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ORIGINAL ARTICLE

Production of anti-human cannabinoid receptor 2 (CB2) monoclonal antibody using non-viral vector induced human CB2 expressing myeloma as an immunogen

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Abstract

Background: Cannabinoid receptor 2 (CB2) of the cannabinoid system is predominantly expressed on immune cells and involved in a diverse range of immune functions. However, the role of CB2 in immunoregulation is still controversial. One of the challenges in the detailed characterization and functional study of CB2 is the lack of CB2-specific monoclonal antibodies (mAbs).

Objective: We aimed to produce mAbs against a native form of human CB2 using human CB2 expressing mouse myeloma cells as immunogens.

Methods: Non-viral vector expression system was used to generate stable human CB2-expressing mouse myeloma cells and were utilized as an immunogen for mouse immunization. Hybridoma technique was employed in the production of mAbs. The produced mAbs were verified by flow cytometry and western blotting.

Results: Using a non-viral vector expression system, myeloma clones, which stable expressed human CB2, were generated and used as immunogen for antibody production. Following mouse immunization process, the anti-CB2 polyclonal antibodies were induced. By hybridoma technique, a mAb against CB2 could be generated. This mAb reacted to CB2-expressing THP-1 cells, but not to non-CB2-expressing SH-SY5Y cells. By western blotting, the generated anti-CB2 mAb reacted with a 42 kDa protein presented in lysates of CB2-expressing THP-1 cells, but not with non-CB2-expressing SH-SY5Y cell lysates.

Conclusion: A new approach using human CB2 expressing myeloma cells as immunogen for production of anti-CB2 mAb was developed. The generated anti-human CB2 mAb is regarded as a valuable tool for CB2 characterization. Moreover, the developed technique can be applied to produce other antibodies of interest.

Key words: Cannabinoid system, Cannabinoid receptor, Non-viral vector expression system, Monoclonal antibody production, Anti-cannabinoid receptor 2 antibody

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Introduction

The endocannabinoid system is a biological system in the body that regulates a variety of physiological conditions through ligand-receptor interactions.[1] Two primary endocannabinoid receptors have been identified, namely cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2).[1-3] These receptors are seven transmembrane cell surface receptors which belong to the G protein-coupled receptor family.[3] N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2AG) have been described as endogenous ligands for CB1 and CB2, respectively.[4,5] It is well documented that the endocannabinoid system regulates a wide variety of neuronal and immunological processes, including memory, neurogenesis, appetite, stress/anxiety, analgesia, thermoregulation sleep and immune cell functions.[6] CB1 was demonstrated to play a role in the modulation of neurological functions, while CB2 regulates the immune system.[7-9]

CB2 is mainly expressed on immune cells. The expression level of CB2 on leukocytes is different, depending on the type of immune cells, activation stage and the type of stimuli.[4,5] Several reports indicated that CB2 is implicated in a variety of immune modulatory functions. The binding of CB2 with endocannabinoids was demonstrated to affect cell signaling pathways, cell migration, cytokine production, proliferation and apoptosis.[4,9-11] CB2 engagement by phyto-endocannabinoids could either inhibit or promote immune cell functions.[12,13] Currently, several CB2 selective agonists have been synthesized.[14-16] Some of these synthetic agonists were demonstrated to modulate the immune function and have therapeutic potential for several disorders.[15,17,18] We recently demonstrated that a synthetic CB2 selective agonist, GW833972A, could attenuate cell-mediated immunity.[19] The detailed mechanism of CB2 involvement in the immune regulation, however, requires further investigation.

A monoclonal antibody (mAb) is an antibody that has high specificity and low cross-reactivity.[20] Currently, mAbs are an important tool for several applications, including research, diagnostics, and therapeutics.[20-22] However, the production of mAb specific to a molecule of interest remains a complex and challenging process, particularly, those specific to cell membrane proteins.[23,24] One of the main limitations in the production of mAbs to cell surface molecules is obtaining sufficient amounts of membrane protein antigens for use as an immunogen.[25] Additionally, an important limitation is the conformational structure of the immunogen. The native conformation of the membrane protein is critical for its function, but it is often difficult to preserve the correct conformational structure during the immunogen preparation process. mAbs that recognize the denatured form of the protein, rather than the native form, may not recognized the native protein.[26] This type of mAb cannot be used for the functional study of a protein of interest.

In this study, we aimed to generate mAb against the native form of human CB2. We designed an alternative method for the preparation of CB2 in native form and its use as an immunogen for mAb production. We first generated mouse myeloma cells that stable expressed human CB2 proteins on their surface using non-viral vector system. These cells were then used as immunogen to immunize mice for the production of anti-CB2 mAbs. By this approach, polyclonal and monoclonal antibodies against human CB2 could be developed.

Materials and Methods

Preparation of stable human CB2-expressing mouse myeloma cells

Stable human CB2-expressing mouse myeloma cells were established using the non-viral vector expression system. In brief, the nucleotide sequences of human CB2 were obtained from the NCBI database (Gene ID: 1269) and subsequently codon optimized to increase the efficiency of gene expression in mice. The codon optimized sequences were synthesized and inserted into the pcDNA3.1(+)-N-6His vector by GenScript (Nanjing, China). After plasmid construction, the nucleotide sequences were verified using Sanger sequencing. The pcDNA3.1(+)-N-6His harbored gene encoding human CB2 was named CB2 expression vector.

To establish stable human CB2-expressing mouse myeloma cells, 5 μg of CB2 expression vectors were transfected into 2 × 10^6 mouse P3-X63Ag8.653 myeloma cells by electroporation using the Amaxa Nucleofactor II machine (Lonza; Basel, Switzerland). After electroporation, the transfected cells were cultured in IMDM medium supplemented with 10% heat-inactivated FBS, 40 μg/mL gentamicin, and 2.5 μg/mL amphotericin B (10% FBS-IMDM) in a 5% CO_2 incubator at 37°C. To obtain stable CB2 expressing cells, geneticin (G418) drugs at 400 μg/mL were added into the transfected cells to select and maintain the CB2-histidine tag expressing mouse myeloma cells. Two days after cultivation, the expression of CB2-histidine tag fusion proteins on the myeloma cells was determined by surface immunofluorescence staining using PE-conjugated mouse anti-6His mAb (BioLegend; San Diego, CA, USA) and analyzed by flow cytometry.

To obtain the stable human CB2-expressing mouse myeloma clone, PE-positive transfected cells were sorted by BD FACS Melody Cell Sorter (BD Bioscience; San Jose, CA, USA). Then, the sorted cells were cultured in 10% FBS-IMDM containing 400 μg/mL geneticin in a humidified atmosphere of 5% CO_2 at 37°C.

In order to achieve a single-cell clone, a limiting dilution technique was performed. The sorted cells were plated into 96 well flat plates at 5 cells in 100 μL of 10% FBS-IMDM containing 10% BM condimed H1, and 400 μg/mL geneticin per well. The cells were cultured in a 5% CO_2 incubator for 2 weeks. After cultivation, the cells in each well were screened under inverted microscopy (Olympus; Tokyo, Japan). The well-contained single-cell clone was selected to verify the CB2-histidine tag expression by surface immunofluorescence staining and flow cytometric analysis.

The clone that expressed the CB2-histidine tag at high levels was chosen and cultivated to serve as the immunogen for the production of mAbs. The obtained CB2-expressing mouse myeloma cells were grown and frozen in liquid nitrogen.

Mouse immunization

The stable human CB2-expressing mouse myeloma cells were thawed and cultured in 10% FBS-IMDM for use as an immunogen. Before each immunization, the CB2 expression was confirmed by surface immunofluorescence staining using PE-conjugated anti-6His mAb and flow cytometry.

BALB/c mice (n = 2; female, age 6-8 weeks, weight 20 g) were intraperitoneally immunized with 2×10^7 CB2-expressing mouse myeloma cells in PBS 300 µL at 2-week intervals for three times. At before immunization and 1 week after the 3rd immunization, blood was collected from the mice tails. Sera were isolated from the collected blood to verify the presence of anti-CB2 polyclonal antibodies (pAbs) by surface immunofluorescence staining. After finishing the project, mice had no clinical symptoms, and no tumor or ascites were observed. The mice weight was increased according to their growth. The spleens look normal size.

Hybridoma production

An immunized mouse that contained anti-CB2 pAbs was sacrificed. The mouse spleen was harvested and minced to get the spleen cells. The spleen cells were fused with mouse Sp2/mIL-6 myeloma cells using 50% (w/v) polyethylene glycol (Sigma Aldrich; St. Louis, MO, USA). The fused cells were plated into 96 well flat plates at 5×10^4 cells in 100 µL per well in HAT medium (Sigma Aldrich) and cultured in a humidified atmosphere of 5% CO_2 at 37°C for 5 days. The HT medium (Sigma Aldrich) was then added to the culture. After cultivation for 7 days, the cells in each well were observed under inverted microscopy. The well-contained hybridoma cells were selected. The culture supernatants from the selected wells were harvested to verify the presence of anti-CB2 antibodies by surface and intracellular immunofluorescence staining using un-transfected myeloma cells, stable CB2-expressing myeloma cells, and CB2-expressing THP-1 cells. Subsequently, a limiting dilution technique was performed to obtain a single hybridoma clone. The culture supernatants of the single-cell clones obtained were then harvested to investigate the anti-CB2 mAb using immunofluorescence staining with THP-1 cells. The positive single-cell clone was selected and allowed to grow.

To ensure the activity of the produced anti-CB2 mAbs, the culture supernatant of each single hybridoma clone was immunofluorescence stained with THP-1 and SH-SY5Y cells. Furthermore, western blotting with THP-1 and SH-SY5Y cells was performed to confirm the specificity of the produced mAbs.

Immunofluorescence staining and flow cytometric analysis

For surface immunofluorescence staining, cells were incubated with 10%AB serum in PBS containing 1% BSA and 0.1% NaN_3 (FACS) buffer for 30 minutes at 4°C to block Fc receptors. For intracellular immunofluorescence staining, cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature. The fixed cells were permeabilized with 0.1% saponin, 5% FCS, and 0.1% NaN_3 in PBS to allow the antibodies to access the intracellular targets. After that, the cells were blocked by incubation with 10% AB serum in 0.1% saponin, 5% FCS, and 0.1% NaN_3 in PBS for 30 minutes at 4°C.

Then, the blocked cells were stained with pre-immunized sera, post-immunized sera, or culture supernatant of hybridoma cells for 30 minutes at 4°C. After washing, the bound antibodies were detected by staining with FITC conjugated goat anti-mouse IgG (H + L) F(ab')2 (Sigma Aldrich) for 30 minutes at 4°C. The stained cells were analyzed by BD Accuri flow cytometry (BD Bioscience, San Jose, CA, USA).

Western blotting

Cells at 5×10^7 cells/mL were lysed using sonication in a non-reducing buffer. The cell lysate was boiled for 5 minutes. Then, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed under non-reducing conditions. The cell lysate was loaded onto polyacrylamide gel, along with a protein ladder. The gel was electrophoresed for 2 hours to separate the proteins. After that, the separated proteins were transferred onto the PVDF membrane using electroblotting for 1.5 hours. A membrane-containing cell lysate was cut into strips. The non-specific binding sites on the membrane strips were blocked with 5% skim milk in PBS for 1 hour at room temperature. The strip containing cell lysate was incubated overnight at 4°C with tested mAb. The membrane strips were washed with 0.1%Tween PBS 3 times. The bound antibodies were detected by staining with HRP-conjugated rabbit anti-mouse immunoglobulins antibodies at a dilution of 1:5,000 (DakoCytomation; Glostrup, Denmark) for 1 hour at room temperature. After washing, the proteins were visualized by incubating the membrane strips with a chemiluminescent substrate (Thermo Fisher Scientific; Waltham, MA, USA). The signals were detected using the ChemiDoc imaging system (Bio-Rad; Hercules, CA, USA).

Ethics approval

This study has been approved by the Chiang Mai University Animal Care and Use Committee (CMU-ACUC; AUP Code 2565/MC-0007) and the Institutional Biosafety Committee (IBC), Chiang Mai University (CMUIBC A-0564017), Chiang Mai University, Chiang Mai, Thailand.

Results

Generation of stable mouse myeloma cells expressing human cannabinoid receptor 2 (CB2) for use as an immunogen

In order to generate hybridoma cell-producing mAb to human CB2, we first established mouse myeloma cells expressing human CB2 for use as an immunogen. The human CB2 gene sequence was retrieved from the NCBI database (Gene ID: 1269). The obtained CB2 gene was codon optimized for gene expression in mouse cells. Upon codon optimization, the codon usage bias was upgraded the CAI (Codon Adaptation Index) from 0.80 to 0.94 as shown in Supplemental Material. This enhancement in codon usage adaptation might result in a remarkable increase in translation efficiency in mouse cells. Furthermore, the amino acid sequence translated from the optimized nucleotide sequence was aligned with the reference amino acid sequence of reported human CB2, and a 100% identical match was found.

The optimized CB2 gene was then cloned into the pcDNA3.1 + N-6HIS vector after the polyhistidine tag (His-tag). The vector used contains human cytomegalovirus immediate-early (CMV) promoter for mammalian cell expression as well as a neomycin resistance gene for the selection of stable expression cells (**Figure 1A**). The achievement of CB2 gene cloning into the pcDNA3.1 + N-6HIS vector was verified by restriction enzyme analysis. The DNA bands in the restriction enzyme digestion condition were observed at sizes expected (**Figure 1B**), indicating the successful insertion of the CB2 gene into the vector. Moreover, the nucleotide sequence of the CB2 gene in the constructed vector was verified by DNA sequencing. By NCBI database blasting, 100% identical match was found. These results indicated that the human CB2 gene was successfully cloned into the pcDNA3.1 + N-6HIS vector and named the CB2 expression vector.

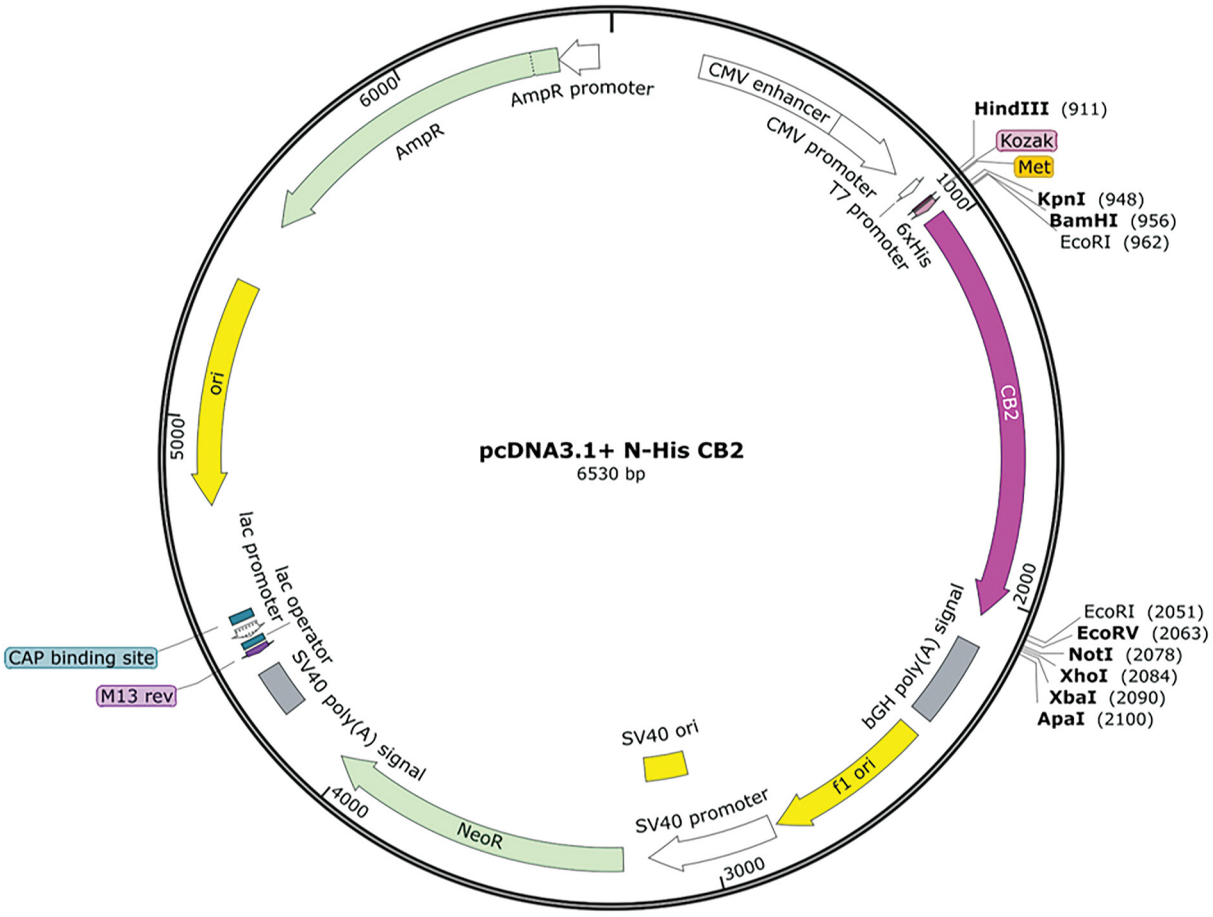

Figure 1. Graphical map of the designed CB2 expression vector and verification of the constructed CB2 expression vector by restriction enzyme analysis. (A) The map of the pcDNA3.1 + N-6HIS CB2 expression vector created by SnapGene software is shown. The human CB2 gene (pink arrow) was cloned after the polyhistidine tag (6xHis). (B) The constructed CB2 expression vector was digested using *Hin*dIII and *Xho*I restriction enzymes. The vector without digestion (lane 1) and after restriction enzymes digestion (lane 2) were separated in 1% agarose gel. Sizes of standard DNA markers (bp) are shown on the right. Bands of the digested constructed vector at around 5357 bp and 1173 bp are shown in lane 2.

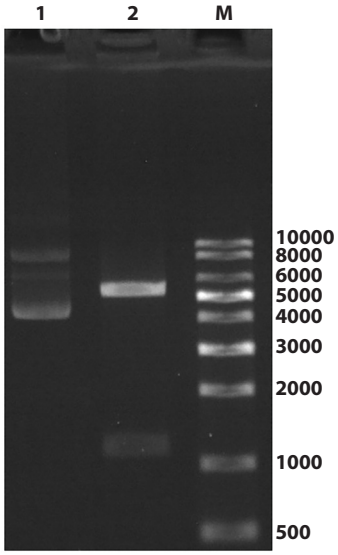

Figure 1. (Continued)

Stable expression of human CB2 on mouse myeloma cells

To validate whether the constructed CB2 expression vector could express the CB2 protein, COS-7 cells were transfected with the constructed vector. After 2 days of transfection, the transfected cells were surface stained with PE-conjugated anti-His mAb. As shown in **Figure 2A**, the transfected cells, but not un-transfected cells, showed positive reactivity. Meanwhile the isotype-matched control mAb was negative with both transfected and un-transfected cells. We further confirmed by staining the transfected cells with commercial rabbit anti-human CB2 pAb which was reported to react with the intracellular domain of human CB2. The rabbit anti-human CB2 pAb showed positive reactivity with the transfected COS-7 cell 56%, but 17.6% with un-transfected cells (**Figure 2B**). As human CB2 and monkey CB2 have high homology, in **Figure 2B**, high background presented in untransfected COS cells (Monkey kidney cell line) with rabbit anti-CB2 polyclonal antibodies might come from the cross-reaction of the antibody used. In our staining, the THP-1 cells, which expressed CB2 protein, were used as a positive control. As expected, THP-1 cells showed a positive signal with rabbit anti-human

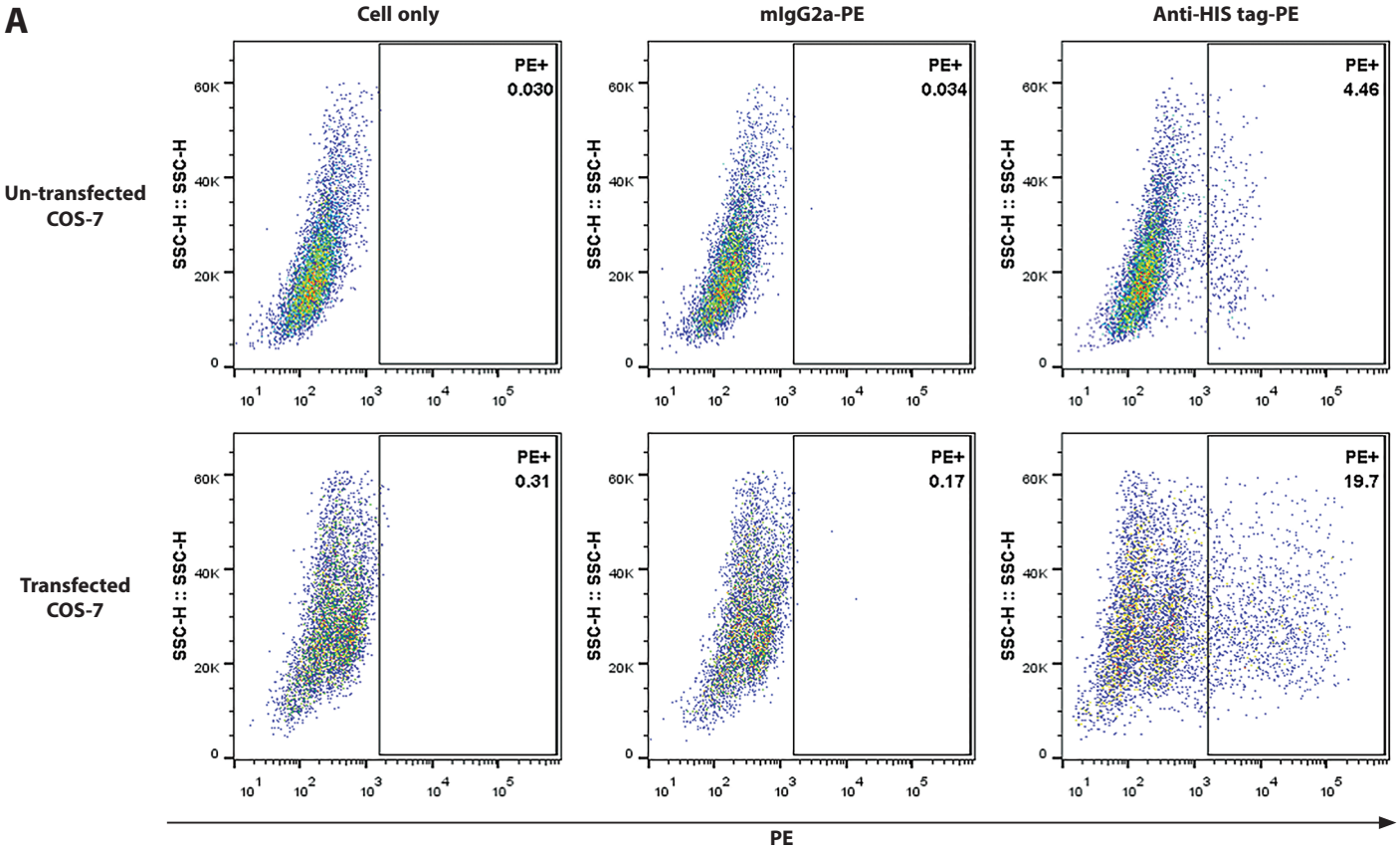

Figure 2. Validation of human CB2 expression in transfected COS-7. COS-7 cells were transfected with the constructed CB2 expression vector or kept in an un-transfected condition. (A) The transfected and un-transfected cells were surface stained with PE conjugated anti-His tag mAb (Anti-His tag-PE) or isotype-matched control mAb (mIgG2a-PE). (B) The transfected, un-transfected cells, and (C) CB2-expressing THP-1 cell line were intracellular immunofluorescence stained with rabbit anti-human CB2 antibody or without antibody followed by PE-conjugated goat anti-rabbit IgG antibody. The staining conditions are indicated and the % of cells in each quadrant is shown.

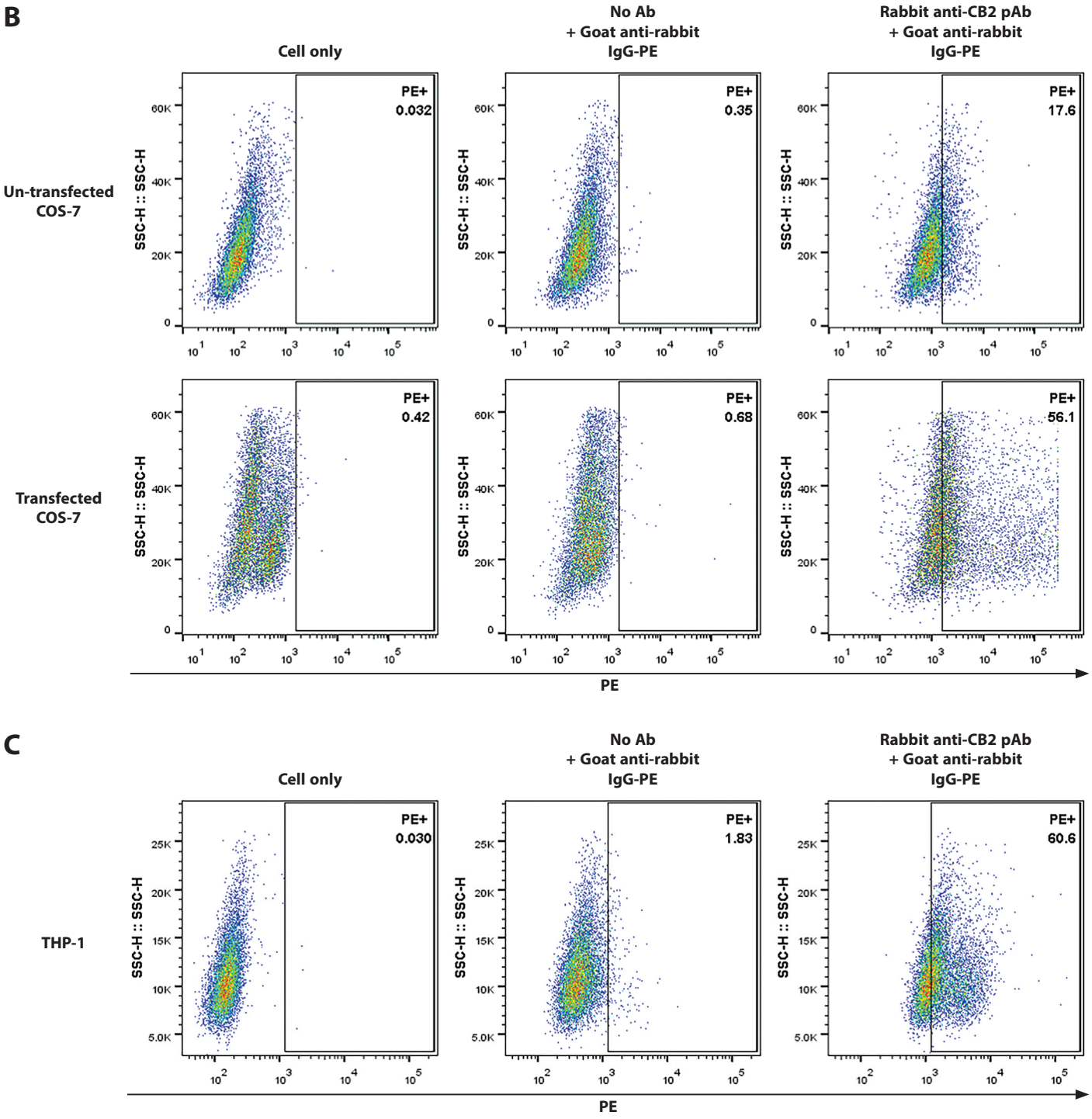

Figure 2. (Continued)

CB2 pAb (**Figure 2C**). These results indicated that the constructed vector could express human CB2 protein. This vector was further utilized for the preparation of stable CB2-expressing myeloma cells.

To obtain human CB2-expressing myeloma cells, the constructed vector was transfected into mouse myeloma cells. Then, the transfected myeloma cells were stained with PE conjugated anti-His mAb and subjected to single-cell cloning using cell sorter and limiting dilution. As shown in **Figure 3A**, several CB2-expressing single clones were obtained. A myeloma clone named 1B4, which was highly positive with 100% expression of human CB2, was selected, grown, and kept in liquid nitrogen.

To investigate whether the expression of CB2 protein on the selected myeloma clone was stable, myeloma clone 1B4 was thawed and cultured in the presence of the G418 drug for one month. The expression of the CB2 proteins was determined and cells that still expressed the CB2 proteins were found (**Figure 3B**). These results indicated that we successfully generated the stable human CB2-expressing myeloma cells. These myeloma cells were used for mouse immunization for mAb production.

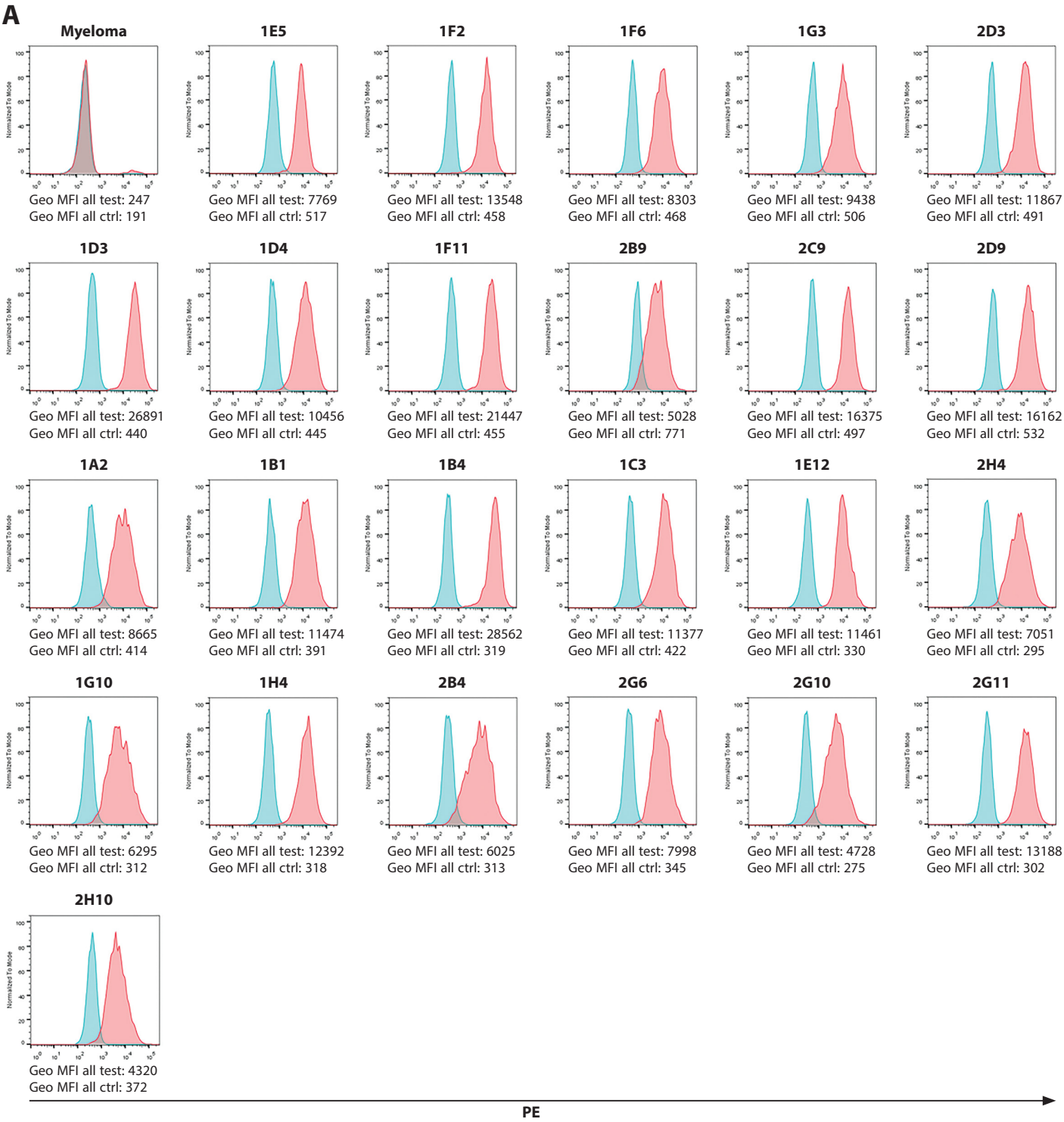

Figure 3. Establishment of stable human CB2 expressing myeloma cells. The CB2 expression vector was transfected into mouse myeloma cells and stained with PE conjugated anti-His mAb. The PE-positive cells were sorted and the limiting dilution was performed. (A) Twenty-four myeloma cell clones and un-transfected myeloma cells (as control) were stained with PE conjugated anti-His mAb (red peaks) or isotype-matched control mAb (blue peaks). The expression levels (Geometric MFI; Geo MFI) are also shown. The clone named 1B4 was selected for further experiments. (B) CB2-expressing myeloma cell clone 1B4 was cultured for one month. The cells were stained with PE conjugated anti-6HIS mAb and isotype matched control mAb by surface immunofluorescence staining and flow cytometric analysis in comparison with un-transfected myeloma cells (Myeloma). The overlay histogram plots represent the PE conjugated anti-6HIS mAb stained cells (red peaks) and isotype matched control mAb stained cells (blue peaks).

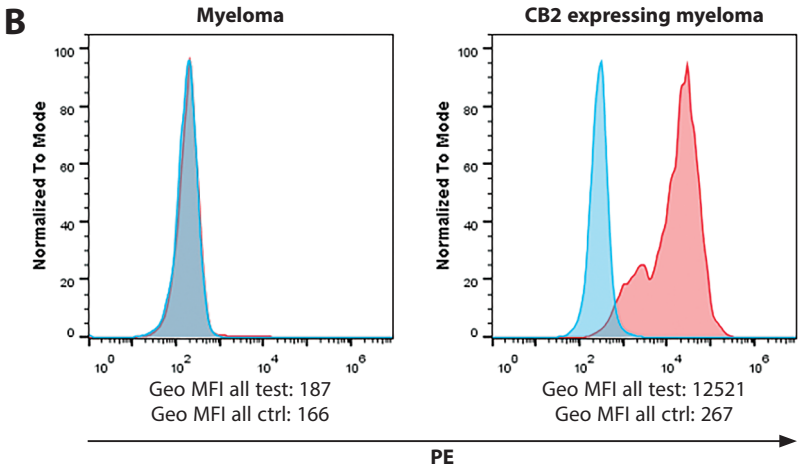

Figure 3. (Continued)

Production of monoclonal antibody against human cannabinoid receptor 2 (CB2) using human CB2-expressing mouse myeloma cells as an immunogen

The generated stable human CB2-expressing myeloma cells were immunized into 2 BALB/c mice for 3 immunizations. The presence of the CB2 specific pAbs in the post-immunized sera was determined. We surface immunofluorescence stained CB2-expressing THP-1 and non-CB2-expressing SH-SY5Y cells with the pre- and post-immunized sera. The THP-1 cell line and SH-SY5Y cell line are naturally express CB2 and non-CB2 expressed cells, respectively. As shown in **Figure 4**, the post-immunized sera from both immunized mice showed positive reactivity with THP-1 cells in comparison with pre-immunized sera. In contrast, the tested sera were negative with SH-SY5Y cells (**Figure 4**). These results suggested that, by our immunization strategy, the anti-CB2 pAbs were induced and the mice were ready for hybridoma production.

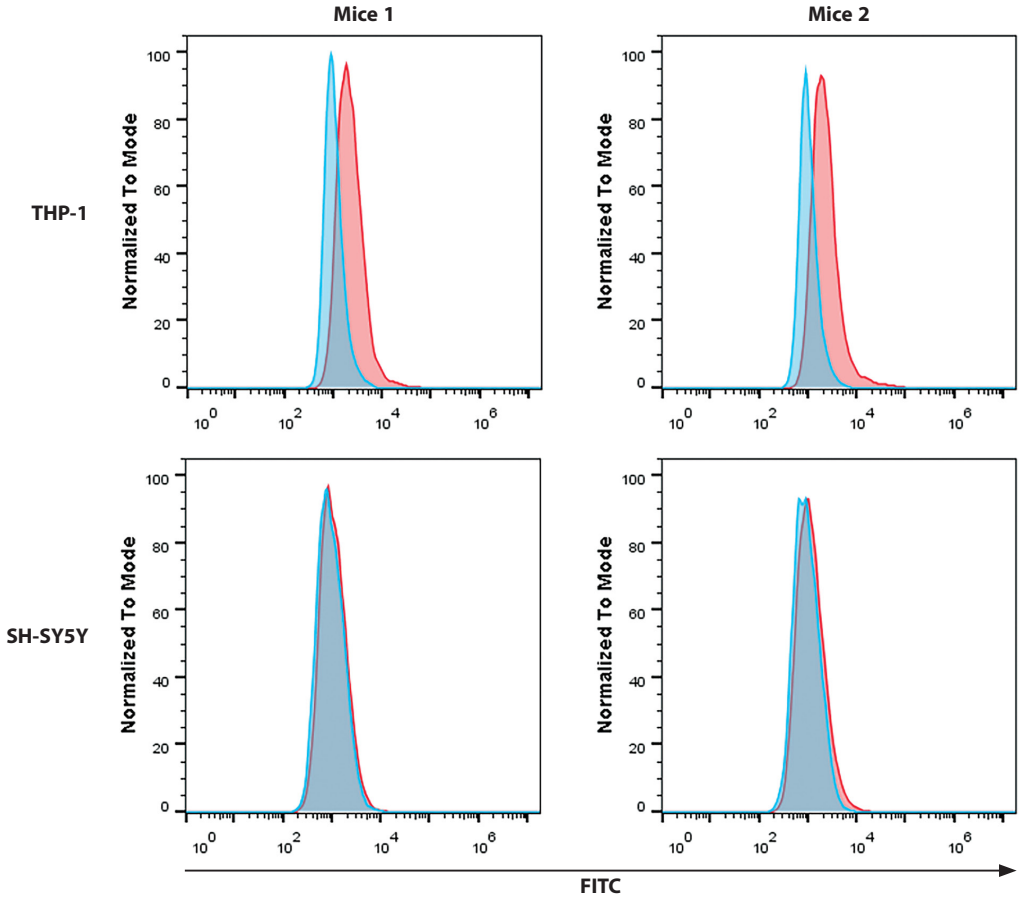

Figure 4. Immunofluorescence surface staining of mouse immunized sera with THP-1 and SH-SY5Y cells. The CB2-expressing THP-1 and non-CB2-expressing SH-SY5Y cells were surface immunofluorescence stained with pre- or post-immunized sera (dilution 1:1000) of 2 immunized mice. The overlay histogram plots represent the pre-immunized sera (Blue peaks) and post-immunized sera (red peaks).

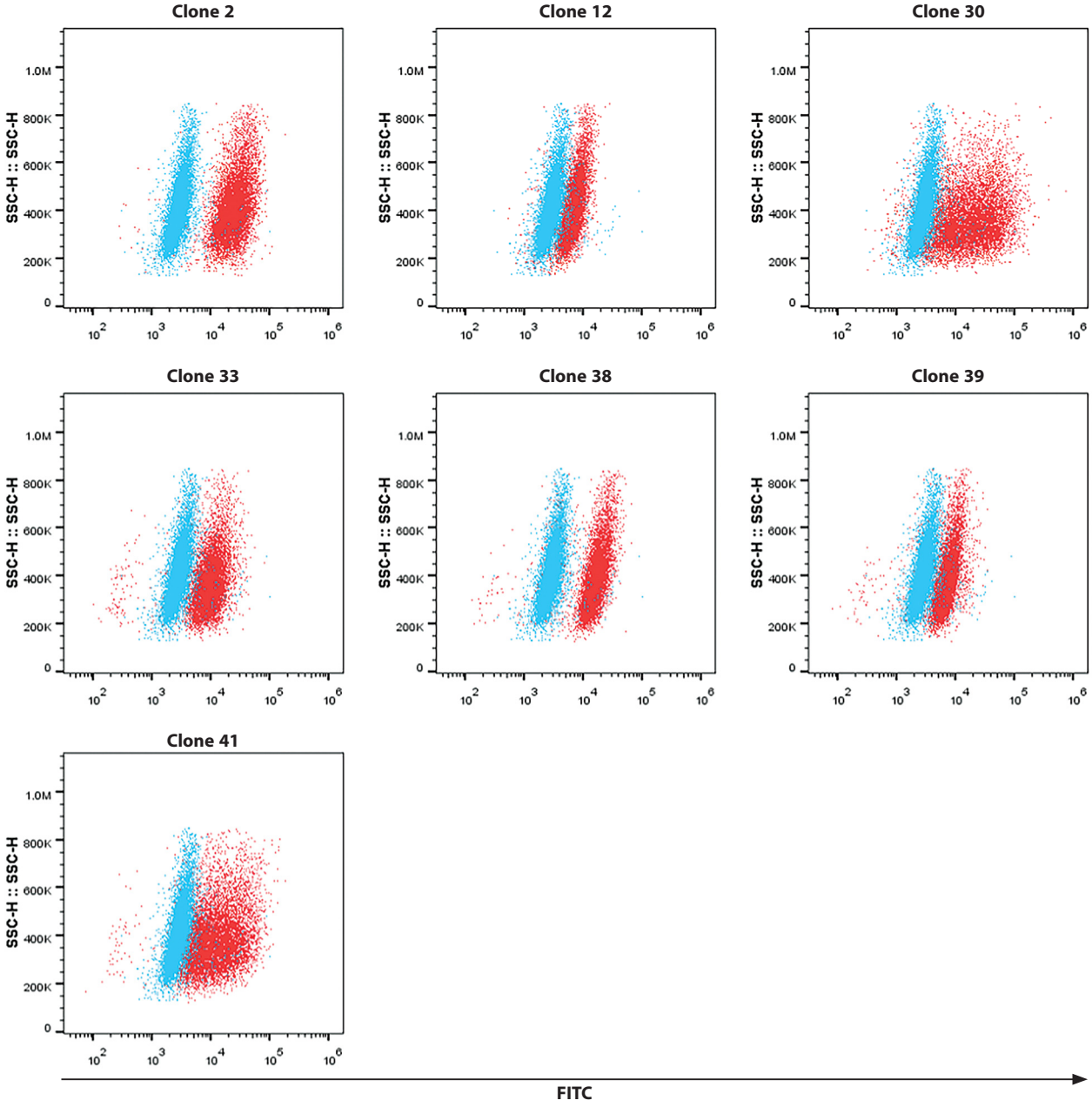

Figure 5. Intracellular immunofluorescence staining of culture supernatants with THP-1 cells. Culture supernatants harvested from hybridoma-containing wells or medium (negative control) were intracellular immunofluorescence stained with CB2-expressing THP-1 cells. Seven culture supernatants that showed positivity reactivity are shown. The overlay scatter plots represent the staining of culture supernatants (red dots) and medium control (blue dots).

An immunized mouse was sacrificed and the spleen cells were fused with myeloma cells using standard hybridoma techniques. The culture supernatants of all hybridoma-containing wells were determined for anti-CB2 antibody production by surface immunofluorescence staining using CB2-expressing myeloma cells, myeloma cells (as control), and CB2-expressing THP-1 cells. All 41 culture supernatants were negative by surface immunofluorescence staining (data not shown). We then performed intracellular staining using CB2-expressing THP-1 cells. As shown in **Figure 5**, 7 culture supernatants showed positive results, but in different patterns.

The hybridoma clones from the 7 positive wells were named WKCR1- WKCR7, respectively. To confirm the specific activity of all obtained mAbs, the culture supernatant of each hybridoma clone was intracellular immunofluorescence stained with CB2-expressing THP-1 and non-CB2-expressing SH-SY5Y cells. As shown in **Figure 6A**, the culture supernatant from hybridoma clone WKCR2 reacted to CB2-expressing THP-1, but reacted weakly to non-CB2-expressing SH-SY5Y cells. The rest of the hybridoma clones reacted to both CB2-expressing and non-CB2-expressing cells indicating that they were not anti-CB2 mAbs. **Figure 6B** shows the surface and

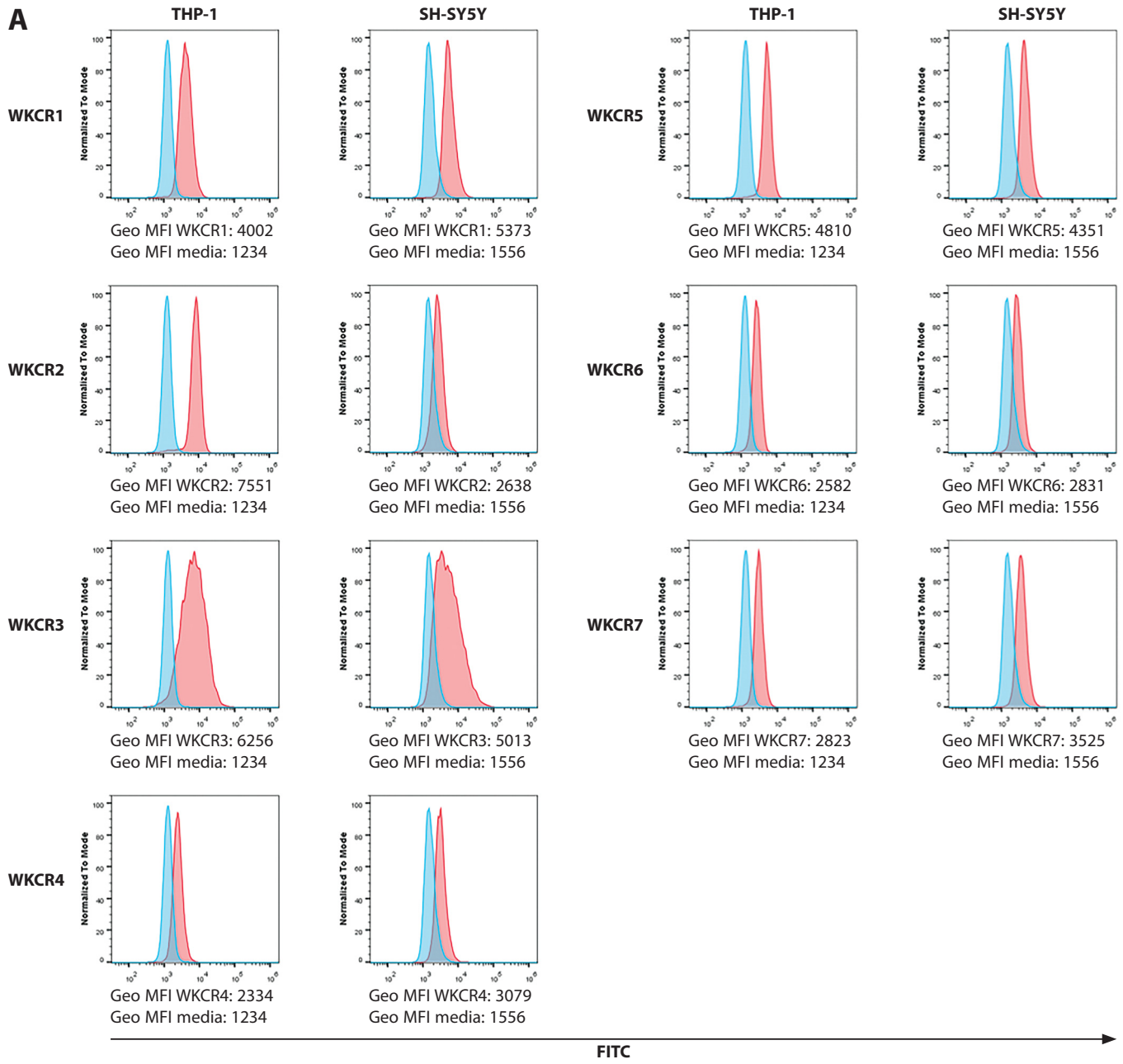

Figure 6. Immunofluorescence staining and western blotting of culture supernatants from hybridoma cell clone WKCR2 with THP-1 and SH-SY5Y cells. (A) CB2-expressing THP-1 and non-CB2-expressing SH-SY5Y cells were intracellularly stained with medium (negative control) and indicated culture supernatants. The overlay histogram plots represent the staining of medium control (blue peaks), and culture supernatants (red peaks). Geometric mean fluorescence intensity values of each condition are expressed under the plots. (B) THP-1 cells were stained with WKCR2 supernatant and media as control by both surface and intracellular immunofluorescence staining. The overlay histogram plots represent the staining of medium control (blue peaks), and culture supernatants (red peaks). (C) Western blotting of culture supernatants clone WKCR2 with SH-SY5Y and THP-1 cell lysate. Conjugate and medium were included as negative control and anti-HSC70 mAb (HSC70) was included as positive control.

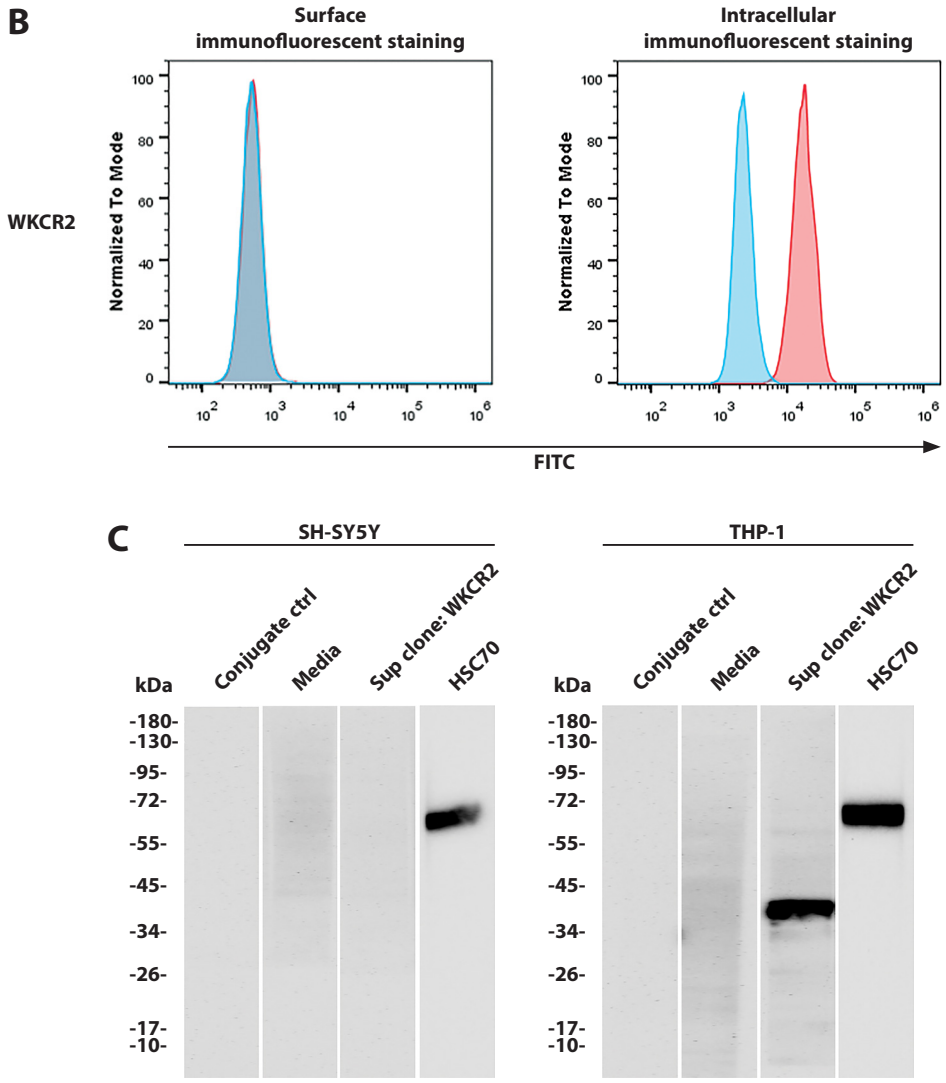

Figure 6. (Continued)

intracellular staining results of clone WKCR2 with THP-1 cells. These results indicated that mAb clone WKCR2 reacted the intracellular part of the CB2 molecule.

Western blotting using THP-1 and SH-SY5Y was carried out to confirm the reactivity of mAb clone WKCR2. A protein band at the molecular weight of 42 kDa appeared in CB2-expressing THP-1, but not in non-CB2-expressing SH-SY5Y cells (**Figure 6C**). This molecular weight was as reported for CB2 protein.[27,28] No bands were observed in media and conjugate controls. Anti-HSC70 mAb was used as positive control and for indicating the protein loading and found a correspondence protein band in both THP-1 and SH-SY5Y.

The isotype of mAb WKCR2 was also determined. The mAb WKCR2 was IgG3 heavy chain with a kappa light chain.

Discussion

Monoclonal antibodies (mAbs) are the antibodies produced by a single B cell clone *in vitro* by hybridoma technique.[29] A mAbs recognize only one epitope on an antigen. It, therefore, has a high degree of specificity and is preferred for applications that require high specificity and low cross-reactivity.[20] In addition, hybridoma cells are immortal and can grow continuously *in vitro*. This makes mAbs able to be produced on a large scale. Currently, mAbs are versatile and have become a valuable tool in research, diagnostics, and therapeutics.[30,31] Despite the many advantages of mAbs, it is important to note that the production of mAb remains a complex process. Particularly, the production of mAbs that are specific to the native form of cell membrane proteins.[23,24]

In this study, we aimed to produce mAbs specific to the native form of human cannabinoid receptor 2, CB2. CB2 was expressed, particularly, on immune cells and was reported to be involved in immune regulation.[9,11] The functions of CB2, however, were reported controversial.[32,33] The generated mAbs, therefore, can be used to characterize and perform a functional study of the CB2. The generated anti-CB2 mAbs may be applied as a therapeutic agent.

For the production of mAbs against human CB2, the preparation of CB2 immunogens is a crucial step.[34] As CB2 is a multi-spanning membrane bound protein, it is difficult to isolate and purify in sufficient amounts from the cell membrane for use as an immunogen. In addition, the immunogen must be in the native conformational structure. The native conformation of the membrane protein is critical for its function, but it is often difficult to preserve the correct conformational structure during the immunogen preparation process. This, therefore, can lead to the production of mAbs that recognize the denatured form rather than the native form. This type of mAbs may not be employed to study the native CB2 function.[26]

In order to obtain mAbs that can bind to native human CB2 proteins, an alternative method for immunogen preparation was developed in this study. In our findings, we established mouse myeloma cells that stable expressed human CB2 on their membrane and were used as immunogens for the production of anti-human CB2 mAb. A non-viral expression vector capable of expressing the human CB2 protein in mammalian cells was constructed. The expression vector pcDNA3.1 + N-6HIS was used in this study. The vector pcDNA3.1 + N-6HIS contains human cytomegalovirus immediate-early (CMV) promoter for high-level expression in a wide range of mammalian cells and neomycin resistance gene for the selection of cells harboring the vectors and expressing the protein of interest. The nucleotide sequences of human CB2 were obtained from the NCBI database. The codon optimization of the nucleotide sequence was performed for the production of human CB2 protein in mouse myeloma cells. A stop codon TAA was added at 3′ end. The CB2 gene was flanked with the sequence of EcoRI restriction enzymes at 5′ and 3′ ends. The synthesized CB2 gene was then cloned into expression vector pcDNA3.1 + N-6HIS at EcoRI site after polyhistidine tag (His-tag). The constructed vector was, then, transfected into mouse myeloma cells for generating stable human CB2-expressing mouse myeloma cells, upon G418 (Geneticin) treatment. In this study, by FACS-sorting and limiting dilution of the transfected myeloma cells, we successfully generated mouse myeloma clones that stable expressed human CB2 by the non-viral expression vector. The CB2-expressing myeloma cells could be grown and used as an immunogen. By this approach, we could overcome the limitation of the lack of a native form of CB2 for mouse immunization. However, it is noted that the folded and displays biological activity of the expressed CB2 were not verify in this study.

Upon immunization into BALB/c mice, we speculated that the human CB2 expressed on mouse myeloma cells would be foreign proteins and induced specific antibody responses in the immunized mice. In addition, the post-translational modifications that occur in mouse myeloma cells ensure that CB2 expressed has a conformational structure similar to its native structure. The generated CB2-expressing myeloma cells were used as immunogens to immunize mice. By this approach, polyclonal antibodies against human CB2 were, indeed, induced in the immunized mice.

By hybridoma technique, the spleen cells of an immunized mouse were fused with myeloma cells. Several mAb secreted by the generated hybridoma cell which reacted to CB2-expressing THP-1 cells were obtained. Surprisingly, the flow cytometric profiles of each mAb were different indicating the mAbs might react to different molecules. The non-CB2-expressing SH-SY5Y cells were used to confirm the specificity of the produced mAbs. Among those mAbs, only one mAb, namely WKCR2, was proven to be specific for CB2 by immunofluorescence and western blotting techniques. This monoclonal antibody was positive with native CB2 positive THP-1, but negative with CB2 negative SH-SY5Y. This might indicate that the produced monoclonal antibody reacted to CB2, not 6 His tag. Additionally, as SH-SY5Y cells express CB1, this observation also confirms the specificity of the produced antibody with CB2 but not CB1.

The rest of the mAb clones reacted to CB2-expressing THP-1 but also to non-CB2-expressing SH-SY5Y cells. These results suggested that these mAbs are not specific to CB2, but specific to other proteins. These proteins might contain in myeloma cells that used as immunogen and could induced immune responses in BALB/c. So, we could generate the hybridoma producing also protein expressed in SH-SY5Y. The generated anti-human CB2 mAb clone WKCR2 reacted to the cytoplasmic domain of CB2, but not to the extracellular domain region, as it showed positive reactivity only when stained intracellularly. This might be because the extracellular part of the CB2 receptor of humans and mice are similar.[35] Therefore, the human CB2 extracellular domain expressed on immunized myeloma cells will not be a foreign antigen in mice.

It is worth to note that most commercial antibodies against human CB2 are polyclonal antibodies. Monoclonal antibody against CB2 is very limited in the markets. Nevertheless, most of them did not clearly inform the cross-reactivity with CB1 that shares 40% homology with CB2. In this study, we produced our anti-CB2 mAb. Our in-house produced anti-CB2 mAb specifically reacts to CB2, but not to CB1. Although the generated anti-CB2 mAb reacts to intracellular part of CB2 molecule, it is still very useful. This mAb can be used for the detection of CB2 expression in any cells by intracellular staining, and western blotting.

Taken together, in this study, we introduce a new approach for the preparation of cell surface immunogen. This technique allows large scale production of immunogen in the conformational structure. By this approach together with the hybridoma technique, a mAb against human CB2 was obtained. This mAb is valuable for use as a research tool for the characterization of human CB2. To the best of our knowledge, this kind of mAb is still unavailable in the market.

Conflict of interests

The authors declare that they have no conflict of interests.

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Author contributions

- WK and NT provided a conceptualization and funding acquisition.
- KJ, NT, CP, WL, KK, RC, and SP performed the experiments.
- KJ, NT, and WK performed data analysis and interpretation.
- KJ drafted the original manuscript.
- WK and NT reviewed and edited the final version of manuscript.

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