



UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

**Phylogenetic, Molecular and Functional Characterization
of New Interleukin-1 Family Members
in Teleost Fish**

**Caracterización Filogenética, Molecular y Funcional
de Nuevos Miembros de la Familia de la Interleuquina-1
de Peces Teleósteos**

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INDEX

ABBREVIATIONS	1
SUMMARY	6
INTRODUCTION	9
1. Cytokines: messengers of the immune system	10
2. The interleukin family	12
3. IL-1 β	15
4. IL-1 β expression and regulation	16
5. IL-1 receptors	17
6. IL-1 β processing mechanism	19
7. Inflammatory caspases: caspase-1	21
8. Caspase-1 activators	22
9. The intracellular receptors NLR (NOD-LRR) family	23
10. IL-1 β release mechanisms	29
11. Intracellular bacteria and the role of pyroptosis	31
OBJECTIVES	35
CHAPTER I: Evolution of inflammasome functions in vertebrates:	
Inflammasome and caspase-1 trigger fish macrophage cell death	
but are dispensable for the processing of IL-1β	37
Abstract	38
1. Introduction	39
2. Materials and methods	41
2.1 Animals	41
2.2. Cell culture and treatment	41
2.3. Cell viability	42
2.4. Caspase-1 activity assay	42
2.5. Western blot	43
2.6. Bactericidal assay	43
2.7. Cell transfection	43
2.8. Statistical analysis	44
3. Results	45
3.1. Infection of seabream head-kidney leukocytes with invasive and	

non-virulent bacteria leads to the processing and release of mature IL-1 β	45
3.2. Seabream caspase-1 fails to process IL-1 β	48
3.3. Classical NLRP3 inflammasome activators fail to activate caspase-1 and IL-1 β processing in seabream macrophages	49
3.4. Invasive <i>S. typhimurium</i> promotes caspase-1 activation and cell death in seabream	50
4. Discussion	52
 CHAPTER II: A <i>Salmonella</i>-zebrafish infection model to study the role of inflammasome in the clearance of intracellular bacteria	 55
Abstract	56
1. Introduction	57
2. Materials and methods	59
2.1. Animals	59
2.2. Cell culture and treatments	59
2.3. Caspase-1 activity assay	59
2.4. Zebrafish microinjection, chemical treatment and infection	60
2.5. Analysis of gene expression	60
2.6. Statistical analysis	61
3. Results and discussion	62
3.1. The T3SS is <i>S. typhimurium</i> is required for its virulence in zebrafish	62
3.2. PYCARD is required for the activation of caspase-1 in zebrafish but dispensable for <i>S. typhimurium</i> clearance	63
3.3. Caspase-1 is dispensable for <i>S. typhimurium</i> clearance but an unidentified caspase is required	67
 CHAPTER III: Identification and functional characterization of a new IL-1 family member, IL-1Fm2, in most evolutionary advanced fish	 70
Abstract	71
1. Introduction	72
2. Materials and methods	74
2.1. Identification of teleost IL-1Fm2 and sequence analysis	74

2.2. Animals	76
2.3. Production of recombinant seabream IL-1Fm2	77
2.4. Western blot	77
2.5. Experimental infections	77
2.6. Cell culture and treatments	78
2.7. Respiratory burst assay	78
2.8. Cell viability	79
2.9. RT-qPCR	79
2.10. Statistical analysis	79
3. Results	80
3.1. Molecular characteristics of seabream IL-1Fm2	80
3.2. IL-1Fm2 is exclusively present in most evolutionary advanced teleost fish	83
3.3. IL-1Fm2 is ubiquitously expressed in <i>S. aurata</i> and weakly induced after infection	86
3.4. IL-1Fm2 is weakly induced by <i>S. aurata</i> phagocytes after PAMP activation	87
3.5 Recombinant <i>S. aurata</i> IL-1Fm2 activates phagocytes	88
4. Discussion	92
CONCLUSIONS	95
REFERENCES	97
PUBLICATIONS	122
RESUMEN EN ESPAÑOL	124
Introducción	125
Objetivos	129
Materiales y métodos	131
Resultados y discusión	138
1. Evolución de las funciones del inflammasoma en vertebrados: el inflammasoma y la caspasa-1 desencadenan muerte celular en macrófagos de dorada, pero son dispensables para el procesamiento de la IL-1 β	139
2. <i>Salmonella</i> como modelo de infección en pez cebra para estudiar	

el papel del inflamasoma en la eliminación de bacterias intracelulares	140
3. Identificación y caracterización funcional de un nuevo miembro de la familia de la IL-1, IL-1Fm2, en los peces teleósteos más avanzados evolutivamente	140
Conclusiones	144

ABBREVIATIONS

Ac-YVAD-CHO	Caspase-1 specific inhibitor
Ac-YVAD-CMK	Caspase-1 specific inhibitor
AG	Acidophilic granulocytes
Alum	Aluminium salt
ASC	Apoptosis-associated speck-like protein
ATP	Adenosine 5'-triphosphate
AP-1	Activator protein-1
BSA	Bovine serum albumin
BzATP	2', 3'-O-(4-benzoylbenzoyl) adenosine 5'-triphosphate
eATP	Extracellular ATP
CARD	Caspase recruitment domain
6xHis	6xHistidin epitope
G	Gill
CEBP	CCAAT/enhancer binding protein
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate
COX-2	Ciclooxygenase-2
CSF	Colony stimulating factor
DAB	3, 3'-diaminobenzidine tetrahydrochloride
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis (2-aminoethyl ether)-N, N, N', N'-tetraacetic acid
F	Direct primer
FBS	Fetal bovine serum

FGSD	Fish-specific genome duplication
gDNA	Genomic DNA
GM-CSF	Granulocyte monocyte-colony stimulating factor
HEK293	Human embryonic kidney 293 cells
HEPES	4-(2-hydroxyethyl) piperazine-1- ethanesulfonic acid
HK	Head kidney
ICE	IL-1 β converting enzyme or caspase-1
IFN	Interferon
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1Ra	IL-1 receptor antagonist
IL-1RacP	IL-1 receptor accessory protein
IL-1RI	Type I interleukin-1 receptor
IL-1RII	Type II interleukin-1 receptor
L	Liver
L-15	Leibovitz's culture medium
LDH	Lactate dehydrogenase
LLO	Listeriolysin O
LPS	Lipopolysaccharides
LRR	Leucine rich repeats
MACS	Magnetic-activated cell sorting
MAF	Macrophage activating factor
MAPK	Mitogen-activated protein kinase
mDAP	Gamma-d-glutamyl-meso-diaminopimelic acid
MDP	Muramyl dipeptide
mRNA	Messenger RNA
MSU	Monosodium urate

MØ	Macrophages
MO	Morpholino
MOI	Multiplicity of infection
NAIP	NLR family, apoptosis inhibitory protein
ND	Non determined
NF-κB	Nuclear factor-κB
n.s	Non-significant
nt	Nucleotides
NLR	Nucleotide-binding domain and leucine rich repeat containing
NLRC4	NLR family CARD domain-containing protein 4
NLRP1	NLR family, pyrin domain containing 1
NLRP3	NLR family, pyrin domain containing 3
ORF	Open reading frame
Pam3CSK ₄	Synthetic tripalmitoylated lipopeptide
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Peritoneal exudate
PGN	Peptidoglycan
PI	Propidium iodide
P2X7R	P2X7 receptor
Poly I:C	Polyinosinic:polycytidylic acid
ProIL-1β	IL-1β precursor
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PYD	Pyrin domain
Q-VD-OPh	Pan-caspase inhibitor

R	Reverse primer
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcription
RTS-11	Trout mononuclear phagocyte cell line
S	Spleen
SAF-1	Seabream fibroblast cell line
sRPMI	RPMI-1640 culture medium supplemented with 0.35% NaCl
SP1	Transcription factor SP1
SPI	<i>Salmonella</i> pathogenicity island
T	Thymus
T3SS	Type 3 secretion system
TCA	Trichloroacetic acid
TdT	Terminal deoxynucleotidyl transferase
TIR	Toll/IL-1R domain
TLR	Toll-like receptors
TNF	Tumor necrosis factor
UTR	Untranslated region
VaDNA	<i>Vibrio anguillarum</i> genomic DNA
XTT	2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt
ZF	Zebrafish
Z-YVAD-CHO	Caspase-1 specific substrate
Z-VAD-FMK	Pan-caspase inhibitor

SUMMARY

During the development of this Doctoral Thesis, we have tried to characterize the processing and release mechanism of IL-1 β in the bony fish gilthead seabream *Sparus aurata*. Stimulation or infection of seabream macrophages (M ϕ) led to the caspase-1-independent processing and release of IL-1 β . In addition, several classical activators of the NLRP3 inflammasome failed to activate caspase-1 and to induce the processing and release of IL-1 β . Furthermore, the processing of IL-1 β in seabream M ϕ is not prevented by caspase-1 or pan-caspase inhibitors, and recombinant seabream caspase-1 failed to process IL-1 β . However, the pharmacological inhibition of caspase-1 impaired *Salmonella typhimurium*-induced cell death. These results suggest a role for the inflammasome and caspase-1 in the regulation of pyroptotic cell death in fish and support the idea that its use as a molecular platform for the processing of pro-inflammatory cytokines arose after the divergence of fish and tetrapods.

Secondly, we aimed to characterize the role of the adaptor molecule PYCARD (ASC) and caspase-1 in the clearance of intracellular bacteria in zebrafish. For this purpose, we have developed a salmonella-zebrafish infection model. Using this model, we found the genetic depletion of PYCARD impaired the activation caspase-1 in zebrafish larvae infected with wild type *S. typhimurium*. However, PYCARD-depletion or pharmacological inhibition of caspase-1 did not have a significant impact in the clearance of *S. typhimurium*. In contrast, pharmacological inhibition of all caspases using a pan-caspase inhibitor increased zebrafish susceptibility of *S. typhimurium* infection. Collectively, these results indicate that *S. typhimurium* possesses different redundant mechanisms to activate inflammatory caspases through different components of the inflammasome and suggest the existence of another inflammatory caspase involved in *S. typhimurium* clearance in zebrafish.

Finally, we have identified a new member of the IL-1 family (IL-1Fm2) that is exclusively present in species belonging to the most evolutionarily advanced group of teleost fish (Series Percomorpha), including the gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and tilapia (*Oreochromis niloticus*) (Perciformes); medaka (*Oryzias latipes*, Beloniformes); stickleback (*Gasterosteus aculeatus*, Gasterosteiformes); platyfish (*Xiphophorus maculatus*, Cyprinodontiformes) and Japanese flounder (*Paralichthys olivaceus*, Pleuronectiformes). However, IL-1Fm2 seems to be absent in pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*, Tetraodontiformes), which also belong to the percomorphs. The expression pattern of

gilthead seabream IL-1Fm2 revealed that although it was hardly induced by pathogen-associated molecular patterns (PAMPs), the combination of PAMPs and IL-1Fm2 synergistically induced its expression in macrophages. In addition, recombinant IL-1Fm2 was able to activate the respiratory burst of seabream phagocytes and to synergistically induce the expression of IL-1 β , TNF α and IL-10 when combined with PAMPs. These results demonstrate an important role of IL-1Fm2 in the regulation of fish immune responses.

INTRODUCTION

1. CYTOKINES: MESSENGERS OF THE IMMUNE SYSTEM

Cytokines are proteins (usually glycoproteins) with a low molecular weight (usually no more than 8-25kDa) that regulate all the important biological processes, including cell growth and activation, inflammation, tissue repair, fibrosis and morphogenesis. They are considered as a protein family from a functional point of view, since not all of them are chemically related (Feldmann, 1996). However, some cytokines share a high homology (about 30%), like interleukin-1 α (IL-1 α) and IL-1 β , or tumoral necrosis factor- α (TNF α) and TNF β . In addition, there are subfamilies with a really high structural homology (about 80%), like the interferon α (IFN α) subfamily with about 20 members.

Cytokines mediate effector phases in both innate and acquired immunity (Abbas et al., 1995). In the innate immunity, cytokines are produced mainly by mononuclear phagocytes and so are usually called monokines. Monokines are produced by mononuclear phagocytes in response to microorganisms and upon T-cell antigen stimulation as part of acquired immunity. However, most of the cytokines involved in acquired immunity are produced by activated T lymphocytes and these molecules are referred to as lymphokines. Lymphokines present a double function, either regulating the proliferation and differentiation of different lymphocytes populations or participating in the activation and regulation of inflammatory cells (mononuclear phagocytes, neutrophils and eosinophils). Both lymphocytes and mononuclear phagocytes produce other cytokines known as colony stimulating factors (CSFs), which stimulate the proliferation and differentiation of immature leukocytes in the bone marrow. Some other cytokines known as chemokines are chemotactic for specific cell types.

Although cytokines are made up of a diverse group of proteins, they share some features (Abbas et al., 1995):

- > They are produced during the effector stages of the innate and acquired immunity, and regulate the inflammatory and immune response.
- > Their secretion is brief and auto-limited. In general, cytokines are not stored as pre-formed molecules, and their synthesis is initiated by a new genetic transcription.

- > A particular cytokine may be produced by many different cellular types.
- > A particular cytokine may act on different cell types.
- > Cytokines usually produce different effects on the same target cell, simultaneously or not.
- > Different cytokines may produce similar effects.
- > Cytokines are usually involved in the synthesis and activity of other cytokines.
- > Cytokines perform their action by binding to specific and high affinity receptors present on the target cell surface. This action can be autocrine, paracrine or endocrine.
- > The expression of cytokine receptors is regulated by specific signals (other cytokines or even the same one).
- > For many target cells, cytokines act as proliferation factors.

Despite the importance of cytokines in the regulation and coordination of all the effector phases in the immune response, little attention has been paid to the phylogeny of cytokine in vertebrates compared to the phylogeny of other aspects of the immune system, specially immunoglobulins and transplant rejection. More recently, several cytokines have begun to be cloned and characterized within non-mammalian vertebrates (Bird et al., 2002a).

In fish, cytokines are grouped into growth factors (Grondel et al., 1984; Lawrence, 1996; Yin et al., 1997), pro-inflammatory cytokines (Jang et al., 1995 a, b; Fujiki et al., 2000, Zou et al., 1999 a, b), chemokines (Fujiki et al., 1999 a, b; Daniels et al., 1999; Laing et al., 2002), immunosuppressive or anti-inflammatory cytokines (Sumathy et al., 1997; Harms et al., 2000; Laing et al., 1999) and IFNs (Congleton et al., 1996; Collet et al., 2002; Hansen et al., 2002). Among these molecules, the pro-inflammatory cytokine interleukin-1 (IL-1) is particularly important and was the first non-mammalian IL-1 to be cloned. Its discovery in teleosts dates the evolution of this molecule to approximately 350 million years ago, since then; evolutionary pressure has rendered this molecule capable of many biological activities. IL-1 bioactivity has been extensively studied in

mammals, where the molecule has a pleiotropic activity (Dinarello, 1997), affecting nearly every cell type (Auron, 1998).

2. THE INTERLEUKIN FAMILY

IL-1 is the common name for a diverse family of proteins, of which IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1ra) and IL-18 are the most representative and studied, although several newly discovered molecules show a clear homology to this group (Dinarello, 1997; Dinarello, 1999; Busfield et al., 2000; Smith et al., 2000; Lin et al., 2001; Pan et al., 2001; Debets et al., 2001).

IL-1 is a major mediator of inflammation and in general initiates and/or increases a wide variety of non-structural function-associated genes that are characteristically expressed during inflammation, particularly other cytokines. It is one of the key mediators of the body's response to microbial invasion, inflammation, immunological reactions and tissue injury. Both *in vivo* and *in vitro* experiments have shown that IL-1 α and IL-1 β have similar, if not identical, multiple biological effects (Oppenheim et al., 1986; Dinarello, 1991; Dinarello, 1994; Dinarello, 1996; Dinarello, 1997) and both forms affect nearly every cell type and share a common receptor on target cells. However it has been shown that the endogenous roles of IL-1 α and IL-1 β differ, being IL-1 β but not IL-1 α a potent activator of the humoral immune response (Nakae et al., 2001). Both IL-1 α and IL-1 β are produced by many different cell types (Oppenheim et al., 1986), including neutrophils, natural killer cells, B-lymphocytes, T-lymphocytes and cells of the central nervous system. However the main producing cells are blood monocytes and tissue macrophages (Lepe-Zúniga and Gery, 1984; Dinarello et al., 1986; Dinarello, 1988; Arend et al., 1989), which are an important source because of their strategic locations, ability to synthesize large amounts of IL-1 and to process the IL-1 precursor more effectively than other cells.

Members of the IL-1 family belong to what is now known as the β -trefoil superfamily due to the presence of 12 β -sheets in their mature protein structure which folds to form a trefoil-like structure (Fig. 1) (Murzin et al., 1992; Nicola, 1994). IL-1 α , IL-1 β and IL-1ra contain the IL-1 family signature pattern or motif, taken from a

conserved region in the C-terminal section with the following consensus pattern; [FC]-x-S-[ASLV]-x(2)-P-x(2)-[FYLV]-[LI]-[SCA]-T-x(7)-[LIVM] (Hofmann et al., 1999).

A novel IL-1 family member (nIL-1Fm) has recently been discovered in rainbow trout (Wang et al., 2009). The unique gene organization of nIL-1Fm, together with its location in the genome and low homology to known family members, suggests that this molecule is not homologous to known IL-1Fm. Nevertheless, it contains a predicted C-terminal β -trefoil structure, an IL-1 family signature encoded by the last exon, a potential IL-1 converting enzyme cut site, and its expression level is clearly increased following infection, or stimulation of macrophages with LPS or IL-1 β . A thrombin cut site is also present and may have functional relevance. Notably, the C-terminal recombinant protein antagonized the effects of recombinant rainbow trout IL-1 β on inflammatory gene expression in a trout macrophage cell line, suggesting it is an IL-1 β antagonist. Modeling studies have confirmed that nIL-1Fm has the potential to bind to the trout IL-1RI and, therefore, may be a novel IL-1 receptor antagonist.

Due to the importance of IL-1 in the immune system of mammals and the evidence suggesting its biological activity in the immune system of non-mammalian vertebrates, work has focussed on isolating this gene in bird, amphibian and fish, and non-mammalian IL-1 β sequences are now available for all three (Table 1). Many of these genes have been obtained using a homology cloning approach. To date, no sequence data are available for IL-1 α or IL-1ra other than for mammals, whilst IL-18 has been described in chicken (Schneider et al., 2000) and in rainbow trout (Zou et al., 2004).

Table 1. Vertebrate IL-1 β sequences

Species	Common Name	Gene	GenBank Accession number
<i>Homo sapiens</i>	Human	IL-1 β	NM_000576
<i>Gallus gallus</i>	Chicken	IL-1 β	NM_204524
<i>Xenopus laevis</i>	African Clawed Frog	IL-1 β	NM_001085605
<i>Melanogrammus aeglefinus</i>	Haddock	IL-1 β	AJ550166
<i>Gadus morhua</i>	Atlantic Cod	IL-1 β	AJ535730
<i>Psetta maxima</i>	Turbot	IL-1 β	AJ295836
<i>Salmo salar</i>	Atlantic Salmon	IL-1 β IL-1 β 2 IL-1 β 3 IL-1 β 4	AY617117 AGKD01000000 HE817773 HE817774

Species	Common Name	Gene	GenBank Accession number
<i>Oncorhynchus mykiss</i>	Rainbow Trout	IL-1 β IL-1 β 2 IL-1 β 3 nIL-1Fm	NM_001124347 AJ245925 AJ557021 AJ555869
<i>Oplegnathus fasciatus</i>	Rock Bream	IL-1 β	FJ155360
<i>Salvelinus alpinus</i>	Arctic Char	IL-1 β	AM084227
<i>Latris lineata</i>	Striped Trumpeter	IL-1 β	FJ532282
<i>Dicentrarchus labrax</i>	European Seabass	IL-1 β	AJ269472
<i>Siniperca chuatsi</i>	Mandarin Fish	IL-1 β	AY647430
<i>Paralichthys olivaceus</i>	Japanese Flounder	IL-1 β nIL-1Fm	AB070835 AB720987
<i>Hippoglossus hippoglossus</i>	Atlantic Halibut	IL-1 β	FJ769829
<i>Rachycentron canadum</i>	Cobia	IL-1 β	AY641829
<i>Pagrus major</i>	Red Seabream	IL-1 β	AY257219
<i>Tetraodon nigroviridis</i>	Spotted Green Pufferfish	IL-1 β nIL-1Fm	AJ574910 CAF99823
<i>Cynoglossus semilaevis</i>	Half-Smooth Tongue Sole	IL-1 β	FJ816103
<i>Sparus aurata</i>	Gilthead Seabream	IL-1 β	AJ277166
<i>Chionodraco hamatus</i>	Crocodile Icefish	IL-1 β	AJ566198
<i>Lateolabrax japonicus</i>	Japanese Sea Perch	IL-1 β	EF513753
<i>Acanthopagrus latus</i>	Yellowfin Seabream	IL-1 β	AY669059
<i>Dentex dentex</i>	Common Dentex	IL-1 β	AJ459239
<i>Diplodus puntazzo</i>	Sharpsnout Seabream	IL-1 β	AJ459238
<i>Ictalurus punctatus</i>	Channel Catfish	IL-1 β IL-1 β 2	ABB46471 ABB46472
<i>Conger myriaster</i>	Whitespotted Conger	IL-1 β	AB291072
<i>Cyprinus carpio</i>	Carp	IL-1 β IL-1 β 2-1 IL-1 β 2-2	AB010701 AJ401030 AJ401031
<i>Carassius auratus</i>	Goldfish	IL-1 β 1 IL-1 β 2	AJ419848 AJ419849
<i>Danio rerio</i>	Zebrafish	IL-1 β nIL-1Fm	FM210810 XP_685720
<i>Salmo trutta fario</i>	Brown Trout	IL-1 β	AY853170
<i>Oryzias latipes</i>	Medaka	nIL-1Fm	XM_004084753
<i>Scyliorhinus canicula</i>	Smaller Spotted Catshark	IL-1 β	AJ295947
<i>Triakis scyllium</i>	Banded Houndshark	IL-1 β	AB074142
<i>Oreochromis niloticus</i>	Tilapia	IL-1 β nIL-1Fm	XM_003460625 XM_003445804
<i>Takifugu rubripes</i>	Fugu	IL-1 β	AB704199

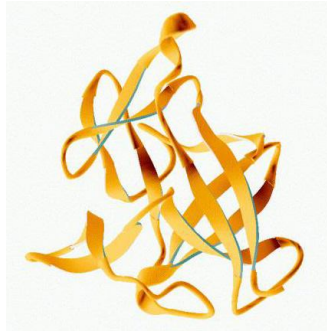


Fig. 1 Three-dimensional structure of IL-1 β where the β -trefoil structure can be observed (adapted from Murzin et al., 1995).

3. IL-1 β

The alignment of vertebrates IL-1 β amino acid sequences shows higher homology in regions containing the secondary structure of the IL-1 β molecule. This suggests the presence of 12 β -sheets in each of these genes, giving the proteins similar folding patterns and making each a member of the β -trefoil family (Nicola, 1994; Bird et al., 2002a).

The secondary structure dictates how the molecule will fold. The way in which it folds decides its tertiary structure and is important for it to interact with the receptor and induce a signal in the target cell. The use of site-specific mutagenesis has allowed the identification of amino acids (Arg⁴, Leu⁶, Phe⁴⁶, Ile⁵⁶, Lys⁹³, Lys¹⁰³ and Glu¹⁰⁵) in human mature IL-1 β , which are essential for binding to type I IL-1 receptor (IL-1RI) (Labriola-Tompkins et al., 1991). These amino acids form a cluster localised in one region of the IL-1 β molecule three-dimensional structure.

Notably, they are all strongly conserved in mammal IL-1 β proteins but not in the non-mammalian sequences known to date.

The IL-1 family signature is conserved in the IL-1 β sequences of mammals, birds and amphibians, but is only partially conserved in fish. So, a new family signature has been proposed taking in count all IL-1 β sequences: [FCL]-x-S-[ASLV]-x(2)-[PSR]-x(2)-[FYLV]-[LIV]-[SCAT]-T-x(7)-[LIVMK]. Although the sequences of IL-1 β share the family signature with IL-1 and probably show the presence of β -sheets, the homology of the nucleotide or amino acid sequence among vertebrate molecules is quite low and varies considerably between fish species (Bird et al., 2002a), due to the great phylogenetic diversity existing within this group of animals.

4. IL-1 β EXPRESSION AND REGULATION

The role of IL-1 β in the immune response has been partly elucidated by expression studies. In mammals, IL-1 β is produced in response to many stimuli, including bacterial LPS, numerous microbial products, cytokines (TNF, IFN- γ , GM-CSF and IL-2), T-cell/antigen-presenting cell interactions and immune complexes (Stylianou and Saklatvala, 1998). Similar studies have revealed that bird, amphibian and fish IL-1 β show a similar expression pattern to its mammalian counterpart. In chicken, IL-1 β is quickly induced in blood monocyte-derived macrophages, reaching optimal levels within 1 h after LPS treatment (Weining et al., 1998). The *Xenopus* IL-1 β transcript was inducible *in vivo* following injection with LPS (Zou et al., 2000a). In fish, IL-1 β expression studies have usually been performed by RT-PCR or Northern blot and it has been shown that *in vivo* and *in vitro* treatment with LPS is able to induce IL-1 β mRNA in all the species tested, including carp, trout, seabass and catshark (Zou et al., 1999a, b, 2000b; Engelsma et al., 2001; Scapigliati et al., 2001; Bird et al., 2002b). In trout many expression studies have been developed. Thus, it is known that stimulation with 5 μ g/ml LPS induces the expression of IL-1 β 1 h after stimulation, reaching maximum levels after 4 h. In addition, culture temperature has a marked effect on IL-1 β expression, because a temperature increase acts as a positive regulator in IL-1 β synthesis (Zou et al., 2000b). Moreover, IL-1 β expression levels of the trout mononuclear phagocyte cell line RTS-11 are up-regulated after LPS treatment (Brubacher et al., 2000). In seabream, IL-1 β mRNA accumulation is induced in leukocytes after bacterial challenge (Chaves-Pozo et al., 2004) or *in vitro* stimulation with different PAMPs (Sepulcre et al., 2007). The protein is also accumulated in leukocytes activated with LPS or bacterial DNA, as detected by western blot using a polyclonal antibody to seabream (Pelegrín et al., 2004).

The promoters of several non-mammalian IL-1 β genes have also been cloned and characterized, such as those of trout (Wang et al., 2002) and catshark (Bird et al., 2002b). In fish, homologous positive and negative regulatory elements for transcription factors have been found. A TATA box is present 24–27 bp upstream of the transcription start site in all species examined. An NF- κ B element and a more upstream enhancer have also been identified (Hiscott et al., 1993; Goto et al., 1999). IL-1 β is a strong inducer of NF- κ B and is thought to positively auto-regulate its own synthesis, which

appears to hold true in fish since the addition of trout recombinant IL-1 β to trout macrophages induced IL-1 β expression (Wang et al., 2002). Preliminary studies using the trout IL-1 β promoter confirm that the NF- κ B transcription factor site is required for expression of the trout IL-1 β gene (Wang et al., 2002). Other potential elements for transcription activators in the trout and catshark promoters include sites for AP1, SP1 and CEBP.

5. IL-1 RECEPTORS

Each of the interleukins, IL-1 α , IL-1 β and IL-1Ra, elicits biological effects by binding specific receptors (IL-1Rs). These receptors belong to IL-1 receptors similar to the Toll superfamily (IL-1/TLR) and are found in vertebrates, insects and plants as part of the host defence and inflammation. There are two primary IL-1 binding receptors, type I (IL-1RI) and type II (IL-1RII). Both receptors possess an extracellular region composed of three immunoglobulin-like domains responsible for IL-1 binding, but differ considerably in the cytoplasmic portion. The IL-1RI has a Toll–interleukin-1 receptor (TIR) domain involved in signal transduction, while IL-1RII lacks the TIR domain, and is unable to deliver biological signals (Heguy et al., 1993; Li and Quin, 2005).

The binding of IL-1 to IL-1RI is not sufficient to initiate the signal transduction, and its association with the IL-1 receptor accessory protein (IL-1RacP) is necessary. The resulting high affinity complex initiates the signal transduction via signalling cascades, such as the NF- κ B pathway, p38 mitogen-activated protein kinase (MAPK) and Jun N-terminal kinase, leading to the induction of a large number of proinflammatory and immune genes (Ridley et al., 1997; May and Ghosh, 1998; Ninomiya-Tsuji et al., 1999).

IL-1RII can perform its role either on the cell surface or as a soluble receptor after it is shed from the plasma membrane (Orlando et al., 1997), acting as a molecular trap for IL-1 and functioning as a negative regulator of the IL-1 system (Colotta et al., 1993, 1994). Thus, it is a decoy receptor capable of recognizing its ligand with high affinity and specificity, but which is structurally incapable of signalling (Colotta et al., 1993). The concept of a high-affinity binding molecule for IL-1 which is expressed on the cell

surface, released in soluble form, and prevents its ligand from interacting with the true receptor on the same cell, was without precedent in cell biology. Its existence, together with the IL-1Ra, a pure polypeptide antagonist, that binds the receptor in a similar manner to IL- α and IL-1 β , but does not induce signal transduction, emphasizes the need for the tight control of the IL-1 system, which mediates potentially devastating local and systemic inflammatory reactions (O'Neill and Dinarello, 2000) (Fig. 2).

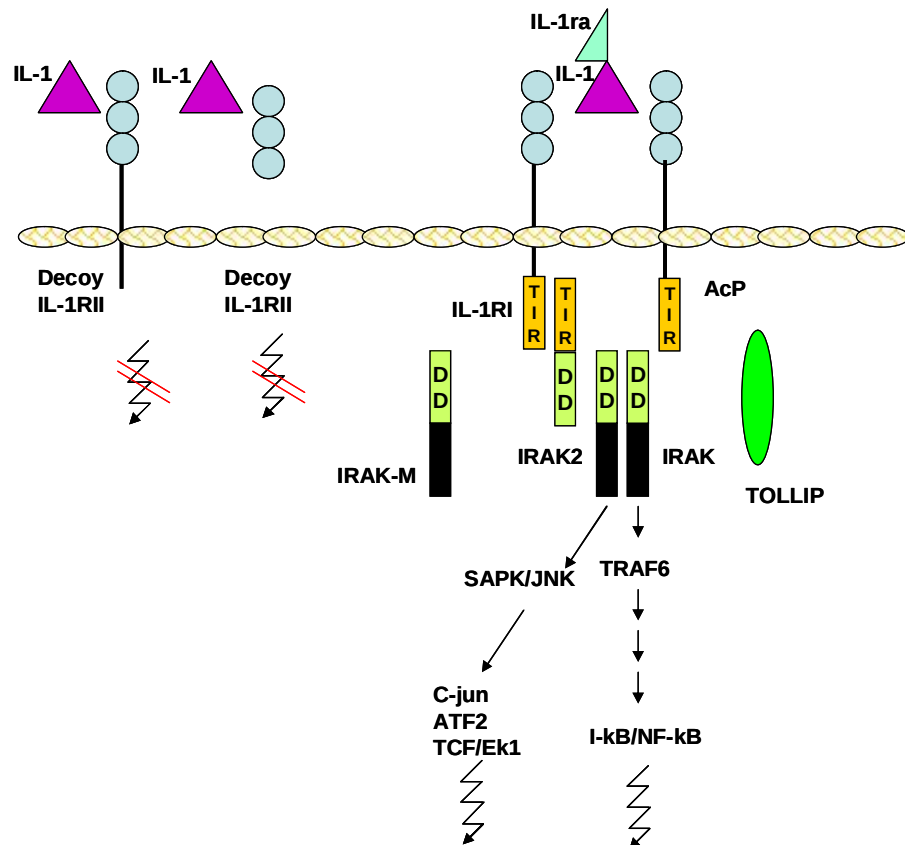


Fig. 2 Domain organisation of interleukin-1 receptors. The cytosolic TIR domain of the IL-1RI is responsible for signal transduction. The decoy IL-1RII lacks the TIR domain and is structurally incapable of signalling.

IL-1RI homologue genes have been sequenced in chicken (Guida et al., 1992), salmon (*Salmo salar*) (Subramaniam et al., 2002) and carp (*Cyprinus carpio*) (Metz et al., 2006). The homologue gene for the IL-1RacP in salmon has also been cloned (Stansberg et al., 2005). In chicken, it has been shown that this is a functional receptor because IL-1 activity is blocked in the presence of the recombinant receptor, IL-1 activity decreases when using a specific antibody that blocks the receptor and this receptor transduces the signal in target cells for IL-1 (Klasing and Peng, 2001). In salmon, IL-1RI and IL-1RacP genes are expressed in different tissues and its

expression is increased after LPS stimulation and IL-1RI is able to interact with IL-1RAcP (Subramaniam et al., 2002; Stansberg et al., 2005). Finally, an homolog gene for IL-1RII has been identified in rainbow trout and gilthead seabream, showing that its expression is up-regulated in head kidney leukocytes upon *in vitro* stimulation with PAMPs or recombinant cytokines as well as in challenged animals (Sangrador-Vegas et al., 2000; López-Castejón et al., 2007a). In addition, co-immunoprecipitation assays have also demonstrated that the IL-1RII is able to physically interact with IL-1 β in the gilthead seabream (López-Castejón et al., 2007b).

6. IL-1 β PROCESSING MECHANISM

Most secretory proteins present an N-terminus signal peptide composed of 13-30 hydrophobic amino acids. This signal leads to the synthesis of the protein to the endoplasmic reticulum and the synthesized protein remains in the reticulum lumen, where the signal peptide is removed (Milstein et al., 1972; Blobel and Dobberstein, 1975a, b). The signal peptide can also be found up to 12 amino acid residues upstream the protein N-terminus, as is the case with ovoalbumin, where the signal peptide remains present in the mature protein (Tabe et al., 1984). Translocation of proteins to the endoplasmic reticulum usually occurs simultaneously to their translation. However, in yeast, synthesized peptides can translocate to the endoplasmic reticulum with the help of different proteins, like Heat Shock Protein 70 (Chirico et al., 1988; Deshaies et al., 1988). Then, secretory proteins move to the Golgi apparatus where they are glycosylated and finally stored in secretory vesicles, which, by exocytosis, fuse to the plasma membrane and release its cargo to the extracellular media (Palade, 1975).

However IL-1 β lacks the signal peptide and so is synthesized in the cytosol (Auron et al., 1984; March et al., 1985) and does not follow this classical secretion reticulum-Golgi route (Matsushima et al., 1986; Bakouche et al., 1987; Rubartelli et al., 1990). The secretion route for this molecule remains unknown, although many studies are being developed in order to shed light on this mechanism. Bacterial products (e.g., lipopolysaccharide, LPS) and host-derived inflammatory factors cause the synthesis of IL-1 β in the form of a biologically inactive procytokine (molecular mass 31 kDa) that remains dispersed in the cytosol until a second stimulus drives the processing and

ICE
↓

Species	Sequence
Human	EEPIFFDTWDN - - EAYVH - - DAPVRS LNCTLR RD SQQ KS LVMSG P - - - -
Mouse	EEPILCDSWDDDDNLLVC - - DVPTRQ LHYRL RD EQQ KS LVLSD P - - - -
Chicken	-EPVTFORLESSYAGAPA - - FRYTRS QSFDI FD INQ KC FVLES P - - - -
Xenopus	EEEIGFSQAKETYASAST - - YRYQRA TTCRI KD TSN KC FVMQK FH - - E N
Seabream	EERTVFER - TAKPAQ - - - - Y TYNFQ SLYSV MD SEQ RH LVRVP N - - - - S
Seabass	EEKIVFERGTTPTAQ - - - - Y SKRRE VOC SV TD SEK RS LVLVP N - - - - S
Trout	EEHIVLELESAPPASRRAGFSSTSQYECSVTDSENKCWVLMNE - - - - A
Carp	EEERLVKPLNETPIYS - - - - - KTS LTLQC TI CDKYK KTMVQ SNKLSDEP

* * * *

The IL-1 β sequences from teleost fish have an Asp residue localized in a position that would produce a 22 kDa protein, which is considered as a possible cleavage site for caspase-1 within these species. Thus, it has been shown that LPS stimulation of the seabream fibroblast cell line SAF-1 followed by ATP activation leads to the release of a 22 kDa IL-1 β molecule. However, this stimulus does not lead to the release of any IL-1 β molecule in seabream leukocytes (Pelegrín et al., 2004) and extracellular ATP fails to activate seabream P2X7 receptor (López-Castejón et al., 2007b). Therefore, to date

there are insufficient data to confirm that the enzyme caspase-1 is responsible for this processing in seabream. Interestingly, it has recently been found that zebrafish (*Danio rerio*) inflammatory caspase A and caspase B (Vojtech et al., 2012) and European seabass (*Dicentrarchus labrax*) caspase-1 (Reis et al., 2012) can process IL-1 β .

7. INFLAMMATORY CASPASES: CASPASE-1

Caspases are a family of cystein proteases that cleave at an aspartic (Asp) residue (Stennicke, 1998) and that develop key roles in the apoptosis and proteolytic activation of cytokines (Nicholson, 1999; Boatright et al., 2003; Nadiri et al., 2006). In humans, the caspase family includes 13 members, whose functions seem to correlate with their phylogenetic relationship (Lamkamfi et al., 2002). Cell death caspases are initiators (caspase-2, -8, -9, and -10) and executioners (caspase-3, -6, and -7) of apoptosis (Nadiri et al., 2006). Initiator caspases sense death signals, and activate more downstream executioner caspases, which cleave cellular substrates, mediating the changes associated with apoptosis. Human inflammatory caspases include caspase-1, -4, -5, and -12. In mice, caspase-5 is absent but they have an additional inflammatory caspase, caspase-11, which has probably arisen by tandem gene duplication of caspase-4 (Nadiri et al., 2006). The arrangement, proximity, as well as the exon-intron structure of the inflammatory caspases, suggest that they originated from the same ancestral gene. Both inflammatory and apoptotic caspases are synthesized as inactive zymogens and share a common conserved structure composed of a prodomain and a catalytic region (Lamkamfi et al., 2002; Nadiri et al., 2006).

Caspase-1 is one of the best characterized inflammatory caspases. Originally, caspase-1 was found in an attempt to purify the enzyme responsible for IL-1 β processing (Thornberry et al., 1992), although later it was also shown to be able to activate IL-18 and IL-33 (Gracie et al., 2003; Dinarello, 2005). Caspase-1, like other pro-inflammatory caspases, contains an N-terminal caspase-recruitment domain (CARD), and a catalytic region composed of both a large (p20) and a small (p10) subunits with a conserved Gln-Ala-Cys-X-Gly active site sequence (where X is Arg, Gln or Gly) found in the large subunit. There are other structural proteins that present this CARD domain, like the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) that binds to caspase-1, producing the oligomerization of

pro-caspase-1 within the inflammasome, facilitating the caspase auto-cleavage into the p20 and p10 subunits (Mariathasan et al., 2004; Stehlik et al., 2003). These p10 and p20 subunits, once released, form an active heterotetramer that acts as a very efficient IL-1 β -converting enzyme.

Although pro-caspase-1 is constitutively expressed, it remains inactive in the cytoplasm until inflammatory cells, like monocytes or macrophages, receive the appropriate stimulus. In these cells, K⁺ release induces a rapid and strong activation of caspase-1, triggering the processing and release of the mature IL-1 β (Gudipaty et al., 2003). In addition, it has recently been shown that caspase-11 (also known as caspase-4) is critical for caspase-1 activation and IL-1 β production in macrophages infected with *Escherichia coli*, *Citrobacter rodentium* or *Vibrio cholerae* (Kayagaki et al., 2011).

Interestingly, functions for caspase-1 different for those known to date have recently been identified. Thus, it has been shown that caspase-1 can stimulate the biogenesis of the membrane to repair the damage produced by the pore-forming toxins, promoting cell survival as a mean of resisting infection by pathogenic bacteria (Gurcel, et al., 2006). It has also been shown that caspase-1 takes part in the NF- κ B activation via TLR2 and TLR4 (Miggin et al., 2006). These results highlight the importance of this enzyme to regulate different aspects of the immune response.

Despite the importance of caspase-1 in inflammation, the information on the presence and activity of this enzyme in fish is scant. Two inflammatory caspases have been found in the zebrafish (*Danio rerio*) with a highly conserved catalytic domain, and with an N-terminal pyrin domain (PYD) rather than the typical CARD of inflammatory caspases (Lamkamfi et al., 2002; Huising et al., 2004). In fact, the function of these fish caspases appears to be related to the regulation of apoptosis rather than inflammation, since they are able to induce apoptosis when transfected into mammalian cells and are essential for morphogenesis of the jaw and gill-bearing arches of fish larvae (Masumoto et al., 2003). The first caspase-1 homologue of fish showing the N-terminal CARD domains has been identified in gilthead seabream (López-Castejón et al., 2008) but whether this caspase-1 is able to process IL-1 β remains to be elucidated.

8. CASPASE-1 ACTIVATORS

It is known that stimuli such as bacterial products, ATP or nigericin, which alter the intracellular ionic milieu and result in cytosolic acidification, lead to caspase-1 activation (Kuida et al., 1995; Li et al., 1995). However, it is not clear how these signals converge on caspase-1 activation. Characterization of the inflammasome as the macromolecular complex required for caspase-1 activation solved a large part of the puzzle (Martinon et al., 2002).

The inflammasome resembles in many ways the apoptosome, which is assembled during mitochondrial apoptosis to activate caspase-9 (Adams et al., 2000). In both cases, the caspases are brought into close proximity in response to an activating signal via a scaffolding molecule. Apoptosis protease activating factor 1 (APAF-1) is such a molecule in the apoptosome. Through its CARD domain it binds the CARD domain of caspase-9 and results in its activation in response to cytochrome c release from the mitochondria. In the case of the inflammasome, members of the NLR (NOD-leucine-rich repeat) family act as regulators of caspase-1 activity (Nadiri et al., 2006).

9. THE INTRACELLULAR RECEPTORS NLR (NOD-LRR) FAMILY

It is known that cells are able to detect the presence of pathogens by means of the toll like receptors (TLR) present in the cell membrane. The TLRs belong to the pathogen recognition receptors (PRR) (Janeway and Medzhitov, 2002), because they recognize pathogen-associated molecular patterns (PAMPs) that are shared by many pathogens but not by the host cell.

This recognition occurs through leucine-rich repeats (LRRs) in the extracellular domains that are implicated in ligand binding and auto-regulation (Kawai and Akira, 2006). PAMPs represent vital molecules for microbial survival and are therefore unlikely to vary in their structures because any major changes would be disadvantageous. Such molecules include bacterial structural components, such as LPS, peptidoglycans (PGN) or viral RNA, and are specifically recognized by their corresponding TLR (Medzhitov, 2001). Recognition of PAMPs by TLRs results in the activation of different intracellular signalling cascades, generally leading to the activation of NF- κ B, activator protein-1 (AP-1) and type I IFN. Moreover, TLRs are

key inducers of the proinflammatory cytokines IL-1 β and IL-18, but do not directly contribute to the activation of inflammatory caspases (Takeda and Akira, 2005).

Apart from TLRs, other PRR families have been identified. A very important one is the NLR receptor family. The NLR family is a group of intracellular receptors that are able to detect pathogens and danger signals in the cytosol and which present three characteristic structural domains. The NLR family is characterized by the presence of a central nucleotide-binding and oligomerization (NACHT) domain, which is commonly flanked by C-terminal leucine-rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. The C-terminus is a leucine-rich repeat domain considered to be the sensing motif able to recognize different ligands, as in TLRs. The intermediary NACHT (NBS or NOD) domain is essential for the oligomerization and activation of NLRs. Oligomerization of the NACHT domain is a pre-requisite for transduction of the signal mediated by the third N-terminal effector domain, which may be a pyrin domain (PYD) or a caspase recruitment domain (CARD). The diversity of effector domains allows the NLRs to interact with a wide array of binding partners and to activate multiple signalling pathways, which is the feature that differentiates members of this receptor family, in which the most studied are NOD1, NOD2, NLRP1, NLRP3, IPAF (also called NLRC4) and AIM2 (Fig. 4).

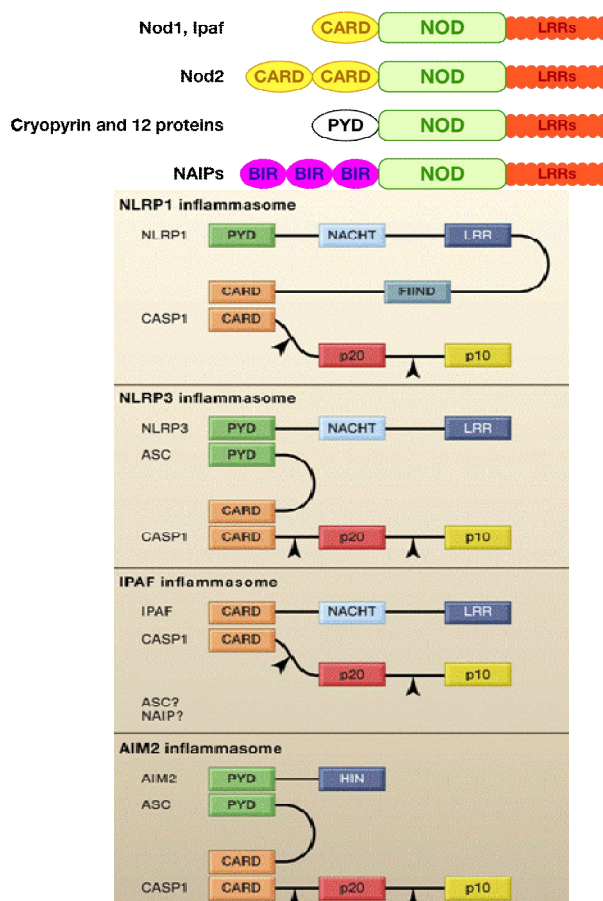


Fig. 4 Members of the NLR family.

The majority of the NLR proteins are constituted by a C-terminal LRR domain, an intermediate oligomerization NOD (NACHT) domain and an effector CARD, PYD or BIR N-terminal domain (adapted from Schroder et al., 2010).

The NLR proteins are normally present in the cytoplasm in an inactive, auto-repressed form. The LRRs fold intramolecularly back onto the NACHT domain, thereby inhibiting spontaneous oligomerization (and activation) of the NLR protein. Upon direct or indirect binding of a PAMP to the LRR, the molecule undergoes a conformational rearrangement, exposing the NACHT domain and thereby triggering oligomerization. In turn NLRs expose the effector domains. Through a homotypic interaction, CARDs and PYDs recruit CARD- and PYD- containing effector molecules, bringing them into close proximity with each other and leading to their activation (Tschoopp et al., 2003). These proteins are the ideal molecular platform needed for the activation of inflammatory caspases (Fig. 5).

Activating ligands for NOD1 and NOD2 are subcomponents of PGN, namely D- γ -glutamyl-meso-DAP(mDAP) and muramyl dipeptide (MDP), respectively (Girardin et al., 2003). NOD1 and NOD2 activate NF- κ B through the recruitment and oligomerization of receptor-interacting protein 2 (RIP2), resulting in the activation of the I κ B kinase complex (Bertin et al., 1999; Ogura et al., 2001). NOD2 has an essential role as an intracellular sensor of PGN in the intestinal immunity, since it has been found that mutations in NOD2 correlate with Crohn's disease (CD) (Martinon et al., 2004). At present, there are no clear links between NOD1 or NOD2 and caspase-1 activation. It seems however that despite the independent activation of NLR and TLR, the signalling cascades triggered by their activation might be similar and possibly involved in redundant functions.

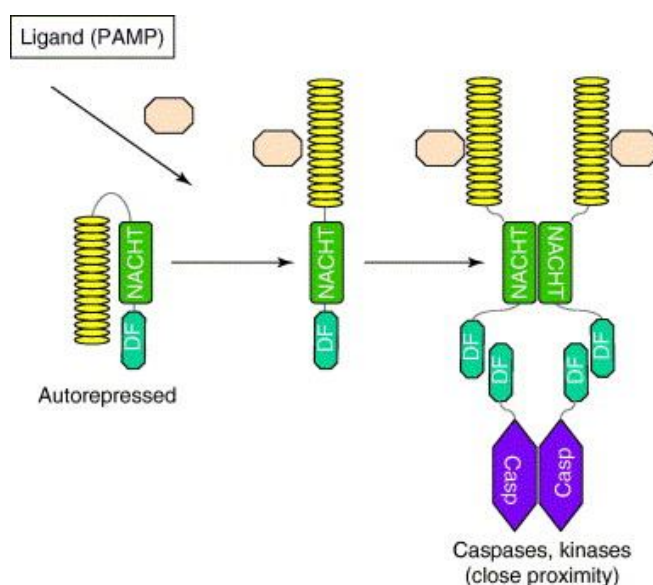


Fig. 5 Proposed mechanism of NLR activation. Upon binding to a ligand, the auto-repressed NLR protein undergoes a conformational change, which allows the NACHT oligomerization domain to be exposed (adapted from Martinon and Tschoopp, 2005).

In contrast, other members of the NLR family, such as NLRPs or IPAF, are involved in caspase-1 activation (and the consequent formation of the inflammasome) (Agostini et al., 2004; Poyet et al., 2001). One of the characteristics shared by NLRPs proteins is their ability to recruit the adaptor protein ASC with a C-terminal CARD and a N-terminal PYD domain. ASC is able to form PYD-PYD interactions with the NLR, recruiting caspase-1 through CARD-CARD interactions, activating it (Martinon et al., 2004). IPAF, however, interacts directly with caspase-1 through its CARD domain and, therefore, ASC is not required in this process (Fig. 6).

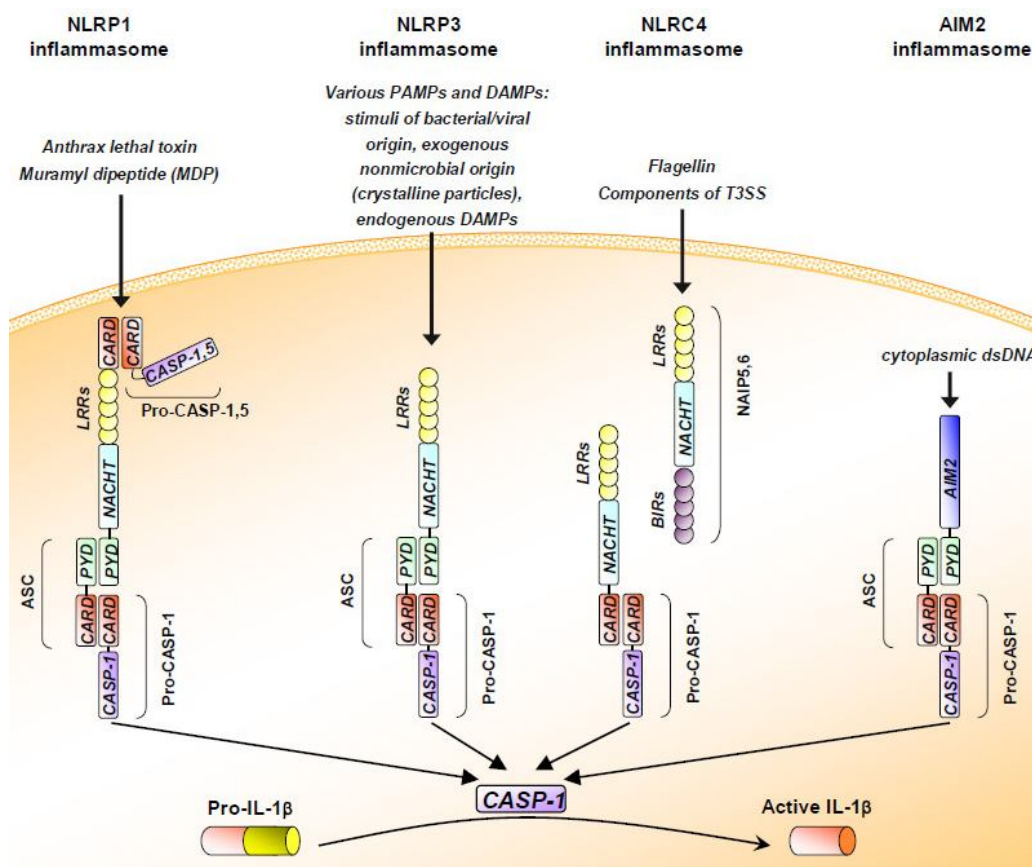


Fig. 6 Caspase-1 activation pathways. A NLR protein oligomerizes in response to an inflammatory stimulus and this allows caspase-1 activation either directly or through the adaptor molecule ASC (adapted from Contassot et al., 2012).

To date different inflammasome activating factors have been identified, but the mechanisms that trigger its activation and consequent IL-1 β processing and release are not well understood (Table 2).

Table 2. Ligands leading to caspase-1 activation through inflammasome formation.

Inflammasome	Ligand
NLRP3	ATP, Nigericin
	P2X7/ATP
	Bacterial RNA, Uric acid crystals
	Pore-forming bacteria: <i>Staphylococcus aureus</i> , <i>Listeria monocytogenes</i>
NLRP1	<i>Bacillus anthracis</i> (Anthrax lethal toxin)
IPAF (NLRC4)	<i>Salmonella typhimurium</i>
	<i>Legionella pneumophila</i>
Aim2	dsDNA

It is now generally accepted that activation and release of IL-1 β requires two distinct signals (Burns et al., 2003): a first signal leading to the synthesis of the proIL-1 β , and a second one involved in caspase-1 activation and IL-1 β release. What constitutes these signals *in vivo* during an infection or an autoinflammatory response is not known for certain. However, *in vitro* studies suggest that the first signal responsible for the proIL-1 β accumulation in the cytosol can be triggered by TLR activation. As regards the second signal, which activates caspase-1 and the inflammasome, different investigations indicate that a physiological signal is extracellular ATP (eATP), since eATP is able to induce the rapid processing and the massive release of IL-1 β by binding the purinergic receptors P2X7 (Hogquist et al., 1991; Rubartelli et al., 1993; Hickman et al., 1994; Perregaux and Gabel, 1994; Di Virgilio, 1995; Di Virgilio et al., 2001). The channel model proposes that extracellular ATP from microbial pathogens activates the P2X7 receptor and allows the efflux of intracellular potassium ions (K⁺) resulting in NLRP3 activation (Petrelli et al., 2007 and Franchi et al., 2007). Also, phagocytosis of crystals leads to lysosomal swelling and damage. The lysosomal perturbation together with the release of cathepsin B, a lysosomal cysteine protease, results in the activation of the NLRP3 inflammasome (Cassel et al., 2008). Even ROS generated during mitochondrial damage and oxidized mitochondrial DNA (mtDNA) produced during apoptosis lead to activation of NLRP3 (Zhou et al., 2009). This phenomenon was negatively regulated by the anti-apoptotic protein Bcl2, suggesting a link between apoptosis and inflammasome activation (Beal, 2003) (Fig.7).

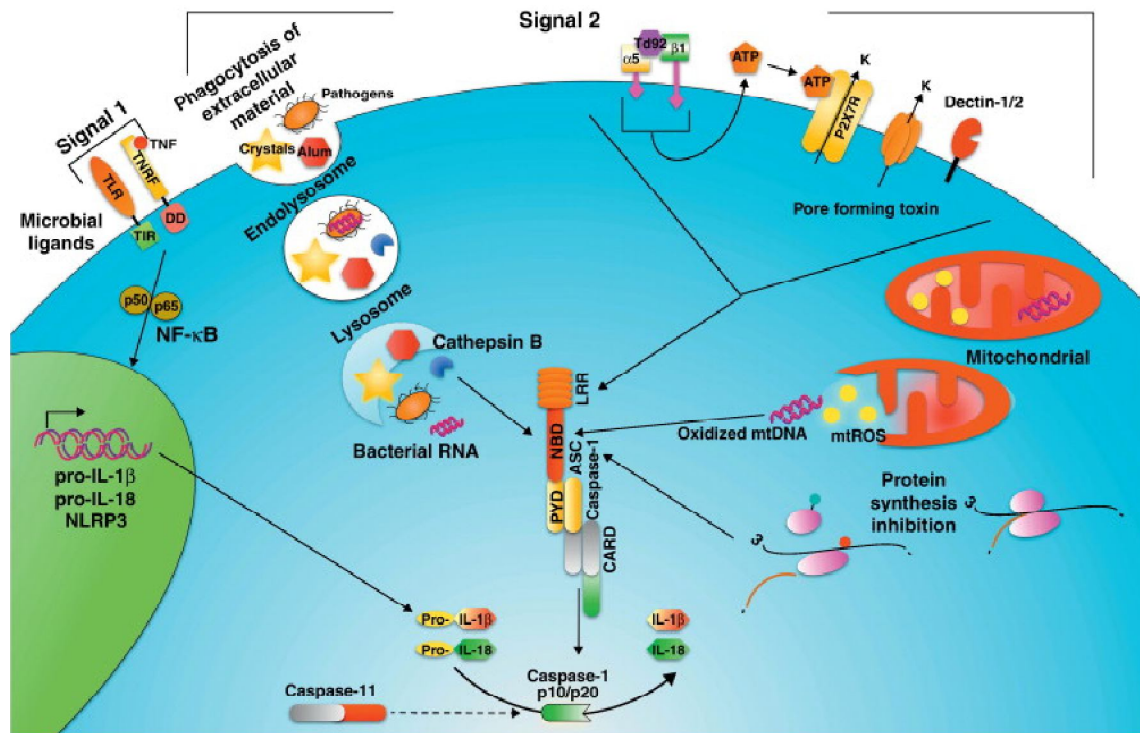


Fig. 7 Caspase-1 activation by multi-signal process. Signal 1 occurs when TNF or a TLR ligand binds its cognate receptor resulting in the translocation of NF- κ B into the nucleus where expression of NLRP3 and the immature (pro-) forms of IL-1 β and IL-18 are induced. Signal 2 is the activation of NLRP3 resulting in recruitment and cleavage of pro-caspase-1 to its active form leading to cleavage of the immature inflammatory cytokines. At least three distinct NLRP3 activation pathways have been identified. Phagocytosis of extracellular particulates and pathogens results in lysosomal destabilization and release of cathepsin B and bacterial mRNA which trigger NLRP3 activation. A decrease in intracellular K⁺ has been shown to result in activation of NLRP3. K⁺ efflux occurs by engagement of extracellular ATP with the P2X7R or directly through bacterial pore-forming toxins. ROS generated during mitochondrial damage and oxidized mitochondrial DNA (mtDNA) produced during apoptosis lead to activation of NLRP3. Td92, a surface protein of *Treponema denticola*, can interact with the α 5 β 1 integrin resulting in ATP release and K⁺ efflux. Inhibition of ribosomal function and protein synthesis can also direct NLRP3 activation, and this mechanism may involve lysosomal destabilization, K⁺ efflux and ROS. Caspase-11 has been defined upstream of caspase-1 during NLRP3 inflammasome activation.

The relevance of the NLR proteins, NLRP or IPAF, lies in the fact that infection with bacterial pathogens, both *in vitro* and *in vivo*, does not require ATP to trigger activation of the inflammasome. This finding might reflect the possibility that bacterial infection simultaneously induces the signals that are individually activated by eATP and TLR stimulation. In fact, it might be that pore formation in cell membranes caused by

bacterial secretion systems triggers an ion flux in the cell that is mimicked by the activity of eATP (Mariathasan and Monack, 2006).

10. IL-1 β RELEASE MECHANISMS

As we have already mentioned, IL-1 β is a leaderless protein that does not appear to be contained within classical exocytotic vesicles. Thus, exactly how it is externalized has long been a mystery, although in recent years different works have shed light on this issue, suggesting a vesicular mechanism. However, two different models have been proposed by Rubartelli and co-workers and Surprenant and colleagues, respectively (Fig. 8). Rubartelli suggests that IL-1 β is accumulated in endocytic vesicles (secretory lysosomes) together with caspase-1; then, the P2X7 receptor-induced loss of intracellular K⁺ activates phosphatidylcholine-specific phospholipase C, which, in turn, causes an increase in cytosolic Ca²⁺, Ca²⁺-dependent phospholipase A₂ activation, and exocytosis of the IL-1 β -containing lysosomes. According to this model, caspase-1 activation and IL-1 β processing are triggered by the K⁺ loss-stimulated activation of a Ca²⁺ independent phospholipase A₂ within the lysosomes (Andrei et al., 2004). In the model proposed by Surprenant, however, upon P2X7 receptor-mediated macrophage activation, IL-1 β is packaged into small plasma membrane blebs that are released into the extracellular space as microvesicles ranging in size from 200 nm to 1 μ m. These microvesicles are akin to the tissue factor-containing microparticles released from various cell types (Morel et al., 2004) and different from the large plasma membrane blebs that are also produced in cells stimulated via the P2X7 receptor (McKenzie et al., 2001; Morelli et al., 2003). Microvesicle budding and release are preceded by phosphatidylserine flip and loss of membrane asymmetry (McKenzie et al., 2001), a change in the membrane phospholipid structure that might have a relevant signalling function (Elliott et al., 2005).

Stimuli other than ATP, which trigger the IL-1 β processing and release can be found, all of them able to induce a K⁺ efflux in the cell; these includes the K⁺ ionophore nigericine, hipotonic stress and some pathogenic bacteria (Bhakdi et al., 1990; Perregaux and Gabel, 1994; Cheneval et al., 1998). Other stimuli leading to IL-1 β release are those which increase the Ca²⁺ permeability of the cell, such as Ca²⁺

ionophores and interaction of IL-1 β producing cells with CD8⁺ T lymphocytes (Rubartelli et al., 1990; Gardella et al., 2001). Therefore, many different signals leading to caspase-1 activation and IL-1 β processing and release can be found (Fig.7). However, despite all the work into the different molecules and mechanisms involved in this process, the mystery is still far from being solved.

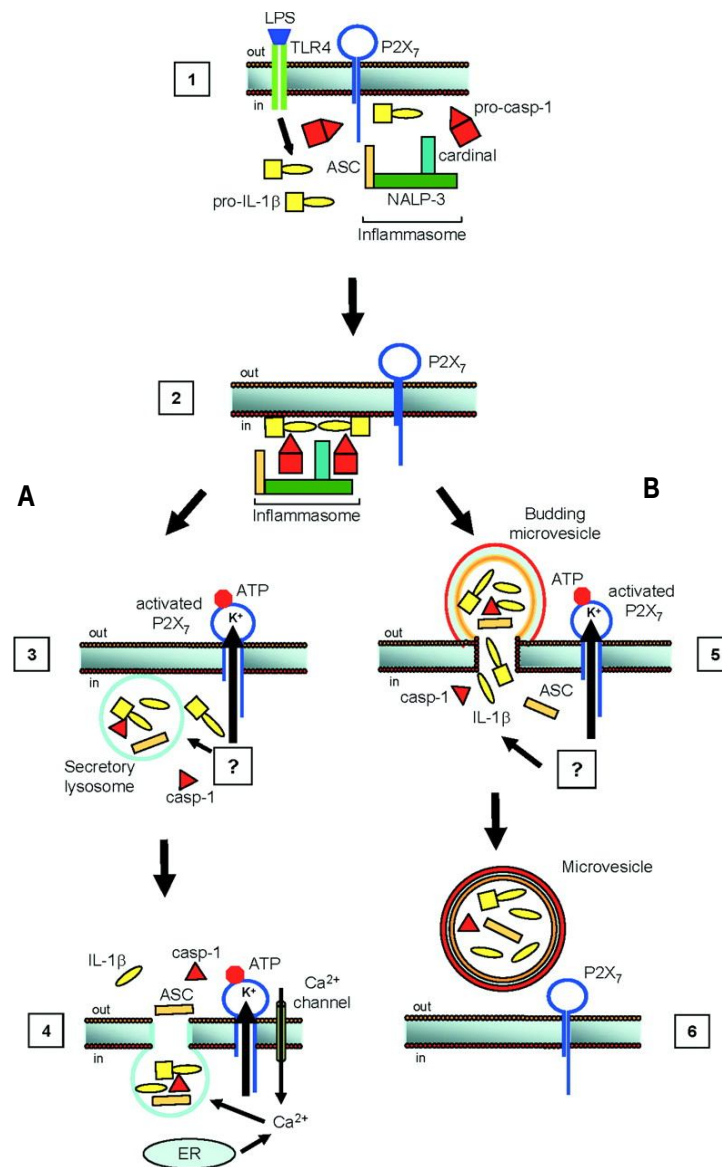


Fig.8 Proposed models for P2X7-stimulated IL-1 β cleavage and release. Rubartelli proposes that IL-1 β is released in secretory lysosomes and depends on the activation of phospholipase A₂ (A). Surprenant, however, proposes that IL-1 β is released within microvesicles shed from the plasma membrane (B) (adapted from Ferrari et al., 2006).

In non-mammalian vertebrates the mystery is even greater. There are very few studies about this process in fish, amphibian and bird. It has been shown that eATP (up to 5mM) does not promote phosphatidylserine externalization, cell death or IL-1 β release in seabream leukocytes while, in the fibroblast cell line SAF-1, ATP rapidly induces phosphatidylserine externalization and the release of IL-1 β -containing microvesicles (Pelegrín et al., 2004).

The gilthead seabream P2X7 receptor has been cloned and functionally characterized (López-Castejón et al., 2007a). Both ATP and the ATP analogue BzATP showed lower potency in the activation of gilthead seabream P2X7 receptor compared with other non-mammalian P2X7 receptors. Despite phosphatidylserine externalization and cell permeabilization in seabream leukocytes after the addition of high BzATP concentrations, IL-1 β remained unprocessed within the cell. However, activation of rat P2X7 receptors ectopically expressed in HEK293 together with human caspase-1 led to the specific secretion of unprocessed seabream IL-1 β . In contrast, neither seabream nor zebrafish P2X7 receptors induced the secretion of mammalian or fish IL-1 β when expressed in HEK293, while a chimeric receptor harboring the ATP-binding domain of seabream P2X7 and the intracellular region of its rat counterpart did so. These findings indicate that P2X7 receptor-mediated activation of caspase-1 and release of IL-1 β result from different downstream signalling pathways and suggest that although the mechanisms involved in IL-1 β secretion are conserved throughout evolution, distinct inflammatory signals have been selected for the secretion of this cytokine in different vertebrates.

11. INTRACELLULAR BACTERIA AND THE ROLE OF PYROPTOSIS

Recent studies have shown the involvement of pyroptosis, a proinflammatory cell death mode that requires caspase-1 activity, as a critical mechanism by which inflammasomes contribute to host responses against gram-negative bacteria pathogens *in vivo*. Pyroptotic cell death has mainly been characterized in myeloid cells infected with pathogenic bacteria such as *Shigella flexneri*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Bacillus anthracis*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Francisella tularensis* (Chen et al. 1996; Hilbi et

al. 1998; Jones et al. 2010; Lamkanfi & Dixit 2010; Miao et al. 2010a; Terra et al., 2010). Pyroptosis is also implicated in clearance of the gram-positive pathogen *Bacillus anthracis* *in vivo* (Terra et al. 2010).

This genetically programmed cell death mode differs morphologically from apoptosis in that it features cytoplasmic swelling and early plasma membrane rupture (Lamkanfi & Dixit 2010). The consequent release of the cytoplasmic content into the extracellular space is thought to render pyroptosis proinflammatory, whereas apoptosis is generally considered an immunologically silent cell death mechanism (Lamkanfi 2011; Taylor et al. 2008). However, apoptosis and pyroptosis also share several biochemical features such as the requirement for caspase activity (albeit the caspases involved differ), condensation of the nuclear compartment and oligonucleosomal fragmentation of genomic DNA (Lamkanfi & Dixit 2010). Although the biochemical pathway by which caspase-1 activation induces pyroptosis largely remains to be elucidated, this cell death mode proceeds independently of IL-1 β and IL-18 (Lamkanfi et al., 2008; Miao et al., 2010a; Monack et al., 1996, 2001).

In vivo, pyroptosis may represent a mechanism that prevents intracellular replication of infectious agents by eliminating the infected macrophages and dendritic cells altogether. By releasing their intracellular content into circulation, pyroptotic cells may simultaneously target surviving bacteria for destruction by phagocytes and neutrophils and alert other immune cells to imminent danger (Miao et al. 2010a). Altogether, pyroptosis is emerging as an intriguing inflammasome-mediated host defense mechanism against intracellular pathogens.

NLRC4 is important for the activation of caspase-1 in macrophages infected with pathogenic bacteria. The activation of caspase-1 by these pathogenic bacteria requires a functional bacterial secretion system that has been suggested as a link between bacterial pathogenicity and NLRC4 activation (Franchi et al., 2009). These secretion systems, which include the type 3 secretion system (T3SS) and type 4 secretion system (T4SS), act as molecular needle-like structures that inject effector proteins into the cytosol of host cells and are critical for pathogen colonization. Flagellin, the main component of the flagellum, is important for activation of the NLRC4 inflammasome (Miao et al., 2006; Franchi et al., 2006). It had been thought that NLRC4 is activated in macrophages via the leakage of small amounts of flagellin through a T3SS (for example, *S. enterica*

and *P. aeruginosa*) or T4SS (for example, *L. pneumophila*) during infection (Sun et al., 2007). However, *Shigella flexneri*, an aflagellated pathogenic bacterium, also induces activation of the NLRC4 inflammasome through the T3SS (Suzuki et al., 2007), and proteins that form the basal body rod component of the T3SS, such as PrgJ, can activate the NLRC4 inflammasome.

PrgJ-like proteins contain regions structurally homologous to the carboxy-terminal portion of flagellin (Miao et al., 2010), (Franchi et al., 2006; Lightfield et al., 2008).

How does NLRC4 sense different structures such as flagellin and PrgJ-like proteins? Distinct Naip (NLR family apoptosis inhibitor protein) proteins link flagellin and PrgJ-like proteins to NLRC4, flagellin binds Naip5 and Naip6 and PrgJ-like proteins interact with Naip2 (Zhao et al., 2011; Kofoed et al., 2011). Although the mechanism by which Naip proteins activate NLRC4 remains unclear, one model proposes that flagellin or PrgJ-like proteins bind to the LRRs of Naip proteins to induce a conformational change in the latter; this in turn induces the activation of NLRC4 (Fig.9)

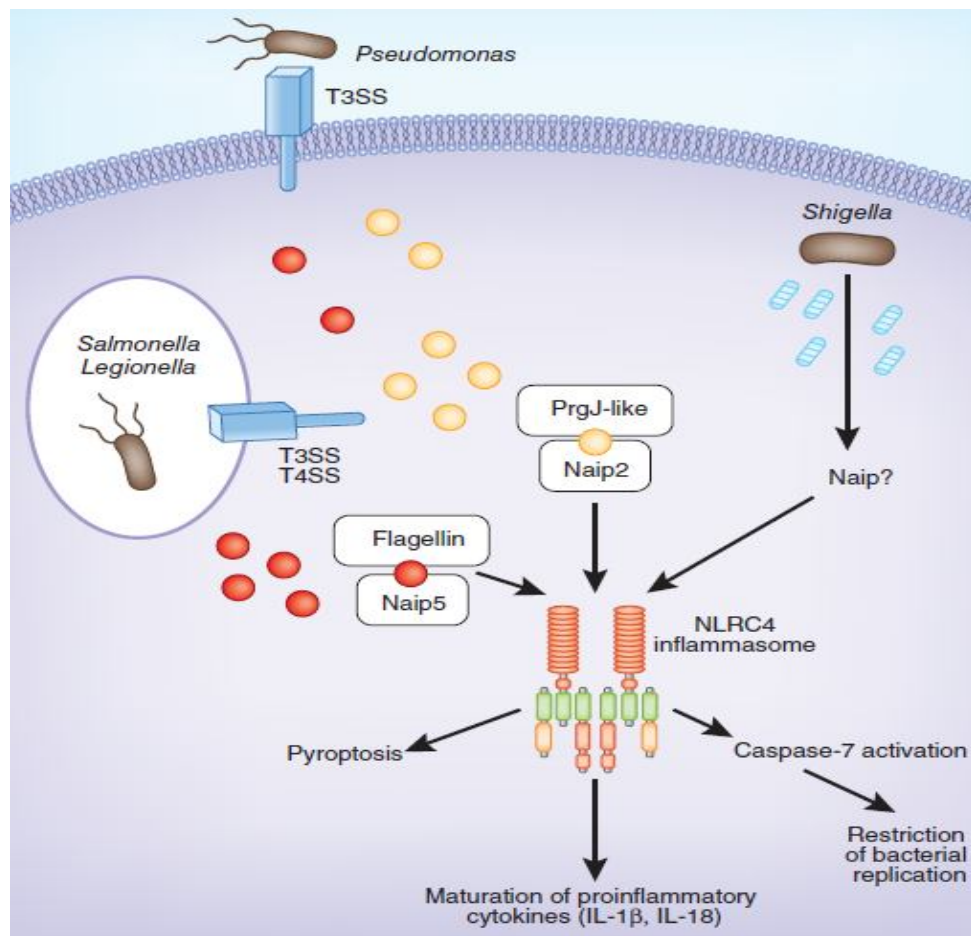


Fig.9 The NLRC4 inflammasome. Infection of macrophages with various Gram-negative bacteria, including *S. enterica*, *L. pneumophila* and *P. aeruginosa*, activates caspase-1 via NLRC4. A critical step is the cytosolic delivery of flagellin or PrgJ-like proteins through bacterial T3SS or T4SS. Flagellin is recognized by Naip5 or Naip6 (not shown here), whereas PrgJ-like proteins are recognized by Naip2. *S. flexneri* activates the NLRC4 inflammasome independently of flagellin through an unknown microbial product. Activation of caspase-1 via NLRC4 leads to the processing and release of IL-1 β and IL-18, the processing of caspase-7 and the induction of other cellular activities that are poorly understood. Adapted from Franchi et al., 2012.

OBJECTIVES

The specific objectives of the present work are:

1. Characterization of the mechanism of processing and release of the gilthead seabream IL-1 β .
2. Characterization of the role played by pyroptosis in the clearance of *S. typhimurium* infection in teleosts.
3. Characterization of the roles played by the inflammasome adaptor molecule PYCARD and caspase-1 in the clearance of *S. typhimurium* infection in zebrafish.
4. Molecular, phylogenetic and functional characterization of a new member of the IL-1 family (IL-1Fm2) identified in teleosts.

Chapter I: Evolution of inflammasome functions in vertebrates: Inflammasome and caspase-1 trigger fish macrophage cell death but are dispensable for the processing of IL-1 β .

ABSTRACT

Members of the nucleotide binding and oligomerization domain-like receptors (NLRs) and the PYD and CARD domain containing adaptor protein (PYCARD) assemble into multiprotein platforms, termed inflammasomes, to mediate in the activation of caspase-1 and the subsequent secretion of IL-1 β and IL-18, and the induction of pyroptotic cell death. While the recognition site for caspase-1 is well conserved in mammals, most of the non-mammalian IL-1 β genes cloned so far lack this conserved site. We report here that stimulation of seabream macrophages (M ϕ) with flagellin or bacterial DNA or infection with invasive or non-invasive bacteria led to the caspase-1-independent processing and release of IL-1 β . In addition, several classical activators of the NLRP3 inflammasome failed to activate caspase-1 and to induce the processing and release of IL-1 β in seabream M ϕ . Furthermore, the processing of IL-1 β in seabream M ϕ is not prevented by specific caspase-1 or pan-caspase inhibitors, and recombinant seabream caspase-1 also failed to process proIL-1 β . However, the infection of seabream M ϕ with wild type *Salmonella typhimurium*, but not with a non-invasive isogenic strain, resulted in caspase-1 activation. Notably, the pharmacological inhibition of caspase-1 impaired *S. typhimurium*-induced cell death in seabream. These results suggest a role for the inflammasome and caspase-1 in the regulation of pyroptotic cell death in fish and support the idea that its use as a molecular platform for the processing of pro-inflammatory cytokines arose after the divergence of fish and tetrapods.

1. INTRODUCCION

The inflammasomes are cytosolic multiprotein platforms required for the activation of inflammatory caspases, namely caspase-1 (Lamkanfi and Dixit, 2011) and caspase-11 (also known as caspase-4) (Kayagaki et al., 2011). Genetic studies in mice have revealed at least four inflammasomes of distinct composition, namely those containing the nucleotide-binding and oligomerization domain-like receptors (NLRs) NLRP1B, NLRP3 and NLRC4, and a recently characterized inflammasome complex assembled around the HIN-200 protein absent in melanoma 2 (AIM2) (Lamkanfi and Dixit, 2011). An adaptor protein, PYD and CARD domain containing (PYCARD, also known as ASC), which recruits NLRs carrying the pyrin homologous domain (PYD) and procaspase-1 through PYD and CARD, respectively, is responsible for the formation of some inflammasomes and also for the activation of procaspase-1 (Taniguchi et al., 2007). However, two elegant recent studies have demonstrated a further complex step, at least in the NLRC4 inflammasome, where another family of intracellular receptors called NAIPs was directly involved in the binding and recognition of specific virulence-associated molecules (Zhao et al., 2011; Kofoed et al., 2011).

Once activated, caspase-1 modulates inflammatory and the host defense responses by processing the pro-inflammatory cytokines IL-1 β and IL-18 into their biologically active forms, which is a prerequisite for their secretion (Gu et al., 1997; Ghayur et al., 1997; Li et al., 1995; Kuida et al., 1995). In addition to secreting IL-1 β and IL-18, caspase-1 and -11 contributes to the host defense through an inflammatory cell death program known as pyroptosis, which occurs in myeloid cells infected with bacterial pathogens such as *Salmonella typhimurium*, *Francisella tularensis* and *Bacillus anthracis* (Lamkanfi et al., 2011; Miao et al., 2010; Terra et al., 2010; Jones et al., 2010).

Although mammalian IL-1 β is relatively well characterized, little information is available on IL-1 β from lower vertebrates. Identification of the first non-mammalian sequences has resulted in an even more puzzling scenario, since the fish, amphibian and bird IL-1 β genes cloned so far lack a conserved caspase-1 recognition site (Bird et al., 2002). We have determined the molecular identity and tissue localization of IL-1 β in the teleost fish gilthead seabream (*Sparus aurata* L.) (Pelegri n et al., 2004), one of the

most commonly cultivated fish for human consumption. Like its mammalian counterpart, we found that endotoxin challenge led to a significant increase in IL-1 β expression in seabream M ϕ and that it accumulates intracellularly (Pelegri n et al., 2004). In contrast, the classical activator of the NLRP3 inflammasome ATP (Mariathasan et al., 2006) fails to provoke IL-1 β release from endotoxin-stimulated seabream leukocytes (Pelegri n et al., 2004) despite phosphatidylserine externalization and cell permeabilization in seabream leukocytes treated with ATP (L pez-Castej n et al., 2007a). In addition, neither seabream nor zebrafish P2X7 receptors induced the secretion of mammalian or fish IL-1 β when expressed in HEK293 cells, while a chimeric receptor harboring the ATP-binding domain of seabream P2X7 and the intracellular region of its rat counterpart did so ATP (L pez-Castej n et al., 2007a). These findings indicate that P2X7 receptor-mediated activation of caspase-1 and the release of IL-1 β result from different downstream signaling pathways, and suggest that, although the mechanisms involved in IL-1 β secretion are conserved throughout evolution, distinct inflammatory signals have been selected for the secretion of this cytokine in different vertebrates.

To throw light on the evolutionary history of the inflammasome and its role in the regulation of the inflammatory response in primitive vertebrates, an immunologically tractable teleost fish species was studied, namely the gilthead seabream (*S. aurata*, Perciformes). We found that stimulation of seabream leukocytes with flagellin or bacterial DNA, or infection with either invasive or non-invasive bacteria, lead to the processing and release of proIL-1 β into an 18 kDa mature form. Interestingly, although pharmacological inhibition of caspase-1 had no effect on the processing of IL-1 β , they impaired *S. typhimurium*-induced cell death in seabream.

2. MATERIALS AND METHODS

2.1 Animals

Healthy specimens (150 g mean weight) of the hermaphroditic protandrous marine fish gilthead seabream (*S. aurata*) were maintained at the Oceanographic Centre of Murcia (Spain) in 14 m³ running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Trouvit, Burgos, Spain). Fish were fasted for 24 hours before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (approval n° #333/2008) for the use of laboratory animals.

2.2 Cell culture and treatments

Seabream head kidney (bone marrow equivalent in fish) leukocytes obtained as described elsewhere (Sepulcre et al., 2002) were maintained 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) and 100 I.U./ml penicillin and 100 µg/ml streptomycin (P/S, Biochrom). Some experiments were conducted using purified cell fractions of MØ and acidophilic granulocytes (AG), the two professional phagocytic cell types of this species (Sepulcre et al., 2002; Roca et al., 2006). Briefly, AGs were isolated by MACS using a mAb specific to gilthead seabream AG (G7) (Sepulcre et al., 2002). MØ monolayers were obtained after overnight culture of G7⁺ fractions in FCS-free medium and their identity was confirmed by the expression of the M-CSFR (Roca et al., 2006).

Seabream MØ, AG and total leukocytes from seabream head kidney were stimulated for 16 h at 23°C with 50 µg/ml phenol-extracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) or 100 ng/ml flagellin (Invivogen) in sRPMI supplemented with 0.1% FCS and P/S (Sepulcre et al., 2007). These PAMPs were found to be the most powerful in the activation of gilthead seabream professional phagocytes (Sepulcre et al., 2007). In some experiments, cells were then washed twice with sRPMI, and incubated for 1 hr with 5 mM ATP (Sigma-Aldrich), 1 µM nigericin (Sigma-Aldrich), hypotonic buffer (36.75 mM NaCl, 0.5 mM KCl, 0.5 CaCl₂, 0.25 mM MgCl₂, 3.25 mM glucose, 2.5 mM HEPES, pH 7.3, 90 mOs), 500 µg/ml MSU crystals

(Invivogen) or 40 µg/ml aluminum crystals (aluminum hydroxide and magnesium hydroxide, Thermo Scientific). For infection experiments, *Salmonella enterica* serovar *typhimurium* SL1344 and the isogenic derivative strain SB169 (sipB::aphT) (Lara-Tejo and Galán, 2009) (provided by Drs. F. García del Portillo and Jorge Galán) and *Escherichia coli* strain 3616 and its isogenic derivative 3617 expressing recombinant listeriolysin O (LLO) from *Listeria monocytogenes* (provided by Dr. D. Higgins) (Higgins et al., 1999) were used. Overnight cultures in Luria-Bertani medium (LB) were diluted 1/5, grown at 37°C with shaking for 3 h, diluted in sRPMI and added to leukocytes to a MOI of 10. After 2 h, 10 µg/ml gentamycin was added to limit the growth of extracellular bacteria and the infected leukocytes were incubated for different lengths of time. In some experiments, leukocytes were pre-treated for 1 h with 100 µM of the caspase-1 inhibitors Ac-YVAD-CMK or Ac-YVAD-CHO, or 50 µM of the pan-caspase inhibitor Z-VAD-FMK (all from Calbiochem-Merck).

Cell supernatants from control and stimulated/infected leukocytes were collected after overnight incubation, unless otherwise indicated, clarified with a 0.45 µm filter and concentrated by precipitation with 20% trichloroacetic acid (Sigma-Aldrich).

2.3 Cell viability

Aliquots of cell suspensions were diluted in 200 µl PBS containing 40 µg/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples was analyzed by using flow cytometry (BD Biosciences).

2.4 Caspase-1 activity assay

The caspase-1 activity was determined with the fluorometric substrate Z-YVAD-AFC (caspase-1 substrate VI, Calbiochem) as described previously (López-Castejón et al., 2008). In brief, cells were lysed in hypotonic cell lysis buffer [25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), 1:20 protease inhibitor cocktail (Sigma), pH 7.5] on ice for 5, 10 min. For each reaction, 2×10^7 seabream leukocytes or 10 µg protein from MØ extracts were incubated for 90 min at 23°C with 50 µM YVAD-AFC and 50 µl of reaction buffer [0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2 M HEPES, 20% sucrose, 29 mM DTT, pH 7.5]. In some experiments, the caspase-1 inhibitors Ac-

YVAD-CMK or Ac-YVAD-CHO were also added at a final concentration of 100 μ M. After the incubation, the fluorescence of the AFC released from the Z-YVAD-AFC substrate was measured with a FLUOstart spectrofluorometer (BGM, LabTechnologies) at an excitation wavelength of 405 nm and an emission wavelength of 492 nm.

2.5 Western blot

Cells were lysed at 4°C in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and a 1:20 dilution of the protease inhibitor cocktail P8340 from Sigma-Aldrich). The protein concentrations of cell lysates were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard. Cell extracts and concentrated supernatants were analyzed on 15% SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad). The blots were developed using a 1:5000 dilution of a rabbit monospecific antibody to gilthead seabream IL-1 β (López-Castejón et al., 2007a) or antibodies to human/mouse IL-1 β (3ZD, Biological Resources Branch, NCI), and enhanced chemiluminescence (ECL) reagents (GE Healthcare) according to the manufacturer's protocol. Membranes were then reprobed with a 1:5000 dilution of an affinity purified rabbit polyclonal to histone H3 (#ab1791, Abcam).

2.6 Bactericidal assay

Leukocytes were lysed with 0.2% saponin (Sigma) and the number of surviving bacteria were enumerated by plating cell extracts on LB-agar plates containing the appropriate antibiotics.

2.7 Cell transfection

Seabream caspase-1 and proIL-1 β were cloned into pcDNA3.1/V5-His-TOPO and pcDNA4His/Max expression vectors (Invitrogen) for the expression of C-terminal V5/His6-tagged or N-terminal His/Xpress proteins, respectively (López-Castejón et al., 2007b; López-Castejón et al., 2008). Other expression constructs were rat P2X7ee (Wilson et al., 2002), human proIL-1 β (Siders et al., 1995) and human caspase-1 (Miura et al., 1993). Plasmid DNA was prepared using the Mini-Prep procedure (Qiagen). DNA pellets were resuspended in water and further diluted, when required, in PBS. Transfections were performed with a cationic lipid-based transfection reagent (LyoVec, Invivogen) according to the manufacturer's instructions. Briefly, HEK293 cells were plated in 6-well plates (400,000 cells/well) together with 100 μ l of transfection reagent

containing 0.15 µg of the rat P2X7-ee, 1.5 µg of either the human or seabream proIL-1β expression constructs and 1.5 µg of the human or seabream caspase-1 expression vector. Forty-eight hours after transfection, cells were washed twice with serum-free medium and incubated for 30 min with 1 mM ATP to activate the P2X7 receptor (López-Castejón et al., 2007a).

2.8 Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups. The differences between two samples were analyzed by the Student *t* test.

3. RESULTS

3.1 Infection of seabream head-kidney leukocytes with invasive and non-virulent bacteria leads to the processing and release of mature IL-1 β

To evaluate the production, processing and release of fish IL-1 β , seabream head-kidney leukocytes pre-stimulated with bacterial DNA or flagellin, two powerful pathogen-associated molecular patterns (PAMPs) for this specie able to drastically induce the expression of the IL-1 β gene and to promote the intracellular accumulation of IL-1 β (Sepulcre et al., 2007; Olavarria et al., 2010; Sepulcre et al., 2011), were infected with wild type *S. typhimurium* SL1344 and the non-invasive isogenic derivative strain SB169, which harbors a mutation in the sipB translocation machinery component (sipB::aphT) of the *Salmonella* pathogenicity island 1 (SPI1)-encoded type 3 secretion system (T3SS) (Lara-Tejo and Galán, 2009). The SPI1 T3SS can be readily recognized by caspase-1 in mammalian cells by detecting of inadvertently translocated flagellin or PrgJ rod protein to the cytosol mediated by NLRC4 (Zhao et al., 2011; Kofoed et al., 2011). Unexpectedly, although stimulation of leukocytes with PAMPs for 16 h led to the processing of proIL-1 β to a ~18 kDa IL-1 β mature form (mIL-1 β) that mainly remained cell-associated, infection of PAMP-stimulated leukocytes with both wild type and non-invasive *S. typhimurium* strains promoted the released of mature IL-1 β (Fig. 1A, B). Similarly, wild type *E. coli* and an isogenic derivative strain expressing recombinant LLO from *L. monocytogenes* (Higgins et al., 1999) were similarly effective in the processing and release of IL-1 β (Fig. 1C). We next sought to determine whether PAMPs alone might also induce the secretion of IL-1 β and found that this was the case (Fig. 1D), although at lower levels than bacteria (Figs. 1A-C). As these results suggest the caspase-1-independent processing and release of IL-1 β in this species, we stimulated seabream leukocytes with ATP (Fig. 2A) and nigericin (Fig. 2B), two classical activators of mammalian NLRP3 inflammasome (Mariathasan et al., 2006), and found that both activators also failed to trigger IL-1 β processing and release in seabream leukocytes. Interestingly, the pharmacological inhibition of caspase-1 failed to inhibit the processing and secretion of IL-1 β in seabream leukocytes in response to *S. typhimurium* infection (Figs. 2B, 2C), even though this inhibitor is able to fully inhibit caspase-1 activity in seabream leukocytes (López-Castejón et al., 2008). These results were further confirmed with the pan-caspase inhibitor Z-VAD-FMK, which also failed to affect IL-1 β processing and release (Fig. 2C).

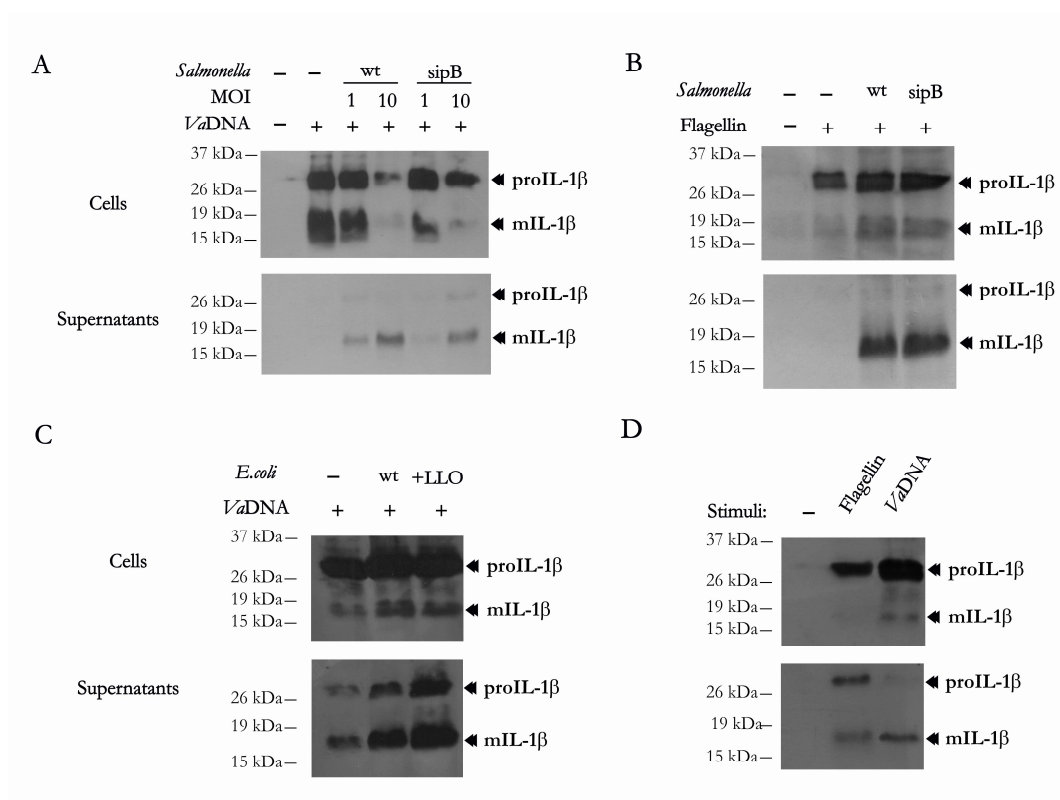


Fig. 1 Infection of seabream leukocytes with invasive and non-virulent bacteria leads to the processing and release of mature IL-1 β . Seabream head-kidney leukocytes were stimulated for 16 h with 50 μ g/ml bacterial genomic DNA (*VaDNA*) (A, B, D) or 1 μ g/ml flagellin (B, D) and then infected with a MOI of 1 (A) or 10 (B-D) of wild type and sipB mutant strain of *S. typhimurium* (A, B) or wild type *E. coli* and its isogenic derivative expressing recombinant LLO (C). Two hours after the infection, gentamycin (10 μ g/ml) was added to limit the growth of extracellular bacteria and the infected leukocytes were incubated for 24 h. Cell lysates (5×10^6 cells) and concentrated supernatants obtained from 10^7 cells were probed with a monospecific polyclonal antibody to seabream IL-1 β . Migration positions for the mature and pro-cytokine forms are indicated. Results are representative of five independent experiments.

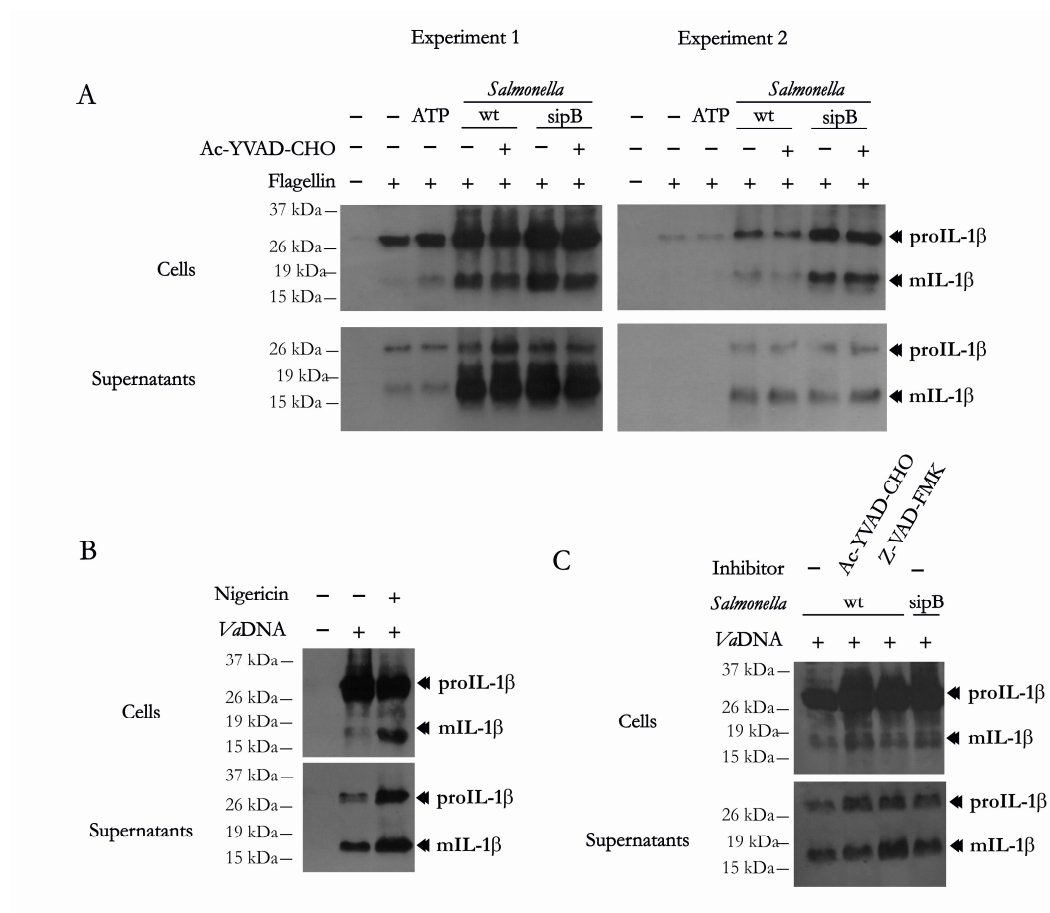


Fig. 2 The processing and release of IL-1 β is caspase-1 independent in seabream leukocytes.

Seabream head-kidney leukocytes pre-treated for 1 h with 100 μ M of the caspase-1 inhibitors Ac-YVAD-CHO or 50 μ M of the pan-caspase inhibitor Z-VAD-FMK (A, C) were stimulated for 16 h with 1 μ g/ml flagellin (A) or 50 μ g/ml bacterial genomic DNA (VaDNA) (B, C). Leukocytes were then infected with a MOI of 10 of wild type *S. typhimurium* and its isogenic sipB mutant derivative (A, C) or stimulated with 5 mM ATP or 1 μ g/ml nigericin for 1 h. Infected cells were treated with gentamycin as described in Legend to Figure 1 and incubated for 24 h. Cell lysates (5×10^6 cells) and concentrated supernatants obtained from 10^7 cells were probed with a monospecific polyclonal antibody to seabream IL-1 β . Migration positions for the mature and pro-cytokine forms are indicated. Two representative experiments are shown in A, while the results presented in B and C are representative of multiple independent experiments.

3.2 Seabream caspase-1 fails to process IL-1 β

We next studied whether seabream caspase-1 ectopically expressed in HEK293 cells was able to process seabream IL-1 β following activation. Seabream caspase-1 was unable to process seabream proIL-1 β , and activation of the rat P2X7 receptor led to the secretion of proIL-1 β . In sharp contrast, human caspase-1 processed human proIL-1 β in both non-stimulated and ATP-stimulated cells, while activation of the P2X7 receptor with ATP led to the release of precursor and mature IL-1 β forms (Fig. 3). In accordance with our previous results (López-Castejón et al., 2008) activation of the rat P2X7 receptor with ATP resulted in the activation of caspase-1 at similar levels to that observed in its human counterpart (Fig. 3).

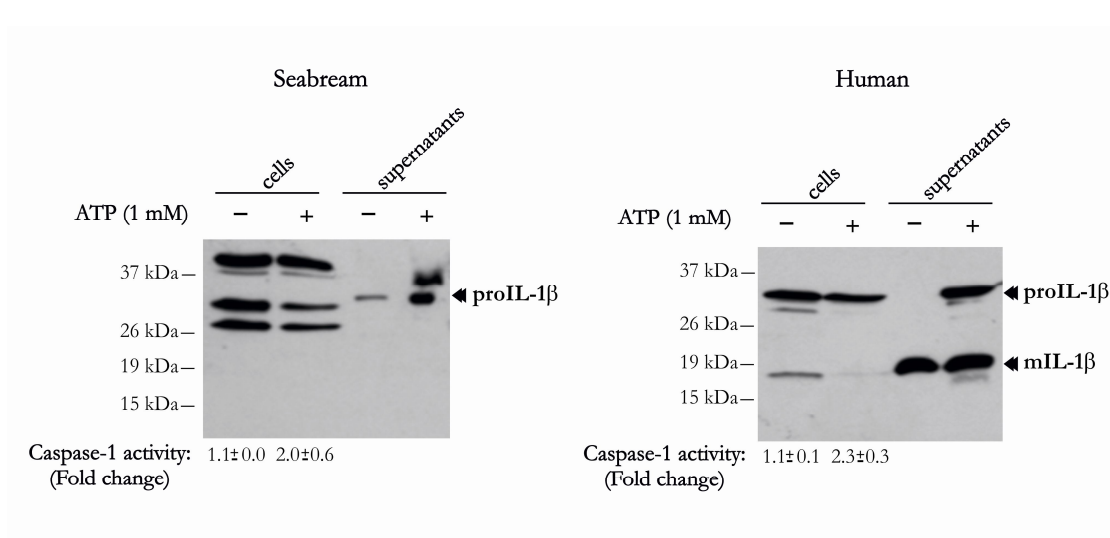


Fig. 3 Seabream caspase-1 fails to process seabream IL-1 β . HEK293 cells were transiently transfected with the seabream (left panel) or human (right panel) caspase-1 and the rat (r) P2X7 expression vectors. After 2 days, cells were stimulated with 1 mM ATP for 30 min and cell lysates (50 μ g/ml) and concentrated supernatants obtained from $\sim 10^6$ cells were probed with antibodies specific to human or seabream IL-1 β . Migration positions for the mature and pro-cytokine forms are indicated. Results are representative of two independent experiments. In parallel, the caspase-1 activity was determined with the fluorogenic substrate Z-YVAD-AFC in cell lysates obtained from 10^6 transfected cells. The basal fluorescence of cells transfected with the rP2X7 alone and non-treated with ATP was used to normalize all samples. The data represent the mean $\pm$ S.E. of two independent experiments.

3.3 Classical NLRP3 inflammasome activators fail to activate caspase-1 and IL-1 β processing in seabream M ϕ

As the relevance of the inflammasome and caspase-1 in the processing and release of IL-1 β by mammalian neutrophils has been little studied and neutrophils may show inflammasome/caspase-1-independent mechanisms to process IL-1 β (Greten et al., 2007), we next examined the activation of caspase-1 and the processing/release of IL-1 β in purified seabream M ϕ . The results show that ATP, K⁺ efflux (nigericin) and several crystals, including MSU and aluminum, all failed to activate caspase-1 in seabream M ϕ (Fig. 4A). Similarly, none of these stimuli was able to promote the processing and release of IL-1 β , although some of them decreased the release of mature IL-1 β (Fig. 4B). Interestingly, a hypotonic solution, which activated caspase-1 in seabream and mouse M ϕ through the activation of NLRP3 inflammasome (Compan et al, 2012) was also unable to trigger the processing of IL-1 β (Figs. 4A, 4B).

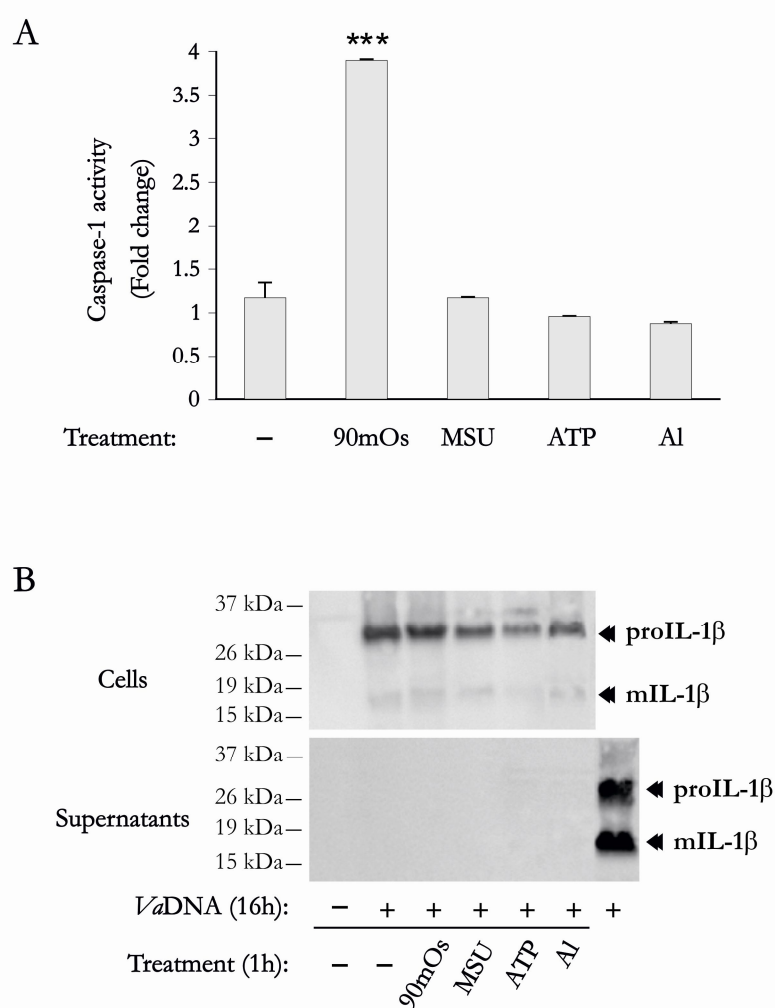


Fig. 4 Classical NLRP3 inflammasome activators fail to activate caspase-1 and IL-1 β processing in seabream M $\emptyset$. Seabream head-kidney M $\emptyset$ were stimulated with 50 μ g/ml VaDNA for 16 h, washed twice and then incubated for 1 h with a hypotonic buffer (90 mOs), 500 μ g/ml MSU crystals, 5 mM ATP or 40 μ g/ml aluminum crystals. (A) The caspase-1 activity of cell extracts (10 μ g) was measured with the caspase-1 substrate Z-YVAD-AFC. The data represent the mean $\pm$ S.E. of three independent experiments. ***P<0.001. (B) The cell extracts (40 μ g) and concentrated supernatants (from 400 μ g cell extract) were also probed with a monospecific polyclonal antibody to seabream IL-1 β . Migration positions for the mature and pro-cytokine forms are indicated and the supernatant from M $\emptyset$ incubated for 16 h with VaDNA (before washing) is shown as a positive control.

3.4 Invasive *S. typhimurium* promotes caspase-1 activation and cell death in seabream

We next analyzed the impact of wild type and sipB mutant strains of *S. typhimurium* in seabream leukocytes and M $\emptyset$. The results show that the wild type strain induced the rapid activation of caspase-1 in infected M $\emptyset$, while the isogenic mutant strain failed to do so (Figs. 5A, B). However, both bacterial strains were able to trigger the processing and release of IL-1 β at similar levels (Fig. 5C). However, wild type *S. typhimurium* induced leukocyte death at higher levels (Fig. 5D) and showed higher proliferative capacity in leukocytes (Fig. 5E) than its sipB mutant isogenic derivative. In addition, pharmacological inhibition of caspase-1 impaired *S. typhimurium*-induced cell death (Fig. 5F).

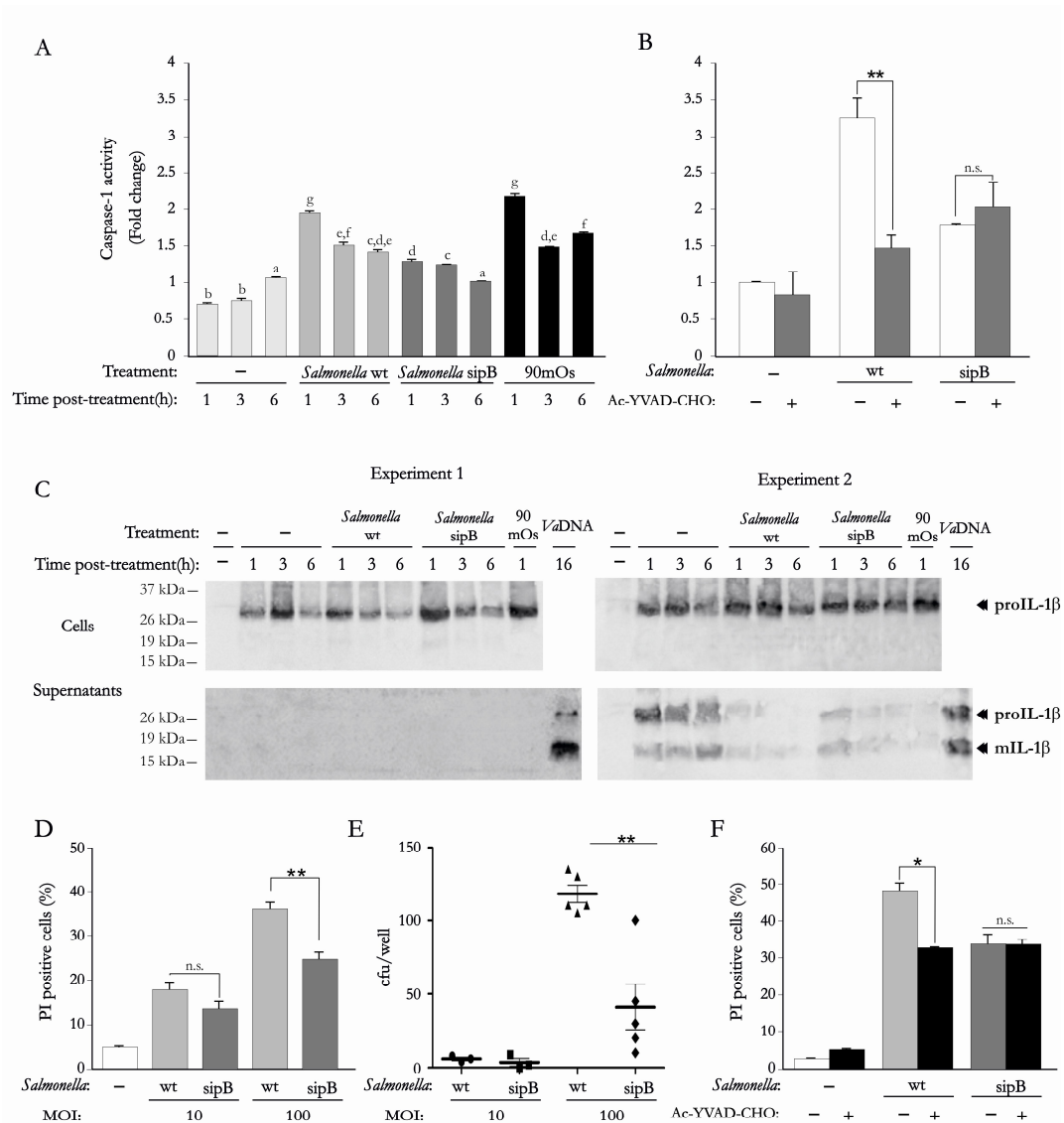


Fig. 5 Invasive *S. typhimurium* promotes caspase-1 activation and cell death in seabream. Seabream head-kidney MØ (A-C) and total leukocytes (D-F) pre-stimulated for 16 h with 50 µg/ml bacterial genomic DNA (VaDNA) in the presence or absence of 100 µM of the caspase-1 inhibitor Ac-YVAD-CHO were infected with a MOI of 10 (A-E) or 100 (D-F) of wild type *S. typhimurium* and its isogenic sipB mutant derivative. Infected cells were treated with gentamycin as described in Legend to Figure 1 and incubated for 24 h. (A, B) The caspase-1 activity of cell extracts from MØ (10 µg) was measured with the caspase-1 substrate Z-YVAD-AFC. The basal fluorescence of non-infected MØ was used to normalize all samples. The data represent the mean ± S.E. of two independent experiments. (C) The cell extracts (40 µg) and concentrated supernatants (obtained from 400 µg cell extracts) were also probed with a monospecific polyclonal antibody to seabream IL-1β. Migration positions for the mature and pro-cytokine forms are indicated. The supernatant from MØ incubated for 16 h with VaDNA (before washing) is shown as a positive control. (D, F) The percentage of PI positive leukocytes was measured by flow cytometry. Values are means ± S.E. of three independent experiments. (E) The numbers of intracellular bacteria were determined by plating them LB agar. Data represent two pooled independent experiments. Each dot represents the number of CFU per leukocyte culture from a single fish. The mean ± S.E. of the CFU for each group are also shown. Different letters denote statistically significant differences ($p < 0.05$) among groups according to a Tukey test of comparison of means. The groups marked with “a” in Figure 5A did not show statistically significant differences from controls. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s.: non-significant.

4. DISCUSSION

In recent years, many studies have reported the crucial role of the inflammasome as a molecular platform involved in the sensing of microbial presence in the cytosol and the subsequent activation of caspase-1 (Lamkanfi et al., 2011; Kayagaki et al., 2011). Once activated, caspase-1 modulates inflammatory and host defense responses by processing the pro-inflammatory cytokines IL-1 β and IL-18 into their biologically active forms, which is a prerequisite for their secretion (Gu et al., 1997; Ghayur et al., 1997; Li et al., 1995; Kuida, et al., 1995). In sharp contrast, we found that, although PAMP stimulation leads to the intracellular accumulation of IL-1 β in seabream leukocytes (Pelegrín et al., 2004), the classical activator of the NLRP3 inflammasome ATP (Mariathasan et al., 2006) fails to provoke IL-1 β release from PAMP-stimulated seabream leukocytes (Pelegrín et al., 2004), despite phosphatidylserine externalization and cell permeabilization in these cells following ATP stimulation (López-Castejón et al., 2007a). As these obvious differences in the processing and secretion of IL-1 β between fish and mammals hinge on the presence of a functional P2X7 receptor in fish and since ATP was seen to be less potent in seabream P2X7 receptors than in mammalian rP2X7 (EC_{50} ~2 mM vs. ~100 μ M) (López-Castejón et al., 2007a), the present study used several activators of mammalian NLRP3 inflammasomes, including nigericin, cell swelling, MSU and aluminum. None of these stimuli was able to trigger the processing and secretion of IL-1 β in seabream. However, stimulation of seabream leukocytes and purified MØ with prolactin (Olvarria et al., 2010) and PAMPs, and infection with non-invasive and invasive bacteria (this study) promoted the processing of proIL-1 β to a ~18 kDa mature form, which was rapidly released. Notably, these stimuli also failed to promote the activation of caspase-1, with the exception of invasive *S. typhimurium*, which activates NLRC4 in mammals (Zhao et al., 2011; Kofoed et al., 2011; Miao et al., 2010) and cell swelling, which activates NLRP3 in fish and mammals (Compan et al., 2012). In addition, the pharmacological inhibition of caspase-1 failed to inhibit the processing of IL-1 β by seabream leukocytes and recombinant seabream caspase-1 was unable to process seabream IL-1 β when expressed and activated in HEK293 cells, further indicating that IL-1 β is processed through a caspase-1-independent mechanism by professional phagocytes in this species. Taken together, these results suggest, therefore, that the processing and release of IL-1 β in early vertebrates is coupled to its synthesis, while a more sophisticated “two step”

mechanism, which involves the inflammasome-dependent activation of caspase-1, evolved after the divergence of fish and tetrapods about 450 million years ago.

Despite the crucial importance of the inflammasome in sensing intracellular pathogens, little information exists concerning this gene family in non-mammalian vertebrates. A recent phylogenetic study has discovered three distinct NLR subfamilies in teleost fish by mining genome databases of various species: the first subfamily (NLR-A) resembles mammalian NODs, the second (NLR-B) resembles mammalian NALPs, while the third (NLR-C) appears to be unique to teleost fish (Laing et al., 2008). In zebrafish, while NLR-A and NLR-B subfamilies contain five and six genes, respectively, the third subfamily is strikingly large, containing several hundred NLR-C genes, many of which are predicted to encode a C-terminal B30.2 domain (Laing et al., 2008). Although genetic depletion of zebrafish NOD1 and NOD2 orthologues belonging to subfamily NLR-A have been found to reduce the ability of embryos to control systemic infection (Oehlers et al., 2011), the functional relevance of this extended array of NLR genes of zebrafish needs to be investigated.

The inability of caspase-1 to process IL-1 β in seabream questions the relevance of caspase-1 and the inflammasome in the regulation of the immune response of early vertebrates. However, in addition to secreting IL-1 β and IL-18, it has been more recently found that caspase-1 and -11 contribute to the host defense through the clearance of infected immune cells by inducing pyroptotic cell death (Lamkanfi et al., 2011; Miao et al., 2010; Terra et al., 2010; Jones et al., 2010). Indeed, an interesting study have very recently found that a *S. typhimurium* strain persistently expressing flagellin was cleared by the cytosolic flagellin-detection pathway through the activation of caspase-1 by the NLRC4 inflammasome; however, this clearance was independent of IL-1 β and IL-18 but closely dependent on caspase-1-induced pyroptotic cell death of the infected M ϕ (Miao et al., 2010). Strikingly, bacteria released from pyroptotic M ϕ exposed them to uptake and rendered them susceptible to killing by reactive oxygen species in neutrophils (Miao et al., 2010). Similarly, the activation of caspase-1 cleared unmanipulated *Legionella pneumophila* and *Burkholderia thailandensis* through cytokine-independent mechanisms (Miao et al., 2010). These results were later confirmed in *Listeria monocytogenes* and *Francisella novicida*, where mutations that caused bacteriolysis in the M ϕ cytosol promote pyroptosis through activation of PYCARD-dependent AIM2 inflammasome, suggesting that pyroptosis is also triggered

by the bacterial DNA released during cytosolic lysis (Sauer et al., 2010; Peng et al., 2011) to clear these intracellular bacteria (Sauer et al., 2011). Consistent with these results, we found that invasive *S. typhimurium*, but not a non-invasive isogenic derivative strain harboring a mutation in the sipB translocation machinery component of the SPI1-encoded T3SS (Lara-Tejo et al., 2009), was able to promote the activation of caspase-1. This, in turn, resulted in leukocyte cell death. As the SPI1 T3SS can be readily recognized by caspase-1 in mammalian cells through the detection of flagellin or PrgJ rod protein inadvertently translocated to the cytosol mediated by NLRC4 (Zhao et al., 2011; Kofoed et al., 2011) our results suggest a role for the inflammasome and caspase-1 in the regulation of pyroptotic cell death and the clearance of intracellular bacteria in fish.

In conclusion, our results support the idea that the use of the inflammasome as a molecular platform for the processing of pro-inflammatory cytokines arose after the divergence of fish and tetrapods. Although our data argue against a role for caspase-1 in the processing of IL-1 β in seabream, they do not rule out the possibility that caspase-1 might mediate the processing of other cytokines, such as IL-18, in fish. Furthermore, we hypothesize that, because of the relatively less elaborated and restrictive adaptive immune response of early vertebrates (Lieschke and Trede, 2009), fish would display a more sophisticated intracellular sensing system than mammals, strengthening their ability to clear intracellular pathogens through the induction of pyroptotic cell death.

Chapter II: A *Salmonella*-zebrafish infection model to study the role of inflammasome in the clearance of intracellular bacteria.

ABSTRACT

The nucleotide-binding domain leucine-rich repeats (NLRs) constitute a family of cytosolic pattern-recognition receptors (PRRs), which are the responsible for the activation of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β), IL-18 and IL-33, and the induction of a new form of cell death called pyroptosis. The activation of these cytokines and the induction of pyroptosis required the inflammatory caspase-1, which is activated via a multiprotein platform called the inflammasome. The activation of several inflammasomes require the adaptor molecule PYD and CARD domain containing (PYCARD, also called ASC), which binds to NLR proteins through its PYD domain and to pro-caspase-1 by its CARD domain, being necessary this coalition to convert pro-caspase-1 into its active form. To clarify the role of the inflammasome and caspase-1 in bacterial infections, we have developed a *Salmonella*-zebrafish infection model. Using this model, we found the genetic depletion of PYCARD impaired the activation caspase-1 in zebrafish larvae infected with wild type *S. typhimurium*. However, PYCARD-depletion or pharmacological inhibition of caspase-1 did not have a significant impact in the clearance of *S. typhimurium*. In contrast, pharmacological inhibition of all caspases using a pan-caspase inhibitor increased zebrafish susceptibility of *S. typhimurium* infection. Collectively, these results indicate that *S. typhimurium* possesses different redundant mechanisms to activate inflammatory caspases through different components of the inflammasome and suggest the existence of another inflammatory caspase involved in *S. typhimurium* clearance in zebrafish.

1. INTRODUCTION

Pattern-recognition receptors (PRRs) are expressed by different cell types, including macrophages, monocytes, dendritic cells, epithelial cells and neutrophils, and sense signals that are given by different effectors like microbial pathogens and/or cellular stress. The nucleotide-binding domain leucine-rich repeats (NLRs) constitute a family of cytosolic PRRs, which are the responsible for the activation of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β), IL-18 and IL-33. The activation of all these cytokines required their processing by caspase-1, which is activated via a multiprotein platform called the inflammasome (reviewed by Lamkanfi and Dixit, 2011). The NLR family, pyrin domain containing 3 (NLRP3) inflammasome has been implicated in the response against different danger signals, such as crystal particles (aluminum salts, monosodium ureate, cholesterol and asbestos) (Franchi et al., 2008). The activation of the NLRP3 inflammasome requires the adaptor molecule PYD and CARD domain containing (PYCARD, also called ASC), which binds to NLR proteins through its PYD domain and to pro-caspase-1 by its CARD domain, being necessary this coalition to convert pro-caspase-1 into its active form (Taniguchi and Sagara, 2007). More recently, it has been reported that guanylate binding protein 5 (GBP5) is necessary for the specific activation of the NLRP3 inflammasome by live bacteria and their cell wall components but not by crystalline agents or double-stranded DNA (Shenoy et al., 2012). Another inflammasome, called the NLR family, CARD domain containing 4 (NLRC4, also known as IPAF) inflammasome is activated in response to *Salmonella enteric* sv. *typhimurium* (*S. typhimurium*) and other flagelled pathogens (Zhao et al., 2011; Kofoed and Vance, 2011), and is the responsible for the processing of pro-inflammatory cytokines and a new form of cell death called pyroptosis, which is mediated by caspase-1 (Lamkanfi and Dixit, 2011; Miao et al., 2010; Terra et al., 2010; Jones et al., 2010). Notably, pyroptosis is required for the clearance of *S. typhimurium*, while the processing of IL-1 β seems to be dispensable (Miao et al., 2010). More recently, the inflammasomes has also been shown to generate a storm of eicosanoids through the activation of cytosolic phospholipase A₂ in resident peritoneal macrophages (von Moltke et al., 2012).

The phylogenetic aspects of the inflammasome have not been investigated. In the zebrafish (*Danio rerio*) various NLR-specific expansions have occurred and no full orthologues of mammalian NLRs have been identified and functionally characterized

(Laing et al., 2008). A recent study suggested that the ability of the inflammasome to process IL-1 β is conserved across vertebrates, since it was found that infection of zebrafish leukocytes with *Francisella noatunensis* promotes caspase-1 activation and the processing of IL-1 β and the inflammatory caspases caspase A and caspase B are able to process *in vitro* IL-1 β (Vojtech et al., 2012). However, the implication caspases in the processing of fish IL-1 β has been dismissed in others reports. Thus fish, amphibian and bird IL-1 β genes cloned so far lack a conserved caspase-1 recognition site (Bird et al., 2002) and the caspase-1 is mediating the induction of pyroptosis after *S. typhimurium* infection in gilthead seabream macrophages but it is dispensable for the processing of the IL-1 β (Angosto et al., 2012).

To clarify the role of the inflammasome and caspase-1 in bacterial infections, we have developed a *Salmonella*-zebrafish infection model. Using this model, we found the genetic depletion of PYCARD impaired the activation caspase-1 in zebrafish larvae infected with wild type *S. typhimurium*. However, PYCARD-depletion or pharmacological inhibition of caspase-1 did not have a significant impact in the clearance of *S. typhimurium*. In contrast, pharmacological inhibition of all caspases using a pan-caspase inhibitor increased zebrafish susceptibility of *S. typhimurium* infection.

2. MATERIALS AND METHODS

2.1 Animals

Zebrafish (*Danio rerio*) of the AB, TL and WIK genetic backgrounds were kindly provided by the Zebrafish International Resource Center and maintained as described in the Zebrafish handbook (Westerfield, 2001). The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (approval n° #333/2008) for the use of laboratory animals.

2.2 Cell culture and treatments

For infection experiments, we used *Salmonella enterica* serovar *typhimurium* wt 12023, and the isogenic derivative mutant strains for SPI-1 (prgH020::Tn5lacZY), SPI-2 (ssaV::aphT) and SPI-1/SPI-2 (prgH020::Tn5lacZY ssaV::aphT) (provided by Prof. D. Holden). Overnight cultures in Luria-Bertani medium (LB) were diluted 1/5, grown at 37°C with shaking for 3 h, diluted in PBS and injected to zebrafish larvae to a MOI of 10 (yolk sac) or 250 (Cuvier's duct).

2.3 Caspase-1 activity assay

The caspase-1 activity was determined with the fluorometric substrate Z-YVAD-AFC (caspase-1 substrate VI, Calbiochem) as described previously (López-Castejón et al., 2008). In brief, cells were lysed in hypotonic cell lysis buffer [25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), 1:20 protease inhibitor cocktail (Sigma), pH 7.5] on ice for 5, 10 min. For each reaction, 2×10^7 seabream leukocytes or 10 µg protein from MØ extracts were incubated for 90 min at 23°C with 50 µM YVAD-AFC and 50 µl of reaction buffer [0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2 M HEPES, 20% sucrose, 29 mM DTT, pH 7.5]. In some experiments, the caspase-1 inhibitors Ac-YVAD-CMK or Ac-YVAD-CHO were also added at a final concentration of 100 µM. After the incubation, the fluorescence of the AFC released from the Z-YVAD-AFC substrate was measured with a FLUOstart spectrofluorometer (BGM, LabTechnologies) at an excitation wavelength of 405 nm and an emission wavelength of 492 nm.

2.4 Zebrafish microinjection, chemical treatment and infection

A splice-blocking morpholino targeting the E2/I2 boundary of zebrafish PYCARD (**NM_131495**) was designed by and purchased from Gene Tools (5'-AGTGATTCGCTTACTCACCATCAGA-3'). It was solubilized in water (2 mM), mixed in microinjection buffer (0.5 x Tango buffer and 0.05 % phenol red solution) and microinjected (0.5-1 nl, 7 ng) into the yolk sac of one-cell-stage embryos using a Narishige IM300 microinjector.

In some experiments, larvae were transferred to fresh E3 medium containing 1% DMSO with or without the pan-caspase inhibitor Q-VD-OPh (50 μ M; Sigma-Aldrich) (Walters et al., 2009; Yang et al., 2012) or the caspase-1 inhibitor Ac-YVAD-CMK (100 μ M, Peptanova) before infection. The inhibitors were replaced every 24 h.

Larvae were infected with an LD₅₀ of wild type and mutant strains of *S. typhimurium*. Briefly, bacteria were injected in the yolk sac or Cuvier's duct of 60 hours post-fertilization (hpf) larvae and then monitored every 24 h over a 5-day period for clinical signs of disease and mortality. The numbers of surviving bacteria were also determined by plating them in LB with the appropriate antibiotics or in Salmonella-Shigella Agar, and determining the numbers of CFU/larvae. Bacteria were isolated from infected zebrafish larvae essentially as described previously (van der Sar et al., 2003).

2.5 Analysis of gene expression

Total RNA was extracted from cell pellets with TRIzol Reagent (Life Technologies) following the manufacturer's instructions and treated with DNase I, Amplification grade (1 unit/ μ g RNA, Invitrogen). The SuperScript III RNase H⁻ Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT₁₈ primer from 1 μ g of total RNA at 50 °C for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min 60°C and 15 s at 95°C. For each mRNA, gene expression was corrected by the ribosomal protein S11 (*rps11*). Used primers are in Table 1.

Table 1: Primers used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

Gene	Accession number	Name	Nucleotide sequence(5'→3')	Use
<i>rps11</i>	NM_213377	F1	GGCGTCAACGTGTCAGAGTA	RT-qPCR
		R1	GCCTCTTCTCAAAACGGTTG	
<i>il1b</i>	NM_212844	F5	GGCTGTGTGTTTGGAATCT	RT-qPCR
		F5	TGATAAACCAACCGGGACA	
<i>tnfa</i>	NM_212859	F2	GCGCTTTTCTGAATCCTACG	RT-qPCR
		R2	TGCCCAGTCTGTCTCCTTCT	
<i>inf1-2</i>	AB158361	F5	CTATGGGCGATCAAGGAAAA	RT-qPCR
		R3	CTTTAGCCTGCCGTCTCTTG	

2.6 Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups. The differences between two samples were analyzed by the Student *t* test.

3. RESULTS AND DISCUSSION

3.1 The T3SS of *S. typhimurium* is required for its virulence in zebrafish

Due to its clinical importance, *S. typhimurium* is an interesting model organism for the study of host–pathogen interaction on the molecular level. It is known that the virulence of *S. typhimurium* is linked to the pathogenicity islands termed *Salmonella* pathogenicity islands (SPIs). Different studies have demonstrated that two different SPIs, called SPI-1 and SPI-2, contain a large number of genes encoding type 3 secretion systems (T3SSs). The function of SPI-1 appears to be required for the initial steps of systemic infections (Galán et al., 1989) and, although the molecular functions of SPI2 has not been characterized in detail, SPI-2 mutants are severely attenuated in virulence in a mouse model of systemic infection and fail to proliferate in infected host organs, like the liver and spleen (Shea et al., 1999).

Infection of zebrafish larvae with wild type (WT) *S. typhimurium* resulted in a high mortality using two different routes of infection: the yolk sac (data not shown) and the Cuvier's duct (Fig. 1A). In contrast, double mutant for SPI-1 and SPI-2 showed reduced virulence, while single SPI-1 or SPI-2 mutants had an intermediate virulence (Fig.1A).

We next examined the ability of these strains of *S. typhimurium* to promote the activation of caspase-1 in zebrafish larvae. The results show that WT *S. typhimurium* induced the activation of caspase-1, while single or double SPI mutants failed to do so (Fig. 1B). Therefore, although both SPI-1 and SPI-2 contribute to *S. typhimurium* virulence in the zebrafish, mutation of any of them impaired the ability of this bacterium to activate caspase-1. Collectively, these results demonstrate the usefulness of this model to ascertain the role of SPI in *S. typhimurium* virulence in a whole vertebrate organism in the absence of adaptive immunity.

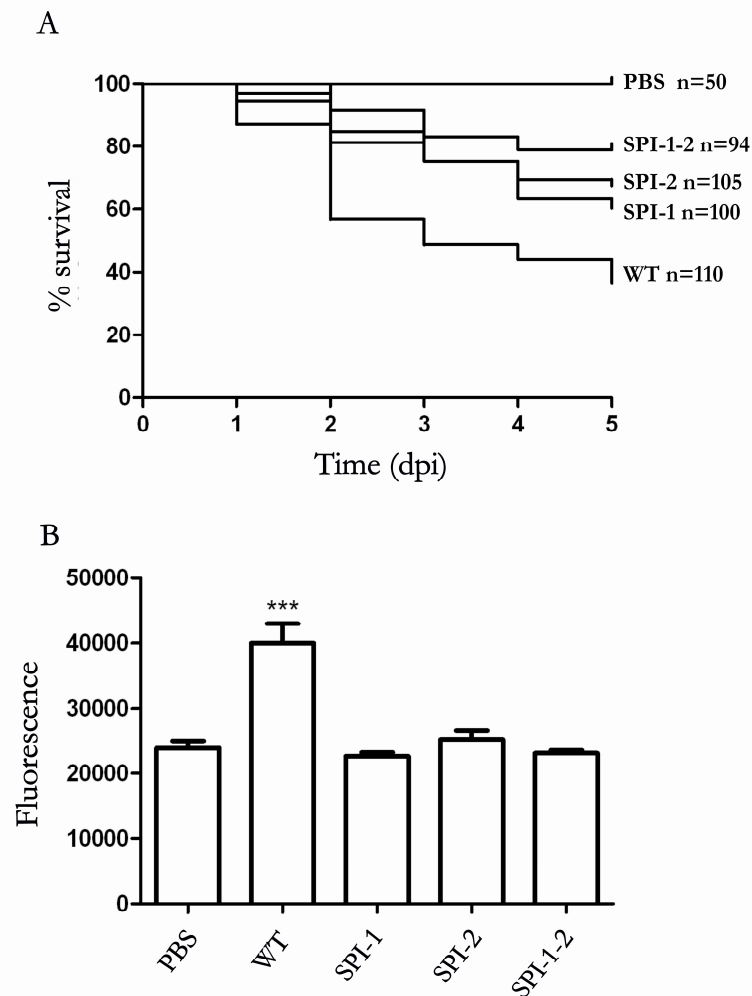


Fig. 1 The T3SS of *S. typhimurium* is required for its virulence in zebrafish and to activate caspase-1. (A) Zebrafish larvae were infected at 2 dpf with the different strains of *S. typhimurium* in the Cuvier's duct with a MOI of 250 and checked for 5 days for clinical signs of disease and mortality. (B) Caspase-1 activity was measured in protein extracts from whole zebrafish larvae 24 post-infection with the different strains of *S. typhimurium*. *** $P < 0.001$ according to a Student *t* test.

3.2 PYCARD is required for the activation of caspase-1 in zebrafish but dispensable for *S. typhimurium* clearance

As PYCARD is an adaptor protein required for the formation of some inflammasomes and the activation of pro-caspase-1 (Taniguchi and Sagara, 2007), we examined its role in the activation of caspase-1 in zebrafish using morpholino-mediated gene knockdown. RT-PCR analysis pointed to the high efficiency of the splice-blocking morpholino used against PYCARD, since it was able to alter the splicing of *pycard* transcripts up to 6 days post-fertilization (dpf) (Fig. 2A). The altered splicing of the *pycard* transcripts resulted in three smaller amplification products than the one observed in samples injected with a standard control morpholino. The higher product contained a

deletion of exon 2 that resulted in a predicted truncated PYCARD protein lacking the caspase recruitment domain (CARD) and, therefore, may act as a dominant negative form (Martinon et al., 2002). The smaller transcript contained a deletion of exons 2, 3 and 4 that resulted in a predicted PYCARD protein lacking the linker between the PYD and CARD domains, its activity being unpredictable. Finally, we failed to sequence the medium size *pycard* transcript but, judging from its size, it should contain a deletion of exon 2 and 3 or exon 3 and 4 that would result in PYCARD proteins with a shortened linker between PYD and CARD.

Genetic depletion of PYCARD in zebrafish had no effects on larval development but significantly reduced endogenous (Fig. 2B) and *S. typhimurium*-induced caspase-1 activity (Fig. 2B). However, we observed that PYCARD deficiency did not significantly alter the resistance of larvae to either WT or SPI-1/SPI-2 mutant *S. typhimurium* (Fig. 2C).

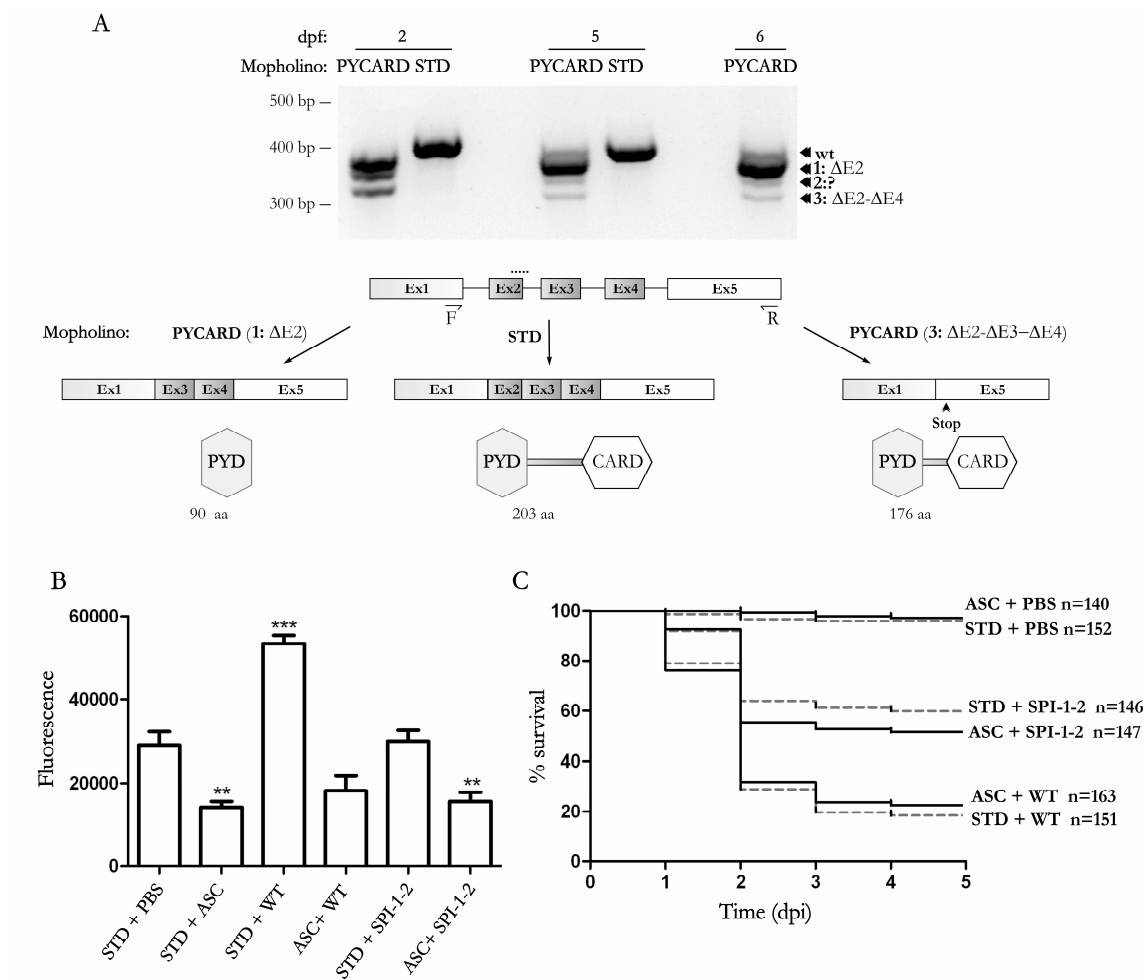


Fig. 2 PYCARD is required for the activation of caspase-1 in zebrafish but did not alter the resistance of larvae to *S. typhimurium* infection. Zebrafish one-cell embryos were microinjected with 7 ng of standard control (STD) or PYCARD morpholinos and infected with ~250 cfu of wild type *S. typhimurium* or its SIP-1/SPI-2 mutant at 60 hpf in the Cuvier's duct. (A) RT-PCR analysis of PYCARD morpholino-induced altered splicing of *pycard* transcript at the indicated days post- fertilization (dpf) and schematic representations of wild type and altered spliced transcripts and the predicted truncated Pycard proteins generated. Samples from embryos injected with a standard control morpholino (STD) are shown for comparison. The primer pairs (F and R) used for amplification and the annealing of MO are indicated with arrows and a dashed line, respectively. (B) The caspase-1 activity of larval extracts (50 μ g) from non-infected larvae at 24 hpf or from infected larvae at 24 hpi was measured with the caspase-1 substrate Z-YVAD-AFC. The data represent the mean $\pm$ S.E. of two independent experiments. (C) Survival assay of STD or PYCARD morphants infected with WT or SPI-1-2 mutant *S. typhimurium*. ** $P < 0.01$ and *** $P < 0.001$ according to a Student *t* test.

These results prompted us to examine the gene expression profile of control and PYCARD morphants larvae following infection with WT and SPI-1/SPI-2 *S. typhimurium*. As expected, infection with the WT bacterium increased the mRNA levels of the gene encoding the pro-inflammatory cytokines IL-1 β , TNF α and, to some extent, IFN γ 1-2 (Fig. 3). However, the double mutant strain failed to increase the transcript levels of these genes (Fig.3). Notably, PYCARD depletion had no significant effects in the induction of IL-1 β transcript levels but significantly impaired the induction of those of TNF α (Fig. 3). Collectively, these results further support the key relevance of the T3SS of *S. typhimurium* for its virulence in zebrafish and at the same time that PYCARD is dispensable for the sensing and clearance of this bacterium. Similar results have been obtained using a mouse model of *S. typhimurium* infection (Lara-Tejero et al., 2006).

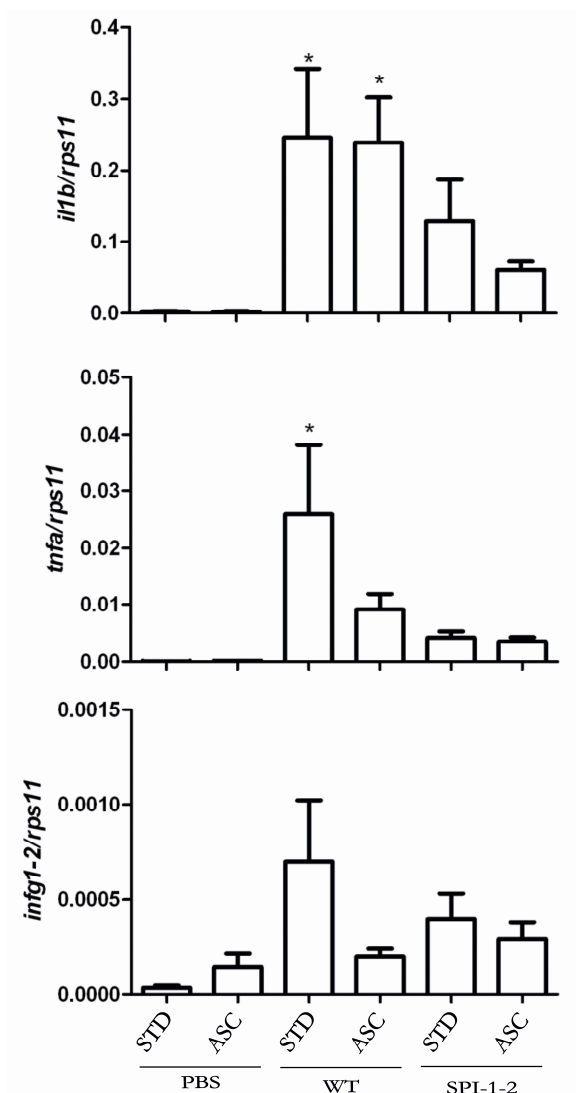


Fig. 3 Gene expression of larvae injected with the STD and ASC morpholino and infected with the *Salmonella* WT and the SPI-1-2. IL-1 β , TNF α and Inf γ 1-2 mRNA expression was measured in zebrafish larvae after 24 h post infection. The results were normalized using the ribosomal protein S11 gene (*rps11*). *P<0.05 according to a Student *t* test.

3.3 Caspase-1 is dispensable for *S. typhimurium* clearance but an unidentified caspase is required

As PYCARD was not required for the clearance of *S. typhimurium*, we next study the role of caspase-1. Unfortunately, no orthologues of caspase-1 has been identified to date in the zebrafish. Therefore, we used the specific caspase-1 inhibitor Ac-YVAD-CMK, which was able to inhibit the activity of recombinant caspase-1 of the teleost fish gilthead seabream (*Sparus aurata*) (López-Castejón et al., 2008). While pharmacological inhibition of caspase-1 had no effects in zebrafish larvae susceptibility to *S. typhimurium* (Fig. 4A), pharmacological inhibition of all caspases resulted in increased susceptibility to the infection (Fig. 4B). These results indicate that caspase-1 is dispensable for *S. typhimurium* clearance in the zebrafish and strongly suggest an important role for another caspase that need to be identified.

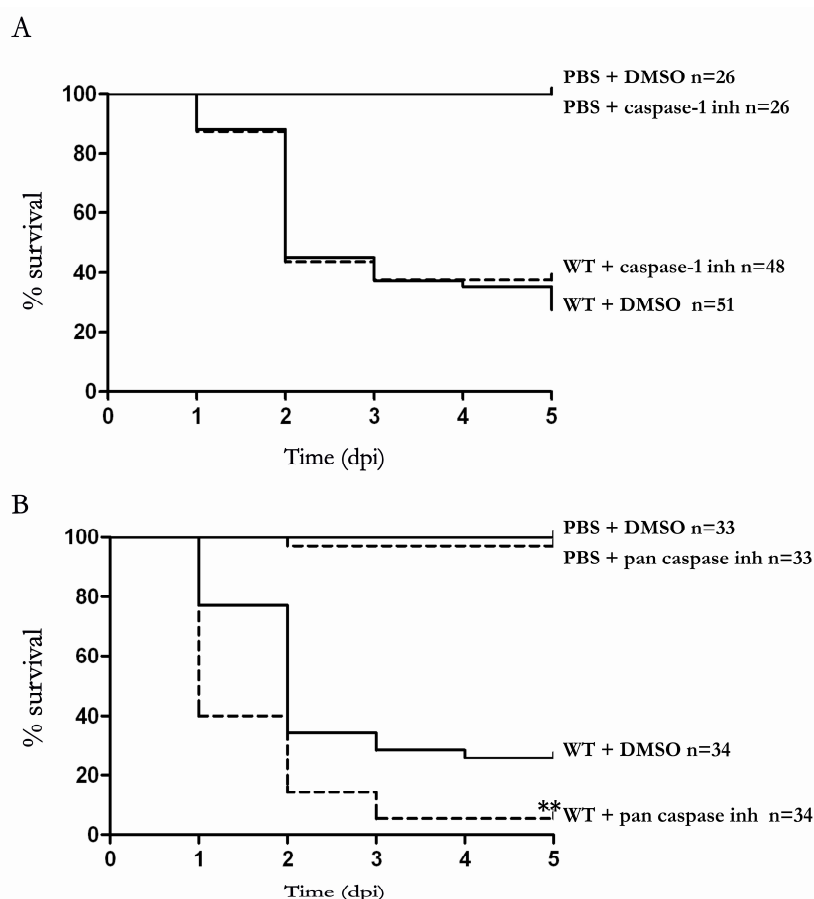


Fig. 4 Survival assay of larvae infected with *S. typhimurium* in the presence of a specific caspase-1 or a general caspase inhibitor. Zebrafish larvae of 48 h were infected with *S. typhimurium* in the Cuvier's duct (MOI=250) in the presence of 100 μ M of the specific caspase-1 inhibitor Ac-YVAD-CMK (A) or 50 μ M of the pan-caspase inhibitor Q-VD-Oph (B). The survival was checked and scored for 5 days. **P<0.01 according to a Log rank test.

The role of PYCARD and caspase-1 in *S. typhimurium* clearance is controversial. An earlier study showed that caspase-1 deficiency confers resistance to oral infection with the *S. typhimurium* (Monack et al., 2000). As this bacterium induces pyroptotic death of macrophages upon infection and because in activated macrophages this rapid death apparently occurs with release of proinflammatory cytokines, it was proposed that caspase-1 was required for *S. typhimurium* to colonize the Peyer's patches and cross the intestinal barrier (Monack et al., 2000). However, other studies have demonstrated that the ability of *S. typhimurium* to kill macrophages in a caspase-1–dependent manner is not required for bacteria to cross the intestinal barrier and become systemic, and that caspase-1 deficiency leads to an increased susceptibility to infection (Lara-Tejero et al., 2006). In addition, neither, NLRP3, PYCARD nor NLRC4 is critical for *S. typhimurium* clearance in mice (Lara-Tejero et al., 2006). Collectively, these results, combined with our data obtained in zebrafish, indicate that *S. typhimurium* possesses different redundant mechanisms to activate caspase-1 through different components of the inflammasome.

One of the most interesting observations of this study is that although caspase-1 seems to be dispensable for *S. typhimurium* clearance in zebrafish, the pan-caspase pharmacological inhibitor results in a higher susceptibility of infected larvae. This result suggests that another caspase is involved in *S. typhimurium* clearance in zebrafish. Caspase-11 has recently found to regulate the activation of the inflammasome in a PYCARD-independent manner (Kayagaki et al. 2011). Interestingly, caspase-11 is required to protect against bacteria that escape the vacuoles and enter into the cytosol and this protection is independent of NLRP3, NLRC4, or ASC (Aachoui et al., 2013). Furthermore, caspase-1-deficient mice are more susceptible to infection with *S. typhimurium* than mice lacking both caspase-1 and caspase-11 (Broz et al., 2012). This phenotype was accompanied by higher bacterial counts, the formation of extracellular bacterial microcolonies in the infected tissue and a defect in neutrophil-mediated clearance (Broz et al., 2012). Therefore, it is tempting to speculate on the existence of a caspase-11 homologue in zebrafish that would be involved in the resistance to *S. typhimurium*. To this end, we have identified a putative caspase-11 homologue in zebrafish (NW_003334571) which upon reciprocal BLASTP analysis against human and mouse protein databases returned caspase-1 (E=1e-60) and caspase-11 (E=1e-58) as

the most significant hits. Gain and loss of function experiments are in progress to ascertain the role of this caspase in zebrafish immune response against *S. typhimurium*.

**Chapter III: Identification and functional
characterization of a new IL-1 family member, IL-
1Fm2, in most evolutionary advanced fish.**

ABSTRACT

The IL-1 family consists of eleven members that play an important role as key mediators in inflammation and immunity. Here, we report the identification of a new member of the IL-1 family (IL-1Fm2) that is exclusively present in species belonging to the most evolutionarily advanced group of teleost fish (Series Percomorpha), including the gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*) and tilapia (*Oreochromis niloticus*) (Perciformes); medaka (*Oryzias latipes*, Beloniformes); stickleback (*Gasterosteus aculeatus*, Gasterosteiformes); platyfish (*Xiphophorus maculatus*, Cyprinodontiformes); and Japanese flounder (*Paralichthys olivaceus*, Pleuronectiformes). However, IL-1Fm2 seems to be absent in pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*, Tetraodontiformes), which also belong to the Percomorpha. The expression pattern of gilthead seabream IL-1Fm2 revealed that although it was hardly induced by pathogen-associated molecular patterns (PAMPs), the combination of PAMPs and IL-1Fm2 synergistically induced its expression in macrophages and granulocytes. In addition, recombinant IL-1Fm2 was able to activate the respiratory burst of seabream phagocytes and to synergistically induce the expression of IL-1 β , TNF α , IL-8 and IL-10 when combined with PAMPs. Finally, although gilthead seabream IL-1Fm2 did not show a conserved caspase-1 processing site, macrophages processed IL-1Fm2 before being released. However, both pan-caspase and caspase-1 inhibitors failed to inhibit its processing and release. These results demonstrate an important role of IL-1Fm2 in the regulation of fish immune responses, shed light into the evolution of the IL-1 family in vertebrates and point to the complexity of this cytokine family.

1. INTRODUCTION

The IL-1 family consists of eleven members with five of them relatively well characterized, namely IL-1 α , IL-1 β , IL-1ra, IL-18 and IL-33 (Dinarello, 2011). They play an important role as key mediators in inflammation, microbial invasion, immunological reactions and tissue injury (Dinarello, 2011). IL-1 β was the earliest cytokine to be discovered and cloned in fish (Zou et al., 1999a; Zou et al., 1999b) and has now been found in many bony fish, including salmonids, cyprinids, gadoids, perciforms and anguilliforms, and cartilaginous fish (Secombes et al., 2011; Bird et al., 2002).

Although fish IL-1 β has some similarities to its mammalian counterparts, there are also differences, the most intriguing being the absence of a conserved caspase-1 processing site in the non-mammalian vertebrate IL-1 β genes (Bird et al., 2002a). In mammals, caspase-1 is activated in a cytosolic molecular platform called the inflammasome (von Moltke et al., 2012; Lamkanfi et al., 2012). Interestingly, while it has recently been found that zebrafish (*Danio rerio*) inflammatory caspase A and caspase B (Vojtech et al., 2012) and European seabass (*Dicentrarchus labrax*) caspase-1 (Reis et al., 2012) can process IL-1 β , caspase-1 is not involved in the processing of gilthead seabream (*Sparus aurata*) IL-1 β (Angosto et al., 2012). In addition, seabream macrophages fail to respond to classical NLRP3 activators, such as ATP, nigericin and alum and monosodium urate crystals (Angosto et al., 2012; Compan et al., 2012; López-Castejón et al., 2007a). Even most puzzling is the observation that invasive *Salmonella typhimurium*, which activates NLRC4 inflammasome in mammals (Kofoed et al., 2011; Zhao et al., 2011), and cell swelling, which activates NLRP3 inflammasome in mammals and fish (Compan et al., 2012), both are able to activate caspase-1 in gilthead seabream but also fail to trigger the processing and secretion of IL-1 β (Angosto et al., 2012; Compan et al., 2012).

In some primitive fish species, such as salmonids and cyprinids, more than one IL-1 β gene has been found (Secombes et al., 2011; Bird et al., 2002a), but this is not common to all species of these phylogenetic groups, since the zebrafish, which is also a cyprinid, has a single IL-1 β gene. However, the finding of several IL-1 β genes in some of the species is not totally unexpected, as they have a tetraploid ancestry and are likely to have multiples of certain genes. In addition, the presence of multiple copies of IL-1

family genes is not restricted to fish because a number of novel homologue members of the IL-1 family have also been found in humans (Dinarello et al., 2011).

Here, we report the identification of a new member of the IL-1 family (IL-1Fm2) that is exclusively present in species belonging to the most evolutionarily advanced group of teleost fish (Series Percomorpha), including the gilthead seabream, European seabass (*Dicentrarchus labrax*) and tilapia (*Oreochromis niloticus*) (Perciformes); medaka (*Oryzias latipes*, Beloniformes); stickleback (*Gasterosteus aculeatus*, Gasterosteiformes); platyfish (*Xiphophorus maculatus*, Cyprinodontiformes); and Japanese flounder (*Paralichthys olivaceus*, Pleuronetiformes). Interestingly, on investigation of the genomes, IL-1Fm2 seems to be absent in pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*, Tetraodontiformes), which also belong to Percomorpha. The expression pattern of gilthead seabream IL-1Fm2 and its biological activity demonstrate its importance in the regulation of the immune response of fish.

2. MATERIALS AND METHODS

2.1 Identification of teleost IL-1Fm2 and sequence analysis

A partial sequence of gilthead seabream IL-1Fm2 was identified by BLAST searching in the EST database of NCBI (accession number **AM957809**). A 3'-RACE strategy using the primers indicated in Table 1 was used to obtain the full coding sequence (Garcia-Castillo et al., 2002), which was deposited in GenBank with accession number **HF936678**. The deduced IL-1Fm2 protein was analyzed for similarity with other known sequences using the BLAST program within the NCBI server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). A direct comparison between two sequences was performed using the ALING program, while multiple sequence alignment was carried out with the CLUSTALW program, from the European Bioinformatics Institute (<http://www.ebi.ac.uk/>). A phylogenetic tree was constructed based on the amino acid sequence alignments using the CLUSTALW program that was displayed using the TreeView X program (Page, 1996). The domains of the protein deduced from the nucleotide sequence were determined by comparison with the PFAM database (Finn et al., 2010). Finally, synteny analysis was performed by searching the available genomes and using GENSCAN (<http://genes.mit.edu/GENSCAN.html>) (Burge and Karlin, 1997) to predict gene sequences within them. The accession numbers of all genes analyzed are indicated in Table 2.

Table 1: Primers used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

Gene	Accession number	Name	Nucleotide sequence(5'→3')	Use
<i>il1fm2</i>	AM957809	F ₁	GAAGGAGGTCAGTCCGAAC	3'RACE
		F ₂	CCATCACCAACAACCTGCAC	
		ORF-F ₂	ATGAGTGCCTTCCATCTG	Cloning
		ORF-R ₂	GTTTAATTTGAACTGGTGAGACGT	
		F	CCATCACCAACAACCTGCAC	RT-PCR
		R	ACGGTCCATGTCGTCATCTT	
<i>il1b</i>	AJ277166	F ₂	GGGCTGAACAACAGCACTCTC	RT-PCR
		R ₃	TTAACACTCTCCACCCTCCA	

<i>tnfa</i>	AJ413189	F ₁	TCGTTTCAGAGTCTCCTGCAG	RT-PCR
		R ₃	CATGGACTCTGAGTAGCGCGA	
<i>il10</i>	FG261948	F	TGGAGGGCTTTCTGTCAGA	RT-PCR
		R	TGCTTCGTAGAAGTCTCGGATGT	
<i>il8</i>	AM765841	F2	GCCACTCTGAAGAGGACAGG	RT-PCR
		R2	TTTGTTGTCTTTGGTCGAA	
<i>rps18</i>	AM90061	F	AGGGTGTGGCAGACGTTAC	RT-PCR
		R	CTTCTGCCTGTTGAGGAACC	

Table 2: European Nucleotide Archive (ENA) accession numbers for the genes used. The genes identified in this study are in red font.

Species	Common Name	Gene	ENA Accession number
<i>Homo sapiens</i>	Human	IL-1 β	NM_000576
<i>Gallus gallus</i>	Chicken	IL-1 β	NM_204524
<i>Xenopus laevis</i>	African clawed frog	IL-1 β	NM_001085605
<i>Melanogrammus aeglefinus</i>	Haddock	IL-1 β	AJ550166
<i>Gadus morhua</i>	Atlantic cod	IL-1 β	AJ535730
<i>Psetta maxima</i>	Turbot	IL-1 β	AJ295836
<i>Salmo salar</i>	Atlantic salmon	IL-1 β IL-1 β 2 IL-1 β 3 IL-1 β 4	AY617117 AGKD01000000 HE817773 HE817774
<i>Oncorhynchus mykiss</i>	Rainbow trout	IL-1 β IL-1 β 2 IL-1 β 3 nIL-1Fm	NM_001124347 AJ245925 AJ557021 AJ555869
<i>Oplegnathus fasciatus</i>	Rock bream	IL-1 β	FJ155360
<i>Salvelinus alpinus</i>	Arctic char	IL-1 β	AM084227
<i>Latris lineata</i>	Striped trumpeter	IL-1 β	FJ532282
<i>Dicentrarchus labrax</i>	European seabass	IL-1 β IL-1Fm2 nIL-1Fm	AJ269472 HF936682 AM984981
<i>Siniperca chuatsi</i>	Mandarin fish	IL-1 β	AY647430
<i>Paralichthys olivaceus</i>	Japanese flounder	IL-1 β nIL-1Fm IL-1Fm2	AB070835 AB720987 AB720985
<i>Hippoglossus hippoglossus</i>	Atlantic halibut	IL-1 β	FJ769829
<i>Rachycentron canadum</i>	Cobia	IL-1 β	AY641829
<i>Pagrus major</i>	Red seabream	IL-1 β	AY257219
<i>Tetraodon nigroviridis</i>	Spotted green pufferfish	IL-1 β nIL-1Fm	AJ574910 CAF99823
<i>Cynoglossus semilaevis</i>	Half-smooth tongue sole	IL-1 β	FJ816103

<i>Sparus aurata</i>	Gilthead seabream	IL-1 β IL-1Fm2	AJ277166 HF936678
<i>Chionodraco hamatus</i>	Crocodile icefish	IL-1 β	AJ566198
<i>Lateolabrax japonicus</i>	Japanese sea perch	IL-1 β	EF513753
<i>Acanthopagrus latus</i>	Yellowfin seabream	IL-1 β	AY669059
<i>Dentex dentex</i>	Common dentex	IL-1 β	AJ459239
<i>Diplodus puntazzo</i>	Sharpsnout seabream	IL-1 β	AJ459238
<i>Ictalurus punctatus</i>	Channel catfish	IL-1 β IL-1 β 2	ABB46471 ABB46472
<i>Conger myriaster</i>	Whitespotted conger	IL-1 β	AB291072
<i>Cyprinus carpio</i>	Carp	IL-1 β IL-1 β 2-1 IL-1 β 2-2	AB010701 AJ401030 AJ401031
<i>Carassius auratus</i>	Goldfish	IL-1 β 1 IL-1 β 2	AJ419848 AJ419849
<i>Danio rerio</i>	Zebrafish	IL-1 β nIL-1Fm	FM210810 XP_685720
<i>Salmo trutta fario</i>	Brown trout	IL-1 β	AY853170
<i>Oryzias latipes</i>	Medaka	nIL-1Fm IL-1Fm2	XM_004084753 XM_004074606
<i>Scyliorhinus canicula</i>	Smaller spotted catshark	IL-1 β	AJ295947
<i>Triakis scyllium</i>	Banded houndshark	IL-1 β	AB074142
<i>Callorhinchus milii</i>	Elephant shark	IL-1 β	JK859830
<i>Gasterosteus aculeatus</i>	Stickleback	IL-1 β nIL-1Fm IL-1Fm2	HF936681 HF936680 HF936679
<i>Oreochromis niloticus</i>	Tilapia	IL-1 β nIL-1Fm IL-1Fm2 IL-1 β a IL-1 β b	XM_003460625 XM_003445804 XM_003452995 GR616979 FF279762
<i>Takifugu rubripes</i>	Fugu	IL-1 β nIL-1Fm	AB704199 HF936683
<i>Xiphophorus maculatus</i>	Platyfish	IL-1 β nIL-1Fm IL-1Fm2	JH557117.1 JH556701.1 JH557184

2.2 Animals

Healthy specimens (300 g mean weight) of the hermaphroditic proterandrous marine fish gilthead seabream were kept at the Spanish Oceanographic Institute (Mazarrón, Murcia) in 14 m³ running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/hour) with natural temperature and photoperiod, and fed

twice a day with a commercial pellet diet (Skretting). Fish were fasted for 24 hours before sampling. The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and the Bioethical Committee of the University of Murcia (approval nº #537/2011) for the use of laboratory animals.

2.3 Production of recombinant seabream IL-1Fm2

Recombinant seabream IL-1Fm2 was produced in *Escherichia coli*. Briefly, the open reading frame of IL-1Fm2 encoding the putative mature protein (residues 118-270) was synthesized, cloned in vector E3, produced as an N-terminal 6xHis fusion protein in *E. coli*, obtained from inclusion bodies with 6 M guanidine hydrochloride and purified by Ni-HiTrap column (GenScript Corporation).

2.4 Western blot

Cells were lysed at 4°C in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, and a 1:20 dilution of the protease inhibitor cocktail P8340 from Sigma-Aldrich). The protein concentrations of cell lysates were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard. Cell extracts (40 µg protein) and supernatants (clarified with a 0.45 µm filter and concentrated by precipitation with 20% trichloroacetic acid, Sigma-Aldrich) were analyzed on 15% SDS-PAGE and transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad). The blots were developed with a rabbit monospecific antibody to seabream IL-1β (1:5000) (Mulero et al., 2008) or a purified mouse mAb generated against recombinant sbIL-1Fm2 (6D1E4, 1:5000, GenScript Corporation), and enhanced chemiluminescence (ECL) reagents (GE Healthcare) according to the manufacturer's protocol. Membranes were then reprobed with a 1:5000 dilution of an affinity purified rabbit polyclonal to histone H3 (#ab1791, Abcam).

2.5 Experimental infections

Fish were injected intraperitoneally with 1 ml of phosphate-buffered saline (PBS) alone or containing a sublethal dose (10^8) of exponentially growing *V. anguillarum* R82 cells (serogroup 01) (Chaves-Pozo et al., 2004). Head kidney, spleen, thymus, liver, peritoneal exudate cells, gills and blood were obtained 4 h after bacterial challenge and processed for subsequent quantitative PCR (qPCR) (see below).

2.6 Cell culture and treatments

Seabream head-kidney leukocytes (bone marrow equivalent of fish) obtained and cultured as described elsewhere (Sepulcre et al., 2002) were stimulated for 4 h with 50 µg/ml phenol-extracted genomic DNA from *V. anguillarum* ATCC19264 cells (VaDNA) (Sepulcre et al., 2007). Some experiments were conducted using purified acidophilic granulocytes obtained by MACS (22) and macrophages monolayers (Roca et al., 2006). Macrophages and acidophilic granulocytes were stimulated for 0.5 to 24 h at 23°C with 50 µg/ml VaDNA, 1 ng/ml flagellin (Invivogen), 25 µg/ml poly I:C (Invivogen), 10 µg/ml lipopolysaccharide from *Escherichia coli* (EcLPS, Sigma), 10 µg/ml *Porphyromonas gingivalis* LPS (PgLPS, Invivogen), 10 µg/ml muramyl dipeptide (MDP, Sigma) (Sepulcre et al., 2007; Sepulcre et al 2009).

To examine the effect of recombinant seabream IL-1Fm2 on the macrophages and acidophilic granulocytes, they were stimulated for 3 (macrophages) or 24 h (acidophilic granulocytes) at 23°C with 50 µg/ml VaDNA and 10 ng/ml recombinant seabream IL-1Fm2. cDNA was produced from the cells and expression of IL-1β, IL-1Fm2, TNFα, IL-8 and IL-10 was then analyzed using qPCR. Protein levels of IL-1Fm2 were also measured within macrophage extracts and supernatants, using the antibody. In some experiments, macrophages were pretreated for 1h with the pan-caspase inhibitor Q-VD-OPh (50 µM; Sigma-Aldrich) or the specific caspase-1 inhibitor Ac-YVAD-CMK (100 µM, Peptanova) (Angosto et al., 2012; López-Castejón et al., 2008).

2.7 Respiratory burst assays

Respiratory burst activity was measured as the luminol-dependent chemiluminescence produced by acidophilic granulocytes after different stimulation times (Sepulcre et al., 2007). This was brought about by adding 100 µM luminol and 1 µg/ml PMA (both from Sigma-Aldrich), while the chemiluminescence was recorded every 117 seconds for 1 h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of quadruple readings, expressed as the slope of the reaction curve from 117 to 1170 seconds, from which the apparatus background was subtracted.

2.8 Cell viability

Aliquots of cell suspensions were diluted in 200 μ l PBS containing 40 μ g/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from triplicate samples was analyzed by using flow cytometry (BD Biosciences).

2.9 RT-qPCR

Total RNA was extracted from tissues (head kidney, spleen, thymus, liver, peritoneal exudate cells, gills, blood, brain, gut and skin) with TRIzol Reagent (Life Technologies) following the manufacturer's instructions and treated with DNase I, Amplification grade (1 unit/ μ g RNA, Invitrogen). The SuperScript III RNase H⁻ Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT₁₈ primer from 1 μ g of total RNA at 50 °C for 50 min. qPCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min 60°C and 15 s at 95°C. For each mRNA, the gene expression was normalized to the ribosomal proteins S18 content in each sample using the Pfaffl method (Pfaffl et al., 2001). The primers used are shown in table 1. In all cases, each PCR was performed with triplicate samples and repeated with at least two independent samples.

2.10 Statistical analysis

Data were analyzed by analysis of a Tukey multiple range test to determine differences between groups using GraphPad Prism 5.01.

3. RESULTS

3.1 Molecular characteristics of seabream IL-1Fm2

The IL-1Fm2 cDNA consisted of 1,383 nucleotides including a 813 bp single open reading frame (ORF), with a 77 bp 5' untranslated region (5'UTR) and a 493 bp 3'UTR containing a poly(A) tail and a putative polyadenylation signal AATAAA located 11 nucleotides upstream of the poly(A) tail (Fig. 1). The translation of the ORF predicted a 270 amino acid polypeptide (calculated molecular mass: 30.6 kDa) that lacked a signal peptide and a conserved caspase-1 cut site (Figs. 1, 2). A potential glycosylation site (NXT) was predicted at residues 106-108 (Fig. 1).

A BLASTP search vs. non-redundant protein sequence databases retrieved as most significant hits several teleost genes annotated as IL-1 β -like (E-value < 10^{-64} , 45-48 % amino acid identity) followed by teleost IL-1 β genes (E-value < 10^{-43} , 32-39 % amino acid identity) and mammalian IL-1 β genes (E-value < 10^{-15} , 27-31 % amino acid identity). Notably, seabream IL-1Fm2 showed 26.4 % and 47.1 % amino acid identity and similarity, respectively, with seabream IL-1 β . In addition, analysis of the domains with PFAM showed that the seabream IL-1Fm2 had an IL-1 propeptide domain (**PF02394**) from residues 6 to 100 and an IL-1 domain (**PF00340**) between positions 149-269. A multiple alignment generated by CLUSTALW showed the highest amino acid conservation in the predicted β -strands regions (Fig. 2). In addition, the IL-1 family signature, [FC]-x-S-[ASLV]-x(2)-P-x(2)-[FYLV]-[LI]-[SCA]-T-x(7)-[LIVM] (**PDOC00226**), was also well conserved (Figs. 1 and 2). These results taken together confirm that gilthead seabream IL-1Fm2 belongs to the IL-1 family.

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-77
acaacagtcgagtggaagcgctcagagagaccggcagggagaacaatctttcaaattgaaaca
-15                                     45
gtttacatcgagaca atgagtgcccttccatctgtctgacgcttttagacagcccaactgaa
                      M S A F H L S D A L D S P T E 105
gtagatgaagaagaatttgaaacccgctgcctcagcttgacgcactgtgacatgcaggat
V D E E E F E T R C L S L T H C D M Q D 165
gttcatgataagaccttcagacttgaggacggactggacctgggtgggttaccacacaaccg
V H D K T F R L E D G L D L V V T H N P 225
atgagcttgacgtgtgtagttaacctgatgctggcgggtgaaccggctcaagaagtcctt
M S L Q C V V N L M L A V N R L K K S L 285
cctcgctgtggcaaggggctcagcgatgatgagctctgctgctgatcctggacagcctg
P R C G K G L S D D E L C C V I L D S L 345
gtggaagattccattgtgaagacgagtgagaacttcaccgtgggagagaaaaggagtatg
V E D S I V K T S E N F T V G E K R S M 405
ttcaaacgttttggcagcgtgaatctgtgcactctgtgtgacacttcaaagaaagacgtc
F K R F G S V N L C T L C D T S K K D V 465
atctgcgtatcagaggaaatgaaactgcaggccatcacgctgaaaggaggtcactgcgaa
I C V S E E M K L Q A I T L K G G H C E 525
cgcaaagtgaatttttagactgtccaagtacatcgacacctgtcagtcggagggtcagccg
R K V N F R L S K Y I D T C Q S E G Q P 585
gtcgttctgtccatcaccaacaacttgacatttctctgctgatgaaagacggccgacct
V V L S I T N N L H I S C S M K D G R P 645
gtgttgaaatctggaggaatgcagtgaggcgaaattacaaacaatcaacaaagatgacgac
V L N L E E C S E A K L Q T I N K D D D 705
atggaccgttttcttctacaggacagacaaggtctctctcacatcacctttgagtcg
M D R F L F Y R T D K G L S H I T F E S 765
gttaaataatcgcggtgtgttcacgacactgtgaggggtgaagagcagcctgtggagatg
V K Y R G W F I S T C E G E E Q P V E M+12
tgtgaagcggacgccaccagacgtctcaccagtttcaaattaaac taaaaacgttttgta
C E A D A T R R L T S F K L N -813
ttcagattgaatgtcaccacaatgtgtgtaacttaagccttttatattatgttatgatgct
+132
tttttttttattttcatctatcattgaatcacttttctctgttttttaactcataagctgt
+192
tttaaaaagtgaatttctccagtggtgatgcaattttattctgcatcaccactagatggcg
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+312
cttgtttaactttatataatttctactcactgctaaaacatagtttttttctttggcag
+372
agaaagtcgtca cttgatattactccaaactactgattaaattattttcacctattcggt
+432
attgtaaatacaattcaattcatttctctactgtctgt ttttacaagtgagtttttgtgt
+493
gttctgttttgttgca atggaaa aataaaa cttttaaaaaaaaaaaaaaaaaaaaaaa aaaaa

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Fig. 1 Molecular characteristics of gilthead seabream IL-1Fm2. Full-length cDNA sequence and deduced amino acid sequence of gilthead seabream IL-1Fm2. The start and stop codons, the polyadenylation signal (AATAAA) in the 3' UTR, and a potential glycosylation site (NFT) are highlighted in grey. The IL-1 family signature [FC]-x-S-[ASLV]-x(2)-P-x(2)-[FYLV]-[LI]-[SCA]-T-x(7)-[LIVM] (**PDOC00226**), is underlined. ENA Accession Number is **HF936678**.

H.Sapiens	IL-1β	-----	MAEVPKLAASEMAYYSNEDDLFF	EADGPKQMKCSFDQ	-----	LDLCPDLGG	47																		
G.gallus	IL-1β	-----	MAFVPDLVLSSSL	--SEETFY	----GPSCLC	--L----	QKKPRLDSEHTVD	42																	
X.laevis	IL-1β	-----	MALVPDLSSIPMEGYSGDDMF	--YSDSPSGMKDDMGDAQWQSSTHCSLDIH			52																		
O.mykiss	IL-1β	-----	MDFESNYSLIK	-----	NT---	SESAAWSSKL	-----	PQG	26																
S.aurata	IL-1β	-----	MESEM	-----	TC---	NVREMWSSKM	-----	PEG	20																
C.carpio	IL-1β	-----	MACHEYVHQLDLSEAFETDSAIYSDSADSDELCDPDPQ	---	SMS	-----	CQCDM	-----	H-D	48															
D.rerio	IL-1β	-----	MAGCQYEVTTIAPKNLWETDSAVYS	---	SDEMDCSDPL	---	AMVGY	--FTF	-----	YKCSYRCDM	HEG	55													
S.aurata	IL-1Fm2	-----	MSAFHLSDALDSPTEV	---	DEEEF	---	ETRCLSLTHCDMDQDVHDKTFRL	-----	EDG	46															
O.niloticus	IL-1Fm2	-----	MCDIDLSQLDCSP	---	EEA	---	IKSTCFDMKEVESEIFRL	-----	NEG	38															
P.olivaceus	IL-1Fm2	-----	MSDFDLSQLAESPLES	---	EEKGF	---	KSFCFDKTDVPDEVINL	-----	DTE	41															
O.latipes	IL-1Fm2	MMTTRMALQVFSICHFLLLFLNVCG	----	RMSLNDFDLSQALDSEPE	-----	DSFCFMDADEKEKFEL	-----	EDN	63																
												↓													
H.Sapiens	IL-1β	IQLRISDHHSYKGRQAASVVVAMDKLRKM	LV--PCPQTFQENDLSTFFPFIFEEPIFFDTWDNEAYVHD	----	APV	<u>RSLNCTLRD</u>	SSQ	131																	
G.gallus	IL-1β	VQVTVRKGGRARSFRAAIVLVAMTKLLR	--R--PSRDFADSDLSALLEEVE	--PVTFQRLE	--SSYAGAPAF	--RYTR	SQSFDIFD	INQ	124																
X.laevis	IL-1β	VQITHGK--GSLHSFRKAVLVVAVEKLR	-----	GKERFFGDEDLGLLDSIFVEEEIGFSQAK	--ETYASASTY	--RYQRATTCTRIKD	TSN	134																	
O.mykiss	IL-1β	LDLEV--SHHPITMRHIANLIAMERLKGG	E-GVTMGTEFKDKDLNLFLESAAVEEHIVLESAAPPASRRRAAGFSST			SQYECSTVD	SEN	113																	
S.aurata	IL-1β	LGLEI--AHHPITMKSVTVIAMIERLKG	VLDSPRGTEFKDENLNLILESAAVEERTVFERTAKPAQ	-----	YTYN	FSQSLYSVMD	SEQ	102																	
C.carpio	IL-1β	IKLEI--SSPHSMRQVNNIIIAVERLKHI	KN--MSSGKPCDEELGFILENVEERLVKPLNETPIY	-----	SKTS	LTQLCTICD	KYK	128																	
D.rerio	IL-1β	IRLEM--WTSQHKMKQLVNVIIALNRMKHI	KP--QS--TEFGEKEVLDMANVIAQEREVNVVDSVPSY	-----	NKTK	NVLQCTICD	QYK	134																	
S.aurata	IL-1Fm2	LDLVV--THNPMSLQCVNMLAVNRLKKS	LP--RCGKGLSDDELCCVILDSLVEDSVIKTSENFTVGEKRSMEKRFK			SVNLCTLD	TSK	132																	
O.niloticus	IL-1Fm2	LELMV--SHNRRTMQSVALLVLGNRMKKP	LT--QSDRKLSDDDVMNII	--DSVVKETV	----	WPPTKGTKRDFLRAN	SEKALITCD	NSQ	119																
P.olivaceus	IL-1Fm2	LDLRI--SRNPGSMKGAATLLLANRMKNV	LS--Q--	KGQSDSECRMLMSVIETITVKTFFENNSIGERRLDFRRLS		S--WECSLTD	QNN	124																	
O.latipes	IL-1Fm2	LDLIV--SYNRRTLKMMVNLMTINKMQMP	PS--EGSSDLQGDGICSTMVHEHVKETVVCNDENVSSGERRVSFTRTG			SRTEFKLSD	MSQ	149																	
								:	:	:	:														
												:	:	:	:	:									
H.Sapiens	IL-1β	<u>KSLVMS</u>	G-----	<u>PYELKALHL</u>	QGQDMEQQ	<u>VVFSMSF</u>	V--QGE	---	ESND	<u>KIPVALGLK</u>	<u>EK</u>	<u>NLYLSCVLK</u>	D-DK	----	<u>PTLQLESVD</u>	<u>PKN</u>	--	205							
G.gallus	IL-1β	KCFVLE	S-----	PTQLVALHL	QGPSSSQK	VRNLNIAL	YRPRGPRGSAGT			QMPVALGK	GYKLYMSCVMS	G-TE	---	PTLQLEAD	VMR	--	203								
X.laevis	IL-1β	KCFVMQ	K--FHENAQLVALQL	QGANIQR	EKVSMAF	YATQPH	---	QGGSKRPVALGLA	GK	NLYLSCRAT	E-DGQDS	PKLYLEES	NIK	--	216										
O.mykiss	IL-1β	KCWVLM	-----	NEAMELHAMML	QGGSSYHK	VHLNLS	YVTPVP	--I	ETEARPVALGIK	GS	NLYLSCSKS	G-GR	----	PTLHLEEA	DKDQL	192									
S.aurata	IL-1β	RHLVRV	----	PNSMELHAVML	QGGTGNCQ	VQLNMTAT	YLPPTP	--S	A-EAVTVTLCK	DT	NLYLSCHKE	G-DD	----	PSLHLEAVD	DKDSL	180									
C.carpio	IL-1β	KTMVQS	NKLSDEP	LHLKAVTL	SAGAMQYK	VQFSMT	FVSS	-A--T	QKEAPVCLGIS	NS	NLYLACTQL	DGSS	----	PVLILKEAS	GSVNT	211									
D.rerio	IL-1β	KSLVLS	----	GGSPHLQAVTL	RAGSSDLK	VRFSMT	YASPSA	--P	ATSAQPVCLGIS	KS	NLYLACSPA	EGSA	----	PHLVLEIS	GSLET	214									
S.aurata	IL-1Fm2	KDLIV	-L----	SEEMKLQAITL	KGHCERK	VNFKMSR	YIDTQC	--S	EGQPVVLS	-I	TNNLHISCSMK	D-GR	----	PVLNLEES	E-AKL	207									
O.niloticus	IL-1Fm2	KDLV	-L----	SGELQQAITL	QGGNCHRK	VNFKMSR	YIPVSP	--PGDS	PGLIVVLS	-I	NNNLYMSCRME	A-DK	----	VVLFLEQYH	D----	193									
P.olivaceus	IL-1Fm2	KGIICK	----	SKDLKLLAITL	TAADYIHK	VKFKMGT	YGSP	----	GIGQTVVLSII	NNHLYISCTMN	G-DI	----	AEKLEES	S-AEDL	198										
O.latipes	IL-1Fm2	RHIVHF	----	SEMKLKAITL	KAGYCHLK	VNFTMSR	YNPTSC	--S	NNSGMTVLLS	-I	TKNLHLSCTKT	D-KE	----	VILNLERRR	P-RAA	226									
								:	:	:	:	:	:	:	:	:									
																		:	:	:	:	:	:	:	:
H.Sapiens	IL-1β	--	<u>IPKKKMEKREVFENKI</u>	----	<u>EINN</u>	----	<u>KLEFESAQFPNWFYISTS</u>	<u>Q--</u>	<u>AENMPVFLG</u>	<u>GT</u>	<u>KGGQD</u>	<u>I-TDFTMQFVS</u>	<u>S-----</u>	269											
G.gallus	IL-1β	--	DIUSVELTRFIFYRLDSP	--	TEG	----	<u>TTRFESAAPFGWFICTSL</u>	<u>L--</u>	<u>QPRQPVGI</u>	<u>TNQPDQVN</u>	<u>I-ATYKLSGR</u>	-----	267												
X.laevis	IL-1β	--	DVKGEDLNRFIFMKSQDGLNE	TS	----	<u>TNSFESVAFPGWFYISTS</u>	<u>Q--</u>	<u>RENELV</u>	<u>QM</u>	<u>VHQKNQEA</u>	<u>I-KDFNLFSVI</u>	-----	283												
O.mykiss	IL-1β	KSIS	QQSDMVRFIFYRRNTGVDI	ST	----	<u>LESASFNRWFISTD</u>	<u>MOQDY</u>	<u>TKPV</u>	<u>DMC</u>	<u>QKAAPNR</u>	<u>L-TFTFIQRHN</u>	-----	260												
S.aurata	IL-1β	LRIT	PGSDMARFLFYKHVTGLNN	ST	----	<u>LVSVFPSNWFYISTA</u>	<u>E--</u>	<u>ENNKPV</u>	<u>DMC</u>	<u>QESARRH</u>	<u>RIFKFLPPKE</u>	<u>VEGEC</u>	253												
C.carpio	IL-1β	IKAGDPN	--	DSLFLFRKETGTRY	NT	----	<u>FESVYKPGWFISTA</u>	<u>F--</u>	<u>DDNEKV</u>	<u>EMN</u>	<u>QMPTTTR</u>	<u>T--</u>	<u>NFTLEDQKRI</u>	----	276										
D.rerio	IL-1β	IKAGDPN	GYDQLLFRKETGSSI	NT	----	<u>FESVKCPGWFISTA</u>	<u>Y--</u>	<u>EDSQV</u>	<u>EM</u>	<u>DRKDKTERI</u>	<u>I--</u>	<u>NFELQDKVRI</u>	----	281											
S.aurata	IL-1Fm2	QTINK	DDMDRFLFYRTDKGLSH	IT	----	<u>FESVKYRGWFISTC</u>	<u>-EG</u>	<u>EEQPV</u>	<u>EM</u>	<u>CEADATRR</u>	<u>L-TSFKLN</u>	-----	270												
O.niloticus	IL-1Fm2	-DILT	DRMERFLFYRKTGTISL	TT	----	<u>FESVKFRGWYISTS</u>	<u>-QQ</u>	<u>ENQPV</u>	<u>EL</u>	<u>CKVDAASRL</u>	<u>L-TNFRVN</u>	-----	255												
P.olivaceus	IL-1Fm2	KVIC	SDGTNDRFLFLRETGVNV	KT	----	<u>FESVKCRGWYISTS</u>	<u>YEK</u>	<u>EEKPV</u>	<u>EM</u>	<u>CKVDSVSR</u>	<u>V-FSFKTS</u>	-----	262												
O.latipes	IL-1Fm2	ADSSSRNAASAEASRIKIAGVPH		QHSPA	QCI	<u>YPEKSERGSRITPLPPR</u>	<u>TNQTE</u>	----					280												

Fig. 2 Molecular characteristics of teleost IL-1Fm2 proteins. Multiple alignment of the teleost IL-1Fm2 proteins with known vertebrate IL-1β proteins. Identical (*) and similar (., or :) residues identified in all the proteins are indicated. The IL-1 family signature [FC]-x-S-[ASLV]-x(2)-P-x(2)-[FYLV]-[LI]-[SCA]-T-x(7)-[LIVM] (**PDOC00226**), is underlined. The head shows the caspase-1 cut site of human IL-1β sequence. The residues that form the secondary structure of 12 β-sheets in human IL-1β are highlighted in grey. The accession numbers are indicated in Table 2.

3.2 IL-1Fm2 is exclusively present in most evolutionary advanced teleost fish

Using the gilthead seabream IL-1Fm2 sequence as query in TBLASTN search of different fish genomes, we identified a IL-1Fm2 orthologue only in several teleost species belonging to the Percomorpha Series, including the Orders Perciformes [*D. labrax* ([HF936682](#)) and *O. niloticus* ([XM 003452995](#))], Beloniformes (*O. latipes*, [XM 004074606](#)), Gasterosteiformes (*G. aculeatus*, [HF936679](#)), Cyprinodontiformes (*X. maculatus*, [JH557184](#)), and Pleuronectiformes (*P. olivaceus*, [AB720985](#)). However, IL-1Fm2 seemed to be absent in Tetraodontiformes (*T. rubripes* and *T. nigroviridis*), which also belong to the Percomorpha. All IL-1Fm2 sequences formed a separate clade from teleost IL-1 β and nIL-1Fm genes (Fig. 3). Notably, the IL-1Fm2 from *P. olivaceus* has very recently been identified and named novel IL-1 β -like1 (nIL-1 β -like1) (Taechavasonyoo et al., 2013). Collectively, these results further support that IL-1Fm2 is a new member of the IL-1 family.

Analysis of the *O. latipes*, *X. maculatus*, *O. niloticus* and *G. aculeatus* genomes revealed the IL-1Fm2 locus in chromosome 12, and scaffolds JH557184, GL831259 and 293, respectively. We also performed synteny analysis and found that the IL-1Fm2 locus of the four species showed in common most of the genes, including CNMM3, NEU3, CNNM4, SEC14L2, UBE2L3, CKMT2, RASGRF2, PURB, HESX1 and POLM (Fig. 4). Importantly, the genes CNMM4 and PURB were syntenically conserved in the teleost IL-1 β and IL-1Fm2 loci. These results suggest that IL-1 β and IL-1Fm2 probably arose from the fish-specific genome duplication (FSGD) (Meyer et al., 2005).

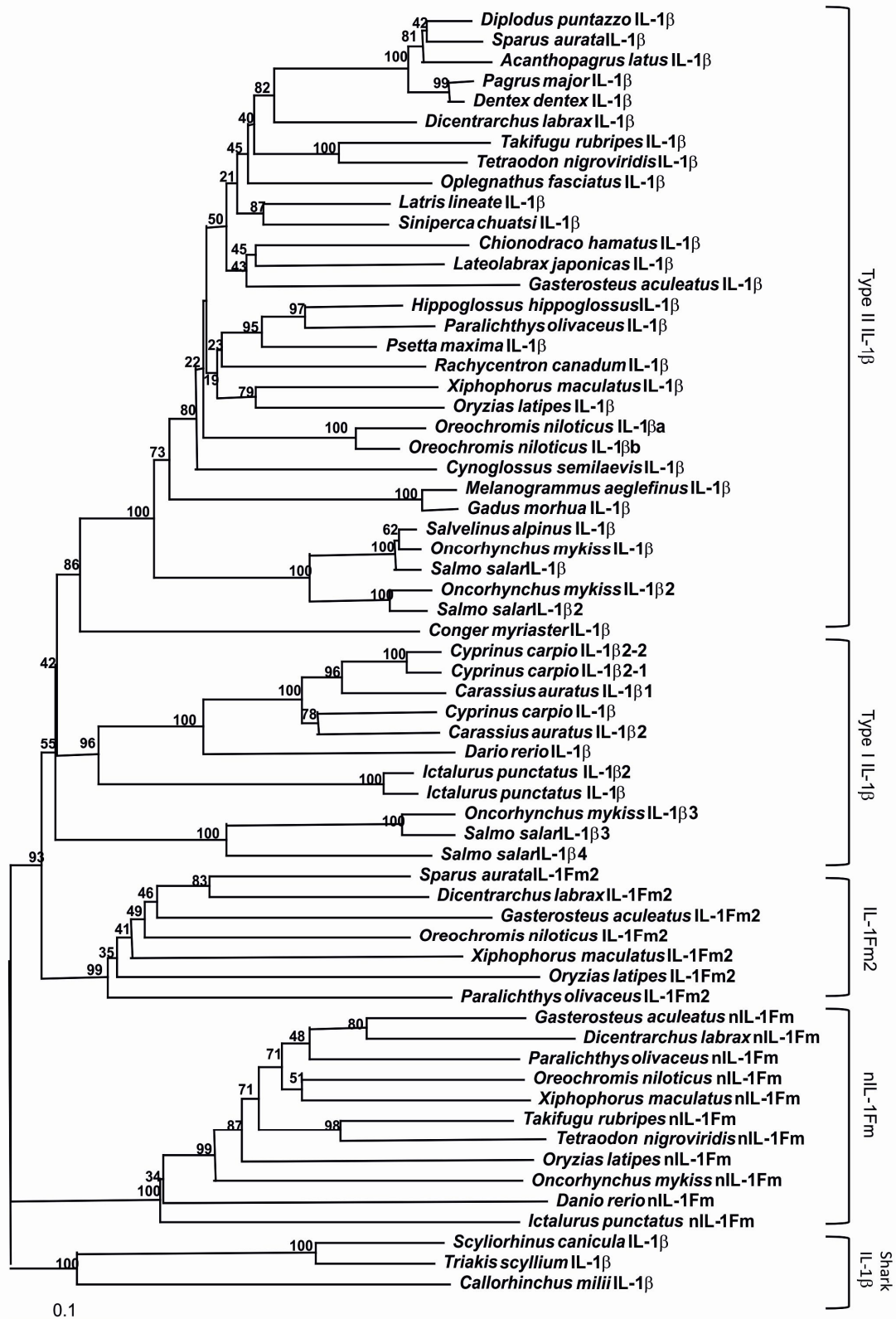


Fig. 3 Phylogenetic relationships of teleost IL-1Fm2. Phylogenetic tree of fish IL-1 family members. The tree was generated by the cluster algorithm using amino acid sequences. Numbers shown are percentages of 100 bootstrap replicates in which the same internal branch was observed. The horizontal lines are drawn proportional to the inferred phylogenetic distances. Vertical lines have no significance. The accession numbers are indicated in Table 2.

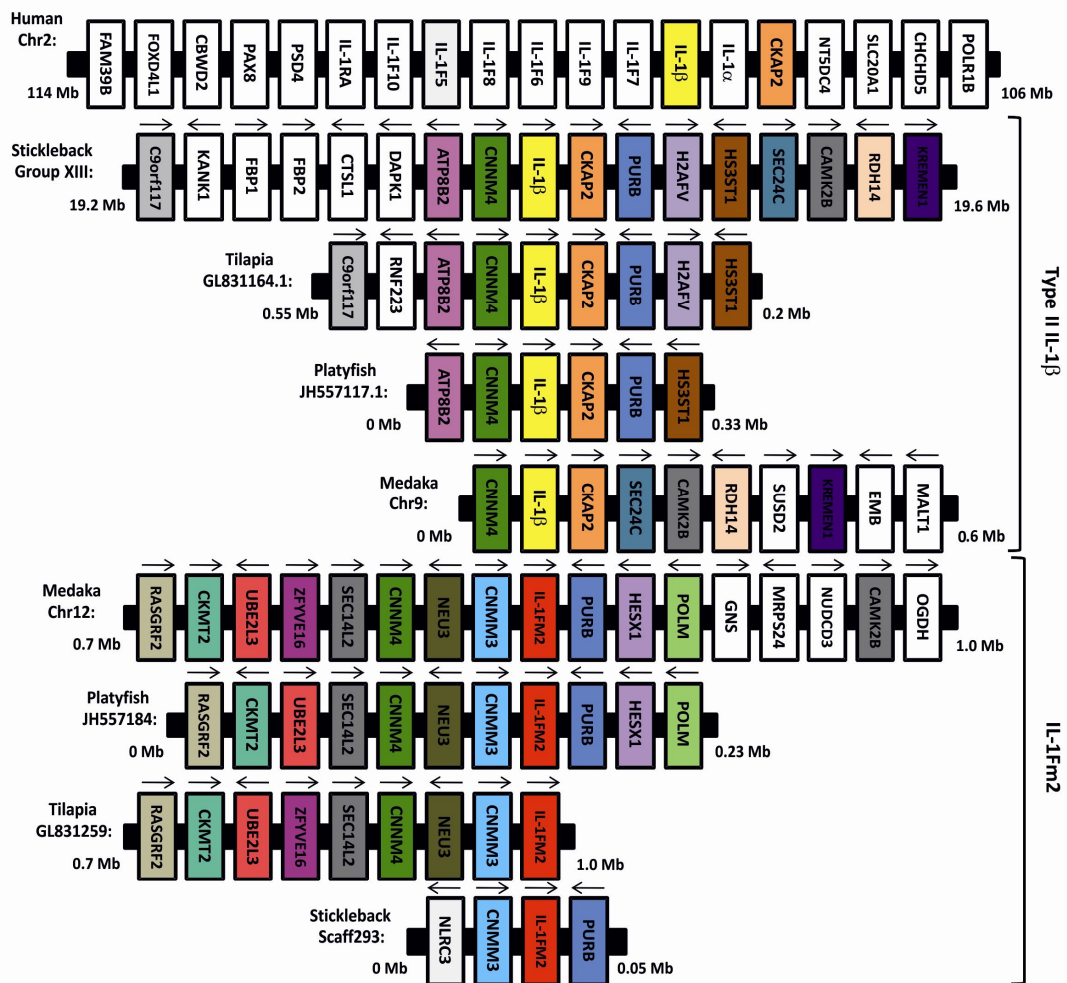


Fig. 4 Synteny analysis of teleost type II IL-1 β and IL-1Fm2 loci. Schematic diagrams showing the syntenically conserved genes in the type II IL-1 β and IL-1Fm2 loci in stickleback (*G. aculeatus*), tilapia (*O. niloticus*), platyfish (*X. maculatus*) and medaka (*O. latipes*). The human IL-1 β locus is also shown for comparison. Conserved genes are indicated in different colors. The direction of gene transcription is indicated with arrows.

3.3 IL-1Fm2 is ubiquitously expressed in *S. aurata* and weakly induced after infection

Although seabream IL-1Fm2 was constitutively expressed in all tissues examined, the lower expression was found in the main organs involved in the immune response, namely the spleen, head-kidney and blood as well as the skin and peritoneal exudate (Fig. 5A). Conversely, the highest expression was found in intestine, liver, gill, brain and thymus. We next examined the *in vivo* expression of IL-1Fm2 during a bacterial infection and found that the mRNA levels of IL-1Fm2 increased slightly in the tissues showing the lowest basal expression, namely head-kidney, spleen, peritoneal exudate and blood, but decreased within the liver, gills and thymus (Fig. 5B).

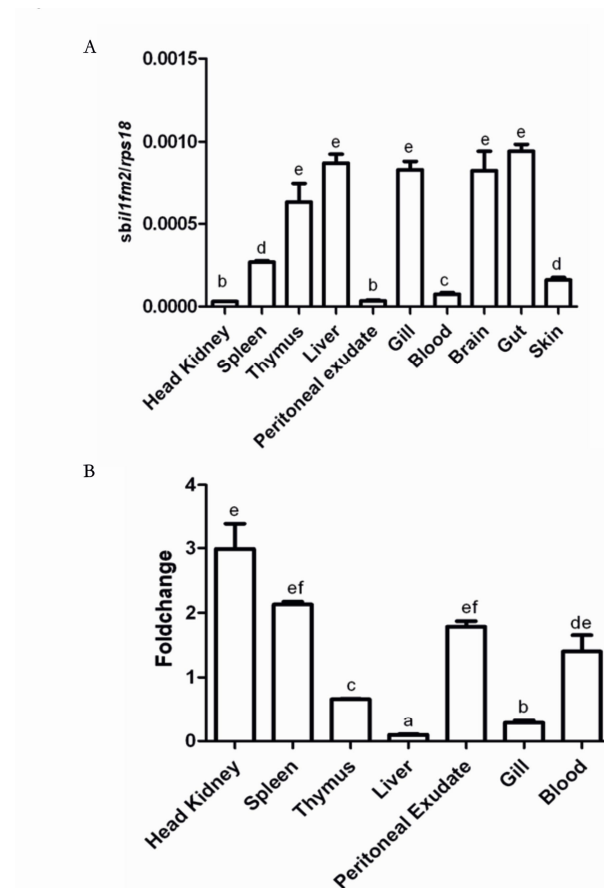


Fig. 5 IL-1Fm2 is ubiquitously expressed in gilthead seabream and weakly induced after infection. The mRNA levels of IL-1Fm2 were determined by RT-qPCR in the indicated immune tissues of control adult specimens (A) and at 4 h after challenge with 10^8 cells of *V. anguillarum* (B). Gene expression is normalized against ribosomal protein S18 (A, B) and is shown as relative to the mean of uninfected fish (B). Each bar represents the mean $\pm$ SEM of triplicate biological samples. Different letters denote statistically significant differences between the groups according to a Tukey test. The groups marked with “a” did not show statistically significant differences from non-infected fish.

3.4 IL-1Fm2 is weakly induced by *S. aurata* phagocytes after PAMP-activation

The two professional phagocytic cell types of the gilthead seabream were purified and stimulated with optimal concentrations of different PAMPs (Sepulcre et al., 2007). It was found that none of the PAMP used, with the exception of bacterial DNA and *E. coli* LPS, was able to increase the mRNA levels of IL-1Fm2 in acidophilic granulocytes (Fig. 6A) and macrophages (Fig. 6B). These results contrast the drastic induction (>1000 fold) of IL-1 β in the same samples (Sepulcre et al., 2007), suggesting that the expression of IL-1 β and IL-1Fm2 genes is differentially regulated.

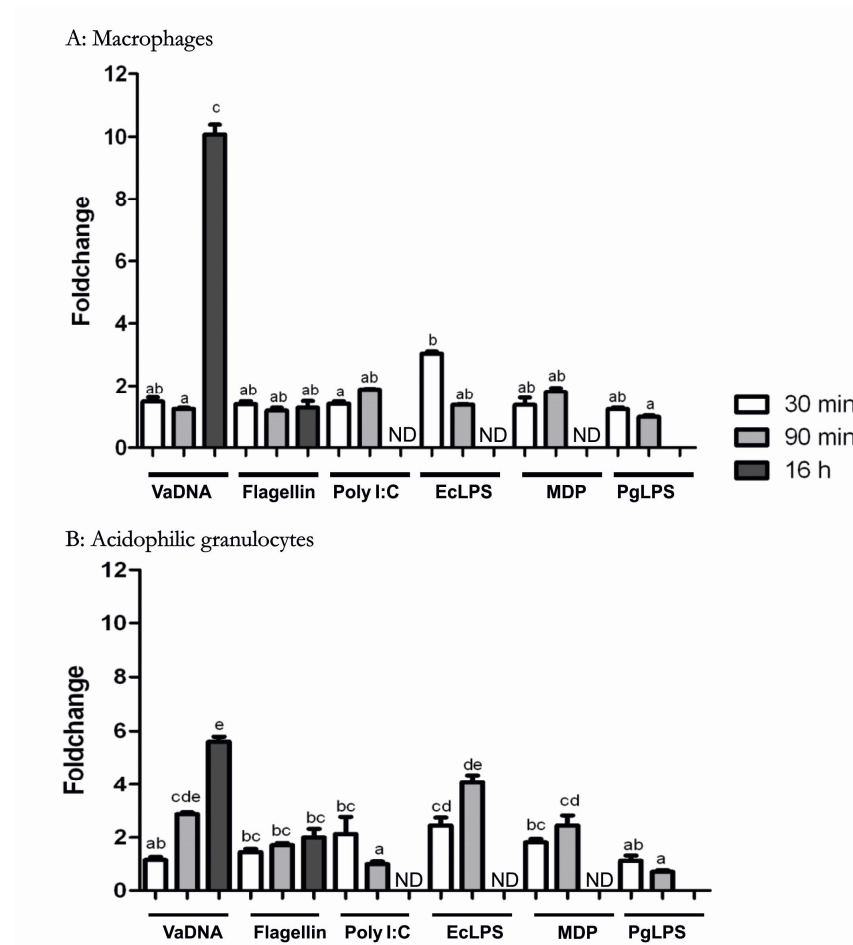


Figure 6: IL-1Fm2 is weakly induced by gilthead seabream phagocytes after PAMP-activation. The mRNA levels of IL-1Fm2 were determined by RT-qPCR in macrophages (A) and acidophilic granulocytes (B) incubated for the indicated times in medium alone or containing 50 μ g/ml VaDNA, 1 μ g/ml flagellin, 25 μ g/ml poly I:C, 10 μ g/ml EcLPS, 10 μ g/ml MDP or 10 μ g/ml PgLPS. Gene expression is normalized against ribosomal protein S18 and is shown as relative to the mean of non-stimulated cells. Each bar represents the mean $\pm$ SEM of triplicate biological samples. Different letters denote statistically significant differences between the groups according to a Tukey test. The groups marked with “a” did not show statistically significant differences from control cells. ND = not determined.

3.5 Recombinant *S. aurata* IL-1Fm2 activates phagocytes

The putative mature seabream IL-1Fm2 was produced in *E. coli* and four different mAbs were generated (Figs. 7A, B). The recombinant IL-1Fm2 was able to prime in a dose-dependent manner the respiratory burst of head-kidney leukocytes triggered by PMA (Fig. 7C), while they had no effects on cell viability (data not shown). However, no additive or synergistic effects were observed with the combination of IL-1Fm2 and bacterial DNA (Fig. 7C). This priming effect was specific, since the purified 6D1E4 mAb neutralized IL-1Fm2 activity, while the mAb 10E2G6, which was raised against zebrafish IL-1 β , failed to do so (Fig. 7D). In addition, the seabream IL-1Fm2 activity was completely abolished by heat treatment (Fig. 7D).

We next examined the effect of recombinant seabream IL-1Fm2 in purified macrophage monolayers (Fig. 8A) and sorted acidophilic granulocytes (Fig. 8B). The results showed that while recombinant IL-1Fm2 had a very weak effect, if any, on the expression of the gene encoding IL-1 β , IL-1Fm2, TNF α , IL-8 and IL-10 when used alone, it showed a powerful synergistic effect with bacterial DNA by increasing the mRNA levels of all the genes analyzed. Collectively, these results indicate that the crosstalk between TLR and IL-1Fm2 signaling regulates the fish inflammatory response.

The strong induction of IL-1Fm2 expression by the combination of bacterial DNA and recombinant IL-1Fm2 led us to examine IL-1Fm2 protein levels in cell extracts and supernatants from macrophages co-stimulated with this combination. It was found that bacterial DNA and recombinant IL-1Fm2 strongly increased the intracellular accumulation of IL-1 β (Fig. 9A), whereas it moderately increased a polypeptide of the expected size for the precursor form of IL-1Fm2 (approx. 30 kDa) (Fig. 9B), confirming the gene expression studies. Strikingly, the 6D1E4 mAbs also reacted with a polypeptide of about 18 kDa present in the supernatants of bacterial DNA/IL-1Fm2-stimulated macrophages (Fig. 9B), which might represent the matured form of IL-1Fm2. This polypeptide was synthesized by macrophages, since recombinant IL-1Fm2 showed a faster electrophoretic mobility (Fig. 9B). Interestingly, the pharmacological inhibition of all caspases, using the pan-caspase inhibitor Q-VD-OPh, or caspase-1, using the specific caspase-1 inhibitor Ac-YVAD-CMK, both failed to inhibit the processing and

release of IL-1Fm2 (Fig. 9C), as it has already been shown for gilthead seabream IL-1 β (Angosto et al., 2012).

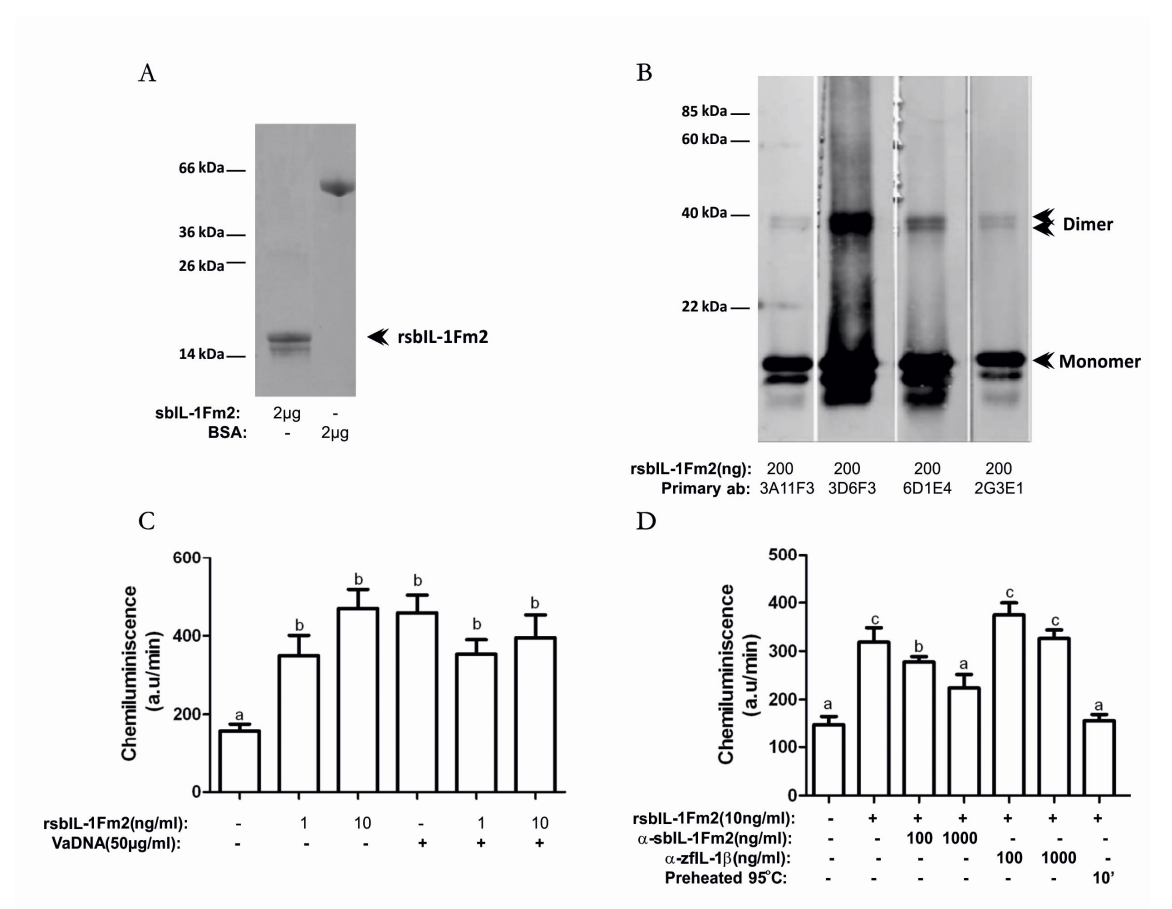


Fig. 7. Recombinant gilthead seabream IL-1Fm2 activates the respiratory burst of leukocytes. (A) SDS-PAGE analysis of IL-1Fm2 recombinant protein (rsbIL-1Fm2). Lane 1: BSA (2 μ g), lane 2: rsbIL-1Fm2 (2 μ g). (B) Western blot analysis of rsbIL-1Fm2 (200 ng/lane) using four different mAbs. The monomer and putative dimers are indicated. (C, D). Respiratory burst triggered by PMA of gilthead seabream head-kidney leukocytes primed for 16 h with 50 μ g/ml VaDNA and/or 1-10 ng/ml rsbIL-1Fm2 (untreated or preheated at 95°C for 10 min) in the presence of 100-1000 ng/ml purified 6D1E4 mAb against rsbIL-1Fm2 or purified 10E2G6 mAb against zebrafish IL-1 β (zflIL-1 β). Data are presented as mean $\pm$ SEM and are representative of three independent experiments. Different letters denote statistically significant differences between the groups according to a Tukey test. The groups marked with “a” did not show statistically significant differences from control cells.

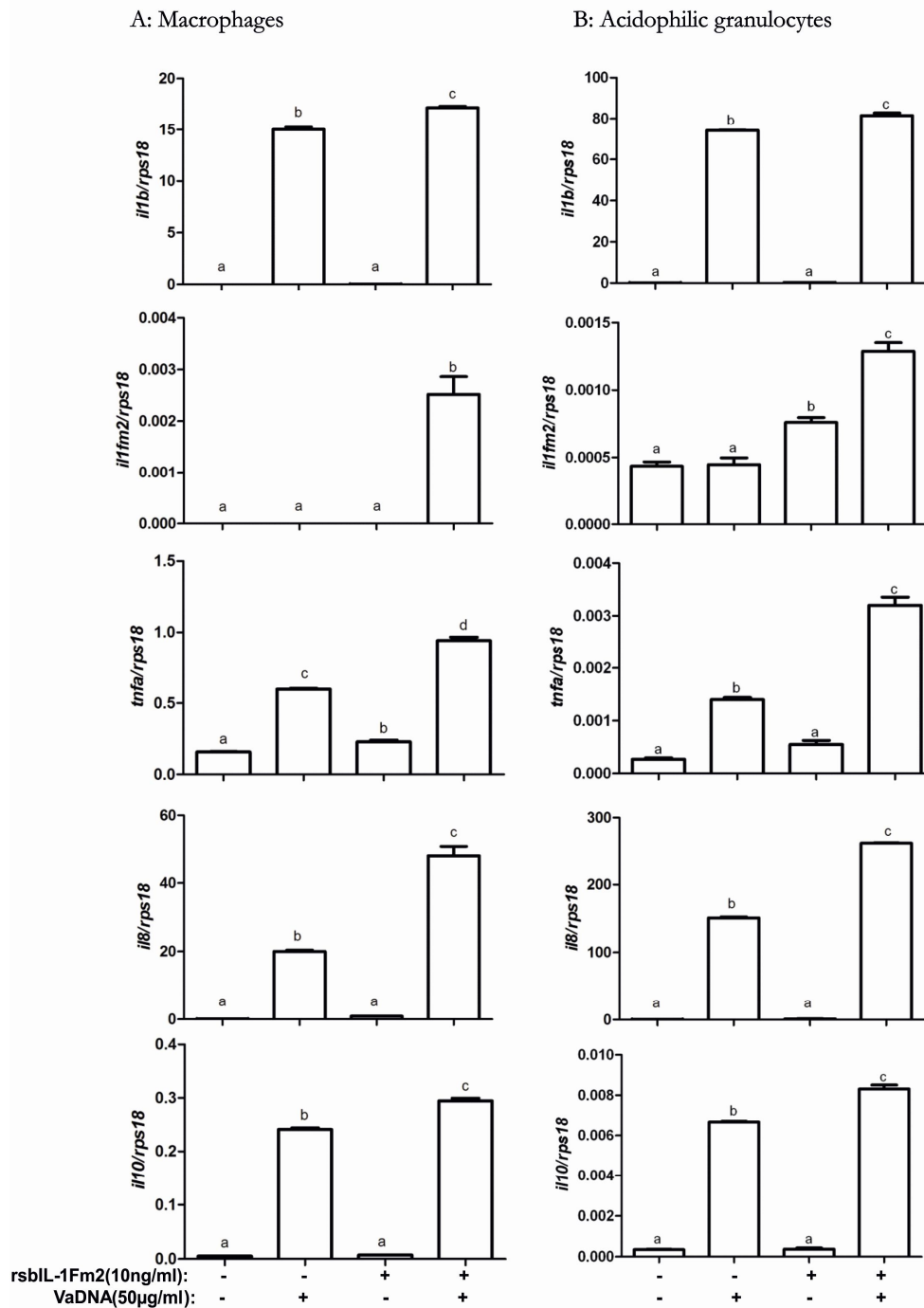


Fig. 8 Recombinant gilthead seabream IL-1Fm2 regulates the expression of pro- and anti-inflammatory genes in phagocytes. The mRNA levels of IL-1 β , IL-1Fm2, TNF α , IL-8 and IL-10 were determined by RT-qPCR in macrophages (A) and acidophilic granulocytes (B) incubated for 3 (macrophages) or 24 h (acidophilic granulocytes) with 50 μ g/ml VaDNA and/or 10 ng/ml recombinant seabream IL-1Fm2 (rsbIL-1Fm2). The results are expressed as the mean $\pm$ SEM of triplicate samples. Different letters denote statistically significant differences between the groups according to a Tukey test. The groups marked with “a” did not show statistically significant differences from control cells.

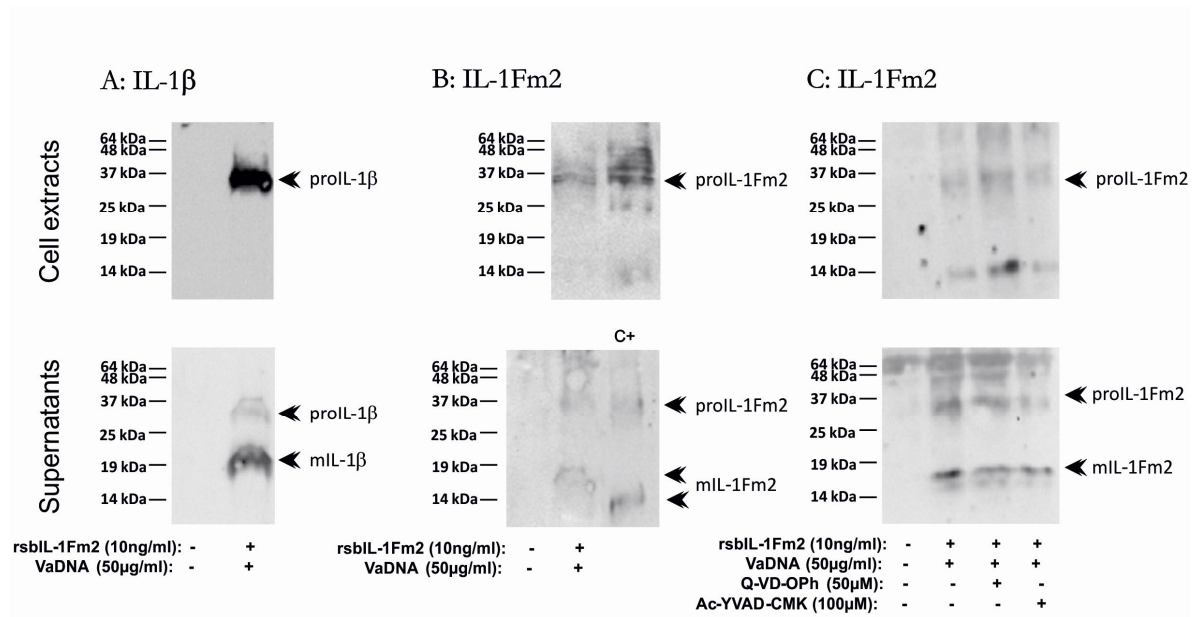


Fig. 9 Gilthead seabream IL-1Fm2 is processed by a caspase-independent mechanism. Macrophages pre-treated for 1 h with 100 μ M of the caspase-1 inhibitor Ac-YVAD-CMK or 50 μ M of the pan-caspase inhibitor Q-VD-OPh were stimulated for 16 h with 50 μ g/ml VaDNA and 10 ng/ml rsbIL-1Fm2. Cell lysates from unstimulated and VaDNA/rsbIL-1Fm2-activated macrophages (30 μ g protein) and concentrated supernatants (corresponding to 100 μ g protein from cell extracts) were probed with a monospecific polyclonal antibody to sbIL-1 β (A) or the mAb 6D1E4 against rsbIL-1Fm2 (B, C). Forty ng of rsbIL-1Fm2 were also probed with the 6D1E4 mAb (B, lower panel, C+). Migration position for the pro-cytokine and mature forms are indicated. The results are representative of two independent experiments.

4. DISCUSSION

In this study, we have identified a new member of the IL-1 family, IL-1Fm2, which is present in most evolutionarily advanced teleost fish, the Series Percomorpha. Surprisingly, after searching their genomes it appears that IL-1Fm2 is absent in pufferfishes, Order Tetraodontiformes. The conservation of synteny of several genes across teleost IL-1 β and IL-1Fm2 loci supports the notion that both IL-1 members arose from the FSGD that occurred approx. 350 mya (Meyer et al., 2005). The secondary loss of many genes after this event (Meyer et al., 2005), might explain the absence of IL-1Fm2 in pufferfishes. The present clarifies the evolution of the IL-1 family in teleost fish, since the recent discovery of IL-1 β 3 gene and IL-1 β 4 pseudogene in salmonids (rainbow trout, *Oncorhynchus mykiss*, and Atlantic salmon, *Salmo salar*) led to the proposal of two types of IL-1 β gene in all teleost fish analyzed, including Perciformes (tilapia) and Beloniformes (medaka) (Husain et al., 2012), both belonging to the Percomorpha. The new IL-1 member identified in tilapia and medaka were named IL-1 β 2 and were classified as type I teleost IL-1 β (Husain et al., 2012). However, the phylogenetic analysis of these two new members together with the ones identified in gilthead seabream and European seabass (this study) and Japanese flounder (Taechavasonyoo et al., 2013) demonstrates that all of them form a separated clade from type I teleost IL-1 β and constitute a subclade together with the nIL-1Fm, which functions as an IL-1 antagonist in rainbow trout (Wang et al., 2009). Therefore, we propose the name IL-1Fm2 for the genes identified in the present study. Furthermore, our phylogenetic analysis also shows that the two new IL-1 members described in the Japanese flounder as IL-1 β -like1 and IL-1 β -like2 (Taechavasonyoo et al., 2013) are indeed the IL-1Fm2 and nIL-1Fm orthologues, respectively, of this species.

The weak inducibility of gilthead seabream (this study) and Japanese flounder (Taechavasonyoo et al., 2013) IL-1Fm2 genes in infected animals and in leukocytes stimulated *in vitro* also supports that IL-1Fm2 is a new member of the IL-1 family rather than an IL-1 β paralogue belonging to the type I teleost IL-1 β . In fact, although rainbow trout IL-1 β 3 gene (type I) was induced at lower levels than IL-1 β 2, and to some extent IL-1 β 1 (both type II), upon macrophages stimulation with PHA (Husain et al., 2012), all type I IL-1 β genes reported so far, including zebrafish (Galindo-Villegas et al., 2012; López-Munoz et al., 2009; López-Munoz et al., 2011) and common carp (*Cyprinus carpio*) (Engelsma et al., 2001) (Cypriniformes), catfish (*Ictalurus punctatus*,

Siluriformes) (Wang et al., 2006) and conger eel (*Conger myriaster*, Anguilliformes) (Tsutsui et al., 2007), are heavily induced in infected animals and/or stimulated leukocytes.

In mammals, IL-1 β needs to be processed to exert biological activity by caspase-1, which is activated in a cytosolic molecular platform called the inflammasome (von Moltke et al., 2012; Lamkandi et al., 2012). However, all non-mammalian IL-1 β genes reported so far lack a conserved caspase-1 recognition site (Bird et al., 2002). Although gilthead seabream IL-1Fm2 does not show a conserved caspase-1 processing site, gilthead seabream macrophages processed IL-1Fm2 before being released. However, both pan-caspase and caspase-1 specific inhibitors fail to inhibit its processing; despite they are able to inhibit apoptosis in fish (Sepulcre et al., 2011; Walters et al., 2009; Yang et al., 2012) and the activity of recombinant gilthead seabream caspase-1 (López-Castejón et al., 2008), respectively. Similarly, the processing and release of gilthead seabream IL-1 β is caspase-1-independent (Angosto et al., 2012). In sharp contrast, it has been found that zebrafish inflammatory caspase A and caspase B (Vojtech et al., 2012) and European seabass caspase-1 (Reis et al., 2012) can process IL-1 β in these species. Nevertheless, classical NLRP3 and NLRC4 inflammasome activators also fail to trigger the processing and secretion of IL-1 β (Angosto et al., 2012; López-Castejón et al., 2007). Therefore, the mechanism of processing and release of fish IL-1 family members deserves further investigations.

Although the information on the biological activity of bony fish cytokines is scant, IL-1 β was one the best cytokine characterized in these animals. In rainbow trout, recombinant IL-1 β and derived peptides were found to promote leukocyte recruitment *in vitro* (Peddie et al., 2001) and induce potent inflammatory response when injected i.p. that results in enhanced resistance to a experimental bacterial (Hong et al., 2003) and viral (Peddie et al., 2003) infections. Similarly, recombinant IL-1 β from the phylogenetically distant European seabass also shows pro-inflammatory effects and induced the proliferation of thymocytes (Buonocore et al., 2005). Although IL-1Fm2 genes seem to be phylogenetically close to nIL-1Fm, which at least in rainbow trout acts as an IL-1 antagonist, recombinant gilthead seabream IL-1Fm2 seems to be pro-inflammatory, as it is able to activate the respiratory burst of phagocytes and to induce the expression of several genes encoding pro-inflammatory mediators, such as IL-1 β ,

TNF α and IL-8. Strikingly, IL-1Fm2 and PAMPs synergistically induce its own expression and that of IL-10 gene, suggesting a key role for this cytokine in the resolution of inflammation and the polarization of the fish adaptive immune responses.

In conclusion, we have identified a new IL-1 family member that is present in most phylogenetically advanced teleost fish and that plays an important role in the regulation of the immune responses of this group of animals. The identification of IL-1Fm2 shed light into the evolution of the IL-1 family in vertebrates and point to the complexity of this cytokine family.

CONCLUSIONS

The results obtained in this work lead to the following conclusions:

1. The inflammasome and caspase-1 are dispensable for the processing of IL-1 β in gilthead seabream.
2. The inflammasome and caspase-1 regulate pyroptotic cell death and the clearance of *S. typhimurium* in gilthead seabream.
3. PYCARD is required for the activation of caspase-1 in zebrafish larvae but dispensable for *S. typhimurium* clearance.
4. Caspase-1 is dispensable for *S. typhimurium* clearance in zebrafish larvae. However, another caspase, that need to be identified, is involved in this process.
5. IL-1Fm2 is a new member of the IL-1 family rather than an IL-1 β orthologue belonging to the teleost type I IL-1 β . In addition, IL-1Fm2 is exclusively present in most evolutionarily advanced teleosts, the Series Percomorpha, although is absent in pufferfishes, Order Tetraodontiformes, which also belong to the Percomorpha.
6. IL-1Fm2 is hardly activated by PAMPs in macrophages and acidophilic granulocytes of the gilthead seabream. However, recombinant IL-1Fm2 and bacterial DNA synergistically induces its own expression and that of genes encoding IL-1 β , TNF α , IL-8 and IL-10.
7. Recombinant IL-1Fm2 and bacterial DNA promote the intracellular accumulation of precursor IL-1Fm2 and the release of an 18 kDa processed form by gilthead seabream macrophages. In addition, the processing and release of IL-1Fm2 is caspase-independent in gilthead seabream macrophages.

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RESUMEN EN ESPAÑOL

INTRODUCCIÓN

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Las citoquinas son moléculas que desarrollan papeles clave en la regulación de la respuesta inmunitaria (Balkwill y Burke, 1989; Vilcek, 1998). La citoquina IL-1 β es un mediador de la inflamación muy importante y en general inicia y/o aumenta la expresión de una amplia variedad de genes que se expresan característicamente durante la inflamación, especialmente otras citoquinas. Es uno de los mediadores claves de la respuesta del organismo a la invasión microbiana, inflamación, reacciones inmunológicas y heridas en los tejidos; y la producen diferentes tipos celulares, aunque los más importantes son los macrófagos y los monocitos (Dinarello, 1994; Dinarello 1997). Esto explica el gran interés que ha despertado el estudio de esta citoquina, intentando comprender cada vez mejor tanto los mecanismos implicados en su producción y liberación como los mecanismos que regulan su actividad biológica.

Uno de los mayores enigmas existentes en torno a esta citoquina es su mecanismo de liberación. La IL-1 β es una citoquina secretora que carece de péptido señal y por tanto no sigue la vía clásica de secreción, retículo endoplasmático-aparato de Golgi (MacKenzie y col., 2001). En mamíferos, la IL-1 β se acumula en los macrófagos como un precursor inactivo, tras la estimulación con diferentes ligandos derivados de patógenos (PAMPs). El ATP extracelular, a través de los receptores purinérgicos P2X7, inicia un mecanismo de procesamiento post-traducciona l de la citoquina precursora y su consecuente liberación (Cerretti y col., 1992; Solle y col., 2001).

La caspasa-1 es una cisteín-proteasa cuya diana es un residuo aspártico, aunque es una enzima bastante promiscua (Stennicke, 1998). Esta caspasa pertenece al grupo de las caspasas inflamatorias, y se produce como una proenzima inactiva, que tras un procesamiento proteolítico, genera dos subunidades, una grande p20 y una pequeña p10, que se activan mediante oligomerización con otras proteínas formando una plataforma molecular denominada inflamasoma (Mariathasan y col., 2004; Stehlik y col., 2003). La caspasa-1 tiene la habilidad de cortar proteolíticamente y activar el precursor de la IL-1 β (Cerretti y col., 1992). Se ha demostrado recientemente que la caspasa-11 (también conocida como caspasa-4) es crítica para la activación de caspasa-1 y la producción de IL-1 β en macrófagos infectados con *Escherichia coli*, *Citrobacter rodentium* o *Vibrio cholerae* (Kayagaki N y col., 2011). A pesar de la importancia de la caspasa-1 en inflamación poco se sabe de su papel en peces. El primer homólogo de

caspasa-1 en pez mostrando el dominio N-terminal (CARD) fue identificado en dorada (López-Castejón y col., 2008), aunque si esta caspasa-1 tiene algo que ver en el procesamiento de la citoquina proinflamatoria IL-1 β en peces está aún por demostrar.

A la pregunta de cómo se activa la caspasa-1, la caracterización del complejo macromolecular denominado inflamasoma ha sido clave para dar respuestas. Miembros de la familia de los NLRs actúan como reguladores de la actividad de la caspasa-1. Estos NLRs están formados por un dominio C-terminal que son repeticiones ricas de leucina, que se consideran los sensores de las distintas señales, un dominio intermedio denominado NACHT, esencial para la oligomerización y activación de estos NLRs, y la oligomerización de este dominio es un pre-requisito para la transducción de la señal por un tercer dominio, el N-terminal, que puede ser un dominio PYD o un dominio de reclutamiento de caspasa (CARD). Dentro de los NLRs más estudiados se encuentran los denominados NLRP3 y NLRC4. El NLRP3 se activa en respuesta a una gran diversidad de señales originadas por distintos estímulos como flujos de K⁺, y el NLRC4 por flagelina y/o componentes del sistema de secreción T3 (T3SS).

Cuando se habla de la activación del inflamasoma en respuesta a infección de bacterias Gram-negativas como *Salmonella typhimurium* hay que hablar de la muerte celular que requiere la activación de caspasa-1 denominada piroptosis, que elimina los macrófagos infectados, liberando el contenido citosólico siendo destruido el contenido por fagocitos y neutrófilos.

La IL-1 β es una citoquina que también está presente en especies de vertebrados no mamíferos, como aves, anfibios y peces. En este caso el misterio es aún mayor y hasta el momento, su mecanismo de producción y liberación es completamente desconocido en estas especies. Inesperadamente se ha visto que la mayoría de las secuencias de IL-1 β encontradas en vertebrados no mamíferos hasta el momento carecen de un punto de corte específico para caspasa-1 en la posición esperada, entre ellas la IL-1 β de dorada (Bird y col., 2002).

Un nuevo miembro de la familia IL-1 (nIL-1Fm) ha sido caracterizado en trucha Wang y col., 2009) que aunque cumple las características moleculares para ser un miembro de la familia de la IL-1, distintos estudios han demostrado que se une al

receptor I de la IL-1 (IL-1RI) de trucha, estos datos indican que se podría tratar un antagonista del receptor de la IL-1.

OBJETIVOS

En el presente trabajo se proponen los siguientes objetivos concretos:

1. Caracterización del mecanismo de procesamiento y liberación de la IL-1 β de dorada.
2. Caracterización del papel desempeñado por la piroptosis en la respuesta de los peces teleósteos frente a *S. typhimurium*.
3. Caracterización del papel desempeñado por la molécula adaptadora del inflammasoma PYCARD (ASC) y la caspasa-1 en la respuesta del pez cebra frente a *S. typhimurium*.
4. Caracterización molecular, filogenética y funcional de un nuevo miembro de la familia de la IL-1 identificado en peces teleósteos (IL-1Fm2).

MATERIALES Y MÉTODOS

1. ANIMALES

Para el desarrollo de la presente Tesis Doctoral se han utilizado ejemplares de dorada (*Sparus aurata* L.), de un peso medio de 150-300 g, pez teleósteo marino hermafrodita de carácter protándrico, procedentes de las instalaciones del centro Oceanográfico de Mazarrón (I.E.O. Mazarrón, Murcia). Los ejemplares estaban en acuarios de 14 m³ con temperatura natural y fotoperiodo. Los peces fueron alimentados 2 veces al día con pienso comercial (Skretting). Se obtuvieron diferentes órganos y células tal como se detalla a continuación en cada apartado específico.

Peces cebra (*Danio rerio*) de las líneas genéticas AB, TL y WIK fueron cedidas por el centro internacional de recursos del pez cebra (ZIRC, Oregón, EEUU) y mantenidos como se describe en el manual del pez cebra (Westerfield, 2000).

Los experimentos desarrollados cumplen con la directiva de la Unión Europea (86/609/EU) y han sido aprobados por el Comité de Bioética de la Universidad de Murcia (nº #333/2008).

2. CULTIVOS CELULARES Y TRATAMIENTOS

Leucocitos totales de riñón cefálico de dorada obtenidos como ya se ha descrito previamente (Sepulcre y col., 2002) se cultivaron en sRPMI [medio de cultivo RPMI-1640 (Gibco) ajustado a la osmolaridad del suero de dorada (353.33mOs) con 0'35% de NaCl] suplementado con 5% de suero bovino fetal (SBF, Gibco) y 100 U.I./ml de penicilina y 100 µg/ml de estreptomicina (P/S, Biochrom). Para algunos experimentos se usaron fracciones celulares purificadas de los dos tipos de células fagocíticas profesionales en esta especie, macrófagos (células adherentes) y granulocitos acidófilos (aislados mediante MACS con el anticuerpo monoclonal específico G7) (Sepulcre y col., 2002; Roca y col., 2006).

Macrófagos, granulocitos acidófilos y leucocitos totales de riñón cefálico de dorada fueron estimulados durante 16 h a 23°C con 50 µg/ml de DNA genómico de células de *Vibrio anguillarum* ATCC19262 o con 100 ng/ml de flagelina (Invivogen), 25 µg/ml de poli I:C, 10 µg/ml de lipopolisacárido (LPS) de *Escherichia coli*, 10 µg/ml de LPS de *Porphyromonas gingivalis* (PgLPS), 10 µg/ml de muramildipéptido (MDP)

(todos de Sigma-Aldrich) y 1-10 ng/ml de proteína recombinante de dorada IL-1Fm2 en medio sRPMI suplementado con 0.1% de suero bovino fetal y P/S (Sepulcre MP y col., 2007).

En algunos experimentos, las células se lavaron 2 veces con medio sRPMI e incubadas durante 1 h con 5 mM de eATP (Sigma-Aldrich), 1 μ M de nigericina (Sigma-Aldrich), un tampón hipotónico (36.75 mM NaCl, 0.5 mM KCl, 0.5 CaCl₂, 0.25 mM MgCl₂, 3.25 mM glucose, 2.5 mM HEPES, pH 7.3, 90 mOs), 500 μ g/ml de cristales de monoureato sódico (MSU) (Invivogen) o 40 μ g/ml de cristales de aluminio (Thermo Scientific)

Para los ensayos de infección se usaron *Salmonella enterica serovar typhimurium* (*S. typhimurium*) 12023 y sus derivados isogénicos prgH020::Tn5lacZY (mutante SPI1), ssaV::aphT (mutante SPI2) y prgH020::Tn5lacZY ssaV::aphT (doble mutante SPI1/SPI2) (cedidas por el Prof. D. Holden, *E.coli* 3661 y su derivado isogénico 3617 que expresa la listeriolisina recombinante de *Listeria monocytogenes* (cedida por el Dr D. Higgins) (Higgins DE y col., 1999). Cultivos en Luria-Bertani (LB) durante toda la noche fueron diluidos 1/5 y crecidos a 37°C en agitación durante 3 h. Las bacterias fueron suspendidas en medio sRPMI y añadidos a los leucocitos a una multiplicidad de infección (MOI) de 10 y de 100. Después de 2 h se añadió 10 μ g/ml de gentamicina para limitar el crecimiento extracelular de la bacteria y los leucocitos infectados fueron incubados a diferentes intervalos de tiempo. En algunos experimentos, los leucocitos fueron pretratados durante 1 h con inhibidor específico de caspasa-1, Ac-YVAD-CMK o Ac-YVAD-CHO (Calbiochem o Peptanova) a 100 μ M o con inhibidores generales de caspasas Z-VAD-FMK o Q-VD-OPh (Sigma-Aldrich) a 50 μ M.

Los sobrenadantes de las células de leucocitos control e infectados fueron recogidos después de la incubación durante toda la noche, filtrados con un filtro de 0.45 μ m y concentrados por precipitación con 20% de ácido tricloroacético (TCA) (Sigma-Aldrich).

3. MICROINYECCIÓN E INFECCIÓN DE PEZ CEBRA

Se diseñó un morfolino contra la unión entre el exón 2 y el intrón 2 de la molécula PYCARD de pez cebra (NM_131495) (GeneTools (5'-

AGTGATTCGCTTACTCACCATCAGA-3'). Se solubilizó en agua (2mM) y se mezcló en tampón de microinyección (0.5 x tampón Tango y 0.05 % rojo fenol) y fue microinyectado (0.5-1 nl, 7ng) en el saco vitelino en embriones de una célula, usando un microinyector Narishige IM300.

Las larvas fueron infectadas con una dosis letal 50 (LD50) de *S. typhimurium* silvestre y mutantes. Las bacterias se inyectaron en el saco vitelino o en el conducto de Cuvier 60 horas después de la fertilización y posteriormente fueron revisadas cada 24 horas durante 5 días para ver signos clínicos de enfermedad y mortalidad.

4. VIABILIDAD CELULAR

Alícuotas de suspensiones celulares fueron diluidas en 200 µl de PBS que contenía 40 µg/ml de yoduro de propidio. El número de células rojas fluorescentes (células muertas) de las muestras por triplicado fueron analizadas por citometría de flujo (BD Bioscience).

5. ENSAYOS DE ACTIVIDAD CASPASA-1

Para determinar la actividad de la enzima caspasa-1 se utilizó el sustrato fluorogénico Z-YVAD-AFC (Calbiochem) según el protocolo del fabricante. Las células se lisaron en tampón de lisis hipotónico [25 mM de ácido 4-(2-hidroxietil)-1-piperazineetanosulfónico (HEPES), 5 µM de ácido [etilenebis(oxonitrilo)]tetraacético (EGTA), 5 mM de ditioteitol (DTT), el cocktail inhibidor de proteasas (1:20, Sigma), pH 7.5] en hielo durante 10 min. Para cada reacción, se incubaron 50 µl del lisado celular obtenido de 10^6 células HEK293, 10^6 células SAF-1 o 2×10^7 leucocitos de dorada durante 90 min a 37°C (células HEK293) ó a 23°C (para las células SAF-1 y leucocitos de dorada) con 50 µM de Z-YVAD-AFC y 50 µl de tampón de reacción [0.2% de ácido 3-[(3-colamidopropil)dimetilamonio]-1-propanosulfónico (CHAPS), 0.2 M de HEPES, 20% de glucosa, 29 mM de DTT, pH 7.5]. En algunos experimentos se usaron los inhibidores Ac-YVAD-CMK o Ac-YVAD-CHO a una concentración final de 100 µM. Finalmente, se midió la fluorescencia del grupo AFC liberado del sustrato Z-YVAD-AFC con un espectrofluorímetro FLUOstar (BGM, LabTechnologies) a una

longitud de onda de excitación de 405 nm y a una longitud de onda de emisión de 492 nm.

6. ANALISIS POR WESTERN BLOT

Extractos celulares y sobrenadantes concentrados se analizaron en geles de poliacrilamida (SDS-PAGE) al 15% y se transfirieron a una membrana de nitrocelulosa durante 50 min a 200 mA. Las membranas se revelaron usando anticuerpos contra la IL-1 β de dorada (López-Castejón y col., 2007), contra la IL-1 β de humano y de ratón (3ZD, Biological Resources Branch, NCI) y un anticuerpo purificado contra la proteína recombinante de dorada IL-1Fm2 (6D1E4, GenScript Corporation) y finalmente los reactivos quimioluminiscentes ECL (Amersham Biosciences) siguiendo el protocolo proporcionado por el fabricante.

7. ENSAYO BACTERICIDA

Los leucocitos fueron lisados con saponina al 0.2% (Sigma-Aldrich) y el número de bacterias fue cuantificada sembrando los extractos celulares en placas de LB-agar conteniendo los antibióticos adecuados.

8. VECTORES DE EXPRESIÓN Y TRANSFECCIÓN

La caspasa-1 y la proIL-1 β de dorada fueron clonadas en los vectores de expresión pcDNA 3.1/V5 His-TOPO y pcDNA4 HIS/Max (Invitrogen), respectivamente (López-Castejón y col., 2007 y López-Castejón y col., 2008). Otras construcciones de expresión fueron el receptor P2X7 de rata (Wilson HL y col., 2002), la proIL-1 β humana (Siders WM y col., 1995) y la caspasa-1 humana (Miura y col., 1993). El ADN plasmídico fue preparado usando el procedimiento de Mini-prep (Qiagen). Las transfecciones se llevaron a cabo usando como agente de transfección LyoVec (Invivogen) según las instrucciones del fabricante. En breve, se pusieron 400.000 células HEK293 por pocillo en placas de 6 pocillos junto a 100 μ l de agente de transfección conteniendo las siguientes cantidades de los siguientes vectores de

expresión: 0.15 µg de P2X7 de rata, 1.5 µg de proIL-1β de humano y de dorada y 1.5 µg de caspasa-1 de humano y de dorada. 48 h después de la transfección las células se lavan dos veces en medio libre de suero y se incuban durante 30 min con 1mM de ATP para activar el receptor P2X7 (López-Castejón y col., 2007a).

9. IDENTIFICACIÓN DE LA IL-1Fm2 DE TELEÓSTEOS Y ANÁLISIS DE LA SECUENCIA

La secuencia parcial de la IL-1Fm2 de dorada fue identificada por una búsqueda BLAST (número de acceso AM957809). Se utilizó la estrategia de amplificación rápida del extremo 3' del cDNA para obtener la secuencia codificante, que se depositó en el GenBank con número de acceso **HF936678**. La proteína IL-1Fm2 fue analizada por similitud con otras secuencias conocidas usando el programa BLAST del servidor del NCBI. Una comparación directa entre dos secuencias se realizó usando el programa ALING, mientras que para el alineamiento de múltiples secuencias se usó el programa CLUSTALW del Instituto de Bioinformática Europeo (EBI). El árbol filogenético fue construido basándose en el alineamiento de amino ácidos usando el TREE VIEW X. Finalmente, los dominios de la proteína fueron deducidos usando la secuencia de nucleótidos sobre la base de datos del PFAM.

10. PRODUCCIÓN DE LA PROTEÍNA RECOMBINANTE IL-1Fm2 DE DORADA

La proteína recombinante IL-1Fm2 fue producida en *E. coli*. En breve, el marco de lectura abierto de la IL-1Fm2 que codifica la forma madura de la proteína (residuos del 118 al 270) fue sintetizado, clonado en el vector E3, producido con la proteína 6xHIS fusionado en el extremo amino terminal en *E. coli*, obtenida en cuerpos de inclusión y purificada en columnas Ni-Hi (GenScript Corporation).

11. CUANTIFICACIÓN DE PROTEÍNA

La concentración de proteína de los lisados celulares se cuantificó mediante el método colorimétrico con el reactivo BCA (Pierce) usando albúmina de suero bovino (BSA) como estándar.

12. ANÁLISIS DE LA EXPRESIÓN GÉNICA

El RNA total se extrajo de los diferentes órganos , suspensiones celulares y larvas de pez cebra con TriZol (Invitrogen) siguiendo las instrucciones del fabricante y a continuación fue tratado con DNasa I libre de RNasa (Fermentas). La retrotranscriptasa Superscript III RNasa H⁻ (Invitrogen) se usó para sintetizar el cDNA con un cebador oligo-dT₁₈ a partir de 1 µg de RNA total a 50°C durante 50 minutos.

La PCR a tiempo real fue realizada en un aparato ABI PRISM 7500 (Applied Biosystems) usando SYBR Green (Applied Biosystems). Las mezclas de reacción fueron incubadas durante 10 min a 95°C, seguido de 40 ciclos de 15s a 95°C, 1 min a 60°C y finalmente 15 s a 95°C, 1 min a 60°C y 15 s a 95°C. Para cada ARN, la expresión génica fue corregida por la proteína ribosómica S18 (*rps18*) para dorada y S11 (*rps11*) para pez cebra.

RESULTADOS Y DISCUSIÓN

1. EVOLUCIÓN DE LAS FUNCIONES DEL INFLAMASOMA EN VERTEBRADOS: EL INFLAMASOMA Y LA CASPASA-1 DESENCADENAN MUERTE CELULAR EN MACRÓFAGOS DE DORADA, PERO SON DISPENSABLES PARA EL PROCESAMIENTO DE LA IL-1 β .

En este capítulo se estudió como *S. typhimurium* y su derivado isogénico SipB que tiene una mutación que afecta al sistema de secreción tipo 3 (T3SS) de esta bacteria, eran capaces de promover la liberación de la forma madura de la IL-1 β (mIL-1 β) en leucocitos de dorada previamente activados con diferentes patrones moleculares asociados a patógenos (PAMPs). La estimulación con PAMPs fue capaz de provocar la liberación de la mIL-1 β , aunque a un nivel menor que la infección con la bacteria. Por otra parte, activadores clásicos del inflamasoma NLRP3, como son el ATP y la nigericina (Mariathasan y col., 2006), no fueron capaces de provocar el procesamiento y liberación de IL-1 β en leucocitos de dorada. El uso de un inhibidor farmacológico específico para caspasa-1 no fue capaz de inhibir el procesamiento ni la liberación de la IL-1 β a pesar de que este inhibidor inhibe la actividad caspasa-1 en leucocitos de dorada (López-Castejón y col., 2008). Este resultado se confirmó usando un inhibidor general de caspasas que también fue incapaz de inhibir el procesamiento y liberación de la IL-1 β de dorada. Además, la caspasa-1 de dorada expresada ectópicamente en células HEK293 fue incapaz de procesar la forma precursora de la IL-1 β (proIL-1 β) de dorada, aunque la activación con ATP del receptor P2X7 de rata previamente transfectado en estas células fue capaz de liberar la proIL-1 β . Todo lo contrario ocurrió cuando las células HEK293 fueron transfectadas con la caspasa-1 y la proIL-1 β humana, dónde el procesamiento de la IL-1 β ocurrió tanto en células no activadas como activadas con ATP.

Se estudió también la capacidad de activadores clásicos del inflamasoma para inducir la actividad caspasa-1 en macrófagos de doradas. Activadores clásicos del inflamasoma tipo NLRP3, como ATP, un flujo de iones K⁺ provocado por el ionóforo nigericina y cristales de MSU y aluminio, no fueron capaces de inducir la actividad caspasa-1 ni de provocar el procesamiento y la liberación de IL-1 β . Por lo contrario, una solución hipotónica, que es capaz de activar caspasa-1 en macrófagos de ratón a través del inflamasoma NLRP3 (Compan y col., 2012) fue capaz de inducir la actividad

caspasa-1 en macrófagos de dorada, pero incapaz de desencadenar el procesamiento de la IL-1 β de dorada.

Por último, se estudió la capacidad de *S. typhimurium*, que es un activador del inflamasoma tipo NLRC4 en macrófagos de mamíferos, de inducir la actividad caspasa-1 en macrófagos de dorada. La forma silvestre de *S. typhimurium* fue capaz de inducir la actividad caspasa-1 en macrófagos infectados en tiempos cortos, mientras que la forma mutante en T3SS, SipB, no fue capaz de provocar esta actividad. Sin embargo, sendas bacterias fueron capaces de desencadenar el procesamiento y liberación de IL-1 β al mismo nivel. Además, la forma silvestre de *S. typhimurium* provocó la muerte celular de los macrófagos y se replicó mejor que su forma mutante. Es interesante destacar que la muerte celular provocada por la forma silvestre de *S. typhimurium* fue reducida de forma estadísticamente significativa por la inhibición farmacológica de caspasa-1, lo que indica que esa muerte celular es dependiente de caspasa-1 y, por tanto, se denominaría piroptosis.

Todos estos resultados nos indican la incapacidad de la caspasa-1 de procesar la IL-1 β en dorada, pero nos muestran un papel importante en la eliminación de bacterias intracelulares a través de la inducción de muerte celular dependiente de caspasa-1 o piroptosis. Además, apoyan la idea de que el uso del inflamasoma y la caspasa-1 para la el procesamiento de citoquinas proinflamatorias surgió después de la divergencia entre vertebrados y tetrápodos. No obstante, el hecho de que estos resultados indiquen que caspasa-1 no es responsable del procesamiento de la IL-1 β de dorada, no descartan que juegue un papel en el procesamiento de otras citoquinas proinflamatorias en dorada, como la IL-18.

2. SALMONELLA COMO MODELO DE INFECCIÓN EN PEZ CEBRA PARA ESTUDIAR EL PAPEL DEL INFLAMASOMA EN LA ELIMINACIÓN DE BACTERIAS INTRACELULARES.

En este capítulo usando el pez cebra como modelo, se estudió el efecto de la infección de larvas usando distintas estirpes de *S. typhimurium*. Se usaron la forma silvestre y varias estirpes isogénicas mutantes en diferentes componentes del T3SS. En concreto, una estirpe con una mutación en SPI-1, necesaria para los pasos iniciales de

infecciones sistémicas (Galán y col., 1989); otra estirpe con una mutación en SPI-2, que aunque no está muy caracterizada se cree que una mutación en SPI-2 hace que pierda su virulencia y su capacidad proliferativa en distintos órganos (Shea y col., 1999); y por último, una doble mutante para SPI-1 y SPI-2. Ensayos de infección en larvas de pez cebra con estas estirpes mostraron que la forma silvestre produce una gran mortalidad, la doble mutante SPI-1-2 tiene una mortalidad reducida, y las mutantes para SPI-1 y SPI-2 presentan una virulencia intermedia comparada con la silvestre y la doble mutante. Sin embargo, de estas estirpes sólo la silvestre fue capaz de inducir la actividad caspasa-1, por lo que aunque SPI-1 y SPI-2 contribuyen a la virulencia, cualquiera de estas mutaciones impide la inducción de la caspasa-1 por la bacteria. Estos resultados nos indican la utilidad de este modelo para determinar el papel del T3SS de *S. typhimurium* en un organismo vertebrado en ausencia de inmunidad adaptativa.

Se estudió también el papel de PYCARD (llamada así por sus dominios PYD y CARD), molécula adaptadora necesaria para la actividad de varios inflammasomas, en este modelo. Para ello se hizo uso del silenciamiento génico transitorio usando un morfolino específico que alteraba el procesamiento del ARNm de PYCARD. El uso de este morfolino resultó en una proteína sin el dominio CARD y por tanto sin función. Aunque el silenciamiento génico de PYCARD no afectó al desarrollo de las larvas, se redujo tanto la actividad caspasa-1 constitutiva como la inducida por la infección con *S. typhimurium* silvestre. Sin embargo, el silenciamiento de PYCARD no afectó a la resistencia de las larvas a la infección por las distintas estirpes de la bacteria. Con estos resultados se decidió analizar la expresión génica de las larvas deficientes para PYCARD. En el caso de las larvas control, la expresión de citoquinas proinflamatorias IL-1 β y TNF α , se indujo tras la infección con la estirpe silvestre, pero no con la doble mutante. En el caso de las larvas deficientes para PYCARD, no se observó ningún efecto en la expresión de la IL-1 β aunque sí redujo la inducción de TNF α . Estos resultados nos indican la importancia del T3SS para la virulencia de *S. typhimurium* y que PYCARD no juega un papel determinante en la detección y eliminación de esta bacteria, tal como se ha observado usando el ratón como modelo (Lara-Tejero y col., 2006).

Teniendo en cuenta que PYCARD no desempeña un papel muy importante en la eliminación de *S. typhimurium*, se analizó la función de la caspasa-1. Ante la ausencia

de un homólogo claro de caspasa-1 en pez cebra, se usó un inhibidor específico de caspasa-1, no observándose diferencias en la mortalidad en presencia del inhibidor. Sin embargo, se observaron diferencias significativas cuando se usó un inhibidor general de caspasas. Así, las larvas tratadas con este último inhibidor fueron más susceptibles a la infección por la estirpe silvestre que las larvas control. Estos resultados indican que la caspasa-1 no juega un papel determinante en la eliminación de *S. typhimurium*, aunque sugieren la participación de otra caspasa que tiene que ser determinada.

3. IDENTIFICACIÓN Y CARACTERIZACIÓN FUNCIONAL DE UN NUEVO MIEMBRO DE LA FAMILIA DE LA IL-1, IL-1Fm2, EN LOS PECES TELEÓSTEOS MÁS AVANZADOS EVOLUTIVAMENTE.

Se identificó una secuencia parcial en la base de ESTs del Archivo de Nucleótidos Europeo (ENA) que fue completada por RACE. Su análisis molecular indicó que presentaba las características de los miembros de la familia de la IL-1. Así, una búsqueda con BLAST dio como resultados más relevantes los genes de las IL-1 β de peces teleósteos, seguidos de los de mamíferos. Un análisis de dominios usando Pfam, reveló un dominio de pro-péptido IL-1 entre los residuos 6 y 100 y un dominio IL-1 entre los residuos 149 y 269. Además, un alineamiento múltiple, usando CLUSTALW, mostró un mayor grado de conservación en los residuos que forman la estructura secundaria típica de las IL-1 β . Finalmente, la firma de la familia IL-1, [FC]-x-S-[ASLV]-x(2)-P-x(2)-[FYLV]-[LI]-[SCA]-T-x(7)-[LIVM], estaba muy bien conservada.

Usando la secuencia obtenida en dorada, se buscaron secuencias similares en genomas de distintas especies, encontrándose en la mayoría de los pertenecientes a la Serie Percomorfa, en concreto en los Ordenes Perciformes, Beloniformes, Gasterosteiformes, Cyprinodontiformes y Pleuronectiformes. Un análisis filogenético de todas las secuencias encontradas, indica que forman un grupo diferenciado de las IL-1 β de peces. Por tanto, es un nuevo miembro de la familia de la IL-1 presente exclusivamente en peces más avanzados evolutivamente y que decidimos denominar miembro 2 de la familia de la IL-1 (IL-1Fm2) para distinguirlo de nIL-1Fm, que ha sido recientemente descrito en trucha arcoíris (Wang et al., 2009) y parece estar presente en todas las especies de teleósteos. Por otro lado, el análisis de sintenia demostró que el

locus de la IL-1Fm2 de distintas especies comparte muchos genes y su orden. Además, también se comparten varios genes entre los loci de la IL-1 β y la IL-1Fm2, sugiriendo que la IL-1 β y la IL-1Fm2 surgieron de la duplicación genómica específica de los teleósteos (Meyer y Van de Peer, 2005).

La IL-1Fm2 se expresó ubicuamente en tejidos y órganos de dorada y se induce débilmente después de una infección. Estudios de expresión *in vitro* tanto en granulocitos acidófilos como en macrófagos con distintos PAMPs mostraron una inducción débil en comparación con la inducción provocada en las mismas condiciones para la IL-1 β , lo que indica que la expresión de estos dos genes está regulada de forma diferente.

La proteína recombinante IL-1Fm2, producida en *E. coli*, fue capaz de activar, de una forma dosis dependiente, el estallido respiratorio de leucocitos de dorada. Estos efectos no se vieron aumentados por la adición de ADN bacteriano (agonista del TLR9). Además, el efecto era específico de la proteína recombinante, ya que se logró revertir mediante el uso de un anticuerpo neutralizante contra IL-1Fm2 y mediante pre-tratamiento con calor. Además, la IL-1Fm2 recombinante fue capaz de inducir la expresión de los genes que cifran varias citoquinas pro- (IL-1 β , TNF α , IL-8) y anti-inflamatorias (IL-10) de forma sinérgica con PAMPs. Es interesante destacar que la combinación de IL-1Fm2 recombinante y ADN bacteriano indujo de forma sinérgica la expresión del gen de la IL-1Fm2.

Finalmente, se analizó los niveles de proteína en extractos celulares y sobrenadantes en macrófagos estimulados con la IL-1Fm2 recombinante y ADN bacteriano mediante el uso de varios anticuerpos monoclonales obtenidos frente a la IL-1Fm2 recombinante. Se observó que la forma precursora de la IL-1Fm2 se acumuló en los extractos celulares de macrófagos activados. Además, los anticuerpos reaccionaron con una proteína de aproximadamente 18 kDa en los sobrenadantes, que podría representar la forma madura de la IL-1Fm2. Es interesante resaltar que tanto un inhibidor específico para caspasa-1 como un inhibidor general de caspasas no lograron inhibir el procesamiento ni la liberación de la IL-1Fm2, como ocurre para el caso de la IL-1 β .

En conclusión, la identificación de este nuevo miembro de la familia de la IL-1, IL-1Fm2, ayuda a arrojar luz sobre la evolución de esta familia de citoquinas en vertebrados e indica su gran complejidad.

CONCLUSIONES

Los resultados de este trabajo conducen a las siguientes conclusiones:

1. El inflammasoma y la caspasa-1 no son necesarios para el procesamiento de la IL-1 β en dorada.
2. El inflammasoma y la caspasa-1 regulan la muerte celular por piroptosis y la eliminación de *S. typhimurium* en dorada.
3. PYCARD es requerido para la activación de caspasa-1 en larvas de pez cebra, pero no es necesario para la eliminación de *S. typhimurium*.
4. Caspasa-1 no es necesaria para la eliminación de *S. typhimurium* en larvas de pez cebra. Sin embargo, otra caspasa, que tiene que ser identificada, está involucrada en este proceso.
5. La IL-1Fm2 es un nuevo miembro de la familia de la IL-1 y no un ortólogo de la IL-1 β del tipo I de teleósteos. Además, la IL-1Fm2 está presente exclusivamente en los teleósteos más avanzados evolutivamente, la Serie Percomorfa, aunque está ausente en peces globo, orden Tetraodontiformes, los cuales también pertenecen a la Serie Percomorfa.
6. La IL-1Fm2 se induce muy débilmente por PAMPs en macrófagos y granulocitos acidófilos de dorada. Sin embargo, la IL-1Fm2 recombinante y el ADN bacteriano inducen sinérgicamente la expresión del gen de la IL-1Fm y la de los genes que cifran la IL-1 β , el TNF α , la IL-8 y la IL-10.
7. La IL-1Fm2 recombinante y el ADN bacteriano promueven la acumulación intracelular de la forma precursora de IL-1Fm2 y la liberación de una forma procesada de unos 18 kDa en macrófagos de dorada. Además, este procesamiento y liberación es independiente de las caspasas.

