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ORIGINAL ARTICLE

The ligation of CD4 molecules, expressed on monocytes by an anti-CD4 monoclonal antibody, inhibits T cell activation and monocyte mobility

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Abstract

Background: CD4, a leukocyte surface glycoprotein, is mainly expressed on $CD4^+$ T cells, but is also expressed on monocytes. The difference in the expression level and structure of CD4 on T cells and monocytes predicts the different functions of this molecule in both cell types. Although the function of CD4 on T cells is well characterized, little is known about that expressed on primary monocytes.

Objective: In this study, we investigated the immunoregulation function of CD4 on peripheral blood monocytes.

Methods: CD4 molecule on monocyte was ligated by anti-CD4 monoclonal antibody (mAb), MT4/3. The effect of mAb MT4/3 on T cell proliferation, cytokine production, the expression of monocyte costimulatory molecules, monocyte migration, and macrophage differentiation were investigated. Moreover, the molecular weight of CD4 on peripheral blood monocyte was carried out by Western immunoblotting.

Results: We demonstrated that mAb MT4/3 inhibited anti-CD3 induced T cell proliferation, cytokine production, and the expression of monocyte costimulatory molecules. The ligation of only CD4 on monocytes was sufficient to inhibit T cell activation. Moreover, mAb MT4/3 could inhibit monocyte migration in a transwell migration assay, but not affect the differentiation of monocytes to macrophages. Using purified primary monocytes, the molecular weight of CD4 expressed on monocytes was identified as 55 kDa.

Conclusion: The CD4 molecule expressed on monocytes might play an important role in the regulation of immune responses in both innate and adaptive immunity. Understanding the novel role of CD4 on monocytes in immunoregulation is valuable in the development of new therapeutic approaches.

Key words: CD4, Anti-CD4 monoclonal antibody, T cell regulation, Monocyte, Monocyte function

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Introduction

Monocyte is a crucial leukocyte, which plays important roles in both innate and adaptive immunity.[1,2] Monocytes express several cell surface molecules, including IgG Fc receptors, complement component receptors, chemokine receptors, receptors for adhesion, as well as CD4 molecules.[1-5] Additionally, monocytes also express pattern recognition receptors (PRRs) and LPS receptors for recognizing invading pathogens.[1,6] Due to their surface expressing molecules,

monocytes effectively function through their phagocytic activity and inflammatory responses. Monocytes can localize to tissues and differentiate into macrophages and dendritic cells.[1,2] Monocytes have several functions, including maintaining tissue homeostasis, performing tissue repair, scavenging toxic or foreign materials, and initiating adaptive immune responses.[1,2,7] The function of monocytes and macrophages in the production of a variety of proinflammatory cytokines upon activation, and their ability to present antigen to T cells, bridges the innate immune and adaptive immune response.[1-3,6,8] Although the characteristics and functions of most leukocyte surface molecules expressed on monocytes have been described, the role of CD4 on monocytes is yet to be well defined. Surprisingly, human, but not mouse, monocytes express CD4 molecules.[9] This predicts the important role of human monocytes' CD4 in the immune system.

CD4 is a 55 kDa type I integral membrane protein with N-linked glycosylation that belongs to the immunoglobulin family.[10,11] The CD4 molecule was first identified through expression on a subset of T cells, known as $CD4^+$ T helper cells. On T cells, CD4 was demonstrably involved in stabilizing and increasing the avidity of interactions between T cell receptors (TCR) and peptide–major histocompatibility complex (MHC) class II complexes on antigen-presenting cells (APC) during the antigen presentation process.[12] Upon this activation, TCR confers downstream signaling via CD4 binding lymphocyte-specific protein tyrosine kinase (Lck).[13-15] The expression of CD4 molecules on monocytes was also demonstrated, but the expression level was 10- to 20-fold less than that on $CD4^+$ T cells.[4,5,16] Importantly, the intracellular domain of CD4 molecules of monocytes and T cells interacts differently with Src family kinase, Hck and Lck in relation to monocytes and T cells.[17,18] It was demonstrated that the activation of CD4 on monocytes by MHC class II triggered the differentiation of monocytes into functional mature macrophages via a different signal transduction pathway from T cells.[19] However, the immunologic function of CD4 on monocytes is still limited.

Several anti-CD4 monoclonal antibodies (mAb) were generated in our research center.[20,21] In this study, the generated anti-CD4 mAbs were used to reveal the function of CD4 molecules expressed on monocytes. We demonstrated that the ligation of CD4 molecules on monocytes by an anti-CD4 mAb, MT4/3, could attenuate T cell responses and monocyte migration. Our results can provide a basis for understanding the role of CD4 on monocytes in immunoregulation. This understanding could be useful in the development of new therapeutic approaches.

Materials and Methods

Antibodies and reagents

Anti-CD4 mAb clones MT4 (isotype IgM) and MT4/3 (IgG2a), anti-hemoglobin clone Hb2a (IgM) and clone PB1 (IgG1) were generated in our laboratory.[20,21] Anti-flavivirus envelope protein mAb clone 4G2 (IgG2a) was kindly provided by Dr. Chanya Puttikhun (Molecular Biology of Dengue and Flaviviruses Research Team, National Center for Genetic Engineering and Biotechnology, NSTDA, Thailand). Anti-CD3ε mAb (clone OKT3) was purchased from Ortho Pharmaceuticals (Raritan, NJ, USA). Anti-CD28 mAb (clone L293) were obtained from BD Bioscience (San Jose, CA, USA). Anti-CD4 mAb (clone MEM-241) was from Invitrogen (Carlsbad, CA, USA). Peroxidase conjugated goat anti-mouse IgG light chain specific was from Jackson (West Grove, PA, USA). Fluorescein isothiocyanate (FITC) conjugated anti-CD3, anti-CD14 and anti-CD19 mAbs, R-phycoerythrin (PE) conjugated anti-CD14 and anti-human cytokine mAbs (anti-IL2, -IFN-γ, -IL-10, -IL-17, -TNF-α, -IL-6 mAbs), PerCP conjugated anti-CD14 mAb, and PE-anti-CD163 mAb were purchased from BioLegend (San Diego, CA, USA). FITC labeled anti-CD86, HLA-DR and HLA-ABC mAbs, PE conjugated anti-CD69 mAb, mouse IgG1-PE isotype control antibody and recombinant human monocytic chemoattractant protein-1 (rh MCP-1), recombinant human granulocyte-macrophage colony-stimulating factor (rh GM-CSF), and recombinant human macrophage colony-stimulating factor (rh M-CSF) were purchased from ImmunoTools (Friesoythe, Germany). Carboxyfluorescein succinimidyl ester (CFSE), brefedin A, monensin and percoll were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-FITC Microbeads, Pan Monocyte Isolation Kit, and LD columns were purchased from Miltenyi Biotec (Bergisch, Gladbach, Germany).

Cell preparation

Peripheral blood was collected from healthy donors using sodium heparin as an anti-coagulant. PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation.

Monocyte depleted PBMCs were prepared by depleting monocytes from PBMCs using magnetic bead separation. In short, PBMCs were blocked by their FC receptors with 10% human blood group AB serum and stained with FITC conjugated anti-CD14 mAb. After washing, anti-FITC microbeads were added. After incubation, a magnetic separation process was performed using an LD column according to the manufacturer's instructions (Miltenyi Biotec). The percentage of monocyte contamination was confirmed by flow cytometry and less than 2% of monocytes were obtained.

Monocyte enriched PBMCs were prepared by Percoll gradient centrifugation. In brief, PBMCs were overlaid on Percoll solution (48.5% Percoll and 0.16 M NaCl in ddH_2O) and were centrifugated at 865×g for 40 minutes. After centrifugation, monocyte enriched PBMCs were collected from the middle layer.

Purified monocytes were isolated from PBMCs using the Pan Monocyte Isolation kit. A magnetic separation process was performed using an LD column according to the manufacturer's instructions (Miltenyi Biotec). The purity of $CD14^+$ monocytes was confirmed by flow cytometry, and > 95% purity was obtained.

This study was approved by the ethics committees of the Faculty of Associated Medical Sciences, Chiang Mai University (AMSEC-63EX-008). The written informed consent was obtained from all research participants before entering the study.

Cell proliferation assay

A cell proliferation assay was performed by analysing the reduction in carboxyfluorescein succinimidyl ester (CFSE) fluorescence intensity using flow cytometry. In brief, PBMCs or monocyte depleted PBMCs (1×10^7 cells/mL) were labeled with 1 µM of CFSE at 37°C for 10 minutes. The CFSE labeling was quenched by adding 10% fetal bovine serum (FBS)-RPMI-1640. Then, cells were washed and resuspended in 10% FBS-RPMI-1640. For the PBMC proliferation assay, 1×10^5 cells of CFSE-labeled PBMCs were plated into 50 ng/mL of anti-CD3 mAb (OKT3) immobilized in a 96-well plate or left unstimulated. For monocyte depleted PBMCs, CFSE-labeled monocyte depleted PBMCs were plated at 1×10^5 cells into 200 ng/mL of OKT3 immobilized in a 96-well plate in the presence of 125 ng/mL of anti-CD28 mAb or left unstimulated. Cells were cultured in the presence or absence of anti-CD4 mAbs or isotype-matched control mAbs at 37°C, 5% CO_2 for 5 days and analyzed by flow cytometry (BD Accuri™ C6, BD Biosciences).

For the anti-CD4 mAb pre-treated monocyte co-culture, 1×10^4 purified monocytes were incubated in the presence or absence of anti-CD4 mAbs or isotype-matched control mAbs at 37°C for 30 minutes. Then, cells were washed to remove the excess antibody. After washing, antibody pre-treated monocytes were co-cultured with CFSE-labeled monocyte depleted PBMCs at a ratio of 1:9 in the presence or absence of immobilized OKT3 at 37°C, 5% CO_2 for 5 days and analyzed by a flow cytometer (BD Accuri™ C6, BD Biosciences).

Intracellular cytokine detection

PBMCs were cultured in a 24-well plate at 1×10^6 cells with or without immobilized anti-CD3 mAb OKT3 and soluble anti-CD28 mAb (25 ng/mL) in the presence or absence of 10 µg/mL of anti-CD4 mAb or isotype-matched control mAb. After incubating for 1 hour, 1 µg/mL of brefeldin A and 1 µM of monensin were added to the cells and incubated at 37°C for 5 hours. The production of cytokines was measured by intracellular staining with PE conjugated anti-human IL-2, IL-6, IL-10, TNF-α, or IFN-γ mAb. Moreover, the population of cells that produced cytokines was identified by staining with FITC conjugated anti-CD3 mAb and PerCP conjugated anti-CD14 mAb. The cytokine producing T cells and monocytes were analyzed by a flow cytometer (BD Accuri™ C6, BD Biosciences).

Surface MHC class I, MHC class II, CD80, and CD86 analysis

PBMCs were cultured in a 24-well plate at 1×10^6 cells in the presence or absence of 10 µg/mL of anti-CD4 mAb or isotype-matched control mAb. Cells were stimulated with or without 25 ng/mL of immobilized OKT3 mAb at 37°C, 5% CO_2, for 18 hours. Then, cells were harvested and stained with FITC conjugated anti-CD80, anti-CD86, anti-HLA-DR (MHC class II), or anti-HLA-ABC (MHC class I) mAbs, and PE conjugated anti-CD14 mAb. Using flow cytometry, levels of HLA-ABC, HLA-DR, CD80, and CD86 expression were analyzed on a CD14 positive cell population.

Transwell migration assay

Monocyte enriched PBMCs were resuspended in 1% BSA-RPMI 1640. Cells were placed in the upper chamber of a 5 µm pore size transwell filter (Merck, Darmstadt, Germany) at 1.5×10^6 cells in the presence or absence of 10 µg/mL anti-CD4 mAb or isotype-matched control mAb. MCP-1 (40 ng/mL) in 1% BSA-RPMI 1640 was placed in the lower chamber. After incubating at 37°C for 1 hour, cells that migrated through the filter into the lower chamber were harvested. Then, cells were washed and stained with FITC conjugated anti-CD14 mAb and PE conjugated anti-CD3 mAb. Migrated cells were analyzed by a flow cytometer (BD Accuri™ C6, BD Biosciences) and cell numbers were enumerated using a hemocytometer.

Macrophage differentiation assay

Monocyte enriched PBMCs were added into 24-well plates and incubated for 2 hours at 37°C, 5% CO_2. Lymphocytes were removed from the plates by washing. The adhered monocytes were cultured in the presence or absence of anti-CD4 mAb or isotype-matched control mAb. For macrophage differentiation, cells were supplemented with 90 ng/mL rh GM-CSF or 10 ng/mL rh M-CSF for classical (M1) macrophage or alternative (M2) macrophage differentiation, respectively. The cytokines were re-fed every 2 days. After incubating for 6 days, cells were stained with Alexa Fluor® 488 conjugated anti-CD86 mAb and PE conjugated anti-CD163 mAb, and analyzed using a flow cytometer (BD Accuri™ C6, BD Biosciences).

Biochemical characterization of CD4 molecules on monocytes

Purified monocytes were solubilized on ice with a lysis buffer containing 1% laurylmaltoside and protease inhibitors. Cell lysates were pre-cleared with mouse IgG2a coated beads. Pre-cleared lysates were subjected to 10% SDS-PAGE under non-reducing conditions, and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% BSA in PBS, pH 7.2, at room temperature for 1 hour. The membrane was incubated with 5 µg/mL of anti-CD4 mAb clone MEM-241 (IgG1) or isotype-matched control mAb clone PB1 (IgG1) at room temperature for 1 hour. After incubation, the membrane was washed followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG (light chain) at room temperature for 1 hour. After washing, luminol substrate was added to detect the chemiluminescent signal of the reactive protein bands.

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 9.2.0 (GraphPad Software, CA, USA). The unpaired *t*-test or two-way ANOVA were used as indicated in the Figure legends. Statistical significance was accepted at a p value of < 0.05.

Results

The ligation of CD4 molecules by anti-CD4 mAb MT4/3 alters the proliferation and cytokine production of T cells and the co-stimulatory molecule expression of monocytes

Anti-CD4 mAbs, MT4 and MT4/3, were generated in our research center. We initially determined the effect of these mAbs on anti-CD3 mAb activated PBMCs. As shown in **Figure 1A and 1B**, the reduction of cell proliferation was observed in the presence of mAb MT4/3 at antibody concentrations of 10 and 40 ug/ml, but not for mAb MT4. The results indicate that the ligation of CD4 molecules by mAb MT4/3 could inhibit anti-CD3 activated T cell proliferation. The anti-CD4 mAb clone MT4/3 was selected for further investigation.

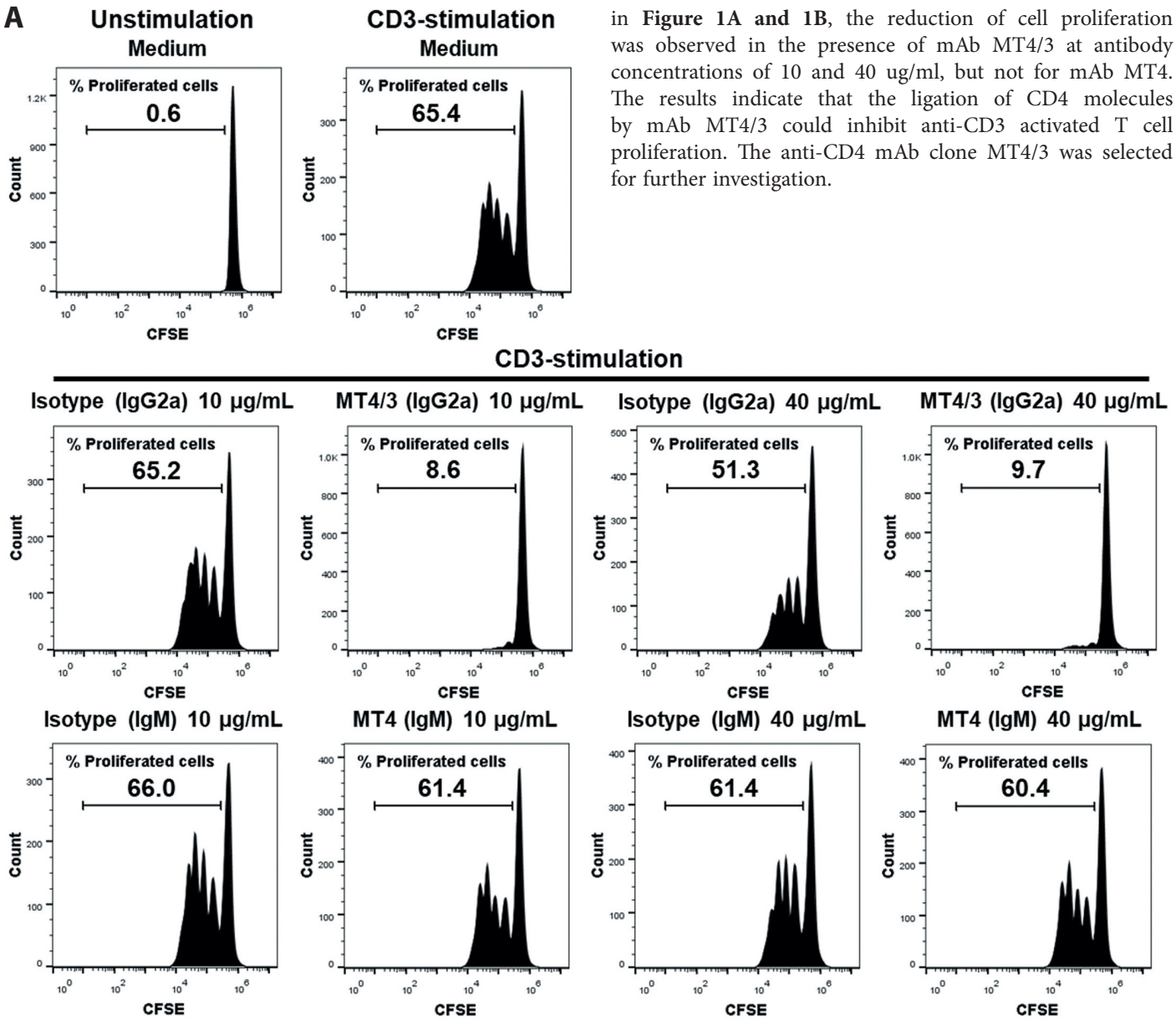
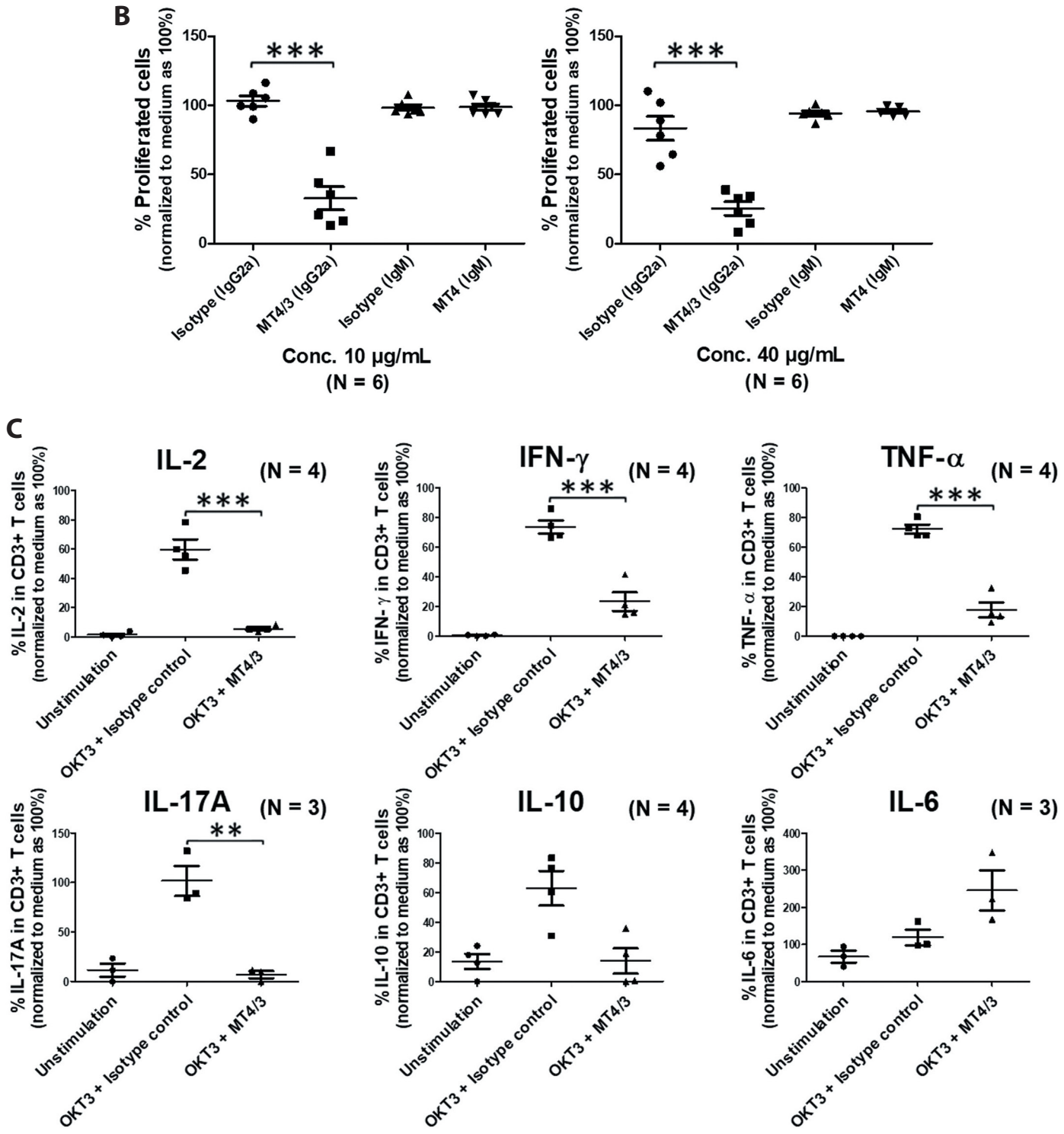


Figure 1. Anti-CD4 mAb, MT4/3, suppresses anti-CD3 mAb activated PBMCs. (A) CFSE labeled PBMCs were stimulated with or without anti-CD3 mAb OKT3 in the presence or absence of anti-CD4 mAbs or isotype-matched control mAbs. A flow cytometric analysis of one representative of the CFSE proliferation data is shown. PBMCs were gated and analyzed for the CFSE intensity of each indicated condition using a histogram plot. The percentage of proliferated cells is indicated. (B) Dot plots demonstrate the percentage of proliferated cells (mean ± SEM) in the indicated condition upon anti-CD3 mAb OKT3 stimulation. Individual data was normalized relative to its medium control as 100%. (C-E) PBMCs were stimulated with anti-CD3 mAb OKT3 in the presence or absence of anti-CD4 mAb or isotype-matched control mAb. $CD3^+$ T cells (C) or $CD14^+$ monocytes (D) were gated and the percentage of cells producing the indicated cytokines (mean ± SEM) is shown. Individual data was normalized relative to its medium control as 100%. The basal level of each cytokine was demonstrated in unstimulating condition, in which PBMCs were incubated without anti-CD3 mAb. (E) The indicated costimulatory molecule expression (MFI ratio) of $CD14^+$ monocytes in the indicated conditions were shown (mean ± SEM). The MFI of indicated molecules was normalized relative to its medium control as 1. An unpaired *t*-test was used for the comparison. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.



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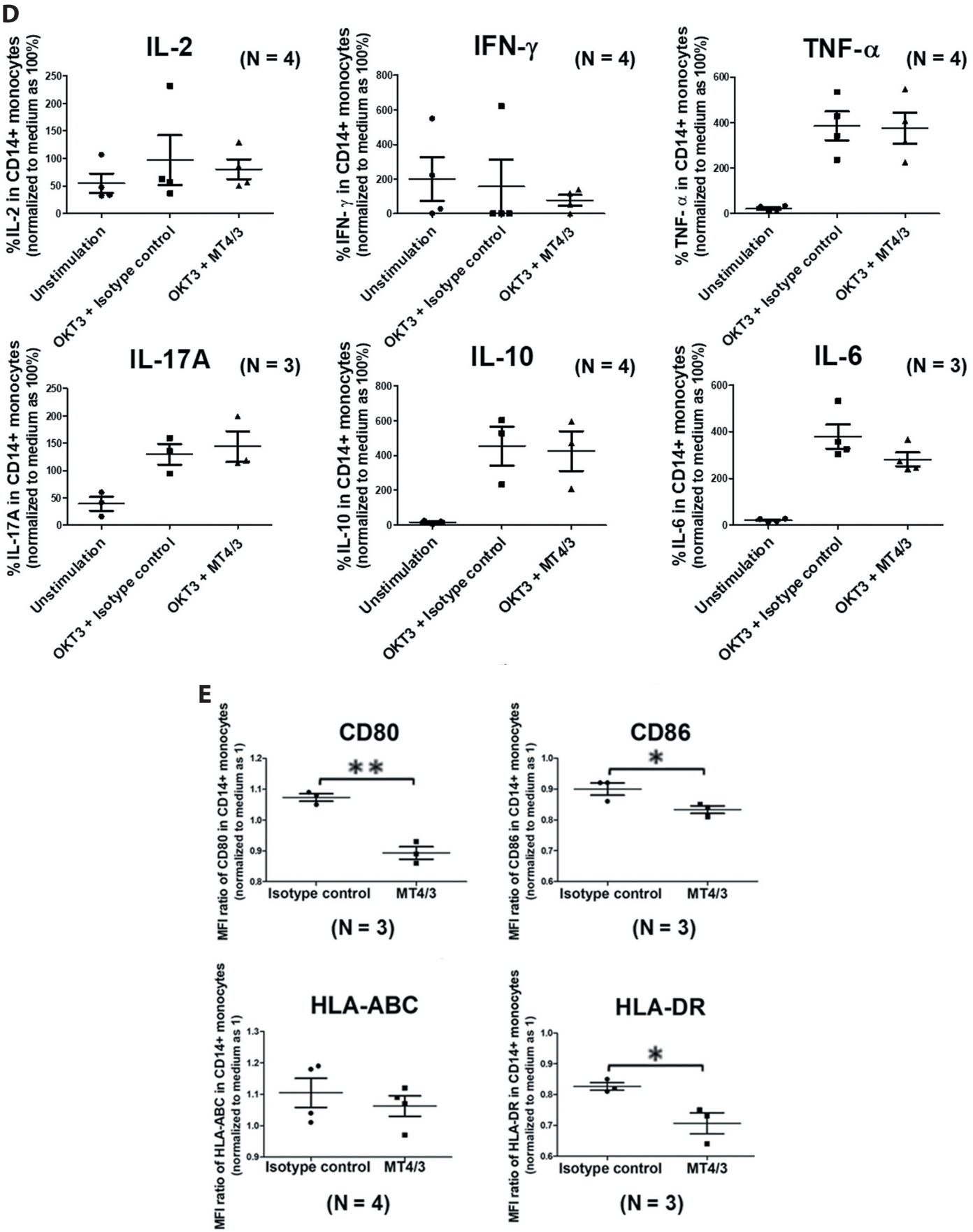


Figure 1. (Continued)

We next determined the effect of mAb MT4/3 on cytokine production by T cells and monocytes. In $CD3^+$ T cells, IL-2, IFN-γ, TNF-α, and IL-17A cytokines were significantly decreased by mAb MT4/3 (**Figure 1C**). IL-10 and IL-6 tended to decrease and increase, respectively, but there was no significant difference between mAb MT4/3 and isotype matched control mAb (**Figure 1C**). In contrast, with $CD14^+$ monocytes, mAb MT4/3 had no significant alteration of the tested cytokine production, including IL-2, IFN-γ, TNF-α, IL-17A, IL-10, and IL-6 (**Figure 1D**).

During T cell stimulation, the co-stimulatory molecules are required for full T cell activation. Since mAb MT4/3 suppresses T cell proliferation and cytokine production, we asked whether ligation of CD4 might alter the expression of co-stimulatory molecules on monocytes. Thus, the effect of mAb MT4/3 on the co-stimulatory molecule expression on monocytes was investigated. Upon the stimulation of PBMCs with anti-CD3 mAb, mAb MT4/3 significantly downregulated CD80, CD86, and HLA-DR expression on monocytes (**Figure 1E**). However, this effect was not observed with the expression of MHC class I molecule, HLA-ABC (**Figure 1E**). Gating strategies for the flow cytometric analysis of **Figure 1** are shown in Supporting Information (**Figure S1**).

The ligation of CD4 molecules on monocytes by anti-CD4 mAb MT4/3 impairs T cell activation

Since PBMCs contain lymphocytes and monocytes, and both cell types express CD4 molecules, we questioned whether the inhibitory effect of mAb MT4/3 was from the ligation of CD4 on T cells or monocytes. To address this question, monocyte depleted PBMCs were used in the experiments. Monocyte depleted PBMCs were activated by anti-CD3 mAb, and anti-CD28 mAb and cell proliferation was determined by a CFSE cell proliferation assay. The gating strategy of the flow cytometric analysis was the same as in Supporting Information (**Figure S1**). The inhibitory effect of mAb MT4/3 (10 ug/mL and 40 ug/mL) on anti-CD3 mAb induced T cell proliferation was abolished in monocyte depleted PBMCs compared with PBMCs (**Figure 2A**), suggesting the involvement of CD4 on monocytes in T cell activation.

To confirm the observed inhibitory effect, monocytes were pre-treated with mAb MT4/3 or mAb MT4. The anti-CD4 mAb pre-pulsed monocytes were reconstituted to autologous monocyte depleted PBMCs, and anti-CD3 induced cell proliferation was determined. The monocytes pre-pulsed with mAb MT4/3, for either 15 or 30 min, could significantly inhibit T cell proliferation (**Figure 2B**). This inhibitory effect was not observed using mAb MT4 pre-pulsed monocytes (**Figure 2B**).

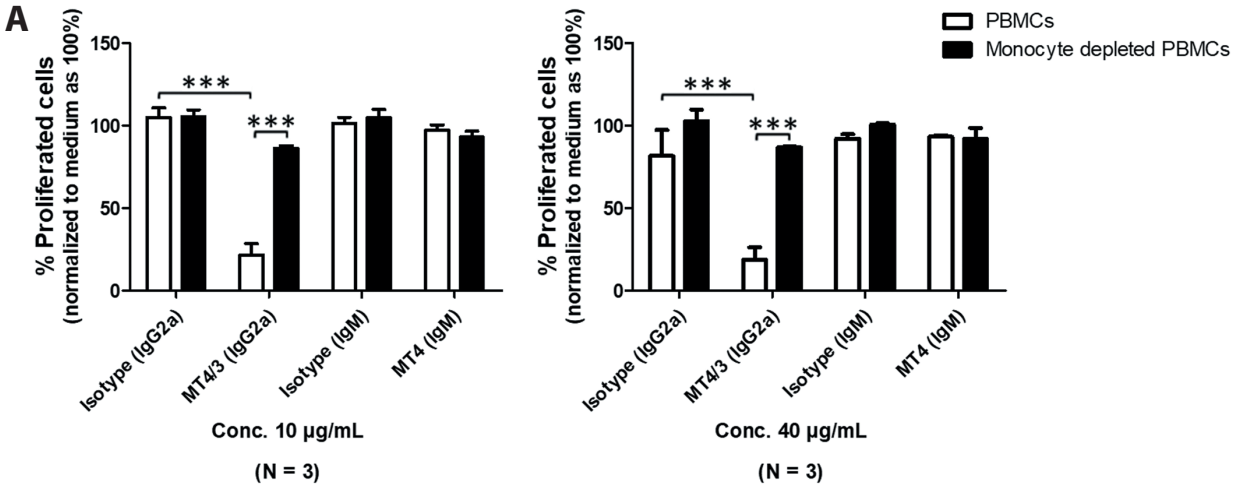


Figure 2. The ligation of CD4 on monocytes downregulates T cell proliferation. (A) PBMCs or monocyte depleted PBMCs were stimulated with anti-CD3 mAb OKT3 in the presence or absence of anti-CD4 mAbs (MT4/3 (IgG2a) or MT4 (IgM)) or isotype-matched control mAbs (Isotype (IgG2a) or Isotype (IgM)). The percentage of proliferated cells was detected by a reduction of CFSE. The percentages of proliferated cells (mean ± SEM) at the indicated conditions of monocyte depleted PBMCs, in comparison with PBMCs, are shown. Individual data was normalized relative to its medium control as 100%. (B) Monocytes were incubated with or without anti-CD4 mAbs MT4/3 (IgG2a), MT4 (IgM), or isotype-matched control mAbs (Isotype [IgG2a] or Isotype [IgM]) for 15 and 30 minutes. The pre-pulsed monocytes were added into monocyte depleted PBMCs at a ratio of 1:9. The percentage of proliferated cells (mean ± SEM) detected by a reduction of CFSE in the indicated conditions is shown.

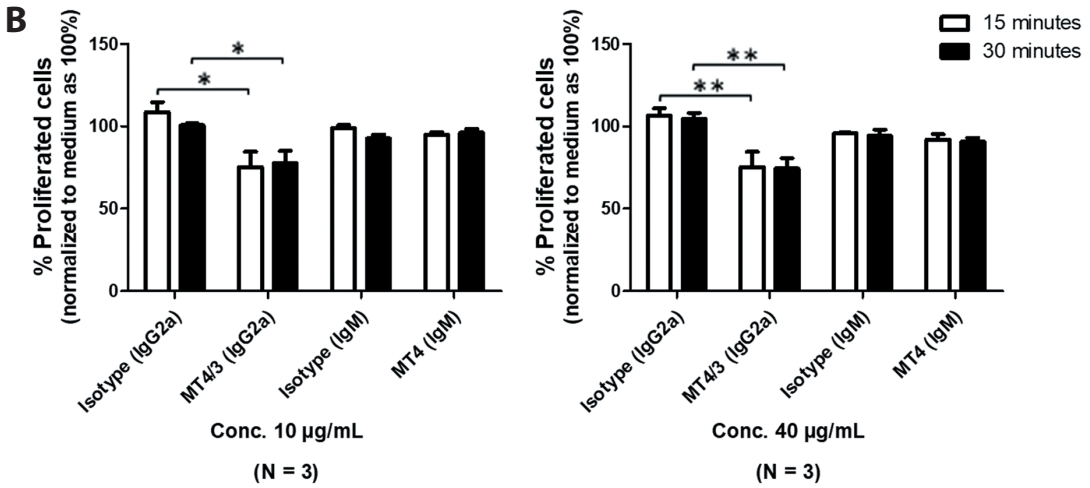


Figure 2. (Continued)

Our results suggested that the ligation of CD4 molecules on monocytes by mAb MT4/3 could regulate T cell activation. The CD4 molecules on monocytes might play important roles in T cell regulation.

The CD4 molecule is involved in monocyte migration but not macrophage differentiation

Migration is one of the crucial functions of monocytes.[1,2] The effect of anti-CD4 mAb MT4/3 on monocyte migration was determined by a transwell migration assay using MCP-1 as a chemotactic factor. MCP-1 induced the migration of monocytes from the upper chamber to the lower chamber. However, this migration was inhibited in the presence of mAb MT4/3, but not the isotype-matched control mAb (**Figure 3A**). This result suggests that in addition to T cells, CD4 on monocytes also plays a role in monocyte migration.

We further determined the role of CD4 on monocytes in the macrophage differentiation process. Monocytes were induced to differentiate M1 or M2 macrophages by GM-CSF or M-CSF, respectively, in the presence or absence of mAb MT4/3. The expression levels of CD86 (M1 marker) and CD163 (M2 marker) were determined. It was found that mAb MT4/3 had no effect on the differentiation of monocytes into M1 and M2 macrophages driven by GM-CSF and M-CSF stimulation, respectively (**Figures 3B and 3C**).

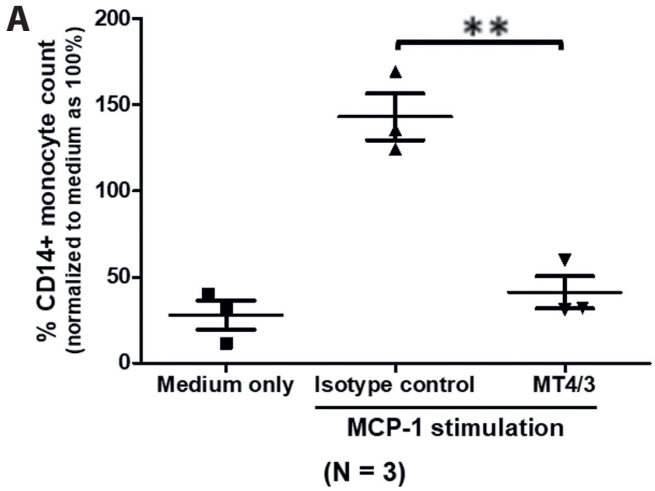
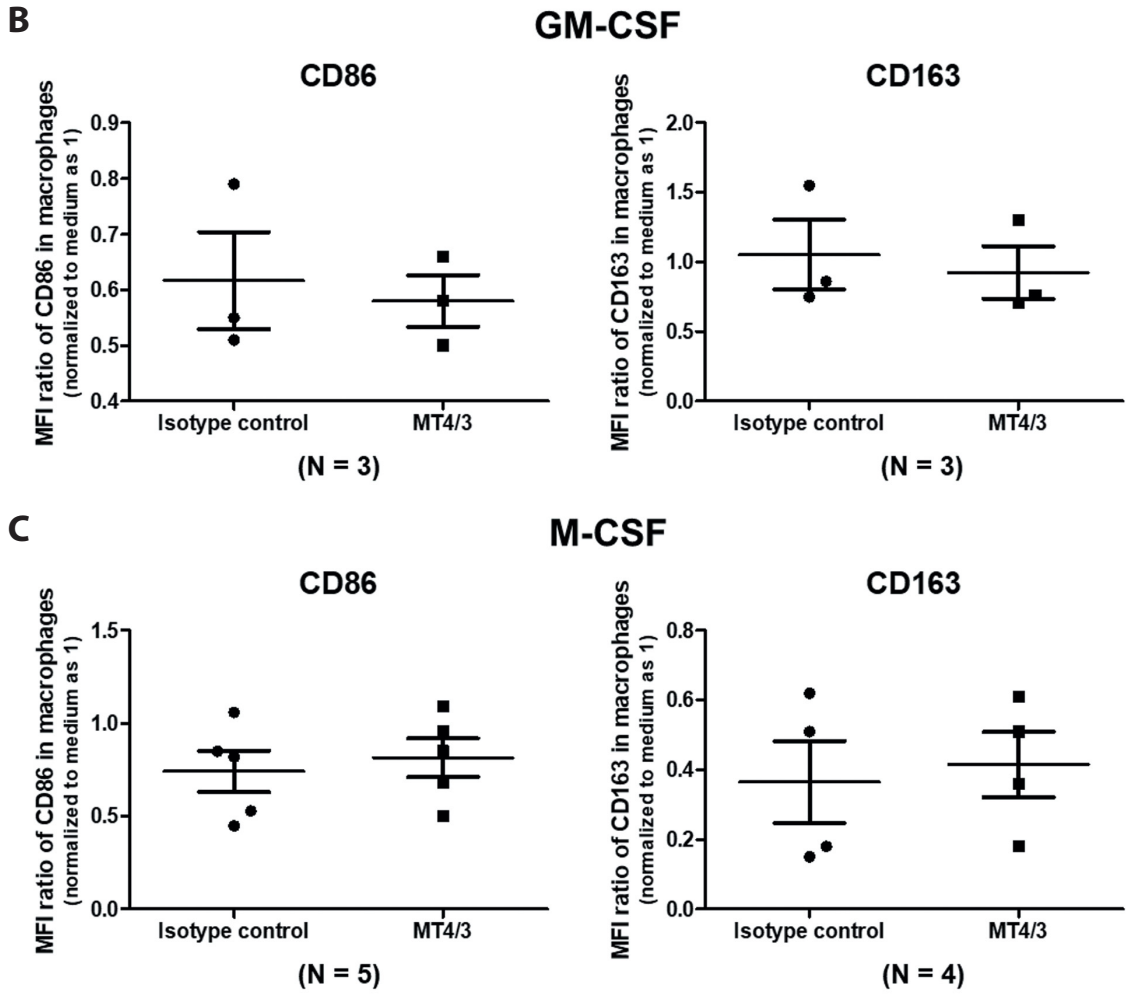


Figure 3. The CD4 molecule on monocytes plays a role in monocyte migration. (A) Monocyte enriched PBMCs (N = 3) were added into the upper chamber of a transwell plate in the presence or absence of anti-CD4 mAb, MT4/3, or isotype-matched control mAb, 4G2. MCP-1 was used as a chemoattractant in the lower chamber of the transwell plate. The absolute number of $CD14^+$ monocytes in the lower chamber was determined. The obtained monocyte counts in each condition were normalized relative to the number of monocytes in the presence of MCP-1, but the absence of mAb MT4/3, as 100%. Mean ± SEM values are indicated. An unpaired *t*-test was used in the comparison. $^{**}P < 0.01$. (B, C) Monocyte enriched PBMCs were cultured in the presence or absence of anti-CD4 mAb MT4/3 or isotype-matched control mAb, and supplemented with GM-CSF (B) or M-CSF (C) for 6 days. The MFI of CD86 and CD163 expression were determined. The MFI of each marker (mean ± SEM) in the indicated conditions was normalized to the medium control as 1.



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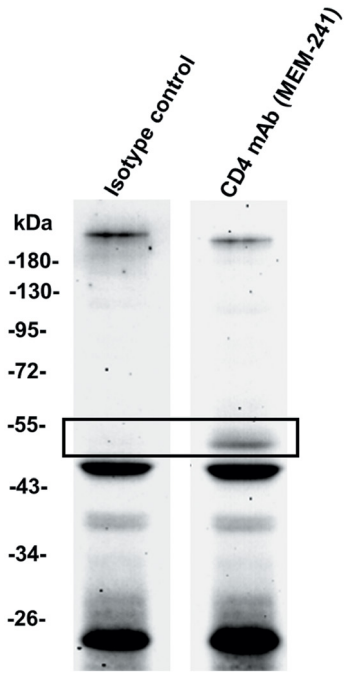

Figure 4. Western immunoblotting of the CD4 molecule expressed on primary monocytes. Purified monocyte lysates were subjected to Western immunoblotting using the anti-CD4 mAb and isotype-matched control mAb. The CD4 protein band is indicated in the black box. The molecular weight markers are illustrated on the left.

The molecular weight of the CD4 molecule expressed on monocytes

Information about the molecular weight (MW) of CD4 expressed on primary monocytes was limited. We determined the MW of CD4 molecules using purified monocytes. Using magnetic separation, a high-purity monocyte population (> 95% purity) was isolated from the PBMCs. The purified monocytes were subjected to Western immunoblotting using anti-CD4 mAb MEM-241. A protein band at 55 kDa was detected (**Figure 4**). The result indicated that monocytes express CD4 molecules with the MW of 55 kDa.

Discussion

CD4 molecules, a type I transmembrane glycoprotein, is expressed on many cell types, including $CD4^+$ T cells and monocytes.[5,16,22,23] While the function of CD4 on T cells is well characterized, little is known about CD4 on monocytes. CD4 molecules are expressed on T cells of various mammalian species and appear to play an important role in the response of T cells to antigens. In striking contrast, the expression of CD4 molecules were found on human monocytes, but not on mouse monocytes or macrophages.[9] This species heterogeneity indicates that T cells and monocytes regulate CD4 antigen expression independently and that CD4 on both cell types may have a different function. In this study, the functions of CD4 on monocytes were investigated, and the undefined functions of CD4 on monocytes were found to contribute to the downregulation of T cell responses and monocyte migration.

We demonstrated that the ligation of CD4 on monocytes by anti-CD4 mAb clone MT4/3 inhibited anti-CD3 induced T cell responses, including cell proliferation, cytokine production, and the co-stimulatory molecule expression of monocytes. The inhibitory effect, however, was not observed by anti-CD4 mAb clone MT4. The different effects of mAbs MT4/3 and MT4 might be due to their different binding epitopes.[20,21] It has been well described that different mAbs display differential functional effects depending on the particular epitope recognized.[24] The epitope recognized by mAb MT4/3 might be the functional epitope, which upon ligation, generates intracellular negative signaling and down-modulation of T cell responses. The distinct effect among mAb MT4/3 and MT4 may also be due to their isotypes. Since mAb MT4 is an IgM isotype, the binding affinity is lower than that of an IgG isotype (mAb MT4/3).[25] The signal transduction mediated through mAb MT4/3 would be better than through mAb MT4, resulting in the regulation of the cellular function of monocytes.

As in our experiments, PBMCs were stimulated with anti-CD3 mAb and T cell responses were determined. The PBMCs contain lymphocytes and monocytes, and both cells express CD4 on their surface. The observed inhibitory effects of mAb MT4/3 may depend on whether the binding of mAb MT4/3 comes from CD4 on T cells or monocytes. To address this inquiry, we carried out experiments using monocyte depleted PBMCs instead of whole PBMCs. The inhibitory effect of mAb MT4/3 on T cell proliferation was obliterated in monocyte depleted PBMCs compared with whole PBMCs. These results indicated that CD4 monocytes affect mAb MT4/3.

To confirm our findings, purified monocytes were pre-treated with mAb MT4/3 and the excess antibodies were washed out. The CD4 ligation mediated by mAb MT4/3, therefore, occurred only on monocytes. The pre-pulsed monocytes were then reconstituted to the monocyte depleted PBMCs. The inhibition effect was observed in the presence of mAb MT4/3 pre-pulsed monocytes, but not with the mAb MT4 pre-pulsed monocytes. These results confirmed that CD4 molecules on monocytes play a functional role in the downregulation of T cell activation. Negative signaling generated by the ligation of CD4 on T cells has previously been reported,[26-28] and we suggest here that the ligation of CD4 by mAb MT4/3 on monocytes is also involved in the hypofunction of T cells. The mAb MT4/3 may function as CD4 molecule agonist or antagonist and affect cell function. Disturbance of the association of CD4 molecules to other membrane proteins is possible and could have caused an alteration of cell function. As demonstrated by Zhen et. al., CD4 molecules could bind to MHC class II molecules. Activation of CD4 via interaction with HMC class II triggered monocyte functions. Binding of mAb MT4/3 may interfere with the binding of MHC class II and CD4 molecules on monocytes, which then decreases monocyte function.

The migration of monocytes into tissue and inflammation sites is a crucial function of the immune response.[1-3] Monocytes express several chemokine receptors, e.g., CCR1, CCR2, CCR5, CXCR1, and CXCR2, which are responsible for their migration to the chemokine gradients.[29,30] Chemokine receptor C-C chemokine receptor type 2 (CCR2) and its ligand, C-C chemokine ligand type 2 (CCL2 or MCP-1), have been found to play a dominant role in the monocyte migration process.[29-31] We, therefore, determined the role of CD4 in monocyte migration using a transwell migration assay. Upon stimulation with MCP-1, we found that the ligation of CD4 by mAb MT4/3 inhibited monocyte migration. We speculated that the ligation of CD4 on monocytes may alter the chemokine receptors on monocytes, resulting in migration inhibition.[2] In T cells, inositol trisphosphate (IP_3) and Ca^{2+}, a second messenger, which provides signals to regulate cytoskeleton rearrangement, were decreased after stimulation by anti-CD4 mAb.[32] These mechanisms may be related to the inhibition of monocyte migration by mAb MT4/3. Controversially, Zhen et al. demonstrated that the ligation of CD4 on monocytes by MHC class II resulted in prolonged Ca^{2+} flux.[19] However, instead of MHC class II, in this study, anti-CD4 mAb was used. The binding sites of MHC class II and mAb MT4/3 may be different and may induce different functional effects. Further study is needed to clarify this hypothesis.

After entering the blood circulatory system, monocytes are recruited into tissues throughout the body and differentiated into macrophages, which play an important role in immune responses or tissue remodeling.[1,2,7] Macrophages are remarkable plastic cells, which can polarize into pro-inflammatory (M1) and anti-inflammatory (M2) subsets.[33] In the macrophage differentiation process, macrophages adopt a specific phenotype and a functional response depending on their stimuli, such as cytokines and signals they encounter in each specific tissue. In our study, GM-CSF and M-CSF were used for the differentiation of monocytes into M1 and M2 macrophages, respectively. CD86 and CD163 were selected as markers for determined M1 and M2 macrophages, respectively.[33] After the ligation of CD4 on monocytes with mAb MT4/3, the expression levels of CD86 and CD163 in both GM-CSF and M-CSF stimulation were not different when compared with isotype-matched control mAb. These results suggest that in our studied condition, the ligation of anti-CD4 mAb on monocytes may not affect the differentiation of monocytes to both M1 and M2 macrophages. This is in discordance with a previous study, which found that the ligation of CD4 on monocytes by MHC class II induced the differentiation of monocytes into macrophages.[19]

A different structure of CD4 expressed on $CD4^+$ T cells and monocytes has been reported.[17,34,35] T cells mainly express the 55 kDa CD4 monomer form, while monocytic cell lines express 110 kDa covalent homodimers and a novel 59 kDa form.[17,23,34] The difference in CD4 expression suggests their different functions in T cells and monocytes. However, to the best of our knowledge, the MW of CD4 molecules on primary monocytes is still yet to be clearly reported. In this study, purified primary monocytes from peripheral blood, with 95% monocytes and less than 5% T cell contamination (**Figure S2**), were subjected to a Western blotting analysis to determine the MW of CD4. Only 55 kDa CD4 molecules were detected. This MW was the same as that reported for CD4 on T cells.[34,35] Our result, in contrast to previous studies,[17] indicated that the same size of CD4 molecules was expressed on monocytes and T cells. The discrepancy in this study and previous studies, however, was not surprising. This study used primary monocytes, whereas monocytic cell lines were employed in previous studies.[17,34,35] The difference in structure of surface molecules between primary leukocytes and hematopoietic cell lines was well recognized.

In conclusion, in this study, the function of CD4 molecules expressed on monocytes were uncovered. We demonstrated that the ligation of CD4 molecules on monocytes, by a specific anti-CD4 mAb, downregulated monocyte migration and T cell responses. Moreover, we demonstrated that the size of CD4 on primary monocytes was similar to that reported for T cells. CD4 molecules expressed on monocytes might play an important role in the regulation of immune responses, and both innate and adaptive immunity. Appropriate anti-CD4 mAb may be utilized in immunotherapy for the treatment of auto-immune diseases or inflammation.

Conflict of interests

The authors declare that they have no conflict of interests.

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Author contributions

- WK and WL provided a conceptualization and funding acquisition.
- PC, SP, NT and WL selected the appropriated methodology, performed the experiments, data analysis and interpretation.
- WL and PC drafted the original manuscript.
- WK reviewed, edited and approved the final version of manuscript.

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Supplemental materials

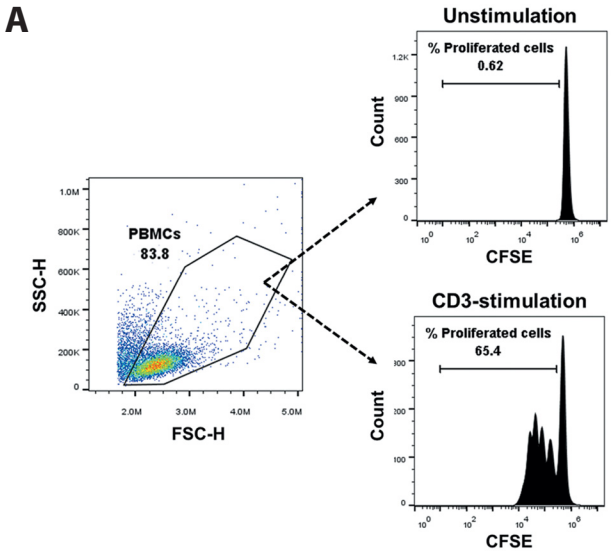

Figure S1. Gating strategies for the analysis of the effect of anti-CD4 monoclonal antibodies on the cell proliferation and cytokine production of T cells and the co-stimulatory molecule expression of monocytes. CFSE labeled PBMCs were stimulated with or without anti-CD3 mAb OKT3 in the presence or absence of anti-CD4 mAbs or isotype-matched control mAbs. (A) PBMCs were gated from FSC-H and SSC-H plots and further analyzed for the CFSE intensity of each study condition using a histogram plot. (B) PBMCs were gated from FSC-H and SSC-H. $CD14^+$ monocytes were gated from SSC-H and anti-CD14-PE plots, and analyzed for mean fluorescent intensity (MFI) of the expression of CD80, CD86, HLA-ABC, and HLA-DR in each study condition. (C) PBMCs were gated from FSC-H and SSC-H to identify $CD3^+$ T cells and $CD14^+$ monocytes. $CD3^+$ T cells were gated from SSC-H and anti-CD3-FITC plots. $CD14^+$ monocytes were gated from SSC-H and anti-CD14-PerCP plots. $CD3^+$ T cells and $CD14^+$ monocytes were analyzed for the percentage of cytokine positive cells (IL-2, IFN-γ, TNF-α, IL-17A, IL-10, and IL-6). The percentage of positive cells was set up from negative isotype-matched control mAb staining.

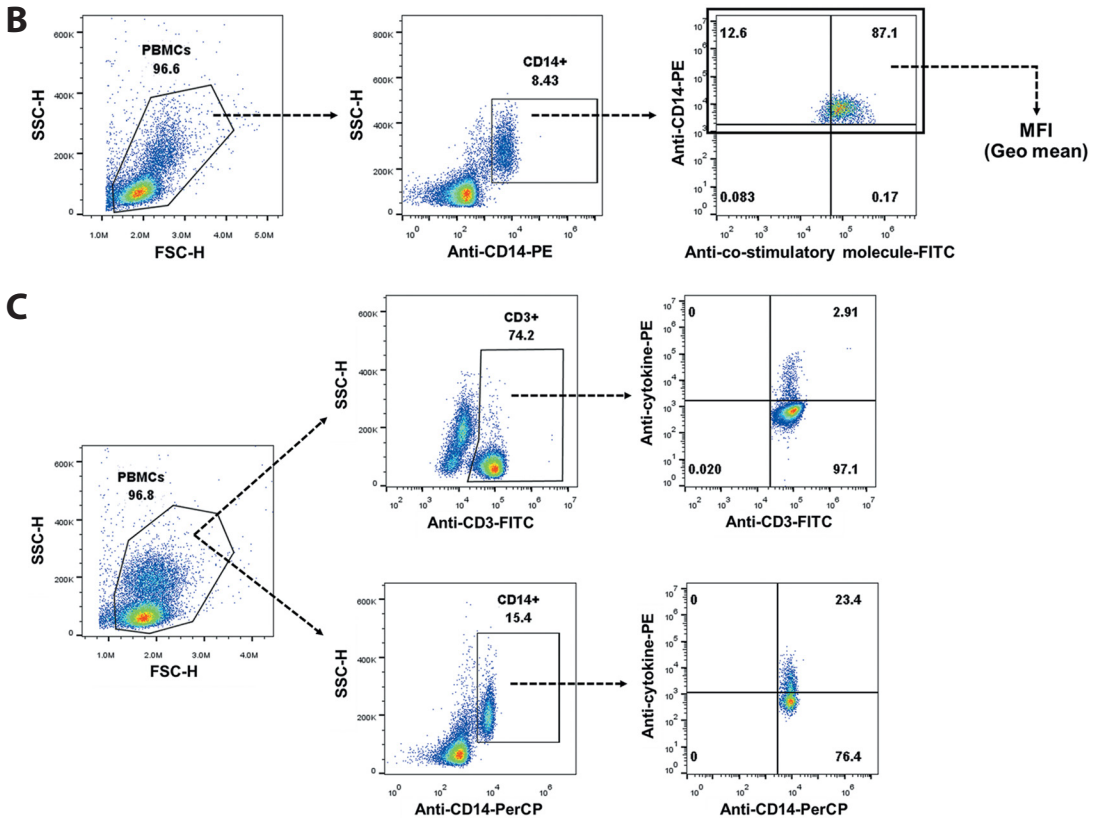

Figure S1. (Continued)

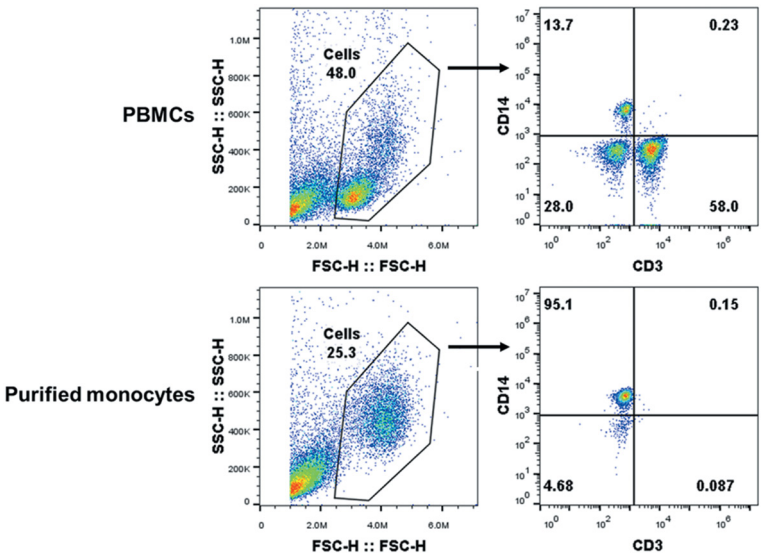

Figure S2. The purity of purified monocytes for Western immunoblotting assay. PBMCs were subjected to isolate monocytes using the Pan Monocyte Isolation kit. The purity of monocytes was determined by immunofluorescence staining with FITC-anti-CD3 and PerCP-anti-CD14. The upper panel and lower panel showed dot analysis of PBMCs and purified monocytes, respectively. Cells were gated from FSC-H and SSC-H and further analyzed with CD3 and CD14 plot. Percentage of each cell population is shown in the plot.