Indian Journal of Experimental Biology
Vol. 51, May 2013, pp. 352-356

Cloning and expression of *Bos indicus* interleukin-4 in mammalian cells

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Received 4 October 2012; revised 25 February 2013

Dendritic cells (DC) which are located at the interface of innate and adaptive immunity are targets of infection by many RNA and DNA viruses. Advances in the *ex vivo* generation of monocyte derived non proliferating dendritic cells have been used for clinical application like immunotherapy. IL-4 cytokine plays essential role in the maturation and generation of DCs. *Bos indicus* interleukin 4 (boIL-4) 408 bp was amplified from PBMC's and cloned in pBSIIKS+ vector. The sequence analysis showed N terminal 69 bp signal sequence and one N-glycosylation site. The phylogenetic tree analysis showed that *Bos indicus* IL-4 is closely related to the ruminant IL-4 and least sharing of genetic line of human and mouse IL-4. The recombinant boIL-4 protein was expressed in CHO cells which secreted a 16 kDa protein which was confirmed by SDS PAGE and western blotting. The rec-boIL-4 protein proliferated the bovine PBMC's, decreased production of nitric oxide in antigen stimulated macrophages, and phagocytosed the micro particles confirming its activity on dendritic cells.

Keywords: Chinese hamster ovary cells, Cytokines, Dendritic cells, Interleukin-4, Nitric oxide

Cytokines are the growth factors which are produced in trace amounts and regulate the immune system. To understand the immune system there is a need to produce these cytokines in sufficient quantities and study their function in defined conditions. The challenge with cytokine research is not merely to understand the variation in biological functions but also to give the therapy to achieve the desired responses. Only handful of cytokines have been studied for their biological applications. IL-4 is one of them which have been researched as an adjuvant to use in combination with vaccines also as immunotherapy in cancer.

Interleukin 4 (IL-4) is a pleotropic cytokine affecting wide range of cell types in immune system[1]. It has many biological roles, including the stimulation of activated B-cell and T-cell proliferation, and the differentiation of CD4+ T-cells into Th2 cells. It is a key regulator in humoral and adaptive immunity[2]. IL-4 induces B-cell class switching to IgE, and up-regulates MHC class II production. IL-4 is also known as B cell stimulatory factor 1 and lymphocyte stimulatory factor 1[3]. It is a glycosylated polypeptide that contains three intra chain disulfide bridges and adopts a bundled four α-helix structure[4].

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Expression of IL-4 has been described in many species[5]. Expression systems using mammalian cells are much more likely to produce functional proteins. In addition the importance and application of IL-4 which is species specific, necessitates cloning and expression of IL-4 from each species. Also Indian breeds of cattle are boasted to be resistant to many diseases. Hence to find any difference in nucleotide or amino acid sequence along with the biological function the work was carried. In the presence of GM-CSF and IL-4, peripheral blood monocytes differentiate *in vitro* into cells with the phenotype and function of dendritic cells, which play a critical role in antigen presentation *in vivo*[6]. Hence attempts have been made to express bovine IL-4 in mammalian CHO cells as secretary protein.

Materials and Method

Cloning of Bovine IL-4 gene—Indian Hallikar cattle were purchased from local farms and maintained in animal experiment station; IVRI. Lymphocytes were cultured in RPMI1640 supplemented with 10 % foetal calf serum. The cells were stimulated with *PHA* (10 µg/mL) at 37 ºC for 18 h in 5% CO_2. Total RNA was extracted from these PBMC's using TRIZOL (Invitrogen) following manufacturer's instructions. First strand (cDNA) synthesis was performed using Super script III 200 units (Invitrogen), oligo$(dT)_{18}$ primer , at 50 ^oC for 60 min. Primer sequences used for amplification of bovine IL-4 were forward

5'GGCGGGTACCATGGGTCTCACCTACCAG3' and reverse 5'GGCGGCGGCCGCTCAACACTTGG AGTATTT CT 3'(Gen Bank accession number; NM_173921). The cDNA was subjected to PCR and products were separated by electrophoresis. Required band was purified using the Hiprep gel extraction kit (Himedia), ligated in KS^+ cloning vector (Invitrogen) by digesting with Kpn I and Not I. The clone was sequenced (Chromus biotech) and the nucleotide sequence analysis was carried using DNA STAR software.

Sub cloning—The bovine boIL—4 gene was released from KS^+ vector by digesting with Kpn I and Not I enzymes and ligated to pcDNA 3.1(Invitrogen) digested with same enzymes. Ligation mixture was transformed in *E. coli Top10* cells (Invitrogen) and cultured in LB media supplemented with ampicillin (50µg/mL). Clone was confirmed by releasing the IL4 gene.

Expression of bovine IL-4 protein—CHO-AA8 maintained in FMD research laboratory (IVRI; Bangalore) was used for expression of IL-4 protein. Cells grown to 80 % confluence were transfected with 5 µg of pcDNA boIL-4 plasmid DNA using cellfection (Roche) in OPTIMEM media and incubated at 37 ºC in 5% CO_2. Supernatant was collected at different time intervals and analyzed for the bovine IL-4 protein expression by measuring the absorbance at 280 nm. The culture supernatant protein was resolved in 15% SDS PAGE[7] and specificity of protein was confirmed by western blotting. Protein transferred PVDF membrane was treated with anti-mouse IL-4 monoclonal antibodies (Serotec) at 1:2000 dilutions and immunodetection was performed using 1:10,000 diluted Goat anti-mouse HRP conjugate (Sigma).

Precipitation and purification of rec boIL-4—The proteins secreted into the media were precipitated at 4 ^oC with ammonium sulphate at 75 % (v/v) saturation as per Abelson[8]. The precipitated proteins were pelleted by centrifugation at 28,000 rpm at 4 ^oC. The pellet was suspended in 1/10th of the original volume in PBS, *p*H 8.0. The protein was dialyzed against 1X PBS using 10 kDa cut off dialysis bag at 4 ^oC for 12 h with repeat change of PBS to remove salts. The protein was lyophilized and aliquots were stored at –70 ^oC for further use.

Biological assay for rec boIL-4

Proliferation of blood lymphocytes by MTT assay—Lymphocytes cell density adjusted to 1×10^6 cells/mL were seeded at rate of 100µL/well in 96-well flat bottom tissue culture plate. Cells were treated with recombinant boIL-4 protein at different concentration starting from 1 ng/well to10 µg/well. Positive control LPS 10 µg/well was included. Cells without treatment were included as negative control. At the end of the incubation, 20 µL of MTT (5 mg/mL) was added to each well and the cells were incubated for another 4 h at 37 °C in 5% CO_2. The resulting formozan crystals were dissolved by adding of MTT solubilization solution (200 µL). Absorbance was measured spectrophotometrically at 570 nm. Background absorbance of multi well plates was measured at 690 nm and subtracted from the 570 nm measurement[10]. Stimulation index (SI) was calculated according to the formula:

SI MTT = OD of stimulated culture/OD of unstimulated culture

where SI MTT is the stimulation index of MTT assay and OD is the optical density.

Inhibition of NO generation by macrophages—PBMC's cell density was adjusted to 1×10^6 cells per mL with 10 % RPMI1640 growth medium and seeded at rate of 200µL/ well in 96 well tissue culture plate. After 12 h of incubation at 37 °C the medium was replaced with fresh growth medium. Only adherent cells were incubated for another 96 h. On 4th day the adherent cells were stimulated with 200 µg/mL of heat inactivated gram negative antigen (*Klebsiella* treated at 65 °C for 1 h) with and without 100 ng/well of rec boIL-4 protein. Positive control LPS (10 µg/well) with and without rec boIL-4 was included. Adherent cells without any treatment were kept as negative control. After 24 h of stimulation, plate was centrifuged at 1500 rpm for 5 min and 80 µL supernatant was collected for nitrate estimation. Equal volume of Griess reagent (Sigma) (250 mg/mL) was added to cell culture supernatant, mixed well and incubated for 10 min at 37 °C and absorbance was measured at 550 nm.

Phagocytosis of microparticles by monocyte derived dendritic cells—PBMC's were collected from whole blood by the method described above and the cell density was adjusted to 1×10^6 cells/mL with 10% RPMI-1640 growth medium and seeded in 6 well plate at the rate 3 mL/well. After 6 h of incubation medium was changed keeping adherent cells. Adherent cells were grown in RPMI 1640 with 10 % FCS and supplemented with rec boIL-4 (100 ng/mL), and bovine GM-CSF 10 ng/mL. Every alternate day medium with cytokines was replaced and incubated for 7 days at 37 °C in CO_2 incubator. Cells were observed

under microscope for morphological changes. These monocyte derived dendritic cells on 7th day were treated with 1 µg of polystyrene latex beads (1 µm size and amine modified to florescent red dye) in RPMI 1640 with 5 % FCS. The incubated cells at 37 °C in 5% CO_2 for 12 h were washed with PBS and observed under the fluorescent microscope.

Results and Discussion

Amplification of bovine IL-4 gene—To establish dendritic cell culture in the laboratory it is necessary to produce the essential bio-reagents from the same species which may be available commercially at high cost and batch standardization is necessary to get repeated results. In the present work, boIL-4 gene of *Bos indicus* (Hallikar breed) was cloned and expressed as recombinant protein in mammalian expression system. The amplified bovine IL4 gene was of 408 bp which is reported in exotic breed of cattle[4]. The purified IL-4 gene fragment was cloned in KS^+ vector (Fig. 1).

Sequence analysis of bovine interleukin-4 gene—Bovine interleukin-4 gene sequence was blasted in http://blast.ncbi.nlm.nih.gov which aligned with IL-4 sequence of different species. However by DNA STAR MEGALIGN analysis by clustral method showed 99.8, 98.8, 94.4, 94.1, 85.1, 75.1 and 58.5 % nucleotide sequence identity with exotic cattle, buffalo, goat, sheep, pig, human and mouse respectively. Similar results were reported earlier[11]. The nucleotide sequence is 408 bp in length along with 69 bp of signal sequence. The amino acid sequence shows asperagine amino acid (N) at position 62 which is the most predicted N-Glycosylation site earlier reported[4]. Change in nucleotide sequence has not contributed to amino acid change. Clustering together of bovine, caprine and ovine sequences is worthy of specific discussion, as they all come under ruminants sharing same genetic line (Fig. 2).

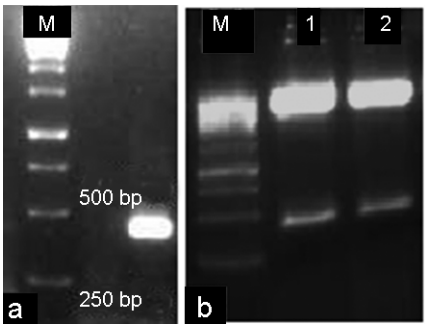

Fig. 1—Cloning of bovine IL-4 gene. (a) Agarose gel (1.2 %) analysis of bovine IL-4 gene amplified from PBMCs; Lane 1: bovine IL-4 amplicon of 408 bp; Lane M: 1 kb DNA ladder (Fermentas); (b) restriction enzyme analysis of pcDNA-IL-4 Plasmid DNA 1.2% agarose gel; LaneM: 1 kb DNA ladder (Fermentas) Lanes 1 and 2: pcDNA-IL-4 Plasmid DNA digested with Kpn I and Not I releasing 426 bp fragment.

(a)

5'ATGGGTCTCA CCTACCAGCT GATCCCAGTG CTGGTCTGCT TACTGGTATG
TACCAGTCAC TTCGTCCATG GACACAAGTG TGATATTACC TTAGCAGAGA
TCATCAAAAC GCTGAACATC CTCACAACGA GAAAGAATTC ATGCATGGAG
CTGCCTGTAG CAGACGTCTT TGCTGCCCCA AAGAACACAA CTGAGAAGGA
AACCTTCTGC AGGGTTGGAA TTGAGCTTAG GCGTATCTAC AGGAGCCACA
CGTGCTTGAA CAAATTCCTG GGCGGACTTG ACAGGAATCT CAACAGCTTG
GCAAGCAAGA CCTGTTCTGT GAATGAAGCC AAGACGAGCA CAAGTACGCT
GAAAGACCTC CTGGAAAGGC TAAAGACGAT **TATGAAGGAG AAATACTCCA**
AGTGTTGA 3'

(b)

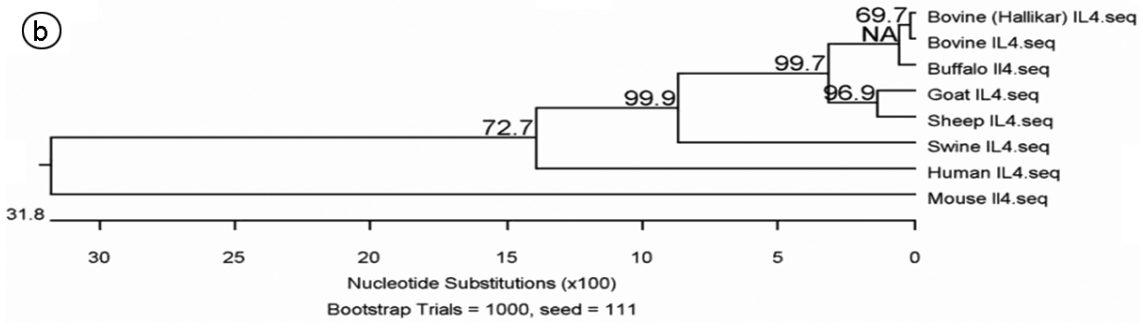

Fig. 2—(a) Nucleotide sequence of Bovine IL-4 cloned in pBSII KS^+ Vector (408 bp). Signal peptide1-69: Sequence shown underlined. Mature peptide 70408 bp nucleotide sequence codes for mature peptide (339 bp), (b): Homology comparison with different species and phylogenetic tree of aligned amino acid sequences.

Expression of bovine interleukin-4 gene in CHO-AA8 cell line—For the purpose of expression in mammalian system pcDNAboIL-4 plasmid DNA was transfected in CHO cells. CHO cell maintenance is useful in both applied and basic biomedical research[12]. The harvested culture supernatant protein concentration increased with increase in time, in control as well as in samples (Table 1). It is expected that the cells secrete their own proteins into the media in addition to foreign protein. In the control culture secretion of host proteins are seen. However in the boIL-4 samples the increase is 3 fold more when compared to control indicating along with host cell protein, boIL-4 foreign protein is also is expressed. Final yield of the crude protein at 48 hrs was 2.049 mg/mL, which is more than the reported expression systems[14].

Characterization of boIL-4 —Culture supernatant containing boIL-4 resolved in denaturing SDS PAGE 15% showed protein band of approximately 16 kDa (Fig. 3) which is absent in the control culture supernatant. The protein reacted to mouse IL-4 antibody whereas, back ground host proteins failed to react with IL-4 antibodies indicating mouse IL-4 antibodies have specifically detected the boIL-4 protein. The molecular weight matches with the reported size of 15-17 kDa *Bos tarus* IL-4 protein[15].

Biological activity of IL4 assay—PBMC's treated with recboIL-4 protein showed dose dependent trend proliferation (Fig. 4). Cells treated with 100 and 500 ng/well showed no significant difference in proliferation. Hence 100 ng/well was considered for other applications. Further recboIL-4 protein reduced the NO production by antigen treated macrophages (Fig. 5). Cells treated with LPS and *Klebsiella* alone show higher nitric oxide level. Cells treated with antigen along with boIL-4 show lower level of nitric oxide than antigen only treated cells. This may be due to IL4 down regulating function on macrophages lead to reduced NO production further bringing Th2 response[5].

Further monocyte derived dendritic cells were produced and grown for 7 days which are further used for research on vaccinology[16]. Here the cells showed

Table 1—Protein concentration at different time intervals

Sl. no	Time (h)	boIL-4 supernatant A 280	Control Supernatant A 280
1	24	0.1040	0.0909
2	36	0.1536	0.1213
3	48	0.2282	0.1542
4	72	0.3034	0.2136
5	96	0.3981	0.3096

CHO cells transfected pcIL-4 Plasmid DNA, the culture supernatant was analyzed for total protein concentration using Nano drop spectrophotometer at 280 nm absorbance.

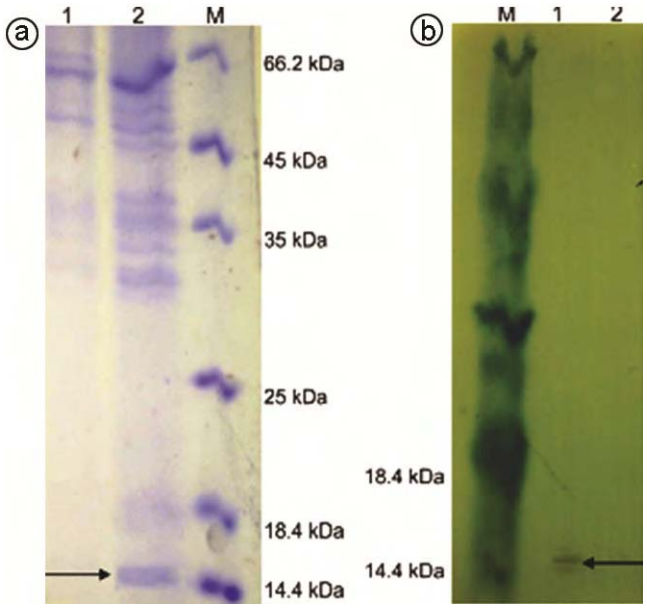

Fig. 3—SDS PAGE (15%) analysis of IL4 protein collected from supernatant of pcboIL-4 transfected CHO cells. (a) Lane 1: Vector transfected Cell control supernatant, Lane 2: bo IL-4 transfected supernatant (48hr), Lane M: protein molecular weight marker; (b): western Blot analysis of IL-4 protein, Lane M: Prestained protein Marker, Lane1: IL-4(16 kDa) protein reacting to monoclonal antibody, Lane2: Cell control supernatant.

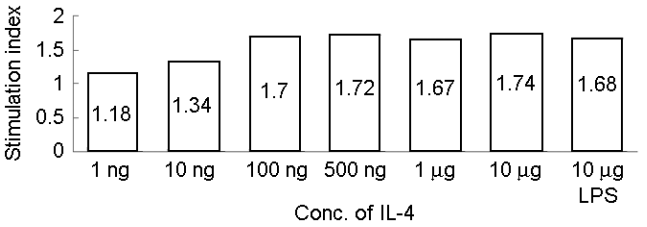

Fig. 4—Lymphocyte proliferation assay by MTT Method. X axis shows different concentration of Bovine IL-4. Y axis shows the stimulation index.

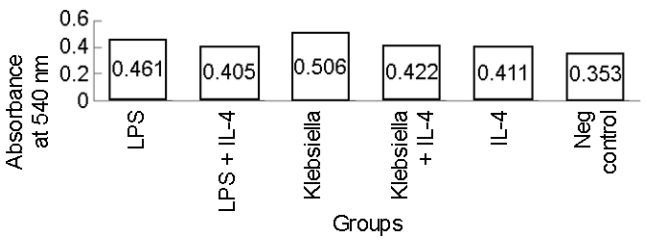

Fig. 5—Macrophage NO inhibition assay. X axis shows different group treatment of macrophages. Y axis shows the absorbance at 540 nm.

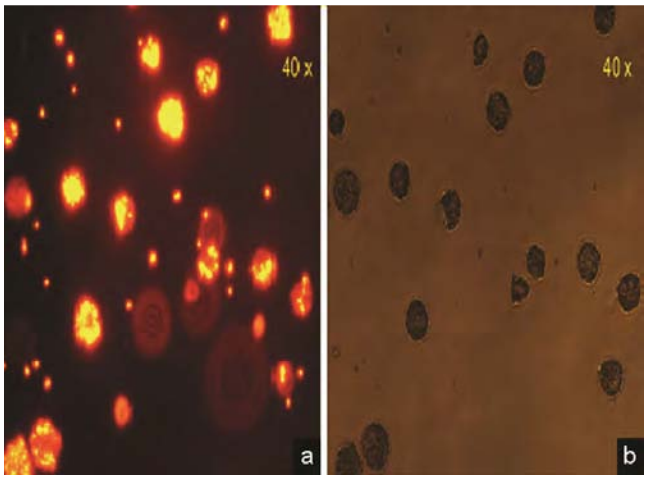

Fig. 6—Phagocytosis of fluorescent red latex beads by monocyte derived dendertic cells on 7th day. (a): Immature dendritic cells showing engulfed florescent red latex beads under green filter. (b) Immature dendritic cells engulfed florescent red latex beads under white light.

small dendrimers and adhered gently on the surface. Adherent monocyte converted dendertic cells when treated with microparticles engulfed microparticles (Fig. 6). Increased phagocytic activity of immature dendritic cells is reported and mature dendritic cells lose this on maturation[17]. Further work is in progress to identify the markers on these DCs along with antigen exposure. The produced recboIL-4 may be used as an adjuvant along with subunit or DNA vaccines.

Acknowledgment

Thanks are due to the Indian Veterinary Research Institute and Indian Council for Agricultural Research for financial assistance and facilities.

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