# Interleukin-1β isolated from a marine fish reveals up-regulated expression in macrophages following activation with lipopolysaccharide and lymphokines.

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**Abstract**

The gilthead seabream IL-1β gene consists of five exons/four introns. The complete coding sequence contains a 102 bp 5' untraslated region (UTR), a single open reading frame of a 762 bp which translates into a 253 amino acid molecule, and a 407 bp 3'UTR with a polyadenilation signal 14 nucleotides upstream of the poly(A)tail. The seabream sequence has highest degree of nucleotide (61.7%) and amino acid (53%) identity with the trout IL-1β sequences. The IL-1β message was detected by RT-PCR in head-kidney, blood, spleen, liver, gill and peritoneal exudate of both non-infected and *Vibrio anguillarum*-challenged fish. More importantly, IL-1β was highly expressed by purified macrophage monolayers and was up-regulated by lipopolysaccharide and lymphocyte-derived macrophage-activating factor stimulation.

## Introduction

Interleukin-1β (IL-1β) is a member of the IL-1 cytokine family having a β-trefoil structure composed of 12 β-sheets (1), and plays a pivotal role in the inflammatory response as well as in the maturation and proliferation of many immune cell types (2). It is also known that IL-1β is synthesised by activated monocytes and macrophages as a precursor with little activity. This precursor is then processed by caspase-1 (also called interleukin-1-converting enzyme, ICE), releasing a mature active peptide (3).

We report the cloning of a IL-1β gene from the marine fish gilthead seabream (*Sparus aurata* L.). IL-1β expression was also studied in different tissues from control and *Vibrio anguillarum*-challenged fish. The up-regulation of IL-1β expression in macrophages following activation with LPS and lymphocyte-derived macrophage-activating factor (MAF) is reported for the first time.

## Results

### Cloning and sequencing

Three overlapping products were obtained by RT-PCR (Fig. 1), which contained the full-length seabream IL-1β cDNA. The gene consisted of 1271 nucleotides including a 762 bp single reading frame, a 102 bp 5' untraslated region (UTR) and a 407 bp 3' UTR, with the latter containing 7 cytokine RNA instability motifs (ATTTA) typical of inflammatory cytokine genes, a 19 bp poly(A)tail and a polyadenylation signal 14 nucleotides upstream of the poly(A)tail (Fig. 2). The translated open reading frame gave a predicted 253 amino acid seabream IL-1β precursor peptide with a molecular weight of about 29 kDa and two potential N-glycosylation sites. Multiple alignments with others IL-1β sequences (Fig. 3) revealed a high level of conservation

within the 12 β-sheets that formed a β-trefoil structure, characteristic of all the members of the IL-1 cytokine family (4). However, the seabream IL-1β precursor, as other non-mammalian molecules sequenced to date, lacked the interleukin-1-converting enzyme (ICE) recognition site.

We found the highest degree of nucleotide identity with the two trout IL-1β sequences (60.5% with type one and 61.7% with type two) and amino acid similarity (67% with type one and 62% with type two). This close relationship with the trout IL-1β molecules was also apparent in the phylogenetic tree analysis, since the seabream IL-1β was close to the trout and carp sequences, and far from mammalian, amphibian and bird IL-1β sequences (data not shown).

Finally, PCR amplifications of genomic DNA, using primers deduced from the IL-1β cDNA sequence, revealed that the seabream IL-1β gene consists of five exons and four introns (Fig. 2).

Expression studies

The IL-1β transcript was detected in all the tissues examined of *V. anguillarum* challenged fish, although expression was clearly weakest in brain and strongest in blood and peritoneal exudate (Fig. 4A). Interestingly, we found a basal expression of the IL-1β transcript in most of the tissues from non-injected fish.

The *in vitro* studies revealed that the IL-1β transcript was found in purified macrophages after only 20 PCR cycles, when it remained absent in total head-kidney leukocytes. This was particularly evident after stimulation of macrophage samples with LPS and/or MAF, since a much stronger expression was detected at both 20 and 30 PCR cycles (Fig. 4B).

**Discussion**

In this study the full IL-1β gene sequence from the marine fish gilthead seabream is reported. The molecule shows a high degree of homology with the three known fish IL-1βs, especially with the trout IL-1β genes. The amino acid regions that form the secondary structure of 12 β-sheets, a feature of the β-trefoil cytokine family (4), were the areas showing the highest homology. In common with inflammatory molecules, the seabream sequence has numerous copies of the ATTTA motif in the 3' UTR, suggesting that its mRNA expression is tightly regulated. Mammalian IL-1β precursor is cleaved by ICE in Asp-X bound (where X is normally a small hydrophobic residue) to yield a 17 kDa carboxyl terminus-derived mature polypeptide which is then transported out of the cells, supporting the hypothesis that IL-1β post-translational processing involves a commitment to cell death (3). However, seabream IL-1β lacks the ICE recognition sequence, as do other non-mammalian IL-1β sequences (5-8). Therefore, the naturally IL-1β cut site and its releasing mechanism are undefined in these animals.

Interestingly, the organisation of the seabream IL-1β gene is different to that in mammals and fish, and comprises only five exons. Mammalian and carp IL-1β genes contain seven exons (9), whereas the trout sequences consist of six exons (10). Undoubtedly, more lower vertebrates Il-1β sequences are needed to fully understand the evolution of the Il-1β family members.

Seabream challenged by *V. anguillarum* show a remarkable variety of tissue-dependent expression changes, as assayed by RT-PCR. The strongest expression was found in peritoneal exudate and peripheral blood cells, which was to be expected since the bacterium was injected intraperitoneally and is a non-virulent strain unable to leave the peritoneal cavity and colonise other fish organs. An unexpected result was that the

IL-1β transcript was also found in some of the tissues examined from non-injected fish, in contrast with earlier findings in trout, where no basal expression of IL-1β was found (6).

In mammals, IL-1β is mainly produced by monocytes and macrophages. However, little is known about the cell types able to produce this molecule in fish. In this study, seabream macrophage monolayers showed a much stronger degree of IL-1β expression than total head kidney cells, suggesting that they are a major source of IL-1β in fish. Interestingly, IL-1β expression by seabream macrophages is enhanced upon LPS and/or MAF stimulation. This is the first demonstration that cytokines produced by activated fish lymphocytes are able to up-regulate IL-1β production by macrophages, suggesting that lymphokines produced during the course of an infection may regulate the production of this pro-inflammatory molecule *in vivo*.

## Material and Methods

### RNA and genomic DNA isolation

Gilthead seabream (*Sparus aurata* L.) head-kidney was extracted as described previously (11). Cell suspensions ($5x10^6$ cells/ml) were stimulated with 10 μg/ml of lipopolysaccharide (LPS, Sigma) for 4 hours at 25ºC. The cells were then centrifuged at 400xg and total RNA extracted from the cell pellets with TRIzol Reagent (Gibco) following the manufacturer's instructions. Genomic DNA was isolated from fresh seabream liver with TRIzol Reagent.

PCR, cloning and sequencing

The SuperScript II RNase $H^-$ Reverse Transcriptase (Gibco) was used to synthesise first strand cDNA with oligo-$dT_{12-18}$ primer (Gibco) from 5 μg of total RNA at 42ºC for 50 min. The cDNA was used in initial PCR with F13 and R primers (Fig. 1) designed against conserved motifs of known IL-1β sequences. PCR products were purified, cloned into the pGEM-T Easy Vector (Promega) and transfected into competent *Escherichia coli* DH-5α cells. Plasmid DNA was isolated and sequenced using a ABI PRISM 377 (Applied Biosystems, Perkin-Elmer).

Based on the partial seabream IL-1β sequence obtained with the product of F13/R primers, several seabream specific primers (Fig. 1) were used to obtain the 3' and 5' ends of the gene by rapid amplification of cDNA ends (RACE)-PCR (8) as well as the full gene by genomic PCR. Generated sequences were analysed using the ALIGN, BLAST2 and CLUSTALW programs (12,13).

Expression studies

Expression of the IL-1β transcript was studied by RT-PCR using F3 and R2 primers (Fig. 1). For *in vitro* experiments, cDNA was extracted from head kidney cells and purified macrophages incubated for 4h at 25ºC in medium alone or containing 10 μg/ml LPS and/or a 1/20 dilution of macrophage-activating factor (MAF) (11). For *in vivo* experiments, cDNA from several relevant tissues was isolated 24 h after challenging fish with $5x10^8$ cells of a non-virulent strain (ATCC 19264) of the fish bacterial pathogen *Vibrio anguillarum* (14).

**Acknowledgements**

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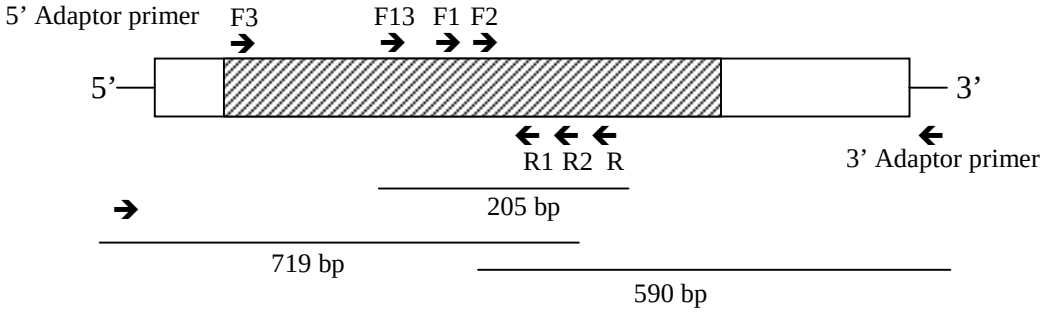
**Figure legends**

**Fig. 1** Primers used to amplify gilthead seabream IL-1β gene and products obtained. R = A/G, Y = C/T.

**Fig. 2** Compiled full-length gilthead seabream IL-1β sequence. The start and stop codons, and RNA instability motif (ATTTA) in the 3' UTR are shown in bold. The polyadenylation signal is underlined. The N-glycosylation sites are boxed. Intron positions are indicated by vertical lines. EMBL accession no. AJ277166.

**Fig. 3** Multiple alignment of the gilthead seabream IL-1β (in bold) with other known IL-1βs. Identical (*) and similar (. or :) residues identified by the CLUSTAL W program are indicated. The arrow shows the caspase-1 cut site seen in all the mammalian sequences. The amino acid regions that form the secondary structure of 12 β-sheets are indicated.

**Fig. 4 A,B** IL-1β *in vivo* (A) and *in vitro* (B) expression assayed by RT-PCR. The results are representative of two independent experiments. *HK* = head kidney. *PE* = peritoneal exudate.




IL-1β untranslated region (509 bp)

IL-1β coding sequence (762 bp)

| Name | Sequence (5’→3’) |
|---|---|
| Forward (F)13 | GGGAAAGAATCTRTACCTGTCYTG |
| Reverse (R) | TGAGAGGTGCTGATGAAC |
| F1 | GCTTGCATCTGGAGGCGGTGG |
| F2 | GGGCTGAACAACAGCACTCTC |
| F3 | ATGCCCGAGGGGCTGGGC |
| R1 | GTCGCTGCCCGGGGTGATCC |
| R2 | CAGTTGCTGAAGGGAACAGAC |
| Oligo-dT adaptor | GGCCACGCGTCGACTAGTAC$(T)_{16}$ |
| Adaptor | GGCCACGCGTCGACTAGTAC |
| β-actin F | ATCGTGGGGCGCCCCAGGCACC |
| β-actin R | CTCCTTAATGTCACGCACGATTTC |

**Figure 1**

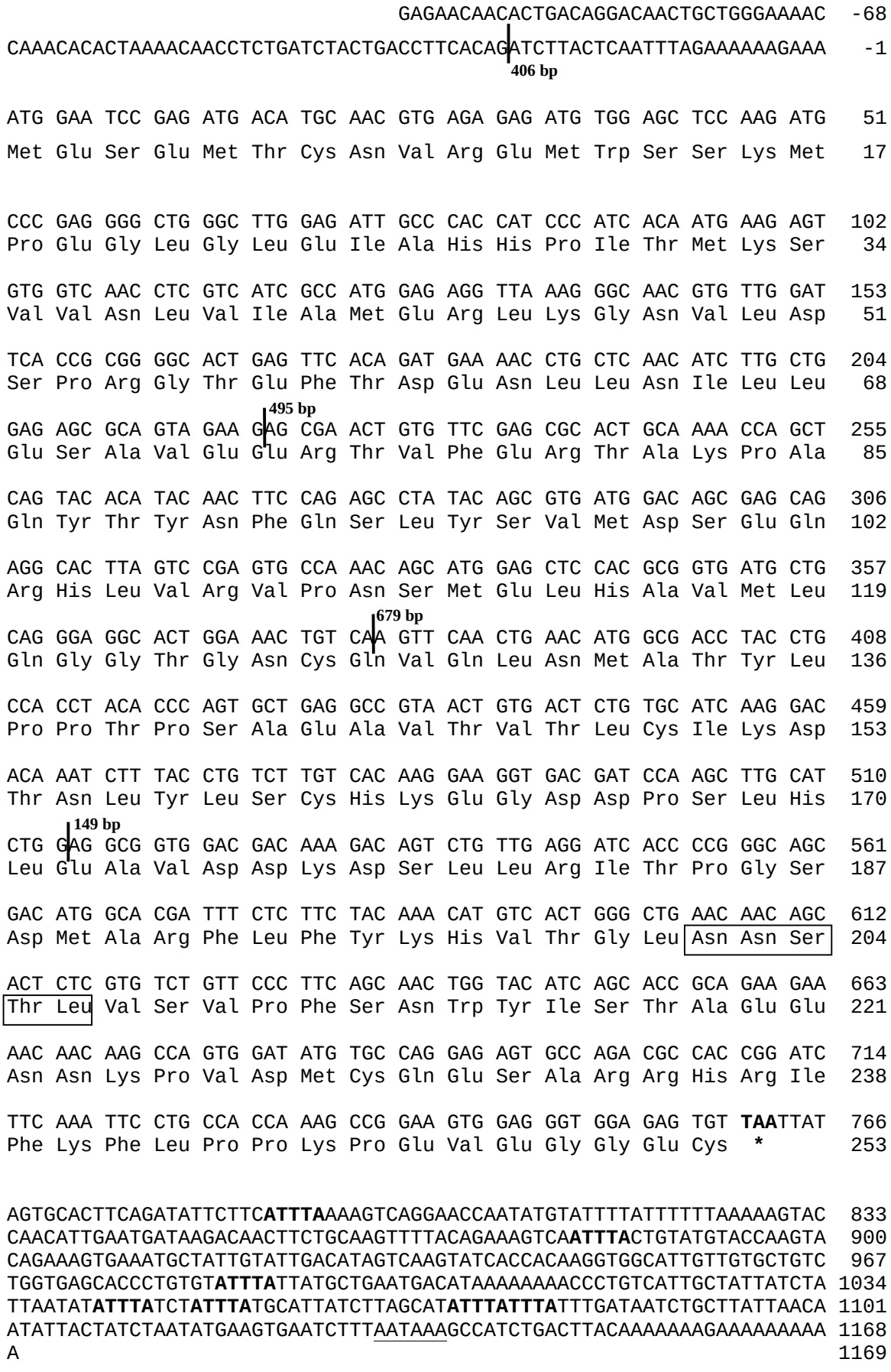

GAGAACAACACTGACAGGACAACTGCTGGGAAAAC -68
CAAACACACTAAAACAACCTCTGATCTACTGACCTTCACAGATCTTACTCAATTTAGAAAAAAGAAA -1
406 bp
ATG GAA TCC GAG ATG ACA TGC AAC GTG AGA GAG ATG TGG AGC TCC AAG ATG 51
Met Glu Ser Glu Met Thr Cys Asn Val Arg Glu Met Trp Ser Ser Lys Met 17
CCC GAG GGG CTG GGC TTG GAG ATT GCC CAC CAT CCC ATC ACA ATG AAG AGT 102
Pro Glu Gly Leu Gly Leu Glu Ile Ala His His Pro Ile Thr Met Lys Ser 34
GTG GTC AAC CTC GTC ATC GCC ATG GAG AGG TTA AAG GGC AAC GTG TTG GAT 153
Val Val Asn Leu Val Ile Ala Met Glu Arg Leu Lys Gly Asn Val Leu Asp 51
TCA CCG CGG GGC ACT GAG TTC ACA GAT GAA AAC CTG CTC AAC ATC TTG CTG 204
Ser Pro Arg Gly Thr Glu Phe Thr Asp Glu Asn Leu Leu Asn Ile Leu Leu 68
495 bp
GAG AGC GCA GTA GAA GAG CGA ACT GTG TTC GAG CGC ACT GCA AAA CCA GCT 255
Glu Ser Ala Val Glu Glu Arg Thr Val Phe Glu Arg Thr Ala Lys Pro Ala 85
CAG TAC ACA TAC AAC TTC CAG AGC CTA TAC AGC GTG ATG GAC AGC GAG CAG 306
Gln Tyr Thr Tyr Asn Phe Gln Ser Leu Tyr Ser Val Met Asp Ser Glu Gln 102
AGG CAC TTA GTC CGA GTG CCA AAC AGC ATG GAG CTC CAC GCG GTG ATG CTG 357
Arg His Leu Val Arg Val Pro Asn Ser Met Glu Leu His Ala Val Met Leu 119
679 bp
CAG GGA GGC ACT GGA AAC TGT CAA GTT CAA CTG AAC ATG GCG ACC TAC CTG 408
Gln Gly Gly Thr Gly Asn Cys Gln Val Gln Leu Asn Met Ala Thr Tyr Leu 136
CCA CCT ACA CCC AGT GCT GAG GCC GTA ACT GTG ACT CTG TGC ATC AAG GAC 459
Pro Pro Thr Pro Ser Ala Glu Ala Val Thr Val Thr Leu Cys Ile Lys Asp 153
ACA AAT CTT TAC CTG TCT TGT CAC AAG GAA GGT GAC GAT CCA AGC TTG CAT 510
Thr Asn Leu Tyr Leu Ser Cys His Lys Glu Gly Asp Asp Pro Ser Leu His 170
149 bp
CTG GAG GCG GTG GAC GAC AAA GAC AGT CTG TTG AGG ATC ACC CCG GGC AGC 561
Leu Glu Ala Val Asp Asp Lys Asp Ser Leu Leu Arg Ile Thr Pro Gly Ser 187
GAC ATG GCA CGA TTT CTC TTC TAC AAA CAT GTC ACT GGG CTG AAC AAC AGC 612
Asp Met Ala Arg Phe Leu Phe Tyr Lys His Val Thr Gly Leu Asn Asn Ser 204
ACT CTC GTG TCT GTT CCC TTC AGC AAC TGG TAC ATC AGC ACC GCA GAA GAA 663
Thr Leu Val Ser Val Pro Phe Ser Asn Trp Tyr Ile Ser Thr Ala Glu Glu 221
AAC AAC AAG CCA GTG GAT ATG TGC CAG GAG AGT GCC AGA CGC CAC CGG ATC 714
Asn Asn Lys Pro Val Asp Met Cys Gln Glu Ser Ala Arg Arg His Arg Ile 238
TTC AAA TTC CTG CCA CCA AAG CCG GAA GTG GAG GGT GGA GAG TGT TAATTAT 766
Phe Lys Phe Leu Pro Pro Lys Pro Glu Val Glu Gly Gly Glu Cys * 253
AGTGCACTTCAGATATTCTTCATTTAAAAGTCAGGAACCAATATGTATTTTATTTTTTAAAAAGTAC 833
CAACATTGAATGATAAGACAACTTCTGCAAGTTTTACAGAAAGTCAATTTACTGTATGTACCAAGTA 900
CAGAAAGTGAAATGCTATTGTATTGACATAGTCAAGTATCACCACAAGGTGGCATTGTTGTGCTGTC 967
TGGTGAGCACCCTGTGTATTTATTATGCTGAATGACATAAAAAAAACCCTGTCATTGCTATTATCTA 1034
TTAATATATTTATCTATTTATGCATTATCTTAGCATATTTATTTATTTGATAATCTGCTTATTAACA 1101
ATATTACTATCTAATATGAAGTGAATCTTTAATAAAGCCATCTGACTTACAAAAAAAGAAAAAAAAA 1168
A 1169

**Figure 2**

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Human          MAEVPKLASEMMAYYSGNEDDLFFEADGPKQMKC--SFQDLDLCPL-DGGIQLRISDHHY 57
Mouse          MATVPELNCEMPPFDS-DENDLFFEVDGPQKMKG--CFQTFDLGCP-DESIQLQISQQHI 56
Sheep          MATVPEPINEVMAYYS-DENELLFEVDGPKQMKS--CTQHLDLGSMGDGNIQLQISHQLY 57
Chicken        MAFVPDLDVLESSSLS--EETFYGPSCLCLQKKP------RLDSEHTTVDVQVTVRKGRG 52
Xenopus        MALVPDLSSIPMEGYSGDDEMFYSDSPSGMKDDMGDAAQWQSSTSHCSLDIHVQITHGKG 60
Trout1         MDFESNYSLIKNTSES---------AAWSSKLPQG-----------------LDLEVSHH 34
Trout2         MEFESNCSLMKNTSAS---------VAWSSKLPQG-----------------LDVEISHH 34
Carp           MAYHKYVHPLDLSEAFETDSAIYSDSADSDELDCP----DPQSMSCQCDMHDIKLELSSH 56
Seabream       ------MESEMTCNVR---------EMWSSKMPEG-----------------LGLEIAHH 28
                                        :                     : :

Human          SKGFRQAASVVVAMDKLRKMLVPCPQT--FQENDLSTFFPFIFEEEP--IFFDTWDN--E 111
Mouse          NKSFRQAVSLIVAVEKLWQLPVSFPWT--FQDEDMSTFFSFIFEEEP--ILCDSWDDDDN 112
Sheep          NKSFRQVVSVIVAMEKLRSR--AYEHV--FRDDDLRSILSFIFEEEP--VIFETSSDE-- 109
Chicken        ARSFRRAAVLVVAMTKLLRR--PRSRD--FADSDLSALLEEVF--EP--VTFQRLESSYA 104
Xenopus        SLHSFRKAVVLVVAVEKLKR--GKERF--FGDEDLLGLLDSIFVEEE--IGFSQAKETYA 114
Trout1         PITMRHIANLIIAMERLKG-GEGVTMGTEFKDKDLLNFLLESAVEEHIVLELESAPPASR 93
Trout2         PITLRCVANLIIAMERLNG-GKGFTLG---RDEGLLNFLLESAVEV---LELESARTEAS 87
Carp           PHSMRQVVNIIIAVERLKHIKNMSSGK--FCDEELLGFILENVIEER------LVKPLNE 108
Seabream       PITMKSVVNLVIAMERLKGNVLDSPRGTEFTDENLLNILLESAVEER------TVFERTA 82
                      ..:::.  .               :. :  ::

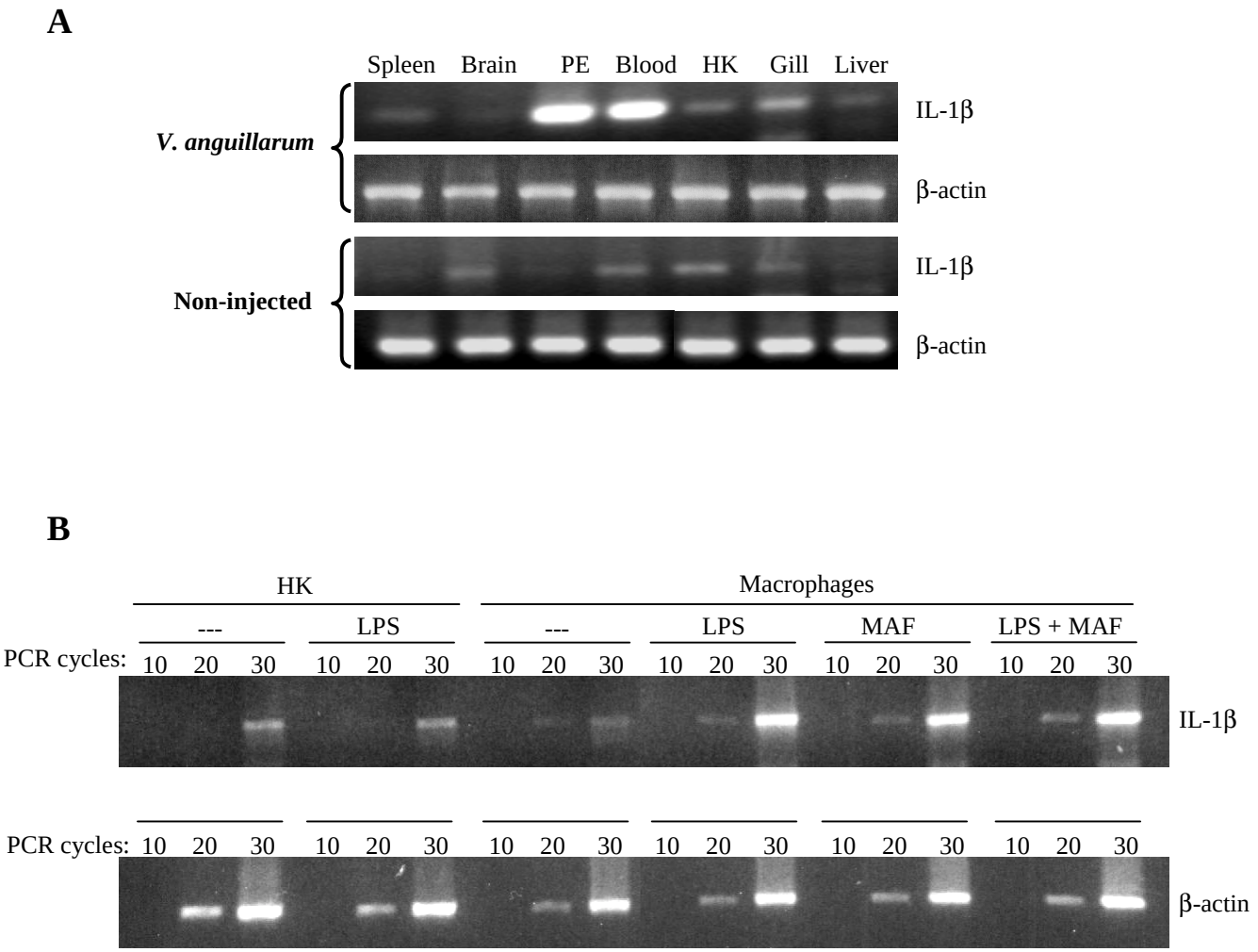
                      ↓
Human          AYVHDAPVRSLNCTLRDSQQKSLVMSGP-----YELKALHLQGQDMEQQVVFSMSFVQ-- 164
Mouse          LLVCDVPIRQLHYRLRDEQQKSLVLSDP-----YELKALHLNGQNINQQVIFSMSFVQ-- 165
Sheep          -LLCDAAVQSVKCKLQDREQKSLVLDSP-----CVLKALHLPSQEMSREVVFCMSFVQ-- 161
Chicken        GAPAFRYTRSQSFDIFDINQKCFVLESP-----TQLVALHLQGPSSSQKVRLNIALYRPR 159
Xenopus        SASTYRYQRATTCRIKDTSNKCFVMQKFH--ENAQLVALQLQGANIQREEKVSMAFYATQ 172
Trout1         RAAGFSSTSQYECSVTDSENKCWVLMNE----AMELHAMMLQGGSSYHKVHLNLSSYVTP 149
Trout2         SRAAFSSKGEYECSVTDSENKCWVLNEG----SMELHAIMLQGGSSYHKVHLNLSTYITP 143
Carp           TPIYSKTSLTLQCTICDKYKKTMVQSNKLSDEPLHLKAVTLSAGAMQYKVQFSMSTFVS- 167
Seabream       KPAQYTYNFQSLYSVMDSEQRHLVRVPN----SMELHAVMLQGGTGNCQVQLNMATYLPP 138
Secondary                 . *  ::  *           * *: * .     :  . ::
Structure             RSLNCTLRD   KSLVMS       YELKALHL        VVFSMSF

Human          ---GEESNDKIPVALGLKEKNLYLSCVLKD---DKPTLQLESVDPKNYPKKK----MEKR 214
Mouse          ---GEPSNDKIPVALGLKGKNLYLSCVMKD---GTPTLQLESVDPKQYPKKK----MEKR 215
Sheep          ---GEERDNKIPVALGIRDKNLYLSCVKKG---DTPTLQLEEVDPKVYPKRN----MEKR 211
Chicken        GPRGSAGTGQMPVALGIKGYKLYMSCVMSG---TEPTLQLEEADVMRDIDSV----ELTR 212
Xenopus        PHQGGS---KRPVALGLAGKNLYLSCRATEDGQDSPKLYLEEISNIKDVKGE----DLNR 225
Trout1         ---VPIETEARPVALGIKGSNLYLSCSKSG---GRPTLHLEEVADKDQLKSISQQSDMVR 203
Trout2         ---VPSETKARPVALGIKGSNLYLSCITSE---GTPTLHLEEVADKEQLKSINHESDMVR 197
Carp           ---SATQKEAQPVCLGISNSNLYLACTQLDG--SSPVLILKEASGSVNTIKAG--DPNDS 220
Seabream       ---TP-SAEAVTVTLCIKDTNLYLSCHKEG---DDPSLHLEAVDDKDSLLRITPGSDMAR 191
Secondary                .* * :   :**::*         * * *:
Structure             DIKPVALGLK  NLYLSCVLK      PTLQLESVD

Human          FVFNK----IEINNKLEFESAQFPNWYISTSQAE--NMPVFLGGTKGGQDITDFTMQFVS 268
Mouse          FVFNK----IEVKSKVEFESAEFPNWYISTSQAE--HKPVFLG-NNSGQDIIDFTMESVS 268
Sheep          FVFYK----TEIKNTVEFESVLYPNWYISTSQIE--EKPVFLGRFRGGQDITDFRMETLS 265
Chicken        FIFYRLDSPTEGTTR--FESAAFPGWFICTSLQP--RQPVGITNQPDQVNIATYKLSGR- 267
Xenopus        FIFMKSQDGLNETSTNSFESVAFPGWYISTSQRE--NELVQMVHQKNQEAIKDFNLFSVI 283
Trout1         FLFYR---RNTGVDISTLESASFRNWFISTDMQQDYTKPVDMCQKAAPNRLTTFTIQRHN 260
Trout2         FLFYK---QDTGVDISTLESAHYRNWFISTALQQDNTKMVNMCQRATLNRNTTFTIQRHN 254
Carp           LLFFR---KETGTRYNTFESVKYPGWFISTAFDD--WEKVEMN-QMPTTRTTNFTLEDQK 274
Seabream       FLFYK---HVTGLNNSTLVSVPFSNWYISTAEE--NNKPVDMCQESARRHRIFKFLPPKP 246
Secondary      ::* :           : *  : .*:*.*         * :           :
Structure      FVFNK----IEI  KLEFESA    WYISTS     NMPVFLG       ITDFTMQFVS

Human          S------ 269
Mouse          S------ 269
Sheep          P------ 266
Chicken        -------
Xenopus        -------
Trout1         -------
Trout2         -------
Carp           RI----- 276
Seabream       EVEGGEC 253
```

**Figure 3**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Spleen
Brain
PE
Blood
HK
Gill
Liver
V. anguillarum
IL-1β
β-actin
Non-injected
IL-1β
β-actin
B
HK
Macrophages
---
LPS
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LPS
MAF
LPS + MAF
PCR cycles:
10 20 30 10 20 30 10 20 30 10 20 30 10 20 30 10 20 30
IL-1β
PCR cycles:
10 20 30 10 20 30 10 20 30 10 20 30 10 20 30 10 20 30
β-actin

**Figure 4**