國 立 交 通 大 學

生化工程研究所

碩 士 論 文

開發具有誘發特異性免疫反應之微脂體複合體與其特性研究

Development and characterization of a lipo-poly-complex in the induction of specific immune responses

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中文摘要

抗原呈現細胞 (antigen presenting cell; APC) 利用 MHC 分子將病源體之抗原和 T 細胞細胞膜表面的 TCR 交互作用後，並配合共同刺激因子，可有效地開啟專一性的 T 細胞免疫反應。目前了解樹突細胞 (dendritic cell; DC) 是抗原呈現細胞中，呈現抗原之能力最具有效率的。根據一些相關樹突細胞的研究，利用樹突細胞能有效吞噬抗原、呈現抗原，及活化 T 細胞的能力，研究人員藉此已將其發展成為一個很好的疫苗平台。但是，在其他研究中也發現到樹突細胞會受到病源體相關因子影響，降低了樹突細胞之免疫功能。於是便發展人工化抗原呈現細胞來解決樹突細胞所遭遇到問題。本篇論文研究成功建立了一個具有免疫調節功能的新穎之微脂體。LPPC (Lipo-PEI-PEG Complex) 是本實驗室開發出來的微脂體，因為它具有穩定吸附蛋白的能力，並且能維持該蛋白的活性。因此，利用 LPPC 新穎的特性，將具有可以調節免疫反應的單株抗體或 MHC 分子給予 LPPC 吸附後，賦予 LPPC 擁有抗原呈現細胞的能力。

研究結果顯示，LPPC 吸附 anti-CD3 和 anti-CD28 單株抗體後，仍保有原來活性可刺激老鼠脾臟細胞和人類周邊單核球在細胞增生及細胞激素 (cytokine) 有較高表現。此外 LPPC 本身可以促進抗原呈現細胞對抗原之吞噬反應，也可以刺激免疫細胞在前發炎反應激素表現及呈現抗原能力。因此 LPPC 在免疫功能上具有佐劑一樣效應，可增強免疫細胞對抗原的反應。而當 LPPC 吸附來自於樹突細胞富含許多 MHC 分子和共同刺激分子的膜蛋白時，一樣也保有該蛋白分子的活性可誘發動物體內專一性的免疫反應。此外 LPPC 吸附帶有特定抗原的人類 MHC I (HLA-A2) 分子和 anti-CD28 單株抗體後，蛋白分子在 LPPC 平台上仍然可以誘發動物體內專一性免疫反應。因此 LPPC 在本研究中，可以靈活變動地賦予免疫功能之優勢去引發專一性的免疫反應，LPPC 未來將可以發展成一個很好的免疫調控平台。

Development and characterization of a lipo-poly-complex in the induction of specific immune responses

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ABSTRACT

Antigen presenting cells (APCs) can efficiently elicit specific T cell immune responses by presenting pathogen-derived peptides on the major histocompatibility complexs (MHCs) to T cell receptor (TCR) on the surface of T cells. Currently, dendritic cells (DCs) have been discovered as the most efficient APCs. Based on these findings, DCs have been developed as a good bio-reagent to activate host's adaptive immunity by up-taking antigen, presenting antigen and stimulating T-cells. However, DCs would be inactivated *in vivo* by certain pathogen-derived antigens. Thus, artificial antigen presenting cells were developed to resolve this problem. In this study, a novel immuno-regulatory liposome was developed. Lipo-PEI-PEG-complex (LPPC) was a novel liposome could strongly absorb proteins on its surface and the bound proteins could maintain their activities. By these characters, LPPC was designed to adsorb certain monoclonal antibodies or MHC molecules which have the abilities to regulate immune responses and the immuno-LPPCs were used as APC.

The results showed that the LPPC adsorbed anti-CD3 and anti-CD28 monoclonal antibodies could increase the proliferation and cytokine secretions (IL-2, IFN-γ and TNF-α) of mouse splenocytes and human peripheral blood mononuclear cells (PBMC). In addition, LPPC can promote the abilities of APCs to up take antigen, induce the proinflammatory cytokine expressions, and present antigen. Thus, LPPC could provide a good adjuvant effect. Besides, the LPPC coated with membrane proteins of DCs performed as APCs to stimulate specific T-cell immune responses. Moreover, antigen-loaded HLA-A2 molecules and anti-CD28 monoclonal antibody were adsorbed on LPPC and they also maintained their specific activities, and induced the specific immune responses. The immuno-LPPC displays its flexible character and advantage to regulate immunity by combining certain immuno-regulatory antibodies and specific-antigen MHC molecules. Therefore, the immuno-LPPC may be developed as a good immunoregulatory platform.

Acknowledgement

燈塔的夜晚，苦悶孤寂，一個人在實驗室敲打著鍵盤，拼湊這兩年來瑣碎片段的記憶，嗅著濃烈咖啡因的味道，喝下這兩年來辛苦沉重的心情，回憶如走馬燈在腦海跑了好幾回，猶記得兩年多前，風塵僕僕孤身一人帶著豪情壯志揮軍北上，直闖風城裡腫瘤免疫絕學之深奧領域中，盼望能學得一招半式，可以為自己和清水家鄉揚名立萬，誰知層出不窮的實驗瓶頸，接踵而來的課業煩惱。 幸好，當初遇到了自詡小諸葛的廖光文老師，讓我見識到如何靈活運用實驗室的資源及多方面審視和分析每個人實驗結果，這些『非常人』之撇步，另外廖老師認真指導加上不厭其煩地傳授資質駑鈍的我，使我可以快速累積和汲取實質經驗。 一天，霎時任督打通茅塞頓開，領悟到免疫絕學之奧妙，終於在今年六月順利通過廖老師和師叔姐們的考驗，成為一個文武雙全的交大碩士生。 當然這求學拜師過程中還是需要其他師兄弟和姊妹們的幫忙，像彥谷學長和于鈴學姐提攜指教，公主們小莉小溫啾咪吐嘲求進步，姵姵小貓咪細心照顧幫忙，馬馬靜敏筱葳維瞳切磋砥礪等眾人幫忙合作下，成就了今天的我，在下心中滿懷感激之情。此外還有已畢業的學長姐們和其他實驗室有緣人們指教，真是很謝謝大家。 『懂得屈膝，跳得更高』

家弘與有緣人共勉之

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Abbreviations

APCs	antigen-presenting cells
CTLs	Cytotoxic T lymphocytes
DCs	dendritic cells
LFA	leukocyte function-associated antigen
ICAM	intercellular adhesion molecule
IFN-γ	interferon-γ
CEA	carcinoembryonic antigen
rAAV	recombinant adeno-associated virus
VEGF	Vascular endothelial growth factor
HPV	human papillomavirus
HIV	human immunodeficiency virus
SIV	simian immunodeficiency virus
IL-2	interleukin-2
IL-1β	interleukin-1 β
IL-6	interleukin-6
IL-8	interleukin-8
IL-10	interleukin-10
TNF-α	Tumor necrosis factor-α
MHC	major histocompatibility complex
PBS	phosphate buffer saline
mAb	Monoclonal antibody
LPPC	Lipo-PEI-PEG-Complex
PEI	polyethyleneimine

PEG	polyethylene glycols
TCR	T cell receptor
PBMC	peripheral blood mononuclear cell
DLS	dynamic light scattering
HP	Helicobacter pylori
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
HSP	Heat shock protein
LPS	lipopolysaccharide
TEM	Transmission electron microscopy
BSA	Bovine serum albumin
HLA-A2	Human leukocyte antigen-A2
MART-1	melanoma-associated antigen recognized by T cell-1
hTERT	human telomerase reverse transcriptase

Chapter 1 Introduction

A major aim in immunotherapy is to generate specific cell-mediate immune responses to regulate host immunity. Efficiently resist pathogens and virus that relies on T cell immune responses (1). The optimal T-cell stimulation requires engagement of the T-cell receptor (TCR) through the major histocompatibility complex (MHC) bound to peptide, together with at least one interaction of a costimulatory molecule with an appropriate ligand on the T cell (2, 3).

The most potent and best-investigated costimulatory molecules are B7-1 and B7-2, which bind to CD28 (4, 5) on the T cell and induce cell proliferations. In contrast, B7 molecules might also bind cytotoxic T lymphocyte antigen 4 (CTLA-4) molecules on activated T cells and induce apoptosis in those T cells. Additional interactions might also regulate T-cell stimulation, including T-cell subtype differentiation, induction of maximal proliferation and prevention of apoptosis. Potential molecules of these interactions are other costimulatory molecules such as ICOS, 4-1BBL and OX40 (6-8).

Another major group of molecules are the adhesion molecules, which include leukocyte function-associated antigen (LFA) and intercellular adhesion molecule (ICAM) (9, 10). These molecules increase the interaction time between the T cell and APC, and enhance efficient activation (11). As discuss above, over the past decade many

new costimulatory molecules have been identified, offering new insights into T-cell activation and regulation.

Naive T cells are activated to produce armed efficient T cells the first time they encounter their specific antigen in the form of a peptide:MHC complex on the surface of an activated antigen presenting cell (APC). Antigen presentation by APCs, most notably macrophages and dendritic cells (DCs), and infected B cells is critical for induction of specific T cells in the form of an adaptive immune response (12). Further, the induction of T cell-mediated immunity is controlled by antigen-presenting DCs, potent stimulators of specific T cell immunity (13). DCs in essence act as nature's adjuvants and play an important role to generate adaptive immunity. They present immunogenic epitopes of antigens in the context of MHC class I and class II molecules in association with costimulatory molecules, and efficiently activate both cytotoxic T cells and T helper cells (14).

DCs are both efficient and specialized in antigen presentation, and they control the magnitude, quality, and memory of the ensuing immune response. Because of the exceptional ability of DCs to activate T-cell immunity in response to microbial pathogens and tumor cells, these cells have been exploited as ex vivo and in vivo tools for immunotherapy. For example, Dr. Lu demonstrate that a therapeutic vaccine made of inactivated SIV-pulsed DCs can elicit effective cellular and humoral immune responses against SIV, allowing the control of SIV replication in the secondary lymphoid tissues and the reduction of cell-associated viral

DNA and cell-free viral RNA in blood of SIV-infected macaques (15). In addition, HSP105-pulsed BM-DC vaccine could induce specific T cells to inhibit the growth of intestinal tumors overexpressing HSP105 (16). Moreover, Dr. Aldrich utilized rAAV with human tumor antigen, carcinoembryonic antigen (CEA), of gene to infect DC for induction of specific immunity (17). In another study, the coadministration of DNA vaccines encoding HPV16 E7 with siRNA targeting key proapoptotic proteins successfully prolongs the lives of DCs, enhances antigen specific $CD8^+$ T-cell responses, and elicits potent antitumor effects against an E7-expressing tumor model (18).

As development of DC-based application, the challenges of these therapies need to be improved. Several virus- and nonvirus-based transduction methods have been used for DC-based therapy. However, all strategies result in different levels of gene expression depending on the transduction efficiency. Therefore, purification of expressing APCs is needed to avoid non-expect interaction (11). In addition, some articles indicated that pathogens or pathogens-derived factors, such as *Candida albicans*, *Mycobacterium tuberculosis*, mycobacterial LAM, and secretions of *Candida* respectively impact on efficiency of DCs and immune cells, affect cytokine expression and impair surface marker of DC (19-22). Moreover, mature DCs express high levels of costimulatory molecules such as B7.1 and B7.2, which could be contact to CTLA-4, an immuno-inhibitory ligand that suppresses T cell activation (23, 24). In addition, many tumors secrete immunosuppressive factors such as TGF-β, IL-10, or VEGF, which affect the function of DCs to influence

the efficiency of DC vaccine (11, 25-27).

As an alternative strategy, artificial antigen-presenting cell systems (aAPC) have been recently developed and are rapidly expanding. They encompass both cellular-based and acellular-based technologies (28). In cellular-based, they utilized genetic methods that transduced into murine fibroblast cells to express immunoregulatory molecules, such as antigen-loaded MHC molecules and B7 molecules (29). In addition, a nonspecific cell-based aAPC (K32 cells) has also been developed, that were transfected with the costimulatory molecule 4-1BBL and the low-affinity Fc gamma receptor CD32 (30).

In acellular-based, they employed chemical method to conjugate immunoregulatory molecules on nanoparticles, bead or liposome. For example, a acellular aAPC was developed that can be used to induce and expand clinically relevant amounts of highly enriched peptide-specific T cells based on HLA-A2–Ig molecules and anti-CD28 monoclonal antibody (mAb) coupled to a magnetic bead (31, 32). In this strategy, peptide resident in the HLA–Ig molecule with any HLA-A2-restricted antigenic peptide can be modified. Thus, a single batch of HLA-A2–Ig-based aAPCs can be loaded with various different antigenic peptides for expansion of cells with different antigenic specificities. These artificial APC-based strategies certainly diminished the effect of immunosuppression.

Our laboratory has developed a novel liposome, Lipo-PEI-PEG

complex (LPPC), that was a biodegradable liposome with the characters that could strongly and rapidly adsorb proteins on its surface, and these proteins could maintain their activities. The purpose of this study, that manufacture artificial antigen presenting cells, or APC-liked liposome, which were combined the liposome with immunostimulatory molecules to develop as an immunoregulatory platform. Therefore, we exploited LPPC combined with immunostimulatory molecules as artificial antigen presenting cells to activate immunity. Here, we added anti-CD3 and anti-CD28 monoclonal antibodies (mAbs), or addition of DCs' membrane proteins, or addition of specific peptide-HLA-A2 complex for the LPPC adsorption. The results showed that LPPC indeed exhibited ability of enhancement of the cell proliferations and cytokine secretions of human peripheral blood mononuclear cells (PBMCs) and murine splenocytes *in vitro*. We also showed that the LPPC with immuno-molecules induced specific immune responses *in vivo*. Moreover, LPPC might have the potential of an adjuvant that enhance immune responses of APCs. In this study, we indeed demonstrated that LPPC showed quickness and good flexibility to construct an immunoregulatory platform as an artificial antigen presenting cell.

Chapter 2 Material and method

2.1 Material

2.1.1 Reagent

The following reagents and chemicals were obtained as indicated: RPMI 1640, Fetal Bovine Serum (FBS), and BSA from Invitrogen. Penicillin/ streptomycin/ amphotericin (PSA) from Biological industries. NaCl, Tris-HCl, Triton X-100, from Amresco. Ficoll-Paque^{TM} Plus from GE healthcare. Propidium iodide (PI) from CE. EDTA and chloroform from TEDIA. NaOH, H_3PO_4, KH_2PO_4, Na_2HPO_4, tween 20, $KHCO_3$, NaN_3, and KAc from SHOWA. KCl from Scharlau. Na_2HPO_4 from J. T. Baker. Urea from USP.

2.1.2 Cell lines

P338D1 (Mouse macrophage-like cell line; ATCC number: CCL-46.)

2.1.3 Antibody

The following antibodies were obtained as indicated: Mouse anti-human CD3 (OKT3) and Mouse anti-mouse CD3 (2C11) were kindly provided from Dr. Steve R. Roffler (ACADEMIA SINICA, Institute of BioMedical Sciences). Anti-human CD28 monoclonal antibody, and anti-mouse CD28 monoclonal antibody were purchased from Biolegent.

2.1.4 Kits

The following kits were obtained as indicated: Human IL-1β, IL-6,

IL-8, IL-2, IFN-γ and TNF-α ELISA kit, and mouse IL-4, IL-10, IL-2, IFN-γ, and TNF-α ELISA kit from R&D. MTT assay kit from Promega.

2.1.5 Animals

Six-eight weeks old female BALB/C mice were purchased from the National Laboratory Animal Center, Taipei, Taiwan, R.O.C. Six-eight weeks old female C57BL/6-Tg (HLA-A2.1) mice were kindly provided from Dr. Shih-Jen Liu (National Health Research Institutes).

2.1.6 Others

Peptide-HLA-A2 monomer and one identify epitope of HPV type 16 E7 protein (YMLDLQPETT) were kindly from Dr. Shih-Jen Liu (National Health Research Institutes).

2.2 Method

2.2.1 LPPC preparation

Briefly, added each DOPC and DLPC 500μl (50 mg/ml) into the round bottom flask, and then added 1000μl methanol into the same container and mix well. The mixture was placed the container of lipid mixture to the rotary evaporator (37℃, without vacuum treatment, minimum rotary speed) until dry (about 2 days). Hydrated the lipid film by steam (about 37℃) for 2~3 hours. Added 5ml aqueous medium (containing 0.675g PEI and 0.22g PEG in 5 ml filtered DDW) gently to the container of dry lipid and agitating gently. The container was vortexed violently for 10 minutes. After vortexed, the LPPC was placed at RT overnight. The turbid medium of LPPC extruded through 200nm mesh

nine times. The product stored into the container to 4℃ refrigerator.

2.2.2 Adsorption characters of LPPC

Timing

Added 50 μg BSA-FITC into 40 μg/ml LPPC solution, and then centrifuged at 10,000 rpm for 5 min at different time. The fluorescence of LPPC pellet was estimated by Spectrofluorometer.

Capacity

Added different amounts of BSA into 40 μg/ml LPPC solution, and then centrifuged at 10,000 rpm for 5 min at 20 minutes. The amounts of BSA onto LPPC were measured by using coomassie plus reagent.

Competition

40 μg/ml LPPC prior to adsorb 50 μg BSA-FITC completely, and added different folds of BSA for competition in 20 minutes. And then centrifuged at 10,000 rpm for 5 min, the fluorescence of LPPC pellet was estimated by Spectrofluorometer. Positive control was the fluorescence of the LPPC solution without adding BSA. Negative control was the fluorescence of the LPPC alone solution.

2.2.3 PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were separated from human white blood cell solution by using Ficoll-Paque™ Plus. Dilute human white blood cells with equal volume of PBS. Add Ficoll-Paque PLUS (6 ml) to the 15 ml centrifuge tube and carefully layer the diluted blood sample (8 ml) on Ficoll-Paque PLUS. Centrifuge the tubes at 400g

for 40min at 18℃. Remove the plasma layer and collect the PBMC layer. Wash the cells with 2 volume of PBS for centrifuging at 1500 rpm for 15 min. Discard the supernatant and lyse the red blood cells by ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in DDW) at room temperature for 10min and followed by centrifuging at 1500 rpm for 15 min. Discard the supernatant and wash the cell with 10 ml PBS. Centrifuge for another 15 min. Discard the supernatant and count the cell number. For the cell proliferation of PBMC, cells were plated in a 96-well at 1 x 10^5 per well. For the cytokine profiles of PBMC, cells were plated in a 24-well plate at 4 x 10^5 per well.

2.2.4 Splenocyte isolation

Mice were sacrificed by dislocation and their spleens were quickly harvested in a laminar flow hood. Spleens were placed in a 280 μm-pored mesh and chopped by scissors. 10 ml of RPMI 1640 (Invitrogen Co., USA) supplemented with 10% FBS, 0.2% $NaHCO_3$ and 1% PSA. were slowly added onto the mesh while spleens were being ground until the spleen tissue became white. Single cell suspension was collected in a Petri dish and recovered by centrifugation at 1,200 rpm at 4℃ for 5 min. Supernatant was discarded and 10 ml 1X ACK lysis buffer was added for 5 min at room temperature. 1X ACK buffer can lyse the red blood cells while leaving the rest of the lymphocytes and leucocytes. The mixture was then diluted by 10 ml of RPMI 1640 and cells were recovered by centrifugation at 1,200 rpm at 4℃ for 5 min. After the supernatant was discarded, the cells were rinsed by 10 ml PBS once more. Finally, cells were resuspended in RPMI 1640 and underwent cell calculation by trypan

blue exclusion. For the cell proliferation of splenocyte, cells were plated in a 96-well at 2.5 x 10^5 per well. For the cytokine profiles of splenocyte, cells were plated in a 24-well plate at 1 x 10^6 per well.

2.2.5 The cytotoxicity of LPPC to PBMCs or splenocytes

PBMC (1×10^5 cells per well) or splenocyte (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates and then except for control treated with different condition. After 72 hr, the cells were centrifuged at 400g for 15 min. Removed the medium, and added 100 μl MTT working solution per well. And then, the 96-well culture plates were put back incubator with 5% CO_2 at 37℃ for 4 hr. The supernatant was removed, and added 100 μl DMSO to dissolve the purple crystal. Put plates on the shaker for 10 min. The optical density was determined by a microplate reader (Tecan) set to 595 nm and the data were analyzed by Magellan5 software.

2.2.6 The activities of monoclonal antibodies adsorbed on LPPC

In this study, anti-CD3 monoclonal antibody (2C11 or OKT3) was utilized as first signal for activation of T cell, and the other monoclonal antibody, anti-CD28 as second signal was for optimal activation of T cell. PBMC (1×10^5 cells per well) or splenocyte (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates and then except for control treated with different condition. 40 μg LPPC pre-adsorbed 100 μg BSA, and then adsorbed with 2.4 μg anti-CD3 mAb or with 2.4 μg anti-CD3 and 2.4 μg anti-CD28 mAb into 100 μl volume. After centrifuged, 2.5 μl LPPC complex treated PBMCs or splenocytes for

72hrs. By using MTT assay, and then the cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of PBMC alone or splenocyte alone.

2.2.7 The stability of immunostimulatory monoclonal antibodies adsorbed on LPPC in RPMI

40 μg LPPC previously adsorbed 100 μg BSA, and then adsorbed with 2.4 μg anti-CD3 mAb or 2.4 μg anti-CD3 and 2.4 μg anti-CD28 mAb into 100 μl volume. After centrifuged, put the LPPC complex into RPMI solution in 37 ℃ for 30 minutes. After 30 minutes, the solution was centrifuged divide into LPPC pellet and the supernatant. The LPPC pellet was resuspend into 100 ul DDW. The 2.5 μl LPPC complex and the 2.5 μl supernatant respectively treated PBMCs (1×10^5 cells per well) or splenocytes (2.5×10^5 cells per well) in 96-well culture plate, and estimated the cell proliferation of immune cells for investigating the efficiency of monoclonal antibodies on LPPC. By using MTT assay, and then the stimulation index was calculated as (O.D. value of sample –O.D. value of PBMC alone or splenocytes alone) / O.D. value of PBMC alone or splenocytes alone.

2.2.8 The dose-effect of monoclonal antibodies adsorbed on LPPC in immune cells

Cell proliferation

PBMC (1×10^5 cells per well) or splenocyte (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates and then except for control treated with different condition. Addition different amounts of

immunostimulatory monoclonal antibodies were combined with 1 μg LPPC to stimulate the proliferation of immune cells, and the cell numbers was counted by MTT assay at 72 hrs. The cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of PBMC alone or splenocyte alone.

Cytokines secretion

PBMC (4×10^5 cells per well) or splenocyte (10^6 cells per well) were dispensed into 24-well culture plates and then except for control treated with different condition. 4 μg LPPC adsorbed different amounts of monoclonal antibodies respectively to treat PBMCs or splenocytes. And the supernatants were collected at 24h and 72 h and frozen at −80 ℃. Supernatants concentrations of TNF-α, IL-2, and IFN-γ were measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*).

Pro-inflammatory cytokine profiles secretion

PBMC (4×10^5 cells per well) was dispensed into 24-well culture plates and then except for control treated with different condition. 4 μg LPPC treated PBMCs and then the supernatants were collected at 24 h, 48 h and 72 h and frozen at −80℃. Supernatants concentrations of IL-1β, IL-6, and IL-8 were measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*).

2.2.9 The comparison of activities of monoclonal antibodies on LPPC

PBMC (1×10^5 cells per well) or splenocyte (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates for monitoring cell proliferation. 40 μg LPPC previously adsorbed 100 μg BSA, and then adsorbed with 2.4 μg anti-CD3 mAb or 2.4 μg anti-CD3 and 2.4 μg

anti-CD28 mAb into 100 μl volume. After centrifuged, 2.5 μl LPPC complex respectively treat PBMCs or splenocytes, and comparing to the same amount mAb that free from added into solution. The cell numbers was counted by MTT assay at 72 hrs. The cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of PBMC alone or splenocyte alone.

On the other hand, PBMC (4×10^5 cells per well) or splenocyte (10^6 cells per well) were dispensed into 24-well culture plates for monitoring cytokines secretion. 40 μg LPPC previously adsorbed 100 μg BSA, and then adsorbed with 6 μg anti-CD3 mAb or 6 μg anti-CD3 and 6 μg anti-CD28 mAb into 100 μl volume. After centrifuged, 10 μl LPPC complex respectively treat PBMCs or splenocytes, and comparing to the same amount mAb that free from added into solution. And the supernatants were collected at 24h and 72 h and frozen at −80 ℃. Supernatants concentrations of TNF-α, IL-2, and IFN-γ were measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*).

2.2.10 The uptake protein ability of P338D1

50 μg BSA-FITC as a green fluorescence protein was previously adsorbed by 150 μg LPPC or was not adsorbed, and then respectively co-cultured two hours with 5×10^5 mouse macrophage, P338D1. Added 100 μl trypan blue to quench the green fluorescence from BSA-FITC that was not uptaken or only adhered to cell surface, and FACS analysis was performed. In addition, 50 μg BSA-FITC as a green fluorescence protein was previously adsorbed by 150 μg LPPC or 10 μg LPPC, and then

respectively co-cultured two hours with 5 $\times 10^5$ mouse macrophage, P338D1.

2.2.11 DC harvest

Balb/C mice were sacrificed by dislocation. Make a long transverse cut through the skin in the middle of the abdominal area. Reflect skin from the hindquarters and the hind legs. Removed the feet, and then removed all muscle from the femurs and tibiae. Separate the legs from the body at the hip joint (one leg each time). Transfer the bones to a 15 mL centrifuge tube containing cold RPMI. Place the bones in a 10 cm bacterial dish containing 70 % ethanol for less 2~5 min for disinfection, then washed with RPMI. Separate femurs and tibiae. Cut both ends of the bone with scissors and the marrow flushed with RPMI10 using a Syringe with a 25 G needle. Collect cell suspension in a 10 cm bacterial dish. Clusters within the cell suspension were disintegrated by vigorous pipetting. Transfer the cell suspension to a 15-mL centrifuge tube. Centrifuges at RT, 300g for 5 min and then discard the supernatant. Add 2 mL of ACK lysis buffer to lyse red cells for 45 sec. The mixture is then added with 10 mL of RPMI10and centrifuges at RT, 300g for 5 min to wash out ACK. Discard the supernatant, and then suspend the cell pellet and then add with 10 mL of RPMI. Transfer the suspension to another tube to remove the settled debris and clumps. Centrifuges at RT, 300g for 5 min and discard the supernatant. Count cell number and then BM leukocytes were seeded at 2.5×10^6 per 100 mm dish in 10 mL R10 medium containing 200 U/mL rmGM-CSF. At day 3, another 10 mL RPMI10 medium containing 200 U/mL rmGM-CSF were added to the plates. At days 6, half of the

culture supernatant was collected (10 mL/dish), centrifuged at RT, 300g for 5 min, and the cell pellet resuspended in 10 mL fresh RPMI10 containing 200 U/mL rmGM-CSF/dish, and given back into the original plate. At days 8, half of the culture supernatant was collected (10 mL/dish), centrifuged at RT, 300g for 5 min, and the cell pellet resuspended in 10 mL fresh R10 containing 200 U/mL rmGM-CSF/dish, and given back into the original plate. At day 9 or 10, non-adherent cells were collected by gentle pipetting. Cells were centrifuged at 300g for 5 min at RT, and resuspended in 10 mL fresh R10 (10^6 per mL) into a fresh 100 mm tissue culture plastic dish containing 100 U rmGM-CSF and 0.5 μg/mL LPS (–20℃, A11, 100 μg/mL). Cells were then cultured for 1 or 2 days for further experiment (complete maturation). The mature dendritic cells were checked by staining with anti-mouse CD11 conjugated PE and analyzed by flow cytometry.

Purification of DC membrane protein

Harvested DC cells (1×10^7 cells) were by centrifuging the cell suspension or culture at 900g for 10 min at 4℃. Resuspend the cell pellet in 10 ml PBS buffer and centrifuged at 900g for 10 min at 4℃. Resuspend the cells in 10 ml HEPES-KOH buffer. Homogenize the cells on ice to fine homogenate using an appropriate cell homogenizer. The cells were centrifuged at 9000g for 15 min at 4℃. Transfer the supernatant into fresh ultracentrifuge tubes and discard the pellet. The fresh ultracentrifuge tubes centrifuged at 50000g at 4℃. Discard the supernatant, briefly air dry, and save the membrane pellet. The membrane pellet was dissolve in the PBS buffer. The concentration of membrane proteins was analyzed by

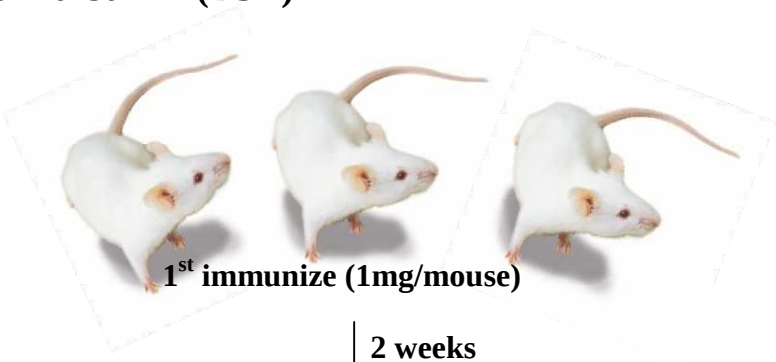
using commassie plus test.

Dendritic cell surface marker staining

10^6 DCs were centrifuged the cells at 4000 rpm for 5min. Resuspend the cells with 500 μl staining buffer (0.5% skim milk in PBS). Stain the cells with antibody on ice in the dark for 30min. After washing the cells with 500 μl staining buffer, centrifuge the cells at 4000 rpm for 5min. Repeat again. Analyze the cells on FACScan with dot plots with quadrant line. Figure 11 indicated that the surface marker expression of DCs, such as CD11c, MHC II, and CD86.

2.2.12 The experimental strategy of animal immunization

Bovine serum albumin (BSA)



1st immunize (1mg/mouse)

↓ **2 weeks**

1st boost (1mg/mouse)

↓ **1 week**

2nd boost (1mg/mouse)

↓ **1 week**

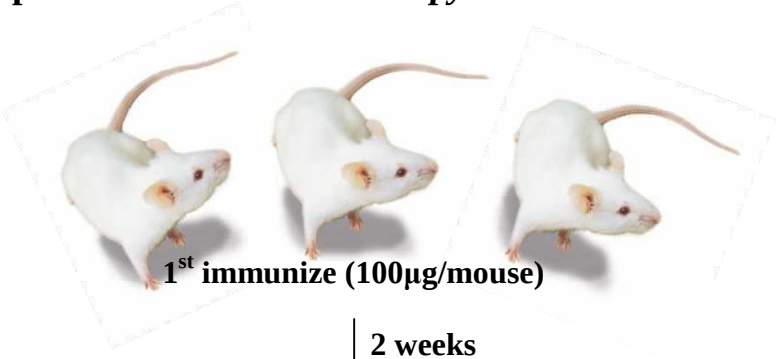
3rd boost (1mg/mouse)

↓

Experiment

Six- to eight-weeks old female BALB/c mice were purchased from the National Laboratory Center and housed in a temperature- and light-controlled room (12L:12D) at the Animal Maintenance Facility of National Chiao Tung University. The mice were first immunized by subcutaneously (s.c.) injection of 1mg/100µl BSA emulsified in CFA. The mice were boosted by subcutaneously (s.c.) injection of 1mg/100µl BSA emulsified in IFA, and all mice were sacrificed postchallenge. The experiment strategy of mice immunization followed above the protocol. The negative group was injected with 100 µl PBS alone.

Heat shock protein 60 of *Helicobacter pylori*



1st immunize (100μg/mouse)

↓ **2 weeks**

1st boost (100μg/mouse)

↓ **1 week**

2nd boost (100μg/mouse)

↓ **1 week**

3rd boost (100μg/mouse)

↓

Experiment

Six- to eight-weeks old female BALB/c mice were purchased from the National Laboratory Center and housed in a temperature- and light-controlled room (12L:12D) at the Animal Maintenance Facility of National Chiao Tung University. The mice were first immunized by subcutaneously (s.c.) injection of 100μg/300μl HpHsp60 emulsified in CFA. The mice were boosted by subcutaneously (s.c.) injection of 100μg/300μl HpHsp60 emulsified in IFA, and all mice were sacrificed postchallenge. The experiment strategy of mice immunization followed above the protocol. The negative group was injected with 300 μl PBS alone.

HPV E7 epitope

1st immunize (30μg /mouse)

↓ **2 weeks**

1st boost (30μg /mouse)

↓ **1 week**

Experiment

Six-eight weeks female C57BL/6-Tg(HLA-A2.1) mice were kindly provided from Dr. Shih-Jen Liu (National Health Research Institutes) and housed in a temperature- and light-controlled room (12L:12D) at the Animal Maintenance Facility of National Chiao Tung University. The mice were first immunized by subcutaneously (s.c.) injection of 30μg/100μl YML peptides emulsified in CFA. The mice were boosted by subcutaneously (s.c.) injection of 30μg/100μl YML peptides emulsified in IFA, and all mice were sacrificed postchallenge. The experiment strategy of mice immunization followed above the protocol. The negative group was injected with 100 μl PBS alone.

2.2.13 The enhancement of antigen presentation of APCs by LPPC

Cell proliferation

First, 100 μg BSA proteins were adsorbed by 40 μg LPPC into 100 μl volume. Splenocytes that isolated from were prior immunized by BSA (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates for monitoring cell proliferation. The 2.5 μl LPPC-complex co-cultured with splenocytes, and MTT assay was used to estimate at 72 hrs. The cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of splenocyte alone. Negative control was the splenocytes from naive mice.

Cytokines secretion

First, 100 μg BSA proteins were adsorbed by 40 μg LPPC into 100 μl volume. Splenocytes that isolated from were prior immunized by HpHSP60 (10^6 cells per well) were respectively dispensed into 24-well culture plates for monitoring cytokines secretion. The 10 μl LPPC-complex co-cultured with splenocytes, and the supernatants were collected at 24h and 72 h and frozen at −80 ℃. Supernatants concentrations of TNF-α, IL-2, IL-10, IL-4, and IFN-γ were measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*).

2.2.14 Membrane proteins with specific antigen coated LPPC

Cell proliferation

50 μg membrane proteins contained BSA or HpHSP60 antigens isolated from DCs and 100 μg BSA proteins were for 40 μg LPPC adsorption into 100 μl volume. Splenocytes that isolated from were prior

immunized by HpHSP60 (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates for monitoring cell proliferation. The 2.5 μl LPPC-complex co-cultured with splenocytes, and MTT assay was used to estimate at 72 hrs. The cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of splenocyte alone. Negative control was the splenocytes from naive mice.

Cytokines secretion

50 μg membrane proteins contained BSA or HpHSP60 antigens isolated from DCs and 100 μg BSA proteins were for 40 μg LPPC adsorption into 100 μl volume. Splenocytes that isolated from were prior immunized by HpHSP60 (10^6 cells per well) were respectively dispensed into 24-well culture plates for monitoring cytokines secretion. The 10 μl LPPC-complex co-cultured with splenocytes, and the supernatants were collected at 24h and 72 h and frozen at −80 ℃. Supernatants concentrations of TNF-α, IL-2, IL-10, IL-4, and IFN-γ were measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*).

2.2.15 The specific peptide-loaded HLA-A2 adsorbed on LPPC

Cell proliferation

50 μg YML peptide-loaded HLA-A2 molecules and 100 μg BSA proteins were for 40 μg LPPC adsorption into 100 μl volume. Splenocyte that immunized by YML antigen (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates for monitoring cell proliferation. The 2.5 μl LPPC-complex co-cultured with splenocytes, and MTT assay was used to estimate at 72 hrs. The cell proliferation rate

was calculated as O.D. value of sample divide into O.D. value of splenocyte alone. Negative control was the splenocytes from naive mice.

Cytokine secretion

50 μg YML peptide-loaded HLA-A2 molecules and 100 μg BSA proteins were for 40 μg LPPC adsorption into 100 μl volume. Splenocyte that prior immunized by YML antigen (10^6 cells per well) were respectively dispensed into 24-well culture plates for monitoring cytokines secretion. The 10 μl LPPC-complex co-cultured with splenocytes, and the supernatants were collected at 24h and 72 h and frozen at −80 ℃. Supernatants concentrations of TNF-α, IL-2, and IFN-γ were measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*).

2.2.16 The animal immunization of the immuno-LPPC *in vivo*

Six- to eight-weeks old female Balb/c mice were purchased from the National Laboratory Center and housed in a temperature- and light-controlled room (12L:12D) at the Animal Maintenance Facility of National Chiao Tung University. The 200μg LPPC previously adsorbed 250 μg peptide-loaded membrane proteins. And then the mice were immunized by intravenous (i.v.) injection of membrane proteins /LPPC complex and all mice were sacrificed after two weeks. The negative group was injected with 300 μl PBS alone.

Six-eight week female C57BL/6-Tg(HLA-A2.1) mice were kindly provided from Dr. Shih-Jen Liu (National Health Research Institutes) and housed in a temperature- and light-controlled room (12L:12D) at the Animal Maintenance Facility of National Chiao Tung University. The

200μg LPPC previously adsorbed 25 μg peptide-loaded HLA-A2 molecules and 25 μg anti-CD28 mAb. And then the mice were immunized by intravenous (i.v.) injection of LPPC complex and all mice were sacrificed after two weeks. The negative group was injected with 300 μl PBS alone.

2.2.17 The animal immunization efficiency of immuno-LPPC *in vivo*

Cell proliferation

Splenocytes that isolated from were prior immunized by immuno-LPPC or PBS (2.5×10^5 cells per well) were respectively dispensed into 96-well culture plates for monitoring cell proliferation. HpHsp60 or YML peptides (2μg/ml) were co-cultured with splenocytes, and MTT assay was used to estimate at 72 hrs. The cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of splenocyte alone. Negative control was the splenocytes from naive mice.

Cytokine secretion

Splenocytes that isolated from were prior immunized by immuno-LPPC or PBS (10^6 cells per well) were respectively dispensed into 24-well culture plates for monitoring cytokines secretion. HpHsp60 or YML peptides (2μg/ml) were co-cultured with splenocytes, and the supernatants were collected at 24h, 48 h and 72 h and frozen at −80 ℃. Supernatants concentrations of TNF-α, IL-2, IL-10, IL-4, or IFN-γ were measured by Enzyme-Linked ImmunoSorbent Assay (*ELISA*).

2.2.18 Statistical analysis

All figures are expressed as mean ± SD. All data were computed by student-test. All statistical significant was set at $p < 0.05$.

Chapter 3 Result

3.1 The characters of LPPC

As figure 1a shown, the shape of LPPC was approximately round and the particle size was about 200 nm. In addition, the dark shadow of LPPC was hair-like, which might be PEI and PEG polymers. LPPC is a cationic liposome, and it was found that LPPC can adsorb proteins on its surface. Therefore, DLS was utilized to investigate the particle size of LPPC with or without protein adsorption. The results showed that the diameters of LPPCs with protein adsorption were about 358 ± 16 nm, which was larger than the LPPC without protein adsorption (Figure 1b). Besides, the previous results have shown the empty LPPCs can be centrifuged and pelleted (Figure 1c) and the further experiments also indicated the protein adsorption did not affect this character. Because centrifugation is available for LPPC, unbound substances could be easily removed.

3.2 The characters of LPPC for protein adsorption

To understand the kinetic for protein adsorption to LPPC, the fluorescence of BSA-FITC was used to evaluate what time the LPPC need to adsorb proteins to their surface. The results showed that LPPC could adsorb 80% of proteins in ten minutes and reach the maximal adsorption in 20 minutes (Figure 2a). Moreover, the protein binding capacity of LPPC was estimated and the results revealed that the maximal adsorption of 40 μg LPPC was about 160 μg BSA (Figure 2b).

Surprisingly, the pre-adsorbed BSA-FITC on LPPC could not be replaced by the additions of different BSA dose (Figure 2c). The results showed that the pre-adsorbed proteins on LPPC were irreplaceable by the posterior added proteins.

3.3 The activities of immunostimulatory monoclonal antibodies adsorbed on LPPC

3.3.1 The cytotoxicity of LPPC to PBMCs or splenocytes

LPPC could adsorb proteins stably and remain their activities as previous experiments (Table 1). Therefore, to investigate whether LPPC could adsorb immunostimulatory monoclonal antibodies and stimulate immunity was further proceeded. First, the cytotoxicity of LPPC was determined for PBMCs or splenocytes at next experiment. The results indicated that 1 μg LPPC was an appropriate dosage for 10^5 PBMCs or 2.5×10^5 splenocytes, because the cells could survive without toxic damage in this concentration (Figure 3).

3.3.2 The effects of the bound antibodies in a dosage-dependent manner

In this study, anti-CD3 and anti-CD28 monoclonal antibodies (mAbs) were utilized to activate T cell, which were used to determine whether the bound protein on LPPC could remain its biofunction. In order to understand the regulatory phenomenon of monoclonal antibodies on LPPC for activities, the different amounts of immunostimulatory mAbs were adsorbed on LPPC to stimulate the proliferation of PBMCs or

splenocytes. The results showed the cell proliferation rates of PBMCs and splenocytes were higher as anti-CD3 mAbs were increased. It would be more obvious when the anti-CD3 mAbs combined with anti-CD28 mAbs to work on immune cells (Figure 4). Therefore, the bound antibodies on LPPC could activate the cell in a dosage-dependent manner.

Further, whether the secretions of cytokines were triggered by the bound mAbs on LPPC in a dose-dependent manner was investigated and the results indicated that LPPC and the adsorbed immunostimulatory mAbs could stimulate PBMCs or splenocytes to secrete cytokines, such as IL-2, IFN-γ and TNF-α. In addition, the concentrations of cytokines in media were increased as the anti-CD3 mAbs were increased (Figure 5). Moreover, the expressions of cytokines were increased by the addition of anti-CD28 mAbs which could provide the costimulatory signal to enhance the T-cell response as previously reported. Besides, LPPC alone could activate TNF-α secretion of PBMCs and splenocytes but it could not trigger any the IL-2 and IFN-γ secretions (Figure 5). Therefore, the LPPC reagent was investigated further to analyze whether the inductions of other pro-inflammatory cytokine profiles were. The results showed that the LPPC could stimulate IL-1β, IL-6 and IL-8 secretions of immune cells, except for TNF-α expression (Figure 6).

Comparing to the activities of unbound mAbs, the bound mAbs showed there were no significant differences between the cell proliferation and cytokine secretions (IL-2, IFN-γ) (Figure 7). Nevertheless, LPPC with mAbs could enhance TNF-α secretion comparing to unbound mAbs, it should be due to the LPPC's ability to facilitate the TNF-α secretion.

3.4 The stability of immunostimulatory monoclonal antibodies adsorbed on LPPC in RPMI

To investigate the stability of the bound mAbs on LPPC in 37℃, the activities of the dissociated antibodies in the medium were estimated. The results indicated that the antibodies on LPPC pellet remained their partial activities after 37℃ treatment to induce 90% proliferation for PBMCs or splenocytes (Figure 8). In addition, the antibodies in supernatant only displayed low activities to cause inferior proliferative index for PBMCs or splenocytes. These results revealed that the antibodies bound on LPPC would rather stably adhere than dissociate from the particle.

3.5 The enhancement of uptake protein ability by LPPC

The previous results indicated that LPPC alone has the ability to enhance pro-inflammatory cytokine secretions of PBMCs and splenocytes (Figure 5, 6). To understand whether LPPC has an adjuvant effect for the enhancement of antigen uptake by phagocytosis, the phagocytic rate for the fluorescent antigen was evaluated. As to the ability of phagocytosis, LPPC/BSA-FITC complexes indeed enhanced the uptake ability of P338D1 compared to BSA-FITC without LPPC adsorption (Figure 9a). In addition, as the additional amounts of LPPC were increasing, the uptake abilities were enhanced with the more efficiency (Figure 9b) and the results also showed that the phagocytic rates of P338D1 were in a dose-dependent manner (Figure 9c).

As far as the ability of presentation is also concerned, figure 10

showed that the addition of BSA alone could be internalized by APC and present to the specific anti-BSA T-cells, which could trigger and increase the cell proliferation and cytokine secretions (including IL-2, IFN-γ, TNF-α, IL-4 and IL-10). By contrast, the results indicated that BSA adsorbed on LPPC could provide more efficacies to induce cell proliferation and cytokine secretions than BSA without LPPC adsorption (Figure 10).

3.6 The specificities of the LPPC-bound proteins

Certain immunostimulatory molecules on cell membrane, such as MHC or B7 molecules could trigger the specific immune response, so that DCs' proteins on plasma membrane were determined whether they could perform their activities on LPPC surface as same as on the plasma membrane. Before isolated the plasma membrane proteins of DCs, anti-CD11c-PE, anti-MHC II-FITC, and anti-CD86-FITC were used to confirm that the surface markers of DCs performed (Figure 11). The membrane proteins derived from the DCs which were treated with HpHsp60 were bound to LPPC, which could react with the splenocytes derived from the mice had been prior immunized with HpHsp60 to induce the cell proliferation and cytokine secretions (Figure 12). However, neither the splenocytes without Hphsp60-immunized nor the DC's membrane proteins without Hphsp60-treated could induce the cell proliferation or cytokine releases (Figure 12).

Moreover, YMLDLQPETT peptides (YML) derived from HPV E7 protein were loaded into the HLA-A2 molecules to verify the specificity of the LPPC-bound proteins again. The YML-loaded HLA-A2 molecules

on LPPC were interacted with the splenocytes derived from naïve or pre-immunizing E7 mice. The results showed that peptide-loaded HLA-A2 molecules on LPPC indeed remained their specificities to activate the splenocytes of pre-immunizing E7 mice and cause the cell proliferation and cytokine expressions but did not react with the naïve splenocytes (Figure 13). In addition, the anti-CD28 mAbs could facilitate the immune responses for the splenocytes of pre-immunizing E7 mice but have no effect on the naïve splenocytes (Figure 13).

3.7 The efficiency of immunization *in vivo*

The cell proliferations and cytokine expressions of the splenocytes which were i.v. immunized by immuno-LPPC estimated whether the induction of specific immune responses. The induction of the specific anti-HpHsp60 immune responses of splenocytes from membrane proteins /LPPC complex immunized was more efficient than that from HpHsp60 antigen (Figure 14). In addition, the results indicated that the splenocytes form the other immunization methods did not significantly react to HpHsp60.

Furthermore, the splenocytes that the LPPC combined peptide-loaded HLA-A2 molecules with anti-CD28 mAbs immunized were efficiently activated immune responses against YML peptides, such as cell proliferations and cytokine secretions (Figure 15). No apparent immune responses were observed against YML peptides in the other immunization methods.

Chapter 4 Discussion

To summarize, we have established a novel platform for immunoregulation which utilized the LPPC to combine with certain immunostimulatory molecules, such as mAbs and MHC-loaded peptides molecules, and in this study the LPPC display its potential to be an artificial APC. First, the LPPC is easily adsorbing a variety of immunostimulatory proteins and the LPPC-bound proteins can remain their activities. Second, the LPPC have an adjuvant effects to enhance proinflammatory cytokine expression, antigen uptake and presentation of APC. Furthermore, our study provided the evidence that the regulatory molecules/LPPC complexes dramatically increase the specific anti-antigen activities of immune responses *in vivo*. As understanding the mechanism about immune responses, it is proposed that certain immunostimulatory molecules could be applied in this platform to regulate immunity for disease therapy. For example, the combination of microbeads with HLA-A2 monomers, anti-4-1BB antibodies and anti-CD28 antibodies can expand large numbers of antigen specific CTLs (33). Moreover, the addition of anti-LFA-1 mAbs with anti-CD3, anti-CD28 could enhance the T-cell expansion for adoptive cell therapy (34). Therefore, certain ligands or costimulatory molecules might be utilized for adsorption of LPPC to regulate immunity as artificial APCs.

The major defect for DC-based therapy was that the maturation of DCs was weaken by certain immunosuppressive substances such as TGF-β, IL-10, or VEGF which derived from tumor cells (11, 25-27).

Moreover, *Candida albicans*, *Mycobacterium tuberculosis*, the secretions of mycobacterial, and the secretions of *Candida* respectively impact on efficiency of DCs and immune cells, affect cytokine expression and impair surface marker of DC (19-22). Many Comparing to our strategy, previous DC-based therapy strategies were DCs incubated with antigens such as tumor antigen HSP105, HPV E7 protein, breast tumor cell lysate, HIV, or SIV to successfully induce host immunity against tumor or pathogens (15, 16, 35-37). We modified these previous strategies in this study, the membrane proteins of DCs which were prior treated and up-take antigens (HpHsp60) were isolated and were adsorbed on LPPC, later the membrane antigen/LPPC complexes would be used as antigen to immunize animals. The results revealed the LPPC adsorbed membrane proteins could induce specific immune responses efficiently (Figure 13). The modified strategy excludes the usage of alive DC to prevent the above problem.

Moreover, immuno-molecules such MHC molecules are covalently coupled to beads or liposomes and used to expand CTL *ex vivo* (38, 39). However, such chemical modification might impair the original functions or activities of immunoregulatory or targeting molecules (40). In contrast, mAb non-covalently adsorbed on the nanoparticle surface could provide a better activity than covalent coupling (41). In this study, the LPPC provided an ability to strongly adsorb proteins on its surface, which was proposed that the activities of the bound immunoregulatory molecules could maintain higher activities without chemical damage. Moreover, figure 2c and figure 8 indicated that the pre-adsorbed proteins on the

surface of LPPC may be irreplaceable in serum without the dissociated doubt.

On the other hand, the results in this study indicated that the LPPC had the interesting characters, the effects of adjuvant. Figure 9 showed LPPC promoted antigen uptake ability of APC in a dose-dependent fashion. In addition, BSA adsorbed on the LPPC provided a better presentation of APC than free BSA to induce T-cell proliferations and cytokine secretions (Figure 10). Moreover, LPPC showed it could directly induced PBMCs or splenocytes to secret proinflammatory cytokines, IL-1β, IL-6, IL-8 or TNF-α. It is well-known that the function of the proinflammatory cytokines could recruit or activate macrophage or other immune cells (42). Similarly, the adjuvant MPL reagent, a liposome-like reagent could also improve the capacity of APCs by enhancements of antigen uptake and cytokine release (43). Furthermore, their primary adjuvant mechanism of cationic liposomes is to target to the membrane of APCs and to induce the uptake and increased presentation of antigen (44). Together these results indicated that the LPPC can be a good adjuvant.

In vivo experiments, the figure 13 and figure 14 showed that the proteins/ LPPC complexes induced a better specific immune response than free proteins of immunization. We proposed that the LPPC might provide a little positive charge to attract immune cells, and the higher density of the immunoregulatory molecules on the LPPC than free form of molecules might also provide good affinity. Moreover, the LPPC reagent had an adjuvant effect. Therefore, the LPPC should be a good

immune platform of enhancement of immune response.

Many investigators have started to develop artificial APCs and Dr. Mathias Oelke classified artificial APCs according to cell-based or non-cell-based in current approaches for immunotherapy (1). Early, murine MHC class I molecules with the costimulatory molecules B7-1 and ICAM-1 were co-expressed on drosophila cell to activate T cells, which resulted in specific killing for tumor cells *in vitro* and *in vivo* (45). However, the transfected drosophila cells were unstable in mouse body. Another cell-based approach was also used. The NIH/3T3 murine fibroblast cells were transduced with the costimulatory molecule B7-1, ICAM-1 and LFA-3, and a single HLA–peptide complex (HLA-A2) as APCs. These aAPCs have proved that they can induce specific CTLs to against tumor cells when the aAPC were pulsed with tumor antigens MART-1 or hTERT (29, 46). In acellular systems, magnetic beads were usually used to assemble as aAPCs, (31, 47, 48). In addition, latex microspheres (49) or liposomes (32) were also developed as aAPC. Generally, the immunoregulatory molecules on these aAPC are MHC molecules or immunoregulatory antibodies. For instance, HLA-A2–Ig molecules, biotinylated murine MHC class I or MHC class II-peptide molecules have been combined with costimulatory molecules such as anti-CD28 antibodies or B7 molecules, which were coupled on particle surface to induce and expand antigen-specific T cells (31, 32, 49).

In this study, the LPPC adsorbing with anti-CD3 and anti-CD28 mAbs or peptide–loaded HLA-A2 provided immunoregulatory function

to stimulate T cells, similar to other acellular-based APCs. But the difference between LPPC and other aAPC is that LPPCs do not need chemical modification and complicated purification. In addition, LPPC can also adsorb membrane proteins to trigger the specific T cells responses (Figure 11 and 13). As our knowledge, no other aAPC coupled with DC's membrane proteins to induce specific immunity. Moreover, figure 11 and 12 showed that neither DC's membrane proteins nor peptide-loaded HLA-A2 could induce stronger immune responses than these antigens alone except these molecules combined with LPPC. Figure 13 also revealed that LPPC/ DC's membrane proteins complex could enhance antigen-induced Th-1 cytokine releases but its abilities to induce Th-2 cytokine releases were as same as antigen alone immunization. Therefore, LPPC should be developed as a convenient and efficient immunoregulatory platform to mediate host immunity.

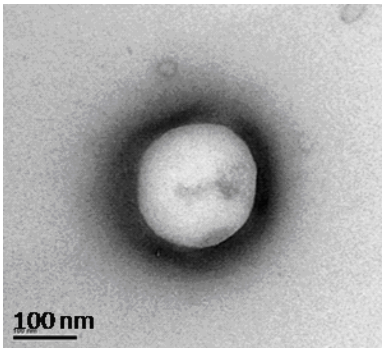
Interestingly, the results showed that LPPC alone could stimulate immune cells to release pro-inflammatory cytokines (Figure 5 and 6). Its mechanism may result from PEI, one component of LPPC, has been proved it was an adjuvant effects to enhance the expressions of both Th1 and Th2 cytokines (50). However, LPPC alone could only induce the proinflammatory cytokine releases including TNF-α (Figure 5), IL-1β, IL-6 and IL-8 (Figure 6) but it had no effects on the releases of Th-1 and Th-2 cytokines including IL-2 (Figure 5a and 5d) , IFN-γ (Figure 5b and 5e), IL-4 (Figure 10) and IL-10 (Figure 10). Because the proportion of PEI in LPPC is 3%, only 1.62 μg PEI would interact with immune cells in our study. In contrast, more than 33.75 μg PEI was used in reaction

(50), the difference between the dosages may result in different immunoregulation (51, 52). Except for the adjuvant effect, LPPC preferentially enhance Th-1 responses. Th-1 responses such as Th1-$CD4^{+}$T cell or CTL have been demonstrated that they are important and necessary against malignant tumors or intracellular pathogens. Thus, the scientists attempted to design the strategies of vaccination by enhancement of Th-1 immunity (53-55). According to our results, LPPC seem to be a good adjuvant which can be developed as the vaccines against tumor or intracellular pathogens.

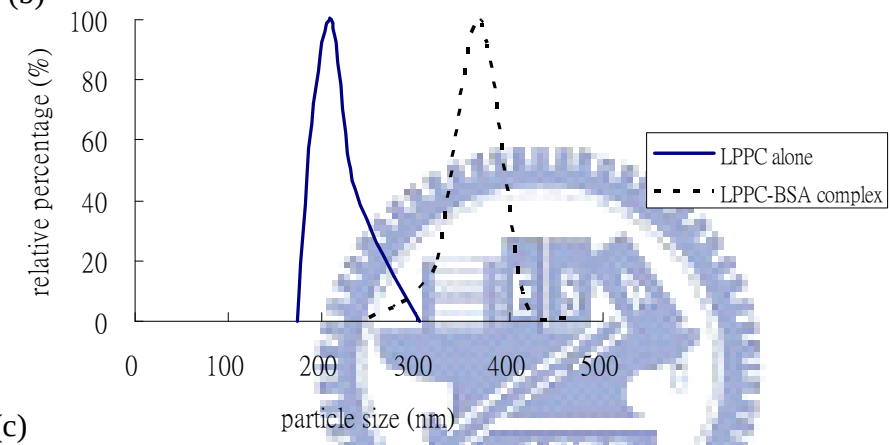
In conclusion, we demonstrated that LPPC could show its flexible and convenient characters as an artificial APC that stimulate immune responses and induce specific immunity *in vitro* and *in vivo*. The immuno-LPPC has immuno-function by adsorbing certain immunoregulatory molecules. Except activation of immunity, the LPPC may also combine with immunosuppressive molecules or apoptosis ligand, such as CTLA-4 and FasL, to provide different function to suppress abnormal immunity (11, 56). In the future, the immuno-LPPC may have broad functional immuno-regulatory abilities to treat human diseases by mediating host immunity.

Figure 1

(a)


100 nm

(b)


relative percentage (%)
100
80
60
40
20
0
0
100
200
300
400
500
particle size (nm)
LPPC alone
LPPC-BSA complex

(c)

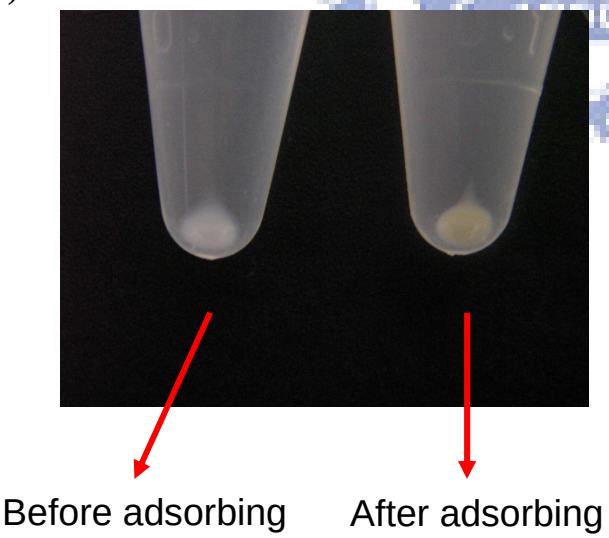
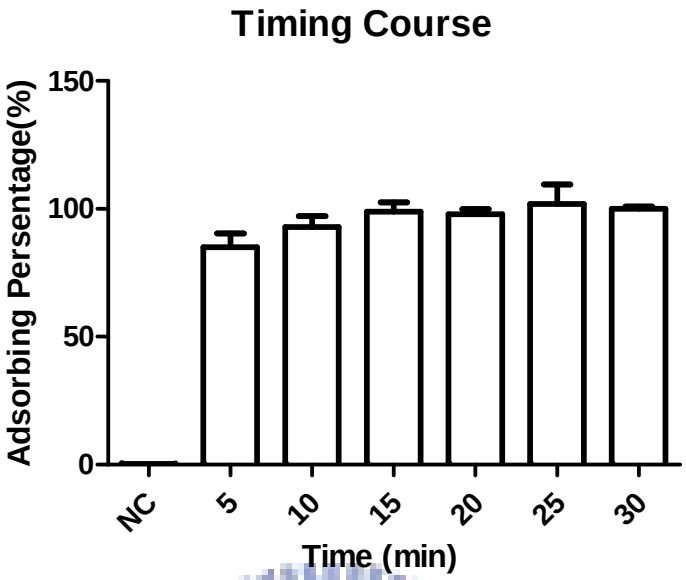

Before adsorbing
After adsorbing

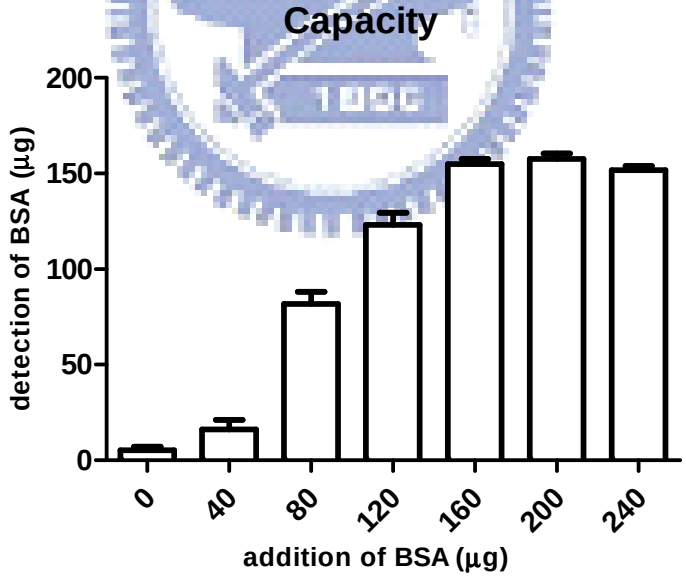
Figure 1. The characters of LPPC. (a) The TEM photo of LPPC; (b) The particle size distribution of LPPC with proteins or without proteins were estimated by utilizing DLS. The average particle size of LPPC without BSA proteins was about 216 ± 4 nm, and the average particle size of LPPC with BSA proteins was about 358 ± 16 nm; (c) The centrifuged property of LPPC with or without proteins.

Figure 2

(a)


Timing Course
Adsorbing Persentage(%)
150
100
50
0
NC
5
10
15
20
25
30
Time (min)

(b)


Capacity
detection of BSA (µg)
200
150
100
50
0
0
40
80
120
160
200
240
addition of BSA (µg)

(c)

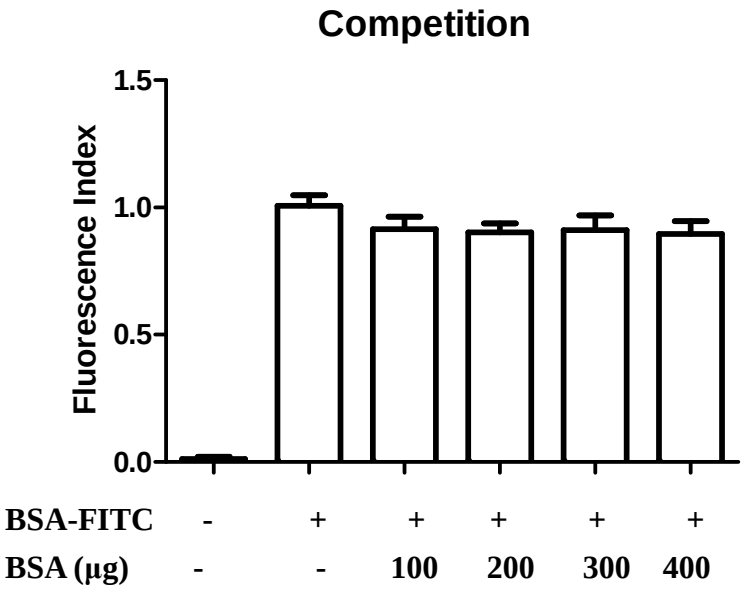


Figure 2. The properties of LPPC adsorption. (a) The time consumption that LPPC adsorbed BSA-FITC by utilizing spectrofluorometer, and twenty minutes was determined for completely adsorption. (b) To understand the quantity of BSA could be adsorbed by LPPC, added different amount of proteins into LPPC solution. The result show that 160 μg BSA was probably the maximal capacity of 40 μg LPPC. (c) Added a great quantity of BSA for competition 50μg BSA-FITC previously adsorbed on 40 μg LPPC, and the result showed that fluorescence index was no significant difference even added 400μg BSA. Results are representative at least two independent experiment.

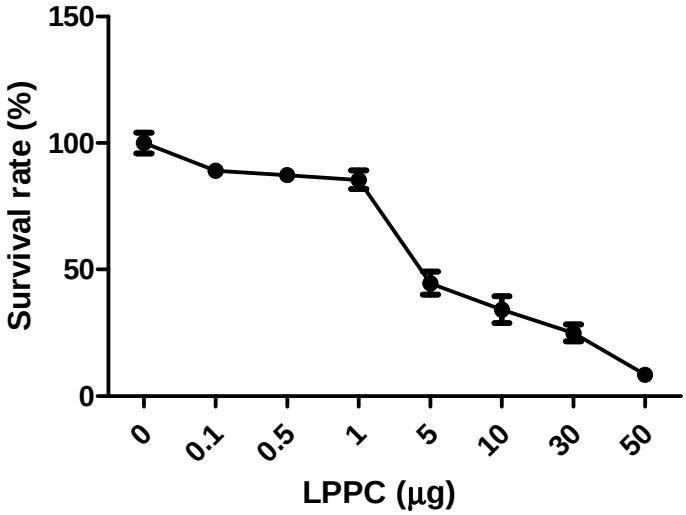
Table 1

Proteins	Assay method
Bovine serum albumin (BSA)	Coomassie Plus Reagent
FITC-conjugated BSA	Spectrofluorometer
Beta-galactosidase	Enzyme activity (PNPG)
HRP-conjugated Antibody	Enzyme activity (TMB solution)
Hp Hsp60	OD 280
Urease B	OD 280

Table 1. The various proteins were adsorbed by LPPC. Added different kinds of proteins into LPPC solution, and centrifuged to estimate various proteins whether adsorbed on LPPC or not. The results were cited from Yen-Ku Liu (NCTU).

Figure 3

(a) PBMC



(b) Splenocyte

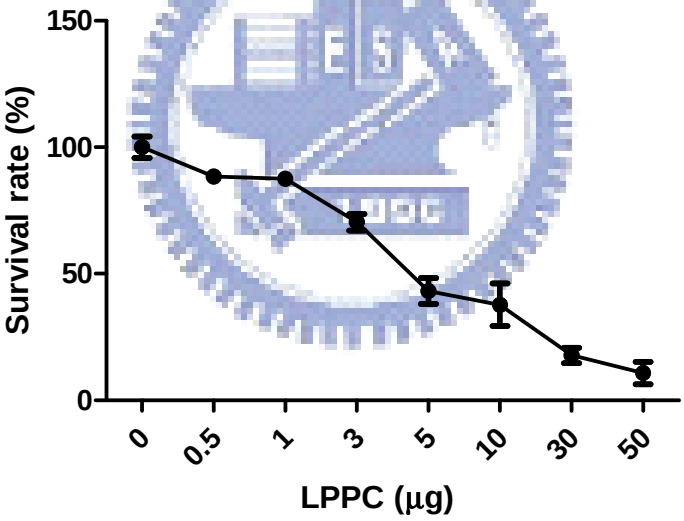
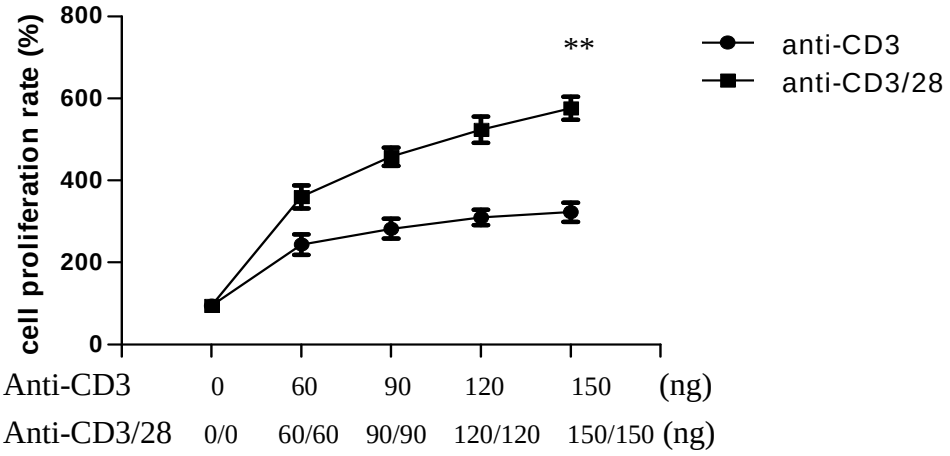


Figure 3. The cytotoxicity of LPPC for PBMC and Splenocyte .
Different amounts of LPPC were respectively added into growth medium containing 10^5 PBMC (a) or 2.5×10^5 splenocytes (b), and incubated in 96-well plate at 72 h. Utilized MTT assay to determine what dosage of LPPC treated immune cells without influenced cells survival. The results showed that 1 μg LPPC was an appropriate amount for PBMC and splenocytes next experiment. Results are representative at least three independent experiment.

Figure 4

(a) PBMC



(b) Splenocyte

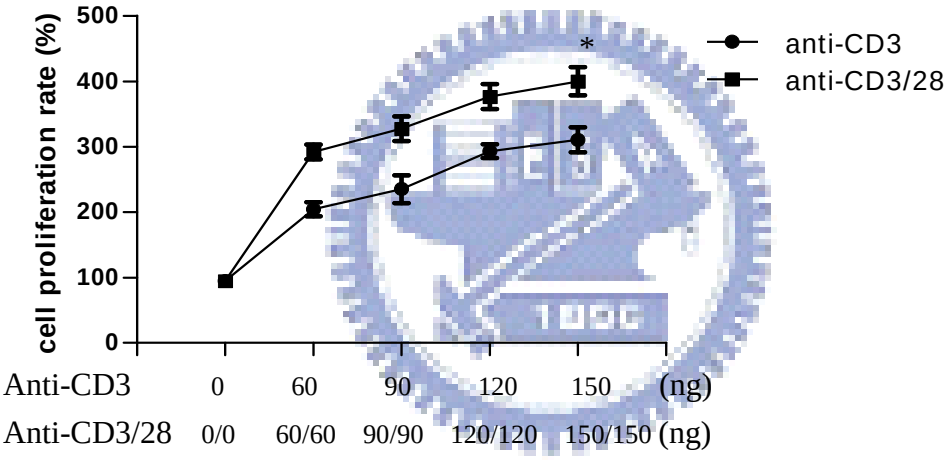
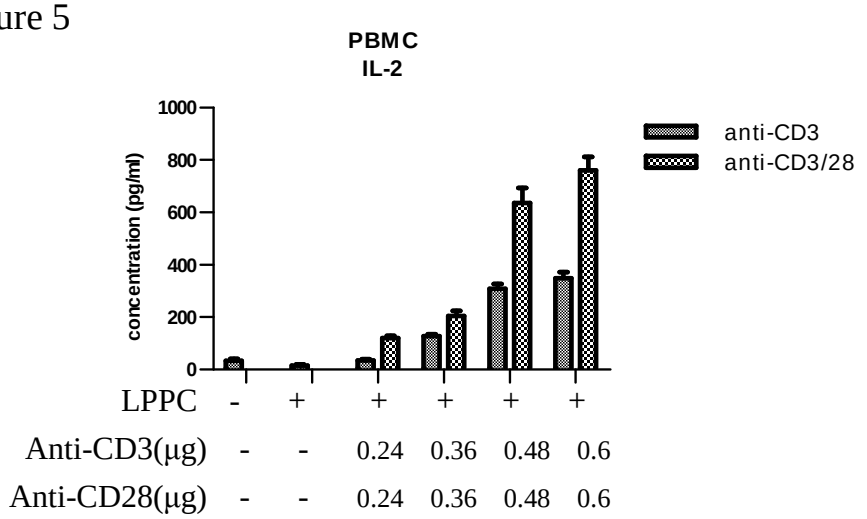


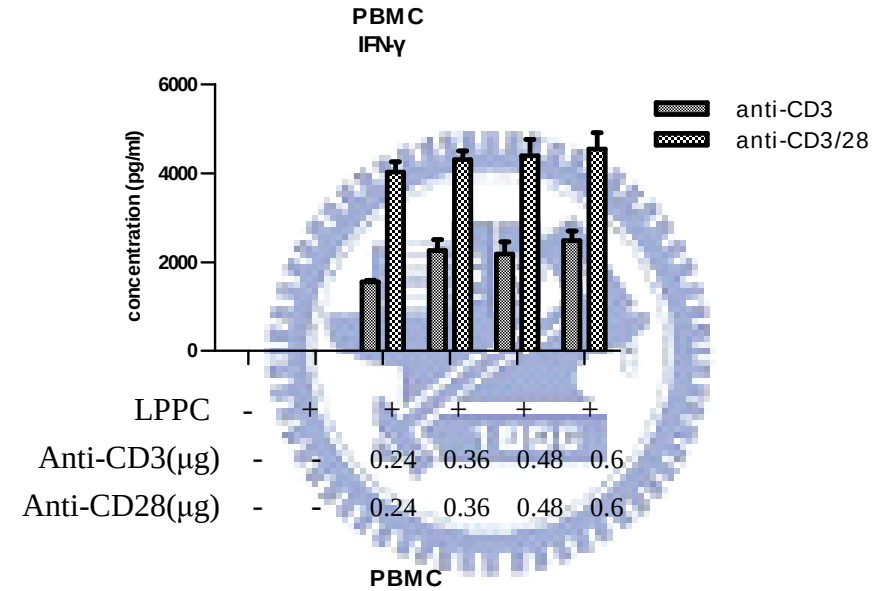
Figure 4. The dose-dependent effect of cell proliferations was stimulated by immunostimulatory monoclonal antibodies on LPPC. For comprehending whether LPPC adsorbed monoclonal antibodies could stimulate immune cells proliferation. Addition different amounts of immunostimulatory monoclonal antibodies were combined with 1 μg LPPC to stimulate the proliferation of immune cells, and the cell numbers was counted by MTT assay. The cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of PBMC alone or splenocyte alone. As the result showed, more amounts of anti-CD3 monoclonal antibody were added, the cell numbers of PBMC (a) and splenocytes (b) were larger, especially combined with anti-CD28 monoclonal antibody. Results are representative at least three independent experiment. (*：$P<0.05$; **：$P<0.01$)

Figure 5

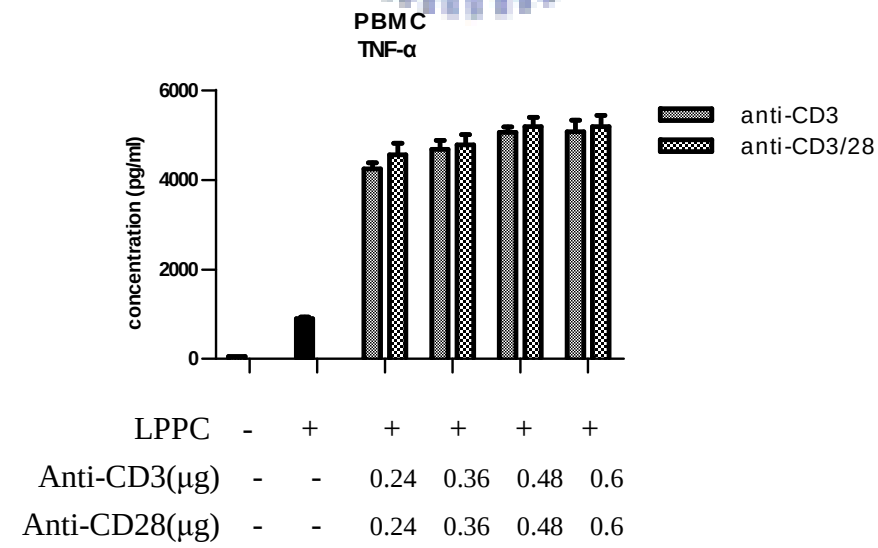
(a)


PBMC
IL-2
concentration (pg/ml)
1000
800
600
400
200
0
anti-CD3
anti-CD3/28
LPPC - + + + + +
Anti-CD3(μg) - - 0.24 0.36 0.48 0.6
Anti-CD28(μg) - - 0.24 0.36 0.48 0.6

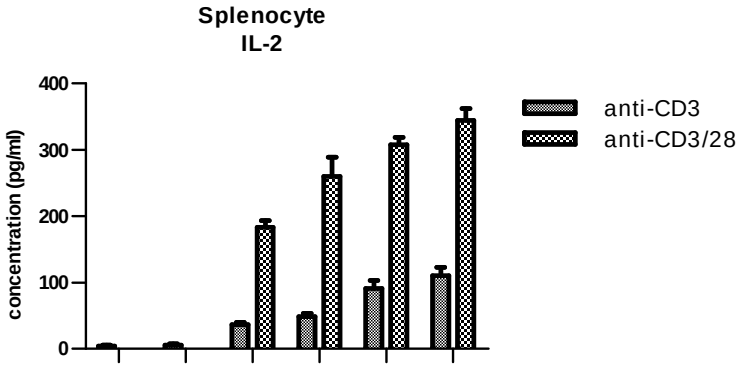
(b)


PBMC
IFN-γ
concentration (pg/ml)
6000
4000
2000
0
anti-CD3
anti-CD3/28
LPPC - + + + + +
Anti-CD3(μg) - - 0.24 0.36 0.48 0.6
Anti-CD28(μg) - - 0.24 0.36 0.48 0.6

(c)

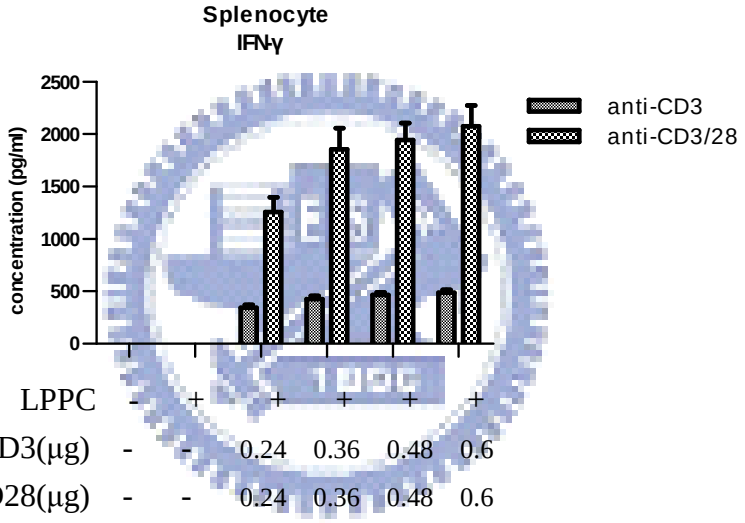

PBMC
TNF-α
concentration (pg/ml)
6000
4000
2000
0
anti-CD3
anti-CD3/28
LPPC - + + + + +
Anti-CD3(μg) - - 0.24 0.36 0.48 0.6
Anti-CD28(μg) - - 0.24 0.36 0.48 0.6

(d)



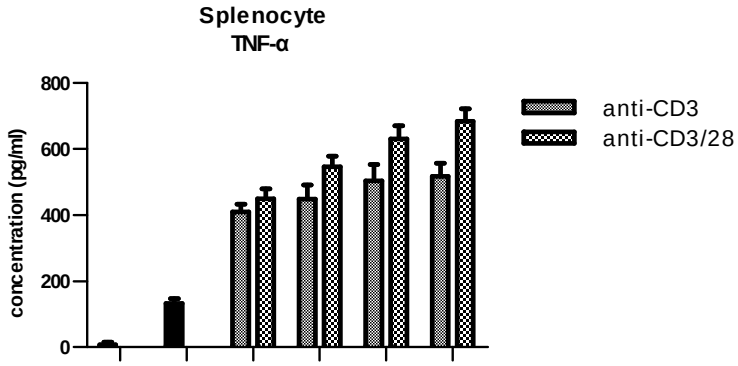
LPPC	-	+	+	+	+	+
Anti-CD3(μg)	-	-	0.24	0.36	0.48	0.6
Anti-CD28(μg)	-	-	0.24	0.36	0.48	0.6

(e)



LPPC	-	+	+	+	+	+
Anti-CD3(μg)	-	-	0.24	0.36	0.48	0.6
Anti-CD28(μg)	-	-	0.24	0.36	0.48	0.6

(f)



LPPC	-	+	+	+	+	+
Anti-CD3(μg)	-	-	0.24	0.36	0.48	0.6
Anti-CD28(μg)	-	-	0.24	0.36	0.48	0.6

Figure 5. The dose-dependent fashion of cytokine profiles secretion was activated by monoclonal antibodies on LPPC. Combined different amounts of immunostimulatory monoclonal antibodies with 4 μg LPPC to treat PBMC or splenocytes, and the cytokines in culture supernatants were measured by ELISA. The results indicated that LPPC adsorbed immunostimulatory monoclonal antibodies could stimulate immune cells' secretion of cytokines, such as IL-2, IFN-γ and TNF-α. And the more dosage of antibodies was added, the higher the concentration of cytokines was monitored. Besides, LPPC alone could activate PBMCs and splenocytes TNF-α cytokine secretion. Results are representative at least three independent experiment.

Figure 6

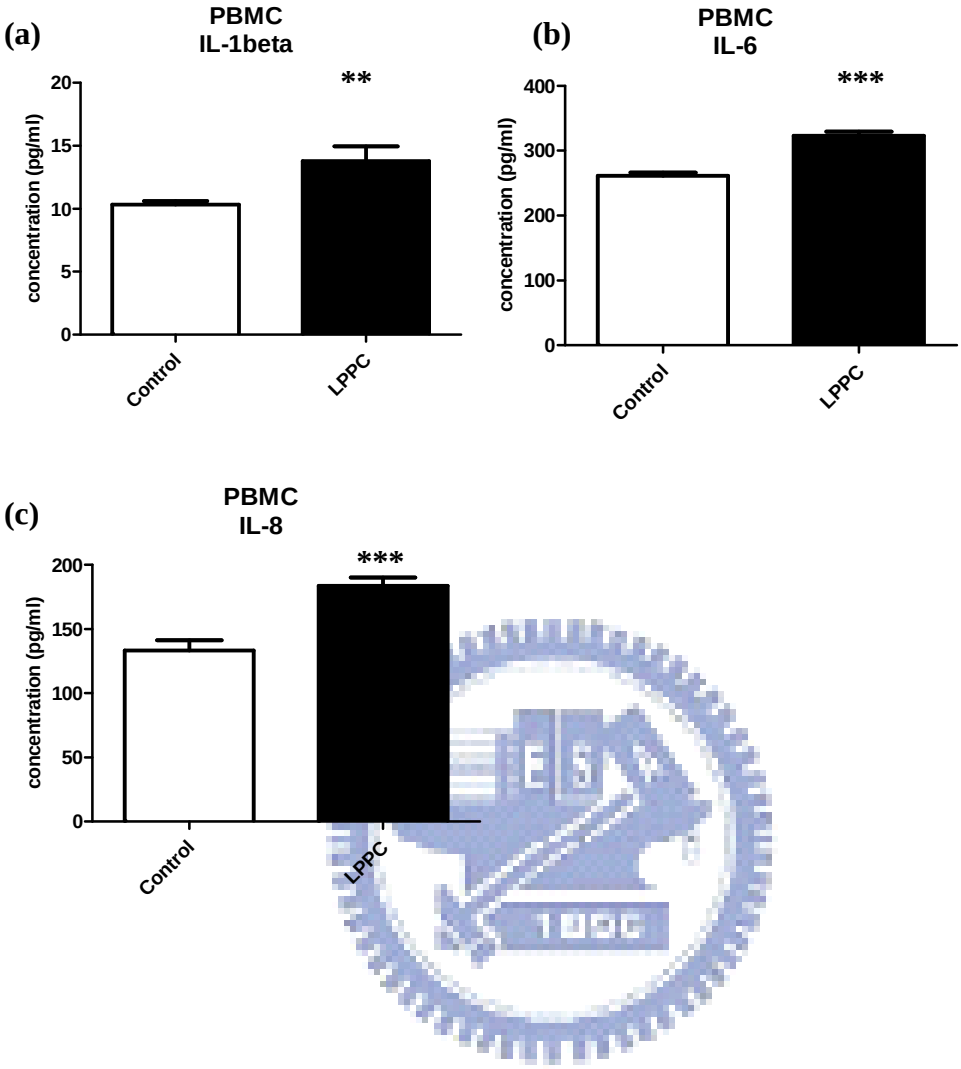


Figure 6. The induction of pro-inflammatory cytokine profiles was by the LPPC. Addition of 4 μg LPPC treated 4 ×10^5 PBMC/well in 24-well plate, and the cytokines in culture supernatants were collected to measure by ELISA. The results indicated that the LPPC could stimulate immune cells' secretion of cytokines, such as IL-1β, IL-6 and IL-8. Results are representative at least three independent experiment. (** : P<0.01; *** : P<0.001)

Table 2

(pg / ml)

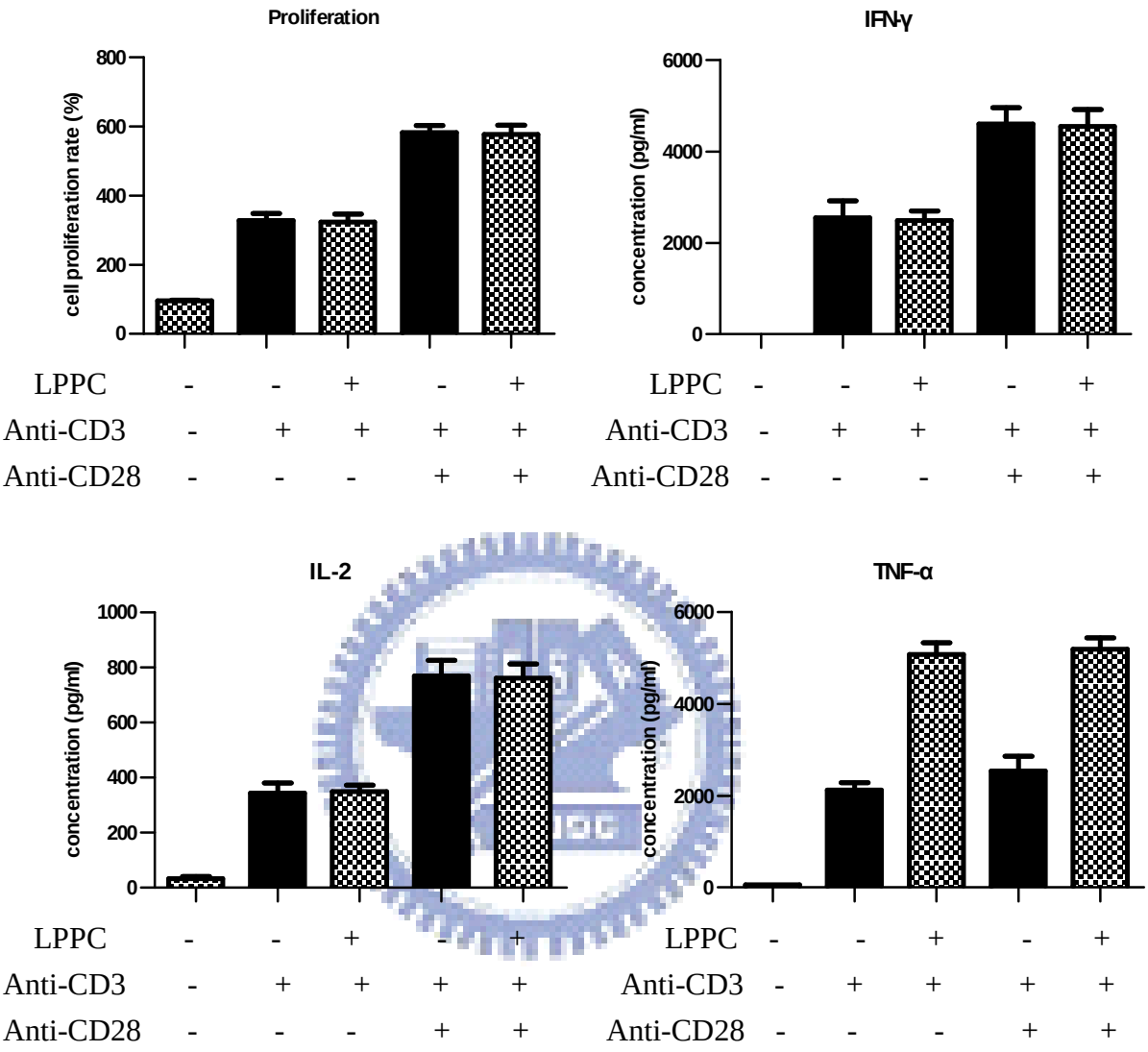
Cytokine / Immune cells		TNF-α	IL-1β	IL-6	IL-8
PBMC (±LPPC)	-	**59 ±7**	**10 ±2**	**260 ±12**	**133 ±19**
	+	**900 ±66 (P<0.0001)**	**14 ±2 ****	**323 ±16*****	**184 ±15*****
Splenocyte (±LPPC)	-	**8 ±5**	**2 ±2**	---	---
	+	**133 ±13*****	**28 ±4 ****	---	---

Cytokine / Immune cells		IL-2	IFN-γ	IL-4	IL-10
PBMC (±LPPC)	-	**32 ±5**	**0 ±2**	---	---
	+	**15 ±3**	**0 ±2**	---	---
Splenocyte (±LPPC)	-	**4 ±2**	**0 ±1**	**2 ±3**	**13 ±4**
	+	**7 ±2**	**0 ±3**	**10 ±5**	**21 ±5**

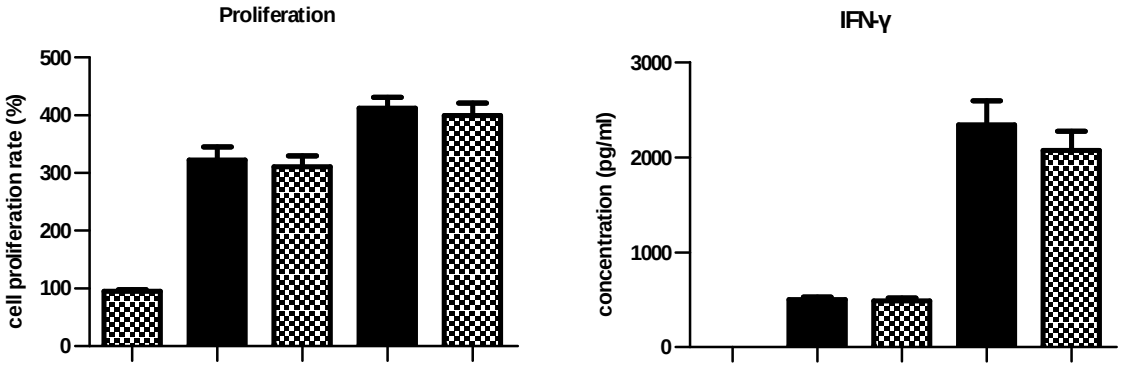
Table 2. The induction of cytokine profiles was by the LPPC. The LPPC could induce the pro-inflammatory cytokine secretions (TNF-α, IL-1β, IL-6, or IL-8), but not Th1 and Th2 related cytokine expressions. (**：P<0.01; ***：P<0.001)

Figure 7

(a) PBMC



(b) Splenocyte



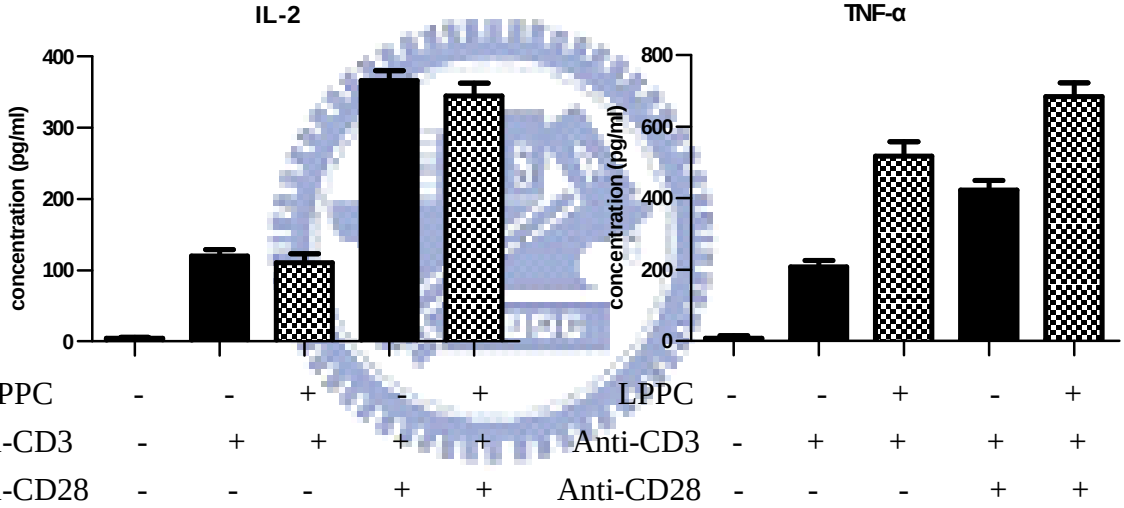
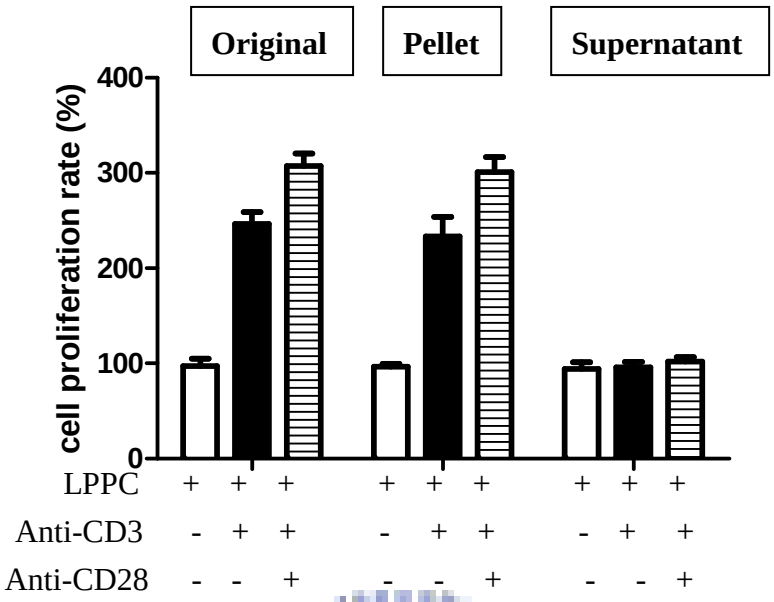


Figure 7. The activities of immunostimulatory monoclonal antibodies on LPPC. LPPC might adsorb the immunostimulatory monoclonal antibodies by electric force and non-covalent force. To compare the activities of monoclonal antibodies with LPPC complexes or without LPPC, the cell proliferations and cytokine secretions were estimated. The results indicated that the activities of monoclonal antibodies were no significant difference between them, such as cell proliferation, IL-2 and IFN-γ secretion. But, LPPC with monoclonal antibodies could enhance TNF-α secretion more than mAbs alone. Results are representative at least three independent experiment. (anti-hCD3 60ng/96well ; anti-hCD28 60ng/96well) (anti-mCD3 60ng/96well ; anti-mCD28 60ng/96well) (anti-hCD3 0.6μg/24well ; anti-hCD28 0.6μg/24well) (anti-mCD3 0.6μg/24well ; anti-mCD28 0.6μg/24well)

Figure 8

(a) PBMC



(b) Splenocyte

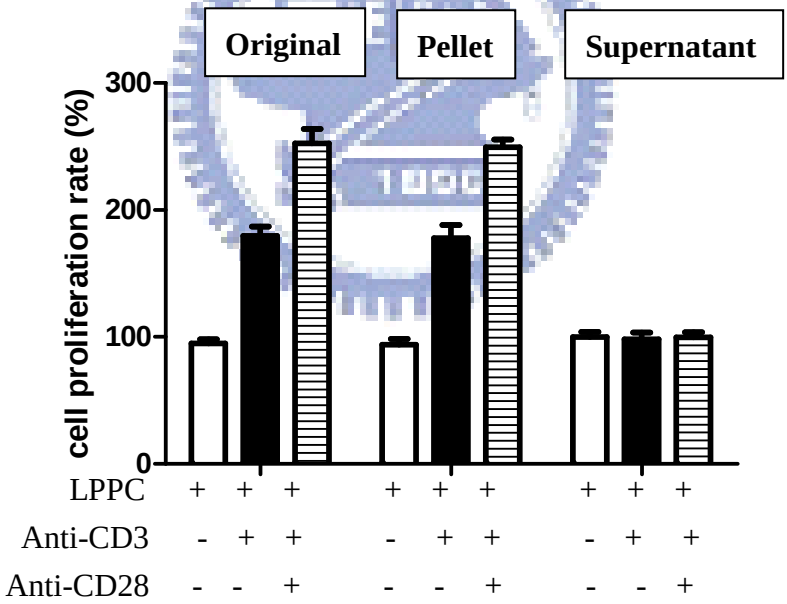
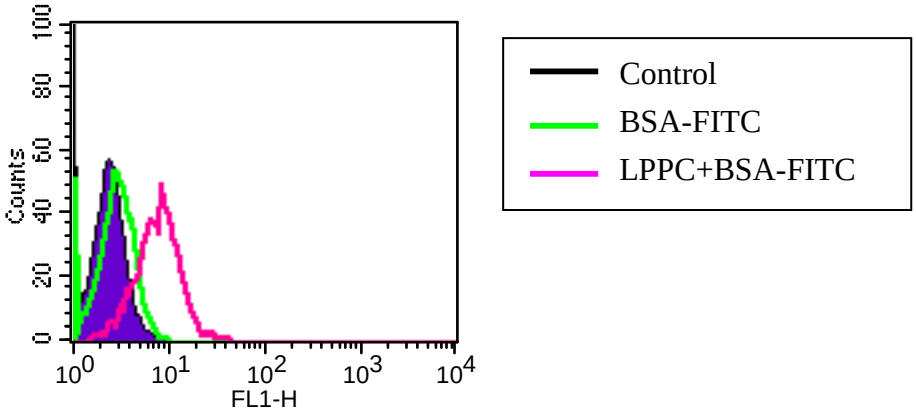


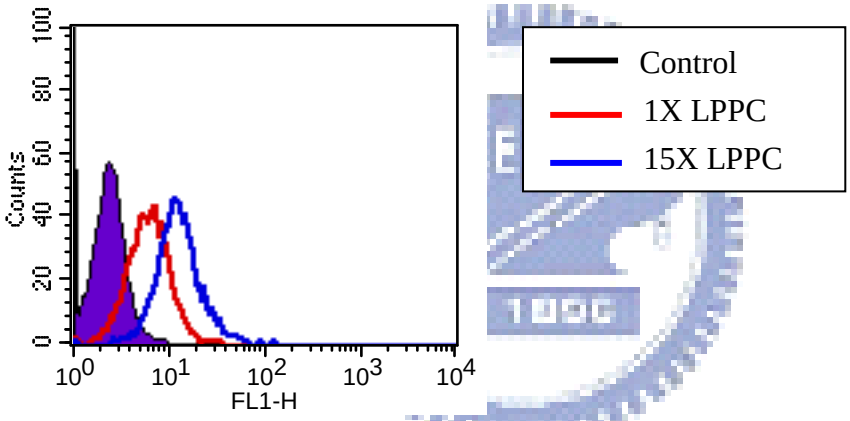
Figure 8. The stability of immunostimulatory monoclonal antibodies on LPPC in RPMI. LPPC previously adsorbed monoclonal antibodies, and then put it into RPMI solution in 37℃ for 30 minutes. After 30 minutes, the solution was centrifuged divide into LPPC pellet and the supernatant. The LPPC pellet and the supernatant respectively treated PBMCs or splenocytes, and estimated the cell proliferation for investigating the stability of monoclonal antibodies on LPPC. By using MTT assay, and then the cell proliferation rate was calculated as O.D. value of sample divide into O.D. value of PBMC alone or splenocyte alone. The results showed that approximately 90% activities of monoclonal antibodies in LPPC pellet, in contrast, the cell proliferation of the supernatant groups were no significant different comparing to the control group. Results are representative at least three independent experiment. (anti-hCD3 60ng ; anti-hCD28 60ng) (anti-mCD3 60ng ; anti-mCD28 60ng)

Figure 9

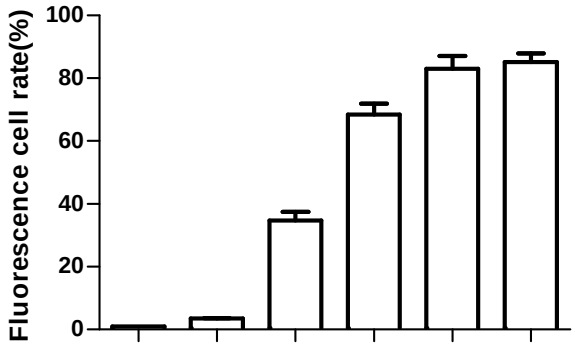
(a)



(b)



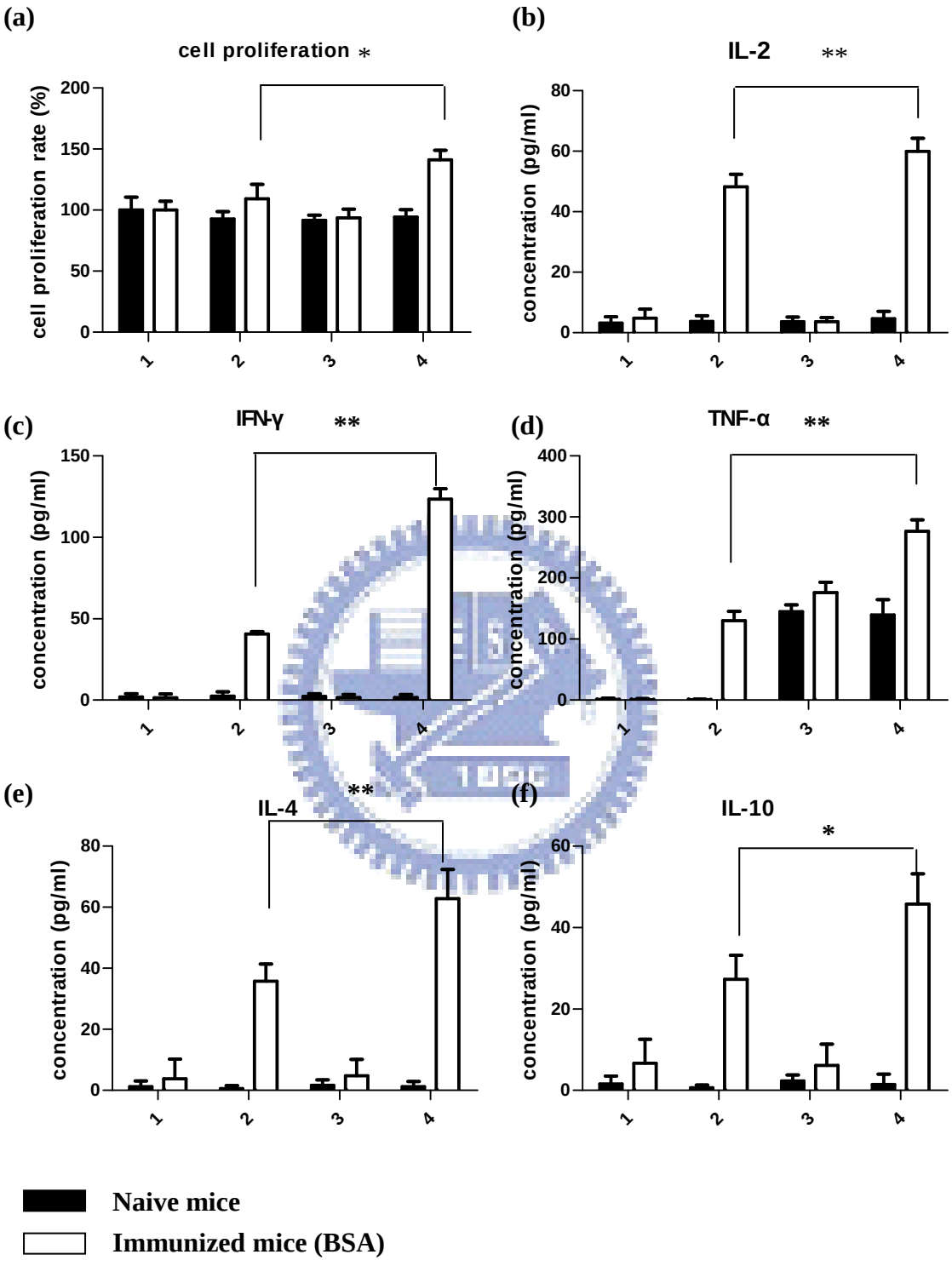
(c)



BSA-FITC	-	+	+	+	+	+
LPPC (μg)	-	-	**20**	**100**	**200**	**300**

Figure 9. The increase of cellular uptake of P338D1 was by the LPPC. To investigate that LPPC might have ability to enhance cellular uptake of antigen presenting cells. LPPC previously coated BSA-FITC co-incubated 5 $\times 10^5$ murine macrophages, P338D1, and then added trypan blue to quench the fluorescence of LPPC complexes without up-taken. (a) The result showed that added the same amounts of BSA-FITC, LPPC could enhance the uptake ability of P338D1. (b) The result indicated that the fluorescence expression of P338D1 from addition fifteen fold amounts of LPPC was higher than that addition one fold amount of LPPC. (c) The fluorescence cell rate of P338D1 was estimated by flow cytometer, and the data showed that LPPC could enhance uptake ability of P338D1 in a dose-dependent manner. Results are representative at least two independent experiment.

Figure 10



1. Splenocyte alone

2. Splenocyte + BSA

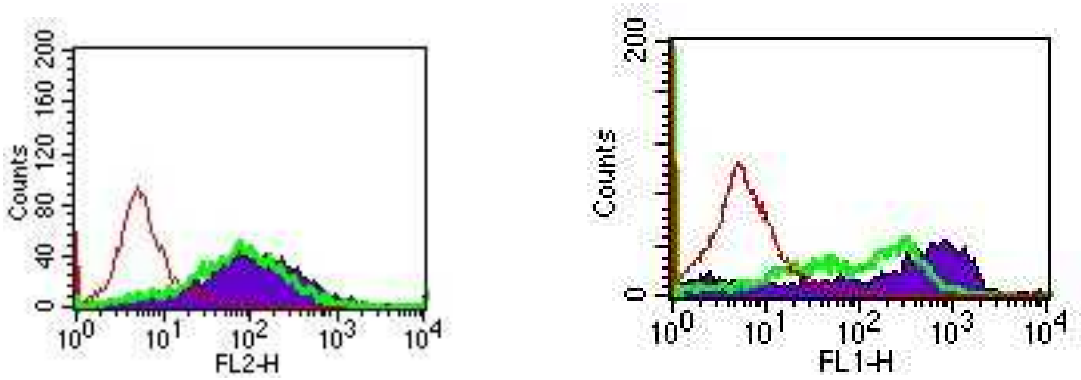
3. Splenocyte + LPPC

4. Splenocyte + LPPC-BSA

Figure 10. The enhancement of antigen presentation of APC was by the LPPC. To further understand whether the other adjuvant effect of the LPPC has, such as the enhancement of antigen presentation of APC, using BSA proteins were mimic as one disease antigen, and then Balb/c mice were prior immunized by s.c. injection with BSA proteins to get anti-BSA T cells. The results showed that as compared with addition of the same amount of BSA, LPPC/ BSA complexes co-cultured with splenocyte that had been immunized by BSA could efficiently activate cell proliferation and induce cytokine secretions. Results are representative at least three independent experiment. (*：P<0.05; **：P<0.01)

Figure 11

(a) CD11c$^+$ (b) MHC II



(c) CD86

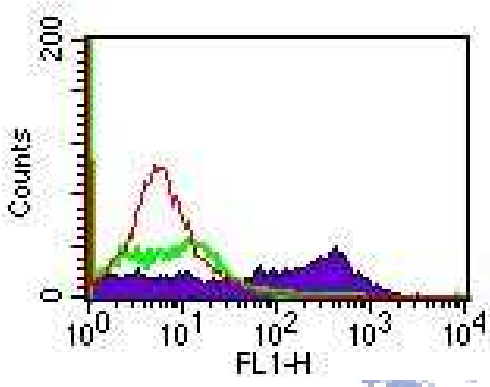


Figure 11. The surface marker expressions of dendritic cells. (a) The average CD11c$^+$ expression of cells were about 80%, and it indicated that the population of the cells were 80% DCs; (b) The MHC II expression of the cells(c) The costimulatory molecules CD86expression. Figure 11b and 11c indicated that high level expression of DCs' surface markers which treated with HpHsp60 and LPS were actually mature. Black line was that DCs were harvested with HpHsp60 and LPS, and green line was that DCs were harvested without treatment, and Red line was negative control.

Figure 12

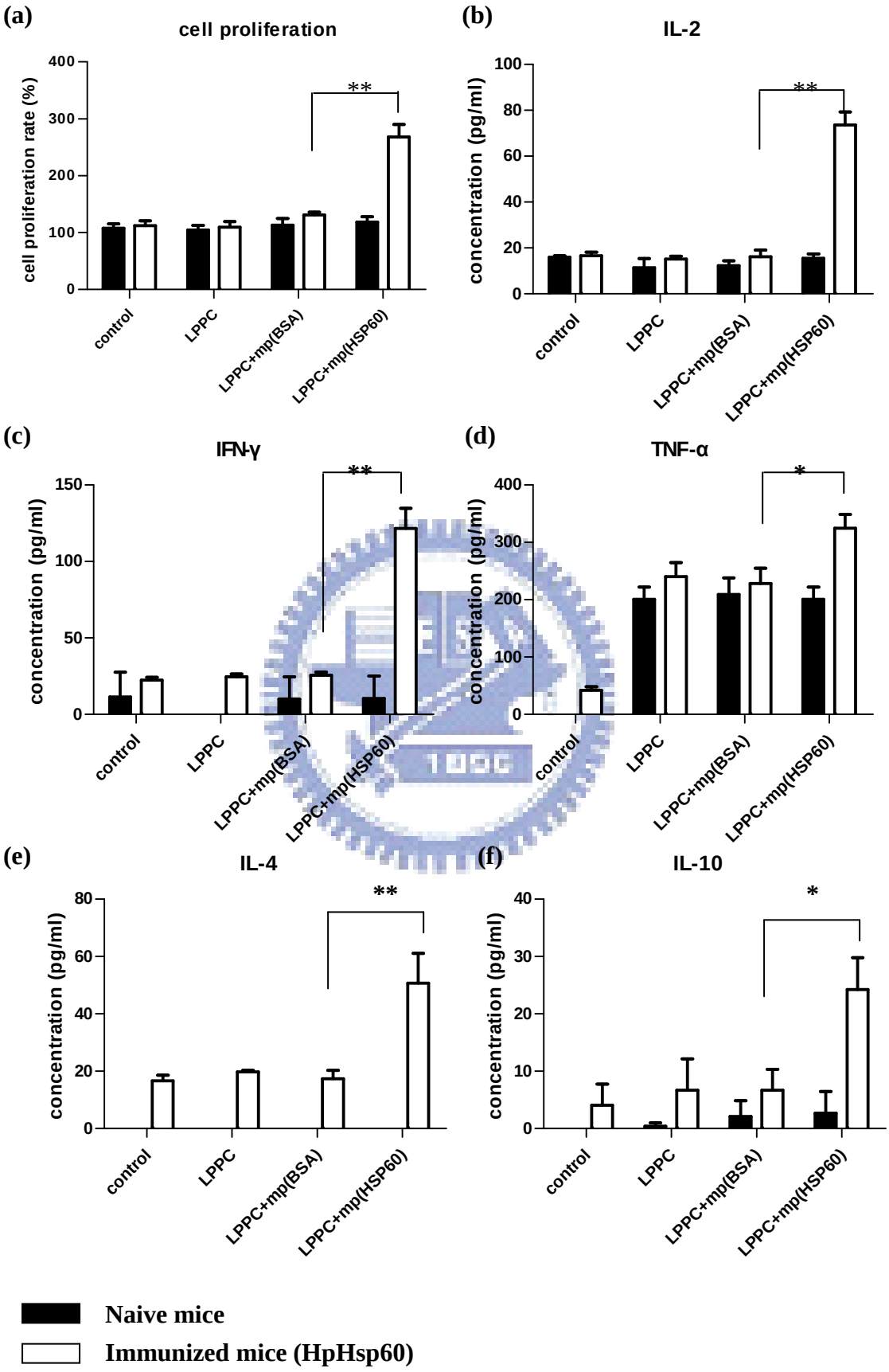

(a)
cell proliferation
cell proliferation rate (%)
400
300
200
100
0
**
control
LPPC
LPPC+mp(BSA)
LPPC+mp(HSP60)
(b)
IL-2
concentration (pg/ml)
100
80
60
40
20
0
**
control
LPPC
LPPC+mp(BSA)
LPPC+mp(HSP60)
(c)
IFN-γ
concentration (pg/ml)
150
100
50
0
**
control
LPPC
LPPC+mp(BSA)
LPPC+mp(HSP60)
(d)
TNF-α
concentration (pg/ml)
400
300
200
100
0
*
control
LPPC
LPPC+mp(BSA)
LPPC+mp(HSP60)
(e)
IL-4
concentration (pg/ml)
80
60
40
20
0
**
control
LPPC
LPPC+mp(BSA)
LPPC+mp(HSP60)
(f)
IL-10
concentration (pg/ml)
40
30
20
10
0
*
control
LPPC
LPPC+mp(BSA)
LPPC+mp(HSP60)
Naive mice
Immunized mice (HpHsp60)

Figure 12. **The specificties of DCs' membrane proteins coated LPPC.** HpHsp60 was as one disease antigen, and Balb/c mice were immunized by s.c. injection with HpHsp60 proteins. And DCs' membrane proteins contained HpHsp60 antigens were isolated for LPPC adsorption. The results showed that LPPC adsorbed membrane proteins with HSP60 antigens could stimulate splenocytes which prior to HSP60 immunized cell proliferation and cytokines secretion compared to LPPC with membrane proteins without HSP60 antigens group. Results are representative at least three independent experiment. (*：P<0.05; **：P<0.01)

Figure 13

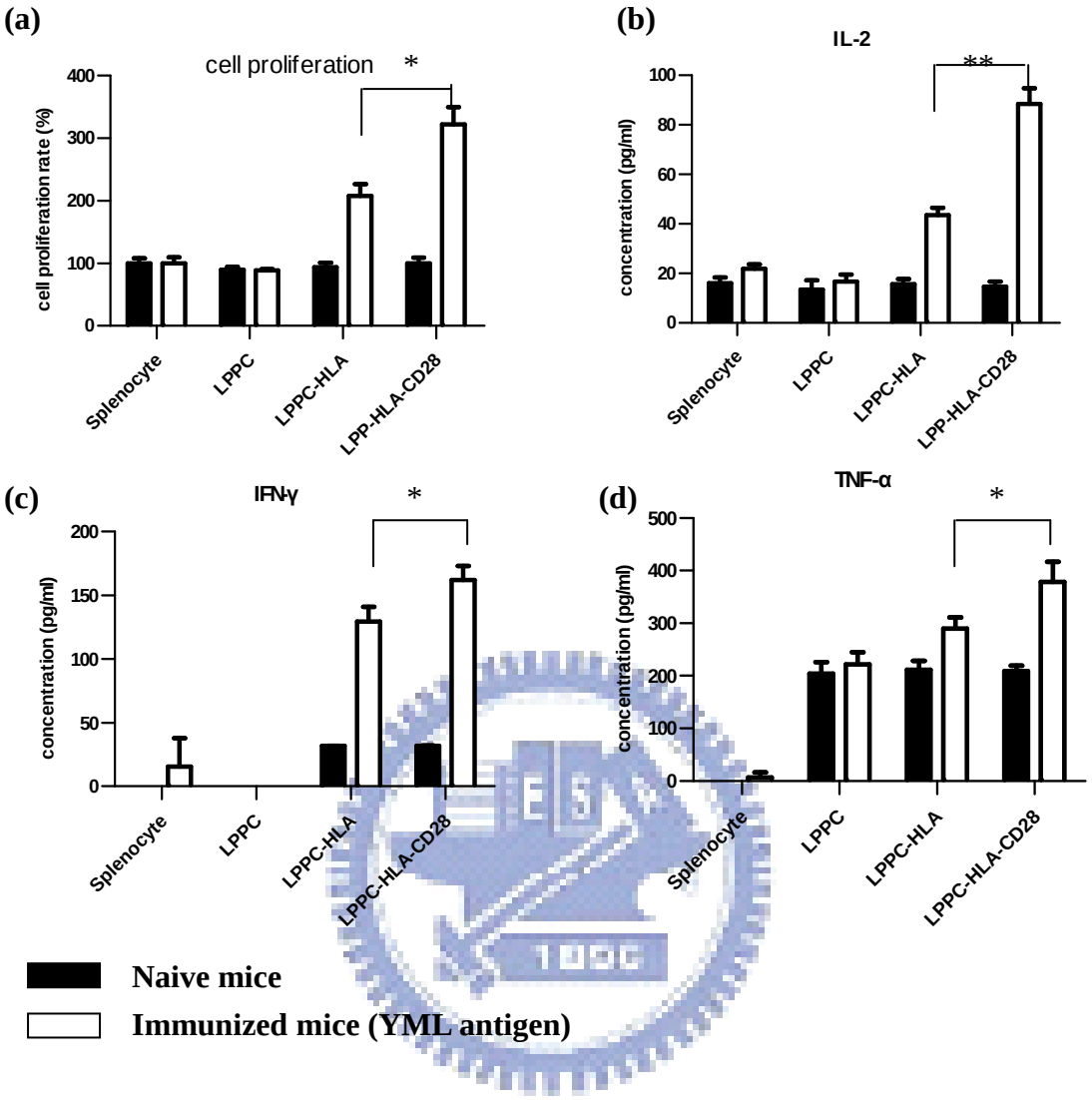


Figure 13. The specificities of antigen-loaded HLA-A2 combined with anti-CD28 monoclonal antibody on LPPC. YML was one identify epitope of HPV E7 protein, and immunized transgenic mice previously. Utilized specific antigen-loaded HLA-A2 on LPPC, and co-cultured with splenocytes from previously immunized transgenic mice. The results showed that LPPC could remain the specific activities of antigen-loaded HLA-A2 to stimulate cell proliferation and cytokines secretion. Moreover, added anti-CD28 monoclonal antibody as the second signal of the optimal T cell activation, the immune responses could be better than that only provided for one signal, antigen-loaded HLA-A2 molecules. Results are representative at least three independent experiment. (*：P<0.05)

Figure 14

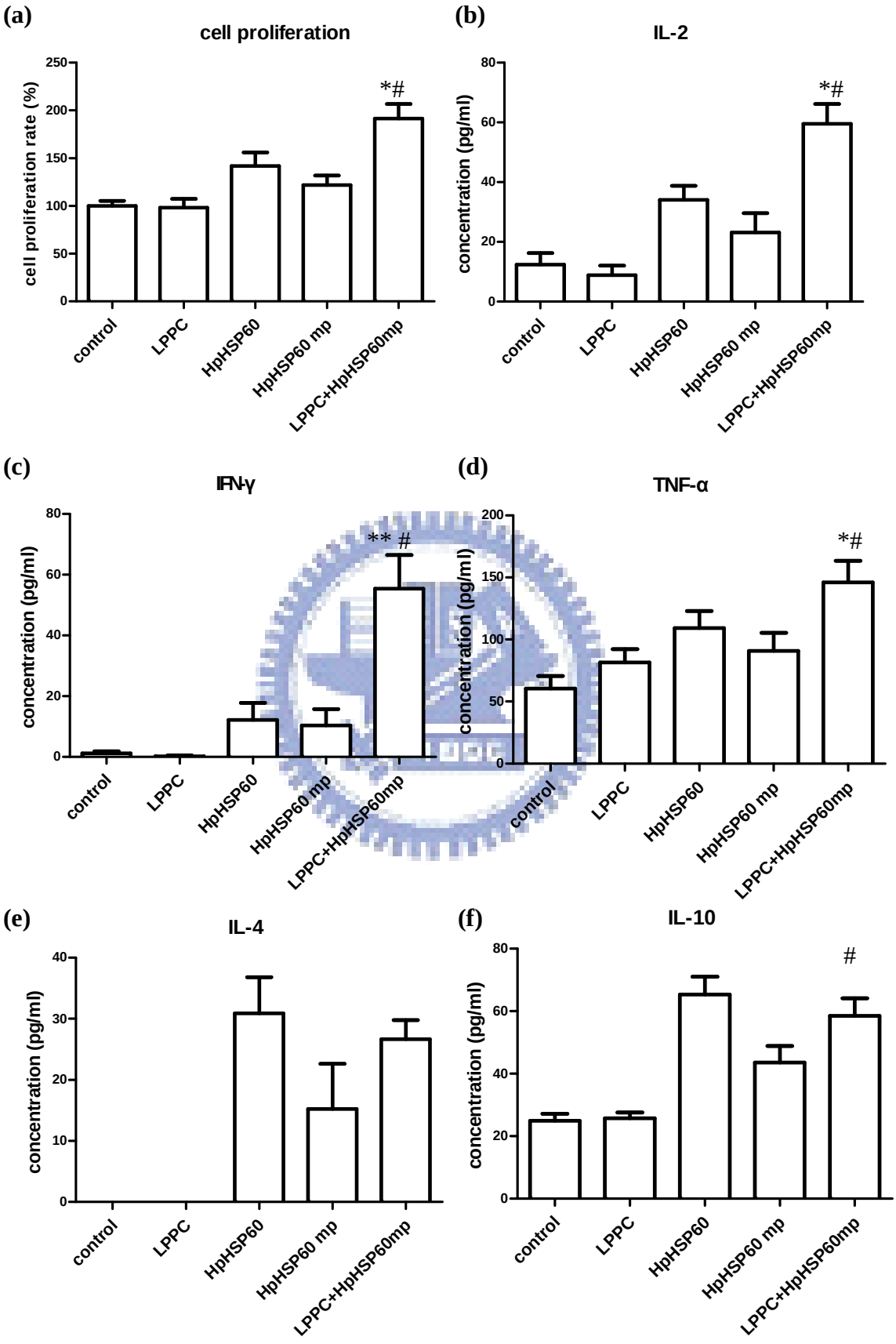

(a)
cell proliferation
cell proliferation rate (%)
250
200
150
100
50
0
*#
control
LPPC
HpHSP60
HpHSP60 mp
LPPC+HpHSP60mp
(b)
IL-2
concentration (pg/ml)
80
60
40
20
0
*#
control
LPPC
HpHSP60
HpHSP60 mp
LPPC+HpHSP60mp
(c)
IFN-γ
concentration (pg/ml)
80
60
40
20
0
** #
control
LPPC
HpHSP60
HpHSP60 mp
LPPC+HpHSP60mp
(d)
TNF-α
concentration (pg/ml)
200
150
100
50
0
*#
control
LPPC
HpHSP60
HpHSP60 mp
LPPC+HpHSP60mp
(e)
IL-4
concentration (pg/ml)
40
30
20
10
0
control
LPPC
HpHSP60
HpHSP60 mp
LPPC+HpHSP60mp
(f)
IL-10
concentration (pg/ml)
80
60
40
20
0
#
control
LPPC
HpHSP60
HpHSP60 mp
LPPC+HpHSP60mp

Figure 14. The animal immunization of the bound membrane proteins derived from DCs on LPPC. HpHsp60 was as one antigen and incubated with DCs for uptake and presentation. In addition, DCs' membrane proteins contained HpHsp60 antigens were isolated for LPPC adsorption. Balb/c mice were immunized by i.v. injection with the membrane proteins /LPPC complex. After two weeks, the mice were sacrificed and the splenocytes were isolated to incubate with 2 μg/ml HpHSP60. The results showed that splenocytes from mice that i.v. injection with the membrane proteins /LPPC complex could activate immune responses efficiently comparing to the other groups. (*：$P<0.05$ when compared to HpHsp60 group, and **：$P<0.01$; #：$P<0.01$ when compared to HpHsp60 mp group) Results are two independent experiments.

Figure 15

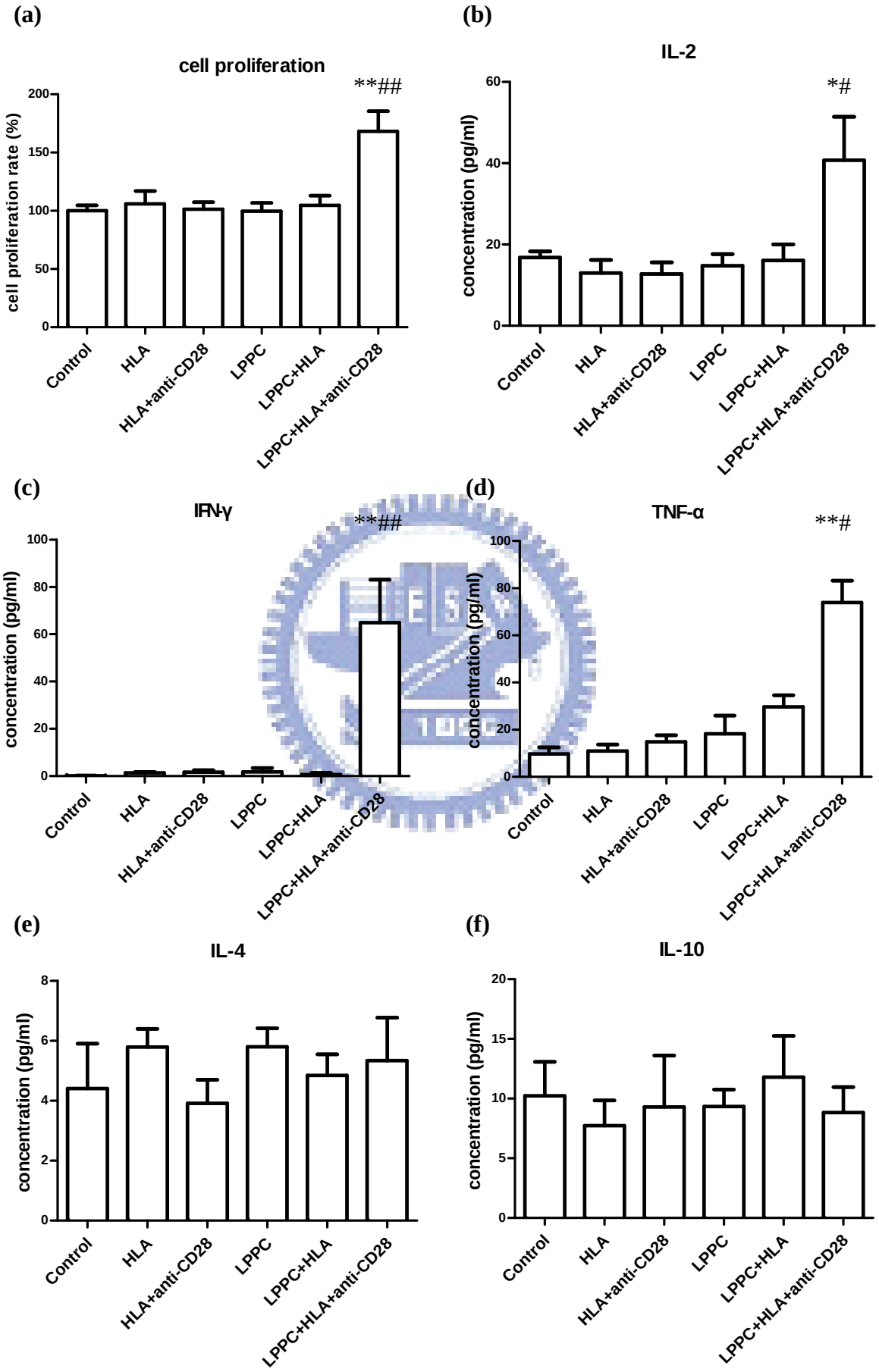

(a)
cell proliferation
**##
cell proliferation rate (%)
200
150
100
50
0
Control
HLA
HLA+anti-CD28
LPPC
LPPC+HLA
LPPC+HLA+anti-CD28
(b)
IL-2
*#
concentration (pg/ml)
60
40
20
0
Control
HLA
HLA+anti-CD28
LPPC
LPPC+HLA
LPPC+HLA+anti-CD28
(c)
IFN-γ
**##
concentration (pg/ml)
100
80
60
40
20
0
Control
HLA
HLA+anti-CD28
LPPC
LPPC+HLA
LPPC+HLA+anti-CD28
(d)
TNF-α
**#
concentration (pg/ml)
100
80
60
40
20
0
Control
HLA
HLA+anti-CD28
LPPC
LPPC+HLA
LPPC+HLA+anti-CD28
(e)
IL-4
concentration (pg/ml)
8
6
4
2
0
Control
HLA
HLA+anti-CD28
LPPC
LPPC+HLA
LPPC+HLA+anti-CD28
(f)
IL-10
concentration (pg/ml)
20
15
10
5
0
Control
HLA
HLA+anti-CD28
LPPC
LPPC+HLA
LPPC+HLA+anti-CD28

Figure 15. The animal immunization of the LPPC combined HLA-A2 molecules with anti-CD28 mAb. Utilized YML peptide-loaded HLA-A2 and anti-CD28 mAb bound to LPPC, and then transgenic mice were immunized by i.v. injection with the the immuno-LPPC complex. After two weeks, the mice were sacrificed and the splenocytes were isolated to incubate with 2 μg/ml YML peptides. The results showed that splenocytes from mice that i.v. injection with the immuno-LPPC complex could activate immune responses efficiently comparing to the other groups, such as cell proliferations and Th-1 cytokine secretions, but not Th-2 cytokine. (*：$P<0.05$ when compared to HLA and anti-CD28 mAb group, and **：$P<0.01$; #：$P<0.05$ when compared to LPPC with HLA group and ##：<0.01) Results are two independent experiments.

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