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Cloning and Characterization of a New Isoform of Mouse Interleukin-18

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Abstract Interleukin-18 (IL-18) is a novel proinflammatory cytokine with potent interferon (IFN)-γ inducing activity that plays an important biological role in the enhancement of the activity of natural killer cells and cytotoxic T lymphocytes. In this study, we have identified a novel short form of IL-18 in mouse, named IL-18s. IL-18s might be an alternative splicing variant of IL-18 and its cDNA contains a 57 bp in-frame deletion. Like IL-18, IL-18s is also widely expressed in mouse tissues. It was suggested that IL-18s might have a caspase-1-dependent mechanism for maturation and secretion similar to that of IL-18: when transfected in COS-7 cells, pro-IL-18s (22 kDa) could be detected, and the mature IL-18s (16 kDa) could also be detected when combined with caspase-1. We observed that recombinant mouse IL-18s did not show any IL-18-like function, and IL-18s could enhance the ability of IL-18 to increase IFN-γ production by approximately 40% in mouse splenocytes. This effect was observed primarily at relative low concentrations of IL-18, suggesting that IL-18s might regulate the activity of IL-18 in the physiological conditions.

Key words IL-18; isoform; IL-18s; IFN-γ; caspase-1; factor Xa

Interleukin-18 (IL-18), originally identified as an interferon (IFN)-γ inducing factor, was first isolated from the livers of mice stimulated with *Propionibacterium acnes* and lipopolysaccharide (LPS) [1]. IL-18 is a potent pro-inflammatory cytokine that induces IFN-γ production in T cells and natural killer (NK) cells [2], enhances the Fas ligand and perforin-mediated T cell and NK cell cytotoxicity [3–8], and plays a critical role in the T-lymphocyte helper type 1 response [8]. IL-18 also induces expression of GM-CSF, inflammatory cytokines (e.g., tumour necrosis factor-α, IL-1β and IL-13), and chemokines, such as IL-8, macrophage inflammatory protein (MIP)-1α and MIP-1β [2,10–12].

IL-18 is structurally related to IL-1β and has been shown to be a member of the IL-1 superfamily [13,14]. Furthermore, the IL-18R complex consists of a ligand-binding subunit (IL-18Rα) and a signaling subunit (IL-18Rβ), both of which are members of the IL-1R family [15–17]. Like IL-1β, IL-18 is synthesized in its precursor form, proIL-18, which requires caspase-1 cleavage to become a biologically active molecule [18–20]. However, unlike IL-1β, *IL-18* mRNA is expressed constitutively in many types of cells and IL-18 protein is stored in the producing cells as a biologically inactive precursor, which is partly due to the particular structure and characteristics of the *IL-18* gene [21–24]. LPS can induce the upregulation of *IL-18* mRNA and the secretion of mature IL-18 by the activation of caspase-1 [20–22]. In addition, the activation and secretion of IL-18 are regulated by caspase-1-independent mechanisms [18,21,25].

Recently, proIL-18 was observed combining with IgM in human blood plasma [26], however its physiological function is still not clear. Furthermore, a 51 bp in-frame deletion in the leading sequence of *IL-18* was also reported in rainbow trout [31]. *IL-18s* cDNA has been reported in rats with a 57 bp in-frame deletion [32], but further reports are not available.

In our study, a mouse *IL-18* isoform named *IL-18s*, 57 bp shorter than the normal *IL-18*, was cloned. We found that the *IL-18s* mRNA was constitutively expressed in a

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variety of tissues accompanying *IL-18*. The biochemical properties of IL-18s were further studied to find that IL-18s had a similar mechanism for maturation and secretion from the mammalian cells, which was caspase-1-dependent. The cloned *IL-18s* cDNA was then expressed in *Escherichia coli*, and recombinant mouse IL-18s did not exhibit any IL-18-like function. When IL-18 and IL-18s were both added to mouse splenocytes, we observed that IL-18s could modestly enhance the ability of IL-18 to induce IFN-γ.

Materials and Methods

Mice and cell lines

BALB/c female mice 6–8 weeks of age were obtained from the Animal Center of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The COS-7 cell line was provided by the Cell Bank of Type Culture Collection, Chinese Academy of Sciences. COS-7 cells were cultured in Dulbecco's modified Eagle's medium and fresh isolated splenocytes were cultured in RPMI 1640 medium in a humidified atmosphere with 5% CO_2 at 37 ºC. Both media were supplemented with 10% fetal bovine serum, 2 mM *L*-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Reagents

LPS (a phenol-extracted preparation from *E. coli* 055: B5) was obtained from Sigma (St. Louis, USA), dissolved in water at 1 mg/ml, and stored at −20 ºC. DOTAP liposomal transfection reagent and protease inhibitor cocktails were purchased from Roche (Mannheim, Germany). Caspase inhibitor I (Z-VAD-FMK) was purchased from Calbiochem (San Diego, USA). Mouse IL-18 (pep-IL-18) and polyclonal rabbit antimurine IL-18 antibody were provided by PeproTech (Rocky Hill, USA). The enzyme-linked immunosorbent assay (ELISA) kit for mouse IFN-γ was purchased from R&D Systems (Minneapolis, USA). Gel was analyzed by a gel imaging system (FR-200; Furi Tech, Shanghai, China).

Reverse transcription-polymerase chain reaction (RT-PCR)

Specific primers (**Table 1**) were synthesized to amplify the full-length cDNA sequences of IL-18 and caspase-1. Total RNA was extracted from spleen of healthy BALB/c mouse using Trizol (Invitrogen, San Diego, USA) according to the manufacturer's instructions and reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, USA) in the presence of oligo($dT_{15–18}$). All products amplified by PCR were directly cloned into pCR4TOPO (Invitrogen) for sequencing.

IL-18s expression in mouse

To determine tissue distribution of *IL-18s* mRNA in healthy mouse, total RNA was extracted from organs including brain, small intestine, heart, liver, spleen, kidney, lung and pancreas. RT-PCR was performed using the primers 18q1/18q2, specifically detecting IL-18 and IL-18s expression. The PCR reaction programs were as follows: 94 ºC for 4 min; 94 ºC for 25 s, 58 ºC for 20 s, 72 ºC for 20 s,

Table 1 Primers used to amplify full-length cDNA sequences of interleukin (IL)-18 and caspase-1

Point	Primer	Sequence (5′→3′)
IL-18 cloning [9]	18C1 (upstream)	ACCTTCCAAATCACTTCCTC
	18C2 (downstream)	CAGGCGAGGTCATCACAAGG
Caspase-1 cloning	ICE-1 (upstream)	CTGCGGTGTAGAAAAGAAACG
	ICE-2 (downstream)	GGCACGATTCTCAGCATAGG
IL-18 detection	18q1 (upstream)	GGCTGTGACCCTCTCTGTGAA
	18q2 (downstream)	TGGCAAGCAAGAAAGTGTCCT
IL-18s propiece cloning	18sT1 (upstream)	AAC**GGATCC**ATGGCTGCCATGTCA
	18T2 (downstream)	AAAGTTACGTCCTTCGATGTCTCCATT
IL-18 propiece cloning	18T1 (upstream)	AAC**GAATTC**ATGGCTGCCATGTCA
	18T2 (downstream)	AAAGTTACGTCCTTCGATGTCTCCATT
Mature piece cloning	18T3 (upstream)	GGAGACATCGAAGGACGTAACTTTGGC
	18T4 (downstream)	AAC**CTCGAG**CTAACTTTGATGTAA

Restriction sites are shown in bold and factor Xa site mutation sequences are underlined.

30–35 cycles; and a final extension at 72 ºC for 10 min. To detect IL-18s protein in mouse, mouse splenocytes were obtained as described previously [27] and lysed in buffer containing protease inhibitors and caspase inhibitor. The lysate was immunoprecipitated with rabbit antimurine IL-18 antibody and resolved by 12%–15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The gel was transferred to nitrocellulose membrane and incubated first with primary antibodies (1:1000) at 4 ºC for 12–16 h, then with peroxidase-conjugated mouse anti-rabbit IgG. The membrane was developed by enhanced chemiluminescence.

Construction of eukaryotic expression plasmids and transient expression of IL-18s

The full-length murine *proIL-18s*, *proIL-18* or *caspase-1* cDNA was subcloned into pcDNA3 with C-end myc-tag (pcDNA3-proIL-18s, pcDNA3-proIL-18, pcDNA3-caspase-1) by introducing the *Bam*HI (or *Eco*RI for proIL-18)/*Xho*I site at the 5′ or 3′ end. All the constructs were confirmed by DNA sequencing.

COS-7 cells were transfected with pcDNA3-proIL-18s (or pcDNA3-proIL-18) alone or in combination with pcDNA3-caspase-1 in 35 mm dishes by DOTAP transfection according to the manual. In brief, 2 μg of DNA was diluted to a concentration of 0.1 μg/μl in HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4); and 15 μl DOTAP reagent was diluted to a final volume of 50 μl with HBS. Then the DNA solution was gently mixed with the DOTAP solution and incubated for 10–15 min at room temperature. The COS-7 cells were washed with serum-free Opti-MEMI (Gibco BRL, Gaithersburg, USA), plated in 1 ml Opti-MEMI containing the DOTAP/DNA mixture for 6 h, then washed and incubated with fresh Opti-MEMI medium supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. After 24 h the cells were fixed with 4% paraformaldehyde, and stained with mouse anti-myc monoclonal antibody and fluorescein-isothiocyanate (FITC)-labeled secondary antibody for immunofluorescence detection. Finally, the nuclei were stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI). The fluorescence of FITC and DAPI were observed using a confocal microscope. For Western blot detection, COS-7 cells were directly lysed in loading buffer or immunoprecipitation buffer containing protease inhibitors followed by immunoprecipitation with anti-IL-18 antibody 24 or 48 h post-transfection. The culture supernatants were collected and concentrated 200-fold by ultrafiltration (Centricon-10; Amicon, Beverly, USA) or concentrated 10-fold by immunoprecipitation with anti-IL-18 antibody. Then the supernatants, immunoprecipitates and cell lysates were detected by Western blot.

Construction of prokaryotic expression plasmids

The murine *proIL-18*s and *proIL-18* cDNA was used for generating a mutation of the caspase-1 cleavage site into a factor Xa site. Introduction of mutation in *proIL-18s* was performed by a two-step PCR reaction [28,29]. The propiece of IL-18s cDNA (or IL-18) was generated using primers 18sT1/18T2 (or 18T1/18T2 for IL-18) containing the *Bam*HI site (or *Eco*RI for *IL-18*) before the open reading frame (ORF). The mature piece of *IL-18s* or *IL-18* cDNA was generated by primers 18T3/18T4 containing the *Xho*I site after the ORF. The 123 bp propiece and 446 bp mature piece (503 bp for *IL-18*) of the *IL-18s* cDNA (or *IL-18* cDNA) were resolved by electrophoresis in 1% agarose and purified. These two cDNA pieces were mixed at 1:1 and used as the template for the second PCR step to generate a complete murine *IL-18s* cDNA in which the caspase-1 site is mutated into the factor Xa site. The IL-18s, IL-18s (Xa), and IL-18(Xa) cDNAs were ligated into the pGEX-4T-1 to form pGEX-proIL-18s, pGEX-proIL-18s(Xa) and pGEX-proIL-18(Xa), respectively, with the use of *Bam*HI/*Xho*I (for IL-18s) or *Eco*RI/*Xho*I (for IL-18). All the constructs were confirmed by DNA sequencing.

Protein expression and purification

pGEX-proIL-18s, pGEX-proIL-18s(Xa) or pGEX-proIL-18(Xa) was transformed into *E. coli* BL21(DE3) competent cells and expressed as described previously [29,30]. In brief, 10 ml overnight culture from a fresh single colony of BL21 was added into 490 ml of 2×YT medium containing 100 μg/ml ampicillin and grown at 37 ºC until its A_{600} reached 0.6. Protein expression was induced by isopropyl β-*D*-thiogalactopyranoside (IPTG; a final concentration of 0.1 mM). Then the culture was further incubated with shaking at 25 ºC for 6 h. For preparation of the fusion proteins, bacteria were harvested by centrifugation (3500 *g* for 10 min at 4 ºC) and rapidly resuspended in buffer A (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% Triton X-100). Lysozyme was added to a final concentration of 1 mg/ml. Cells were lysed on ice by mild sonication and the mixture was kept on ice for 15–20 min. The soluble proteins were obtained by centrifugation at 20,000 *g*, 4 ºC for 20 min, mixed with 1 ml glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Uppsala, Sweden), and incubated with mild shaking at 4 ºC for 1 h. Then the beads were pelleted by centrifugation (500 *g* for 5 min), washed twice with 10 volumes of buffer A, twice with 10 volumes of buffer B (buffer A containing 300 mM NaCl), and twice with 10 volumes of cleavage

buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM $CaCl_2$).

Cleavage of the fusion protein and purification of murine IL-18s

The fusion protein GST-proIL-18s, GST-proIL-18s(Xa) or GST-proIL-18(Xa) was incubated (22 ºC, 3–12 h) with the appropriate amount of thrombin protease (for proIL-18s) or factor Xa (for mature IL-18s and IL-18) in 1 ml cleavage buffer according to the manual (Amersham Pharmacia Biotech) with constant shaking on a rotating wheel. Once the digestion was completed, the supernatant was collected by centrifugation (500 *g* for 5 min at 4 ºC), and washed three times with 1 ml ice-cold phosphate-buffered saline. All the eluates were mixed, and clarified by centrifugation (12,000 *g*, 10 min) and ultrafiltration (Centricon-10). The purified protein concentration was measured using Bradford reagent (Bio-Rad, Hercules, USA) and stored at –70 ºC. The fusion proteins before digestion and the concentrated elutes were resolved by 12%–15% SDS-PAGE followed by Coomassie blue staining.

Bioassay of murine IL-18s

Mouse spleen cells were used to assay murine IL-18 as described previously [27]. Spleen cells were washed twice and cultured in RPMI 1640 medium in 48-well culture plates. Murine proIL-18s or mature IL-18s was added alone or in combination with pep-IL-18. Spleen cells were stimulated with 0.5 μg/ml LPS. The culture was incubated for 24 h at 37 ºC and the supernatants were then assayed for mIFN-γ by ELISA.

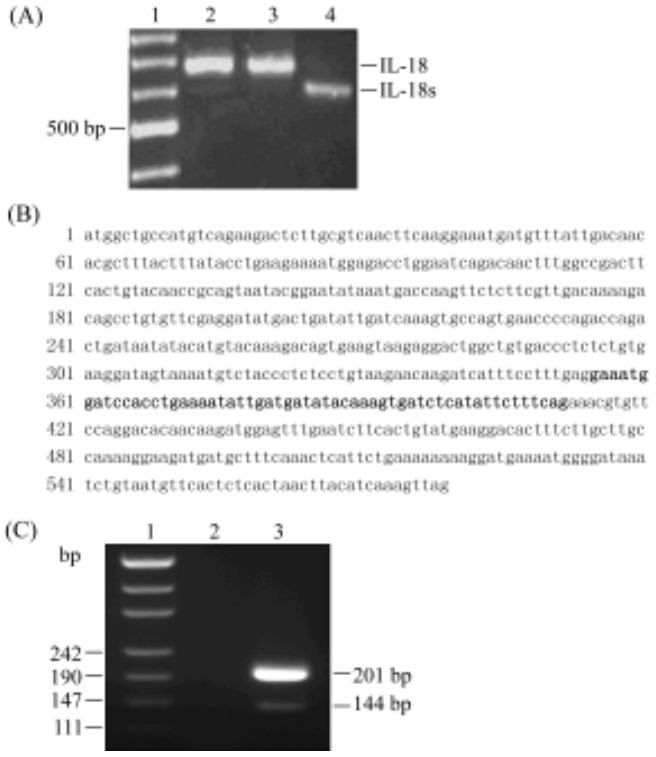

Fig. 1 Sequence of a novel short form of interleukin-18 (*IL-18s*) cDNA and confirmation of the deletion in *IL-18s* by reverse transcription-polymerase chain reaction (RT-PCR)

(A) Cloning of *IL-18* and *IL-18s*. The RNA was extracted from mouse splenocytes, and *IL-18* and *IL-18s* were amplified by RT-PCR. 1, 100 bp DNA ladder marker; 2, the full length of *IL-18* (676 bp) and *IL-18s* cDNA (619 bp); 3 and 4, the full-length cDNA of *IL-18* and *IL-18s*, respectively. (B) *IL-18* and *IL-18s* cDNA nucleotide sequences. Nucleotide positions are numbered on the left of the cDNA sequence. The deleted sequence is shown in bold. (C) Confirmation of the presence of the deletion in *IL-18s* by RT-PCR. The RNA was extracted from mouse splenocytes and specific primer pair (18q1/18q2) was used for the amplification of *IL-18* and *IL-18s*. 1, molecular mass marker; 2, negative control; 3, the PCR products of *IL-18* (201 bp) and *IL-18s* (144 bp).

Results

Molecular cloning of a novel mouse *IL-18* isoform

When cloning the mouse *IL-18* cDNA by RT-PCR from spleen cells, two distinct products were detected [**Fig. 1 (A)**]. Subsequent DNA sequencing identified these two cDNAs: one is identical to the *IL-18* cDNA, and the other contains a 57 bp in-frame deletion that results in a 19 amino acid deletion in the C-terminal portion of the putative protein, accordingly named IL-18s. The 57 bp deletion, corresponding to nucleotide 355–411 of the full-length *IL-18* cDNA, occurs at the 5′ end of exon 7 of the *IL-18* gene [**Fig. 1(B)**]. For the reconfirmation of the existence of *IL-18s*, a specific primer pair 18q1/18q2 was used and a similar result (a strong band for *IL-18*, and a faint band for *IL-18s*) was reproduced by RT-PCR [**Fig. 1(C)**].

Expression of *IL-18s* mRNA and protein in mouse

As shown in **Fig. 2(A)**, both *IL-18* and *IL-18s* were expressed in all the selected organs with a similar expression pattern detected previously in spleen [**Fig. 1(C)**], and a comparatively high expression of IL-18s was observed in pancreas. Similarly, two protein bands were observed in the lysates of fresh isolated splenocytes. The major band (approximately 24 kDa) corresponds to proIL-18, and the minor band (approximately 21–22 kDa), consistent with the predictive size, might correspond to proIL-18s [**Fig. 2(B)**].

Expression of IL-18s in COS-7 cells

To determine the expression and distribution of IL-18 and IL-18s, we investigated the subcellular localization of

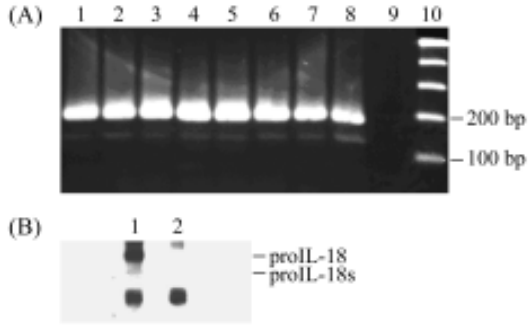

Fig. 2 Expression of a novel short form of interleukin-18 (*IL-18s*) in mouse

(A) Tissue distribution of *IL-18s* mRNA expression. 1, heart; 2, liver; 3, lung; 4, kidney; 5, brain; 6, small intestine; 7, spleen; 8, pancreas; 9, negative control; 10, 100 bp DNA ladder marker. (B) The putative expression of proIL-18s protein in splenocyte. The cell lysates from freshly isolated splenocytes were immunoprecipitated with or without anti-IL-18 antibody (lanes 1 and 2, respectively). The immunoprecipitates were resolved by 12%–15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, and electrotransferred onto nitrocellulose membranes. The membranes were then probed with antibodies against IL-18.

them in COS-7 cells transfected with pcDNA3-IL-18 and pcDNA3-IL-18s observed by confocal microscopy. IL-18 was clearly detected in the cell cytoplasma only in the transfected cells and IL-18s localized similarly in the cytoplasma [**Fig. 3(A)**]. Both IL-18 and IL-18s showed as uneven dotted-distribution in the cytoplasma.

IL-18s shares the same leader sequence and potential caspase-1 cleavage site with IL-18. Thus we wanted to determine whether caspase-1 cleaves proIL-18s and facilitates the secretion of mature IL-18s. COS-7 cells were transfected with pcDNA3-IL-18s alone or in combination with pcDNA3-caspase-1. pcDNA3-IL-18 was used as a positive control in parallel. Twenty-four hours after transfection, the cells were directly lysed. The subsequent immunoblotting results revealed that a polypeptide of approximately 22 kDa corresponding to proIL-18s was recognized by anti-murine IL-18 antibody, and a 24 kDa band corresponding to proIL-18 was also detected [**Fig. 3(B)**]. When co-transfected with pcDNA3-

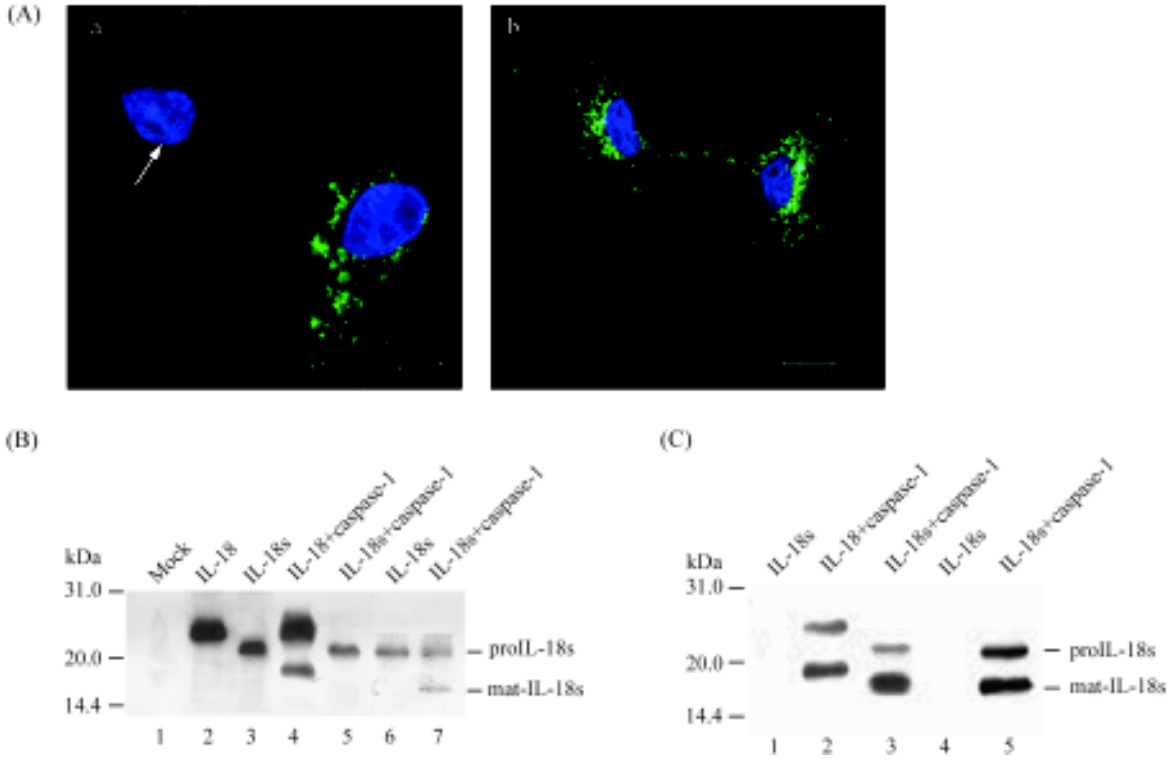

Fig. 3 Eukaryotic expression of a novel short form of interleukin-18 (IL-18s)

(A) Confocal microscopy analysis of the cellular distribution of IL-18 and IL-18s in COS-7 cells. COS-7 cells were transiently transfected with myc-tagged IL-18 and IL-18s expression plasmids, and stained with mouse anti-myc monoclonal antibody and fluorescein-isothiocyanate (FITC)-labeled secondary antibody 24 h later. In these merged images, IL-18 (a) or IL-18s (b) is shown as green and nuclei as blue. The non-transfected cell as negative control is indicated by an arrow. (B) The expression of IL-18s in COS-7 cells compared with IL-18. From lane 1 to lane 5, the cells were directly lysed with loading buffer. Lane 1 contained lysates from mock transfected cells and COS-7 cells were transfected with an expression plasmid for IL-18 (lane 2), IL-18s (lane 3) alone, or in combination with the expression plasmid encoding caspase-1, respectively (lanes 4 and 5). In lanes 6 and 7, cells were transfected with IL-18s and IL-18s+caspase-1, respectively, and gently lysed followed by immunoprecipitation. Molecular mass markers are shown on the left. (C) The expression of IL-18s in media compared with IL-18. COS-7 cells were transfected with expression plasmids. Media were directly concentrated 200-fold by ultrafiltration (lanes 1–3) or concentrated 10-fold followed by immunoprecipitation (lanes 4 and 5) and analyzed by immunoblotting.

caspase-1, the mature IL-18 (approximately 18 kDa) but not the mature IL-18s, could be detected in the cell lysates [**Fig. 3(B)**]. When the cells were lysed in immunoprecipitation buffer and immunoprecipitated with anti-IL-18 antibody, both the pro-IL-18s and mature IL-18s could be detected [**Fig. 3(B)**].

For the secretion of IL-18s, the culture was analyzed by immunoblotting 72 h post-transfection. A band (approximately 16 kDa) for mature IL-18s could be detected either in concentrated preparations or in the immunoprecipitated protein preparations [**Fig. 3(C)**] when pcDNA3-IL-18s was co-transfected with pcDNA3-caspase-1. The expression pattern with a strong band of mature IL-18s and a faint band of proIL-18s is similar to that of IL-18 [**Fig. 3(C)**]. All these data suggest that caspase-1 can cleave proIL-18s and facilitate the secretion of mature IL-18s.

Expression, purification of GST-proIL-18s and production of mouse IL-18s

We constructed proIL-18 and proIL-18s expression vectors in which the caspase-1 cleavage site was replaced with the factor Xa site [29]. Expression of the GST-proIL-18s and GST-proIL-18 was induced by IPTG in *E. coli*. After induction, a band of approximately 43 kDa corresponding to the fusion protein was specifically bound to glutathione-Sepharose beads (**Fig. 4**). After extensive washing, the beads were digested with factor Xa or thrombin protease. All the purified proteins were analysed by SDS-PAGE. **Fig. 4** shows the purified proteins of mature IL-18s, proIL-18s and mature IL-18.

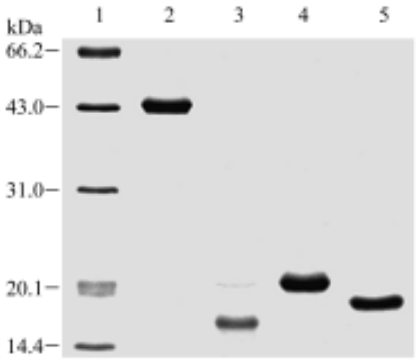

Fig. 4 Prokaryotic expression and purification of a novel short form of interleukin-18 (IL-18s) and IL-18 in mouse

Escherichia coli containing the expression plasmids for IL-18 and IL-18s were cultured and induced as described in "Materials and Methods". The supernatants of the bacterial sonicates were affinity-purified on glutathione agarose and samples subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (12%–15%). 1, molecular mass marker; 2, affinity-purified GST-proIL-18s fusion protein; 3–5, purified mouse IL-18s (16 kDa), proIL-18s (22 kDa) and IL-18 (18 kDa), respectively, after the cleavage of fusion proteins. The gel was stained with Coomassie blue.

IL-18s lacks IL-18-like IFN-γ-inducing activity

We evaluated whether IL-18s, like IL-18, could stimulate IFN-γ production in mouse splenocytes. As expected, pep-IL-18 markedly stimulated IFN-γ production in a dose-dependent manner [**Fig. 5(B)**], whereas the mature IL-18s did not stimulate IFN-γ production at the concen-

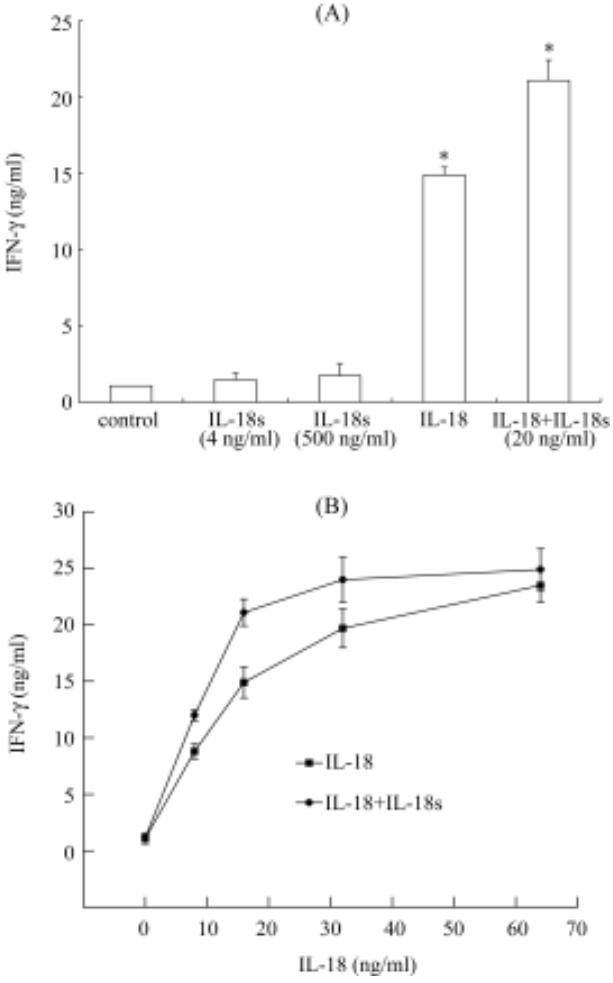

Fig. 5 Novel short form of interleukin-18 (IL-18s) enhances IL-18-induced interferon (IFN)-γ production

(A) Mature IL-18s at 5×series dilution (0.8–500 ng/ml) was added to the spleen cells in 24-well plates alone or combined with IL-18 (16 ng/ml) and stimulated with lipopolysaccharide (LPS). After 24 h, the supernatants were collected and detected with enzyme-linked immunosorbent assay (ELISA). The control was only stimulated with LPS. The mean of three separate experiments is shown. **P*< 0.05 vs. control group. (B) IL-18 at 2×series dilution (8–64 ng/ml) was added to the spleen cells in 24-well plates alone or combined with IL-18s (20 ng/ml). After 24 h, the supernatants were collected and detected with ELISA. The mean of three separate experiments is shown.

tration of 0.8–500 ng/ml (5×series dilution) [**Fig. 5(A)**]; similar results were obtained for proIL-18s (data not shown).

Ability of IL-18 to induce IFN-γ production enhanced by IL-18s

In order to determine whether IL-18s affects the activity of IL-18, the mouse splenocytes were stimulated with 2×series diluted pep-IL-18 (8–64 ng/ml) alone or combination with IL-18s (20 ng/ml). As shown in **Fig. 5 (B)**, at low concentrations of IL-18, IL-18s increased the production of IL-18-induced IFN-γ. At 16 ng/ml of IL-18, the production of IFN-γ was increased from 14.9 ng/ml to 21.1 ng/ml (an approximately 40% increase) ($P<0.01$). When the concentration of IL-18 increased to 64 ng/ml, this effect was not clearly observed ($P>0.05$). Furthermore, the proIL-18s did not affect the activity of IL-18 (data not shown). These results suggest that IL-18s might enhance the activity of IL-18 when the concentration of IL-18 is comparatively low.

Discussion

Here we reported that a short form of IL-18, termed IL-18s, 57 bp shorter than IL-18, was constitutively expressed in a wide range of mouse tissues including spleen, liver, lung, heart, brain, kidney, pancreas and gut. The expression of IL-18s was not markedly affected by LPS in splenocytes or peritoneal macrophage (data not shown).

The deleted 57 bp fragment, corresponding to nucleotide 355–411 of the full-length *IL-18* cDNA, is located at the 5′ end of exon 7 of the *IL-18* gene [**Fig. 1(B)**]. The presence of the dinucleotide $A^{410}G^{411}$ at the 3′ end of the 57 bp deletion suggests that *IL-18* and *IL-18s* appear to be from the same pro-mRNA and IL-18s is an alternative splicing variant, which is in good accordance with the eukaryotic splice donor-acceptor site sequence AG/GU [23]. This dinucleotide AG is conserved not only in mouse, but also in rat and human, which might suggest that *IL-18s* exists in different species. Our results provided new evidence indicating the wide existence of *IL-18s* mRNA. We also found that, as well as a 24 kDa band corresponding to proIL-18, an approximately 21–22 kDa band could be detected in the fresh isolated splenocytes, possibly corresponding to proIL-18s. Furthermore, Pirhonen *et al.* revealed a protein (approximately 22 kDa) existed in human macrophage [33]. All of these evidences suggest that IL-18s might widely exist, and is constitutively expressed at the levels of both mRNA and protein like IL-18.

The proIL-18 requires caspase-1 cleavage to become a biologically active molecule. Caspase-1 can enhance the maturation of proIL-18 and facilitate the export of the mature IL-18 from cells. But the caspase-1-dependent mechanism for the maturation and secretion of IL-18 has not yet been clarified, although a small amount of proIL-18 can be secreted from cells independent of caspase-1 [18–22]. In the present study, when IL-18s was transiently expressed in COS-7 cells combined with caspase-1, the mature IL-18s protein (approximately 16 kDa) could not be detected in the cell lysates [**Fig. 3(B)**], but could be detected in the cell lysates by immunoprecipitation and in the cell culture supernatants. We suggest that the possible reasons for IL-18s secretion might be: first, the process of caspase-1-dependent maturation and caspase-1-enhanced secretion may be closely-linked; second, the secreted mature IL-18 could bind to the surface of COS-7 cells, but the mature IL-18s could bind very weakly (data not shown), so there must be some secreted mature IL-18 counted as an intracellular part and identified in the cell lysates; third, the transient expression level of IL-18s was relatively low compared with that of IL-18; fourth, it might be a problem of recognition limitation by the anti-IL-18 antibody; and finally, the mature IL-18s detected in cell lysates with the method of immunoprecipitation might be partly due to the further digestion of caspase-1 during the process of cell lysis and immunoprecipitation.

IL-18 has a 12-β-pleated sheet structure and an IL-1 signature-like sequence (136–160 amino acids), which is a characteristic of the IL-1 family [2,14]. For the deletion of 19 amino acids (119–137 aa), the IL-1 signature-like sequence in IL-18s is not integrated: the first conserved amino acid F136 is deleted and the eighth β-strand also partly deleted, which may influence the configuration of IL-18s. Furthermore, it was reported that the residues K79, E84 and D98 of mouse IL-18 are important for binding IL-18Rβ to the binary complex and for biological activity [34]. In IL-18s, the residue D98 is deleted and the other two residues are very close to the 19 deleted amino acids, which suggests that the deletion in IL-18s may greatly affect the interaction with IL-18R. Consistent with this, neither proIL-18s nor mature IL-18s induced any IFN-γ production. Although IL-18s could weakly bind to the IL-18R extracellular domain (data not shown), it might fail to recruit the IL-18Rβ chain to form a functionally active ternary complex with the IL-18Rα chain similar to that of IL-1F7b [35].

Until now, the function of IL-18s has not been clearly known. IL-18s slightly enhances the ability of IL-18 to

induce IFN-γ, primarily at relatively low concentrations of IL-18, which might be part of its function. We presume that IL-18s might act as a naturally expressed modulator of IL-18 activity *in vivo*. Under appropriate stimuli, the concentrations of IL-18 and IL-18s increase in local microenvironments and more IFN-γ is induced. When IL-18s reaches its working concentration, the concentration of IL-18 may be too high for IL-18s to further enhance the activities of IL-18. Increasing levels of IFN-γ induce further expression of IL-18BPs, which slows down the increasing activity of IL-18 and gradually decreases the concentration of active IL-18. When IL-18 descends to a relatively low concentration, IL-18s might exert its function and enhance the production of IFN-γ. To further understand the function of this novel IL-18 isoform, more researches based on these primary results are needed.

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