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**Characterization of the immunomodulatory property of a
palmitated form of the host defence peptide, Indolicidin**

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**CHARACTERIZATION OF THE IMMUNOMODULATORY
PROPERTY OF A PALMITATED FORM OF THE HOST
DEFENCE PEPTIDE, INDOLICIDIN**

by

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中文摘要

Cathelicidin 和 Defensin 是兩種主要的宿主防禦肽 (Host defence peptides, HDPs) 族群。Cathelicidin 的族群成員的序列長度約在十二到八十個氨基酸之間，因此具有不同的分子量大小。Indolicidin (Indo) 是 Cathelicidin 家族的成員之一，其序列中含有十三個氨基酸，序列為：ILPWKWPWWPWR。從序列中可以得知，Indo 本身富含了脯氨酸和色氨酸兩種氨基酸。如同其它的 Cathelicidin 成員，Indo 同樣也具有對抗微生物和免疫調節的功能。Indo 之所以讓我感到興趣並成為我的研究主題的原因，是因為它可以有效的刺激動物體內的免疫細胞，產生和發炎反應相關的一些細胞素，例如：TNF- α ，以進一步地活化免疫反應。

Tripalmitoyl-cysteinyl-seryl-(lysyl)₃-lysine, Pam₃CysSerLys₄ (P₃CSK₄)，是第二型類鐸受器的配體，由一段肽序列(CSK₄)接上三個棕櫚酸分子(Pamitic acid, Pam)所組成。從這樣的想法開始，或許利用棕櫚酸化的方式，將棕櫚酸接到 Indo 上，也能夠依循相同的機制，刺激第二型類鐸受器，以產生發炎相關的細胞素，最後活化免疫系統。

關於這類型的研究，目前還沒有被研究人員發表相關報導至文獻中。在我的研究當中，我成功的將一個棕櫚酸分子接到了Indo上，然後再透過「高效液相色譜儀」(High-performance liquid chromatography, HPLC) 和「液相層析質譜儀」(Liquid chromatography-mass spectrometry, LC-MS) 將 Indo 純化並定性。在動物實驗當中，福馬林去活化後的禽流感病毒 (Formalin-inactivated H5N1, FI-H5N1) 被當作是實驗中的測試抗原，並和利用化學方式合成好的 Indo 和 Pam-Indo 混合後，以肌肉注射的方式打入BALB/c (H-2^d) 老鼠當中以評估肽如何去影響測試抗原 (antigen, FI-H5N1) 所產生的免疫效應。

老鼠以不同的接種配方免疫之後，FI-H5N1-coated Enzyme Linked Immunosorbant Assay (FI-H5N1-coated ELISA) 被用來測量老鼠血清中，針對 FI-H5N1 的抗體濃度。實驗結果顯示，在打入 FI-H5N1 和 Pam-Indo 接種配方的老鼠組別當中，老鼠血清之中針對 FI-H5N1 的抗體表現量顯著地降低；然而，在打入了 FI-H5N1 和 Pam 或 Indo 的老鼠組別中，卻看不到同樣的抑制現象。

B 細胞要活化以防禦抗原，例如：H5N1，是需要「CD4⁺ 的輔助型T 細胞」的幫助才能達成。而CD4⁺的輔助型T細胞的活化，則需要和「抗原呈現細胞」(Antigen presenting cells, APCs) 產生交互作用才能達成。因此，了解 Pam-Indo 如何影響 APCs 就成為了下一個要被探討的重要問題。為了驗證這個概念，我將老鼠大腿骨中的骨髓細胞取出，在含有「巨噬細胞集落刺激因子」(Granulocyte macrophage colony stimulating factor, GM-CSF) 的培養液當中培養，約八到十天後，即可產生未成熟的骨髓樹突細胞。接下來將產生的骨髓樹突細胞加入含有脂多醣 (lipopolysaccharide, LPS) 和 Pam, Indo, Pam + Indo 或 Pam-Indo 的培養基中培養。實驗結果顯示，Pam-Indo 可以有效的抑制，被脂多醣刺激後的樹突細胞產生 CD40 和 CD86 分子。而這樣子的抑制現象，在含有 Pam, Indo 或 Pam + Indo 的組別當中並沒有看到。

綜合以上的實驗結果可以證明，Pam-Indo 可以有效促進耐受性未成熟樹突細胞的生成，進而抑制老鼠免疫系統針對FI-H5N1的抗體反應。

ABSTRACT

Cathelicidins and Defensins are the two main classes of host defence peptides (HDFs). Cathelicidins contain peptides of different molecular sizes, which can range from 12 amino acids to around 80 amino acids long. Indolicidin (abbreviated as Indo from here on) is a 13 amino acid member of the cathelicidins with the sequence, ILPWKWPWWPWRR. From the sequence, it can be seen that the peptide is rich in proline and tryptophan. Like many other cathelicidins, Indo also possesses anti-microbial as well as immunomodulatory properties. The special feature of the immunomodulatory property of Indolicidin is that it has been shown to stimulate cells to produce inflammatory cytokines.

Tripalmitoyl-cysteinyl-seryl-(lysyl)₃-lysine, P₃CSK₄ (Pam₃CysSerLys₄), is known to be a toll-like receptor-2 (TLR-2) ligand. So, pamilation of Indo may allow it to target TLRs to elicit inflammatory immune responses. These studies have not been reported in the literature. In this research project, I have prepared Pam-Indo by linking monopalmitic acid to Indo, purified it, and characterized it using mass spectrometry.

The potential immunomodulatory property of Pam-Indo was evaluated by testing how it could affect the generation of immune responses against formalin-inactivated H5N1 influenza virus (FI-H5N1) in BALB/c (H-2^d) mice. The results of the studies I have carried out showed that mice immunized with Pam-Indo generated much lower level of antibodies against FI-H5N1 as compared to mice of the same age administered with FI-H5N1, or FI-H5N1 formulated in Pam (monopalmitic acid), Indo, or Pam + Indo.

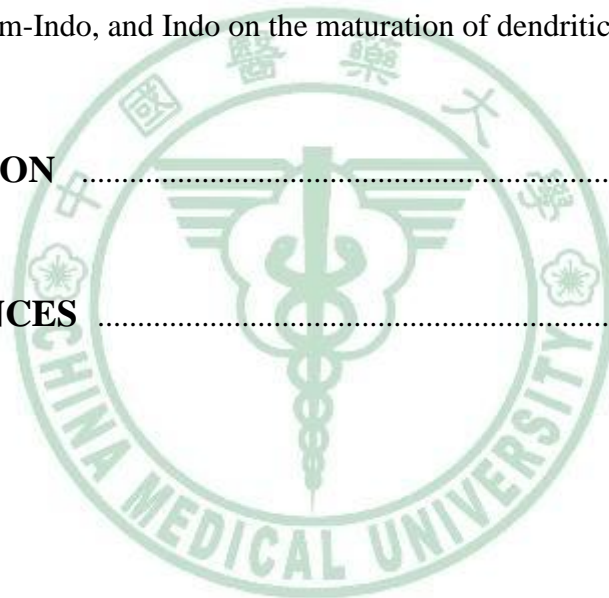
FI-H5N1-coated Enzyme Linked Immunosorbant Assay (ELISA) was used to detect the serum anti-FI-H5N1 antibodies in these mice. B cell response to an antigen (FI-H5N1) requires immunological help from CD4⁺ T cells, and how effective FI-H5N1-specific CD4⁺ T cells are generated in turn relies on how well antigen-presenting cells (APCs) present the H5N1 antigen to them. I, therefore, investigated how Pam-Indo affects APCs. To do this, I had cultured bone marrow monocytes in the presence of mouse (granulocyte macrophage colony stimulation factor, GM-CSF) to convert them to become immature myeloid dendritic cells (imDCs). Pam-Indo was found to suppress maturation of DC following stimulation with lipopolysaccharide (LPS). Expression of the CD86 and CD40 costimulatory molecules are downregulated in LPS-treated imDCs in the presence of Pam-Indo.

This effect was not seen in LPS-stimulated DCs exposed to Indo or, Pam. The results suggest Pam-Indo mediates the generation of tolerogenic imDCs to suppress the induction of antibody responses against FI-H5N1.

TABLE OF CONTENTS

ACKNOWLEDGEMENT	I
ABSTRACT IN CHINESE	III
ABSTRACT	V
TABLE OF CONTENTS	VII
LIST OF FIGURES	IX
LIST OF ABBREVIATIONS	X
1. INTRODUCTION AND OBJECTIVES	1
1.1 Host defence peptides	1
1.2 The mammalian immune system	2
1.3 Toll-like receptors	4
1.4 Modulation of Immune response	5
1.5 Study objectives	6
2. MATERIALS AND METHODS	7
2.1 Preparation of formalin-inactivated influenza H5N1 virus	7
2.2 Indolicidin, and palmitated indolicidin synthesis, purification and characterization	7
2.3 Mice immunization	10
2.4 FI-H5N1-coated direct Enzyme Linked Immunosorbent Assay (ELISA)	10
2.5 ELISPOT assay	11

2.6	Preparation of bone marrow cell derived dendritic cells, and surface staining studies	13
2.7	Statistical analysis	14
3.	RESULTS	15
3.1	Characterization of the synthesized Indo and Pam-Indo	15
3.2	Immunomodulatory property studies of Pam-Indo, and Indo in BALB/c (H-2 ^d) mice	15
3.3	Effect of Pam-Indo, and Indo on the maturation of dendritic cells	17
4.	DISCUSSION	18
5.	REFERENCES	31



LIST OF FIGURES

Figure 1. Secondary structures of cathelicidins.	21
Figure 2. Cells of the innate and adaptive immune compartment are derived from hematopoiesis of a pluripotent stem cell	22
Figure 3. TLRs are expressed in different cell types of the innate immune compartments	23
Figure 4. Interaction between an DC (an APC) and a CD4 ⁺ T cell	24
Figure 5. Reverse-phase HPLC and LC/MS analysis of Indolicidin	25
Figure 6. Reverse-phase HPLC and LC/MS analysis of Pam-Indo	26
Figure 7. Anti-H5N1 antibody responses generated in BALB/c (H-2 ^d) mice immunized with Pam-Indo, or Indolicidin formulated in Pam	27
Figure 8. ELISPOT assay of IL-4- and IFN- γ -secreting T cells in the spleens of individual BALB/c (H-2 ^d) mice immunized with Pam-Indo, or Indolicidin formulated in Pam	28
Figure 9A. Effect of Pam-Indo on CD40 expression in LPS-stimulated murine BM cell derived imDCs	29
Figure 9B. Effect of Pam-Indo on CD86 expression in LPS-stimulated murine BM cell derived imDCs	30

LIST OF ABBREVIATIONS

APC : antigen presenting cells

ANC : acetone

DIC : N,N'-diisopropylcarbodiimide

DMF : dimethylformamide

Fmoc : 9H-fluoren-9-ylmethoxycarbonyl

GM-CSF : granulocyte macrophage colony stimulation factor

HBTU : O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate

HDP : host defence peptides

HPLC : high pressure liquid chromatography

HOBT : hydroxybenzotriazole

Indo : Indolicidin

LC/MS : liquid chromatography-mass spectrometric analysis

NMM : N-methyl morpholine

Pam : palmitic acid

PMNs : polymorphonuclear leukocytes

SPPS : solid phase peptide synthesis

TFA : trifluoroacetic acid

TIS : trisopropylsilane

TLRs : toll-like receptors

1. INTRODUCTION AND STUDY OBJECTIVES

1.1 Host defence peptides

During infection, lymphoid cells of the mammalian immune system produce compounds called host defence peptides (HDPs) to provide the first line of protection. For this reason, HDPs are also called antimicrobial peptides. Most HDPs are cationic in nature. For this reason, HDFs are also called cationic antimicrobial peptides. HDFs differ greatly in their molecular sizes, and therefore adopt different conformations. The four secondary structures they could adopt are: amphiphilic α helix, β -pleated sheet that is usually stabilized by more than two disulphide bonds, a loop for a HDF bearing two cysteine residues, or extended (Figure 1). The three classes of HDPs known are the defensins, cathelicidins, and histatins. The former two classes have been most studied. For defensins, individual members are characterized by its high content of positively-charged amino acids, and a six-cysteine motif. Mammalian defensins are comprised of α and β defensins. α defensins have been shown to have broad antimicrobial activity against Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses. β defensins, on the other hand, are active against Gram-negative bacteria and yeast [1]. Since my project is on Indolicidin, which is a member of cathelicidins, I will turn to review this area of the literature

Cathelicidins are comprised of many types of peptides with varying molecular sizes that can be between 12 – 80 amino acids long [2]. Due to their large differences in sizes, HDFs can adopt different secondary structures (Figure 1). Indolicidin is a member of cathelicidins isolated from bovine neutrophils. The amino acid composition of Indolicidin reveals it contains 39% tryptophan and 23% proline. They are, therefore, rich in these residues [3]. For its small size, Indolicidin adopts an

extended conformation [4]. The exact mechanism associated with the antimicrobial activity of Indolicidin is still unknown, except the peptide has been suggested to be able to penetrate into the lipid bilayers of cell membranes of many organisms and cell types [5-7]. In any case, under experimental conditions, Indolicidin has been shown to be able to kill bacteria [3], fungi [7], protozoa [8], and HIV-1 [9].

Immunomodulatory property is the other property of Indolicidin. One *in vitro* studies found that human 16HBE4o⁻ bronchial epithelial cells cultured in the presence of Indolicidin produce the proinflammatory cytokine, interleukin 8 (IL-8). However, Indolicidin would also significantly inhibit LPS-stimulated human 16HBE4o⁻ bronchial epithelial cells to release TNF- α [1]. Bronchial (airway) epithelium cells would first encounter microbes entering through the respiratory system. Their responses to the agent through cytokines they secret could influence the generation of innate and adaptive immune responses against the agent [10]. These mechanisms have been suggested to prevent severe lung infections from occurring despite microbes continue to enter through our airways [11-12]. Besides these studies, Indo has not been tested to see how it may affect cells of both the innate and adaptive immune compartments.

1.2 The mammalian immune system

To understand how Indo may immunomodulate cells in the innate and adaptive immune compartments, it is important to know the immune system. The cells of the immune system of an adult mammal are mainly from the cells present in the bone marrow. In the bone marrow, these cell types undergo cellular differentiation process called hematopoiesis to become cells of the immune system (Figure 2). The innate immune compartment contains leucocytes with cytoplasmic granules. The main

leucocytes are the polymorphonuclear leukocytes (PMNs), and they produce the HDPs in responses to microorganisms. The minor groups of leucocytes are the eosinophils and basophils. Tissue macrophages, and blood monocytes are also cell types present in the innate immune system.

The adaptive immune system contains the thymus- and bone marrow-derived lymphocytes. They are therefore called T and B cells, and they are agranular. These cells are found in the blood. The way cells of the innate and adaptive immune system respond to invading microorganisms are very different. The neutrophils and macrophages/monocytes are phagocytic cells. They engulf the microbes, and degrade them using their intracellular enzymes. B cells express surface immunoglobulins (abbreviated as Igs). They become activated when their membrane-bound Igs recognized the antigen conformation of the microorganisms. T lymphocytes do not recognize the organisms directly. They need to interact with antigen presenting cells (APCs) that have captured (by phagocytosis, or through recognition by TLRs) the microorganisms. Microorganisms are degraded (processed) inside the APCs, and the peptides generated from the microbial proteins would associate with the Major Histocompatibility Complex (MHC) class 1 and class 2 molecules on the APCs, and be recognized by the T cell receptors (TCRs) of the $CD8^+$ and $CD4^+$ T cells, respectively. Whether $CD8^+$ and $CD4^+$ T cells can be activated, or how well they can become activated would depend very much on the nature of the antigen presented by the APCs. The antigens of the microbe may be processed in such a way that peptides are presented by the APCs to generate effective immune responses. But for some reason, these events may not happen so that APCs after microorganism captured are rendered ineffective to present antigens to the $CD8^+$ or $CD4^+$ T cells, or both. Under this condition, APCs are said to be tolerogenic.

1.3 Toll-like receptors (TLRs)

TLRs belong to a class of surface receptors expressed on APCs. They are also called pattern recognition receptors (PRRs) in the literature to imply they recognize molecular patterns of pathogens (ie. PAMPs, which stands for pathogen-associated molecular patterns of the pathogens). In human and mice, at least ten different types of TLRs have been discovered, and they are shown in Figure 3. Common structural features are found in these TLRs. They all contain a leucine-rich repeat extracellular domain that binds the ligands. Their transmembrane region is short; and the cytoplasmic domain is well conserved among the different TLRs. Most of the TLRs in their monomeric forms could recognize their ligands. But others will form heterodimers with other TLR monomers before they can recognize the ligands. For example, TLR2 heterodimerizes with TLR4, or it can form a heterodimer with TLR6 to recognize similar but non-identical ligands. So, diacetylated lipopeptide is recognized by TLR2/TLR4 heterodimer, and triacetylated lipopeptide binds to the TLR2/TLR6 heterodimer. Ligand binding to TLR leads to signal transduction. In this process, proteins called adaptor molecules are recruited. Different adaptor molecules can be recruited depending on how the TLR is activated when ligand binds to it. So, adaptor molecules called Myeloid differentiation primary response gene (88) (MYD88) is recruited when TLRs except TLR2 is activated. MyD88 in turn recruits other messengers to amplify the signal. NF κ B is finally activated to trigger the transcription of the interleukin-1 (IL-1) gene [13]. Another adapter molecule called TIR domain-containing adaptor protein (TIRAP) is recruited when TLR2/TLR4 is activated to turn-on the transcription of the IL-6 gene [14]. The cytokines produced by APCs through TLR activation can regulate lymphocytes of the adaptive immune system.

1.4 Modulation of Immune response

Generation of immune responses to antigens can be modulated, and adjuvants are compounds that are well mentioned in the literature to be able to do this. In Latin, adjuvant means to “help”. So, the addition of an adjuvant to a vaccine preparation is to help the antigen component in the vaccine to produce a stronger immune response. The antigen part of the vaccine is a component of a microbe, or the whole microbe which has been inactivated by formalin treatment for example. How strong the immune responses are generated depend on how the antigen is processed to produce the antigenic peptides that can associate with Major Histocompatibility Complex (MHC) class 1 and 2 molecules by antigen presenting cells (APCs). The peptide/MHC class 1, and peptide/MHC class 2 complexes are then presented to the T cell receptors (TCRs) expressed on CD8⁺ and CD4⁺ T cells, respectively. How well antigens are taken up by antigen presenting cells, and the way adjuvant affect this process can affect the antigen presenting properties of the APCs, myeloid dendritic cells (mDCs), for example can be made immunogenic, or tolerogenic. Immunogenic mDCs are characterized by their ability to present the processed antigens they captured in the form of antigenic peptides in association with the MHC class 1 and class 2 molecules to CD8⁺ and CD4⁺ T cells to convert them into effector cells. In contrast, tolerogenic mDCs are unable to do so. One difference between immunogenic and tolerogenic mDCs is the level of costimulatory molecules, such as CD40, and CD80/86 they express. Costimulatory molecules of mDCs have to interact with the CD28 molecules to induce the activation of CD8⁺ and CD4⁺ T cells.

For a preventive vaccine to work well, it needs to generate immunogenic mDCs so that strong CD8⁺ and/or CD4⁺ T cell memory can be produced. This way, the vaccinated individuals when infected by the same pathogen will be able to produce effective protective immune responses against the evading pathogen.

The well known information about adjuvants can enhance immune responses has attracted a lot of interest to develop new adjuvants, and studying how they work. Up to now, only very few adjuvants have been approved by the Federal Drug Administration (FDA) to be used in human vaccines. Alum is the most common one, and it has been used since 1930s. More recently, an adjuvant called MF59TM, which is a detergent stabilized in oil-in-water emulsion has been approved to formulate influenza virus vaccine for the elderly [15]. Also, another adjuvant called AS04, which is a combination of alum and Monophosphoryl Lipid A (MPL), has been approved to formulate hepatitis B, and HPV vaccines [16-17].

1.5 Study objective

In my research project, I have investigated the immunomodulatory property of a mono-palmitated form of Indolicidin (Pam-Indo). Studies of this compound has not been reported. The moiety chosen to be linked to Indolicidin is monopalmitic acid (CH₃(CH₂)₁₄COOH, abbreviated as Pam). Previous reports showed that the tri-palmitated peptide Pam₃CSK₄ is known to be a ligand of TLR-2. *In vitro* studies have shown that Pam₃CSK₄ binds to TLR-2, and triggers protein kinase 1/2 (ERK1/2) signal transduction to drive murine dendritic cells to release multiple cytokines, including IL-1, IL-4, IL-6, and IL-7 [18]. So, the overall immunomodulatory effect produced by Pam-Indo may reflect the responses generated by the APCs against Pam and Indolicidin.

2 MATERIALS AND METHODS

2.1 Preparation of formalin-inactivated influenza H5N1 virus

The experimental antigen used in this study project is formalin-inactivated influenza H5N1 virus (FI-H5N1). This antigen is provided by Dr. Alan Hu using a method he has developed for the production of the FI-H5N1 vaccine [19], which is now in phase 1 clinical trial in Taiwan.

2.2 Indolicidin, and palmitated indolicidin synthesis, purification and characterization

Indolicidin expressed in bovine neutrophils has the amino acid sequence, ILPWKWPWWPWR. This peptide was synthesized at 0.1-mmol scale using the Fmoc-based Solid phase peptide synthesis (SPPS) procedure described by Merrifield [20] using the PS3 synthesizer (Protein technologies, USA). In this method, Indolicidin was synthesized from the carboxyl-terminus. All the amino acids used to synthesize the peptide were purchased from Novabiochem Inc. (Darmstadt, Germany). The first reaction in the synthesis was to deprotect, ie. removed the Fmoc (9H-fluoren-9-ylmethoxycarbonyl) group from the first arginine, Fmoc- Arg(Pbf), attached onto the NovaTGF resin purchased from Novabiochem (Darmstadt, Germany). This was done by treatment with a 4.5% N-methyl morpholine (NMM) in distilled dimethylformamide (DMF, Sigma-Aldrich, Missouri, USA) solution for 30 min. The second amino acid was activated by O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) so that it could be coupled to the deprotected arginine. Coupling was done with 20% piperidine (Sigma-Aldrich,

Missouri, USA) in DMF for 1.5 hr. In the next coupling step, the Fmoc group attached to the second arginine was again deprotected, and the third amino acid, a tryptophan, was activated to allow the synthesis to continue. These steps were repeated, and double coupling was performed at the Arg, Trp, and Pro positions were attached residues to optimize chain elongation. When synthesis was completed, the last amino acid, which was Isoleucine (I) was deprotected. The peptide was cleaved from the resin by a cleavage cocktail trifluoroacetic acid (TFA)/ trisopropylsilane (TIS) purchased from Sigma-Aldrich (Missouri, USA) in water in a ratio of 94:3:3 (v/v) for 2 hours at RT. Crude Indolicidin was then mixed with excess volume of ice-cold ether (Sigma-Aldrich, Missouri, USA) to get it precipitated out from solution. Indo precipitate was collected on the filter of a G4 buchner funnel (ATG, Tokyo , Japan). The filter with Indo precipitate on it was placed in a lyophilizer to dry. Dried Indo was then transferred to clean glass vial and stored in a -20°C freezer.

Indolicidin synthesized was subjected to reverse-phase HPLC (High Pressure Liquid Chromatography) analysis to check for its purity. This was done by loading 1000 µL of the Indolicidin in the solution of H₂O /acetone (1:1, v/v) (ACN, Sigma-Aldrich, Missouri, USA) was injected to load the peptide onto a preparative C18 reverse phase column (Waters, Massachusetts, USA, 10×100 mm, 5-µm particle size). Elution of Indolicidin was done by applying a solution of 0–100% water/ACN gradient in the presence of 0.1% TFA into the column at a flow rate of 1 mL per min over a period of 40 mins. Elution profile was monitored by absorbance measurement at 206-214 nm. Indolicidin eluted as a single peak was collected, lyophilized in a freeze-dryer (FreeZone Plus 4.5, Labconco, USA).

To check if Indolicidin made was authentic, the peptide was dissolved in H₂O / ACN (1:1, v/v), and analyzed using a LC/MS liquid chromatography-mass spectrometer (Agilent, 6410 serials, USA).

Preparation of Pam-Indo involves synthesis of another batch of Indolicidin. For this batch, three additional amino acids were coupled to the Indolicidin chain. These residues were KSS. So, the sequence of the final product intended to be synthesized was KSS-ILPWKWPWWPWRR. To allow the palmitoyl to couple to the K residue of this peptide, the Fmoc- Lys(Pbf) was deprotected from the the K residue, and palmitoyl was activated by Hydroxybenzotriazole (HOBt). In the coupling step, N,N'-diisopropylcarbodiimide (DIC) in DMF was used as coupling reagent to get monopalmitic acid to link to the deprotected K residue. Coupling procedure was repeated twice to increase the efficacy of coupling during the synthesis procedure. Palmitated Indolicidin (Pam-Indo) was finally cleaved with the cleavage cocktail, trifluoroacetic acid (TFA)/ trisopropylsilane(TIS) in water (94:3:3 ratio, v/v), and lyophilized. Dried Pam-Indo was reconstituted in H₂O/ACN (1:1), and analyzed using reverse-phase HPLC, and subjected to LC/MS mass spectrometric analysis as described for Indolicidin,.

2.3 Mice and immunization

Seven-week-old female BALB/c (H-2^d) mice were purchased from the National Laboratory Animal Breeding and Research Center (Taiwan). Before used in experiments, mice were maintained for 7-10 days in the animal care facility certified by our Institute's animal care and use committee before they were immunized. For immunization, mice were divided into groups of 6 each, and individually administered intramuscular with one of the following inoculums: FI-H5N1, FI-H5N1 formulated in Pam-Indo, FI-H5N1 formulated in Indo, and FI-H5N1 formulated in Pam. Each of the animals was boosted three weeks later with their priming inoculums. Blood samples were collected by tail-bleeding, and spleens were removed from these mice 14 days post boost to determine the humoral and cellular responses they have generated.

2.4 FI-H5N1-coated direct Enzyme Linked Immunosorbent Assay (ELISA)

FI-H5N1-coated ELISA was used to detect the presence of anti-H5N1 antibodies in the immune sera collected from the individual experimental mice. To carry out the assay, individual wells of a 96-well Maxisorp ELISA plate (Nunc, Denmark) was coated with 1 µg HA equivalent of the FI-H5N1 preparation in a pH 9.6 coating buffer, (Sigma). The ELISA plates were incubated at 4°C overnight. Each well of the assay plate was washed three times, each time with 200.0 µL per well of the wash buffer [PBS, pH 7.2 (Gibco), containing 0.05% Tween 20 (Sigma)]. 200.0 µL of blocking buffer (5.0% skim milk prepared in PBS, pH 7.2) was then added to each experimental wells to inhibit non-specific binding. After 2 hr of blocking at room temperature, the assay wells were washed three times, each time with 250.0 µL of the wash buffer using an ELISA washer (ELx405, BioTek, USA). 100.0 µL of varying

dilutions of the individual immune sera were added to each of the FI-H5N1-coated well. Each serum dilution was tested in duplicates. The ELISA plate was left at room temperature for 2 hours to allow binding of the antibodies (if they are present in serum) to the immobilized influenza virus antigens. The experimental wells were then washed 4 times in wash buffer, before 100.0 μ L of a HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, Pennsylvania, USA) diluted 1 in 10,000 in assay buffer was added for detection. The wells were washed five times to remove the unbound antibodies 1 hr later. The plate was then patted dry on multi-layers of paper towel, and 50.0 μ L of TMB peroxidase substrate (SureBlue™, KPL, Gaithersburg, USA) was added to the individual assay wells. The plate was left in a closed drawer for 20 min to allow colour development to occur. The reaction was stopped by the addition of 50.0 μ L of 1.0 N HCl to each well. Absorbance readings representing specific binding of antibodies to the coated viral antigens were measured at OD 450 nm with an ELISA reader (Spectra Max M2 model, USA).

2.5 ELISPOT assay

ELISPOT assays were performed to determine the frequencies of interferon-gamma (IFN- γ)- and interleukin-4 (IL-4)-producing H5N1-specific CD4⁺ cells in the spleens of the experimental mice. The assays were done by first coating individual wells of a Multiscreen HTS™ IP plate (Millipore) with 10.0 μ g of an anti-mouse IFN- γ (clone AN-18), or 10.0 μ g of an anti-mouse IL-4 (clone 11B11, eBioscience), all purchased from eBioscience (San diego, USA), prepared in PBS, pH 7.2 (Gibco/ Invitrogen, USA). After overnight incubation at 4°C, each of the assay wells were washed three times, each time with 200 μ L of serum-free RPMI 1640 (Gibco/ Invitrogen, USA), and blocked by pipetting 200 μ L of Culture medium (CM) into it. CM is RPMI-1640

containing 10% heat-inactivated FBS (Gibco/ Invitrogen, USA), 2 mM L-glutamine, 25 mM HEPES (Gibco/ Invitrogen, USA), 0.05 mM 2-mercaptoethanol and 1% Penicillin / Streptomycin (Gibco/ Invitrogen, USA). The plates were washed three times 2 hr later, each time with 200.0 μ L of serum-free RPMI 1640. 100.0 μ L of the splenocyte suspension (prepared as described in the next subsection) were then aliquoted into each of the assay wells. 100.0 μ L of CM containing 1 μ g FI-H5N1 (HA equivalent) were then added to the individual splenocyte cultures. 100.0 μ L of CM with no FI-H5N1 were added to separate cultures as negative controls. The plates were incubated in a 37°C incubator equilibrated with 5.0% CO₂ for 3 days. Cells were then dumped off, and the reagents together with the procedures provided by eBioscience (California, USA) were used and undertaken to detect the spots that represent the presence of cytokine-producing cells on the PVDF membrane. These steps involved washing the assay wells 3 times, each time with PBS/0.05% Tween 20. A 100.0 μ L solution containing 5.0 μ g per mL of biotinylated secondary anti-IFN- γ (clone R4-62A) or anti-IL-4 antibodies (clone BVD6-24G2) both purchased from eBioscience (California, USA) prepared in assay buffer (PBS, pH 7.2 containing 10% FBS), was added to each well. The plates were incubated at room temperature for 2 hr. The washing steps were repeated, before 100.0 μ L of avidin-horseradish peroxidase enzyme (Cat.No. 00-4100-94, eBioscience, USA) at 1 in 250 dilution (prepared in assay buffer) was added to each assay wells. The plates are washed again three times with PBS/0.05% Tween 20 followed by three times more with PBS alone. One hundred microliter of the aminoethylcarbazole staining solution (AEC, Sigma Aldrich, USA) are added to each well to develop the spots. The reaction was stopped after 20min by flooding the wells under running tap water. The spots are counted by CTL-ImmunoSpot analyzer® (CTL, Ohio, USA) with the aid of the Immunospot software version 3.0 (provided by the manufacturer).

2.6 Bone marrow cell culture to generation of immature myeloid dendritic cells, and surface staining studies

The cultures were set up to evaluate whether Pam-Indo may immunomodulate the *in vitro* differentiation of murine bone marrow cells (BMCs) into immature myeloid dendritic cells (imDCs) and the maturation of imDCs that were generated. BMCs were obtained by flushing the femora and tibiae bone of adult BALB/c (H-2^d) with serum-free RPMI 1640 (Gibco/ Invitrogen, USA) using a 26-gauge needle (Terumo, Tokyo, Japan). BMCs collected in a 50.0 mL sterile centrifuge tube (Corning, Massachusetts, USA) were washed once with RPMI 1640 by centrifugation at 1,000 rpm for 10 min. The cell pellet was resuspended at 2×10^7 per mL in CM [RPMI 1640 supplemented with 10.0% fetal bovine serum (FBS, Gibco/Invitrogen, USA), and 1x penicillin /streptomycin mix]. 1.0 mL of the BMC suspension was then seeded into individual wells of a 24-well tissue culture plate (Corning, Massachusetts, USA). The BMCs were allowed to be converted to imDCs according to the protocol described by Lutz et al [21] (This was done by adding 0.5 mL of CM containing 20.0 ng of mouse granulocyte macrophage colony stimulating factor (GM-CSF, Biovision, California, USA) with/without 5 to 40 µg of Pam-Indo or Indo to the individual BMC cultures. Cultures were kept in a 37 °C incubator equilibrated with 5.0 % (v/v) CO₂. On days 3 and 7 after culture set up, 0.5 mL of the culture medium was carefully removed, and the same amount of fresh CM supplemented with GM-CSF was added back. Cells were collected on day 10. A portion was phenotypically characterized for its expression of costimulatory the CD40 and CD86 molecules. The remaining cells collected from the cultures set up without Pam-Indo or Indo added were stimulated with 5.0 µg of LPS for 24 hr in the presence/absence of varying concentration of

Pam-Indo and Indo, respectively. The cells were then again stained for their surface expression of CD40 and CD86 molecules.

2.7 Statistical analysis

The unpaired Student's *t*-test was used to determine if the differences in specific responses observed between groups of mice were significant. A value of $p < 0.05$ was considered significant.



3. RESULTS

3.1 Characterization of the synthesized Indo and Pam-Indo

Indo synthesized using the FMOC chemistry was purified by reverse phase HPLC on a C18 column. Figure 5A showed that Indolicidin was eluted as a single peak at 18.8 min. These results showed that synthesis of Indo had proceeded smoothly, and a highly purified grade of the peptide was obtained. Indo was found to have a mass of 1908 Da when it was analyzed in a LC/MS mass spectrometric (Fig 5B). This is the expected mass for Indo.

Pam-Indo synthesized by coupling Pam to Indo using Fmoc chemistry was also analysed using the reverse phase HPLC on a C18 column. Figure 6A showed Pam-Indo was eluted as a single peak at 27.3 min. LC/MS mass spectrometric analysis of Pam-Indo found that it had a mass of 2449 Da (Figure 6B), and this was its expected mass.

3.2 Immunomodulatory property studies of Pam-Indo, and Indo in BALB/c (H-2^d) mice

To investigate the immunomodulatory properties of Pam-Indo and Indo, these compounds were individually mixed with FI-H5N1, and each of the formulations was injected into BALB/c (H-2^d) mice. Blood samples were collected from each of the experimental mice, and assayed for the presence of antibodies binding to FI-H5N1 immobilized on ELISA wells. Results obtained from these experiments showed that antibodies reacting against FI-H5N1 were not detected in the prebled serum samples of the unimmunized mice. High titers of serum anti-FI-H5N1

antibodies were measured in mice injected intramuscularly with 3.07×10^7 pfu of FI-H5N1. The antibody titers varied between 1.0×10^5 and 1.1×10^3 for the 6 mice in this group (Fig 7). Mice injected with FI-H5N1-Indo formulation, or FI-H5N1-Pam formulation mounted about the same level of anti-FI-H5N1 antibody responses. However, antibodies reacted against FI-H5N1 were detected at much lower level in mice immunized with FI-H5N1 formulated in Pam-Indo. The antibody titers in this group of mice range from 2×10^3 to 4×10^3 . So, they were about forty-fold lower than the levels in mice immunized with FI-H5N1, FI-H5N1 formulated in Indo, or FI-H5N1 formulated in Pam. Another group of mice injected with FI-H5N1 formulated in a mixture of Indo, and Pam also produced much less FI-H5N1-reactive antibodies. The mean anti-FI-H5N1-specific antibody titer measured in this group of mice was 6.35×10^3 , which was slightly higher than the values detected in the FI-H5N1/Pam-Indo-formulation immunized group. The results obtained from these studies showed that immunomodulatory property of Indo requires Pam to be present. Conjugating Pam to Indo to create a composite structure is a more potent immunomodulatory agent than a mixture of Indo and Pam.

A well known immunological phenomenon is that B cells responding to antigens require T helper cells. When they receive “help”, B cells activated by antigens can differentiate to become antibody-producing (plasma) cells [22]. To see how Pam-Indo influenced this mechanism, T cell responses to FI-H5N1 were analyzed. The experiments were done by removing spleens from each of the experimental mice, and the splenocytes were cultured with FI-H5N1 to evaluate the numbers of T cells that are stimulated to produce IFN- γ , and IL-4. These cytokines are produced by antigen specific T-helper cells called Th1 and Th2 lymphocytes, respectively. The results obtained from these experiments were shown in Figure 8. The numbers of

“immuno-spots” representing lymphocytes secreting IFN- γ scored in mice immunized with FI-H5N1 were between 76, and 179. IL-4-secreting cells in this group of mice varied between 69, and 174. For mice immunized with FI-H5N1 formulated in Pam-Indo, the numbers of IFN- γ -, and IL-4-secreting cells detected were between 15 and 56, and 10 and 31, respectively. These numbers are significantly lower than those scored in the FI-H5N1-immunized mice. The numbers of T cells secreting these cytokines between mice immunized with Pam-Indo formulated FI-H5N1 and Indo formulated FI-H5N1 are about the same. Mice immunized FI-H5N1 formulated in Pam generated about equal numbers of IFN- γ -, and IL-4-secreting cells as compared to FI-H5N1 immunized mice. These results suggest Pam-Indo, or a mixture of Pam and Indo immunomodulated to generate less number of FI-H5N1-specific CD4⁺ T cells. Fewer numbers of FI-H5N1-specific Th2 T cells generated had caused lower level of antibody responses elicited against FI-H5N1.

3.3 Effect of Pam-Indo, and Indo on the maturation of dendritic cells.

FI-H5N1-specific CD4⁺ T cells are generated from naïve T cells (those that have not contacted with antigen before) following their interaction with APCs expressing the MHC class 2 molecules associated with H5N1 peptides. In the presence of IL-12 secreted by APCs, the activated CD4⁺ could develop into Th1 cells. If IL-4 is present at a high level, the activated CD4⁺ T cells would differentiate into Th2 cells. With these immunological concepts in mind, the effect of Pam-Indo on the activation of APCs was investigated. The experiments were done by flushing out bone marrow cells from the femur and tibiae bone of BALB/c (H-2^d) mice. The bone marrow cells were then cultured in the presence of murine GM-CSF to generate immature myeloid

dendritic cells (imDCs). Lipopolysaccharide (LPS) stimulation would cause the activation of imDCs to express higher level of the costimulatory molecules. It was found that Pam-Indo affected LPS-activation of imDCs since the expression of CD40 and CD86 molecules are significantly downregulated. The effect was dependent on the amount of Pam-Indo added to the LPS-stimulated imDC cultures. Pam-Indo at greater than 10.0 $\mu\text{g/mL}$ was required to suppress CD40. Significant suppression of CD86 could be achieved at much lower Pam-Indo concentration, at 2.5 $\mu\text{g/mL}$.

4. Discussion

Discovery and development of new adjuvants/immunomodulatory agents has been and still is an important area in preventive and therapeutic vaccine research and development. HDPs have microorganism killing property, and possess immunomodulatory properties. In the study described in this thesis, I have selected to investigate the immunomodulatory property of Indo. Indo has been shown to stimulate bronchial epithelial cell lines to produce the proinflammatory cytokine, IL-8. However, whether Indo may similarly stimulate cells of the innate and adaptive immune compartments have not been investigated.

To study the immunomodulatory property of Indo, Indo was conjugated to Pam thinking that the palmitoyl component of Pam-Indo could facilitate interaction with APCs, therefore allowing Pam-Indo to be more readily captured by the APCs. Indo used to make Pam-Indo was made using Fmoc chemistry. The peptide was analyzed using reverse-phase HPLC, and LC/MS, and confirmed to be authentic. Pam-Indo was also synthesized using Fmoc chemistry, and confirmed to be authentic using reverse-phase HPLC, and LC/MS.

The experimental system used to evaluate the immunomodulatory property of Pam-Indo involves analyzing immune responses generated in BALB/c (H-2^d) mice immunized with FI-H5N1 formulated in Pam-Indo. Immune responses generated in the same strain of mice immunized with FI-H5N1 alone, or formulated in Indo, a mixture of Pam and Indo, as well as Pam, are also evaluated for comparison. FI-H5N1 immunization is found to generate high titers of anti-HI-H5N1 antibodies. The mean numbers of FI-H5N1-specific Th1 and Th2 T cells detected in this group of mice are 102, and 138, respectively. Indo, or Pam does not affect the generation of the FI-H5N1-specific antibody and CD4⁺ T cell responses. However, Pam-Indo, or a mixture of Pam and Indo was able to do so. Mice immunized with FI-H5N1 formulated in Pam-Indo, or Pam plus Indo are found to mount much lower antibody, as well as Th1 and Th2 responses against FI-H5N1. The lower FI-H5N1-specific responses generated in mice immunized with FI-H5N1 in Pam-Indo, or Pam and Indo are therefore, due to lower numbers of FI-H5N1-specific Th2 T cells that are generated.

Naïve CD4⁺ T cells require to interact with APCs to become activated CD4⁺ T cells. This is a well known immunological concept. Therefore, induction of H5N1-specific CD4⁺ T cells may be influenced by the effect of Pam-Indo, or Pam plus Indo on APCs. The results from *in vitro* stimulation of bone marrow cell-derived imDCs in LPS showed that Pam-Indo could inhibit the maturation of imDCs through suppressing the expression of the CD40 and CD86 molecules. So, it is the less effective presentation of H5N1 peptide/MHC class 2 complex by the APCs to the naïve CD4⁺ T cells that result in lower numbers of H5N1-specific Th1 and Th2 cells that are generated.

In summary, Pam-Indo is found to be an APC immunomodulatory agent. The observations gathered from the studies described in this thesis suggest Pam-Indo can be further explored to evaluate its therapeutic potential for autoimmunity, transplantation, and allergies.



FIGURES

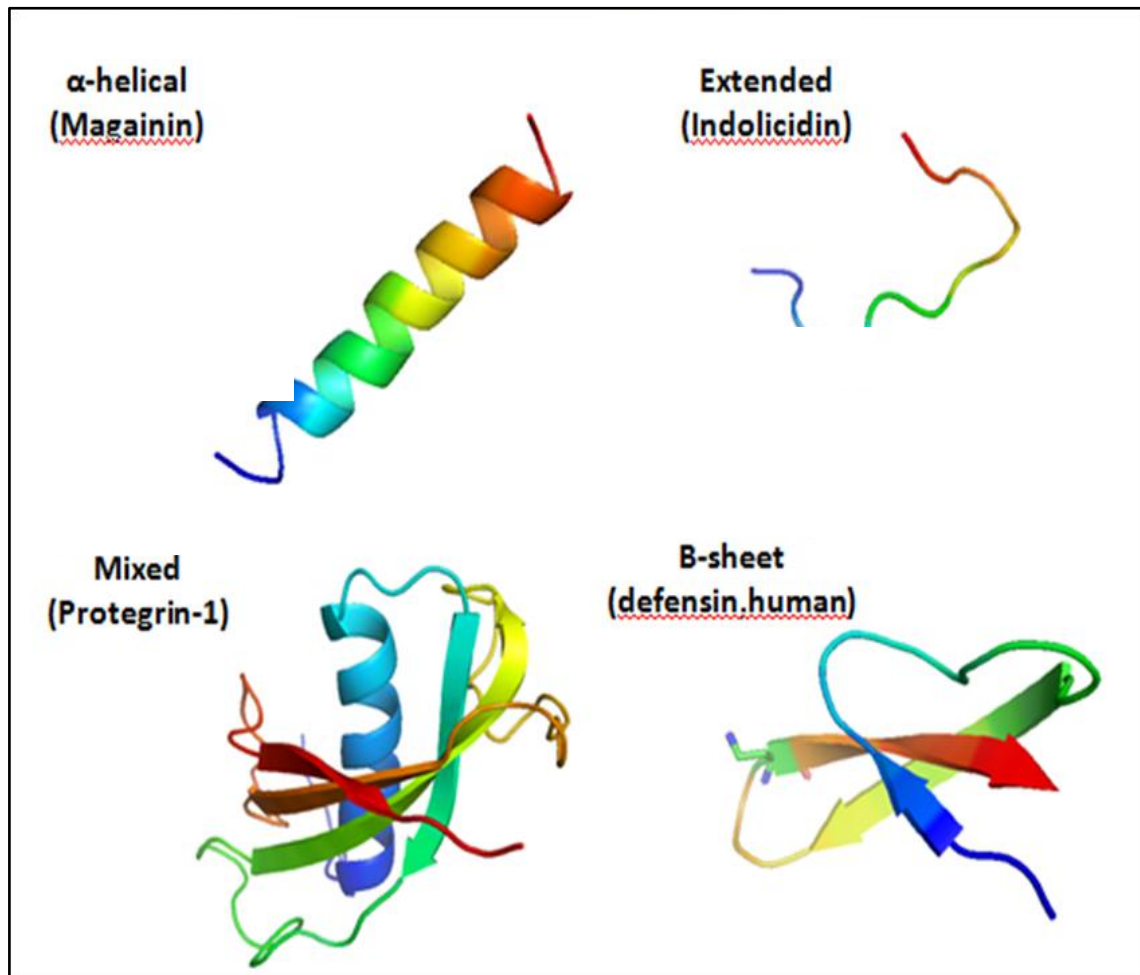


Figure 1. Secondary structures of cathelicidins. Molecular sizes of cathelicidins can vary from 12-80 amino acids. Depending on their sizes, they are known to adopt different secondary structures. Indolicidin is a short peptide containing 13 amino acids, and it adopts an extended conformation. Proline (P), and tryptophan (W) are rich in the sequence of Indolicidin.

Adapted from :

http://upload.wikimedia.org/wikipedia/en/e/ef/Various_AMPs.png

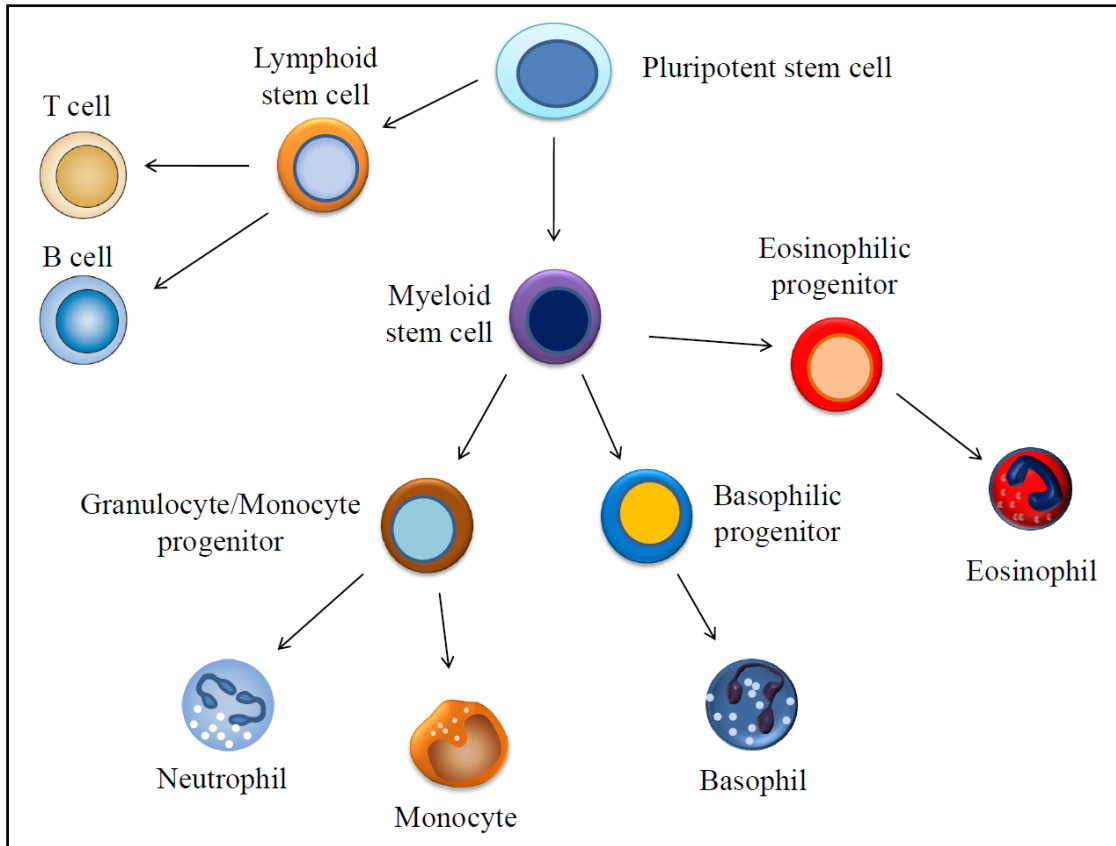


Figure 2. Cells of the innate and adaptive immune compartment are derived from hematopoiesis of a pluripotent stem cell. The pluripotent stem cell differentiates to give rise to a lymphoid, or a myeloid stem cell. The lymphoid stem cell then goes through further differentiation to become a T or a B cell. T and B cells are the main cells in the adaptive immune compartment. The myeloid stem cell gives rise to different progenitor cells, and each of them form a neutrophil, monocyte, basophil, or an eosinophil. These cells are in the innate immune compartment.

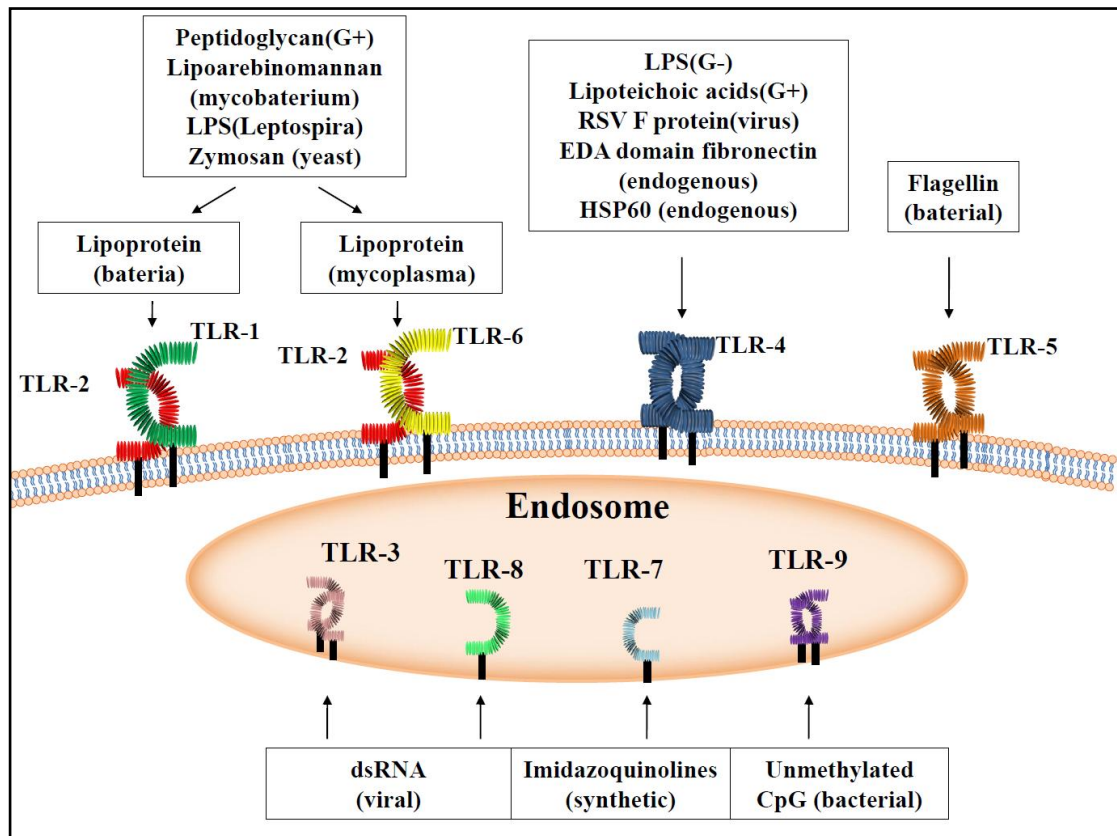


Figure 3. TLRs are expressed in different cell types of the innate immune compartments. Each TLR has a leucine-rich repeat extracellular domain for binding the respective ligands shown. The transmembrane regions of the TLRs are short, and their cytoplasmic domains are well conserved. Many of the TLRs could bind their respective ligands when they are in their monomeric forms. Others will form heterodimers with other TLR monomers before they can recognize the ligands. For example, TLR-2 heterodimerizes with TLR-6 to recognize lipoprotein. Ligand-TLR interaction results in the production of cytokines, and these cytokines would regulate immune responses generated against antigens.

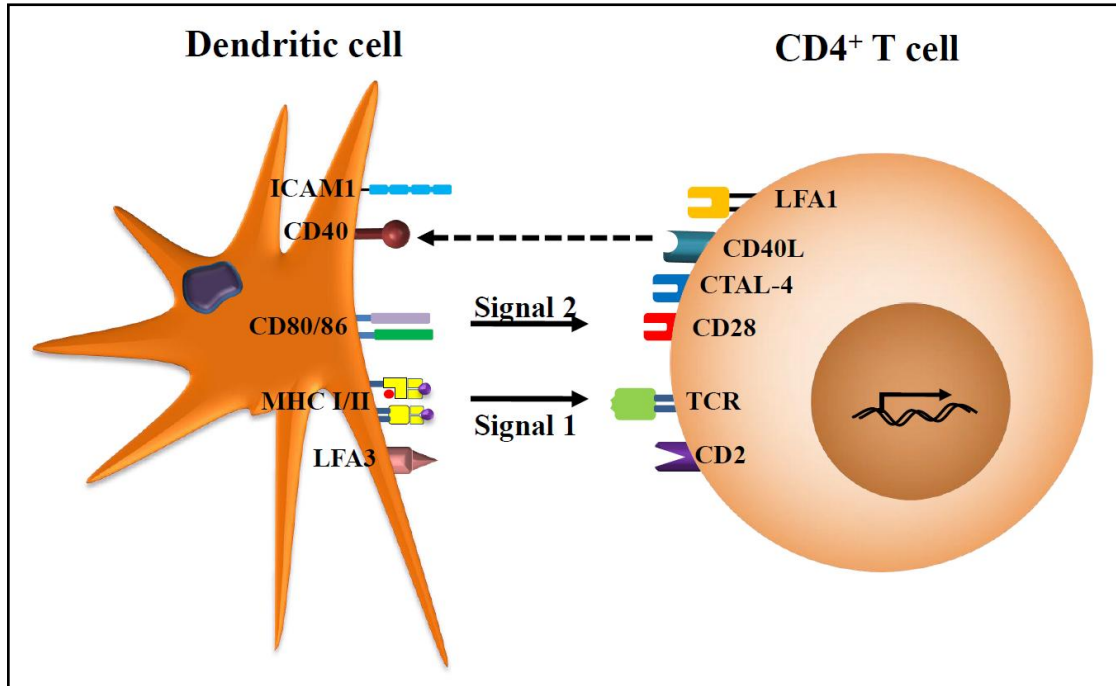


Figure 4. Interaction between a DC (an APC) and a CD4⁺ T cell. A peptide derived through intracellular degradation (processing) of an antigen (microbe) is presented in association with MHC class II molecule to the TCR of a T cell (CD4⁺ T cell as shown) to generate signal 1. Partially activated CD4⁺ T cell expresses CD40L to interact with CD40 on the DC to cause the DC to express higher level of the CD80 and CD86 costimulatory molecules to interact with CD28 on the T cell to generate signal 2. The strength of signal 2 will determine how the T cell may be activated.

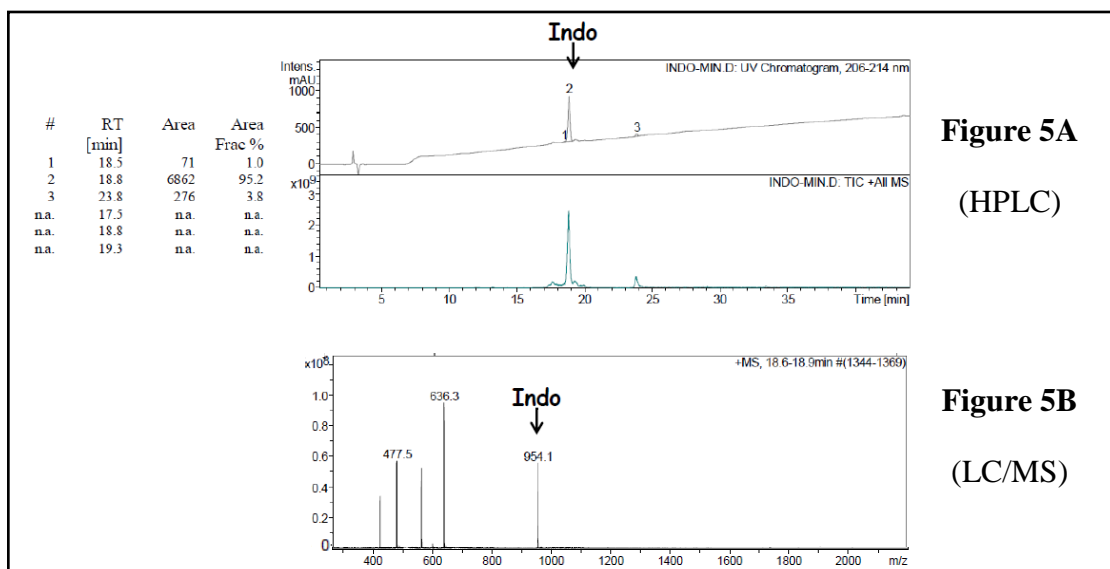


Figure 5A
(HPLC)

Figure 5B
(LC/MS)

Figure 5. Reverse-phase HPLC and LC/MS analysis of Indolicidin. Indolicidin was synthesized using Fmoc chemistry, and analyzed as described in subsection 2.2. Indolicidin was found to elute as a single sharp peak at 18.8 min (Fig 5A), and a mass of 1908 Da (Fig 5B).

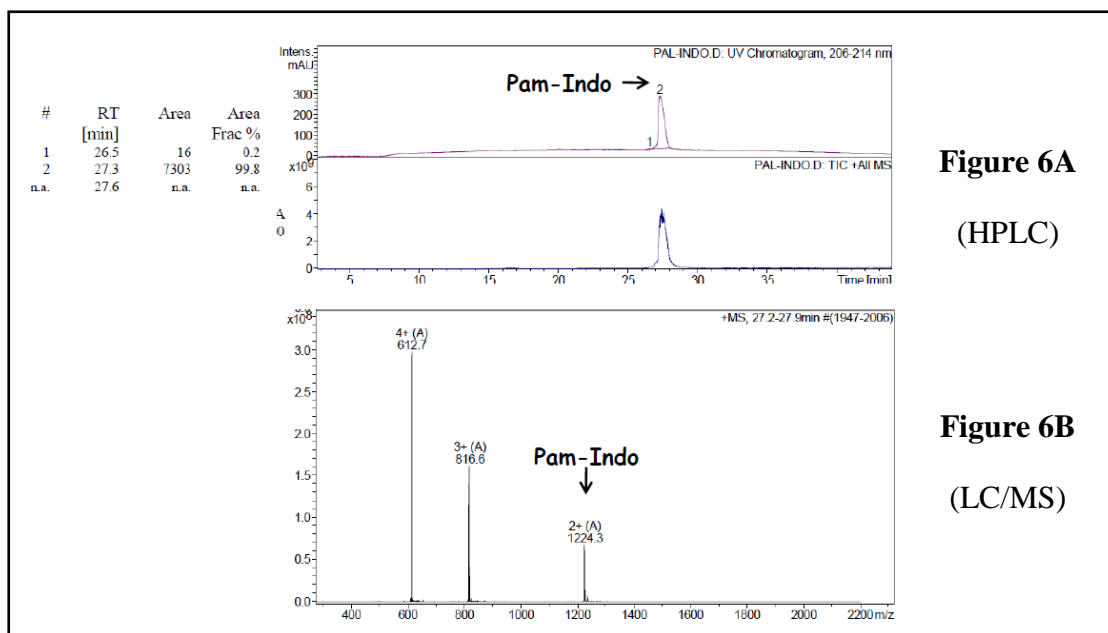


Figure 6. Reverse-phase HPLC and LC/MS analysis of Pam-Indo. Pam-Indo was synthesized using Fmoc chemistry, and analyzed as described in subsection 2.2. Pam-Indo was found to elute as a single sharp peak at 27.3 min (Fig 5A), and a mass of 1908 Da (Fig 5B).

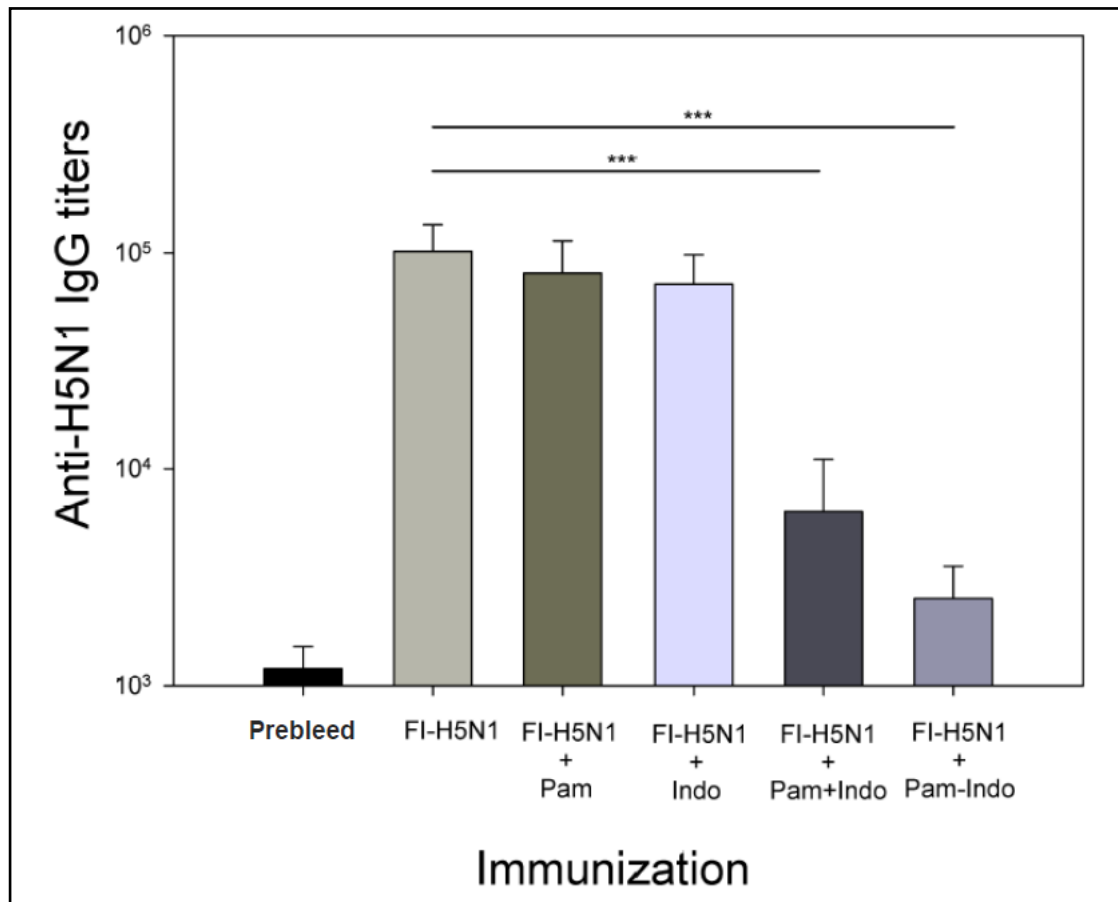


Figure 7. Anti-H5N1 antibody responses generated in BALB/c (H-2^d) mice immunized with FI-H5N1 formulated in, Indo, Pam, or Pam-Indo. Each mouse was prebled before immunization. At 14 days after the second immunization, each mouse was bled and assayed for serum antibodies in the FI-H5N1-coated ELISA. Results shown represented anti-H5N1-specific antibody titers of 6 mice in each experimental group. Statistically significant differences are represented by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

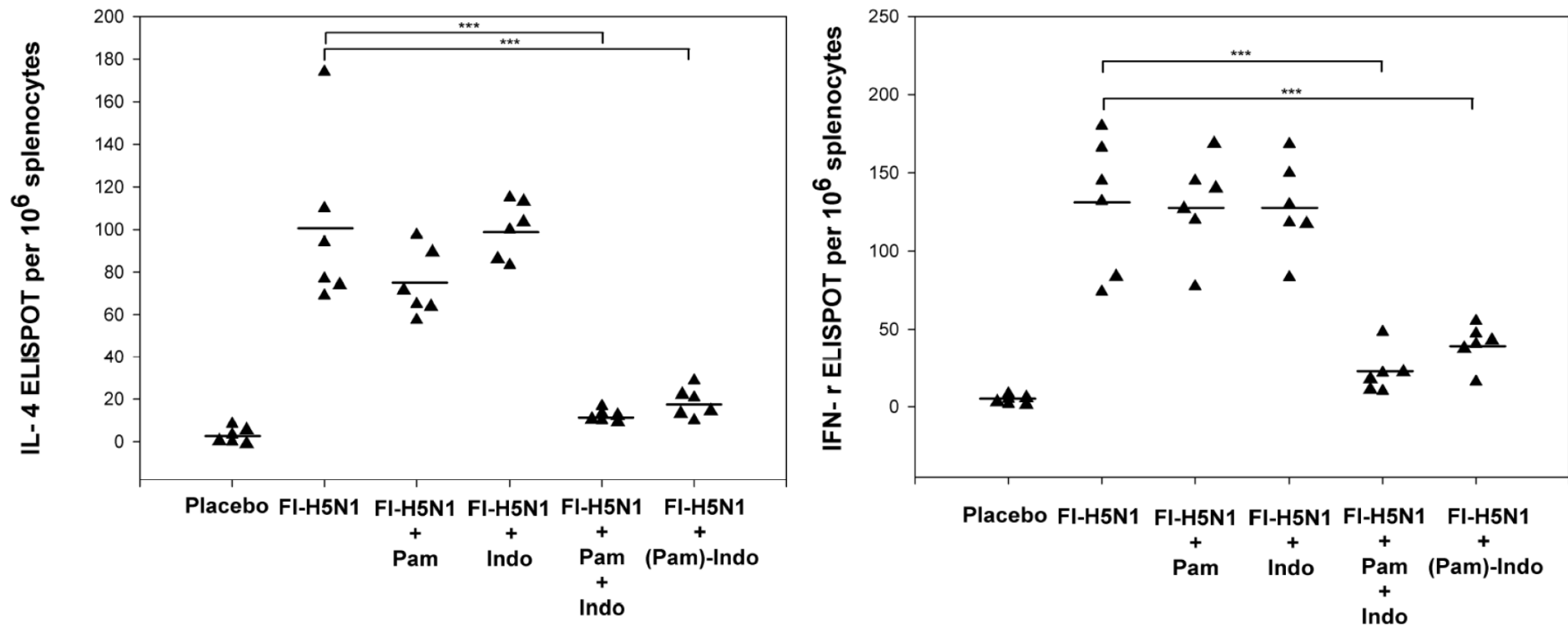


Figure 8. ELISpot assay of IL-4- and IFN- γ -secreting T cells in the spleens of individual BALB/c (H-2^d) mice immunized with FI-H5N1 formulated in Pam, Indo, Pam mixed with Indo, or Pam-Indo. The spleens of each of the experimental mouse was removed, and cultured in anti-IL-4, or anti-IFN- γ coated ELISpot wells, in the presence or absence of FI-H5N1 (equivalent to 1.0 μ g of HA). After 3 days of culture, the assay wells were washed, and the respective detection antibodies were added. Results shown represented the ELISpot score of each mouse. Statistically significant differences are represented by: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

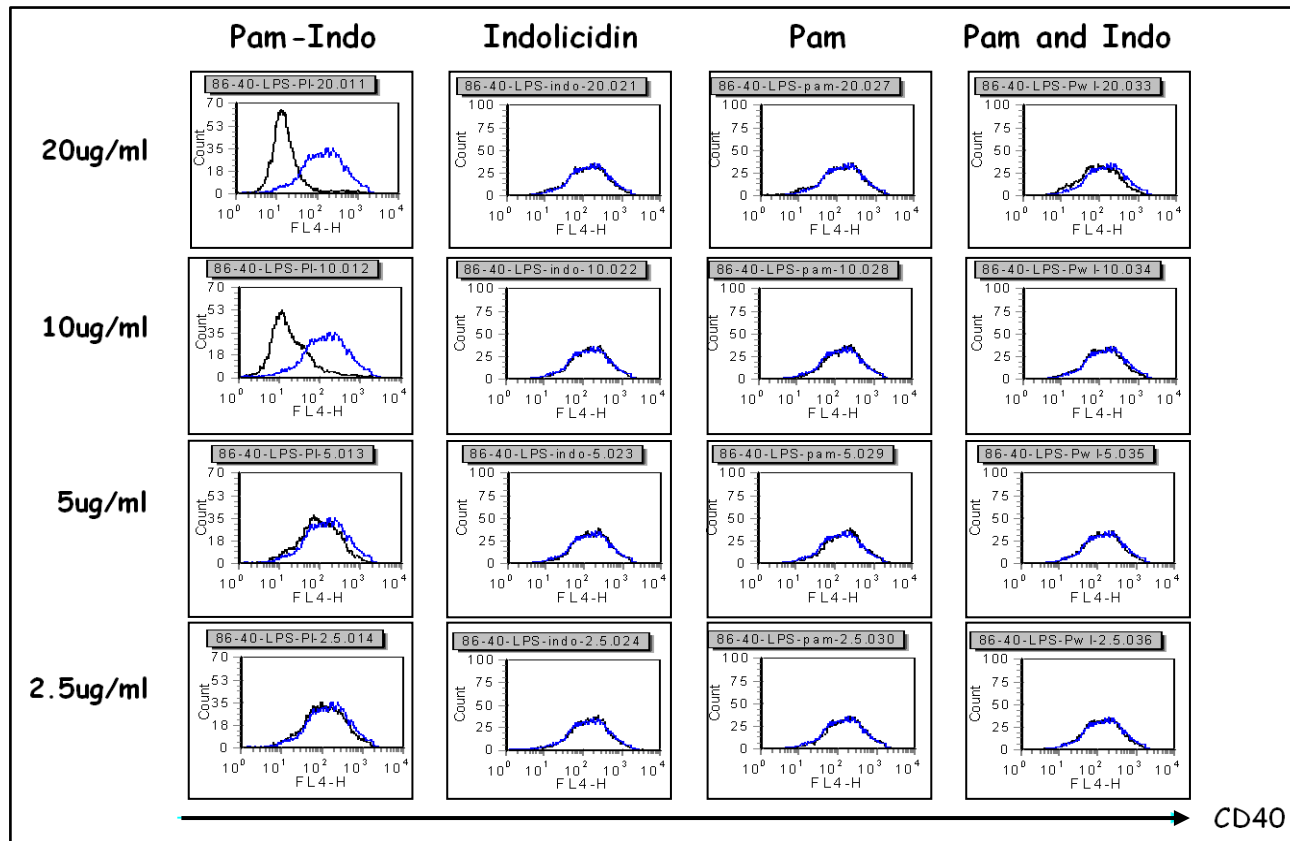


Figure 9A Effect of Pam-Indo on CD40 expression in LPS-stimulated murine BM cell derived imDCs. BM cells are removed the femur and tibiae bones of adult BALB/c (H-2^d) mice. BM cells were then cultured at 1×10^6 in each well of a 24-well tissue culture plate in CM containing 20.0ng of murine GM-CSF for 8-10 days. Fresh CM plus GM-CSF was added to the BM cultures every 3 days. imDC generated were stimulated with LPS (5.0 μ g per mL) with Pam-Indo, Indo, or Pam. BM cells not stimulated are negative controls. Two days later, cells were stained with APC-conjugated anti-CD40 (clone IC10, eBioscience) antibodies. Results showed CD40 expression as analyzed by Flow Cytometry.

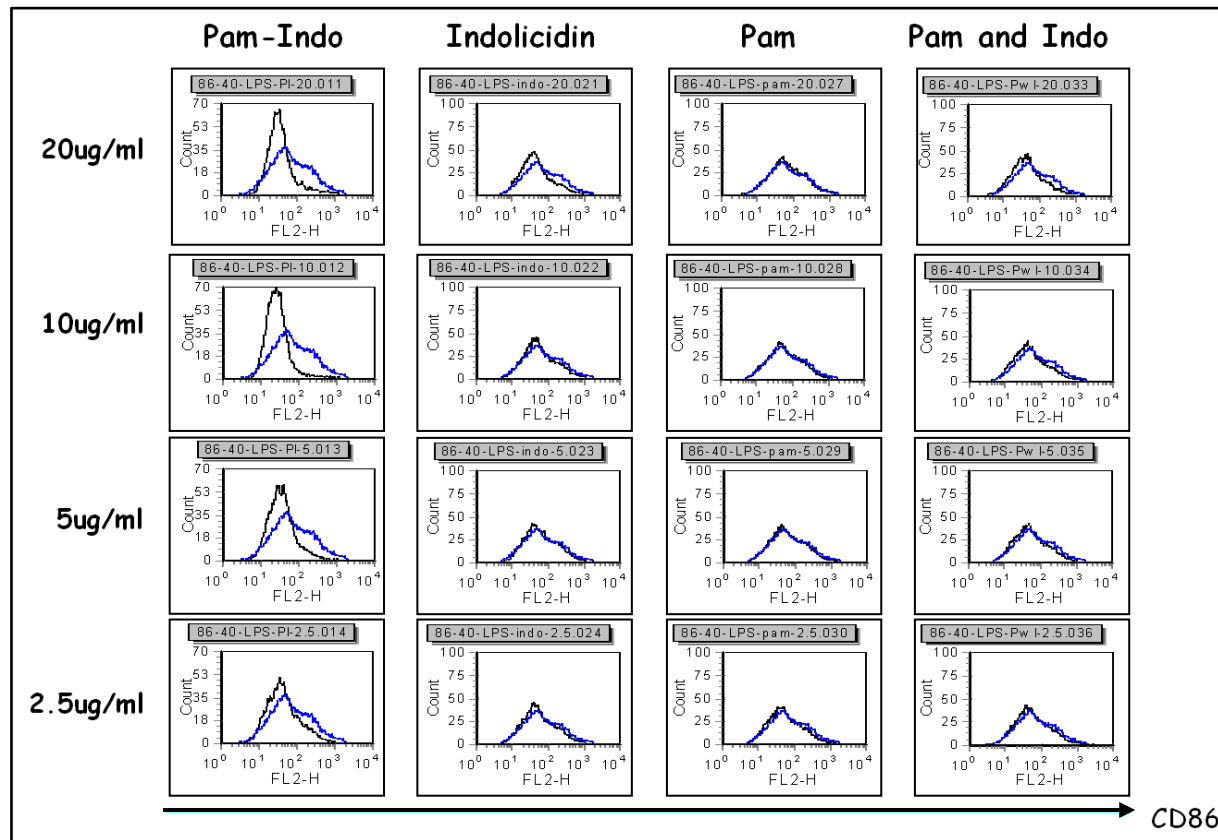


Figure 9B Effect of Pam-Indo on CD86 expression in LPS-stimulated murine BM cell derived imDCs. Figure legends are essentially the same as outlined in Fig. 9A, except anti-mouse CD86-specific monoclonal antibodies were used for staining.

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