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Large scale purification and characterization of recombinant human autotaxin/lysophospholipase D from mammalian cells

Yuanda Song[1,2], Emily Dilger[1], Jessica Bell[1], William A Barton[1] & Xianjun Fang[1,]*

[1]Department of Biochemistry and Molecular Biology, Virginia Commonwealth University School of Medicine, Richmond, VA 23298, USA, [2]Department of Biochemistry, Central South University School of Biological Sciences and Technologies, Changsha, Hunan 410013, P.R. China

We utilized a mammalian expression system to purify and characterize autotaxin (ATX)/lysophospholipase D, an enzyme present in the blood responsible for biosynthesis of lysophosphatidic acid. The human ATX cDNA encoding amino acids 29-915 was cloned downstream of a secretion signal of CD5. At the carboxyl terminus was a thrombin cleavage site followed by the constant domain (Fc) of IgG to facilitate protein purification. The ATX-Fc fusion protein was expressed in HEK293 cells and isolated from conditioned medium of a stable clone by affinity chromatography with Protein A sepharose followed by cleavage with thrombin. The untagged ATX protein was further purified to essential homogeneity by gel filtration chromatography with a yield of approximately 5 mg/liter medium. The purified ATX protein was enzymatically active and biologically functional, offering a useful tool for further biological and structural studies of this important enzyme. [BMB reports 2010; 43(8): 541-546]

INTRODUCTION

Autotaxin (ATX)/NPP-2 was first isolated as a tumor cell motility-stimulating factor from conditioned medium of a human melanoma cell line (1, 2). It was later identified to be lysophospholipase D (LysoPLD) present in the blood that converts lysophosphatidylcholine (LPC) to lysophosphatidic acid (LPA) (3, 4). Many biological functions of ATX are attributable to its LysoPLD activity to synthesize LPA, a multi-functional lipid mediator that acts on cell surface G protein-coupled receptors (3, 4).

Recent studies demonstrated that ATX possesses a signal peptide sequence at the amino terminus, followed by two somatomedin-B-like domains, a catalytic domain, and a nuclease-like domain at the carboxyl terminus (2, 5). The catalytic domain of ATX functions as both phosphodiesterase which cleaves phosphodiester bonds at the 5' end of oligonucleotides, and lysophospholipase which catalyzes production of LPA from LPC (5-7). The affinity of ATX for LPC is much higher than that for nucleotides, suggesting a preference for LPC as a physiological substrate over nucleotides (3, 4). In contrast to ATX, other members of the NPP family such as NPP-1 and NPP-3 do not exhibit any LysoPLD activity. Thus ATX is evolutionarily divergent from other NPP proteins and has been thus far only enzyme known to synthesize LPA extracellularly (3, 4). Homozygous deletion of ATX leads to embryonic lethality at E9.5 due to impaired vessel formation in the yolk sac and embryo proper (8, 9). Blood LPA levels in ATX heterozygous mice are about half of those present in wild type littermates, indicating that ATX is a major LPA-generating enzyme in the blood circulation (8, 9).

Accumulating evidence implicates ATX in the development and progression of cancers and other pathophysiological conditions (10, 11). Therefore, ATX has become an appealing therapeutic target to attenuate LPA production and LPA associated biological functions. However, the progress to identifying pharmacological inhibitors/activators of ATX has been hampered by the lack of detailed structure-activity relationship studies that require large quantities of pure and integral ATX protein.

RESULTS

Expression of recombinant human ATX in HEK293 cells

We took advantage of the pSS-Fc plasmid that had been engineered to carry a CD5 signal sequence (CD5-SS) (12). The human ATX cDNA encoding aa 29-915 was cloned downstream of CD5-SS. A thrombin cleavage site and the IgG Fc were located in frame downstream of the ATX cDNA in the final expression vector pSS-ATX-Fc as illustrated in Fig. 1. We chose HEK293 cells for overexpression of ATX as the cell line is highly transfectable and easy to be maintained for stable expression of exogenous genes (13). The ATX fusion protein was expressed in a number of stable clones (ATX-2, ATX-4, and ATX-8) as seen from immunoblotting analysis of cell lysates (Fig. 2A). To confirm the efficient secretion of ATX protein, we

*Corresponding author. Tel: 804-828-0787; Fax: 804-828-1473; E-mail: xfang@vcu.edy

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collected culture supernatants from the ATX-expressing clones. After precipitation with Protein A sepharose, high levels of ATX-Fc was detected in the supernatants (Fig. 2B). The yield of ATX-Fc in culture supernatants was so high that the protein present in 50 μl culture supernatants could be visualized by direct staining of SDS-PAGE with Coomassie brilliant blue (Fig. 2C).

Purification of ATX from culture supernatants

We collected 7.5 liters conditioned medium from ATX-2. The secreted ATX-Fc was first isolated by running through a Protein A sepharose column. The protein bound to the column was eluted with 100 mM glycine buffer, followed by immediate neutralization with Tris-HCl pH 8.0 to prevent potential denaturing. Fractions of 3 ml were collected, and 10 μl from each fraction was analyzed by SDS-PAGE. As shown in Fig. 3A, the predominant protein band was of approximately 150 Kd as expected for ATX-Fc (the IgG Fc was of about 30 Kd in size). A minor band migrated at the size of 50-60 Kd was likely the heavy chain of the bovine IgG from FBS. Fractions 2-5 were pooled for further purification. The total amount of ATX-Fc isolated from 7.5 liters of supernatants was typically 50-100 mg as estimated by photo spectrometry at A280 and by the Bradford assay.

To remove the Fc tag, thrombin (29 units) was added to the pooled fractions and incubated at 4°C overnight. ATX-Fc was completely digested without affecting the integrity of untagged ATX (Fig. 3B). The solutions were then loaded to a Protein A sepharose column again to trap Fc. The step was repeated at least three times to have complete separation of Fc from free ATX. Indeed, after these repeated cycles, the major protein remaining in the flow through was untagged ATX (Fig. 3B).

An aliquot of the untagged ATX was further purified by Superdex 200 gel filtration chromatography with FPLC (Unicorn 5.10). The UV monitor showed one major peak (data not shown) and SDS-PAGE analysis confirmed the predominant ATX protein in the peak fractions (Fig. 3C). However, minor bands of lower molecular weights (60-75 kD), likely representing nominal degradation of ATX, were detectable in the samples. It has been reported that ATX is sensitive to cleavage between Arg340 and Lys341, giving a truncated form of 66 kD (14). The final yield of the purified ATX after the gel filtration chromatography with FPLC was approximately 5 mg per liter of conditioned medium.

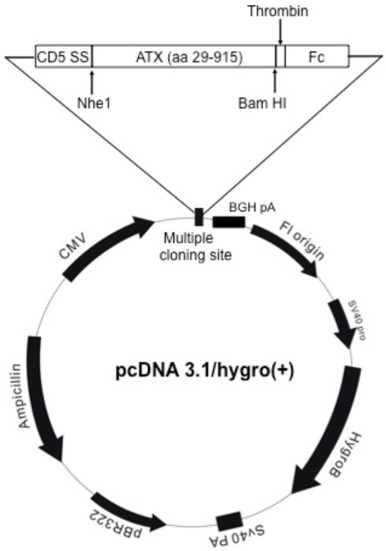

Fig. 1. Construction of the pSS-ATX-Fc plasmid. The human ATX cDNA was inserted downstream of the CD5 SS and upstream of the IgG Fc. Between ATX and Fc was a pre-engineered thrombin cleavage site.

Enzymatic activity of ATX

To confirm that the purified ATX was enzymatically active, we examined LysoPLD activity by quantifying choline released from the substrate LPC (3, 4). As shown in Fig. 4A, ATX re-

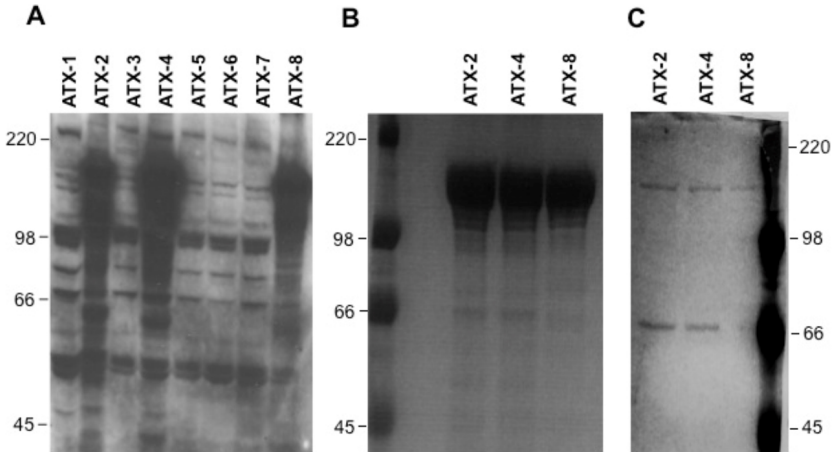

Fig. 2. Stable expression of the ATX-Fc fusion protein in HEK293 cells. The pSS-ATX-Fc plasmid was transfected into HEK293 cells. Stable transfectants was cloned as stable lines. Shown was immunoblotting analysis of the ATX-Fc protein in cell lysates of stable cell lines (A). The secretion of the fusion protein in three positive clones was confirmed by precipitation with Protein A sepharose beads from 10 ml of serum-free conditioned medium (B) or by direct analysis of 50 μl of conditioned medium with SDS-PAGE (C). The gels in (B) and (C) were stained with Coomassie brilliant blue.

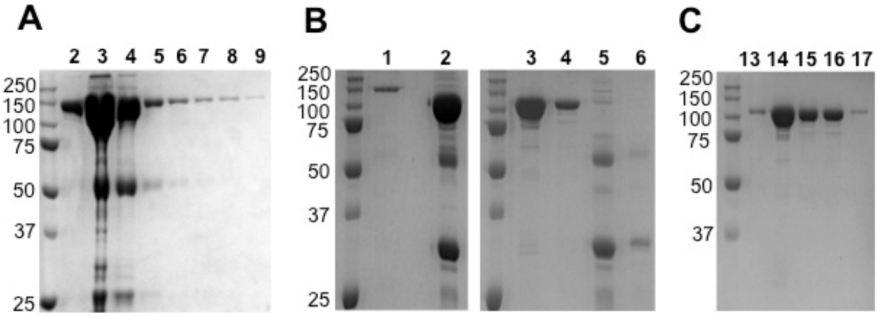

Fig. 3. Purification of ATX. (A) Isolation of the ATX-Fc fusion protein from culture supernatants. The conditioned medium of ATX-2 cells was passed through Protein A sepharose. The protein was eluted with 100 mM glycine pH 3.0 and neutralized with 1 M Tris HCl pH 8.0. Samples of fractions were analyzed by SDS-PAGE. (B) Removal of the Fc tag. Thrombin was added to the pooled fractions of ATX-Fc. The samples before (lane 1) and after (lane 2) thrombin digestion were analyzed by SDS-PAGE (left). ATX and Fc were separated by passing through Protein A sepharose. The Fc tag bound to the column was eluted with glycine pH 3.0. The flow through solutions (lane 3, 4) and proteins eluted from the Protein A column (lane 5, 6) were analyzed by SDS-PAGE (right). (C) Further purification of ATX by Superdex 200 gel filtration (FPLC). An aliquot of ATX was loaded onto a gel filtration column and fractionated in HBS. Shown was SDS-PAGE analysis of peak fractions 13-17.

leased choline from LPC in a dose-dependent manner (left). Similarly, ATX drove choline release from SPC (Fig. 4A, right), confirming the ability of ATX to produce S1P in addition to LPA. We further analyzed LysoPLD activity of the purified ATX and conditioned medium by examining conversion of radioactively labeled LPC to LPA. The ATX protein (1-100 ng) or the conditioned medium was incubated with ^{14}C-labeled LPC. TLC analysis of lipid extracts confirmed the formation of LPA and cPA from LPC in the presence of ATX-containing culture supernatants or purified ATX protein (Fig. 4B).

Biological functions of purified ATX

ATX exerts pleiotropic biological effects through synthesis and actions of LPA. One characteristic biological function of ATX is its motility-stimulating effect on tumor cells (3, 4). To determine whether the purified ATX was biologically functional driving migration of tumor cells, we examined the migratory response of the human ovarian cancer cell line SKOV-3 to different doses of ATX in the presence or absence of LPC. As shown in Fig. 4C, ATX indeed stimulated migration of SKOV-3 cells. The ability of ATX to drive cell migration was enhanced by the presence of the ATX substrate LPC (5 μM) (Fig. 4C).

LPA stimulates production of cytokines such as IL-8 and IL-6 in cancer cell lines (15). To examine whether purified ATX is also capable of driving cytokine production, we incubated SKOV-3 with varying doses of the purified ATX in the presence or absence of 5 μM LPC. The culture supernatants were collected for analysis of IL-8 levels by ELISA. As shown in Fig. 4D, ATX increased IL-8 production in SKOV-3 cells. The presence of LPC enhanced the ability of ATX to stimulate IL-8 production in these cells. The results together suggested that the purified ATX protein was biologically functional.

DISCUSSION

In this report, we took advantage of a mammalian expression system to express, purify and characterize ATX. The full length human ATX is a pre-pro-protein (5, 6). The N-terminal, hydrophobic domain (aa 1-27) is proteolytically cleaved between Gly27 and Phe28 or somewhere close to Gly27-Phe28 by a signal peptidase of the secretory pathway (6, 7). The N-terminal region could be also cleaved between Arg35 and Ala36 by Furin, a ubiquitous subtilisin-like proprotein convertase (6). Thus ATX is secreted from mammalian cells as a mature form lacking the N terminal, hydrophobic sequences. In several previous studies, ATX protein was purified from *E. Coli.*, the *Sf9* insect cells and bovine serum (3, 16, 17). These protein samples lack either appropriate post-transcriptional modifications associated with ATX maturation or lack sufficient purity necessary for detailed structural and biological studies. Our effort in this study provides a high yield of homogenous ATX protein naturally secreted from mammalian cells.

The purified ATX protein is enzymatically active. In the *in vitro* assays, the protein catalyzed choline release from LPC to generate LPA or from SPC to form S1P, suggesting that ATX is capable of synthesizing both LPA and S1P. Consistent with Tsuda *et al*. (16), we also observed ATX-mediated formation of cyclic PA (cPA) from LPC, especially in the presence of high doses of ATX (Fig. 4B). In these assays, the enzymatic activity of ATX was relatively low with detectable activity at $>$10 ng ATX. This was probably because the reactions were not optimized or limited by negative feedback from ATX products as described previously (18). It is also possible that the enzymatic activity of ATX may be regulated by some co-factors that were absent from the final protein preparations.

The purified ATX protein mimics LPA in stimulation of cell migration and cytokine production in cancer cell lines.

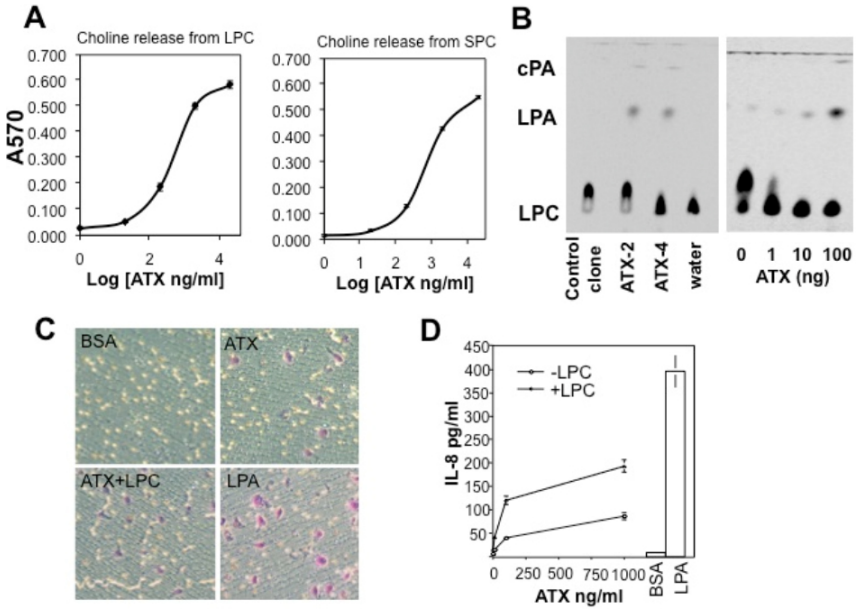

Fig. 4. Enzymatic and biological activities of purified ATX. (A) Purified ATX catalyzes choline release from LPC or SPC in a dose-dependant manner. ATX protein were incubated with 1 mM 1-palmitoyl LPC (left) or SPC (right) at 37°C for 1 hour. The choline release was quantified by the photometric analysis of an oxidized hydrogen donor. Shown are plots of the absorbance at 570 nM of the reactions by the log of the ATX concentrations. (B) ATX converts radio-labeled LPC into LPA and cPA. 1-(^{14}C)-palmitoyl-LPC was incubated with medium from a control clone, supernatants from two ATX clones or with indicated concentrations of purified ATX at 37°C for 1 hour. The lipid contents were extracted from the reactions and separated by TLC. (C) ATX promotes cell migration. Serum-free medium containing LPA (1 μM) or ATX (100 ng/ml) with or without LPC (1-oleoly, 5 μM) was loaded to the lower chambers of transwells. SKOV-3 cells (5×10^4 cells/0.5 ml) were placed to the upper chambers. Shown were microscopic photographs (30X) of migrated cells stained with crystal violet. (D) ATX stimulates IL-8 production. SKOV-3 cells cultured in 6-well plates were starved and stimulated for 16 hours with LPA (5 μM) or indicated concentrations of ATX in the presence or absence of LPC (1-oleoly, 5 μM). IL-8 in culture supernatants was determined by ELISA.

Altering LPA levels in animals or in culture is practically difficult as LPA is degraded quickly (3, 4). The availability of large quantities of pure ATX protein offers an ideal approach to manipulate LPA levels *in vitro* and *in vivo* and to investigate the biological responses to changes in extracellular concentrations of LPA.

In summary, we have used human cells to express and purify recombinant ATX protein structurally analogous to the native ATX protein. The quantities and purities of the protein exceeded those prepared from other previously described expression and purification approaches (1, 3, 4, 16, 17). The enzymatically active and biologically functional ATX protein is well suited for further structural, pharmacological and biological studies.

MATERIALS AND METHODS

Reagents

1-oleoly (18 : 1) LPA, 1-palmitoyl (16 : 0) and 1-oleoly (18 : 1) LPC, and SPC were obtained from Avanti Polar Lipids (Alabaster, AL). Anti-ATX polyclonal rabbit antibody was kindly provided by Dr. J. Aoik (Tohoku University, Aoba-ku, Sendai, Japan). Fetal bovine serum (FBS) was from Atlanta Biologicals (Lawrenceville, GA). All other tissue culture reagents were obtained from Invitrogen Inc. (Carlsbad, CA). The Quantikine IL-8 ELISA kits were obtained from R & D systems (Minneapolis, MN). The nProtein A sepharose beads were from GE Healthcare (Piscataway, NJ).

Western blot

Cells were lysed with SDS sample buffer. Conditioned medium or purified proteins were dissolved in SDS sample buffer using 2XSDS. After boiling for 5 min, proteins in 1XSDS sample buffer were resolved by SDS-PAGE, transferred on to Immobilon (PVDF) and immunoblotted with antibodies according to the manufacturers. Immunocomplexes were visualized with an ECL® (enhanced chemiluminescence) detection kit (GE Healthcare).

Construction of pSS-ATX-Fc

The plasmid pSS-Fc was derived from modification of pcDNA3.1/Hygro(+) (12). In pSS-Fc, the CD5 signal sequence (SS), a thrombin cleavage site and IgG Fc were inserted in frame into the multiple cloning site of pcDNA3.1/Hygro(+). The ATX

cDNA (2664 bp, aa 29-915) was amplified by PCR from an NPP-2 clone (ATCC). The PCR product was digested with Nhe I and Bam HI and then ligated into pSS-Fc to make pSS-ATX-Fc (Fig. 1). The DNA sequences of ATX cDNA, CD5 SS, the thrombin site and the subsequent Fc were confirmed by automatic sequencing.

Expression of pSS-ATX-Fc in HEK293 cells

pSS-ATX-Fc was transfected into HEK293 cells. Stable transfectants were selected with 150 μg/ml hygromycin. Individual hygromycin-resistant colonies were isolated by ring cloning and expanded sequentially in 24-well, 6-well and 60-mm plates. The ATX-expressing clones were maintained in high glucose DMEM supplemented with 7.5% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml).

To obtain large volumes of conditioned medium, the cells of the ATX-2 clone were first seeded in 145-mm dishes and then sub-cultured to 2.0 liter roller-bottles (Cambrex BioSci, Inc., Walkersville, MD) with 250 ml complete medium, and incubated at 37°C at approximately 6 RPM. When the cells reached approximately 70% confluence, the culture medium was replaced with DMEM containing 1% FBS, and incubated at 3 RPM for 36-40 hours before harvesting. The bottles of culture were refed fresh DMEM and incubated for another 36-40 hours. The conditioned medium were pooled and clarified by centrifugation at 4°C. p-APMSF (0.1 μM) was added to the supernatants following centrifugation.

Purification of ATX

The conditioned medium (7.5 L) was passed through a column of 2 ml Protein A sepharose beads equilibrated with Hepes-buffered saline (HBS: 10 mM Hepes, pH 7.4 and 150 mM NaCl). The column was then washed extensively with HBS, and eluted with 100 mM glycine pH 3.0. The eluted fractions were immediately neutralized with 1 M Tris-HCl pH 8.0. The peak fractions of ATX were pooled and the ATX-Fc fusion protein was digested overnight with 29 units of thrombin (Novagen, Madison, WI) on a slow rocking shaker. The digestion was confirmed by SDS-PAGE. When the reaction was complete, it was terminated by adding 0.2 μM p-APMSF. The cleaved fusion tag was removed by passing through a Protein A sepharose column.

The untagged ATX was concentrated to approximately 10 mg/ml using Amicon ultra centrifugal filter (50,000 MWCO). The protein supplemented with 10% glycerol was quickly frozen in liquid nitrogen and stored in −80°C. For further purification, aliquots of the untagged ATX were loaded onto a 100 ml Superdex 200 gel filtration column and fractionated in HBS.

LysoPLD activity assay

The LysoPLD activity of ATX was analyzed by measuring choline released from LPC or SPC (3). Briefly, 1 mM palmitoyl LPC or SPC was incubated in a reaction buffer (100 mM Tris-HCl pH 9.0, 500 mM NaCl, 5 mM $MgCl_2$ and 0.05 %Triton X-100) in the presence of indicated doses of ATX protein for 1 hour at 37°C. The choline released was measured using an enzymatic photometric assay involving choline oxidase, horseradish peroxidase, and TOOS reagent (N-ethyl-N-(2-hydoroxy-3-sulfoproryl)-3-methylaniline) as a hydrogen donor (19).

The LysoPLD activity was also determined by analyzing the conversion of radio-labeled LPC to LPA. 1-(^{14}C) palmitoyl-LPC (Amersham Biosciences) (0.05 μCi) and cold palmitoyl-LPC (1 μM final concentration) were reconstituted in a reaction buffer (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 50 mM Tris-HCl, pH 8.0) and sonicated before addition of 2 mg/ml BSA. The reaction was initiated by adding conditioned medium, ATX protein or vehicle, and then incubated at 37°C for 1 hour. The reaction solutions were made acidic by adding 0.02 M acetic acid and lipid contents were extracted three times with 1-butanol as previously described (18). The lipid extracts were dried out with speed vacuum and resuspended in 25 μl 1-butanol. The resulting samples were separated by thin layer chromatography (TLC) on silica gel-60 plates in chloroform/methanol/ammonium hydroxide (60 : 35 : 8).

Cell migration assay

The migration of SKOV-3 cells was assayed using transwell chambers (pore size 8 μm) (BD Biosciences, Bedford, MA) (20). The inserts were precoated with type I collagen. Serum-starved cells were loaded to the upper chamber. ATX, ATX+LPC, or LPA was added to the lower chambers. The ligand solution and cell suspension were prepared in serum-free RPMI 1640 medium containing 0.05% BSA. The cells were incubated for 6 hours. Nonmigrated cells were removed from the top filter surface with a cotton swab. Migrated cells attached to the underside of the transwells were washed with PBS and stained with crystal violet and photographed under a microscope.

ELISA of IL-8 production

Culture supernatants of cells treated without or with ATX, ATX+LPC, or LPA were collected and analyzed for IL-8 concentrations by ELISA using the IL-8 ELISA kit (D8000C, R&D Systems).

Acknowledgements

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