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Molecular and functional characterization of zebrafish Gbp4: a new inflammasome component

Caracterización molecular y funcional de Gbp4 de pez cebra: un nuevo componente del inflamasoma

Dña. Sylwia Dominika Tyrkalska

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The work of science is to substitute facts for appearances, and demonstrations for impressions.

John Ruskin

Zadanie nauki polega na tym, by zastąpić wizję faktami, a wrażenia dowodami.

John Ruskin

ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
ºC	Celsius degrees
Ab	Antibody
AD	*Anno Domini*
AGs	Acidophilic granulocytes
AIM2	Absent in melanoma 2
Ala	Alanine
ALRs	AIM2-like receptors
ANOVA	Analysis of variance
AP-1	Activator protein 1
Arg	Arginine
As	Antisense
Asp	Aspartic acid
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	Adenosine triphosphate
auf	Arbitrary units of fluorescence
BCA	Bicinchoninic acid
Bcl2	B-cell lymphoma 2
BSA	Bovine serum albumin
C+	Positive control
C-terminal	Carboxi-terminal
CARD	Caspase Activation and Recruitment Domain
CD	Crohn´s Disease

cDNA	Complementary DNA
CEBP	CCAAT-enhancer-binding protein
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
COX	Cyclooxygenase
CSFs	Colony stimulating factors
CSIC	Consejo Superior de Investigaciones Científicas
CTL	Cytotoxic T cells
Cxcr2	Chemokine (C-X-C Motif) receptor 2
Cys	Cysteine
Da	Dalton
DAMP	Damage-associated molecular pattern molecules
DAPI	4',6-diamidino-2-phenylindole
DM	Double mutant
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpf	Days post-fertilization
dsDNA	Double-stranded DNA
DsRed	Red fluorescent protein from *Discosoma sp.*
DTT	Dithiothreitol
eATP	extracellular ATP

ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ENA	European nucleotide archive
EU	European Union
eGFP	Enhanced GFP
F	Forward primer
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
FFP_ITN	FishForPharma Initial Training Network
Fli	Friend leukemia integration
Gbp	Guanylate binding protein
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Guanosine monophosphate
GTP	Guanosine-5'-triphosphate
h	Hours
h	Human
H.	Hamilton

H3	Histone 3
HEK293	Human Embryonic Kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK	Head kidney
HMGB1	High-mobility group protein 1
hpf	Hours post-fertilization
hpi	Hours post-infection
IκB	Nuclear factor-κappa B inhibitor
IACUC	Institutional animal care and use committee
ID	Identification
IFN	Interferon
Ig	Immunoglobulin
iGLuc	Protease activity reporter assay
IHNV	Infectious hematopoietic necrosis virus
IL	Interleukin
IL-1RI	Type I IL-1 receptor
IL-1ra	IL-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
Ile	Isoleucine
IND	Indomethacin
IPAF	Ice protease-activating factor
IRGs	Immunity-related GTPases

ISDCI	International Society of Developmental and Comparative Immunology
JNK	c-Jun N-terminal kinase
LAF	Lymphocyte-activating factor
LB	Luria Bertani Broth
LC-MS/MS	Liquid Chromatography-tandem Mass Spectometry
Leu	Leucine
LPS	Lypopolisaccharide
LRR	Leucine-rich repeat
Ly	Lymphocytes
Lys	Lysine
Lyz	Lysozyme C
MΦ	Macrophages
mAbs	Monoclonal antibodies
MAPK	Mitogen-activated protein kinase
MDP	D-γ-glutamyl-meso-DAP(mDAP) and muramyl dipeptide
MHC	Major histocompatibility complex
MO	Morpholino
MOI	Multiplicity of infection
Mpeg1	Macrophage expressed protein 1
Mpx	Myeloperoxidase
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA

myc	Myelocytomatosis
N-terminal	Amino-terminal
NADPH	Nicotinamide adenine dinucleotide phosphate
NAIP	Neuronal apoptosis inhibitor protein
NACHT	Neuronal apoptosis inhibitor protein
NBS	Nucleotide binding site
NF-κB	Nuclear factor kappa B
NLRs	NOD-like receptors
NLRCs	NLR-CARD containing family
NLRPs	NLR-PYRIN containing family
NOD	Nucleotide – binding oligomerization
ns	Not significant
p	p-value
P2X7	P2X purinoceptor 7
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGN	Peptidoglycan
PhD	*Philosophiae doctor*
Phe	Phenylalanine
PI	Propidium iodide
PIs	Principal investigators
PMSF	Phenylmethanesulfonylfluoride

PRRs	Pattern recognition receptors
PTG	Prostaglandin
PTGS	Prostaglandin-endoperoxide synthase
PYD	Pyrin domain
R	Reverse primer
RIG-1	Retinoic acid-inducible gene 1
RIP	Receptor interacting protein
RLRs	RIG-1 like receptors
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rps11	Ribosomal protein S11
RT	Reverse transcription
qPCR	Quantitative PCR
RTS-11	Mononuclear phagocyte cell line of rainbow trout
S. Typhimurium	*Salmonella* Typhimurium
SCV	*Salmonella*-containing vacuole
SDS-PAGE	*SDS*-poliacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
S.E.M	Standard error of the mean
SIF	*Salmonella* induced filaments
SP-1	Specificity protein 1
SPI	*Salmonella* Pathogenicity Island

Std	Standard control
subsp.	Subspecies
T3SSs	Type III Secretion System
Tg	Transgenic
Th	Helper T cell
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TXAs	Thromboxanes
USA	United States of America
wt/vol	Weight/Volume
ZDM	Zebrafish Disease Models
ZIRC	Zebrafish international resource center
ZFNs	Zinc finger nucleases

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SUMMARY

Summary

The nucleotide-binding domain leucine-rich repeats (NLRs) constitute a family of cytosolic pattern recognition receptors (PRRs), which are the responsible for the caspase-1-mediated processing and activation of pro-inflammatory cytokines, such us interleukin-1β (IL-1 β) and IL-18 , and the induction of new form of cell death called pyroptosis. NLRs achieve these functions by forming multiprotein signaling platforms, called inflammasomes, which alert the immune system about the presence of infection or tissue damage. Here we report that zebrafish Gbp4, an IFNγ-inducible GTPase harboring a C-terminal CARD domain, is expressed in neutrophils and is required for the inflammasome-dependent clearance of *Salmonella* Typhimurium *in vivo*. Despite the presence of the CARD domain, Gbp4 required the universal inflammasome adaptor Asc for mediating its antibacterial function. In addition, the GTPase activity of Gbp4 was indispensable for inflammasome activation, neutrophil recruitment and *S.* Typhimurium clearance. Reconstitution of Gbp4-Asc complexes in human embryonic kidney 293 cells revealed a macromolecular complex with an outer ring of Asc and an core of Gbp4. Mechanistically, Gbp4 was essential for the inflammasome-dependent release of prostaglandins, of which PGD2 is associated with the Gbp4-mediated bacterial clearance. Our results therefore point to GTPase-binding proteins as the key inflammasome adaptors required for prostaglandin biosynthesis and intracellular bacterial clearance by neutrophils *in vivo*.

INTRODUCTION

1. Immunity

Immunity is the balanced state of having adequate biological defenses to fight with the infection, disease, or other unwanted biological invasion caused by microorganisms (viruses, bacteria, fungi, protozoa and multicellular parasites) and macromolecules (proteins and polysaccharides), excluding the pathological result of such a reaction such as allergy or autoimmune diseases (Beck and Habicht, 1996). The immune system is composed of cells and molecules that are responsible for immunity, and the collective and coordinated response against mentioned foreign substances. Due to the wide variety of infectious agents, which our organism finds, it requires a variety of immune responses to combat each type of infection (Male, 2006).

The immune response starts with the recognition of the pathogen or foreign material, and ends with the development of a mechanism able to remove it (Male, 2006). The immune response can be divided into two branches: innate which is natural, and adaptive, which is acquired, specific, long-lasting and requires the recognition of specific "non-self" antigens (Pancer and Cooper, 2006). The fundamental difference between them is that the adaptive response is highly specific for a particular pathogen and is more effective with each successive encounter with the same pathogen. Therefore, we can say that the two key features of the adaptive immune response are specificity and memory (Male, 2006).

Innate immune response includes physical barriers, like skin, phagocytic cells, such as macrophages, neutrophils or dendritic cells, eosinophils, natural killer cells and finally various blood molecules (complement and acute phase proteins) (Zen and Parkos, 2003; Male, 2006; Mollen *et al*., 2006; Abbas *et al.*, 2015), acting as the first line of defense against infection until the specific response is triggered.

Adaptive response includes lymphocytes (Ly) and secreted antibodies. It appears exclusively in vertebrates and allows for a stronger immune response as well as immunological memory (Abbas *et al.*, 2015; Pancer and Cooper, 2006). The Ly are derived from hematopoietic stem cells in the bone marrow (Janeway and Medzhitov, 2002) and are capable of specifically recognizing individual pathogens. This group is divided into two main categories of cells: T Ly (T cells) and B Ly (B cells). B cells are responsible for the recognition and combating extracellular pathogens and their products through their secretions, called antibodies. They have the binding property with high

specificity to a target of foreign molecule, called an antigen (Sproul *et al*., 2000). T cells, in turn, have a large number of activities. T helper Ly (Th) have regulatory functions and are involved in the development and production of antibodies (Ab) by B cells or interacting with phagocytic cells helping them to destroy the pathogens phagocytosed before. These cells have no cytotoxic activity and do not kill infected cells or clear pathogens directly. Another type of T Ly are cytotoxic T cells (CTL) that recognize and destroy virus-infected cells (or cells infected with other pathogens), damaged or dysfunctional and tumour cells (Harty *et al*., 2000).

The immune system as a whole represents a very complex interacting network and acts in an integrated and coordinated way. The communication between the innate and adaptive immune systems involves cell-cell interactions in relation to antigen presentation or soluble molecules, such as cytokines or chemokines. These are, not necessarily, mutually exclusive interactions. However, the cross-talk between the innate and adaptive systems may be bidirectional (Getz, 2005). There is considerable interaction between Ly and phagocytes (Male, 2006). For example, some phagocytes capture and degrade antigens and present them to T cells. The presentation of the antigen, which is attached to the surface to major histocompatibility complex receptor (MHC), occurs in a form suitable for both of them (Holtmeier and Kabelitz, 2005). This process is called antigen presentation. In response, the Ly secrete soluble factors (cytokines), which activate phagocytes to destroy the pathogens that they have phagocytosed before. The result of these interactions is that the majority of immune responses against pathogens consist of a wide variety of innate and adaptive components. In the early stages of infection, the innate response predominates but Ly subsequently begin to generate the adaptive response.

1.1. Immune system of teleost fish

In teleost fish, the immune system shows similar characteristics to those of birds and mammals, displaying cellular and humoral responses that have the features of specificity and memory (Van Muiswinkel, 1995), although there are some important differences (Uribe *et al*., 2011). Teleost is the first animal group that have an innate and adaptive immune system well-structured and differentiated. Its innate response comprises physical barriers (epithelium and mucosa), cellular effectors (phagocytic cells and nonspecific cytotoxic cells) and humoral factors (complement and other acute phase

proteins), whereas adaptive response comprises a cellular (Ly) and humoral (Ab) components (Uribe *et al*., 2011). However, despite their similarities with other vertebrate immune systems, there are clear differences as fish depend more heavily on innate defence mechanisms, mainly in low temperature conditions (the fish are poikilothermic) since the adaptive immune response is dependent on the temperature (Cuchens and Clem, 1977; Avtalion and Clem, 1981; Abruzzini *et al*., 1982; Clem *et al*., 1984; Clem *et al*., 1991; Clem *et al*., 1985). In contrast to higher vertebrates, fish are free-living organisms from early embryonic stages of life and depend on their innate immune system to survive (Rombout *et al*., 2005).

The organs and tissues of the immune system in teleosts have been classified, as in mammals, in primary and secondary organs (Zapata *et al.*, 1996). Fish lack bone marrow; its function takes the kidney, a primary organ that is the largest site of hematopoiesis until adulthood. Kidney consists of two parts: the anterior or cephalic (head kidney, HK), with mainly hematopoietic function, and subsequent or posterior, basically with excretory function. Regarding to secondary lymphoid organ, the spleen is the most important but presents few Ly, although may increase in number by administration of an antigen. The spleen in zebrafish remains a small organ that contains large amounts of erythroblasts at 30 days post fertilization. At three months, when lymphoblasts are evident in the spleen, emerging ellipsoids are involved in the capture of antigen. A similar developmental pattern has been described for other teleost spleen, such as that of the Atlantic salmon, grouper and catfish (dos Santos *et al*., 2000; Petrie-Hanson and Ainsworth, 2000).

1.1.1. Innate immune system

The first line of the defence of fish against invasion of microorganisms consists of physical and chemical barriers, such as scales, flakes, gills, skin and its secretions (mucus) (Ingram, 1980; Shepard, 1994; Ellis, 2001). The most important function of the mucus is to prevent attachment and inhibit the entry of bacteria, fungi or parasites to the epithelial surfaces and digest microorganisms, thanks to a battery of lytic enzymes, such as lysozyme and lectins, complement proteins, antibacterial peptides and immunoglobulin M (IgM) (Alexander and Ingram, 1992; Rombout *et al*., 1993; Aranishi and Nakane, 1997; Boshra and Sunyer, 2006; Saurabh and Sahoo, 2008).

Innate cellular response of the fish includes a variety of leukocytes, which include phagocytes (monocytes/ MΦ and granulocytes) and nonspecific cytotoxic cells (Secombes, 1996). Phagocytes are very important in innate immunity by its capacity to eliminate viruses, bacteria and parasites because their function is less influenced by the temperature (Rowley *et al*., 1988; Secombes and Fletcher, 1992; Blazer, 1991; Sepulcre *et al*., 2002; Lange and Magnadottir, 2003; Magnadottir *et al*., 2005). Moreover, they can be the initiator of activation and regulation of the specific immune response (Clem *et al*., 1985; Clem *et al*., 1991; Vallejo *et al*., 1992). The process of phagocytosis in fish is almost the same as described for mammalian leucocytes, ending with two mechanisms responsible for the killing of phagocytized microorganisms. The first one is the production of reactive oxygen intermediates (ROS) with a rapid and abrupt increase in the rate of oxygen consumption, known as respiratory burst, being independent on mitochondrial respiration. The second one is the production of nitric oxide (NO) and other nitrogen reactive intermediates (RNS). It is further known that ROS produced by phagocytes of fish have bactericidal activity (Sharp and Secombes, 1993; Skarmeta *et al.*, 1995). In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules, which in the presence of halide and hydrogen peroxide kills bacteria by halogenation of the bacterial cell wall. Moreover, these cells use lysozymes and other hydrolytic enzymes of lysosomes for pathogen elimination (Fischer *et al*., 2006). On the other hand, the nonspecific cytotoxic cells of fish are morphologically distinct from the large granular lymphocytes of mammals, whereas, they are suggested to be functionally similar (Evans and Jaso-Friedmann, 1992). These cells are able to eliminate a range of spontaneously xenogeneic targets, including parasites in fish and traditional targets of natural killer cells in mammals (Hasegawa *et al*., 1998). Differently than the natural killer cells of mammals, the nonspecific cytotoxic cells of catfish are not granulated, small Ly that are commonly found in lymphoid tissues, but are rarely found in the blood (Shen *et al*., 2002).

Beside cellular effectors described above, there are a wide variety of substances (humoral effectors) that act on the innate defence of the fish (Alexander and Ingram, 1992). These molecules may be classified into different groups due to its function. There are bacterial growth inhibitors such as: transferrin, antiproteases and ceruloplasmin; viral replication inhibitors such as interferon; inhibitors of bacterial toxins; lysines such as lysozyme and chitinase; agglutinins and precipitins such as

lectins and C-reactive protein, and finally complement components. In teleosts, as well as in higher vertebrates, the complement system can be activated in three different ways: the classical pathway, which is triggered by Ab binding to the cell surface (Holland and Lambris, 2002), the alternative pathway, which is independent of antibodies and is activated directly by foreign microorganisms, and the lectin pathway, which is activated by the binding of a protein complex consisting of mannose/mannan-binding lectin in bacterial cells (Sakai, 1992). However, the mechanisms and molecules involved in this system in teleosts are not well understood, with the exception of the genetic sequence of the mannose-binding lectin protease that is associated with serum (Matsushita *et al*., 1998; Nikoskelainen *et al*., 2002). The complement system performs several functions important for the humoral clearance of the pathogens, such as leukocyte chemotactic activity (Lamas and Ellis, 1994), opsonisation (Sakai, 1984a), inactivation of certain toxins (Von Eschen and Rudbach, 1974; Ellis, 1980; Sakai, 1984b), the bactericidal activity (Sakai, 1983), cytotoxicity and viral inactivation (Sakai, 1992), are the most remarkable.

1.1.2. Adaptive immune system

Antibodies are the key mediators of the adaptive immune response, together with its producing cells, Ly. The main, predominant immunoglobulin in teleosts is the IgM class. It is a tetramer and contains eight antigenic combining sites (Acton *et al*., 1971). Some teleosts have a monomer of IgM in their serum, but the factors leading to its expression are still unknown (Wilson and Warr, 1992). *In vitro* studies, using conjugated monoclonal antibodies (mAbs) against specific antigenic determinants on the surface of Ly and functional immunological assays, have shown that fish have two cell populations, which are equivalent to B and T cells of mammals. mAbs against immunoglobulin M (IgM) of teleost serum are able to react with only one of the Ly population (Lobb and Clem, 1982; DeLuca *et al*., 1983; Secombes *et al*., 1983; Navarro *et al.*, 1993), which suggest that the surface of Igs may be a marker for B cell-like cells and allow the isolation of the two cell populations of lymphocytes of teleost: Ig+ e Ig-. Further, due to the functional studies, it has been shown that these two populations in fish (Ig+ and Ig- Ly) have the functional characteristics of the B and T Ly of mammals, respectively (DeLuca *et al*., 1983; Sizemore *et al*., 1984; Miller *et al*., 1986; Marsden *et al.*, 1995). Moreover, IgD as a second immunoglobulin isotype (Edholm *et al*., 2011) and IgT as a third one (Danilova *et al*., 2005; Hansen *et al*., 2005; Uribe *et al*., 2011)

have been also identified in fish. Although the functional relevance of IgD remains to be determined, as in mammals, the teleost-specific IgT seems to be specialized in mucosal immunity. Therefore, those antibodies would play the functional role as the equivalent of mammalian IgA (Zhang *et al*., 2010; Xu *et al*., 2013).

The immunological response of the skin and gills is important because they are in direct contact with the environment. Therefore, teleost antibodies are found not only in intestine (Rombout *et al*., 1986; Jones *et al*., 1999), bile (Jenkins *et al*., 1994) and systemically in the plasma, but also in the skin (Hatten et al., 2001; Cain et al., 2000) and gill mucus (Lumsden *et al*., 1993). Moreover, specific antibodies can be produced without generating a systemic response (Uribe *et al*., 2011).

Likely mammalian, adaptive immune system of teleost presents memory before a second exposure to an antigen (Arkoosh and Kaattari, 1991; Whittington *et al*., 1994; Van Muiswinkel, 1995). After a first contact with the antigen, primary response develops with the production of a specific titer of antibodies in the serum that is increased with the further contact with the same antigen (secondary response). The whole process is dependent on the temperature.

As previously mentioned, the initiation of the adaptive immune response is controlled by MΦ which functions consist of degrading and presenting antigens together with MHC proteins to Ly so that they can recognize the determine antigen (Clem *et al*., 1985; Vallejo *et al.*, 1992). However, the recent studies in zebrafish shown the existence of a population of dendritic cells with antigen-presenting properties (Lugo-Villarino *et al*., 2010).

1.2. Regulatory molecules of the immune response

1.2.1. Cytokines

Cytokines are proteins, usually glycoproteins with a low molecular weight that does not exceed 8-25 kDa. They regulate all the important biological processes, including cell growth and activation, inflammation, tissue repair, fibrosis and morphogenesis and take part in cell signalling. They are released by cells and affect the behavior of other cells, and sometimes the releasing cell itself. They are considered as a protein family from a functional point of view, since not all of them are chemically and

structurally related (Marini, 2006). However, there are some cytokines that share a high homology (about 30%), like interleukin-1β (IL-1β) and IL-1α, or tumoral necrosis factor α (TNF-α) and β (TNFβ). In addition, there are subfamilies with a really high structural homology (about 80%), like the interferon α (IFNα) subfamily with about 20 members.

Cytokines operate as the intermediators in both innate and adaptive immunity and are produced by a broad range of cells (Abbas *et al.,* 2015). In the innate immunity, cytokines are produced mainly by mononuclear phagocytes (monocytes and MΦ), so they are usually called monokines. They are produced in the response to pathogens and upon T-cell antigen stimulation as part of adaptive immunity. Nevertheless, most of the cytokines that are involved in adaptive immunity are produced by activated T Ly and named as lymphokines. Lymphokines have many roles exerting cross regulatory or inhibitory effects (Seder, 1994), including the regulation of the proliferation and differentiation of different Ly populations and participating in the activation and regulation of inflammatory cells such as mononuclear phagocytes, neutrophils and eosinophils. Both Ly and mononuclear phagocytes produce other cytokines known as colony stimulating factors (CSFs), which activate the intracellular signalling to stimulate the proliferation and differentiation of immature leukocytes in the bone marrow and induce the haematopoiesis (Cheers *et al*., 1988). Finally, the other type of cytokines that induce directed chemotaxis for specific cell types are known as chemokines. They are considered to have pro-inflammatory properties and can be induced during an immune response to recruit cells of the immune system to a site of the infection. However, they can also be considered homeostatic being involved in control of the migration of cells during normal processes of tissue maintenance or development (Lee *et al*., 2000).

Although cytokines are made up of a diverse group of proteins, they share some features listed below (Abbas *et al.,* 2015):

- The production of the cytokines occurs during the effector stages of the innate and adaptive immunity to regulate the inflammatory and immune response.
- The secretion of the cytokines is brief and auto-limited. They 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.
- However, a particular cytokine may act on different cell types.
- Cytokines usually produce different effects on the same target cell, simultaneously or not.
- The effect produced by different cytokines can be similar.
- The synthesis and activity of cytokines is dependent on other cytokines.
- The action of cytokines is due to their binding to specific and high affinity receptors, which are present on the target cell surface. This action can be autocrine, paracrine or endocrine.
- The cytokine receptors expression is modulated by specific signals that arrive from other cytokines or even the same ones.
- For many target cells, cytokines act as proliferation factors.

In fish, cytokines are grouped into growth factors (Grondel and Harmsen, 1984; Lawrence, 1996; Yin *et al.,* 1997), pro-inflammatory cytokines (Jang *et al*., 1995a; Jang *et al*., 1995b; Zou *et al*., 1999a; Zou *et al*., 1999b; Fujiki *et al*., 2000), chemokines (Daniels *et al*., 1999; Fujiki *et al*., 1999; Laing *et al*., 2002), immunosuppressive or anti-inflammatory cytokines (Sumathy *et al*., 1997; Laing *et al*., 1999; Harms *et al*., 2000) and IFNs (Congleton and Sun, 1996; Collet and Secombes, 2002; Hansen and La Patra, 2002).

1.2.2. Lipid mediators

Lipids, a group of naturally occurring molecules, in addition functioning as an energy source and as structural components of the cell membrane, act as effectors and second messengers in a variety of biological processes (Cabral, 2005; Fahy *et al*., 2009; Subramaniam *et al*., 2011). The structural composition of lipid mediators can differ exerting multiple effects on cellular functions associated with homeostasis, immune response and inflammation.

Bioeffector lipids are produced as a result of cutting the phospholipids located in cell membrane by the action of phospholipases. These enzymes are activated in response to a variety of extracellular stimuli such as bacterial peptides, cytokines,

growth factors and mechanical trauma. The activity of lipid mediators as second messengers may be intracellularly or extracellularly. The extracellular signalling can occur through G-protein coupled receptors. The lipid bioeffectors are able to act as immune effector molecules, complementing the activities of pro-inflammatory and anti-inflammatory non-lipid immune modulators.

Arachidonic acid is the precursor of the immune active lipids, collectively called eicosanoids. These signaling molecules are made by oxidation of 20-carbon fatty acids (Fahy *et al*., 2009; Subramaniam *et al*., 2011). They are divided into several groups including prostanoids, leukotrienes, lipoxins and endocannabinoids. Major producer of eicosanoids bioeffectors are cells derived from the myeloid lineage (platelets, monocytes, macrophages, neutrophils and mast cells) (Harizi and Gualde, 2002), with the exception of leukotrienes that are produced by other non-immune cells. There are several enzymes that regulate cellular levels of arachidonic acid. They keep it in esterified form until it is mobilized by phospholipases. The inflammatory stimuli are needed for the induction of the translocation of cytosolic phospholipase to the endoplasmic reticulum and nuclear membrane to release arachidonic acid from the lipid membrane of these cell organelles.

An important group of eicosanoids are prostanoids, which includes prostaglandins (PTG), thromboxanes (TXA) and prostacyclins. These molecules are synthesized *de novo* by the action of two cyclooxygenase isoforms, COX-1 (PTGS1) or COX-2 (PTGS2) from arachidonic acid released from cell membrane. This process is based on the catalysis of the conversion of the free essential fatty acids to prostanoids by a two-step process. These enzymes act upstream of a variety of isomerases whose action culminates in the production of PTGA2, PTGD, PTGE2, PTGI2 and TXA. COX-1 play important role in the housekeeping prostaglandin production which occurs on a continuous basis in the organism. However, COX-2 is involved in the synthesis of pro-inflammatory prostaglandins and its expression is very low or undetectable in the majority of cells and increases significantly after stimulation with LPS, IL-1 or TNFα, particularly in immune cells (Smith *et al*., 1996; Woodward *et al*., 2008) (Figure 1).

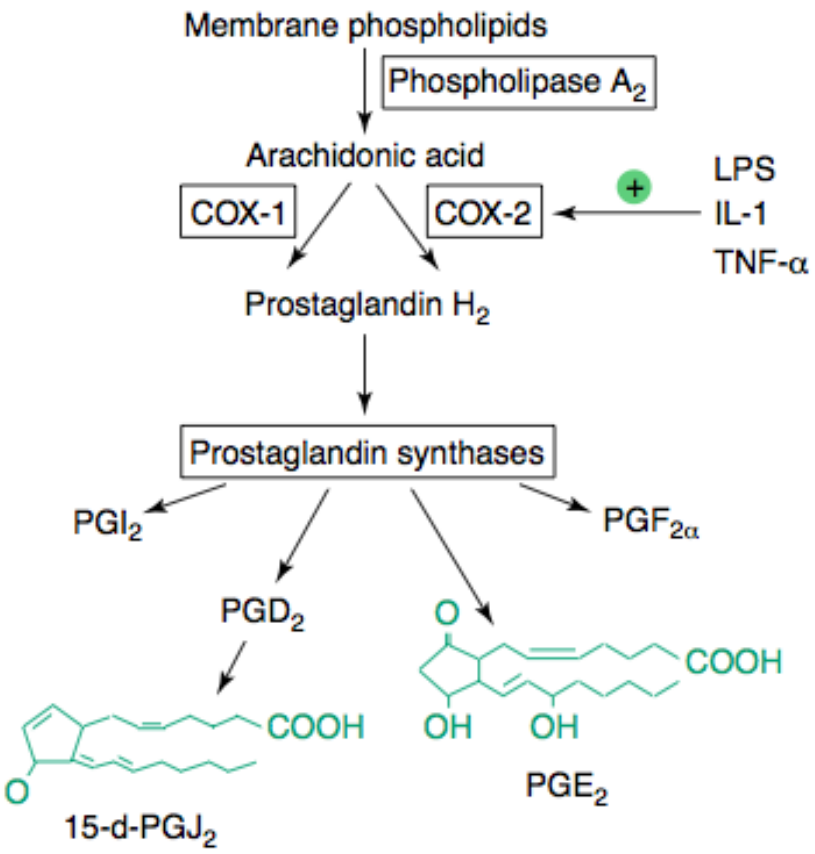


Figure 1. Pathway of prostaglandin production. Cytosolic PLA2 releases arachidonic acid from membrane phospholipids and COX enzymes convert it to PGH2, the common precursor for all PGs. PGH2 is then converted into one of the active PG by specific terminal synthases such as PGE synthases, PGF synthases, PGD synthase and PGI synthase. PGD2 converts spontaneously into active PGJ2 (Harris *et al*., 2002).

The name *prostaglandin* derives from the prostate gland. When it was first isolated from seminal fluid in 1935 by the Swedish physiologist Ulf von Euler (Von Euler *et al*., 1935), and independently by M.W. Goldblatt (Goldblatt, 1935), it was believed to be part of the prostatic secretions. However, it was later shown that many other tissues secrete prostaglandins for various functions. The structural differences between prostaglandins are associated with their different biological activities. A given prostaglandin may have different and even opposite effects on different tissues. The ability of the same prostaglandin to stimulate a reaction in one tissue and inhibit the same reaction in another tissue is determined by the type of receptor to which the prostaglandin binds. In this way, they can act as anti-inflammatory or pro-inflammatory mediators depending on the context. Prostaglandins can act as autocrine effectors or on target cells present in the neighborhood of the range of their secretion. They differ from

endocrine hormones because they are not produced at a specific site but in many places throughout the human body (Sheddan and Mulzer, 2006). Specific prostaglandins are named with a letter, which indicates the type of ring structure, followed by a number, which indicates the number of double bonds in the hydrocarbon structure (Woodward *et al*., 2008; Claar *et al*., 2015).

1.3. The innate immune system receptors

The innate immune system detects the presence of microbes and initiates mechanisms to eliminate potentially infectious threats. Microbial detection is achieved through germline-encoded pattern-recognition receptors (PRRs) that survey both the extracellular and intracellular space for conserved microbial determinants that work as indicators of the infection (Kumar *et al*., 2011). Most PRRs can be classified into two main classes: membrane-bound receptors and unbound intracellular receptors. The first class consists mainly of the TLRs, which are found at the cell surface or on endocytic compartments, hence they survey for the presence of microbial ligands in the extracellular space and within endosomes. The second class consists of leucine-rich repeat (LRR)-containing (or NOD-like) receptors (NLRs), RIG-1 like receptors (RLRs), the AIM2-like receptors (ALRs). They are located in the cytoplasm, where they sense the presence of intracellular pathogens (Kumar *et al*., 2011; Brubaker *et al*., 2015).

1.3.1. Toll – like receptors (TLRs)

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. As mentioned before the TLRs belong to the PRRs (Janeway and Medzhitov, 2002; Janeway *et al*., 2005), that can recognize pathogen-associated molecular patterns (PAMPs) which are shared by many pathogens but not by the host cells. That mechanism triggers the intracellular signaling pathways, which results in releasing of pro-inflammatory cytokines, chemokines, interferons (IFNs), and prostanoids, as well as lead to the expression of co-stimulatory molecules (Akira *et al*., 2006; Chen *et al*., 2015).

TLRs are single, membrane-spanning, non-catalytic receptors usually expressed in "guardian cells", such as macrophages and dendritic cells. They are believed to function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having different ligand specificity. The

recognition of structurally conserved molecules derived from microbes occurs through leucine-rich repeats (LRRs) in the extracellular domains that are involved in ligand binding and auto-regulation (Kawai and Akira, 2006). PAMPs represent vital molecules important 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 signaling cascades, generally leading to the activation of NF-κB, activator protein-1 (AP-1) and type I IFN. Moreover, TLRs are key inducers of the pro-inflammatory cytokines IL-1β and IL-18, but do not directly contribute to the activation of inflammatory caspases (Takeda and Akira, 2005).

In zebrafish, 15 Tlrs have been identified, including Tlr 1, 2, 3, 4a/b, 5a/b, 7, 8a/b, 9, 14, 19, 20a, 21 and 22 (Meijer *et al*., 2004; Palti, 2011).

1.3.2. NOD-like receptors (NLRs) and Inflammasome

Apart from TLRs, other PRR families have been identified. A very important one is the NLR receptor family, which was described 15 years ago (Inohara *et al*., 1999; Inohara *et al*., 2000; Girardin *et al*., 2001; Hoffman *et al*., 2001; Albrecht *et al*., 2003). NLRs include 22 members identified in humans and more than 30 in mice (Ting *et al*., 2008; Schroder and Tschopp, 2010). The NLR family is a group of intracellular receptors that are able to detect pathogens that enter to the cell via phagocytosis or pores, and DAMPs that are associated with cell stress or danger signals occurring in the cytosol. The NLRs present three characteristic structural domains: a central nucleotide-binding and oligomerization (NACHT) domain, which is commonly flanked by C-terminal LRRs and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains. The C-terminal LRR domain is 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 PYD or CARD (Shaw *et al*., 2008; Franchi *et al*., 2009). The diversity of effector domains allows the NLRs to interact with a wide variety of binding partners and to activate multiple signalling pathways. The most

studied receptors belonging to this family are NOD1, NOD2, NLRP1, NLRP3, NLRC4 (formerly IPAF) and AIM2.

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 (Tschopp *et al*., 2003). These proteins are the ideal molecular platform needed for the activation of inflammatory caspases.

Based on the domains present in N-terminal region, the NLRs are classified in two subgroups: NLR*C*s (*C*ARD), and NLR*P*s (*P*YRIN). The NLR members NOD1 and NOD2 belong to the NLRC subgroup as they contain an amino-terminal CARD domain. NOD1 contains a single CARD domain, whereas NOD2 has two of them (Ogura *et al*., 2001; Moreira and Zamboni, 2012). 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.*, 2003a; Girardin *et al*., 2003b). NOD1 and NOD2 activate NF-κB through the recruitment and oligomerization of receptor-interacting protein 2 (RIP2), resulting in the activation of the IκB 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 and Tschopp, 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.

In contrast, other members of the NLR family, such as NLRPs and NLRCs, are involved in caspase-1 activation and the consequent formation of the inflammasome, a multiprotein complex (Poyet *et al*., 2001; Agostini *et al*., 2004; Baroja-Mazo *et al*., 2014). 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 define NLR, recruiting caspase-1 through CARD-CARD interactions, leading to its activation (Martinon and Tschopp, 2004; Sahoo *et al*., 2011). NLRC4, however, can interact directly with caspase-1 through its CARD domain and, therefore, ASC is probably not required in this process (Latz *et al*., 2013) (Figure 2).

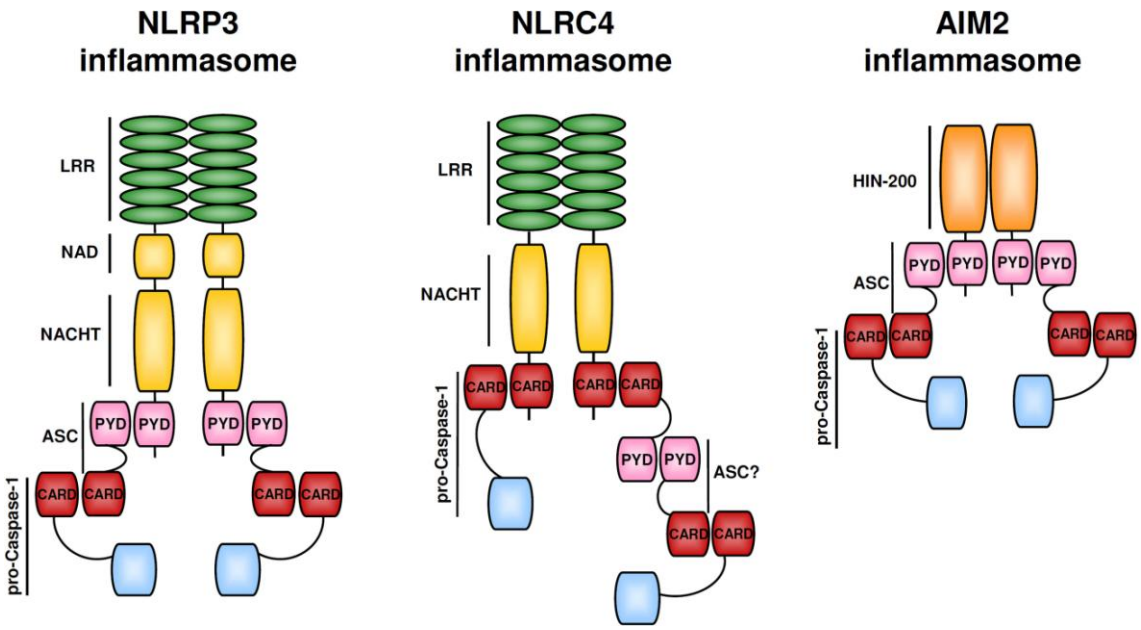


Figure 2. Inflammasome constituents. The NLRP3 inflammasome consists of NLRP3, ASC, and caspase-1. NLRC4 can directly interact with pro-caspase-1, but maximal NLRC4 inflammasome activation might require ASC. The AIM2 inflammasome is composed of AIM2, ASC, and caspase-1. Domains: CARD, caspase-recruitment domain; HIN-200 domain; LRR, leucine-rich repeat; NACHT, nucleotide-binding, and oligomerization domain; PYD, pyrin domain (Eitel *et al*., 2010).

Moreover, based on previously published results, a model for unified prion-like polymerization mechanism for the assembly of ASC-dependent inflammasome was proposed. Stimulation of NLRP3 or AIM2 induces a conformational change that result in the oligomerization or close apposition of their individual PYRIN domains (PYD). Subsequently, these oligomers, through PYRIN-PYRIN interactions recruit multiple ASC proteins resulting in prion nucleation, which is otherwise prevented from occurring spontaneously due to a high-energy barrier. ASC prions rapidly template other ASC molecules resulting in the formation of large polymers and through CARD-CARD interactions, the ASC polymers recruit multiple caspase-1 molecules, bringing

them into close proximity to induce their autocleavage and activation (Cai *et al*., 2014; Lu *et al*., 2014). The PYRIN domains of ASC acts as the building block of the filaments and the CARDs are flexibly linked to induce pro-caspase-1 polymerization and activation (Figure 3). Relative few sensor molecules are sufficient to induce polymerization of relative large numbers of ASC and caspase-1 molecules and also the process is self-propagating, this mechanism allows robust signal amplification. The principle of prion-like polymerization seems to be conserved in signal transduction because a fungal NOD-like pattern recognition receptor also induces conversion of a fungal prion (Cai *et al*., 2014; Ruland, 2014).

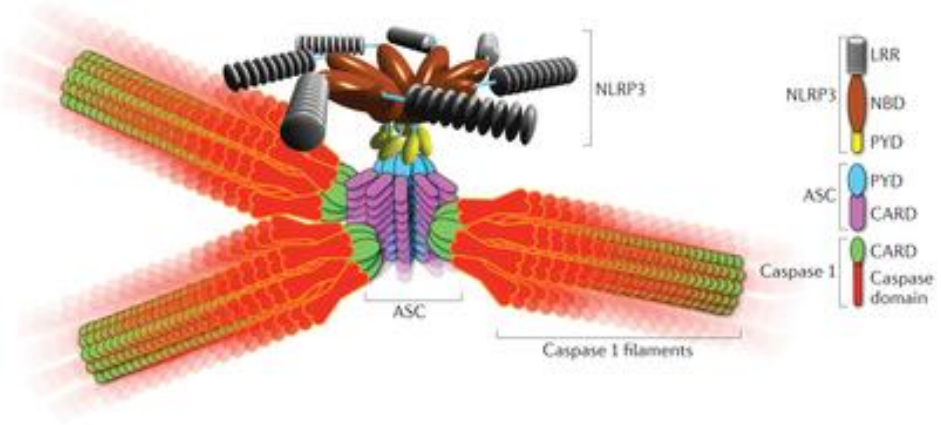


Figure 3. Model of inflammasome assembly. Up-stream sensing proteins, such as NLRP3, oligomerize upon activation to form a platform of PYDs that induces ASC filament assembly through PYD/PYD interactions. Multiple ASC^{CARD} molecules cluster to promote caspase-1 filament formation through CARD/CARD interactions. Proximity induced dimerization of the caspase domain activates the enzyme followed by auto-cleavage (Lu *et al.*, 2014).

It is thought that the number of inflammasome complexes forming in a cell is determined by the number of NLRs being activated. Each NLR initiating its own inflammasome assembly is independent of one another. However, it was shown that the important foodborne pathogen *Salmonella* enterica serovar Typhimurium (*S.* Typhimurium) simultaneously activates at least two NLRs, whereas only a single inflammasome complex is formed in a macrophage. Both NLRC4 and NLRP3 are simultaneously present in the same inflammasome, where both NLRs are required to drive IL-1β processing within the *Salmonella*-infected cell and to regulate and control

the bacterial burden in mice which is essential to surviving the infection (Man *et al*., 2014).

The direct activation of NLRs by PAMPs has not been demonstrated yet. For instance, the NLRC4 inflammasome is activated in response to two distinct bacterial proteins: flagellin (Franchi *et al*., 2006; Miao *et al*., 2006) and PrgJ (Miao *et al*., 2010a; Miao *et al*., 2010b), a conserved component of pathogen-associated type III secretion systems. However, the physical interaction between both PAMPs and NLRC4 has never been shown. The homologues of NLRC4, NAIP5 and NAIP6 (NLR family, apoptosis inhibitory protein 5 and 6), has been implicated in activation of NLRC4 acting as the activating proteins (Molofsky *et al*., 2006; Ren *et al*., 2006; Zamboni *et al*., 2006; Lightfield *et al*., 2008; Lightfield *et al*., 2011). NAIP5, as well as, NAIP6 directly and specifically interact with flagellin, which determins the inflammasome-stimulation activities of different bacterial flagellins. NAIP5 engagement by flagellin promotes a physical NAIP5–NLRC4 association, rendering full reconstitution of a flagellin-responsive NLRC4 inflammasome in non-macrophage cells. The related NAIP2 functioned analogously to NAIP5, serving as a specific inflammasome receptor for T3SS rod proteins such as *Salmonella* PrgJ and *Burkholderia* BsaK (Kofoed *et al*., 2011; Zhao *et al*., 2011).

In addition, post-translational modifications of NLRs, such as phosphorylation (Qu *et al*., 2012), ubiquitylation (Juliana *et al*., 2012; Py *et al*., 2013) and even proteolysis (D'Osualdo *et al*., 2011; Finger *et al*., 2012; Frew *et al*., 2012), have been suggested to be necessary for the activation of certain NLR sensors. Therefore, the modification of NLRs by host enzymes could be crucial for their activation and, at the same time, could represent a novel target for pharmacological intervention strategies.

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

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

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

It is now generally accepted that activation and release of IL-1β, in mammals, requires two distinct signals (Burns *et al*., 2003): a first signal leading to the synthesis of the proIL-1β by activation of the transcription factor NF-κB (Latz *et al.*, 2013), and a second one involved in caspase-1 activation and IL-1β release (Figure 4). 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-1β accumulation in the cytosol can be triggered by TLR activation. Different investigations indicate that the second signal, which leads to the activation of caspase-1 and inflammasome, being a physiological signal is extracellular ATP (eATP), since eATP is able to induce the rapid processing and the massive release of IL-1β 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 activates the P2X7 receptor and allows the efflux of intracellular potassium ions (K^+) resulting in NLRP3 activation (Franchi *et al*., 2007; Petrilli *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*., 2010). This phenomenon was negatively regulated by

the anti-apoptotic protein Bcl2, suggesting a link between apoptosis and inflammasome activation (Beal, 2003).

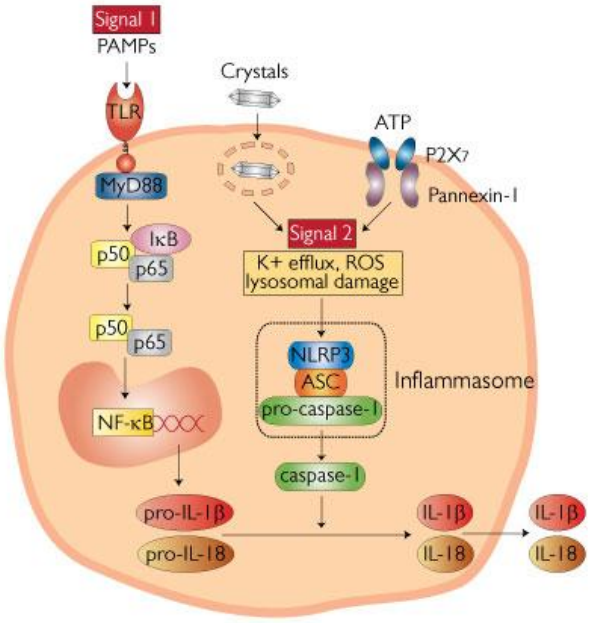


Figure 4. Production, maturation and release of IL1β require two distinct signals. The first signal leads to synthesis of pro-IL-1β and other components of the inflammasome, such as NLRP3 itself; the second signal results in the assembly of the NLRP3 inflammasome, caspase-1 activation and IL-1β secretion. Adapted from: http://www.invivogen.com/review-nlrp3-inflammasome.

The relevance of the NLR proteins, NLRP or NLRC, 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 *et al*., 2006; Mariathasan and Monack, 2007).

1.4. IL-1β, a member of the interleukin family

IL-1 is the common name for a diverse family of proteins (11 cytokines), of which IL-1α, IL-1β, 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; Debets *et al*., 2001; Lin *et al*., 2001; Pan *et al*., 2001). Members of that

family induce a complex network of pro-inflammatory cytokines and via expression of integrins on the surface of leukocytes and endothelial cells, regulate and initiate inflammatory responses (Dinarello, 2011).

Interleukin 1 was discovered by Igal Gery in 1972 (Gery *et al*., 1972; Gery and Waksman, 1972; Gery and Handschumacher, 1974). He named it lymphocyte-activating factor (LAF) because it was a lymphocyte mitogen. It was not known until 1985 that interleukin 1 consists of two distinct proteins, now called interleukin-1 alpha (IL-1α) and interleukin-1 beta (IL-1β) (March *et al.*, 1985). IL-1α is a protein that in humans is encoded by the *IL1A* gene (March *et al*., 1985; Nicklin *et al*., 1994), and IL-1β, also known as catabolin, by the *IL1B* gene (Auron *et al.*, 1984; March *et al*., 1985; Clark *et al*., 1986; Bensi *et al*., 1987).

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-1α and IL-1β 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-1α and IL-1β differ, being IL-1β but not IL-1α a potent activator of the humoral immune response (Nakae *et al.*, 2001). Both IL-1α and IL-1β 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 MΦ (Lepe-Zuniga 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 (Murzin *et al*., 1992; Nicola, 1994). The alignment of vertebrates IL-1β amino acid sequences shows higher homology in regions

containing the secondary structure of the IL-1β 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.*, 2002b).

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 (Arg^{4}, Leu^{6}, Phe^{46}, Ile^{56}, Lys^{93}, Lys^{103} and Glu^{105}) in human mature IL-1β, 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-1β molecule three-dimensional structure. Notably, they are all strongly conserved in mammal IL-1β proteins but not in the non-mammalian sequences known to date.

The IL-1 family signature is conserved in the IL-1β 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-1β sequences: [FCL]-x-S-[ASLV]-x(2)-[PSR]-x(2)-[FYLIV]-[LIV]-[SCAT]-T-x(7)-[LIVMK]. Although the sequences of IL-1β 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.*, 2002b), due to the great phylogenetic diversity existing within this group of animals.

IL-1α and IL-1β bind to the same receptor molecule, which is called IL-1RI. There is a third ligand of this receptor – the Interleukin 1 receptor antagonist (IL-1Ra), which does not activate downstream signaling, so it acts as an inhibitor of IL-1α and IL-1β signaling by competing with them for binding sites of the receptor (Weber *et al.*, 2010; Dinarello, 2011). IL-1α or IL-1β bind first to the first extracellular chain of IL-1RI, that recruits the IL-1 receptor accessory protein (IL-1RAcP), which serves as a coreceptor and is necessary for signal transduction and it is also needed for activation of IL-1RI by IL-18 and IL-33 (Weber *et al.*, 2010). Those signaling pathways lead to activation of many transcription factors, such as NF-κB, AP-1, c-Jun N-terminal kinase (JNK) and p38 MAPK (Simi *et al.*, 2007; Weber *et al.*, 2010).

The role of IL-1β in the immune response has been partly elucidated by expression studies. In mammals, IL-1β 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-1β show a similar expression pattern to its mammalian counterpart. In chicken, IL-1β is quickly induced in blood monocyte-derived MΦ, reaching optimal levels within 1 h after LPS treatment (Weining *et al*., 1998). The *Xenopus* IL-1β transcript was inducible *in vivo* following injection with LPS (Zou *et al*., 2000a). In fish, IL-1β 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-1β mRNA in all the species tested, including carp, trout, seabass and catshark (Zou *et al*., 1999a; Zou *et al*., 1999b; Zou *et al*., 2000b; Scapigliati *et al*., 2001; Engelsma *et al*., 2001; Bird *et al*., 2002a). In trout many expression studies have been developed. Thus, it is known that stimulation with 5 μg/ml LPS induces the expression of IL-1β 1 h after stimulation, reaching maximum levels after 4 h. In addition, culture temperature has a marked effect on IL-1β expression, because a temperature increase acts as a positive regulator in IL-1β synthesis (Zou *et al*., 2000b). Moreover, IL-1β expression levels of the trout mononuclear phagocyte cell line RTS-11 are up-regulated after LPS treatment (Brubacher *et al*., 2000). In seabream, IL-1β 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 Ab to seabream (Pelegrín *et al*., 2004).

The promoters of several non-mammalian IL-1β genes have also been cloned and characterized, such as those of trout (Wang *et al*., 2002) and catshark (Bird *et al*., 2002a). 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-κB element and a more upstream enhancer have also been identified (Hiscott *et al*., 1993; Goto *et al*., 1999). IL-1β is a strong inducer of NF-κB and is thought to positively auto-regulate its own synthesis, which appears to be true in fish since the addition of trout recombinant IL-1β to trout macrophages induced IL-1β expression (Wang *et al*., 2002). Preliminary studies using

the trout IL-1β promoter confirm that the NF-κB transcription factor site is required for expression of the trout IL-1β gene (Wang *et al*., 2002). Other potential elements for transcription activators in the trout and catshark promoters include sites for AP1, SP1 and CEBP.

1.5. Inflammatory caspases: Caspase-1

Caspases are a family of cystein proteases that cleave after aspartate residues (Asp) (Stennicke and Salvesen, 1998; Miao *et al*., 2011). Although caspase – mediated processing can result in substrate inactivation it may also generate active signaling that participates in ordered processes (McIlwain *et al*., 2013) and develop key roles in the apoptosis and proteolytic activation of cytokines (Nicholson, 1999; Boatright and Salvesen, 2003; Nadiri *et al*., 2006). In humans, the caspase family includes 13 members, whose functions seem to correlate with their phylogenetic relationship (Lamkanfi *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 of 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, situated in the cytosol and share a common conserved structure composed of a prodomain and a catalytic region (Lamkanfi *et al*., 2002; Nadiri *et al*., 2006; Miao *et al*., 2011).

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-1β processing (Thornberry *et al*., 1992), although later it was also shown to be able to activate IL-18 (Gracie *et al*., 2003; Martinon and Tschopp, 2004; Dinarello, 2005). While Caspase-1 was once thought to also process IL-33, this is no longer believed to be the case (Cayrol *et al*., 2009; Luthi *et al*., 2009; Talabot-Ayer *et al*., 2009; Ali *et al*., 2010). 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 that binds to capase-1, producing the oligomerization of pro-caspase-1 within the inflammasome, facilitating the caspase auto-cleavage into the p20 and p10 subunits (Stehlik *et al*., 2003; Mariathasan *et al*., 2004). These p10 and p20 subunits, once released, form an active heterotetramer that acts as a very efficient IL-1β-converting enzyme (Guey *et al*., 2014).

Although pro-caspase-1 is constitutively expressed in resting cells, it remains inactive in the cytoplasm until inflammatory cells, like monocytes or macrophages, receive the appropriate stimulus (Martinon *et al*., 2002). In these cells, K^+ release induces a rapid and strong activation of caspase-1, triggering the processing and release of the mature IL-1β (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-1β 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-κB activation via TLR2 and TLR4 (Miggin and O'Neill, 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. Inflammatory caspases have so far only been found in vertebrates (Martinon and Tschopp, 2004). In zebrafish and pufferfish, two subgroups of inflammatory caspases with a highly conserved caspase domain were discovered. The first subgroup is formed by Caspy-1 and Caspy-2 with highly conserved catalytic domain, and with an N-terminal pyrin domain (PYD) rather than the typical CARD of inflammatory caspases (Lamkanfi *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 gilthed seabream (López-Castejón *et al*., 2008) but this caspase-1 is unable to process IL-1β (Angosto et al., 2012).

It is known that stimuli such as bacterial products, ATP or nigericin, which alter the intracellular ionic milieu and result in cytosolic acidification and many others, 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).

1.6. Pyroptosis, a caspase-1 dependent form of programmed cell death

Many forms of the programmed cell death pathways are critical for organogenesis, development, immunity or even the maintenance of homeostasis in multicellular organisms. Pyroptosis, a highly pro-inflammatory form of cell death, is an essential innate immune response to prevent the spread of the intracellular infection. Pyroptosis is induced by the activation of pro-inflammatory caspases within inflammasomes (Boucher *et al*., 2015).

The existence of this unusual cell death was first seen in 1992, when the Sansonetti laboratory observed that murine macrophages infected with the Gram-negative bacterium, *Shigella* flexneri, were undergoing a form of cell death which was similar to apoptosis (Zychlinsky *et al*., 1992). Afterwards, the Falkow laboratory made similar observations in cells infected with a closely related pathogen, *Salmonella* Typhimurium, reporting the presence of DNA degradation, changes in nuclear morphology and finally vacuole formation (Monack *et al*., 1996). In addition to these apoptosis-like features, Cookson and co-workers reported that cell death by such infected cells also presented features similar to other death type: necrosis (Brennan and Cookson, 2000). These cells formed membrane pores of 1-2.5 nm and displayed swelling and Ca^{2+} influx, leading to membrane rupture and the extracellular release of cellular contents. This unusual form of cell death induced by pathogens, containing

hallmarks of both: apoptosis and necrosis (Brennan and Cookson, 2000), was found to be dependent on the proteolytic activity caspase-1 (Monack *et al*., 1996) (Figure 5).

The term 'pyroptosis' was coined by Cookson and Brennan in 2001 to simply distinguish this form of cell death from apoptosis and necrosis (Cookson and Brennan, 2001). It has only been described in macrophages and dendritic cells (Edgeworth *et al*., 2002; Fink and Cookson, 2007), although there is some evidence of caspase-1 activity in other cell types (Feldmeyer *et al*., 2007).

As mentioned before, many features of pyroptosis seem to be very similar with apoptosis, but upon further investigation are found to be different. Firstly, during pyroptosis, cells suffer the DNA damage (Zychlinsky *et al*., 1992) and become positive in the TUNEL assay (Chen *et al*., 1996; Brennan and Cookson, 2000). However, the nuclear morphology of pyroptotic cells is distinct from apoptotic cells (Brennan and Cookson, 2000; Watson *et al*., 2000), and DNA laddering is not always present (Watson *et al*., 2000). In apoptosis occurs the irreversible condensation of chromatin that localizes to the nuclear membrane. The nucleus then breaks up in the process of karyorrhexis (Majno and Joris, 1995). In contrast, examination of the nucleus in pyroptotic cells reveals chromatin condensation, but the nucleus remains intact and karyorrhexis does not occur (Zychlinsky *et al*., 1992; Watson *et al*., 2000).

A second feature that is common between pyroptosis and apoptosis is the positive staining with annexin V. Annexin V binds phosphatidyl serine that is normally present in the inner leaflet of the cell membrane. During apoptosis, phosphatidyl serine translocates to the outer leaflet, which results in positive cell surface staining with annexin V. This feature discriminates apoptotic from live cells (Koopman *et al*., 1994). During pyroptosis, pores open in the cell membrane, which permits annexin V to enter the cell and stain the inner site of the membrane. In contrast, membrane impermeant dyes such as 7-aminoactinomycin (7-AAD) or propidium iodide (PI) stain pyroptotic cells by entering through the pores, but do not stain apoptotic cells (Brennan and Cookson, 2000; Edgeworth *et al*., 2002; Fink and Cookson, 2007; Silveira and Zamboni, 2010). An additional consequence of pore formation in pyroptosis is cellular swelling (Fink and Cookson, 2007). Conversely, apoptotic cells shrink. Thus, finally the mechanisms resulting in positive annexin V staining are different between apoptosis and pyroptosis.

Another apparent similarity between pyroptosis and apoptosis is the formation of spherical membrane bound structures. During apoptosis, the cell blebs, dividing itself into spherical membrane-bound structures known as apoptotic bodies (Majno and Joris, 1995). During pyroptosis, increased osmotic pressure results in large spherical protrusions of the membrane as it rips away from the cortical cytoskeleton. Electron micrographs of the pyroptosis protrusions revealed spherical structures, so they were initially thought to be apoptotic blebs (Zychlinsky *et al*., 1992; Chen *et al*., 1996). However, continued rapid swelling of these protrusions causes fusion and rupture. This results in the release of cytosolic contents to the extracellular space, commonly measured by assaying for lactate dehydrogenase release (Brennan and Cookson, 2000). This does not occur during apoptosis, since apoptotic bodies are not undergoing rupture, but are cleared by phagocytosis.

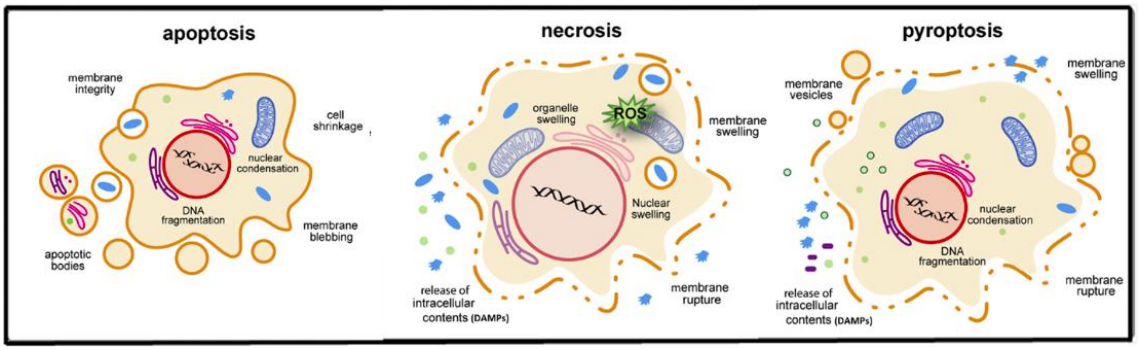


Figure 5. Three key modes of the cell death. In classic apoptosis, the retention of plasma membrane integrity and the formation of apoptotic bodies render it an immunologically silent death mode. Whereas, cells dying by necrosis or pyroptosis secrete pro-inflammatory cytokines and release their cytoplasmic content, into the extracellular space (Guo *et al*., 2014).

Several important human pathogens establish an intracellular niche within host cells, where they can proliferate and protect themselves from soluble immune mediators. Several studies have demonstrated clear functions for inflammasomes in controlling the replication of *Salmonella* inside of the host cell, through cytokine activation and pyroptosis induction (Miao *et al*., 2010b; Franchi *et al*., 2012a; Chen *et al*., 2014). Pyroptosis of infected cells is appearing as an important mechanism of microbial clearance (Miao *et al*., 2010b), in addition to host defense mechanisms coordinated by inflammasome dependent IL-1β (Chen *et al*., 2014) and IL-18 (Raupach *et al*., 2006; Franchi *et al*., 2012a). Macrophage pyroptosis releases *Salmonella* from its intracellular replicative niche, and therefore increases its susceptibility to neutrophil-

mediated extracellular destruction. This mechanism also appears to be very effective in limiting the replication of other intracellular pathogens, including *Legionella* and *Burkholderia* (Miao *et al*., 2010b). Additionally to removing the bacterial replicative niche, pyroptosis also releases multiple alarmins (Oppenheim *et al*., 2007; Said-Sadier *et al*., 2012) such as ATP, IL-33 (Moussion *et al*., 2008) and HMGB1 (Scaffidi *et al*., 2002) to alert, inform about the presence of the pathogen and recruit immune cells to the site of infection (Bergsbaken *et al*., 2009). The oligomeric form of the adaptor protein - ASC can also act as an unusual alarmin released during pyroptosis that can propagate inflammasome signaling in surrounding macrophages (Baroja-Mazo *et al*., 2014; Franklin *et al*., 2014). Pyroptotic cells are also reported to undergo lysosomal exocytosis (Bergsbaken *et al*., 2011) to release lysosomal antimicrobial peptides (Mansour *et al.*, 2014) that facilitate the clearance of pathogens by recruiting neutrophils. The ability to undergo pyroptotic death is not unique to immune cells. For example, pyroptosis of the gut epithelium promotes the extrusion of infected cells into the gut lumen, preventing *Salmonella*, and probably other pathogens, from transversing the epithelial barrier to invade the host (Knodler *et al*., 2014; Sellin *et al*., 2014).

1.7. The guanylate binding proteins family (GBPs)

The guanylate-binding proteins (GBPs) were first identified in the late 1970s, and within a short period of time, investigators were aware that GBPs possessed unique properties, in particular the ability to bind GMP agarose. Since then, much study has gone into understanding their mechanism of induction by interferons (IFNs) and other cytokines, and they have been used extensively as markers for IFN responsiveness in both cells and whole organisms. In time, we learned that GBPs had the unusual ability to hydrolyze GTP to both GDP and GMP. More recently, we have begun to appreciate their novel structure, one that suggests unique mechanisms of GTP binding and hydrolysis and unique forms of regulation. In addition, we have begun to unravel some of their functions and to separate these functions into those functions that do and those that do not require GTPase activity (Vestal, 2005) (Figure 6).

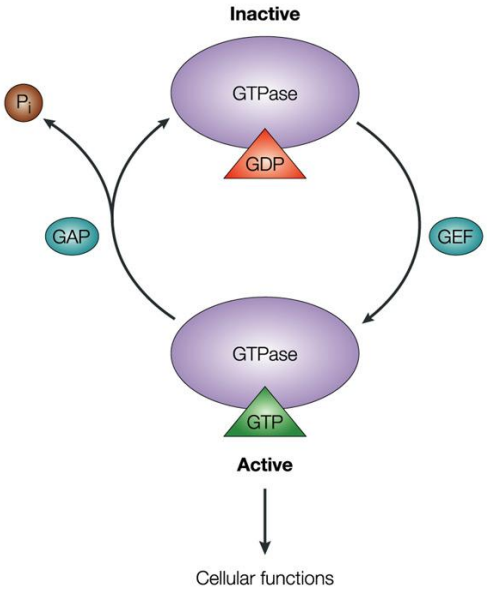


Figure 6. GTP-binding proteins (GTPases). To perform their function, GTPases cycle between GDP-bound inactive states and GTP-bound active states. Guanine Nucleotide Exchange Factor (GEF) converts GTPase to GTP-bound form. GTPase-activating protein (GAP) promotes GTPase activity facilitating its return to GDP-bound form (Taylor *et al*., 2004).

GTPases are classified into three groups: the small 47-KDa immunity-related GTPases (IRGs), the Mx proteins (MX1, MX2), and the large 65- to 67-kDa GTPases. Guanylate-binding proteins (GBP) fall into the last class and function in cell-autonomous immunity, that is, mechanisms that allow host cells to kill pathogens or restrict their replication, and have even been associated with the activation of inflammasomes (Haldar *et al*., 2013; Meunier *et al*., 2014). They are structurally related to the dynamins (Selleck *et al*., 2013). In humans, there are seven GBPs (hGBP1-7) while the mouse contains 13 including two alternative splice isoforms (Kresse *et al*., 2008; Kim *et al*., 2011). Structurally, human GBP1 consists of two domains: a compact globular N-terminal domain harbouring the GTPase function, and an alpha-helical finger-like C-terminal domain. Human GBP1 is secreted from cells without the need of a leader peptide, and has been shown to exhibit antiviral activity against Vesicular stomatitis virus and Encephalomyocarditis virus, as well as being able to regulate the inhibition of proliferation and invasion of endothelial cells in response to IFN-γ (Prakash *et al*., 2000; Naschberger *et al*., 2006; Tripal *et al*., 2007).

It is also known that GBP proteins regulate the canonical inflammasome response. Similar to ASC, IFN-inducible GTPases can form protein multimers (Prakash *et al*., 2000). It was recently shown that tetrameric GBP5 binds to NLRP3 and thereby promotes the assembly of an ASC-caspase-1 multimer (Shenoy *et al*., 2012). In addition, cluster of GBPs is required for the full activity of the non-canonical caspase-11 inflammasome during infections with vacuolar Gram-negative bacteria (Meunier *et al*., 2014). As the noncanonical inflammasome lacks ASC and NLRP3, GBPs can promote its activation by a different mechanism. A detailed understanding of this mechanism will require the identification of specific components of the noncanonical inflammasome, which may include GBP complexes serving as platforms for the oligomerization of caspase-11(Pilla *et al*., 2014).

2. The zebrafish

2.1. Description, distribution, taxonomy, ecology and reproduction

Zebrafish (*Danio rerio*) is a small shoaling cyprinid fish, which size reaches maximum 60mm (Figure 7). Although details of the distribution are not entirely clear, *D. rerio* may be widely distributed in shallow, slow-flowing, tropical freshwaters on the Indian subcontinent. Their natural range is centered around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh, and Nepal. They are most commonly encountered in shallow ponds and standing water bodies with visibility to a depth of approximately 30 cm, often connected to rice cultivation (Spence *et al*., 2008). Zebrafish ows its name for the characteristic stripes running along the body and the fins.

Figure 7. Adult zebrafish. Adapted from http://www.renalgenes.org/zebrafish.html.

Taxonomically, the zebrafish (*Danio rerio*) is a derived member of the genus *Danio*, of the family Cyprinidae, order Cypriniformes. For many years it was referred

to, in scientific literature *Brachydanio rerio,* until its reassignment to the genus *Danio* (Mayden *et al.*, 2007).

Zebrafish are omnivorous, feeding primarily on zooplankton and insects, although phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scales, arachnids, detritus, sand, and mud have also been reported from gut content analyses (Spence *et al*., 2008).

Zebrafish are promiscuous and breed seasonally during monsoon season, which occur from April to August (spawning has also been recorded outside wet season, suggesting that breeding may be seasonal as a result of food availability). Mating behavior is also heavily influenced by photoperiod, as spawning begins immediately at first light during breeding season and continues for about an hour. In order to initiate courtship about 3 to 7 males chase females and try to lead female towards a spawning site by nudging her and/or swimming around her in a tight circle or figure eight. Spawning sites consists of bare substrate that tends to be well vegetated. In captivity, gravel spawning sites are preferred to silt spawning sites. In the wild, zebrafish breed in silt-bottomed habitats. When a breeding pair reaches the spawning site, the male aligns his genital pore with the female's and begins to quiver, which causes the female to release her eggs and the male to release his sperm. The female releases 5 to 20 eggs at a time. This cycle repeats for about an hour. While the presence of female pheromones is required for initiation of courtship behavior in the male, male gonadal pheromones are required by the female for ovulation to occur. There is limited evidence for male-male competition and female mate preference (Spence *et al*., 2006).

Zebrafish lay non-adhesive eggs without preparing a nest, and are considered to be group spawners and egg scatterers. Although time to hatch depends on water temperature, most eggs hatch between 48 and 72 hours after fertilization. Chorion thickness and embryo activity also impact incubation time. Zebrafish are approximately 3 mm upon hatching and are immediately independent. They are able to swim, feed, and exhibit active avoidance behaviors within 72 hours of fertilization (Engeszer *et al*., 2004; Engeszer *et al*., 2007a; Engeszer *et al*., 2007b).

2.2. The zebrafish as a vertebrate research model

For many decades, zebrafish has been both a very popular aquarium fish and an important research model in several fields of biology (notably, toxicology and developmental biology). Since it was first used in a scientific laboratory 30 years ago, its popularity in biomedical research has significantly increased due to their unquestionable advantages respect other vertebrate models. The development of zebrafish as a model organism for modern biological investigation began with the pioneering work of George Streisinger and colleagues at the University of Oregon (Streisinger *et al*., 1981; Dooley and Zon, 2000; Briggs, 2002).

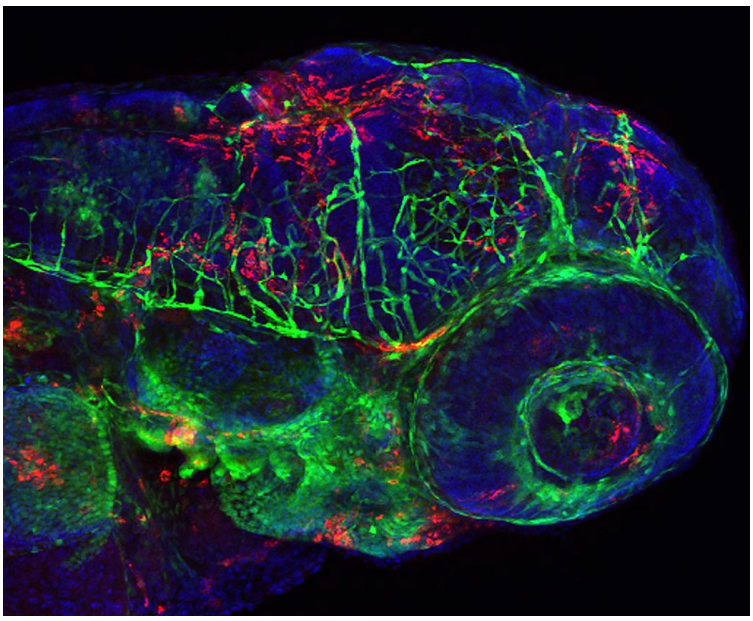


Figure 8. Transgenic *fli*:eGFP larvae 72hpf. Notice the transparency of the larvae, which easily allows the in vivo study of the infection using fluorescence microscopy (IHNV virus in red, blood vessels in green, adjacent tissues in blue). Adapted from: http://www.fishforpharma.com/result.

The use and importance of zebrafish in biological research has exploded and diversified to the point that these fish are extremely important vertebrate models in an extraordinary array of research fields (Vascotto *et al*., 1997), due to multiple advantages:

- Small size. Low maintenance cost and small space needed.
- Robust fish. High resistance to pathogens.
- High fecundity and large production of embryos (around 200 eggs/female/week) makes phenotype-based forward genetics doable.

- Short generation time (for a vertebrate). Typically 3 to 4 months, making it suitable for selection experiments.
- Zebrafish eggs are large relative to other fish (0.7 mm in diameter at fertilization time), optically transparent and externally developed following fertilization, making them easily accessible to embryonic manipulation and imaging.
- Transparency of zebrafish embryos, together with the large availability of transgenic lines, let *in vivo* tracking of cells (Figure 8).
- Rapid development, which is very similar to the embryogenesis in higher vertebrates including humans, the precursors develop to all major organs within 36 hours, and larvae display food seeking and active avoidance behaviour within five days after fertilization (2 to 3 days after hatching).
- As a vertebrate, zebrafish has special value as a model of human disease and for the screening of therapeutic drugs (Chakraborty *et al*., 2009) and is often more tractable for genetic and embryological manipulation and cost effective than other vertebrate models such as mice (Trede *et al*., 2004).
- Easy to transfer among different labs by transporting their eggs.
- The zebrafish genome has now been completely sequenced, making it an even more valuable research organism.
- As a vertebrate, zebrafish shares considerable genetic sequence similarity with humans.
- It is relatively easy to knockdown specific genes by using morpholinos and overexpressing proteins by mRNA or plasmids.
- Use of reverse genetics approaches using zinc finger nucleases (ZFNs) (Meng *et al*., 2008) and a transposon strategy (Kawakami, 2004) for generating transgenic zebrafish, which help in analyzing new roles of additional genes in larval and adult zebrafish.
- Existence of a centralized online resource for the zebrafish research community (http://zfin.org), making easier the work with this model.

All these advantages have led to the increased interest of scientists using zebrafish as an animal model research in the last years. In the past, it was a major vertebrate model especially in the developmental and genetic research (Hill *et al*., 2005), whereas now, the zebrafish gains also growing importance in other fields. Nowadays, it has been proposed as an excellent model for the study of the immune system (Renshaw and Trede, 2012), hematopoiesis (Martin *et al.*, 2011), vascular development (Isogai *et al.*, 2009; Gore *et al*., 2012; Quaife *et al*., 2012), neurogenesis (Schmidt *et al.,* 2013) and cancer research (Stern and Zon, 2003; Mione and Trede, 2010), among others. Some researchers have even used zebrafish to investigate the genetic basis of vertebrate behaviour (Dooley and Zon, 2000; Miklósi and Andrew, 2006; Spence *et al*., 2008; Norton and Bally-Cuif, 2010) and development (Bruneel and Witten, 2015). Moreover, zebrafish has become a popular model in pharmacological studies and drug screening (Langenheinrich, 2003).

3. *Salmonella enterica* serovar Typhimurium

Infectious diseases, such as Salmonellosis, are responsible for one-third of all mortality worldwide and have become a significant public health threat in both developed and developing countries. Innate immunity is very important, for mobilization of the host defenses that eliminate pathogens. *Salmonella* is a global food-borne pathogen that infects and replicates within macrophages of both humans and animals and accounts for 28 million cases of enteric fever and gastroenteritis in all over the world each year (Fàbrega and Vila, 2013; Man *et al*., 2014). A critical step in disease pathogenesis for many clinically important bacteria is their ability to infect and survive within host cells. Control of its infection is difficult due to the bacterium's high tolerance to environmental stress, widespread distribution, multiple drug resistance, and adaptability (Chen *et al*., 2013).

3.1. *Salmonella* Typhimurium discovery and description

The discovery of the genus *Salmonella* goes back to 1885. At that time Daniel Elmer Salmon, an American veterinary pathologist, and Theobald Smith, his assistant, had been searching for the cause of common hog cholera. Smith isolated a new species of bacteria, at that time formerly called *S. cholerae-suis*, from ill pigs and proposed it as the casual agent. However, although Smith was the actual discoverer, Salmon claimed

credit for the discovery, and the organism was subsequently named after him. Later research revealed that this organism rarely causes enteric symptoms in pigs and was therefore not the agent they were looking for (Schultz, 2008).

The genus *Salmonella*, which is closely related to the genus *Escherichia*, is composed of Gram-negative, non-spore-forming, rod-shaped bacteria, which belong to the Enterobacteriaceae family. These microorganisms can range from around 0.7 to 1.5 μm in diameter and 2 to 5 μm in length. They are facultative anaerobes and show predominantly peritrichous motility. This genus refers to primary intracellular pathogens leading to different clinical manifestations in the development of infection in humans (Murray, 1999; Coburn *et al*., 2007).

3.2. *Salmonella* scientific classification

The genus *Salmonella* includes three species: *Salmonella enterica*, *Salmonella bongori*, and *Salmonella subterranean* (Su and Chiu, 2007). The type species, *S. enterica*, is further classified into six subspecies: *enterica* (subsp. I), *arizonae* (subsp. IIIa), *diarizonae* (subsp. IIIb), *houtenae* (subsp. IV), *indica* (subsp. VI), and *salamae* (subsp. II) (Su and Chiu, 2007). *Salmonella* strains belong to over 50 serogroups which are based on the O antigen, and to over 2500 serotypes (each having a unique combination of somatic O, and flagellar H1 and H2 antigens). Most of these serotypes belong to one single *Salmonella* subspecies, *enterica*, and are associated with >99% of *Salmonella* caused diseases in humans (Su *et al*., 2004). Genome sequencing and comparative genomic analysis showed a close similarity of core regions from various *Salmonella* genomes, together with evidence of recombination and rearrangement, genomic degradation, pseudogenes, and clonal diversity both within and among the serotypes (Chiu *et al*., 2005; Liu *et al*., 2009). Genomic comparisons of host-restricted (*S*. Typhi, *S*. Paratyphi, and *S*. Gallinarum) and host adapted (*S*. Typhimurium and *S*. Enteridis) serotypes revealed that genomic degradation is a common evolutionary mechanism for host adaptation and increased its pathogenicity (Chiu *et al*., 2005; Liu *et al*., 2009). Continuous genetic re-assortment in *Salmonella*, leading to increased virulence and the emergence of resistance to multiple drugs, is of significant public health concern.

3.3. *Salmonella* as an intracellular pathogen and its virulence

Type III secretion systems (T3SSs) are multiprotein structures which are found in a big group of Gram-negative bacterial pathogens, which transport proteins across the two membranes of the bacterial cell and a third membrane of the target host cell (Cornelis and Van Gijsegem, 2000). They are associated with the pathogenicity at molecular level. The identification of T3SS has provided new insight into the molecular factors and mechanisms underlying bacterial pathogenesis (Kaur and Jain, 2012). *Salmonella enterica* encodes two different T3SSs, which are responsible for the performance of a range of virulence functions during the infection. The *Salmonella* Pathogenicity Island 1 (SPI-1) T3SS is still activated in extracellular bacteria and translocates effectors through the host cell plasma membrane (Figure 9). Those actions lead to localized actin polymerization, membrane ruffling and entry of bacteria into the host cell (Galán, 2001), where they remain in a membrane-bound compartment known as the *Salmonella*-containing vacuole (SCV). The second T3SS, also encoded by a pathogenicity island (SPI-2), is activated already in intracellular bacteria, probably in response to low [Ca^{2+}] and [Fe^{2+}], acidic pH and low osmolarity (Lee *et al*., 2000; Zaharik *et al.*, 2002; Garmendia *et al*., 2003) in the SCV. SPI-2 gene expression begins at 1 h after uptake of bacteria by host cells, and increases with the time, over the next few hours (Cirillo *et al*., 1998; Uchiya *et al*., 1999; Beuzón *et al.*, 2000; Knodler *et al*., 2002; Kuhle and Hensel, 2002; Garmendia *et al*., 2003). The expression of SPI-2 genes and associated effectors is controlled by the SsrA-SsrB, two component of regulatory system, which are encoded within SPI-2 (Cirillo *et al*., 1998).

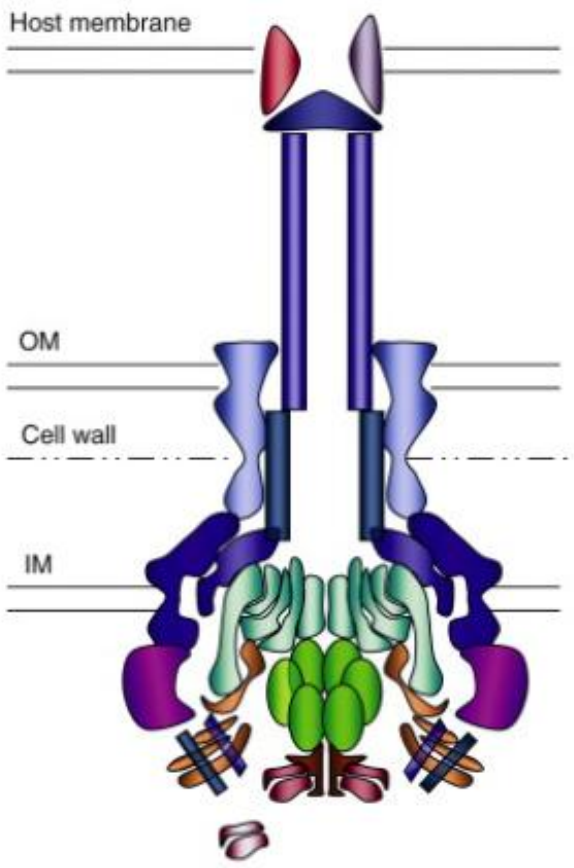


Figure 9. Scheme of Type 3 Secretion System SPI-1. The basal body of the T3SS spans the inner membrane (IM), the periplasm and the outer membrane (OM) of the bacterium. The extracellular part of the T3SS comprises pilin subunits that form the pilus and translocon proteins that form a pore in the host plasma membrane (IM –inner membrane, OM- outer membrane) (Sun and Gan, 2010).

The SPI-2 T3SS secretes three proteins – SseB, SseC and SseD – that are required for translocation of the effector proteins into and across the vacuolar membrane (Nikolaus *et al*., 2001), and therefore they are considered to be components of a translocon. The translocated effectors are encoded both within and outside SPI-2, and enable intracellular replication of bacteria in macrophages, epithelial cells and fibroblasts (Hensel *et al*., 1995; Ochman *et al*., 1996; Cirillo *et al*., 1998; Hensel *et al*., 1998; Beuzón *et al*., 2000; Beuzón *et al*., 2002). Usually, SCVs form *Salmonella* induced filaments (SIF). This process can control the integrity of the SCV membrane and its expansion, which is necessary for bacterial cell division. It is also possible that by controlling vesicular fusion on the SCV, these bacterial proteins ensure delivery of nutrients to the SCV, thereby facilitating bacterial replication. However, in some cases the bacteria do not have the time or the capability to modify the vacuole leading to the fusion of the SCV with phagolysosome triggering intra-vacuole destruction or

autophagy. In other cases, *Salmonella* damages the SCV membrane triggering vacuole destruction, allowing bacteria to escape into the cytosol, where they can be destroyed, particularly in activated macrophages, or multiply extensively especially in epithelial cells (Wiedemann *et al*., 2014) (Figure 10).

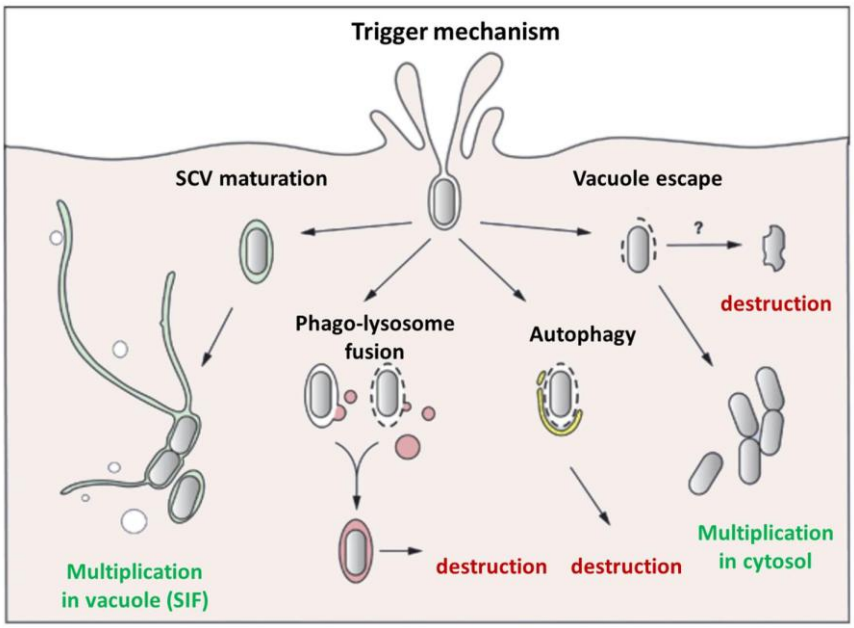


Figure 10. Different behaviors of internalized *Salmonella*. The majority of *Salmonella* strains, internalized within an animal cell by the trigger mechanism mediated by the T3SS-1, are enclosed in a canonical SCV where they can multiply, and form SIF, which allow delivery of nutrients. When bacteria do not have the time or the capability to modify the vacuole or simply escapes from the vacuole into the cytosol it undergoes the destruction or autophagy (Wiedemann *et al*., 2014).

4. The zebrafish – *Salmonella* Typhimurium infection model

Recently the zebrafish embryo system has appeared as a new model to study innate immunity in vertebrates, which is offering several advantages that complement mammalian model systems (Davis *et al.*, 2002; van der Sar *et al*., 2003; Ward *et al.*, 2003). Moreover, zebrafish became a valuable model for host – microbe interaction studies, affording researchers with the opportunity to survey large population of hosts and to visualize microbe – host associations at a cellular level in living animals (Sullivan and Kim, 2008; Milligan-Myhre *et al*., 2011). To complement the investigations of infection diseases, zebrafish model is a unique tool for studying the function of phagocytic cells (Torraca *et al*., 2014). The major cell types of the innate immune system, macrophages and neutrophils, being main phagocytes, develop during the first days of embryogenesis prior to the maturation of lymphocytes that are required

for adaptive immune responses. The high efficiency of infections and chemical treatments in zebrafish that can be performed even at a large scale, allows identification of novel microbial virulence factors and high throughput compound screens to investigate disease mechanisms (Miller and Neely, 2005; Lieschke and Currie, 2007). Moreover, the zebrafish system is particularly suitable for large-scale forward and reverse genetic screens aimed at the identification of genes with novel functions in development of the immune system or in the immune response (Trede *et al*., 2008; Pase *et al*., 2009).

Many infection systems for study viral and bacterial disease in zebrafish have been developed during last years (Phelps and Neely, 2005; Lesley and Ramakrishnan, 2008; Meeker and Trede, 2008). Although many of the zebrafish pathogen models were developed to address human infectious disorders, the results of these studies should provide important clues for the development of effective vaccines and prophylactic measures against bacterial and viral pathogens in economically important fishes (Sullivan and Kim, 2008). The most common and relevant human pathogens used in zebrafish infection models are: *Mycobacterium* (Cambier *et al*., 2014; Torraca *et al*., 2015), *Salmonella* Typhimurium (Medina and Royo, 2013; de Oliveira *et al*., 2015), *Burkholderia* (Mesureur and Vergunst, 2014), *Staphylococcus aureus* (Hepburn *et al*., 2014; McVicker *et al*., 2014), *Shigella flexneri (Mostowy et al., 2013; Mazon Moya et al.*, 2014) and *Candida albicans* (Gratacap *et al*., 2014; Wang *et al*., 2015). There are different routes of the infection depending on what is the purpose of the study. The most common are: duct of cuvier, yolk sac circulation valley, caudal vein and blood islands for used to study phagocytosis (Benard *et al*., 2012). The hindbrain ventricle (Phennicie *et al*., 2010) and tail muscle injection (Lin *et al*., 2009) are used for macrophage migration. The otic vesicle (Le Guyader *et al*., 2008) is usually used for neutrophil migration and notochord is used for *M*. marinum mutants injection being permissive compartment for its growth that are strongly attenuated when injected in other tissues (Benard *et al*., 2012). Finally yolk sac injection is used for systemic infection (van der Sar *et al*., 2003; Benard *et al*., 2012) (Figure 11).

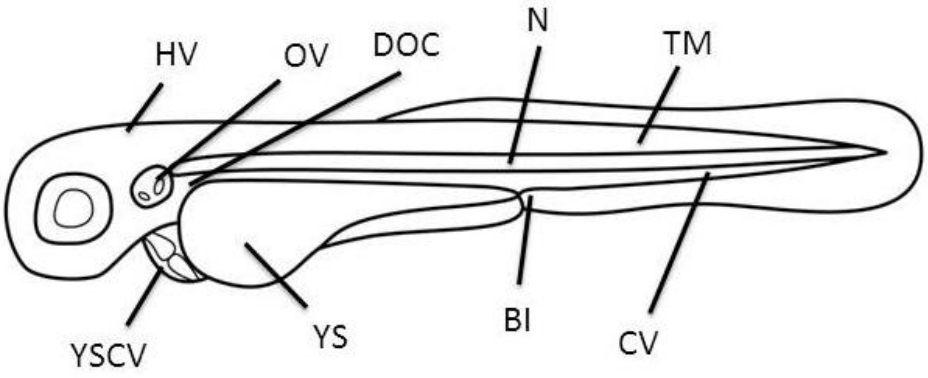


Figure 11. Locations in zebrafish explored as sites for infections (HV- Hindbrain, OV – Otic Vesicle, DOC –Duct of Cuvier, N – Notochord, TM – Tain Muscle, YSCV – Yolk Sac Circulation Valley, YS –Yolk Sac, BI – Blood Islands, CV – Caudal Vein).

An infection model for well-studied human pathogen in zebrafish embryos which has been previously established is *S.* Typhimurium (Stockhammer *et al*., 2009). Bacterial virulence mechanisms of *Salmonella* being a complex phenotype, is fully manifested only during host pathogen interactions in vivo and studying a natural infection in an animal which develops signs of disease similar to those observed in humans is essential for a complete understanding of *Salmonella* pathogenesis. The symptoms in human patients closely resemble signs of disease observed in primate models as mouse or calf, but the high cost and scarcity of these animals has prevented the widespread use of those models (Santos *et al*., 2001). However, nowadays the replacement of mammalian systems by zebrafish, having similar biological processes, brings less cost-intensive and more efficient alternatives.

OBJECTIVES

The specific objectives of the present work are:

1. Establish a zebrafish – *Salmonella* Typhimurium infection model to study inflammasome activation, assembly and function.
2. Characterize the role of flagellin of *S.* Typhimurium in the infection mechanism in zebrafish.
3. Characterize the role of zebrafish Gbp4 in inflammasome activation, assembly and clearance of *S.* Typhimurium.
4. Characterize the role of neutrophils in the Gbp4-dependent clearance of *S.* Typhimurium in zebrafish.
5. Characterize the role played by the Gbp4 inflammasome in prostaglandin production and *S.* Typhimurium clearance.

MATERIALS AND METHODS

1. Animals

Wild-type zebrafish (*Danio rerio* H. Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center (ZIRC, Oregon, USA) and mated, staged, raised and processed as described in the zebrafish handbook (Westerfield, 2000). The transgenic lines *Tg(mpx:eGFP)i114*, with green fluorescent neutrophils (Renshaw *et al.*, 2006), were provided by Prof. Stephen A. Renshaw. The transgenic line *Tg*(*mpeg1:eGFP*)*gl22*, with green fluorescent macrophages (Ellett *et al*., 2011), was provided by Prof. Graham J. Lieschke., and the *Tg(lyz:DsRED2)nz50* (Hall *et al*., 2007) by Prof. Phil Crosier.

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU) and IACUC. Experiments and procedures were performed as approved by the Bioethical Committee of the University of Murcia (approval number #537/2011).

2. DNA constructs

The genes encoding zebrafish wild type Gbp4, the GTPase-dead mutant Gbp4KS→AA, Gbp4ΔCARD, the double mutant Gbp4KS→AA/ΔCARD, zfiGLuc and GLuc were synthesized by GenScript Corporation. The cmv/sp6:gbp4 and cmv/sp6:gbp4-mCherry constructs were generated by MultiSite Gateway assemblies using LR Clonase II Plus (Life Technologies) according to standard protocols and using Tol2kit vectors described previously (Kwan *et al*., 2007). The zebrafish Asc-Myc construct in the pcDNA3 backbone was previously described (Masumoto *et al*., 2003).

3. Morpholinos and mRNA injection

Specific morpholinos (Gene Tools) were resuspended in nuclease-free water to 1 mM, 2 mM or 3 mM (Table 2). *In vitro*-transcribed RNA was obtained following manufacturer's instructions (mMESSAGE mMACHINE kit, Ambion). Morpholinos and RNA were mixed in microinjection buffer (0.5x Tango buffer and 0.05 % phenol red solution) and microinjected into the yolk sac of one-cell-stage embryos using a microinjector (Narishige) (1 nl per embryo). The same amount of MOs and/or RNA were used in all experimental groups. The efficiency of the MOs was checked by RT-PCR and Western blot.

Gene	ENA or Ensembl ID	Target	Sequence (5'→3')	Concentration (mM)	Reference
gbp4	ENSDARG00000068857	e1/i1	GCTGTTTGTGTGTCTCTAACCTGTT	0.1	This work
Pycard (asc)	ENSDARG00000040076	e2/i2	AGTGATTCGCTTACTCACCATCAGA	1.68	This work
		atg/5´UTR	GCTGCTCCTTGAAAGATTCCGCCAT	0.6	This work
cxcr2	ENSDARG00000054975	atg/5´UTR	ACTCTGTAGTAGCAGTTTCCATGTT	0,3	Deng *et al.*, 2013

Table 2. Morpholinos used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). ENA, European Nucleotide Archive (http://www.ebi.ac.uk/ena/).

4. Chemical treatments

In some experiments, 2 dpf embryos were manually dechorionated and treated by bath immersion with the Ptgs inhibitor indometacin (10 µM, Sigma-Aldrich), the caspase-1 inhibitor Ac-YVAD-CMK (100 µM, Peptanova) or the pan-caspase inhibitor Q-VD-OPh (50 µM, SM Biochemicals LLC) diluted in egg water supplemented with 1% DMSO, or with 10 µM of 16, 16 dimethyl PGE2, 16, 16 dimethyl PGD2, d12PGJ2 or 15dPGJ2 (all from Cayman Chemical) diluted in egg water supplemented with 0,01%-0,2% methyl acetate (Sigma-Aldrich).

5. Infection assay

For most infection experiments, *S.* Typhimurium 12023 (wild type) and the isogenic derivative mutants for SPI-1 (prgH020::Tn5lacZY), SPI-2 (ssaV::aphT) and SPI-1/SPI-2 (prgH020::Tn5lacZY ssaV::aphT) (kindly provided by Prof. D. Holden) were used. For some experiments the *S.* Typhimurium strains were used: 14028s (wild type), its isogenic derivative mutant fliC/fljB, and the FliCON, which persistently expresses the flagellin protein FliC (Miao *et al.*, 2006; Miao *et al.*, 2010a) (kindly provided by Dr. E.A. Miao). For otic vesicle infections, *S.* Typhimurium 12023 expressing DsRedT3 (de Oliveira *et al.*, 2015) was used.

Overnight cultures in Luria-Bertani medium (LB) were diluted 1/5 in LB with 0.3 M NaCl, incubated at 37 ºC until 1.5 optical density at 600 nm was reached, and

finally diluted in sterile PBS. Zebrafish one-cell embryos were injected with MOs and/or mRNAs. Larvae of 2 dpf were decorionated manually, anesthetized in embryo medium with 0.16 mg/ml tricaine and 10-50 bacteria (Yolk sac) (Figure 12), 100 bacteria (Otic vesicle) or 250 (Cuvier's duct) per larvae were microinjected. Larvae were allowed to recover in egg water at 28-29 ºC, and monitored for clinical signs of disease or mortality over 5 days (Figure 12). All the survival assays have been performed three times, using 40-100 larvae per treatment in each independent experiment. Each graph shows the mean result of the three experiments, having an accumulated sample size of 120-300 larvae per treatment.

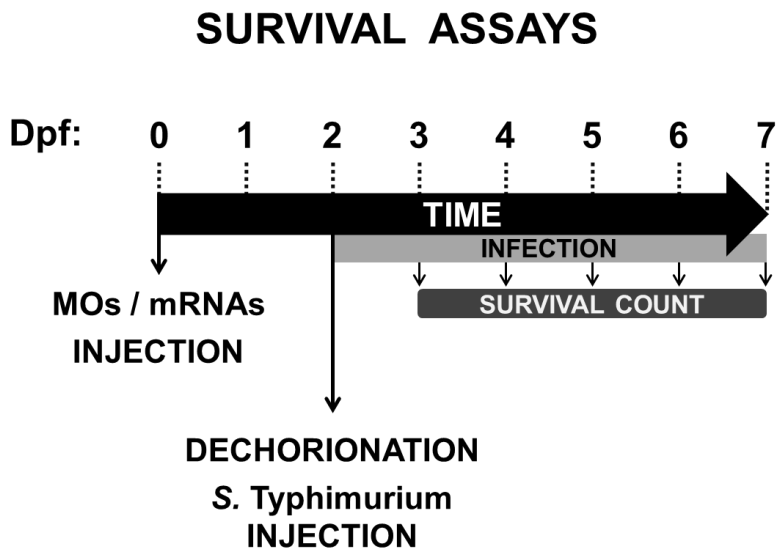


Figure 12. Survival assay. Scheme showing the experimental procedure used for the survival assays.

6. Caspase-1 activity assay

Zebrafish one-cell embryos were injected with MOs and/or mRNAs. 2 dpf larvae were dechorionated and infected with different strains of *S.* Typhimurium into the yolk sac (MOI=50). 3 dpf larvae were collected and used to measure the caspase-1 activity (Figure 13). 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, larvae 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-Aldrich), pH 7.5] on ice for 10 min. For each reaction, 70 µg of proteins were incubated for 90 min at 23ºC 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]. After the incubation, the fluorescence of the AFC released from the Z-YVAD-AFC substrate was measured with a FLUOstar spectofluorometer (BGM, LabTechnologies) at an excitation wavelength of 405 nm and an emission wavelength of 492 nm. All the caspase-1 activity assays have been performed three times, using 25-40 larvae per treatment in each independent experiment. Only one of the three graphs are shown in each case as a representative result.

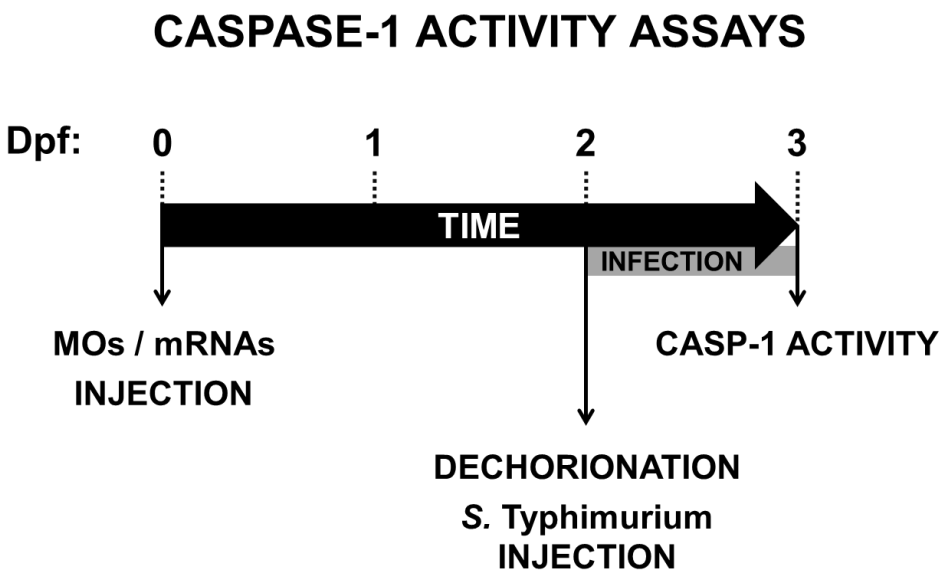


Figure 13. Caspase-1 activity assay. Scheme showing the experimental procedure used for the caspase-1 activity assays.

7. Live imaging of zebrafish larvae

At 72 hpf, larvae were anesthetized in tricaine (0.16 mg/ml) and mounted in 1% (wt/vol) low-melting-point agarose (Sigma-Aldrich) dissolved in egg water. Images were captured using an epifluorescence LEICA MZ16FA stereomicroscope equipped with green and red fluorescent filters, while animals were kept in their agar matrixes at 28.5 °C. All images were acquired with the integrated camera on the stereomicroscope and were used for subsequently counting the number of neutrophils (*mpx:eGFP*).

8. Neutrophil recruitment assay and cell death analysis

To study neutrophil recruitment and pyroptotic cell death in a localized site of *S.* Typhimurium infection, 2 dpf larvae were anesthetized in embryo medium with 0.16 mg/ml tricaine and mounted in 1% low melting point agarose supplemented with 0.16 mg/ml tricaine. 0.5 nL of PBS or *S.* Typhimurium:DsREDT3 suspension (100 bacteria/larva), supplemented with phenol red, was then injected into the otic ear vesicle. Embryo medium with 0.16 mg/ml tricaine solution was added, in order to maintain the embryos hydrated during the experiments. Images of the otic area were taken at 1.5, 3, 4.5 and 6 hour post-infection (hpi) using a Leica MZ16F fluorescence stereo microscope, treated with ImageJ software (http://rsb.info.nih.gov/ij/) and neutrophil counts determined. For the analysis of pyroptotic cell death, 2 μM of the green fluorescent dye YO-PRO (Life Technologies) was injected in the otic vesicle at 3 hpi and images were taken 1 h later as above.

9. Luminiscence

Embryos were microinjected as described above with 200 pg of in vitro transcribed zfiGLuc or GLuc RNA. After 48h, larvae were infected with wild type *S.* Typhimurium and whole larval extracts obtained at 24 hpi as described previously (Alcaraz-Pérez *et al*., 2008). Larval extracts were then combined 1:1 with distilled water containing 4.4 μM coelenterazine (Sigma-Aldrich) to achieve a final concentration of 2.2 μM. The luciferase signal was then measured on a Luminometer Optocomp II (MGM Instruments). All the luminescence assays have been performed three times, using 20 larvae per treatment in each independent experiment. Only one of the three graphs are shown in each case as a representative result.

10. Flow cytometry

At 72 hpf, approximately 300 to 500 *Tg(mpx:eGFP)* and *Tg*(*mpeg1:eGFP*) larvae were anesthetized in tricaine (0.16 mg/ml), incubated at 28°C for 90 min with 0.077 mg/ml Liberase (Roche) and the resulting cell suspension passed through a 30 μm cell strainer. Sytox (Life Technologies) was used as a vital dye to exclude dead cells. Flow cytometric acquisitions were performed on a FACSCALIBUR (BD) and cell sorting was performed on a Coulter (Epics Altra). Analyses were performed using FlowJo software (Treestar).

11. Analysis of gene expression

Total RNA was extracted from whole embryos/larvae, larvae heads or sorted cell suspensions with TRIzol reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, amplification grade (1 U/μg RNA; Invitrogen). SuperScript III RNase H Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo(dT) primer from 1 μg 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 normalized to the ribosomal protein S11 (*rps11)* content in each sample Pfaffl method (Pfaffl, 2001). The primers used are shown in Table 3. In all cases, each PCR was performed with triplicate samples and repeated at least with two independent samples.

Gene	ENA ID	Name	Sequence (5'→3')	Use
Asc	NM_131495	F	ATTTTGAGGGCGATCAAGTG	RT-PCR
		R	GCATCCTCAAGGTCATCCAT	
rps11	NM_213377	F1	GGCGTCAACGTGTCAGAGTA	RT-qPCR
		R1	GCCTCTTCTCAAAACGGTTG	
gbp4	NM_001082945	F	ACTGGGAGATGTGGAAAAGGGCG	
		R	CCATAGCCTTGTTGTCGATCACCCC	
Il1b	NM_212844	F5	GGCTGTGTGTTTGGGAATCT	
		R5	TGATAAACCAACCGGGACA	
Gfp	EF591490	F1	ACGTAAACGGCCACAAGTTC	
		R1	**AAGTCGTGCTGCTTCATGTG**	
cxcl8-l1	XM_001342570	F1	GTCGCTGCATTGAAACAGAA	
		R1	CTTAACCCATGGAGCAGAGG	
ptgs2a	NM_153657	F1	TGGATCTTTCCTGGTGAAGG	
		R1	GAAGCTCAGGGGTAGTGCAG	
ptgs1	AY028584	F	TTTTGCTGCTGAGTGTGTCC	
		R	CGAACACAGATCCCTTGGTT	
Tnfa	NM_212859	F2	GCGCTTTTCTGAATCCTACG	
		R2	TGCCCAGTCTGTCTCCTTCT	
Il10	NM_001020785	F2	ATTTGTGGAGGGCTTTCCTT	
		R2	AGAGCTGTTGGCAGAATGGT	

Table 3. Primers used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). ENA, European Nucleotide Archive (http://www.ebi.ac.uk/ena/).

12. Protein determination

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

13. Western blot

Cell extracts from (60 µg) from dechorionated and deyolked zebrafish larvae obtained at 2 dpf using 200 µL 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 SigmaAldrich) were resolved on 10% SDS-PAGE, transferred for 50 min at 200 mA to nitrocellulose membranes (BioRad), probed with a 1/200 dilution of four different mAbs to zebrafish Gbp4 generated using the SEALTM technology (Abmart), and developed with enhanced chemiluminescence (ECL) reagents (GE Healthcare) according to the manufacturer's protocol. Membranes were then reprobed with a 1/5,000 dilution of a commercial rabbit antibody to histone 3 (#ab1791, Abcam), as an appropriate loading control.

HEK293 transfected cells (600.000) were lysed in CHAPS buffer (20 mM HEPESKOH (pH 7.5), 5 mM MgCl2, 0.5 mM EGTA, 0.1 mM PMSF, 0.1 % CHAPS), separated in 10-8 % SDS-PAGE gel and transferred to nitrocellulose membranes, which after blocking with 5% nonfat milk were incubated overnight at 4ºC with the primary antibodies rabbit polyclonal against GFP (1:1000 dilution; sc-8334; Santa Cruz) and the anti c-myc (1:5000 dilution, 46-0603; Invitrogen). For detection, corresponding horseradish peroxidase conjugated secondary antibodies (1:5000 dilution; Amersham) were used.

14. Inflammasome reconstitution assays in HEK293 cells

HEK293T cells (CRL-11268; American Type Culture Collection) were maintained in DMEM:F12 (1:1) supplemented with 10% FCS, 2 mM Glutamax and 1% penicillin-streptomycin (Life Technologies). 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 24-well plates (120,000 cells/well)

together with 25 µl transfection reagent containing a total of 250 ng of total plasmid DNA. The Gpb4-GFP and the Asc-myc constructs were used at a ratio of 1:1. For immunofluorescence assays, HEK293T cells were seeded on poly-L-lysine coated coverslips, washed twice with PBS, fixed with 4% formaldehyde in PBS for 15 min at room temperature, and then washed three times with PBS. Nonspecific binding in cells was blocked with 1% bovine serum albumin (Sigma) and cells were permeabilized with 0.2% saponin (Fluka) in PBS for 30 min at room temperature. After that, cells were incubated for 1h at room temperature with the mouse monoclonal primary antibody against c-myc (1:1000 dilution; 46-0603; Invitrogen). Cells were washed and then were incubated for 1 h at room temperature with Alexa Fluor 647 donkey anti-mouse IgG (1:200 dilution; A-31571; Life technologies), then were rinsed in PBS and mounted on slides with ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Images were acquired with a Nikon Eclipse Ti microscope equipped with a 20xS Plan Fluor objective (numerical aperture, 0.45), a 40xS Plan Fluor objective (numerical aperture, 0.60) and a 60x Plan Apo Vc objective (numerical aperture, 1.40) and a digital Sight DS-QiMc camera (Nikon) with a Z optical spacing of 0.2 um and 387-nm/447-nm, 472-nm/520-nm and 650-nm/668-nm filter sets (Semrock). Images were deconvolved using ImageJ software, and maximum-intensity projections images are shown in the results.

15. Statistical analysis

Data are shown as mean ± SEM and they were analysed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups. The survival curves were analysed using the log-rank (Mantel-Cox) test. All the experiments were performed at least three times, unless otherwise indicated. The sample size for each treatment is indicated in the graph or in the figure legend or in corresponding section in M&M. Statistical significance was defined as $p < 0.05$.

RESULTS

1. Zebrafish Gbp4 has two domains, GBP and CARD, and is highly expressed in neutrophils but not in macrophages

Bioinformatic analysis looking for proteins with CARD domains as potential novel inflammasome components were previously performed in our laboratory. The results of those screenings led us to focus on two genes present in the zebrafish genome that encode for two proteins belonging to the GBP family: Gbp3 and Gbp4. Both proteins contain a GBP domain located at N-terminal, and a CARD located at C-terminal (Figure 14). When we compared zebrafish Gbp3 and Gbp4 with human GBP5, a protein already shown to be part of the inflammasome in mammals, despite lacking a CARD domain, we obtained 23%/35% and 36%/53% of amino acid homology/identity respectively. Taking that into consideration, we decided to continue our study with Gbp4, the protein that showed a higher homology with human GBP5, since it would be also more probable that they share common functions.

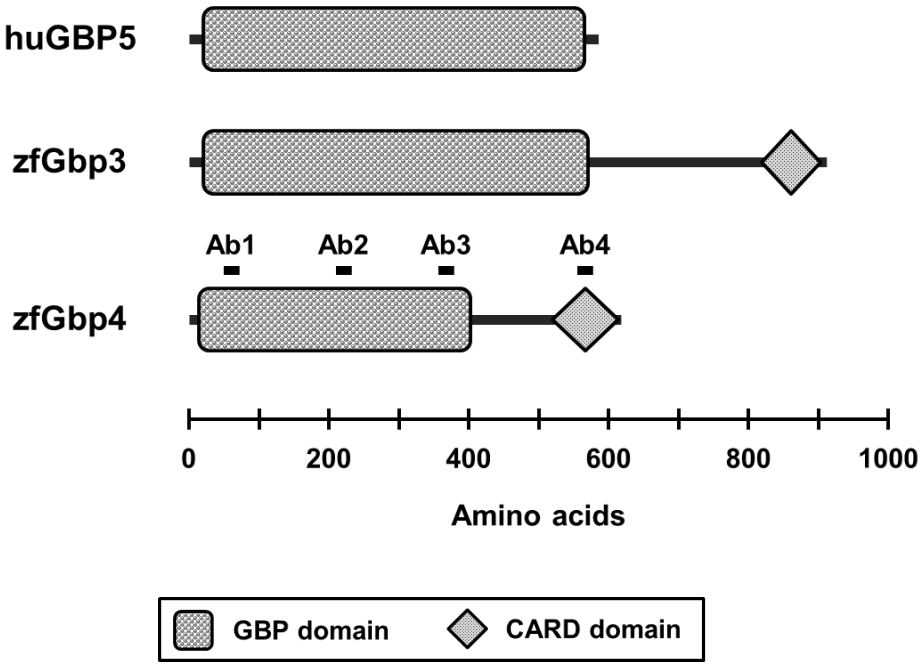


Figure 14. Diagrams showing the domain organization of human GBP5, zebrafish Gbp3 and zebrafish Gbp4. The guanylate-binding protein domains (GBP) (Pfam accession number PF02263) are shown as grey boxes, while the caspase recruitment domains (CARD) (Pfam accession number PF00619) are shown as grey rhombus. The position of each domain is indicated with respect to a ruler. The binding sites for the different anti-Gbp4 antibodies used in this thesis are indicated.

The first step was to analyze the gene expression profile of zebrafish Gbp4 in the main cell types involved in the innate immune response: neutrophils and macrophages.

For that, we isolated neutrophils and macrophages by FACS-sorting from the *mpx*:GFP (Renshaw *et al*., 2006) and *mpeg1*:GFP (Ellett *et al*., 2011) zebrafish transgenic lines, respectively. We found that Gbp4 transcripts were highly enriched in neutrophils (Figure 15A), while they were hardly detected in macrophages (Figures 15B). In addition, the yolk sac infection with *S.* Typhimurium had negligible effects in the mRNA levels of Gbp4 in both neutrophils (Figure 15A) and macrophages (Figure 15B). Both types of cells showed increased mRNA levels of *il1b* gene upon infection, confirming the inflammation (Figures 15).

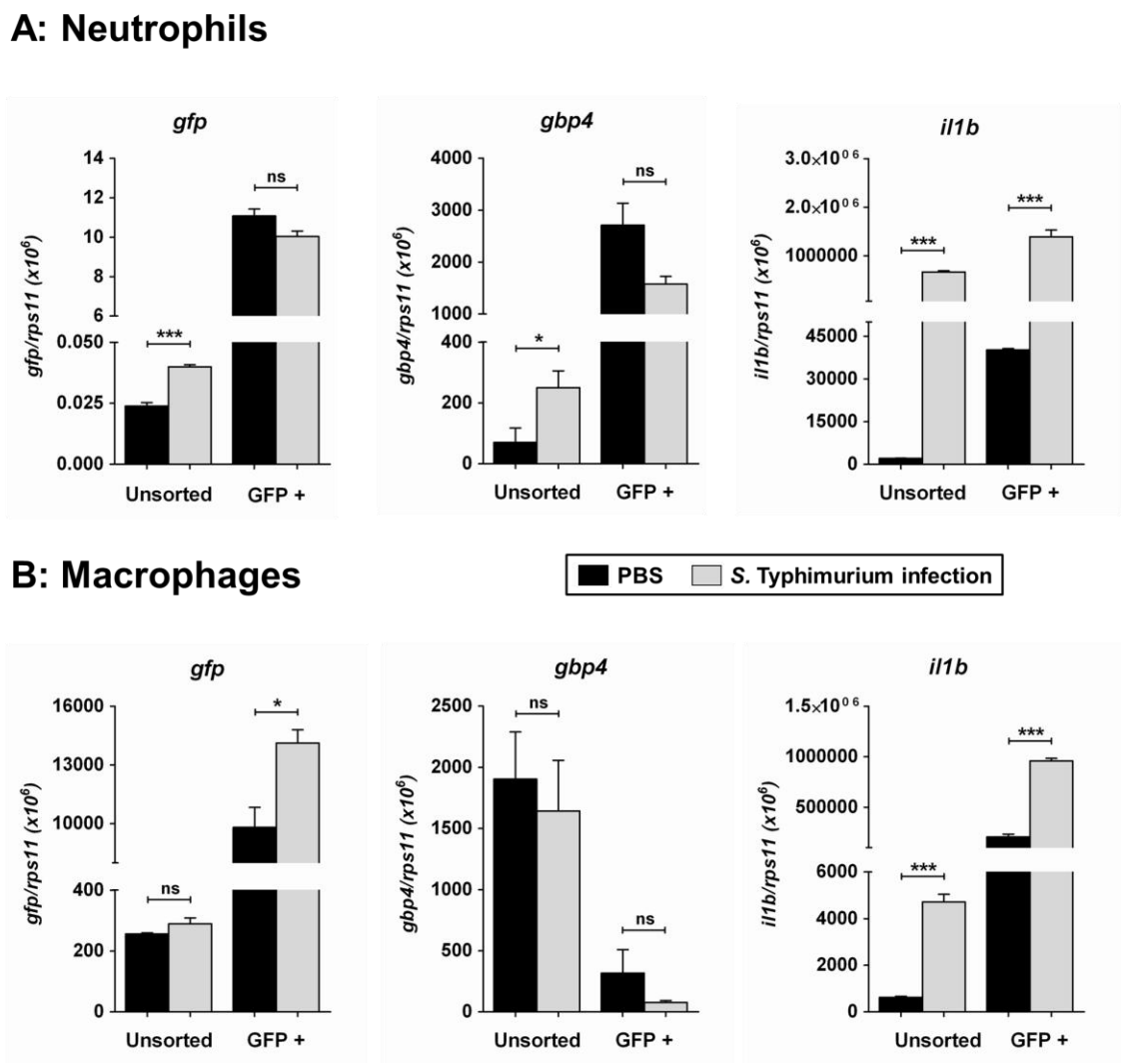


Figure 15. Gene expression levels of *gfp*, *gbp4* and *il1b* in FACS-sorted neutrophils (A) or macrophages (B). 48 hpf zebrafish larvae were infected with *S.* Typhimurium into the yolk sac. The mRNA levels were determined by RT-qPCR from whole 3dpf larvae. Gene expression was normalized against *rps11*. ns, not significant. *p<0.05; ***p<0.001.

Next, we wanted to study the function of Gbp4 by analyzing the effects of its

genetic inhibition, using a morpholino (MO)-mediated gene knockdown strategy to target the E1/I1 and alter the splicing of *gbp4* mRNA (Figure 16).

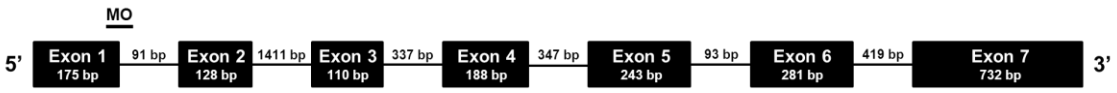


Figure 16. Diagram showing the exons/introns organization of zebrafish *gbp4*. The position where the Gbp4 MO binds to the pre-mRNA, between the exon 1 and the intron 1, is indicated. *gbp4* has seven exons and six introns, and the sizes of all them are shown.

Before working with the Gbp4 MO, we checked its efficiency by western blot using four different mAbs, targeting the GBP domain as well as the CARD (Figures 14 and 17). The results obtained showed a strong reduction of Gbp4 protein in the Gbp4 morphants compared to controls, demonstrating that the Gbp4 MO was working properly (Figure 18).

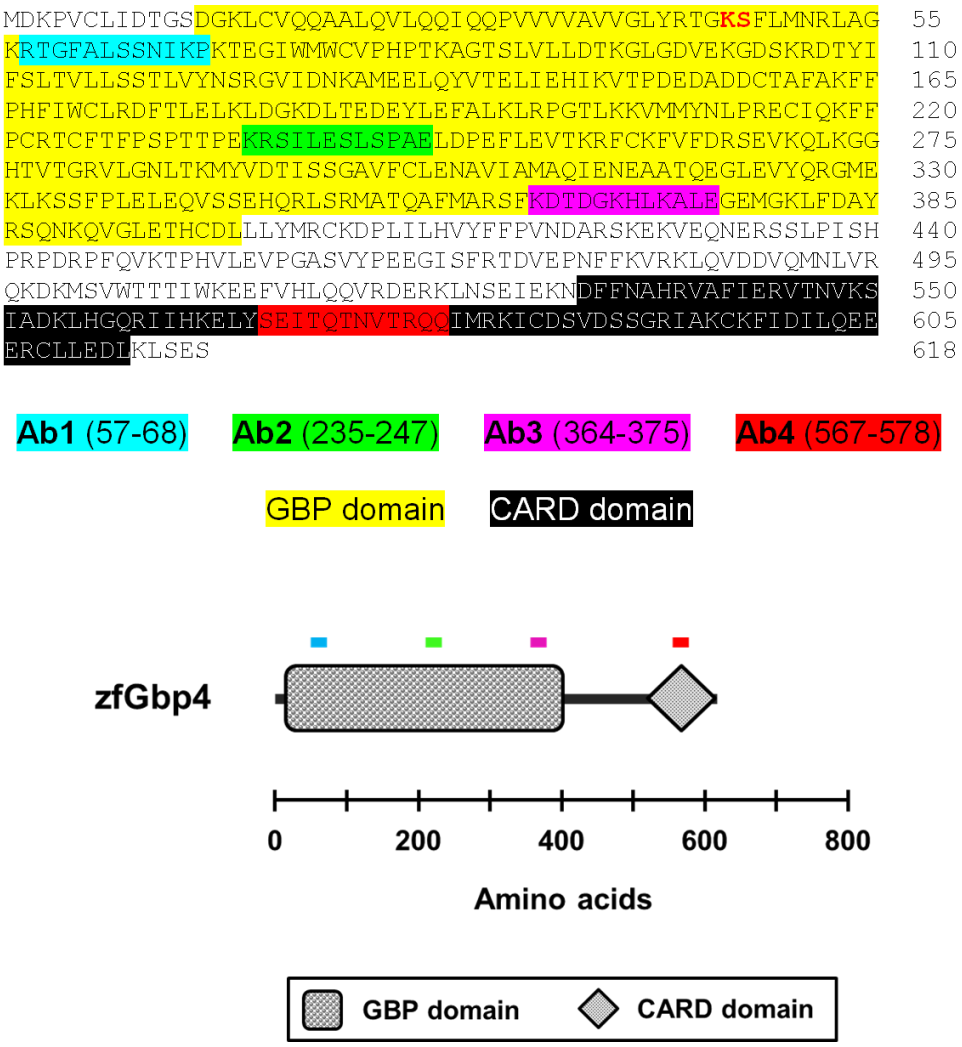


Figure 17. Four different anti-Gbp4 antibodies bind to different parts of the protein. Three of the antibodies, Ab1, Ab2 and Ab3, bind to the Gbp-domain, while the antibody Ab4 binds to the CARD. The GBP domains (Pfam accession number PF02263) are shown as grey boxes, while the CARD domains (Pfam accession number PF00619) are shown as grey rhombus. The position of each domain is indicated with respect to a ruler. Binding sites are shown in different colours.

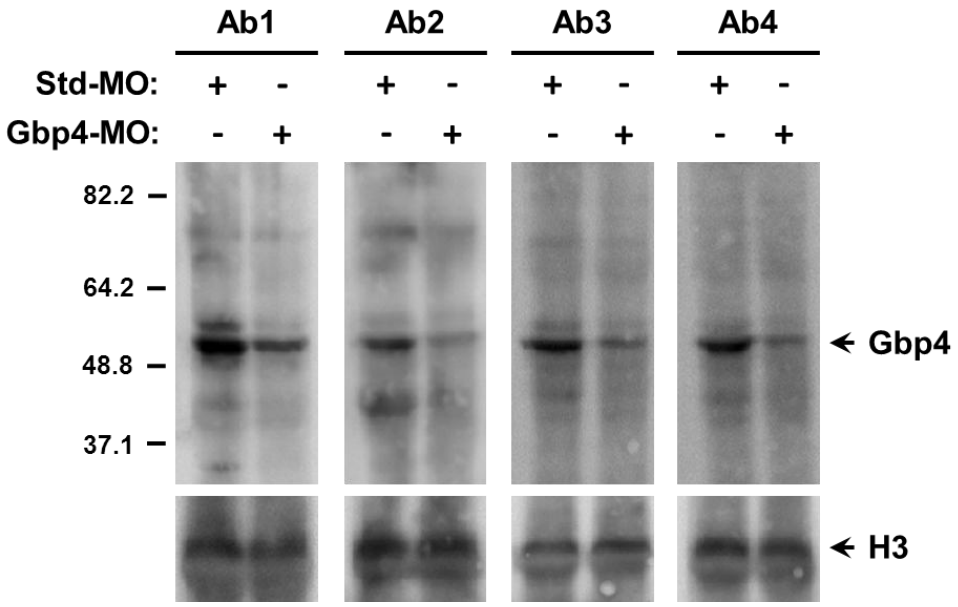


Figure 18. Validation of the MO to Gbp4 by Western blot. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs. Total proteins were isolated from dechorionated and deyolked larvae at 3 dpf (n=50), and the efficiency of Gbp4 MO was validated using western-blot with four different antibodies. An anti-histone 3 (H3) antibody was used for the loading control.

As mentioned in the introduction, the inflammasome activation results in the activation of caspase-1. Thus, we used the caspase-1 activity levels as a mirror of inflammasome activation. Caspase-1 activity was evaluated using a fluorometric substrate, Z-YVAD-AFC, which has previously shown to be processed by fish native and recombinant caspase-1 (López-Castejón *et al*., 2008; Compan *et al*., 2012). Firstly, we found that the 24 hpf larvae presented basal caspase-1 activity. Moreover, the results showed a dose-dependent inhibition of basal caspase-1 activity in larvae injected with increasing concentrations of the Gbp4 MO compared to controls (Figure 19A). No developmental defects or mortality were observed in Gbp4 morphant animals injected with 0.5 pg/egg MO (data not shown), so this dose was chosen to be used in the following experiments of the present thesis. Curiously, the inhibition of caspase-1 activity observed in Gbp4-deficient larvae was similar to that found in larvae deficient in the inflammasome adaptor protein Asc, suggesting that both proteins could play a similar role (Figure 19B).

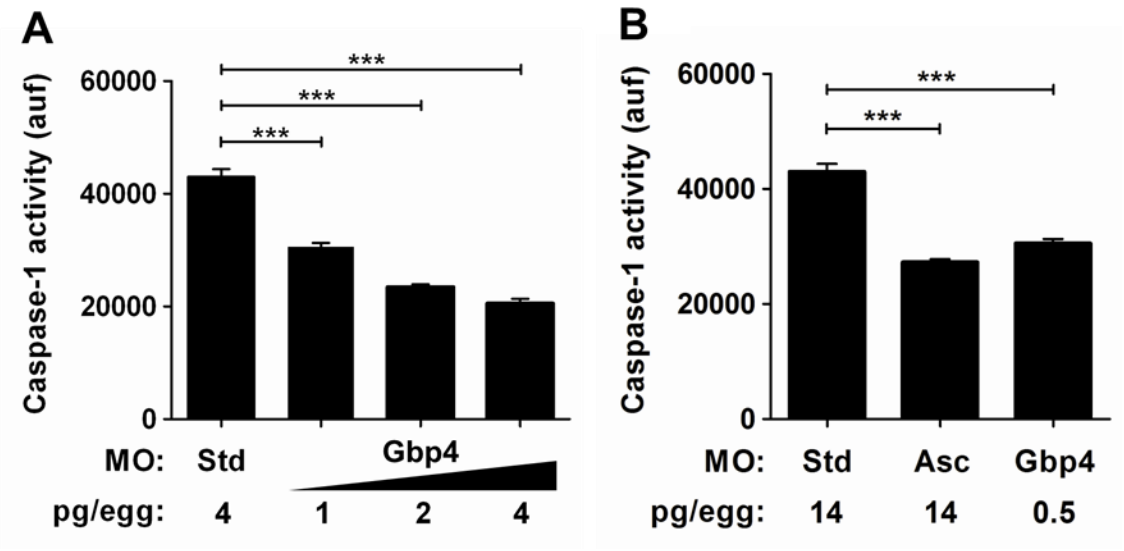


Figure 19. Basal caspase-1 activity levels were decreased in Gbp4-deficient larvae. Zebrafish one-cell embryos were injected with standard control (Std), Gbp4 or Asc MOs. Caspase-1 activity levels were measured, observing that basal levels of caspase-1 activity decreased in a dose-dependent manner in Gbp4-deficient larvae **(A)** to levels similar to those found in Asc-deficient larvae **(B)**. ***p<0.001.

2. Zebrafish Gbp4 is required for the inflammasome-dependent resistance to *S.* Typhimurium

Due to its clinical importance, *S.* Typhimurium has been widely used as a model organism for the study of host-pathogen interactions and, in particular, to the role of the inflammasome in the clearance of intracellular bacteria. As mentioned before, the virulence of *S.* Typhimurium is linked to their two pathogenicity islands, termed SPI-1 and SPI-2, which contain a large number of genes encoding a T3SS. The function of SPI-1 appears to be required for the initial steps of systemic infections (Galán and Curtiss, 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). We have previously shown that infection of zebrafish larvae with wild type (WT) *S.* Typhimurium resulted in a high mortality using two different routes of infection: the yolk sac and the Cuvier's duct (Angosto, 2013). Notably, the percentage of surviving larvae at the day five after infection did not exceed the 40%. In contrast, a syngenic double mutant for SPI-1 and SPI-2 showed reduced virulence. However, single SPI-1 or SPI-2 mutants had an intermediate virulence and survival rate (Angosto, 2013). Notably, WT *S.* Typhimurium induced the activation of caspase-1,

while single or double SPI mutants failed to do so. The levels of caspase-1 activity of all the mutants were similar to the basal levels of the controls (Angosto, 2013). These results demonstrated the usefulness of this model to ascertain the role of *S.* Typhimurium SPI in bacterial virulence in a whole vertebrate organism in the absence of adaptive immunity. So, we next infected Gbp4-deficient fish with WT *S.* Typhimurium and found an increased susceptibility to the infection compared with their control siblings (Figure 20A).

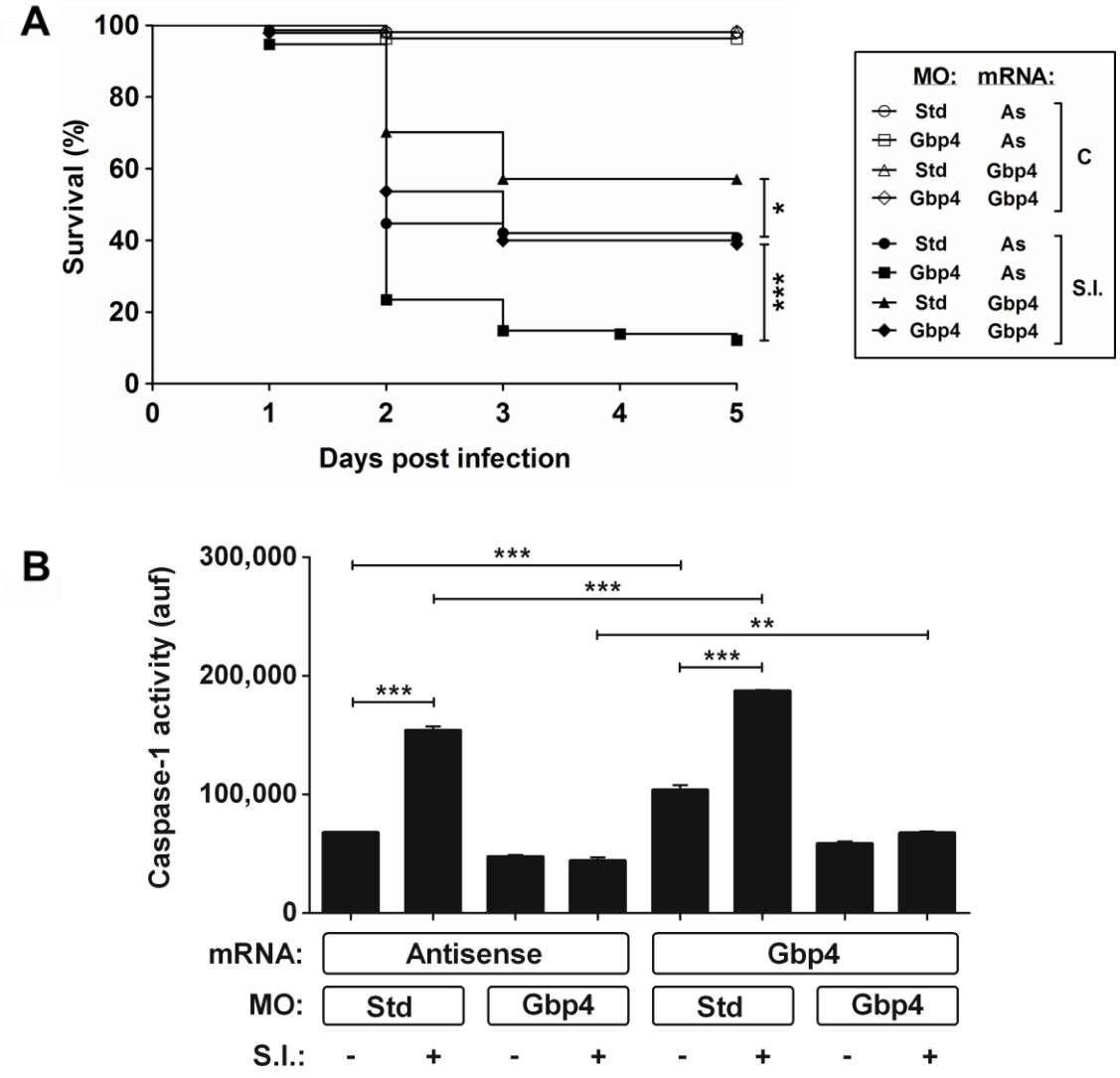


Figure 20. Overexpression of Gbp4 increased the resistance and caspase-1 activity in larvae infected with *S.* Typhimurium. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, in combination with antisense (As) or Gbp4 mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

The caspase-1 activity levels obtained in this experiment (Figure 20B) agreed with the results found in the survival assay (Figure 20A), since we also observed impaired caspase-1 activity in larvae deficient in Gbp4 in response to the infection. In addition, bacterial susceptibility and caspase-1 activity were both reversed by injection of non-targetable Gbp4 mRNA. In this case Gbp4 mRNA was able to rescue the effect of MO Gbp4. More interestingly, forced overexpression of Gbp4 mRNA alone increased resistance of the fish to the infection (Figure 20A). Caspase-1 activity levels were also increased upon the infection (Figure 20B). Going further, the results obtained in these experiments confirmed the specificity of the MO, and revealed the crucial role played by Gbp4 in the resistance against *S.* Typhimurium.

In contrast, both survival (Figure 21A) and caspase-1 activity (Figure 21B) assays showed that Gbp4 overexpression did not affect fish resistance to the SPI-1/SPI-2 *S.* Typhimurium mutant. Thus, no significant differences were found in the survival rates comparing larvae overexpressing Gbp4 to controls after *S.* Typhimurium double mutant infection (Figure 21A). Following a similar pattern, the caspase-1 activity levels did not increase upon the SPI-1/SPI-2 mutant infection (Figure 21B).

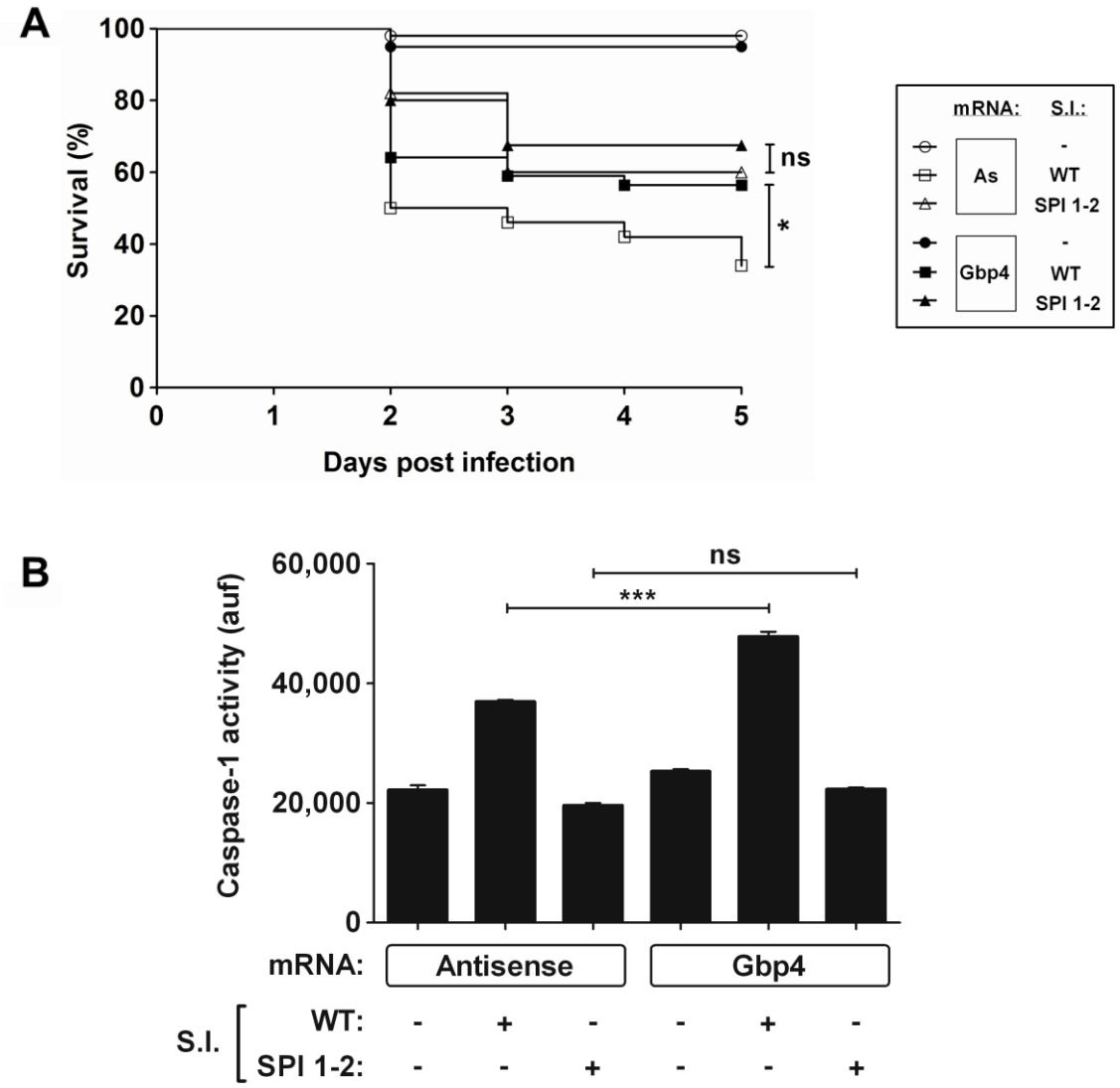


Figure 21. Overexpression of Gbp4 did not increase the larvae resistance and the caspase-1 activity levels upon infection with the double mutant of S. Typhimurium for the T3SS (SPI 1-2). Zebrafish one-cell embryos were injected with antisense (As) or Gbp4 mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. Two different strains of *S.* Typhimurium were used: the wild type (WT) and the mutant SPI 1-2, which presents a double mutation in the T3SS. S.I., *S.* Typhimurium infection. ns, not significant; *p<0.05; ***p<0.001.

Notably, the detection of *S.* Typhimurium in zebrafish was largely dependent on flagellin, a globular protein that arranges itself in a hollow cylinder to form the filament in a bacterial flagellum. The strain that persistently expresses the flagellin protein FliC (FliCON) (Miao *et al.*, 2010a) showed reduced virulence, hence higher resistance of the larvae (Figure 22A), and was most powerful at inducing caspase-1 activity (Figure 22B).

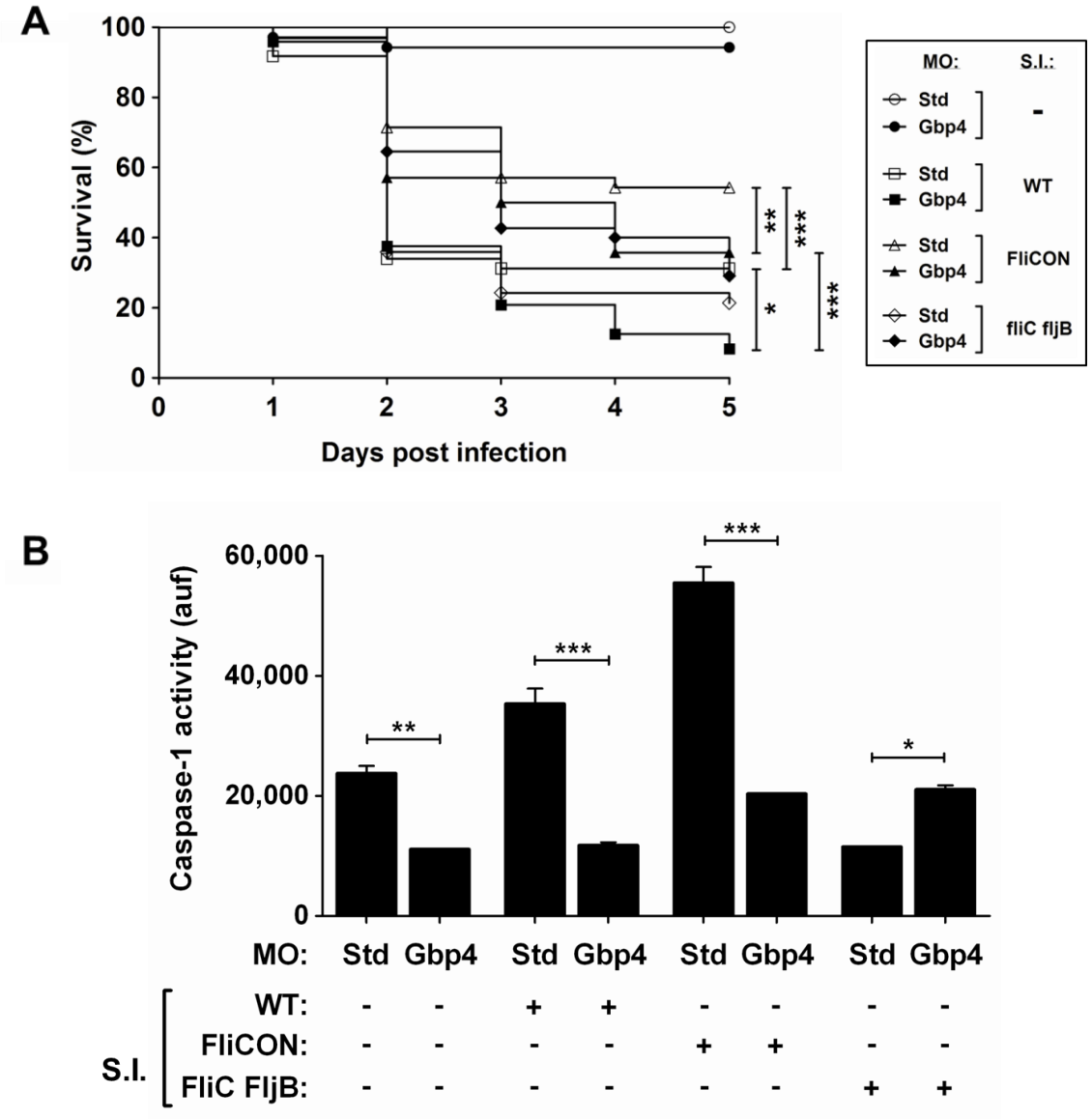


Figure 22. *S.* Typhimurium recognition and clearance in zebrafish was dependent on flagellin. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. Three different strains of *S.* Typhimurium were used: the wild type (WT), the FliCON and he mutant FliC/FljB, which present differences in the flagellin production. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

However, the syngenic mutant strain lacking flagellin (FliC/FljB) (Miao *et al.*, 2006) behaved as the WT strain (Figures 22), suggesting that WT *S.* Typhimurium repressed flagellin expression after infection, as occurs in mammals (Cummings *et al.*, 2006). In addition, all these responses were clearly dependent on Gbp4, since its depletion resulted in the inhibition of the resistance of fish to S. Typhimurium infection (Figure 22A), as well as in the decrease of the caspase-1 activity levels (Figure 22B).

Although a homolog of mammalian caspase-1 has never been identified in the zebrafish genome to date, it has been reported that caspy, a caspase harboring an N-terminal pyrin domain (PYD), which preferentially cleaves AcYVAD-AMC, a caspase-1 substrate, is able to interact and co-localize to the speck with zebrafish Asc when ectopically expressed in HEK cells (Masumoto *et al*., 2003).

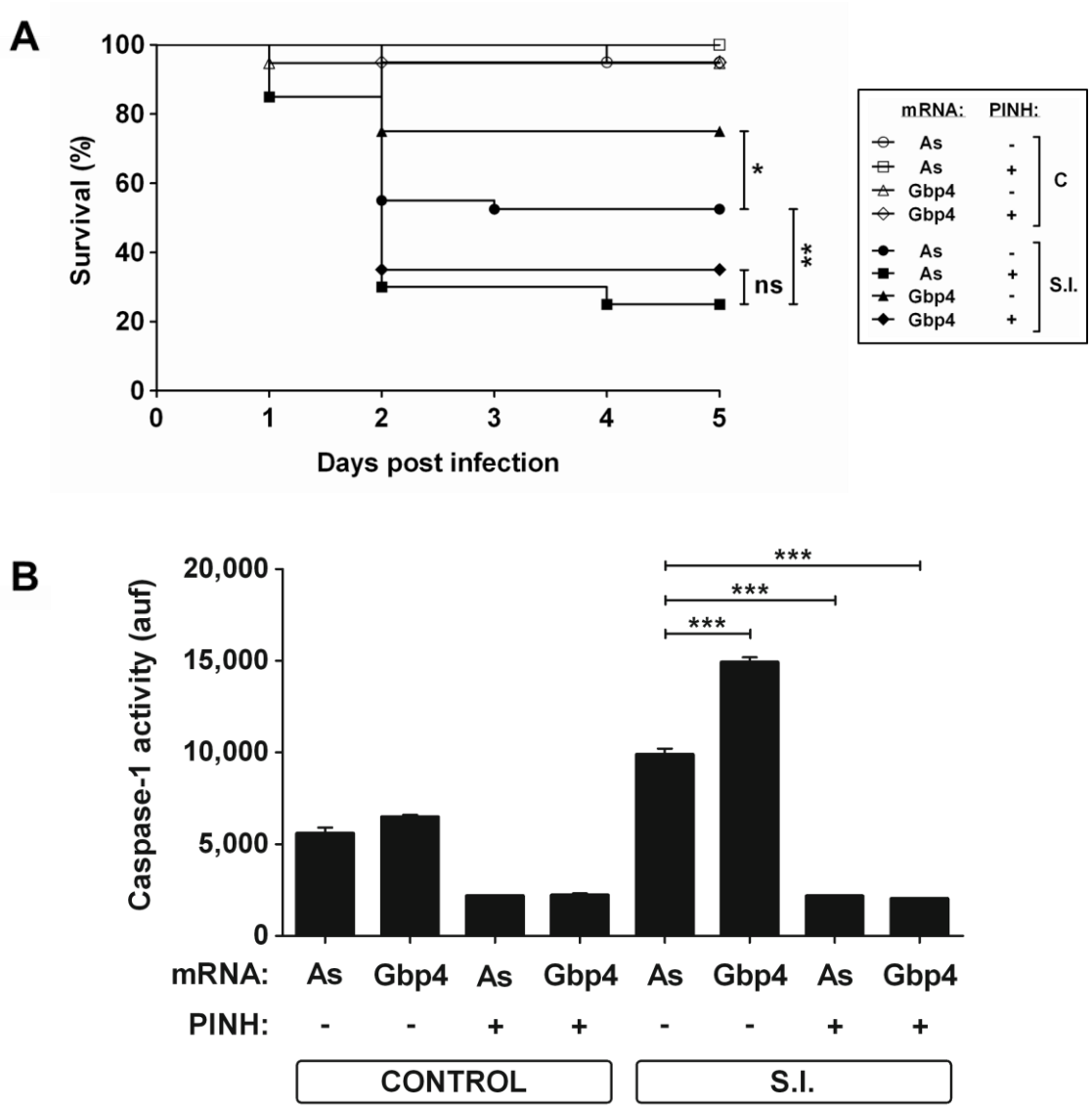


Figure 23. Chemical inhibition of caspases abrogated the high resistance and decreased *in vivo* the caspase-1 activity caused by overexpression of Gbp4. Zebrafish one-cell embryos were injected with antisense (As) or Gbp4 mRNAs, and treated by immersion in 50 µM of a general inhibitor of caspases (PINH) or vehicle alone (DMSO). Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

So that, we used both a pan-caspase (Figure 23) and a specific caspase-1

inhibitor (Figure 24), which has been shown to inhibit the activity of fish recombinant caspase-1 (López-Castejón *et al*., 2008; Compan *et al*., 2012), observing that bath treatment with both inhibitors resulted in increased susceptibility of zebrafish larvae to *S.* Typhimurium infection and, more importantly, fully abrogated the Gbp4-mediated increased resistance to the infection (Figures 23A and 24A).

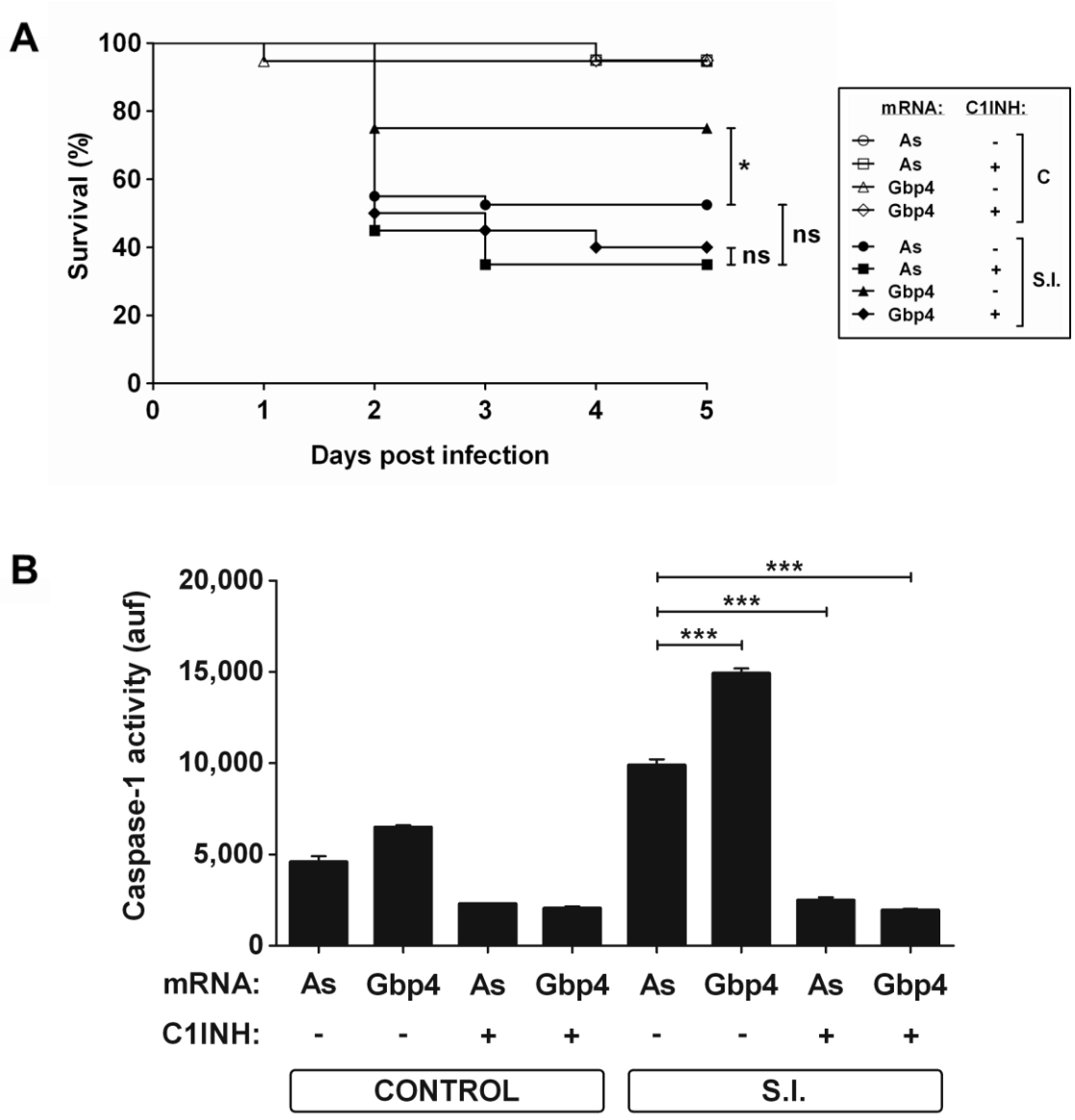


Figure 24. Pharmacological inhibition of caspase-1 abrogated the high resistance to infection and decreased caspase-1 activity *in vivo* caused by overexpression of Gbp4. Zebrafish one-cell embryos were injected with antisense (As) or Gbp4 mRNAs, and treated by immersion in 100 µM of a specific inhibitor of caspase-1 (C1INH) or vehicle alone (DMSO). Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

As expected, both inhibitors strongly decreased the caspase-1 activity levels *in vivo*, even their basal levels (Figures 23B and 24B).

The presence of a CARD in zebrafish Gbp4 suggests that it may directly recruit and activate caspase-1 without the need of the adaptor Asc. Something similar occurs in the case of the mammalian NLRC4, which also possesses the CARD domain and is able to directly recruit and activate capase-1 (Latz *et al*., 2013). Taking that into consideration, we knocked down Asc using a splice-blocking MO that targeted the E2/I2 boundary of *asc* RNA (Angosto, 2013).

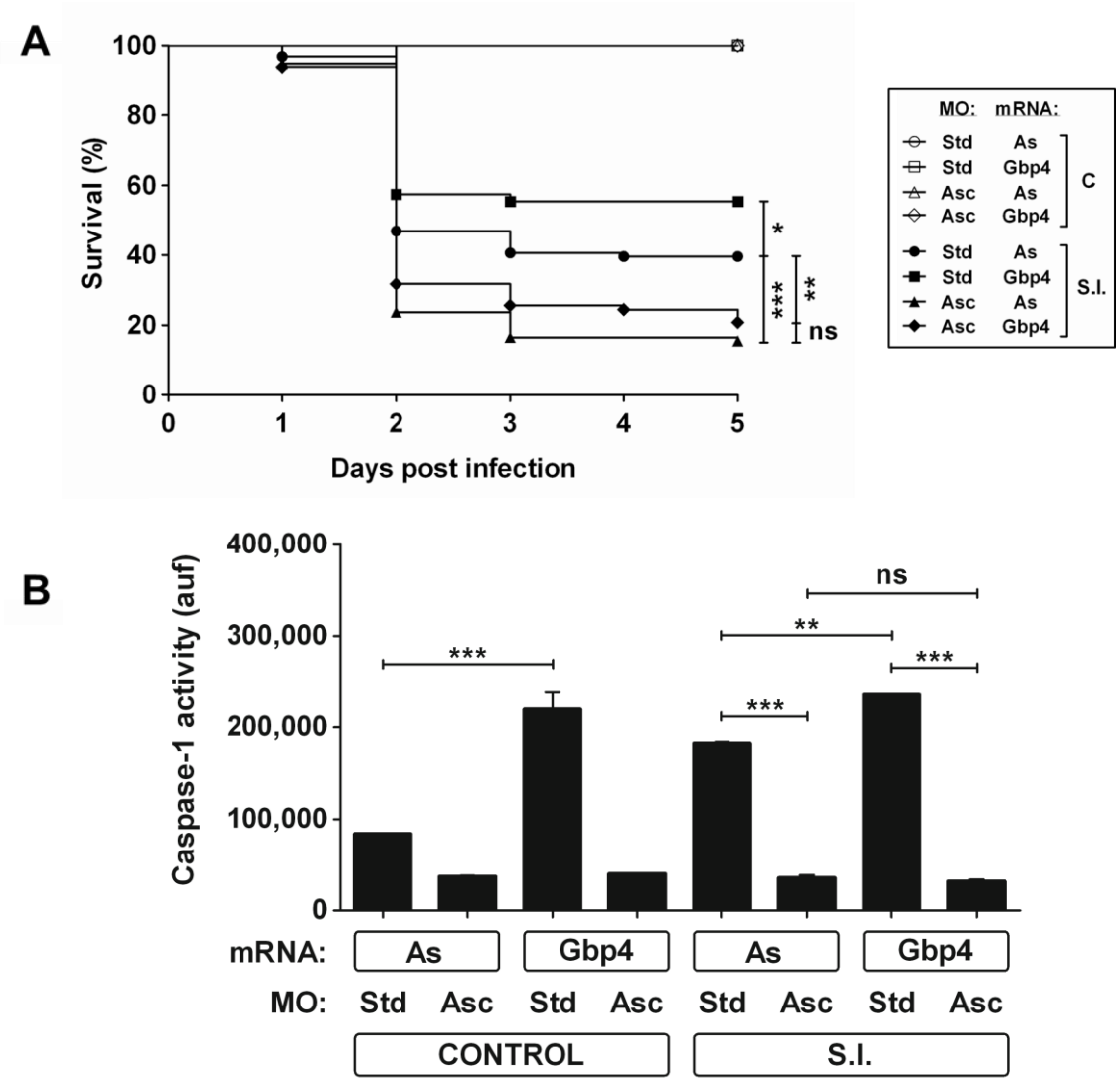


Figure 25. Genetic inhibition of Asc abrogated the Gbp4-induced resistance and caspase-1 activity upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Asc MOs, in combination with antisense (As) or Gbp4 mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

Genetic depletion of Asc in zebrafish had no apparent effects on larval development, but significantly reduced basal (Figure 19B) and *S.* Typhimurium- and Gbp4-induced caspase-1 activity (Figure 25B). Moreover, we observed that Asc deficiency drastically increased the susceptibility of larvae to *S.* Typhimurium and, strikingly, completely abrogated the Gbp4-mediated increased infection resistance (Figure 25A). Thus, the fact that Gbp4 mRNA was unable to rescue the effect of Asc MO suggests that Gbp4 requires Asc for activating the inflammasome.

Next, we wanted to confirm the phenotype and the results obtained using the

Asc MO. For that, we used another morpholino targeting Asc (Asc-ATG MO). This new MO directly inhibits the translation of the mRNA into protein. Asc-ATG MO binds to the 5'-untranslated region (5'-UTR) of the mRNA, and can interfere with the progression of the ribosomal initiation complex from the 5' cap to the start codon. In this way, the translation of the coding region of the targeted transcript is avoided.

The results obtained with the Asc-ATG MO confirmed what we had observed before using the Asc MO, including the increased susceptibility to *S.* Typhimurium infection (Figure 26A) and the reduction of caspase-1 activity levels (Figure 26B). The fact that the overexpression of Gbp4 was unable to rescue the effects of the Asc-ATG MO also agreed with the results previously obtained with the Asc MO (Figure 26). In addition, as we found using the Asc MO, the overexpression of Gbp4 in the Asc-ATG morphants did not result neither in the increase of the resistance to *S.* Typhimurium infection (Figure 26A) nor in the increase of caspase-1 activity levels in infected larvae (Figure 26B).

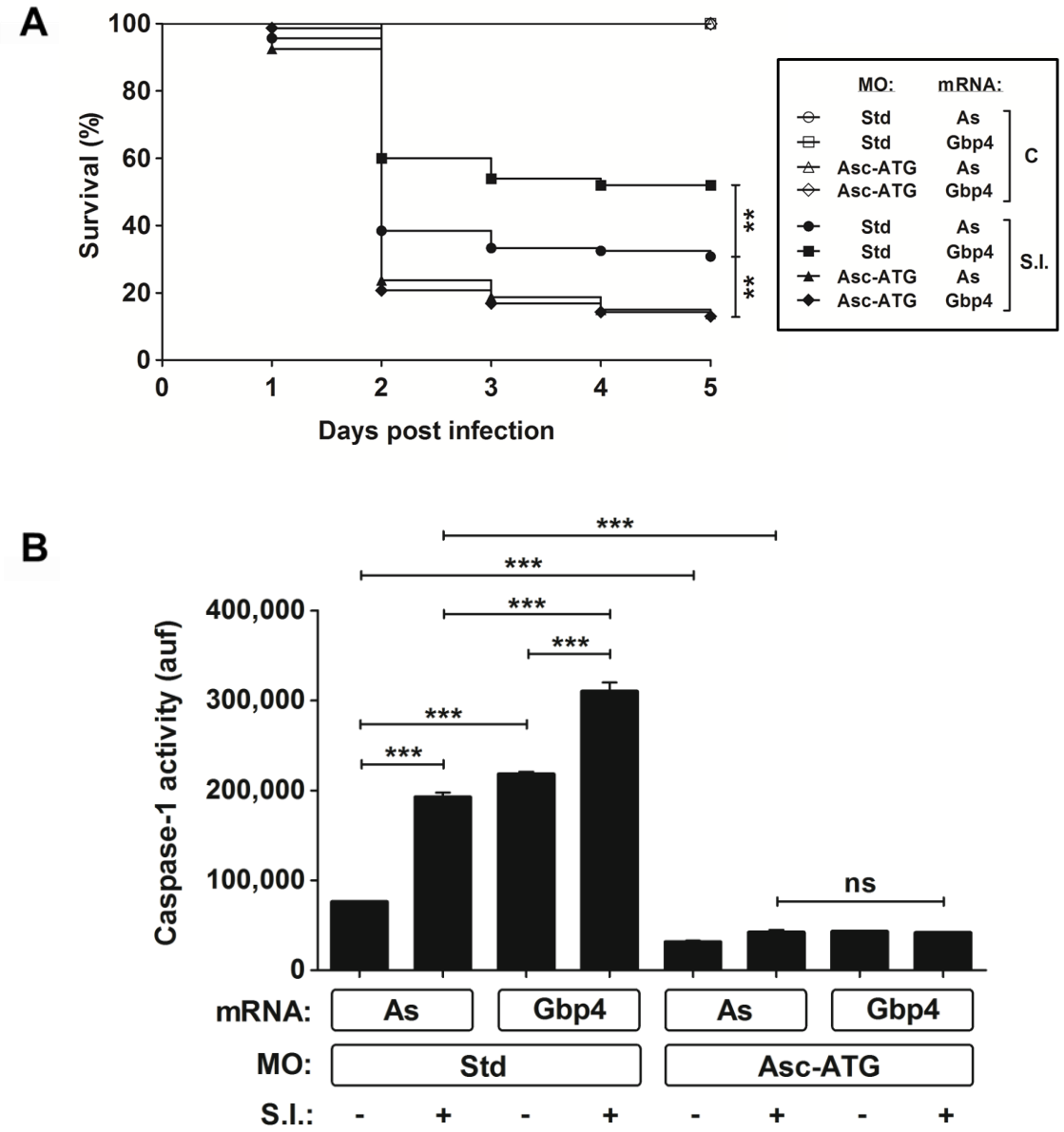


Figure 26. Asc-ATG MO also decreased the resistance of fish and caspase-1 activity levels upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Asc-ATG MOs, in combination with antisense (As) or GBP4 mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. ns, not significant; **p<0.01; ***p<0.001.

These results led us to study the impact of forced overexpression of Asc and found that resulted in strongly increased larval resistance to the infection (Figure 27A) and caspase-1 activity (Figure 27B), these effects being largely independent of Gbp4. In this case, the overexpression of Asc was able to rescue the effect of the Gbp4 MO (Figures 27).

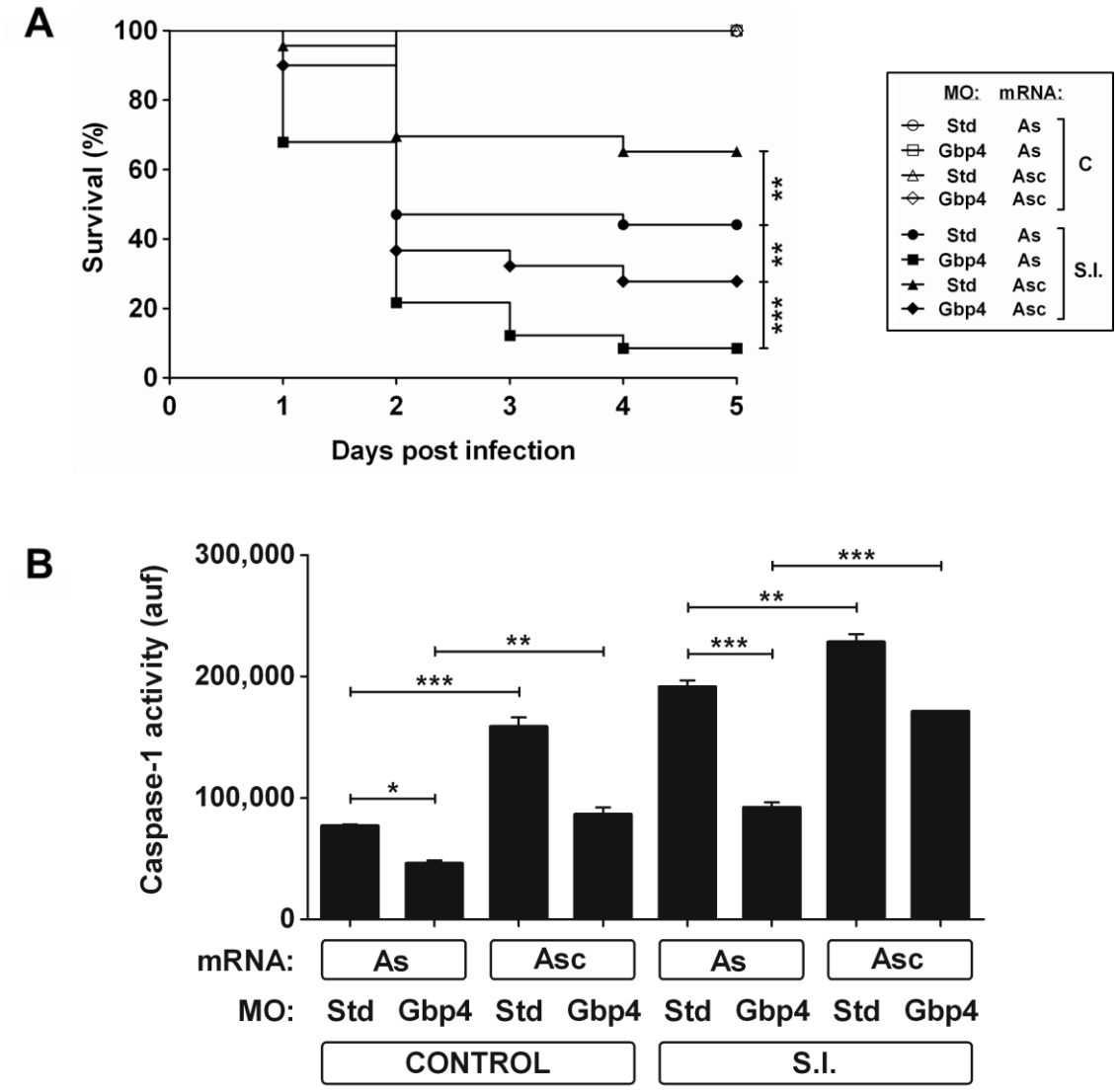


Figure 27. Forced expression of Asc increased the resistance and caspase-1 activity levels of Gbp4-deficient larvae. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, in combination with antisense (As) or Asc mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

The relevance of Asc in the clearance of *S.* Typhimurium was further confirmed by the ability of a mutant zebrafish Asc, harboring a C-terminal GFP instead of its CARD, to behave as DN by increasing larval susceptibility to *S.* Typhimurium (Figure 28). Collectively, these results demonstrate that Gbp4 acts upstream Asc to induce the activation of the inflammasome.

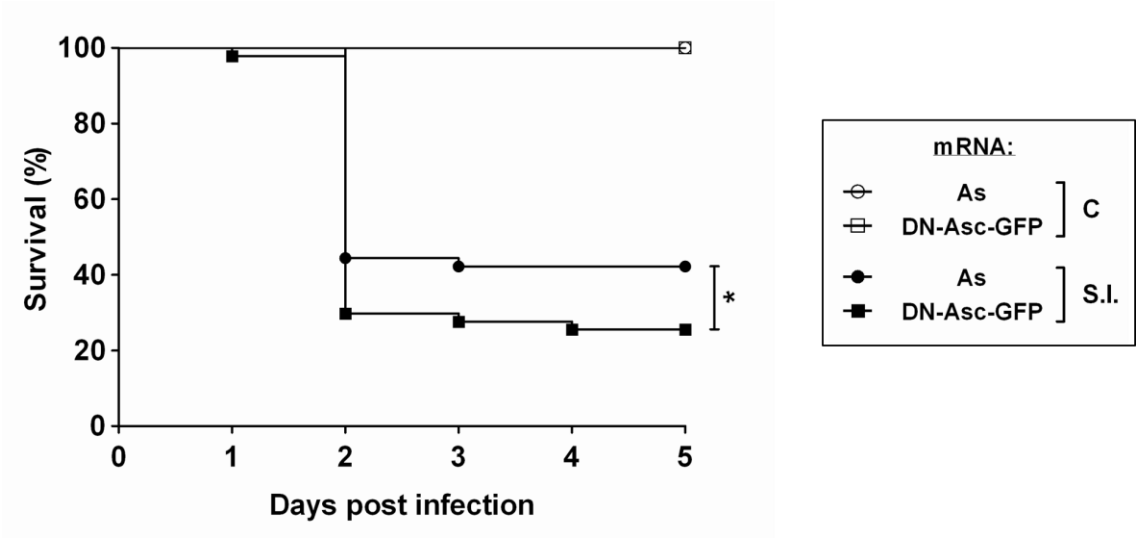


Figure 28. Overexpression of a dominant negative form of Asc confirmed the phenotype of Asc deficient larvae. Zebrafish one-cell embryos were injected with antisense (As) or DN-Asc-GFP mRNAs. Survival assays were performed as described in Figure 12. S.I., *S.* Typhimurium infection. *p<0.05.

3. The GTPase activity of Gbp4 is indispensable for the inflammasome-dependent resistance to *S.* Typhimurium

Although mammalian GBP1 displays GTPase-dependent tetramerization, both recombinant WT GBP5 and the GTPase-deficient mutant (GBP5KS→AA) formed tetramers and were equally able to promote Asc multimerization (Shenoy *et al*., 2012). However, ASC assembly was abolished when two tetrameric GBP5 mutants were used, suggesting that tetrameric GBP5 promotes Asc oligomerization (Shenoy *et al*., 2012). Then we also generated a zebrafish Gbp4 mutant form deficient in its GTPase activity (Gbp4KS→AA) and, unexpectedly, we found that it was not only unable to increase the resistance of zebrafish to *S.* Typhimurium infection, but rather it behaved as a DN that increased susceptibility to the infection (Figure 29A) and concomitantly inhibited caspase-1 activity (Figure 29B).

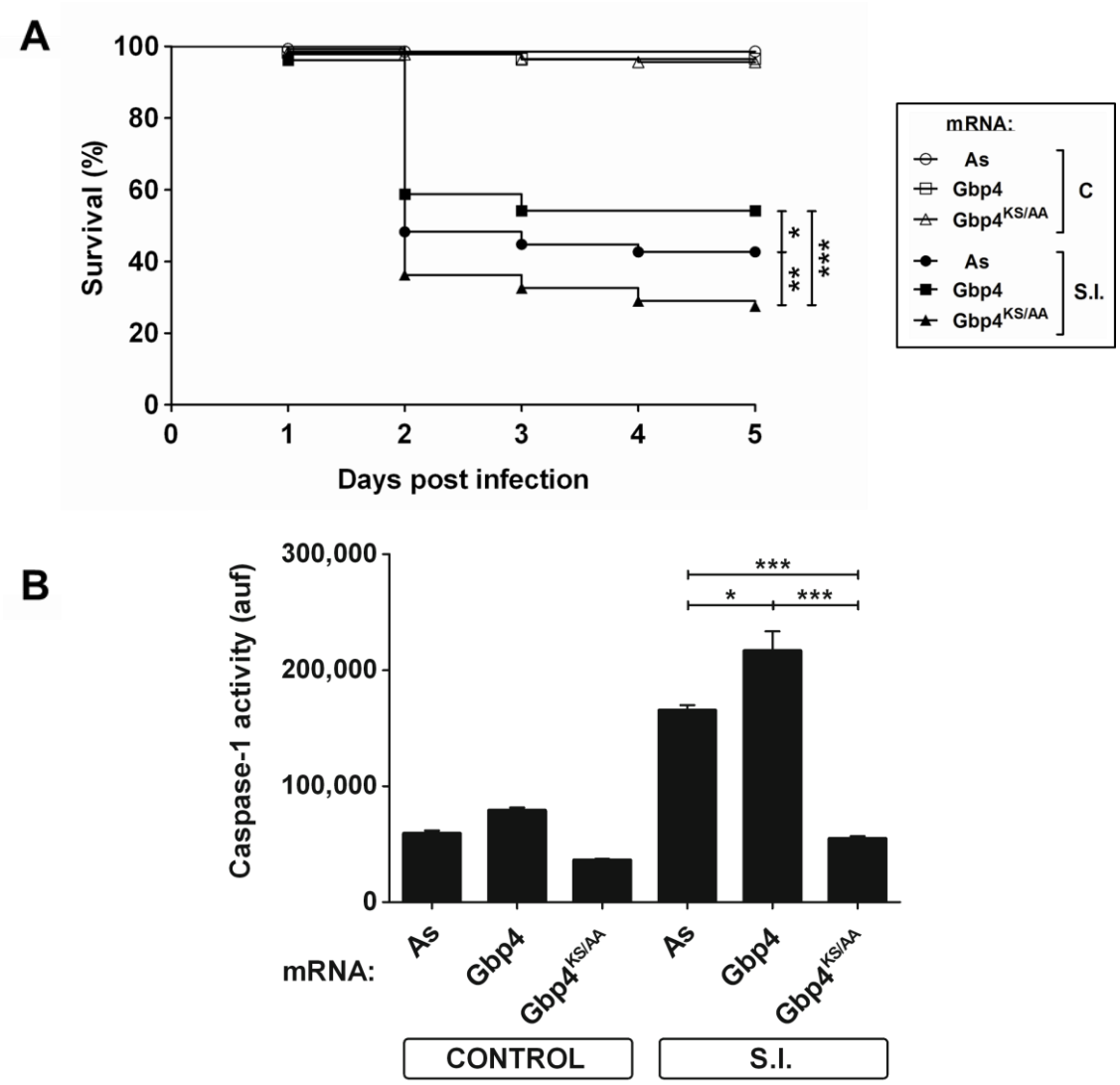


Figure 29. Forced expression of a Gbp4 mutant form deficient in its GTPase activity resulted in high susceptibility and decreased caspase-1 activity levels upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with antisense (As), Gbp4, or Gbp4KS/AA mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

After that, we decided to test the impact of a Gbp4 mutant devoid of its CARD (Gbp4ΔCARD) and observed that although it was able to partially rescue the higher susceptibility of Gbp4-deficient larvae, it failed to increase their infection resistance when expressed alone (Figure 30A). In addition, it hardly reversed the caspase-1 activity observed in Gbp4-deficient larvae upon infection (Figure 30B).

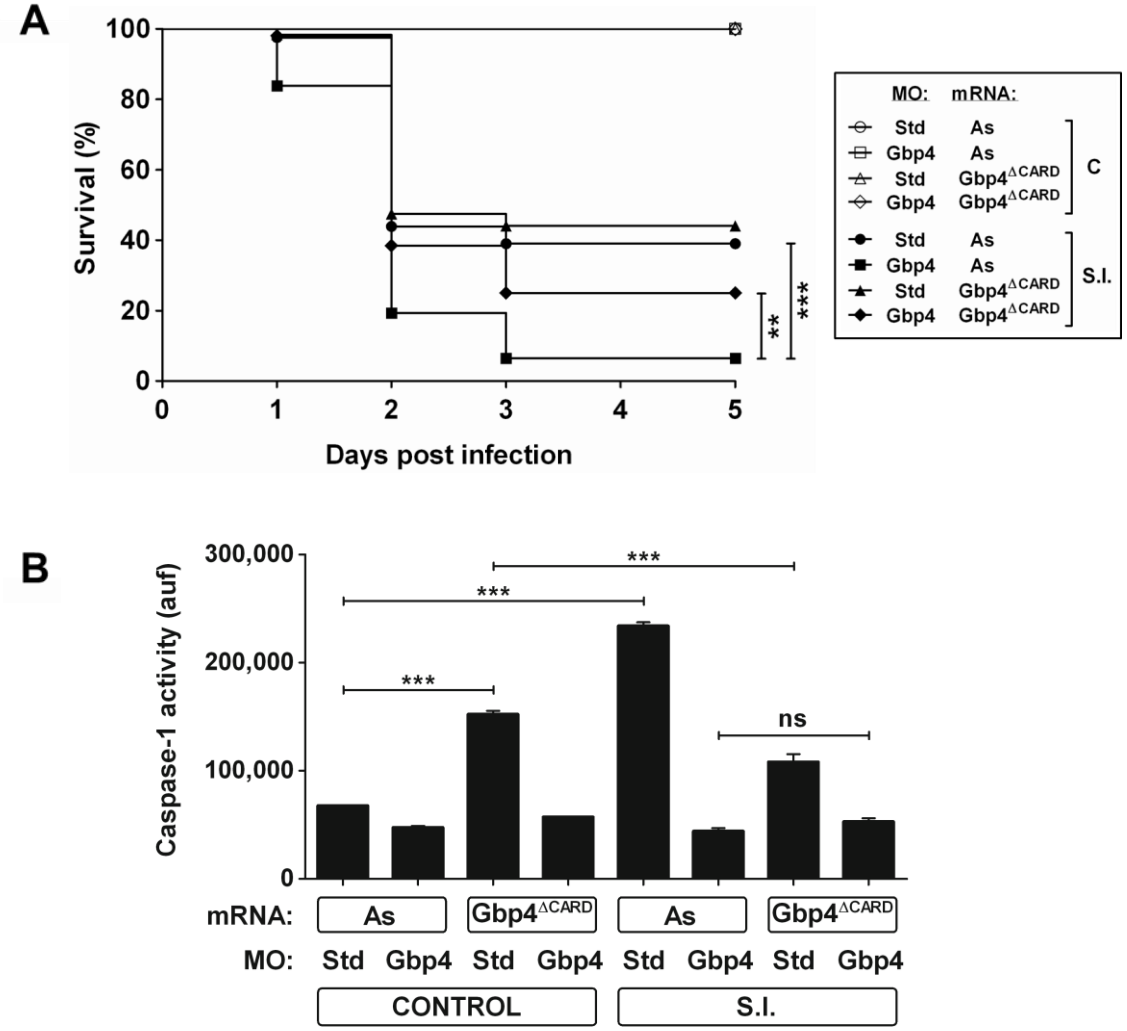


Figure 30. Forced expression of a CARD mutant form of Gbp4 partially rescued the susceptibility and hardly reversed the caspase-1 activity of Gbp4-deficient larvae upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, in combination with antisense (As) or Gbp4ΔCARD mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. ns, not significant; **p<0.01; ***p<0.001.

On the other hand, a double mutant (DM) form devoid of both GTPase activity and the CARD behaved as the GTPase-deficient mutant (Figure 31). Moreover, thisdouble mutants showed higher susceptibility upon the infection compared to controls (Figure 31A). What is more, their caspase-1 activity levels were not increased after the infection and were comparable with the basal levels of not infected controls (Figure 31B).

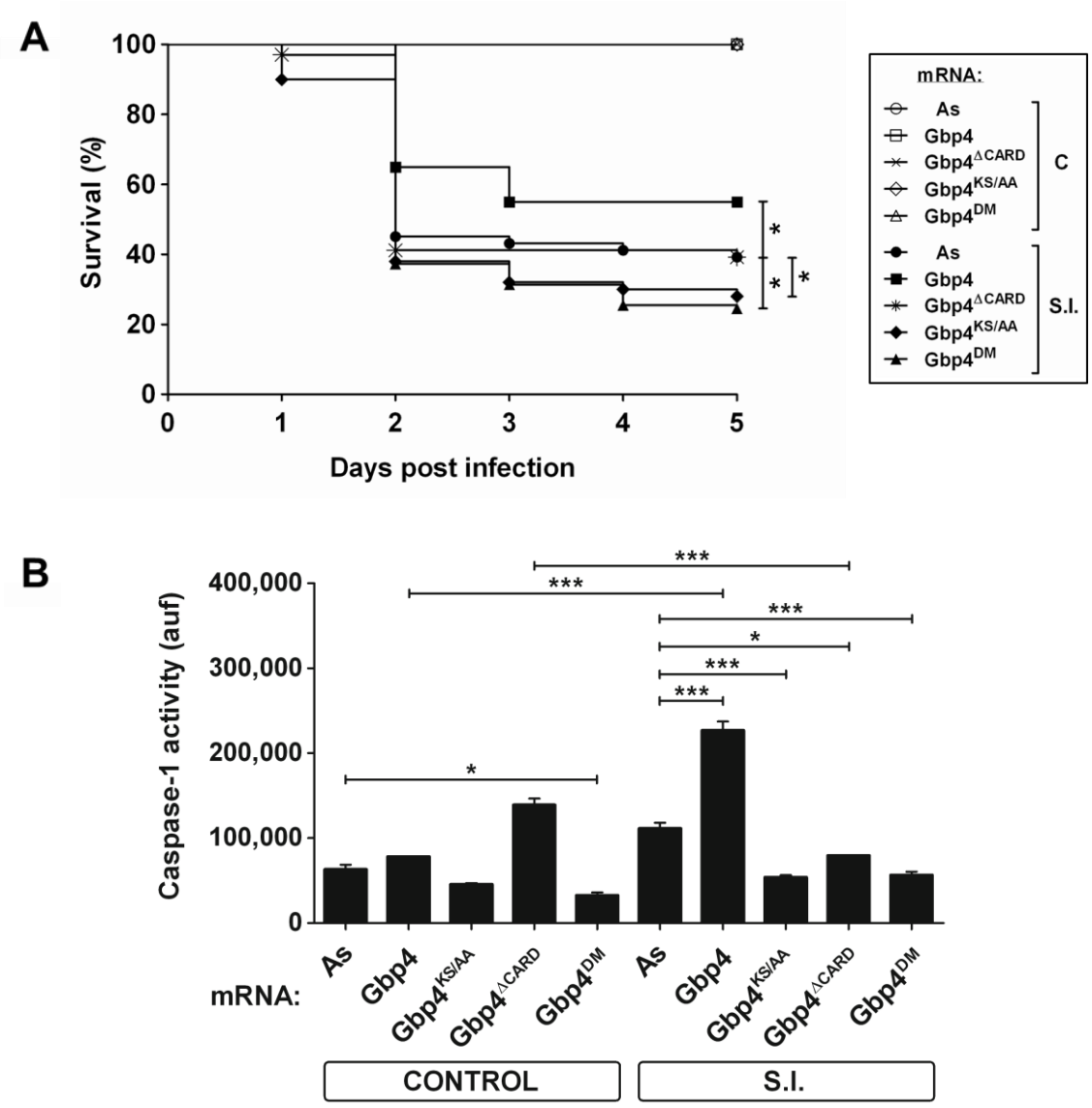


Figure 31. Forced expression of a double mutant form of Gbp4 did not rescue the susceptibility and the caspase-1 activity levels of Gbp4-deficient larvae upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with antisense (As), Gbp4, Gbp4ΔCARD, Gbp4KS/AA, or Gbp4DM mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; ***p<0.001.

Strikingly, mouse GBP5 was able to fully reverse the high susceptibility to *S.* Typhimurium (Figure 32A) of Gbp4-deficient larvae and their lower caspase-1 activity levels in non-infected conditions (Figure 32B). However, it was unable to increase the larval infection resistance when expressed alone, even though it increased caspase-1 activity in non-infected animals (Figure 32B).

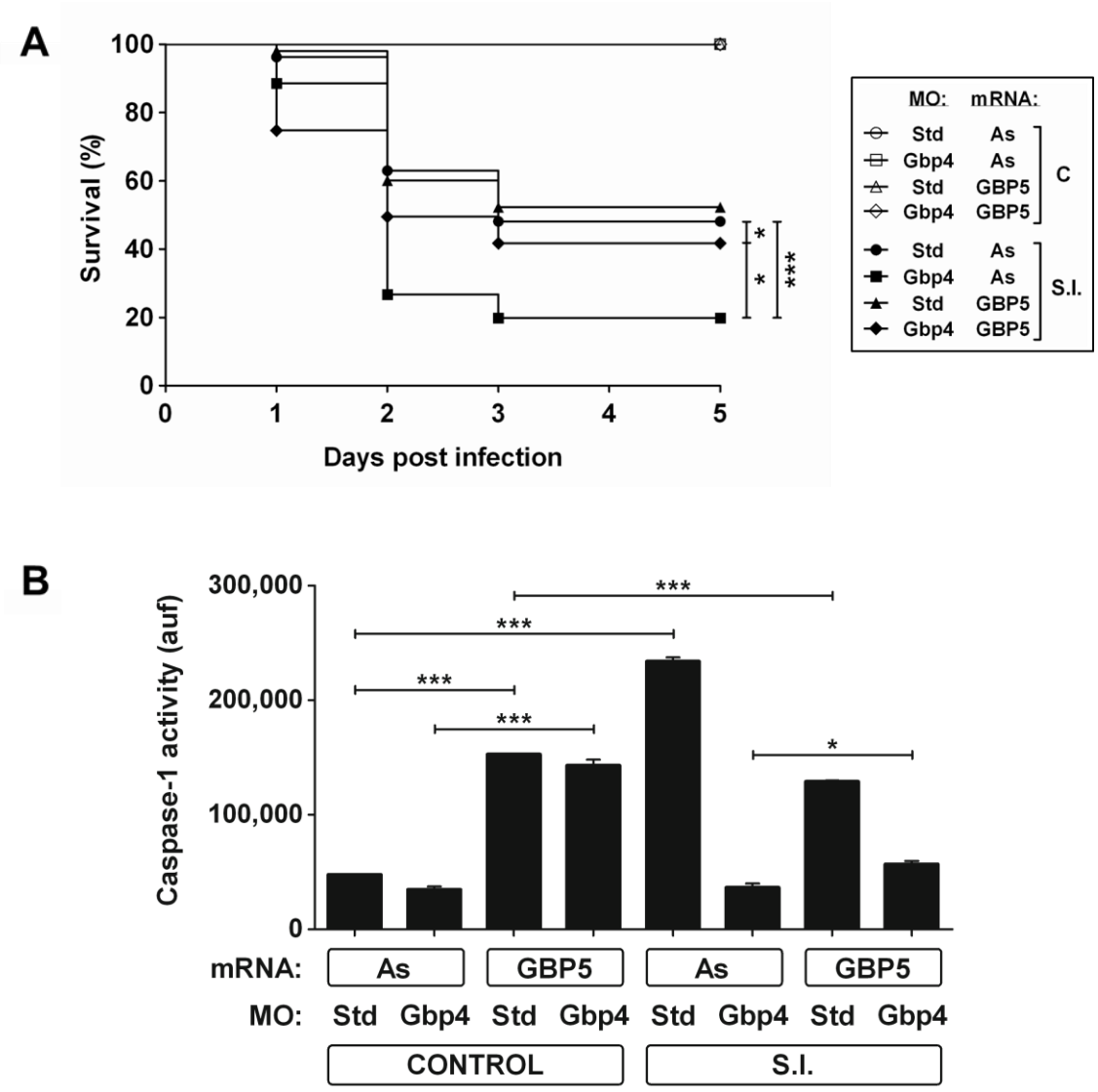


Figure 32. Forced expression of mouse GBP5 rescued the susceptibility but was unable to increase the caspase-1 activity levels of Gbp4-deficient larvae upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, in combination with antisense (As) or GBP5 mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; ***p<0.001.

Furthermore, all these effects required zebrafish Asc (Figures 33), as reported in mammals (Shenoy *et al.*, 2012). Overexpression of mouse GBP5 was not able to rescue the high susceptibility due to Asc-deficiency (Figure 33A), while the decreased levels of caspase-1 activity in larvae deficient in Asc were not improved by the overexpression of GBP5 (Figure 33B).

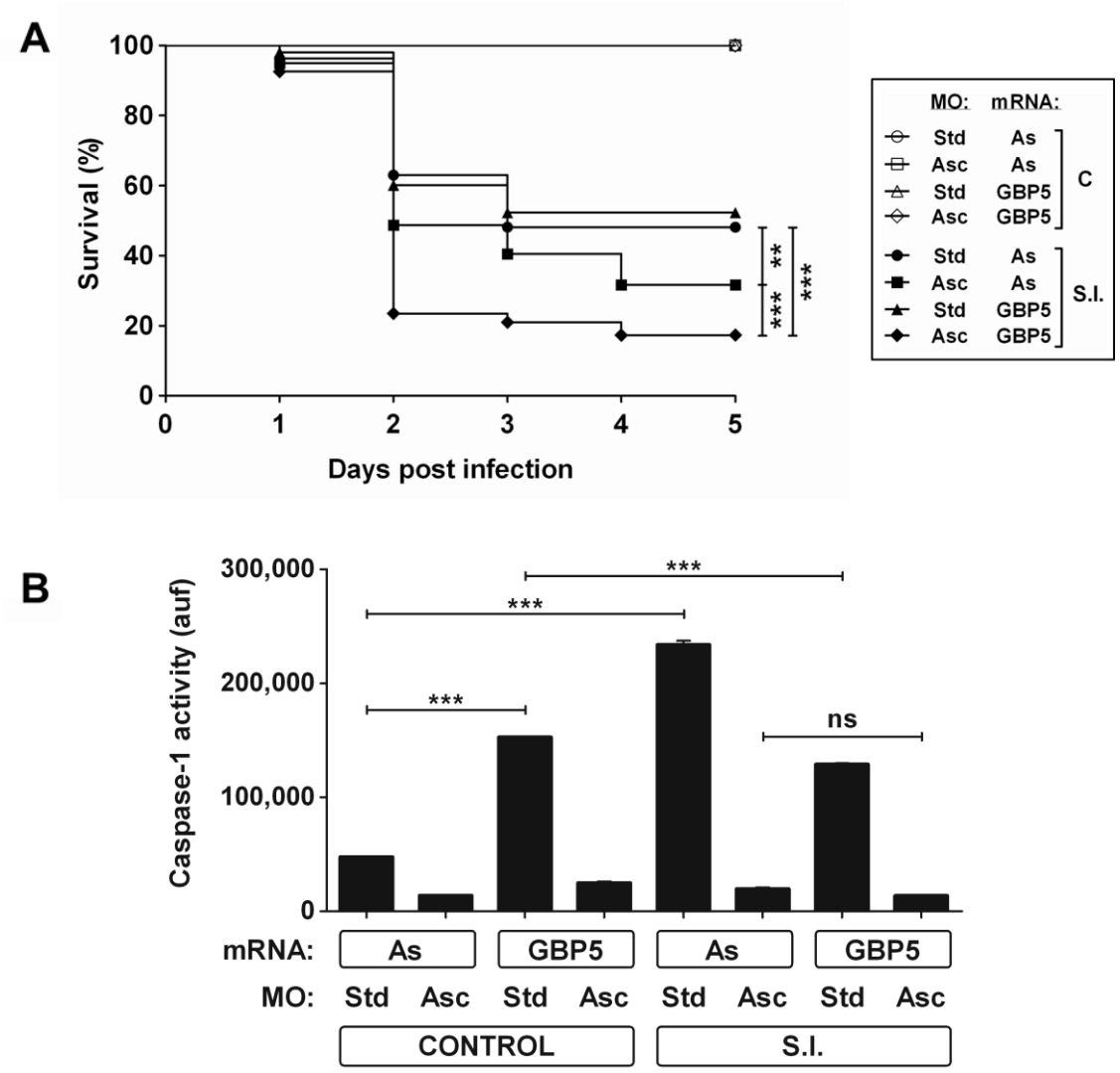


Figure 33. Mouse GBP5 was unable to rescue the lost resistance and did not increase the caspase-1 activity in Asc-deficient larvae upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Asc MOs, in combination with antisense (As) or GBP5 mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. ns, not significant. **p<0.01; ***p<0.001.

Notably, mouse GBP5KS→AA failed to rescue the hypersusceptibility to *S.* Typhimurium (Figure 34A) and to restore caspase-1 activity levels (Figure 34B) in Gbp4-deficient larvae. In addition, it did not show a DN effect, in contrast to zebrafish Gbp4KS→AA (Figure 29).

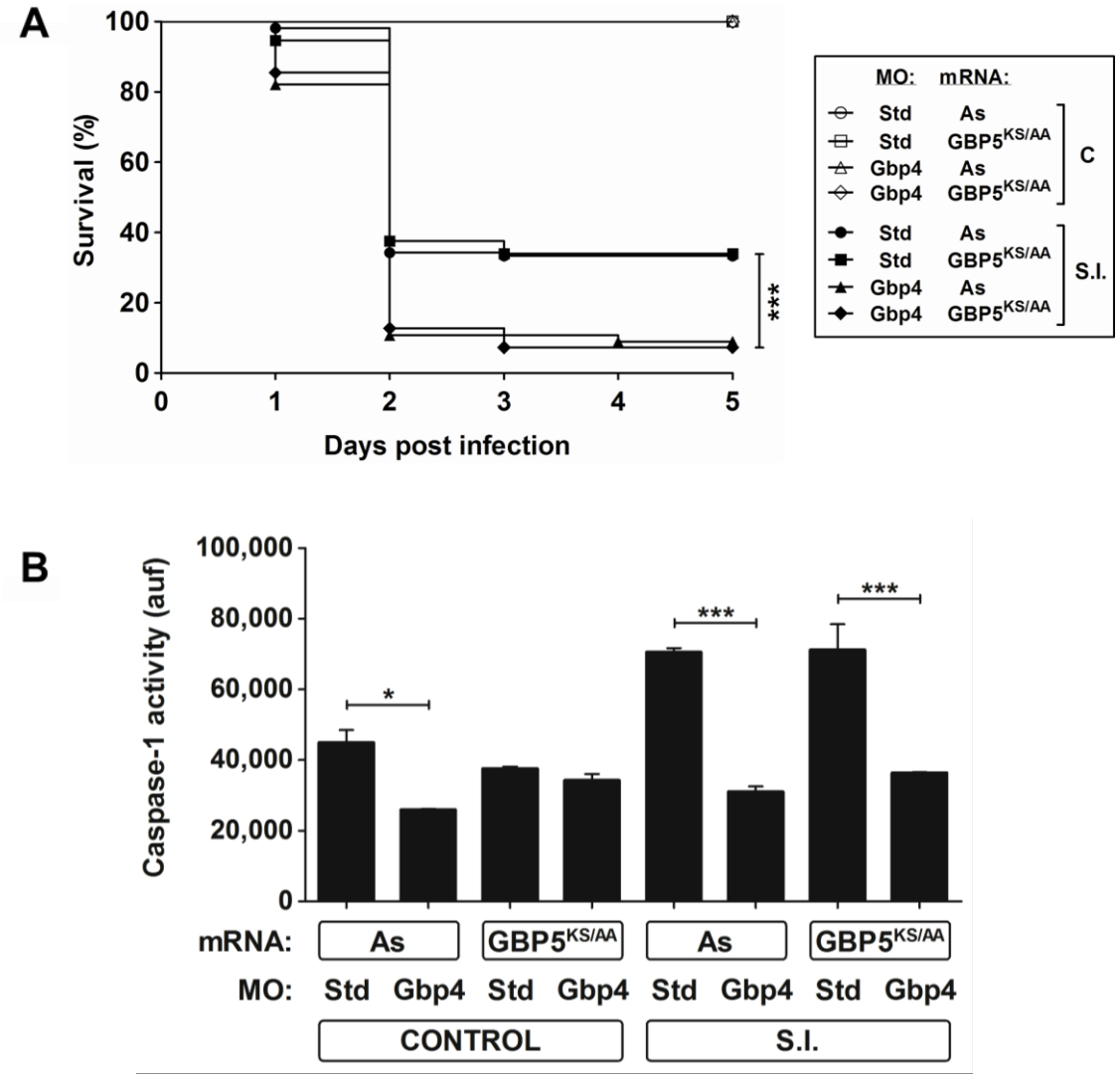


Figure 34. The GTPase mutant of mouse GBP5 was unable to rescue the susceptibility and caspase-1 activity levels in Gbp4-deficient larvae upon the *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, in combination with antisense (As) or GBP5KS/AA mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; ***p<0.001.

Collectively, these results indicate that mammalian GBP5 behaved as zebrafish Gbp4ΔCARD, and suggest that the CARD of zebrafish Gbp4 conferred its ability to promote caspase-1-mediated resistance to intracellular bacteria. Supporting this hypothesis, the expression of increasing amounts of WT Gbp4 promoted a dose-dependent activation of caspase-1 in both control and infected larvae (Figure 35).

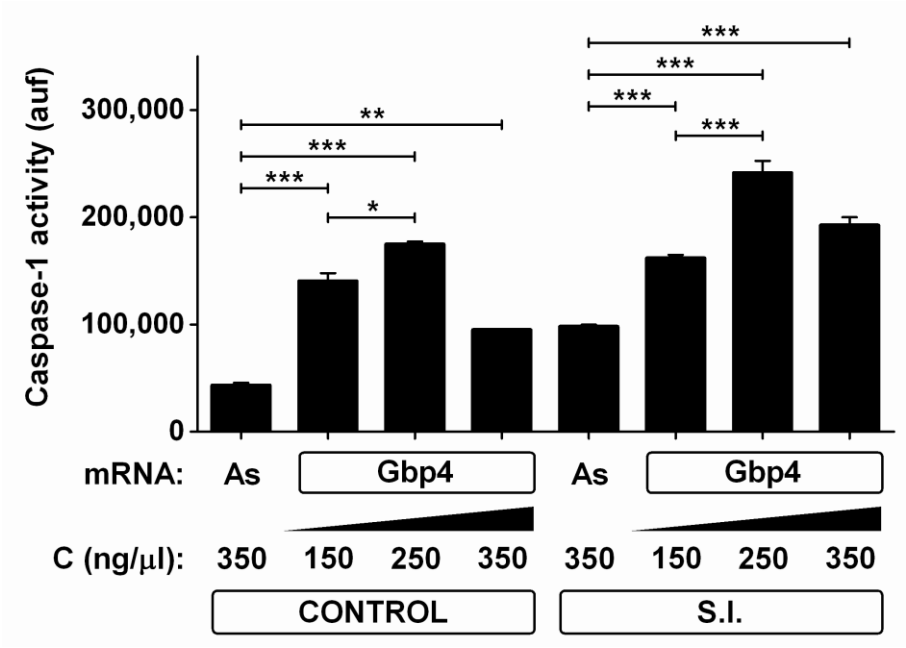


Figure 35. Increasing amounts of Gbp4 mRNA caused dose-dependent activation of caspase-1 activity upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with antisense (As) or increasing concentrations of Gbp4 mRNAs. Caspase-1 activity assay was performed as described in Figure 13. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

However, as expected, Gbp4KS→AA and Gbp4DM impaired the activation of caspase-1 in both conditions (Figures 36 and 37), confirming their DN effect.

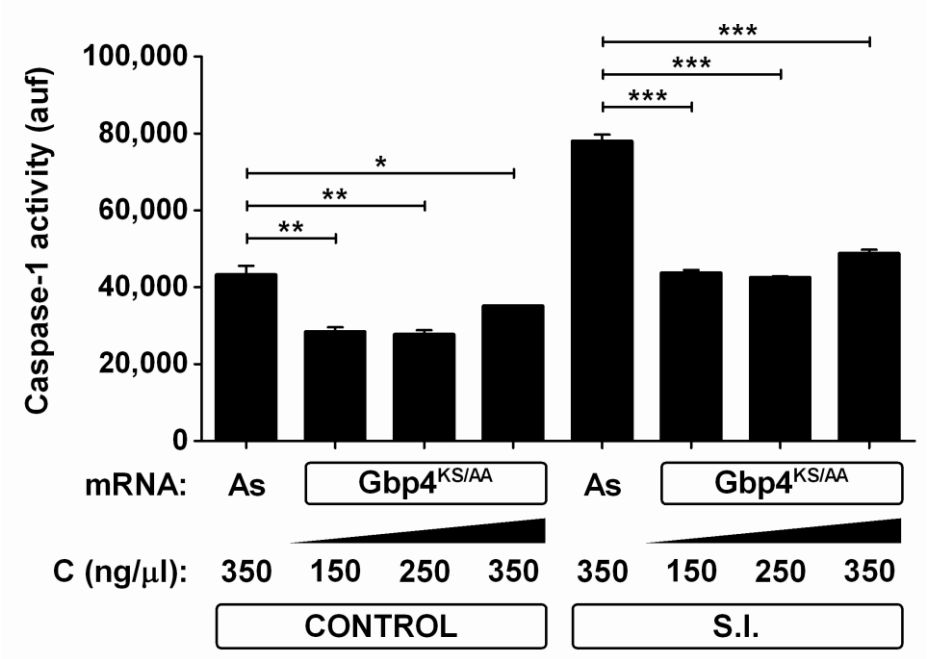


Figure 36. Increasing amounts of Gbp4KS→AA mRNA impaired activation of caspase-1 activity upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with antisense (As) or increasing concentrations of Gbp4KS/AA mRNAs. Caspase-1 activity assay was performed as described in Figure 13. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

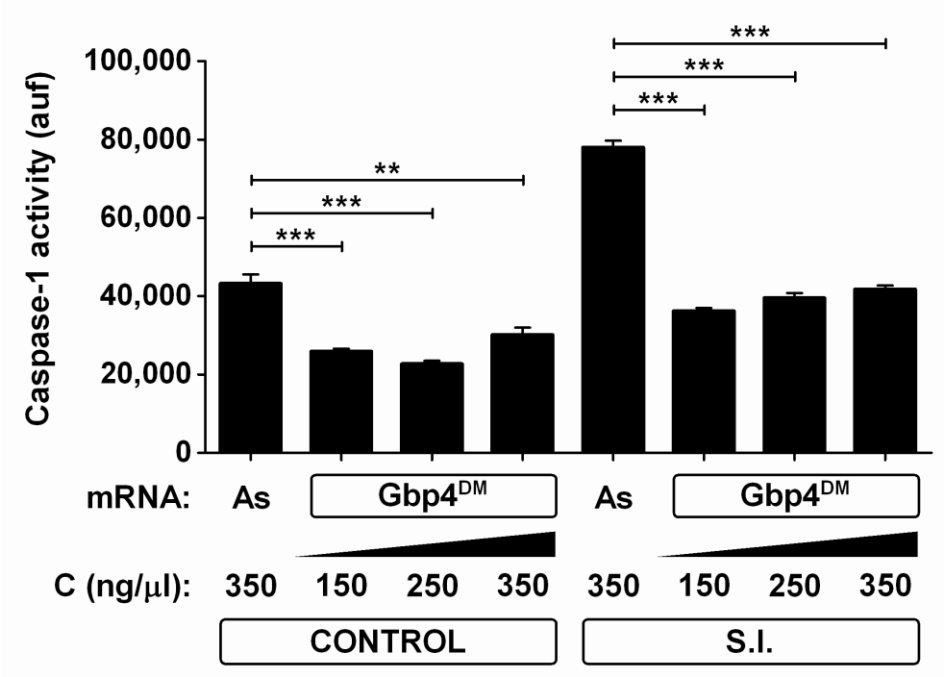


Figure 37. Increasing amounts of Gbp4DM mRNA also impaired activation of caspase-1 activity upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with antisense (As) or increasing concentrations of Gbp4DM mRNAs. Caspase-1 activity assay was performed as described in Figure 13. S.I., *S.* Typhimurium infection. **p<0.01; ***p<0.001.

However, Gbp4△CARD promoted a dose-dependent caspase-1 activation in non-infected larvae, while inhibited its activation upon infection (Figure 38).

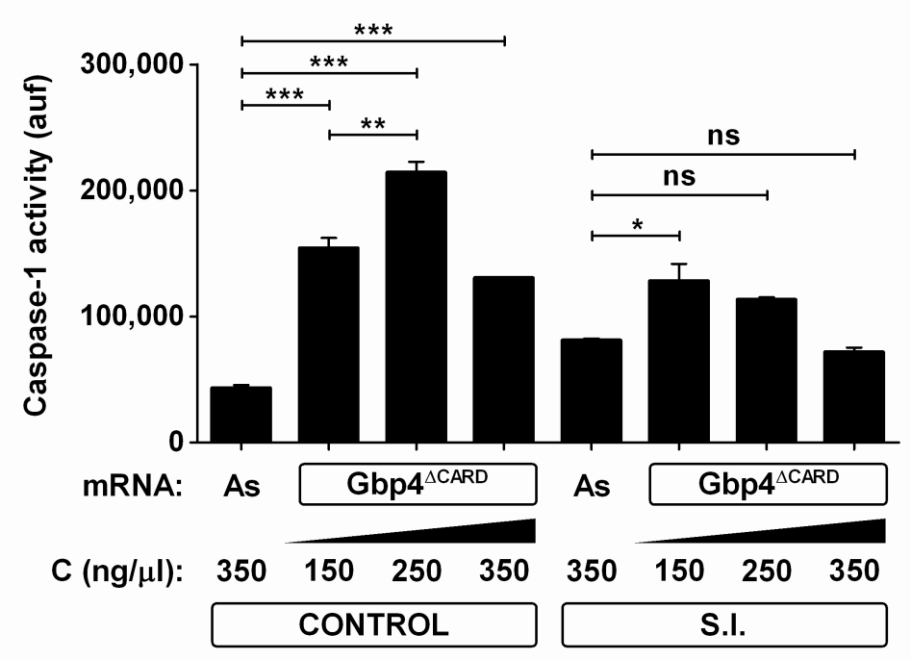
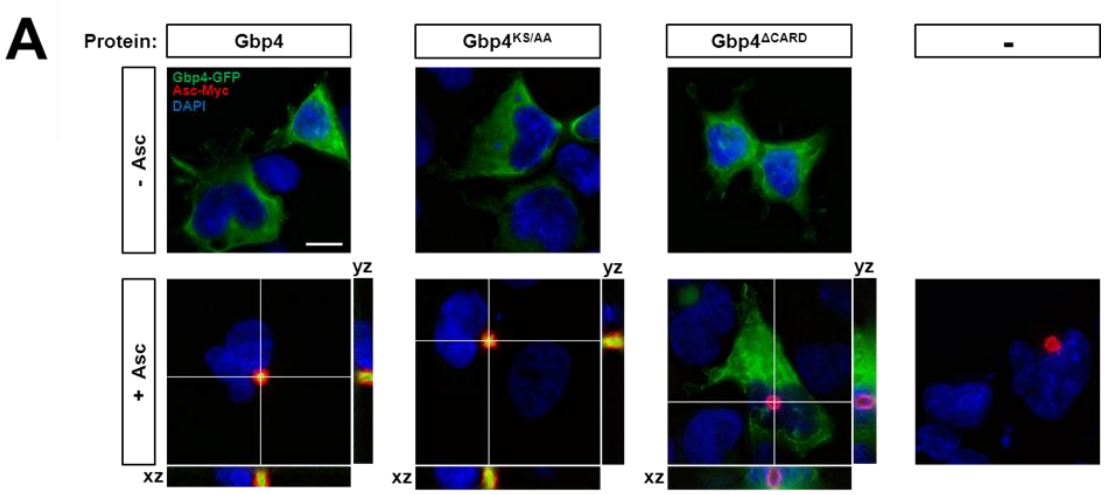
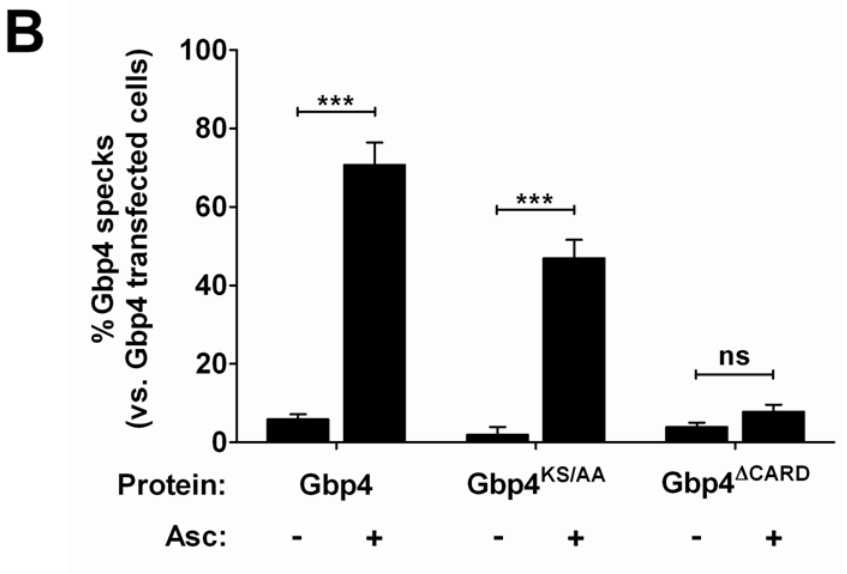


Figure 38. Increasing amounts of Gbp4△CARD mRNA caused dose-dependent inhibition of caspase-1 activation upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with antisense (As) or increasing concentrations of Gbp4ΔCARD mRNAs. Caspase-1 activity assay was performed as described in Figure 13. S.I., *S.* Typhimurium infection. ns, not significant; *p<0.05; **p<0.01; ***p<0.001.

Those results suggest that not only WT Gbp4, but also the GTPase-deficient mutant, were able to interact with Asc and then trigger or block inflammasome assembly, caspase-1 activation and *S.* Typhimurium clearance. We tested this idea by reconstituting Gbp4-Asc complexes in human embryonic kidney (HEK) 293 cells, which lack each of these components. WT Gbp4 and GTPase-deficient mutant fused to mCherry were found to localize to the specks in the presence of Asc, while they were diffusely distributed in the cytosol in the absence of Asc (Figure 39).


A
Protein:
Gbp4
Gbp4KS/AA
Gbp4ΔCARD
-
Gbp4-GFP
Asc-Myc
DAPI
- Asc
+ Asc
yz
xz


B
% Gbp4 specks
(vs. Gbp4 transfected cells)
100
80
60
40
20
0
***
***
ns
Protein:
Gbp4
Gbp4KS/AA
Gbp4ΔCARD
Asc:
- + - + - +

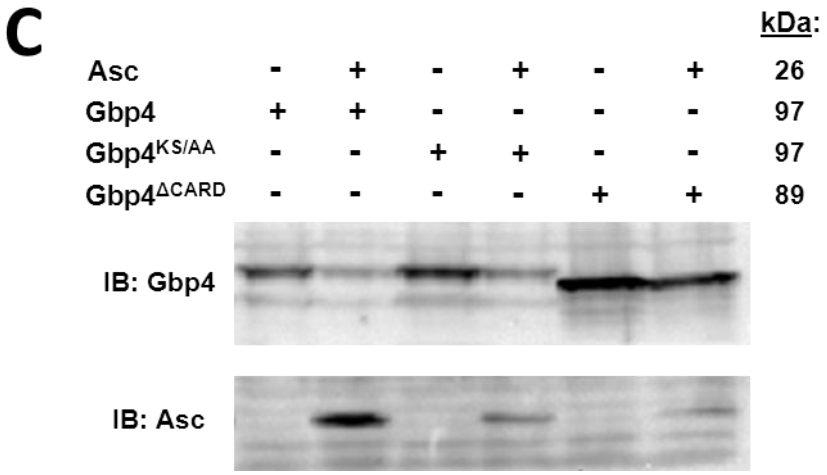

C
kDa:
Asc - + - + - + 26
Gbp4 + + - - - - 97
Gbp4KS/AA - - + + - - 97
Gbp4ΔCARD - - - - + + 89
IB: Gbp4
IB: Asc

Figure 39. Both WT Gbp4 and the GTPase mutant were able to form specks in the presence of Asc in the HEK293 cell line. (A) HEK293 cells were transfected with zebrafish Gbp4-GFP, Gbp4KS/AA-GFP or Gbp4ΔCARD-GFP in the presence and absence of zebrafish Asc-myc. Fixed cells were then immunolabeled with anti-Myc antibodies (Asc, Red). Representative frontal (xy) and lateral (xz and z) views of green (Gbp4), red (Asc) and blue (DAPI, nuclei) maximum-intensity projection images of HEK293 cells. Scale bars, 10 µm. **(B)** Percentage of Gbp4 specks in relation to the total number of Gbp4 transfected cells. ns, not significant; ***p<0.001. **(C)** Transfected cells were lysed and anti-GFP and anti-c-myc antibodies were used to validate the transfection assays by detecting Gbp4 and Asc by western-blot. The mass weights for all the proteins are indicated.

In addition, the CARD-deficient Gbp4 mutant was found to be diffusely distributed in the cytosol independently of Asc (Figures 39A and 39B). Strikingly, a macromolecular complex with an outer ring of Asc and a central core of either WT or GTPase-deficient mutant Gbp4 was observed (Figure 39A). Anti-Myc and Anti-GFP antibodies were used to check the correct working of the transfection assays by western blot. As expected, we observed different bands belonging to all the transfected proteins (Figure 39C).

4. Neutrophils mediate the Gbp4-dependent resistance to *S*. Typhimurium

As Gbp4 is highly expressed in neutrophils and these cells are essential for *S*. Typhimurium clearance in zebrafish, we next examined the impact of Gbp4 in neutrophil development and functions. The genetic depletion of Gbp4 resulted in the reduction of the number of neutrophils at 3 dpf, being that effect rescued by its overexpression (Figure 40).

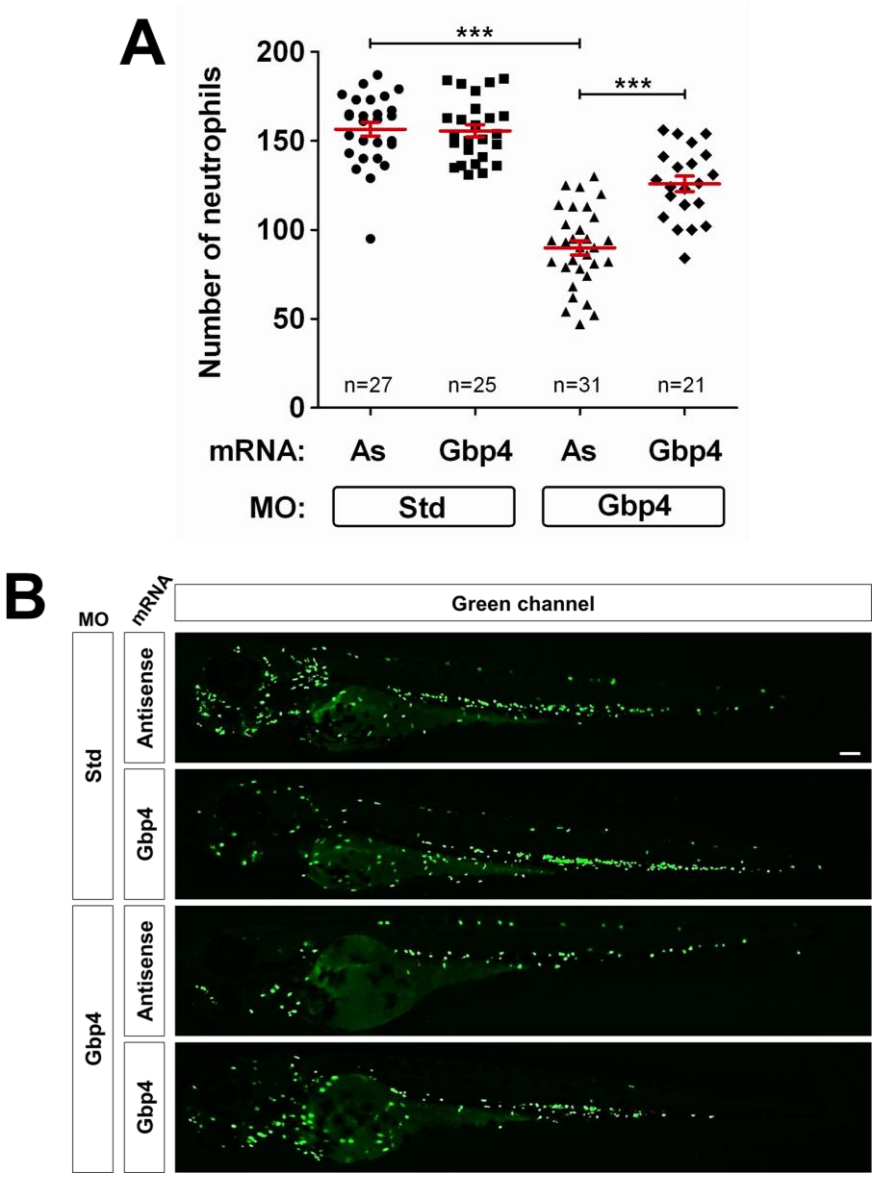


Figure 40. Neutrophil number depended on Gbp4. Zebrafish *mpx:eGFP* one-cell embryos were injected with standard control (Std) or Gbp4 MOs, in combination with antisense (As) or Gbp4 mRNAs. **(A)** The total number of neutrophils was counted in whole larvae for all the treatments, and represented as the mean ± SEM. The sample size (n) is indicated for all the treatments. **(B)** Representative images of green channels of the different treatments showing the neutrophil number and distribution in all cases. ***p<0.001 Scale bars 100 µm.

Expression of either Gbp4KS→AA or the double mutant Gbp4KS→AA/ΔCARD phenocopied the effect of the MO on neutrophil numbers (Figure 41). In both cases the total numbers of neutrophils were significantly lower comparing with control fish.

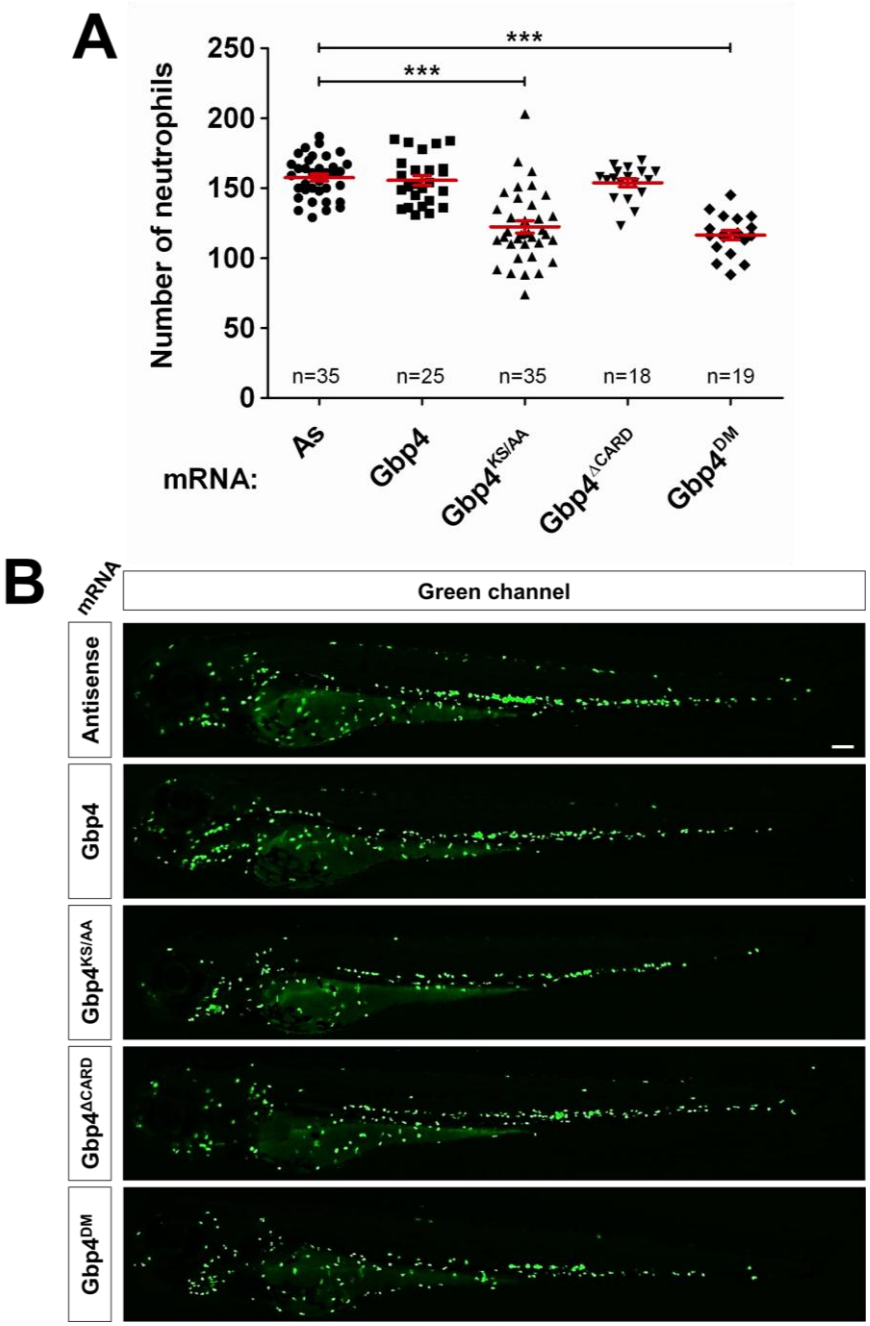


Figure 41. GBP4KS→AA mutant and Gbp4 DM had lower number of neutrophils. Zebrafish *mpx:eGFP* one-cell embryos were injected with antisense (As), Gbp4, Gbp4ΔCARD, Gbp4KS/AA, or Gbp4DM mRNAs. **(A)** The total number of neutrophils was counted in whole larvae for all the treatments, and represented as the mean ± SEM. The sample size (n) is indicated for all the treatments. **(B)** Representative images of green channels of the different treatments showing the neutrophil number and distribution in all cases. ***p<0.001 Scale bars 100 µm.

However, WT Gbp4 and Gbp4ΔCARD (Figure 41), as well as mouse WT GBP5 and GBP5KS→AA (Figure 42), did not affect the number of neutrophils, ruling

out that the Gbp4-dependent resistance to *S.* Typhimurium infection was related to neutrophil numbers.

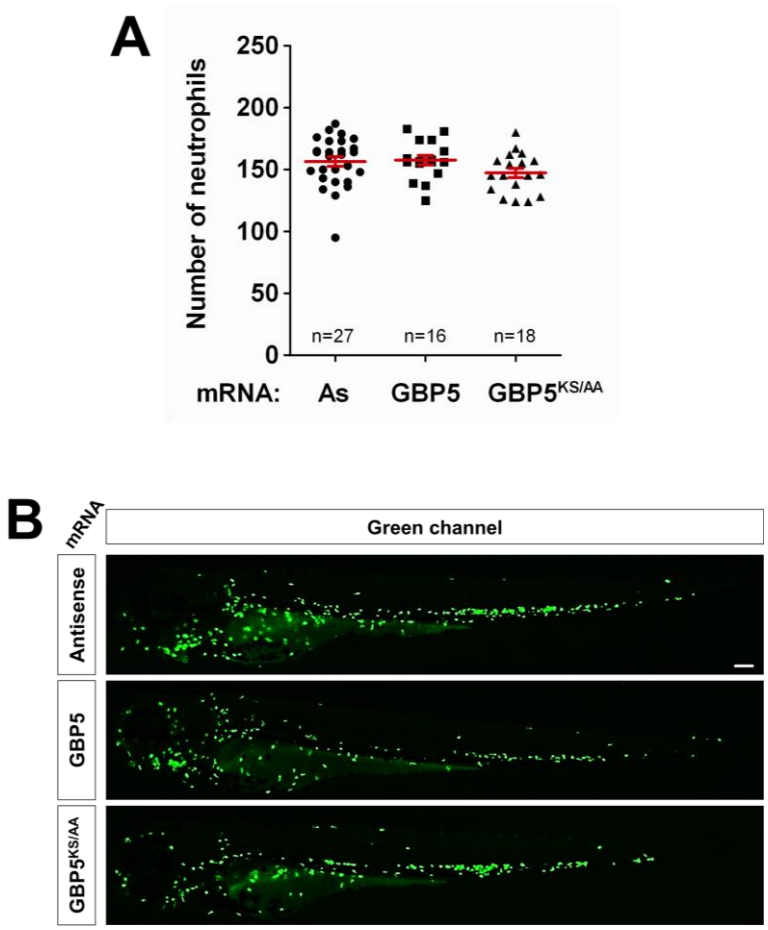


Figure 42. Forced expression of mouse GBP5 or GBP5KS→AA did not affect the number of neutrophils. Zebrafish *mpx:eGFP* one-cell embryos were injected with antisense (As), GBP5, or GBP5KS/AA mRNAs. **(A)** The total number of neutrophils was counted in whole larvae for all the treatments, and represented as the mean ± SEM. The sample size (n) is indicated for all the treatments. **(B)** Representative images of green channels of the different treatments showing the neutrophil number and distribution in all cases. Scale bars 100 µm.

These results prompted us to blocked neutrophil recruitment to the infection foci using a specific MO for Cxcr2, which is responsible for IL-8-dependent neutrophil recruitment to *S.* Typhimurium in zebrafish (Deng *et al.*, 2013).

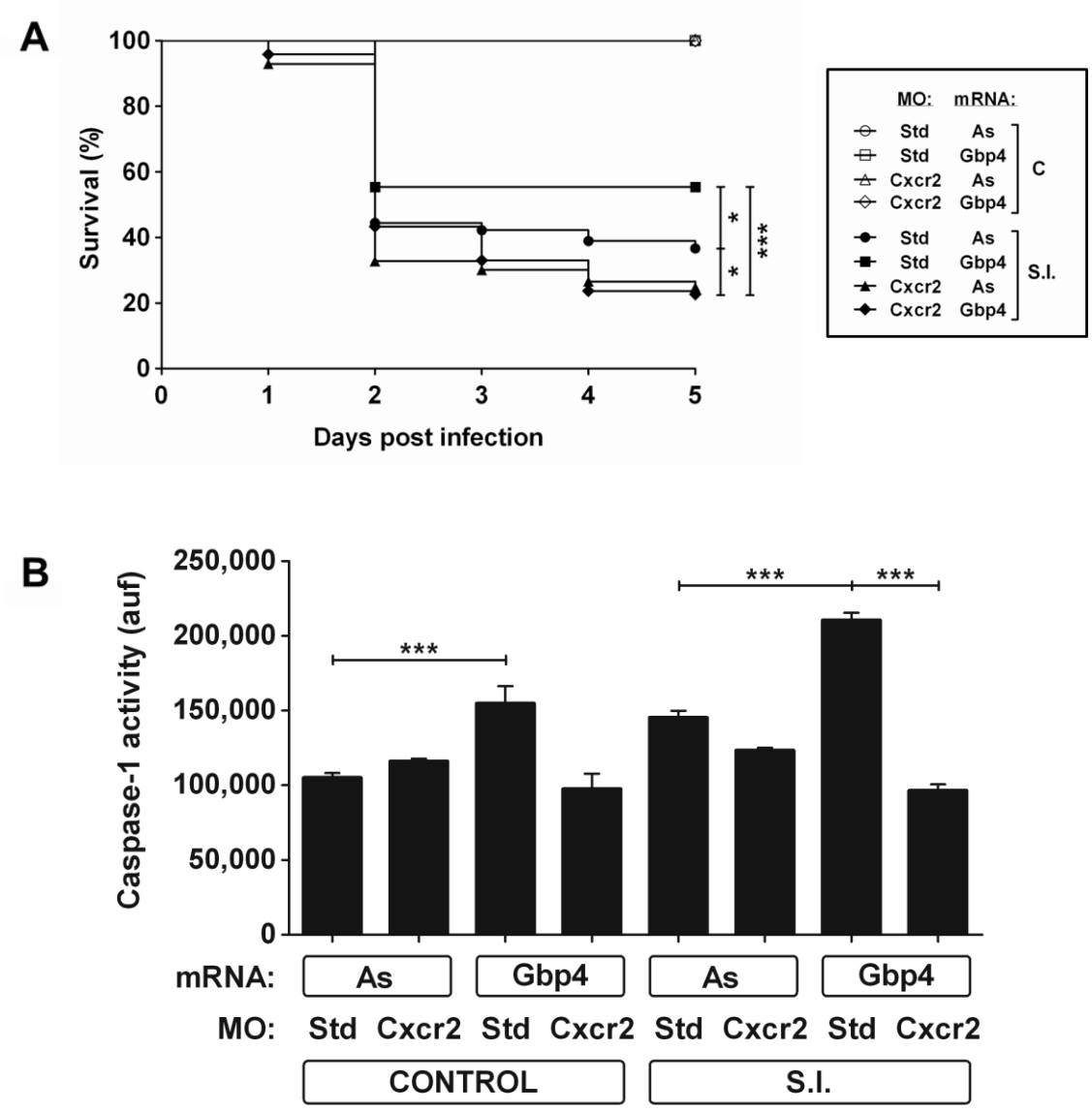


Figure 43. Blockage of the IL-8 receptor abrogated Gbp4-mediated resistance and decreased caspase-1 activity upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Cxcr2 MOs, in combination with antisense (As) or Gbp4 mRNAs. Survival **(A)** and caspase-1 activity assays **(B)** were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; ***p<0.001.

The results showed that Cxcr2-deficiency resulted in higher susceptibility to the infection and, interestingly, it fully overcame both Gbp4- (Figure 43A) and Asc-induced (Figure 44A) infection resistance. In contrast, the overexpression of Gbp4 or Asc in Cxcr2-deficient larvae did not affect the caspase-1 activity levels (Figures 43B and 44B).

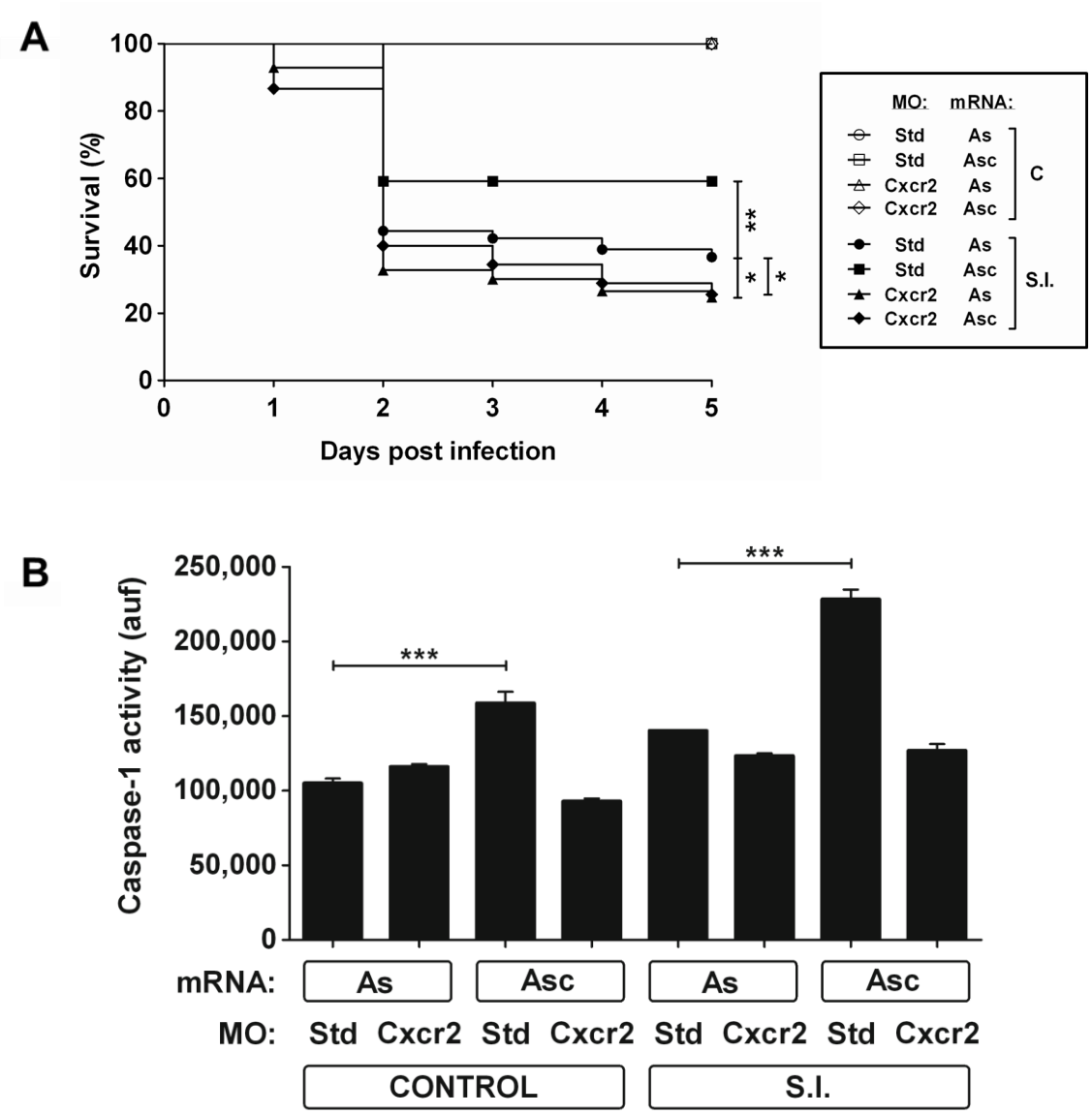


Figure 44. Blockage of the IL-8 receptor abrogated Asc-mediated resistance and decreased caspase-1 activity levels upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Cxcr2 MOs, in combination with antisense (As) or Asc mRNAs. Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

Furthermore, neutrophil recruitment to *S.* Typhimurium, using a localized ear infection model (de Oliveira *et al.*, 2015), was severely impaired in larvae forced to express Gbp4KS→AA while potentiated in larvae forced to overexpress WT Gbp4 (Figure 45).

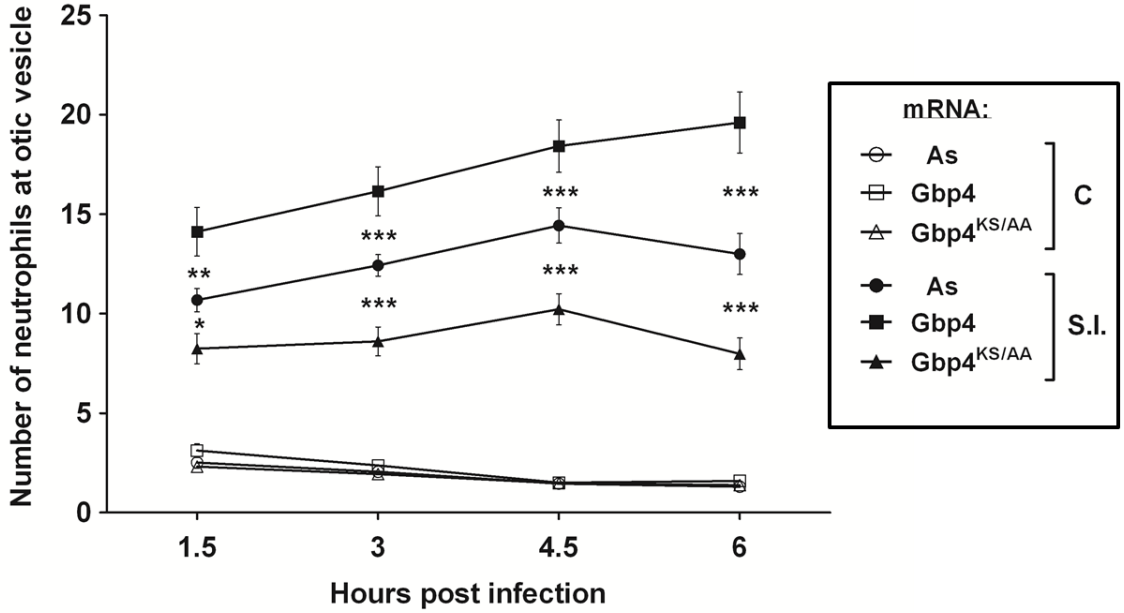
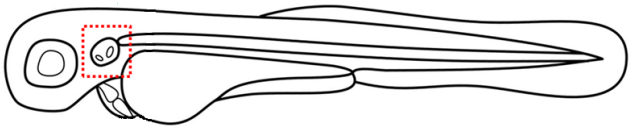
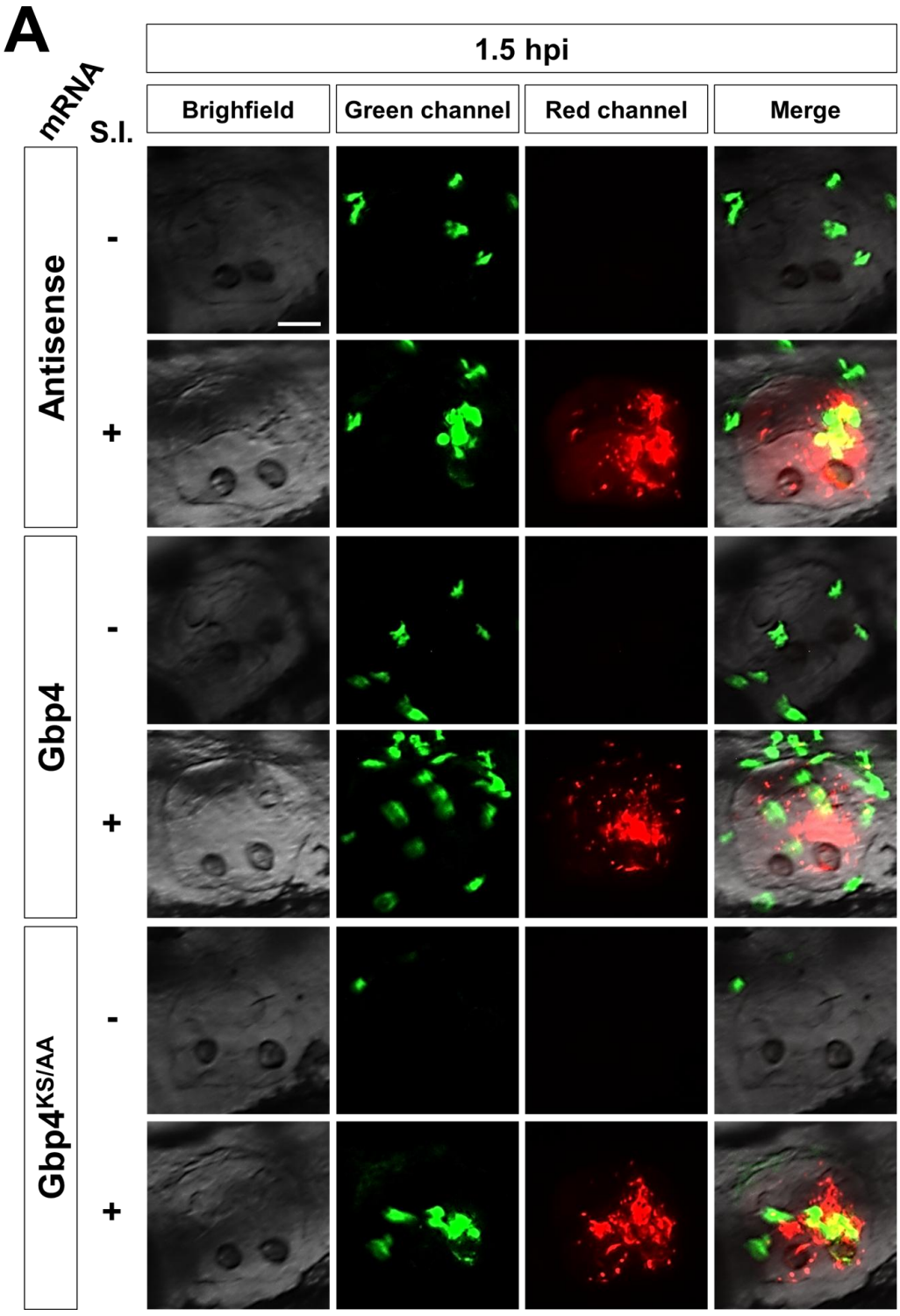
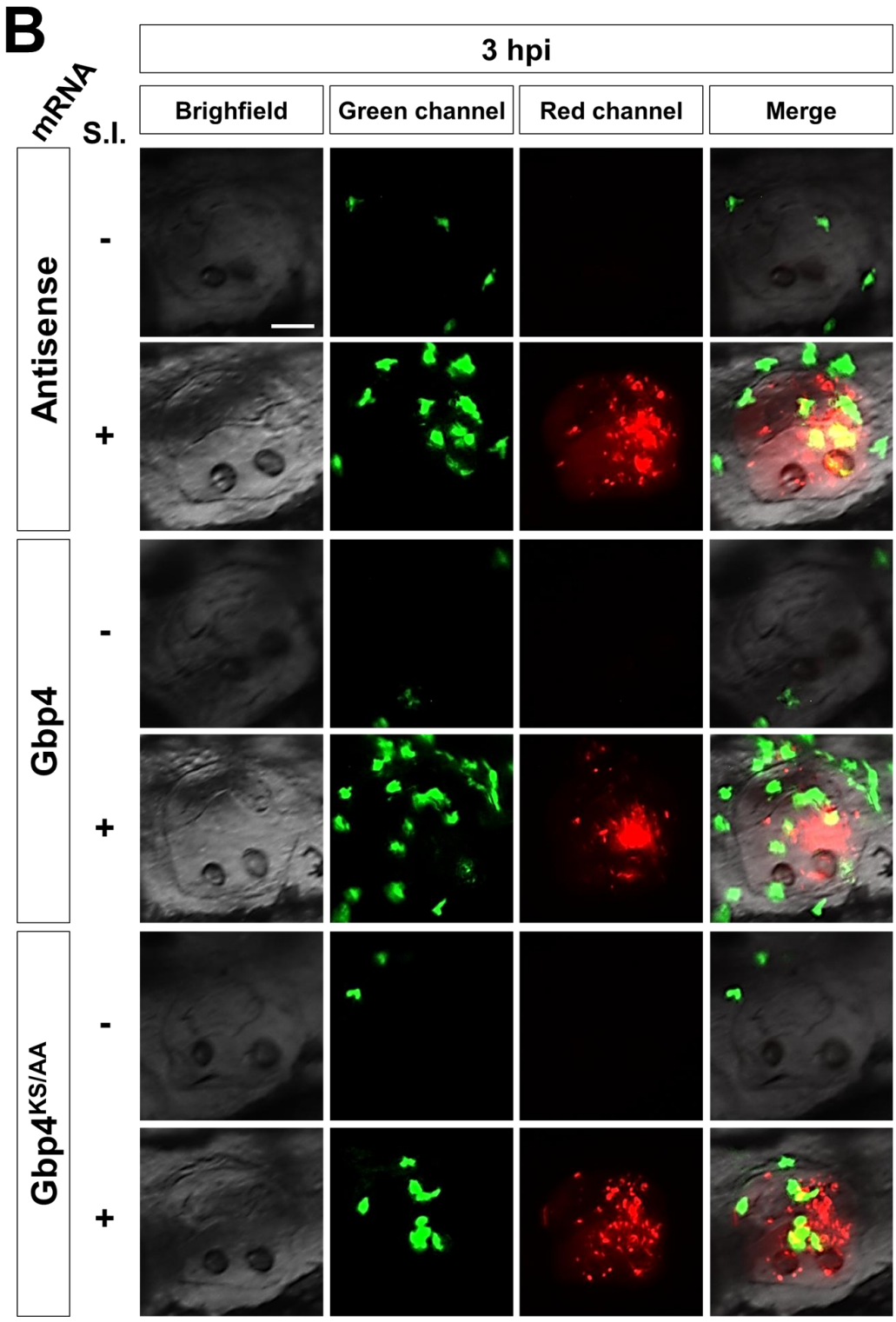


Figure 45. Gbp4 regulated neutrophil recruitment to the infection site. Zebrafish *mpx:eGFP* one-cell embryos were injected with antisense (As), Gbp4, or Gbp4KS/AA mRNAs. *S.* Typhimurium infection was performed at 2 dpf into the otic vesicle (MOI=100), and the number of neutrophils recruited to the infection site was counted for the different treatments at 1.5, 3, 4.5 and 6 hours post-infection. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

The number of neutrophils recruited to the infection site was counted at 1.5 (Figure 46A), 3 (Figure 46B), 4.5 (Figure 46C), and 6 (Figure 46D) hours post infection (hpi). The maximum number of recruited cells, both in controls and larvae overexpressing Gbp4KS→AA, was detected at 4.5 hpi, and from that time it started to decrease. However, the peak of maximum recruitment was found at 6 hpi in fish overexpressing WT Gbp4. As expected, the injection of PBS worked as a good negative control, since it did not cause any recruitment of neutrophils.




A
1.5 hpi
mRNA
S.I.
Brighfield
Green channel
Red channel
Merge
Antisense
Gbp4
Gbp4KS/AA
-
+
-
+
-
+


B
3 hpi
mRNA
S.I.
Brighfield
Green channel
Red channel
Merge
Antisense
Gbp4
Gbp4KS/AA
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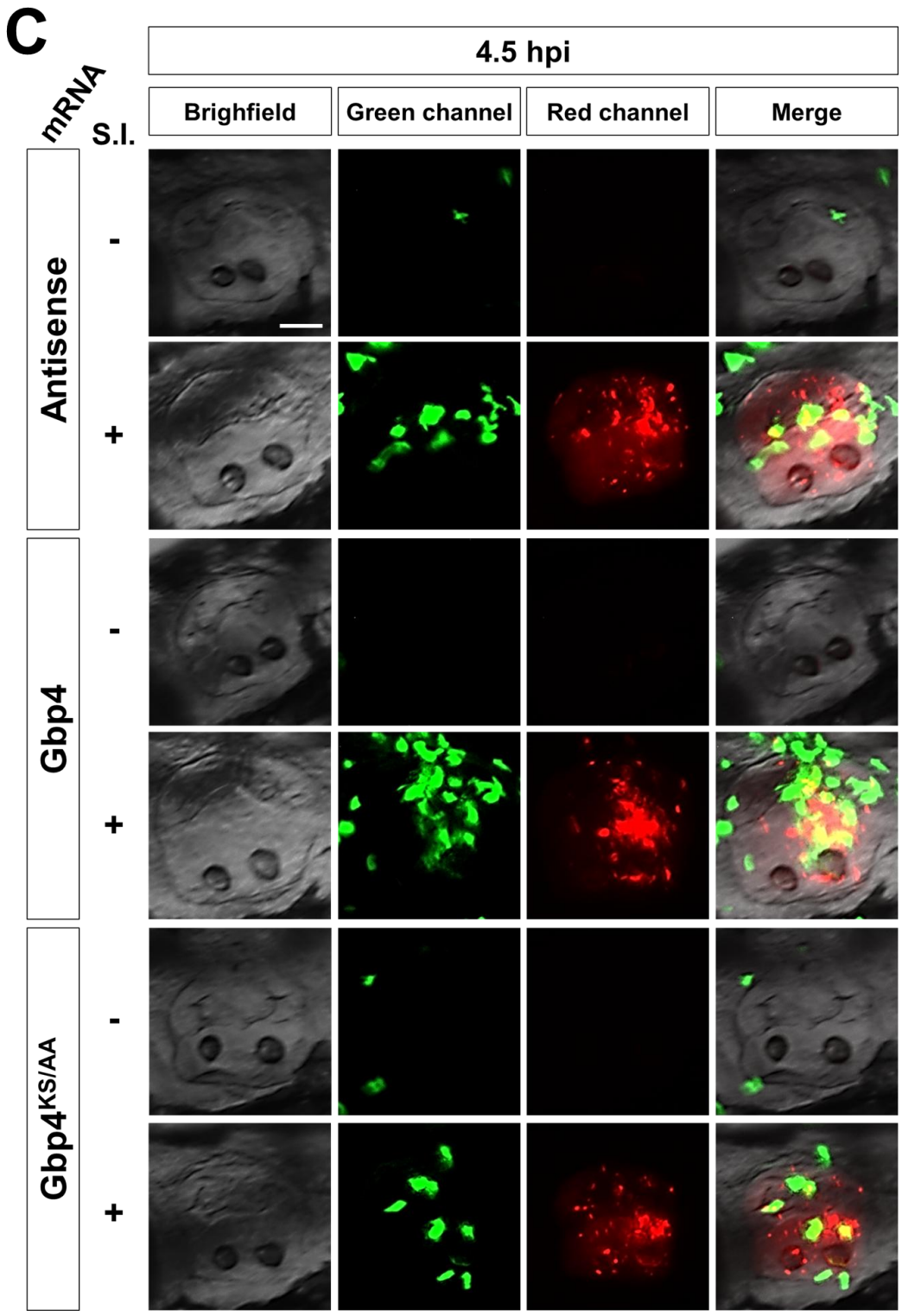
C
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mRNA
S.I.
Brighfield
Green channel
Red channel
Merge
Antisense
Gbp4
Gbp4KS/AA
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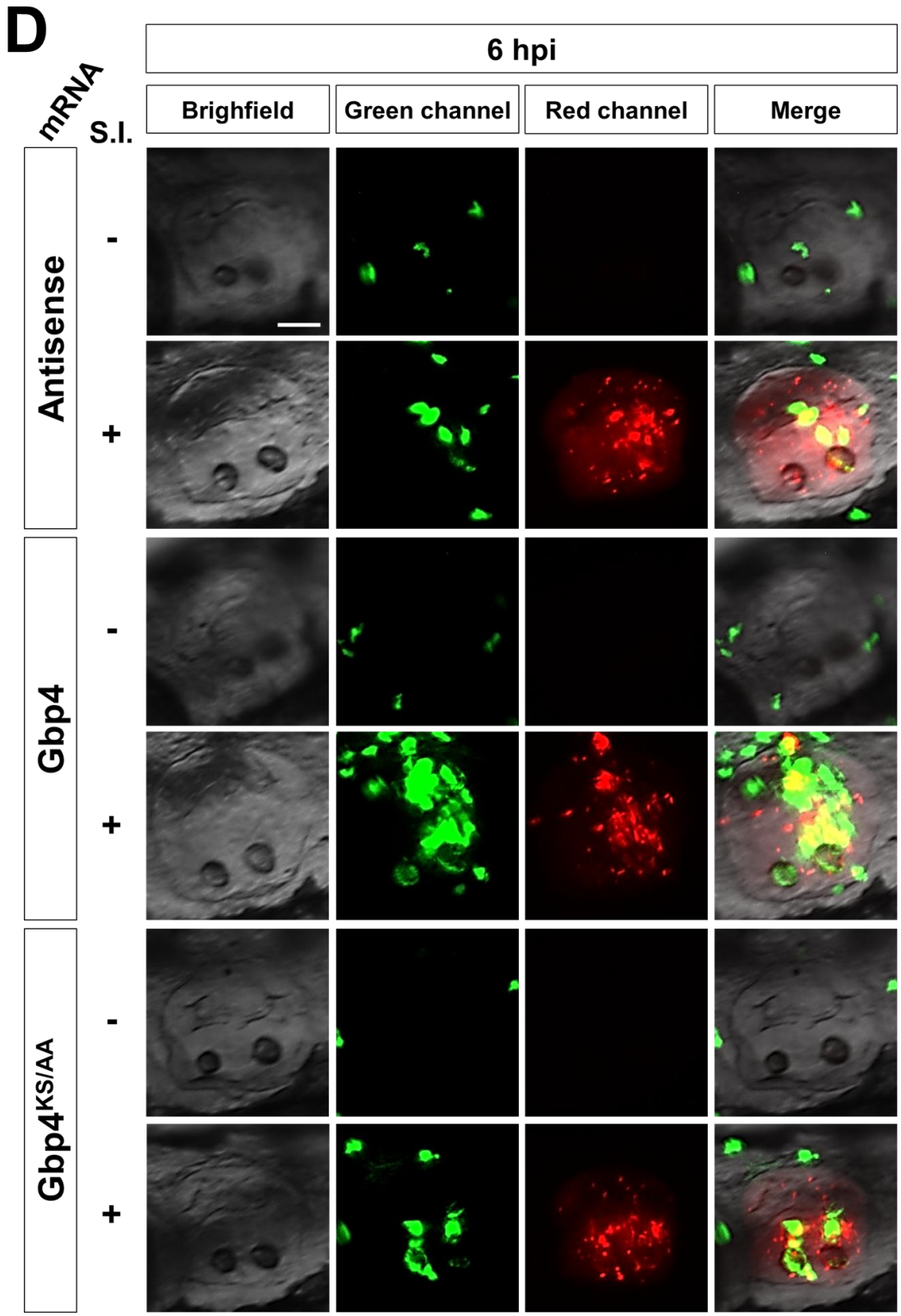
D
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Gbp4KS/AA
-
+

Figure 46. Representative pictures of control, Gbp4- and Gbp4KS→AA- overexpressing larvae for neutrophil recruitment at 1.5 hpi (A), 3hpi (B), 4.5 hpi (C), and 6 hpi (D) upon *S*. Typhimurium infection. Representative images of the different treatments injected in Figure 65. Neutrophils express the green fluorescent protein GFP, while *S*. Typhimurium expresses the red fluorescent protein DsRed. Images are focused on the otic vesicle. S.I., *S*. Typhimurium infection. Scale bars 50 µm.

Notably, RT-qPCR analysis showed that forced expression of WT Gbp4 resulted in a quicker and stronger immune response to *S*. Typhimurium (i.e. higher transcript levels of *cxcl8* and *ptgs2a* genes at 1 hpi), which led to the resolution of the infection-associated inflammatory response at 24 hpi (i.e. lower/basal transcript levels of *il1b*, *tnfa*, *cxcl8*, *ptgs1* and *ptgs2a*) (Figures 47-49).

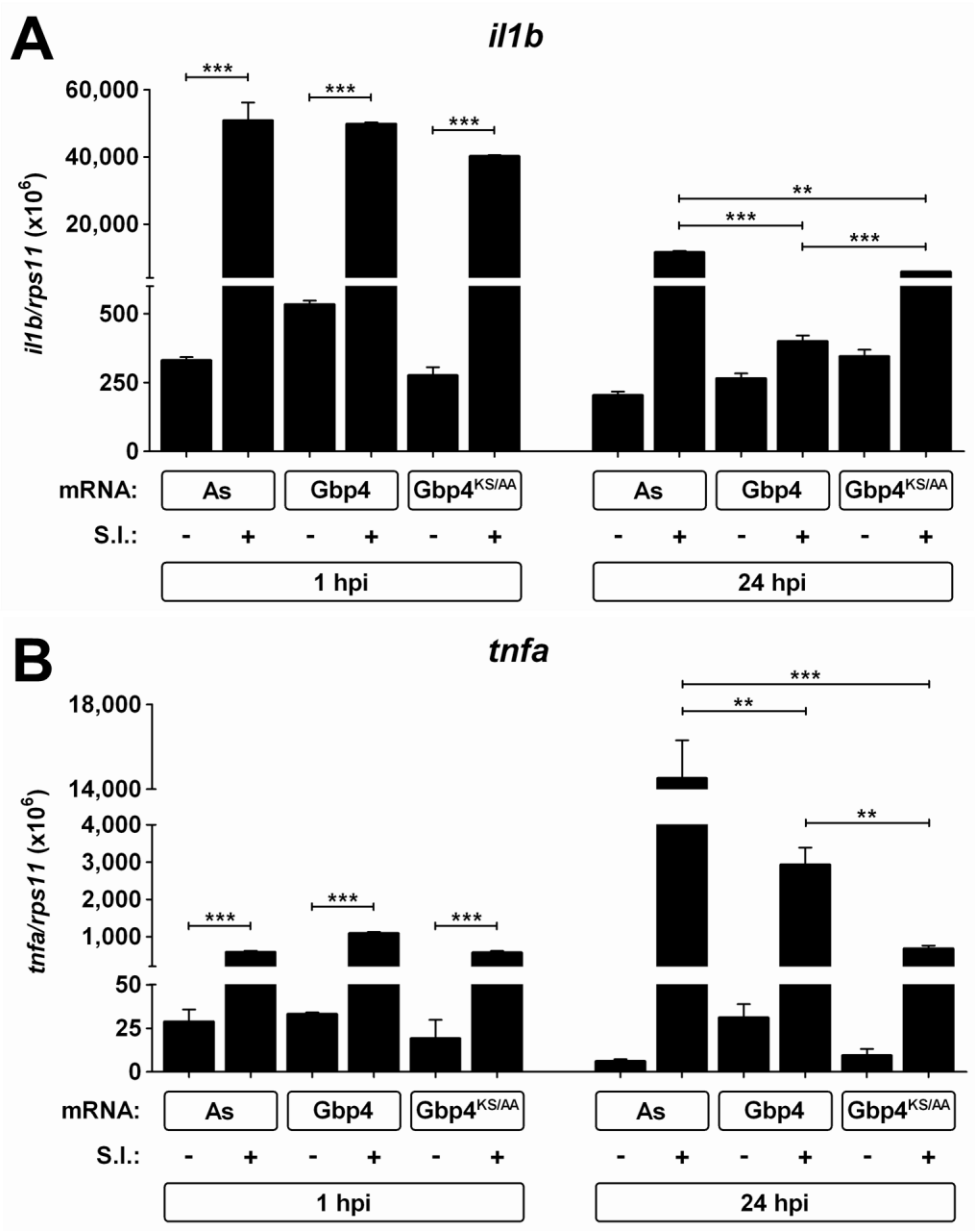


Figure 47. Expression levels of *il1b* (A) and *tnfa* (B) in the head of zebrafish infected with *S.* Typhimurium in the otic vesicle. Zebrafish one-cell embryos were injected with antisense (As), Gbp4 or Gbp4KS/AA mRNAs. The mRNA levels were determined by RT-qPCR from the heads at 1hpi and 24 hpi. Gene expression was normalized against *rps11*. S.I., *S.* Typhimurium infection. **p<0.01; ***p<0.001.

Unexpectedly, however, expression of GTPase-deficient Gbp4 also led to lower transcript levels of genes encoding proinflammatory molecules, probably reflecting in this case the impaired recruitment of neutrophils to the infection site (Figure 47-49).

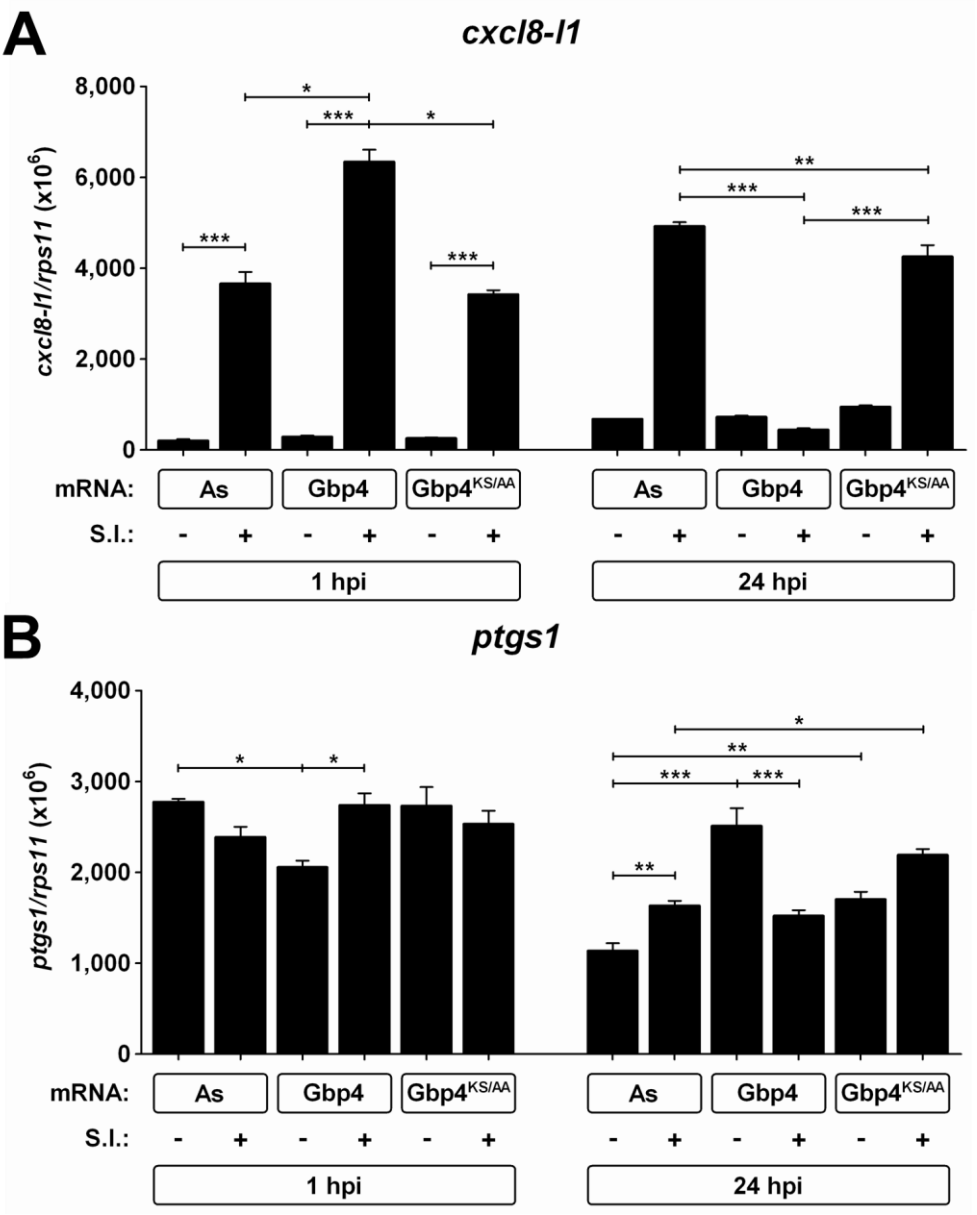


Figure 48. Expression levels of *cxcl8-l1* (A) and *ptgs1* (B) in the zebrafish head infected with *S.* Typhimurium in the otic vesicle. Zebrafish one-cell embryos were injected with antisense (As), Gbp4 or Gbp4KS/AA mRNAs. The mRNA levels were determined by RT-qPCR from the heads at 1hpi and 24 hpi. Gene expression was normalized against *rps11*. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

Collectively, these results indicate a pivotal role of Gbp4 in neutrophil homeostasis and functions in zebrafish and support the crucial role of these cells in the clearance of intracellular bacteria, as occurs in mammals (Miao *et al*., 2010a).

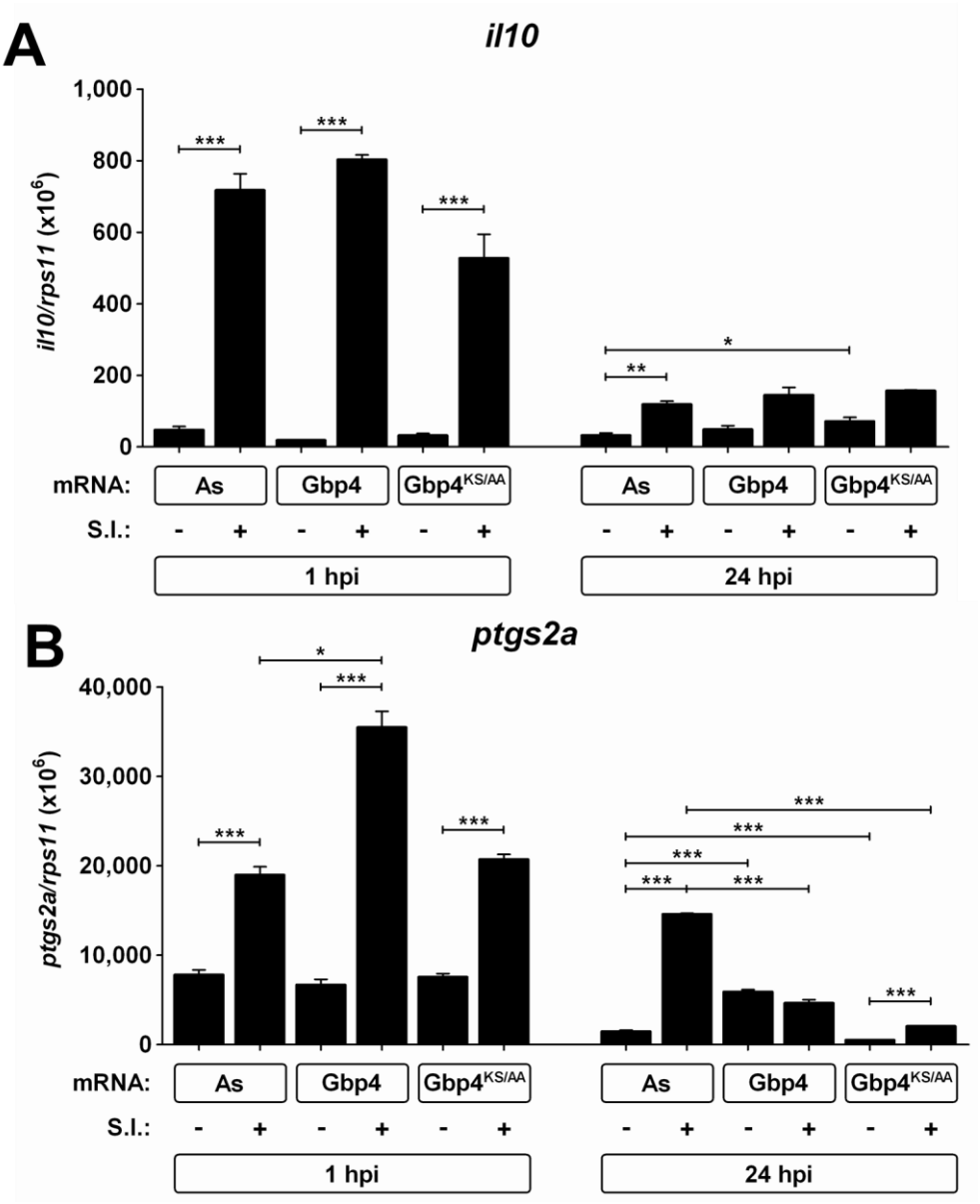


Figure 49. Expression levels of *il-10* (A) and *ptgs2a* (B) in the zebrafish head infected with *S.* Typhimurium in the otic vesicle. Zebrafish one-cell embryos were injected with antisense (As), Gbp4 or Gbp4KS/AA mRNAs. The mRNA levels were determined by RT-qPCR from the heads at 1hpi and 24 hpi. Gene expression was normalized against *rps11*. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

il1b mRNA levels decreased in larvae overexpressing Asc at 24hpi, confirming the quick resolution of the inflammation. Larvae forced to express Asc responded in a quick and strong manner to *S.* Typhimurium infection localized in the otic vesicle and resolved the infection-associated inflammatory response (Figure 50), agreeing with the result previously found in larvae overexpressing Gbp4.

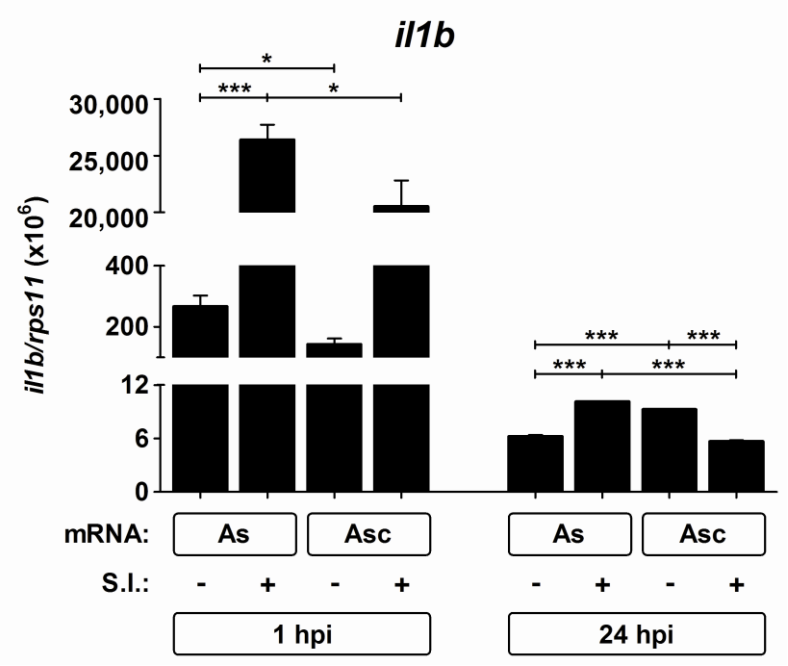


Figure 50. Expression levels of *il1b* in the head of larvae overexpressing Asc and infected with *S.* Typhimurium in the otic vesicle. Zebrafish one-cell embryos were injected with antisense (As) or Asc mRNAs. The mRNA levels were determined by RT-qPCR from the heads at 1hpi and 24 hpi. Gene expression was normalized against *rps11*. S.I., *S.* Typhimurium infection. *p<0.05; ***p<0.001.

5. The Gbp4-mediated resistance to *S.* Typhimurium is independent of IL-1β processing and pyroptotic cell death

The involvement of caspase-1 in the processing of IL-1β in fish is controversial (Angosto *et al*., 2012; Compan *et al*., 2012; Angosto *et al*., 2013) and in fact, all fish IL-1β sequenced to date lack a conserved caspase-1 processing site (Bird *et al*., 2002b). Although we have previously shown that *S.* Typhimurium fails to promote IL-1β processing in the teleost fish gilthead seabream (Angosto *et al*., 2012), we investigated whether *S.* Typhimurium infection triggered IL-1β processing in zebrafish.

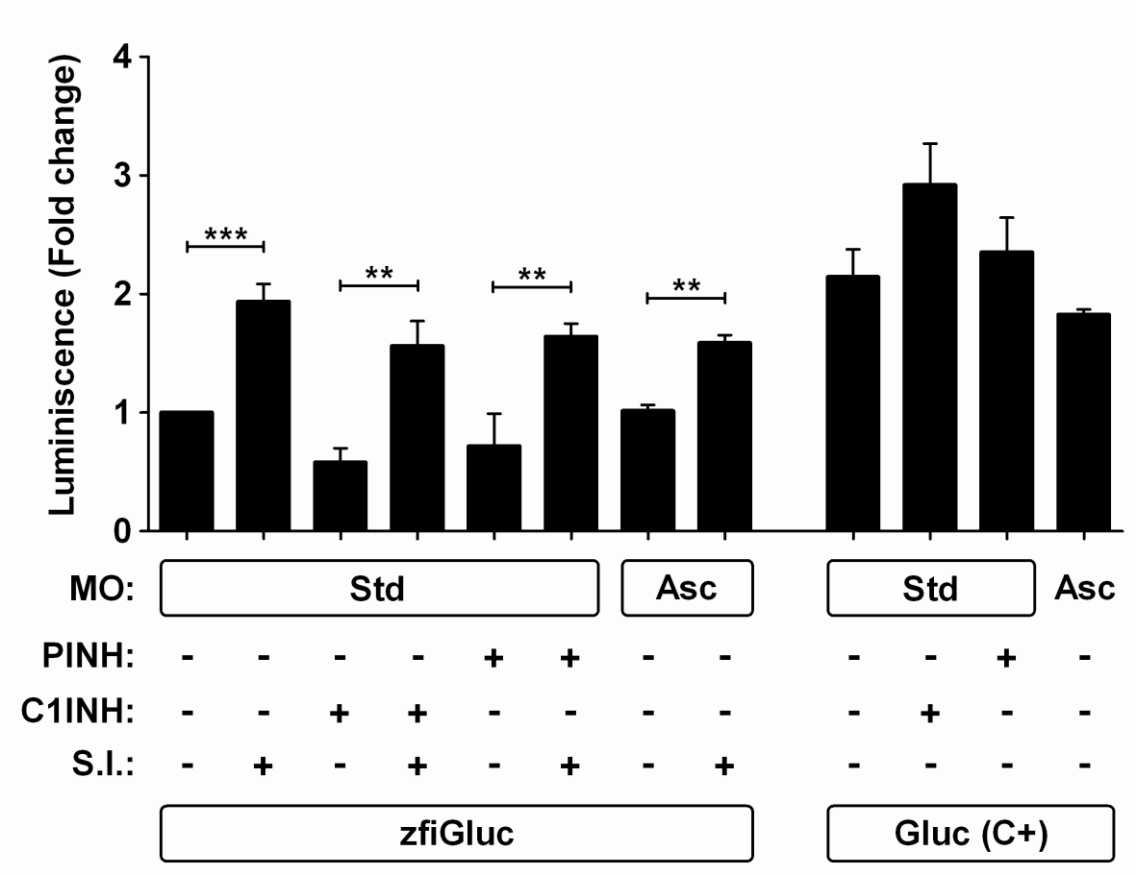


Figure 51. Il-1β processing was Asc- and caspase-1- independent. Zebrafish one-cell embryos were injected with standard control (Std) or Asc MOs with the coinjection of zfiGluc mRNA and treated by immersion in 50 µM of a general inhibitor of caspases (PINH), 100 µM caspase-1 (C1INH) or vehicle alone (DMSO). Positive control (C+) was injected with Gluc mRNA. Protease activity reporter assay was performed at 24 hpi. S.I., *S*. Typhimurium infection. **p<0.01; ***p<0.001.

Unfortunately, processed IL-1β was not detected by western blot in whole larval extracts using several mAbs (data not shown), suggesting a rapid elimination of the mature cytokine *in vivo*.

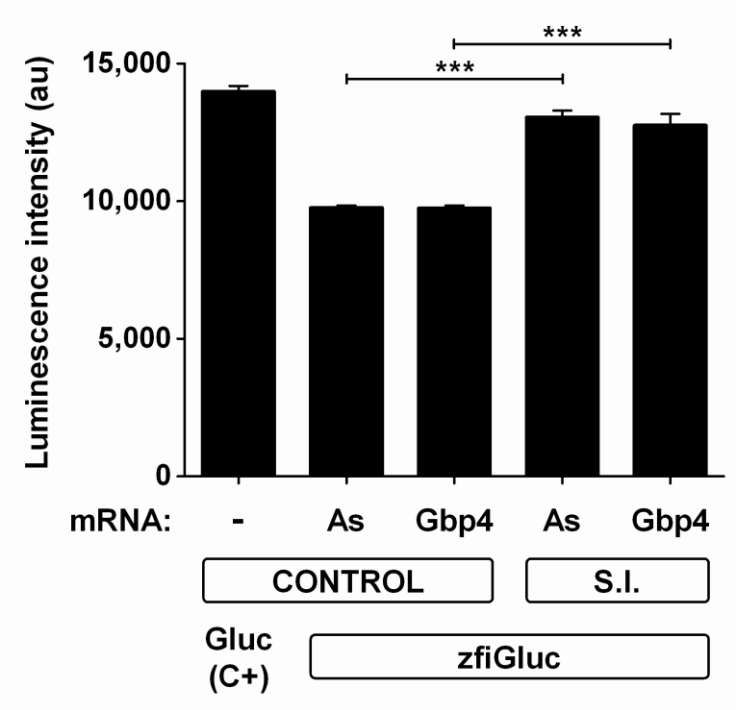


Figure 52. Il-1β processing was not dependent on Gbp4. Zebrafish one-cell embryos were injected with antisense (As) or Gbp4 mRNAs with the coinjection of zfiGluc mRNA. Positive control (C+) was injected with Gluc mRNA. Protease activity reporter assay was carried out 24 hpi. S.I., *S.* Typhimurium infection. ***p<0.001.

Therefore, we used a recently developed luciferase-based inflammasome and protease activity reporter assay (iGLuc), based on the biological activity of a pro-IL-1β-*Gaussia* luciferase fusion construct, in which pro-IL-1β- dependent formation of protein aggregates renders GLuc enzyme inactive (Bartok *et al.*, 2013). Infection of larvae expressing a RNA encoding a fusion between zebrafish pro-IL-1β and GLuc (zfiGLuc) resulted in increased luciferase activity independently of Asc (assayed in Asc-deficient fish) and caspase-1 activity (assayed in the presence of caspase-1 or pan caspase inhibitors) (Figure 51).

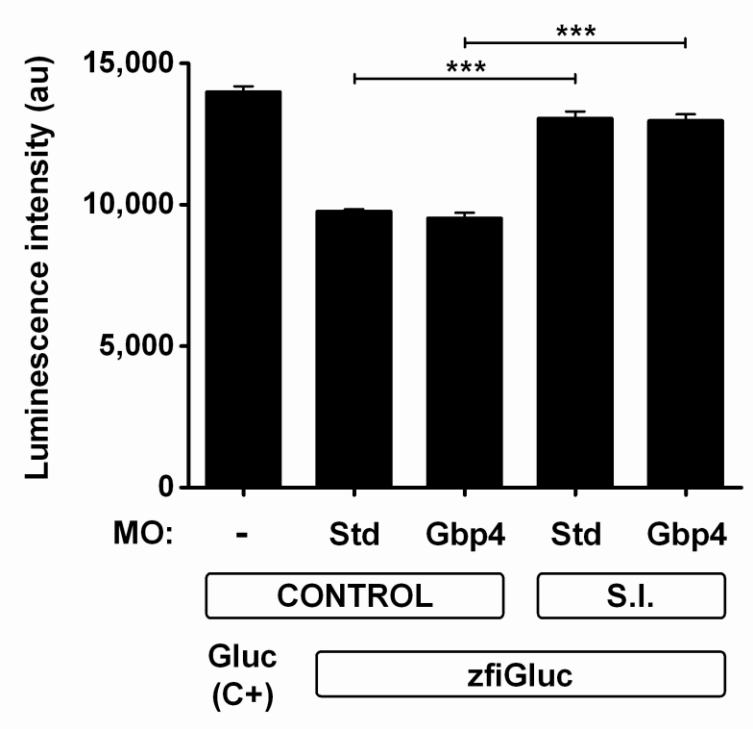


Figure 53. IL-1β processing was not abrogated in Gbp4-deficient larvae. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs with the coinjection of zfiGluc mRNA. Positive control (C+) was injected with Gluc mRNA. Protease activity reporter assay was carried out 24 hpi. S.I., *S.* Typhimurium infection. ***p<0.001.

As expected, the luciferase activity of native GLuc was unaffected by Asc deficiency or the presence of caspase inhibitors (Figure 51), confirming the specificity of the assay in whole zebrafish larvae. Similarly, Gbp4 levels did not affect the luciferase activity in fish expressing zfiGLuc (Figures 52 and 53).

We then analyzed whether *S.* Typhimurium infection resulted in pyroptotic cell death. As we have shown, infection of macrophages from the teleost fish gilthead seabream with wild type *S.* Typhimurium, but not with its derivative isogenic SPI-1 mutant strain, triggers pyroptosis (Angosto *et al.*, 2012). However, we did not find significant cell death of neutrophils in a localized ear infection model, assayed by YO-PRO uptake (Figure 54) and annexin V staining (data not shown).

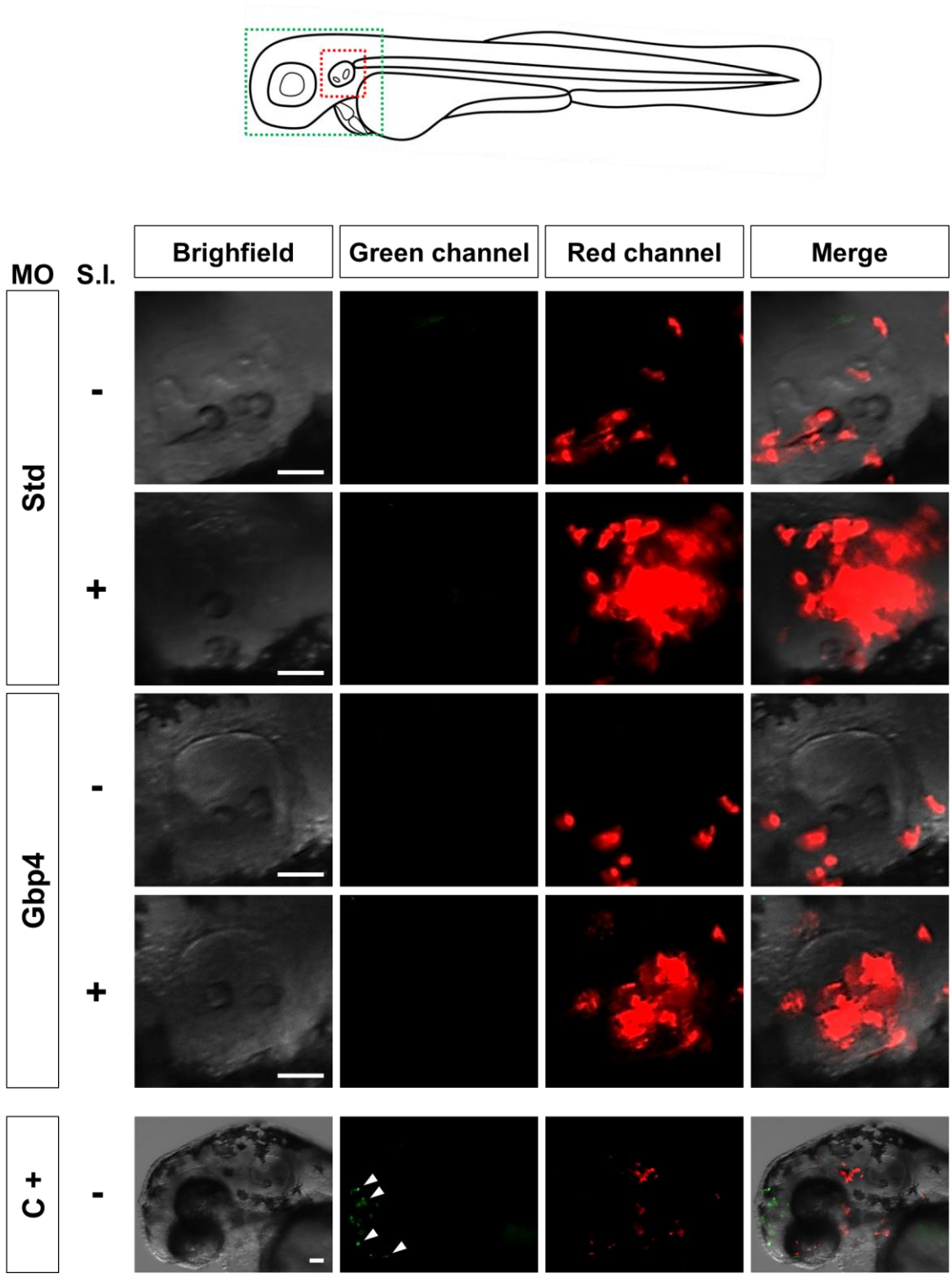


Figure 54. *S.* Typhimurium infection in the otic vesicle did not cause the programmed form of cell death called pyroptosis neither in controls nor in Gbp4-deficient larvae. Zebrafish *lys:dsRED* one-cell embryos were injected with standard control (Std) or Gbp4 MOs. *Salmonella* infection was performed in the otic vesicle (MOI=100) and 3 hpi YO-PRO compound was injected in the site of the infection to visualize dead cells. The pictures of each larvae was taken at 4.5 hpi at the fluorescent microscope and representative pictures for each treatment are shown. Some larvae showed a group of unidentified cells in the head dying during larval development (C +). Scale bars: 50 µm.

6. Gbp4-dependent resistance to *S*. Typhimurium is associated with prostaglandin biosynthesis

The above results led us to analyze whether Gbp4/inflammasome/caspase-1-mediated resistance to *S*. Typhimurium involved the production of eicosanoids, since it has been shown in mammals that the activation of the NLRC4 inflammasome results in the generation of a pathological storm of eicosanoids (von Moltke *et al*., 2012).

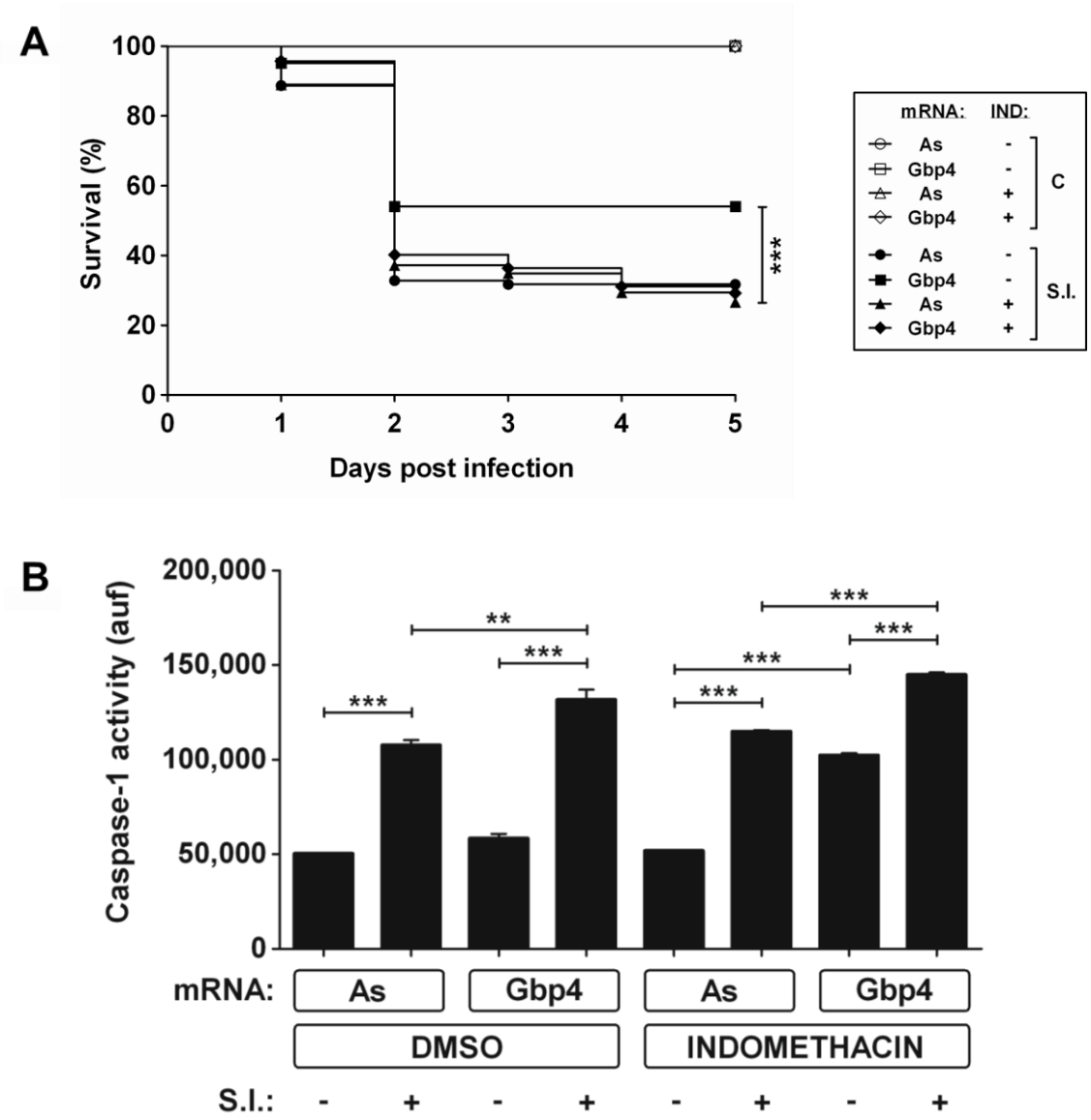


Figure 55. Indomethacin abrogated Gbp4-dependent resistance but did not change the caspase-1 activity levels upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with antisense (As) or Gbp4 mRNAs, and treated by immersion in 10 µM of a general inhibitor of prostaglandins (Indomethacin, IND) or vehicle alone (DMSO). Survival (A) and caspase-1 activity (B) assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. **p<0.01; ***p<0.001.

Pharmacological inhibition of the key enzymes in prostaglandin biosynthesis, prostaglandin-endoperoxide synthases (Ptgs), also known as cyclooxygenases (Cox), fully reversed the higher infection resistance of larvae forced to express Gbp4 (Figure 55A) but, unexpectedly, the resistance of larvae forced to express Asc was unaffected (Figure 56A).

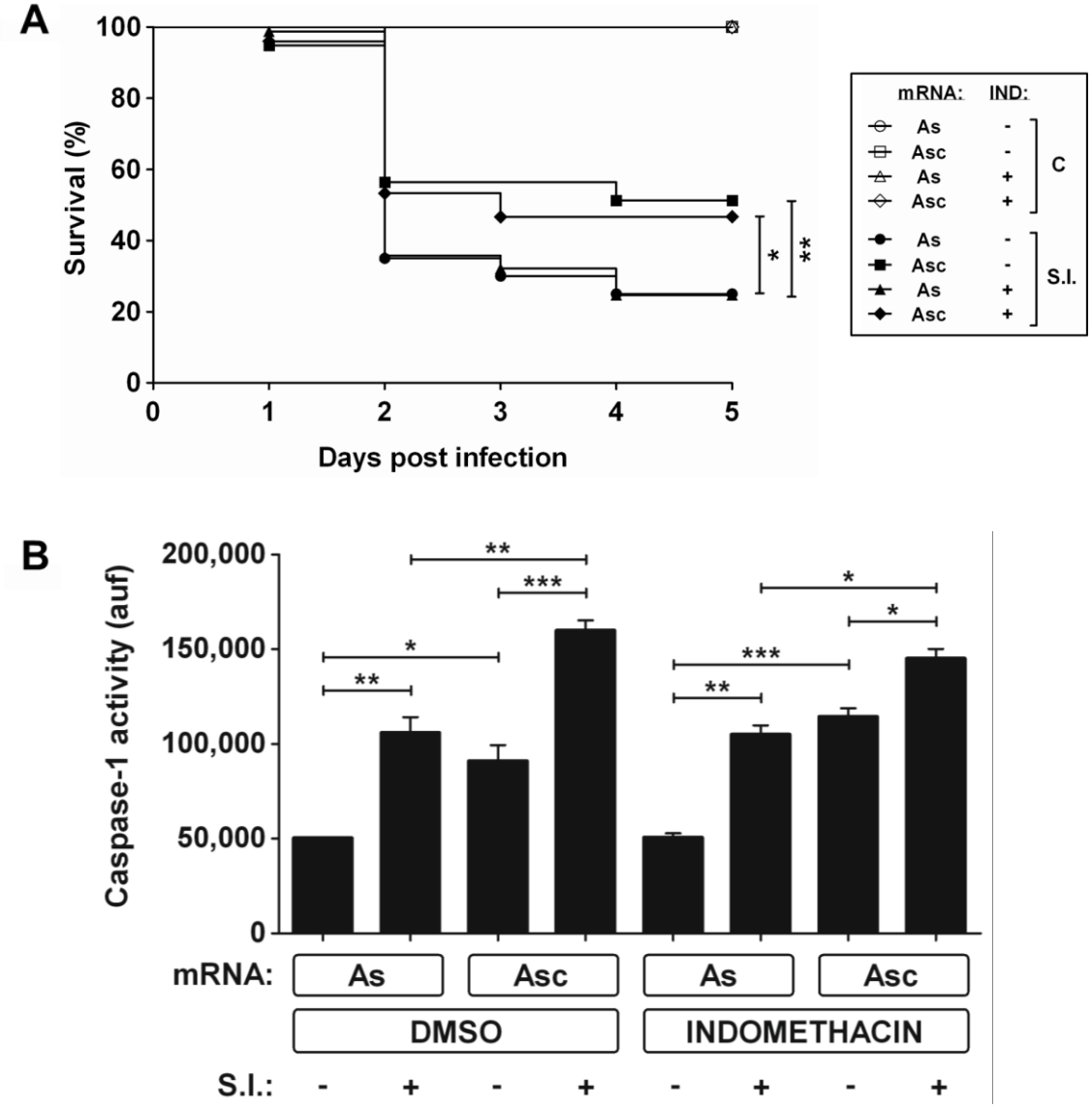


Figure 56. Indomethacin did not abrogate Asc-dependent resistance and did not change the caspase-1 activity levels upon *S.* Typhimurium infection. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, and treated by immersion in 10 µM of a general inhibitor of prostaglandins (Indomethacin, IND) or vehicle alone (DMSO). Survival **(A)** and caspase-1 activity **(B)** assays were performed as described in Figures 12 and 13 respectively. S.I., *S.* Typhimurium infection. *p<0.05; **p<0.01; ***p<0.001.

In addition, Ptgs inhibition did not affect the susceptibility of WT larvae (Figures 55A and 56A) and caspase-1 activity (Figures 55B and 56B), suggesting that prostaglandin production was downstream Gbp4-dependent inflammasome activation.

While the exogenous addition of PGE2 (Figure 57), 15dPGJ2 (Figure 58) or 12PGJ2 (Figure 59) further increased or had no effect on the susceptibility to *S.* Typhimurium infection in both WT and Gbp4-deficient larvae, PGD2 partially rescued

the higher susceptibility of Gbp4- deficient larvae, while it had no effect on their WT siblings (Figure 60).

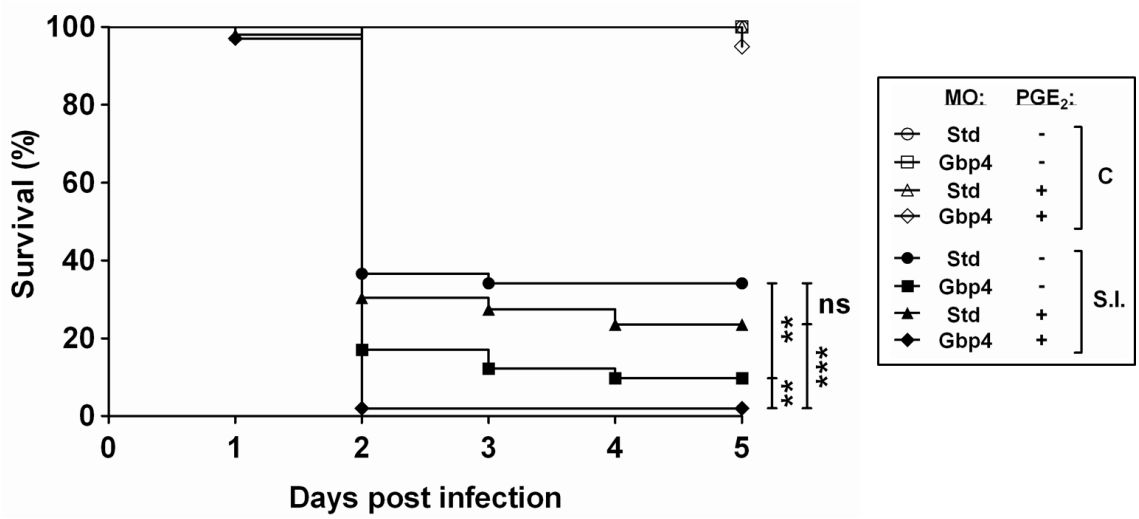


Figure 57. Increased resistance of larvae overexpressing Gbp4 was not dependent on PGE_2 production. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, and treated by immersion in 10 µM 16,16-dimethyl-prostaglandin E2 (PGE2) or vehicle alone (methyl acetate). Survival assays were performed as described in Figure 12. S.I., *S.* Typhimurium infection. ns, not significant; **p<0.01; ***p<0.001.

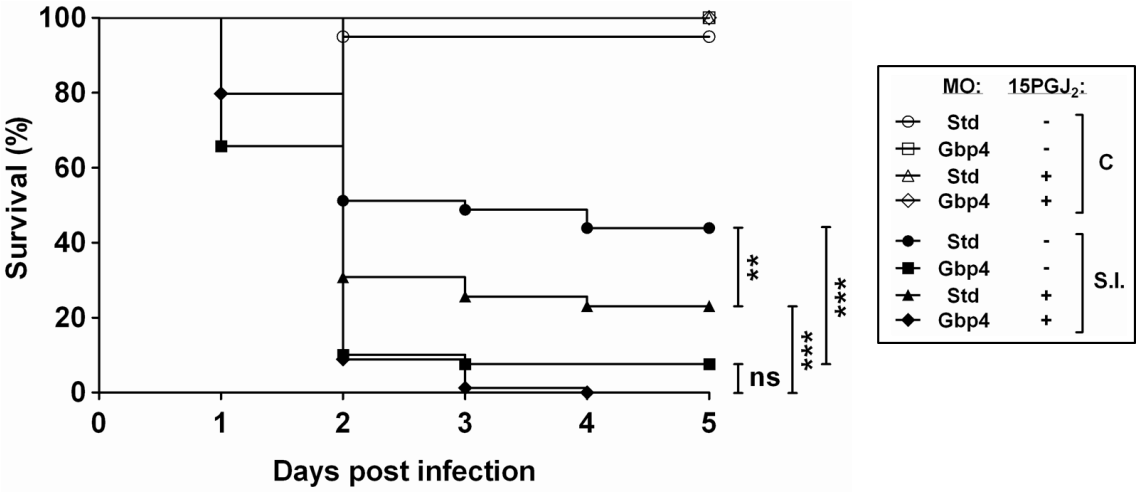


Figure 58. Increased resistance of larvae overexpressing Gbp4 was not dependent on $15PGJ_2$ production. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, and treated by immersion in 10 µM 15-deoxy-delta-12,14-prostaglandin J2 (15PGJ2) or vehicle alone (methyl acetate). Survival assays were performed as described in Figure 12. S.I., *S.* Typhimurium infection. ns, not significant; **p<0.01; ***p<0.001.

Collectively, these results demonstrate the critical role of Gbp4 in the inflammasome-mediated induction of prostaglandin biosynthesis and the clearance of intracellular bacteria by neutrophils *in vivo*.

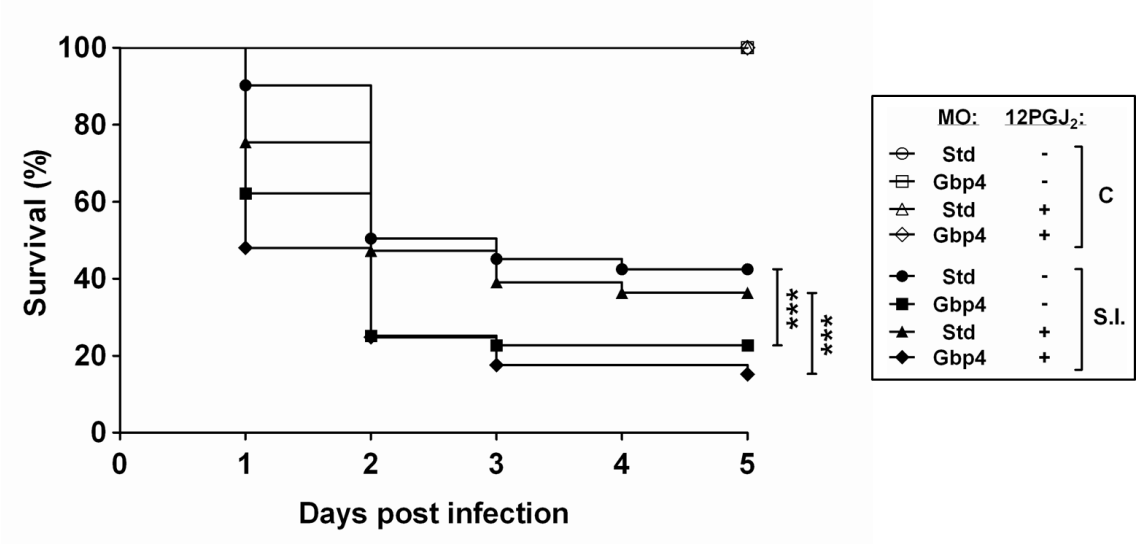


Figure 59. Increased resistance of larvae overexpressing Gbp4 was not dependent on $12PGJ_2$ production. Zebrafish one-cell embryos were injected with standard control (Std) or Gbp4 MOs, and treated by immersion in 10 µM 12-prostaglandin J2 (12PGJ2) or vehicle alone (methyl acetate). Survival assays were performed as described in Figure 12. S.I., *S.* Typhimurium infection. ***p<0.001.

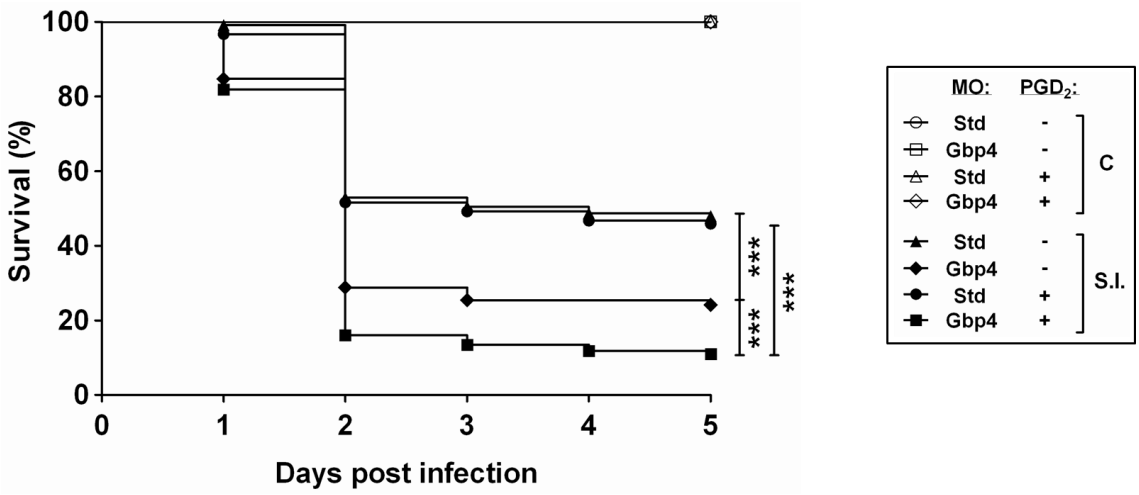


Figure 60. Increased resistance of larvae overexpressing Gbp4 was partially dependent on higher PGD_2 production. Zebrafish one-cell embryos were injected with antisense (As) or Gbp4 mRNAs, and treated by immersion in 10 µM 16,16-dimethyl-prostaglandin D2 (PGD2) or vehicle alone (methyl acetate). Survival assays were performed as described in Figure 12. S.I., *S.* Typhimurium infection. ***p<0.001.

DISCUSSION

We discovered here a novel role of GBPs in the inflammasome-dependent biosynthesis of prostaglandins and the clearance of intracellular bacteria by neutrophils *in vivo*. Zebrafish Gbp4 seems to be required for neutrophil homeostasis and although this observation deserves further investigation, the critical role of this protein in the control of *S.* Typhimurium infection is independent of this effect, since forced expression of WT Gbp4 did not affect neutrophil numbers while resulted in inflammasome/asc/caspase-1-dependent increased infection resistance. The key role played by neutrophils in *S.* Typhimurium resistance in zebrafish larvae is further supported by the failure of forced expression of both Gbp4 and Asc to increase bacterial resistance of animals deficient in Cxcr2, which mediates neutrophil but not macrophage recruitment to sterile wound, bacterial pathogens, including *S.* Typhimurium, and transformed cells (de Oliveira *et al*., 2013; Deng *et al*., 2013; Freinsinger *et al*., 2013; de Oliveira *et al*., 2014; de Oliveira *et al*., 2015). The role of neutrophils and macrophages in the clearance of intracellular bacteria *in vivo* is controversial, probably reflecting the complexity and heterogeneous nature of the interactions between intracellular pathogens and host innate immune cells (Bumann, 2015). While an earlier study showed that NLRC4-dependent pyroptotic cell death of macrophages releases the intracellular pathogens into the extracellular environment rendering them susceptible to neutrophil-mediated destruction (Miao *et al*., 2010a), very recent studies have elegantly demonstrated that neutrophils are crucial for the clearance of *S.* Typhimurium by inflammasome-independent mechanisms, i.e. oxidative stress generated through NADPH oxidase and myeloperoxidase (Burton *et al*., 2014; Franchi *et al*., 2012b). However, although experimental evidences supporting a role for cell-autonomous neutrophil inflammasome-dependent mechanisms on intracellular bacterial clearance is still lacking, a recent study has shown that these cells are able to sustain IL-1β production during acute *S.* Typhimurium infection thank to their unique resistance to pyroptotic cells death upon NLRC4 inflammasome activation (Chen *et al*., 2014). The evolutionary conserved resistance of zebrafish neutrophils to pyroptotic cell death upon strong activation of caspase-1 by *S.* Typhimurium infection and/or forced expression of Gbp4 and Asc further points out to the relevance of neutrophils in the clearance of intracellular bacterial pathogens in vertebrates.

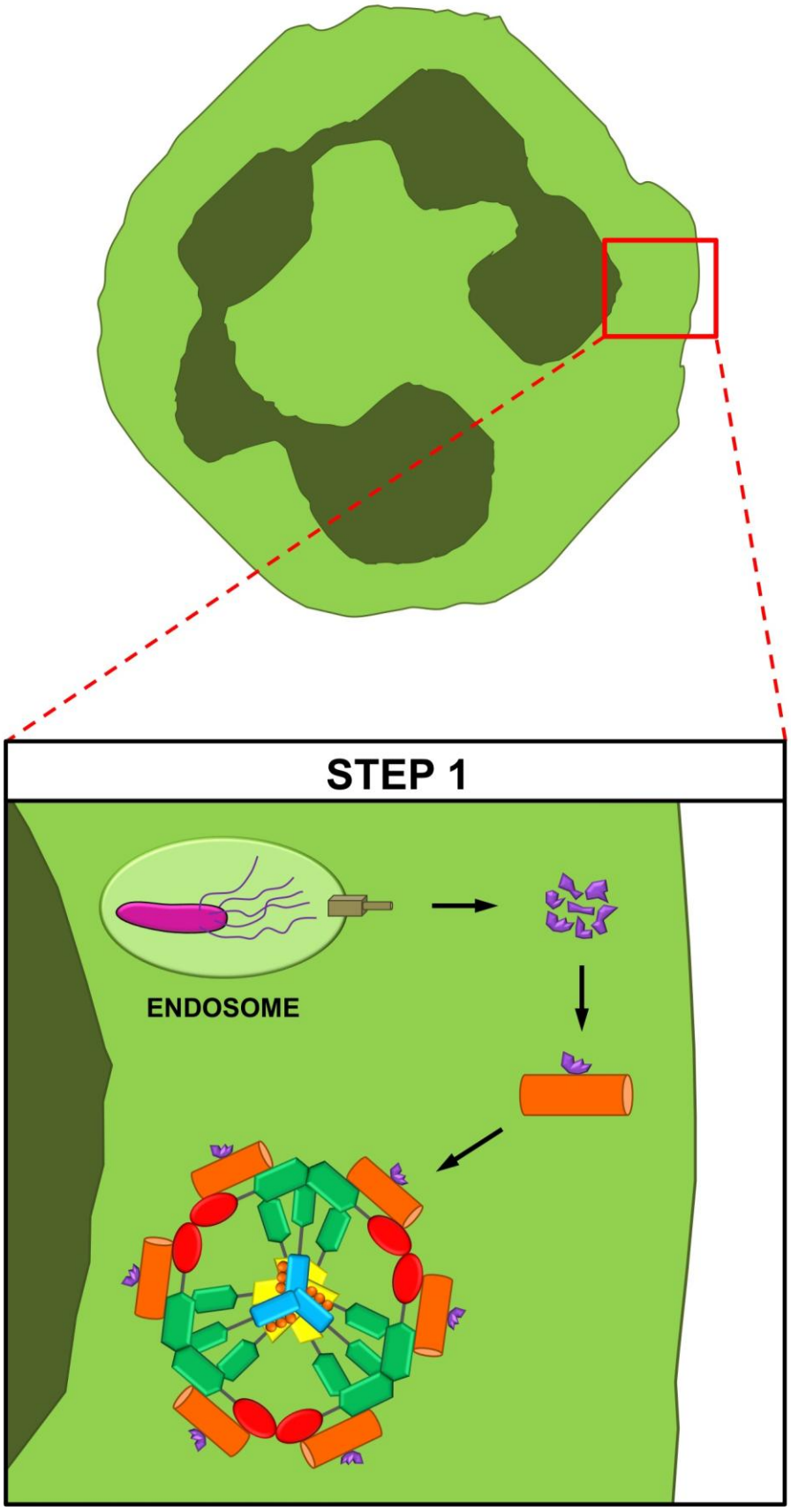
Another interesting observation from the zebrafish - *S.* Typhimurium infection model used in this doctoral thesis is the ability of Gbp4 to regulate neutrophil

recruitment to the infection foci. The altered neutrophil recruitment to the infection site observed in larvae forced to express WT and GTPase-deficient Gbp4 may indirectly resulted from the strong/poor inflammatory response to the infection and the quick/delayed clearance of the bacterium, as judged from the immune-related gene expression profile. Nevertheless, our results do not rule out a direct inhibition of neutrophil migration as a result of inflammasome-mediated changes in actin polymerization, as it has recently been observed *ex vivo* in mouse macrophages infected with *S.* Typhimurium (Man *et al*., 2014). This discrepancy may be the result of the differential cellular outcomes triggered upon inflammasome activation in neutrophils and macrophages, as occur with their disparate sensitivity to pyroptotic cell death (Chen *et al*., 2014). This deserves further investigation and the zebrafish will be a unique model to visualize at real-time the impact of inflammasome activation on cytoskeleton reorganization during neutrophil/macrophage recruitment and bacterial uptake and killing.

Gbp4 contains a C-terminal CARD, which in principle allows direct interaction with CARD-containing targets, omitting the need for a PYD/CARD-containing Asc adaptor. However, our epistasis analysis unequivocally demonstrates that Gbp4-dependent clearance of *S.* Typhimurium and caspase-1 activation both require Asc and are highly dependent on bacterial flagellin. In addition, our *in vivo* results were further confirmed by reconstituting Gbp4-Asc complexes in HEK293 cells where both WT and GTPase-deficient mutant were found to form a macromolecular complex with Asc while CARD-deficient Gbp4 was diffusely distributed in the cytosol independently of Asc and hardly rescued the high infection susceptibility of Gbp4-deficient larvae. These results are not unexpected, since NLRC4, which also has an N-terminal CARD, requires ASC for NLRC4 inflammasome speck formation, caspase-1 activation and efficient IL-1β processing (Proell *et al*., 2013). Strikingly, the Gbp4-Asc macromolecular complex observed here is rather similar to the ASC complex that comprises NLRC4, NLRP3, caspase-1, caspase-8, and pro-IL-1β, which has recently been described in macrophages infected with *S.* Typhimurium (Man *et al*., 2014).

Another interesting, but unexpected, observation of this study is the ability of the GTPase-deficient Gbp4, but not mouse GBP5, to behave as DN, despite being able to localize with Asc specks. Although ASC assembly is abolished in tetramerization GBP5 mutants, suggesting that tetrameric GBP5 promotes Asc oligomerization, both WT and

GTPase-deficient mutant GBP5 seem to form tetramers and are equally able to promote Asc multimerization (Shenoy *et al*., 2012). This is consistent with our *in vivo* study where forced expression of mouse GTPase-deficient GBP5 had no effect on bacterial resistance and caspase-1 activity, while WT GBP5 was able to rescue the high bacterial susceptibility and reduced caspase-1 activity of Gbp4-deficient larvae. These results suggest that Gbp4 would interact with a putative NLR sensor, which remains to be identified, likely though its GTPase domain, and Asc through its CARD, while GBP5 exclusively interacts with the PYD of NLRP3 through its GTPase domain (Shenoy *et al*., 2012). More importantly, the GTPase activity of Gbp4 is dispensable for inducing the oligomerization of Asc but crucial for the subsequent activation of caspase-1. Therefore, we propose a two-step model where (i) the presence of flagellin in the cytosol promotes the interaction of Gbp4 with Asc through CARD domains and (ii) the hydrolysis of GTP by Gbp4 results in a conformational change in this complex that allow the oligomerization and activation of caspase-1 (Figure 61).


STEP 1
ENDOSOME

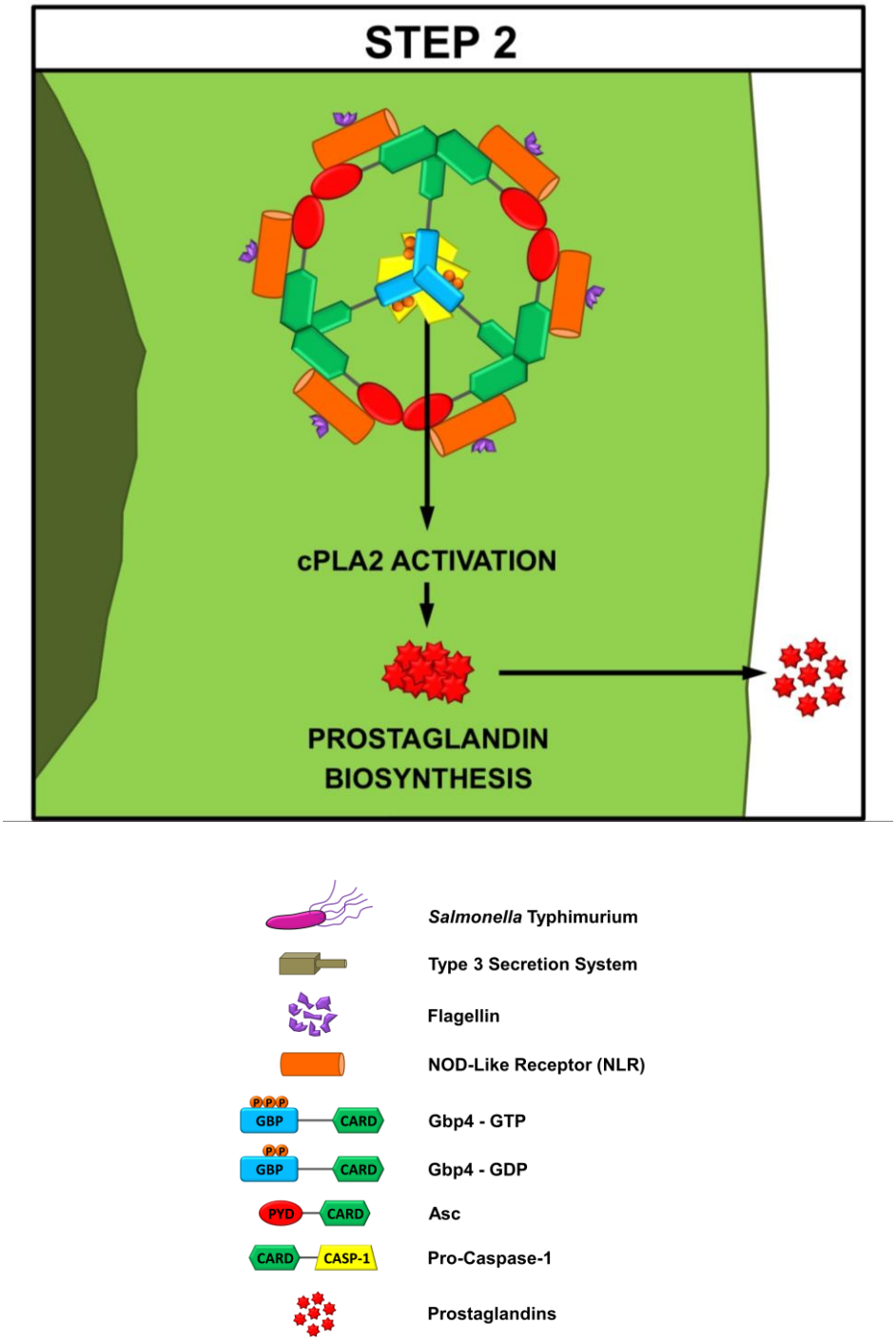


Figure 61. Proposed two-step model illustrating Gbp4-dependent inflammasome assembly and prostaglandin production upon *S.* Typhimurium infection. Step 1: *S.* Typhimurium, an intracellular bacterium, is taken up by neutrophils, localized to the SCV and using its T3SS translocates bacterial proteins to the cytosol. Flagellin, being one of those bacterial proteins, can be recognized by cytosolic NLRs, probably NLRP3 and/or NLRC4. Consequently, the NLRs induce the assembly of a Gbp4 and Asc through CARD domains and the subsequent recruitment of pro-caspase-1. **Step 2:** the hydrolysis of GTP to GDP or GMP by Gbp4 results in a conformational change in the inflammasome complex that allows the activation of caspase-1 via its autocleavage. As the result, caspase-1 induces prostaglandin biosynthesis, probably through the activation of cPLA2, as occurs in mammals (von Moltke *et al*., 2012).

Although it is widely accepted that inflammasome activation promotes bacterial pathogen clearance through the processing of pro-inflammatory cytokines IL-1β and IL-18 (Franchi *et al*., 2012b; Cai *et al*., 2012; Shimada *et al*., 2011; Wegiel *et al*., 2014) and the induction of pyroptotic cell death of macrophages (Aachoui *et al*., 2013; Miao *et al*. 2010a), the relative contribution of each pathway has been a matter controversy. In addition, some studies have even shown that strategies that inhibit inflammasome activation or downstream cytokine signaling resulted in enhanced bacterial clearance and diminished pathology (Cochen *et al*., 2013). We have not found any evidence of neutrophil cell death during infection of larvae with *S.* Typhimurium, being this in agreement with a recent study reporting that neutrophils do not undergo pyroptotic cell death upon NLRC4 activation (Chen *et al*., 2014). Furthermore, although we failed to detect mature IL-1β in whole larvae, the results obtained with the luciferase-based reporter assay iGLuc (Bartok *et al*., 2013) does not support a role for the inflammasome or caspase-1 in the processing of IL-1β in zebrafish, supporting our previous studies in fish macrophages (Angosto *et al*., 2012) and the absence of a conserved caspase-1 processing site in lower vertebrate IL-1βs (Angosto *et al*., 2013; Bird *et al*., 2002b). Nevertheless, our data strongly support that Gbp4-mediated resistance to *S.* Typhimurium is associated to the production of prostaglandins, in agreement with the ability of NLRC4 inflammasome to generate a storm of eicosanoids in resident mouse peritoneal macrophages (von Moltke *et al*., 2012). Although a particular cocktail of prostaglandins likely favors bacterial clearance, PGD2 seems to be particularly important in the resistance to *S.* Typhimurium, while 15dPGJ2, and to some extend PGE2, had a negative effect on bacterial resistance. Curiously, the relevance of prostaglandins in particular, and eicosanoids in general, in the clearance of intracellular bacteria has received little attention, despite that a metabolomic study recently showed that *S.* Typhimurium infection disturbs eicosanoid metabolism in mice (Antunes *et al*., 2011). Based on these *in vivo* data, the same authors performed an *in vitro* study and found that exogenous addition of $15dPGJ_2$ reduces macrophage colonization by *S.* Typhimurium, reactive nitrogen species production, and the expression of gene encoding both pro- and anti-inflammatory cytokines (Buckner *et al*., 2013), and therefore making difficult to predict the impact of this prostaglandin *in vivo*. Importantly, it is the fact that pharmacological inhibition of prostaglandin synthesis only affected the infection resistance of larvae forced to express Gbp4 but not of those forced to express Asc. Although these results deserve further investigations, we

speculate that Gbp4 in zebrafish, and likely GBP5 in mammals, is required for the inflammasome-mediated production of prostaglandins (Figure 61).

In summary, we report here a unique model to study the impact of inflammasome in inflammation and infectious disease progression with clear complementarities with the mouse model, among others the possibility to study the local inflammation activation and pathogen responses simultaneously in a whole organism, and the possibility of genetic and chemical screenings. Using this model, we uncovered a previously unappreciated role of neutrophils in the clearance of *S.* Typhimurium infection *in vivo* through Gbp4/Asc inflammasome-dependent biosynthesis of prostaglandins.

CONCLUSIONS

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

1. Zebrafish is an excellent model to study inflammasome activation and function upon *S.* Typhimurium infection.

2. Intracellular recognition of *S.* Typhimurium in zebrafish highly depends on flagellin production.

3. Gbp4-dependent inflammasome activation improves zebrafish larvae resistance and increases caspase-1 activity upon *S.* Typhimurium infection.

4. A Gbp4 mutant lacking GTPase activity, as well as a double GTPase and CARD mutant, behave as dominant negatives by increasing the susceptibility to *S.* Typhimurium and, in parallel, decreasing caspase-1 activity.

5. A Gbp4 mutant lacking the CARD domain acts as mammalian GBP5; that is, rescues the higher susceptibility of Gbp4-deficient larvae but fails to increase caspase-1 activity upon *S.* Typhimurium infection in zebrafish.

6. Gbp4 regulates neutrophil recruitment to the site of the infection. In addition, the Gbp4-dependent clearance of *S.* Typhimurium is mediated by neutrophils.

7. The Gbp4-dependent inflammasome activates prostaglandin biosynthesis, which in turn promotes *S.* Typhimurium clearance.

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RESUMEN EN CASTELLANO

1. Introducción

La inmunidad es el estado de equilibrio que se alcanza como consecuencia de tener las defensas adecuadas para luchar contra cualquier infección, enfermedad, u otra invasión no deseada, causadas por microorganismos (virus, bacterias, hongos, protozoos, parásitos multicelulares) o macromoléculas (proteínas y polisacáridos), excluyendo el resultado patológico, tales como las alergias y las enfermedades autoinmunitarias (Beck and Habicht, 1996). El sistema inmunitario es un conjunto de estructuras y procesos biológicos que protegen a un organismo contra las enfermedades. Tanto células como moléculas forman parte del sistema , que es el responsable de la inmunidad, y sus respuestas colectivas y coordinadas constituyen la respuesta inmunitaria (Male, 2006). Debido a la enorme variedad de agentes infecciosos que pueden atacar hoy en día nuestro organismo, se requiere una variedad de respuestas inmunitarias para combatir cada tipo de infección (Small, 2006).

La respuesta inmunitaria, que comienza con el reconocimiento del patógeno o sustancia extraña y termina con el desarrollo de mecanismos capaces de eliminarlos (Male, 2006), se puede dividir en dos ramas: respuesta inmunitaria innata, natural o inespecífica que actúa rápidamente como primera línea de defensa antes de que se desencadene la respuesta adaptativa, e incluye fundamentalmente barreras físicas, células fagocíticas, eosinófilos, células *natural killer* y varios tipos de moléculas circulantes (Male, 2006; Abbas *et al.*, 2015; Mollen *et al.*, 2006) y la respuesta inmunitaria adaptativa, adquirida o específica (aparece más tarde y supone una respuesta altamente específica frente a un patógeno en particular, siendo especificidad y memoria sus dos características más destacables (Male, 2006). A pesar de que aparecen en momentos diferentes y de que presentan características distintas, las respuestas inmunitarias innata y adaptativa actúan de forma integrada y coordinada (Male, 2006).

El sistema de peces teleósteos presenta características similares al de otros vertebrados, incluyendo mamíferos (Van Muiswinkel, 1995), siendo el primer grupo animal que desarrolló ambos sistemas, innato y adaptativo, bien estructurados y diferenciados. Sin embargo, presenta algunas diferencias como su mayor dependencia de los mecanismos de defensa innatos (Uribe *et al*., 2011), sobre todo a bajas temperaturas, puesto que la respuesta inmunitaria adaptativa depende de ese parámetro (Cuchens and Clem, 1977; Avtalion, 1981; Abruzzini *et al.*, 1982; Clem *et al.*, 1984,

1985, 1991). Además, los peces carecen de médula ósea, actuando la parte anterior del riñón (riñón cefálico) como principal órgano hematopoyético.

El funcionamiento del sistema inmunitario está modulado por la acción de una serie de moléculas reguladoras, fundamentalmente citoquinas (Abbas *et al.*, 2015), que regulan casi todos los procesos biológicos, incluyendo el crecimiento celular y su activación, la inflamación, la reparación de tejidos, la fibrosis, la morfogénesis y también participan en la señalización celular. Por otra parte, existen los mediadores lipídicos (Smith *et al.*, 1996; Harizi and Gualde, 2002; Cabral, 2005), que además de funcionar como fuente de energía y como componentes estructurales de la membrana celular, actúan también como efectores y segundos mensajeros en una variedad de procesos biológicos, complementando las actividades proinflamatorias y antiinflamatorias de los inmunomoduladores no lipídicos. Los bioefectores lipídicos más relevantes para el desarrollo de la presente tesis doctoral son las prostaglandinas, que son producidas por diferentes tejidos como en respuesta a estímulos extracelulares, tales como péptidos bacterianos, citoquinas y factores de crecimiento, y presentan diversas funciones.

El sistema inmunitario innato es capaz de detectar la presencia de microorganismos y de desencadenar mecanismos capaces de eliminar dichas amenazas potencialmente infecciosas. Esa detección se logra gracias a receptores capaces de reconocer específicamente patrones moleculares asociados a patógenos (PAMPs), que están presentes tanto en el espacio extracelular como intracelularmente, y que reconocen determinados componentes microbianos conservados que funcionan como indicadores de infección (Kumar *et al*., 2011). La mayoría de receptores que reconocen PAMPs (PRRs), pueden clasificarse en dos grupos: receptores de membrana (TLRs) y receptores solubles intracelulares (*leucine-rich repeat (LRR)-containing (or NOD-like)* receptores (NLRs), *RIG-1 like* receptores (RLRs) y *AIM2-like* receptores (ALRs)). Los TLRs se encuentran en la superficie celular o dentro de la célula en la membrana de los endosomas, mientras que los receptores solubles se encargan de detectar la presencia de patógenos en el citoplasma (Kumar *et al*., 2011; Brubaker *et al*., 2015). De todos estos receptores, los más relevantes para este trabajo son los NLRs, que presentan tres dominios estructurales: uno central de unión a nucleótidos y oligomerización (NACHT) que es comúnmente flanqueado por repeticiones C-terminales ricos en leucina (LRR), que detectan diferentes ligandos, y por dominios N-terminales de reclutamiento de la

caspasa (CARD) o dominios Pyrin (PYD). Esta diversidad de dominios efectores permite que los NLRs puedan interactuar con una amplia variedad de moléculas y que, por tanto, puedan activan múltiples vías de señalización.

Los NLRs están normalmente presentes en el citoplasma en una forma inactiva, con los LRRs plegados intramolecularmente en el dominio NACHT, inhibiendo de este modo su posible oligomerización espontánea y la consiguiente activación. La unión directa o indirecta de un PAMP al LRR desencadena su reorganización conformacional, exponiendo el dominio NACHT y provocando de ese modo su oligomerización. A través de una interacción homotípica, los CARDs y PYDs reclutan otros dominios CARD y PYD de otras moléculas efectoras, poniéndolos en proximidad y activando la consiguiente respuesta (Tschopp *et al*., 2003). Estas proteínas actúan como plataformas moleculares, llamadas inflamasomas, y que son necesarias para la activación de las caspasas inflamatorias, en particular de la caspasa-1. Dicha caspasa es capaz de integrarse en el inflamasoma en forma inactiva, facilitando así su posterior autoescisión y activación. Hay muchos tipos de inflamasomas, siendo NLRC4 o NLRP3 de mamíferos los dos más conocidos. A pesar de la importancia de la caspasa-1 en la inflamación, la información sobre la presencia y la actividad de esta enzima en peces es muy escasa. Hasta el día de hoy solo se han encontrado caspasas inflamatorias en los vertebrados, mientras que en pez cebra solo han sido identificados dos subgrupos de caspasas inflamatorias con sus dominios característicos conservados (Martinon and Tschopp, 2004).

El resultado de la activación del inflamasoma en los mamíferos es el procesamiento de moléculas proinflamatorias, principalmente de IL-1β (Thornberry *et al*., 1992). La IL-1β es un importante mediador de la inflamación, que normalmente induce la expresión de una gran variedad de genes que participan en el proceso inflamatorio. Esta citoquina es uno de los principales mediadores de la respuesta inmunitaria frente a invasiones microbianas, inflamación, reacciones inmunológicas y lesiones en tejidos (Oppenheim *et al*., 1986; Dinarello, 1991; Dinarello, 1994; Dinarello, 1996; Dinarello, 1997). Otro resultado de la activación del inflamasoma es la muerte celular programada llamada piroptosis, que es crucial para varios procesos tales como la organogénesis, el desarrollo, la inmunidad, o incluso el mantenimiento de la homeostasis en los organismos multicelulares. La piroptosis es una forma altamente proinflamatoria de muerte celular, que supone una respuesta esencial para la inmunidad

innata puesto que impide la propagación de infecciones intracelulares. Hasta ahora sólo se ha demostrado que tiene lugar en los macrófagos y en las células dendríticas (Edgeworth *et al*., 2002; Fink y Cookson, 2007), aunque hay evidencias de la actividad de la caspasa-1 en otros tipos celulares (Feldmeyer *et al*., 2007).

Durante los últimos han sido identificadas algunas proteínas de la familia GBP que regulan la respuesta inflamatoria participando en la activación del inflamasoma. La activación del inflamasoma se basa en la formación de multímeros de ASC, una proteína adaptadora (Broz y Monack, 2011; Franchi *et al.*, 2012a). Dichos multímeros de forman una plataforma central para la formación de varios tipos de inflamosomas. Se ha demostrado que GBP5 tetramérico se une al NLRP3, y fomentando así el ensamblaje de un multímetro de ASC - caspasa-1 (Shenoy *et al*., 2012).

El pez cebra (*Danio rerio* H), perteneciente a la familia Cyprinidae, presenta una serie de características que le han convertido en un excelente organismo modelo de vertebrados, siendo importante en investigación en multitud de áreas del conocimiento (Vascotto *et al.*, 1997). De entre sus principales ventajas destacan su pequeño tamaño, su elevada fecundidad (unos 200 huevos por hembra a la semana), su corto tiempo de generación, su rápido desarrollo, el conocimiento de la secuencia completa de su genoma, el desarrollo extrauterino de sus embriones (facilitando una manipulación sencilla), la facilidad con la que puede manipularse la expresión de genes específicos, y, sobre todo, su transparencia, lo que permite el seguimiento y estudio *in vivo* y a tiempo real de células individuales trabajando con líneas transgénicas. Todas estas ventajas han hecho posible que el pez cebra se establezca como un organismo modelo crucial en el estudio del sistema inmunitario (Renshaw and Trede, 2012), de la hematopoyesis (Martin *et al.*, 2011), del desarrollo vascular (Quaife *et al.*, 2012; Gore *et al.*, 2012), de la neurogenesis (Schmidt *et al.*, 2013), del cáncer (Mione and Trede, 2010), del comportamiento (Miklósi and Andrew, 2006; Spence *et al.*, 2008; Norton and Bally-Cuif, 2010) y, lo que es más importante para la presente tesis doctoral, de la infección. Este modelo permite estudiar en detalle las interacciones hospedador/patógeno, pudiendo tener una gran población de hospedadores y visualizar a los patógenos a nivel celular (Sullivan and Kim, 2008; Milligan-Myhre *et al*., 2011). Dentro del campo de las enfermedades infecciosas, el modelo de pez cebra es una herramienta única para el estudio de la función de las células fagocíticas (Torraca *et al*., 2014). Tanto las infecciones como los tratamientos químicos se pueden realizar a gran escala en el pez

cebra, permitiendo así la identificación de nuevos factores de virulencia microbiana y el escrutinio de posibles nuevos tratamientos para desentrañar los mecanismos de las enfermedades (Miller y Neely, 2005; Lieschke y Currie, 2007). Muchos de los modelos de infección en pez cebra para el estudio de las enfermedades virales y bacterianas han sido desarrollados durante los últimos años (Phelps y Neely, 2005; Lesley y Ramakrishnan, 2008; Meeker y Comercio, 2008).

Un modelo de infección con un patógeno humano bien estudiado en embriones de pez cebra es *S.* Typhimurium (Stockhammer *et al.*, 2009). Sus mecanismos de virulencia solo se manifiestan totalmente durante las interacciones hospedador-patógeno *in vivo.* Por tanto, para un mejor entendimiento de la patogénesis de *Salmonella* es esencial disponer de un modelo de infección natural en un animal que desarrolle signos de la enfermedad similares a los observados en los seres humanos. Algunos modelos animales, como ratones o primates, presentan síntomas muy similares a los que aparecen en pacientes humanos, pero el elevado coste de esos modelos y su escasez en algunos casos ha impedido su uso generalizado (Santos *et al*., 2001). Sin embargo, hoy en día el pez cebra puede reemplazar a esos modelos de mamíferos ya que sus procesos biológicos son similares, ofreciendo una alternativa menos costosa y más eficiente.

2. Objetivos

Los objetivos específicos de este trabajo son:

1. Establecimiento de un modelo de infección de *Salmonella* Typhimurium en pez cebra para estudiar la activación, el ensamblaje y el funcionamiento del inflamasoma.
2. Caracterización del papel de la flagelina de *S.* Typhimurium en el mecanismo de infección en pez cebra.
3. Caracterización del papel de Gbp4 de pez cebra en la activación y ensamblaje del inflamasoma, así como en la eliminación de *S.* Typhimurium.
4. Caracterización del papel de los neutrófilos en la eliminación de *S.* Typhimurium dependiente de Gbp4 en pez cebra.
5. Caracterización del papel de Gbp4 en la producción de prostaglandinas dependiente del inflamasoma, y del de las prostaglandinas en la eliminación de *S.* Typhimurium.

3. Materiales y métodos

3.1. Animales

Los peces cebra silvestres utilizados para la realización de la presente Tesis Doctoral fueron obtenidos del *Zebrafish International Resource Centre* (ZIRC, Oregón, EEUU). Su cuidado, manejo, mantenimiento, cruce, procesamiento, etc.., se llevó a cabo según se describe en el manual del pez cebra (Westerfield, 2000). Las líneas transgénicas de pez cebra utilizadas en este trabajo fueron obtenidas de otros laboratorios, gracias a la colaboración de los doctores Stephen A. Renshaw, Graham J. Lieschke y Phil Crosier.

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 #537/2011).

3.2. Construcciones de ADN

Los genes de pez cebra que codifican para Gbp4 silvestre, el mutante de GTPasa Gbp4KS→AA, el mutante del dominio CARD Gbp4ΔCARD, el doble mutante Gbp4KS→AA/ΔCARD, zfiGLuc y GLuc, fueron sintetizados por GenScript Corporation. Las construcciones de cmv/sp6:gbp4 y cmv/sp6:gbp4-mCherry fueron generadas por MultiSite Gateway, ensambladas usando LR Clonase II Plus (Life Technologies), según el protocolo estándar y usando los vectores Tol2kit previamente descritos (Kwan *et al*., 2007). La construcción de Asc-Myc de pez cebra en el plásmido pcDNA3 estaba previamente descrita (Masumoto *et al*., 2003).

3.3. Microinyección de morfolinos y ARNm

Los morfolinos utilizados en este trabajo fueron diseñados y comprados en *Gene Tools*, y resuspendidos en agua libre de nucleasas a una concentración de 1mM, 2mM y 3mM. Los ARNs fueron obtenidos mediante transcripción *in vitro* siguiendo las instrucciones del fabricante (mMESSAGE mMACHINE kit, Ambion). Los morfolinos y los ARNs fueron mezclados en tampón de microinyección (tampón Tango 0.5x y 0.05% de solución de rojo fenol) y microinyectados dentro del vitelo de embriones de 1 célula usando un microinyector (Narishige) (1 nl por embrión). En todos los grupos

experimentales se utilizó la misma cantidad total de morfolinos y/o ARNs. La eficacia de los morfolinos se comprobó mediante las técnicas de RT-PCR y Western blot.

3.4. Tratamientos químicos

En algunos experimentos, los embriones fueron descorionados manualmente 48 horas después de la fertilización, y posteriormente incubados a 28ºC mediante inmersión en baño con una concentración de 10 μM del inhibidor de las prostaglandinas indometacina (Sigma-Aldrich), 100 μM del inhibidor de la caspasa-1 Ac-YVAD-CMK (Peptanova) o 50 μM del inhibidor general de las caspasas Q-VD-OPh (SM Biochemicals LLC) diluído en egg water y con un 1% de DMSO o con 10 μM de 16,16 dimetil PGE2, 16, 16 dimetil PGD2, d12PGJ2 o 15dPGJ2 (todos de Cayman Chemical) diluído en egg water y con un 0,01%-0,2% de metil acetato (Sigma-Aldrich).

3.5. Ensayos de infección

En la mayoría de los experimentos de infección, *S.* Typhimurium 12023 (silvestre) y los mutantes isogénicos derivados de SPI-1 (prgH020::Tn5lacZY), SPI-2 (ssaV::aphT) y SPI-1/SPI-2 (prgH020::Tn5lacZY ssaV::aphT) (facilitado por Prof. D. Holden) fueron utilizados. En algunos experimentos, las estirpes de *S.* Typhimurium fueron utilizadas: 14028s (silvestre), sus mutantes isogénicos derivados fliC/fljB, que no expresa flagelina, y FliCON, que continuamente expresa la flagelina FliC (Miao *et al*., 2006; Miao *et al*., 2010a) (facilitado por Dr. E.A. Miao). En las infecciones en el oído, *S.* Typhimurium 12023 expresando DsRedT3 (de Oliveira *et al*., 2015) fue utilizada.

Los cultivos de bacterias cultivados durante toda la noche en caldo de cultivo Luria-Bertani (LB) fueron diluidos 1/5 en LB con 0.3 M NaCl, incubados a 37 ºC hasta alcanzar una densidad óptica de 1,5 medida en un espectrofotómetro a una longitud de onda de 600 nm, y, finalmente, diluidos en PBS estéril. Embriones de pez cebra en el estado de una sola célula fueron microinyectados con morfolinos y/o ARNs. Dos días después de la fertilización, las larvas fueron descorionadas manualmente, anestesiadas con 0.16 mg/ml de tricaína, y las infecciones fueron llevadas a cabo microinyectando 10-50 bacterias (vitelo), 100 bacterias (oído) o 250 bacterias (conducto de Cuvier) por larva. Las larvas se recuperaron en agua a 28-29ºC, y fueron monitorizadas para seguir los signos clínicos de la enfermedad o su mortalidad durante 5 días. Todos los ensayos

de supervivencia se han realizado tres veces, usando 40-100 larvas por tratamiento en cada experimento independiente. Cada gráfica muestra el resultado medio de los tres experimentos, teniendo un tamaño de muestra acumulado de 120-300 larvas por tratamiento.

3.6. Ensayos de actividad caspasa-1

Embriones de pez cebra en el estado de una sola célula fueron inyectados con morfolinos y/o ARNs. Dos días después de la fertilización, las larvas fueron descorionadas manualmente e infectadas en el vitelo con diferentes estirpes de *S.* Typhimurium (MOI=50). Tres días después de la fertilización, las larvas fueron recogidas y utilizadas para la medida de la actividad de la caspasa-1. La actividad caspasa-1 fue determinada con el substrato fluorogénico Z-YVAD-AFC (caspase-1 substrate VI, Calbiochem) como ya había sido descrito previamente (López-Castejón *et al*., 2008). A continuación, las larvas fueron lisadas en tampón de lisis [25 mM de ácido 4-(2-hidroxietil)-1-piperazineetanosulfónico (HEPES), 5 μM de ácido [etilenebis (oxonitrilo)] tetraacético (EGTA), 5 mM de ditrioteitol (DTT), cocktail inhibidor de proteasas (1:20, Sigma), pH 7'5] en hielo durante 10 min. Para cada reacción, se incubaron 70 μg de las proteínas durante 90 minutos a 23ºC con 50 μM YVAD-AFC y 50 μl del tampón de reacción [0'2% de ácido 3-[(3-colamidopropil) dimetilammonio]-1-1propanosulfonico (CHAPS), 0'2 M de HEPES, 20% de glucosa, 29 mM de DTT, pH 7'5]. 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. Todos los ensayos de actividad caspasa-1 fueron realizados tres veces, utilizando 25-40 larvas por tratamiento en cada experimento independiente. Solo una de las tres gráficas se muestra en cada caso como un resultado representativo.

3.7. Toma de imágenes de larvas de pez cebra

Las larvas fueron anestesiadas en tricaína (0.16 mg/ml) 72 horas después de la fertilización, y montadas en agarosa de bajo punto de fusión (Sigma-Aldrich) al 1% (peso/volumen) disuelta en *egg water*. Las imágenes fueron capturadas usando un estereomicroscopio de epifluorescencia LEICA MZ16FA equipado con filtros para fluorescencia verde y roja, mientras que los animales fueron mantenidas en sus matrices

de agar a 28 °C. Todas las imágenes fueron adquiridas con la cámara integrada en el microscopio estéreo y posteriormente fueron utilizadas para el recuento de los neutrófilos (*mpx:eGFP*).

3.8. Reclutamiento de neutrófilos y análisis de la muerte celular

Para el estudio del reclutamiento de neutrófilos y la muerte celular (piroptosis) en un lugar localizado de infección con *S.* Typhimurium, dos días después de la fertilización las larvas fueron anestesiadas con 0’16 mg/ml de tricaína y montadas en 1% agarosa con bajo punto de fusión suplementada con 0’16 mg/ml de tricaína. 0’5 nl de PBS o de suspensión de *S.* Typhimurium:DsREDT3 (100 bacterias/larva), suplementado con rojo fenol, fueron inyectados en el oído de larvas de pez cebra. Agua con 0’16 mg/ml de tricaína fue añadida para mantener los embriones hidratados durante el experimento. Imágenes del área del oído fueron tomadas a las 1’5, 3, 4’5 y 6 horas después de infección usando un estereomicroscopio de epifluorescencia LEICA MZ16FA. Posteriormente las imágenes fueron tratadas con el software ImageJ (http://rsb.info.nih.gov/ij/) y se llevó a cabo el recuento de los neutrófilos. Para el análisis de la muerte celular (piroptosis), 2 μM del compuesto verde fluorescente YO-PRO (Life Technologies) fue inyectado en el oído 3 horas después de infección, y las imágenes fueron tomadas 1 hora después como anteriormente.

3.9. Ensayos de luminescencia

Los embriones fueron microinyectados como se ha descrito anteriormente con 200 pg de los ARNs zfiGLuc o GLuc. Después de 48 horas, las larvas fueron infectadas con *S.* Typhimurium silvestre y los extractos de larvas completas obtenidos a las 24 horas después de la infección como se ha descrito previamente (Alcaraz-Pérez *et al*., 2008). Los extractos de las larvas fueron entonces combinados 1:1 con agua destilada conteniendo 4’4 μM coelenterazine (Sigma-Aldrich) para llegar a una concentración final de 2’2 μM. La señal de la luciferasa fue entonces medida en un Luminómetro Optocomp II (MGM Instruments). Todos los ensayos de luminiscencia han sido realizados tres veces, usando 20 larvas por tratamiento en cada experimento independiente. Solo una de las tres graficas se muestra en cada caso como un resultado representativo.

3.10. Citometría de flujo

Entre 300 y 500 larvas de las líneas transgénicas *Tg(mpx:eGFP)* y *Tg(mpeg1:GFP)* fueron anestesiadas en tricaína (0.16 mg/ml) 72 horas después de la fertilización, incubadas con 0’077 mg/ml de Liberase (Roche) a 28ºC durante 90 minutos, y la suspensión celular resultante fue pasada a través de filtros de 30 µm de tamaño de poro.

Los experimentos de citometría de flujo se llevaron a cabo usando un FACSCALIBUR (BD), mientras que para el aislamiento de células (*sorting*) se utilizó un Coulter (Epics Altra). Los resultados fueron analizados con el software FlowJo (Treestar).

3.11. Análisis de expresión génica

El ARN total fue extraído de larvas/embriones completos, cabezas de las larvas o de suspensiones celulares usando TriZol (Invitrogen) y siguiendo las instrucciones del fabricante. A continuación, dicho ARN fue tratado con DNasa I libre de RNasa (Invitrogen). La retrotranscriptasa Superscript III RNasa H^- (Invitrogen) fue utilizada para sintetizar el ADNc con un cebador oligo-dT_{18} a partir de 1 µg de ARN total a 50ºC durante 50 minutos.

Para la PCR a tiempo real se usó un equipo ABI PRISM 7500 (Applied Biosystems), utilizando SYBR Green (Applied Bioystems). Las mezclas de reacción fueron incubadas durante 10 minutos a 95ºC, seguido de 40 ciclos de 15 segundos a 95ºC, 1 minuto a 60ºC y finalmente 15 segundos a 95ºC, 1 minuto a 60ºC y 15 segundos a 95ºC. Para cada ARN, la expresión génica fue corregida por ARN de la proteína ribosómica S11 (*rps11*). En todos los casos, cada PCR fue realiza por triplicado y repetida al menos con dos muestras independientes.

3.12. Determinación de las proteínas

La concentración de las proteínas de los lisados celulares fue estimada con el ensayo de BCA (Pierce) usando BSA como el estándar.

3.13. Western blot

Los extractos de las células (60 µg) de las larvas de pez cebra descorionadas y desviteladas obtenidos a 2 días después de la fertilización usando 200 µL de tampón de lisis (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% NP-40, y 1:20 dilución del coctel de inhibidores de las proteasas P8340 de SigmaAldrich) fueron resueltos en 10% SDS-PAGE, transferidos durante 50 minutos a 200 mA a una membrana de nitrocelulosa (BioRad), incubados con la dilución de 1/200 de los cuatro anticuerpos diferentes para Gbp4 de pez cebra generados usando el SEALTM Technology (Abmart), y revelados con los reactivos de quimioluminiscencia ECL (GE Healthcare) según el protocolo del fabricante. Las membranas fueron entonces incubadas con la dilución de 1/5.000 del anticuerpo comercial producido en conejo para detectar la histona 3 (#ab1791, Abcam), como control de carga.

Células transfectadas de HEK293 (600.000) fueron lisadas en el tampón CHAPS (20 mM HEPESKOH (pH 7.5), 5 mM MgCl2, 0.5 mM EGTA, 0.1 mM PMSF, 0.1 % CHAPS). Los extractos proteicos fueron separados en 10-8 % SDS-PAGE gel y transferidos a una membrana de nitrocelulosa, que después del bloqueo con 5% de leche desnatada fueron incubados durante la noche a 4ºC con los anticuerpos policlonales primarios de conejo anti GFP (dilución 1:1.000; sc-8334; Santa Cruz) y anti c-myc (dilución 1:5000; 46-0603; Invitrogen). Para la detección, se utilizó un anticuerpo secundario conjugado a peroxidasa de rábano (dilución 1:5.000; Amersham).

3.14. Ensayo de reconstitución del inflamasoma en células HEK293

Células HEK293T (CRL-11268; American Type Culture Collection) fueron mantenidas en DMEM:F12 (1:1) suplementado con 10% FCS, 2 mM Glutamax y 1% penicilina-estreptomicina (Life Technologies). El ADN plasmídico fue preparado utilizando el kit de Mini-Prep (Qiagen). Los precipitados de ADN fueron resuspendidos en agua y entonces diluidos, cuando fue necesario, en PBS. Las transfecciones fueron realizadas con un reactivo de transfección basado en lípidos catiónicos (LyoVec, Invivogen) según las instrucciones del fabricante. En breve, las células de HEK293 fueron cultivados en las placas de 24 pocillos (120,000 células/pocillo) junto con 25 µl del reactivo de transfección conteniendo 250 ng de ADN plásmido total. Los plámidos de Gpb4-GFP y Asc-myc fueron utilizados a una relación de 1:1. Para el ensayo de

immunofluorescencia, las células HEK293T fueron cultivadas en los cubreobjetos cubiertos con poly-L-lysina, lavados dos veces con PBS, fijados con 4% de formaldehido en PBS durante 15 minutos a temperatura ambiente, y entonces lavados tres veces con PBS. La unión inespecifica en las células fue bloqueada con 1% albúmina de suero bovino (Sigma) y las células fueron permeabilizadas con 0.2% saponina (Fluka) en PBS durante 30 minutos a la temperatura ambiente. Después de eso, las células fueron incubadas durante 1h a temperatura ambiente con el anticuerpo primario monoclonal del ratón anti c-myc (1:1000 dilución; 46-0603; Invitrogen). Las células fueron lavadas, incubadas durante 1 hora a la temperatura ambiente con el anticuerpo de burro anti ratón IgG Alexa Fluor 647 (1:200 dilución; A-31571; Life technologies), lavados en PBS, y montados en cubreobjetos con ProLong Diamond Antifade Mountant con DAPI (Life Technologies). Las imágenes fueron adquiridas con el microscopio Nikon Eclipse Ti equipado con el objetivo de 20xS Plan Fluor (apertura numérica, 0.45), el objetivo 40xS Plan Fluor (apertura numérica, 0.60) y el objetivo 60x Plan Apo Vc (apertura numérica, 1.40) y la cámara digital Sight DS-QiMc (Nikon) con el Z espaciamiento óptico de 0.2 um y 387-nm/447- nm, 472-nm/520-nm and 650-nm/668-nm juegos de filtros (Semrock). Las imágenes fueron deconvolucionados usando ImageJ software, y la intensidad máxima de proyecciones de las imágenes se muestra en el apartado de los resultados.

3.15. Análisis estadístico

Los datos se muestran como media ± SEM y fueron analizados por análisis de la varianza (ANOVA) y el test del rango múltiple Tukey para determinar las diferencias entre los grupos. Las curvas de supervivencia fueron analizadas usando el log-rank (Mantel-Cox) test. Todos los experimentos fueron realizados por lo menos tres veces, a menos se indique lo contrario. El tamaño de la muestra para cada tratamiento está indicado en la gráfica o en la leyenda de la gráfica o en la sección correspondiente en los Materiales y Métodos. La significancia estadística se definió como $p < 0'05$.

4. Resultados

4.1. La proteína Gbp4 de pez cebra está compuesta por dos dominios funcionales, GBP y CARD, y presenta altos niveles de expresión en los neutrófilos pero no en los macrófagos

Diversos análisis bioinformáticos buscando proteínas que contengan el dominio CARD como potenciales componentes del inflamasoma, fueron previamente realizados en nuestro laboratorio. Los resultados de esa búsqueda nos llevó a centrarnos en dos genes presentes en el genoma del pez cebra, que cifran dos proteínas que pertenecen a la familia de GBP: Gbp3 y Gbp4. Ambas proteínas contienen un dominio GBP situado en N-terminal, y un dominio CARD situado en C-terminal. Cuando se compararon Gbp3 y Gbp4 de pez cebra con GBP5 humano, una proteína que ya se sabe que forma parte del inflamasoma en mamíferos a pesar de no presentar dominio CARD, se obtuvo 23% / 35% y 36% / 53% de homología/ identidad de aminoácidos respectivamente. Por tanto, decidimos continuar nuestro estudio con Gbp4, la proteína que muestra una homología mayor en comparación con GBP5 de humano, ya que sería también más probable que compartan funciones.

El primer paso fue analizar el perfil de expresión de Gbp4 de pez cebra en los principales tipos de células que participan en la respuesta inmunitaria innata: los neutrófilos y los macrófagos. Para ello, aislamos neutrófilos y macrófagos mediante FACS usando las líneas transgénicas *mpx: GFP* (Renshaw *et al*., 2006) y *mpeg1*:GFP (Ellett *et al*., 2011), respectivamente. Encontramos que los transcritos de Gbp4 fueron altamente enriquecidos en los neutrófilos, mientras que apenas se detectaban en los macrófagos. Además, la infección con *S.* Typhimurium tuvo efectos insignificantes en los niveles de ARNm de Gbp4 en ambos tipos celulares. Sin embargo, ambos tipos celulares mostraron un aumento de los niveles de ARNm de gen *il1b* después de la infección, confirmando la inflamación.

A continuación, quisimos estudiar la función de Gbp4 mediante el análisis de los efectos de su inhibición genética, utilizando un morfolino (MO), que nos permiten silenciar la expresión de genes específicos alterando su procesamiento. Antes de trabajar con el Gbp4 MO, comprobamos su eficacia por Western blot utilizando cuatro mAbs diferentes que se unen tanto al dominio GBP como al CARD. Los resultados obtenidos mostraron una fuerte reducción de la proteína Gbp4 en animales inyectados con este

morfolino comparando con los controles, demostrando que el Gbp4 MO estaba funcionando correctamente.

Como se ha mencionado en la introducción, la activación del inflamasoma da lugar a la activación de la caspasa-1. Por lo tanto, hemos utilizado los niveles de la actividad de la caspasa-1 como un espejo para medir el nivel de activación del inflamasoma. La actividad caspasa-1 se evaluó utilizando un sustrato fluorogénico Z-YVAD-AFC (López-Castejón *et al*., 2008; Compan *et al*., 2012). Los resultados mostraron una inhibición dependiente de la dosis de actividad basal de la caspasa-1 en las larvas inyectadas con concentraciones crecientes de Gbp4 MO en comparación con los controles. No observamos defectos en el desarrollo ni en la mortalidad en los animales inyectados con Gbp4 MO 0’5 pg / huevo, por lo que esa dosis fue la elegida para ser utilizada en los experimentos de la presente tesis. Curiosamente, la inhibición de la actividad caspasa-1 en larvas deficientes en Gbp4 fue similar a la encontrada en larvas deficientes en la proteína adaptadora del inflamasoma Asc, lo que sugiere que ambas proteínas podrían desempeñar un papel similar.

4.2. Gbp4 es necesaria para la resistencia a *S*. Typhimurium en pez cebra

Debido a su importancia clínica, *S*. Typhimurium ha sido ampliamente utilizada como un organismo modelo para estudiar las interacciones hospedador-patógeno y, en particular, para el estudio del papel del inflamasoma en la eliminación de las bacterias intracelulares. Como se ha mencionado antes, la virulencia de *S.* Typhimurium está vinculada a sus dos islas de patogenicidad, llamados SPI-1 y SPI-2, que contienen un gran número de genes que codifican un T3SS. La infección de larvas de pez cebra con *S*. Typhimurium silvestre resultó en una alta mortalidad utilizando dos rutas diferentes de infección: el saco vitelino y el conducto de Cuvier. En contraste, un doble mutante singénico para SPI-1 y SPI-2 mostró virulencia reducida. Sin embargo, los mutantes de SPI-1 o SPI-2 tenían una tasa de virulencia intermedia. Notablemente, *S.* Typhimurium silvestre indujo la activación de la caspasa-1, mientras que todos los mutantes de SPI no lo hicieron.

Estos resultados demostraron la utilidad de este modelo para determinar el papel de *S*. Typhimurium SPI en la virulencia bacteriana en un organismo vertebrado completo en ausencia de la inmunidad adaptativa. Por tanto, en el siguiente paso infectamos peces deficientes en Gbp4 con *S*. Typhimurium silvestre y encontramos una

mayor susceptibilidad a la infección en comparación con los controles. Los niveles de actividad caspasa-1 obtenidos en este experimento apoyaban los resultados encontrados en el ensayo de supervivencia, ya que también se detectó una alteración de la actividad caspasa-1 en larvas deficiente en Gbp4 en respuesta a la infección. Además, tanto la susceptibilidad de las larvas como la actividad caspasa-1 fueron rescatadas por la sobreexpresión de Gbp4. Más interesante, la sobreexpresión forzada de ARNm de Gbp4 aumentó la resistencia de los peces a la infección y los niveles de la actividad de la caspasa-1 se incrementaran sobre la infección. Yendo más lejos, los resultados obtenidos en estos experimentos confirmaron la especificidad del MO, y reveló el papel crucial de Gbp4 en la resistencia contra *S*. Typhimurium.

Por otra parte, tanto la supervivencia como los ensayos de la actividad de la caspasa-1 mostraron que la sobreexpresión de Gbp4 no afectó a la resistencia de los peces después de la infección de los mutantes de SPI-1 / SPI-2 de *S*. Typhimurium. No se encontraron diferencias significativas en la supervivencia de las larvas que sobreexpresaron Gbp4 comparando con los controles después de la infección con doble mutante de *S*. Typhimurium. Siguiendo un patrón similar, los niveles de la actividad de la caspasa-1 no aumentó en la infección con el mutante SPI-1 / SPI-2, pero llegó a los niveles endógenos.

En particular, la detección de *S*. Typhimurium en pez cebra dependía mucho de la flagelina. La cepa que expresa la flagelina persistentemente FliC (FliCON) (Miao *et al.*, 2010a) mostró una virulencia reducida, por lo tanto, las larvas tenían mayor resistencia y la bacteria era más potente en la inducción de la caspasa-1. Sin embargo, la cepa singénica mutante que no produce flagelina (FliC / fljB) (Miao *et al*., 2006) se comportara como la cepa silvestre, lo que sugiere que la expresión de la flagelina de *S.* Typhimurium silvestre se reprime después de la infección, como ocurre en los mamíferos (Cummings *et al*., 2006). Además, todas estas respuestas fueron claramente dependientes de Gbp4, ya que la reducción resultó en la inhibición de la resistencia de los peces con la infección de *S*. Typhimurium, así como en la disminución de los niveles de la actividad de la caspasa-1.

Aunque no se ha identificado ningún homólogo de la caspasa-1 de mamíferos en el genoma del pez cebra, si se ha encontrado que caspy, que preferencialmente corta AcYVAD-AMC, el sustrato de la caspasa-1, es capaz de interactuar y colocalizar en

agregados con Asc cuando es expresada ectópicamente en HEK (Masumoto *et al*., 2003). Por tanto decidimos utilizar tanto un inhibidor general de caspasas como un inhibidor específico de caspasa-1, que se ha demostrado que inhibe la actividad de la caspasa-1 recombinante en los peces (López-Castejón *et al*., 2008; Compan *et al*., 2012). Los resultados mostraron que el tratamiento con ambos inhibidores resultó en un aumento de la susceptibilidad de las larvas de pez cebra a la infección por *S*. Typhimurium y, más importante, neutralizó totalmente el aumento de la resistencia mediada por Gbp4 a la infección. Como se esperaba, ambos inhibidores disminuyeron fuertemente los niveles de actividad caspasa-1 *in vivo*, incluso sus niveles basales.

La presencia del dominio CARD en Gbp4 de peces sugiere que pueden reclutar y activar directamente a la caspasa-1, sin la necesidad de la proteína adaptadora Asc. Algo similar ocurre en el caso del NLRC4 de mamíferos, que también posee el dominio CARD y que es capaz de reclutar y activar directamente la caspasa-1. Así, silenciamos Asc mediante un MO que evitaba el procesamiento del ARNm de *asc*. Como ya lo hicimos para el Gbp4 MO, el primer paso fue comprobar la eficacia del Asc MO, para lo que se realizó un análisis mediante RT-PCR que mostraba que el Asc MO era capaz de alterar el splicing de los transcriptos de *asc* hasta 6 días después de la fertilización (Angosto, 2012).

La inhibición genética de Asc en pez cebra no tuvo efectos aparentes sobre el desarrollo larvario, pero redujo significativamente los niveles de actividad caspasa-1 basales e inducidos por *S*. Typhimurium. Por otra parte, se observó que la deficiencia de Asc aumentaba drásticamente la susceptibilidad de las larvas a la infección de *S*. Typhimurium y, sorprendentemente, eliminaba por completo la mayor resistencia mediada por Gbp4 a la infección. Por lo tanto, el hecho de que la sobreexpresión de Gbp4 no pudiese rescatar los efectos del Asc MO sugiere que GBP4 podría ser una proteína dependiente de Asc.

A continuación, quisimos confirmar el fenotipo y los resultados obtenidos. Por eso, utilizamos otro morfolino para Asc (Asc-ATG MO). Este nuevo MO inhibe directamente la traducción del ARNm, mientras que no hay diferencias en los niveles de los transcriptos de *asc*. Los resultados obtenidos con el Asc-ATG MO confirmó lo que habíamos observado antes utilizando el Asc MO, incluyendo el aumento de la susceptibilidad a la infección con *S*. Typhimurium y la reducción de los niveles de la

actividad de la caspasa-1. El hecho de que la sobreexpresión de Gbp4 no pudiese rescatar a los efectos de Asc-ATG MO también estaba de acuerdo con los resultados obtenidos anteriormente con el Asc MO. Además, como hemos visto con el Asc MO, la sobreexpresión de Gbp4 en los de Asc-ATG no resulto ni en el aumento de la resistencia en la infección con *S.* Typhimurium ni en el aumento de los niveles de actividad caspasa-1 en larvas infectadas.

Sin embargo, la sobreexpresión de Asc aumentó considerablemente la resistencia de las larvas a la infección y los niveles de actividad caspasa-1, siendo esos efectos independientes de Gbp4. La relevancia de Asc en la eliminación de *S.* Typhimurium fue confirmada por la capacidad del mutante de Asc, que tiene un GFP en el C-terminal en lugar del dominio CARD, ya que se comportaba como un dominante negativo (DN) aumentando la susceptibilidad de las larvas infectadas con *S.* Typhimurium. En conjunto, estos resultados demuestran que Gbp4 actúa aguas arriba de Asc, induciendo la activación del inflamasoma.

4.3. La actividad GTPasa de Gbp4 es indispensable para la resistencia a *S.* Typhimurium dependiente del inflamasoma

Siguiendo el ejemplo del grupo de MacMicking (Shenoy *et al.*, 2012), generamos una forma mutante de Gbp4 de pez cebra deficiente en su actividad GTPasa (Gbp4KS → AA) y, de forma inesperada, encontramos que no sólo era incapaz de aumentar la resistencia de las larvas en la infección por *S.* Typhimurium, sino que también se comportaba como un dominante negativo que aumentaba la susceptibilidad a la infección y concomitantemente inhibía caspasa-1.

Después de eso, decidimos probar el efecto de un mutante de Gbp4 que no tuviese el dominio CARD (Gbp4△CARD), y observamos que a pesar de que era capaz de rescatar parcialmente la alta susceptibilidad de las larvas deficientes en Gbp4, no lograba incrementar su resistencia a la infección cuando se expresaba solo. Además, casi no invertía la actividad de la caspasa-1 observada en larvas deficientes en Gbp4 después de la infección. Por otra parte, un doble mutante (DM) desprovisto tanto de la actividad GTPasa como del dominio CARD se comportaba como el mutante de GTPasa en cuanto a sus niveles de supervivencia y de actividad caspasa-1.

Sorprendentemente, GBP5 de ratón era capaz de revertir completamente la alta

susceptibilidad de las larvas deficientes en Gbp4 a la infección con *S.* Typhimurium, pero reducía los niveles de actividad caspasa-1 en condiciones de infección. Sin embargo, era incapaz de aumentar la resistencia de las larvas a la infección cuando se expresaba solo, a pesar de que aumentaba los niveles de actividad caspasa-1 en animales no infectados. Además, todos estos efectos requerían Asc de pez cebra, al igual que ha sido descrito también para los mamíferos (Shenoy *et al*., 2012). La sobreexpresión de GBP5 de ratón no fue capaz de rescatar la alta susceptibilidad de las larvas deficientes en Asc, mientras que los niveles reducidos de actividad caspasa-1 no aumentaron como consecuencia de la sobreexpresión de GBP5. Es interesante destacar que GBP5KS → AA de ratón no rescató la hipersusceptibilidad a *S.* Typhimurium, y tampoco los niveles de actividad caspasa-1 en larvas deficientes en Gbp4. Además, no mostró un efecto de DN, en contraste con el Gbp4KS → AA de pez cebra. En conjunto, estos resultados indican que GBP5 de mamíferos se comportó como GBP4 △ CARD de pez cebra, y sugieren que el dominio CARD de Gbp4 de pez cebra confirió su capacidad de promover la resistencia mediada por la caspasa-1 a las bacterias intracelulares. Apoyando esta hipótesis, la sobreexpresión de concentraciones crecientes de Gbp4 silvestre promovió un incremento de la actividad caspasa-1 dosis-dependiente tanto en el control como en las larvas infectadas. Sin embargo, como se esperaba, Gbp4KS → AA y Gbp4DM reducen la activación de la caspasa-1 en ambas condiciones, confirmando su funcionamiento como DN. GBP4 CARD indujo una activación dosis-dependiente de la actividad caspasa-1 en larvas no infectadas, mientras que inhibió la activación después de la infección.

Estos resultados sugieren que no sólo Gbp4 silvestre, sino también el mutante deficiente en GTPasa, fueron capaces de interactuar con ASC y luego desencadenar o bloquear el ensamblaje del inflamasoma, activación de caspasa-1 y eliminación de *S.* Typhimurium. Dicha hipótesis fue comprobada mediante la reconstitución de los complejos Gbp4-Asc en células de riñón embrionario humano (HEK293), que no tienen ninguno de estos componentes de forma natural. Gbp4 silvestre y el mutante deficiente en GTPasa fusionados con mCherry colocalizaron en agregados en presencia de Asc, mientras que estaban distribuidos difusamente en el citosol en ausencia de Asc. Además, los mutantes deficientes en el dominio CARD de Gbp4 fueron distribuidos de manera difusa en el citosol independientemente de Asc. Sorprendentemente, se observó un complejo macromolecular con un anillo exterior de Asc y el centro con Gbp4

silvestre o mutante deficiente en GTPasa. Anticuerpos anti-GFP y anti-Myc fueron utilizados para comprobar el correcto funcionamiento de los ensayos de transfección por western blot. Como esperábamos, se observaron diferentes bandas pertenecidas a todas las proteínas transfectadas en cada caso.

4.4. Los neutrófilos median la resistencia a *S*. Typhimurium dependiente de Gbp4

Dado que Gbp4 se encuentra altamente expresada en los neutrófilos, y que estas células son esenciales para la eliminación de *S.* Typhimurium en pez cebra, quisimos comprobar el impacto de Gbp4 en el desarrollo y funcionamiento de los neutrófilos. La inhibición genética de Gbp4 resultó en la reducción del número de neutrófilos a 3 días después de la fertilización, siendo rescatado ese efecto por su sobreexpresión. La expresión de ambos, Gbp4KS → AA y el doble mutante Gbp4KS→ AA / ΔCARD, tuvo el mismo efecto que el MO en el número de los neutrófilos. En ambos casos, el número total de neutrófilos fue significativamente menor comparado con el control. Sin embargo, Gbp4 silvestre y Gbp4 Δ CARD, así como GBP5 silvestre y GBP5KS → AA de ratón, no afectaron a la cantidad de neutrófilos, descartando que la resistencia dependiente de Gbp4 a la infección por *S.* Typhimurium esté relacionada con el número de neutrófilos.

El siguiente paso fue bloquear el reclutamiento de neutrófilos a los focos de infección usando un MO específico para Cxcr2, que es responsable del reclutamiento de neutrófilos dependiente de IL-8 en pez cebra. Los resultados mostraron que el bloqueo de Cxcr2 resultó en una mayor susceptibilidad a la infección y, curiosamente, desapareció completamente la resistencia a la infección inducida tanto por Asc como por Gbp4. En contraste, la sobreexpresión de Gbp4 o Asc en larvas deficientes en Cxcr2 no afectó a los niveles de actividad caspasa-1.

Por otra parte, realizamos experimentos de reclutamiento de neutrófilos a un lugar de infección con *S.* Typhimurium, usando un modelo de infección localizada en el oído (de Oliveira *et al*., 2015). El patrón de reclutamiento se vio muy afectado en larvas que expresaban Gbp4KS → AA, donde se redujo, mientras que fue potenciado en larvas que sobreexpresaban la forma silvestre de Gbp4. En particular, el análisis mediante RT-qPCR mostró que la sobreexpresión de Gbp4 silvestre resultó en una respuesta inmune más rápida y más fuerte ante *S.* Typhimurium (es decir, los niveles de transcripción de

más altos de los genes de *cxcl8* y *ptgs2a* 1 hora después de infección), lo que causó la resolución de la infección 24 horas después de infección (es decir, los niveles de los transcriptos bajos / basales de *il1b*, *tnfa*, *cxcl8*, *ptgs1* y *ptgs2a*).

Inesperadamente, sin embargo, la expresión del mutante deficiente en GTPasa de Gbp4 (Gbp4KS → AA) llevó a un descenso en los niveles de transcripción de genes que cifran moléculas proinflamatorias, probablemente relacionado en este caso con los problemas de reclutamiento de los neutrófilos al sitio de la infección. La disminución de los niveles de ARNm de *il1b* en larvas que sobreexpresaban Asc 24 horas después de infección, confirmó la resolución rápida de la inflamación.

Las larvas que sobreexpresaban Asc respondieron de una manera rápida y fuerte a la infección por *S.* Typhimurium localizada en el oído, apoyando el resultado encontrado anteriormente en larvas que sobreexpresaban Gbp4 silvestre. En conjunto, estos resultados indican un papel fundamental de Gbp4 en la homeostasis y funcionamiento de los neutrófilos en pez cebra, y apoyan el papel crucial de estas células en la eliminación de las bacterias intracelulares, como ocurre en los mamíferos (Miao *et al*., 2010a).

4.5. La resistencia mediada por Gbp4 a la infeccion con *S*. Typhimurium es independiente tanto del procesamiento de IL-1β como de la muerte celular por piroptosis

Desafortunadamente, IL-1β procesada no fue detectada por Western blot en extractos totales de larvas utilizando varios mAbs (datos no mostrados), lo que sugiere una rápida eliminación de la citoquina madura *in vivo*. Por lo tanto, se utilizó un ensayo desarrollado recientemente basado en la actividad biológica de una construcción de fusión de pro-IL-1β-Gaussian luciferasa, en la que la formación de los agregados de las proteínas fusionadas mantienen a la enzima inactiva hasta el procesamiento de la pro-IL-1β (Bartok *et al*., 2013). Como esperábamos, la actividad luciferasa nativa de Gluc no se vio afectada por la deficiencia en Asc ni por la presencia de inhibidores de las caspasas, confirmando la especificidad del ensayo en larvas de pez cebra (Thesis Doctoral de D. Agosto). Del mismo modo, los niveles de Gbp4 no afectaron a la actividad luciferasa en los peces que expresaban zfiGLuc.

A continuación, los resultados nos llevaron a analizar la muerte celular por piroptosis en respuesta a la infección con *S.* Typhimurium. Como hemos demostrado anteriormente, la infección de los macrófagos de dorada con la estirpe silvestre de *S.* Typhimurium, pero no con el mutante SPI-1, desencadena la piroptosis (Angosto *et al*., 2012). Sin embargo, no hemos encontrado un incremento de la muerte celular de los neutrófilos en un modelo de infección localizada en el oído, realizado mediante tinción con YO-PRO y captación y Anexina V (datos no mostrados).

4.6. La resistencia a la infección con *S.* Typhimurium dependiente de Gbp4 está asociada con la biosíntesis de prostaglandinas

Los resultados anteriores nos llevaron a analizar si la resistencia mediada por Gbp4 / inflamasoma /caspasa-1 a *S.* Typhimurium estaba relacionada con la producción de eicosanoides, ya que se ha demostrado en mamíferos que la activación de NLRC4 da lugar a la generación de una tormenta patológica de eicosanoides (von Moltke *et al*., 2012). La inhibición farmacológica de las enzimas clave en la biosíntesis de prostaglandinas, las endoperoxidasas-sintetasas de prostaglandinas (Ptgs), también conocidas como ciclooxigenasas (Cox), neutralizaba totalmente el incremento en la resistencia a la infección que se observaba en las larvas que sobreexpresaban Gbp4 silvestre, pero inesperadamente la resistencia de las larvas que sobreexpresaban Asc no se vio afectada. Además, la inhibición de Ptgs no afectó a la susceptibilidad de las larvas silvestres ni a la actividad caspasa-1, sugiriendo que la producción de prostaglandinas tenía lugar aguas abajo de la activación del inflamasoma dependiente de Gbp4.

Mientras que la adición exógena de PGE2, 15dPGJ2 o 12PGJ2 aumentó o no tuvo ningún efecto sobre la susceptibilidad a la infección por *S.* Typhimurium tanto en las larvas silvestres como en deficientes en Gbp4, la adición de PGD2 rescataba parcialmente la alta susceptibilidad de las larvas deficientes en Gbp4, mientras que no tuvo efecto en larvas silvestres. En conjunto, estos resultados demostraban el papel fundamental de Gbp4 en la inducción de la biosíntesis de las prostaglandinas mediada por el inflamasoma, así como en la eliminación de las bacterias intracelulares por los neutrófilos *in vivo*.

5. Discusión

En la presente tesis doctoral descubrimos y describimos un nuevo papel de las GBPs en la biosíntesis de las prostaglandinas dependiente del inflamasoma, así como en la eliminación de las bacterias intracelulares por los neutrófilos *in vivo*. El Gbp4 de pez cebra parece ser necesario para la homeostasis de los neutrófilos, y aunque esta observación merece una investigación más profunda, el papel fundamental de esta proteína en el control de la infección por *S.* Typhimurium es independiente de ese efecto, ya que la sobrexpresión de Gbp4 silvestre no afectó al número total de neutrófilos, mientras que si resultó en un incremento de la resistencia a la infección dependiente del inflamasoma / asc / caspasa-1. El papel clave de los neutrófilos en la resistencia a *S.* Typhimurium en larvas de pez cebra se apoya en el hecho de que la sobreexpresión de ambos, Gbp4 y Asc, no tiene efecto en animales deficientes en Cxcr2. Cxcr2 media el reclutamiento de neutrófilos pero no de macrófagos a las heridas estériles, a patógenos bacterianos, (incluyendo *S.* Typhimurium) y a células transformadas (de Oliveira *et al*, 2013; Deng *et al*, 2013; Freinsinger *et al*, 2013; de Oliveira *et al*, 2014; de Oliveira *et al*, 2015). La función de los neutrófilos y los macrófagos en la eliminación de las bacterias intracelulares *in vivo* es controvertida (Bumann, 2015). Estudios muy recientes han demostrado elegantemente que los neutrófilos son cruciales para la eliminación de *S.* Typhimurium por mecanismos independientes del inflamasoma, concretamente el estrés oxidativo generado por las enzimas NADPH y mieloperoxidasa (Burton *et al*, 2014; Franchi *et al*, 2012b.). Un estudio reciente ha demostrado también que los neutrófilos son capaces de sostener la producción de IL-1β durante una infección aguda con *S.* Typhimurium, gracias a su resistencia a la muerte celular por piroptosis tras la activación del inflamasoma de tipo NLRC4 (Chen *et al*., 2014). Dicha resistencia a la piroptosis, conservada evolutivamente por los neutrófilos de pez cebra tras una potente activación de la caspasa-1 por la infección por *S.* Typhimurium y/o por la sobreexpresión de Gbp4 y Asc, muestra la importancia de los neutrófilos en la eliminación de los patógenos bacterianos intracelulares en los vertebrados.

Otra observación interesante del modelo de infección de pez cebra - *S.* Typhimurium utilizado en esta tesis doctoral es la capacidad de Gbp4 para regular el reclutamiento de los neutrófilos a los focos de infección. El reclutamiento alterado de neutrófilos al sitio de infección observado en larvas con sobreexpresión de Gbp4

silvestre y mutante deficiente de actividad GTPasa, resultaron indirectamente en la fuerte/débil respuesta inflamatoria a la infección y rápida / retrasada eliminación de la bacteria, según los perfiles de expresión génica. Sin embargo, nuestros resultados no descartan una inhibición directa de la migración de neutrófilos como resultado de la polimerización de actina mediada por el inflamasoma, ya que recientemente se ha observado *ex vivo* en macrófagos de ratón infectados con *S*. Typhimurium (Man *et al*., 2014). Esto merece una investigación más profunda, y el pez cebra será un modelo único para visualizar en tiempo real el impacto de la activación del inflamasoma en la reorganización del citoesqueleto durante el reclutamiento de los neutrófilos / macrófagos y la captación y eliminación de las bacterias.

Gbp4 contiene un dominio CARD en la zona C-terminal que, en principio, permite la interacción directa con otras proteínas que también constan de un dominio CARD, omitiendo la necesidad de proteínas adaptadora Asc. Sin embargo, nuestro análisis demuestra inequívocamente que la eliminación de *S*. Typhimurium dependiente de Gbp4 y la activación de la caspasa-1 requieren Asc, y que son altamente dependientes de la flagelina bacteriana. Además, nuestros resultados *in vivo* fueron confirmados por la reconstitución de agregados Gbp4-Asc en células HEK293, donde ambos Gbp4, silvestre y mutante de GTPasa, formaron un complejo macromolecular con Asc mientras que el mutante sin el dominio CARD aparecía distribuido difusamente en el citosol independientemente de Asc, y apenas rescató la alta susceptibilidad a la infección de las larvas deficientes en Gbp4. Sorprendentemente, el complejo macromolecular Gbp4-Asc observado aquí es bastante similar a los complejos ASC - NLRC4, NLRP3, caspasa-1, caspasa-8, y pro-IL-1β, que recientemente han sido descritos en los macrófagos infectados con *S*. Typhimurium (Man *et al*., 2014).

Otra interesante, pero inesperada, observación de este estudio es la capacidad de Gbp4 deficiente en GTPasa, pero no del mismo mutante de GBP5 de ratón, de comportarse como DN, a pesar de ser capaz de localizar con los speck de Asc. Aunque el ensamblaje de ASC se evita en la tetramerizacion de los mutantes de GBP5, sugiriendo que GBP5 tetramérico causa la oligomerización de Asc, ambos, el silvestre y GBP5 mutante de GTPasa, parece que forman tetrámeros y son igualmente capaces de promover la multimerización de Asc (Shenoy *et al*., 2012). Esto es consistente con nuestro estudio *in vivo* donde la sobreexpresión del mutante de GBP5 de ratón deficiente en GTPasa no tuvo ningún efecto sobre la resistencia a las bacterias ni sobre

la actividad caspasa-1, mientras que el GBP5 silvestre fue capaz de rescatar la elevada susceptibilidad y los bajos niveles de actividad caspasa-1 de larvas deficientes en Gbp4. Estos resultados sugieren que Gbp4 interactuaría con un NLR sensor putativo, que sigue pendiente de identificar, probablemente por su dominio de GTPasa, y con Asc a través de su dominio CARD, mientras que GBP5 de ratón interactuaría exclusivamente con el PYD de NLRP3 a través de su dominio GTPasa (Shenoy *et al*., 2012). Más importante aún, la actividad GTPasa de Gbp4 es prescindible para la inducción de la oligomerización de Asc, pero crucial para la activación de la caspasa-1 posteriormente. Así, proponemos un modelo de dos etapas en el que (i) la presencia de flagelina en el citosol promueve la interacción de Gbp4 con Asc a través de los dominios CARD y (ii) la hidrólisis de GTP por Gbp4 resulta en un cambio conformacional en este complejo que causa la oligomerización y la activación de la caspasa-1.

Aunque esta aceptado que la activación del inflamasoma promueve la eliminación de los patógenos bacterianos a través del procesamiento de las citoquinas proinflamatorias IL-1β e IL-18 (Franchi *et al.*, 2012b; Cai *et al.*, 2012; Shimada *et al.*, 2011; Carbón *et al.*, 2014) y la inducción de muerte celular por piroptosis de macrófagos (Aachoui *et al*., 2013; Miao *et al*., 2010a), la contribución relativa de cada ruta ha tenido gran controversia. No hemos encontrado ninguna evidencia de la muerte celular de los neutrófilos durante la infección de las larvas con *S.* Typhimurium, apoyando un reciente estudio que muestra que los neutrófilos no se ven sometidos a muerte celular tras la activación de NLRC4 (Chen *et al*., 2014). Además, no hemos logrado detectar IL-1β madura en larvas completas, y los resultados obtenidos con el ensayo basado en la luciferasa iGLuc (Bartok *et al*., 2013) están en contra de la función del inflamasoma o de la caspasa-1 en el procesamiento de IL-1 β en pez cebra, apoyando nuestros estudios anteriores en macrófagos de peces (Angosto *et al.*, 2012) y la ausencia de un sitio conservado de procesamiento para la caspasa-1 en la IL-1β de los vertebrados inferiores (Angosto *et al*., 2013; Bird *et al*., 2002b). Sin embargo, nuestros datos apoyan firmemente que la resistencia a la infección con *S.* Typhimurium mediada por Gbp4 está asociada a la producción de prostaglandinas, estando de acuerdo con la capacidad del inflamasoma NLRC4 para generar una tormenta de eicosanoides en los macrófagos peritoneales de ratón (von Moltke *et al*., 2012).

Aunque un cóctel de prostaglandinas favorece la eliminación de bacterias, la prostaglandina PGD2 en particular parece ser importante en la resistencia a *S.*

Typhimurium, mientras que 15dPGJ2 o PGE2, tuvieron un efecto negativo en la resistencia bacteriana. Curiosamente, la relevancia de las prostaglandinas en particular, y de los eicosanoides en general, en la eliminación de las bacterias intracelulares ha recibido poca atención hasta ahora, a pesar de los estudios metabolómicos que mostraban que la infección por *S.* Typhimurium altera el metabolismo de eicosanoides en ratón (Antunes *et al*., 2011). Es importante destacar el hecho de que la inhibición farmacológica de la síntesis de las prostaglandinas sólo afectó a la resistencia a la infección de larvas que sobreexpresaban Gbp4, pero no de larvas que sobreexpresaban Asc. Aunque estos resultados merecen más estudios, especulamos que tanto Gbp4 de pez cebra como GBP5 de mamíferos probablemente son requeridos para la producción de prostaglandinas mediada por el inflamasoma.

Resumiendo, describimos aquí un modelo único para estudiar el impacto del inflamasoma en la inflamación y la progresión de las enfermedades infecciosas, claramente complementario con el modelo de ratón. Entre otras ventajas, destacan la posibilidad de estudiar las respuestas de activación de la inflamación y las respuestas frente a patógenos simultáneamente en un organismo completo, así como la posibilidad de realizar escrutinios genéticos y químicos. El uso de este modelo, anteriormente poco apreciado, nos ha llevado al descubrimiento del papel de los neutrófilos en la eliminación de la infección por *S.* Typhimurium *in vivo* dependiente de la biosíntesis de prostaglandinas, a través del inflamasoma en un en el que participan Gbp4 y Asc.

6. Conclusiones

Los resultados obtenidos en este trabajo nos han permitido extraer las siguientes conclusiones:

1. El pez cebra es un excelente modelo para estudiar la activación y la función del inflamasoma en respuesta a una infección por *S.* Typhimurium.

2. El reconocimiento intracelular de *S.* Typhimurium en pez cebra depende altamente de la producción de flagelina.

3. La activación del inflamasoma dependiente de Gbp4 mejora la resistencia de las larvas de pez cebra e incrementa la actividad caspasa-1 después de la infección con *S.* Typhimurium.

4. Tanto Gbp4 mutante que carece de la actividad GTPasa, como el doble mutante al que también le falta el dominio CARD, se comportan como dominantes negativos, incrementando la susceptibilidad a *S.* Typhimurium y, en paralelo, reduciendo la actividad caspasa-1.

5. El mutante de Gbp4 sin el dominio CARD actúa, al igual que GBP5 de mamíferos, rescatando la elevada susceptibilidad de las larvas deficientes en Gbp4, pero no aumenta la actividad caspasa-1 después de la infección con *S.* Typhimurium en pez cebra.

6. Gbp4 regula el reclutamiento de neutrófilos al lugar de infección y, además, la eliminación de *S.* Typhimurium dependiente de Gbp4 esta mediada por los neutrófilos.

7. El inflamasoma dependiente de Gbp4 activa la biosíntesis de las prostaglandinas, que a su vez fomentan la eliminación de *S.* Typhimurium.

ANNEXE I

Participation in publications during the PhD

1. Candel S, de Oliveira S, López-Muñoz A, García-Moreno D, Espín-Palazón R, **Tyrkalska SD**, Cayuela ML, Renshaw SA, Corbalán-Vélez R, Vidal-Abarca I, Tsai HI, Meseguer J, Sepulcre MP, Mulero V (2014). Tnfa Signaling Through Tnfr2 Protects Skin Against Oxidative Stress–Induced Inflammation. Plos Biology. 12 (5): e1001855.

2. Candel S, Sepulcre MP, Espín-Palazón R, **Tyrkalska SD**, de Oliveira S, Meseguer J, Mulero V (2015). Md1 and Rp105 regulate innate immunity and viral resistance in zebrafish. Dev Comp Immunol 50 (2): 155-165.

3. **Tyrkalska SD**, Candel S, Angosto D, Garcia-Moreno D, Sepulcre MP, Martín-Sánchez F, Pelegrín P, Mulero V (2015). Zebrafish guanylate binding protein 4 mediates *Salmonella* Typhimurium clearance *in vivo* through the inflammasome-dependent production of prostaglandins. (under review)

ANNEXE II

Contributions to scientific conferences during the PhD

1. **Tyrkalska SD,** Angosto D, Candel S, Sepulcre MP, García-Goreno D, López-Muñoz A, Mulero V. A zebrafish-salmonella infection model provides new insights into the role of the inflammasome. (Zebra)Fish immunology workshop, Wageningen (Holland), 21-25 April 2013. Poster.

2. **Tyrkalska SD,** Angosto D, Candel S, Sepulcre MP, García-Moreno D, López-Muñoz A, Mulero V. Identification of new inflammasome components in zebrafish. Zebrafish disease models 6 (Zdm6), Murcia (Spain), 14-17 July 2013. Poster.

3. Candel S, de Oliveira S, García-Moreno D, Espín R, **Tyrkalska SD**, Cayuela ML, Renshaw SA, Meseguer J, Sepulcre MP, Mulero V. Tnfr2 deficiency triggers skin inflammation in the zebrafish via the production of h_2o_2 and the activation of the nf-κb signaling pathway. Zebrafish disease models 6 (Zdm6), Murcia (Spain), 14-17 July 2013. Oral presentation.

4. Garcia-Moreno D, Bernabe M, Gómez-Abenaza E, **Tyrkalska SD**, Cayuela ML, Mulero V. Generation of zebrafish mutants using talen and crispr-cas technologies: a valuable tool to study hematopoiesis, inflammation and cáncer. Zebrafish disease models 6 (Zdm6), Murcia (Spain), 14-17 July 2013. Poster.

5. Angosto-Bazarra D, Garcia-Moreno D, López-Muñoz A, **Tyrkalska SD**, Sepulcre Cortéz MP, Meseguer Peñalver J, Mulero Méndez V. The zebrafish-salmonella infection model provides new insight into the function of the inflammasome. Zebrafish disease models 6 (Zdm6), Murcia (Spain), 14-17 July 2013. Poster.

6. **Tyrkalska SD**, Candel S, Angosto D, Sepulcre MP, García-Moreno D, López-Muñoz A, Mulero V. Identification of a new inflammasome components in zebrafish involved in the clearance of *Samlonella* Typhimurium. Semmelweis symposium 2013 "Molecular mechanisms and therapeutic targets in inflammatory diseases", Budapest (Hungary), 7-9 November 2013. Poster.

7. Candel S, de Oliveira S, García-Moreno D, López-Muñoz A, Espín R, **Tyrkalska SD,** Cayuela ML, Renshaw SA, Corbalán R, Vidal-Abarca I, Meseguer J, Sepulcre MP, Mulero V. Identification of new therapeutic targets

for the treatment of skin inflammatory diseases using the zebrafish. Semmelweis symposium 2013 "Molecular mechanisms and therapeutic targets in inflammatory diseases", Budapest (Hungary), 7-9 November 2013. Poster.

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10. Valera A, **Tyrkalska SD**, Mingming H, Meseguer J, Mulero V. Identification of new inflammasome components: zebrafish Wdr90 is tightly regulated by Gbp4 and inflammasome activation. 13th ISDCI International Society of Developmental and Comparative Immunology 2015, Murcia (Spain), 28 June - 03 July 2015. Poster.

11. Mingming H, **Tyrkalska SD**, Valera A, Meseguer J, Mulero V. Identification of new inflammasome components: zebrafish reticulon 3 is regulated by Gbp4 and inflammasome activation. 13th ISDCI International Society of Developmental and Comparative Immunology 2015, Murcia (Spain), 28 June - 03 July 2015. Poster.

12. Candel S, Garcia-Moreno D, **Tyrkalska SD**, Meseguer J, Mulero V. Identification and characterization of evolutionary conserved Md1 splice variants in zebrafish. 13th ISDCI International Society of Developmental and Comparative Immunology 2015, Murcia (Spain), 28 June - 03 July 2015. Poster.

ANNEXE III

Research stays in other laboratories during the PhD

2013

Host institution:

Name: Institute of Biology.
Faculty of Science.
Leiden University.

Country: Leiden. Netherlands.

Responsable person in the Host: Dr. Annemarie H. Meijer.

Stay period:

From: October 9, 2013.

To: November 29, 2013.

Duration: 8 weeks.

2014

Host institution:

Name: Academic Unit of Respiratory Medicine. Department of Infection and Immunity. The Medical School. The University of Sheffield.

Country: Sheffield. South Yorkshire. UK.

Responsable person in the Host: Dr. Stephen A. Renshaw.

Stay period:

From: October 05, 2014.

To: November 16, 2014.

Duration: 6 weeks.

ANNEXE IV

Zebrafish transgenic lines used in this work

Line	Labelled cells	Reference
Tg(mpx:eGFP)i114	Neutrophils	Renshaw *et al.*, 2006
Tg(lyz:dsred)nz50	Neutrophils	Hall *et al.*, 2007
Tg(mpeg1:eGFP)gl22	Macrophages	Ellett *et al.*, 2011

ANNEXE V

Species mentioned in this work

FISH

Danio rerio (Hamilton, 1822) Zebrafish

Oncorhynchus mykiss (Walbaum, 1792) Rainbow trout

Sparus aurata (Linnaeus, 1758) Seabream

MAMMALS

Homo sapiens sapiens, Linnaeus, 1758 Human

Mus musculus, Linnaeus, 1758 Mouse

BACTERIA

Salmonella enterica Typhimurium, Salmon 1885