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A genetically engineered mouse/human chimeric antibody targeting CD99 enhances antibody-dependent cellular phagocytosis against human mantle cell lymphoma Z138 cells

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ABSTRACT

Background: Mantle cell lymphoma (MCL) is an aggressive form of B-cell non-Hodgkin lymphoma. The elimination of MCL cells via phagocytosis is essential for cancer eradication. Therefore, discovering novel targeted antibodies that can induce phagocytosis is needed. We have demonstrated that our in-house-produced mouse anti-CD99 mAb clone MT99/3 could induce potent anticancer activities against MCL cell lines in both *in vitro* and *in vivo* mouse xenograft models. Nevertheless, for use in humans, the mouse mAb needs to be transformed into a mouse/human chimeric mAb that contains a human Fc region to activate human immune effector functions, especially macrophage-mediated phagocytosis. Antibody-dependent cellular phagocytosis (ADCP) mediated by mouse/human chimeric mAb MT99/3 against MCL has not been previously reported.

Objective: This study aimed to genetically engineer a mouse/human chimeric antibody against human CD99 derived from mouse mAb MT99/3 and to evaluate its effect in mediating the ADCP mechanism for eradicating MCL cells *in vitro* using monocyte-derived macrophages.

Materials and methods: The expression plasmid to produce chimeric anti-CD99 antibody, ChAbMT99/3, was constructed by fusing the variable domains of mouse mAb MT99/3 with the constant domains of human IgG1 and the constant domains of kappa light chain. ChAbMT99/3 was expressed in the stable human expression system based on HEK293T cells. ChAbMT99/3 was purified from the culture supernatant of ChAbMT99/3-expressing HEK293T cells using Protein G chromatography. The purity and structure of ChAbMT99/3 were verified by SDS-PAGE and western blotting. The binding specificity and activity were determined by staining with cells expressing recombinant and native human CD99. The anticancer activity of ChAbMT99/3 in mediating the ADCP mechanism against MCL cell line Z138 using human monocyte-derived macrophages was evaluated.

Results: We successfully constructed the plasmid to produce ChAbMT99/3. Human HEK293T cells stably expressing ChAbMT99/3 were established. The ChAbMT99/3-expressing HEK293T cells could secrete ChAbMT99/3 into the culture supernatant. The high purity and complete IgG structure of ChAbMT99/3 were obtained from the purification process. Crucially, this chimeric antibody retained its binding reactivity to recombinant and native human CD99. In addition, the produced ChAbMT99/3, upon binding to MCL cells, significantly enhanced ADCP against MCL cell line Z138 in a dose-dependent manner.

Conclusion: The production of a mouse/human chimeric antibody against human CD99 derived from mouse mAb MT99/3 was successful. The engineered antibody could mediate ADCP activity against MCL cells. The produced ChAbMT99/3 might be a promising therapeutic candidate for MCL treatment.

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Introduction

Mantle cell lymphoma (MCL) is a rare and aggressive form of B-cell non-Hodgkin lymphomas (NHLs). MCL is genetically characterized by the *translocation of t(11;14) (q13;q32)*, leading to the overexpression of cyclin D1 and subsequent dysregulation of the cell cycle.[1] MCL accounts for 3-10% of all NHL cases, with a higher prevalence in middle-aged to older patients.[2] The treatment for MCL depends on age, overall performance status, and underlying co-morbidities.[3] Standard regimens include intensified chemotherapy in combination with rituximab, a chimeric antibody against CD20.[4] Most MCL patients respond to rituximab-based regimens, and remarkable clinical efficacy has been observed. However, patients often relapse due to drug resistance.[5] Therefore, novel targeted antibodies need to be established as therapeutic options for mantle cell lymphoma.

In the current landscape of cancer treatment, monoclonal antibodies (mAbs) have emerged as a promising therapeutic option due to their specificity and diverse mechanisms of action.[6,7] The Fc region of an antibody plays a crucial role in activating immune effector functions.[6] One of the essential Fc-related mechanisms for cancer eradication is antibody-dependent cellular phagocytosis (ADCP).[8] This mechanism is involved in activating Fc-gamma receptors (FcγRs) on macrophage surfaces by Fc region of antibody that recognizes cancer cells, resulting in the internalization and degradation of the cancer cells.[9] Several therapeutic antibodies demonstrated a significant increase in macrophage-mediated phagocytosis.[10] These antibodies are recognized as effective drugs in various cancers. However, it has been reported that MCL cells upregulate the "don't eat me" CD47 molecule, leading to resistance to macrophage-mediated phagocytosis.[11] Hence, therapeutic antibodies that mediate phagocytosis against MCL are still required.

As CD99 expression on MCL was reported, we have investigated the anticancer activities of mouse anti-CD99 mAb clone MT99/3 against MCL.[12] We demonstrated that the mAb MT99/3 could activate host immune effectors via antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) mechanisms *in vitro*, leading to the killing of MCL cell lines. Moreover, it could inhibit the growth of MCL in a mouse xenograft model *in vivo*.[12] These results indicated the potential of mAb MT99/3 in treating mantle cell lymphoma. However, the anticancer activity of mAb MT99/3 in the eradication of MCL cells by the ADCP mechanism has not been investigated.

For human use, mouse mAb is not appropriate. The mouse mAb, upon treatment, will be a foreign substance and induce adverse effects.[13,14] Therefore, the transformation of mouse mAb into a mouse/human chimeric mAb is required. The chimeric mAb contains a human Fc region, while the variable domains are still a mouse mAb to retain its binding reactivity. The transformed antibody could also be used to investigate the effect of an antibody in mediating phagocytosis by macrophage.

In this study, we generated a genetically engineered mouse/human chimeric antibody against human CD99 named ChAbMT99/3 by fusing the variable domain of mouse mAb MT99/3 with the constant domains of human IgG1 and kappa light chain. We evaluated the ADCP mediated by the generated chimeric antibody ChAbMT99/3 against the MCL cell line Z138. Our results demonstrated the potential of ChAbMT99/3 as an applicable therapeutic option for eradicating mantle cell lymphoma by phagocytosis.

Materials and methods

Antibodies

The anti-human CD99 mAb clone MT99/3 (mouse IgG2a) was generated in our laboratory.[15] PE/cyanine7-conjugated anti-CD3 mAb, PE-conjugated anti-CD19 mAb, PE-conjugated anti-CD56 mAb, PerCP-conjugated anti-CD14 mAb were purchased from BioLegend (San Diego, CA, USA). PE-conjugated anti-CD4 mAb, and APC-conjugated anti-CD8 mAb were purchased from BD Bioscience (San Jose, CA, USA)

Cell lines

Mantle cell lymphoma Z138 cells were obtained from JCRB cell bank, Osaka, Japan (gift from Prof. Dr. Seiji Okada, Kumamoto University, Kumamoto, Japan). Jurkat E6.1 cells were purchased from the American Type Culture Collection (ATCC). Both cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics. CD99-expressing myeloma cells were generated in-house.[16] Myeloma and CD99-expressing myeloma cells were cultured in a 10% FBS-IMDM medium. HEK293T cells were cultured in a 10% FBS-DMEM medium. All cell lines were maintained at 37 °C in a 5% CO_2 incubator.

Construction of expression plasmid for chimeric antibody against human CD99

The plasmid pTRIOZ-hIgG1 (InvivoGen, San Diego, CA, USA) was utilized for construction. Nucleotide sequences of the variable heavy chain (VH) and variable light chain (VL) obtained from mouse mAb MT99/3-producing hybridoma clone were codon-optimized. These sequences were designed to be inserted into the pTRIOZ-hIgG1 plasmid using SnapGene software (GSL Biotech LLC, San Diego, CA, USA). The codon-optimized VH chain sequences were synthesized with AgeI and NheI restriction sites, while VL chain sequences had SgrAI and BsiWI sites at the 5′ and 3′ ends, respectively. The VL and VH genes were cloned into the plasmid pTRIOZ-hIgG1 in front of genes for constant kappa light chain (CL) and constant heavy chain (CH), respectively, using specific restriction enzymes. The expression plasmid for ChAbMT99/3 was named pTRIOZ-ChAbMT99/3. After construction, the VH and VL nucleotide sequence was verified using Sanger sequencing. The codon optimization, gene synthesis, construction, and verification of the pTRIOZ-ChAbMT99/3 plasmid were carried out by GenScript Biotech (Nanjing, China).

Transformation and verification of the constructed plasmid

Using the heat-shock method, the constructed plasmid pTRIOZ-ChAbMT99/3 was transformed into competent *Escherichia coli (E. coli)* DH5-α. Plasmid-harboring *E. coli* were selected by spreading onto LB agar (BD Biosciences) containing 25 µg/mL of zeocin. A single colony was selected and inoculated into LB broth (BD Biosciences) with zeocin. Plasmids were purified using the QIAprep Spin Miniprep Kit (QIAGEN, Germany) according to the manufacturer's instructions. Purified plasmids were verified by digestion with *Bam*HI and *Eco*RI, and the size was determined by agarose gel electrophoresis.

Transfection and verification of antibody expression in transfected HEK293T cell line

The plasmid pTRIOZ-ChAbMT99/3 was transfected into the HEK293T cells. In brief, HEK293T cells (2×10^5) were seeded into a 6-well plate (Corning Inc., NY, USA) and cultured for 2 days. Transfection was performed by mixing 0 ng (as control) or 500 ng of plasmids with 3 µL of Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer's procedure. Then, cells were incubated at 37°C in a 5% CO_2 incubator for 3 days. The transfected cells were intracellular immunofluorescence stained to detect antibody expression. In brief, transfected cells (1×10^5) were fixed with 4% paraformaldehyde for 15 mins at room temperature (RT), then permeabilized using 0.1% saponin. Then, the cells were blocked the Fc receptors with 10% FBS and stained with Alexa Fluor 488-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (negative control) (Life technology, Eugene, OR, USA), for 30 mins on ice. The stained cells were assessed by flow cytometry (Accuri™ C6 plus flow cytometer, BD Biosciences).

Additionally, culture supernatant of un-transfected and transfected HEK293T cells was collected for testing binding reactivity on CD99-positive Jurkat E6.1 cells. In brief, Jurkat E6.1 cells (5×10^5) were incubated with 10% FBS, followed by staining with culture supernatant. Then, Alexa Fluor 488-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories) was added, with subsequent analysis using the Accuri™ C6 plus flow cytometer.

Establishment of HEK293T cells stably expressing chimeric anti-CD99 antibody

The transfected HEK293T cells were seeded at a density of 100 cells per well in 150 µL of 10% FBS-DMEM with 100 µg/mL of zeocin in a 96-well plate. Every 3 days, a medium with zeocin at a concentration of 300 µg/mL was added into the wells at a volume of 50 µL. Subsequently, surviving cells in the selected wells were harvested to determine the expression of the chimeric antibody using intracellular immunofluorescence staining.

Production and purification of chimeric antibody against human CD99

The selected ChAbMT99/3-expressing HEK293T clone was cultured in a 75 cm^2 T-flask and maintained in 10% FBS-DMEM with 100 µg/mL of zeocin. Upon reaching 80% confluence, the cells were washed with DMEM. The culture medium was changed to 293-SFM II media (Invitrogen) with 100 µg/mL of zeocin. After that, cells were cultured by frequent mixing for 5 days. The culture supernatant was harvested, centrifuged, and filtered through a 0.2 µm filter. Antibody purification was performed using a Hitrap protein G column with an ÄKTA start purification system, followed by buffer exchange with PBS. Concentrations of the purified antibody were determined by a Nanodrop® 2000 spectrophotometer. Twenty micrograms of protein per condition were used to assess the purity and structure by SDS-PAGE and western blotting. For western blotting, the proteins from SDS-PAGE were transferred to PVDF membranes. The antibody structure was determined using HRP-conjugated goat anti-human IgG, Fcγ fragment-specific antibodies (Jackson ImmunoResearch Laboratories), followed by detection with SuperSignal™ West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA).

Verifying the binding reactivity of the purified chimeric antibody against human CD99

Myeloma cells and CD99-expressing myeloma cells (5×10^5), were incubated with 10% FBS. Then, cells were stained with the purified ChAbMT99/3 or an isotype-matched control Ab (human IgG). After washing, Alexa Fluor 488-conjugated goat anti-human IgG antibody was added. The stained cells were then analyzed using the Accuri™ C6 plus flow cytometer.

Mantle cell lymphoma Z138 cells were incubated with 10% FBS. The cells were stained with ChAbMT99/3 and mAb MT99/3 at final concentrations of 0.2, 1 and 5 µg/mL. Consequently, cells were incubated with Alexa Fluor 488-conjugated goat anti-human IgG antibody for the detection of bound ChAbMT99/3 or Alexa Fluor 488-conjugated goat anti-mouse Igs antibody for the detection of bound mouse mAb MT99/3 (Invitrogen, Eugene, OR, USA). The stained cells were analyzed using the Accuri™ C6 plus flow cytometer.

For human peripheral blood mononuclear cells (PBMCs) staining, PBMCs were prepared using Ficoll-Hypaque gradient centrifugation. PBMCs were blocked with 20% human AB serum and then incubated with biotinylated ChAbMT99/3 or a biotinylated human IgG (isotype-matched control Ab). After washing, cells were stained with FITC-conjugated streptavidin (BioLegend) along with antibodies for the identification of $CD4^+$ T cells ($CD3^+CD4^+$), $CD8^+$ T cells ($CD3^+CD8^+$), B cells ($CD19^+$), NK cells ($CD3^-CD56^+$), NKT cells ($CD3^+CD56^+$) and monocytes ($CD14^+$). The stained cells were analyzed using the Accuri™ C6 plus flow cytometer.

Determination of binding affinity of chimeric antibody against human CD99

To determine the binding affinity of ChAbMT99/3 and compare it with its parental mAb MT99/3, streptavidin-coated biosensors (Pall Life Sciences, Port Washington, NY, USA) were employed to immobilize the biotinylated CD99 extracellular domain (ECD) peptide.[17] The loading step involved pre-wetting the biosensor tip with 0.05% Tween -PBS (PBST), followed by immersion in a solution containing 10 µg/mL of CD99 ECD peptide for 120 sec. The excess peptide was subsequently removed by immersing the tip in PBST. For the association step, the peptide-coated biosensors were incubated with a serial two-fold dilution of ChAbMT99/3 or mouse MT99/3, ranging from 10 µg/mL to 0.625 µg/mL, for 120 sec. This step was followed by dissociation in PBST for 300 sec. The entire experiment was conducted using an Octet® BLI System (Pall ForteBio), and the acquired data were analyzed using ForteBio data analysis software version 9.0 to determine the equilibrium dissociation constant (K_D) of antibodies.

Monocyte-derived macrophages

Monocyte-enriched PBMCs were prepared using Percoll gradient centrifugation. In brief, PBMCs at a concentration of 1×10^7 cells/mL were overlaid on Percoll working solution (GE Healthcare). Cells were centrifuged at 895×g, 40 min, with break-off at RT. The ring of monocyte-enriched PBMCs was collected. The cell numbers were counted using Turk's solution. The percentages of monocytes were determined by flow cytometry. The monocyte-enriched PBMCs were plated in a 24-well plate at 5×10^5 monocytes per well and incubated at 37°C in a 5% CO_2 incubator for 1 hr. Thereafter, non-adherent cells were removed by washing with PBS. The adherent cells were stimulated with human macrophage colony-stimulating factor (M-CSF) in 10% FBS-RPMI at 50 ng/mL, 400 µL for 6 days. Of note, fresh medium containing M-CSF was renewed every 2 days.

Assay for antibody-dependent cellular phagocytosis

Z138 cells used as target cells were labeled with CFSE at a concentration of 2 µM. CFSE-labeled Z138 were incubated with a final concentration of isotype-matched control Ab (human IgG) and ChAbMT99/3 at 0.2, 1, or 5 µg/mL or medium without antibody. After incubation at RT for 15 mins, the excess antibodies were removed by centrifugation at 2100×g for 4 mins. The antibody-treated Z138 cells were co-cultured with monocyte-derived macrophages (effector cells) at an effector-to-target ratio (E:T) of 1:4 and incubated at 37 °C in a 5% CO_2 incubator for 4 hs. Next, the cells were harvested and stained with PerCP-conjugated anti-CD14 mAb. The percentage of phagocytosis was assessed by flow cytometry.

Statistical analysis

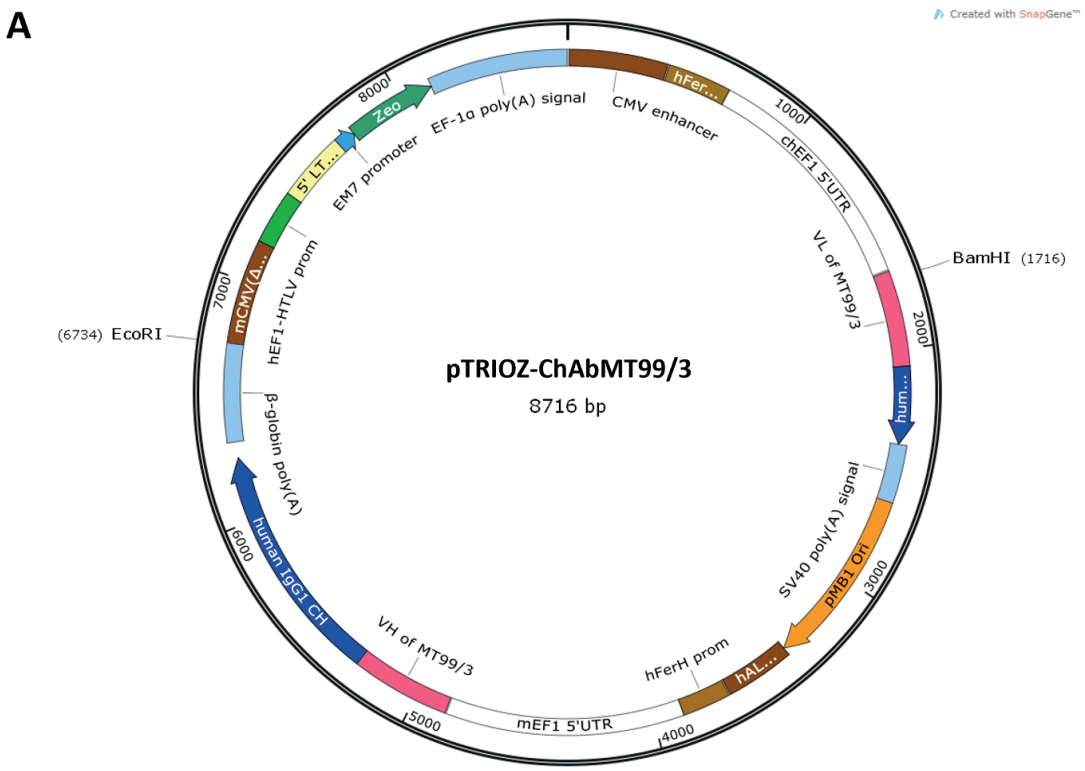
The data are expressed as mean±SD. The data were analyzed by One-way ANOVA with Tukey's multiple comparisons using GraphPad Prism Version 9.5.1 (GraphPad Software, San Diego, CA). $p<0.05$ was considered statistically significant.

Results

Construction of expression plasmid for chimeric anti-CD99 antibody production

The variable domains of mouse mAb MT99/3 were genetically engineered by fusing them with the constant domains of human IgG1 and kappa light chain. The VH and VL genes of the mouse mAb MT99/3 were codon-optimized, synthesized, and cloned into heavy/light chain cassettes of plasmid pTRIOZ-hIgG1, as illustrated in Figure 1A. DNA sequencing of VH and VL genes in the constructed plasmid pTRIOZ-ChAbMT99/3 was performed to validate the nucleotide sequences after synthesis and cloning. DNA sequencing results were translated into amino acid sequences before alignment with original amino acid sequences derived from mouse mAb MT99/3. As expected, amino acid sequences of the synthesized VH and VL genes showed a 100% identity match to the parental sequences (Figure 1B). The results confirmed that the plasmid pTRIOZ-ChAbMT99/3 was successfully constructed.

Plasmids were transformed into competent *E. coli* to amplify the constructed pTRIOZ-ChAbMT99/3, and zeocin was used for clonal selection. Purified plasmids were verified by digestion with *Bam*HI and *Eco*RI. DNA bands at approximately 3698 bp and 5018 bp were observed (Figure 1C), corresponding to the predicted sizes. The result indicated that the amplified plasmid could be further used for antibody expression.



B Amino acid sequence of variable heavy chain

Score	Expect	Method	Identities	Positives	Gaps
287 bits(734)	3e-105	Compositional matrix adjust.	136/136(100%)	136/136(100%)	0/136(0%)

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Sbjct  1    MKCSWVIFFLMAVVTGVNSDVQLQQSGAELVKPGASVRLSCTLSGFNIKDTYIHWVNQRP  60

Query  61   EQGLEWIGRIDPGNGNIKYDPKFQGKATITADTSSNKVYLHLSSLTSEDTAVYYCARSGG  120
            EQGLEWIGRIDPGNGNIKYDPKFQGKATITADTSSNKVYLHLSSLTSEDTAVYYCARSGG
Sbjct  61   EQGLEWIGRIDPGNGNIKYDPKFQGKATITADTSSNKVYLHLSSLTSEDTAVYYCARSGG  120

Query  121  YDFDYWGQGTTLTVSS  136
            YDFDYWGQGTTLTVSS
Sbjct  121  YDFDYWGQGTTLTVSS  136
```

Amino acid sequence of variable light chain

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270 bits(691)	7e-99	Compositional matrix adjust.	131/131(100%)	131/131(100%)	0/131(0%)

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C

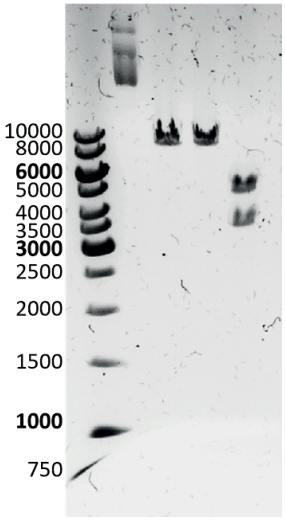


Figure 1. *Expression plasmid for chimeric anti-CD99 antibody production. A: a graphical map of plasmid pTRIOZ-ChAbMT99/3 was created by SnapGene software. Genes encoding variable heavy chain (VH) and variable light chain (VL) against human CD99 were cloned into the heavy/light chain cassettes of plasmid pTRIOZ-hIgG1, pink color, B: amino acid sequence alignment of VH and VL in the constructed plasmid pTRIOZ-ChAbMT99/3. Parental VH/VL amino acid sequences of mAb MT99/3 (Query) were aligned with those of ChAbMT99/3 (Sbjct), C: agarose gel electrophoresis of the constructed plasmid pTRIOZ-ChAbMT99/3 after amplification. The plasmids were digested with restriction enzymes BamHI (lane 2), EcoRI (lane 3), BamHI and EcoRI (lane 4), or without enzyme (lane 1). Sizes of standard markers (base pair) are shown on the left.*

Establishment of HEK293T stably expressing chimeric anti-CD99 antibody

To generate ChAbMT99/3-expressing human cells, HEK293T cells were transfected with the constructed pTRIOZ-ChAbMT99/3 plasmid. Three days post-transfection, intracellular immunofluorescence staining with Alexa Fluor 488-conjugated anti-human IgG antibody was performed and showed positive reactivity with transfected cells (Figure 2A). The results demonstrated the successful expression of ChAbMT99/3 in human HEK293T cells.

In addition, the culture supernatant of transfected HEK293T cells also exhibited positive reactivity with CD99-positive Jurkat E6.1 cells, while the culture supernatant of un-transfected cells showed no reactivity (Figure 2B). The results indicated that ChAbMT99/3 produced by HEK293T cells could be secreted into the culture supernatant. Subsequently, to establish HEK293T stably expressing ChAbMT99/3, transfected cells were cultured in a medium containing zeocin. Transfected cells survived in zeocin treatment, while un-transfected cells perished within 5 days. These transfected cells were collected from 20 wells to assess the intracellular expression of ChAbMT99/3. Cells in all selected wells showed positive reactivity with Alexa Fluor 488-conjugated anti-human IgG antibody (Figure 2C). However, expression levels varied among tested wells (Figure 2C). A transfected HEK293T clone 2F6 demonstrating the highest geometric MFI of the homogenous pattern was selected for ChAbMT99/3 production.

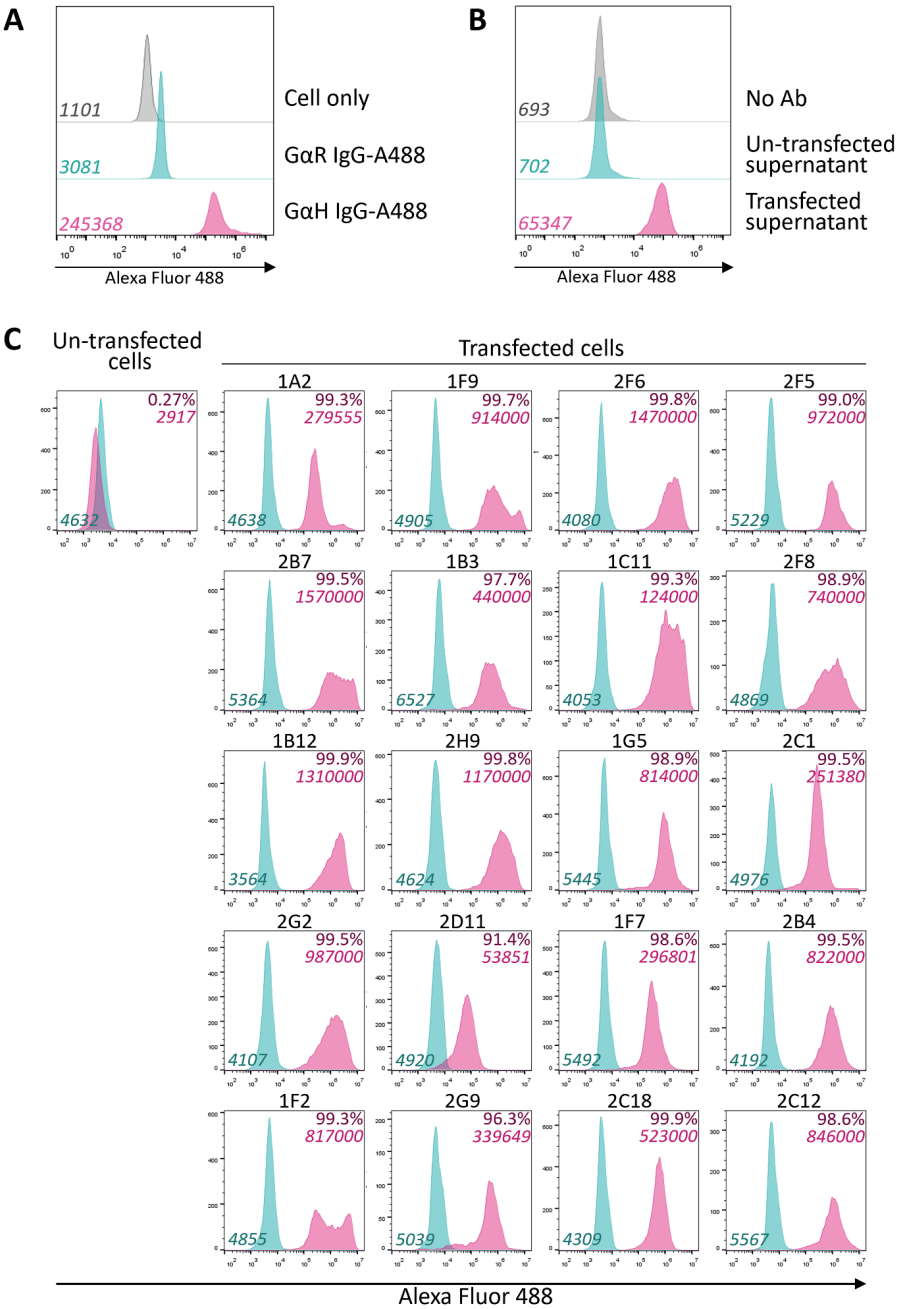


Figure 2. *Expression of chimeric anti-CD99 antibody in transfected HEK293T cells. A: human HEK293T cells were transfected with plasmid pTRIOZ-ChAbMT99/3. The transfected cells were performed intracellular staining with Alexa Fluor 488-conjugated goat anti-human IgG (GαH IgG-A488), Alexa Fluor 488-conjugated goat anti-rabbit IgG (GαR IgG-A488), or without antibodies (Cell only), B: culture supernatants obtained from the un-transfected or transfected HEK293T cells, or medium (No Ab) were subjected to stain Jurkat E6.1 cells followed by Alexa Fluor 488-conjugated goat anti-human IgG antibody, A-B: geometric mean fluorescence intensity (MFI) is indicated in the overlay histogram plots, C: un-transfected and transfected cells after zeocin selection were intracellularly stained using Alexa Fluor 488-conjugated goat anti-human IgG antibody (depicted in pink) or Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (displayed in green). The geometric MFI values and the percentage of positive cells are indicated in the overlay histogram plots.*

Large-scale production and purification of chimeric anti-CD99 antibody

To increase the amount of ChAbMT99/3, the HEK293T expressing stable ChAbMT99/3 clone 2F6 was cultured in a serum-free medium containing zeocin. The culture supernatant was purified using a protein G column. Approximately 1 mg of antibody was obtained from approximately 200 mL of the culture supernatant. The purity and structure of the purified ChAbMT99/3 were assessed using SDS-PAGE and western blotting. By SDS-PAGE, high purity of the purified ChAbMT99/3 was obtained. Protein bands were observed at approximately 55 kDa and 25 kDa under reducing conditions, corresponding to the heavy and light chains of the antibody, respectively. In non-reducing conditions, a protein band at approximately 150 kDa, representing the whole structure of the human IgG antibody, was detected (Figure 3A). For western blot analysis, positive bands were observed at 55 kDa under reducing conditions and 150 kDa under non-reducing conditions, which were the expected positions for the heavy chain and the whole structure of IgG antibody, respectively (Figure 3B). However, the major bands appeared above 180 kDa, suggesting a multimeric form where two or more antibody molecules were linked via inter-chain disulfide bonds (Figure 3A-B). The results indicated the successful production and purification of ChAbMT99/3 with high purity. The structure of ChAbMT99/3 was drawn as an intact molecule of mouse/human chimeric antibody harboring human IgG1 constant domains, as shown in Figure 3C.

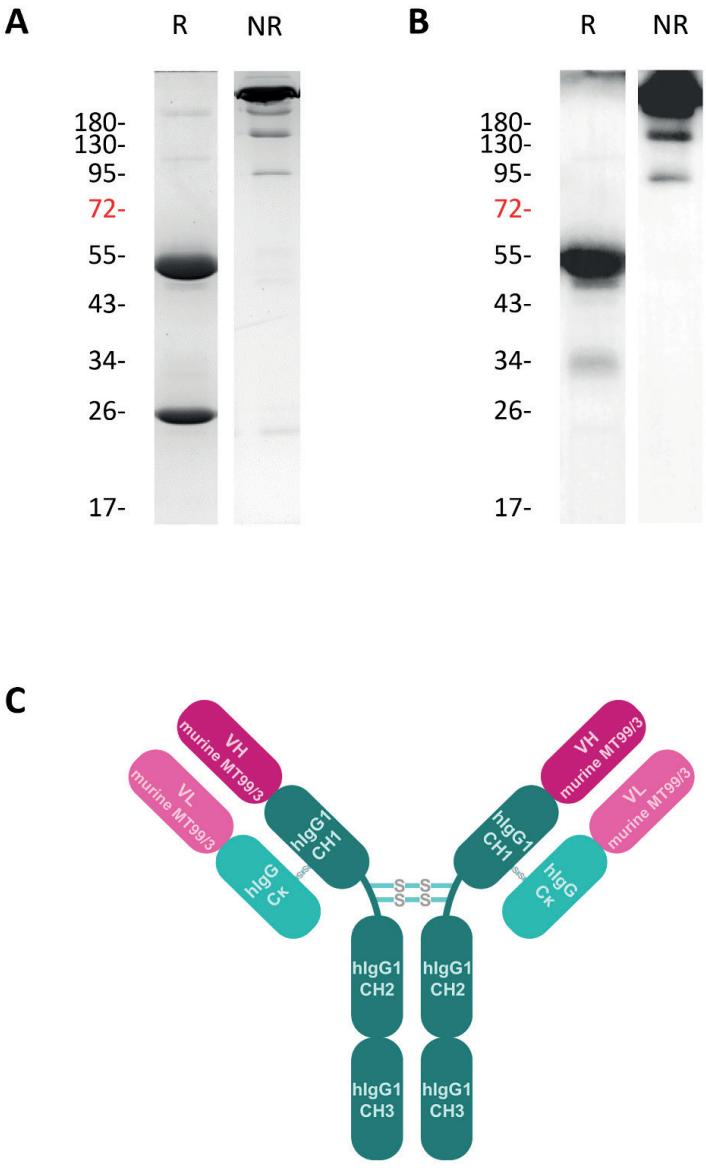


Figure 3*. The purity and structure of purified ChAbMT99/3. A: purified ChAbMT99/3 was subjected to 10% SDS-PAGE under reducing (R) and non-reducing (NR) conditions. Protein bands were stained with Coomassie blue, B: Western blotting was analyzed using HRP-conjugated goat anti-human IgG, Fcγ fragment specific Abs, C: schematic drawing of ChAbMT99/3 structure. VH and VL from murine-derived mAb MT99/3 (pink color) combined with the constant domain of human IgG1 and the constant domain of the kappa light chain (green color).*

Binding reactivity of chimeric anti-CD99 antibody

To confirm the binding specificity of ChAbMT99/3 against human CD99, ChAbMT99/3 was stained with recombinant human CD99-expressing myeloma cells. The results showed positive reactivity with CD99-expressing myeloma cells and negative reactivity with normal myeloma cells (Figure 4A). These results confirmed that the ChAbM99/3 retained its binding specificity to human CD99. Subsequently, to assess the binding activity of ChAbMT99/3 against native human CD99, surface staining of ChAbMT99/3 with MCL cell line Z138 and human PBMCs was carried out. ChAbMT99/3 could recognize Z138 cells that expressed native human CD99 on cell surfaces comparable with mouse mAb MT99/3 (Figure 4B). Moreover, the fluorescence intensity was increased in a dose-dependent manner (Figure 4B). For PBMCs, ChAbMT99/3 showed positive reactivity with all subpopulations in different patterns of CD99 expression levels, as previously reported.[18] In that order, the highest levels of CD99 expression were found in NKT cells, NK cells, monocytes, T cells, and B cells (Figure 4C). Notably, the produced ChAbMT99/3 demonstrated good binding activity to the native form of human CD99, suggesting a potential tool for studying anticancer activities. Moreover, BLI data revealed that the binding affinity of ChAbMT99/3 compared with the parental mouse mAb MT99/3 using CD99 EDC peptide exhibited very similar K_D values, $4.17x10^{-10}$ M and $4.62x10^{-10}$ M, respectively (Figure 5). This suggested that the binding capability of the produced ChAbMT99/3 and the parental mouse MT99/3 with CD99 molecule was comparable.

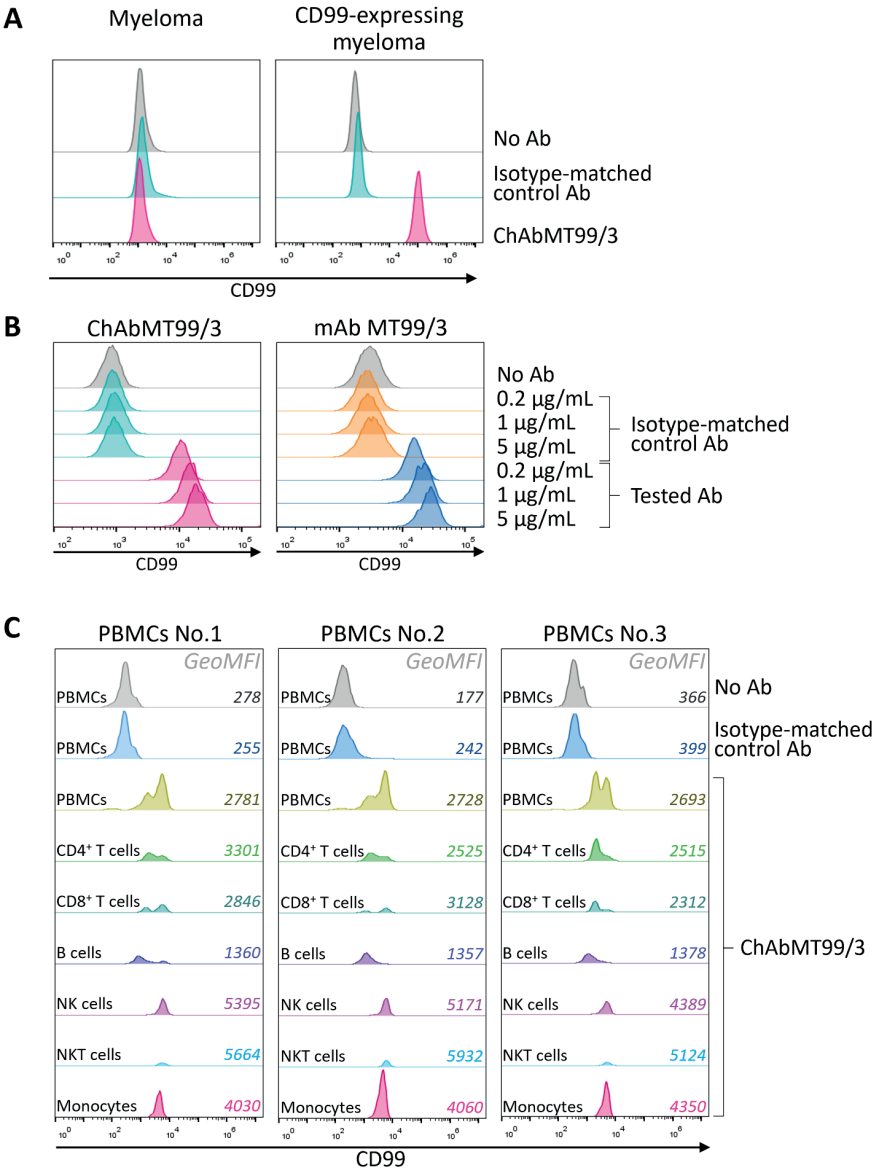
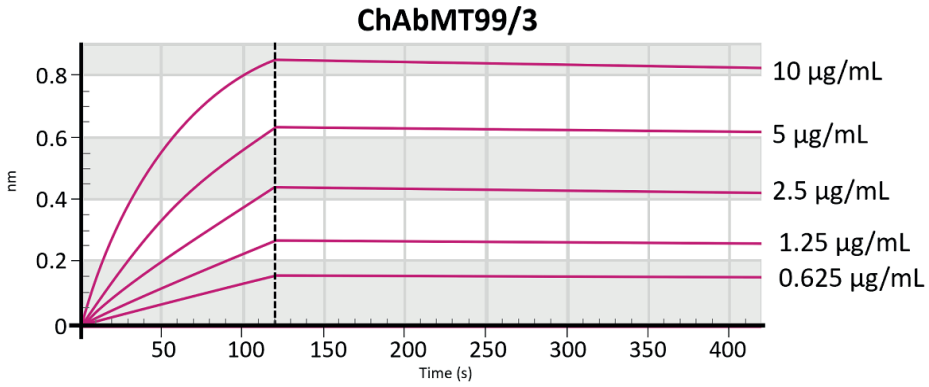
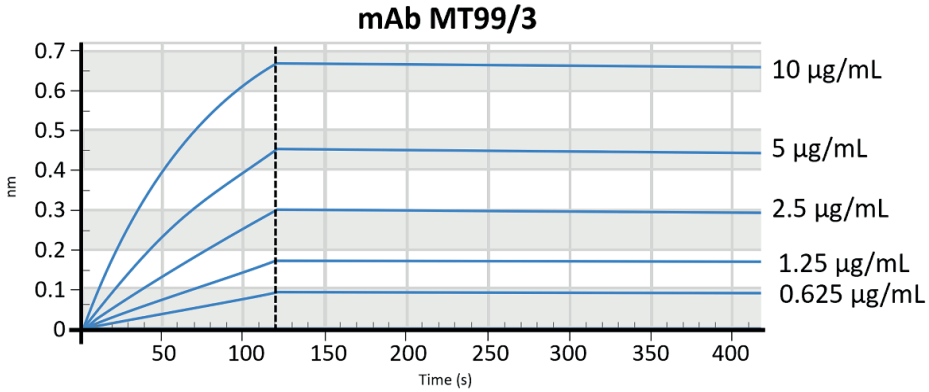


Figure 4. *Binding reactivity of chimeric anti-CD99 antibody. A: Myeloma cells and CD99-expressing myeloma cells were stained with 10 µg/mL of ChAbMT99/3, an isotype-matched control antibody (human IgG), or without antibody (No Ab), B: human mantle cell lymphoma Z138 cell line was stained with the indicated concentrations of ChAbMT99/3, mAb MT99/3, isotype-matched control Ab, or without antibody (No Ab), C: human PBMCs (3 donors) were stained with 10 µg/mL of biotinylated ChAbMT99/3, a biotinylated isotype-matched control antibody (human IgG), or without antibody (No Ab). The antibody binding on the cell surface was detected by specific conjugates. The numbers shown in the histogram indicate geometric MFI.*



K_D (M)	k_a (1/Ms)	k_d (1/s)
4.17×10^{-10}	2.5×10^{5}	1.04×10^{-4}



K_D (M)	k_a (1/Ms)	k_d (1/s)
4.62×10^{-10}	1.75×10^{5}	8.08×10^{-5}

Figure 5. *The binding affinity of ChAbMT99/3 and mouse mAb MT99/3. Biotinylated CD99 ECD peptides were loaded onto a streptavidin-coated biosensor and associated with the indicated doses of ChAbMT99/3 or mouse mAb MT99/3, followed by dissociation in PBST. The graphs are shown in the fitting view. k_a: the association rate constant, k_d: the dissociation rate constant, K_D: the equilibrium dissociation constant (k_d/k_a).*

ChAbMT99/3 enhances macrophages-mediated phagocytosis on mantle cell lymphoma

Phagocytosis is an essential mechanism for the elimination of lymphoma cells.[19,20] Thus, ADCP mediated by ChAbMT99/3 against MCL was evaluated. Z138 cells were treated with various concentrations of ChAbMT99/3, isotype-matched control antibody, or medium without antibody. After that, the treated cells were co-cultured with monocyte-derived macrophages. The results demonstrated that ChAbMT99/3 could enhance the eradication of Z138 cells by mediating the ADCP mechanism in a dose-dependent manner (Figure 6), correlated with antibody binding on the cell surface (Figure 4B). The macrophage-mediated phagocytosis in the ChAbMT99/3 treatment at 1 and 5 µg/mL concentrations was significantly increased compared with the isotype-matched control Ab treatment (Figure 6B). These results indicated the potential of ChAbMT99/3 as a therapeutic option for eradicating MCL by the ADCP mechanism.

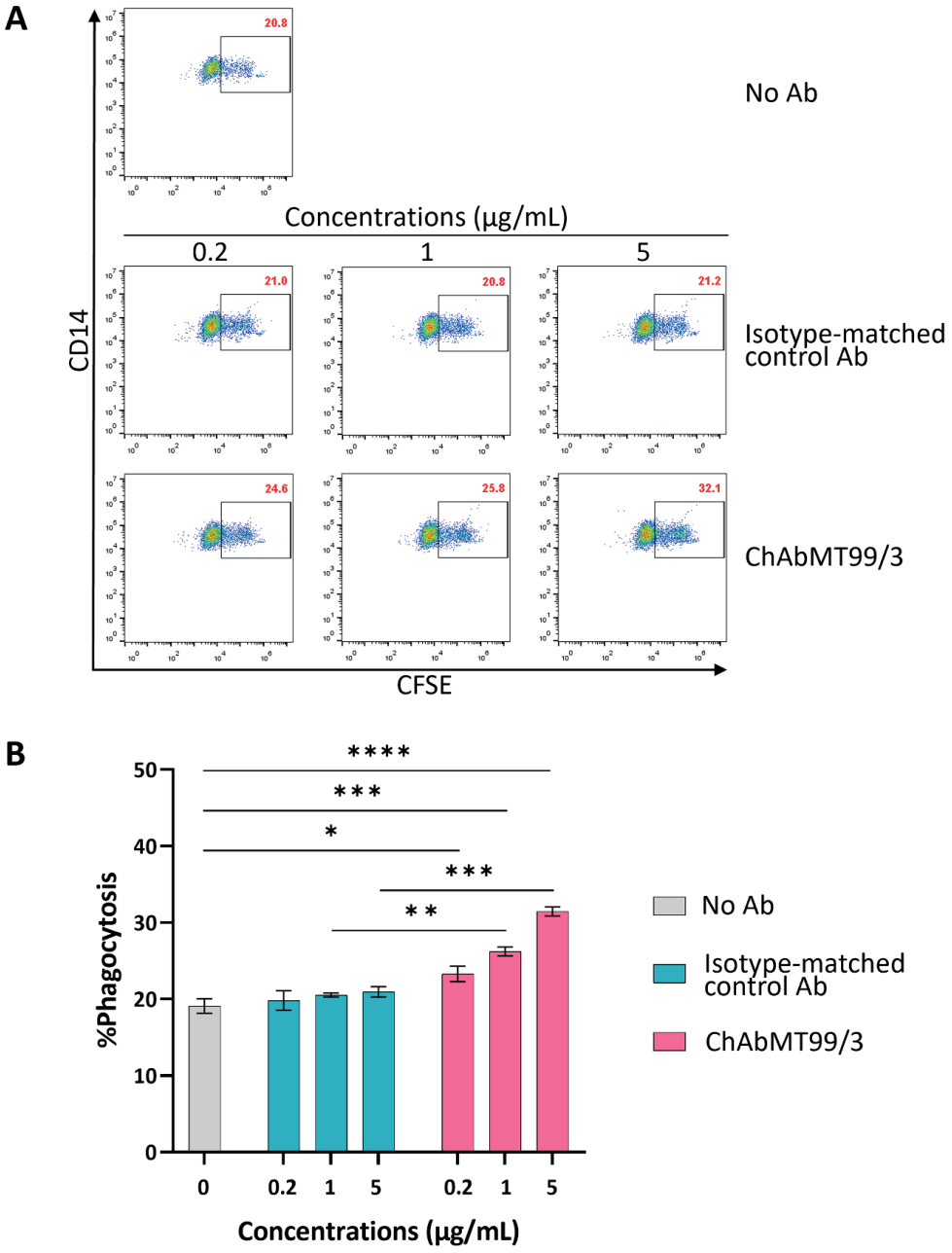


Figure 6. *ADCP mechanism mediated by ChAbMT99/3. A-B: Monocyte-derived macrophages (3 donors) were used as effector cells and incubated with CFSE-labelled Z138 in the presence of isotype-matched control Ab (human IgG) or ChAbMT99/3 at 0.2, 1 and 5 µg/mL or medium (0 µg/mL, No Ab). The percentages of phagocytosis ($CD14^+CFSE^+$) were analyzed by flow cytometry. $CD14^+$ cells were gated for further analysis of double positive of $CD14^+CFSE^+$ cells. A: Dot plots are shown as a representative of flow cytometric data. B: The bar graphs are shown as mean ± SD. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical comparison. *P<0.05, **P<0.01, ***P<0.001 ****P<0.0001 represent statistically significant values.*

Discussion

Mantle cell lymphoma is an aggressive type of mature B-cell lymphoma. It can resist rituximab by inducing the internalization of CD20 molecules, consequently decreasing levels of CD20 expression on the cancer cell surface after rituximab treatment.[21] Therefore, discovering novel antibodies is crucial for improving therapeutic efficacy in mantle cell lymphoma. Our previous results indicated that the in-house produced anti-CD99 mAb clone MT99/3 is a promising antibody for treating mantle cell lymphoma. The mouse mAb MT99/3 could mediate MCL eradication by inducing ADCC and CDC mechanisms *in vitro* and anticancer activities *in vivo*.[12] However, the ADCP mechanism of mAb MT99/3 has not been investigated. The Fc region of mouse mAb was inappropriate for activating certain Fc receptors on human immune effectors.[22] Moreover, for clinical use, mouse mAb is inappropriate. After being introduced into the human body, the mouse mAb acts as a foreign antigen, effectively triggering an immune response. The reactions against mouse mAb will cause several adverse effects and destroy the treated antibody. This will reduce the efficacy of treatment.[13, 14] A chimeric antibody was requested for

clinical use to increase humanness and reduce its potential to cause an immune response.

Therefore, in this study, we engineered the mouse mAb MT99/3 into the mouse/human chimeric antibody named ChAbMT99/3, which contained the human Fc region. This engineered antibody was evaluated for the ADCP mechanism against MCL using human monocyte-derived macrophages. The pTRIOZ-hIgG1 plasmid containing the IgG1 constant domain and zeocin resistance genes was employed to construct expression plasmid pTRIOZ-ChAbMT99/3. The VH and VL genes of mouse mAb MT99/3 were cloned upstream of constant heavy chain and constant light chain genes, respectively. Therefore, this antibody consisted of the variable domain of mouse mAb MT99/3 connecting the constant domains of human IgG1 and kappa light chain. Human IgG1 exhibits the highest affinity for FcγR binding compared with other subclasses, so it is a potent activator of the ADCP mechanism.[23,24] Notably, human IgG1 antibodies commonly serve as the framework for Fc engineering strategies to boost immune effector functions.[25,26] HEK239T cells were employed as host cells in this study as they have been widely used for manufacturing research-grade proteins, with the benefit of producing fully human post-translational modifications (PTMs).[27] Moreover, the use of HEK293T avoids problems of possible immunogenicity caused by the presence of non-human PTMs.[28] Additionally, the ease of transfectability and relatively high protein productivity of HEK293T contribute to its popularity for the production of recombinant proteins.[29] In this study, HEK293T cells were transfected with pTRIOZ-ChAbMT99/3 using Lipofectamine. Flow cytometry analysis showed 100% transfectability. Stable cells with high antibody expression levels were obtained after zeocin drug selection. The purified ChAbMT99/3 was achieved from the culture supernatant using protein G affinity chromatography. High purity and full IgG structure of ChAbMT99/3 were obtained. These results demonstrated that a genetically engineered mouse/human ChAbMT99/3 was successfully produced in a stable human expression system. Furthermore, the produced ChAbMT99/3 retained its binding specificity to human CD99, showing positive reactivity with human CD99-expressing myeloma cells. Importantly, ChAbMT99/3 could recognize native human CD99 on the cell surface of MCL cell line Z138, the same as its parental mouse mAb MT99/3. However, indirect immunofluorescence assay could not be directly used to compare the binding capability of ChAbMT99/3 and mouse mAb MT99/3 with CD99 due to differences in conjugates. Therefore, we conducted BLI to measure the binding affinity of both antibodies using CD99 ECD peptide.[17] Our findings revealed that the K_D values of ChAbMT99/3 and mouse mAb MT99/3 were very close. This suggested that the binding affinities of ChAbMT99/3 and mouse mAb MT99/3 were comparable. Moreover, the ChAbMT99/3 could recognize CD99 on human PBMCs in the same pattern as other antibodies against CD99.[18]

Macrophage-mediated ADCP is one of the most essential mechanisms in cancer eradication.[8] The upregulation of CD47, the "don't eat me" molecule, in mantle cell lymphoma, hinders the elimination of cancer cells via phagocytosis.[11] Several studies demonstrated that anti-CD47 mAbs must interfere with the "don't eat me" signal and restore phagocytosis against MCL.[11,30] Therefore, antibodies that are capable of inducing the ADCP mechanism are pivotal. In our experiment, surprisingly, ChAbMT99/3 could significantly enhance the macrophage-mediated phagocytosis of an MCL cell line Z138 in a dose-dependent manner. The increase in phagocytosis was correlated with the number of bound antibodies on the surface of target cells. Furthermore, in the context of ADCP, the engagement of "eat-me" and "don't eat me" signals is pivotal.[31,32] ChAbMT99/3, which recognized CD99 on target cells, might alter these signals by downregulating "don't eat me" molecules or upregulating "eat me" molecules, resulting in higher sensitivity of MCL toward macrophage-mediated ADCP. Moreover, the internalization rate upon antibody binding with an antigen on target cells activates immune functions.[33,34] ChAbMT99/3 might induce a slow internalization rate and retain many bound antibodies on the cell surface to activate the Fc receptors on macrophages. However, the mechanism by which ChAbMT99/3 could overrule the function of CD47 expressed in mantle cell lymphoma is still unknown. Nevertheless, the combination of rituximab with anti-CD47 mAb exhibited the synergistic effect of phagocytosis.[11, 30] Therefore, the combination treatment of ChAbMT99/3 and anti-CD47 mAbs may be better than a single treatment and is attractive for further investigation.

Conclusion

The results demonstrated the successful production of a genetically engineered mouse/human chimeric antibody targeting human CD99 using a stable human expression system based on HEK293T cells. Notably, the produced chimeric antibody derived from mouse mAb MT99/3 could retain its binding reactivity to recombinant and native human CD99. Interestingly, the ChAbMT99/3 significantly enhanced antibody-dependent cellular phagocytosis against mantle cell lymphoma. Consequently, ChAbMT99/3 might be a promising therapeutic option for eradicating mantle cell lymphoma by the ADCP mechanism. However, further investigations are necessary to explore its potential in other anti-cancer activities.

Conflict of interest

The authors declare no conflict of interest.

Ethics approval

The study was approved by the ethics committees of the Faculty of Associated Medical Sciences, Chiang Mai University (AMSEC-66EX-014).

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References

[1] Campo E, Raffeld M, Jaffe ES. Mantle-cell lymphoma. Semin Hematol. 1999; 36(2): 115-27.

[2] Dabaja B, Ha CS, Cox JD. Chapter 34 - Leukemias and Lymphomas. In: Cox JD, Ang KK, editors. Radiation Oncology (Ninth Edition). Philadelphia: Mosby; 2010. p. 875-911.

[3] Hanel W, Epperla N. Emerging therapies in mantle cell lymphoma. J Hematol Oncol. 2020; 13(1): 79. doi: 10.1186/s13045-020-00914-1

[4] Fischer L, Jiang L, Bittenbring JT, Huebel K, Schmidt C, Duell J, *et al*. The addition of rituximab to chemotherapy improves overall survival in mantle cell lymphoma-a pooled trials analysis. Ann Hematol. 2023; 102(10): 2791-801. doi: 10.1007/s00277-023-05385-1

[5] Roué G, Sola B. Management of Drug Resistance in Mantle Cell Lymphoma. Cancers (Basel). 2020; 12(6): 1565. doi: 10.3390/cancers12061565

[6] Zahavi D, Weiner L. Monoclonal Antibodies in Cancer Therapy. Antibodies (Basel). 2020; 9(3): 34. doi: 10.3390/antib9030034

[7] Grossbard ML, Press OW, Appelbaum FR, Bernstein ID, Nadler LM. Monoclonal Antibody-Based Therapies of Leukemia and Lymphoma. Blood. 1992; 80(4): 863-78. doi: 10.1182/blood.V80.4.863.863

[8] Cao X, Chen J, Li B, Dang J, Zhang W, Zhong X, *et al*. Promoting antibody-dependent cellular phagocytosis for effective macrophage-based cancer immunotherapy. Sci Adv. 2022; 8(11): eabl9171. doi: 10.1126/sciadv.abl9171

[9] Kamen L, Myneni S, Langsdorf C, Kho E, Ordonia B, Thakurta T, *et al*. A novel method for determining antibody-dependent cellular phagocytosis. J Immunol Methods. 2019; 468: 55-60. doi: 10.1016/j.jim.2019.03.001

[10] Van Wagoner CM, Rivera-Escalera F, Jaimes-Delgadillo NC, Chu CC, Zent CS, Elliott MR. Antibody-mediated phagocytosis in cancer immunotherapy. Immunological Reviews. 2023; 319(1): 128-41. doi: 10.1111/imr.13265

[11] Chao MP, Alizadeh AA, Tang C, Myklebust JH, Varghese B, Gill S, *et al*. Anti-CD47 Antibody Synergizes with Rituximab to Promote Phagocytosis and Eradicate Non-Hodgkin Lymphoma. Cell. 2010; 142(5): 699-713. doi: 10.1016/j.cell.2010.07.044

[12] Takheaw N, Sittithumcharee G, Kariya R, Kasinrerk W, Okada S. Anti-human CD99 antibody exerts potent antitumor effects in mantle cell lymphoma. Cancer Immunol Immunother. 2021; 70(6): 1557-67. doi: 10.1007/s00262-020-02789-0

[13] Carter P. Improving the efficacy of antibody-based cancer therapies. Nature Reviews Cancer. 2001; 1(2): 118-29. doi: 10.1038/35101072

[14] Khan FH. Chapter 25 - Antibodies and Their Applications. In: Verma AS, Singh A, editors. Animal Biotechnology. San Diego: Academic Press; 2014. p. 473-90.

[15] Kasinrerk W, Tokrasinwit N, Moonsom S, Stockinger H. CD99 monoclonal antibody induce homotypic adhesion of Jurkat cells through protein tyrosine kinase and protein kinase C-dependent pathway. Immunology Letters. 2000; 71(1): 33-41. doi: 10.1016/S0165-2478(99)00165-0

[16] Takheaw N, Laopajon W, Chuensirikulchai K, Kasinrerk W, Pata S. Exploitation of human CD99 expressing mouse myeloma cells as immunogen for production of mouse specific polyclonal antibodies. Protein Expr Purif. 2017; 134: 82-8. doi: 10.1016/j.pep.2017.02.015

[17] Takheaw N, Kotemul K, Chaiwut R, Pata S, Laopajon W, Rangnoi K, *et al*. Transcriptome Analysis Reveals the Induction of Apoptosis-Related Genes by a Monoclonal Antibody against a New Epitope of CD99 on T-Acute Lymphoblastic Leukemia. Antibodies. 2024; 13(2): 42. doi: 10.3390/antib13020042

[18] Shi J, Zhang Z, Cen H, Wu H, Zhang S, Liu J, *et al*. CAR T cells targeting CD99 as an approach to eradicate T-cell acute lymphoblastic leukemia without normal blood cells toxicity. Journal of Hematology & Oncology. 2021; 14(1): 162. doi: 10.1186/s13045-021-01178-z

[19] Glennie MJ, French RR, Cragg MS, Taylor RP. Mechanisms of killing by anti-CD20 monoclonal antibodies. Molecular Immunology. 2007; 44(16): 3823-37. doi: 10.1016/j.molimm.2007.06.151

[20] Overdijk MB, Verploegen S, Bögels M, van Egmond M, Lammerts van Bueren JJ, Mutis T, *et al*. Antibody-mediated phagocytosis contributes to the anti-tumor activity of the therapeutic antibody daratumumab in lymphoma and multiple myeloma. MAbs. 2015; 7(2): 311-21. doi: 10.1080/19420862.2015.1007813

[21] Beers SA, French RR, Chan HT, Lim SH, Jarrett TC, Vidal RM, et al. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. Blood. 2010; 115(25): 5191-201. doi: 10.1182/blood-2010-01-263533

[22] Doevendans E, Schellekens H. Immunogenicity of Innovative and Biosimilar Monoclonal Antibodies. Antibodies (Basel). 2019; 8(1): 21. doi: 10.3390/antib8010021

[23] Yu J, Song Y, Tian W. How to select IgG subclasses in developing anti-tumor therapeutic antibodies. Journal of Hematology & Oncology. 2020; 13(1): 45. doi: 10.1186/s13045-020-00876-4

[24] Kretschmer A, Schwanbeck R, Valerius T, Rösner T. Antibody Isotypes for Tumor Immunotherapy. Transfus Med Hemother. 2017; 44(5): 320-6. doi: 10.1159/000479240

[25] Wirt T, Rosskopf S, Rösner T, Eichholz KM, Kahrs A, Lutz S, *et al*. An Fc Double-Engineered CD20 Antibody with Enhanced Ability to Trigger Complement-

Dependent Cytotoxicity and Antibody-Dependent Cell-Mediated Cytotoxicity. Transfus Med Hemother. 2017; 44(5): 292-300. doi: 10.1159/000479978

[26] Carter PJ. Potent antibody therapeutics by design. Nat Rev Immunol. 2006; 6(5): 343-57. doi: 10.1038/nri1837

[27] Dumont J, Euwart D, Mei B, Estes S, Kshirsagar R. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. Crit Rev Biotechnol. 2016; 36(6): 1110-22. doi: 10.3109/07388551.2015.1084266

[28] Tan E, Chin CSH, Lim ZFS, Ng SK. HEK293 Cell Line as a Platform to Produce Recombinant Proteins and Viral Vectors. Front Bioeng Biotechnol. 2021; 9: 796991. doi: 10.3389/fbioe.2021.796991

[29] Abaandou L, Quan D, Shiloach J. Affecting HEK293 Cell Growth and Production Performance by Modifying the Expression of Specific Genes. Cells. 2021; 10(7): 1667. doi: 10.3390/cells10071667

[30] Aroldi A, Mauri M, Ramazzotti D, Villa M, Malighetti F, Crippa V, et al. Effects of blocking CD24 and CD47 'don't eat me' signals in combination with rituximab in mantle-cell lymphoma and chronic lymphocytic leukaemia. J Cell Mol Med. 2023; 27(20): 3053-64. doi: 10.1111/jcmm.17868

[31] Khalaji A, Yancheshmeh FB, Farham F, Khorram A, Sheshbolouki S, Zokaei M, et al. Don't eat me/eat me signals as a novel strategy in cancer immunotherapy. Heliyon. 2023; 9(10): e20507. doi: 10.1016/j.heliyon.2023.e20507

[32] Liu Ye, Wang Y, Yang Y, Weng L, Wu Q, Zhang J, *et al*. Emerging phagocytosis checkpoints in cancer immunotherapy. Signal Transduction and Targeted Therapy. 2023; 8(1): 104. doi: 10.1038/s41392-023-01365-z

[33] Liao-Chan S, Daine-Matsuoka B, Heald N, Wong T, Lin T, Cai AG, *et al*. Quantitative assessment of antibody internalization with novel monoclonal antibodies against Alexa fluorophores. PLoS One. 2015; 10(4):e 0124708. doi: 10.1371/journal.pone.0124708

[34] Jin H, D'Urso V, Neuteboom B, McKenna SD, Schweickhardt R, Gross AW, *et al*. Avelumab internalization by human circulating immune cells is mediated by both Fc gamma receptor and PD-L1 binding. OncoImmunology. 2021; 10(1): 1958590. doi: 10.1080/2162402X.2021.1958590