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Identification of CD4 isoforms by two anti-CD4 monoclonal antibodies

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ABSTRACT

Background: CD4 isoforms expressed on leukocyte surface have been reported. The function of the CD4 isoforms is, however, unknown. Several studies are conducted aiming to uncover the functions of the CD4 isoforms which will lead to a better understanding of the immune responses.

Objectives: To identify CD4 isoforms expressed on leukocyte surface using two anti-CD4 monoclonal antibodies (mAbs) clones MT4 and MT4/3.

Materials and methods: Anti-CD4 mAbs MT4 and MT4/3 were purified by affinity chromatography. Specificity of the obtained purified mAbs were verified by 293T transfection and cell depletion experiment. Cellular distribution profiles of mAbs MT4 and MT4/3 were determined by immunofluorescence and flow cytometry. Identification of CD4 isoforms was performed by confocal microscopic analysis.

Results: Anti-CD4 mAbs MT4 and MT4/3, generated in our research center, were purified and confirmed their specificity by CD4-DNA transfection and cell depletion experiment. Cellular distribution profiles obtained from mAbs MT4 and MT4/3 were similar to those obtained using standard anti-CD4 mAb. By confocal microscopic analysis, mAbs MT4 and MT4/3 were demonstrated to recognize different CD4 molecule expressed on cell surface.

Conclusion: Anti-CD4 mAbs MT4 and MT4/3 were demonstrated to react with different CD4 isoforms. To the best of our knowledge, this is the first report showing that CD4 isoforms could be determined by specific mAbs. These mAbs will be an important tool for employing in characterization of structure and function of the CD4 isoforms.

Introduction

CD4 is a cell surface glycoprotein expressed on a sub-population of T-lymphocytes named helper T lymphocytes or $CD4^+$ lymphocytes.[1] As was described for $CD4^+$ lymphocytes, CD4 molecule is a 55 kDa glycoprotein. It is a member of the immunoglobulin (Ig) superfamily which contains four-Ig-like extracellular domains, D1-D4.[2-4] The oligomers of CD4 molecules have been described.[2, 5-10] CD4 molecules play very important roles in $CD4^+$ lymphocyte activation. This molecule was demonstrated to stabilize the interaction of TCR on T lymphocytes and peptide-MHC class II complexes on antigen presenting cells (APCs) and mediate intracellular T cell signaling.[2, 11, 12] Upon T cell activation, large multiprotein clusters of CD4, TCR and CD3 molecules are formed and provide a focal point for intracellular signal transduction resulting in T cell activation and induction of the adaptive immunity.[3, 13-17]

In addition to T lymphocyte, CD4 molecules are expressed on several cell types, including monocytes, macrophages, Langerhans cells, dendritic cells, eosinophils, megakaryocytes and mast cells.[3, 18-23] However, the expression of CD4 on non-lymphatic cells is considerably lower than

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those on $CD4^+$ lymphocytes. It was reported that monocytes express CD4 molecules 10-20 folds less than those expressed on lymphocytes.[24, 25] Regarding to the molecular interaction, it is well documented that, on $CD4^+$ lymphocytes, CD4 function as the receptor for MHC class II, IL-16 and HIV gp 120.[3, 26] However, the function of CD4 molecules on other cells is not yet clearly known. Neither the interaction nor the signaling capability of CD4 on non-T cells is fully revealed.

Monocytes are phagocytes which typically represent approximately 10% of peripheral blood mononuclear cells (PBMCs). In addition to its specific marker CD14, all monocytes express CD4 molecules on their surface.[27, 28] However, the CD4 molecules expressed on $CD4^+$ T lymphocytes and monocytes are significant different.[29] In contrast to the well-documented 55 kD CD4 monomer in lymphocytes, monocytes express two CD4 monomers, i.e. 55 kD and 59 kD isoforms.[29] The 59 kD CD4 isoform appears to be distended through disulfide disruption.[29] The functions of 59 kD isoforms, however, is still unknown. CD4 isoforms expressed on T lymphocytes, nevertheless, is still mystery. Several ongoing researches are conducted to uncover the characteristics and functions of the CD4 isoforms. This will, subsequently, lead to the better understanding of the immune responses.

In the present report, by using our generated anti-CD4 mAbs MT4 and MT4/3, we demonstrated that these mAbs recognized different CD4 molecules expressed on leukocyte surface. The mAbs MT4 and MT4/3 could differentiate CD4 isoforms

Materials and methods

Antibodies, reagents and cell lines

Anti-CD4 mAb clones MT4 and MT4/3,[30, 31] anti -hemoglobin clone Hb1b, anti-phage clone 13M1F, anti-RBCs clone WK3 and FITC labelled anti-hemoglobin Bart's clone PB1 were produced in our laboratory. FITC-conjugated anti-CD4 mAb (clone Leu3a) and BD FACS™ lysing solution were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Anti-CD31 mAb (clone LC/70A) were purchased from DAKO (Glostrup, Denmark). FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H+L) antibodies was obtained from Merck Millipore (Darmstadt, Germany). Hitrap IgM Sepharose column and Protein G Sepharose column were obtained from GE Healthcare Bio-Sciences (Piscataway, NJ, USA). Lipofectamine® 2000 reagent for transfection experiments and goat anti-mouse IgM (μ-chain specific) conjugated with Alexa Flour 488 were obtained from Invitrogen (Carlsbad, CA, USA). Goat anti-mouse IgG (Fc_γ specific) conjugated with Cy™3 was purchased from Jackson ImmunoResearch (West Grove, PA, USA). Ficoll-Hypaque solution (IsoPrep) was purchased from Robbins Scientific Corporation (Sunnyvale, CA, USA). Dynabeads™ M-280 Streptavidin, ProLong™ Gold Antifade Mountant and Hybridoma-Serum Free Media (SFM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). 7-Amino-actinomycin D (7AAD) was purchased from BioLegend (San Diego, CA, USA).

293T cells were maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 40 mg/mL gentamicin and 2.5 mg/mL amphotericin B (10%FBS-DMEM) and cultured in a humidified atmosphere of 5% CO_2 at 37 °C.

Production and purification of anti-CD4 mAb clones MT4 and MT4/3

Anti-CD4 mAb clones MT4 and MT4/3 were generated in our laboratory.[30, 31] To produce the purified anti-CD4 mAbs, the hybridoma producing anti-CD4 mAb clones MT4 and MT4/3 were thawed and cultured in 10%FBS-DMEM at 37 °C in a 5% CO_2 incubator. The hybridoma cells were, then, adapted for culturing in Hybridoma-Serum Free Media (SFM) by gradually increasing the ratio of Hybridoma-SFM to 10%FBS-DMEM from 0:100 to 25:75, 50:50, 75:25 until 100% Hybridoma-SFM. After adaptation, the hybridomas were grown for 1 week in 10 cm dish using SFM system. The culture supernatants of hybridoma culture were then collected and tested for the activity of antibody using indirect immunofluorescence technique. Then, the anti-CD4 mAb clones MT4 and MT4/3 were purified from the collected supernatants.

For purification of mAb MT4 (IgM isotype), the culture supernatant was added to the Hitrap IgM Sepharose column and affinity purified using Aktaprime fraction collector (GE Healthcare Bio-Sciences). The unbound mAbs were washed out and the bound mAbs were eluted using 20 mM sodium phosphate buffer pH 7.5. For purification of mAb MT4/3 (IgG2a isotype), the culture supernatant was added to the protein G Sepharose column. The unbound mAbs were washed out. Then, the bound mAbs were eluted using 0.1M citric acid buffer pH 3.0. The eluates were collected and neutralized with neutralizing buffer (2M Tris-HCl pH 8.0) and dialyzed against PBS for overnight. The concentration of mAbs MT4 and MT4/3 were measured by OD280 reading and stored at -20 °C.

Transfection of 293T cells

For 293T cell transfection, plasmid encoding CD4 protein[31] was transfected into 293T cells using lipofectamine mediated transfection. Lipofectamine 2000 reagent was used for plasmid transfection according to manufacturer instruction (Invitrogen). Briefly, 293T cells ($4x10^5$ cells/well) were plated into 6 wells-plate and incubated at 37 °C in a 5% CO_2 incubator for overnight. The plasmid DNA-liposome complex is prepared at ratio 1 μg of DNA : 7 μL of lipofectamine reagent in DMEM and incubated at room temperature for 5 minutes. The mixture was then slowly added into 293T cells and further incubated at 37 °C in a 5% CO_2 for 72 hours to allow expression of the corresponding proteins. After incubation, transfected cells were collected for further staining with mAbs.

Cell depletion experiment

For mAbs coated magnetic bead preparation, streptavidin superparamagnetic beads (Dynabeads™ M-280 Streptavidin) were washed three times with phosphate buffered saline (PBS). Then, 20 μg of biotinylated mAbs MT4 or MT4/3 in PBS were added into 1 mg of the washed M280 beads and incubated on rotator for 30 minutes at room temperature. Afterward, the beads were washed four times with PBS

containing 0.1% BSA (0.1% BSA-PBS). The mAbs coated M280 beads were adjusted to $8x10^8$ beads/mL with 0.1% BSA-PBS containing 0.02%NaN_3 (1%FCS-PBS-0.02%NaN_3) and stored at 4 °C.

For cell depletion, 5 µL of M280-MT4 or M280-MT4/3 beads in 0.1%BSA-PBS-0.02%NaN_3 were added into 200 µL of K3EDTA blood in 1.5 mL microcentrifuge tube and incubated on a rotator for 30 minutes at room temperature. After incubation, the tubes were placed in magnetic stand and let stand for 5 minutes at room temperature. Then, the non-adherent cells were transferred into new tube. The cell depleted blood was then stained with FITC-conjugated anti-CD4 mAb by lysed whole blood staining. The stained cells were then analyzed by flow cytometer (BD Accuri™ C6; Becton Dickinson). The data were analyzed by FlowJo software.

Peripheral blood mononuclear cells (PBMCs), red blood cells (RBCs), and platelets preparation

For PBMCs preparation, heparinized blood was mixed with PBS at 1:1 ratio. The diluted blood was, then, overlaid onto Ficoll-Hypaque solution and then spun at 400 g, 25 °C for 30 minutes with break-off setting. After centrifugation, the PBMCs were harvested from white ring at the interphase of Ficoll-Hypaque and plasma layer. The cells were counted by hemocytometer and adjusted to $1x10^7$ cells/mL with FACS buffer (1%FCS-PBS-0.02%NaN_3).

For RBC preparation, whole blood samples were washed with FACS buffer for 3 times. Packed RBCs were then adjusted to 0.3% (v/v) cell suspension with FACS buffer.

For platelets preparation, platelets were isolated from ACD blood by centrifugation at 200 g for 20 minutes at 25 °C. Platelet-rich plasma (PRP) fraction was collected and centrifuged at 800 g for 20 minutes at 25 °C to obtain platelet pellet. The obtained platelets were resuspended with Tyrode's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.90 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 5 mM HEPES, 5 mM Glucose, and 1% BSA) and counted with Rees and Ecker' solution by hemocytometer and adjusted to $1x10^7$ cells/mL with FACS buffer.

Indirect immunofluorescent assay

PBMCs ($5x10^5$ cells/mL; 50 µL), RBCs (0.3% RBCs; 50 µL) or platelets ($5x10^5$ cells/mL; 50 µL) were incubated for 30 minutes at 4 °C with 10% human AB serum to block the non-specific Fc receptor mediated binding of the antibodies. Blocked cells were then incubated for 30 minutes at 4 °C with mAbs MT4 or MT4/3, or isotype matched control antibodies (Hb1b; IgM and 13M1F; IgG2a). Then, the cells were washed twice with FACS buffer and FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H+L) antibodies were added and incubated for another 30 minutes at 4 °C. After washing, cells were fixed with PBS containing 1% paraformaldehyde. The stained cells were then analyzed by flow cytometer (BD Accuri™ C6 or FACSort; Becton Dickinson). The data were analyzed by FlowJo software.

Lysed whole blood staining

Fifty microliters of K3EDTA blood samples were incubated with anti-CD4 mAbs clone MT4 or MT4/3 for 30 minutes at room temperature. Samples were then washed twice with FACS buffer and incubated with the FITC-conjugated goat $F(ab')_2$ anti-mouse IgG (H+L) antibodies. After 30 minutes incubation, 1 mL of lysing solution (BD FACS™ Lysing Solution; Becton Dickinson) was added and mixed well then let stand at room temperature in dark for 15 minutes for lysis of RBCs. The remained WBCs were washed twice with FACS buffer and final stained with 7-Aminoactinomycin D (7AAD) for 10 minutes to separate WBCs from the remained non-lysed RBCs. The stained cells were then analyzed by flow cytometer (BD Accuri™ C6 or FACSort; Becton Dickinson) and the data were analyzed by FlowJo software.

Confocal microscopic analysis

PBMCs were isolated from heparinized whole blood by Ficoll-Hypague density gradient centrifugation. $1x10^6$ of PBMCs were incubated with mAbs MT4 or MT4/3 or isotype matched control antibodies (Hb1b; IgM and 13M1F; IgG2a) for 30 minutes at 4 °C. Goat anti-mouse IgM (µ-chain specific) conjugated with Alexa Flour 488 (Invitrogen) and goat anti-mouse IgG (Fc_γ specific) conjugated with Cy™3 (Jackson ImmunoResearch) for detecting mAb MT4 or MT4/3, respectively, were added and incubated for 30 minutes. After incubation, the cells were washed twice and fixed with 2% paraformaldehyde and resuspended with 10 µL of PBS.

For slide mounting, ten microliters of stained cells were plated on 0.01% poly-L-lysine pre-coated 10 mm diameter cover slips and incubated for 20 minutes to let the cells adhere on cover slip. 10 µL of ProLong Gold antifade reagent (Thermo Fisher Scientific) were dropped on glass slide. Then, cells coated cover slips were slowly place on droplet of ProLong Gold antifade reagent by preventing air bubbles formation. After let the slide dry for overnight, the cover slips were seal with nail polish and stored at 4 °C until determined by confocal microscope (Zeiss, Thornwood, NY, USA).

Human Ethics

This study was approved by the ethics committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (AMSEC-61EM-022).

Results and Discussion

Purification of anti-CD4 monoclonal antibody clones MT4 and MT4/3

Two anti-CD4 mAbs, named MT4 (IgM isotype) and MT4/3 (IgG2a isotype), were generated in our research center. In order to characterize the mAbs MT4 and MT4/3 reactivity, the purified form of these mAbs were required. Hybridoma producing anti-CD4 mAb clones MT4 and MT4/3 were adapted to grow in SFM. The antibody containing culture supernatants were subjected for antibody purification using affinity chromatography. The obtained purified mAbs were verified for their purity by SDS-PAGE analysis. As shown in Figure 1, the purified mAbs showed the bands at the expected sizes of IgM for MT4 and IgG for MT4/3, in both reducing and non-reducing conditions. In the purified

mAbs, undesired proteins were observed at very low level. The data indicated that the purified mAbs obtained were suitable to be used in further experiments.

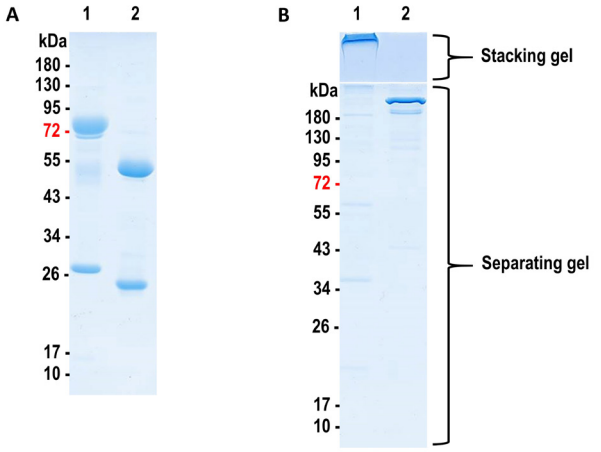

Figure 1. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified monoclonal antibodies MT4 and MT4/3. Purified mAbs MT4 and MT4/3 were mixed with reducing or non-reducing buffer and boiled at 95 °C for 5 minutes. Electrophoresis was done in a 10% SDS-PAGE gel at 120 V, 25 mA. Gel was stained with Coomassie Brilliant Blue. (A) In reducing conditions, bands at 75 and 27 kDa and 50 and 25 kDa were observed for mAb MT4 (lane 1) and mAb MT4/3 (lane 2), respectively. (B) In non-reducing conditions, >180 kDa bands was observed for MT4 (lane1) and MT4/3 (lane 2). The positions of molecular mass markers in kDa are indicated on the left*

Specificity of anti-CD4 monoclonal antibody clones MT4 and MT4/3

As we aimed to use these mAbs for determination of CD4 molecule expressed on leukocyte surface, the specificity of these mAbs were firstly verified. CD4-DNA[31] was transfected into 293T cells. The transfected cells were then used to determine the specificity of anti-CD4 mAbs MT4 and MT4/3. As shown in Figure 2, both mAbs MT4 and MT4/3 strongly reacted to CD4-transfected cells, but not to untransfected cells. The CD4 transfected cells were confirmed for CD4 molecules expression as they were positive with commercial anti-CD4 mAb (Becton Dickinson) (Figure 2). The results confirmed that the mAbs MT4 and MT4/3 are specific for CD4 molecule.

We further determined whether the mAbs MT4 and MT4/3 recognized the native CD4 molecules expressed on peripheral blood lymphocytes using cell depletion experiment. Mabs MT4 and MT4/3 coated magnetic beads were prepared. The mAb-coated magnetic beads were then used to deplete the correspondence cells from whole blood. The cell depleted blood samples were then subjected for determination of $CD4^+$ lymphocytes by using standard anti-CD4 mAb. As shown in Figure 3, both MT4- and MT4/3-coated magnetic beads could deplete $CD4^+$ lymphocytes from the peripheral blood leukocytes. The results indicated that mAbs MT4 and MT4/3 were specific for CD4 molecules and could react to both recombinant CD4 proteins and native CD4 molecules expressed on cell surface.

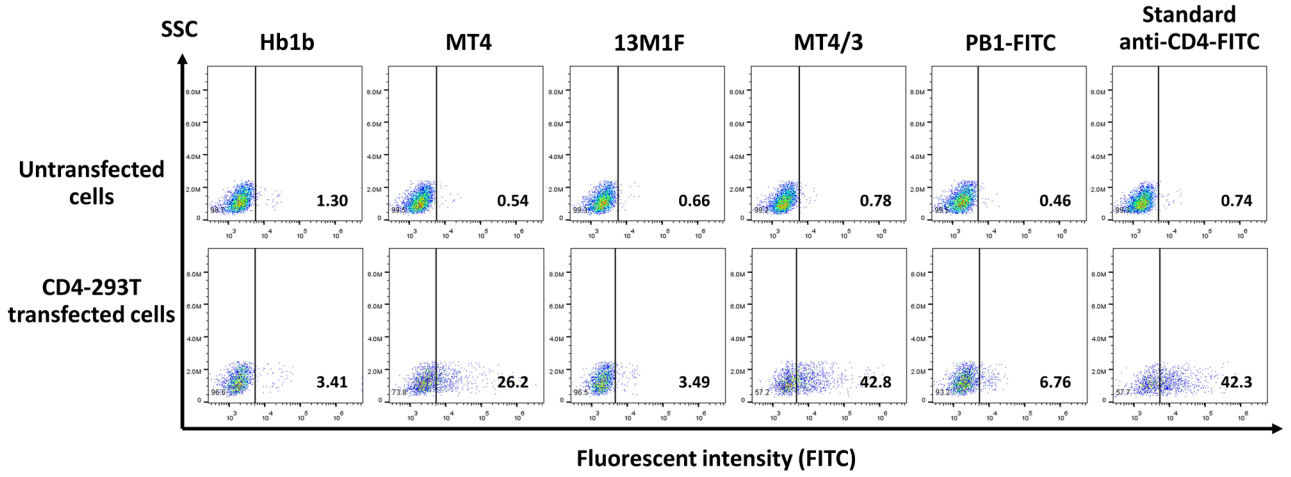

Figure 2. *Immunofluorescence analysis of monoclonal antibodies MT4 and MT4/3 with CD4-DNA transfected cells. The 293T cells were transfected with plasmid encoding CD4 protein (CD4-DNA). The CD4-293T transfected or untransfected cells were stained with mAbs MT4, MT4/3, or isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a) by indirect immunofluorescence staining and analyzed by flow cytometer. In addition, the CD4-293T transfected cells were confirmed for expression of CD4 molecules by staining with standard anti-CD4 mAb labeled with FITC (standard anti-CD4-FITC) or isotype matched control mAb (PB1-FITC) and analyzed by flow cytometer.*

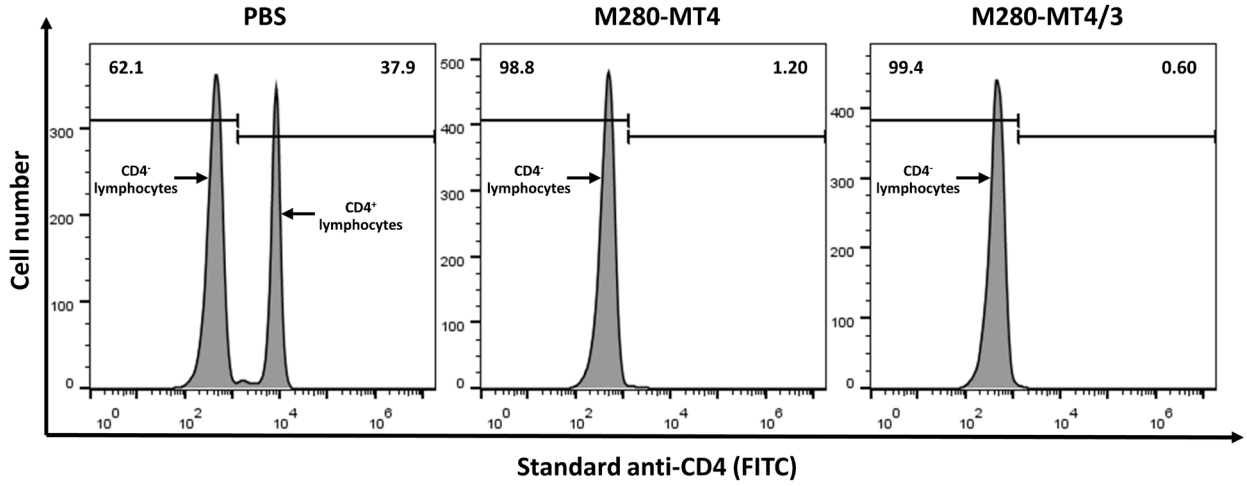

Figure 3. *Determination of the specificity of monoclonal antibodies MT4 and MT4/3 by cell depletion. CD4+ cells in whole blood samples were depleted by mAb MT4-coated magnetic beads (M280-MT4) or mAb MT4/3-coated magnetic beads (M280-MT4/3) or PBS. The CD4+ cells depleted whole blood samples were stained with standard anti-CD4-FITC and 7-AAD by lysed whole blood staining and analyzed by flow cytometer. The lymphocyte population was gated according to their granularity (SSC) and 7-AAD staining. The percentage of CD4+ cells and CD4- cells (as indicated) in the CD4⁺ cells depleted whole blood by using PBS (control), M280-MT4 or M280-MT4/3 are shown in histograms.*

Reactivity of mAbs MT4 and MT4/3 with peripheral blood leukocytes

The reactivity of mAbs MT4 and MT4/3 with various peripheral blood leukocytes were determined. By lysed whole blood staining, the mAbs MT4 and MT4/3 (but not isotype matched control mAbs) reacted to a population of lymphocytes (Figure 4). The number of MT4⁺ lymphocytes and MT4/3⁺ lymphocytes were similar to those obtained by using standard anti-CD4 mAb (n=10) (Table 1).

In monocyte staining, the mAbs MT4 and MT4/3 reacted to monocyte population (n=10), but in different patterns (Figure 5). In all subjects, the mAb MT4 showed weaker reactivity compare to mAb MT4/3. It is worth noting that monocytes in some subjects (for example; sample No. 10) were negative with mAb MT4 at all mAb concentrations tested but were positive with mAb MT4/3 (Figure 5). The saturated concentration of the mAbs used was demonstrated to be 20 µg/mL or 40 µg/mL depend on individual blood samples (Figure 5). These results indicated that the different reactivity pattern between mAbs MT4 and MT4/3 with monocytes was not affected by insufficient antibodies as at concentration of 80 µg/mL the mAb MT4 still showed negative reactivity with monocytes. The results are in accordance to our previous reports.[30, 32]

The reactivity of mAbs MT4 and MT4/3 with red blood cells and platelets were also determined. In all subjects tested (n=5), RBCs and platelets were negative with mAbs MT4 and MT4/3 (Figure 6A and B).

The reactivities of mAbs MT4 and MT4/3, as was compared to the standard anti-CD4 mAb and the reported CD4 expression profiles,[33, 34] confirmed that the both mAbs MT4 and MT4/3 are anti-CD4 mAbs.

Table 1 Percentage of CD4⁺ lymphocytes determined by monoclonal antibodies MT4, MT4/3 or FITC labeled standard anti-CD4 mAb. Ten different whole blood samples were stained with mAbs MT4, MT4/3, or FITC labeled standard anti-CD4 mAb by lysed whole blood staining. The percentages of CD4⁺ cells in lymphocyte population are shown.

Sample Number	% Positive cells		
	Standard anti-CD4 mAb	mAb MT4	mAb MT4/3
1	29.4	29.8	31.2
2	41.8	38.4	40.8
3	32.0	32.2	35.4
4	34.5	35.3	33.8
5	41.5	38.9	39.4
6	39.8	34.5	37.6
7	45.4	43.9	44.6
8	39.7	39.2	39.1
9	39.0	37.0	37.2
10	38.0	32.5	36.9

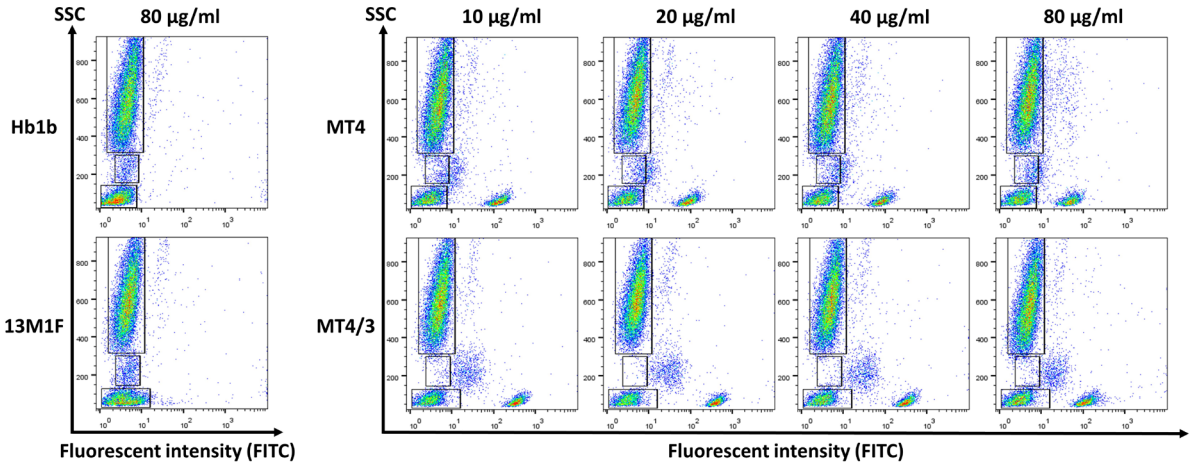

Figure 4. *Immunofluorescence analysis of the reactivity of monoclonal antibodies MT4 and MT4/3 with peripheral blood leukocytes. Whole blood samples were stained with various concentrations of mAbs MT4, MT4/3, or isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a) and 7-AAD by lysed whole blood staining. The leukocyte population was gated according to their granularity (SSC) and 7-AAD staining. Granularity (SSC) and FITC fluorescence were plotted to show the reactivity of mAbs MT4 and MT4/3 at concentration of 10, 20, 40, or 80 µg/mL to each leukocyte population. The reactivity of isotype matched control mAbs (Hb1b and 13M1F) at 80 ug/mL are shown and marked by rectangles. One subject is shown as representative of 10 studied subjects.*

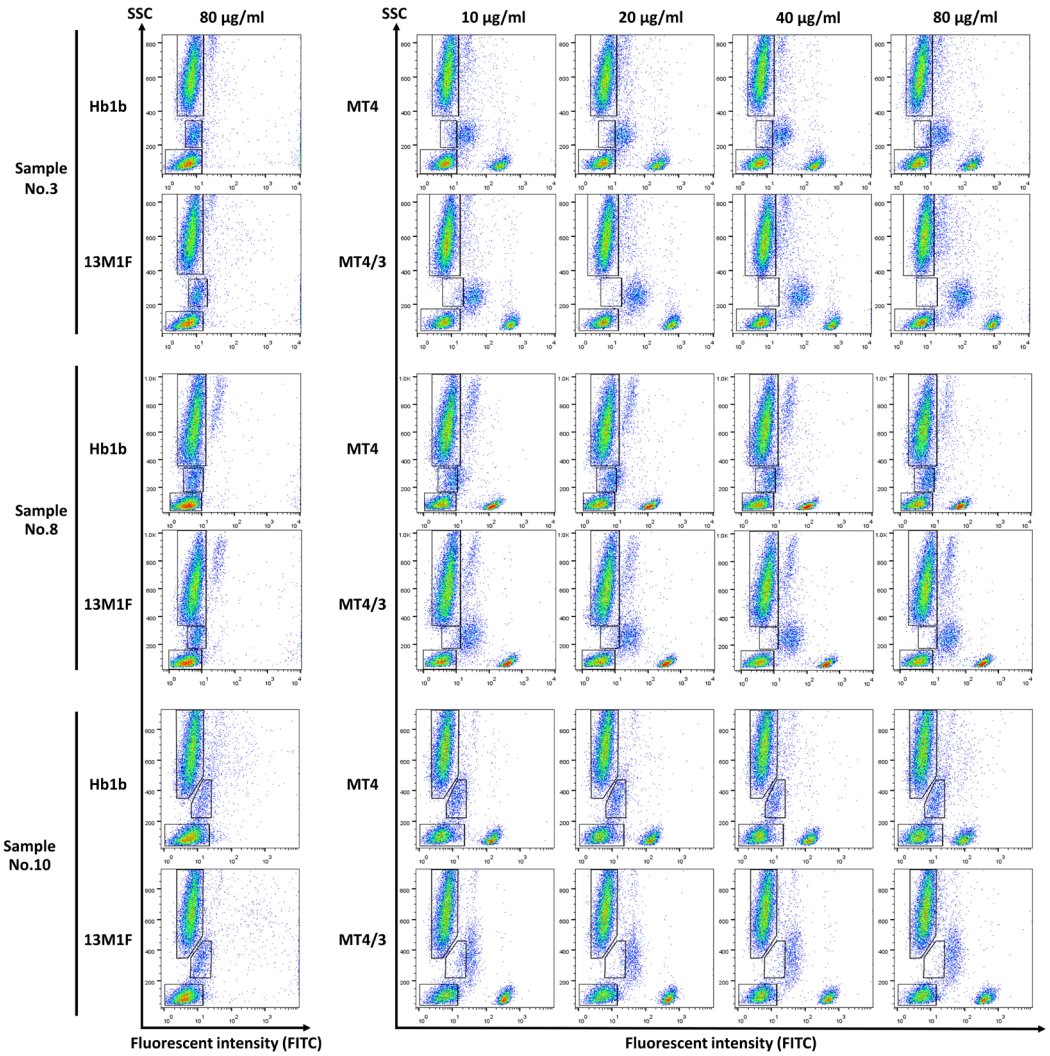

Figure 5. *The reactivity of monoclonal antibodies MT4 and MT4/3 with peripheral blood leukocytes of three study subjects. Whole blood samples were stained with mAbs MT4, MT4/3, or isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a) and 7-AAD as was described in Figure 4. The leukocyte population was gated according to their granularity (SSC) and 7-AAD staining. Granularity (SSC) and FITC fluorescence were plotted to show the different reactivity of mAbs MT4 and MT4/3 to monocyte population of different blood samples. The mAb MT4 shows positive, weak positive, and negative reactivities with monocytes from sample No. 3, No. 8, and No. 10, respectively. Whereas, mAb MT4/3 shows positive reactivity with monocytes from all tested samples at any concentrations. The fluorescence intensities of isotype matched control mAbs for all cell populations are shown and marked by rectangles. Three subjects are shown as representative of 10 studied subjects.*

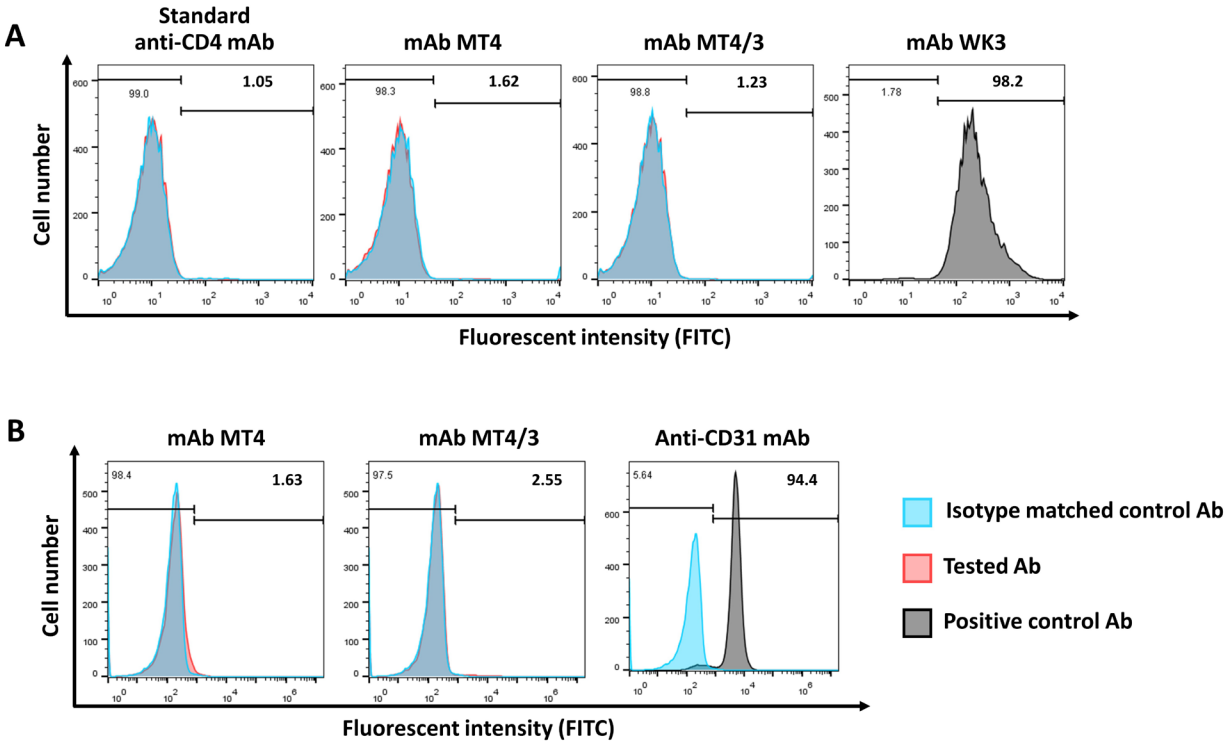

Figure 6. *Immunofluorescence analysis of the reactivity of monoclonal antibodies MT4 and MT4/3 with red blood cells and platelets. (A) RBCs and platelets (B) were stained with mAbs MT4, MT4/3, isotype matched control mAbs (Hb1b; IgM and 13M1F; IgG2a), standard anti-CD4-FITC, or mAb WK3 (as positive control for RBCs), or anti-CD31 mAb (as positive control for platelets) by indirect immunofluorescence staining and analyzed by flow cytometer. The RBCs or platelets were gated and the cells expressing molecule recognized by the tested mAbs are shown in red histogram. The isotype matched control and positive control mAbs are shown as light blue histograms and grey histogram, respectively.*

Monoclonal antibodies clones MT4 and MT4/3 recognize different CD4 isoforms

CD4 isoforms expressed on leukocyte surface have been reported.[29] As described above, the reactivities of mAbs MT4 and MT4/3 with monocytes were different. We speculated that mAbs MT4 and MT4/3 perhaps recognized different CD4 isoforms. Co-localization of the molecule recognized by mAbs MT4 and MT4/3 were, then, determined by confocal microscopic analysis. It was found that upon staining with mAbs MT4 and MT4/3, co-localization of the two mAbs were observed (Figure 7B and C). The non-colocalized molecules recognized by either mAb MT4 or MT4/3, however, were also detected (Figure 7B and C). Interestingly, cell that expressed only molecules that recognized by mAb MT4 was identified (Figure 7B).

These results demonstrated that, on cell surface, epitopes that recognized by mAbs MT4 and MT4/3 are on the same molecule or on different molecules but they are very closely expressed on cell surface, therefore, caused co-localization. However, some CD4 molecules contain epitopes that can be recognized by either mAb MT4 or MT4/3 and are expressed separately, thus caused non-co-localization. Some cells, nonetheless, expressed CD4 molecules contain only epitope that recognized with mAb MT4. These CD4 molecules are not carried the epitope recognized by mAb MT4/3. Our results indicated that, by mAbs MT4 and MT4/3, CD4 isoforms could be identified.

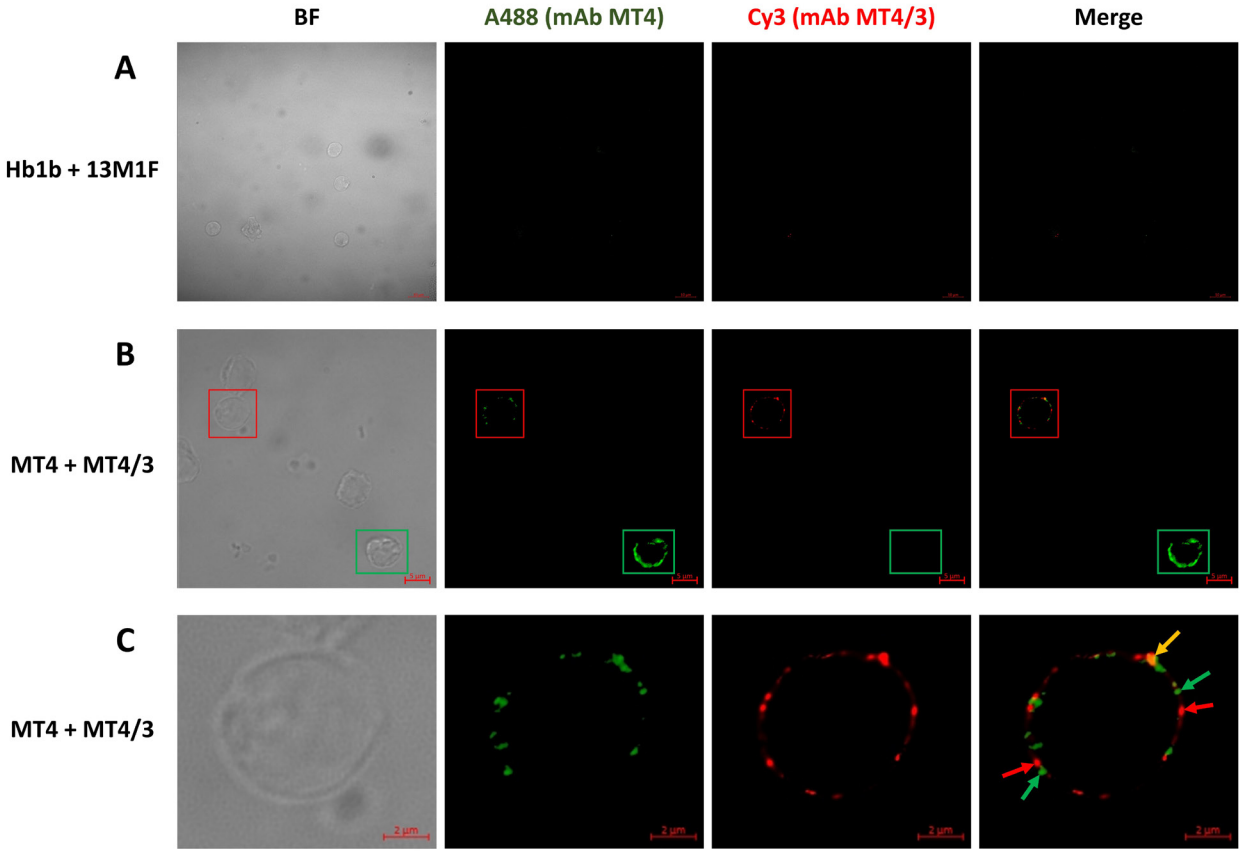

Figure 7. *Confocal microscopic analysis of the reactivity of monoclonal antibodies MT4 and MT4/3. PBMCs were double stained with mAbs MT4 and MT4/3 or Hb1b and 13M1F (isotype matched control mAbs) and following by Alexa Flour 488-anti-mouse IgM (μ-chain specific) and Cy™3-anti-mouse IgG (Fcγ specific) for detecting mAbs MT4 and MT4/3, respectively. The stained cells were analyzed by confocal microscope (100x). The mAbs MT4 and MT4/3 double positive (red rectangle) and MT4 single positive cells (green rectangle) are shown (B) while the isotype matched control mAbs are negative (A). The MT4 and MT4/3 double positive cell (from A, red rectangle) was magnified to observe in detail by ZEN 2.3 software (C). In this cell, the non-colocalized and colocalized cell surface molecules recognized by mAbs MT4 or MT4/3 are demonstrated. Non-colocalization of the molecules recognized by mAbs MT4 or MT4/3 are marked by green and red arrowheads, respectively. The co-localization of the molecules recognized by mAbs MT4 and MT4/3 are marked by yellow arrowhead.*

Discussion

The structure and function of CD4 molecules expressed on $CD4^+$ lymphocytes have been well characterized. However, little is known about CD4 molecules of non-lymphocytic cells. There are some, but few, reports demonstrated that the structure of CD4 molecules on lymphocytes and monocytes were different.[29] Lymphocytes express monomeric CD4, whereas monocytes express dimeric form.[6, 8] Furthermore, it was demonstrated that lymphocytes express a 55 kD monomeric CD4 molecules, while monocytes co-express two monomeric isoforms, 55 and 59 kD species.[29] The 59 kDa isoform was demonstrated as a transition-state, structural -intermediate in the formation of disulfide-linked homodimers.[29] The function of CD4 expressed on lymphocytes and monocytes were also different. Tyrosine kinase of CD4 on lymphocytes and monocytes were dissimilar, in which CD4 of lymphocytes were associated with Lck, but of monocyte were associated with Hck.[26, 29, 35] Heterogeneity in both structure and functions in the CD4 molecules of lymphocytes and monocytes was concluded. The existence of isoforms of CD4 molecules were also postulated.

In our research center, two anti-CD4 mAbs, named MT4 and MT4/3, were generated.[30, 31] These mAbs reacted with both recombinant CD4 proteins and native CD4 expressed on leukocytes. The cellular reactivity profiles of mAbs MT4 and MT4/3 were determined and found similar compared to the standard anti-CD4 mAb. However, when analyzed in detail, some differences in the reactivity of mAbs MT4 and MT4/3 were evidenced. In some subjects, monocytes were negative or weakly positive when stained with mAb MT4, but positive with mAb MT4/3 (this study and [32]). As CD4 isoforms have been reported,[29] we asked whether mAbs MT4 and MT4/3 bind to different isoforms. Co-localization, using confocal microscopic analysis, between mAbs MT4 and MT4/3 were conducted. The co-localization of both tested mAbs were found on cell surface suggesting that epitopes recognized by both mAbs may be present on a CD4 molecule. In addition, it can also be presumed that mAbs MT4 and MT4/3 reacted with different epitopes which located in different CD4 isoforms, but these isoforms were closely expressed on cell surface. Interestingly, within any positive cell, there are some CD4 molecules that reacted

with mAb MT4 or MT4/3, but without co-localization. The results indicated that mAbs MT4 and MT4/3 reacted with its epitopes expressed on distinct CD4 isoforms and expressed at different area on the cell surface. Surprisingly, very rare cells were detected positively only with mAb MT4. This observation indicated that this cell expressed only CD4 isoform that recognized by mAb MT4. Taken together, we demonstrated that our generated anti-CD4 mAbs MT4 and MT4/3 reacted with different CD4 isoforms expressed on leukocyte surface. Based on our findings, we postulated that CD4 isoforms are existence and be detected by mAbs MT4 and MT4/3. The molecular mechanism of the uncovered isoforms, however, is still unknown.

In conclusion, we reported here that the CD4 isoforms are existence and could be identified by our generated anti-CD4 mAbs, MT4 and MT4/3. To the best of our knowledge, this kind of anti-CD4 mAb has never been reported. Our anti-CD4 mAbs that can differentiate CD4 isoforms will be a valuable tool for characterization in detail of the structure and function of CD4 isoforms.

Conflict of interest

The authors declare that they have no competing interests.

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