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Triggering of CD99 on monocytes by a specific monoclonal antibody regulates T cell activation

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ABSTRACT

CD99, a leukocyte surface glycoprotein, has been implicated in many cellular processes including cell adhesion, cell migration and T cell activation. Our previous study demonstrated the anti-CD99 monoclonal antibody (mAb) clone MT99/3 inhibited T cell activation; however, the mechanism is unclear. In this study, we demonstrated that CD99 expressed on monocytes played a role in the inhibition of T cell activation. Anti-CD99 mAb MT99/3 downregulated the expression of costimulatory molecule CD86, but upregulated IL-6, IL-10 and TNF-α production by monocytes. The inhibitory effect of mAb MT99/3 required cell to cell contact between monocytes and lymphocytes. The soluble mediators produced by monocytes alone were insufficient to induce hypo-function of T lymphocytes. In summary, we demonstrated that ligation of CD99 on monocytes by anti-CD99 mAb MT99/3 could mediate T cell hypo-responsiveness. These findings provide the first evidence of the role of CD99 on monocytes that contributes to T cell activation.

1. Introduction

CD99 is a cell surface molecule abundantly expressed on hematopoietic, non-hematopoietic and cancer cells [1–3]. This molecule is a heavily glycosylated type I integral membrane protein consisting of an extracellular domain, a transmembrane domain and a short cytosolic domain [4]. Although the full functionality of CD99 is still unclear, this molecule has been implicated in many cellular processes including cell adhesion, cell migration and cell apoptosis [5–7]. CD99 has been described as a T cell co-stimulator by translocation of T cell receptor (TCR) complex into the lipid raft and enhancement of tyrosine phosphorylation of TCR-ζ mediated T cell activation [8]. In addition, it has been reported that co-ligation of CD99 and CD3 resulted in activation of resting peripheral blood T cells and specifically inducing Th1-type cytokine production [9]. CD99 engagement induced the upregulation of molecules involving T cell activation including TCR, MHC class I and MHC class II surface expression via accelerated intracellular transportation from Golgi complex [10]. From the published data, CD99 molecule has been characterized as a multifunction molecule which plays an important role in many cellular events [5,11–13].

In addition to the published information, we have demonstrated that CD99 was associated with MHC class I, MHC class II and tetraspanin protein CD81 which are accessary molecules for T cell activation [14]. Upon T cell activation, CD99 was translocated into the immunological synapse. Moreover, we observed that, upon activation of peripheral blood mononuclear cells with anti-CD3 mAb, the proliferation of T cells was inhibited by anti-CD99 mAb clone MT99/3 [14]. However, the underlying mechanism in these phenomena is unknown. In this study, we have defined the mechanism involved in inhibition of T cell activation by anti-CD99 mAb clone MT99/3. In the present study, we demonstrated that monocytes were an important player in the control of T cell activation upon ligation with anti-CD99 mAb MT99/3. We suggested that the mAb MT99/3 might react with a functional epitope of CD99 molecule. Ligation of this mAb to its specific epitope may trigger and signal monocytes to do their function on T cell regulation.

2. Materials and methods

2.1. Antibodies

Anti-CD99 mAb clone MT99/3 (isotype IgG2a) was generated in our

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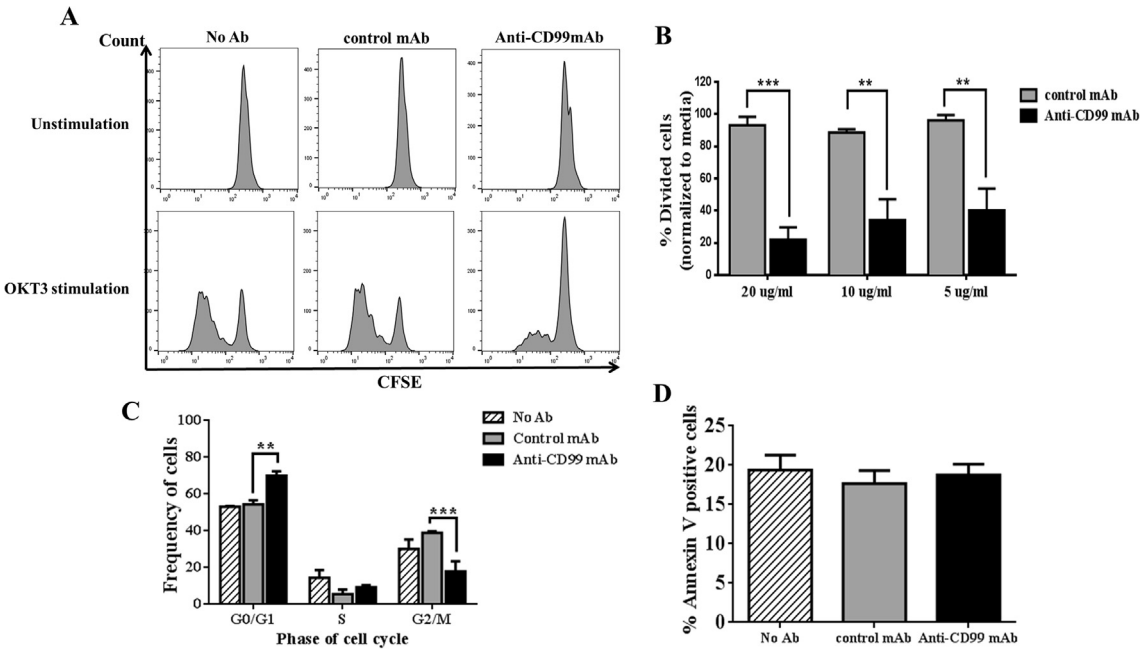

Fig. 1. Ligation of CD99 by anti-CD99 mAb clone MT99/3 inhibits anti-CD3 mAb induced T cell proliferation. (A) CFSE-labeled PBMCs were stimulated with anti-CD3 mAb OKT3 or kept unstimulated in the presence of anti-CD99 mAb MT99/3 or isotype-matched control mAb. Cell proliferation was determined by flow cytometry. Histograms demonstrate CFSE proliferation profile of unstimulated PBMCs (upper panel) and OKT3 stimulated PBMCs (lower panel) in the presence of the indicated mAb, as illustrated. One subject is shown as representative of 4 studied subjects. (B) CFSE-labeled PBMCs (n = 4) were stimulated with anti-CD3 mAb OKT3 in the presence of various concentrations of anti-CD99 mAb MT99/3 or isotype-matched control mAb. The proliferation was analyzed by flow cytometry. The percentage of cell divide in the presence of the indicated mAb was calculated by normalizing relative to medium control as 100%. The bar graphs represent mean ± SE. Two-way ANOVA followed by Tukey's test was used for comparison. (C) PBMCs (n = 3) were incubated with anti-CD3 mAb OKT3 in the presence or absence of indicted mAbs. After cultivation, cells were stained with propidium iodide and analyzed by flow cytometry. Frequency of cell in each phase of cell cycle was calculated by Flowjo software. The bar graphs represent mean ± SE and Two-way ANOVA followed by Tukey's test was used for comparison. (D) PBMCs (n = 3) were cultured with anti-CD3 mAb OKT3 and the indicated antibodies for 5 days. Cells were stained with Annexin V-FITC and PI and analyzed by flow cytometry. The representative graph (mean ± SE) shows percentage of apoptosis of three samples. $^{**}P < 0.01$; $^{***}P < 0.005$.

laboratory [5]. Isotype-matched control mAb 4G2 (anti-dengue viral protein; IgG2a) was obtained from Dr. Prida Malasit (Division of Medical Molecular Biology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand). Isotype-matched control mAb 13 M (anti-phage protein; IgG2a) was produced in our laboratory. The anti-CD3 mAb clone OKT3 (IgG2a) was purchased from Ortho Pharmaceuticals (Raritan, NJ, USA). Anti-CD28 mAb clone L293, FITC-conjugated anti-CD3 mAb, PE-conjugated anti-CD25 mAb and PE-conjugated anti-PD1 (CD279) mAb were purchased from BD Bioscience (San Jose, CA, USA). PerCP-conjugated anti-CD3 mAb and PerCP-labeled anti-CD14 mAb were purchased from BioLegend (San Diego, CA, USA). FITC-conjugated anti-CD86, -HLA-ABC (MHC class I) and -HLA-DR (MHC class II) mAbs, PE-conjugated anti-CD69 mAb and PE-conjugated mouse IgG1 isotype control antibody were purchased from ImmunoTools (Friesoythe, Germany). HRP-conjugated anti-human immunoglobulins Ab and HRP-conjugated anti-mouse immunoglobulins Ab were obtained from Dako (Santa Clara, CA, USA).

2.2. CD99 recombinant protein production

A CDM8-derived receptor globulin (Rg) expression plasmid encoding CD147Rg, the extracellular domain of CD147 fused to the DNA coding for CH2-CH3 of human IgG1, was kindly provided by Prof. Dr. Hannes Stockinger (Medical University of Vienna, Vienna, Austria) [15]. CD99Rg was constructed by replacing the extracellular domain sequence of CD147 by that of CD99, which was amplified by PCR using the plasmid DNA encoding full length of CD99 cDNA as template [5]. The DNA sequencing of the constructed vector was performed to confirm CD99 specific sequence (Fig S1-A and S1-B). The plasmid CD99Rg was transfected into monkey kidney cell line COS7 using lipofectamine 2000 (Invitrogen, CA, USA) according to manufacturer instruction. The CD99Rg was purified from COS7 cell lysate by affinity chromatography on a HiTrap Protein G HP (GE Healthcare, Uppsala, Sweden), based on the comprising human IgG-Fc part.

To check the characteristics of CD99Rg, western blotting analysis was performed. In brief, lysate of plasmid CD99Rg transfected cells or purified CD99Rg were subjected for protein separation by 10% SDS-PAGE followed by transferring to nitrocellulose membranes. The membranes were blocked with skimmed milk and incubated with anti-CD99 mAb clone MT99/3 or HRP-conjugated anti-human immunoglobulins Ab. The anti-CD99 mAb incubated membrane was further incubated in HRP-conjugated anti-mouse immunoglobulins Ab. The chemiluminescent detection system was employed for specific protein detection (Fig. S1-C and S1-D).

2.3. Cells isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors by Ficoll-Hypaque gradient centrifugation (IsoPrep; Robbins Scientific Corporation, Sunnyvale, CA, USA). The monocyte-depleted PBMCs were prepared using Percoll solution (48.5% Percoll and 0.16 M NaCl in ddH_2O). Briefly, PBMCs were overlaid onto Percoll solution and centrifuged at 865 ×*g* at for 40 min (GE Healthcare, Uppsala, Sweden). The pellets under Percoll solution were collected as monocyte-depleted PBMCs. The purity of monocyte-depleted PBMCs was checked by flow cytometry (FACSort flow cytometer, BD Biosciences) and it was found that < 2% monocytes (CD14 + cells) were contained in the obtained monocyte-depleted PBMCs. Purified T cells (CD3 + T cells) and purified monocytes (CD14 + cells) were isolated from PBMCs by magnetic antibody cell sorting (MACS) system using Pan T Cell Isolation Kit and Pan Monocyte Isolation Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany), respectively. The purity of the

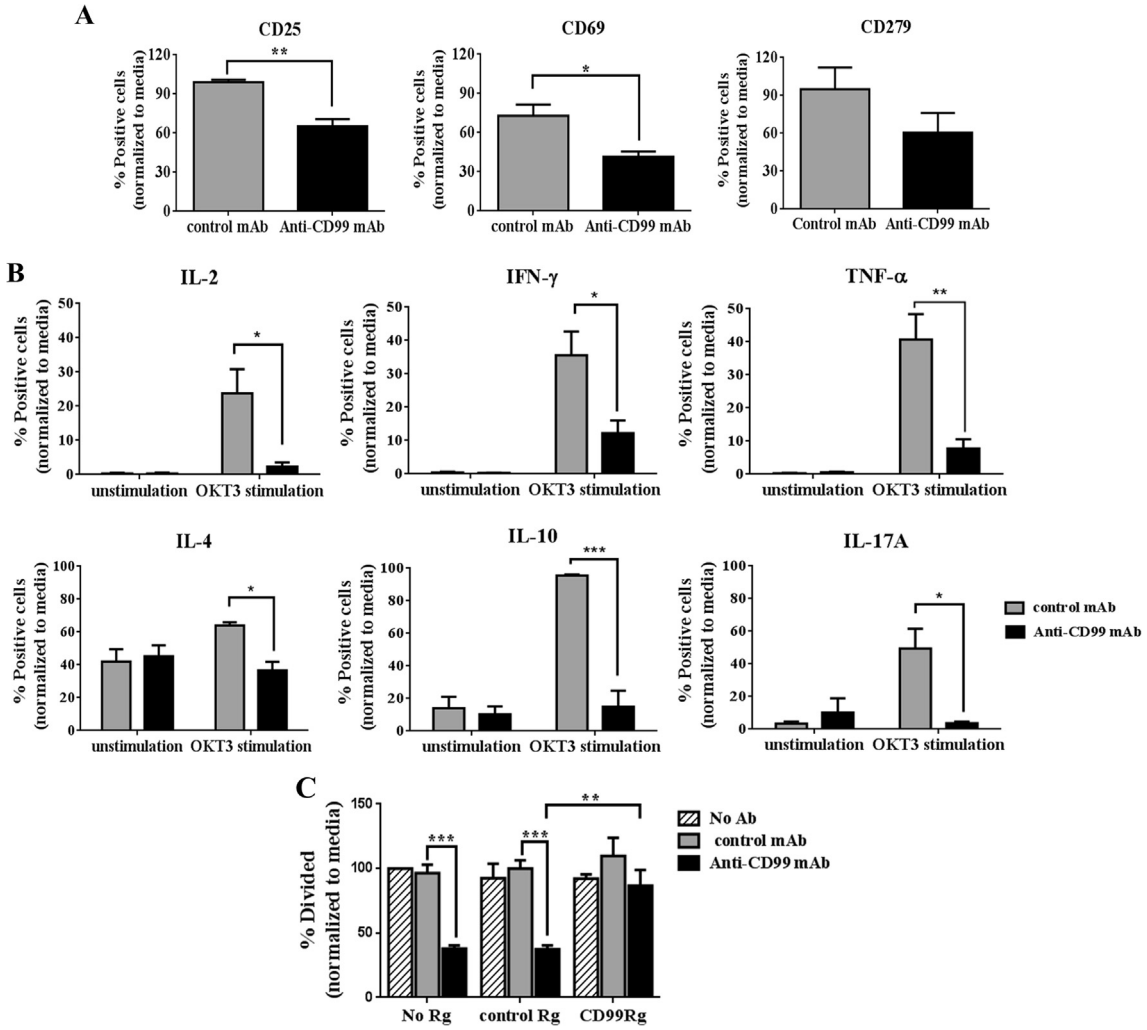

Fig. 2. Anti-CD99 mAb clone MT99/3 inhibited T cell activation and cytokine production. (A) PBMCs (n = 3) were incubated with anti-CD3 mAb OKT3 in the presence or absence of indicated mAbs. The surface expression of CD25, CD29 and PD-1 (CD279) was analyzed by flow cytometry. Each individual data was plotted as percentage of positive cell normalized to its medium control. The bar graphs represent mean ± SE. Two-way ANOVA followed by Tukey's test was used for comparison. (B) PBMCs (n = 3) were stimulated with anti-CD3 mAb OKT3 or kept unstimulated in the presence or absence of the indicated mAbs. After incubation for 6 h in the presence of protein transporter inhibitors, cells were collected and stained with FITC anti-CD3 mAb and PE anti-cytokine antibodies or PE isotype-matched control mAb. The CD3 + T cells were gated and subjected to determine cytokine expressing cells. Each data was normalized to its medium control in activated condition. All plots are shown in mean ± SE and Unpaired *t*-test was used for comparison. (C) CFSE labeled PBMCs (n = 3) were stimulated with anti-CD3 mAb OKT3 in the presence or absence of the mAbs or recombinant proteins or mixture of mAb and recombinant proteins as indicated. After 5 days of cultivation, cell proliferation was determined by flow cytometry. The percentage of cell divide in the indicated conditions was calculated by normalizing relative to medium control as 100%. The bar graphs represent mean ± SE and Two-way ANOVA followed by Tukey's test was used for comparison. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

purified T cells and monocytes obtained was more than 90%.

2.4. Proliferation assay by carboxyfluorescein succinimidyl ester dilution technique

The carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich, St. Louis, MO, USA) dilution technique was employed for cell proliferation assay as previously described [14]. The CFSE labeled PBMCs (CFSE-PBMCs) at concentration of $1x10^6$ cells/ml were added into anti-CD3 mAb OKT3 (25 ng/ml) coated plate for activation. The CFSE labeled monocyte-depleted PBMCs (CFSE monocyte-depleted PBMCs) were activated by immobilized OKT3 (50 ng/ml) and soluble anti-CD28 mAb (100 ng/ml).

For CFSE-labeled purified T cells (CFSE-T cells) and purified monocyte cultivations, cells at ratio 2.5:1 (T cells: monocytes) were used in either co-cultivation or separated cultivation by Transwell (0.4 μm) (Corning®, NY, USA). For co-cultivation, CFSE-T cells and purified monocytes were added into OKT3 coated plate (50 ng/ml) plus soluble anti-CD28 mAb (100 ng/ml). In the separated cultivation, CFSE-T cells were stimulated with OKT3 coated plate at lower chamber whereas purified monocytes were added into top chamber of Transwell in the presence of anti-CD28 mAb. The anti-CD99 mAb clone MT99/3 and isotype-matched control mAb (10 μg/ml) were added into each well.

For neutralization assay, CFSE labeled PBMCs were added into anti-CD3 mAb immobilized plate. The recombinant protein CD99Rg or CD147Rg (recombinant protein control) or anti-CD99 mAb or isotype-matched control mAb were added into each well. The mixture of antibody and recombinant protein were added into well. Cells were cultured at 37 °C in 5% CO_2 incubator for 5 days and cell proliferation was analyzed by flow cytometry.

For antibody pre-pulsed monocytes assay, purified monocytes were pre-incubated with or without anti-CD99 mAb MT99/3 for 30 min at room temperature. Cells were washed to remove excess antibodies. Antibody pre-pulsed monocytes were incubated with CFSE-T cells at ratio 1:2.5 in an OKT3-immobilized plate. Cells were cultured at 37 °C

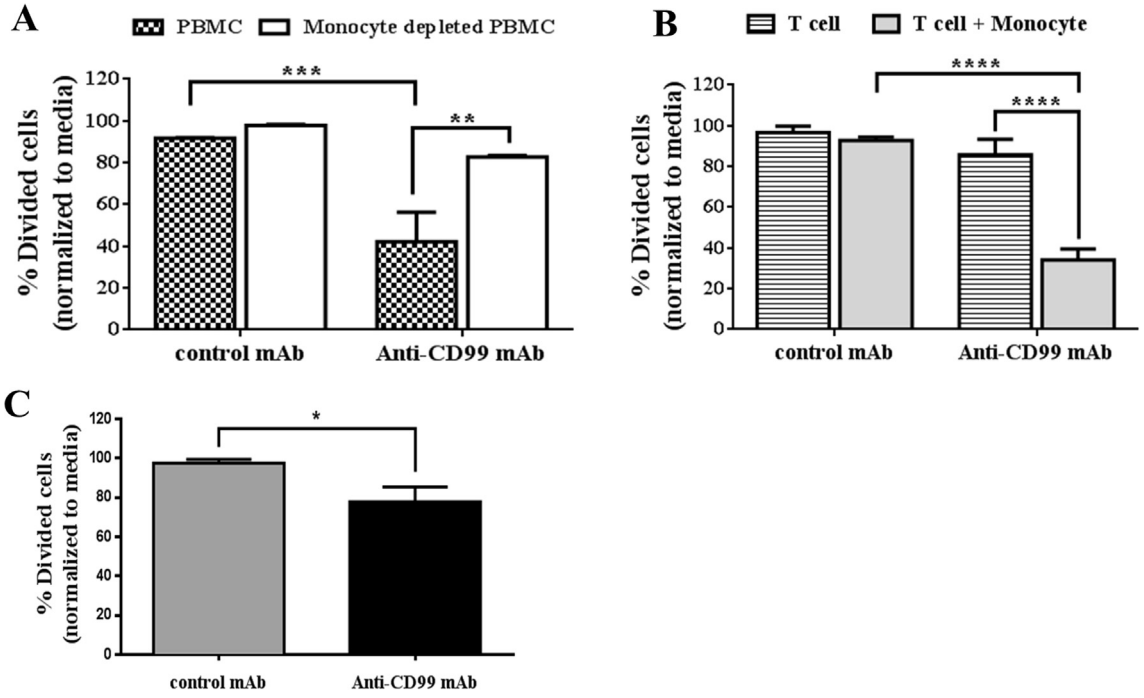

Fig. 3. Ligation of CD99 on monocyte by anti-CD99 mAb clone MT99/3 impairs T cell proliferation. Proliferation assay was performed in PBMCs and monocyte depleted PBMCs (A), purified T cells and purified T cells co-cultured with purified monocytes (B) in the presence or absence of anti-CD99 mAb. The reduction of CFSE was determined by flow cytometry and demonstrated as percentage of cell divide (n = 3). The data was shown in mean ± SE. Two-way ANOVA followed by Tukey's test was used for comparison. (C) Purified monocytes were incubated with or without indicated mAbs. After washing, mAb MT99/3 pre-pulsed monocytes were added into well containing activated T cells. The proliferation was analyzed by flow cytometry (n = 6). Unpaired *t*-test was used for comparison. The data was shown in mean ± SE of % divided cell, normalized to its medium control as 100%. In all figures, $^{*}p < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}p < 0.001$.

in 5% CO_2 incubator for 5 days and harvested to determine the reduction of CFSE using a flow cytometer (BD Accuri™ C6; BD Biosciences).

2.5. Cell cycle assay

PBMCs were cultured with immobilized anti-CD3 mAb OKT3 in the presence or absence of 10 μg/ml of anti-CD99 mAb MT99/3 or isotype-matched control mAb. After 3 days of cultivation, cells were harvested and fixed with 70% ethanol on ice for 30 min. Then, cells were stained with 1 ml of propidium iodide (PI) solution (20 μg/ml PI, 0.1% TritonX-100, 2 mM EDTA, 8 μg/ml RNase). The cell cycle was assessed by flow cytometry and analyzed using cell cycle analysis tool FlowJo software.

2.6. Cell surface marker analysis

For activation-associated marker detection, PBMCs were stimulated with anti-CD3 mAb OKT3 coated plate in the presence or absence of anti-CD99 mAb MT99/3 or isotype-matched control mAb. Cells were stained with PE-conjugated anti-CD69 mAb, anti-CD25 mAb or anti-PD1 (CD279) mAb after cultivation for 1, 3 and 5 days, respectively. Cells was analyzed by flow cytometry.

For double antibody staining of monocytes, PBMCs were treated as described above for 18 h. Cells were stained with PerCP-conjugated anti-CD14 mAb and FITC-conjugated anti-CD86 mAb, anti-MHC class I mAb or anti-MHC class II mAb. By flow cytometric analysis, CD14 + monocytes were gated and subjected to analysis for the expression of molecules of interest on their surface.

2.7. Intracellular cytokine assay

PBMCs were stimulated with anti-CD3 mAb OKT3 or kept unstimulated in the presence or absence of anti-CD99 mAb and isotype-matched control mAb. After incubation for 1 h, 1 μg/ml of brefedin A solution and 1 μM monensin (Sigma-Aldrich, St. Louis, MO, USA) were added and continuously incubated at 37 °C in a 5% CO_2 incubator for 4 h. The cells were fixed with 4% paraformaldehyde-PBS at RT for 15 min, subsequently permeabilized with 0.1% saponin solution (PBS containing 0.1% saponin, 5% FBS, 0.02% NaN_3) on ice for 15 min. Cells were stained with anti-human interleukin 2 (IL-2), IL-4, IL-10 or IL-17A, tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ) mAbs conjugated with phycoerythrin (PE) and FITC-conjugated anti-CD3 mAb to define T cell population. For monocyte cytokine production, cells were double stained with PE-conjugated anti-human IL-6, IL-10 and TNF-α and PerCP-conjugated anti-CD14 mAb. The intracellular cytokines in T cells and monocytes were assessed using a flow cytometer (BD Accuri™ C6; BD Biosciences).

2.8. Cell apoptosis assay

The PBMCs were stimulated with immobilized anti-CD3 mAb OKT3 in the presence or absence of anti-CD99 mAb or isotype-matched control mAb (10 μg/ml) at 37 °C in a 5% CO_2 incubator for 5 days. After cultivation, cells were collected and stained with FITC-conjugated Annexin V and PI solution. The apoptotic cells were determined using a flow cytometer (BD Accuri™ C6; BD Biosciences). Percentage of Annexin V positive cells were calculated as apoptotic cells.

2.9. Statistics

All data was shown in mean ± standard error (SE). Unpaired *t*-test and Two-way analysis of variance (ANOVA) were used and the level of significance was considered at $p < 0.05$. All experiments were performed as biological replication. The number of subjects used in each experiment was indicated in each figure legend (n).

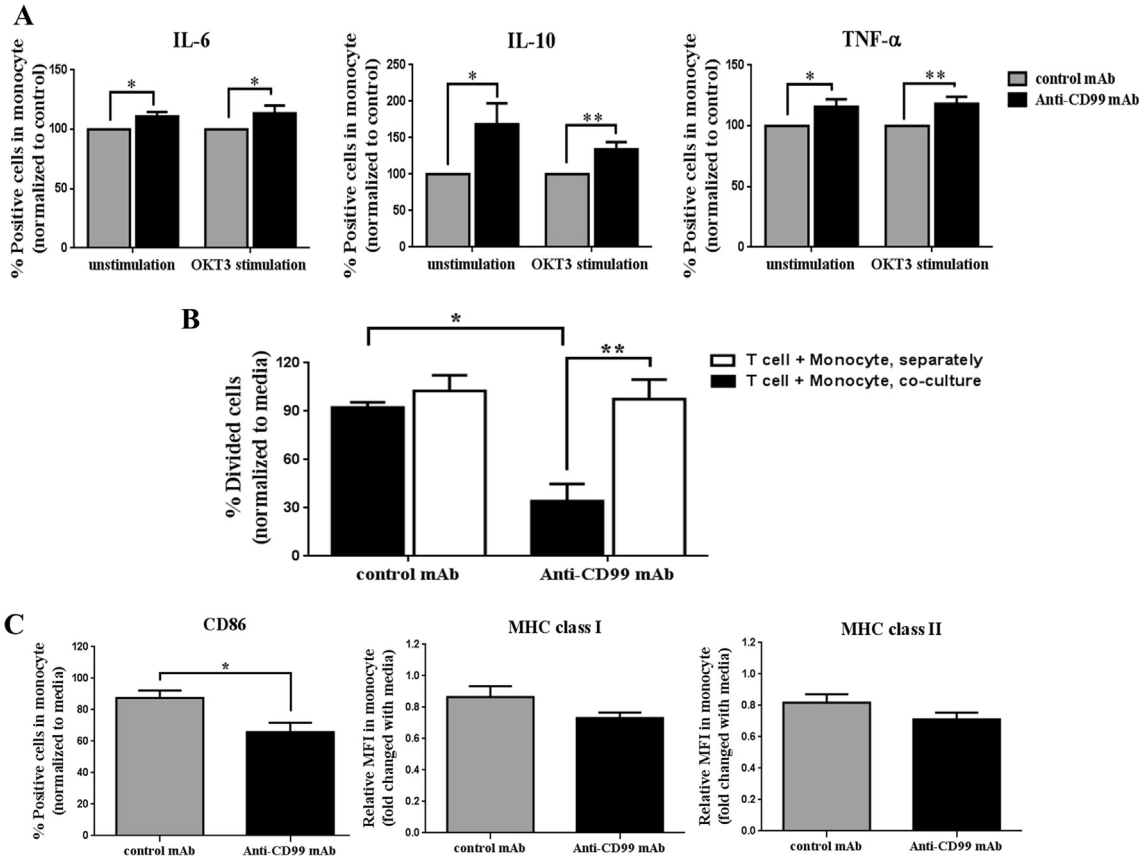

Fig. 4. Effect of anti-CD99 mAb clone MT99/3 on cytokine production and costimulatory molecule expression on monocytes. (A) PBMCs (n = 3) were stimulated with anti-CD3 mAb OKT3 or kept unstimulated in the presence or absence e of indicated mAbs. Cells were intracellularly stained with cytokine specific mAbs and anti-CD14 mAb. The CD14 + monocytes expressing cytokines were analyzed by flow cytometry. The data was shown in mean ± SE. Two-way ANOVA followed by Tukey's test was used for comparison. (B) CFSE-labeled T cells (n = 3) were co-cultured with purified monocytes or culture separately by Transwell chamber in the presence of anti-CD99 mAb MT99/3 or isotype-matched control mAb upon T cell stimulation. Cell proliferation was analyzed by flow cytometry. The data was plotted as a bar graph of percentage of divided cells (mean ± SE) normalized to its medium control as 100%. Two-way ANOVA followed by Tukey's test was used for comparison. (C) PBMCs (n = 3) were stimulated with anti-CD3 mAb PKT3 in the presence or absence of indicated mAb. The expression of CD86, MHC class I and MHC class II on CD14 + monocytes was determined by flow cytometry. Percentage of CD86 positive cells in monocytes was calculated by normalizing with its medium control as 100%. The relative geometric mean fluorescence intensity of MHC class I and MHC class II (GeoMFI of specific marker mAb staining/GeoMFI of isotype-matched control mAb staining) was normalized to medium control as 1. The data was shown in mean ± SE. Unpaired *t*-test was used for comparison. In all figures, $^{*}p < 0.05$, $^{**}p < 0.01$.

2.10. Human ethics

This study was approved by the ethics committee of the Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand (AMSEC-60EX-022).

3. Results

3.1. Anti-CD99 mAb clone MT99/3 inhibits anti-CD3 mAb induced cell proliferation

The effect of anti-CD99 mAb clone MT99/3 on PMBC proliferation upon activation with anti-CD3 mAb clone OKT3 was determined. The results showed that mAb MT99/3 inhibited OKT3 induced cell proliferation, while mAb MT99/3 itself had no effect on unstimulated PBMCs (Fig. 1A). The inhibitory effect mediated by mAb MT99/3 was observed in dose dependent fashion (Fig. 1B). As shown in Fig. 1C, in the presence of mAb MT99/3, cell cycle of OKT3 activated PBMCs was arrested at G0/G1 phase, cell population in the G2/M phase was reduced in the presence of mAb MT99/3 compared to the presence of isotype-matched control mAb.

It has been reported that the ligation of CD99 induced immature T cell line apoptosis [7]. The inhibitory effect of anti-CD99 mAb on cell proliferation may be due to the mAb induced cell apoptosis. Hence, the effect of agonistic anti-CD99 mAb on cell apoptosis was determined by using Annexin V staining assay. It was found that mAb MT99/3 showed no effect on the induction of cell apoptosis (Fig. 1D).

3.2. Ligation of CD99 with anti-CD99 mAb clone MT99/3 blocks the expression of CD25 and CD69 and cytokine production of T cells

To further determine the effect of anti-CD99 mAb on T cells activation, the inhibitory effect of mAb MT99/3 on T cell activation-associated markers, CD25 and CD69, was investigated. The results showed that, upon OKT3 activation, mAb MT99/3 significantly reduced surface expression of CD25 and CD69 (Fig. 2A). The expression of inhibitory molecules on T cell activation, PD-1 (programmed cell death-1; CD279), was also investigated. As was shown in Fig. 2A, expression of PD1 appeared to be reduced upon mAb MT99/3 treatment, however, there was no statistically significant difference compared to isotype matched control mAb.

In addition, the effect of mAb MT99/3 on Th1 cytokine (IL-2, IFN-γ and TNF-α), Th2 cytokine (IL-4 and IL-10) and Th17 cytokine (IL-17A) production by T cells was determined. Anti-CD99 mAb MT99/3 had no effect on cytokine production in unstimulated cells (Fig. 2B). However, upon OKT3 activation, mAb MT99/3 suppressed the production of all

cytokines tested including IL-2, IFN-γ, TNF-α, IL-4, IL10 and IL-17A (Fig. 2B). Taken together, our results indicated that anti-CD99 mAb MT99/3 downregulated T activation and Th1, Th2 and Th17 cytokine production.

As anti-CD99 mAb blocked T cell activation, we raised the question whether the inhibitory effect of anti-CD99 mAb is really due to the binding of the mAb MT99/3 to the surface expressed CD99 molecule. To address this question, CD99 recombinant proteins were then produced and used to prove our enquiry. Anti-CD3 mAb OKT3 induced PBMC proliferation assay was performed in cultures with or without mAb MT99/3 in the presence of recombinant proteins, CD99Rg or CD147Rg (recombinant protein control). As shown in Fig. 2C, recombinant proteins added had no effect on OKT3 induced T cell proliferation. As expected, the mAb MT99/3 by itself significantly inhibited cell proliferation (Fig. 2C). However, in cultures containing recombinant protein and mAb MT99/3, CD99Rg but not CD147Rg could abolish the inhibitory effect of mAb MT99/3 (Fig. 2C). These findings illustrated that the inhibitory effect of anti-CD99 mAb clone MT99/3 observed is due to the ligation of mAb MT99/3 to CD99 molecules.

3.3. Monocytes play a key role in anti-CD99 mAb clone MT99/3 inhibiting T cell activation

Since PBMCs contain lymphocyte and monocyte populations, and CD99 molecules were expressed on both cell types (Fig. S2) [1,16], we raised the question whether lymphocytes or monocytes are the primary cells which responded to anti-CD99 mAb ligation and induced the inhibitory effect on T cell activation. To address this question, the inhibitory effect of mAb MT99/3 was determined using OKT3 induced monocyte depleted PMBCs proliferation and compared with untouched PBMCs. As shown in Fig. 3A, the inhibitory effect of mAb MT99/3 was not observed when using monocyte depleted PMBCs but could be observed when using untouched PBMCs. We speculated that monocytes might play role in the inhibition of T cell activation upon anti-CD99 mAb MT99/3 ligation. The role of CD99 on monocytes in regulating T cell activation was investigated by using purified T cell and purified monocytes. As shown in Fig. 3B, upon OKT3 activation, anti-CD99 mAb MT99/3 had no effect on proliferation of purified T cell. However, the inhibitory effect of mAb MT99/3 on purified T cell was restored when purified monocytes were added into the culture.

To ensure that ligation of CD99 on monocytes by mAb MT99/3 resulting in the inhibition of T cell activation, the purified monocytes were pre-incubated with mAb MT99/3 and washed. The mAb MT99/3 pre-pulsed purified monocytes were added into purified T cells upon stimulation with anti-CD3 mAb. The MT99/3 mAb pre-pulsed monocytes could inhibit T cell activation (Fig. 3C). These data suggest that the effect of anti-CD99 mAb MT99/3 on T cell activation might come from the triggering of CD99 molecules on monocytes by specific mAbs and these monocytes then mediate T cell activation.

3.4. Binding of anti-CD99 mAb clone MT99/3 alters cytokine production and CD86 expression of monocyte and results in impairment of T cell function

To investigate the mechanism of the regulation of T cell through ligation of CD99 on monocytes, cytokine production in response to mAb MT99/3 triggering was investigated. IL-6, IL-10 and TNF-α cytokine producing monocytes were significantly increased in the presence of mAb MT99/3 compared with isotype-matched control mAb (Fig. 4A). The effect of mAb MT99/3 on the induction of the production of all cytokines tested by monocytes was observed in either OKT3 stimulated or unstimulated conditions (Fig. 4A).

We then asked the question whether increasing of cytokine production in monocytes was the cause of altered T cell activation. Proliferation assay was done using Transwell platform, which permitted only soluble proteins passing through membrane, and compared with co-cultivation condition. Surprisingly, the inhibitory effect of anti-CD99 mAb MT99/3 on T cell proliferation was abolished in the condition where the purified T cell and purified monocyte were cultured in separate chambers of the Transwell plate (Fig. 4B). In contrast, the inhibitory effect of mAb MT99/3 was observed in the co-cultivation of purified T cells and monocytes in the same well (Fig. 4B). These results indicated that at least contact between lymphocytes and monocytes is required for the inhibition of T cell activation by anti-CD99 mAb. Monocyte soluble mediators alone were not sufficient to regulate T cell activation.

As cell-to-cell interaction between monocytes and lymphocytes was necessary for inhibiting T cell activation, the alteration of the expression of accessary molecules involving T cell activation on monocytes was investigated. As shown in Fig. 4C, in the presence of anti-CD99 mAb MT99/3, the expression of CD86 on monocytes was reduced whereas MHC class I (HLA-ABC) and MHC class II (HLA-DR) were not changed. These finding demonstrated that ligation of CD99 molecules on monocytes by anti-CD99 mAb MT99/3 triggers a change of monocyte costimulation molecule CD86 and cytokine production (IL-6, IL-10 and TNF-α) of monocytes which then regulate T cell activation.

4. Discussion

Our previous studies demonstrated that anti-CD99 mAb clone MT99/3 inhibited T cell proliferation [14]. The results, however, were obtained from the activation of PBMCs with anti-CD3 mAb in the presence of mAb MT99/3. As PBMCs contain CD99 expressing lymphocytes and monocytes [1,16], in the present study, we then investigated whether lymphocytes or monocytes contained in PBMCs play a role in the inhibitory effect induced by anti-CD99 mAb ligation.

We first confirmed the effect of anti-CD99 mAb clone MT99/3 on anti-CD3 induced T cell activation in PBMC culture. Our results demonstrated that, upon anti-CD3 mAb activation of PBMCs, mAb MT99/3 inhibited cell proliferation and cells were arrested at G0/G1 phase. Ligation of CD99 molecules by specific mAbs has been demonstrated to induce cell apoptosis [13,17,18]. This may cause the arrestment of cell proliferation and cell cycle. Hence, the effect of anti-CD99 mAb MT99/3 on cell apoptosis was investigated by using Annexin V staining assay. We found that ligation of CD99 by mAb MT99/3 did not induce cell apoptosis. The T cell inhibitory signal through PD-1/PDL-1/2 checkpoint pathway was also investigated [19]. Upon mAb MT99/3 reduced T cell proliferation, T cells inhibitory molecule PD-1 was not significant altered suggesting that the hypo-responsiveness of T cells was unlikely due to the PD-1/PDL-1/2 checkpoint pathway. The T cell activation-associated markers, CD25 and CD69, however, were decreased in the presence of mAb MT99/3. In addition, we observed the mAb MT99/3 effect on the capability of Th1, Th2 and Th17 cytokine production. Our results, however, were different from the previous reports demonstrated that anti-CD99 mAb clone DN16 or 3B2/TA8 stimulated T cell activation and increased Th1 cytokine production [8,9]. The dissimilar effect of anti-CD99 mAb on T cell activation might result from the different clone of anti-CD99 mAb used in the experiments. Indeed, our anti-CD99 mAb clone MT99/3 was shown to induce Jurkat T cell homotypic aggregation via protein kinase C-dependent pathway [5] whereas anti-CD99 mAb clone DN16 was induced through protein kinase C-independent pathway [20]. We, therefore, suggested that our produced anti-CD99 mAb clone MT99/3 recognized the distinct functional epitope of CD99 from mAbs used in previous reports [8,9]. The inhibition of T cell activation mediated by mAb MT99/3 might function through a different mechanism than other reported mAbs.

As we demonstrated that mAb MT99/3 inhibited T cell activation, we raised the question whether the observed results came from the interaction between the Fab part of mAb MT99/3 and CD99 expressed on cell surface. The recombinant CD99 proteins were then generated and employed to address the question. We found that the recombinant

CD99 proteins could abrogate the inhibitory effect of mAb MT99/3. The results confirmed that the inhibitory effect of mAb MT99/3 must have come from the specific interaction of anti-CD99 mAb to its epitope on CD99 molecule.

Monocytes are cells which facilitate adaptive immunity [21]. Several molecules including soluble and membrane bound molecules of monocytes have been reported to be involved in the regulation of T cell activation [22–25]. As CD99 is abundantly expressed on monocytes [6,16], we queried whether CD99 molecules expressed on monocytes are involved in the regulation of T cell activation. We demonstrated, in fact, that monocytes were necessary in the inhibitory effect of mAb MT99/3 on T cell activation. The mAb MT99/3 itself could not inhibit anti-CD3 induced monocyte depleted and purified T cell activation. The addition of purified monocytes into purified T cell culture could restore the inhibitory effect. In addition, mAb MT99/3 pre-pulsed monocytes, in the absence of mAb MT99/3, could initiate T cell hypo-function.

The full activation of T cells requires TCR/CD3 stimulation and other signals which are mediated by accessary molecules expressed on monocytes [22,26–30]. Receiving incomplete signals, T cells enter a hypo-responsive state [29,30]. Several molecules including soluble and membrane bound molecules of monocytes have been reported to regulate T cell activation [22–25]. We therefore investigated the effect of mAb MT99/3 on cytokines produced by monocytes. Upregulation of IL-6, IL-10 and TNF-α by monocytes during mAb MT99/3 ligation was observed. TNF-α has been reported to be an autocrine feedback loop that increased the production of IL-6 and IL-10 on monocytes [31]. IL-10 has been reported to directly inhibit T cell activation [32]. We speculated that the mAb MT99/3 induced monokines may play a role in the observed inhibitory effect. We then investigated whether the observed inhibitory effect of mAb MT99/3 was due to the cytokines of monocytes. Purified monocytes and T cells were separately cultured in Transwell and the effect of mAb MT99/3 was compared to the co-culture condition. Surprisingly, the inhibitory effect could be observed only in co-culture condition. Soluble factor alone was not enough to inhibit T cell activation.

Several accessary molecules expressed on monocytes were demonstrated to be involved in the regulation of T cell activation [26,33,34]. As cell to cell contact was demonstrated to be essential for inhibition of T cell activation by MT99/3, effect of mAb MT99/3 on co-stimulatory molecules CD86 and MHC molecules expressed on monocytes was then determined. Down-regulation of the expression of CD86, but not MHC class I and II on monocyte, during mAb MT99/3 ligation was demonstrated. We hypothesized that ligation of CD99 molecules on monocytes by anti-CD99 mAb increase the production of cytokines, these cytokines might act as autocrine that led to the down-regulation of CD86 expression on monocytes surface [34,35]. Reduction of CD86 molecule, an accessary molecule, would mediate T cell hypo-function [36,37]. Although soluble factor alone was not enough to inhibit T cell proliferation, upregulation of IL-10 in monocytes by anti-CD99 mAb might be a synergistic effect with the downregulation of CD86 for T cell modulation.

In conclusion, we first reported the functional role of CD99 on monocytes in the regulation of T cell activation. Downregulation of costimulatory molecule expression and increased cytokine production in monocytes, upon ligation with anti-CD99 mAb MT99/3, was demonstrated and might result in decreased T cell activation. The knowledge obtained in this study might provide better understanding of the roles of CD99 participation in immune response.

Conflict of interest

Authors declare no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.10.012.

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