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NOTE

Expression of bovine (*Bos indicus*) interleukin-18 in *Escherichia coli* and its biological activity

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ABSTRACT

IL-18 modulates immune functions by inducing IFN-γ production and promoting Th1 immune responses. In the present study, we amplified and cloned the sequence (582 bp) encoding full-length bovine IL-18 from PBMC stimulated with PHA. The nucleotide and the deduced amino acid sequence of *Bos indicus* IL-18 showed an identity of 86–98% compared with IL-18 sequences of other ruminants. The insert was subcloned into a pET 32a vector and expressed in *Escherichia coli* as a fusion protein and the matured protein was obtained by caspase I treatment. The specificity of these proteins was confirmed by western blotting. The biological activity of the purified protein was analyzed by its ability to induce IFN-γ production in PBMC measured by ELISA and qPCR.

Key words biological activity, *Bos indicus*, expression, interleukin (IL-18).

IL-18, an enhancer of IL-12-driven Th1 immune response in hosts, is important in developing immunity against intracellular pathogens. IL-18 was originally termed IFN-γ-inducing factor because of its ability to induce IFN-γ production by Th1 cells and NK. Besides its IFN-γ-inducing activity, IL-18 has been found to have other biological functions, such as stimulation of Th1 cell development, upregulation of GM-CSF and IL-2Rα, decreased IL-10 expression in T cells and the modulation of immunoglobulin secretion by B cells (1–5). The pro-inflammatory activity of IL-18 is mediated by the production of nitric oxide, prostaglandins (6, 7) and inflammatory cytokines, such as TNF-α and IL-1β. IL-18 induces chemokines (IL-8, MIP-1, MCP-1) and recruits monocytes and macrophages to the site of infection (2, 8). IL-18 is secreted as a precursor molecule of 24 kDa which gets cleaved into an 18-kDa mature molecule which is mediated by caspase I (6). IL-18 from several domestic animals, such as buffalo, sheep, pig, horse, goat, antelope, ox and cow, has been characterized (7–12). In the present communication, we report the cloning and expression of the full-length bovine (*Bos indicus*) IL-18 cDNA in the prokaryotic system and its biological activity *in vitro*.

PBMC (5×10^6 cells) isolated from cattle (Indian local Hallikar breed, *Bos indicus*) by density gradient centrifugation using Histopaque 1.077 (Sigma India, Bangalore, India) grown in RPMI-1640 medium were stimulated with PHA (10 μg/ml) for 16 hr. Total RNA was extracted from stimulated cells using TRIZOL reagent (Invitrogen, Carlsbad, CA) and reverse transcription was carried out using an oligo dT primer. cDNA was used to amplify the bovine IL-18 sequence using IL-18L (5′ GGCGGAATTCATGGCTGCAGAACAAGT AGA 3′) and reverse IL-18-R (5′ GGCGGCG GCCGCCTAGTTCTG-GTTTTGAACAGT 3′) primers designed using the IL-18 sequence of *Bos taurus* (Accession No. EU276078). The

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List of Abbreviations: GM-CSF, granulocyte macrophage–colony stimulating factor; IFN-γ, interferon gamma; IL, interleukin; IL-18, interleukin-18; mAb, monoclonal antibody; MCP-1, monocyte chemoattractant protein; MIP-1, macrophage inflammatory protein; NK, natural killer cells; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; qPCR, quantitative polymerase chain reaction; TNF, tumor necrosis factor.

purified PCR product was digested with *Eco*RI and *Not*I and ligated to *Eco*RI- and *Not*I-digested pBSSK$^+$ vector. The cloned insert was subjected for sequencing in an automated DNA sequencer and the sequence data submitted to Gen Bank (Accession No. FJ985771). The nucleotide and the deduced amino acid sequence of the cloned gene was aligned with the sequences of different species of domestic animals and humans (available in the public database) using the Meg Align (DNASTAR version 1.82) program and phylogenetic analysis was carried out using MEGA version 2.1 (13).

The IL-18 sequence was subcloned into a pET 32a vector (Novagen USA) at *Eco*RI and *Not*I sites (pET-bo-IL18). The pET-IL18 plasmid was transformed into *E. coli* strain BL-21(DE3) PLys for protein expression. Cells were grown 37°C to $OD_{600} = 0.8$ and the expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 hr at 30°C. Bacterial lysate was used for SDS-PAGE analysis under denaturing conditions to visualize the protein band. Histidine-tagged fusion protein was purified on a nickel-chelating agarose affinity column according to the manufacturer's instructions under denaturing conditions (Invitrogen). Purified IL-18 was renatured by dialysis and the endotoxin was removed by treating with Triton X 114 using a two-phase extraction method (14) and the endotoxin quantity was measured by Limulus amebocyte lysate assay kit (LAL; Biocompare, Kidlington, UK). The purified proteins were analyzed by SDS-PAGE and western blotting using anti-human mAb (2 μg/ml; R&D Systems, Minneapolis, MN). The purified protein was treated with 1 U/20 ng protein human recombinant caspase I (Biovision, Mountain View, CA). The digested protein was used for measurement of biological activity.

The induction of IFN-γ from PBMC by IL-18 protein was used as an assay for IL-18 biological activity. Initially, the dose of IL-18 required for optimal stimulation of PBMC was determined by using various doses (50–400 ng) and the 200 ng dose was found to be optimal. PBMC isolated from cattle were stimulated with recombinant IL-18 protein (200 ng/ml) and IFN-γ gene expression in PBMC was then measured by real-time PCR. Total RNA extracted from PBMC using TRIZOL was used to synthesize cDNA using an oligo dT primer. PCR reaction mixture contained 1× SYBR green reaction mix (Applied Biosystems Carlsbad, CA) and 300 nm of both IFN-γ-specific primers IFN-L (5′ tggatatcatcaagcaagacatgtt 3′) and IFN-R (5′ aggtcattcatcactttgatgagttcat 3′). Real-time PCR was carried out using the ABI prism 7300 (Applied Biosystems) with conditions consisting of an initial step at 50°C for 2 min and 95°C for 2 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The transcribed RNA from the plasmid pcDNA-IFN-γ (containing full sequence of bovine IFN-γ) was used in the standard curve. The IFN-γ-RNA copy number was calculated from the quantified RNA (optic density at 260 nm) and serial dilutions of RNA (with 1×10^2 to 1×10^7 copies of RNA) were used for standard curve preparation.

Production of IFN-γ by bovine whole blood stimulated with bovine recombinant IL-18 was carried out using a bovine IFN-γ ELISA kit (Mabtech, Nacka Strand, Sweden) according to the manufacturer's instructions (15). Heparinized whole blood was incubated with 200 ng/ml purified IL18 in a six-well plate for 24 hr at 37°C in a CO_2 incubator. The plate was centrifuged at 800 $\times g$ for 10 min at 4°C. Supernatant (100 μl) was pipetted out from each well into a new 96-well plate. IFN-γ standard known amounts in duplicate were included and the quantity of IFN-γ in the unknown samples was estimated from the standard curve obtained from the known standards. Data are expressed as mean ± SEM.

Bovine IL-18 has an open reading frame of 582 bp encoding a protein of 193 amino acids with a predicted molecular weight of 24 kDa. The sequence alignment showed that bovine precursor IL-18 also has a signal peptide of 35 amino acids at the N-terminal followed by a mature peptide of 157 amino acids. Bovine IL-18 has high sequence homology with that of other ruminants, as it shares 94% and 92% amino acid identity with IL-18 of bubaline and nilgai, respectively (AC NO-AY401033, AY842499). It was shown to have 91%, 90%, 85% and 82% amino acid identity with carpine, ovine, porcine and equine IL-18 sequences, respectively (AY605263, AJ401033, U68701.1). However, it shares approximately 76–78% amino acid identity with human, fox, feline, and canine proteins (BC007461, EF581884, AB056857, Y11133). Phylogenetic analysis of the nucleotide sequences of IL-18 of different species of domestic animals is presented as an unrooted tree (Fig. 1). As is evident from the tree, IL-18 sequences of ovine, nilgai and buffalo are clustered together and are distinct from other species, including humans. Sequence comparison of IL-18 of *Bos indicus* with that of other species showed that Glu42 and Lys89 are conserved in all the species in which sequences were compared. These conserved amino acids are shown to be critical to binding

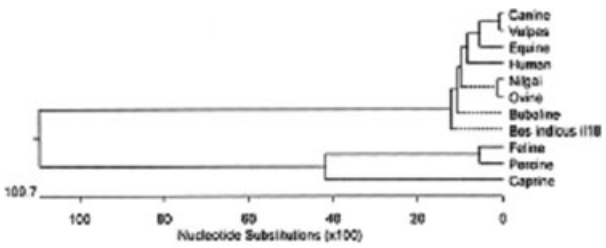

Fig. 1. Phylogenetic analysis of IL-18 cDNA sequences of different animals and human.

not only the IL-18 receptor molecule, but also the IL-18 binding protein, a naturally occurring protein which binds and neutralizes the biological activity of IL-18. Deletion mutants of IL-18 at Glu42 and Lys89 have lost their biological activity (16). Structure analysis of human IL-18 has revealed that three sites are important for receptor binding. From the aligned sequences, it was observed that the residues forming these critical sites were nearly well conserved in all the domestic animals. They include R49, D53, M69, D71 and D168, which form site I; K40, L41, K44, R94 and R140, which form site II; and 115K, 120K and D134, which form site III.

Bovine IL-18 insert was subcloned into a pET 32a vector, transformed into BL-21 (DE3) PLys cells and the expression of the gene was induced by IPTG. An additional band of 42 kDa was observed in cases where lysate from induced bacteria corresponded to the IL-18 fusion protein. Histidine-tagged protein was purified from the lysates by a nickel column. Precursor IL-18 undergoes proteolytic cleavage by IL-1β converting enzyme to generate a mature bioactive 18 kDa molecule. We expressed the IL-18 in soluble form and purified the protein by a nickel-nitrilotriacetic acid (Ni-NTA) column. Purified IL-18 was renatured by dialysis and the endotoxin was removed by treating with Triton-X114 and the endotoxin content in the purified product was found to be 0.09 EU/μg protein (endotoxin unit). The fusion protein of 42 kDa was expressed initially and the 18 kDa matured protein was obtained after caspase I. Fusion protein from the crude lysate, purified protein and the mature protein reacted with IL-18 anti-human monoclonal antibodies in an immunoblot assay (Fig. 2a,b). As IL-18 is a potent inducer of IFN-γ, we studied the level of IFN-γ-specific mRNA

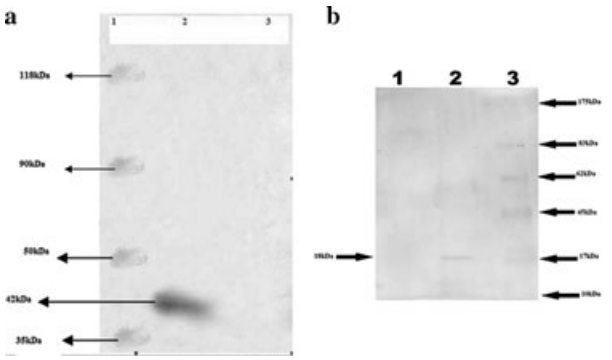

Fig. 2. (a) Immunoblotting of recombinant bovine precursor IL-18. Immunoblotting of recombinant IL-18 using anti-human IL-18 mAb. Lane 1, prestained molecular weight marker; lane 2, purified IL-18; lane 3, BL-21 cell control. (b) Immunoblotting of recombinant bovine mature IL-18 after treatment with caspase I. Immunoblotting of recombinant IL-18 using anti-human IL-18 mAb. Lane 1, BL-21, cell control; lane 2, mature IL-18; lane 3, prestained molecular weight marker.

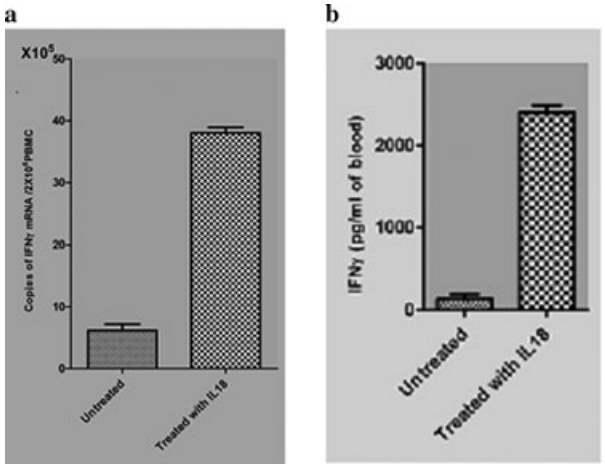

Fig. 3. (a) Biological activity of recombinant bovine IL-18. Briefly, 2×10^6 bovine PBMC were incubated with purified recombinant protein 200 ng/ml for 24 hr. Copies of bovine IFN-γ mRNA in cells were measured by real time RT-PCR. ■, PBMC; ▩, PBMC with mature bovine IL-18. (b) Biological activity of bovine IL-18. IFN-γ measured by whole blood ELISA. ■, Blood without IL-18 protein treatment; ▩, blood treated with mature bovine IL-18.

production in the presence of recombinant IL-18 protein by qPCR to assay the biological activity of IL-18. Quantitation of IFN-γ showed a 36-fold increase in copy number in IL-18-treated cells compared to the basal level (Fig. 3a). Quantification of IFN-γ secreted by the stimulated cells (whole blood) was carried out by ELISA and showed a 35-fold increase compared to the basal level (Fig. 3b). Quantities of IFN-γ were 120 pg/ml and 2400 pg/ml in the unstimulated and IL-18-stimulated cells, respectively. Stimulation of IFN-γ by IL-18 measured by qPCR is more or less similar to values observed in ELISA.

These studies have shown that IL-18 expressed in *E. coli* is biologically active. Recently, several researchers have used IL-18 as an effective vaccine adjuvant not only in mice, but also in non-human primates (4, 17). Biologically active bovine recombinant IL-18 protein expressed in the prokaryotic system will be useful in investigating the role of this cytokine as a therapeutic agent or a vaccine adjuvant in bovines.

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