國立臺灣大學醫學院分子醫學所

碩士論文

Institute of Molecular Medicine

College of Medicine

National Taiwan University

Master Thesis

玻尿酸藉由結合CD44活化中性白血球的分子機轉研究

Studies on the Molecular Basis of CD44 and Hyaluronan in Neutrophils Activation

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中華民國 102 年 7 月

July, 2013

國立臺灣大學碩士學位論文
口試委員會審定書

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本論文係林佳慧君（學號 R00448011）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 102 年 7 月 17 日承下列考試委員審查通過及口試及格，特此證明

口試委員：

（簽名）

（指導教授）

系主任、所長 （簽名）

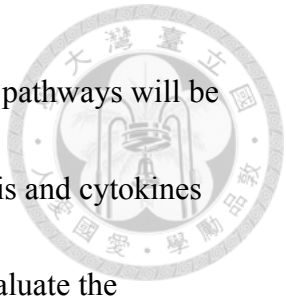
中文摘要

CD44 是一個具有多重功能且位於細胞表面的醣蛋白質，其功能涉及細胞之間的相互作用、細胞附著、遷移、增殖和血管新生，而且在大多數的哺乳動物細胞中皆有表現。玻尿酸（hyaluromic acid; HA）是 CD44 一個非常重要的配體，廣泛分佈在整個結締組織、上皮細胞或神經組織中。CD44 與玻尿酸結合會活化其下游訊息傳遞路徑而引發許多作用。在之前的研究中已經證明 CD44 具有誘導淋巴細胞活化、再循環和吸引淋巴細胞的功能。一些研究報告更指出 CD44 和玻尿酸的結合可以調控發炎反應、拮抗組織損傷和修復由發炎所造成的細胞傷害、減少發炎因子的釋放和增加幹細胞遷移。然而 CD44 與玻尿酸結合後對於中性白血球的影響目前仍不清楚。在我們初步的研究中，發現了一些有趣的結果。首先，脂多醣（LPS）和 anti-CD44 會誘導多核型白血球 (PMN)中 CD44 的表現量增加。而玻尿酸的刺激可以增加多核型白血球的吞噬作用並誘導細胞骨架的重組。CD44 與玻尿酸的結合能誘導 IL-8 的產生。接下來我們更發現，玻尿酸可以誘導 MAP kinase (p38)、ERK1/2 磷酸化增加。在未來的研究方面，我們將添加不同的促發炎因子，觀察多核型白血球 CD44 的表現量。並使用抑製劑阻斷訊息傳遞路徑研究其對多核型白血球的吞噬作用和 cytokine 產生的影響以及所扮演的角色。此外，我們也將使用玻尿酸酶分解玻尿酸，以研究 CD44-HA 的結合對於多核型白血球的影響。最後，我們藉由降低CD44的表現量確認玻尿酸與CD44結合對於發炎反應的調解具有重要性。

關鍵字：CD44、玻尿酸、MAP kinase（p38）、ERK1/2、多核型白血球吞噬作用、細胞骨架的重組

Abstract

CD44 is a multifunctional cell-surface glycoprotein that is involved in cell-cell interaction, cell adhesion, migration, proliferation and angiogenesis and is widely expressed in a large number of mammalian cell types. Hyaluronic acid (HA), one of important ligand of CD44, is distributed widely throughout connective, epithelial, and neural tissues. After CD44-HA binding initiates CD44 downstream signaling pathways, the signaling induces multiple functions. Previous studies have demonstrated that CD44 induces the lymphocyte activation, recirculation, and homing. Some paper have shown that CD44-HA interaction could regulate inflammation, tissue injury and repair through regulating inflammatory cell recruitment, release of inflammatory cytokines, and stem cell migration. However, the effects of CD44-HA interaction on neutrophils remained unclear. In our preliminary study, several interesting results were found. First, we found that the LPS and anti-CD44 could increase the expression of CD44 on PMN. HA could also enhance the phagocytosis activity and cytoskeleton rearrangement of PMN and its binding to CD44 could induce the interlukin-8 (IL-8) production. Furthermore, we found that HA could induce phosphorylation of MAP kinase (p38), and ERK1/2 signaling pathways. In the future, we will test the effect of different pro-inflammatory



cytokines on the surface expression of CD44 on PMN. The signaling pathways will be selectively blocked for the observation of their effects on phagocytosis and cytokines production of PMN. In addition, the hyaluronidase will be used to evaluate the CD44-HA interaction on PMN. Lastly, the knockdown of CD44 expression in PMN will be carried out to define the CD44-independent mechanisms by which HA can mediate inflammation.

keywords：CD44、hyaluronic acid、MAP kinase (p38)、ERK1/2、PMN phagocytosis、cytoskeleton rearrangement

目　錄

Chapter 1. Introduction

CD44 is a multifunctional cell-surface glycoprotein and widely expressed in a large number of mammalian cell types. The most abundant standard isoform of human CD44 protein (CD44s) contains 363 amino acids and its molecular weight is approximately 37 kDa. The structure of CD44s consists of three regions, a C-terminal cytoplasmic domain, to which numerous signaling molecules bind directly or indirectly on activation of the ligand binding, a transmembrane domain, and an extracellular domain which is the so-called link modules of hyaluronan (HA) binding proteins (1, 2) (Figure 1A). The variant isoforms of CD44 (CD44v) inserts an alternatively spliced exons within the extracellular domain, a membrane proximal domain, which can alter the binding affinity for HA and confer interaction with alternative ligands. The CD44 isoform go through the post-translational modification with a molecular weight of about 80 kDa (2). The CD44s is widely expressed in a large nimber of mammalian cell types, and the expression of CD44v is detectable in hematopoietic cells (3), particularly in MNCs (4) and in reactive lymph node cells (3, 5). The principal and important ligand of CD44 is hyaluronic acid (HA), a ubiquitous component of the extracellular matrix (ECM). But it can also interact with other ligands, such as osteopontin (6), collagens (7, 8), and fibronectin (9). HA is a linear, polymeric glycosaminoglycan composed of repeating

disaccharides D-glucuronic acid and *N*-acetyl-D-glucosamine linked by a glucuronidic β (1→3) bond (10, 11) (Figure 1B). HA is widely distributed throughout connective, epithelial, and neural tissues.

CD44-HA interactions mediate cell adhesion and migration in a variety of physiological and pathophysiological processes, including tumour metastasis, wound healing and leukocyte extravasation at sides of inflammation (11, 12, 13, 14). Previous studies have demonstrated that CD44 induces the lymphocyte activation, recirculation, and homing (15, 16). According to Paul W. *et al.* have indicated that the role of CD44 in regulating HA interactions depends on the cell types, and the effect and mechanism of CD44-HA interaction on macrophage have reported (11, 17). However, the effects and mechanism of CD44 binding on neutrophils remain unclear.

CD45, as leukocyte common antigen (LCA), is glycoproteins uniquely expressed on the surface of all leukocytes and their hemopoietic progenitor cells. CD44 is also a one of common LCA on neutrophils. CD45 is a family of high molecular weight transmembrane protein tyrosine phosphatase (PTPase) expressed on all nucleated haematopoietic cells (18). Many authors have reported the possible roles of CD45 and its isoforms in T and B cell differentiation (19), natural killer T cells and cytotoxic T lymphocyte functions (20), cytokine production by MNC (21) and TCR-associated signalling in T cells (22).

Previous studied have shown that the lower CD44 expression in immunodeficiency animal model or disease patient. However, the expression of CD44 is higher in inflammation condition (1, 2). CD44 has a vital role in involving in immune response. So we want to know the role and mechanism of CD44-HA interaction on PMN. To figure out the relationship of CD44 and CD45, and the effects and mechanism of CD44 binding to HA on neutrophils. In the present study, we found that anti-CD44 could increase the expression of CD44 on PMN. CD44-HA interaction could induce IL-8 production through MAP kinase (p38), and ERK1/2 signaling pathways. In addition, it could enhance phagocytosis and cytoskeleton rearrangement of the PMN.

Chapter 2. Materials and methods

2.1 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 in 37℃ (molecular wight: 425000-575000) and incubated at room temperature for 30 minutes. The cell suspension was gently layered over Ficoll-Hypaque density gradient solution (specific gravity 1.077; GE Healthcare, Waukesha, Wisconsin, USA) and centrifuged at 500 x *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. These cells were then rinsed twice with PBS and re-suspended in RPMI 1640 (Gibco/BRL, Grand Island, New York, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine (hereafter referred to as complete medium). The viability of PMN and MNC were detected by trypan blue dye and the cell concentration was adjusted to 2×10^6/ml in complete medium.

2.2 Cell culture

Human promyelocytic leukemia cell line (HL-60) was maintained in complete medium at 37℃ in a humidified atmosphere containing 5% CO_2. HL-60, MNC and PMN (2×10^6

cells/ml) were treated with LPS (20ng/ml), h-IgG (1μg/ml), anti-CD44 (1μg/ml), anti-CD45 (1μg/ml), anti-CD3 (1μg/ml)/ anti-CD28 (1μg/ml), and hyaluronic acid (HA 1mg/ml and 2mg/ml) at 37℃, then cultured and harvested at the indicated time points. Induction of differentiation was obtained by seeding the cells at a concentration of 5 x 10^5/ml in the presence of DMSO (Microbiological Associates, Rockville, Md.) at a final concentration of 1.3% (v/v) for 5 days. After exposure, the cells were resuspended in DMSO-free medium. Next, differentiated-HL60 were treated with LPS (20ng/ml), anti-CD44 (1μg/ml), and hyaluronic acid (HA 1mg/ml and 2mg/ml) at 37℃, then cultured and harvested at the indicated time points.

2.3 Detection of CD44 expression treated with inflammatory molecule by flow cytometry

MNC and PMN were treated with anti-CD3 (1μg/ml)/ anti-CD28 (1μg/ml) or LPS (20ng/ml) at 37℃, before being cultured and harvested at the indicated time points. The cells were washed twice with PBS. The cells were then fixed with 4% paraformaldehyde for 30 minutes at room temperature and incubated with anti-CD44-FITC overnight to detect the CD44 expression. The percentage (%) and mean fiuorescence intensity (MFI, denoted by mean channel number) of CD44 expression were determined by FACSort flow cytometry (Becton Dickinson) at wave

length 488nm excitation.

2.4 Cytokine measurements

After treatment with HA and pro-inflammatory molecule at indicated time points, the cells were centrifuged at 800 x *g* for 10 minutes for the detectection of cytokine concentrations in cell culture supernatant. The concentrations of cytokines including IL-8 were quantified using their respective ELISA kits (R&D Systems, Minneapolis, Minnesota, USA).

2.5 Detection of PMN phagocytosis-enhancing activity of HA by flow cytometry

Fluoresbrit carboxylate microspheres (0.75 μm in diameter, Polyscience Inc.) were washed with PBS in advance twice and opsonized by incubation with fresh human serum at 37℃ for 2 hours. Fresh prepared PMN (2×10^6 cells/ml) were treated with LPS (20ng/ml), h-IgG (1μg/ml), anti-CD44 (1μg/ml), anti-CD45 (1μg/ml), and HA (1mg/ml and 2mg/ml) at 37℃ for 1 hours. The mixture was let reacting with opsonized beads (1×10^8 beads/ml) at 37℃ for 1 hour in 5% CO_2-95% air. After incubation and wash by PBS twice, 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 of 488nm excitation.

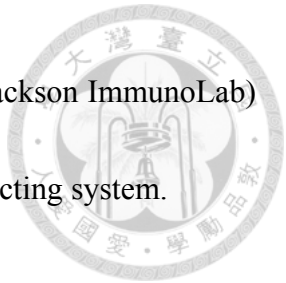
2.6 Preparation of whole cell extraction

After treatment with HA or control medium at different time points at 37℃, the cells were centrifuged at 800 x *g* for 10 minutes followed by wash with cold PBS. After centrifugation at 800 x *g* for 10 minutes, the pelleted cells were lysed with cold RIPA buffer (25 mM 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 lysates were centrifuged at 10,000 x *g* at 4℃ for 20 minutes to remove the debris and the supernatants were applied for Western blot. The protein concentration of the cell extraction was measured using the BCA Protein Assay (Pierce).

2.7 Western blotting

Proteins were separated by 10% SDS-PAGE and transfer to polyvinylidene fluoride (PVDF) membrane (Millipore Inc.) in a Mini Trans-Blot cell (Bio-Rad) for 2 hours at 350 mA. 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 overnight at 4℃. After washing the membrane three times with TBST buffer each for 5 minutes, the

complexes were detected by HRP-conjucated-secondary antibody (Jackson ImmunoLab) and ECL Western Blotting Substrate (Pierce) chemifluorescence detecting system.



2.8 Immunofluorescence microscopic observation of cytoskeleton change in activated-PMN

After stimulation at 37℃ by HA or LPS for 2 hours, the activated-PMN were wash with PBS and then fixed for 30 minutes at room temperature with a solution of 4% paraformaldehyde followed by washing with PBS. Then mixture was centrifuged and the supernatant was discarded. The cells were resuspended in 200 μL of PBS. 5 to 10 μL of the cell suspension was smeared above a gelatin-coated slide. Placed the slide on a hot plate (low heat setting), and allowed the liquid to evaporate. The PMN were permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature, and washed three times with PBS. The blocking solution (10% FBS in PBS) was added to inhibit the non-specific binding. PMNs were stained with fluorescent phalloidin (1:200) for visualization of actin filament at room temperature for 30 to 60 minutes. The slides were washed three times with PBS and stained with DAPI (4’,6-diamidino-2-phenylindole, 1:1000) at room temperature for 5 minutes. After three times washes with PBS, the slides were mounted with glycerol and gently covered the cover slip avoiding producing the air bubbles. The cells were visualized the

cytoskeleton was observed using a fluorescence microscope (Olympus, Japan).

2.9 Statistical analysis

The statistical analyses were performed using the analysis of variance (ANOVA).

Statistical significance was defined as $p < 0.05$.

Chapter 3. Results

3.1 The expression of CD44 on MNC and PMN

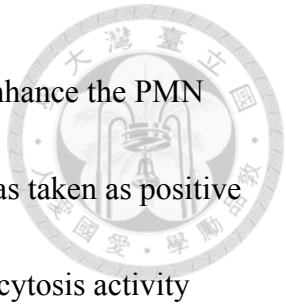
Previous investigations have shown that during inflammation, CD44 expression is upregulated on hematopoietic and parenchymal cells (23, 24), and it plays a crucial role in an variety of inflammatory diseases including murine models of inflammatory bowel disease, collagen- and proteoglycan-induced arthritis, cutaneous inflammation, experimental autoimmune encephalomyelitis, and IL-2-induced vascular leak syndrome (25, 26, 27, 28). To understand whether the inflammatory molecules could enhance the CD44 expression on MNC and PMN, we used pro-inflammatory molecules anti-CD3/ anti-CD28 on MNC. The results indicated that co-stimulated anti-CD3/ anti-CD28 as well as LPS provided a strong pro-inflammatory signal on PMN (29, 30). As determined and demonstrated by flow cytometry data, anti-CD3/ anti-CD28 had significantly enhanced the percentage of CD44 expression on MNC compared to the control (Figure 2A). On the other hand, LPS also slightly increased the CD44 expression on PMN (Figure 2B). Accordingly, we concluded that pro-inflammatory cytokines could increase the CD44 expression on PMN.

3.2 HA induced IL-8 production on PMN

Next, we determined whether CD44-HA interaction could induce the PMN into the activation condition and enhance inflammation. We used LPS, anti-CD44, anti-CD45 and HA to stimulate the PMN and then detect the interleukin-8 (IL-8) concentration, which is a one of the neutrophil-specific CXC subfamily of chemokines. IL-8 is also a potent neutrophil chemotactic and activating factor and is a primary pro-inflammatory cytokine produced by many cells (31, 32, 33, 34). We could demonstrate that LPS increases the IL-8 production and HA has significantly increased IL-8 concentration, compared to the controls. However, anti-CD44 and anti-CD45 might have little increasing effect on IL-8 production. Therefore, CD44-HA interaction could promote PMN to go inyo the activated-form.

3.3 HA enhanced PMN phagocytosis activity

Previous studied have shown that the role of CD44 in binding, ingestion (phagocytosis), and clearance of apoptotic cells (35) as well as microbial pathogens (36). A direct antibody ligation of CD44 on macrophages enhances the subsequent uptake of apoptotic cells (37, 38). To further confirm the CD44-HA interaction could enhance the PMN phagocytosis, we used HA to stimulate PMN and Fluoresbrit carboxylate microspheres



to determine the PMN phagocytosis by flow cytometry. LPS could enhance the PMN phagocytosis compared to the controls (49.23% to 75.57%) and so was taken as positive controls. The CD44-HA interaction significantly increased the phagocytosis activity (49.23% to 93.01%), to an extent of even greater than LPS stimulation. Anti-CD44 (49.23% to 62.87%) and anti-CD45 (49.23% to 63.57%) also stimulated the PMN phagocytosis (Figure 4), but this increase was smaller than that exerted by LPS and HA. These results of anti-CD45 were consistent with previous reports (39). It suggested that CD44-HA interaction could enhance the PMN phagocytosis activity.

3.4 HA induces cytoskeleton rearrangement on PMN

Previous investigations have indicated that the cytoplasmic domain of CD44 has a linker protein- ankyrin specific binding site (36, 40). Ankyrins proteins are a family of adaptor proteins that mediate the attachment of integral membrane proteins to the spectrin-actin based membrane cytoskeleton (41). To understand if CD44-HA interaction on PMN could initiate the CD44 downstream signaling pathway and induce cytoskeleton rearrangement, the cells were treated with HA. After treatment with HA, we used phalloidin to stain actin filaments and observed the aggregation of actin filaments. We found that not only HA could induce cytoskeleton rearrangement but also anti-CD44 and anti-CD45 could partially induce the actin aggregation (Figure 5). These

results indicated that CD44 on PMN could mediate the cytoskeleton change and CD44-HA interaction could enhance cytoskeleton rearrangement to achieve its biological function.

3.5 HA induced phosphorylation of MAPK and ERK1/2 on PMN and MNC

Previous studied have demonstrated that CD44-HA interaction could activate several signaling pathways (42, 43). To further understanded the molecule mechanism of the CD44-HA interaction on activated-PMN and activated-MNC, we observed the phosphorylation of signal transduction. In Fig 6A and 6B, you can see that HA could increase the phosphorylation of MAPK (P38) and ERK1/2 on MNC. On the other hand, CD44-HA interaction could also significantly increase the phosphorylation of MAPK (P38) and ERK1/2 on PMN (Figure 6C and 6D). These results indicated that HA could induce and activate MAPK (P38) and ERK1/2 signaling pathway.

3.6 HA abolished to induce IL-8 production on differiated-HL60 cells

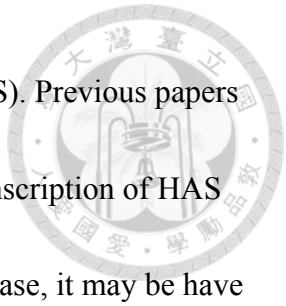
According to Liu J. *et al.* have shown that down-regulation of CD44 contributes to the differentiated-HL60 cells (44). To investigate the effect of CD44-HA interaction on the differentiation of HL-60 cells, we treated with DMSO to induce cells differentiation and detect IL-8 production after treatment with HA. We found that LPS increased the IL-8

production, however HA and anti-CD44 abolished to induce IL-8 production on differiated-HL60 cells (Figure 7). Therefore, it indicated that HA is specific binding to CD44 and induce downstream signaling pathway.

Chapter 4. Discussion

1. The role of hyaluronic acid in tissue and cell

Hyaluronic acid, a ubiquitous component of the extracellular matrix (ECM), is widely distributed throughout connective, epithelial, and neural tissues. CD44 is expressed in numerous cell types. Previous papers have shown that CD44 is involved in and mediated many biological functions. In normal condition, HA is enriched in ECM. How the cell determines and regulates HA binding to CD44 further prevents HA induced downstream signaling pathway from activating PMN or MNC is unknown. Otherwise, many authors have reported that the CD44 molecule plays a central role in the development of collagen- or proteoglycan (cartilage delivered)-induced arthritis (45, 46, 47, 48, 49). The expression of CD44 and extractable HA is increased in the arthritic inflamed joint tissues, suggesting that they are associated with the inflammatory process (50). Hyaluronic acid is primarily used to increase mobility of the joints, clear eye vision, combat signs of aging such as wrinkles, relief fibromyalgia and help wound heal. The phenomenon is obviously contrary to our current results showing that HA could induce tissue inflammation. So, it might have another mechanism underlying the mediation and regulation of the CD44-HA interaction. There have several possibilities



to affect the CD44-HA interaction: one is hyaluronan synthases (HAS). Previous papers have indicated that inflammation molecules, TNFα, increases the transcription of HAS and the expression on cell membrane (51). And another is hyaluronidase, it may be have some mechanism to regulate it enzyme activity or concentration in cells to increase or decrease the concentration of HA. There have the papers shown that the HA oligomer compete for endogenous polymeric HA, thus replacing high affinity, multivalent and cooperative interactions with low affinity, low valency receptor interactions (52). The last is the expression of CD44, according to figure 7 that down-regulation of CD44 abolished CD44-HA activation. And these possibilities need further improve and understand its regulation and detail mechanism.

2. The relationship of CD44 and CD45

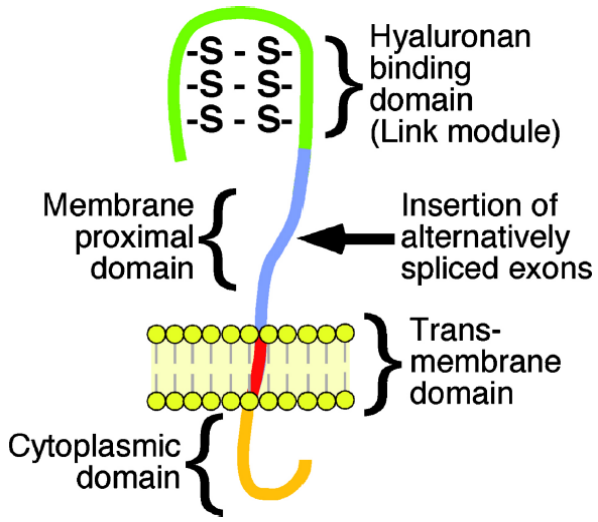
CD45, as leukocyte common antigen, is a family of high molecular weight transmembrane protein tyrosine phosphatase (PTPase) expressed on all nucleated haematopoietic cells (17). Many authors have reported the possible roles of CD45 and its isoforms in cell differentiation (18), cytokine production (20) and TCR-associated signalling (21). Recent studied have shown that CD45, negatively regulatory role for CD45 in CD44 signaling leading to actin rearrangement and cell spreading in activated thymocytes and T cells. According to Yu C. L. *et al.* 2002 and our present data the CD45 and CD44 had similar function on PMN. The relationship of CD44 and CD45 mediating and regulating PMN remains to be confirmed.

Chapter 5. Conclusions and Future directions

This study showed the effect of CD44-HA interaction on PMN. First, we found that pro-inflammatory molecule (LPS and anti-CD3/anti-CD28) could increase the expression of CD44. Next, we showed that HA could activate PMN and increase the IL-8 production. We also demonstrated CD44-HA interaction not only could enhance the phagocytosis activity but also induced cytoskeleton reorganization. We observed that CD44-HA interaction could induce phosphorylation of MAPK and ERK1/2 signaling pathway. Last, we investigated that HA abolished induce the IL-8 production on the differentiation of HL60 cells induced by DMSO. In the future, we are going to use signaling transduction inhibitor to block the signaling pathway and to investigate the effects of phagocytosis as well as cytokines production on PMN. In addition, we will use the hyaluronidase to investigate the CD44-HA interaction on PMN. Last, we will knock down the CD44 in PMN expression to define the CD44-independent mechanisms by which HA can mediate inflammation.

Chapter 6. Figures

A



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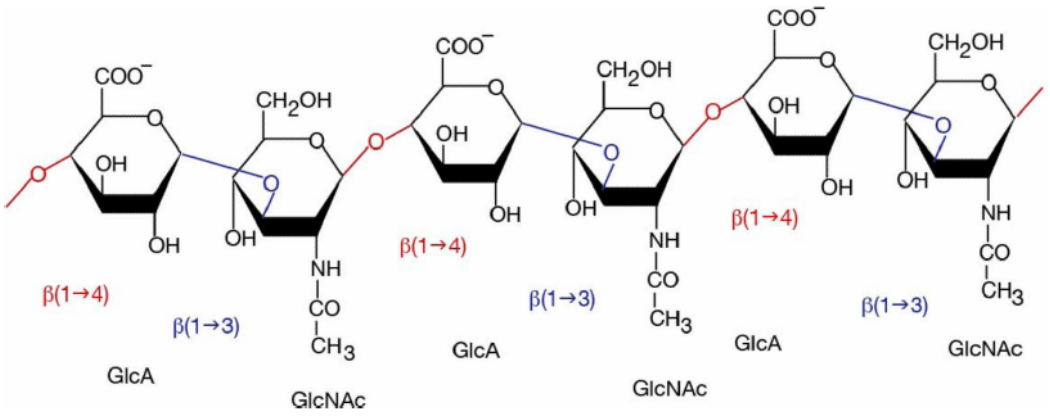
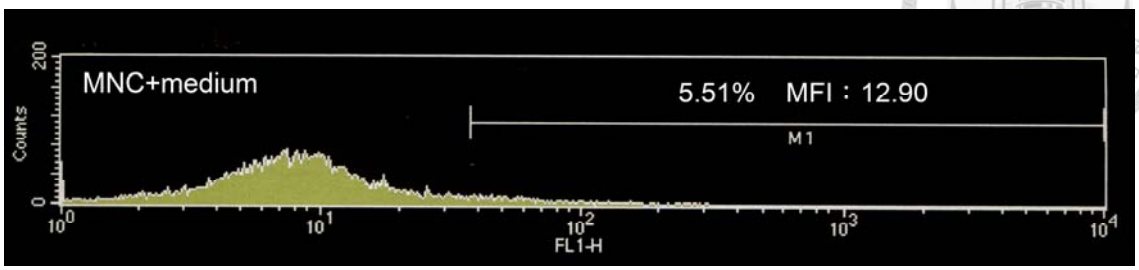
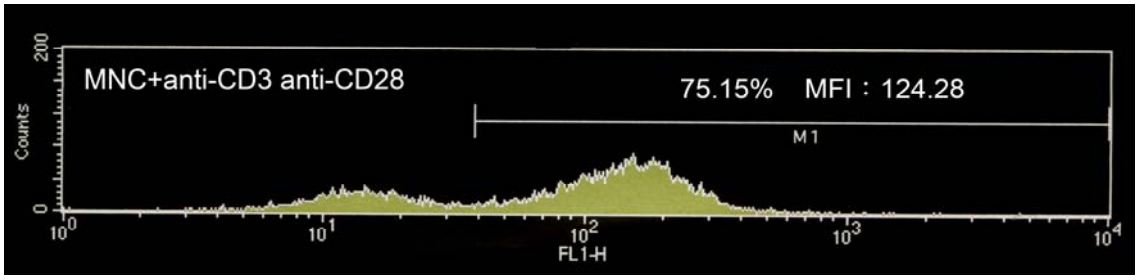


Figure 1 Domains of CD44 and the structure of hyaluronic acid (HA). (A) The major domains of the standard isoform of human CD44 are shown. They consist of three regions, a C-terminal cytoplasmic domain which signals molecules directly or indirectly to binding region, a hydrophobic transmembrane domain, and a extracellular

domain, which is the so-called link modules of hyaluronan binding proteins. However, the variant isoforms of CD44 inserts a alternatively spliced exons within the extracellular domain, a membrane proximal domain, which can alter the binding affinity for HA and confer interaction with alternative ligands. (B) Hyaluronic acid is composed of repeating polymeric disaccharides D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) linked by a glucuronidic β (1→3) bond.

A





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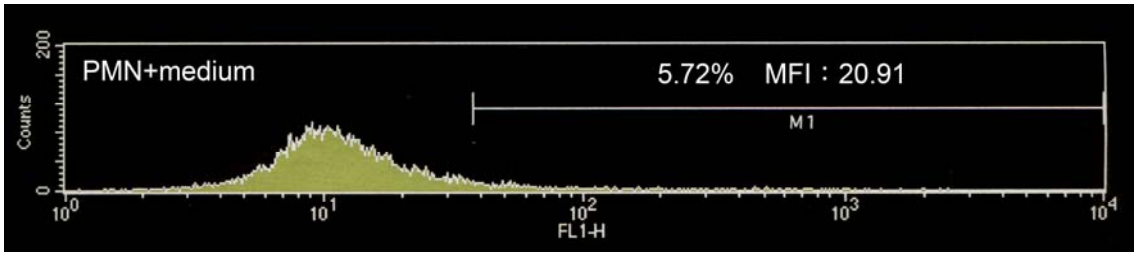


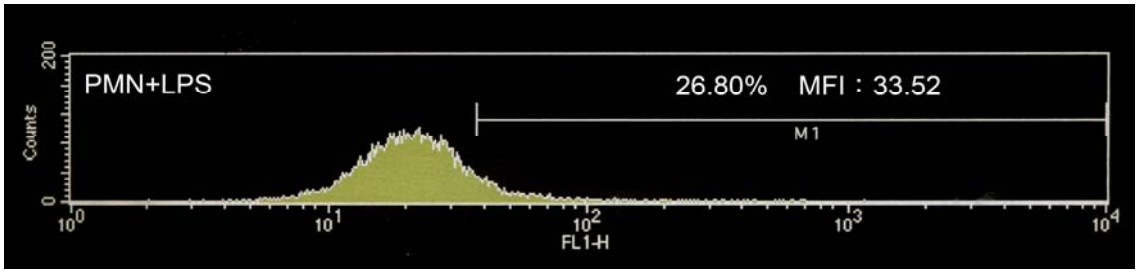


Figure 2 Pro-inflammatory molecule enhances the expression of CD44 on MNC and PMN. (A) MNC added with anti-CD3 and anti-CD28, (B) PMN added with LPS and inflammatory molecule could enhance the expression of CD44.

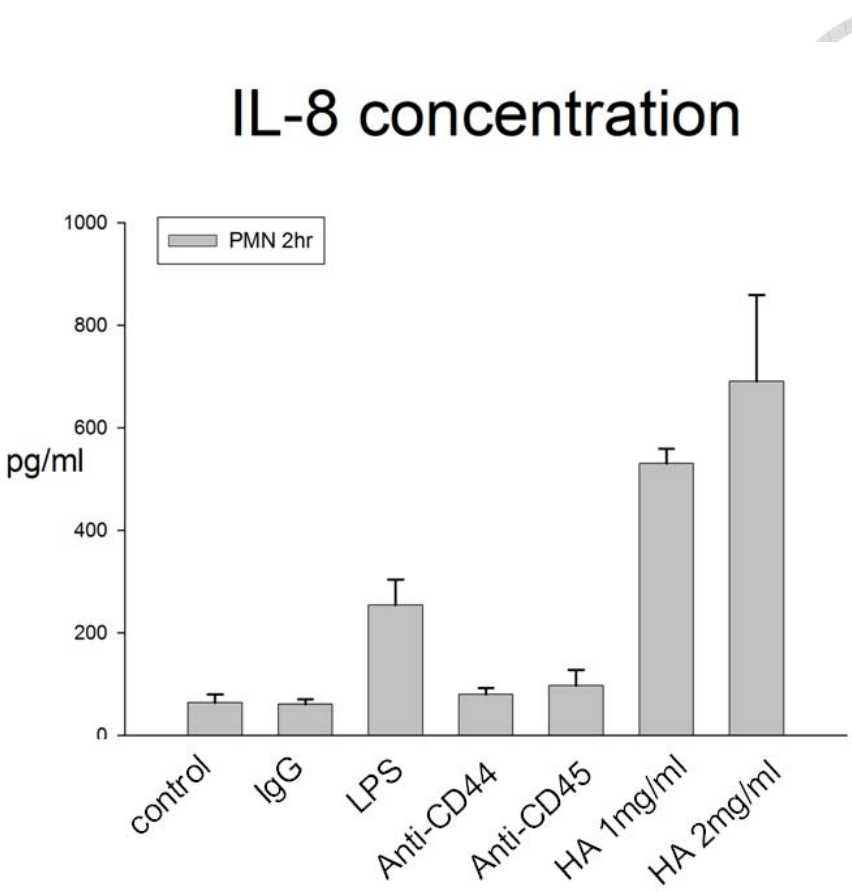
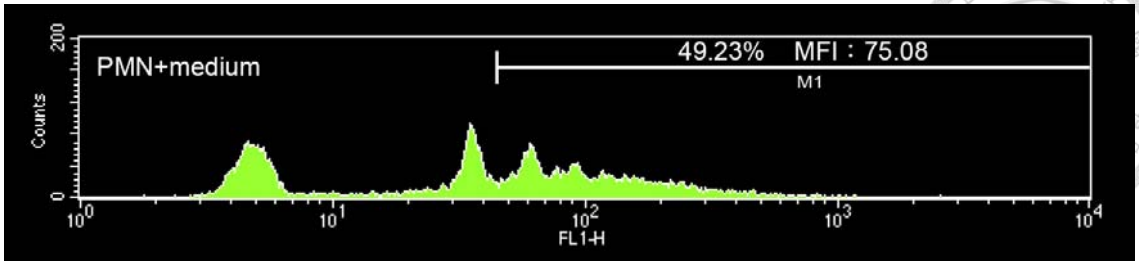
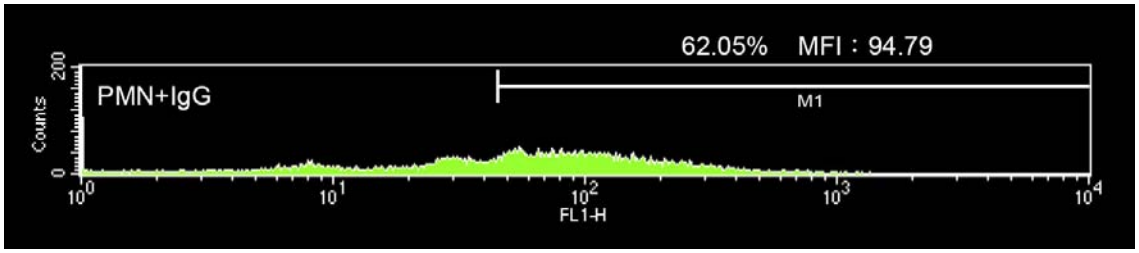
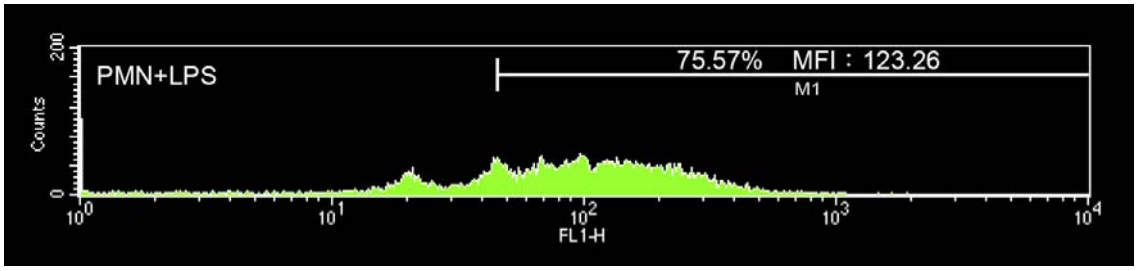
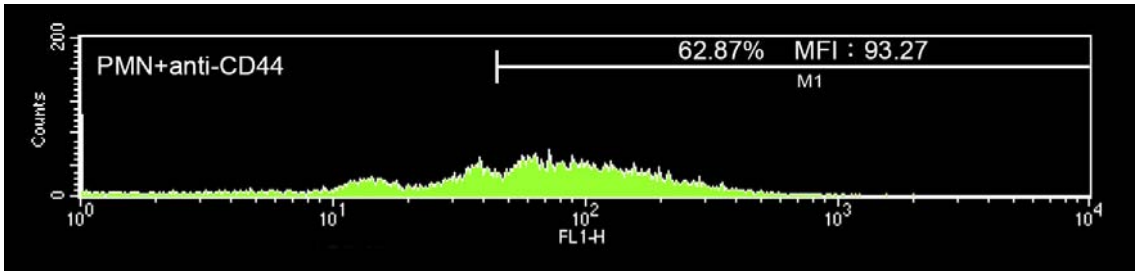


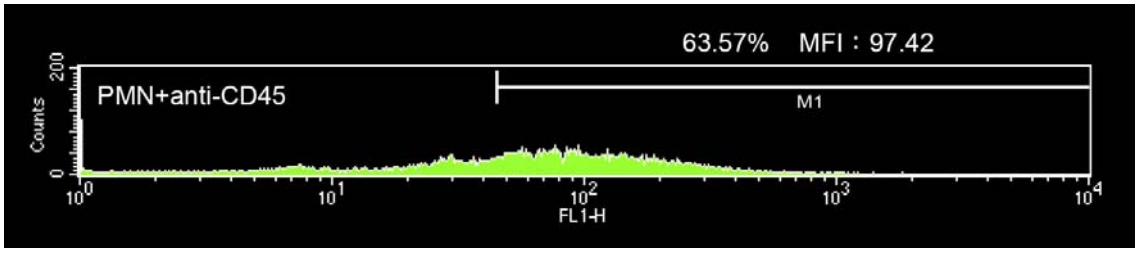
Figure 3 HA induced IL-8 production. The detailed procedures were described in "Materials and Mathods" LPS as positive control. HA could induce the IL-8 production by PMN. However, anti-CD44 or anti-CD45 had little effect on IL-8 concentration.


PMN+medium
49.23% MFI : 75.08
M1
Counts
FL1-H


PMN+IgG
62.05% MFI : 94.79
M1
Counts
FL1-H


PMN+LPS
75.57% MFI : 123.26
M1
Counts
FL1-H


PMN+anti-CD44
62.87% MFI : 93.27
M1
Counts
FL1-H


PMN+anti-CD45
63.57% MFI : 97.42
M1
Counts
FL1-H

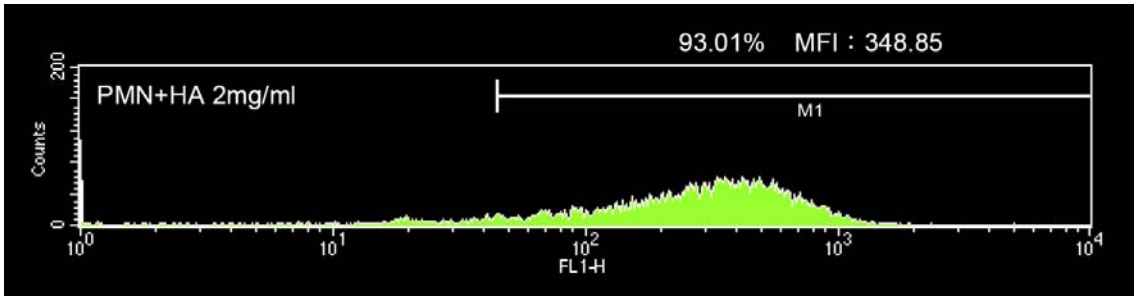
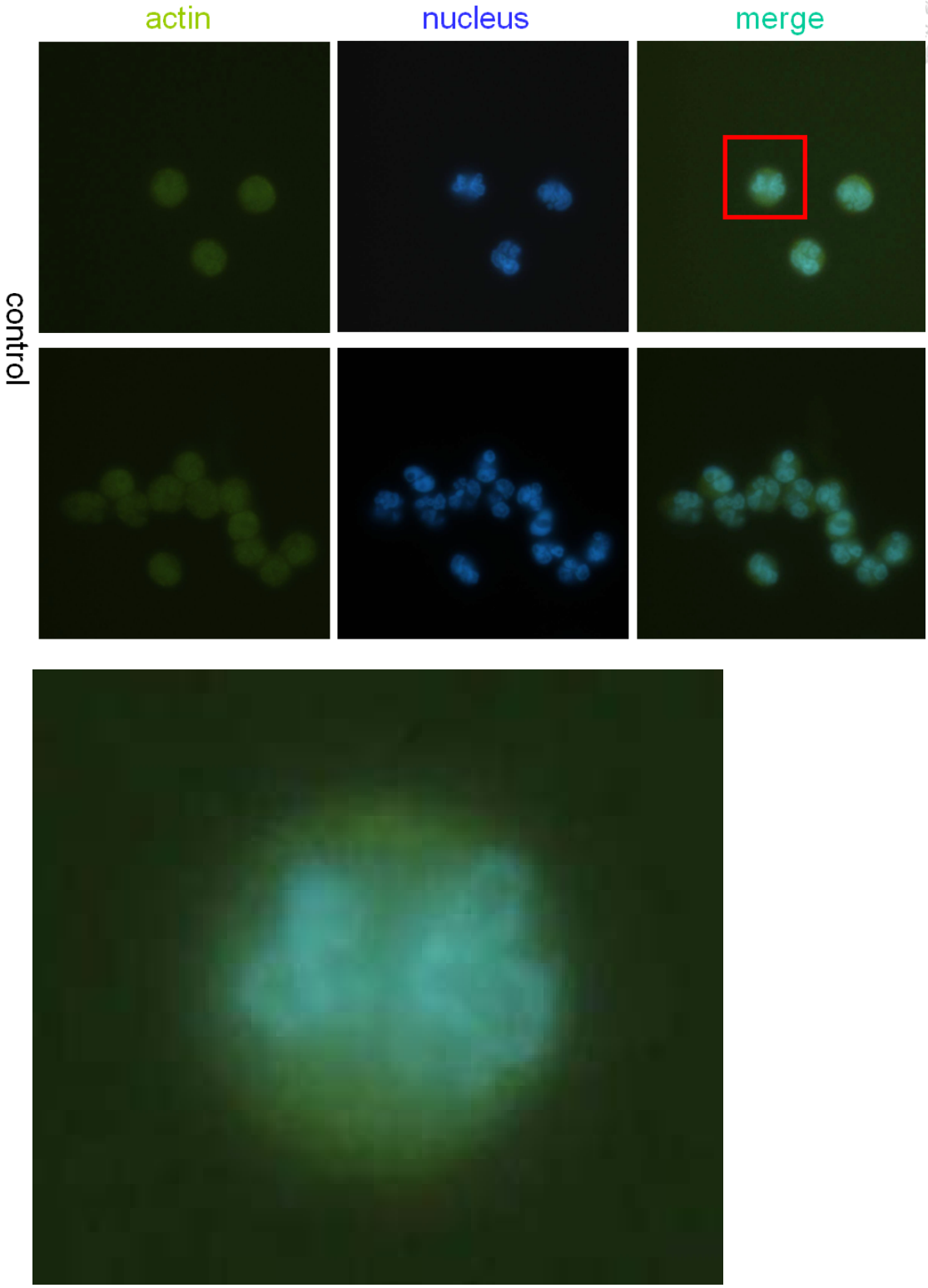
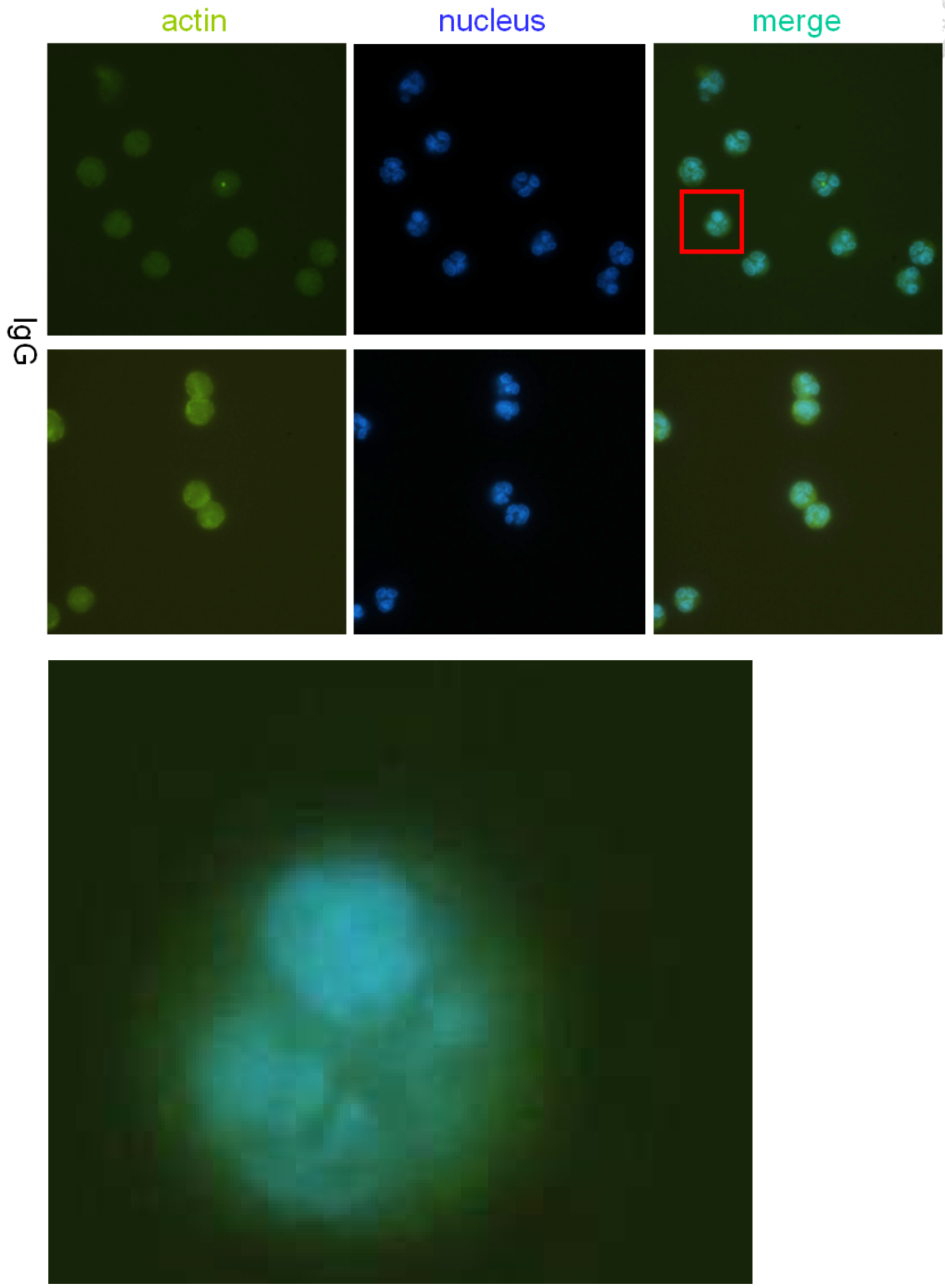

PMN+HA 2mg/ml
93.01% MFI : 348.85
M1
Counts
FL1-H

Figure 4 HA enhanced PMN phagocytosis activity. LPS enhances the phagocytosis and is used as positive control. HA had greater enhancement of phagocytosis compared to control and LPS. And anti-CD44 and anti-CD45 induced increase in phagocytosis, compared to the controls to an extent less them that by LPS.

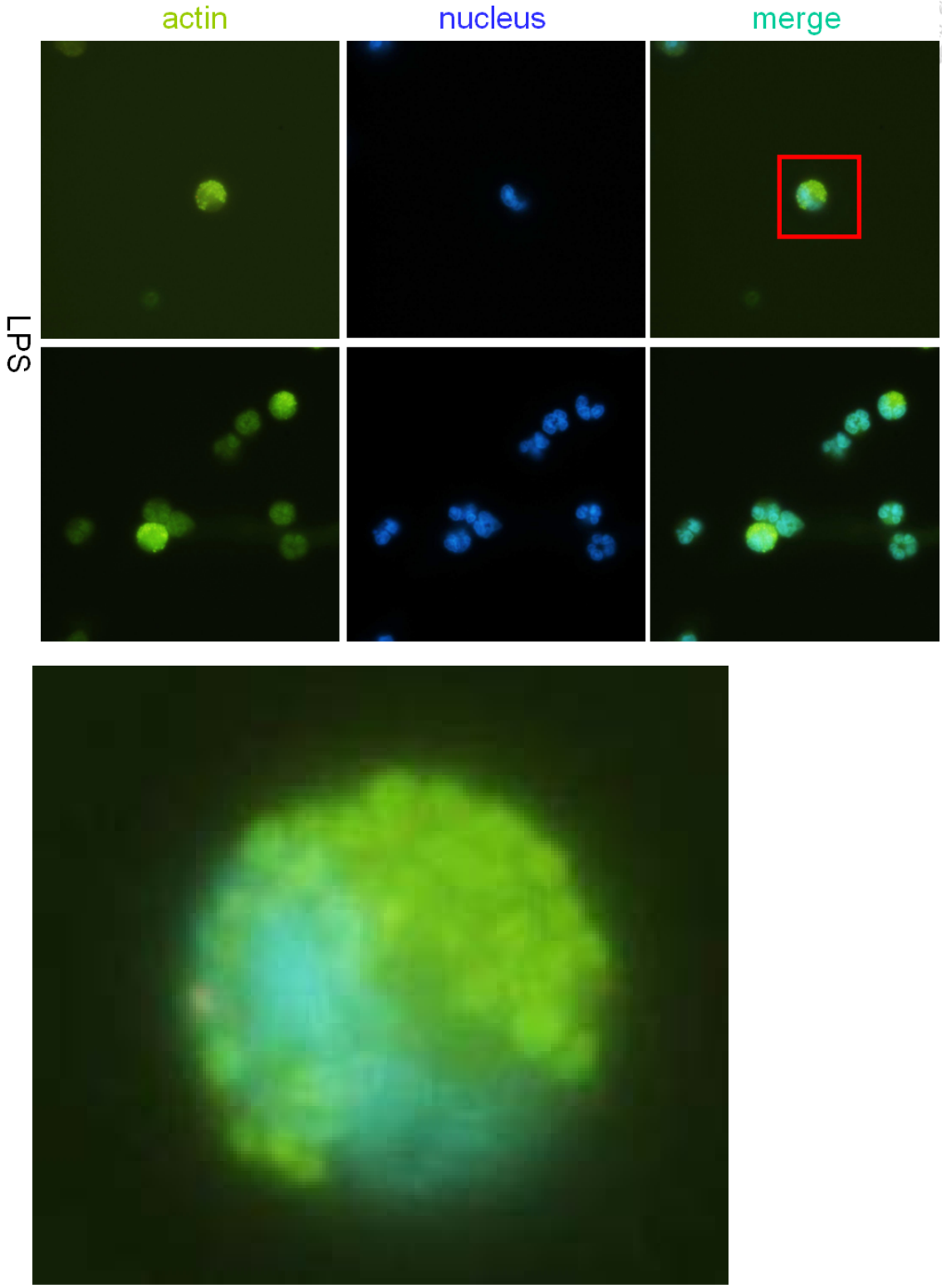
A



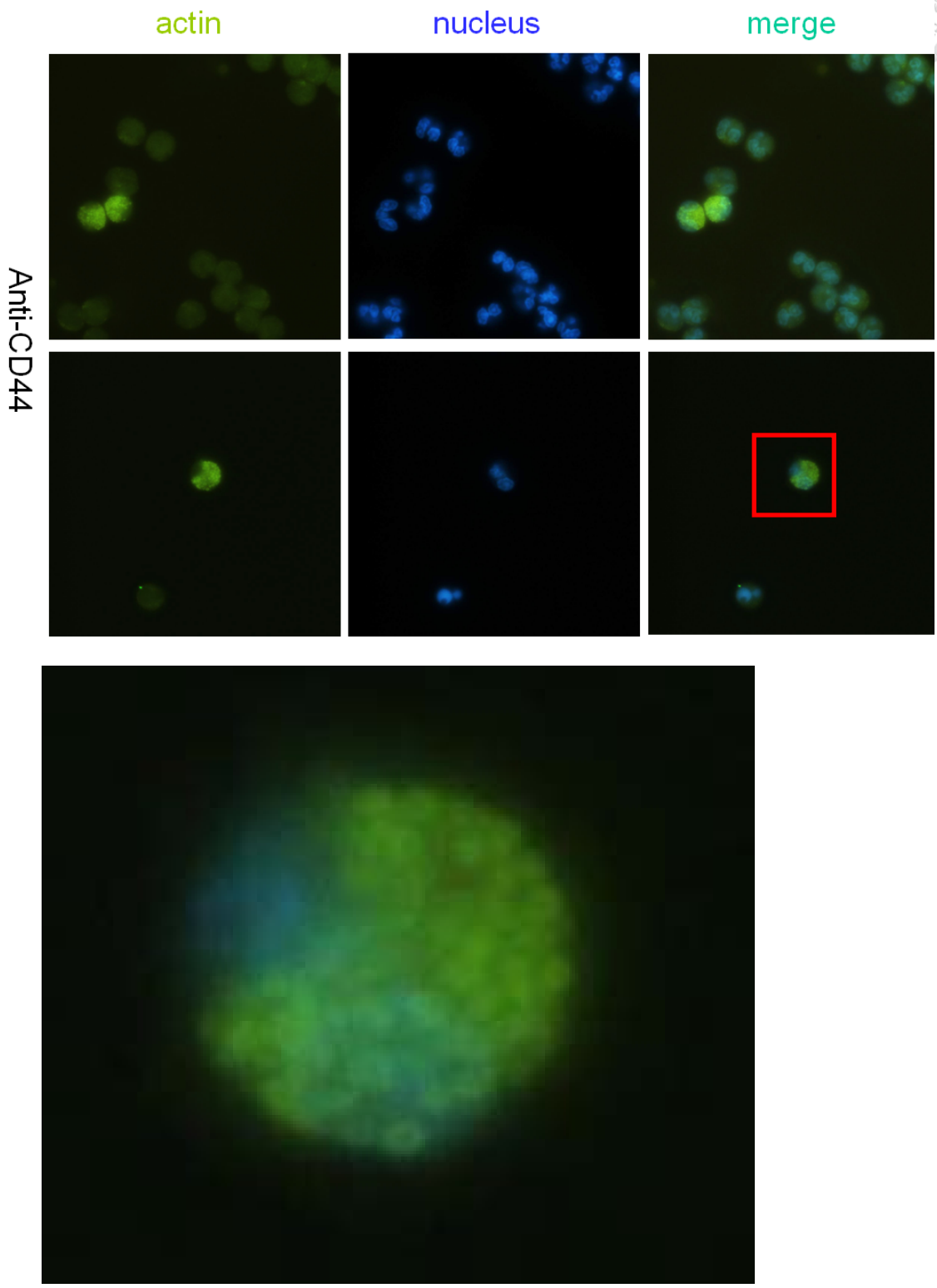
B



C



D



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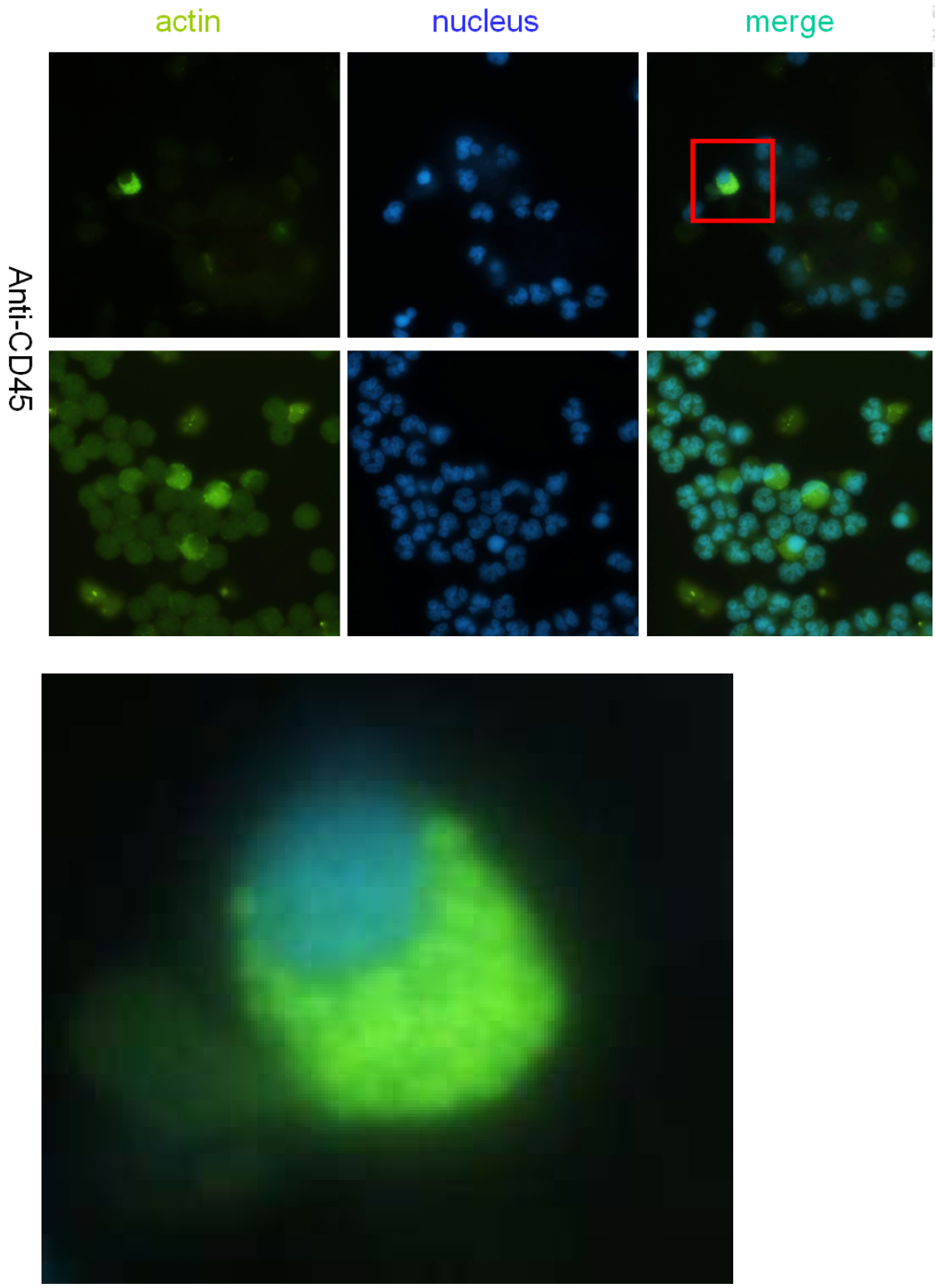

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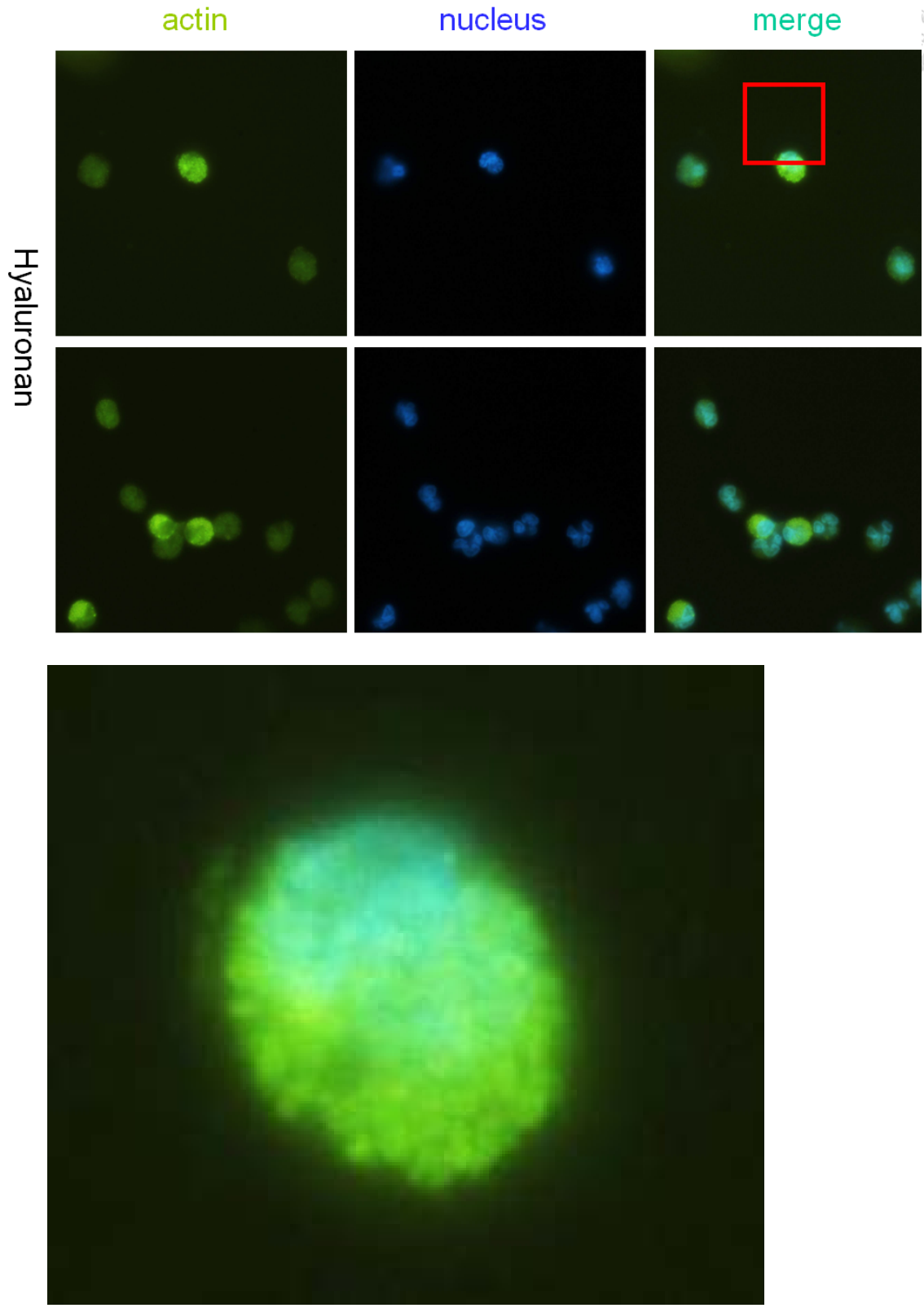


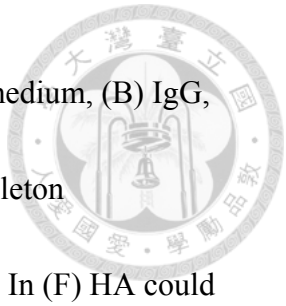
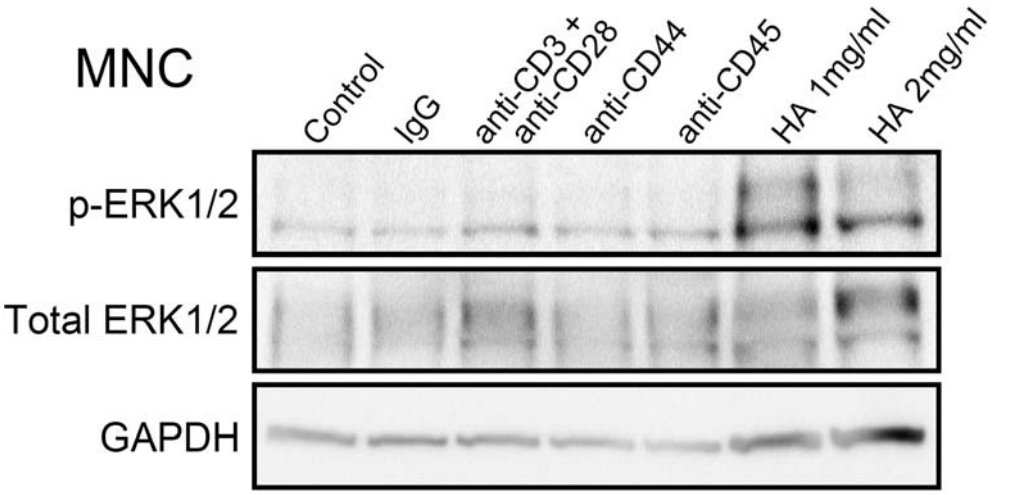
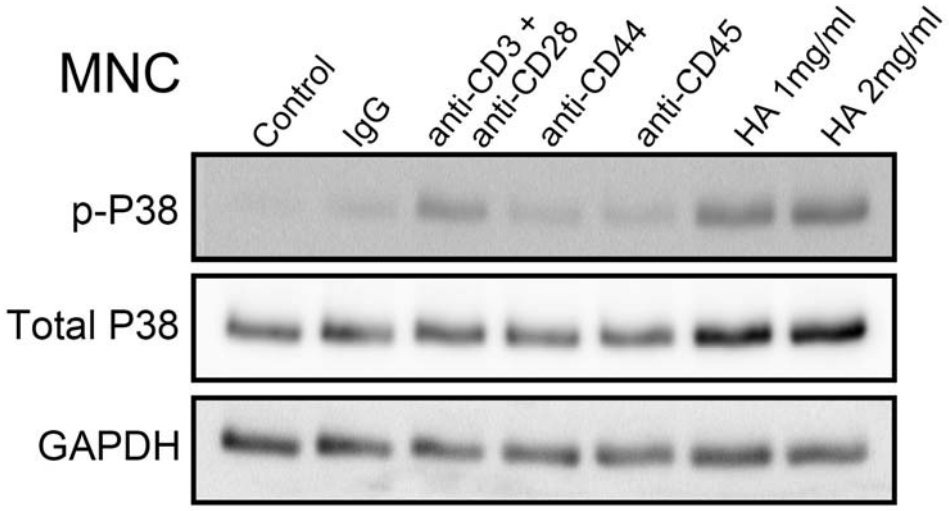


Figure 5 HA induced cytoskeleton rearrangement on PMN. (A) medium, (B) IgG, (C) LPS, (D) anti-CD44, (E) anti-CD45, (F) HA. LPS induce cytoskeleton rearrangement and could enhance the actin aggregation phenomenon. In (F) HA could clearly induce the aggregation of actin, compared to the control. (D) and (E) also show the structure of actin aggregation in PMN. HA, anti-CD44 and anti-CD45 are presumed to induce cytoskeleton rearrangement in PMN.

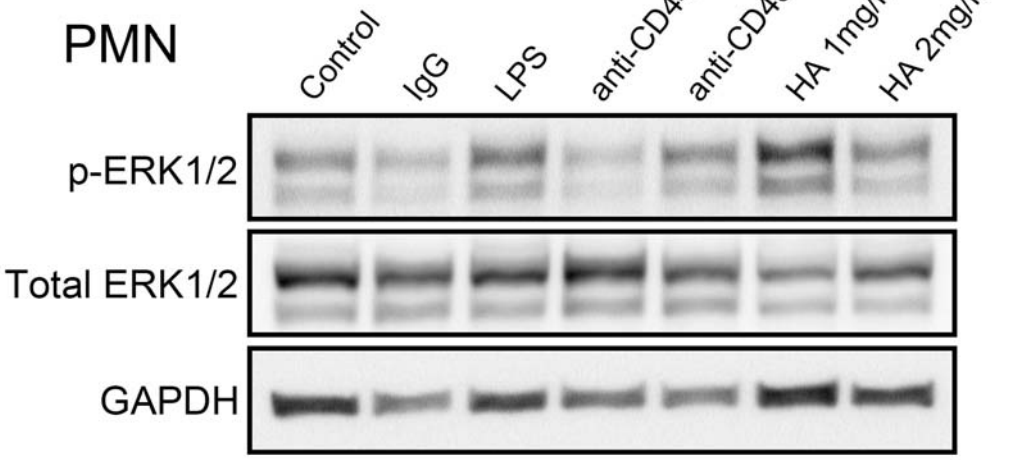
A


MNC
Control
IgG
anti-CD3 +
anti-CD28
anti-CD44
anti-CD45
HA 1mg/ml
HA 2mg/ml
p-ERK1/2
Total ERK1/2
GAPDH

B


MNC
Control
IgG
anti-CD3 +
anti-CD28
anti-CD44
anti-CD45
HA 1mg/ml
HA 2mg/ml
p-P38
Total P38
GAPDH

C



D

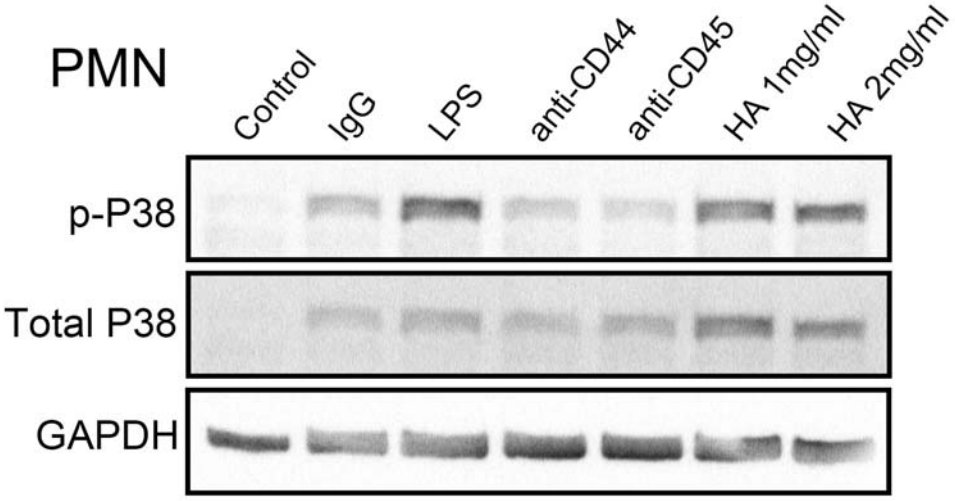


Figure 6 HA induced phosphorylation of MAPK and ERK1/2 on PMN and MNC.

(A) phosphor-ERK1/2, (B) phosphor-P38 on MNC. (C) phosphor-ERK1/2, (D) phosphor-P38 in PMN. In (A) and (B) HA increase the phosphorylation of MAPK and ERK1/2 in MNC. Anti-CD3 and anti-CD28 is positive control. In (C) and (D) HA increase the phosphorylation of MAPK and ERK1/2 in PMN. LPS is positive control.

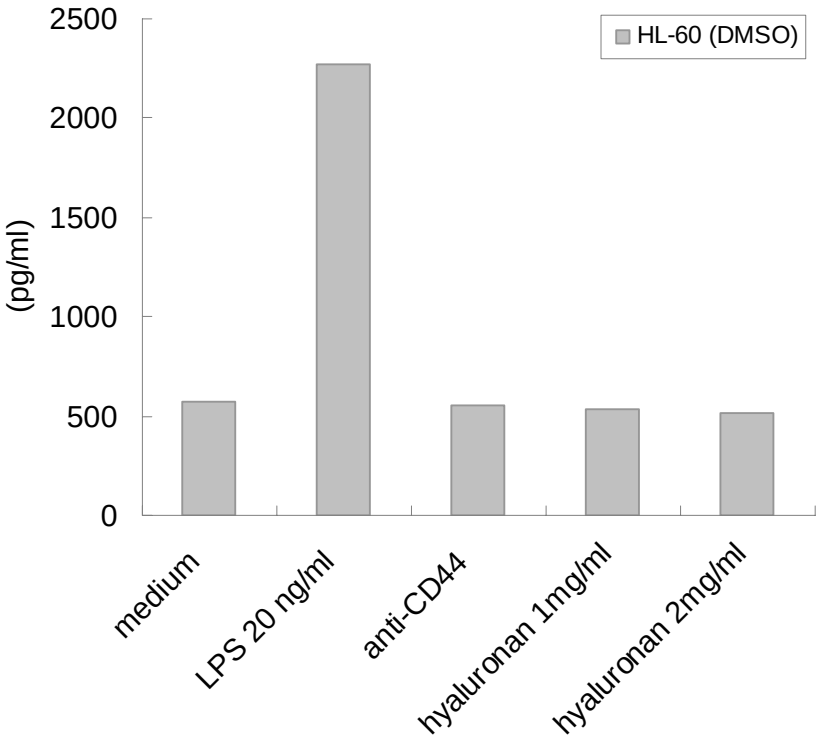


Figure 7 HA abolished to induced IL-8 production on differiated-HL60. The detailed procedures were described in "Materials and Mathods" LPS as positive control. The differentiation of HL60 cells were induced by DMSO contributes to decrease the expression of CD44. HA could abolish to induce IL-8 production on differiated-HL60 as well as anti-CD44.

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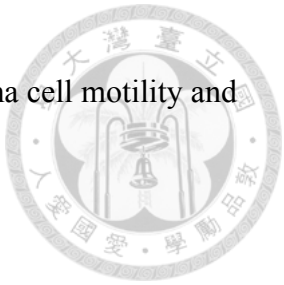

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