Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Research in Veterinary Science 106 (2016) 170–172

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Cloning and characterization of giant panda (*Ailuropoda melanoleuca*) IL-18 binding protein

Yue Yan [a,1], Jiabo Deng [b,1], Lili Niu [b], Qiang Wang [b], Jianqiu Yu [b], Huanhuan Shao [a], Qinghua Cao [a], Yizheng Zhang [a], Xuemei Tan [a,*]

[a] College of Life Sciences, Sichuan University, Key Laboratory for Bio-Resources and Eco-Environment of Ministry of Education, Sichuan Key Laboratory of Molecular Biology and Biotechnology, Chengdu 610064, PR China
[b] Chengdu Zoo, Chengdu 610081, PR China

ARTICLE INFO

Article history:
Received 20 April 2015
Received in revised form 31 March 2016
Accepted 25 April 2016

Keyword:
Giant panda
Interleukin-18
IL-18 binding protein
Bioactivity analysis

ABSTRACT

The giant panda (*Ailuropoda melanoleuca*) is an endangered species. Interleukin-18 (IL-18) plays an important role in the innate and adaptive immune responses by inducing IFN-γ. IL-18 has been implicated in the pathogenesis of various diseases. IL-18 binding protein (IL-18BP) is an intrinsic inhibitor of IL-18 that possesses higher affinity to IL-18. In this study, we cloned and characterized IL-18BP in giant panda (AmIL-18BP) from the spleen. The amino acid sequence of giant panda IL-18BP ORF shared about 65% identities with other species. To evaluate the effects of AmIL-18BP on the immune responses, we expressed the recombinant AmIL-18BP in *Escherichia coli* BL21 (DE3).The fusing protein PET-AmIL-18BP was purified by nickel affinity column chromatography. The biological function of purified PET-AmIL-18BP was determined on mice splenocyte by qRT-PCR. The results showed that AmIL-18BP was functional and could significantly reduce IFN-γ production in murine splenocytes. These results will facilitate the study of protecting giant panda on etiology and immunology.

Interleukin-18 (IL-18) plays an important role in the innate and adaptive immune responses (Dinarello, 1999). IL-18 was originally identified as an IFN-gamma-inducing factor, structurally similar to IL-1, promoting Th1 type immune responses (Okamura et al., 1995; Gillespie and Horwood, 1998; Akira, 2000). Mice deficient in IL-18 have a blunted natural killer cell response and markedly reduced IFN-γ production (Dinarello, 2000).

The activity of IL-18 is via an IL-18 receptor (IL-18R) complex (Born et al., 1998). This IL-18R complex is made up of IL-18 receptor alpha (IL-18Rα, formerly known as IL-1Rrp) and IL-18 receptor beta (IL-18Rβ, formerly known as IL-1RAcPL) to heterodimerize (Torigoe et al., 1997; Kimura et al., 2008). Both receptor chains belong to the IL-1 receptor family, and the binding of IL-18 to its receptor triggers signaling pathways is similar to IL-1 family members (Kojima et al., 1998). Following exposure to IL-18, IL-18Rα recruits the IL-1 receptor activating kinase (IRAK) and TNFR-associated factor-6 (TRAF-6) (Kojima et al., 1998; Akira, 2000). The latter was important because it found to associate with the NF-κB-inducing kinase (NIK) (Malinin et al., 1997).

IL-18 is a pro-inflammatory cytokine that is produced from T lymphocytes and macrophages and enhanced by cytotoxicity of natural killer cells (Dinarello, 1999). It can also cause severe allergic inflammatory reactions (Tsutsui et al., 2004). IL-18BP is a circulating protein with high affinity for IL-18 and can neutralize its bioactivity (Dinarello, 2000; Dinarello et al., 2013). IL-18BP acts as a natural inhibitor of IL-18 induced IFN-γ and suppresses the Th1 response (Aizawa et al., 1999; Dinarello et al., 2003). In fact, the severity of autoimmune diseases appeared to be affected by the relative levels of IL-18 and IL-18BP (Leach et al., 2008; Richards et al., 2014). IL-18BP has been shown to prevent or attenuate disease development in various mouse models (Richards et al., 2014). Yellayi et al. (2010) reported that rhesus macaques IL-18BP was functional and could significantly reduce murine IL-18 and LPS-induced IFN-γ production by murine splenocytes. IL-18BP had been used safely in humans and clinical trials of IL-18BP as well as neutralizing anti-IL-18 antibodies (Dinarello et al., 2013).

As well known the giant panda (*Ailuropoda melanoleuca*) is one of the most endangered animals (Tang et al., 2008). The wild population is estimated <3,000 at present (Zhan et al., 2006). Diseases are probably the greatest threats giant panda, especially virus diseases. Previous studies had shown that canine distemper virus (CDV), canine parvovirus (CPV) and canine coronavirus (CCV) had been detected from the giant pandas (Tan et al., 2007; Qin et al., 2010). IL-18BP shows a great potential for the treatment of allergy (Kimura et al., 2008). There are several reports that IL-18BP can prevent or attenuate the development of disease in animal models (Faggioni et al., 2001; Banda et al., 2003; Raeburn et al., 2002). So it is important to study the immune function genes of IL-18BP of giant panda, which will be helpful to protect giant panda on etiology and immunology.

* Corresponding author.
E-mail address: txmyyf@scu.edu.cn (X. Tan).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.rvsc.2016.04.004
0034-5288/

Therefore, this study aimed to clone and characterize IL-18BP of giant panda.

The cDNA of IL-18BP of giant panda was amplified by RT-PCR. Total RNA was isolated from spleen sample of giant panda (natural death, Chengdu, Sichuan, China) by TRIzol reagent (Invitrogen, CA, USA) according to manufactures' instructions. Remaining DNA was removed by DNase I (Fermentas, Canada). 1 μg of total RNA, Oligo $(dT)_{18}$ and M-MLV reverse transcriptase (Invitrogen, CA, USA) was used in cDNA synthesis. The primers for giant panda IL-18BP were listed in Supplementary Table S1. All primers used for PCR and sequencing were made by Invitrogen (China). The products were ligated to the pMD18-T vector (TaKaRa, Dalian, China) and transformed into *Escherichia coli* JM109 competent cells. The bacteria were grown on LB plates with ampicillin and X-Gal. The plasmids were selected and tested by PCR and enzyme digestion with *Eco*R I and *Pst* I (TaKaRa, China), followed by sequencing (Huada Genomics Technology Co. Ltd., Beijing, China). The recombinant plasmid was named as pMD-AmIL18-BP. The results showed that the open reading frame (ORF) of the giant panda IL-18BP was 567 bp in length and encoded 188 amino acid residues (GenBank accession no. KM232608), which containing 28 amino acid signal peptides. Thus, the mature peptide of AmIL-18BP was 160 amino acids, with the predicted molecular weight of 17.6 kDa. The theoretical pI was 9.23. Secondary structure predicted by Predict Protein and the structure of human IL-18 visualizes that the AmIL-18BP molecule was composed of eight β-strands and three α-helixes. Comparison of the amino acid sequences of AmIL-18BP with human, mouse and Rhesus monkey showed 68.28%, 65.56% and 66.84% sequence identity, respectively. The sequence was conserved among giant panda, rhesus, human, and murine proteins within the middle portion of the amino acid sequence from residues 66 to 130 (Supplementary Fig. S1).To better determine the evolutionary position of AmIL-18BP, a phylogenetic tree was constructed by use of the genome data of other animals. AmIL-18BP clustered with dog IL-18BP (Supplementary Fig. S2).

In order to detect the isoforms of giant panda IL-18BP, RACE amplifications of 3′ ends was performed. Human IL-18BP gene encodes for at least four distinct isoforms (IL18BPa, b, c and d) derived from mRNA splice variants isolated from several cDNA libraries (Hong et al., 2012). IL-18BP sequences differed primarily in their carboxyl termini whereas the N-terminal was identical (Kim et al., 2000; Dinarello, 2000). Different isoforms of the human IL18BP, murine IL-18BP and rhesus IL-18BP shared 100% identity in the 5′-untranslated regions (5′-UTR) (Hong et al., 2012; Dinarello, 2000; Yellayi et al., 2010). So in this study, only 3′ RACE was used to test the polymorphisms of AmIL-18BP in the 3′-UTR. The first PCR were performed with primers of adaptor and 18BP outer-F (Supplementary Table S1) by conditions of 16 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 1 min. Then first PCR amplification products as template, the second PCR were performed with primers of adaptor and 18BP inner-R (Supplementary Table S1) by conditions of 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and extension at 72 °C for 1 min. The sequenced results showed that 3′-UTR was 183 bp with classical polyadenylation signal sequence AATAAA and AmIL-18BP gene had no other type. So we amplified only a single isoform from spleen of giant pandas.

To express and purify functional recombinant AmIL-18BP in *E. coli*, coding fragment of AmIL-18BP was amplified with primers and PCR products were digested with *Bam*HI and *Hind*III (Fermentas, Canada) (Supplementary Table S1). It was subcloned into vector pET-32a (+) (Novagen, USA) to construct prokaryotic expression plasmid pETAmIL18BP. After the sequencing, the recombinant vector was transformed into *E. coli* strain BL21 (DE3). Positive clones were screened on LB broth containing ampicillin (100 μg/mL) and grown with shaking at 150 rpm for overnight at 37 °C. 2 mL culture was transferred into fresh LB medium and continued shaken at 150 rpm at 37 °C for about 2–3 h until the OD600 reached 0.6. IPTG (final concentration of 0.5 Mm) was added to the medium. After additional cultivation at 18 °C for 16 h, the bacteria were harvested by centrifugation at 4000 ×*g* for 10 min, and the pellet resuspended in 20 mM Tris-HCl buffer (pH 8.0).The supernatant was applied to the Ni Sepharose column, and purified using Histidine-6 tag peptide at N-terminus of this prokaryotic expression vector immobilized metal ion affinity chromatography (GE Healthcare, Sweden), following the manufacturer's recommendations. The concentration was determined by BCA Protein Assay Kit (Thermo Scientific, IL, USA). Then the purified samples were store at 4 °C with Protease Inhibitor Cocktail (Sigma-Aldrich, MO, USA). SDS-PAGE was performed with 5% stacking gel and 12%(v/v) resolving gel and the proteins were visualized by Coomassie brilliant blue R-250 (Amresco, USA).

The sequence encoding mature peptide was successfully inserted into the pET-32a (+) vector. After transformed in *E. coli* BL21 (DE3), and induced by IPTG, the supernatant of cell lysate was visualized by SDS-PAGE. Compared with control BL21 (DE3) cells and non-induced cell sharboring pET-AmIL18BP, fusion protein with molecular weight of 35.7 kDa was expressed in soluble form. Histidine-tagged fusion protein was loaded to the nickel affinity HisTrap HP column. After eluted from the column with lotion, a single band was displayed on the gel (Fig. 1). The concentration of the purified PET-AmIL-18BP was approximately 0.5 mg/mL, according to BCA methods.

To investigate the biological activity of AmIL-18BP, cytokine IFN-γ production was measured by real-time qPCR. Splenocytes were isolated from two $C_{57}B_{l6}$ mice, and resuspended at 2.5×10^6 cells/mL in a 200 μL volume. 1×10^6 cells/well were cultured in 24-well plates in RPMI-1640 with 10% FBS in triplicate. Then three groups were treated with recombinant giant panda IL-18 (PET-AmIL-18) (500 ng/mL) and PET-AmIL-18BP (500 ng/mL), and then PET-AmIL-18 (500 ng/mL) and PBS regarded as control groups. All of them were incubate for 24 h at 37 °C with 5% CO_2. Collected the cell at 3 h, 6 h and 24 h. Total RNA was isolated from the cultured spleen lymphocytes using TRIzol reagent. cDNA synthesis and contaminate genomic DNA removing were performed

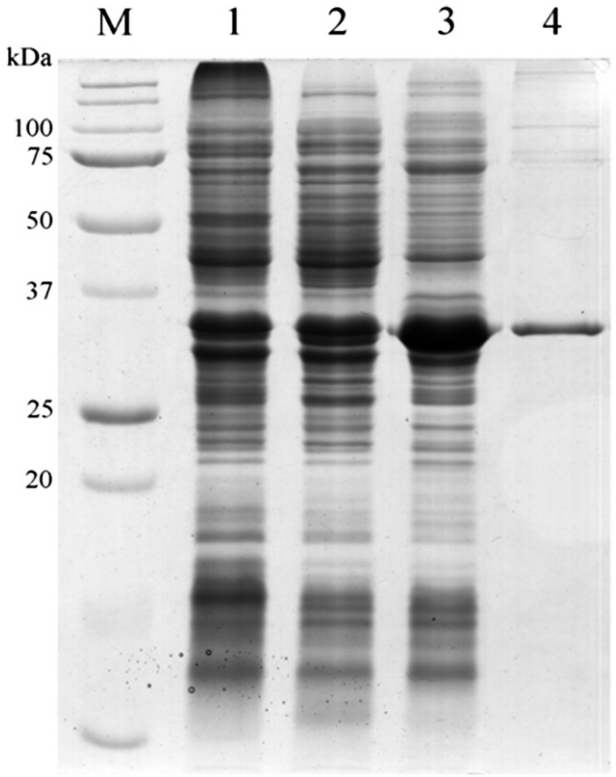

Fig. 1. AmIL-18BP protein expression and purification in *E. coli* BL21 (DE3). M, molecular weight marker; lane 1,cell lysates of *E. coli* BL21(DE3); lane 2, cell lysates of bacteria harboring pET-AmIL18BP without IPTG induction; lane 3, cell lysates of bacteria harboring pET-AmIL18BP with IPTG induction; lane 4, purified AmIL-18BP fusion protein.

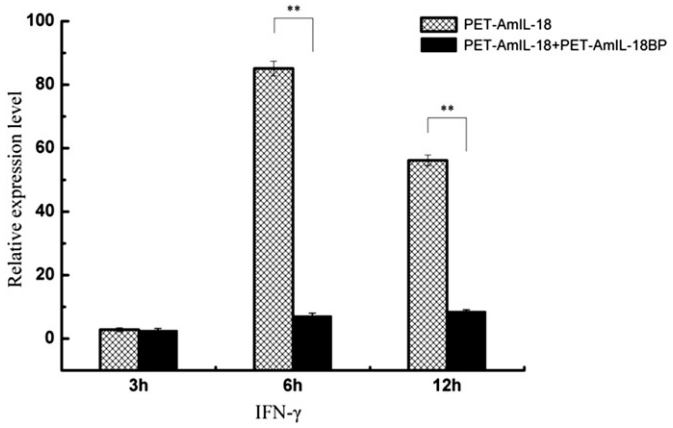

Fig 2. Inhibition of AmIL-18BP in IFN-γ production. Note: The asterisk indicated a statistically significant difference (**$P < 0.01$,*$P < 0.05$) compared with the control.

by PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). Reverse transcription was reacted at 37 °C for 15 min and 85 °C for 5 s. Quantitative real-time PCR (qRT-PCR) was performed to check the concentration of IFN-γ. The PCR condition was 98 °C for 2 min, 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s, followed a gradient temperature from 65 °C to 95 °C, generating a melting curve to validate only an objective product had been amplified. The relative expression levels of these transcripts were normalized to those of GAPDH using Bio-Rad CFX Manager and calculated using Livak methods ($2^{-\Delta\Delta CT}$). Data in response to media were converted to the fold relative to that of the PBS controls. The results were analyzed statistically using SSPS 18.0 software, $P < 0.05$ considered as significantly different between control and treated groups.

Then mice spleen lymphocytes were treated with recombinant protein AmIL-18 and AmIL-18BP. The effect on cytokine IFN-γ production was measured by real-time qPCR. As shown in Fig. 2, together with AmIL-18BP, AmIL-18 significantly reduced the expression level of IFN-γ in mouse lymphocytes, 2–9 fold lower than that of PBS control in the 3–12 h time ($P < 0.01$).

In conclusion, we succeeded cloned, expressed and purified the recombinant giant panda IL-18BP proteins. Our data also demonstrated its biologic function on mouse lymphocytes. These results will be helpful to increase our knowledge of the biology of potential therapeutic value use for suppression of inflammatory responses induced by IL-18, which will promote the study of protecting giant panda on etiology and immunology.

Acknowledgements

This work was supported by Chengdu Giant Panda Breeding Research Foundation (No. CPF-2010-02).

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.rvsc.2016.04.004.

References

Aizawa, Y., Akita, K., Taniai, M., Torigoe, K., Mori, T., Nishida, Y., Ushio, S., Nukada, Y., Tanimoto, T., Ikegami, H., Ikeda, M., Kurimoto, M., 1999. Cloning and expression of interleukin-18 binding protein. FEBS Lett. 445, 338–342.

Akira, S., 2000. The role of IL-18 in innate immunity. Curr. Opin. Immunol. 12, 59–63.

Banda, N.K., Vondracek, A., Kraus, D., Dinarello, C.A., Kim, S.H., Bendele, A., Senaldi, G., Arend, W.P., 2003. Mechanisms of inhibition of collagen-induced arthritis by murine IL-18 binding protein. J. Immunol. 170, 2100–2105.

Born, T.L., Thomassen, E., Bird, T.A., Sims, J.E., 1998. Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. J. Biol. Chem. 273, 29445–29450.

Dinarello, C.A., 1999. Interleukin-18. Methods 19, 121–132.

Dinarello, C.A., 2000. Targeting interleukin 18 with interleukin 18 binding protein. Ann. Rheum. Dis. 59, 17–20.

Dinarello, C.A., Novick, D., Rubinstein, M., Lonnemann, G., 2003. Interleukin 18 and interleukin 18 binding protein: possible role in immunosuppression of chronic renal failure. Blood Purif. 21, 258–270.

Dinarello, C.A., Novick, D., Kim, S., Kaplanski, G., 2013. Interleukin-18 and IL-18 binding protein. Front. Immunol. 4, 289.

Faggioni, R., Cattley, R.C., Guo, J., Flores, S., Brown, H., Qi, M., Yin, S., Hill, D., Scully, S., Chen, C., Brankow, D., Lewis, J., Baikalov, C., Yamane, H., Meng, T., Martin, F., Hu, S., Boone, T., Senaldi, G., 2001. IL-18-binding protein protects against lipopolysaccharide-induced lethality and prevents the development of Fas_Fas ligand-mediated models of liver disease in mice. J. Immunol. 167, 5913–5920.

Gillespie, M.T., Horwood, N.J., 1998. Interleukin-18: perspectives on the newest interleukin. Cytokine Growth Factor Rev. 9, 109–116.

Hong, K., Oh, K., Lee, S., Hong, J., Choi, J., Kwak, A., Kang, D., Kim, E., Jo, S., Jhun, H., Kim, S., 2012. Recombinant Fc-IL-18BPc isoform inhibits IL-18-induced cytokine production. Hybridoma (Larchmt) 31, 99–104.

Kim, S.H., Eisenstein, M., Reznikov, L., Fantuzzi, G., Novick, D., Rubinstein, M., Dinarello, C.A., 2000. Structural requirements of six naturally occurring isoforms of the IL-18 binding protein to inhibit IL-18. Proc. Natl. Acad. Sci. U. S. A. 97, 1190–1195.

Kimura, T., Kato, Z., Ohnishi, H., Tochio, H., Shirakawa, M., Kondo, N., 2008. Expression, purification and structural analysis of human IL-18 binding protein: a potent therapeutic molecule for allergy. Allergol. Int. 57, 367–376.

Kojima, H., Takeuchi, M., Ohta, T., Nishida, Y., Arai, N., Ikeda, M., Ikegami, H., Kurimoto, M., 1998. Interleukin-18 activates the IRAK-TRAF6 pathway in mouse EL-4 cells. Biochem. Biophys. Res. Commun. 244, 183–186.

Leach, S.T., Messina, I., Lemberg, D.A., Novick, D., Rubenstein, M., Day, A.S., 2008. Local and systemic interleukin-18 and interleukin-18-binding protein in children with inflammatory bowel disease. Inflamm. Bowel Dis. 14, 68–74.

Malinin, N.L., Boldin, M.P., Kovalenko, A.V., Wallach, D., 1997. MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. Nature 385, 540–544.

Okamura, H., Tsutsi, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., Akita, K., Namba, M., Tanabe, F., Konishi, K., Fukuda, S., Kurimoto, M., 1995. Cloning of a new cytokine that induces IFN-γ production by T cells. Nature 378, 88–91.

Qin, Q., Li, D., Zhang, H., Hou, R., Zhang, Z., Zhang, C., Zhang, J., Wei, F., 2010. Serosurvey of selected viruses in captive giant pandas (*Ailuropoda melanoleuca*) in China. Vet. Microbiol. 142, 199–204.

Raeburn, C.D., Dinarello, C.A., Zimmerman, M.A., Calkins, C.M., Pomerantz, B.J., McIntyre Jr., R.C., Harken, A.H., Meng, X., 2002. Neutralization of IL-18 attenuates lipopolysaccharide-induced myocardial dysfunction. Am. J. Physiol. Heart Circ. Physiol. 283, 650–657.

Richards, K.H., Doble, R., Wasson, C.W., Haider, M., Blair, G.E., Wittmann, M., Macdonald, A., 2014. Human papillomavirus E7 oncoprotein increases production of the anti-inflammatory interleukin-18 binding protein in keratinocytes. J. Virol. 88, 4173–4179.

Tan, X.M., Tang, Y., Yang, Y.F., Song, H.M., Zhang, Y.Z., 2007. Gene cloning, sequencing, expression and biological activity of giant panda (*Ailuropoda melanoleuca*) interferon-alpha. Mol. Immunol. 44, 3061–3069.

Tang, Y., Tan, X.M., Yue, C.W., Li, C.X., Fan, Z.X., Zhang, Y.Z., 2008. Cloning, sequence, and function analyses of giant panda (*Ailuropoda melanoleuca*) CD9 gene. Mol. Reprod. Dev. 75, 1418–1425.

Torigoe, K., Ushio, S., Okura, T., Kobayashi, S., Taniai, M., Kunikata, T., Murakami, T., Sanou, O., Kojima, H., Fujii, M., Ohta, T., Ikeda, M., Ikegami, H., Kurimoto, M., 1997. Purification and characterization of the human interleukin-18 receptor. J. Biol. Chem. 272, 25737–25742.

Tsutsui, H., Yoshimoto, T., Hayashi, N., Mizutani, H., Nakanishi, K., 2004. Induction of allergic inflammation by interleukin-18 in experimental animal models. Immunol. Rev. 202, 115–138.

Yellayi, S., Braun, S., Domingues, H.G., Kityatana, N., Murali, R., Johnson, R.P., Mansfield, K., Westmoreland, S.V., O'Neil, S.P., 2010. Cloning and characterization of rhesus IL-18 binding protein, a natural antagonist to IL-18. Cytokine 51, 232–239.

Zhan, X.J., Li, M., Zhang, Z.J., Goossens, B., Chen, Y.P., Wang, H.J., Bruford, M.W., Wei, F.W., 2006. Molecular censusing doubles giant panda population estimate in a key nature reserve. Curr. Biol. 16, 451–452.