Production and mechanism of secretion of interleukin-1β from the marine fish gilthead seabream

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Running title: Production of IL-1β in fish

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[1]**Abbreviations:** HK, head-kidney / ICE, IL-1β-converting enzyme / IL-1β, interleukin-1β / LPS, lipopolysaccharide / MAF, macrophage-activating factor / PRR, pattern recognition receptor / PS, phosphatidylserine / sbIL-1β, gilthead seabream IL-1β / PTG-1, protegrin-1 / TCA, trichloroacetic acid / TLRs, Toll-like receptors / VaDNA, *Vibrio anguillarum* genomic DNA.

Abstract

Interleukin-1β (IL-1β[1]) is a secretory cytokine lacking a signal peptide, and does not follow the classical endoplasmic reticulum to Golgi pathway of secretion. Its post-translational processing by IL-1β-converting enzyme (ICE) and subsequent release from activated macrophages requires ATP acting on $P2X_7$ receptors. No information is available on the production and release of fish IL-1β, but the IL-1β gene sequences reported to date lack a conserved ICE recognition site. We show for the first time that lipopolysaccharide (LPS)/macrophage-activating factor (MAF)/bacterial DNA (VaDNA)-primed immune cells of fish accumulate intracellular IL-1β as a ~30 kDa polypeptide (proIL-1β). The combination of LPS and VaDNA was found to be synergistic, suggesting that each ligand is recognized by a different pattern recognition receptor (PRR). More importantly, addition of extracellular ATP does not promote IL-1β secretion by immune cells and fails to induce phosphatidylserine (PS) flip. In contrast, fish SAF-1 fibroblasts shed microvesicles containing a 22 kDa IL-1β form within 30 min of activation with ATP. Notably, the post-translational processing of IL-1β by SAF-1 cells is abrogated by a specific ICE inhibitor.

Keywords: IL-1β/ATP/Cytokines/Leaderless proteins

Introduction

The mammalian interleukin-1β (IL-1β) is a key pro-inflammatory cytokine [1], whose mechanism of release remains enigmatic. Unlike most secreted proteins, it lacks a secretory signal sequence and does not follow the classical endoplasmic reticulum to Golgi route of secretion [2]. IL-1β is mainly produced by activated macrophages as a 31 kDa biologically inactive precursor molecule (proIL-1β) that is proteolytically cleaved to a 17 kDa active form by IL-1β-converting enzyme (ICE, caspase-1) [3]. Cleavage of IL-1β is coupled to its secretion because processed IL-1β can only be found extracellular [2]. Although the levels of basal processing and release of IL-1β are quite low, they are dramatically induced by the presence of extracellular ATP, which can be autocrinally generated by activated monocytes, and which interacts with $P2X_7$ purinergic receptors [4]. This has now been confirmed by results from mice lacking the $P2X_7$ receptors where macrophages primed with LPS are unable to process and to release IL-1β in response to application of ATP [5].

Although mammalian IL-1β is relatively well characterized, little information is available on IL-1β from lower vertebrates. The first non-mammalian sequences reported have even resulted in a more puzzling scenario, since fish, amphibian and bird *IL-1β* genes cloned so far lack a conserved ICE recognition site [6-11]. The production of specific antibodies to fish IL-1β will help, therefore, to understand the evolution of vertebrate IL-1β, as well as to identify mechanisms orchestrating its secretion in fish.

In this work, we report the production of a specific antiserum to IL-1β from a fish (gilthead seabream, *Sparus aurata* L.). The antibody allows us to characterize the inflammatory stimuli that regulate in this species the production, processing and release of IL-1β. To this end, we examined the extracellular signals involved in the secretion of

this cytokine and, to our surprise, found that extracellular ATP failed to induce the secretion of IL-1β by immune cells, whereas it promoted post-translational processing and release of IL-1β within microvesicles in SAF-1 fibroblasts of the same species.

Materials and Methods

Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata* L., Teleostei) were obtained from Culmarex S.A. (Murcia, Spain). They were kept in 260 l running seawater aquaria (flow rate 1500 l/h) at 20ºC with a 12-h L/D cycle, and were fed with a commercial pellet diet (Trouvit) at a feeding rate of 15 g dry diet/Kg biomass of fish/day.

Cloning of gilthead seabream IL-1β into expression vector

The coding region for the gilthead seabream IL-1β (sbIL-1β) was obtained by polymerase chain reaction amplification with F6 (5'-AAGGATCCAATGGAATCCGAGATGACATGC-3') and R5 (5'-AAGGATCCTTAACACTCTCCACCCTCC-3') as primers and LPS- (Sigma) stimulated head-kidney cDNA obtained using protocols described elsewhere [10] as template. The PCR-amplified product was purified and cloned into the *Bam*HI site of the pBluescript SK+ vector, and then subcloned into the *Bam*HI site of the pET15b vector for bacterial expression of the His_6-tagged protein.

Overexpression and purification of sbIL-1β

Escherichia coli BL21(DE3) cells freshly transformed with the pET15b-*sbIL-1β* construct were cultured overnight in LB-ampicillin medium. After dilution into fresh

LB-ampicillin, the cultures were grown at 37ºC to A_{600} of 0.4-0.6 and induced with 1mM isopropyl-D-thiogalactoside (IPTG, Gibco) for 2 h at 37ºC. Protein expression in whole cells extracts was checked by centrifuging 0.1 ml of induced culture (14,000 rpm in a Microfuge), and the cell pellet was lysed by boiling in SDS-loading buffer for analysis by SDS-PAGE [12] and Western blotting using an anti-polyhistidine monoclonal antibody (Sigma). To check the solubility of the expressed protein, the cell pellet (obtained as above) was suspended in buffer A (50 mM Tris, 2 mM EDTA, 200 mM NaCl, 4 mM 2-mercaptoethanol, pH=7.5), sonicated and centrifuged, and the supernatant and pellet were separately analysed by Western blotting. Under these experimental conditions, recombinant His_6-tagged sbIL-1β was found to be mainly insoluble.

Pelleted cells from 0.5 liter induced cultures were suspended in 3 ml of extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 6M guanidine-HCl, pH=7.8) and stirred for 3 h at room temperature. sbIL-1β protein from clarified supernatants was purified employing TALON metal affinity resin and the accompanying purification protocol (CLONTECH).

Polyclonal anti-sbIL-1β antibody

Anti-sbIL-1β rabbit polyclonal antibody was obtained using standard procedures [13]. Immunization was performed with His_6-tagged sbIL-1β excised off SDS-PAGE gels reversibly stained with Zn-imidazole negative staining [14]. The reactivity of the antisera to sbIL-1β was checked by Western blotting (see above).

Cell culture and treatments

Seabream head-kidney leukocytes obtained as described elsewhere [15] were stimulated overnight at 25°C with 0.1-20 μg/ml LPS, a 1/20 dilution of macrophage-activating factor (MAF) [15], 50U of human recombinant tumour necrosis factor alpha (hrTNFα, Sigma) [16] and/or 25-200 μg/ml genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) in sRPMI [RPMI-1640 culture medium (Gibco) adjusted to gilthead seabream serum osmolarity (353.33 mOs) with 0.35% NaCl] supplemented with 5% FCS (Gibco), unless otherwise indicated, and 100 I.U./ml penicillin and 100 μg/ml streptomycin (Biochrom). In some experiments, cells were then washed twice with sRPMI, and incubated for additional time periods between 0.5-3 hr with 5 mM ATP (Sigma) or 10μg/ml PTG-1 (a gift of Dr. CA Gabel) in sRPMI supplemented with 0.1% FCS. Where indicated, cells were pretreated for 60 min with 100 μM of the specific ICE inhibitor YVAD-CHO (Calbiochem) before ATP addition. At the end of the incubation, clarified supernatants were concentrated by precipitation with 20% trichloroacetic acid (TCA, Sigma), and the cells were extensively washed and lysed with boiling SDS-sample loading buffer. Alternatively, clarified supernatants were centrifuged at 100,000 *g* in a JA30.50 rotor (Beckman Avanti J30I) for 90 min at 4°C in the presence of protease inhibitors (Cocktail P8340, Sigma) to collect microvesicles [17].

The gilthead seabream fibroblast cell line SAF-1 [18] was purchased from the ECACC (UK) and grown in Leibovitz's L-15 culture medium (Gibco) supplemented with 15% FCS and penicillin/streptomycin at 25°C. Murine J774 macrophages and peritoneal macrophages from BALB/c mice were grown in RPMI-1640 medium supplemented with 10% FCS and penicillin/streptomycin at 37°C.

Western blot analysis

Cell extracts, concentrated supernatants and microvesicle fractions were analysed on a 15% SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad). The blots were developed using a 1/1000 dilution of polyclonal sbIL-1β antiserum or monoclonal 3ZD anti-mouse/human IL-1β (Biological Resources Branch, NCI) antibody, and enhanced chemiluminescence (ECL) reagents (Amersham Biosciences) according to the manufacturer's protocol. Membranes were then stained with a 0.1% Ponceau solution (Sigma) and/or reprobed with a 1:10,000 dilution of a monoclonal anti-β-actin (A5441, Sigma) to confirm a similar protein loading in all lanes. In some cases membranes were also reprobed with a 1:100 dilution of a goat polyclonal antibody raised against a conserved epitope of cathepsin D from human origin (sc-6486, Santa Cruz Biotechnology, Inc.).

Annexin V binding

PS exposure on the extracellular leaflet of the membrane was determined by flow cytometry using a FITC-Annexin V kit (BD Biosciences) according to the manufacturer's instructions. Cells were also gated by propidium iodide (PI, Sigma), used in this case as a marker of non-viable, necrotic cells.

Protein determination

The protein concentrations of cell lysates, supernatants and microvesicle preparations were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard.

Results

sbIL-1β is intracellularly accumulated by activated leukocytes

A rabbit polyclonal antiserum was generated against purified recombinant His_6-tagged sbIL-1β and used in Western blotting to detect the presence of IL-1β immunoreactivity in lysates from activated seabream leukocytes (Fig. 1). The antiserum reacted strongly against His_6-tagged sbIL-1β and detected a ~30 kDa polypeptide in cell lysates from LPS-stimulated head-kidney cells, but not in those from resting cells (Fig. 1A). In contrast, pre-immune serum was unable to react with any protein from lysates prepared from either LPS-stimulated or resting head-kidney cells (Fig. 1A). The size of the polypeptide that reacted with the sbIL-1β antiserum was similar to that expected for the deduced proIL1-β from the *sbIL-1β* cDNA sequence (10). Addition of lymphocyte-derived MAF alone or in combination with LPS also resulted in the production and intracellular accumulation of sbIL-1β by head-kidney cells, whereas addition of hrTNFα failed to induce the production of sbIL-1β and even reversed the effects of LPS and MAF (Fig. 1B). Notably, the combination of LPS and genomic DNA from the bacterium *Vibrio anguillarum* (VaDNA) was found to be synergistic (Fig. 1C). DNA from salmon testes failed to induce the production of sbIL-1β by head-kidney cells (Fig. 1C), and DNase I-digested VaDNA was not as effective as the non-digested one (Fig. 1D).

We next examined the kinetics of proIL-1β accumulation by seabream head-kidney leukocytes following LPS-stimulation (Fig. 2A). Cells incubated for 24 h in medium containing 5% FCS in the absence of LPS resulted in a weak production of proIL-1β. Nevertheless, the amount of the precursor intracellularly accumulated increased significantly upon LPS addition. The effect of LPS on proIL-1β accumulation was found to be dose-dependent, with greater increases of proIL-1β accumulation

occurring upon raising LPS dosage to higher than 5 μg/ml (Fig. 2B). Similarly, VaDNA was also able to increase in a dose-dependent manner pro-IL1β accumulation (Fig. 2C).

Extracellular ATP does not promote the release of sbIL-1β by immune cells

To determine whether extracellular ATP is capable of stimulating sbIL-1β secretion, a staged assay was used. Cells were stimulated with LPS to initiate proIL-1β synthesis and then treated with ATP. Unexpectedly, addition of 5 mM ATP following LPS-priming of seabream head-kidney cells did not promote proIL-1β post-translational processing and release (Fig. 3A). In sharp contrast, ATP treatment of mouse peritoneal macrophages led to the release of large amounts of the 17kDa IL-1β mature form and a concomitant disappearance of the cell-associated 31 kDa procytokine species (Fig. 3B).

It has been very recently demonstrated that human monocytes release IL-1β by microvesicle shedding from plasma membrane following ATP addition [19]. So we next examined if a similar mechanism might operate in fish. LPS/VaDNA-activated head-kidney cell cultures treated with ATP for up to 3 hr in the absence (Fig. 4) or presence of 5% FCS (not shown) released no significant sbIL-1β within microvesicles. Similarly, PTG-1, an antimicrobial peptide capable of initiating IL-1β post-translational processing and release [20], did not evoke measurable sbIL-1β in microvesicles nor in microvesicle-free fractions (Fig 4). These results were further confirmed by examining the cytokine that remained cell-associated following ATP and PTG-1 treatments (Fig. 4, left panel). We found that the amount of the 30 kDa IL-1β species to be consistently similar for the control, ATP- and PTG-1-treated cells.

Extracellular ATP fails to induce PS flip in seabream immune cells

Annexin V is a high-affinity PS binding protein which is widely used as a marker of cells destined for, or in the execution phase of, cell death by apoptosis because translocation of PS from the inner leaflet of the plasma membrane to the outer (PS flip) is a general feature of such apoptotic cells [21]). MacKenzie et al. [19] have recently shown that human $P2X_7$ receptor activation with ATP for 2 min resulted in a reversible PS flip which is associated with the rapid shedding of microvesicle containing mature IL-1β. We applied, therefore, FITC-conjugated annexin V to gilthead seabream head-kidney leukocytes that had been incubated for 30 min with ATP and then extensively washed. Unexpectedly, ATP addition (5 mM) did not induce a significant PS flip in head-kidney leukocytes: prior to agonist application <3% of cells were annexin-positive, and <2% of cells were annexin-positive after being treated for 30 min with ATP (Table 1). Similar results were obtained with the gilthead seabream fibroblast cell line SAF-1, where <1% of cells were annexin-positive both prior to and after ATP application (Table 1). By contrast, a significant increase in PS translocation (6.8% *vs* 17.4%) was found in murine J774 macrophages after being treated for 30 min with ATP and then washed and stained with FITC-conjugated annexin V (Table 1).

As PS flip upon ATP application for up to 10 min is completely reversed in human HEK293 cells stably expressing the rat $P2X_7$ receptor [19], we applied FITC-conjugated annexin V in the presence of ATP (15 min incubation with ATP alone followed by a second 15 min incubation after the addition of FITC-conjugated annexin V). While the head-kidney leukocytes were still refractive to ATP, about 20% of SAF-1 cells became annexin-positive under these experimental conditions. These results suggest the presence of a $P2X_7$ receptor homologue in SAF-1 cells whose engagement would lead to a fully

reversible PS flip within less than 30 min, and that this is not the case with head-kidney leukocytes.

Extracellular ATP promotes the secretion of a 22 kDa sbIL-1β form by SAF-1 cells

The translocation of PS in SAF-1 cells after extracellular ATP addition, prompted us to examine whether this cell line was able to produce and release IL-1β. VaDNA, but not LPS nor digested VaDNA, stimulated the intracellular accumulation of a 30 kDa sbIL-1β form (Fig. 5A). The addition of ATP to primed cells resulted in a significant reduction of cell-associated sbIL-1β (Fig. 5B, left panel) and a concomitant appearance of a 22 kDa polypeptide in the microvesicle fraction that reacted with the sbIL-1β antiserum (Fig. 5B, middle panel) and could, therefore, represent mature sbIL-1β. The microvesicle fractions showed a particular protein pattern that was completely different from those for cell extracts and supernatants: microvesicle fractions were negative for cathepsin D and β-actin when analyzed by Western blotting (data not shown). Importantly, the 30 kDa sbIL-1β species was detected in the microvesicle fraction when the cells were pre-treated with the specific ICE inhibitor YVAD-CHO (Fig. 5B, middle panel). Finally, sbIL-1β was never detected in the microvesicle-free supernatants (Fig. 5B, right panel), in common with that found in THP-1 monocytes [19].

Discussion

Using a polyclonal antiserum developed against IL-1β of the marine fish gilthead seabream, we have been able to investigate the production and release of fish IL-1β. Firstly, we found that IL-1β is synthesized, but not secreted by head-kidney leukocytes after stimulation with LPS, bacterial genomic DNA and lymphocyte-derived MAF either alone or in combinations. Importantly, LPS and bacterial DNA produced a synergistic

increase of IL-1β intracellular accumulation, suggesting that each ligand would signal through a different PRR. This observation is further supported by the fact that bacterial DNA was able to prime gilthead seabream SAF-1 cells for IL-1β production, whilst LPS was not able to do so. In mammals, Toll-like receptors (TLRs) represent a newly family of PRR that play a major role in pathogen recognition and initiation of inflammatory and immune responses [22]. TLR4 is responsible for recognition of LPS [23], while TLR9 recognizes the CpG motif found in microbial DNA [24]. Fish TLRs have not been cloned nor characterized to date, but a recent study has used the draft of the pufferfish *Fugu rubripes* genome project to predict that this teleost species contains ten *TLR* genes, and that eight of them are orthologues of human *TLR* genes [25]. Interestingly, the pufferfish has two *TLR* genes (*TLR21* and *TLR22*) that are unique to the fish, whereas it lacks *TLR4*. Functional studies are needed, however, to elucidate the role played by each TLR in fish immune response as well as their specificity.

Earlier studies in mammals have demonstrated that extracellular ATP acting on $P2X_7$ purinergic receptors promotes IL-1β post-translational processing and secretion [5,26,27]. Surprisingly, we found in the present study that extracellular ATP failed to promote the post-translational processing and release of proIL-1β in primed gilthead seabream leukocytes. However, we observed that extracellular ATP could induce a rapid release of a mature sbIL-1β form of about 22 kDa from SAF-1 fibroblast cells with a concomitant reduction of the pro-IL1β species that remained cell-associated. Furthermore, we demonstrated that the mature sbIL-1β form is present in a particulate fraction that has been previously showed to contain two types of membrane vesicles: exosomes and microvesicles [28]. Exosomes are derived from exocytosis of endolysosome-related multivesicular bodies, while microvesicles are generated by surface shedding and are associated with PS exposure [28]. The translocation of PS to the

outer leaflet of the cell membrane suggest that sbIL-1β may be released within microvesicles rather than within exosomes. This would be in agreement with previous studies in human THP-1 monocytes [19] and platelets [17], where IL-1β is released by microvesicle shedding.

The mechanism involved in the cleavage of fish IL-1β has also been clarified in this study. This is particularly important in view of the fact that all fish, amphibian and bird *IL-1β* genes sequences cloned so far lack a conserved ICE recognition site [6-11]. We demonstrated that the specific ICE inhibitor YVAD-CHO prevents the processing but not the release of sbIL-1β by primed SAF-1 cells, suggesting that fish IL-1β needs to be post-translationally processed to a 22 kDa polypeptide by an ICE protease before being secreted for it to play its physiological role. It is tempting to speculate on the relevance of a conserved Asp residue (Asp_{60} in gilthead seabream) in the cleavage of fish proIL-1β: this Asp is also found in all known non-mammalian IL-1β sequences, and cleavage at this position would yield a mature polypeptide with a molecular weight of approximately 22 kDa. On the other hand, the presence of ICE enzymes at this level of phylogeny is not surprising; we have found two sequences of the zebrafish *Danio rerio* (EMBL accession numbers AF233434 and AF327410) and one of the pufferfish (SINFRUT00000075787) that show a high degree of homology to mammalian ICE. However, our attempts to identify a gilthead seabream ICE homologue using a homology cloning approach have been unsuccessful to date.

Why gilthead seabream primary immune cells are unable to respond to extracellular ATP must await further studies. This will require the molecular cloning of the fish $P2X_7$ receptor homologue. A BLAST search against the pufferfish genomic database revealed the presence of a predicted transcript (SINFRUT00000054475) that codes for a $P2X_7$ receptor protein showing 46% identity and 61% similarity to the human

$P2X_7$ receptor. Studies are in progress in our laboratory to clone and characterize the gilthead seabream $P2X_7$ receptor. This would facilitate expression studies in primary leukocytes and SAF-1 cells, as well as for searches of proteins that may interact with it. In mammals, $P2X_7$ receptor interacts with several proteins to form a receptor signaling complex that may initiate the cytoskeletal rearrangements observed following receptor activation [29,30]. For example, epithelial membrane protein (EMPs) interact with the C terminus of the $P2X_7$ receptor and are able to induce membrane blebbing, PS flip, and cell death when overexpressed in HEK293 cells [30]. These findings suggest that the interaction of the $P2X_7$ receptor with EMPs may mediate some aspects of the downstream signaling following $P2X_7$ receptor activation. Therefore, the inability of extracellular ATP to promote PS flip, cell death, microvesicle shedding and IL-1β release in gilthead seabream immune cells may be related to the expression and/or activation of any of the components of the $P2X_7$ receptor signaling complex.

In summary, our data suggest that fish IL-1β is intracellularly accumulated following activation and that a second stimulus, such as ATP, is required for post-translational processing by an ICE-like protease and release within microvesicles. However, the inability of extracellular ATP to promote IL-1β secretion in primary immune cells might also indicate the absence of a functional $P2X_7$ receptor signaling complex in these cells as well as the involvement of signals other than ATP in the secretion of IL-1β in fish.

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Figure legends

Figure 1. sbIL-1β precursor is intracellularly accumulated by activated leukocytes. Western blot analysis of whole lysates obtained from $5x10^6$ head-kidney leukocytes incubated for 16 h in the absence or presence of LPS (10 μg/ml), hrTNFα (50U), MAF (1/20 dilution), VaDNA (50 μg/ml), salmon DNA (50 μg/ml) and/or DNase I-digested VaDNA (50 μg/ml). Filters were hybridized with rabbit pre-immune (A) or immune antiserum (A, B, C and D) to His_6-tagged sbIL-1β. The arrow-head in the left panel of A shows the 30 kDa polypeptide from LPS-activated leukocyte whole lysates that reacted with the immune antiserum. Reaction of immune antiserum with purified recombinant His_6-tagged sbIL-1β is also shown in A for comparison.

Figure 2. Kinetics of proIL-1β intracellular accumulation. Immunoblots of whole cell lysates obtained from $5x10^6$ head-kidney leukocytes incubated in medium alone or supplemented with 10 μg/ml LPS for the indicated times (A) or with different concentrations of LPS (B) or VaDNA (C) for 8 h and 16 h, respectively.

Figure 3. ATP fails to promote sbIL-1β release by head-kidney leukocytes. LPS-activated gilthead seabream head-kidney leukocytes (A) and mouse peritoneal exudate macrophages (B) were treated with 5 mM ATP for 30 min. Whole cell lysates ($5x10^6$ cells) and supernatants obtained from $25x10^6$ cells were probed with polyclonal anti-sbIL-1β antiserum (A). Cell lysates ($5x10^5$ cells) and supernatants obtained from 10^6 cells were probed with monoclonal 3ZD anti-mouse/human IL-1β (B). Migration positions for the mature and procytokine forms are indicated.

Figure 4. sbIL-1β is not contained within microvesicles following ATP or PTG-1 addition. LPS/VaDNA-primed head-kidney leukocytes were treated with 5 mM ATP or 10 μg/ml PTG-1 for 3 h. Whole cell lysates ($5x10^6$ cells), and microvesicle and supernatant fractions obtained from $25x10^6$ cells were then resolved by Western blot analysis.

Figure 5. ATP promotes post-translational processing and secretion of sbIL-1β β by SAF-1 cells. (A) IL-1β immunoblot from cells stimulated with 10 μg/ml LPS and/or 50 μg/ml VaDNA for 16 h. A control of cells incubated with 50 μg/ml DNase I-digested VaDNA for 16 h is also included for comparison. (B) VaDNA-primed cells were treated with 5 mM ATP, in the absence and presence of 100 μM YVAD-CHO, for 30 min. Whole cell lysates ($2.5x10^5$ cells), and microvesicle and supernatant fractions obtained from 10^6 cells were then resolved by Western blot analysis. The 30 kDa and the 22 kDa polypeptides that reacted with the anti-sbIL-1β antiserum in the microvesicle fractions from ATP treated cells are indicated by an arrow and arrow-head, respectively.

Table 1. ATP induces PS flip in SAF-1 cells, but not in head-kidney leukocytes. Cells were incubated for 30 min in the absence (control) or presence of 5 mM ATP, and then washed twice and stained with FITC-annexin V (pre-ATP). Alternatively, cells were stained with FITC-annexin V in the presence of ATP (total incubation time 30 min) (ATP). Values are given as the mean ± S.E. of the percentage of annexin-positive cells obtained from duplicate cultures. ND: not determined. HK: head-kidney.

		Control	pre-ATP	ATP
Seabream	HK	2.8±0.1	1.5±0.0	1.4±0.1
	SAF-1	0.7±0.1	0.8±0.2	19.1±3.9
Mouse	J774	6.9±0.1	17.4±3.0	ND

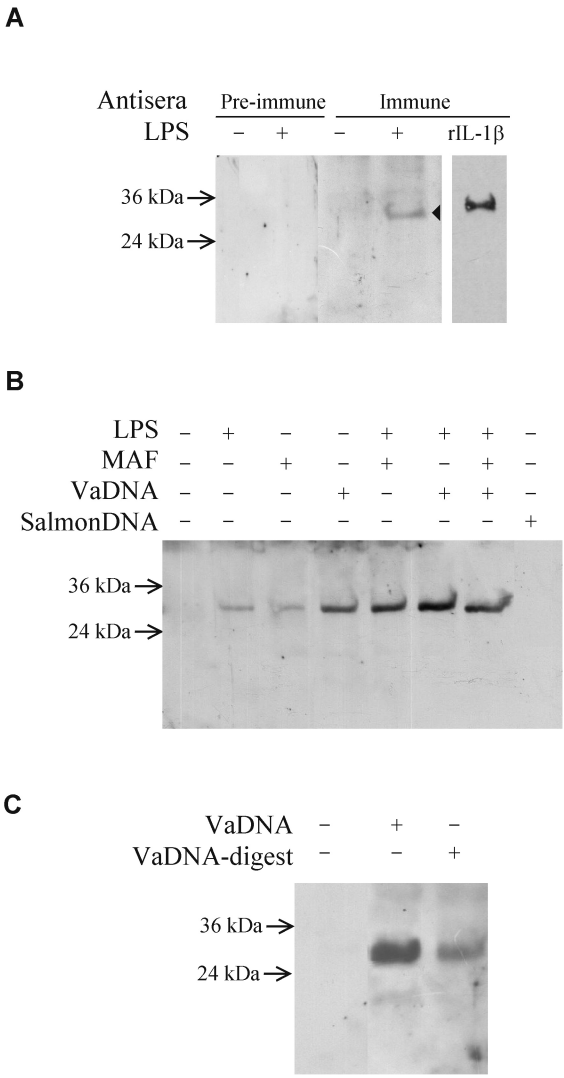


Figure 1: Pelegrín et al.

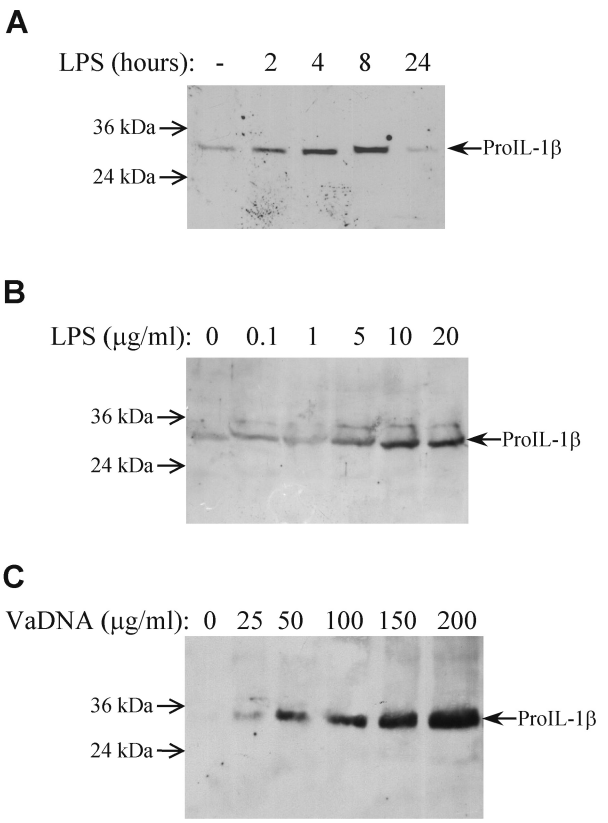
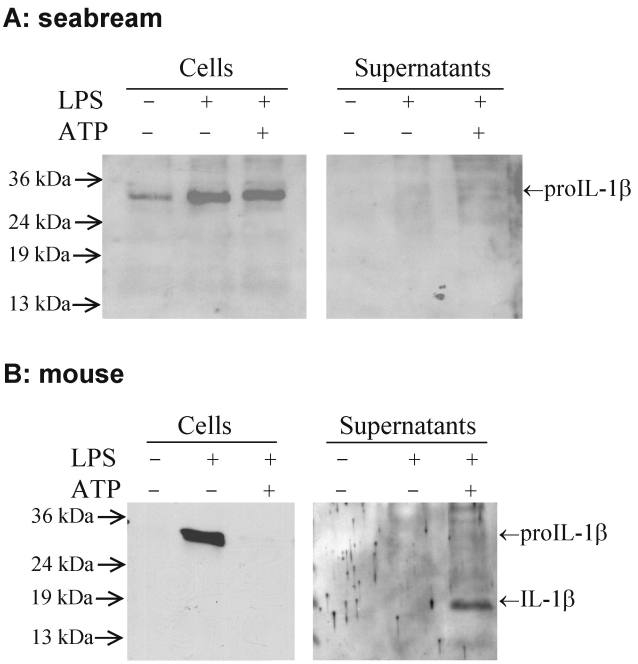


Figure 2: Pelegrín et al.



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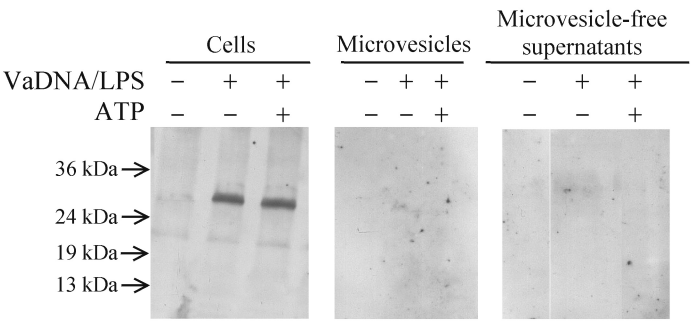

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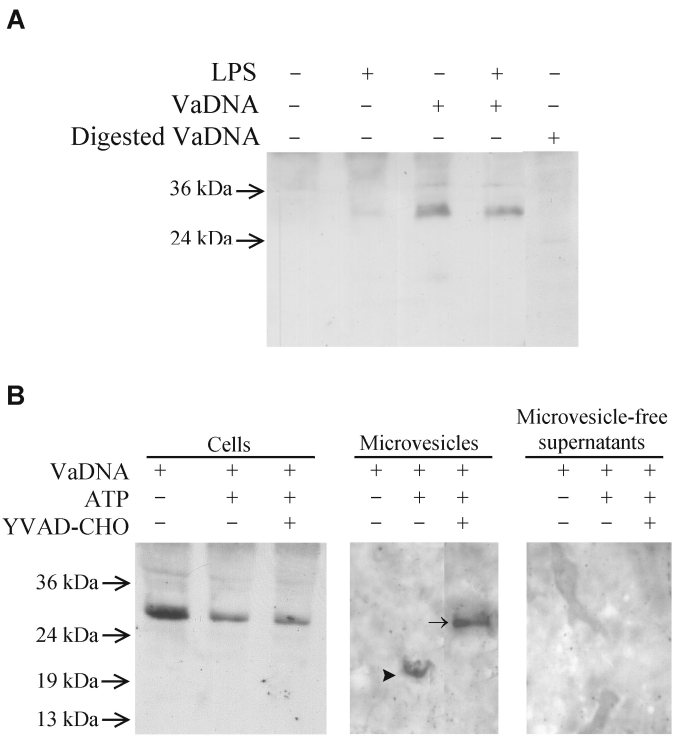

Figure 5: Pelegrín et al.