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Functional and Key Gene Expression Analyses of Chicken Monocyte-derived Dendritic Cells with Recombinant Interleukin 4

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ABSTRACT

The central role of dendritic cells (DCs) as bridging innate and adaptive immunity leads to the expanding use of these cells in the poultry vaccine studies. The most effective way to produce enough DCs is monocyte transformation by combined induction of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF).

In this study full length of chicken IL-4 (cIL-4) cDNA was cloned, characterized and expressed in *Escherichia coli*. Subsequently, the expressed IL-4 was used to induce monocytes-derived DCs (MDDC).

Typical features of DCs such as long membrane protrusions, apparently was dominant only four days after cytokine induction. Analyses of selected key genes' expression also confirmed that most of the monocytes shifted to DCs.

The findings of the present study strongly suggest that the cloning and expression of cIL-4 in the bacterial host without any codon optimization or other modifications could produce mature MDDC in six to seven days.

Keywords: Cloning; Gene expression; Interleukin-4; Monocytes

INTRODUCTION

Dendritic cells (DCs) in innate and adaptive immunity play a pivotal role. They initiate several immune responses including phagocytosis, antigen processing and presenting, exclusively T-cell activation and cytokines secretion. Nowadays, incremental trend in poultry industry is only possible through increasing research and development in chicken vaccines.[1] Shortages of DC in about two decades ago was eliminated by monocytes treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin4 (IL-4) cytokines. Peripheral blood mononuclear cells (PBMCs) contain a few number of DCs; however, they are accepted as a source of monocytes-derived DC (MDDC).[2]

This cellular transition from monocytes to DCs is facilitated by bacterial lipopolysaccharide (LPS), IL-10 and tumor necrosis factor alpha (TNF-α) treatment. Some reports showed Mitogen-activated protein kinase

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(MAPK) and especially nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) are involved in maturation and survival of MDDC.[3]

During the conversion process of monocytes to DCs, a lot of changes in the range but not type of gene expression occur.[4] Since DCs are professional antigen presenting cells, over-expression of both class I and II MHC was not surprising.[3] The longest lifespan of DCs happens within immature state and after meeting the inflammatory signals, they over-express pattern recognition receptors (PRRs),[5,6] and undergo maturation to antigen-presenting cells (APCs).[4]

Many of poultry vaccine researches have been conducted by applying in vitro DCs culture produced from PBMCs.[7] In most of the studies, preparation of chicken DC from monocytes were performed with a combination of both GM-CSF and IL-4.[8,9,10] Herein, first we expressed and purified cIL-4 in *E.coli* system and then used it as the only stimulus for transformation of the monocytes to DC.

MATERIALS AND METHODS

Regents and Media

Chicken PBMCs and MDDCs were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Biochrom, Berlin, Germany) supplemented with 2 mmol/L L-glutamine (Life Technologies, Carlsbad, California, USA), 50 U penicillin/mL and 50 mg streptomycin/mL, fetal calf serum (FBS), kanamycin, gentamycin, nonessential amino acids, sodium pyruvate, and b-mercaptoethanol (Sigma-Aldrich, Missouri, USA)

Cloning of Chicken IL-4 cDNA

Total RNA was extracted from chicken whole blood using Total RNA Isolation Kit (Denazist Asia, Iran) and transcripted into cDNA using RevertAid RT Reverse Transcription Kit (Thermo Fisher, Massachusetts, USA) according to the manufacturer's instructions.

PCR cloning and protein purification procedure were conducted as previous reports.[10,11] The full coding sequence of chicken IL-4(cIL4) was amplified using 5'-CGGGATCCCGGCCGCCATGGGCTCCTCACTGC C-3' as forward primers with Kozak and BamHI restriction site sequences and 5'-CGGGATCCCGGAATTCTCATCACTTATT TTTAGCTA-3' contained EcoRI restriction site and an additional stop codon as reverse primer. Taq DNA Polymerase Master Mix Red (Ampliqon, Denmark) was applied for amplification in cycling programs as follows: 94°C for 3 min, then 94°C for 60 s, 52°C for 55 s, and 72°C for 2 min for 45 cycles, followed by on cycle extension at 72°C for 10 min. The result of PCR with universal primers confirmed directional cloning. The construct was generated using prokaryote system pET28a vector, which has already polyhistidine-tag. The PCR products as well as the vector were digested with EcoRI and BamH1 to insert directionally cIL-4 cDNA in vector. The result of PCR with universal primers was used to confirm directional cloning.

Subsequently, confirmed constructs were transformed into competent *E.coli* DH5α. Individual constructs were isolated from transformants, screened for the correct size of inserted fragment by restriction endonuclease cleavage with BamHI and EcoRI. The constructs also was sequenced using universal primers to obtain full sequence of cIL-4 and to confirm correct directional cloning (Figure 1).

Expressed cIL-4 was purified and quantified using chromatography on Ni2+-NTA agarose column as previously described[12]. Protein concentrations were determined by the Bradford[13] method using Bio-Rad protein assay (Berkeley, California, USA) and bovine serum albumin (BSA) were used as standard.

Homology modeling of cIL-4

A homology model of chicken was generated using the ICM Homology build model option of ICM-Pro (Molsoft L.L.C. San Diego, USA) after its sequence aligned to the human IL-4 structure (PDB ID: 2B8U). Ramachandran plot for modeled cIL-4 also showed all the φ and ψ backbone dihedral angles for each amino acid were in favored and allowed regions.

In Vitro Experimental Design, Isolation of PBMC, and Production of MDDCs

We used three-week-old White Leghorn chicken from a local poultry farm. All procedures used in this study were approved by the animal ethics committee of research institute of modern biological techniques of university of Zanjan, Iran.

Heparinized blood samples of Chicken were pooled together and applied to PBMC isolation according to our previous report, with some modifications. Briefly, each pooled blood sample was diluted 1:2 in Dulbecco's phosphate buffered saline (DPBS) and then

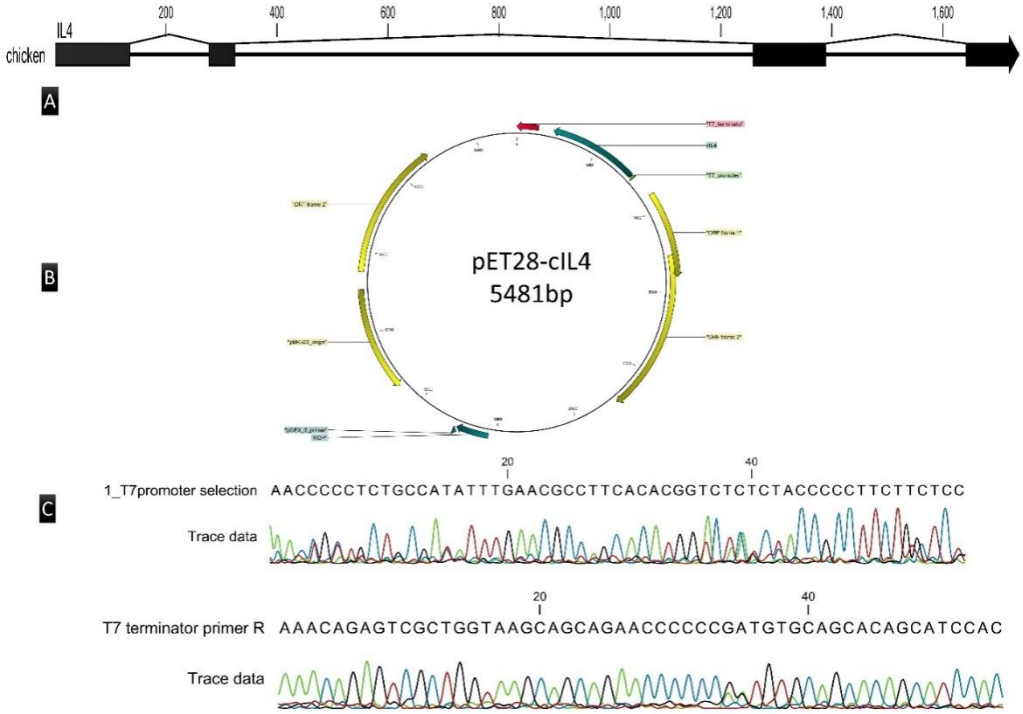

Figure 1. Cloning of chicken IL-4 into *E. coli*: A: The full length of IL-4 in chicken chromosome 13 is 1737bp including 4 exons. (Black boxes and lines represent exons and introns, respectively). B: 411 bp of coding sequence of IL-4 were recovered from cDNA and cloned into pET28a vectors directionally which included a his-tag sequence for target protein purification. C: To confirm directional cloning, the construct was sequenced using two universal primers (T7 promoter F and T7 terminator R).

layered on top of 15 mL Ficoll-Paque plus (Lympholyte, Zierikzee, the Netherlands) and finally centrifuged for 40 min in 1100 gravity (g) in 20˚C. The cloudy white layer was transferred to sterile 50 mL falcon tube then 10 mL PBS was added and finally PBMCs were precipitated by centrifuge in 500 g for 5 min in 4˚C. The isolated PBMC from each sample were then dispensed in 3 mL (10^7 PBMC/mL Roswell Park Memorial Institute medium-fetal bovine serum (RPMI-FBS) volumes into culture plates (3cm diameters) and incubated for 2 h in a 37˚C chamber containing 95% humidity and 5% CO2. Non-adherent lymphocytes were removed and viable monocytes were evaluated using trypan blue (≥99%). The monocytes were counted, transferred and seeded (at a density of 5×10^6 cells/mL medium) in polystyrene tissue culture plates containing RPMI medium with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL penicillin-streptomycine, and 1 µg/mL recombinant cIL-4 for 7 days and every day the medium was replaced with fresh DC medium. Monitoring and harvest of DCs were conducted at day 4 and 7.

RNA Purification and cDNA Synthesis

RNA was purified from about 10^6 monocytes (day1), immature DCs (day4) and MDDCs (day7) with RNA extraction kit (DENAzist Asia, Mashhad, Iran). Library of cDNA was synthesized using 1 mg of the isolated RNA and 10 pM/µL of oligo-dT primer from each cell population using RevertAid Reverse Transcriptase (ThermoFisher, Massachusetts, USA).

qPCR Assays for Cell Transformation Assay

Exon junction or intron-spanning primers were designed using AlleleID 7.83 (Premier Biosoft, USA) to avoid mispairing during PCR for gallus MHCII, ACTB, TGF-β, CD14, and IL-8 (Table 1).

Table 1. Designed quantitative PCR primers used for MDDCs gene expression. All primers were designed using AlleleID version7.83 software with option "exon junction primer search"

Accession Numbers	Forward/ Reverse Sequences	Amplicon
NM_205518.1	5'TTTCTTGGGTATGGAGTC3'	199
	5' GGGCAATGATCTTGATT3'	
NM_001044679.2	5'AGGTATCTGGTCAGGTATGTCTA3'	154
	5' CCACTTCATTCATTCGGTTCTC3'	
NM_205454.1	5'GGGAGGCCAGAGAAAGAA3'	100
	5'CCTGTCGGAAGTCAATGTAA3'	
NM_205498.1	5'CTCTGTCGCAAGGTAGGA3'	175
	5'ACCTCTCTTCCATCCTTTAG3'	
NM_001139478	5'GGGGGGACCTGGAGAAATA3'	153
	5'AGGAGAAGAACCGCAGGA3'	

MDDCs: monocytes-derived dendritic cells

Real time quantitative PCR was performed using 5×HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), according to manufacturer protocols. 20 mL of final volume of qPCR reaction contained 10 pM of each specific forward and reverse primers, 4mLEvaGreen master mix, and 1 mL cDNA template. Efficiency for each primer pair and also a single specific peak of melting analysis (included ramping from 50–99 ˚C, rising 0.5 ˚C/step and waiting 10 s for each step) approved specificity and sensitivity for all reactions (data are not shown).

Statistical Methods

Preprocessing analysis of the qPCR data were calculated using GenEX version 6 software (MultiD Co, Goteborg, Sweden). Normalization was conducted against reference gene ACTB and the minimum of value of cycle of threshold (CTs) between all four repeats. Relative differences in qPCR among each three groups including D1, D4 and D7 of experiment were measured using the Pfaffl method.[14]

The obtained results were expressed as means± standard deviation of means (SD). Relative data (Pfaffl-

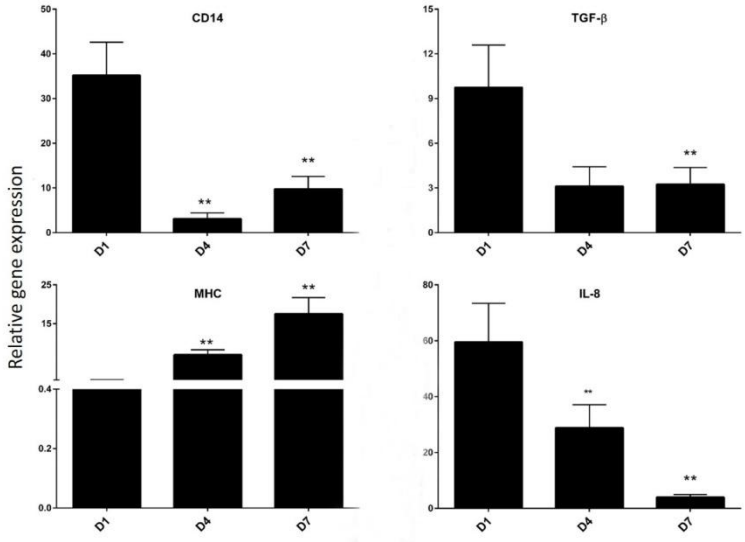

Figure 2. Changing in expression of four key genes during monocytes transforming to dendritic cells. Comparative transcription of CD14, TGF-β, MHC and IL-8 were measured in days 1 (D1), 4 (D4) and 7 (D7) after chicken monocytes were treated with chicken IL-4. Relative expression levels were normalized based on lowest value in 4 replications. Data is shown as means (±SD) from 4 individuals.

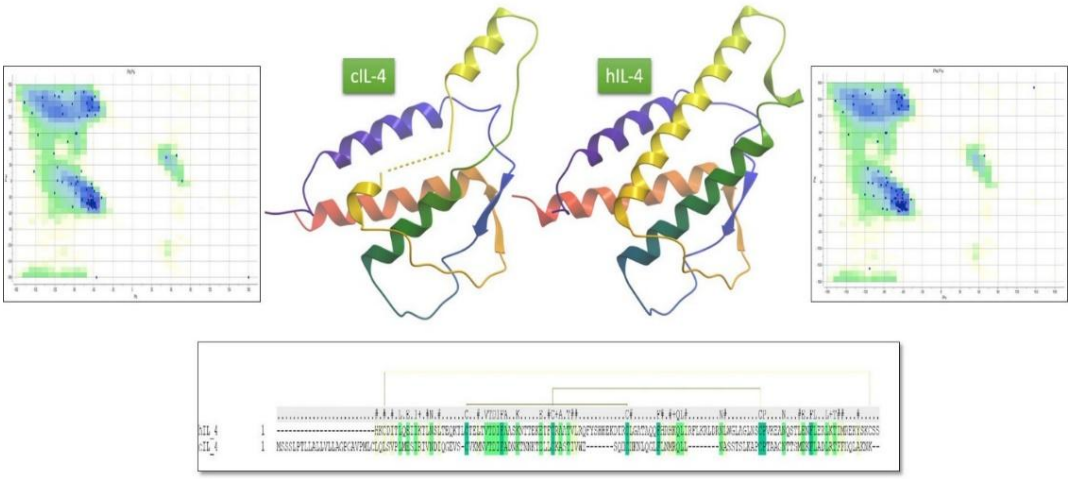

Figure 3. The complete spatial model of chicken IL-4. The human IL-4 crystal structure obtained from protein data bank: www.rcsb.org (accession number: 2B8U) at 1.8 Å resolution was considered to be the optimal template for cIL-4. The 3d file of human IL-4 was obtained from protein data bank: www.rcsb.org (accession number: 2B8U) and is represented in toggle ribbon format. The modeled chicken IL-4 was conducted using complete chicken sequenced coding sequence based on alignment with wild-type of human IL-4 as template in ICM-pro software. Ramachandran plots for both proteins were measured and confirmed accuracy of modeling.

based fold change) were logarithmic transformed to achieve analysis of variance (ANOVA) assumptions and LSD (least significant difference) were used to calculate pairwise comparison in SPSS version 23 (Corporation, New York, USA). A p-value≤0.05 was considered as significant.

RESULTS

In the present study, we expressed and investigated the production of recombinant cIL-4 to provide an adequate resource of MDDC for in vitro vaccine experiments. Analysis of fragments after digestion of pET28a-cIL-4 in agarose gel electrophoresis and sequencing the result using universal primers confirmed directional cloning in *E. coli* was successful (Figure 1).

Modeled recombinant cIL-4 also is confirmed identity in motifs by 3D alignment of chicken and human IL-4. The Ramachandran plot analysis revealed more than 96.5% of the phi-psi angles of residues were in most favored and allowed regions (Figure 3).

Recombinant cIL_4 were used to transform chicken monocytes to mature DCs. We first examined the cellular morphology of monocytes caused by incubation with recombinant cIL-4. Monocytes treated with cIL-4 showed pronounced dendritic morphology particularly cytoplasmic protrusions. This change in morphology indicated that all cellular processes needed for trigging monocytes transformation to DC was conducted by IL-4 only.

DISCUSSION

The main goal of this project was to produce some key biomolecules in poultry vaccine industry in order to achieve self-sufficiency in MDDCs production. GM-CSF and IL-4 are two convenient cytokines for monocytes transformation to chicken MDDCs. We used pET system in *E. coli* to produce recombinant chicken IL-4 and purified IL-4 was used for MDDCs production.

After 4 days of exposing chicken monocytes to cIL-4, monocytes with membrane protrusions were dominant in cell culture.

Chicken DC repository includes chicken epidermal DCs and DCs isolated from caecal tonsil.[15] To cover increasing demand of DC for vaccine researches, recombinant GM-CSF and IL-4 were used to generate chicken MDDC.[10] However, to the best our knowledge, there was no report about using only one cytokine to transform monocytes to DCs. It is widely accepted that 5 to 7 days GM-CSF and IL-4 are needed to generate DCs with special cluster of differentiation (CD) and

cytokines expression from monocytes in vitro.[16]

As DCs orchestrate both adaptive and innate responses, many efforts in vaccine industries have been conducted to use DCs directly as ex vivo immunotherapy or direct targeting of antigen to DCs surface receptors in vitro. Sentinel function of DCs for the recognition and processing of pathogens and a regulatory function to control both innate and adaptive immunity makes them the best target in vaccine development.[17]

It was well established that maturation of MDDC for differentiation need IL-4, GM-CSF, and also a secondary signal such as LPS, TNF-α or IL-3.[18,19] Herein, cellular transformation were used to obtain DC from chicken monocytes and MDCCs were confirmed with gene expression of CD14, TGF-β, MHCII and IL-8 during DC maturation.

CD14 is a part of pattern recognition receptors (PRRs) and it involves in bacterial LPS sensing. In particular, it acts as a co-receptor of toll-like receptor 4 (TLR4). Previous researches have shown that severe reduction or loss of expression of CD14 is a marker for DC maturation.[20] In this study we observed a decrease in CD14 gene expression in day 4 and 7 of monocytes culture; however, there was no steady trend in decline over time (Figure 2). As mature DCs are obtained only after exposure to activation factors like LPS, monocyte-conditioned medium, our MDDCs after 4 and 7 days expressed pronounced amount of CD14 which is absent in mature DCs.[21,22,23]

Treatment of monocytes with IL-4 over-expressed CD14 in day 4 and particularly day 7 which is marker for DCs maturation.[24-27]

Transformation of monocytes to DCs in absence of GM-CSF has been previously reported; however, the resulting cells showed less capacity to T-cell activation and a less secretion of progenitor cell inhibitory cytokines compared to DCs produced with both cytokines.[28]

We successfully cultured chicken monocytes and transformed it to immature MDDC using cIL-4. Subsequently, the cells were characterized according to some morphological and molecular properties.

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