Article

Functional Identification of the Stable Transfection C5aR Cell Line Molt-4

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The complement C5 anaphylatoxin receptor is a member of the seven transmembrane-spanning G protein-coupled receptor superfamily that signals through Gαi and Gα16. C5aR is mostly expressed on neutrophils, macrophages and endothelial cells. C5a and C5aR interaction plays an important role in numerous biological effects such as *in vivo* cytokine storm which results in inflammatory damage. Considering the limitation of collection of human peripheral blood neutrophils and their short half life, the stably transfected cell line for studying the biological effects of C5aR is needed. In this study, we transfected C5aR gene into Molt-4 cell line and examined the function of ectopic C5aR. Our results showed stable expression of the C5aR in Molt-4 cell line and their interaction with human C5a induced ERK1/2 phosphorylation, Ca^{++} influx. This stable transfected cell line may provide a useful tool for studying signal pathways related to C5a and C5aR interplay and antibody development specific for C5aR. *Cellular & Molecular Immunology*. 2007;4(6):461-465.

Key Words: complement, C5a, C5aR, Molt-4

Introduction

The complement system was discovered about a century ago. In the past decade, the interest in complement research has been rekindled because of the discovery of complement receptors and new functional aspects of complement activation products in inflammatory diseases in which C5a plays important roles (1). C5a-C5aR interaction is the major mediator of cytokine storm *in vivo*, which results in severe auto damage (2). The complement C5 anaphylatoxin receptor (C5aR; CD88) is a member of the seven transmembrane-spanning G protein-coupled receptor superfamilies (3). C5L2 is another C5a receptor, which is similar to C5aR but is not G-protein coupled. C5aR signals through Gαi and Gα16, whereas C5L2 has no known signaling activity (4, 5). C5aR expression was originally described on myeloid cells including neutrophils, eosinophils, basophils, and monocytes. More recently, C5aR has also been found expressed on a variety of nonmyeloid cells in many organs, especially in the lung and liver (6, 7).

The up-regulation of C5aR in many inflammatory diseases, such as multiple organ failure (MOF) and sepsis, has been reported recently. Study on the biological effects and the signaling events of C5aR might provide much needed insights into the pathogenesis of inflammatory diseases (8). Unfortunately, because the natural C5aR expressing cells, especially neutrophils, are not easy to operate, experiments aimed at finding the function and the signaling events of C5aR are hard to carry out. To further research the bioactivities of C5aR, in this study, we transfected human C5aR into MOLT-4 cell line and established the anaphylatoxin C5aR expressing cell model MOLT-4. In the phenotype and function, the transfected cell line showed the same ability as neutrophil to induce ERK1/2 phosphorylation which was mediated by Gα16 subunit, Ca^{++} influx after interaction with C5a also happened in a time- and dose-dependent way. Our study here provided an excellent model for studying the biological activity of C5aR.

Materials and Methods

Materials

Expression plasmid pcDNA4.0 was purchased from Invitrogen.

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Received Nov 22, 2007. Accepted Dec 21, 2007.

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Restriction endonucleases *EcoR* I and *Xba* I were purchased from New England Biolads. Midipreps DNA purification kit and RT-PCR kit were purchased from Promega. Square Wave Pulse Generator ECM830 was purchased from BIO-RAD. Recombinant human C5a was purchased from Sigma-Aldrich. PE anti-human CD88 (C5a Receptor) mAb was purchased from BD PharMingen. Fluo-4 NM Calcium Assay Kits was purchased from Invitrogen. Rabbit anti-human phospho-ERK1/2 antibody Kit, anti ERK1/2 antibody and HRP-goat anti-rabbit IgG were purchased from Cell Signaling.

Cell culture and neutrophil isolation

A T-leukemia cell line with the expression of CD3 and MOLT-4, was preserved in our lab and cultured in RPMI 1640 medium supplemented with 10% (vol/vol) FBS, penicillin G (100 U/ml), and streptomycin (100 μg/ml). Neutrophils were isolated from whole blood of healthy volunteers using Ficoll-Paque gradient centrifugation and dextran sediment. Hypotonic red blood cell lysis was achieved using sterile H_2O. After isolation neutrophils were resuspended in Hanks' balanced salt solution (HBSS, Invitrogen) containing 1% fetal calf serum (FCS).

Generation of pcDNA4.0/C5aR constructs and sequencing

The C5aR cDNA was reversely transcripted from extracted total RNA of human neutrophils and were inserted into the plasmid pcDNA4.0. Briefly, the new PCR products encoding C5aR were amplified in the system containing the rTaq polymerase. PCR was performed using the following primers which amply a 1.1 kb section of the human neutrophil C5aR gene. 5' primer, 5'-CTG TCT AGA CCG CAC CAT GGC CCG CTC GTC GCT GAC C-3' and 3' primer, 5'-CCT GCT CTA GTT GGA CGA CAC TGG GAG ATC TGT G-3'. The purified products cut by *EcoR* I and *Xba* I were inserted into the pcDNA4.0 vector to construct the recombinant plasmid pcDNA4.0/C5aR. The recombinant plasmid was identified with the endonucleases cutting and sequencing (Invitrogen, Shanghai).

Transfection pcDNA4.0/C5aR into MOLT-4 cells

Recombinant pcDNA4.0/C5aR and pcDNA4.0 vector control were transfected into MOLT-4 cells by electroblot. Briefly, 1 × 10^7/ml MOLT-4 cells were suspended in 400 μl medium and put into the transfection Pocul. pcDNA4.0/C5aR or pcDNA4.0 were added at the concentration of 30 μg/ml, and 260 voltage, 20 ms pulse length were used to transfect the vectors into target cells (Square Wave Pulse Generator ECM830). Then after incubation at 4°C for 5 min, the cells were transferred into a 24-well plate in 1 ml/well, incubated at 37°C, in 5% CO_2 for 48 h. Finally, Zeocin from 100 to 500 μg/ml was used to select the stably transfcted cell line. The single colony of transfected MOLT-4 cells was picked out after subcloning.

Identification of C5aR expression by flow cytometry

Surface expression of C5aR (CD88) was detected by flow cytometry. Cells were washed and resuspended in buffer with fluorescent monoclonal antibody at 4°C for 30 min. Human neutrophile was used as positive control. Untransfected MOLT-4 cells were used as negative control.

Analysis of C5aR and Gα16 subunit expression by RT-PCR

Total RNA was isolated from 2.0-3.0 × 10^6 neutrophils, MOLT-4/C5aR and MOLT-4 cells respectively using the Trizol method (Life Technologies Inc., Rockville, MD). Didestion of any contaminating DNA was achieved by treatment of samples with RQ1 RNase-free Dnase (Promega, Inc., Madison, WI). Reverse transcription was performed with 5 μg RNA, using the Superscript II RNase H Reverse transcriptase (Gibco BRL, Inc., Grand Island, NY). The primers used for amplifying C5aR were the same as mentioned above. The primers used for amplifying Gα16 were as follows: 5' primer, 5'-AGC CAG GAC CCC TAT AAA GTG ACC ACG-3' and 3' primer, 5'-CGA GAA CGT GAT CGC CCT CAT CTA CC-3'. Gene expressions of C5aR and Gα16 in human neutrophil and Molt-4/C5aR were analyzed by RT-PCR. The RT-PCR product was confirmed by electrophoresis of samples in 1.2% agarose gel.

Analysis of C5a/C5aR signaling by Western blot

Different cells (about 5 × 10^6 each group) were lysed in lysis buffer (50 mM Tris-Cl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1 mM PMSF). Clarified lysates were resolved on 12% SDS-polyacrylamide gels and then transferred to Osmonics nitrocellulose membrane. Phospho-ERK1/2, ERK1/2 were detected by the specific rabbit anti-human phosphoplus ERK1/2 monoclonal and HRP-conjugated secondary antisera. The immunoblots were visualized by chemiluminescence with the ECL kit and the images detected in X-ray films were quantified by densitometric scanning using the Eagle Eye II still video system (Stratagene, La Jolla, CA, USA).

Ca^{++} influx after ligation of C5aR on MOLT-4/C5aR by recombinant human C5a

The cells were spined down and the medium was removed, then we resuspended the pellet in assay buffer to a density of 2.5 × 10^6 cells/ml, pipeted the resuspended cells. About 125,000 cells/50 μl were added into a 96-well plate, and incubated at 37°C for 60 min. Fifty microliters of the 2× dye loading solution was added into each well and incubated at 37°C for 30 min, and then an additional 30 min at room temperature. No washed and loaded 25 μl stimulants were added into each well by a multi-channel pipet, and read immediately by Ca^{++} fluorescence protocol (Perkin Elmer). Untransfected MOLT-4 cells were used as negative control.

Statistical analysis

Experimental conditions were compared by using Student's *t* test for single measurements or ANOVA for repeated measurements. *p* values less than 0.05 were considered significant.

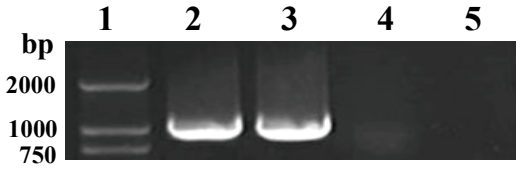

Figure 1. PCR amplification of C5aR gene. C5aR expression in transfected MOLT-4/C5aR cells was identified by RT-PCR. Neutrophils, and Molt-4/pcDNA4.0 as well as untransfected Molt-4 cells were used as positive and negative controls respectively. Lane 1, DNA marker; Lane 2, PCR products with neutrophils; Lane 3, PCR products with Molt-4/C5aR template; Lane 4, PCR products with Molt-4/pcDNA4.0 template; Lane 5, PCR products with untransfected Molt-4.

Results

Transfected Molt-4 cell line expressed the gene of human C5aR

Firstly, we investigated whether the mRNA of C5aR and Gα16 were expressed in MOLT-4/C5aR cell line. The agarose gel electrophoresis (AGE, 10 g/L) showed the size of the PCR products about 1.1 kp and 400 bp long respectively, which were definitely the same size as C5aR gene and Gα16 (Figures 1 and 2). Then, we investigated the cell surface expression of C5aR in MOLT-4/C5aR using flow cytometry. Figure 3 showed that the percentage of transfected C5aR expression on MOLT-4/C5aR was 97.38%, similar to physiological C5aR expression on neutrophils (97.45%); while untransfected MOLT-4 cells did not express C5aR mRNAand protein (Figures 1 and 3).

Human C5a induced ERK1/2 phosphorylation in MOLT-4/C5aR cell line

To test the functional activity of our cell line, we examined the ability of C5a to stimulate the ERK1/2 signaling pathway in MOLT-4/C5aR cells. MOLT-4/C5aR cells were incubated with 100 nM C5a. Stimulation of ERK1/2 phosphorylation was attained 5 min after C5a treatment. As shown in Figure 4, the Western blotting assay showed that a hybridization band was found in recombinant MOLT-4/C5aR cells and human neutrophils, while no band was detected in untransfected

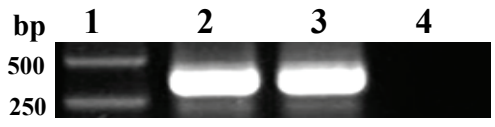

Figure 2. PCR amplification of Gα16 gene. Gα16 expression in transfected MOLT-4/C5aR cells was identified by RT-PCR. MOLT-4 and CHO cells were used as positive and negative control respectively. Lane 1, DNA marker; Lane 2, PCR products with Molt-4; Lane 3, PCR products with Molt-4/C5aR template; Lane 4, PCR products with CHO template.

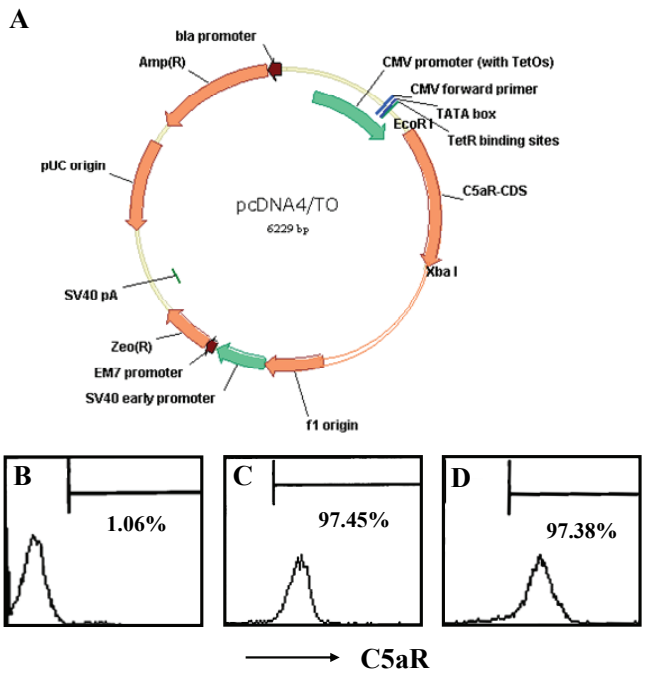

Figure 3. Construction and expression of pcDNA4.0/C5aR in MOLT-4 cells. (A) Schematic structure of C5aR-expressing pcDNA4.0/C5aR vector. The expression of C5aR on untransfected MOLT-4 cells (B), neutrophils (C) and transfected MOLT-4 cells (D) were identified by flow cytometry.

MOLT-4 cells.

Recombinant human C5a can induce Ca^{++} influx in MOLT-4/C5aR cell line

To test whether our cell model functionally worked well, the effects of C5a-C5aR interaction on Ca^{++} influx in MOLT-4/C5aR were detected. The cells were stimulated by recombined human C5a at dose of 0.002~2000 nM respectively. As shown in Figure 5, C5a could induce Ca^{++} influx in MOLT-4/C5aR cells in a time- and dose-dependent manner. Our data suggested that C5a-C5aR interaction could evoke the effect of Ca^{++} influx, while MOLT-4 cells have no such ability.

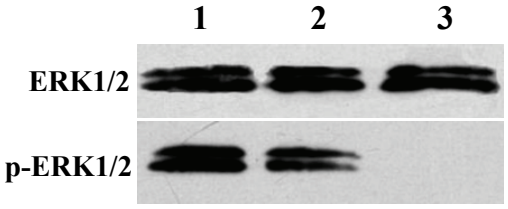

Figure 4. Western bloting detection of ERK1/2 induced by C5a in MOLT-4/C5aR cells. Lane 1, the detection of ERK1/2 in neutrophils; Lane 2, the detection of ERK1/2 in MOLT-4/C5aR cells; Lane 3, the detection of ERK1/2 in untransfected MOLT-4 cells as negative control.

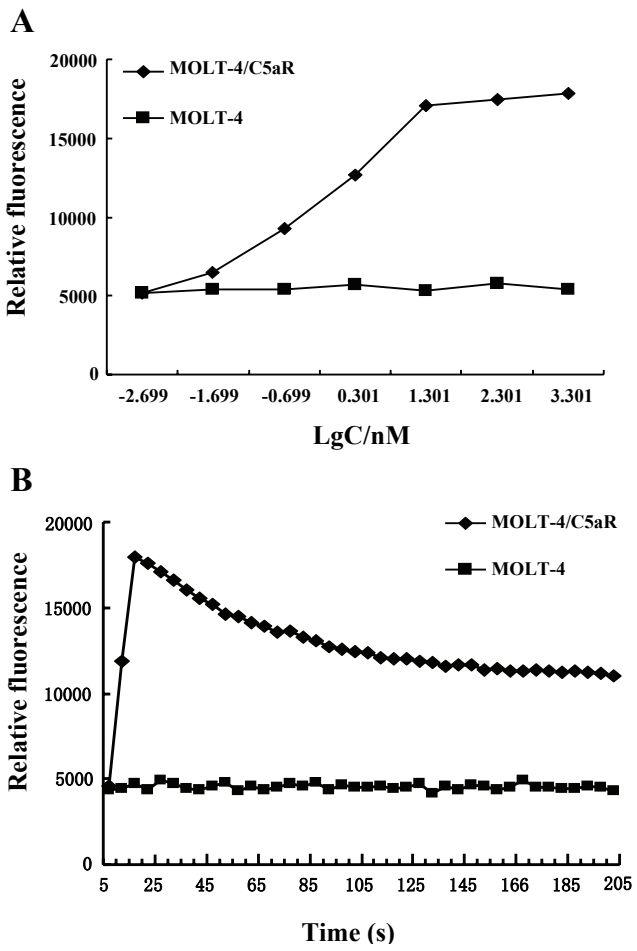

Figure 5. Fluo-4 NM Calcium Assay Kits detection of Ca^{++} influx by C5a stimulation in MOLT-4/C5aR and MOLT-4 cells. (A) The detection of Ca^{++} influx with different dose of C5a stimulation in peak value and untransfected MOLT-4 cells as negative control. (B) The detection of Ca^{++} influx with 20 nM C5a stimulation in MOLT-4/C5aR and untransfected MOLT-4 cells as negative control. The representative data were shown from three independent experiments with similar results.

Discussion

C5aR is a member of the rhodopsin family of seven transmembrane spanning G protein coupled receptors and binding of C5a to this receptor can induce cell-specific activation. C5aR expressed highly in neutrophils, macrophages and endothelial cells. C5aR is also expressed, albeit at a lower level, on non-myeloid cells such as epithelial, endothelial and smooth muscle cells in the liver and lung. It has been reported that C5a can induce the migration and increase phagocytic capability in macrophages and neutrophils (9). Interaction of C5a-C5aR induces many signaling events which mediate the biological effects of target cells (10).

The function of neutrophils is identified in many infection diseases (11-14). However, the isolation procedures of neutrophil are too complicated, time-consuming, and non-specific cell activation always happens. In addition，the survival time of neutrophil *in vitro* is very short. To solve this problem, we established a cell line which stably and functionally expressed C5aR. The expression of C5aR was detected by RT-PCR and flow cytometry; neutrophils and untransfectied MOLT-4 cells were used as positive and negative controls respectively. The results showed that C5aR expressed on MOLT-4/C5aR cell line but not on untransfected MOLT-4 cells.

To further study the function of transfected cell line, C5a was ligated on MOLT-4/C5aR cell and the signaling events were detected. As we know, C5a-C5aR binding could induce the downstream signal transduction which is dependent on Gαi and Gα16. As the MOLT-4 cells involve the C5aR associated Gα16 subunit, it provides a good tool for our study. Western blotting assay showed that ERK1/2 phosphorylation could be induced by C5a in MOLT-4/C5aR cells, suggesting that C5a ligation could induce the activation of downstream signaling events in MOLT-4/C5aR cells, so transfected cell line provided a new means for studying neutrophil signaling transduction.

In summary, we established a C5aR expressing cell line on human MOLT-4 cells, and the expression of C5aR was confirmed at both mRNA and protein levels. Ligation of C5aR on MOLT-4/C5aR cells by recombinant C5a induced Ca^{++} influx in a time- and dose-dependent manner, indicating that MOLT-4/C5aR cell line worked well and functionally useful for the study of the signaling events of C5a-C5aR. Considering the shortcoming of neutrophils and other C5aR expression cells, our system here ensures stable and high expression of C5aR, thus provided an excellent model for further research of the downstream signaling events and the biological effects mediated by C5aR.

Acknowledgements

This work was supported by the grants from the National Key Basic Research Program of China (2007CB512406), the National Natural Science Foundation of China (30571732) and National “863” Fund Grant of China (2006AA02A254).

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