行政院國家科學委員會補助專題研究計畫成果報告

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計畫主持人：余家利

協同主持人：謝松洲

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□赴國外出差或研習心得報告一份

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一、中文摘要

為了決定表現在多形核中性白血球(PMN)細胞膜上的 CD_{45} 異構物(isoform)的生物學活性，我們使用抗 CD_{45}，$CD_{45}RA$ 或 $CD_{45}RO$ 的單株抗體作為代理配體(surrogate ligand)粘合細胞膜上。我們發現正常 PMN 膜上 95.5± 3.2%有 CD_{45}，42.3± 5.8%有 $CD_{45}RA$ 及 96.7%±2.6%有 $CD_{45}RO$ 的存在。當這些分子與其元體結合之後，會明顯增加吞噬作用(66%增加)，主要是由增加細胞表面的 complement receptor type 3 (CR3)。而這些抗體(anti-CD_{45} > anti-$CD_{45}RO$ > anti-$CD_{45}RA$)也會增加 PMN 產生 IL-8。但是只有 anti-$CD_{45}RA$ 及 anti-$CD_{45}RO$ 會增加 PMN 表現 TNF-α mRNA，而這兩種抗體會減少 PMN 的 Protein tyrosine phosphorylation。但是只有 anti-$CD_{45}RO$ 會抑制 Src family protein tyrosine kinase p56lck 的表現。這些結果顯示 CD_{45} isoform 與其特異性抗體結合之後會對 protein tyrosine phosphorylation 及 Src family tyrosine kinase $p56^{lck}$ 產生不同的影響，而刺激 PMN 的不同活性。

關鍵詞：白寫球共同抗體、白寫球吞噬能力、前致炎細胞激素、蛋白質酪氨酸激胺 $p56^{lck}$

Abstract

Keywords:

二、緣由與目的

三、研究報告應含的內容

四、打字編印注意事項

五、參考文獻

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SUMMARY

To determine the biological functions of membrane expressed CD45 isoforms on polymorphonuclear neutrophils (PMN), the monoclonal antibody against CD45, CD45RA or CD45RO was used as surrogate ligand for binding to the molecules on cells. We found 99.5±3.2% CD45, 42.3±5.8% CD45RA and 96.7±2.6% CD45RO molecules were expressed on normal human PMN. The interaction of CD45, CD45RA or CD45RO with its specific antibody on PMN surface markedly enhanced phagocytosis (60% increase) mainly via increased expression of complement receptor type 3 (CR3) on the cells. The production of IL-8 by PMN was significantly augmented by the antibodies (anti-CD45>anti-CD45RO>anti-CD45RA). But only anti-CD45RA and anti–CD45RO enhanced TNF-α mRNA expression in PMN. Both anti-CD45RA and anti- CD45RO decreased protein tyrosine phosphorylation of PMN. However, only anti- CD45RO suppressed Src family protein tyrosine kinase $p56^{lck}$ expression in the cells. These results suggest that the binding of CD45 and its two isoforms with their specific antibodies stimulated different PMN activities by affecting different degree on protein tyrosine phosphorylation and Src family tyrosine kinase $p56^{lck}$.

Keywords: leukocyte-common antigen, PMN phagocytosis, proinflammatory cytokines, protein tyrosine kinase $p56^{lck}$

INTRODUCTION

The CD45 molecule, referred to as "leukocyte-common antigen", is a family of high molecular weight transmembrane protein tyrosine phosphatase (PTPase) expressed on all nucleated hematopoietic cells [1, 2]. CD45 is expressed as one of the eight potential isoforms that vary in molecular weight from 180 (CD45RO) to 220kDa (CD45RA) due to alternative mRNA splicing of up to three exons, 4-6, encoding a variable amino- terminal domain rich in O-linked sugars [3]. In human peripheral $CD4^+$T lymphocytes, CD45RA+ and CD45RO+ subsets have been confirmed as representing the naïve and memory T cells, respectively [4]. Many authors have reported the possible roles of CD45 and its isoforms in T- and B-cell differentiation [5-10], natural killer and cytotoxic T lymphocyte functions [10-12], cytokine production of mononuclear cells [13-15] and TCR-associated signaling in T cells [16, 17]. Recently, CD45 has been demonstrated to be crucial in the activation of $p56^{lck}$ member of the Src family tyrosine kinase during T cell activation [18-23]. Nevertheless, only a few studies have involved the biological roles of CD45 isoforms in granulocytes. Hook et al. [24] reported that monoclonal antibody to CD45 inhibited IgE-mediated histamine release from human basophils. Harvath et al. [25] demonstrated that CD45 epitopes interacted with leukotriene B_4 (LTB_4) and

complement component · C5a receptor-associated molecules to regulate the chemotactic responses of polymorphonuclear neutrophils (PMN).

Mature PMN had long been regarded as the terminally differentiated cells incapable of protein synthesis. But recently, many authors found that PMN could express a variety of important cytokine and chemokine mRNA including IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α, G-CSF, GM-CSF, MIP-1α, MIP-1β and MCP-2 constitutionally or by stimulation [26, 27]. Ontogenically, CD45RO isoform was little expressed on immature myeloid cells but became increasing dense toward terminal stage of myeloid maturation [28]. In contrast, the high molecular weight isoform, CD45RA, was virtually absent from the cell surface in the mature myeloid cells. Instead, there is a cytoplasmic pool of each protein isoform associated with membrane-bound granules throughout myeloid cell differentiation [29]. Nevertheless, only a few studies have involved the biological roles of CD45 isoforms in granulocytes. In the present study, we incubated normal human PMN with antibody against CD45, CD45RA or CD45RO as surrogate ligand and found the interactions exerted a profound modulating effect on PMN functions. The molecular basis of the effects by anti-CD45 isoform antibody on PMN was discussed.

MATERIALS AND METHODS

Isolation of neutrophils, lymphocytes and monocytes from normal human peripheral blood

Heparinized venous blood obtained from normal individuals was mixed with one-quarter of a volume of 2% dextran solution (molecular weight 500 000 daltons) and incubated at room temperature for 30 min. Leukocyte-enriched supernatant was collected and diluted with the same volume of Hanks' balanced salt solution (HBSS). After Ficoll-Hypaque (specific gravity 1.077-1.078) density gradient centrifugation at 300g for 20 min, the mononuclear cells (MNC) were aspirated from the interphase and the PMN were obtained from the bottom. The contaminating red blood cells in PMN suspensions were lysed by incubating with chilled 0.83% NH_4Cl solution at 4^0C for 10 min. The adherent cells (monocytes and macrophages) in MNC suspensions were harvested by scrapping with rubber policeman after incubation at 37℃ in 5% CO_2-95% air for 60 min. The procedure was repeated twice for obtaining the highly pure adherent (monocytes) and non-adherent cells (lymphocytes) from MNC. The concentration of PMN, lymphocytes and monocytes was adjusted to $2x10^6$/ml suspended in 10% fetal bovine serum in RPMI-1640 (10% FBS-RPMI). The viability of three cell populations was greater than 95% confirmed by trypan blue dye

exclusion. The purity of PMN and lymphocytes was ≥95% confirmed by Wright's stain. The purity of monocytes was ≥93% confirmed by non-specific esterase staining kit (Sigma Chemical Company, St. Louis, MO, USA).

Detection of CD45, CD45RA and CD45RO expression on the surface of different cell populations by flow cytometry

Freshly isolated PMN, lymphocytes and monocytes ($1x10^6$/ml) were incubated with 5μl of monoclonal antibody (50μg/ml) against human CD45 (clone J-33, IgG), CD45RA (clone ALB11, IgG), or CD45RO (clone UCHL-1, IgG2a) purchased from Immunotech (Cedex, France) or isotype-matched mouse IgG (sigma) in ice-bath for 30 min. After three washes with PBS, pH7.2, the cell suspensions were stained with FITC-labeled goat anti-mouse IgGs (Jackson ImmunoRsearch Lab. Inc. West Grove, PA, USA) in ice-bath for another 30 min. After several time washes, both percentage (%) and mean fluorescence intensity (MFI#, denoted by mean channel number#) of the positive cells were measured by FACSort flow cytometry (Becton-Dickinson Immunocytometry Systems, Moutain View, CA, USA).

Agglutination test of PMN and MNC with monoclonal antibody against CD45 and isoforms

Fifty microliters of PMN or MNC suspension ($5x10^6$/ml) were incubated with

10μl of anti-CD45, anti-CD45RA or anti-CD45RO monoclonal antibody (50μg/ml) and 40μl of PBS, pH 7.2 in a round-bottomed microwell at room temperature for 4h. The cell agglutination was observed.

Measurement of PMN phagocytosis by flow cytometry

We followed the method reported in our previous study [30]. Briefly, PMN ($1x10^6$/ml) were pre-incubated with 10μl of antibody against CD45 isoforms (50μg/ml) for 45 min. Both percentage and mean fluorescence intensity (MFI#) of PMN engulfing (phagocytosis) fluorescence beads (0.75μm in diameter) were measured by FACSort flow cytometry (Becton-Dickinson).

Measurement of phagocytosis-related membrane receptors by flow cytometry

The indirect immunofluorescence antibody method was used to measure the phagocytosis-relevant membrance receptors expression including complement receptor type 1 (CR1), type 3 (CR3) and IgG Fc receptor type III (FcγRIII) on PMN. Briefly, PMN ($1x10^6$/ml) were pre-incubated with antibody against CD45, CD45RA or CD45RO at room temperature for 60 min. These cells were stained with monoclonal mouse anti-human CR1 (C3bR/CD35, Ancell Corporation, Bayport, MN, USA), anti-CR3 (CD11b, Ancell) or anti-FcγRIII (CD16, Ancell) in ice-bath for 30 min, and then FITC-labeled goat anti-mouse IgGs in ice-bath for 30 min. Both

percentage and MFI# of positive PMN were analyzed by FACSort flow cytometry (Becton-Dickinson).

Preparation of PMN cultured supernatants

A 0.25ml of PMN suspension ($2x10^6$/ml), 0.01ml of individual anti-CD45 isoform antibody (50μg/ml) or mouse IgG (50μg/ml), and 0.24ml of 10% FBS-RPMI were mixed in a conical tube and was incubated at 37℃, in 5% CO_2-95% air for 24h. The cell-free cultured supernatants were harvested and stored at -20^0C until assayed.

Quantitation of IL-8 in the PMN cultured supernatants by ELISA

Commercially available ELISA kit (Quantikine, R & D System, Minneapolis, MN, USA) was used for measuring the concentration of IL-8 in PMN cultured supernatants. The detailed procedures are described in manufacturer's instruction booklet. The minimal detectable concentration of IL-8 was 18.1pg/ml.

Reverse transcription-polymerase chain reaction (RT-PCR) detection of different cytokine mRNA expression in PMN

(a) Total cellular RNA extraction and cDNA synthesis

PMN ($1x10^7$/ml) were incubated with antibody against CD45, CD45RA, CD45RO, or mouse IgG at 37℃ in 5% CO_2-95% air for 2h. The total cellular RNA

was then extracted according to the method of Chomczynski & Sacchi [31]. cDNA was synthesized by priming 1μg/ml of total RNA at 42℃ for 1 h in a final volume of 20μl containing 1μg of oligo-dT primer (Pharmacia Fine Chemicals, Piscataway, NJ, USA), 200nmole of each dNTP (Pharmacia), and MMLV reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, USA) at 200 U/ng RNA.

(b) Amplification of cDNA by PCR

An aliquot of cDNA was amplified by PCR using oligonucleotide paired primers specific for human IL-1β, IL-4, IL-8, TNF-α and IFN-γ. Primers for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used to amplify the cDNA of ubiquitous molecule in the cells as internal control. The nucleotide sequence of these pair primers was demonstrated below:

Human IL-1β: 5'ATG GCA GAA GTA CCT AAG CTC GC 3' (sense)

5'A CAC AAA TTG CAT GGT GAA GTC AGT T 3' (anti-sense)

Human IL-4: 5' CGG CAA CTT TGA CCA CGG ACA CAA GTG GGA TA 3' (sense)

5' ACG TAC TCT GGT TGG CTT CCT TCA CAG GAC AG 3' (anti-sense)

Human IL-8: 5' ATG ACT TCC AAG CTG GCC GTG GCT 3' (sense)

5' T CTC AGC CCT CTT CAA AAA CTT CTC 3' (anti-sense)

Human TNF-α: 5' GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A3' (sense)

5' GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC 3' (anti-sense)

Human IFN-γ: 5' ACC ACA GTC CAT GCC ATC AC 3' (sense)

5'TCC ACC ACC CTG TTG CTG TA3' (anti-sense)

Human G3PDH: 5'ACC ACA GTC CAT GCC ATC AC 3' (sense)

5'TCC ACC ACC CTG TTG CTG TA3' (anti-sense)

A HYBAID OmniGene DNA Thermal Cycler (Teddington, Middlesex, TW, UK) was run for 26 cycles of denaturation at 94℃ for 1 min and annealing/extension at 65 ℃ for 2 min in the case of G3PDH. 35 cycles of denaturation at 95℃ for 1 min and annealing/extension at 60℃ for 2 min were carried out for the cytokines. The cDNA fragments amplified by these sets of primers were 802bp of IL-1β, 344bp of IL-4, 289bp of IL-8, 444bp of TNF-α, and 427bp of IFN-γ. The PCR products were electrophoresed in 1.8% agarose gel with φx174 digested by HaeIII enzyme as

calibration markers.

Detection of protein tyrosine phosphorylation and protein tyrosine kinase $p56^{lck}$ by Western blot

Cell lysates were obtained from anti-CD45, anti-CD45RA, anti-CD45RO or mouse IgG-treated PMN ($5x10^6$/ml) as the usual method. The protein concentration of cell lysates was adjusted to 2 mg/ml and then analyzed in Western blot probed by mouse monoclonal anti-phosphotyrosine (clone PT 20, IgG2b) or anti-$p56^{lck}$ (clone 28, IgG2a) purchased from Transduction Laboratories (Lexington, Kentucky, USA). The antigen-antibody complexes were electro-transferred to nitrocellulose membrane and were detected by an enhanced chemiluminescence (ECL) protein detection system (Amersham, Aylesbury, UK).

Statistical analysis

Results represent mean±SD in the whole study. Statistical significance was assessed by Student's paired t test for multiple comparisons.

RESULTS

Phenotypic expression of CD45 isoforms on normal human neutrophils, lymphocytes and monocytes

To identify the phenotypic expression of CD45 isoforms on normal human neutrophils, lymphocytes and monocytes, the individual cell populations were stained with anti-CD45 isoform antibody and were detected by flow cytometry. We found almost all of the leukocytes expressed CD45. PMN expressed 42.3±5.8% of CD45RA and 96.7±2.6% of CD45RO molecules on the surface. By contrast, lymphocytes expressed 69.2±6.6% of CD45RA and 37.8±5.5% of CD45RO molecules. Monocytes/macrophages expressed 34.1±4.4 % of CD45RA and 76.1±7.6% of CD45RO molecules on the surface.

Agglutinating activity of anti-CD45 isoform antibodies on human PMN and MNC

The agglutinating activity of anti-CD45 isoform antibodies on normal human PMN and lymphocytes was tested. We found only anti-CD45RO caused PMN agglutination (data not shown). It is possible that the immunochemical properties of anti-CD45RO antibody *per se*, or the density of CD45RO on PMN is high enough to be agglutinated by its specific antibody.

Effect of different anti-CD45 isoform antibodies on neutrophil phagocytosis

Preincubation of individual anti-CD45 isoform antibody with PMN for 45min remarkably enhanced PMN phagocytosis in both percentage and mean fluorescence intensity (Fig.1). The enhancement of neutrophil phagocytosis was mainly due to the increased expression of CR3, but not CR1, on PMN surface by the three antibodies. However, anti-CD45RO concomitantly increased the expression of CR1 molecules on PMN (Fig. 2).

Effect of anti-CD45 isoform antibodies on IL-8 production of PMN

PMN had been reported to produce many important cytokines either spontaneously or by stimulation. Among these, IL-8 is most prominent cytokines produced by PMN (26, 27). We found the three anti-CD45 isoform antibodies could activate PMN to produce an increased amount of IL-8. Anti-CD45 was the most effective antibody in this activity (Fig. 3).

Effect of anti-CD45 isoform antibodies on cytokine mRNA expression in PMN

In an attempt to know how many cytokins in human PMN can been elicited by anti-CD45 isoform antibodies, the mRNA of IL-1β, IL-4, IL-8, TNF-α, and IFN-γ in PMN were detected by RT-PCR after incubation with different antibodies for 2h. As shown in Fig.4, normal human PMN could spontaneously express mRNA of IL-1β

and IL-8, but not TNF-α, IL-4 or IFN-γ. After 2h incubation with anti-CD45RA or anti-CD45RO, but not with anti-CD45, PMN were activated to express TNF-α mRNA whereas IL-4 or IFN-γ gene remained non-expression.

Effect of anti-CD45 isoform antibodies on protein tyrosine phosphorylation and $p56^{lck}$ expression in PMN

Phosphorylation of specific tyrosine residues in a protein molecule is the result of activation on their respective protein tyrosine kinase. Functionally, CD45 and its isoforms are tyrosine phosphatase that can dephosphorylate the cytoplasmic phosphoproteins. As expected, many cytoplasmic proteins in PMN have already been phosphorylated in the tyrosine residues since the cells are metabolitically active even in non-stimulated state. After incubation with individual anti-CD45RA and -RO antibodies for 10 min, a remarkable decrease in 180 and 100kDa phosphoproteins was found whereas anti-CD45 was totally ineffective (Fig.5). Interestingly, only anti-CD45RO suppressed the expression of a Src family protein-tyrosine kinase $p56^{lck}$ (Fig.6)

DISCUSSION

CD45 represents a family of transmembrane tyrosine phosphatase with molecular weights (MW) of 220kDa, 205kDa, 190kDa or 180kDa by alternative splicing from a CD45 precursor mRNA [32]. CD45RA with a MW of 210-220kDa and CD45RO with a MW of 170-180 kDa were phenotypically expressed in different helper T cell subsets [33]. Much evidence suggests that cells expressing CD45RA consist of naïve CD4+ cells whereas CD45RO phenotype represents a pool of memory cells [34]. In normal bone marrow myeloid cells, CD45RO was very dimly expressed on immature cells but became increasingly dense during differentiation /maturation of the cells. By contrast, CD45RA disappeared virtually from the cell surface of mature myeloid cells and only 3-15% of PMN expressed surface CD45RA [28]. However, our results are not consistent with this report in that approximately 40% of CD45RA expressed on normal human PMN. Although the real cause for the discrepancy is not clear, our data may suggest that the increased % of CD45RA on PMN may be due to activation of PMN during cell separation that augments the translocation of cytoplasmic CD45RA molecule to the surface membrane. Our preliminary data demonstrated that the cytoplasmic and surface membranous CD45 isoform molecules were increased by bacterial endotoxin stimulation (data not shown). It is quite interesting that anti-CD45RA is a potent PMN activator even greater than

anti-CD45 despite that only 40% of normal PMN expressed the molecule compared to $\geqq$99.5% of CD45. Two possibilities are considered; (i) A low density expression of CD45RA molecules on some of the PMN surface rendered more than half of cells detected as false negative. (ii) Certain unidentified cytokines or mediators, such as IL-8 or TNF-α released from a small population of activated CD45RA (+) PMN after binding with antibody stimulated the translocation of cytoplasmic CD45RA molecule to the surface membrane on the previously negative cells. As a result, all of the PMN expressed CD45RA and then transduced an activation signal into the cells [35].

It is conceivable that two biological functions are clearly dependent on CD45 and its isoforms on lymphocytes. First, the CD45 is a positive regulator on proximal signal transduction following ligation of the T cell receptor and other surface receptors (i.e. CD2 or surface immunoglobulins on B cells) via the tyrosine phophorylation circuit in lymphocytes. The second is the regulation of integrin-mediated cell adhesion by CD45 molecules [3, 36]. A primary target of CD45 is the Src family protein kinase $p56^{lck}$ that associated with CD4 and CD8 [37]. CD45 and isoforms can act as either a positive or negative regulator of the kinase activity of $p56^{lck}$ [36, 38, 39]. Dephosphorylation of the C-terminal inhibitory site (site 1) tyrosine in $p56^{lck}$ molecule by interaction with CD45 molecules changed the kinase confirmation into an active structure. In conjunction with TCR activation, the

dephosphorylated Src family kinase are then phosphorylated on a second stimulatory site (site 2) to become an active form to promote the catalytic function of the kinase in the lymphocytes [40, 41]. In an *in vitro* study, Mustelin et al. [42] reported that the incubation of purified CD45 with $p56^{lck}$ increased the catalytic activity of the kinase more than 2-fold. This finding indicates that expression of CD45 in T lymphocytes is required to positively regulate tyrosine phosphorylation of the carboxyl-terminal tyrosine residue of $p56^{lck}$ in order to maintain the kinase in an active state. Other evidence suggests that this tyrosine residue in site 2 may also be a potential substrate for CD45 dephosphorylation and resume to the inactive form. This dual effect renders CD45 molecule involving in both positive and negative regulation of Src family protein tyrosine kinase. Whether a similar acting mechanism of CD45 isoforms operating in neutrophils as well as in lymphocytes have not been reported yet in the literature.

The main purpose of the present study is to define the biological functions of membrane-expressed CD45 isoforms on PMN. Because the natural ligands for CD45 isoform molecules are not yet found, anti-CD45 monoclonal antibodies have been used as surrogate ligand for this purpose [34]. Accordingly, we incubated human PMN with individual anti-CD45 isoform antibody and detected the functional changes of these cells. Three original findings were observed; (a) The three anti-CD45 isoform

antibodies stimulated PMN phagocytosis mainly via increased expression of CR3 on the cell surface. (b) Anti-CD45RA and anti-CD45RO enhanced both IL-8 and TNF-α gene expression, but anti-CD45 only increased IL-8 production by PMN. (c) Both anti-CD45RA and anti-CD45RO decreased protein tyrosine phosphorylation.but only anti-CD45RO suppressed Src family protein tyrosine kinase $p56^{lck}$ expression in PMN. It is speculated that the binding of CD45 isoform molecules with specific antibody on PMN dephosphorylates the inhibitory site 1 in the C-terminal Src family kinase domain. Autophosphorylation of the stimulatory site 2 in the molecule subsequently occurs in PMN even without antigen or mitogen stimulation since PMN are already committed to activation. But the activated protein tyrosine kinase $p56^{lck}$ with catalytic activity does not underlying dephosphorylation by CD45 isoform. The active form kinase phosphorylates the motifs in certain transcription factors and transduces a positive signal to active PMN. As a consequence, PMN was activated to increase production/expression of IL-8 and TNF-α and phagocytosis. Because CD45 and its two isoforms are functional protein tyrosine phosphatase, decreased total protein tyrosine phosphorylation in PMN after binding with antibody seemed reasonable as in lymphocytes. However, we noted that the binding of CD45, but not CD45RA or CD45RO, with specific antibody did not decrease tyrosine phosphorylation as demonstrated in Fig.5. The real cause of this inconsistency is not

clear. It remains possible that difference in cell physiology between PMN and lymphocytes is responsible for it.

Another interesting finding is that only anti-CD45RO antibody suppressed $p56^{lck}$ expression in PMN. We noted that the monoclonal anti-$p56^{lck}$ antibody was induced by immunizing with a 21.3kDa fragment of the human $p56^{lck}$ N-terminal domain corresponding to amino acid residues 1-191. This epitope is far from the C-terminal catalytic kinase domain of the Lck. Theoretically, there should be no changes of $p56^{lck}$ expression in anti-CD45 isoform antibody-treated PMN. However, in the agglutination test (data not shown), only anti-CD45RO aggregated PMN. It remains possible that the agglutination of PMN by anti-CD45RO antibody reduced the antigenicity of Src kinase $p56^{lck}$. Or, alternatively, the interaction of CD45RO and $p56^{lck}$ accelerates the decay of $p56^{lck}$ molecules. However, further investigation is necessary to confirm it. In conclusion, we are the first to study the biological functions of CD45 and its two isoforms, CD45RA and CD45RO, in PMN. We suggest that the binding of the three molecules and specific antibodies activate PMN by different degree suppression on protein phosphorylation and $p56^{lck}$ protein kinase.

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FIGURE LEGENDS

Fig.1: Effect of antibody against of CD45, CD45RA or CD45RO on neutrophil phagocytosis. Human PMN ($1x10^6$/ml) were pre-incubated with mouse IgG (100μg/ml), LPS (100ng/ml, as positive control) or different antibody (100μg/ml) for 45 min before the measurement of phagocytosis. Both percentage (%) and mean fluorescence intensity (MFI#, denoted by mean channel number #) of positive cells were detected by FACSort flow cytometry. The three antibodies markedly enhanced PMN phagocytosis in both percentage and MFI# compared to mouse IgG control. The same experiment was repeated 5 times with a similar tendency.

Fig. 2: Effect of antibody against CD45, CD45RA or CD45RO on the membrane expression of phagocytosis-related receptors; complement receptor type 1 (CR1), type 3 (CR3), and IgG Fc receptor type III (FcγRIII) on human PMN. PMN ($1x10^6$/ml) were pre-incubated with, LPS (100ng/ml, as positive control), or different antibody (100μg/ml) for 60 min. Both percentage (%) and mean fluorescence intensity (MFI#, denoted by mean channel number #) of positive cells were detected by flow cytometry. All of the three antibodies enhanced CR3 expression but only anti-CD45RO enhanced CR1 expression compared

to mouse IgG (100μg/ml) control. The same experiment was repeated 4 times with a similar tendency.

Fig.3: Effect of anti-CD45 isoform antibodies (100μg/ml) on IL-8 production of normal human PMN ($1x10^6$/ml) after incubation for 24h. Bacterial polysaccharide (LPS 100ng/ml) was used as positive control. "*" denotes $p<0.01$ and "**" denotes $p<0.002$ compared to mouse IgG (100μg/ml) control calculated by Student's paired t test for multiple comparisons.

Fig.4: Effect of antibody against CD45, CD45RA or CD45RO on IL-1β, IL-4, IL-8, TNF-α and IFN-γ mRNA expression of human PMN detected by RT-PCR after incubation of PMN with respective antibody (100μg/ml) for 2h. Lanes 1-6: incubation with mouse IgG (100μg/ml), lanes 7-12: incubation with anti-CD45, lanes 13-18: incubation with anti-CD45RA and lanes 19-24: incubation with anti-CD45RO. Lane 1: G3PDH (452bp, as internal control), lane 2: IL-1β (802bp), lane 3: TNF-α (695bp), lane 4: IL-8 (289bp), lane 5: IL-4 (344bp), lane 6: IFN-γ (452bp), lane 7: G3PDH, lane 8: IL-1β, lane 9: TNF-α, lane 10: IL-8, lane 11: IL-4, lane 12: IFN-γ, lane 13: G3PDH, lane 14: IL-1β, lane 15: TNF-α, lane 16: IL-8, lane 17: IL-4, lane 18: IFN-γ, lane 19: G3PDH, lane 20: IL-1β, lane 21: TNF-α, lane 22: IL-8, lane 23: IL-4 and lane 24: IFN-γ. Obviously, the expression of TNF-α mRNA in PMN was enhanced

by anti-CD45RA and anti-CD45RO antibodies. The same experiment was repeated 3 times with a similar tendency.

Fig.5: Detection of protein tyrosine phosphorylation in different PMN lysates after incubation with antibody (100μg/ml) against CD45, CD45RA or CD45RO for 10 min probed by anti-phosphotyrosine antibody and ECL system in Western blot analysis. Lane 1: incubation with mouse IgG (100μg/ml), lane 2: incubation with LPS (100ng/ml), lane 3: incubation with anti-CD45, lane 4: incubation with anti-CD45RA, and lane 5: incubation with anti-CD45RO. Two bands with 180 and 100kDa were markedly diminished after incubation with anti-CD45RA and anti-CD45RO, but not with anti-CD45. The same experiment was repeated 3 times with a similar tendency.

Fig.6: Detection of Src family protein tyrosine kinase $p56^{lck}$ in different PMN lysates after incubation with antibody (100μg/ml) against CD45, CD45RA or CD45RO for 10 min probed by anti-$p56^{lck}$ antibody and ECL system in Western blot analysis. Lane 1: incubation with mouse IgG (100μg/ml), lane 2: incubation with LPS (100ng/ml), lane 3: incubation with anti-CD45, lane 4: incubation with anti-CD45RA, lane 5: incubation with anti-CD45RO, and lane 6: Jurkat cell lysate as a positive control for $p56^{lck}$ molecule supplied by the

kit. Only anti-CD45RO suppressed $p56^{lck}$ expression in PMN. The same experiment was repeated 3 times with a similar tendency.