Usefulness of recombinant His-ppIL-α and its specific Ab for the analysis of ppIL-1α function

Eri Sata

Nihon University Graduate School of Dentistry,

Major in Orthodontics

(Directors: Profs. Mitsuru Motoyoshi and Masatake Asano)

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This thesis is based on the following article and additional results regarding the immunofluorescence staining (Fig. 6).

Sata E, Takada L, Kaetsu R, Fukasawa M, Ohtsu M, Motoyoshi M, Asano M Establishment of enzyme-linked immunosorbent assay system against propiece interleukin-1α, Journal of Oral Science, in press

Abstract: Interleukin-1α **(**IL-1α) is produced inside the cells in its precursor form (pIL-1α) and enzymatically cleaved to generate mature (mIL-1α) and propiece IL-1α (ppIL-1α). Both pIL-1α and ppIL-1α are thought to be localized in the nucleus due to the presence of nuclear localizing signals (NLS). The research on the function of ppIL-1α is hampered due to the lack of ppIL-1α specific antibody (Ab). In the present study, we attempted to generate anti-ppIL-1α Ab using the recombinant histidine-tagged ppIL-1α (His-ppIL-1α) as an immunogen. The full length IL-1α open leading frame was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA of HeLa cells. The amplified fragment was subcloned to pTrc-His vector (pTrc-pIL-1α) and used as a template for the construction of pTrc-mIL-1α and pTrc-ppIL-1α vectors. Each vector was transformed to *E.coli* strain BL21 and used to generate histidine-tagged recombinant proteins. His-ppIL-1α (0.4 or 0.2 mg/0.5 ml) was mixed with 0.5 ml of Freund's complete adjuvant and subcutaneously injected (5 times with 2 weeks intervals) to two rabbits. After final immunization, total blood was collected from common carotid artery. The anti-serum was obtained and subjected to affinity purification. The reactivity and specificity of the Ab were examined by Western blotting. The antibody successfully recognized not only the His-ppIL-1α but also transfectant-derived green fluorescence protein (GFP)-tagged ppIL-1α as a 43 kDa band. GFP itself was not recognized by the Ab indicating the specific recognition of the Ab. The Ab was biotinylated and used to establish the sandwich enzyme-linked immunosorbent assay (ELISA) system. The serially diluted His-ppIL-1α

was applied to the ELISA system and the standard curve was obtained. The detection limit was 3.1 ng/ml. The cell lysates of GFP and GFP-ppIL-1α transfectants were applied to the ELISA system and revealed that GFP-ppIL-1α transfectants contained 19.2 ng/ml of GFP-ppIL-1α. To further examine the intracellular localization of ppIL-1α, immunofluorescence staining was performed. ppIL-1α cDNA was cloned to the mammalian expression vector pcDNA, transfected to HeLa cells and subjected to immunofluorescence staining. The fluorescence was mainly detected in the nuclei of ppIL-1α transfectant but not in mock transfectant. Slight fluorescence was also detected in the cytoplasm indicating the preferred localization of ppIL-1α in the nucleus.

The Ab and ELISA system allows the functional analysis of ppIL-1α. Moreover, His-ppIL-1α can be applied to in vitro experiments. These experimental tools may further expand our knowledge regarding the biological functions of ppIL-1α.

Keywords: alarmin, ELISA, propiece IL-1α

Introduction

The interleukin (IL)-1 family includes seven agonistic ligand members (1) among which, IL-1α was cloned in 1985 (2). IL-1α is initially synthesized as precursor (pIL-1α) intracellularly. pIL-1α is cleaved by calcium-dependent proteinase calpain to generate N-terminal half propiece IL-1α (ppIL-1α) and C-terminal half mature mIL-1α (1)(Fig. 1). The secretion of mIL-1α triggers various downstream inflammatory reactions through its receptors. On the otherhand, it is reported that pIL-1α and ppIL-1α preferentially localize in the nucleus because of its nuclear localizing sequence (NLS) and upregulate interleukin-6 (IL-6) and interleukin-8 (IL-8) expression by nuclear factor kappa beta (NF-κB) and activator protein -1 (AP-1) activation (3).

Alarmins are molecules that are rapidly released from damaged cells (4) and function as early warning signals to activate the innate and adaptive immune systems. pIL-1α is released from the damaged cells in response to various danger signals, such as hypoxic shock, oxidative stress, and heat shock (5) and recognized as an alarmin. Intriguingly, pIL-1α is released from necrotic cell but not from apoptotic cells (6, 7).

The functional differences between pIL-1α and mIL-1α have been investigated using recombinant proteins (8). The results indicated that both molecules can induce the secretion of IL-6 from the human lung carcinoma cell line, A549 (8). As described above, the roles of not only mIL-1α but pIL-1α is getting clearer. Since mIL-1α is generated by cleavage of pIL-1α, the amount of ppIL-1α

generated must be equivalent to that of mIL-1α released from the cells. However, the roles of ppIL-1α are unknown, because antibodies that can detect ppIL-1α have not been provided. For elucidation of biological functions of ppIL-1α, the recombinant ppIL-1α and its detection systems might be useful. In the present study, the ppIL-1α-specific Ab was obtained by immunizing rabbits with a histidine-tagged recombinant ppIL-1α (His-ppIL-1α) protein. The Ab successfully detected His-ppIL-1α and transfectant-derived ppIL-1α by western blotting. Using biotinylated Ab, the sandwitch enzyme-linked immunosorbent assay (ELISA) system was established. Moreover, the intracellular localization of ppIL-1α was examined by immunofluorescence staining using the Ab. The system allows the analysis of ppIL-1α functions in the extracellular milieu.

Materials and Methods

DNA construction

Full length human IL-1α open reading frame was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using total RNA of HeLa cells. The fragment was cloned to the bacterial expression vector pTrc/His (Invitrogen, Waltham, MA, USA) in a way of T/A cloning by adding the A residue by incubating with Taq polymerase (TaKaRa, Shiga, Japan) and designated as pTrc-pIL-1α vector (Fig. 2a). This vector was used as a template to generate ppIL-1α and mIL-1α, which were then cloned to the same vector and designated as pTrc-ppIL-1α (Fig. 2b) and pTrc-mIL-1α (Fig. 2c), respectively. The constructs were transformed to the *E.coli* strain BL21 (Agilent, Santa Clara, CA, USA) and used for protein synthesis. For transfection experiments, ppIL-1α was cloned to green fluorescence protein (GFP)-containing mammalian expression vector pEGFP-C3 vector (TaKaRa Bio USA, Mountain View, CA, USA) by infusion method (In-Fusion HD Cloning Kit, TaKaRa). The *Eco*RI site was added in the primers (pEGFP-ppIL-1α vector, Fig. 2d). The pEGFP-C3 vector was used as a control vector (Fig. 2e). The pIL-1α, ppIL-1α and mIL-1α was amplified by PCR and cloned to *Eco*RI site of the pcDNA3.1 (TaKaRa) expression vector (Fig. 2f, g, h), respectively. The sequences of all inserts were confirmed and all plasmids were transformed to *E.coli* strain DH5α.

Cell culture and transfection

HeLa cell was maintained with 10% fetal calf serum (FCS)-Dulbecco's minimal essential medium (DMEM) supplemented with penicillin and streptomycin (Sigma).

For transfection, the HeLa cells were seeded (5×10^5/35 mm dish) on the day before the experiments. Each vector was mixed with OPTI-MEM and Plus 3000 reagent (Invitrogen). The Lipofectamine 3000 reagent (Invitrogen) was diluted with OPTI-MEM. Each mixture was mixed and incubated for 15 min at room temperature (RT). The reaction mixture was added to the HeLa cultures and incubated for 18 h at 37℃. The transfectants were washed with PBS and cell lysates were collected using cell lysis buffer (1% Triton X-100/ 10 mM Tris-HCl buffer [pH 8.0]). The samples were centrifuged (10,000 × g, 3 min at 4℃) and supernatants were subjected to western blotting and sandwitch ELISA.

Recombinant protein synthesis and purification

Transformed bacterial cells were incubated in Luria-Bertani (LB) media for 18 h at 37℃. The cells were stimulated with 1 mM of Isopropyl- β- D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO, USA) for another 18 h and harvested (15,000 × *g*, 10 min) using a centrifuge (Beckman, Indianapolice, IN, USA). The cells were suspended in 8 M urea solution (8 M urea; 100 mM sodium dihydrogen phosphate; and 10 mM Tris-HCl [pH 6.3]) and sonicated with a sonication

apparatus (American Laboratory Trading, San Diego, CA, USA). The samples were centrifuged (15,000 × *g*, 10 min) and the supernatants were transferred to new tubes. The nickel-resin (GE Healthcare, Chicago, IL, USA) was added to the samples and rotated for 18 h at 4℃. The samples were centrifuged (10,000 × *g* for 5 min at 4℃) and the supernatants were discarded. The Ni-resin was extensively washed with 8 M urea buffer following which, Ni-resin-bound recombinant proteins were eluted with 250 mM imidazole solution (250 mM imidazole in 8 M urea solution). The obtained proteins were dialysed against phosphate buffered saline (PBS) and used for immunization.

Ab preparation

The prepared recombinant His-ppIL-1α (0.4 or 0.2 mg/0.5 ml) was mixed with 0.5 ml of Freund's complete adjuvant (Rockland Immunochemicals, Limerick, PA, USA). The mixture was subcutaneously injected (5 times with 2 weeks intervals) to two rabbits. After final immunization, total blood was collected from common carotid artery. The blood was incubated at 37℃ for 1 h and further incubated at 4℃ overnight. The samples were centrifuging at 1,200 × *g* for 15 min and the anti-sera was obtained. The anti-sera was subjected to affinity purification. His-ppIL-1α was dialysed against 0.1 M $NaHCO_3$ for over night. The sample was mixed with Affi Gel-10 (BioRad, Helicules, CA, USA) for 1 h at RT. The prepared affinity column was equilibrated by PBS and the

obtained anti-serum was applied. The bound-Ab was eluted with 3 M $MgCl_2$ and dialysed against PBS. This fraction was used as an affinity-purified Ab.

Western blotting

For Western blotting, cell lysates prepared from each transfectant was subjected to 15 or 12% SDS-PAGE. Western blotting was performed as described previously (9). Primary Abs against ppIL-1α (× 10,000) and anti-GFP Ab (× 1,000) (Abcam, Cambridge, UK) were diluted with 1% BSA-PBST (0.1% tween-20/PBS), while the secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) Ab (× 10,000)(Abcam) with 1% BSA-PBST. The bands were detected using an ECL kit (GE Healthcare, Tokyo, Japan).

ELISA

The 96-well plates were coated with anti-ppIL-1α Ab (× 1,000, 50 μl/well) for 18 h at RT. The plate was washed with 0.01% Tween 20-PBS using the automated plate washer (Bio-Rad). Non-specific binding was blocked by incubating the plate with 100 μl of 1% BSA-PBS for 1 h. The blocking buffer was discarded and the samples were applied and incubated for 1 h. After wash, biotinylated anti-ppIL-1α Ab (× 1,000, 50 μl/well) was added to each well and incubated for 1 h. The plates were washed and further incubated with streptavidine-HRP (× 1,000, 50 μl/well, Merck-Millipore,

Temecula, CA, USA) for 30 min. After wash, the color reaction was performed by adding SureBlue (SeraCare, Milford, MA, USA) (200 μl/well) and incubated for 10 min. The reaction was stopped by 2 M H_2SO_4 (50 μl/well) and the optic density (OD) was measured by microplate reader (450 nm) (Bio-Rad).

Immunofluoresence staining

Hela cells (5 × 10^4/cover slip/24 well plate) were transfected with pcDNA or pcDNA-ppIL-1α vectors using the Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham, MA, USA). The transfectants were fixed with 2% paraformaldehyde for 30 min at RT. The cells were washed with PBS and further incubated with 1% triton X-100 solution for 10 min. After wash, non-specific binding was blocked with 1% BSA-PBS for 30 min. The cells were incubated with anti-ppIL-1α Ab (× 100 dilution with 1% BSA-PBS) for 18 h. The cells were washed with PBS and further incubated with goat-anti-rabbit IgG Ab (Jackson Immuno Reserch, Westgrove, PA, USA) for 1 h. The cells were extensively washed with PBS and mounted on glass slides using Fluoroshield with DAPI (Gene Tex, California, USA). Images were taken using a fluorescence microscope (All-in-one Fluorescence Microscope, Keyens, Osaka, Japan).

Statistical analysis

As a result of normality test using statistical software, no normal distribution was found. Therefore,

nonparametric Mann-Whitney *U*-test was performed (Fig. 5b, $n = 7$, each group). Results are presented as mean ± standard deviation (SD). * p value < 0.05 was considered as statistically significant.

Results

Purification of His-ppIL-1α and His-mIL-1α

The purity of His-ppIL-1α and His-mIL-1α was confirmed with Coomasie brilliant blue (CBB) staining. As shown in Fig. 3, each protein was detected as 16 and 17 kDa single bands. His-ppIL-1α migrated slightly faster than His-mIL-1α, and was used for rabbit immunization.

ppIL-1α-specific Ab

The rabbit antisera obtained by immunizing His-ppIL-1α was subjected to affinity purification. The reactivity of the purified Ab was examined with Western blotting. The cell lysates obtained from GFP- and GFP-ppIL-1α transfectants were loaded onto 15% SDS-PAGE and subjected to Western blotting. When the membrane was blotted with anti-GFP Ab, both GFP and GFP-ppIL-1α were detected as 27 and 43 KDa bands (Fig. 4a), respectively. On the other hand, only GFP-ppIL-1α was detected by anti-ppIL-1α Ab (Fig. 4a). Moreover, the cell lysates were prepared from pcDNA (mock), pcDNA-mIL-1α (m), pcDNA-pIL-1α (p) and pcDNA-ppIL-1α (pp) transfectants and subjected to Western blotting (Fig. 4b). As shown, only pIL-1α and ppIL-1α, but mIL-1α, were detected as 34 and 17 kDa bands (Fig. 4b). The results indicated the specific recognition of pIL-1α and ppIL-1α by the Ab.

ELISA

The standard curve was drawn by applying serially diluted His-ppIL-1α (Fig. 5a). The detection limit of the system was 3.1 ng/ml. Using this system, the amount of ppIL-1α in the cell lysates of GFP- and GFP-ppIL-1α-transfectants was measured. As shown in Fig. 5b, GFP-ppIL-1α transfectants contained 19.2 ng/ml of GFP-ppIL-1α.

Immunofluoresence staining

The pcDNA and pcDNA-ppIL-1α transfectants were subjected to immunofluorescence staining. As shown in Fig. 6, no fluorescence was detected in the pcDNA transfectant. Extreamly strong fluorescence was detected in the nuclei of the pcDNA-ppIL-1α transfectant (Fig. 6). Slight fluorescence was also detected in the cytoplasms of the pcDNA-ppIL-1α transfectant.

Discussion

In the present study, the ppIL-1α-specific Ab was obtained using recombinant His-ppIL-1α generated in *E.coli*. The Ab was biotinylated and the sandwitch ELISA system against ppIL-1α was established. Anti-ppIL-1α Ab detected GFP-ppIL-1α but not GFP (Fig. 4) by Western blotting indicating the specific detecteion of ppIL-1α. Up to date, the monoclonal Ab against pIL-1α was generated by Carlsen et al (10). Moreover, Ross et al. reported the identification of nuclear pIL-1α with the monoclonal Ab via flow cytometry (11). However, ppIL-1α ELISA system have never been established.

Most of the research on IL-1α is focused on mIL-1α because it can be secreted outside the cell and evoke immunological reactions. For this reason, the reagents that can detect mIL-1α, but not ppIL-1α, are more commonly available. The biological functions of ppIL-1α in the nucleus have been investigated due to the existence of NLS. Werman et al. demonstrated that ppIL-1α functions as a transcriptional regulator (3). In addition, ppIL-1α can affect the cell growth of various cancer cell lines (12, 13). These studies were conducted mainly by transfection method with the tagged-ppIL-1α and the detection of them can be achieved by Ab against tag molecules. The specific Ab against ppIL-1α might lead to the direct detection of the molecule and facilitate the elucidation of ppIL-1α functions in the body.

The factors released in response to dangerous stimuli are called as alarmins (4). These

molecules inform the surrounding cells about the hazardous situations leading to the occurrence of various reactions. Although IL-1α is recognized as an alarmin (14), it is not elucidated whether ppIL-1α per se can act as an alarmin. Immunofluorescence staining revealed the preferential nuclear localization of ppIL-1α (Fig. 6). The pcDNA-ppIL-1α transfectant can be cultured under the stress conditions such as hypoxic or heat environment. The ELISA system established in this study might allow the measurement of ppIL-1α released from these cells. Further experiments are needed to elucidate the biological functions of ppIL-1α.

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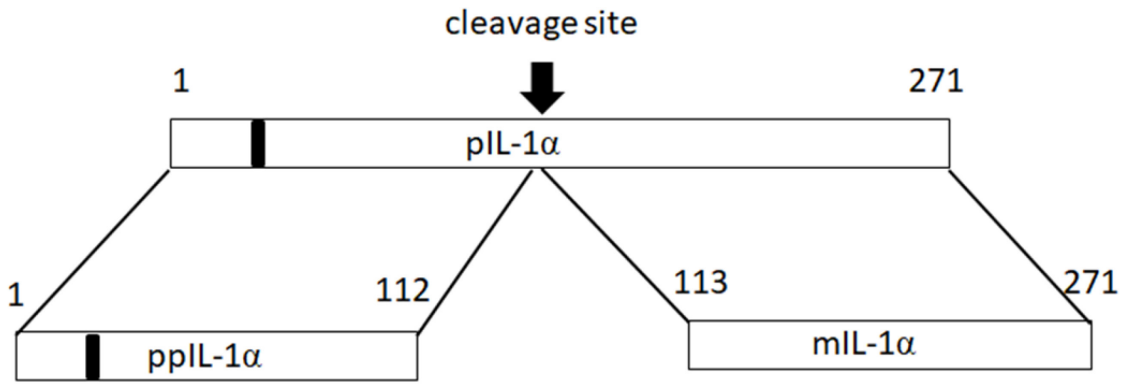

Fig. 1 The schematic structure of pIL-1α, mIL-1α and ppIL-1α. The number represent the amino acid positions (N-terminal amino acid of pIL-1α is 1). The pIL-1α is composed of 271 amino acids. The position between amino acid 112 and 113 was cleaved and resulted in generation of ppIL-1α (1-112) and mIL-1α (113-271). The black bars represent the NLS

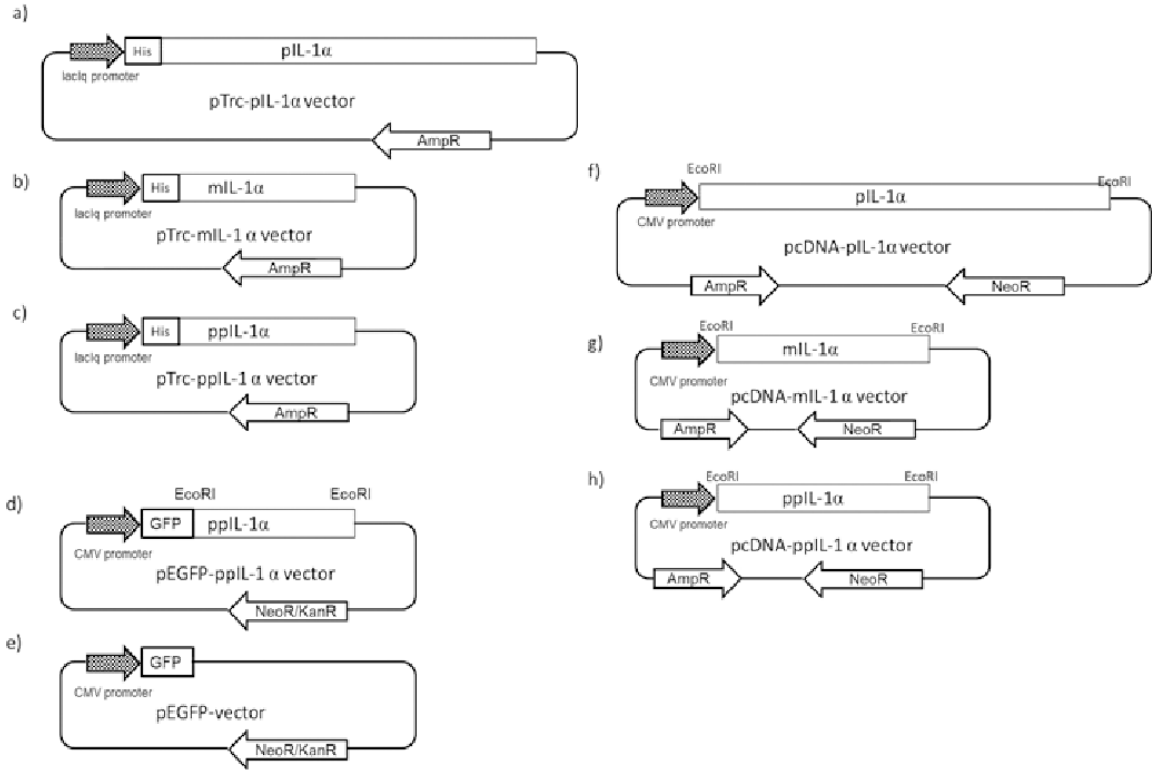

Fig. 2 a) Full length IL-1α cDNA was cloned to the bacterial expression vector pTrc-His and designated as pTrc-pIL-1α vector. The C-terminal (amino acid 1 to 112) and N-terminal halves (amino acid 113 to 271) of the full length IL-1α cDNA were cloned to the same vector and designated as pTrc-mIL-1α (b) and pTrc-ppIL-1α vector (c), respectively. ppIL-1α was cloned to *Eco*RI site of the pEGFP-C3 vector and designated as pEGFP-ppIL-1α vector (d). The empty pEGFP-vector was used as a control vector (e). pIL-1α, mIL-1α and ppIL-1α were cloned to *Eco*RI site of the pcDNA3.1 vector and designated as, pcDNA-pIL-1α (f), pcDNA-mIL-1α (g) and pcDNA-ppIL-1α (h) vector, respectively. Black arrows indicate the promoters (promoter types:

under the arrows). White arrows are the selection markers.

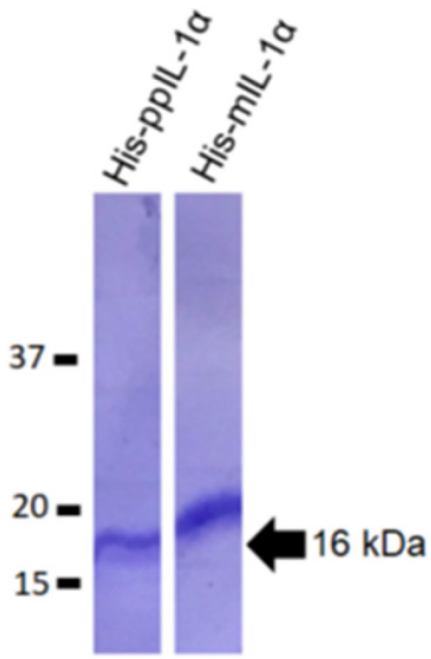

Fig. 3 Purified His-ppIL-1α (left lane) and His-mIL-1α (right lane) were loaded onto SDS-PAGE (15%) and subjected to CBB staining. Each protein was detected as single bands of 16 and 17 kDa. The positions of molecular weight marker are shown (37, 20 and 15 kDa)

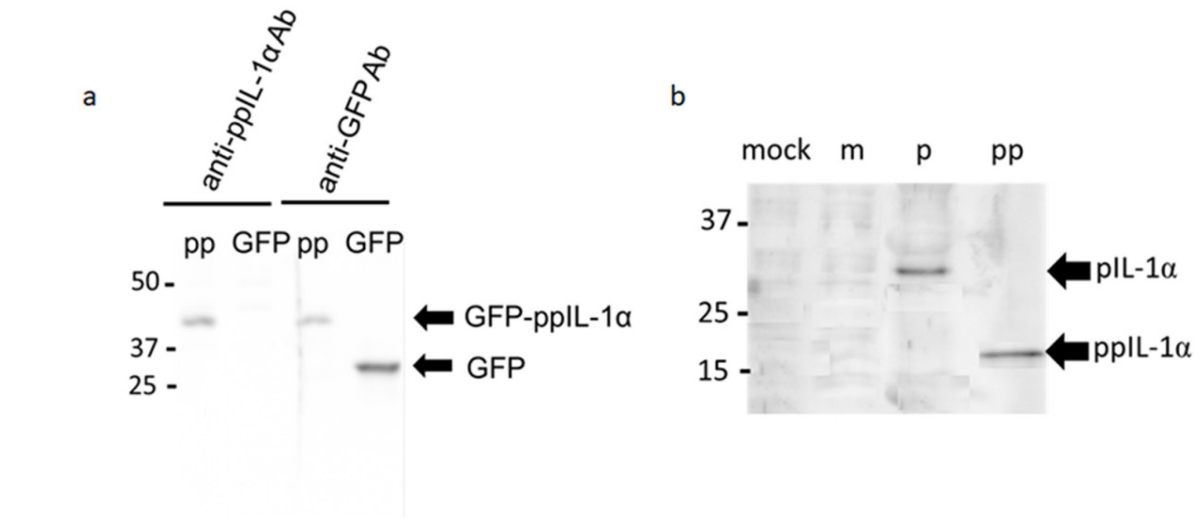

Fig. 4 a) The cell lysates obtained from GFP- or GFP-ppIL-1α transfectants were loaded to 15% SDS-PAGE and subjected to Western blotting. Anti-GFP Ab (× 1,000) and anti-ppIL-1α Ab (× 10,000) were incubated with the membrane followed by HRP-goat anti-rabbit IgG (H+L) Ab. The positions of molecular marker are indicated by the numbers (50, 37 and 25 kDa). b) The cell lystaes were prepared from pcDNA (mock), pcDNA-mIL-1α, pcDNA-pIL-1α and pcDNA-ppIL-1α transfectants, respectively, and subjected to Western blotting (12% SDS-PAGE)(1 st Ab; the Ab obtained in this study (× 10,000), 2 nd Ab; goat anti-rabbit IgG (H+L)(× 10,000). The representative data of three independent experiments were shown.

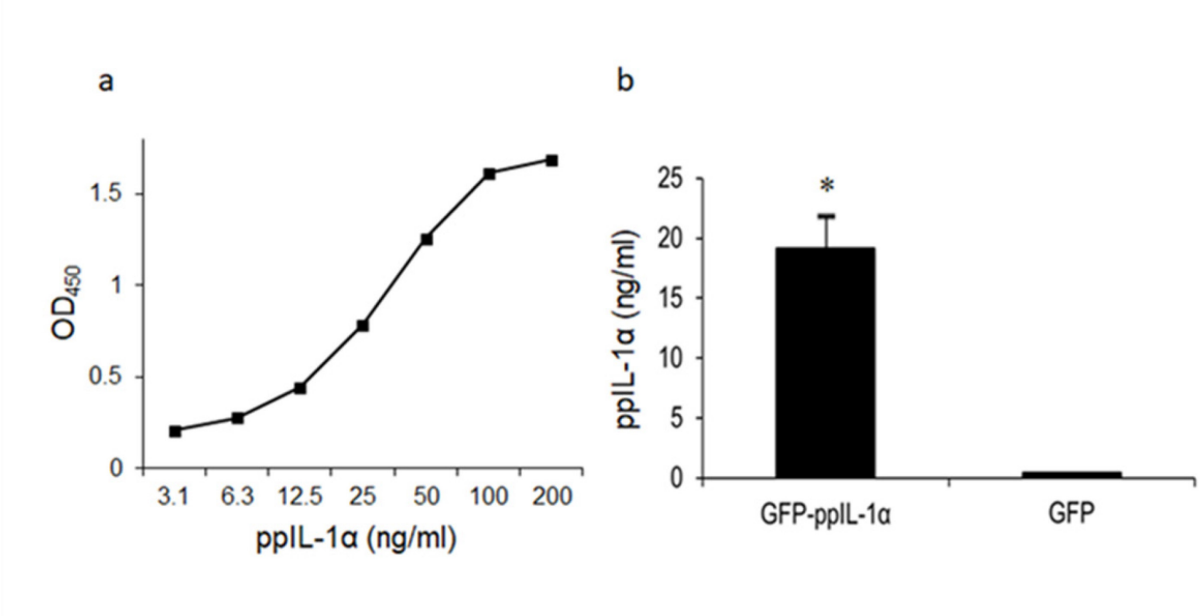

Fig. 5 ELISA for ppIL-1 α

a) Serially diluted His-ppIL-1α was applied to ELISA system and the standard curve was obtained.

b) The cell lysates of GFP- and GFP-ppIL-1α transfectants were subjected to ELISA. The mean ± standard deviations were shown (*$p < 0.05$ was considered as statistical significance). The data are means ± standard deviation of seven independent experiments ($n = 7$, each group).

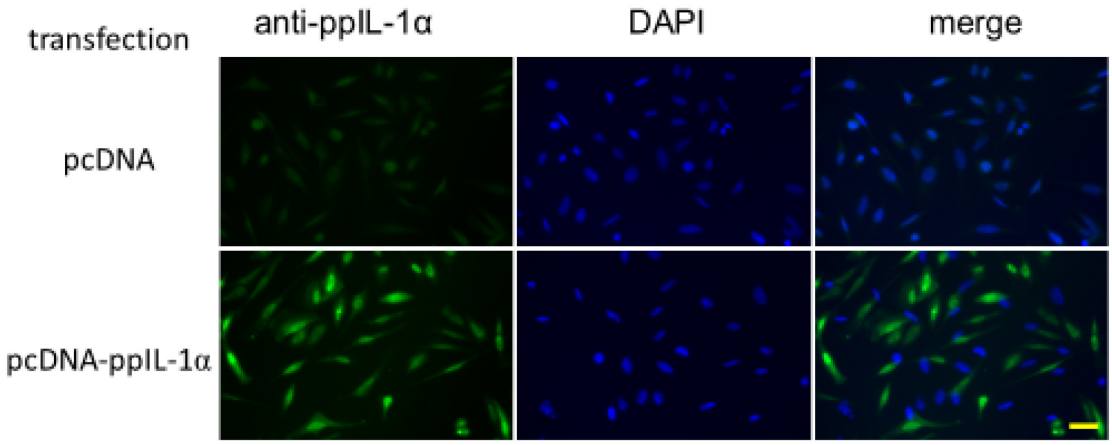

Fig. 6 Immunofluoresence staining.

Both pcDNA (mock) and pcDNA-ppIL-1α transfectants were incubated with rabbit anti-human IL-1α Ab (× 100) followed by goat anti-rabbit IgG Ab (× 100). The representative of at least 3 independent experiments are shown. Scale bar: 20 μm