Veterinary Immunology and Immunopathology 136 (2010) 194–200

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Expression, purification and monoclonal antibodies preparation of recombinant equine mature interleukin-18

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ARTICLE INFO

Article history:
Received 12 December 2009
Received in revised form 14 February 2010
Accepted 1 March 2010

Keywords:
Interleukin-18 (IL-18)
Expression
Bioactivity
mAb
Equine

ABSTRACT

IL-18 is a cytokine originally discovered as an important modulator of immune responses and subsequently shown to be pleiotropic. In this report, we expressed the recombinant equine mature interleukin-18 (rEMIL-18) in *E. coli* and purified it by nickel affinity gel column chromatography. Purified rEMIL-18 had biological activity commensurate with recombinant human IL-18, as determined by its synergistic effect with recombinant human IL-12 (rhIL-12) on the induction of IFN-γ gene expression in equine peripheral blood mononuclear cells (PBMC). Following intraperitoneal (i.p.) immunization of BALB/c mice with rEMIL-18, nine monoclonal antibodies (mAbs) against equine interleukin-18 (EIL-18) were obtained and characterized. These mAbs recognized different epitopes on equine mature interleukin-18 (EMIL-18) protein based on their reactivity with two peptides containing different amino acid sequences and one of these mAbs has neutralization activity against EIL-18 in an IFN-γ-induction assay.

1. Introduction

Interleukin-18 (IL-18), originally characterized as interferon-γ (IFN-γ)-inducing factor (IGIF), was purified from the liver of mice that had been inoculated with *Propionibacterium acnes* and challenged with LPS to induce toxic shock (Okamura et al., 1995). IL-18 is synthesized as a biologically inactive precursor protein, pro-IL-18, which is 24 kDa in size (Okamura et al., 1995). This molecule is cleaved by the proteolytic enzyme caspase-1 (IL-1β converting enzyme, ICE) to produce bioactive mature IL-18 (18 kDa) (Ghayur et al., 1997; Gu et al., 1997; Ushio et al., 1996). Both the 24 kDa precursor and the 18 kDa mature protein are also cleaved by caspase-3 to an inactive form (Akita et al., 1997). IL-18 can induce IFN-γ production by T and B lymphocytes in synergy with IL-12 and increases cytolytic activity of NK cells (Micallef et al., 1996; Okamura et al., 1995; Yoshimoto et al., 1998). IL-18 plays an important role not only in Th1-dominated immunological responses, but also in antigen presentation, allergic inflammation, and mucosal immunity (Bombardieri et al., 2004; Foss et al., 2001; Kohno et al., 1997). These previous findings demonstrate that IL-18 is an important cytokine involved in the development of protective immune responses, particularly cell-mediated immune responses. Therefore, IL-18 may be clinically useful as an anti-microbial and/or anti-tumor agent (Muneta et al., 2000a). There are many expression systems available for the production of recombinant IL-18 protein. Among them, prokaryotic expression systems, eukaryotic expression systems and the baculovirus and insect cell expression

Abbreviations: rEMIL-18, recombinant equine mature interleukin-18; rhIL-12, recombinant human IL-12; EIL-18, equine interleukin-18; IFA, indirect immunofluorescence assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; AcEIL-18, equine IL-18 expression recombinant baculovirus.

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doi:10.1016/j.vetimm.2010.03.002

system have been used successfully for expression of recombinant IL-18 (Muneta et al., 2000a, 2003; Nagata et al., 2002; O'Donovan et al., 2004; Okamura et al., 1995; Ushio et al., 1996; Wu et al., 2004). The production of recombinant equine IL-18 (rEIL-18) has been attempted in mammalian cells and in an insect cell expression system (O'Donovan et al., 2004; Wu et al., 2004). However, these systems presented technical limitations associated with low yield and the production of a mixture of precursor and mature equine IL-18. Currently, there are no commercially available mAbs against equine IL-18 (EIL-18). In this report, we describe the efficient production and purification of rEMIL-18 using a prokaryotic expression system and nickel affinity gel column chromatography. Furthermore, we confirm the biological activity of rEMIL-18 and use the protein to successfully generate EIL-18-specific mAbs.

2. Materials and methods

2.1. Cloning of EIL-18 cDNA and construction of expression vector pET-EMIL-18

Total RNA was isolated with RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacture's instructions from healthy horse PBMC separated by Ficoll-Hypaque (Haoyang, Tianjin, China) following stimulation with 7.5 μg/ml ConA (Sigma, Saint Louis, MO, USA) for 12 h at 37 °C with 5% CO_2. Total RNA was reverse transcribed and the resulting cDNA used as template to amplify the full-length of EIL-18 DNA. Oligonucleotide primers were designed according to the entire coding region of EIL-18 sequences reported in GenBank (accession numbers: Y11131). The sequences of the EIL-18 forward and reverse primers were as follows: forward 5′-ATGGCTGCTGGACCAGTAGAAGAC-3′ and reverse 5′-CTAGTTCTGGTTTT GAACAGTG-3′. The PCR products were inserted into the pMD18-T vector (TaKaRa, Dalian, China) according to the manufacturer's instructions. The positive clones with the correct sequence were named as pMD-EIL-18. The leader peptide sequence of the EIL-18 gene was deleted using NdeI and XhoI site adapters with the forward primer 5′-GGAATTC*CATATG*TACTTTGGCAGGCTTGAAC-3′ and the reverse primer 5′-CCG*CTCGAG*TTAGTTCTGGTTTTGAACAGTG-3′, using the pMD-EIL-18 plasmid as the template. The amplified DNAs were digested with NdeI and XhoI (TaKaRa), and ligated into prokaryotic expression vector, pET-28a (+) (Novagen, Madison, WI, USA) at the NdeI/XhoI site. As a result, the recombinant vector contained equine mature IL-18 (EMIL-18) in frame with a His6-tag to allow purification by nickel affinity gel column chromatography. Clones with the correct inserts were identified using PCR. Finally, the nucleotide sequences of the selected clones were confirmed by sequencing (Shanghai Bioengineering, Shanghai, China). The positive clones were named as pET-EMIL-18.

2.2. Soluble expression and purification of rEMIL-18

The pET-EMIL-18 plasmid was transformed into *E. coli* strain BL21 (DE3) (Novagen). Transformed bacteria were plated on LB-agar supplemented with kanamycin (50 μg/ml) for selection pET-EMIL-18-containing transformants. The transformants were grown overnight at 37 °C in LB supplemented with kanamycin (50 μg/ml) and the bacteria were subcultured the following morning at 37 °C. When the OD_{600} reached 0.6, protein expression was induced with 0.4 mM IPTG overnight at 16 °C. The cells were harvested by centrifugation at 8800 × *g* (F0685 rotor, Beckman) for 10 min at 4 °C, and the bacterial pellet resuspended in 50 mM Tris–HCl (pH 8.0). The bacterial pellet was subjected to supersonic wave with a 5 s pulse at 5 s intervals for 15 min. Then the lysate was centrifuged at 8800 × *g* for 15 min at 4 °C, and the supernatant collected and stored at −20 °C. The bacterial suspension was analyzed by polyacrylamide gel electrophoresis (PAGE) using a 15% acrylamide gel containing 0.1% SDS. The protein bands were visualized by staining with Coomassie brilliant blue R250. Purification of rEMIL-18 was performed according to the protocol described previously (Liu et al., 2008). In brief, the stored supernatant was thawed on ice for 15 min before being mixed with an equal volume of binding buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole) (Novagen). The mixed solution that contained the unpurified rEMIL-18 fused with a His6-tag, was filtered through a 0.45 μm Supor® membrane (Pall-Gelman, NY, USA) and then loaded onto a 10 ml His Bind column (Novagen) which was previously charged with NiSO4 and equilibrated with binding buffer. After extensive washing with binding buffer containing 20 mM imidazole, the fusion protein was eluted with 6 ml elution buffer (20 mM Tris–HCl, pH 7.9, 0.5 M NaCl, 1 M imidazole). 6 ml fractions were collected and SDS-PAGE was performed to identify fractions containing protein with a molecular weight corresponding to His6-tagged rEMIL-18. The peak fractions containing the fusion protein were pooled and dialyzed overnight at 4 °C against PBS. Purified protein samples were stored at −20 °C.

2.3. Western blotting

The proteins on the gel were transferred onto a nitrocellulose membrane (Pall-Gelman). The membrane was blocked for 45 min at room temperature with PBS containing 1% BSA (Sigma), and then incubated for 1.5 h with rabbit antibody against human IL-18 (1:2500) (Boster, Wuhan, China), followed by incubation with an HRP-conjugated anti-rabbit IgG reagent (1:5000) (Sigma). Bound antibody was visualized using diaminobenezidene (DAB).

2.4. Biological activities of rEMIL-18

The biological properties of purified rEMIL-18 were examined using the IFN-γ induction assay previously described (Wu et al., 2004). In brief, equine PBMC were separated from erythrocytes and granulocytes by Percoll (Haoyang) density gradient separation of the peripheral blood of a healthy horse. PBMC (2×10^6/well) were suspended in 2 ml of RPMI 1640 containing 10% fetal bovine serum and 500 ng/ml rEMIL-18 or 500 ng/ml recombinant human IL-18 (rhIL-18) (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) as a positive control. The cells were cultivated with or without 100 pg/ml recombinant human interleukin-12 (rhIL-12) (R & D Systems, MN, USA) for 12 h