addition, serum samples taken from the mice were colorimetrically analyzed for liver and kidney functions. Group of PBS was served as a negative control, while incomplete Freund's adjuvant (IFA, water-in-oil emulsion) as a positive control group for inflammation. No inflammation can be seen in the PBS group after injection (Figure S1A-i), while the recruitment of inflammatory cells to the injection site is observed in the LD-indolicidin group (Figure S1A-ii, filled arrow). Also cell necrosis and calcification is visible surround the injected mass (Figure S1A-ii, open arrow). These findings indicated that i.m. injection of LD-indolicidin induced more or less inflammatory infiltration at the injection site compared with the PBS-treated control group. In the IFA control group, however, inflammation is much more widespread with the influx of lots of polymorphonuclear cells (Figure S1A-iii, filled arrow), and severe necrosis of adipose tissue is observed at the local injection site (Figure S1A-iii, open arrow). There were no histological changes among the three groups in the integrity components of the non-injected muscles, brain, liver, kidney, stomach, intestines, and genital tract that were examined (Figure S2). These observations indicate that the inflammatory tissue response to LD-indolicidin was moderate and restricted at the local injected site. Since IFA is known to cause very extensive tissue inflammations, the adjuvant use of IFA is restricted for the research purposes in laboratory animals. LD-indolicidin on the other hand, causes only mild inflammatory reactions and is prospectively used as adjuvant in vaccines for human use. Furthermore, serum samples from the mice treated with LD-indolicidin were colorimetrically analyzed for liver and kidney functions. The data showed that the serum concentrations of the biochemical indicators such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine (CRE) were in normal range (AST: 70-400 IU/L; ALT: 25-100 IU/L; CRE: 0.2-0.6 mg/dL)[7] and were of no difference between the groups of PBS control and LD-indolicidin (Figure S1B). In summary, the presence of alterative D-form stereoisomer in the native-peptide sequence shows tolerance in mice, which is a feature of great interest for further *in vivo* applications.
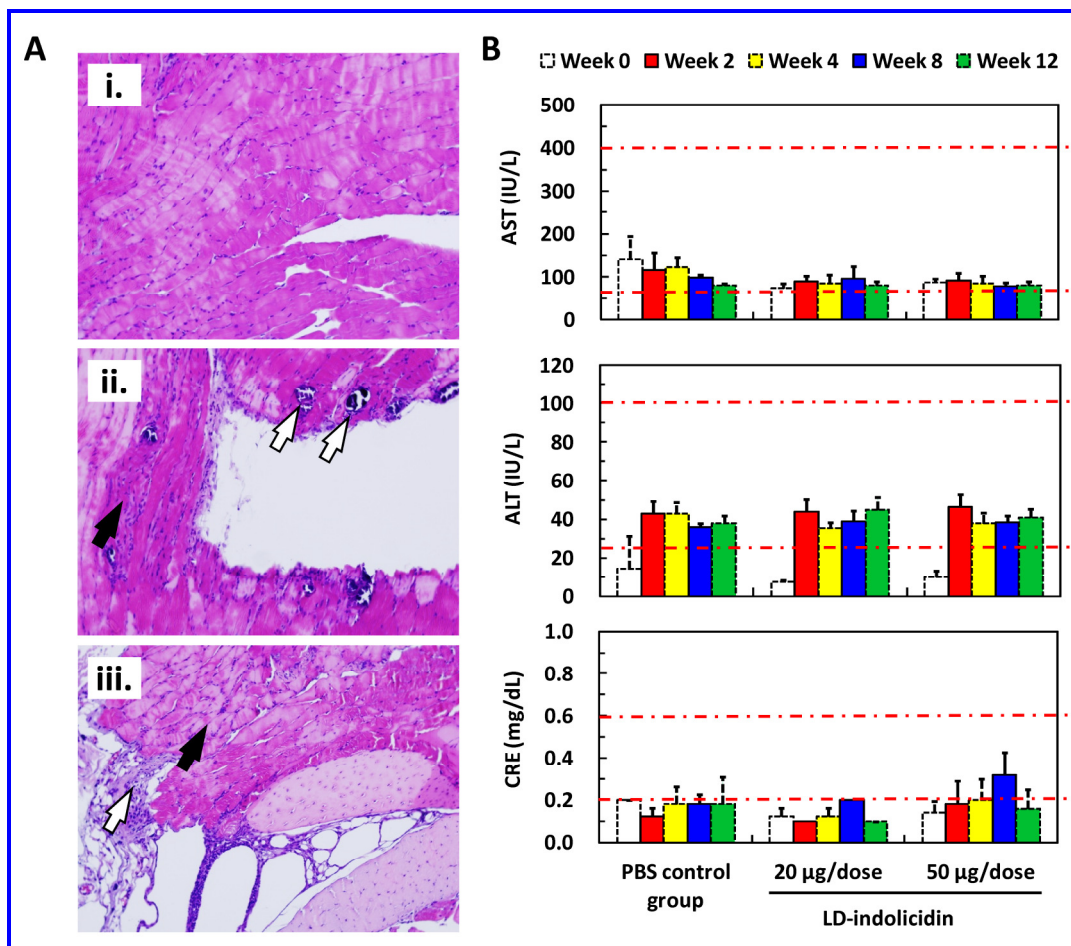
Figure S1. *In vivo* biocompatibility of LD-indolicidin. (A) Histopathological examination of tissue sections of the injection site following a single intramuscular (i.m.) injection of BALB/c mice with i): PBS; ii): LD-indolicidin; iii): IFA emulsion. Muscles at the injection sites were excised, fixed with 10% of formaldehyde overnight and embedded in paraffin. Sections of 4 μm were stained with H&E and examined using an Olympus DP70 microscope (x200 magnification). (B) Serum AST, ALT, and CRE levels of the mice treated with LD-indolicidin. BALB/c mice were injected once i.m. with 20 μg or 50 μg of LD-indolicidin. Serum samples were collected and the concentrations of the biochemical indicators were measured by colorimetric analysis. The data are presented as mean with standard deviation of five mice per group. The dotted horizontal lines represent normal ranges in mice.
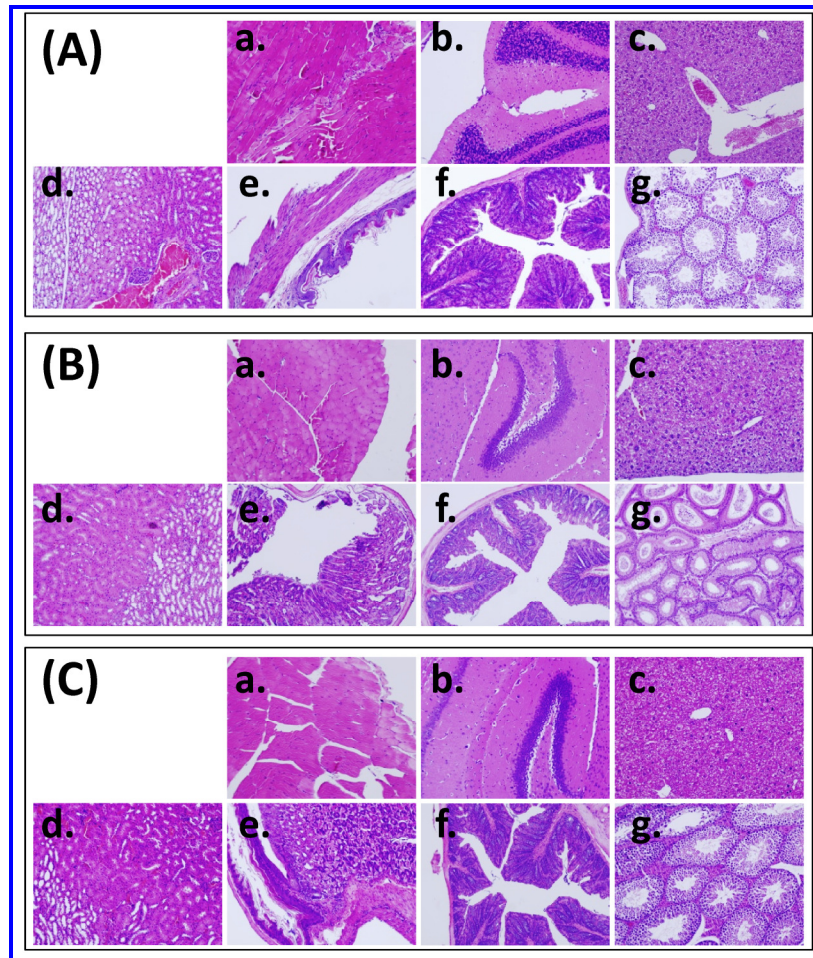
Figure S2. Histopathological examination of tissue sections of the organ integrities following a single intramuscular injection of BALB/c mice with (A) PBS, (B) LD-indolicidin, or (C) IFA emulsion. The organ integrities were excised, fixed with 10% of formaldehyde overnight and embedded in paraffin. Sections of 4 μm were stained with H&E and examined using an Olympus DP70 microscope (x200 magnification). a-g represent the tissues of non-injected muscles, brain, liver, kidney, stomach, intestines, and genital tract, respectively.

2.2. B-cell immunity summary

The antibody assays were performed by single-dose intramuscular vaccination in BALB/c mice with 0.5 µg HA of inactivated H5N1 virus, with or without adjuvant. Analysis of post-vaccination geometric mean titers (GMTs) is aimed at ruling out meaningful differences before and after vaccination. Figure S3 shows the antigen-specific IgG anti-virus titers, hemagglutination inhibition (HI) activity, and viral neutralizing (VN) ability elicited in mice following a single intramuscular dose of H5N1 inactivated virus vaccine. The antigen-specific antibodies, which were induced by the vaccine candidate formulated either with indolicidin or LD-indolicidin, were in the same level as those without formulation. In addition to GMTs, the licensure criteria of pandemic influenza vaccines set out by the US FDA and the European Union CHMP are based on HI activity, which include the mean geometric increase, the percentage of vaccinees who seroconverted, and the proportion of vaccinees achieving an HI antibody titer of 40 or more after vaccination (deemed to be the seroprotective threshold for seasonal vaccines).[8] Interestingly, we find that the seroprotection rate (mice achieving a post-vaccination titer higher than 40) never reached 100% when the mice immunized with inactivated virus alone; nevertheless, the merit of vaccination with peptide-formulated inactivated virus was revealed at the 8th week after administration: the seroprotection reached 100% after formulations.
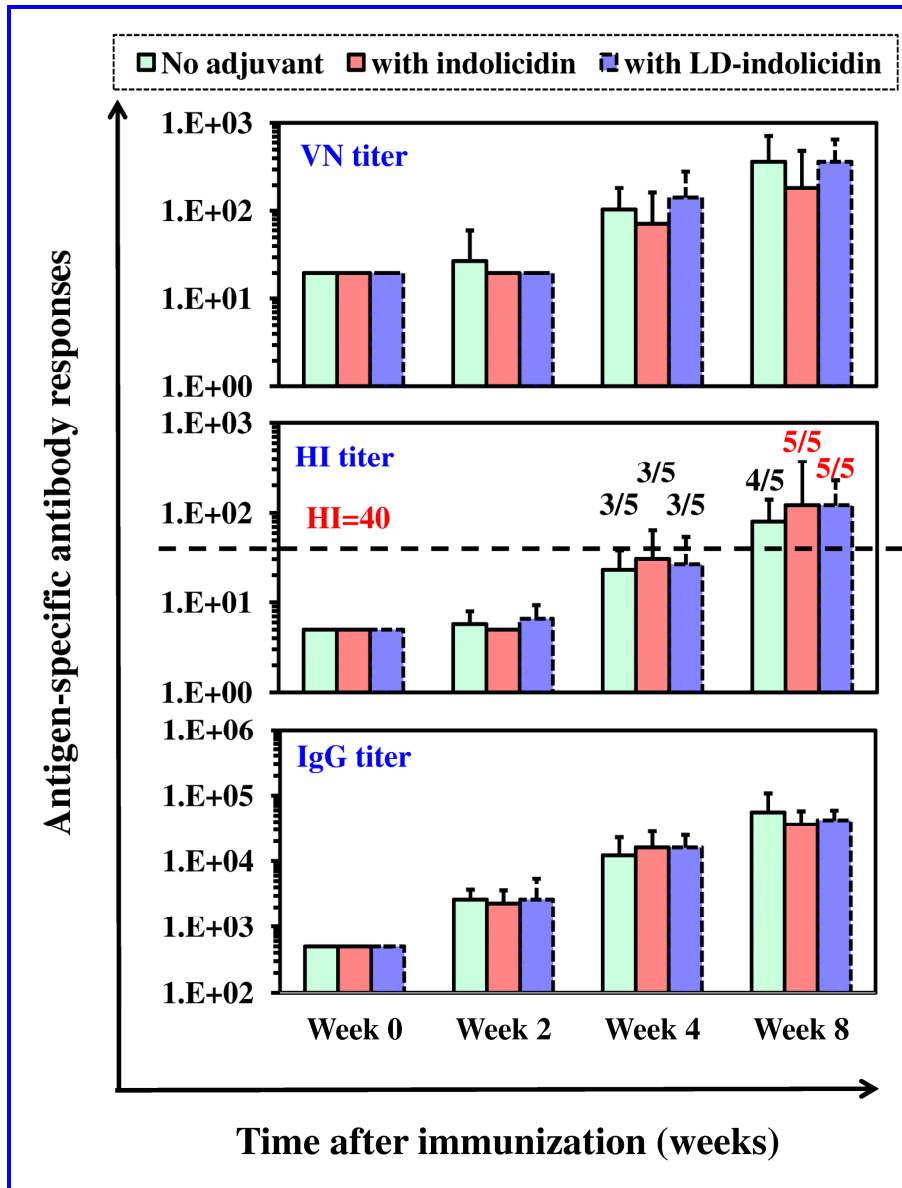
Figure S3. Antigen-specific antibody responses following immunization in mice with inactivated H5N1 virus alone or formulated with indolicidin or LD-indolicidin. BALB/c mice were vaccinated once i.m. with H5N1 inactivated virus 0.5 μg HA. Sera were collected and the antibody titers were determined by ELISA, HI titration, and VN assay. The data are presented as GMTs with standard deviation of five mice per group. The seroprotection was calculated from the proportion of mice achieving a post-vaccination HI titer ≥ 40.

3. Experimental Details

3.1. Peptide synthesis and characterization

Indolicidin and analog peptide were synthesized in-house by solid phase method using an automated peptide synthesizer; model PS-3 from Protein Technologies, Inc., employing the fluorenyl-methoxycarbonyl (Fmoc) group for α-amino group protection. The resin used is derived from NovaSyn TGR resin with the modified Rink linker (Merck, Frankfurter, Germany). Tryptophan and lysine residues were protected with the tert-butoxycarbonyl (tBoc) and arginine residues were protected with 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group. The final de-blocking step was carried out with cocktail (a mixture of TFA/Triisopropylsilane/water = 94:3:3). The crude peptides were recovered by precipitation method with diethyl ether as non-solvent and characterized by analytical reversed-phase HPLC. The purity less than 95% needs to be further purified by reversed-phase HPLC using gradient elution (Waters 600E Multisolvent Delivery System). Mass spectrometry data obtained from Agilent 1100 Series LC/MSD high performance ion trap mass spectrometers to ensure the target peptide is obtained.

3.2. Enzymatic degradation

Enzymatic degradation of indolicidin and LD-indolicidin by trypsin (PIERCE, Rockford) and α-chymotrypsin (Sigma, Steinheim, Germany) was carried out using 200 μg/mL of peptide and 4 μg/mL of enzyme (peptide-to-enzyme weight ratio of 50:1) in PBS at 37oC. Samples of 100 μL were taken after 5, 10, 30, 60, and 120 mins. The enzymatic reaction was terminated by addition of 2.5% (v/v) folic acid. Samples were then analyzed by LC/MSD Trap XCT (Agilent Technologies Inc., CA).

3.3. Mice and ethics statement

Pathogen-free female BALB/c mice (H-2d, 5-week old) were obtained from the National Laboratory

Animal Breeding and Research Center (Taipei, Taiwan) and acclimatized for at least one week at the NHRI animal facility prior to use. All experiments were conducted in accordance with the guidelines of Laboratory Animal Center of NHRI. The animal use protocols have been reviewed and approved by the NHRI Institutional Animal Care and Use Committee (NHRI-IACUC-099073-A).

3.4. *In vivo* biocompatibility

For histological examination, mice were injected intramuscularly (i.m.) in quadriceps with 200 μL of PBS alone (negative control group) or supplemented with 20 μg of LD-indolicidin or a water-in-oil emulsion of PBS in incomplete Freund's adjuvant (IFA, Sigma, Steinheim, Germany) at a PBS/IFA ratio of 1/1 v/v as a positive control group. 14 days after treatment, the tissue of the injection site was excised, sectioned, and stained with hematoxylin and eosin (H&E) by the Pathology Core Laboratory of NHRI for histological examination. Other organ integrities from non-injected skeletal muscles, brain, liver, kidney, stomach, intestines, and genital tract were then removed, stored in the same fixative at room temperature until use, and embedded in paraffin. For biochemistry test, mice were injected once i.m. with 20 μg or 50 μg of LD-indolicidin. Sera were taken from five mice per group at weeks 0, 2, 4, 8, 12 via the submandibular veins and were analyzed in the Laboratory Animal Center of NHRI for measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and creatinine (CRE).

3.5. Vaccine preparation

The vaccine used in this study was the formalin-inactivated whole-virus vaccine NIBRG-14 (kindly supplied by the UK National Institute of Biological Standard and Control, NIBSC), which was derived from a reassorted H5N1 vaccine strain containing modified hemagglutinin (HA) and neuraminidase (NA) from the highly pathogenic avian influenza strain A/Vietnam/1194/2004. The virus was propagated in Madine-Darby canine kidney (MDCK) cells in a serum-free medium microcarrier cell culture system.

Formalin inactivated vaccines were prepared with 0.1% formalin at 37°C for 24 hrs. The HA content was determined by single radial immunodiffusion.

3.6. Immunization and B-cell immunoassays

To investigate the B-cell immunity of indolicidin and analog peptide, the mice were vaccinated once intramuscularly (i.m.) with 0.5 µg HA of H5N1 inactivated virus administrated with or without adjuvant peptide (given as a 20 µg per dose). Following the injection, serum samples were collected from immunized mice via the submandibular veins and the antibody titers were determined by enzyme-linked immunosorbent assay (ELISA), hemagglutination inhibition (HI) titration, and viral neutralizing (VN) assay.

For ELISA immunoassay, the presence of NIBRG-14-specific antibodies in the sera was determined by ELISA. In brief, 100 µL of dilute inactivated virus (1 µg/mL) were coated in 96-well microtiter plates with 0.05 M carbonate buffer (pH 9.6, Sigma, St. Louis, MO) by overnight incubation at room temperature. Coated plates were washed once with PBS containing 0.05% Tween®20 (Sigma, St. Louis, MO) and then blocked with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) in PBS at room temperature for 2 hrs. Diluted sera (starting dilution 1:1000, serial two-fold serum dilutions) from immunized animals were applied to wells at room temperature for 2 hrs. Followed by HRP-conjugated goat anti-mouse IgG (ICN Cappel, Aurora, Ohio, 1:5,000), the assay was developed with substrate solution 2,2'-azino-di(3-ethyl-benzthiazoline-6-sulfonate (ABTS® Peroxidase, KPL, MD) for 20 min at room temperature. Plates were read at 450 nm using an ELISA plate reader (Thermo Multiskan® spectrophotometer, Vantaa, Finland). The titers were determined from the reciprocal of the final dilution that gave an optical of two-fold absorbance of pre-immune sera. For calculation purposes, an undetectable level was scored as a titer equal to 500.

For HI titration, the principle of the HI test is based on the ability of specific anti-influenza

antibodies to inhibit hemagglutination of turkey red blood cells (RBCs) by influenza virus HA. Non-specific inhibitors of agglutination were removed by heat treatment and addition of receptor-destroying enzyme. After pretreatment, serum samples (two-fold dilutions starting with an initial dilution of 1:10) were incubated with four HA units of influenza strain. Turkey RBCs were then added and the inhibition of agglutination was scored. The serum titer was expressed as the reciprocal of the highest dilution that showed complete inhibition of HA. For calculation purposes, an undetectable level was scored as a titer equal to 5.

For VN assay, the 200 $TCID_{50}$ per well of NIBRG-14 virus were incubated with two-fold-diluted mice sera at a starting dilution of 1:40. Mixtures of virus and serum were transferred to monolayers of MDCK cells and incubated at 37°C and 5% CO_2 for 4 days. The neutralizing titer was defined as the reciprocal of the highest serum dilution at which the infectivity of the H5N1 virus' 200 $TCID_{50}$ for MDCK cells was completely neutralized in 50% of the wells. Infectivity was identified by the presence of cytopathy on Day 4 and the titer was calculated using the Reed-Muench method. For calculation purposes, an undetectable level was scored as a titer equal to 20.

3.7. Immunization and T-cell immunoassays

To investigate T-cell immunity, BALB/c mice were primed intramuscularly (i.m.) with candidate vaccines, with or without adjuvant. At day 14, all mice were boosted i.m. with the same vaccine formulations. Seven days after the boost, the mouse spleen was removed aseptically and transferred to a tube containing 1 mL of culture medium (cRPMI) consisting of RPMI 1640 (HyClone, Logan, Utah, USA) containing 2 mM L-glutamin and supplemented with 25 mM HEPES (Gibco, Invitrogen, NY, USA), 0.05 mM 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 10% heat-inactivated foetal bovine serum (FBS, HyClone, Logan, Utah, USA) and 1% antibiotics. Cell suspensions were prepared by mashing the spleen through a cell strainer using a syringe plunger. The resultant suspension was collected in a 50-mL tube

and centrifuged at 1,000 rpm for 5 min. To remove erythrocytes, the cell pellet was resuspended in 5 mL of RBC lysis buffer (Biolegend, San Diego) and incubated at room temperature for 1 min. The reaction was then terminated in 20 mL of cRPMI and the mixture was centrifuged for 5 min. The pellet was then washed twice with cRPMI and resuspended in 5 mL cRPMI. After cell counting with a hemacytometer by the trypan blue dye exclusion, U-bottomed 96-well plates were seeded with 2×10^5 cells in cRPMI at a total volume of 200 µL per well. Cells were stimulated in triplicate in the presence or absence of 0.5 µg HA/mL of inactivated NIBRG-14 virus. Concanavalin A (5 µg/mL, Sigma, Saint Louis) was used as a positive control. Plates were then incubated for 4 days at 37°C and 5% CO_2 in air. Interferon (IFN)-γ- and interleukin (IL)-4-secreting cells were assessed by enzyme linked immunosorbent spot (ELISPOT) assays (eBioscience, San Diego, CA) of cell suspensions for 72 hrs of culture. The data are expressed as the mean plus the standard deviation of triplicate cultures. A two-tailed Student's *t*-test was performed, and results statistical significance ($p<0.05$) compared with no adjuvant group are marked with an asterisk. IFN-γ and IL-4 concentrations in supernatants were measured by ELISA using paired antibodies according to the manufacturer's instructions (DueSet® ELISA Development kit, R&D Systems, Inc., Minneapolis, MN).

References

(1) Huang, M. H.; Leng, C. H.; Liu, S. J.; Chen, H. W.; Sia, C.; Chong, P. Vaccine delivery systems based on amphiphilic bioresorbable polymers and their role in vaccine immunogenicity. In *Immunogenicity*; Villanueva, C. J., Ed.; Nova Science Publishers: New York, 2011; pp 61-90.

(2) Kruse, T.; Kristensen, H. H. Using antimicrobial host defense peptides as anti-infective and immunomodulatory agents. *Expert. Rev. Anti. Infect. Ther.* **2008**, 6, 887-895.

(3) Mutwiri, G.; Gerdts, V.; Lopez, M.; Babiuk, L. A. Innate immunity and new adjuvants. *Rev. Sci. Tech.* **2007**, 26, 147-156.

(4) Yang, D.; Chertov, O.; Bykovskaia, S. N.; Chen, Q.; Buffo, M. J.; Shogan, J.; Anderson, M. Schröder, J. M.; Wang, J. M.; Howard, O. M.; Oppenheim, J. J. Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **1999**, 286, 525-528.

(5) Biragyn, A.; Ruffini, P. A.; Leifer, C. A.; Klyushnenkova, E.; Shakhov, A.; Chertov, O.; Shirakawa, A. K.; Farber, J. M.; Segal, D. M.; Oppenheim, J. J.; Kwak, L. W. Toll-like receptor 4-dependent activation of dendritic cells by beta-defensin 2. *Science* **2002**, 298, 1025-1029.

(6) Yang, D.; Chertov, O.; Oppenheim, J. J. Participation of mammalian defensins and cathelicidins in anti-microbial immunity: receptors and activities of human defensins and cathelicidin (LL-37). *J. Leukoc. Biol.* **2001**, 69, 691-697.

(7) Hall, RL. Clinical Pathology of Laboratory Animals. In *Animal models in toxicology,* 2nd ed.; Gad, S.C., Ed.; CRC Press: New York, 2007; pp 787-830.

(8) Leroux-Roels, I.; Borkowski, A.; Vanwolleghem, T.; Drame, M.; Clement, F.; Hons, E.; Devaster, J. M.; Leroux-Roels, G. Antigen sparing and cross-reactive immunity with an adjuvanted rH5N1 prototype pandemic influenza vaccine: a randomised controlled trial. *Lancet* **2007**, 370, 580-589.