Construction and Expression of a Novel Anti-CD14 Human-Mouse Chimeric Antibody Hm2F9

Di-Ying Shen, Bo-Tao Ning, Yong-Min Tang, and Si-Si Li

Anti-CD14 antibody can inhibit the lipopolysaccharide (LPS)–induced systemic inflammatory response syndrome in case of bacteremia or endotoxemia. To obtain chimeric anti-CD14 antibody, we constructed and expressed a novel chimeric antibody Hm2F9 composed of anti-CD14 single-chain fragment variable (scFv) and the Fc region (the hinge, CH2, and CH3 domains) of human IgG1 in Chinese hamster ovary (CHO) cells based on our previous study of scFv2F9. The Hm2F9 antibody, sized 150 kDa, retained the strong specific antigen-binding ability to the CD14 antigen with a comparable activity (the percentage of positive cells 99.07%) to its parental murine antibody 2F9 (the percentage of positive cells 98.86%). At the same time, Hm2F9 could manifestly block the binding of LPS to CD14, whose positive-cell percentage drops significantly with percentage of 98.63% (from 98.37% to 1.35%). The chimeric antibody Hm2F9 expressed in CHO cells retained high affinity to human CD14 and biological function to LPS.

Introduction

CD14 IS A 55-kDa GLYCOPROTEIN expressed on the surface of monocytes and macrophages (Ziegler-Heitbrock and Ulevitch, 1993), which serves as a receptor for complexes of lipopolysaccharide (LPS) of Gram-negative bacteria (Pugin *et al.*, 1993), peptidoglycan (Dziarski *et al.*, 1998), cell walls (Pugin *et al.*, 1994), lipoteichoic acids of Gram-positive bacteria, and lipoarabinomannan of mycobacteria (Verbon *et al.*, 2001). Recognition of these constituents by CD14 triggers signal transduction through Toll-like receptors (TLRs) 2 and 4, and leads to the release of proinflammatory cytokines, lipid mediators, coagulation factors, and reactive intermediates (Takeuchi *et al.*, 1999). This response that functions to eliminate localized and systemic microbial pathogens may lead to severe sepsis and septic shock (Axtelle and Pribble, 2001). In such cases, inhibiting the innate response to infection by blocking CD14 function may reduce organ pathology and mortality as a result of sepsis and septic shock. Animal studies have shown that anti-CD14 antibodies prevent the deleterious systemic responses that occur in association with LPS administration, and Lau *et al.* reported that rMil2 (anti-CD14 chimeric antibody) virtually abolished the *Escherichia coli*-induced cytokine responses. TNF, IL-1 β , IL-6, and IL-8 were reduced by 71%, 89%, 88%, and 100%, respectively (Schimke *et al.*, 1998; Verbon *et al.*, 2001; Lau *et al.*, 2013). Further, Verbon *et al.* (2001) have shown that treatment with an anti-CD14 antibody (IC14) inhibits the LPS-induced clinical syndrome of fever, chills, and myalgia and reduces cytokine release in healthy subjects.

A novel anti-CD14 single-chain antibody scFv_{2F9} derived from a murine monoclonal antibody ZCH-7-2F9 was generated in our lab previously (Tang *et al.*, 2007). The small size of single-chain fragment variable (scFv) and its rapid clearance from blood are suitable for some applications; however, it can also be a limitation for a wider range of therapeutic uses. And the absence of constant domains may limit the potential interaction with human host immune system (Firer and Gellerman, 2012). Thus, to produce larger, multivalent scFvs with longer serum half-life and effector function, one approach is to add multimerization domains to carboxy-terminus. Several minibody formats, such as scFv-Fc, have been proven to be ideal candidates for immunotherapy (Holliger and Hudson, 2005). Joining scFv to Fc fragment can produce disulfide bonds to yield dimeric molecules; the ability to bind to two antigens greatly increases their functional affinity and accumulates to a higher concentration in target positions (Cao *et al.*, 2009).

In this study, we described the construction, expression in Chinese hamster ovary (CHO) cells, and characterization of an anti-CD14 human-mouse chimeric antibody Hm2F9, where the 2F9 scFv is fused to the Fc domains of human IgG1.

Materials and Methods

Construction of expression vector

The Fc fragment (hinge, CH₂, and CH₃) of human IgG1 was amplified from human peripheral blood lymphocytes using primer pairs Fc-1 (5'-GCGGGAATTCGAGCCCAAATCTTGTA) and Fc-2 (5'-GCGCTCTAGACTACTGCGTGTAGTGGGTGGTT). The just-mentioned two underlined

sequences are corresponding to *EcoRI* and *XbaI* restriction endonucleases and are respectively homologous to plasmid sequences of the multiple cloning sites in pCk (an eukaryotic expressing vector constructed in our lab previously, containing a signal peptide sequence from a murine Ig κ -chain). Thirty-five cycles of polymerase chain reaction (PCR) were performed with incubations for 50 s at 94°C, 55 s at 55°C, and 1 min at 72°C. The purified PCR fragment was initially cloned into pGEM-T-easy vector (Promega) to generate TA-Fc. Then, the TA-Fc was digested with *EcoRI* and *XbaI* and the Fc domain of human IgG1 was cloned into backbone vector pCk to generate vector pHMCH3.

The cDNA coding for the scFv2F9 was amplified from the vector pSegtag2A/scFv2F9 (constructed in our lab previously) (Tang *et al.*, 2007) by primer pairs S1 (5'-GGCCAGCCGG CCCAGGTCCAAGTGCAGCACTTGTGA) and S2 (5'-GA ATTCTTTTATTTCCTCACTTGG). The amplified product was cleaved with *SfiI* and *EcoRI*, and cloned into *SfiI*-*EcoRI*-digested pHMCH3 vector to yield the expression vector pHMCH3-Hm2F9. Finally, the resulting vector was confirmed by restriction endonuclease digestion assay and DNA sequencing.

Transfection and mammalian expression

CHO cells (preserved in our lab) were grown in RPMI-1640 medium (Gibco) with 10% newborn calf serum at 37°C in a humidified atmosphere of 5% CO₂, and split when they reached 70–80% confluence by trypsinization. CHO cells were plated at a density of 1×10^5 cells per well in 24-well plates without penicillin and streptomycin for 24 h before transfection. CHO cells were transiently transfected with purified pHMCH3-Hm2F9 and pCk using LipofectamineTM 2000 Reagent (Invitrogen), according to the manufacturer's protocol. Positive clones were selected in the presence of 600 μ g/mL G418 (BBI), and single-cell clone was isolated by continuous limiting dilution. The highest antibody-producing clone was chosen by flow cytometric analysis (Becton Dickinson) and then grown in RPMI-1640 medium with 10% newborn calf serum. CHO-S-SFM II serum-free medium was used to produce Hm2F9.

Purification of the Hm2F9 antibody

The supernatant was harvested and concentrated with an Amicon ultra-15 centrifugal filter unit (Millipore) and then purified by a Bio-scaleTM mini affi-prep protein A cartridge (Bio-Rad). The eluted fractions were concentrated with an Amicon ultra-15 centrifugal filter unit (Millipore).

SDS-PAGE and western blot analysis

Purified Hm2F9 was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Sambrook *et al.* (Sambrook *et al.*, 2006) with 12% separation polyacrylamide gel and 5% condensed gel; then, one gel was stained with Coomassie blue R250 (Beyotime) and another gel was transferred to PVDF membrane (Beyotime). After blocking with milk for 2 h, the transferred membrane was incubated with 1:1000 dilution of HRP-conjugated goat anti-human IgG1 Fc antibody (Sigma) for 2 h. Specific binding was detected with ECL western blotting detection reagents (Beyotime).

Binding activity of Hm2F9 detected by flow cytometric analysis

Specific binding of Hm2F9 to CD14 was determined by flow cytometric analysis. Human peripheral blood mononuclear cells (PBMCs) expressing CD14 antigen and a negative control line, NALM-6 (preserved in our lab), were used. Approximately 5×10^5 cells were added to each tube and incubated with Hm2F9 and then bound protein was detected with fluorescein-labeled goat anti-human IgG Fc antibody and fluorescein-labeled goat anti-mouse IgG Fab (KPL). The fluorescent signals were analyzed by flow cytometry (FCM) using the CELLQUEST software.

Biological function of Hm2F9 detected by flow cytometric analysis

To determine the binding ability, blocking test against fluorescein-labeled LPS was carried out using the FCM. Four groups, including negative control, isotype group, LPS-FITC group, and Hm2F9+LPS-FITC group, were designed; each tube was labeled with A, B, C, and D. About 5×10^5 PBMCs were added to each tube suspended with 50 μ L of phosphate-buffered saline (PBS). Hm2F9 was added to tube D. After incubation at 4°C away from light for 30 min, tube D was rinsed with PBS two times. Finally, 2.5 μ L of γ 1-FITC was added to tube B and 2.5 μ L of LPS-FITC (Invitrogen) was added to tubes C and D, respectively. Each tube was mixed well and incubated at 4°C away from light for 30 min and rinsed and resuspended with 50 μ L PBS; fluorescence of FITC of each tube was detected through FCM.

Results

Construction of pHMCH3-Hm2F9 expression vector

We constructed a compact antibody (Fig. 1A), generated by joining the human IgG1-Fc fragment (Fig. 1B) with the scFv2F9 (Fig. 1C) and then cloned into a eukaryotic expressing vector pCk to construct vector pHMCH3-Hm2F9; the resulting vector was confirmed by restriction endonuclease digestion assay with *BamHI* and *XbaI* (Fig. 1D), and DNA sequencing.

Expression and purification of the Hm2F9 antibody

CHO cells were transfected with pHMCH3-Hm2F9 by lipofection. After selection under G418 and continuous limiting dilution, a stable and efficient expressing recombinant protein clone was isolated. The recombinant antibody was successfully purified by protein A affinity chromatograph. SDS-PAGE analysis revealed a single band of about 60 kDa corresponding to the predicted size (Fig. 2, lane 1) under reducing condition. Under nonreducing condition, the isolated products showed two bands at about 60 and 150 kDa (Fig. 2, lane 2). Western blotting showed the same result detected with HRP-conjugated goat anti-human IgG1 Fc antibody (Fig. 2, lane 3, 4).

Binding ability of the Hm2F9 antibody

The experiment used FCM to test the binding ability of IgG by indirect stain, and the antibody can partially bind to PBMCs through Fc receptor. To exclude the possibility of unspecific binding to PBMCs through Fc receptor, we added

FIG. 1. Construction of the scFv2F9-Fc fusion gene. (A) Schematic diagram of the scFv2F9-Fc fusion gene; (B) the Fc fragment (690 bp, lane 1) amplified from human peripheral blood lymphocytes; (C) the scFv2F9 fragment (745 bp, lane 1) amplified from the vector pSegtag2A/scFv2F9 (constructed in our lab previously); (D) the scFv2F9-Fc fragment (1429 bp, lane 1) digested with restriction endonucleases *Bam*HI and *Xba*I. Fc; scFv, single-chain fragment variable; lane M, different DNA marker.

γ 1-FITC as isotype control (Fig. 3B). We could see the partially unspecific binding of GAH-Fc-FITC to PBMCs in Figure 3D with low positive cell percentage of 11.86%. While the positive cell percentage of Hm2F9 plus GAH-Fc-FITC was significantly higher than the former, which was

99.48% and 98.73%. The percentage was only 1.77% on the negative control cell line NALM-6 (Fig. 3K).

Biological function of Hm2F9 detected by flow cytometric analysis

The results from Figure 4 showed that Hm2F9 could block the binding of LPS to CD14. After the blocking of Hm2F9 against LPS, the positive cell percentage dropped significantly with percentage of 98.63% (from 98.37% to 1.35%); the mean fluorescence intensity (MFI) also reduced manifestly with percentage of 92.57% (from 98.36 to 7.31) (Fig. 4).

Discussion

In our previous study, we reported a single-chain antibody against CD14 antigen, which was recombined through a linker (Gly₄Ser)₃ between the VH and VL of murine ZCH-7-2F9 antibody. ScFv—only 1/5 size of the parental antibody (Bird *et al.*, 1988) and retains the specific, monovalent, antigen-binding ability—is best suited to tumor targeting due to superior tissue penetration (Adams and Schier, 1999). However, their fast dissociation rates and rapid renal clearances limit the utilization of scFv. A number of modification strategies have been successfully employed to reduce the dissociation rates, showing significant increase in functional affinity. It would be desirable to be able to engineer an scFv into an IgG-like format that combines the affinity and specificity of the scFv with the bivalency and functional effectors of a complete immunoglobulin. The human IgG1 Fc region incorporation into the engineered antibody has several advantages. (1) The antibody can be produced in the form of an IgG-like dimeric molecule with

FIG. 2. SDS-PAGE and western blot analysis of Hm2F9. Protein ladder is shown in the left lane (lane M); Hm2F9 was run under reducing (lane 1) and nonreducing conditions (lane 2) stained with Coomassie blue R250, while western blot analysis of Hm2F9 was run under reducing (lane 3) and non-reducing conditions (lane 4) detected with HRP-conjugated goat anti-human IgG1 Fc antibody. SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

FIG. 3. Flow cytometry analysis of Hm2F9 fusion. Eight groups of PBMCs, including background control (A), isotype control (B), positive control parental murine antibody 2F9-FITC (C), culture supernatant from CHO-pCk plus GAH-Fc-FITC (D), culture supernatant from CHO-Hm2F9 plus GAH-Fc-FITC (E), 2F9 plus GAM-Fab-FITC (F), culture supernatant from CHO-pCk plus GAM-Fab-FITC (G), and culture supernatant from CHO-Hm2F9 plus GAM-Fab-FITC (H); three groups of negative control cell line (NALM-6), including isotype control (I), parental murine antibody 2F9-FITC (J), and culture supernatant from CHO-Hm2F9 (K). Two parameters as percentage of positive cells (%Gate) and mean fluorescence intensity (MFI Geo mean) were used to assess the binding activity of the Hm2F9 antibody. CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells.

FIG. 4. Biological function of Hm2F9 detected by flow cytometric analysis. Four groups, including negative control (A), isotype group (B), LPS-FITC group (C), and Hm2F9+LPS-FITC group (D), were designed. LPS, lipopolysaccharide; PBS, phosphate-buffered saline.

reduced immunogenicity and strengthened affinity to antigen; (2) this can increase the stability of the engineered antibody and prolong the serum half-life (>10 days) through interactions with the neonatal Fc receptor (Ono *et al.*, 2003; Wang *et al.*, 2012), which is particularly important in the nonequilibrium environment of vasculature and tissues; (3) a simpler route for purification through Protein A or Protein G affinity chromatography can be provided.

In this work, we have successfully constructed a eukaryotic expression vector, pHMCH3-Hm2F9, to express an scFv-Fc fusion protein (in which the scFv2F9 was fused to the Fc domains of human IgG1) in CHO cells (Fig. 1A). The Hm2F9 antibody exhibited an excellent biological activity. It retains the strong specific antigen-binding ability of its parental IgG, indicating that the dimeric structure may contribute to strong antigen-binding ability.

Western blot analysis has shown that the fusion protein is a monomer about 60 kDa under reducing conditions (Fig. 2, lane 1), while under nonreducing conditions it is a mixture of monomer and disulfide-bonded 150-kDa dimer (Fig. 2, lane 2). These results suggest that Hm2F9 is a dimeric antibody, which is important to the antigen-binding activity and the structural stability. We have also found that the Fc-mediated dimerization of purified Hm2F9 is not very stable. When the purified fusion protein is concentrated and stored, the forms of the fusion proteins are interconverted. The dimeric form decreased after the purified Hm2F9 was stored at 4°C longer than 2 weeks, and a novel 200-kDa multimer component was generated (data not shown). This observation was consistent with previous studies of an scFv-Fc fusion protein using anti-CD20 antibody (Wu *et al.*, 2001; Cang *et al.*, 2012). Another study of an anti-CEA antibody has shown a concentration dependence of scFv-diabody interconversion (Wu *et al.*, 1996). They thought that cross-pairing of the variable regions is the basis of the multimerization of the scFv-Fc constructs. In contrast, the Hm2F9 fusion protein that was concentrated from the harvested culture supernatant of CHO cells transfected with pHMCH3-Hm2F9 and grown in the CHO-S-SFM II serum-free medium without purification remains stable. The pH of the elution buffer of purification may affect the stability of the Hm2F9 dimer.

In conclusion, we have successfully constructed a universal vector system for the cloning and expression of recombinant antibody containing the human IgG1 Fc domain in CHO cells. Further, the chimeric antibody Hm2F9 expressed retains the comparable affinity to the antigen of its parental antibody, and has the biological function of blocking the LPS to CD14. Further study of its bioimmunological characteristics will be performed in the future. The current research warrants further development for its therapeutic purposes.

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Disclosure Statement

All the authors declare no conflict of interests.

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- Address correspondence to:
Bo-Tao Ning, MD, PhD
Division of Hematology-Oncology
The Children's Hospital of Zhejiang University
School of Medicine
Hangzhou 310003
China
E-mail: ningbotao@126.com
- Yong-Min Tang, MD
Division of Hematology-Oncology
The Children's Hospital of Zhejiang University
School of Medicine
Hangzhou 310003
China
E-mail: y_m_tang@zju.edu.cn

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