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碩士論文

Department of Molecular Medicine
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Master Thesis

由尿液純化而來的 Tamm-Horsfall glycoprotein 活化中性白血球的分子機制研究

Study on the molecular basis of urinary Tamm-Horsfall glycoprotein-induced neutrophils activation

蕭學謙

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本論文係蕭學謙君（學號 R98448008）在國立臺灣大學分子醫學研究所完成之碩士學位論文，於民國 100 年 07 月 23 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

Tamm-Horsfall glycoprotein (THP) 是由亨利式管(Henle's loop)中的上行端及遠曲小管所分泌的醣蛋白，分子量約有 80-90 KD，其醣分子佔全部重量 25-35%，大部分由 sialic acid 組成。THP 被認具有保護泌尿道表皮細胞免於被病原菌入侵的功能。在先前的研究指出，THP 可以和不同的蛋白質結合: 包括免疫球蛋白輕鏈、第一介白素(IL-1), 補體(complement component) 1q,以及腫瘤壞死因子-α(TNF-α)；然而對於 THP 如何影響免疫系統其詳細的機制至今依然不清楚，因此我們將 THP 以各種不同的酵素處理，試圖去了解究竟是 THP 分子外圍的醣側鏈或是其核心蛋白質結構才具有關鍵性的功能；我們發現 THP 經不同醣分解酵素處理之後依然具可以刺激多核型白血球的活性，但是 THP 經蛋白分解酵素處理之後便會失去其功能，所以我們認為 THP 的核心蛋白結構之重要性是大於側鏈的醣分子。接下來我們發現多核型白血球在 p38 的抑制劑(SB 203580)處之後便會失去其吞噬能力，這表示 THP 和脂多糖(lipopolysaccharide)對於多核型白血球吞噬能力的刺激都是需要 p38 路徑參與其中。最後我們進一步發現，THP 具有類似表皮生長因子(EGF)刺激多核型白血球的功能，THP 和表皮生長因子都會活化多核型白血球吞噬能力、導致細胞骨架的變化以及 ERK1/2 的表現；然而一旦我們利用表皮生長因子受體的抑制劑(GW2974)便可以抑制 THP 和表皮生長因子對多核型白血球的活化能力，但卻不會影響脂多糖對多核型白血球的活化。總結,在論文中我們證明了 THP

會活化多核型白血球，推測可能藉由 THP 中 EGF-like domain 來活化 p38 以及 ERK1/2 的訊息傳遞路徑來達成。

關鍵字: Tamm-Horsfall glycoprotein，P38 mitogen-activated protein kinases，多核型白血球吞噬作用，細胞骨架的重組，ERK1/2，EGF 樣結構。

Abstract

Tamm-Horsfall glycoprotein (THP) or uromodulin is produced by renal tubular cells of the ascending limp of Henle's loop. Tamm-Horsfall glycoprotein is a 80-90KDa GPI-anchored protein and contains approximately 25-35% of carbohydrate-side chain in weight with abundant sialic acid. THP is an important defense molecule in protecting urinary tract epithelial cells from microbial invasion. Our previous data has shown that THP can bind to diverse proteins including immunoglobulin light chains, complement component 1q, interleukin-1(IL-1), and tumor necrosis factor-α (TNF-α).In addition, we found that THP could enhance PMN phagocytosis. However, the mechanism phagocytosis-enhancing activity of THP remained unclear. For further elucidating the mechanism for this activity by THP, we purified THP from normal human urine.

At first, THP was cleaved by different carbohydrate-and protein-degrading enzymes and the data demonstrated that the protein-core structure was more important for activating PMN phagocytosis than carbohydrate-side chains. Next, we found that THP could bind to the surface membrane of PMN and induced phosphorylation of MAP kinase (p38),ERK1/2 and NF-κB signaling pathways. Furthermore, p38 inhibitor SB203580 could abolish LPS and THP induced-PMN phagocytosis.

Finally, we found THP and EGF (Epidermal growth factor) exhibited a similar function

on PMN and HL-60(human promyelocytic leukemia cells). Our results demonstrated that EGF and THP could induce PMN phagocytosis via rearrangement of cytoskeletal molecules by increasing expression of cdc42, RhoA and Rac. GW2974 (EGFR inhibitor) could reduce the THP- and EGF-induced PMN phagocytosis and ERK1/2 expression in HL-60. In contrast, GW2974 had no effect on LPS-induced PMN phagocytosis. Putting these results together, we concluded that THP used EGF-like domain to stimulate PMN via EGF singling pathway to phosphorylated p38 and ERK1/2. The EGF-like domain in THP molecule may play an important role in PMN phagocytosis-enhancing activity.

Keywords: Tamm-Horsfall glycoprotein, PMN phagocytosis, P38 mitogen-activated protein kinases, cytoskeleton rearrangement, ERK1/2 ,EGF-like domain

目錄

Chapter 1. Introduction

Tamm-Horsfall glycoprotein (THP) or uromodulin is produced by renal tubular cells in the distal loop of Henle and early distal convoluted tubules of the nephron[1] and we can collect it in normal urine about 30-50mg/24hours[2]. It is an important protein to protect urinary system against pathogens invasion[3]. Some papers indicated that THP deficient mice were highly susceptible to severe urinary bladder and kidney infection[4], and anti-THP antibody was detected in the serum of patients with urinary tract infection[5,6]. Mutations of THP gene result in different kidney disorders characterized by renal salt wasting, hyperuricemia, gout and progressive renal failure[7-9].

Tamm-Horsfall glycoprotein is a 80-90KDa GPI-anchored protein that contains a N-terminal signal peptide, three epidermal growth factor (EGF)-like domains, a domain containing eight conserved cysteine residues(D8C) and a zona pellucida (ZP) domain [10](Fig 1A) .THP contains approximately 25-35% of carbohydrate -side chain with abundant sialic acid[11], and the major glycomoiety structure of THP is N-linked glycans[12]. The high mannose glycans are carried by Asn_{251}[13], that mediate the interaction with type 1 fimbriated *Escherichia coli*[14]. The structure can prevent *E-coli* from binding to urinary epithelial cells. O-linked side chain was also found in THP that showed binding affinity with different molecules[15].

THP has be demonstrated binding to a variant of molecules such as interleukin 1(IL-1)[16],

tumor necrosis factor-α (TNF-α)[17], immunoglobulin light chains[18] and complement component 1 and 1q[19]. Whether the protein core structure or the carbohydrate-side chain is required for the binding affinity remains elucidating. Some papers showed that THP could activate polymorphonuclear netrophils (PMN), lymphocytes and monocytes[20-22] ,but the molecular basis for this activation is not clear. In addition, Saemann et al.[23]reported that THP bound to Toll-like receptor-4(TLR-4) ,and stimulated immune response. In the present study, we found that THP purified from normal urine stimulated PMN phagocytosis via EGF signaling pathway and inducing cytoskeletal related molecules cdc42, RhoA, and Rac participation.

Chapter 2. Materials and methods

1. Purification of Tamm-Horsfall glycoprotein(THP) from normal human urine

Urine was collected from normal individuals in a clean bottle at 4°C. The purification of THP was followed by the report of Hunt and McGiven[24]. Briefly, the batch urine was made up to 0.58M with NaCl and stirred for 30 minutes at 4°C, centrifuged at 2000 ×g for 20 minutes and removed the supernatant. After the 3 cycle of alkaline distilled water pH9.0(adjusted by 1N NaOH) dissolution, 0.58 M NaCl precipitation (centrifuged 16100 ×g at 4°C for 20 minutes), the solution was dialyzed against double-distilled water by Standard Regenerated Cellulose membranes(spectrum,MWCO:8000)at 4°C overnight. The obtained THP were lyophilized, and stored at -70°C until use. For experiments, the purified THP was dissolved in Phosphate buffered saline (PBS, 137mmol/L NaCl, 2.7mmol/L KCl, 8.1mmol/L $Na_2HPO_4 \bullet 2\ H_2O$, 1.76mmol/L KH_2PO_4), pH7.4. The purity and relative molecular weight of THP were detected by 10%sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in reduced state and stain by GelCode Blue Stain (Thermo).

2. Isolation of PMN and MNC from normal human peripheral blood

Heparinized venous blood obtained from normal individuals was mixed with

one-quarter volume of 2% dextran solution (molecular weight:425000-575000) and incubated at room temperature for 20 minutes. The cell suspension was gently layered over Ficoll-Hypaque density gradient solution (specific gravity 1.077) and centrifuged at 300 ×g for 30 minutes. The MNC were aspirated from the interphase whereas the PMN were collected from the bottom. The residual RBC in PMN was lysed in cold 0.85% ammonium chloride solution. The viability of PMN and MNC were detected by trypan blue dye and the cell concentration were adjusted to $2X10^6$/ml in 10% fetal bovine serum (FBS) in RPMI-1640(10%FBS-RPMI)

3. Digestion of THP by different carbohydrate-, glycoconjugate-, and protein-specific degrading enzymes

All incubations were at 37 °C for 16 h. The concentration of the respective enzyme was: neuraminidase (10 units/ml) in 50 mM sodium acetate, pH5.0; β-galactosidase (0.05 units/ml) in 50 mM sodium acetate, pH5.0; proteinase K (0.5 mg/ml) in 10 mM Tris, pH 7.5 with 1 mM $MgCl_2$ carboxypeptidase Y, (enzyme:substrate=1:10) in 0.2 M pyridine-acetate buffer, pH5.6; o-sialoglyco-protein endopeptidase (50 μg/ml) in PBS, pH 7.2. These digested THP products were then heated at 65 °C for 60 min to inactivate the residual enzymes in the mixture. The concentrations of these enzyme-digested THP products were determined by the BCA (bicinchoninic acid) protein assay (pierce).

4. Determination of binding activity of THP with Tumor necrosis factor-alpha (TNF-α)

One hundred microliters of intact THP (5 μg/ml) dissolved in coating buffer(0.1M sodium carbonate, pH9.5) and were incubated in microwells at 4 °C for 24 h. After two washes with washing buffer (PBS with 0.05% Tween-20), the non-specific binding of the microwells was blocked by PBS containing 1% bovine serum albumin (BSA). Human recombinant TNF-α (Amersham Biosciences) at different concentrations of 46.88, 93.75, 187.5, 375, 750, 1000, 1500, 2000, or 3000 pg/ml were added to the microwells at 4 °C and incubation in continuous rotation for 2 hours. The TNF-α binding to THP was measured by commercially available TNF-α ELISA kit (BD OptEIA^{TM}). A Scatchard plot was drawn and analyzed by the Prism statistical program provided by GraphPad Software.

5. Lectin-binding activity of THP by ELISA

One hundred microliters of intact THP (5 μg/ml) dissolved in coating buffer were incubated in microwells at 4 °C for 24 h. After two washes by wash buffer and the non-specific binding of the microwells were blocked by PBS containing 1% BSA, 100 μl of the respective commercially available biotin-conjugated lectins (5 μg/ ml): MAA, SNA-I, GNA, DSA or ConA, were added to the microwells. The mixtures were incubated at room temperature for 1 h. After three washes by wash buffer, horseradish

peroxidase (HRP)-conjugated streptavidin was added and incubated for another 60 min at room temperature. After color development with Tetramethylbenzidine (TMB) and Hydrogen peroxide (The BD Pharmingen^{TM} TMB Substrate Reagent Set), 50μ l of 2N H_2SO_4 was finally added to terminate the reaction. The binding affinity of THP with different lectins was measured at OD450nm absorbance by ELISA reader (Dynex Technologist, Chantilly, VA, USA).

6. Detection of PMN phagocytosis-enhancing activity of THP, enzyme digested products, and protein kinase inhibitors by flow cytometry

Fluoresbrit carboxylate microspheres (0.75 μm in diameter,Polyscience Inc.) were previously opsonized by incubation with fresh human serum at 37 °C for 2 hours. Fresh prepared PMN (2 ×10^6 cells/ml) were pre-treated with PD 98059(50μM), SB-203580 (1μM), and Wortmannin (0.1μM) at 37 °C for

20 minutes and then lipopolysaccharide (LPS 20ng/ml), THP or THP enzyme digested products (10μg/ml) were added and incubated at 37 °C for 30 minutes. The mixture reacted with opsonized beads (1 ×10^8 beads/ml) at 37 °C for 45minutes in 5% CO_2–95% air. After incubation and washed by PBS, PMN were fixed with 4% paraformaldehyde to stop the phagocytosis. The percentage (%) and mean fluorescence intensity (MFI# denoted by mean channel number) of PMN phagocytosis were determined by FACSort flow cytometry (Becton Dickinson) at wave length 488 nm

excitation.

7. Cell culture and preparation of whole cell extraction

Human promyelocytic leukemia cell line (HL-60) was grown in Iscove's Modified Dulbecco's Media (IMDM) medium containing 10%FBS under humid air with 5% CO_2 at 37°C. HL-60(2×10^6 cells/ml) was treated with LPS (20ng/ml), THP (10ug/ml), and epidermal growth factor (EGF, 20ng/ml) at 37°C. After treatment at different time points, the cell were centrifuged at 800 ×g for 5 minutes, the followed by washed with cold PBS. After centrifugation at 800 ×g for 5 minutes, the pelleted cells were lysed with cold RIPA buffer (25mM Tris-HCl pH 7.6,150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail and Phosphatase Inhibitor Cocktail (Roche) and kept on ice for 30 minutes. The cell lysis was centrifuged at 13,500 rpm at 4°C for 15 minutes to remove the debris, and the supernatant were applied for Western blot. The concentration of the cell extraction was determined by the BCA assay (Pierce).

8. Western blotting

Proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Inc.) in a Mini Trans-Blot cell (Bio-Rad) for 2 hours at 350mA. The PVDF membranes were blocked with Tris-buffered saline and Tween 20 (TBST: 50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) containing 1%BSA at

room temperature for 30 minutes, and probed with a specific antibody at 4°C for 16 hours. After washing the membrane twice with TBST, the complexes were detected by HRP-conjugated-secondary antibody (Jackson ImmunoLab) and ECL Western Blotting Substrate (Pierce) chemifluorescence detecting the system.

9. Immunofluorescence microscopic observation of cytoskeletal change in activated-PMN

After stimulation at 37°C by THP or LPS for 60 minutes, the activated PMN were fixed for 30 minutes at room temperature with a solution of 4% paraformaldehyde followed by three washes with PBS. PMN were permeabilize with 0.1% Triton X-100 for 15 minutes at room temperature, and then uadded blocking solution (PBS contained 1%BSA) to block the non-specific binding. PMN were stained with fluorescent phalloidin (each sample dilute 5μl phalloidin into 200μl blocking solution) for visualization of actin filament at 37°C for 30-60 minutes. Washed PMN 3 times with PBS and stained with DAPI (4',6-diamidino-2-phenylindole ,1:1000 dilute) at 37°C for 5 minutes. After 3 time washes with PBS, PMN were transferred to the slide by cytospin technique and were observed under the fluorescence microscope.

10. Cytospin technique

Each sample (total volume 100μl) was pipetted into a plastic chamber, placed in a Cytospin slide centrifuge, and forced by centrifugation (350 ×g for 5 minutes) through a

horizontal tube in the chamber, through a hole in a filter paper, and onto a glass slide. The filter paper placed between the plastic chamber and the glass slide absorbed the supernatant fluid, and cells traveled through the hole in the filter paper and were deposited in a circular area on the slide.

Chapter 3. Result

1. Determination of the binding affinity of THP to TNF-α

Many papers have showed that THP can bind to different molecules[16,17,19,25] . We have also found that THP has binding affinity of serum proteins and cytokines (Figure 2A). We noticed that THP had higher binding affinity with TNF-α compared with other serum proteins. TNF-α is a cytokine involved in the inflammation and causes many of the clinical problems associated with autoimmune disorders such as rheumatoid arthritis[26] and ankylosing spondylitis[27]. Therefore, anti-TNF-α antibody therapy is widely used in these patients [28]. So, we wanted to test if THP may be used as an anti-TNF-α therapeutic agent. To understand the K_d value and the binding domain of THP with TNF-α is a critical problem for further clinic use. As demonstrated in Western blot data, we found THP exhibited dose-response binding capacity with TNF- α (Figure 2B).However, we have also found that the binding affinity of THP with TNF-α is weaker than anti-TNF-α antibody and the enzyme digested THP products show no binding affinity with TNF-α (Figure 2C).However,We could demostrate anti-TNF-α antibody recognizing the TNF-α (Figure 2C, lane 2), but under the same condition, normal THP didn't show binding affinity with TNF-α(Figure 2C, lane 1). We also demonstrated that these three bands of β-galactosidase-treated THP (Figure 2C, lane 7) were non-specifically bound between TNF-α antibody and β-galactosidase-treated THP

compared with Figure 2D, since Figure 2D were probed by anti-TNF-α antibody only. Furthermore, we calculated the K_d and B_{max} value of THP with TNF-α by ELISA (Figure 2E), and the results showed that THP bound to TNF-α with low affinity (K_d~1500 pg/ml, 9×10^{-11}mol/L). Compared to other protein-protein interaction, the K_d value of biotin and streptavidin is about 10^{-14}mol/L which is the most powerful non-covalent binding affinity in nature.

2. Lectin binding activity of THP

THP contained 25-35% carbohydrate side-chains in weight and abundant sialic acid[29], and this unique molecule could patently stimulate PMN phagocytosis[22] ENREF 20. Therefore, we intended to clarify whether the carbohydrate side-chains or the protein core structure is required for the activation of PMN phagocytosis. In figure 3A, THP shows high affinity with DSA(specific for β(1,4)GlcNAc oligomers) and ConA(specific for GlcNAc/branch mannose), but low affinity with GNA(specific for mannose residues), MMA(specific for Siaα(2,3)Gal/GalNAc), and SNA-1(specific for Siaα(2,6)Gal/GalNAc). These data suggest that the major carbohydrate side-chains in THP are β(1,4)GlcNAc oligomers and branch mannose but lacks Siaα(2,3)Gal/GalNAc and Siaα(2,6)Gal/GalNAc. Previous paper also shows THP lacks Siaα(2,6)Gal/GalNAc in the carbohydrate side-chains[13].

3. THP and its enzyme digested product stimulated PMN phagocytosis

To further confirm that the carbohydrate side-chains or the protein structure is necessary for PMN phagocytosis, we used different enzymes to remove the carbohydrate side-chains on cleavage the protein structure. In figure 3B, we confirmed that normal THP could stimulate PMN phagocytosis, and after treatment with carbohydrate digested enzyme such as β-*N*-Acetylglucosaminidase, β-galactosidase, and o-sialoglycoprotein endopeptidase, these enzyme digested THP products remained the activity to still stimulate PMN phagocytosis. However, the carboxypeptidase Y and proteinase K treatment cleaved the THP protein structure (Figure 1B,lanes 5 and 6), and thus the PMN phagocytosis activity was supessed. These results suggested the protein core structure is essential for the PMN phagocytosis activity.

4. PMN phagocytosis activity is p38 dependent.

To further understand the molecule mechanism of the THP-induced PMN phagocytic activity, we used the different protein kinase inhibitors to block the signal transduction. We treated PMN in advance with different kinase inhibitors: SB 203580(p38 inhibitor), PD 98059(MEK1 Inhibitor) and Wortmannin (PI3Ks inhibitor), and then analyzed the PMN phagocytoic activity. We found SB 203580 could effectively decrease non-stimulated, (Figure 4A, decreased from 50% to 30.2%), LPS-induced (Figure 4B, decreased from 68.8% to 43.3%)and THP-induced PMN phagocytosis (Figure 4C, decreased from 64.7% to 36.1%). This result suggested that THP and LPS shared the

same signal pathway in the PMN phagocytosis, and this process is dependent on p38 signaling pathway. We treated PMN with THP and LPS at different time points and analyzed p38 and NF-κB by Western blot (Figure 5A and 5B) .We found that after incubation with THP, the phosphorylated p38 and NF-κB expression levels were higher than untreated-PMN.The treatment of SB 203580 could reduce phosphorylated p38 level dramatically (Figure 5A, the level of phosphorylated p38 decreased by 80%). This result was consistent with the data from the flowcytometry: THP induced PMN phagocytosis is p38 dependent. In figure 5C, HL-60 also exhibited a similar response to THP treatment (phosphorylated p38 was 2-fold increased compared with the untreated-HL-60).

5. THP induced cytoskeleton rearrangement

Phagocytosis is a cellular process of engulfing large particles, and it is an important mechanism to remove pathogens and cell debris of the body. In higher organisms, phagocytic cells such as macrophages and neutrophils are necessary for host defense mechanism against invading pathogens[30]. During phagocytosis, many types of receptors on the cell membrane could bind its target material. These receptors included opsonin receptors(CR1[31] and Fc[32] which bind to C3b and antibody, respectively.) , and Toll-like receptors(Toll-4 can recognize LPS). The small GTP-binding proteins such as Cdc42, Rac and RhoA can change the local cytoskeleton rearrangement[33] to facilitate the

engulfment of pathogens or cell debris. We analyzed these small GTP-binding proteins after treatment with LPS and THP, and found that LPS could increase Cdc42 by 80% (Figure 5D). However, Cdc42, RhoA, and Rac didn't significantly change in response to THP (Figure 5D-5F). Because phagocytosis was combined with local cytoskeleton remodeling, we further investigated this point and found THP could also induced cytoskeleton rearrangement. As shown in Figure 6A, normal PMN expressed smooth cytoskeleton structure and F-actin, which were widely distributed throughout the cytoplasm. However, after treatment with LPS and THP, F-actin aggregated and formed many small puncta in the cytoplasm (Figure 6B and 6C). This result suggested that THP not only induced phagocytosis but also caused cytoskeleton rearrangement.

6. THP and EGF have similar activation on HL-60 and PMN

We have showed that THP can stimulate PMN phagocytosis and this process is p38 dependent and requires intact THP protein core structure. We hypothesized that EGF-like domain in THP processed EGF activity to active PMN because EGF has been demonstrated to enhance TNF-α-induced activation of neutrophils[34] and induce cytoskeleton rearrangement[35]. Our results indicated that after treatment with EGF or THP for 5-15 minutes, ERK1/2, the EGFR downstream signaling molecule, was significantly increased. In contrast to LPS, the ERK1/2 increased in amount until 30 minutes (Figure 7A). We also observed that the Cdc42 expression was increased after

treatment with LPS (average 1.73-fold increase) compared to EGF (average 1.32-fold increase) and THP (average 1.3-fold increase) (Figure 7B). These results suggest that THP has different signaling pathway from LPS in HL-60, but mediate a similar activity with EGF. We further tested if THP and EGF shared a similar signaling in PMN phagocytosis. We used GW2974 (an inhibitor of EGFR and ErbB-2 receptor tyrosine kinase) to inhibit the EGF function. We found that after treatment with GW2974, ERK1/2 was significantly decreased in response to EGF and THP stimulation, but treatment with LPS didn't affect the level of ERK1/2 even after combined incubation with GW2974(Figure 8A). We also analyzed the PMN phagocytosis (Figure 8B, 8C) and found that GW2974 abolished the EGF and THP induced PMN phagocytosis (Figure 8C). In contrast, GW2974 didn't reduce the LPS induced PMN phagocytosis. This result indicated THP and EGF need the same signal pathway to enhance PMN phagocytosis.

Chapter 4. Discussion

1. What is the role of p38 MAP kinase in the phagocytosis?

p38 MAP kinase pathway is crucial for cell to adapt the environmental stress, such as the concentration of nutrients, UV stress, growth factors, and cytokines. There are four p38 MAP kinases in mammals: α, β (also called SAPK3), γ (also called ERK6) and δ (also called SAPK4)[37-39]. p38 inhibitor SB 203580 can directly bind to ATP binding pocket of the p38α and p38β[40], but doesn't affect the p38γ and p38δ[41,42]. Recently, some papers indicated that p38γ regulates cytoskeleton by phosphorylation of SAP97 and triggeres SAP97 dissociation from GKAP[43](guanylate kinase-associated protein) which is located in the cytoskeleton. SB 203580 treatment can reduce the CD54 expression in macrophage[44]. We have showed SB 203580 abolished LPS- and THP- induced PMN phagocytosis, but didn't affect the F-actin aggregation in the LPS-, THP-, and EGF-treated PMN (Figure 6E). In conclusion, we suggest that p38α/β and p38γ are involved in phagocytosis. In addition, LPS, THP, and EGF can active these three p38 isoforms; p38α/β may affect receptors expression which is required for PMN phagocytosis, and p38γ may affect cytoskeleton rearrangement, which is induced by the THP and LPS.

2. EGF-like domain in THP molecule may be recognized by cell surface receptor

This study has demonstrated that THP can stimulate PMN by its protein core structure, and THP can activate p38 and processes EGF-like function. Some papers reported that epidermal growth factor-like domain 7 (EGFL7), which contains two EGF-like domains and is secreted by endothelial cells, modulates Notch signaling and affects neural stem cell renewal[45]. On the other hand, EGF-like domain of P-selectin also plays an important role in ligand recognition and cell adhesion[46]. Therefore, we suggest that EGF-like domains of THP play an important role in PMN phagocytosis by binding to cell surface receptors. However, which receptor and domain of THP can bind each other deserves further confirmation.

3. Different receptors may affect PMN phagocytosis by different signal transduction.

During phagocytosis, antibody-opsonized particles are recognized by surface receptors of the Fc portion of immunoglobulins (FcRs) and the Rho proteins (Cdc42 and Rac) mediate the local cytoskeleton rearrangement[47]. However, after treatment with THP, we found modest increase in Cdc42, RhoA, or Rac by Western blot analysis (Figure 5D-5F). Interestingly, we observed distinct F-actin aggregation in PMN (Figure 6). Accordingly, GTP-bound form Rac and Cdc42 is more reliable to measure the activity. Recently study showed Cdc42 and PI3-kinase cooperation for actin polymerization during phagocytosis[47], and we also found Wortmannin (PI3Ks inhibitor) reduced phagocytosis

in response to LPS (Figure 4B, decreased from 68.8% to 51.2%). Wortmannin didn't reduce THP-treated and untreated PMN phagocytosis (Figure4A and 4C); furthermore, we found Wortmannin could increase (50% to 57% and 64.7% to 68%, respectively.) PMN phagocytosis. This might suggest different stimulation can active different Rho GTPases: LPS induced Cdc42 and PI3K. However, THP transduce alternative signal pathway. p38 may be located at the downstream of Rho GTPases, but the relationship between Rho GTPases and p38 remains to be confirmed.

4. THP protects the urinary epithelial cells from pathogens

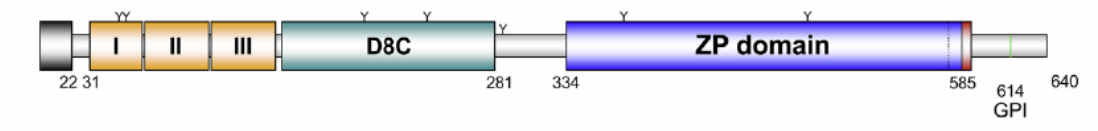
THP is widely distributed along the urinary tract. The previous investigations have showed it can bind to pathogens[14] and remove pathogens in urinary tract. If pathogens penetrate the urinary epithelial cells, these pathogens may be engulfed by THP-stimulated PMN to remove them. We suggest the carbohydrate-side chains of THP may stabilize the THP molecule in urinary tract for protection.

Chapter 5. Conclusion

This study showed the protective role of THP in the urinary system. THP contains 25-35% of carbohydrate -side chains in weight, but we found the protein core structure played an essential role in enhancing PMN phagocytosis. THP and LPS could stimulate PMN phagocytosis and this process required p38.However, p38 inhibitor (SB 203580) didn't affect cytoskeleton remodeling. We thus suggest LPS and THP have different signal pathway, i.e., THP can activate EGF signaling and LPS depends on TLR-4 signaling pathways[36].

Chapter 6. Figures

A. Domain structure of Tamm-Horsfall glycoprotein



B.

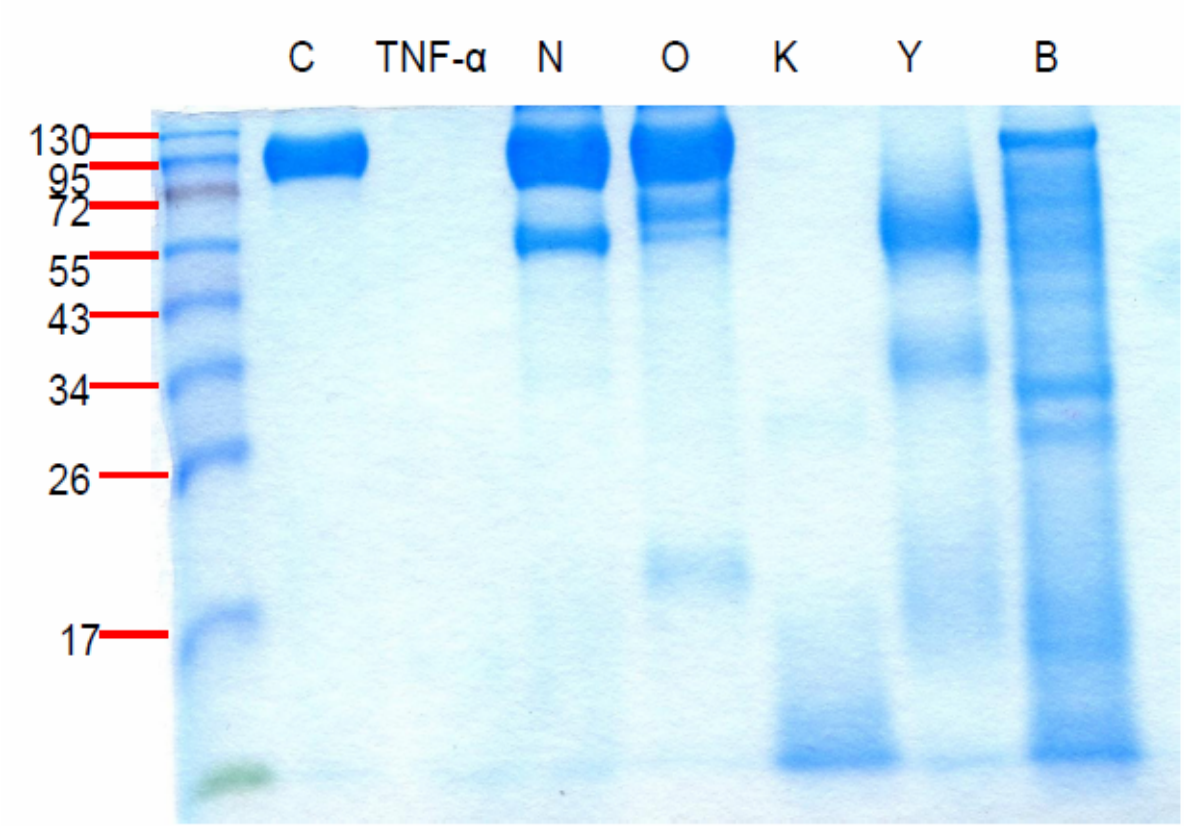
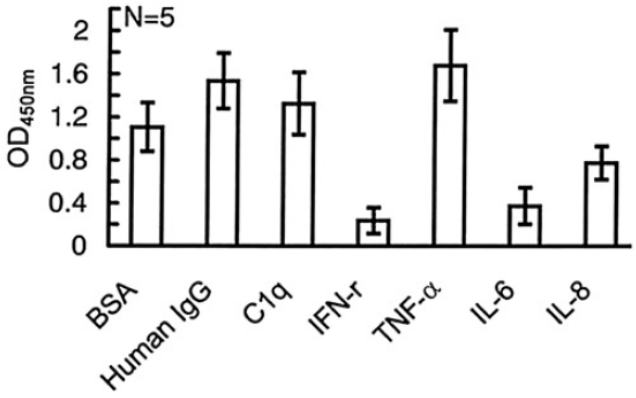


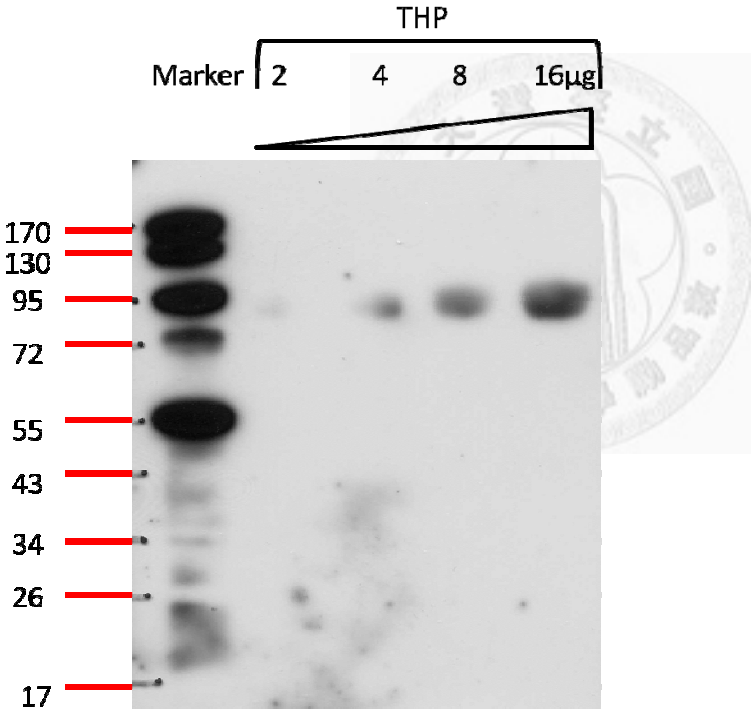
Figure 1 Domain structure of Tamm-Horsfall glycoprotein and its enzyme-digested products in 10% SDS-PAGE analysis. (A) Leader peptide is shown as a black box, EGF-like domains are displayed as orange boxes, D8C domain is shown as a light blue box and ZP domain is shown as a dark blue box. The seven N-glycosylation sites are marked as Y. (B) Intact and different enzyme-digested

THP were stained with Coomasie blue. Intact THP(C); the lines show neuramidase digestion (N); O-sialoglycoprotein endopeptidase digestion (O); carboxypeptidase Y digestion (Y); Proteinase K digestion (K); and β-galactosidase digestion (B).

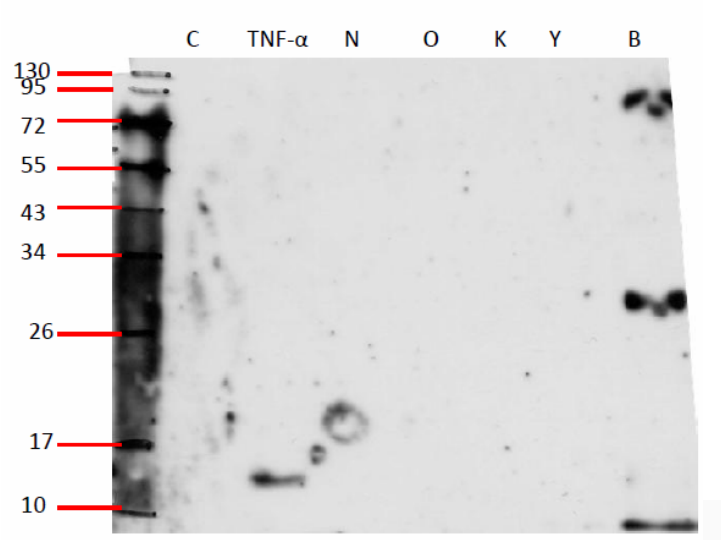
A. Binding activity of THP to different molecules by ELISA



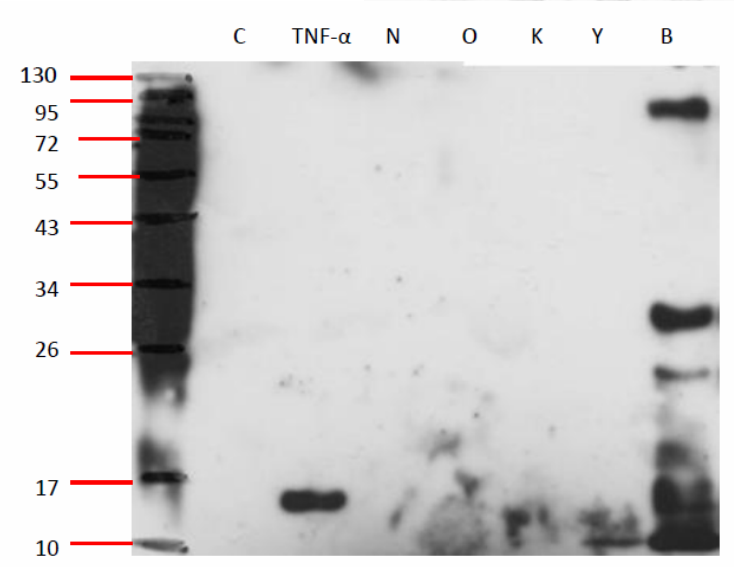
B. Dose –response binding activity of THP to TNF-α by Western blot



C. The binding activity of different enzymes digested THP products with TNF-α by Western blot



D. The binding activity of TNF-α and different enzymes digested-THP products probed by anti-TNF-α antibody by Western blot



E. Binding affinity of THP with TNF-α by ELISA

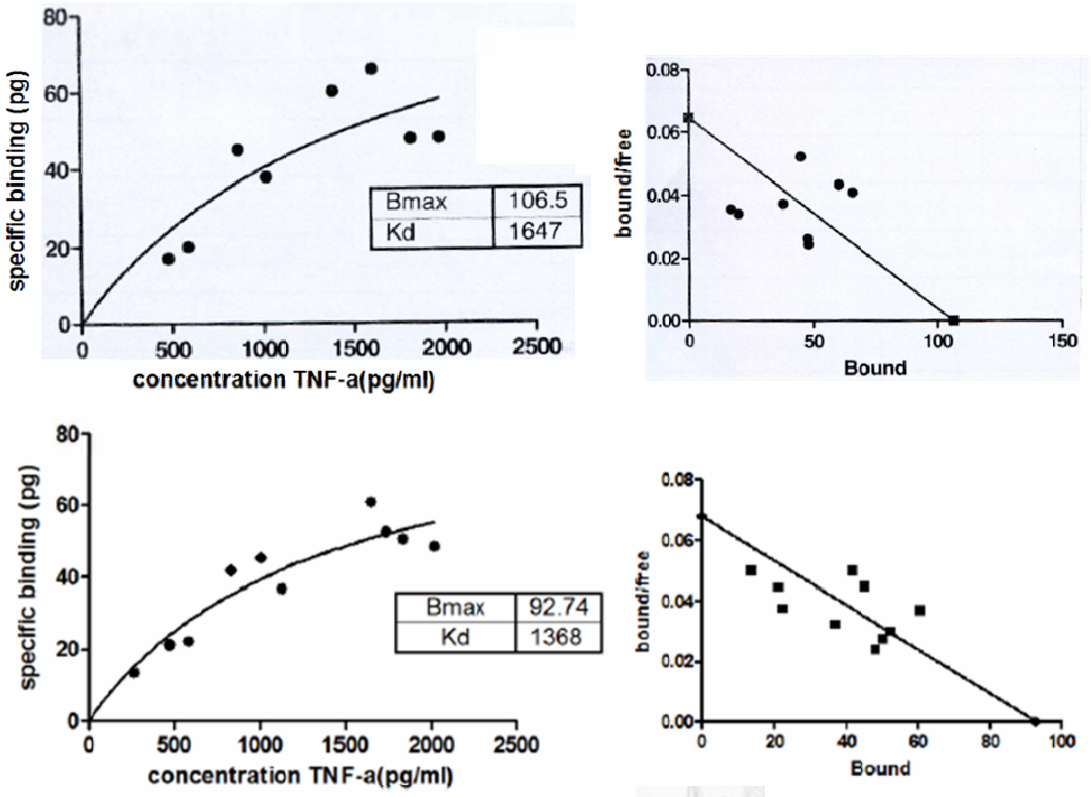
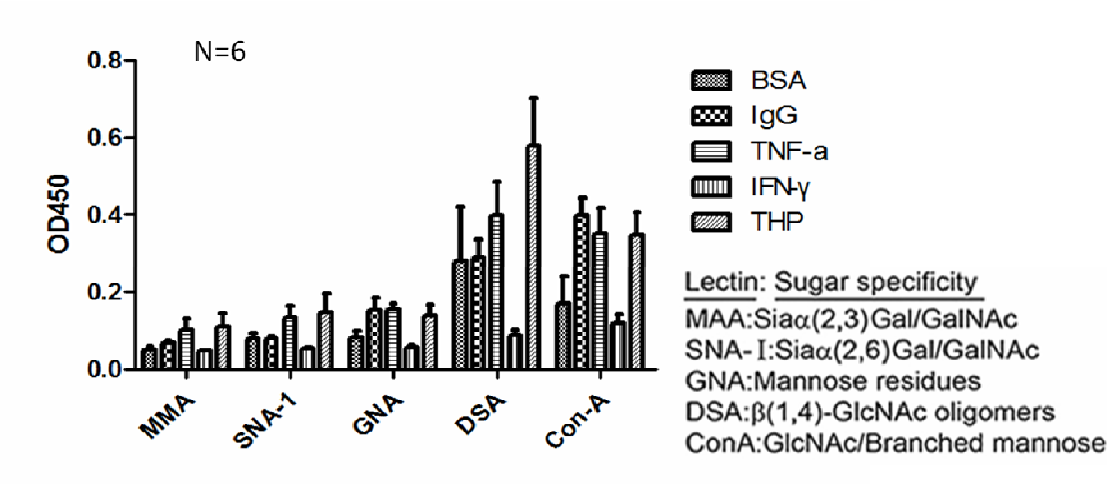


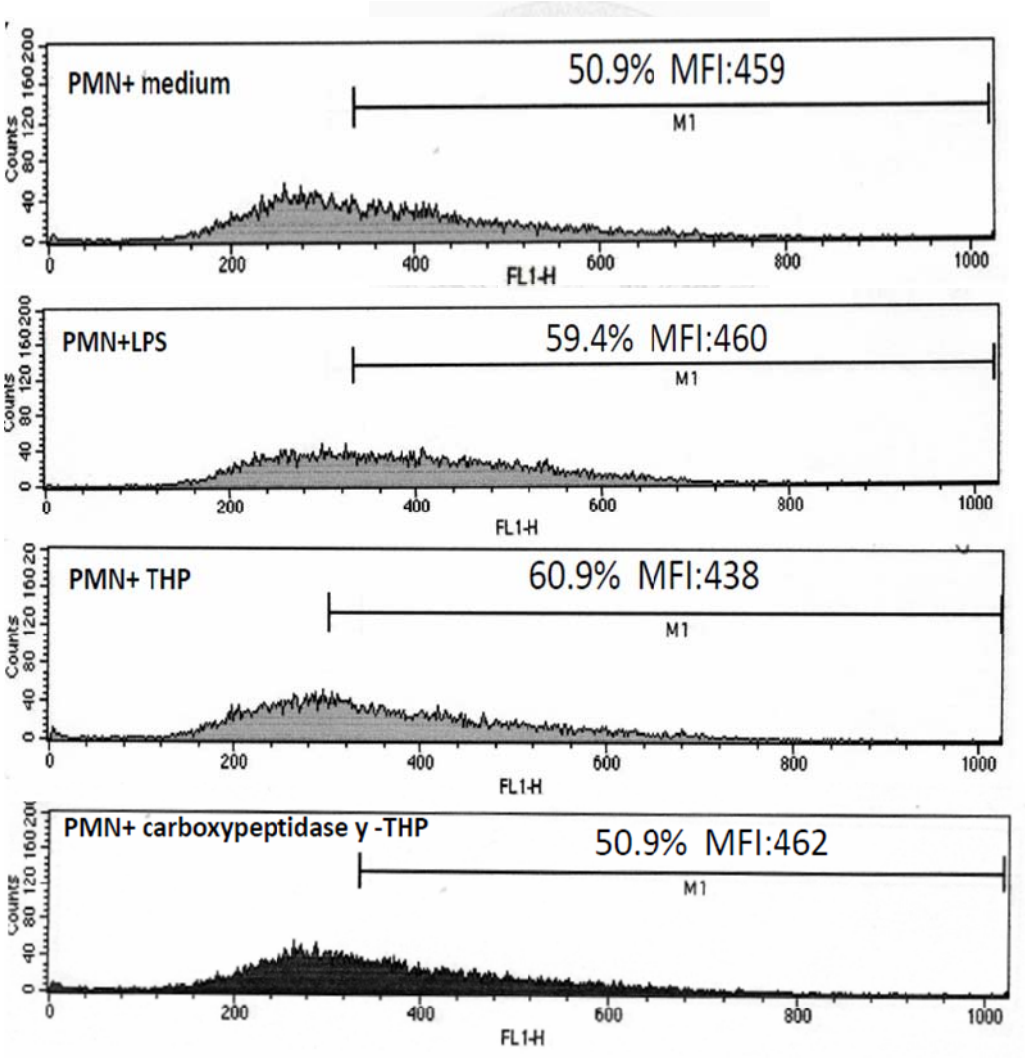
Figure 2 The binding activity of THP with different protein molecules and calculated K_d value with tumor necrosis factor-alpha (TNF-α). (A) The binding capacity of THP with different serum proteins and cytokines by ELSIA (B) Dose – responsive binding of THP from 2 to 16 μg/well with TNF-α by Western blot. (C) The binding of TNF-α with different enzymes-digested THP products probed by HRP-TNF-α. Intact THP denoted by C. Neuramidase digestion denoted by N. O-sialoglycoprotein endopeptidase digestion denoted by O. Carboxypepttidase Y digestion denoted by Y. Proteinase K digestion denoted by K. β-galactosidase digestion denoted by B. TNF-α loading labeled

in TNF-α. (D) With the same condition of Figure 2C except no TNF-α addition and probed by anti-TNF-α antibody. (E) Binding affinity of THP with human recombinant TNF-α. The method for calculating B_{max} and K_d values are described in detail in the “Materials and Methods”.

A. Lectin binding affinity of THP and other mloecules



B. PMN phagocytosis activity



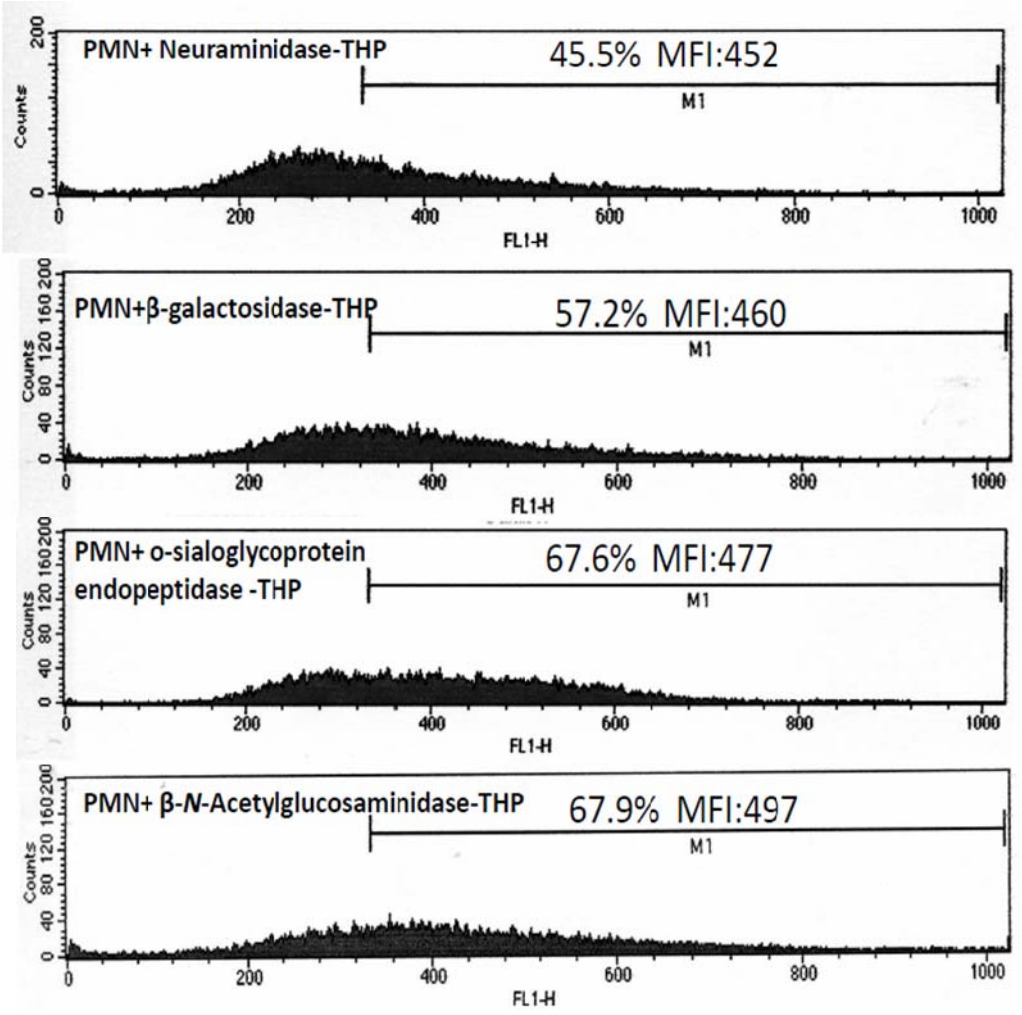
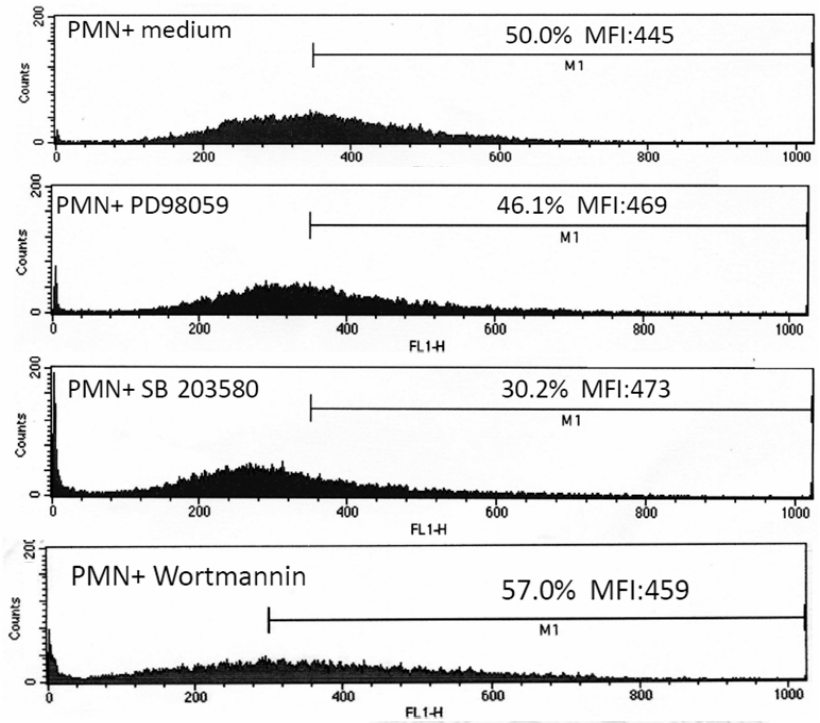
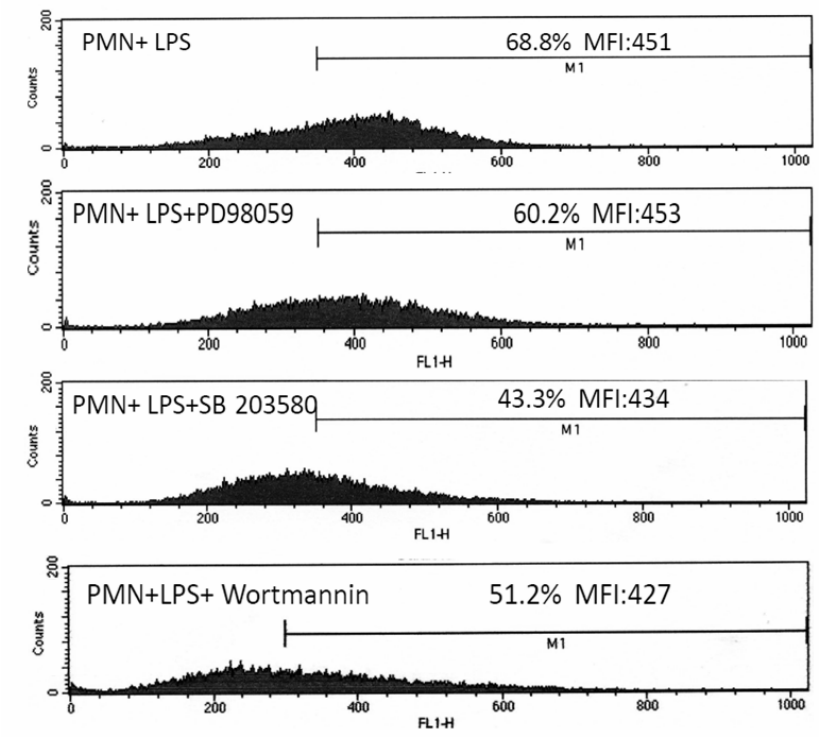


Figure 3 Analysis of the THP carbohydrate side chain by lectins and PMN phagocytosis activities induced by intact THP and its enzyme-digested products. The detailed procedures were described in "Materials and Methods" (A) Lectin binding affinity of THP and other mloecules. (B) PMN phagocytosis activity

A. Affection of the PMN phagocytic activity different kinase inhibitors.



B. Affection of the LPS-induced PMN phagocytic activity by different kinase inhibitors



C. Affection of the THP-induced PMN phagocytic activity by different kinase inhibitors

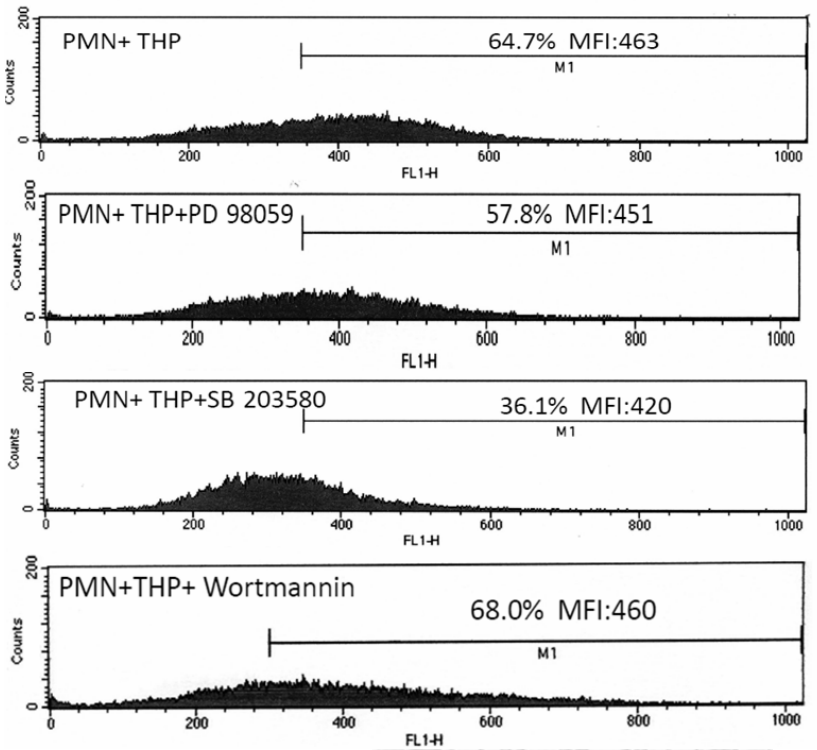
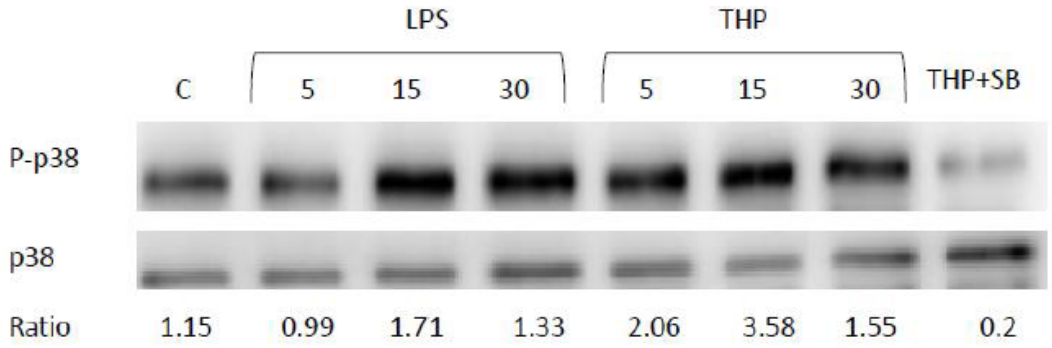
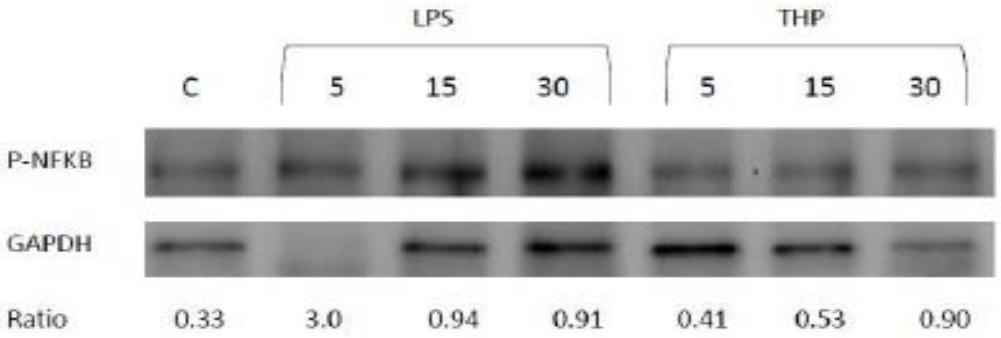


Figure 4 Analysis the signal transduction of THP phagocytosis-enhancing activity by different protein kinase inhibitors. (A)Non-stimulated PMN phagocytic activity. (B) LPS induced PMN phagocytic activity. (C)THP induced PMN phagocytic activity. PMN were pretreated with different kinase inhibitors. PD 98059: a non-competitive inhibitor of mitogen-activated protein kinase kinase 1(MKK1), SB 203580: a p38 inhibitor, and Wortmannin: a specific covalent inhibitor of phosphoinositide 3-kinases (PI3Ks).

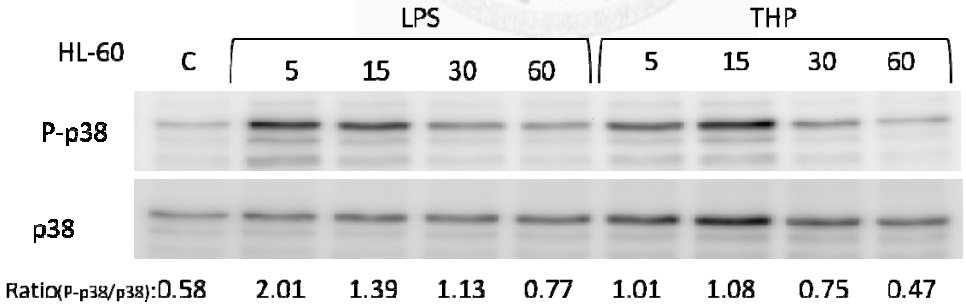
A. THP and LPS stimulated p38 in PMN



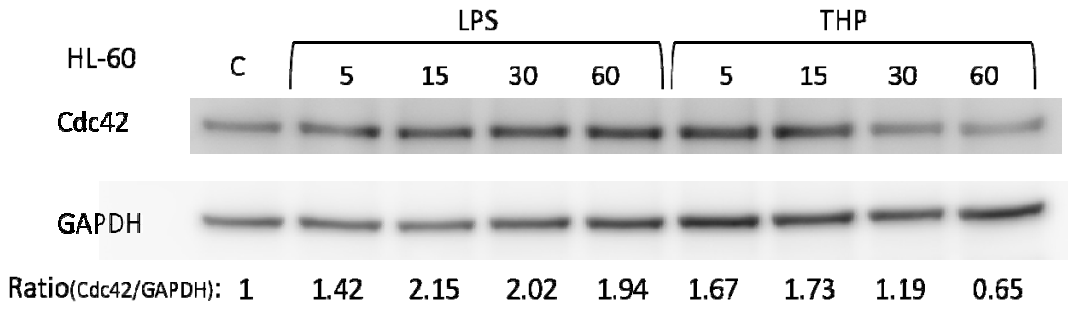
B. THP and LPS stimulated NF-κB in PMN



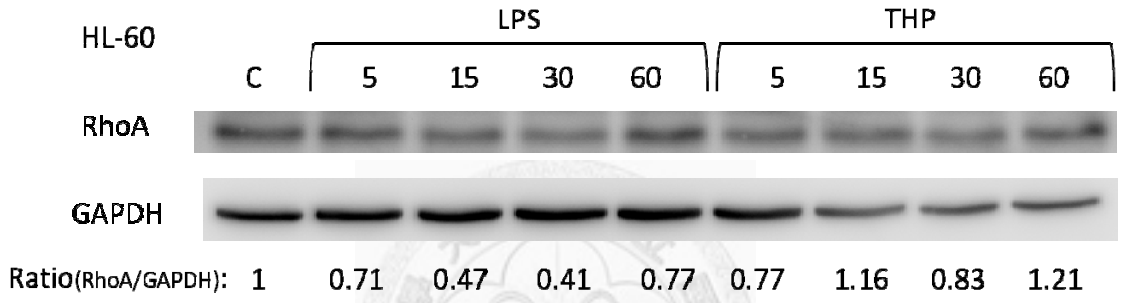
C. THP and LPS stimulated p38 in HL-60



D. Analysis Cdc42 in HL-60



E. Analysis RhoA in HL-60



F. Analysis Rac in HL-60

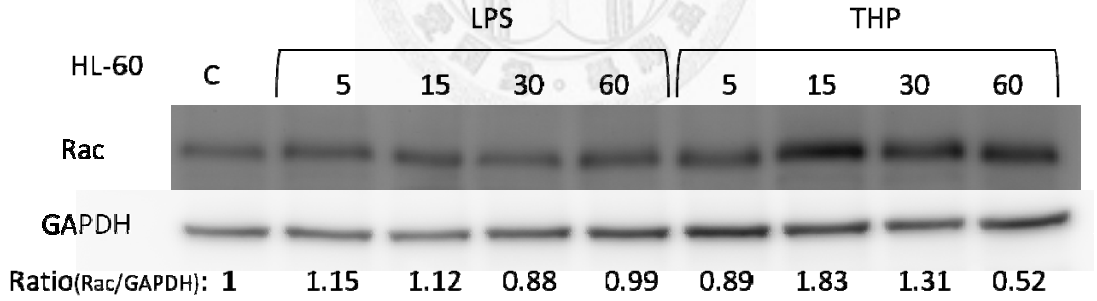
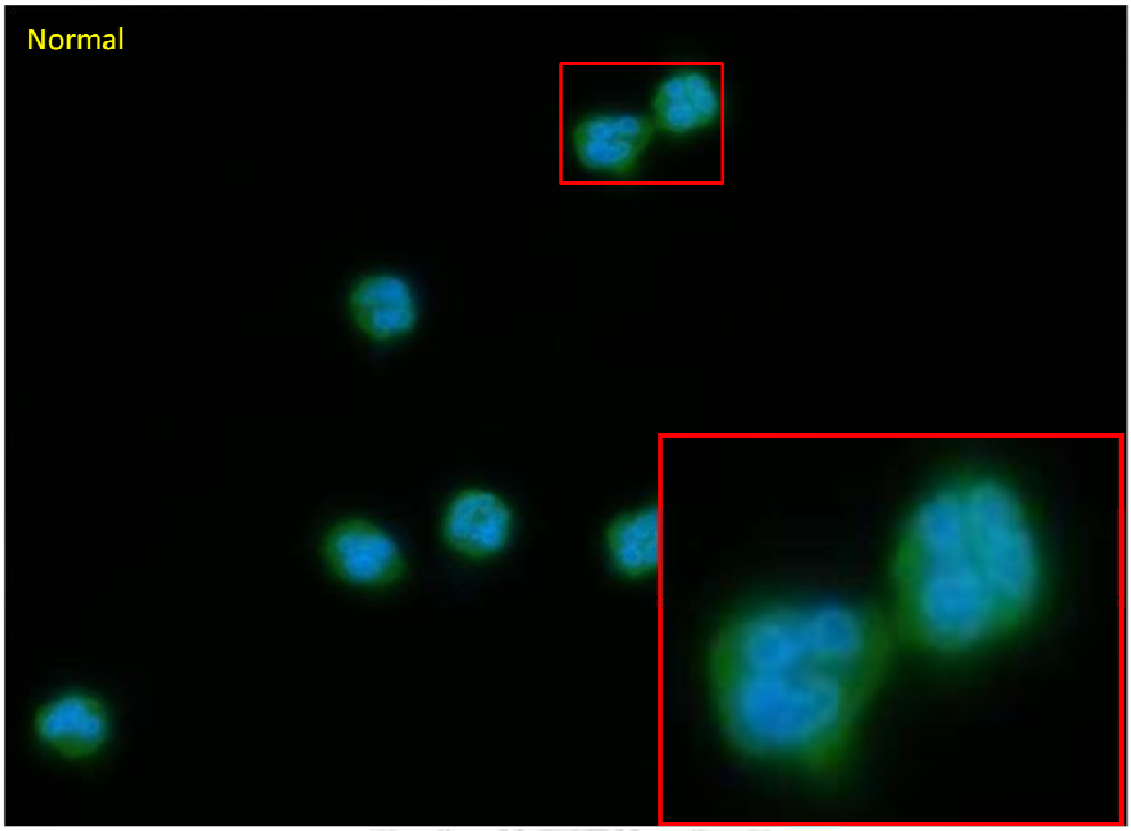
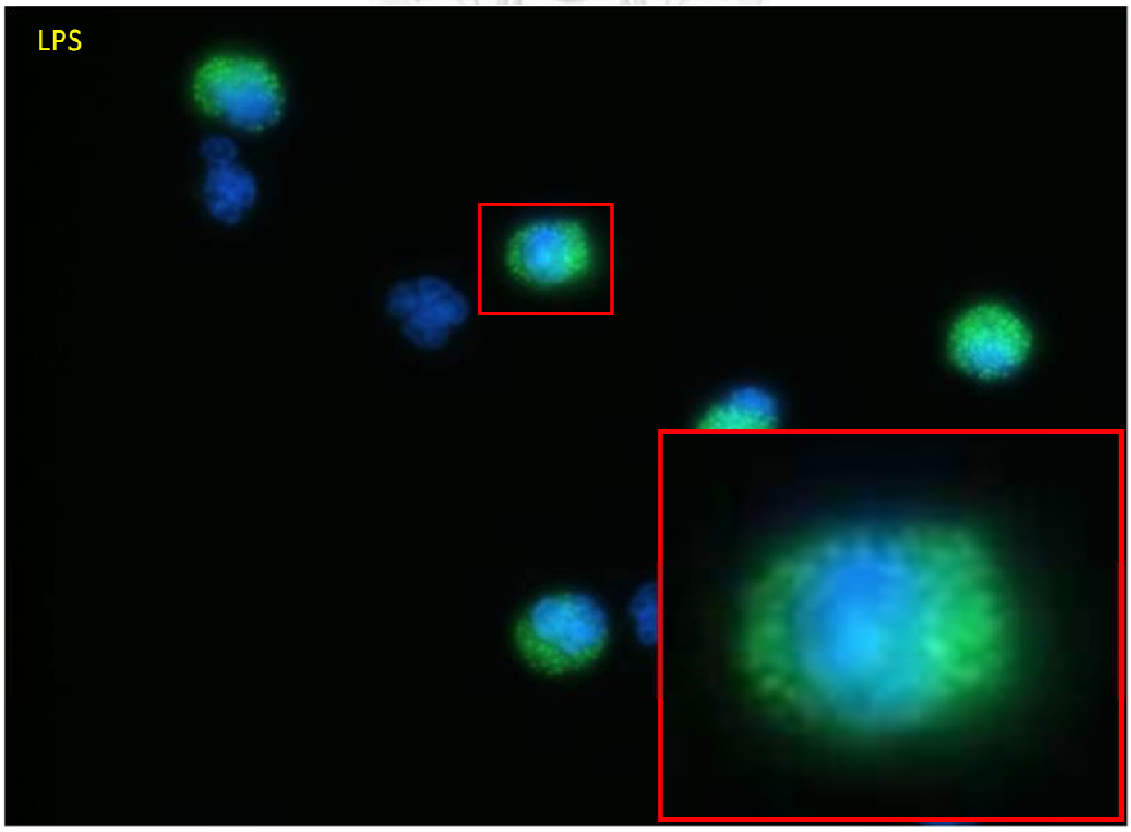


Figure 5 THP and LPS stimulated p38 and NF-κB phosphorylation in PMN and HL-60. PMN were treated with LPS and THP for 5-30 minutes and then analyzed (A) phospho-p38 and (B) phospho-NF-κB in PMN. (C) phosphor-p38 in HL-60, (D) Cdc42 expression in HL-60, (E)RhoA expression in HL-60, (F)Rac expression in HL-60 by western blot.

A.



B.



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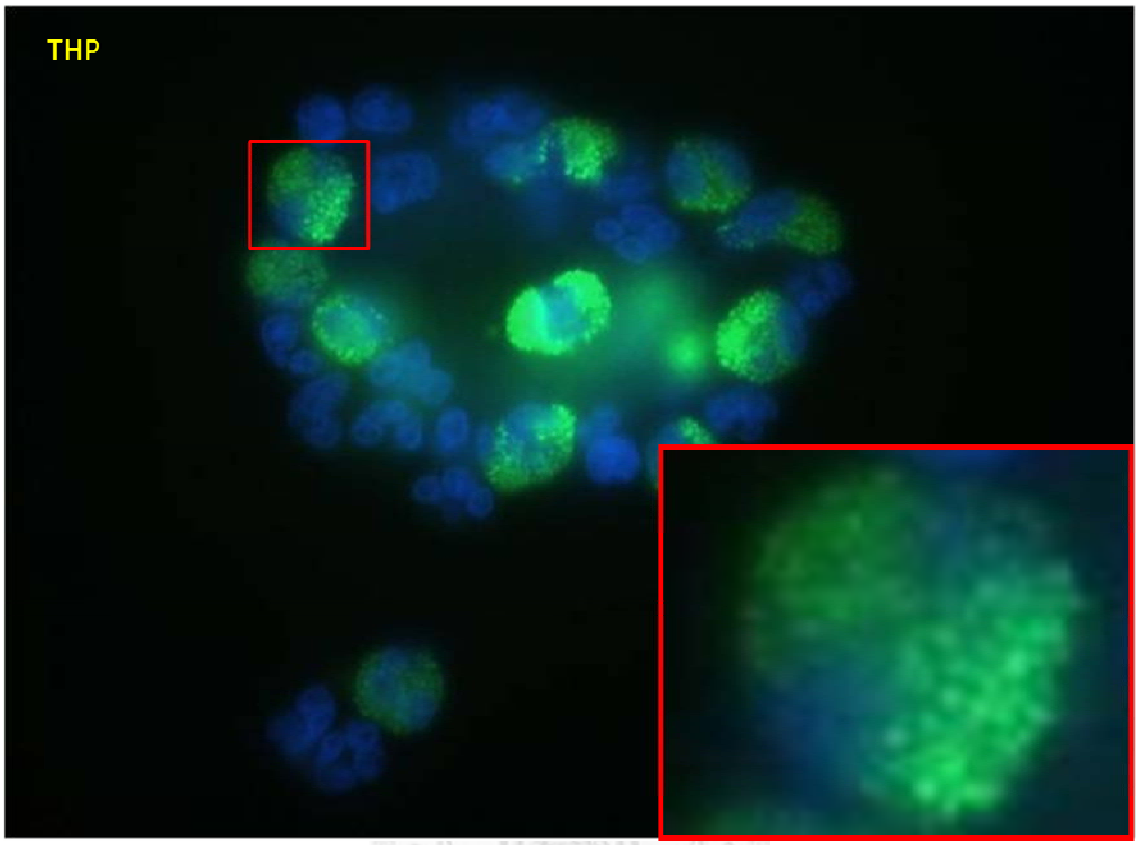

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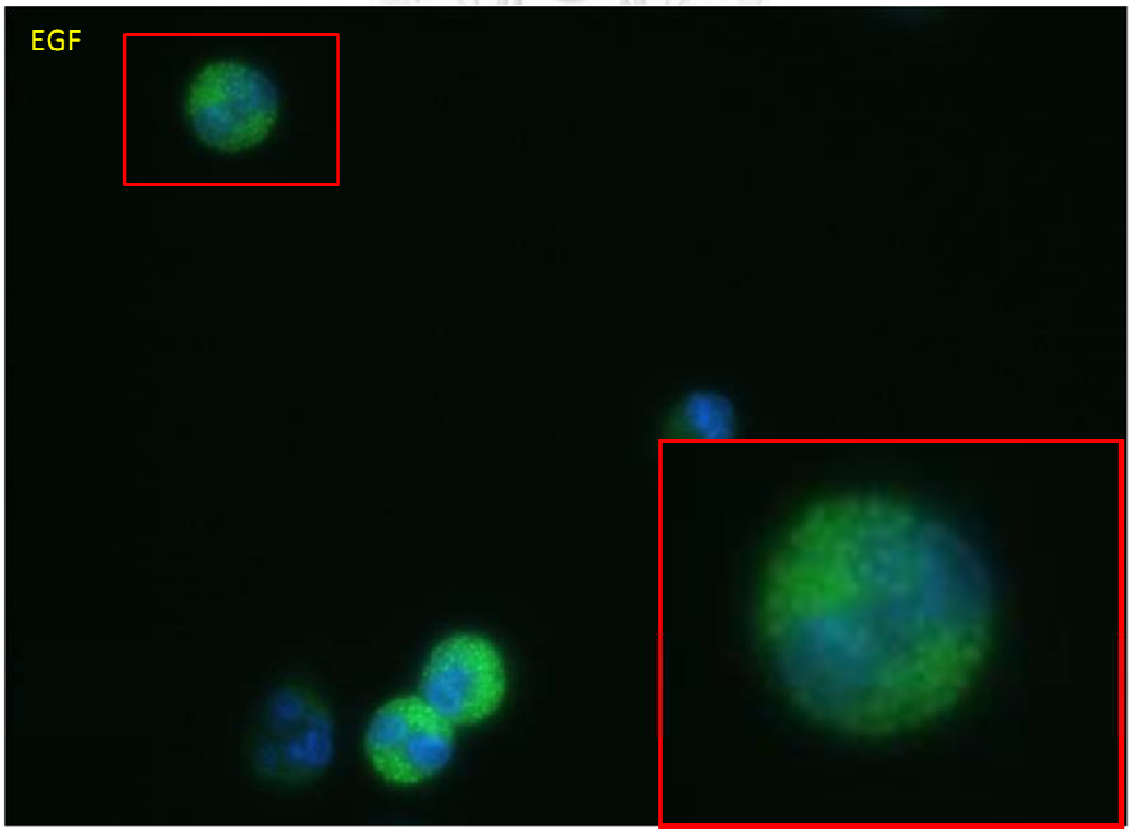
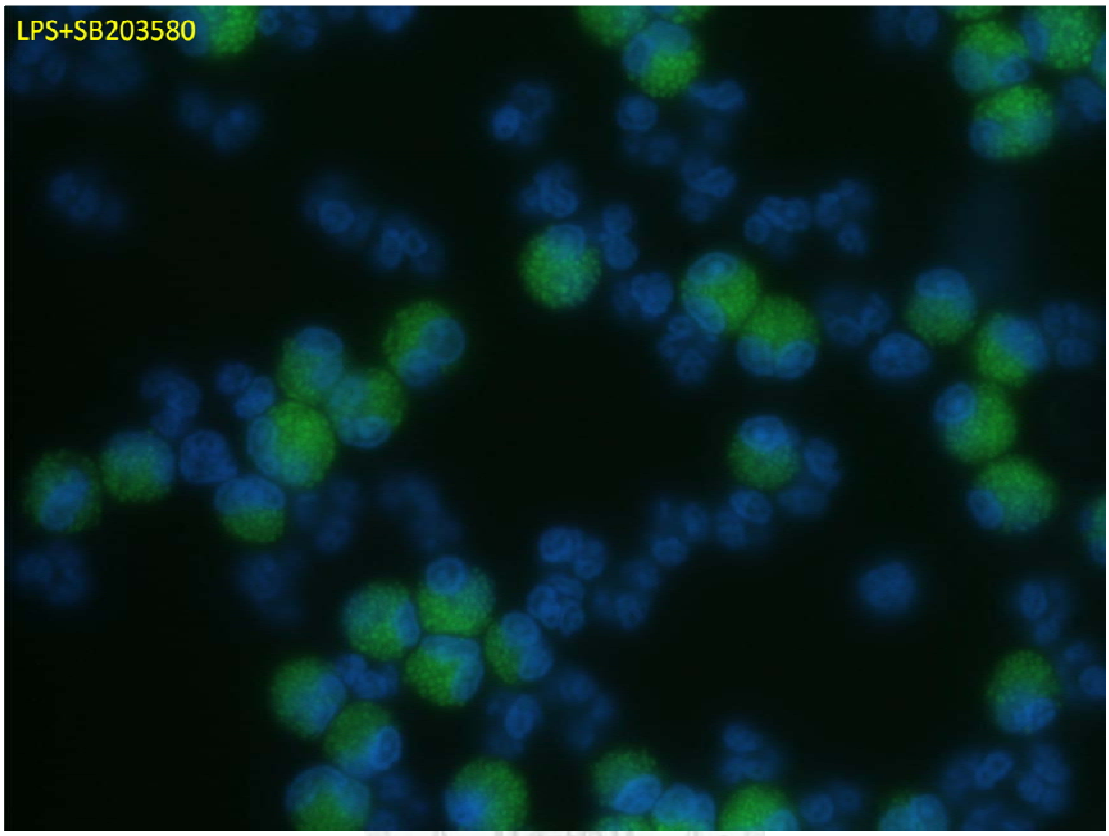


E.



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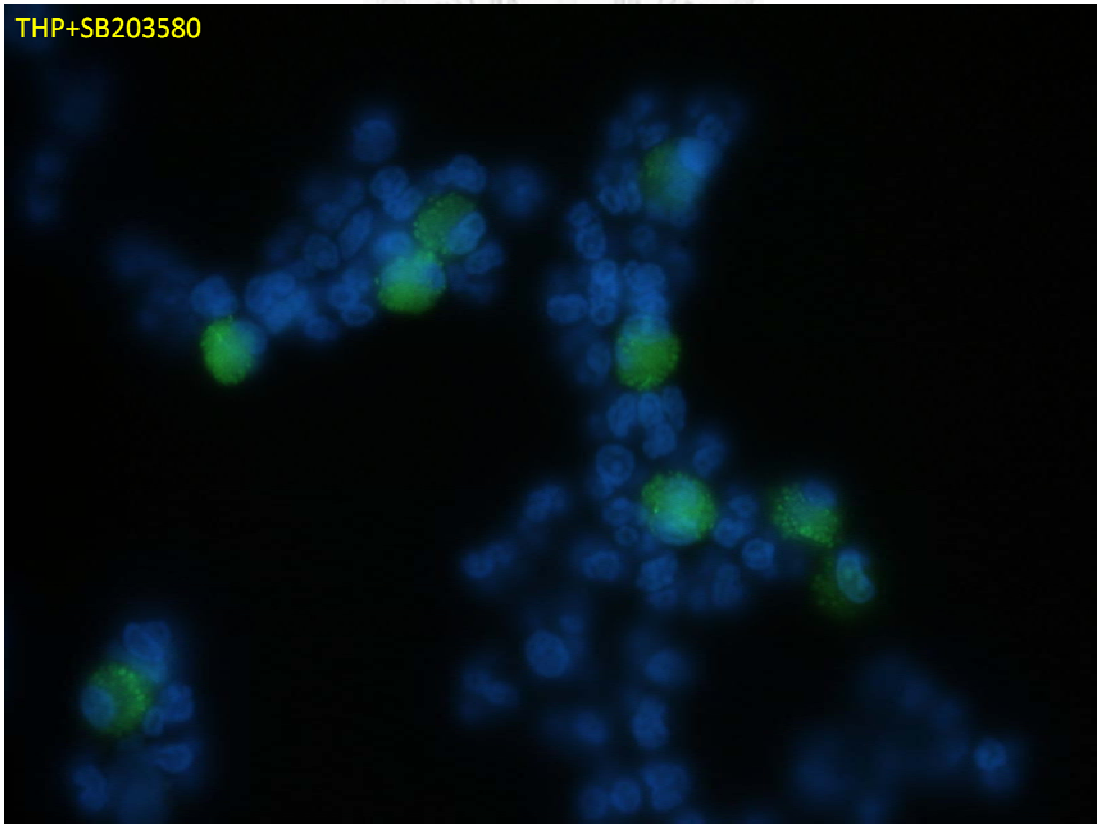


G.

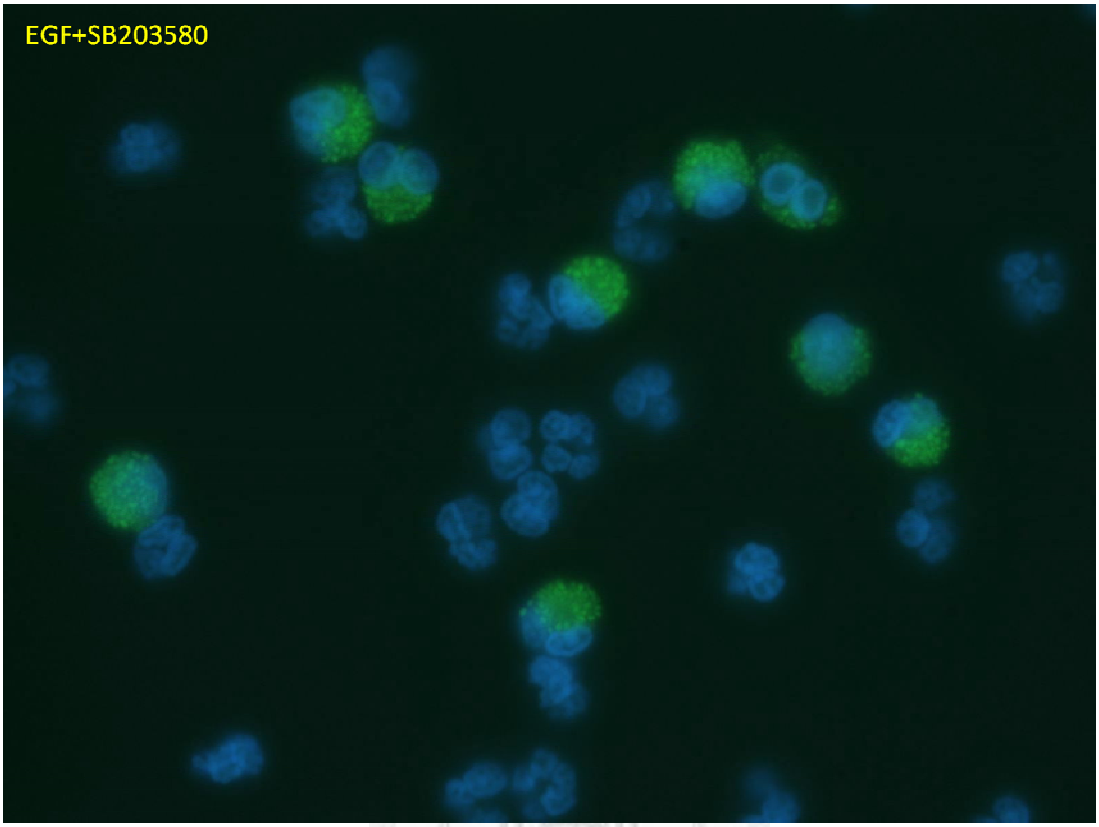
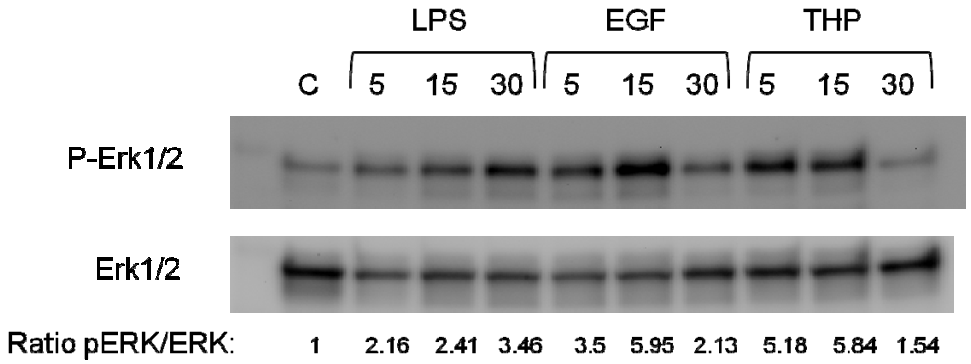


Figure 6 THP and different PMN activation induced cytoskeleton rearrangement.

PMN were incubated with (A) medium, (B) LPS, (C) THP, and (D) EGF for 30 minutes, and stained with fluorescent phalloidin and DAPI. In (E)-(G), PMN were pre-treated with SB 203580(p38 inhibitor) for 20 minutes, and then stimulated with (E)LPS, (F)THP, and (G)EGF respectively; finally, we stained them with fluorescent phalloidin and observed under the fluorescence microscope.

A. Effects of LPS, EGF, and THP on ERK1/2 signaling at different time points



B. Effects of LPS, EGF, and THP on Cdc42 at different time points

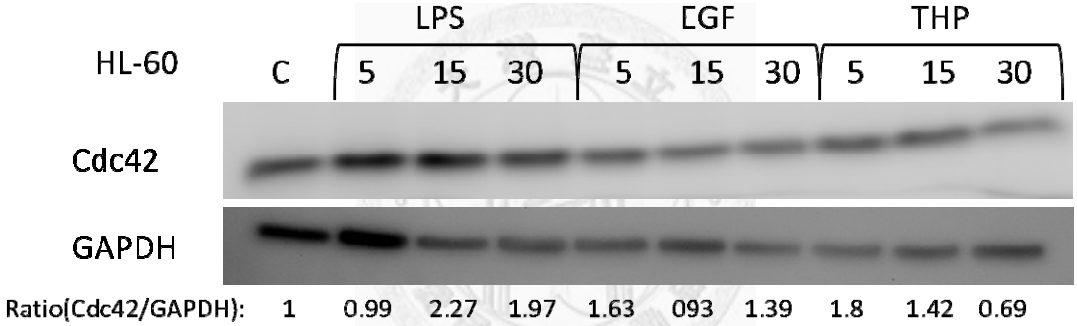
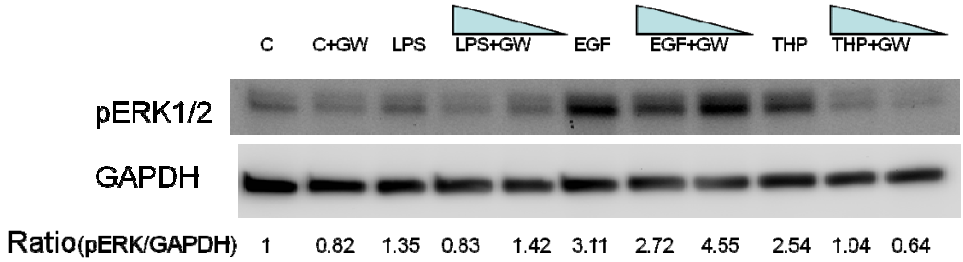
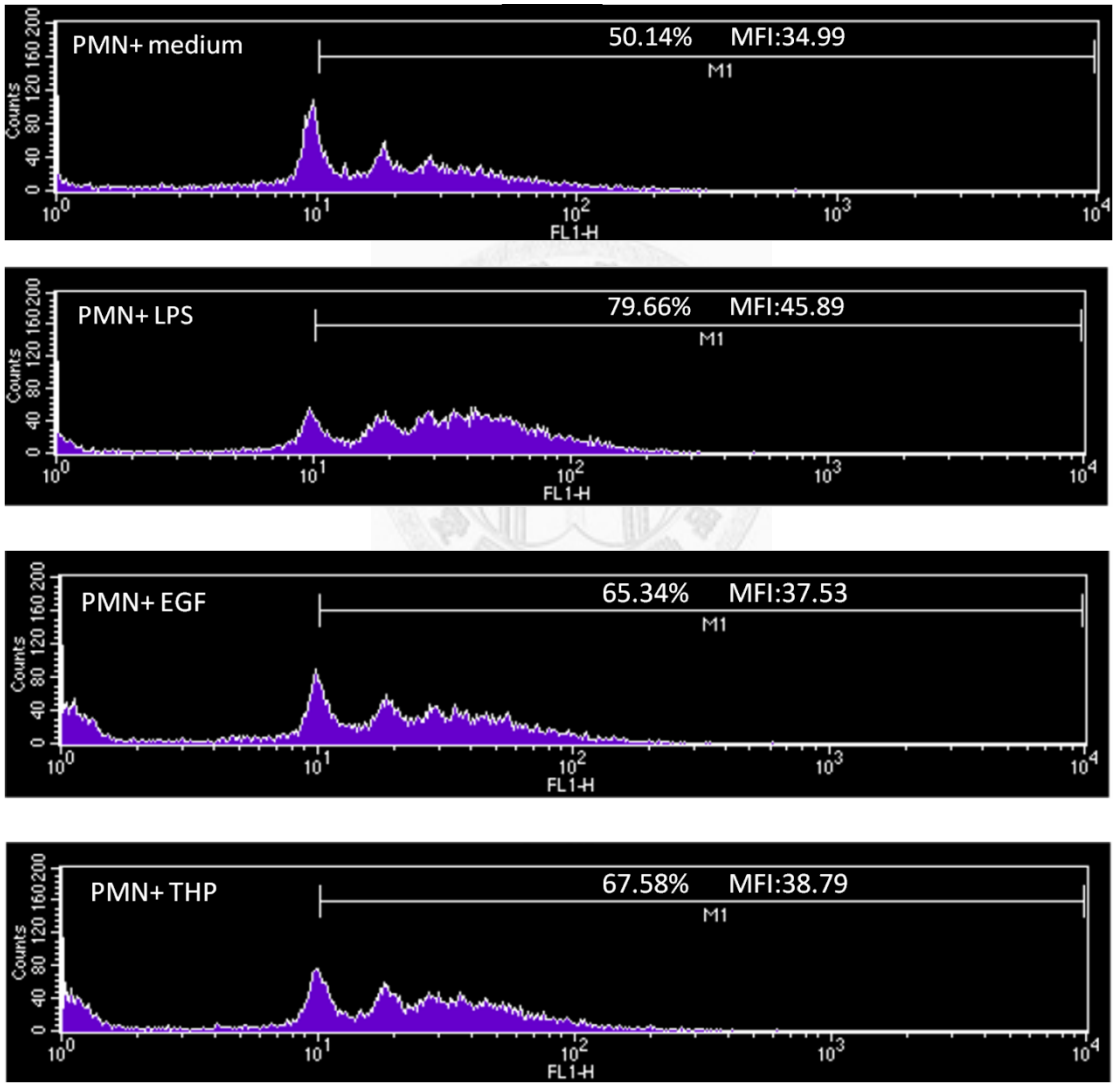


Figure 7 Comparison of ERK1/2 and cdc42 expression in LPS, EGF, or THP stimulated HL-60. HL-60 were treated with LPS, EGF or THP in 5-30 minutes and were analyzed for (A) phospho- ERK1/2 and (B) Cdc42 expression level by Western blot.

A. GW2974(EGFR inhibitor) affected the ERK1/2 signaling



B. LPS, EGF, and THP induced PMN phagocytosis.



C. GW2974 affected LPS, EGF, and THP induced PMN phagocytosis.

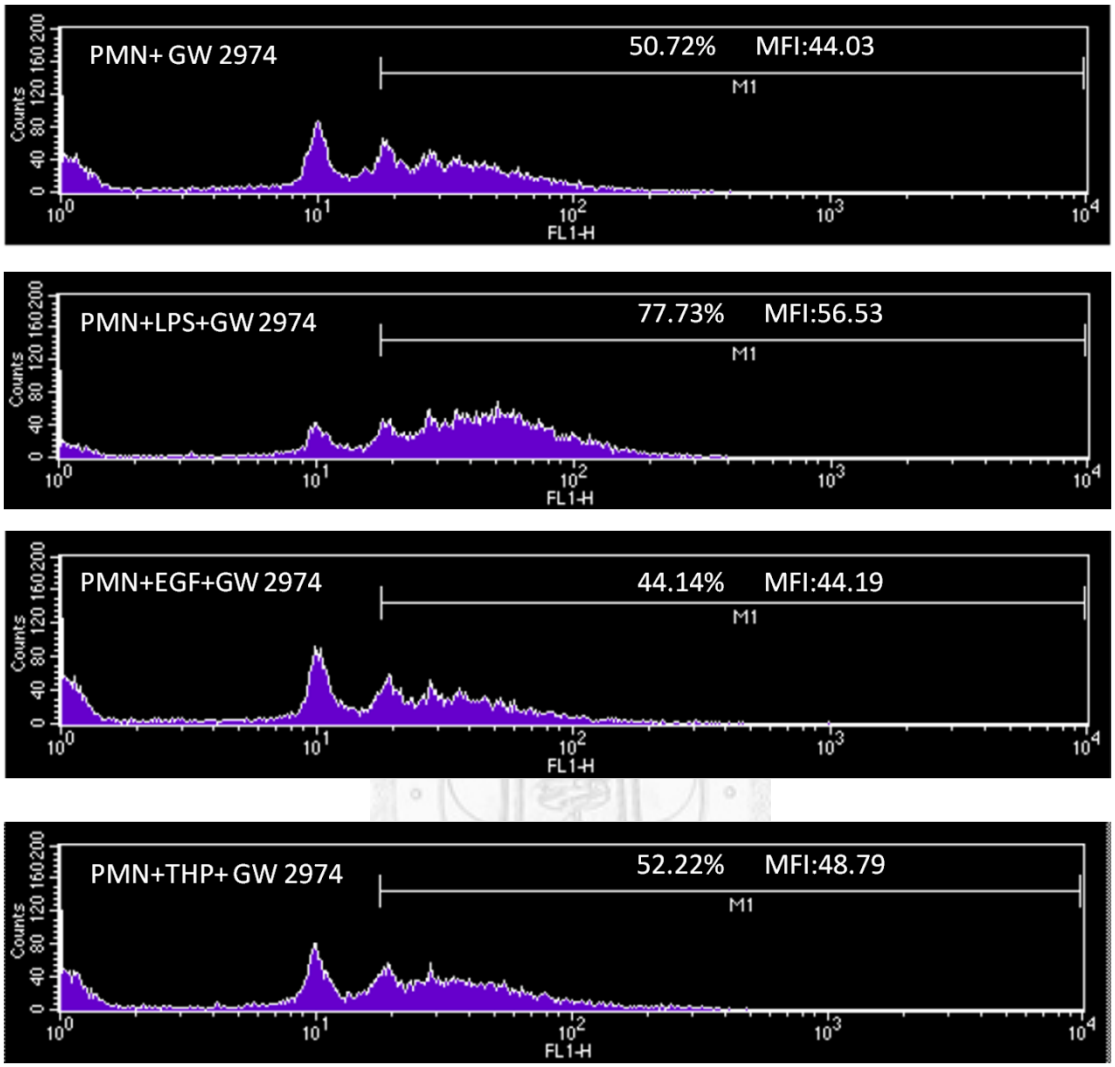


Figure 8 EGFR inhibitor GW2974 decreased phospho-ERK1/2 expression and inhibited THP and EGF but not LPS induced PMN phagocytosis. (A) HL-60 incubated with different concentration of GW2974 for 20 minutes, followed by treatment with LPS, EGF, and THP for 15 minutes. (B) LPS, EGF, and THP stimulated PMN for 30minutes and were analyzed for the PMN phagocytosis

activity. (C) PMN pre-treated with GW2974 for 20 minutes and analyzed for LPS, EGF, or THP induced PMN phagocytic activity.

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