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Nitric Oxide Inducing Function and Intracellular Movement of Chicken Interleukin-18 in Cultured Cells

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Abstract To evaluate the characteristics of chicken interleukin-18 (ChIL-18) in different forms *in vitro*, the ChIL-18 full-length gene (*ChIL-18-F*) and the ChIL-18 presumed mature protein gene (*ChIL-18-M*) were cloned and inserted into the eukaryotic expression vector pCI, to construct recombinant pCI-ChIL-18-F and pCI-ChIL-18-M. The recombinant plasmids were then transferred into chicken splenic lymphocytes (CSLs). Western blot showed that ChIL-18-F, with a molecular weight of 23.0 kDa, was produced in CSLs transfected by pCI-ChIL-18-F; ChIL-18-M, with a molecular weight of 19.5 kDa, was produced in CSLs transfected by pCI-ChIL-18-M. The nitric oxide (NO) level in the transfected CSLs and the culture medium at different time points was further examined under confocal microscopy using 4,5-diaminofluorescein staining. The results showed that both pCI-ChIL-18-F and pCI-ChIL-18-M groups showed significant increase in intracellular and extracellular NO production compared with pCI transfected control cells. These results suggest that both ChIL-18-F and ChIL-18-M could stimulate NO secretion in CSLs. To characterize the intracellular distribution of ChIL-18, *ChIL-18-F* and *ChIL-18-M* were each fused to the enhanced green fluorescent protein gene, and expressed in Vero cells. The results showed that the ChIL-18-F tended to the membranous region in Vero cells, while ChIL-18-M did not. This indicates that the N-terminal 27 amino acid peptide helped ChIL-18 target to Vero cell membranes.

Key words chicken interleukin-18; N-terminal peptide; nitric oxide; splenic lymphocyte; intracellular movement

The interleukin-18 gene (*IL-18*) was first cloned from propioibacteriumacnes-treated and lipopolysaccharide-treated mouse livers in 1995 by Okamura *et al.* [1]. In mammals, IL-18 is a pro-inflammatory cytokine with biological properties similar to those of IL-12. It acts in synergy with IL-12 to promote the production of Th1 cells [2]. As a member of the IL-1 family, the mammalian IL-18 was originally described as an interferon-γ (IFN-γ) inducing factor [3,4]. Other functions of IL-18 include the induction of IL-1β and tumor necrosis factor-α, the enhancement of natural killer cell cytotoxicity and neutrophil activity, as well as the enhancement of Fas ligand expression of Th1 cells [5,6]. As IL-1β, IL-18 is synthesized as a precursor molecule with a typical signal peptide and is cleaved by caspase-1, an intracellular protease, into an active cytokine [7]. Although mammalian IL-18 has been described in many materials, the study of ovipara IL-18 was very limited.

The chicken IL-18 gene (*ChIL-18*) was first cloned from the chicken macrophage cell line HD-11 in 2000 by Schneider *et al.* [8]. ChIL-18 can regulate IFN-γ expression in T cells [9,10], which upregulates the expression of MHC class I molecules, activates macrophages, and stimulates the secretion of nitrogen intermediates, such as nitric oxide (NO) [11]. The production of NO was always used to measure IFN-γ activity, which showed the activity of IL-18. One clone

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strategy for ChIL-18 from Xiaoshan chicken, a local Chinese breed, was established in our laboratory. The ChIL-18 full-length gene (*ChIL-18-F*) was amplified from splenic lymphocytes stimulated with lipopolysaccharide (GenBank accession No. AY628648). The full-length cDNA of Xiaoshan chicken *IL-18* consists of 591 bp and contains the complete open reading frame (ORF). Sequence comparisons revealed that the critical aspartate residue is conserved in ChIL-18, indicating that ChIL-18 may also be cleaved at this residue. Presumed mature ChIL-18 (ChIL-18-M) thus consists of 169 amino acid residues [8].

Human IL-18 was confirmed to be synthesized as a biological inactive precursor (pro-IL-18), which is cleaved by caspase-1 to form a mature cytokine with biological activities [12]. Schneider *et al.* have also shown that the recombinant ChIL-18-M expressed in bacteria can induce IFN-γ synthesis in primary cultured chicken spleen cells [8]. Puechler *et al.* have described a sensitive bioassay that is based on ChIL-18 inducing the release of IFN-γ in a permanent chicken cell line [13]. But there is no report about the eukaryotic expression of ChIL-18-F or ChIL-18-M, nor the NO secretion induction. It is not known whether ChIL-18-F is biologically active, or whether it is converted into ChIL-18-M through the action of caspase-1 in chicken splenic lymphocytes (CSLs).

In this report, the characteristics of ChIL-18-F and ChIL-18-M were analyzed in CSLs. NO secretion was used to evaluate the activities of ChIL-18-F and N-terminal truncated ChIL-18-M. The effects of N-terminal 27 amino acid peptide (NP) on the distribution and subcellular tropism of ChIL-18 were further traced by enhanced green fluorescent protein (EGFP) in Vero cells.

Materials and Methods

Plasmids and cell culture

Eukaryotic expression vector pCI was purchased from Promega (San Luis Obispo, USA). pT-ChIL-18-F containing the complete ORF of Xiaoshan chicken *IL-18* was constructed in our laboratory. Vero cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, USA) supplemented with 5% fetal bovine serum (FBS; Gibco), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 ºC with 5% CO_2.

Construction of recombinant eukaryotic expression plasmids

Assuming that the Xiaoshan ChIL-18-F protein is cleaved at the 27th amino acid residue after the conserved aspartate residue, we used polymerase chain reaction (PCR) to amplify a cDNA fragment encoding an N-terminal truncated form of ChIL-18. The ORF of *ChIL-18-F* and *ChIL-18-M* were amplified with primer pairs P1/P2 and P3/P2 (**Table 1**) from pT-ChIL-18-F respectively. The PCR fragments were each inserted into pCI after digestion with restriction enzymes, resulting in pCI-ChIL-18-F (containing the complete ORF of *ChIL-18*, *ChIL-18-F*) and pCI-ChIL-18-M (containing *ChIL-18-M*).

In another independent experiment, the ORF of *ChIL-18-F* and *ChIL-18-M* were amplified by primer pairs P1/P4 and P3/P4 respectively (**Table 1**). The two fragments were cloned into pEGFP-N1 vector (Clontech, Palo Alto, USA), producing pChIL-18-F-EGFP and pChIL-18-M-EGFP.

Transfection of *ChIL-18* with or without NP coding sequence into CSLs

The CSLs were aseptically isolated from 5-week old specific pathogen free chickens [14], and cultured in RPMI 1640 (HyClone, Logan, USA) supplemented with 5% FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 ºC with 5% CO_2. After 48 h, the CSLs were centrifugated at 1500 *g* for 10 min, and plated at 1×10^7 cells/ml in growth medium without antibiotics. The plasmids pCI-ChIL-18-F and pCI-ChIL-18-M were transferred into resuspended CSLs using Lipofectamine 2000 (Invitrogen, Carlsbad,

Table 1 Sequence of primers used in the experiments

Primer	Nucleotide sequence	Location	Restriction enzyme
P1	CGC*GAATTC*ATGAGCTGTGAAGAGATCG	1–19	*Eco*RI
P2	CCC*GTCGAC*TCATAGGTTGTGCCTTTCA	572–591	*Sal*I
P3	CGC*GAATTC*ATGGCCTTTTGTAAGGATAAAA	82–101	*Eco*RI
P4	CCC*GGATCC*GTAGGTTGTGCCTTTCATT	570–589	*Bam*HI

Restriction enzyme sites are in italic.

USA) as previously described [15].

NO secretion measurement

The transfected CSLs and culture media were collected at different time points for NO production analysis [16]. NO in transfected cells was observed under laser confocal microscopy 510 (Zeiss, Oberkochen, Germany) after staining with 4,5-diaminofluorescein (DAF-2). The nitrate reduction test was used to determine NO secretion with an NO testing kit (Institute of Jiancheng Biological Engineering, Nanjing, China) [17].

Recombinant ChIL-18 expression analysis

The transcriptions of target genes were determined by reverse transcription (RT)-PCR with primers P3 and P4 using the total RNA extracted from cells 24 h after transfection as the template. At different post-transfection time points, the cells were collected, alternately frozen and thawed three times, and centrifugated at 8000 *g*. The supernatant was collected. Western blot and enzyme-linked immunosorbent assay (ELISA) were carried out to determine protein expression, with rabbit anti-ChIL-18 serum as the primary antibody and horseradish peroxidase conjugated goat anti-rabbit IgG (Invitrogen) as the secondary antibody. The ChIL-18 antiserum was prepared in our laboratory from New Zealand white rabbits immunized with recombinant ChIL-18 protein expressed in *Escherichia coli*. The titer of the antibody was up to 1:12,800.

Subcellular tropism of recombinant ChIL-18 with or without NP in Vero cells

After the Vero cells were transfected with pChIL-18-F-EGFP or pChIL-18-M-EGFP, they were examined under laser confocal microscopy 510 for EGFP localization.

Results

NO secretion induced by recombinant ChIL-18

NO secretion in the transfected cells was observed under confocal microscopy 510 by staining with DAF-2, a high sensitivity fluorescent probe that can pass through the cellular membrane and shows a fluorescent loop after association with NO. **Fig. 1** shows that a small number of CSLs displayed fluorescence 3 h post-transfection, and the cell number increased 24 h post-transfection.

NO secretion in the transfected cells and the culture medium was studied further. As shown in **Figs. 2** and **3**, NO levels significantly increased in the pCI-ChIL-18-F and pCI-ChIL-18-M transfection groups compared with the pCI control, both in the cells and in the culture medium.

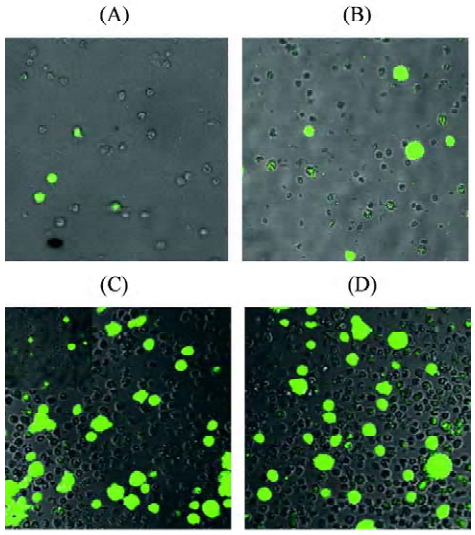

Fig. 1 Nitric oxide secretion in cultured chicken splenic lymphocytes (CSLs) transfected with pCI-ChIL-18-F and pCI-ChIL-18-M

Confocal micrographs show transfected CSLs transiently expressing different plasmids by staining with 4,5-diaminofluorescein. (A) pCI-ChIL-18-F, 3 h post-transfection. (B) pCI, 24 h post-transfection. (C) pCI-ChIL-18-F, 24 h post-transfection. (D) pCI-ChIL-18-M, 24 h post-transfection. Images were taken in the plane where maximum fluorescence appeared. Only a small number of CSLs showed fluorescence 3 h post-transfection (A), and the number of fluorescent cells was increased 24 h post-transfection (C,D). Magnification, 200×.

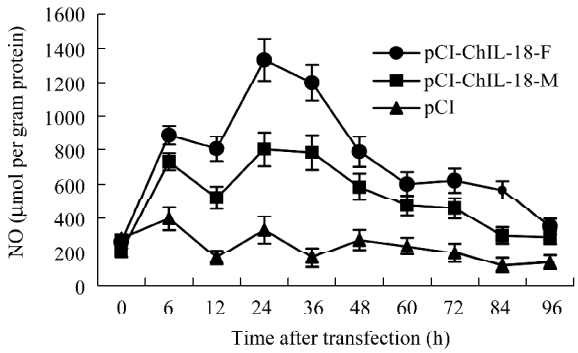

Fig. 2 Nitric oxide (NO) production in transfected chicken splenic lymphocytes (CSLs)

NO production was analyzed in the CSLs transfected with pCI-ChIL-18-F, pCI-ChIL-18-M or pCI. At different post-transfection time points, the cell pellets, after being alternately frozen and thawed three times, were used to determine NO output with the NO testing kit. The difference between the pCI-ChIL-18-F or pCI-ChIL-18-M transfection groups and the pCI control group was significant (P<0.05). Data were expressed in mean±SD (n=3).

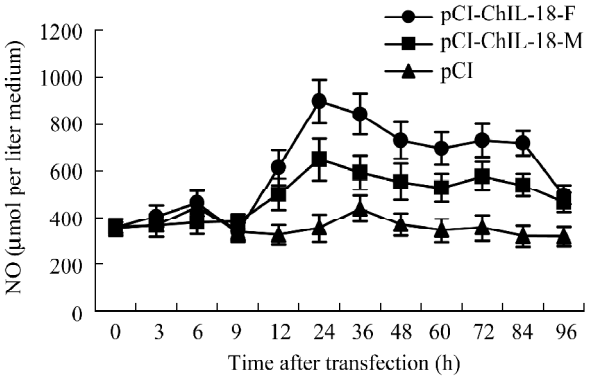

Fig. 3 Nitric oxide (NO) production in culture medium

NO concentration in the culture medium of chicken splenic lymphocytes transfected with pCI-ChIL-18-F, pCI-ChIL-18-F or pCI was determined with the NO testing kit. At different post-transfection time points, the NO output in the culture media were determined. The difference between pCI-ChIL-18-F or pCI-ChIL-18-M transfection groups and the pCI control group was significant (P<0.05). Data were expressed in mean±SD (n=3).

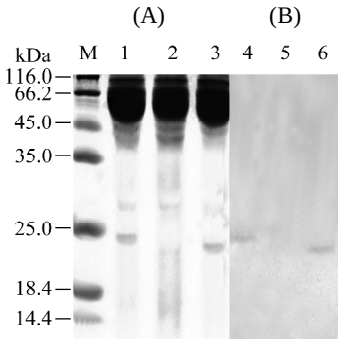

Fig. 4 Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) (A) and Western blot (B) analysis of *ChIL-18* expression in chicken splenic lymphocytes

The cells were collected 24 h post-transfection, lysed and electrophoresed in 15% SDS-PAGE, electro-transferred to nitrocellulose membrane and reacted with rabbit anti-ChIL-18 serum. M, protein marker; 1 and 4, chicken splenic lymphocytes transfected with pCI-ChIL-18-F; 3 and 6, chicken splenic lymphocytes transfected with pCI-ChIL-18-M; 2 and 5, negative controls.

NO levels peaked in the cells and the medium 24 h post-transfection. The NO production curve of both cultured cells and culture medium had a similar trend, except for cell samples 3 h and 9 h post-transfection. The results suggested that both ChIL-18-F and ChIL-18-M had the ability to induce NO secretion.

Recombinant ChIL-18 expression in CSLs

One fragment of DNA of approximately 500 bp was amplified by RT-PCR from each test group 24 h after transfection (data not shown). Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis could also detect specific protein bands (23.0 kDa for ChIL-18-F and 19.5 kDa for ChIL-18-M) at the same time point (**Fig. 4**). Only the band of 23.0 kDa could be seen in *ChIL-18-F* gene transfected CSLs, which suggested that the N-terminal 27 amino acid peptide of ChIL-18-F was not cleaved in CSLs. ChIL-18-F itself was biologically active.

To further examine the expression of ChIL-18-F and ChIL-18-M, the protein outputs at different time points were determined by ELISA. There was no significant difference on the expression between ChIL-18-F and ChIL-18-M (P>0.05). These results showed that ChIL-18-F and ChIL-18-M were both expressed in CSLs.

Expression and localization of recombinant ChIL-18-EGFP

To further investigate the intracellular localization of ChIL-18-F and ChIL-18-M, pChIL-18-F-EGFP and pChIL-18-M-EGFP were transfected into Vero cells. The fused proteins were expressed and observed by laser confocal microscopy 510 (**Fig. 5**). The transfected cells presented a diffusing and uniform fluorescence throughout the cytoplasm and nucleus 10 h post-transfection [**Fig. 5(A–C)**]. No specific intracellular targeting of the fluorescence was observed, and no accumulation occurred in the membranous region or in the peripheral vesicles. The results showed that ChIL-18 fused to EGFP with or without leader peptide was expressed in Vero cells. However, the distribution of fluorescence in the cells transfected with pChIL-18-F-EGFP was different to that produced in the cells transfected with pChIL-18-M-EGFP 24 h post-transfection. The fluorescence of ChIL-18-F appeared to clearly target to the membranous region of the Vero cells [**Fig. 5(F)**], but ChIL-18-M and EGFP did not [**Fig. 5(D, E)**]. This indicated that the N-terminal 27 amino acid peptide induced the intracellular movement of ChIL-18 from the cytoplasm and the nucleus to the membrane.

Discussion

In this work, the NO inducing ability of Xiaoshan ChIL-18-F and ChIL-18-M was determined. Our results also demonstrated that ChIL-18-F and ChIL-18-M are both active in NO induction (P<0.05). Although the full-length gene could help the expression of ChIL-18 in CSLs, there was no statistical difference in the expression level

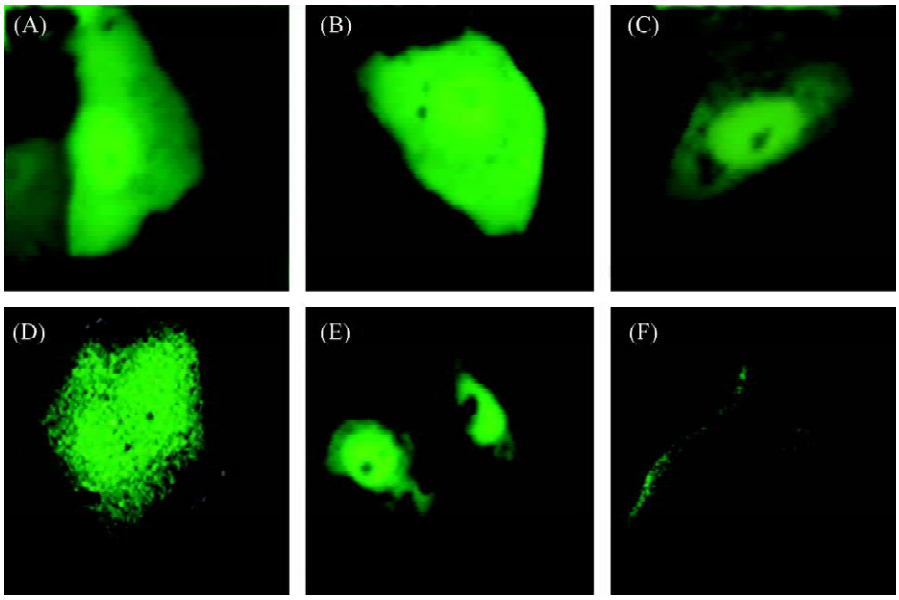

Fig. 5 Localization of enhanced green fluorescent protein (EGFP)-tagged ChIL-18 with or without N-terminal peptide (NP) in cultured Vero cells

Confocal micrographs of transfected Vero cells expressing EGFP-tagged ChIL-18-M (A), EGFP only (B) and EGFP-tagged ChIL-18-F (C) 10 h post-transfection. Confocal micrographs of transfected Vero cells expressing EGFP-tagged ChIL-18-M (D), EGFP only (E) and EGFP-tagged ChIL-18-F (F) 24 h post-transfection. Images were taken in the plane where maximum fluorescence appeared. Transfected cells presented uniform fluorescence throughout the cytoplasm and the nucleus 10 h post-transfection (A–C). But 24 h post-transfection, the fluorescence of the ChIL-18 with NP construct appeared targeted to the membranous region of the Vero cells (F). Magnification, 200×.

of ChIL-18-F and ChIL-18-M (P>0.05). These results indicated that the higher NO output might be related to the magnification effects of expression. On the other hand, as **Fig. 5** showed, the N-terminal 27 amino acid peptide of ChIL-18 was involved in ChIL-18 targeting the membrane of Vero cells. These results suggest that the N-terminal 27 amino acid peptide of ChIL-18 could combine to some membrane proteins of Vero cells.

As the proteins were expressed under the control of same promoter, the corresponding mRNAs should be produced at the same rate, as determined using *ChIL-18*-specific RT-PCR (data no shown). However, time-dependent fluorescence decrease was observed in cells transfected with pChIL-18-F-EGFP or pChIL-18-M-EGFP compared with those transfected with pEGFP-N1. There are several possible reasons: (1) the ChIL-18-EGFP fusion protein is somewhat unstable in Vero cells and might be rapidly degraded; (2) the Vero cell system might not be suitable for efficient expression of ChIL-18; or (3) there are multiple influencing factors for eukaryotic expression systems.

Both mammalian pro-IL-18 and ChIL-18 lack a signal peptide that usually directs proteins to the secretory apparatus of cells. Molecular mechanisms of the release of these two proteins are assumed to be very similar but are not completely understood yet [18]. In this work, we found that ChIL-18 enhances the NO secretion in transfected CSLs, which suggests the increase of IFN-γ output; the intracellular movements of recombinant ChIL-18-F and ChIL-18-M were different, which indicated that the N-terminal 27 amino acid peptide targeted ChIL-18 to the membrane of Vero cells; and ChIL-18-F showed similar functions to ChIL-18-M, which are different to mammalian IL-18. This work provides new facets to the previous description of ChIL-18.

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