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Evidence that fish death after *Vibrio vulnificus* infection is due to an acute inflammatory response triggered by a toxin of the MARTX family

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ABSTRACT

Vibrio vulnificus is an emerging zoonotic pathogen associated with fish farms that is capable of causing a hemorrhagic septicemia known as warm-water vibriosis. According to a recent transcriptomic and functional study, the death of fish due to vibriosis is more related to the inflammatory response of the host than to the tissue lesions caused by the pathogen. In this work, we hypothesize that the RtxA1 toxin (a *V. vulnificus* toxin of the MARTX (Multifunctional Autoprocessing Repeats in Toxin) family) is the key virulence factor that would directly or indirectly trigger this fatal inflammatory response. Our hypothesis was based on previous studies that showed that *rtxA1*-deficient mutants maintained their ability to colonize and invade, but were unable to kill fish. To demonstrate this hypothesis, we infected eels (model of fish vibriosis) by immersion with a mutant deficient in RtxA1 production and analyzed their transcriptome in blood, red blood cells and white blood cells during early vibriosis (0, 3 and 12 h post-infection). The transcriptomic results were compared with those obtained in the previous study in which eels were infected with the *V. vulnificus* parental strain, and were functionally validated. Overall, our results confirm that fish death after *V. vulnificus* infection is due to an acute, early and atypical inflammatory response triggered by RtxA1 in which red blood cells seem to play a central role. These results could be relevant to other vibriosis as the toxins of this family are widespread in the *Vibrio* genus.

1. Introduction

Vibrio vulnificus is a marine zoonotic pathogen capable of causing death by septicemia (vibriosis hereafter) in both humans and fish, with farmed eel and tilapia as the most susceptible hosts [1]. The species is highly variable and is divided into 5 phylogenetic lineages (L) formed by multiple clones and some clonal complexes. Recently, it has been shown that fish farms are playing an important role in the evolution of this species by favoring the emergence of new zoonotic lineages and clonal complexes [2]. Consequently, *V. vulnificus* is a pathogen of undoubted interest for both animal and public health.

Among all fish vibriosis caused by *V. vulnificus*, eel vibriosis is the best studied and characterized. This disease is caused by a clonal complex within L2 (formerly biotype 2) [3]. The pathogen infects trough water, is attracted to the mucus covering the fish gills, attaches to the epithelium where it multiplies and forms a biofilm [4,5]. The accumulated toxins and exoenzymes secreted by the pathogen cause local inflammation that allows it to invade the bloodstream, where it survives, and subsequently cause hemorrhagic septicemia that ends with the death of the eel [6]. Recently, Hernández-Cabanyero et al. demonstrated that the hemorrhagic septicemia caused by *V. vulnificus* is due to an early, acute and atypical inflammatory response against the pathogen in which both red blood cells (RBCs) and white blood cells (WBCs) are involved [7].

The pathogenicity of *V. vulnificus* for eels depends on multiple virulence factors that are expressed in a coordinated manner in response to environmental stimuli [8]. Of these, the MARTX toxin (Multifunctional Autoprocessing Repeats in Toxin; also called RtxA1 toxin) is the most important, as deletion of this gene results in loss of virulence for eels as well as in a significant reduction of virulence for mice (the animal model to test virulence for humans) [9]. Interestingly, this toxin is also involved in triggering an early cytokine storm in mice [10].

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MARTX toxins have been implicated in the death of different eukaryotic cell types (including blood cells) after bacteria-cell contact [9,11]. These toxins are modular proteins of very high molecular weight that have two external modules of constant amino acid sequence and an internal module of variable sequence formed by different domains with specific cytotoxic activity [12]. The toxin is released upon contact with the eukaryotic cell and the external modules bind to the cell membrane to form a pore through which the internal module is translocated into the host cell. The domain that has proteolytic activity (cysteine protease domain; CPD) is then activated after binding the host cell inositol hexakisphosphate (InsP6) and releases each functional domain into the cell, whose activities result in cell death [13]. The specific mechanism of cell destruction depends on the precise combination of functional domains presented by the toxin [12]. Particularly, the toxin involved in virulence for eels contains an actin cross-linking domain (ACD) [10], two alpha/beta hydrolase domains (ABH) that inhibit autophagy and endosomal trafficking [14], and a "makes caterpillars floppy-like" domain (MCF) that induces depolarization of the mitochondrial membrane potential causing activation of cell death [14]. Remarkably, this *rtxA1* gene is duplicated in the chromosome II and the fish virulence plasmid pFv (formerly pVvBt2) in all eel virulent strains of *V. vulnificus* [2,9,15].

In view of the above, we hypothesized that RtxA1 produced after bacterial contact with blood cells would activate the lethal pro-inflammatory response typical of vibriosis. To test this hypothesis, we infected eels with a previously obtained double mutant in *rtxA1* (Δ*rtxA1*; deleted both the plasmid and the chromosomal genes) [9] derived from the strain used by Hernández-Cabanyero et al. (CECT 4999, hereafter R99) [7]. Then, we analyzed the eel transcriptome from whole blood (B), RBCs and WBCs samples following the experimental design of Hernández-Cabanyero et al. [7]. The transcriptomic results were then compared with those previously published for eels infected with the parental strain and finally were validated in functional assays. The results obtained in this work confirm the hypothesis that RtxA1 triggers a dysregulated toxic shock that induces fish death in response to a very rapid and strong cytokine storm (mainly *il1β*, *il17a*, *il20* and *il12β*) and suggest that the interaction between the toxin and RBCs is crucial for the development of this toxic shock. Finally, we obtained evidence for an RNA-based RtxA1-dependent response that opens the door to future studies on the molecular mechanisms regulating fish immune responses.

2. Materials and methods

2.1. Ethic statement

All assays involving animals for scientific use were approved by the Institutional Animal Care and Use Committee and the local authority (Conselleria de Agricultura, Medio Ambiente, Cambio Climático y Desarrollo Rural. Generalitat Valenciana) under the protocol 2016-USC-PEA-00033 type 2. The experiments were carried out in the Aquarium Experimental Plant of the Central Service for Experimental Research of the University of Valencia in Spain (SCSIE-UV) following European Directive 2010/63/EU and the Spanish law 'Real Decreto' 53/2013.

2.2. Bacterial strains, growth media and conditions

The *V. vulnificus* strain R99 (toxin-producing or parental strain) and its derivative double mutant in RtxA1 (Δ*rtxA1*), defective in the two copies of *rtxA1* gene [9] (non-toxin-producing mutant) were used in this study. The parental strain was isolated from kidney of a moribund eel and was demonstrated to belong to the clonal complex of L2 [3]. Both strains were routinely grown on Tryptone Soy Agar with 1 % NaCl (TSA-1) plates or in Luria-Bertani with 1 % NaCl (LB-1) tubes with gentle agitation (100 rpm), at 28 °C for 18 h. Bacterial concentration was checked by drop-plate counting in TSA-1 plates [16]. The bacterial strains were stored in LB-1 supplemented with 20 % glycerol at −80 °C.

2.3. Animal maintenance and infection

Healthy 120 g adult European eels were purchased from a local eel farm that does not vaccinate against *V. vulnificus*. Animal maintenance and experiments were carried out at the Aquarium Experimental Plant of SCSIE-UV in 160 or 60 L tanks, respectively, containing natural seawater (NSW) UV-sterilized and with salinity adjusted to 1 % at 28 °C with aeration and a filtration system.

The eels were divided into two groups, test (two groups, 24 eels each) and control (one group of 12 eels), which were immersed for 1 h in an infectious bath containing 2×10^6 CFU/ml bacteria (parental or mutant) or NSW [4]. After infection, eels were transferred to new tanks containing clean NSW, and skin mucus and blood from four live eels from each group were sampled at 0, 3 and 12 h' post-infection (hpi) [7]. An additional control group of 12 eels was infected with Δ*rtxA1* and maintained for one week to calculate mortality percentage. Blood samples were used for bacterial counting on TSA-1 by drop plate and for transcriptomic analysis while skin mucus was only used for bacterial counting. The same number of eels and infection and sampling protocol was used to confirm transcriptomic evidences by functional analysis.

2.4. Blood collection and sample preparation

Eels were anaesthetized with MS222 (50 mg/L) and 2 ml of blood was extracted with heparinized syringes from the caudal vein. 1 ml of the collected blood (used as whole blood sample [B]) was stored on ice until use and the extra 1 ml was processed to separate RBCs and WBCs according to Lee et al. and Callol et al., [9,17]. Briefly, sampled blood was centrifuged at 800×*g* for 5 min and the pellet containing cells was washed with 1 ml of sterile PBS (Phosphate Buffered Saline, pH 7) and centrifuged again in the same conditions. Then the cells were recovered and resuspended in the same volume of PBS. The suspension was added to 2 ml of Ficoll®-Paque Premium (Sigma-Aldrich) and a density gradient separation was carried out by centrifugation at 720×*g* for 30 min. RBCs and WBCs layers were collected and washed in PBS. The cells for each fraction were observed and counted under the light microscope using a Neubauer chamber. Finally, the collected samples (B, RBCs and WBCs) were treated with 1 ml of NucleoZOL (Macherey-Nagel) for RNA preservation and stored at −80 °C until use.

2.5. RNA extraction

Total RNA from B, RBCs and WBCs samples was extracted using NucleoZOL (Macherey-Nagel) according to manufacturer's instructions. RNA samples were treated with TURBO™ DNase (Ambion) and purified with RNA Cleanup and Concentration Micro Kit RNA (Thermo Scientific) following manufacturer's specifications. RNA integrity and quality were verified with a 2100 Bioanalyzer (Agilent) and only high-quality samples (RNA Integrity Number (RIN) $\geq$ 7.5) were selected and used for transcriptome analysis.

2.6. Microarray hybridization and data analysis

Eel transcriptome in response to *V. vulnificus* Δ*rtxA1* was analyzed according to Hernández-Cabanyero et al., [7]. Briefly, 200 ng of total RNA were used to obtain labeled cDNA using Cy3 Dye (Agilent). The resultant cDNA was hybridized into the eel-specific microarray platform (ID 042990, Agilent) that is enriched in genes from the immune system [18] (accession number GPL16775). The microarray contains 42,403 probes (3 per target) corresponding to each one of the ORFs identified in the eel immune-transcriptome [18]. Then, the oligonucleotide microarrays slides were scanned with Agilent Technologies Scanner, model G2505B. Raw data were obtained with Agilent's Feature Extraction software (Agilent Technologies) and quality reports were checked for

Table 1
Microarray validation. Comparison of fold change (FC) values obtained by microarray and RT-qPCR. In case of RT-qPCR, results were obtained using actin (*act*) as the reference gene and the FC ($2^{-\Delta\Delta Ct}$) for each gene was calculated.

Gene name	Gene acronym	Sample	FC[a]	
			Array	RT-qPCR
Interleukin 10 receptor subunit beta	*il10*	B 3 hpi vs 0 hpi	−1.79 (=)	−1.61 (=)
Interleukin 1 beta	*il1β*	B 12 hpi vs 0 hpi	3.47 (+)	2.28 (+)
Interleukin 10 receptor subunit beta	*il10*	RBCs 12 hpi vs 0 hpi	−1.81 (=)	−1.01 (=)
Interleukin 18	*il18*	WBCs 3 hpi vs 0 hpi	3.2 (+)	2.49 (+)
Interleukin 10 receptor subunit beta	*il10*	WBCs 12 hpi vs 0 hpi	−1.81 (=)	−1.21 (=)

[a] Qualitative classification: = , $-2<X<2$; +, $2 \leq X < 10$; ++, $10 \leq X < 25$.

each array. The extracted data were analyzed with Genespring 14.5 GX software (Agilent technologies) as previously specified [7]. Principal component analysis (PCA) was used as a quality control, to detect any outlier sample, and to describe differences between groups. All samples were analyzed at gene-level using a relative analysis, comparing each sample against a reference sample (0 hpi of each cell type). Statistical analysis available in Genespring software was run and one-way ANOVA ($P < 0.05$) followed by Tukey's pairwise comparisons were performed to describe transcriptomic profile differences along the time for each cell-type in response to the mutant strain. Finally, the obtained transcriptomic data were compared to those obtained after eel's infection with the parental strain [7]. Venn diagrams were created using the free software available at Bioinformatics and Evolutionary Genomics group at Ghent University (http://bioinformatics.psb.ugent.be/webtools/Venn/). Fig. S1 summarizes the experimental design and all the comparisons performed. Results are presented as fold change (FC) mean from three independent biological samples. Transcriptomic data are available at Gene Expression Omnibus (GEO) database with accession number GSE196944.

2.7. RT-qPCR

This analysis was used to validate the microarray results. Table 1 lists the genes, the conditions in which the samples were taken and the control used in each case to calculate the fold induction. Table S1 lists the primers used. RNA samples obtained as specified in the previous sections were submitted to reverse transcription using Maxima H Minus Reverse Transcriptase (Thermo Scientific). Then, qPCR was performed on cDNA using Power SYBR® green PCR Mastermix on a StepOnePlus™ Real-Time PCR System. The CT values were determined with StepOne Software v2.0 to establish the relative RNA levels of the tested genes, referenced to eel actin (*act*) [19]. The fold induction ($2^{-\Delta\Delta Ct}$) for each gene was calculated according to Livak and Schmittgen [20].

2.8. Enzymatic activities in serum

Proteolytic, hemolytic and bacteriolytic activity of serum samples from infected and control animals were evaluated as previously specified by Hernández-Cabanyero et al., [7]. Briefly, serum samples were serially diluted in PBS (1:2 to 1:64) and 5 μl of each dilution were plated on 1 % agarose plates supplemented with either 5 % casein (proteolytic activity) or 1 % bovine erythrocytes (Sigma) (hemolytic activity), or on LB-1 plates containing a continuous layer of the parental strain (bacteriolytic activity). As controls we plated 5 μl of PBS (negative control for all the assays), proteinase K (2.5 mg/ml in PBS, positive control for proteolysis), molecular water (positive control for hemolysis) or lysozyme (1 mg/ml, positive control for bacteriolysis). Plates were

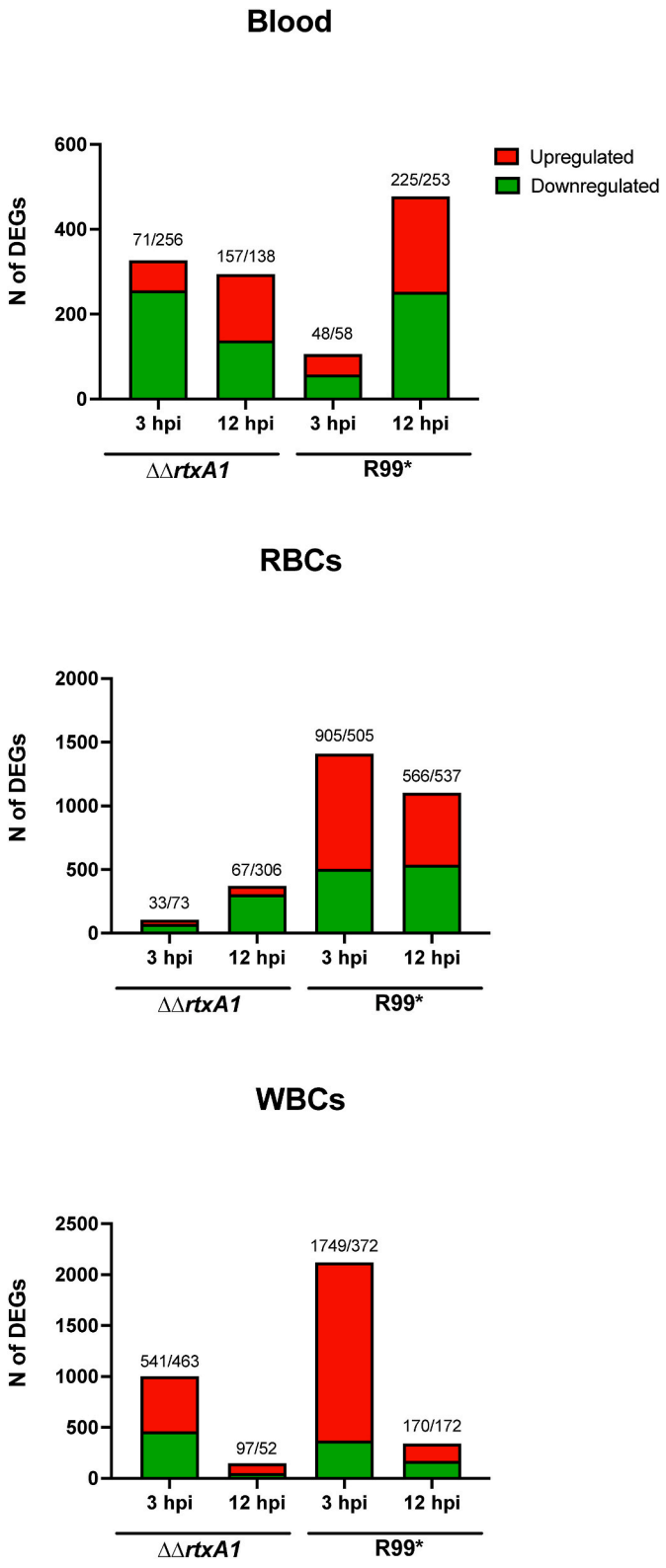

Fig. 1. Magnitude of the eel blood eel immune response against toxin-producing (R99) and non-toxin-producing (Δ*rtxA1*) *V. vulnificus*. Bars represent the total number of differentially expressed genes (DEGs; upregulated [red] or downregulated [green]) per sample (blood, RBCs [red blood cells] and WBCs [white blood cells]) and sampling time relative to time 0. The numbers of up-/downregulated DEGs are indicated at the top of each bar. * Data for infection with the parental toxin-producing strain (R99) were taken from Hernández-Cabanyero et al., [7].

Table 2
Transcriptome of eel blood after infection with non-toxin-producing *V. vulnificus* ΔΔ*rtxA1*. Selection of differentially expressed genes (DEGs) in blood by eels infected with the non-toxin-producing *V. vulnificus* strain (ΔΔ*rtxA1*). DEGs are grouped according to their putative biological function. Fold change (FC) values (rage of values for genes with duplications in the genome) correspond to the comparison between the time indicated at the top of each column (3 hpi; 12 hpi) and basal time (0 hpi). The FC value represents the mean obtained from 3 independent biological samples. Detailed FC values and gene accession number are specified in Table S2.

DEG	FC[a]					
	B[b]		RBCs[c]		WBCs[d]	
	3 hpi	12 hpi	3 hpi	12 hpi	3 hpi	12 hpi
Pathogen detection and antigen presentation systems						
PRR						
tlr20a	–	–	–	–	4.7	–
tlr3	–	–	–	–	4.2	–
tlr13	–	–	–	–	−4.1	–
Antigen presentation						
bl3-7 (MhcI)	55.6	–	–	–	–	–
mhcI	–	–	–	–	2.5	–
AP-1 complex	–	–	–		(2.1, 2)	–
Pathogen control and destruction						
Pathogen growth inhibition						
Mx protein	3.9	14	–	–	(7.9, 6)	–
Hemoglobin subunit	–	–	–	–	(11.7, 2.1)	–
Transferrin receptor (*tfr1*)	–	–	–	–	6.4	–
Complement system						
B/C2	–	10.6	–	–	–	–
Bf-2	–	(10.5, 7.6)	–	–	–	–
C5a receptor	–	6.8	–	–	–	–
C4BPB	–	–	−2.2	–	–	–
C5-2	–	–	–	3.3	–	–
C1q, A subunit	–	–	–	–	4.6	–
C3–H2	–	–	–	–	3.9	–
C7-1	–	–	–	–	3.4	–
C1q, B chain	–	–	–	–	3.1	–
Antibacterial effectors						
Nephrosin (*npsn*)	–	(61, 11)	–	–	–	(-32, 39)
Lbp/Bpi	3.6	(27, 10.8)	–	–	(-7, −8.4)	–
Nitric oxide synthase	−2.5	–	–	–	–	–
Nitric oxide synthase 1 adaptor protein (*nos1ap*)	–	–	–	–	5.5	–
Lectins						
C-type lectin receptor	−2.2	−3	–	–	3.7	–
gal1	–	–	–	–	14	–
gal2	–	–	–	–	8.1	–
gal4	–	–	–	–	2.9	–
Cell death by autophagy						
TNF receptor 14	–	–	–	–	7.8	–
p53	–	–	–	–	4.9	–
p53 apoptosis effector related to PMP-22	–	–	–	–	3.1	–
traf7	–	–	–	–	−2.3	–
Inflammatory response						
Signal transducers and transcriptional factors						
src-family tyrosine kinase SCK	–	10.6	–	–	–	–
traf3	–	3.2	–	–	–	–
c-jun	–	−2.9	–	−3.2	–	–
socs1	–	–	2.5	–	–	–
klf13	–	–	–	−3.4	–	–
map2k6	–	–	–	–	6.7	–
c-fos	–	–	–	–	(6.1, 3.5)	–
p38	–	–	–	–	4.4	–
Kdel receptor 3	–	–	–	–	3.8	–
mapk7	–	–	–	–	−2	–
NF-Kβ inhibitor alpha	–	–	–	–	(-3, 3.2)	–
mapk2	–	–	–	–	–	2.8
erk1	–	–	–	–	–	−2.4
Inflammatory cytokines and related proteins						
IL-1β receptor type 1 soluble	–	6.5	–	–	–	–
Granulin	–	6.2	–	–	–	–
Allograft inflammatory factor-1	–	4.4	–	–	–	–
IL-8 precursor	–	3.8	–	–	–	–
IL-1 receptor type 1	–	3.6	–	–	−2.7	–
il1β	–	3.5	–	–	–	–
Progranulin type I	–	3.3	–	–	–	–
irf3	–	2.3	–	–	7.3	–
irf2A	–	–	–	−2.2	–	–
irf2B	–	–	–	−2.3	–	–
irf7	–	–	–	–	7.5	–
il18A	–	–	–	–	3.2	–
il12β	–	–	–	–	2.6	–
infa	–	–	–	–	2.4	–
il16	–	–	–	–	−2.2	–
Chemokines and receptors						
CC CK3	−2.3	–	–	–	–	–
CK 13	–	–	–	–	4.2	–
CK 19 precursor	–	–	–	–	3	–
C-X-C motif receptor 3	–	–	–	–	−2.5	–
Septicemia markers						
Cyclooxygenase 2 (*cox2*)	–	14.2	–	–	–	–
mmp9 or *gelatinase B*	–	(8.7, 7.4)	–	–	–	–
Leukotriene	–	3.8	–	–	−3	2
Hyaluridase 2 (*hyal2*)	–	4.8	–	–	–	–
Coagulation factors						
Angiotensinogen	−2.2	–	–	–	–	–
Antithrombin protein	−2.1	–	–	–	2.6	–
Coagulation factor VIII precursor	–	5.6	–	–	−7.3	–
Plasminogen	–	–	–	–	−2.3	–
Coagulation factor VIII	–	–	–	–	−9.6	–
Angiogenesis and hematopoiesis						
cldn18	–	–	–	–	5	–
cldn1	–	–	–	–	4.2	–
angpt1	–	–	–	–	4	–
RNA-based *response*						
microRNA 142a (*miR-142a)*	–	–	–	–	2	–
Systemic RNA deficient-1 (*sidt1*)	–	–	–	–	−4.6	–

–: not detected as differentially expressed.
[a] FC: Fold change is represented as a rage of values (X, X) for genes with duplications in the genome.
[b] B: blood.
[c] RBCs: red blood cells.
[d] WBCs: white blood cells.

incubated at 28 °C for 24 h. The maximal dilution of eel serum with positive activity (transparent halo on agarose-casein or agarose-erythrocytes, or inhibition halo of bacterial growth) was determined and considered the titter of proteolytic, hemolytic or bacteriolytic activity, respectively [21].

2.9. Cytokines in serum

The levels of production of selected cytokines (Tnfα and Il1β) were analyzed at protein level by indirect ELISA. Serum samples were obtained from infected and control animals as specified in the previous sections. To avoid interferences in the binding-assay, albumin was depleted from 100 μl of serum samples according to Chen et al., [22]. Protein concentration in the samples was measured and adjusted using Pierce BCA Protein Assay Kit (Thermo Scientific). 96-well polystyrene plates (Thermo Scientific) were coated with 50 μl of serum (containing 5 μg of protein) per well by incubating overnight at 4 °C. Before use, the coated plates were incubated for 45 min at 37 °C with 1 % BSA in PBS. After washing, the plates were incubated for 2 h at room temperature (RT) with 50 μl/well of anti-Tnfα or anti-Il1β, serially diluted in PBS. Plates were washed 3 times with PBS-0.1 % Tween-20 and then incubated with 100 μl/well of peroxidase-labeled goat anti-mouse IgG Ab (Sigma) diluted 1:2000 in PBS for 1 h at room temperature (RT). Finally, plates were washed 3 times as indicated above and incubated with 50 μl/well of the 1-Step ultra TMB-ELISA (Thermo Fisher) as a substrate for the peroxidase reaction for 15 min at RT. Absorbance was measured at 450 nm. The maximal dilution of anti-Tnfα or anti-Il1β with positive detection was determined and considered the titter of Tnfα or Il1β, respectively. The primary antibodies used were obtained against recombinant Tnfα or Il1β from teleost fish, and have been previously validated in fish samples [23–25].

2.10. Statistical analysis

The results presented in this work correspond to the mean of three technical replicates performed in three independent experiments. Statistical analysis was performed using GraphPad Prism 7. Data were analyzed by ANOVA analysis followed by the post-hoc multiple comparison by Bonferroni's method to determine differences between groups ($p < 0.05$).

3. Results and discussion

The hypothesis of this work was that the RtxA1 toxin of *V. vulnificus* is directly or indirectly involved in the activation of a rapid and acute inflammatory response that is the main cause of eel death by hemorrhagic septicemia. To demonstrate this hypothesis, we compared the transcriptomic response of eels infected with a non-toxin-producing mutant with that previously determined for eels infected with the parental toxin-producing strain [7]. We chose blood as the target tissue, because vibriosis is a septicemic disease, and as sampling times, 0 (control), 3 (time at which the toxin is expressed during eel vibriosis and at which the cytokine storm is activated in mice), and 12 (time at which eels start to die) hpi [9,10,26].

3.1. Eel immune response

3.1.1. Immune response against the non-toxin-producing mutant. First, we analyzed the immune response against the non-toxin-producing mutant (Δ*rtxA1*) from the transcriptomic results. Fig. 1 shows the number of differentially expressed genes (DEGs) per sample and sampling time. The amount of DEGs was relatively low regardless of sampling time and cell type analyzed. WBCs showed a greater response than RBCs, especially at 3 hpi, at which time they differentially transcribed about 1000 genes, half of which were upregulated, while RBCs differentially transcribed only 73 genes, most of which were downregulated. Interestingly, the response of WBCs decreased significantly at 12 hpi, at which time only about 150 genes were differentially transcribed, 67 of which upregulated, while RBCs differentially transcribed around 370 with most of them being downregulated. Finally, the number of DEGs detected in B samples were around 350 regardless sampling time being most of them downregulated at 3 hpi and upregulated at 12 hpi (Fig. 1).

Table 1 provides a comparison between FC values obtained by hybridization with the eel-specific microarray and by RT-qPCR. Table S2 shows the DEGs by RBCs and WBCs together with those detected in B samples and Table 2 summarized a selection of them, grouped by putative function. We highlight the following genes and processes involved in this response:

Pathogen detection and antigen presentation systems: after exposure of eel to the mutant strain, RBCs did not differentially transcribe any microbial pattern recognition receptor or major histocompatibility complex (*mhc*) genes. In contrast, genes for Toll-like receptors (Tlrs) and Mhc were differentially upregulated (*tlr20a*, *tlr3* and *mhcI*) and downregulated (*tlr13*) by WBCs all at 3 hpi. In addition, we detected a strongly upregulated *mhcI* gene variant in B samples at 3 hpi (Table 2 and Table S2).

Pathogen growth inhibition, targeting and destruction: we found some DEGs related to pathogen growth inhibition, such as the transferrin receptor 1 (*tfr1*) gene, as well as to pathogen targeting and destruction, such as genes for complement-related proteins. Among them, genes encoding for C3–H2, C5-2, C7-1 and C1qA and B were upregulated by WBCs at 3 hpi, whereas C5-2 was up-regulated by RBCs at 12 hpi and genes for B/C2, Bf-2 and C5a receptor factors were upregulated in B samples at 12 hpi. In addition, genes for three lectins (*gal1*, *gal2* and *gal4*) were upregulated by WBCs at 3 hpi, the gene for Lbp/Bpi protein was upregulated in B samples at 3 hpi, and especially at 12 hpi, and the gene for the Mx protein was upregulated by WBCs at 3 hpi and as well as in B samples at 3 and 12 hpi (Table 2; Supplementary Table S2). Finally, the gene encoding nephrosin (*npsn*), an antibacterial protein in fish [27], was one of the most strongly up- and downregulated in B and WBCs samples, respectively, at 12 hpi (Table 2; Supplementary Table S2).

Cell death: very few genes related to cell death or autophagy were differentially expressed in the eel blood in response to the mutant with the exception of WBCs that upregulated apoptosis-related genes at 3 hpi (*p53*) (Table 2; Supplementary Table S2).

Inflammatory response: we found evidence of an early regulation of different pathways that would lead to a mild pro-inflammatory response. At the signal transduction level, only some genes for Mapk kinases (*map2K6* and *mapK2*) where upregulated by WBCs (3 and 12 hpi, respectively); whereas *mapk7* was downregulated by WBCs at 3 hpi (Table 2; Supplementary Table S2). This slightly activation of Mapk signaling pathways would explain why we did not find a strong activation of genes encoding pro-inflammatory cytokines. In fact, the observed inflammatory response was limited to an early (3 hpi) but low (FC around 2) response by WBCs consisting of the upregulation of two interleukins (*il18A* and *il12β*) and one interferon (*ifna*), followed by a discrete upregulation of genes for Il1β, two receptors for Il1β, Il8 precursor, progranulin and granulin in B samples at 12 hpi (Table 2; Supplementary Table S2). These results suggest that the eels would express a low-grade regulated inflammatory response against a non-toxin-producing *V. vulnificus* strain.

Sepsis markers: we also found three genes considered as markers of the acute phase of disease (i.e., genes encoding coagulation factor VIII, leukotriene, and cyclooxygenase (*cox2*)), and genes related to endothelial damage (i.e., metalloproteinase 9 (*mmp9*)) upregulated at 12 hpi in B samples (Table 2; Supplementary Table S2).

RNA-based response: one of the most striking results of the present study was the upregulation of the microRNA-142a (miR-142a), which was detected in WBCs at 3 hpi (Table 2). microRNAs (miRNAs) regulate target gene expression either by translational repression or degradation of target mRNAs [28]. They are involved in the regulation of various

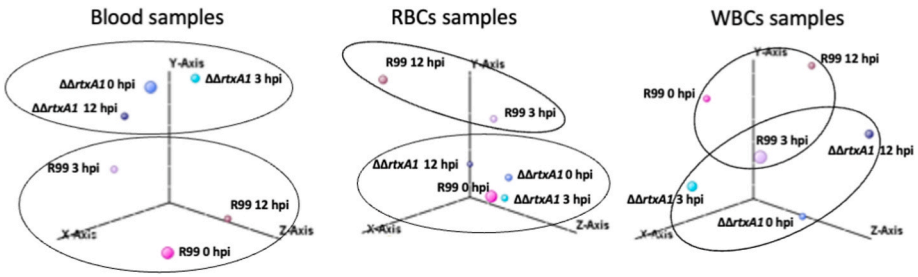

Fig. 2. 3-D plot of principal component analysis (PCA) on transcriptomic data obtained from eels infected with toxin-producing (R99) and non-toxin-producing (ΔΔ*rtxA1*) *V. vulnificus*. Three principal components are depicted, PC1 on the X-axis, PC2 on the Y-axis and PC3 on the Z-axis. PC values for each type of sample were: blood (PC1 = 40.97 %, PC2 = 20.99 % and PC3 = 13.61 %); red blood cells (RBCs) (PC1 = 36.67 %, PC2 = 29.14 % and PC3 = 10.92 %); and white blood cells (WBCs) (PC1 = 45.11 %, PC2 = 21.69 % and PC3 = 12.37 %). The different groups found are encircled by a line.

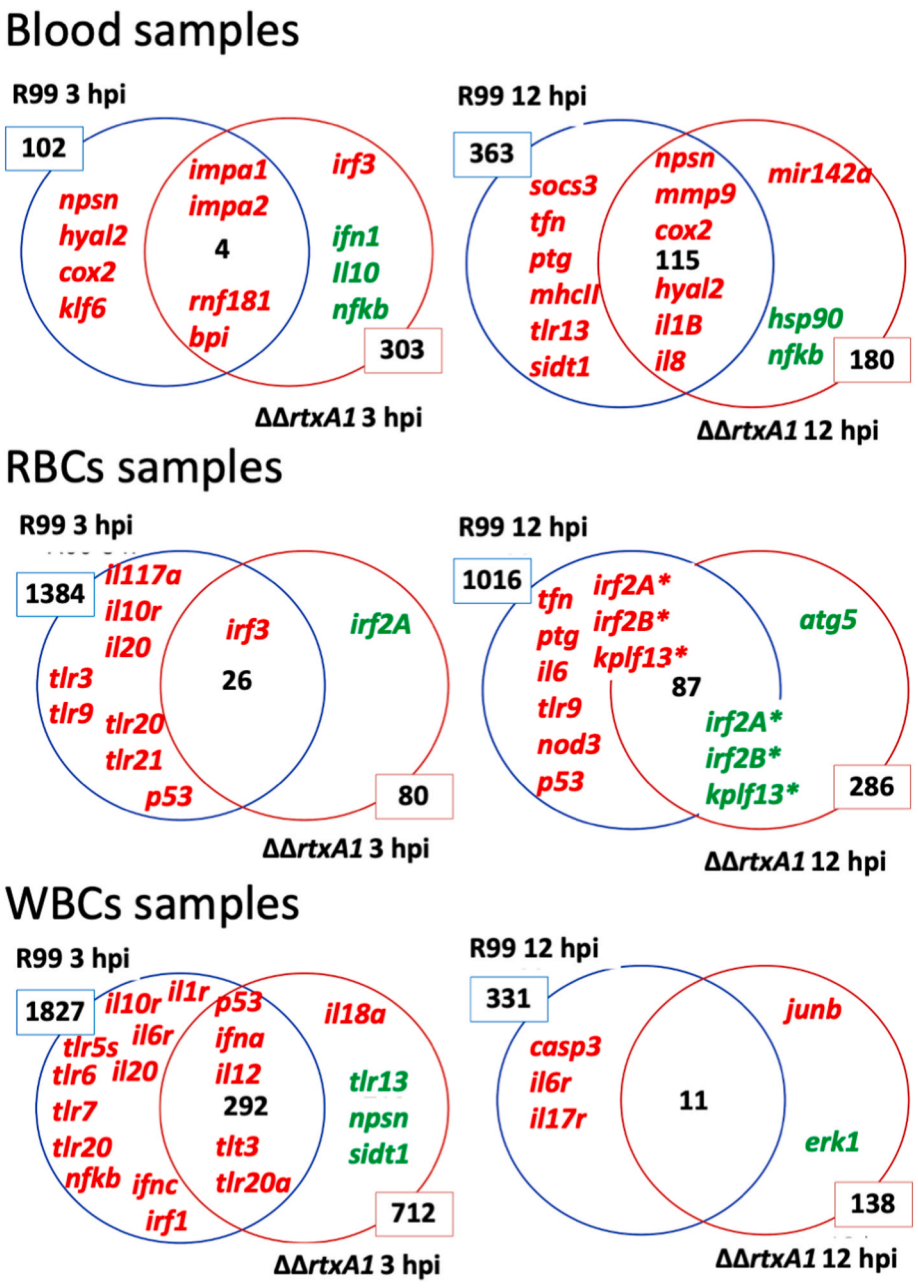

Fig. 3. Venn diagrams showing the overlap of differentially expressed genes (DEGs) by eels infected with toxin-producing (R99) and non-toxin-producing (ΔΔ*rtxA1*) *V. vulnificus*. Only a selection of genes is shown for their relevance to inflammatory response. * represents genes shared between conditions but showing opposite regulation. Samples tested were: whole blood (B), red blood cells (RBCs) and white blood cells (WBCs) at 3 and 12 hpi.

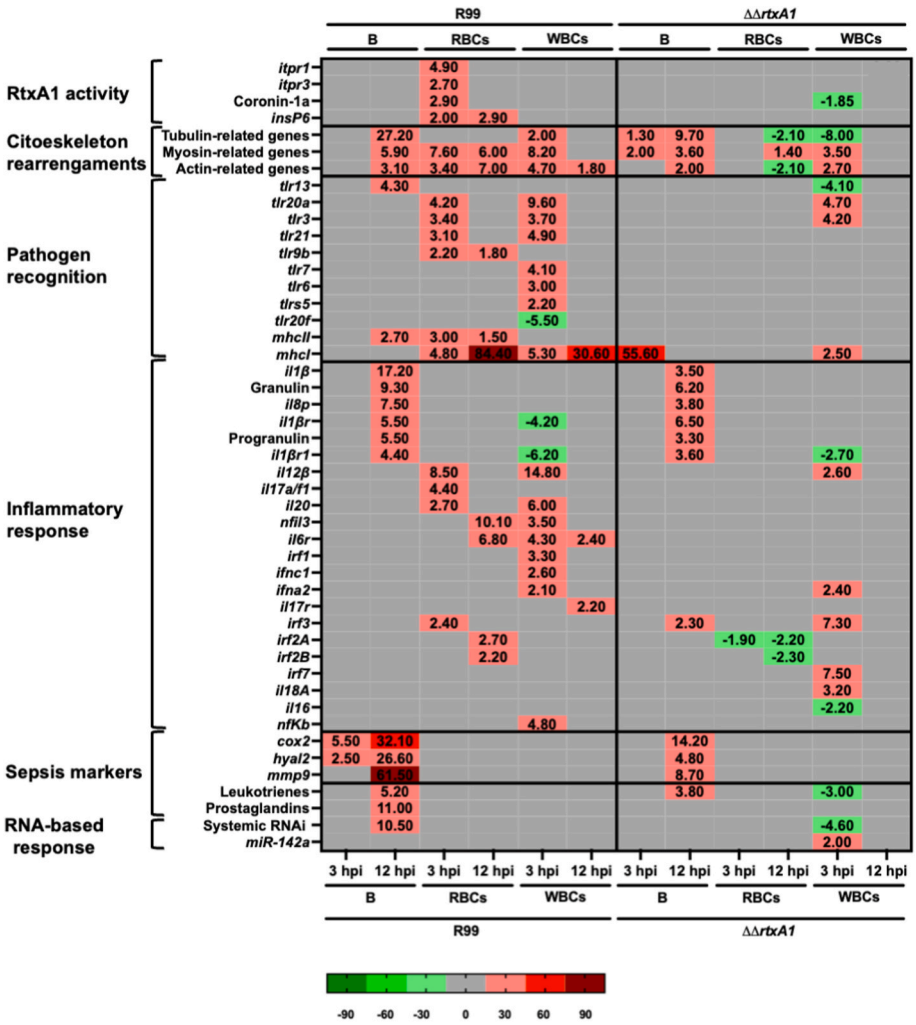

Fig. 4. Heat map showing a selection of RtxA1 toxin-associated immune response marker genes. Samples tested were: whole blood (B), red blood cells (RBCs) and white blood cells (WBCs) at 3 and 12 hpi.

Table 3
TNFα and Il1β detection in eel serum after infection with the toxin-producing strain (R99) and the non-toxin-producing mutant (ΔΔ*rtxA1*). Eels were infected by immersion and the levels of the pro-inflammatory cytokines TNFα and I1β were determined in serum from non-infected eels (control), and eels infected at different hours post infection (hpi) either with the R99 strain or with the Δ*rtxA1* mutant. Results are presented as titter values (maximal dilution of antibody with a positive result in 3 independent biological samples).

Serum sample	TNFα	Il1β
Non-infected	1: 1500	1: 1500
R99 0 hpi	1: 1000	1: 1500
R99 3 hpi	1: 1500	1: 1500
R99 12 hpi	1: 2500	1: 3500
ΔΔ*rtxA1* 0 hpi	1: 1500	1: 1500
ΔΔ*rtxA1* 3 hpi	1: 1500	1: 1500
ΔΔ*rtxA1* 12 hpi	1: 2000	1: 2500

immune-related pathways of both the innate and adaptive immune systems [29]. Concretely, miR-142 has been found to regulate hematopoiesis, inflammation and T cell differentiation in humans and mice [30,31]. Moreover, miR-142 isoforms could target transcripts which are involved in cytokine signaling, such as *socs1* [31], which was upregulated by RBCs in response to the infection with the non-toxin-producing mutant strain (Table 2). Socs1 acts as a negative regulator of cytokine signaling (i.e., inhibition of *stat* and *inf*) [32,33]. Similar results have been reported in grass carp upon *Aeromonas hydrophila* infection in a study where the authors also suggest that miR-142a could be involved in avoidance of bacterial injury in fish [34]. Of special interest was that a gene for a specific transporter of a systemic interference RNA, systemic RNAi deficient-1 (*sid1*) [35], was downregulated at 3 hpi by WBCs in response to the mutant strain (Table 2). RNAi are common to all vertebrates and are involved in ancestral innate defense mechanisms against viral infections [35]. Systemic RNAi are part of the conserved biological response mechanisms to double-stranded RNA and is involved in resistance to endogenous and exogenous pathogenic nucleic acids [36]. Its function in fish innate immunity is completely unknown. Taking all the mentioned results into account, we hypothesized that *V. vulnificus* could activate a response against endogenous RNA that in turn would trigger the cytokine storm.

3.1.1. Comparison of the immune response against the toxin producing and non-producing strains

We unravel the role of the toxin in the activation of a lethal inflammatory response by comparing the transcriptome in blood from eels infected with the toxin-producing strain (data obtained in a previous work [7]) with that of eels infected with the non-toxin-producing mutant (data obtained in the present work).

First, we performed a principal component analysis (PCA) considering the variables strain and sampling time. The analysis explained about 70 % of the total variance and divided the samples into two

Table 4
Proteolytic, hemolytic and bacteriolytic activity of eel serum before (control) and after infection with the toxin-producing strain (R99) and the non-toxin-producing mutant (Δ*Δrtx*A1). Eels were infected by immersion and the lytic activities were determined in serum from non-infected eels (control), and eels infected at different hours post infection (hpi). Results are presented as the titter (inverse of the maximal dilution of serum with a positive result in 3 independent biological samples) of the corresponding activity minus the titter of control sera from non-infected eels.

Serum sample	Proteolytic activity[a]		Hemolytic activity[b]		Bacteriolytic activity[c]	
	Δ*Δrtx*A1	R99	Δ*Δrtx*A1	R99	Δ*Δrtx*A1	R99
0 hpi	0	0	0	6	2	2
3 hpi	0	6	2	6	2	2
12 hpi	0	2	0	2	0	6

[a] Proteolytic activity: evaluated by plating 5 μl of the serum samples and dilutions (serial dilution 1:2 to 1:64 on PBS) on 1 % agarose plates supplemented with 5 % casein. The maximal serum dilution that produced a transparent halo was considered as the titter of this activity.

[b] Hemolytic activity: evaluated by plating 5 μl of the serum samples and dilutions (serial dilution 1:2 to 1:64 on PBS) on 1 % agarose plates supplemented with 1 % erythrocytes (bovine erythrocytes from Sigma). The maximal serum dilution that produced a transparent halo was considered as the titter of this activity.

[c] Bacteriolytic activity: evaluated by plating 5 μl of the serum samples and dilutions (serial dilution 1:2 to 1:64 on PBS) on LB-1 plates inoculated with a *V. vulnificus* lawn. The maximal serum dilution that inhibited bacterial growth was considered as the titter of this activity.

separated groups, one corresponding to eels infected with the toxin-producing strain and the other to those infected with the mutant, with only two exceptions (response against the parental strain by RBCs at 0 hpi and WBCs at 3 hpi) (Fig. 2). The PCA analysis would therefore support the hypothesis that the toxin would trigger a specific immune response. Venn diagrams showing the common and specific DEGs in blood, RBCs and WBCs support the strain-specific response, as most transcripts that change their transcription level in animals infected with the toxin-producing strain were not shared with those differentially expressed in animals infected with non-toxin-producing mutant (Fig. 3).

The number of DEGs in response to the infection with the toxin-producing strain was significantly higher than that obtained in response to the non-toxin-producing mutant, irrespective of sampling time and cell type analyzed (Fig. 1). This effect was particularly striking in the case of RBCs that were virtually unreactive against the non-toxin-producing mutant (106 and 679 DEGs at 3 and 12 hpi, respectively, mostly downregulated) whereas they showed a high response against the toxin-producing strain (1410 and 1103 DEGS, 3 and 12 hpi, respectively, half of them upregulated) (Fig. 1). In fact, several studies have shown that RBCs are the main targets for these toxins *in vitro* [9,37].

Fig. 4 shows the differential expression of a list of selected genes and cellular processes that are putatively related with the RtxA1 toxin activity *in vivo*. Among them, we highlight the following ones:

Activation and potentiation of RtxA1 toxin activity: the gene *insP6* is upregulated by RBCs at 3 and 12 hpi with the toxin-producing strain (Fig. 4). This would allow the resultant InsP6 to bind the toxin CPD domain which in turn would result in the release of the rest of the effector domains [13]. Moreover, we found a gene encoding for coronin-1a, together with some genes belonging to its upstream signaling pathway (i.e., inositol trisphospate receptor genes, *itpr1* and *itpr3*) upregulated by RBCs at 3 hpi only after infection with the toxin-producing strain (Fig. 4). Coronin-1a binds to actin, which leads to cytoskeleton rearrangements and, at the same time, induces calcium mobilization [38–40]. Calcium mobilization in turn potentiates RtxA1 activity, since it has been described that presence of calcium in the environment is required for secretion of the toxin by the bacterium [41,42]. Consequently, RBCs in the presence of the parental strain would overexpress genes compatible with RtxA1 overactivation contributing to aggravate the inflammatory response against *V. vulnificus*.

Cytoskeletal rearrangements: genes related with cytoskeleton changes were mostly found upregulated in the samples taken from eels infected with the toxin-producing strain (Fig. 4). This response could be related to the activity of two of the effector domains of the toxin, the ACD domain, with actin-cross-linking activity, and the MCF domain, with apoptosis activation activity [10,12,43]. In contrast, a minor cytoskeletal-related response, probably related to cell motility and activity (i.e., angiogenesis, diapedesis, etc.) was detected in B samples at 12 hpi infection with the non-toxin producing mutant (Fig. 4).

Pathogen detection and antigen presentation systems: genes encoding TLRs were differentially activated in response to the toxin-producing strain. Among them we highlight two genes encoding the intracellular TLRs that respond to RNA, *tlr7* and *tlr9b,* which were highly upregulated in response to the toxin-producing strain but not by the non-toxin-producing mutant (Fig. 4). This activation has also been observed in mice infected with the same parental strain used in this study [10]. Tlr9 and Tlr3 have been involved in detection of intracellular pathogens by fish, such the hematopoietic necrosis virus (IHNV) and *Edwardsiella* spp. [44–46]. Although *V. vulnificus* is not an intracellular pathogen, the RtxA1 toxin is found in the cytoplasm of target cells where it is activated and releases the functional domains into the cytoplasm [12]. Thus, we propose that the intracellular existence of this toxin could be the cause of the activation of intracellular recognition mechanisms.

Inflammatory response: our results showed that the proinflammatory immune response was clearly related to toxin production. In fact, the number of inflammation-related genes (genes for pro-inflammatory cytokines, chemokines and proteins involved in their production) and their FC value was much higher in immune cells challenged with the toxin-producing strain than in cells challenged with the non-toxin-producing mutant (Fig. 4).

Sepsis markers: although we found upregulated some genes encoding markers of sepsis (i.e., genes related to tissue damage and acute phase of infection) in eels infected with the non-toxin-producing mutant, the FC value for these genes was much lower than that found in samples from eels infected with the toxin-producing strain (Fig. 4). For example, the FC values for *mmp9*, *cox2* and leukotriene-encoding genes were up to six times higher in the blood of animals infected with the toxin-producing strain (Fig. 4). Accordingly, genes for prostaglandins were not found to be differentially expressed in response to infection with the mutant strain but were differentially upregulated in response to infection with the toxin-producing strain (Fig. 4).

RNA-based response: one of the most interesting results of the comparative transcriptome analysis was the discovery of an RNA-based response related to the toxin activity *in vivo*. Previously, Hernández-Cabanyero et al., highlighted that a gene for a systemic RNAi (*sid1*) was upregulated in eels infected with the toxin-producing strain. In that study, the authors only detected the transcription of such gene in response to the toxin-producing strain and never in the non-infected animals. Therefore, they proposed *sid1* as a marker for vibriosis which was validated as such in *in vivo* experiments [7]. The authors hypothesized that there would be an RNA-based immune response underlying the cytokine storm caused by *V. vulnificus* that would be related to the toxin. According to this hypothesis, we showed in this work that this gene was downregulated by eels infected with the non-toxin-producing strain (Table 2 and Figs. 3 and 4). Moreover, the gene for the microRNA miR-142a, which was not differentially expressed in response to the toxin-producing strain [7], was upregulated by WBCs at 3 hpi with the non-toxin-producing mutant (Table 2 and Figs. 3 and 4). Consequently, our results show that infected eels develop an RNA-based response that is dependent on toxin production. Thus, in its absence the infected eels would produce a microRNA (miR-142a) that would likely attenuate the inflammatory response and, in its presence, a systemic RNAi that would be associated with a lethal inflammatory response. A relationship between systemic RNAi and cytokine storm has already been described in

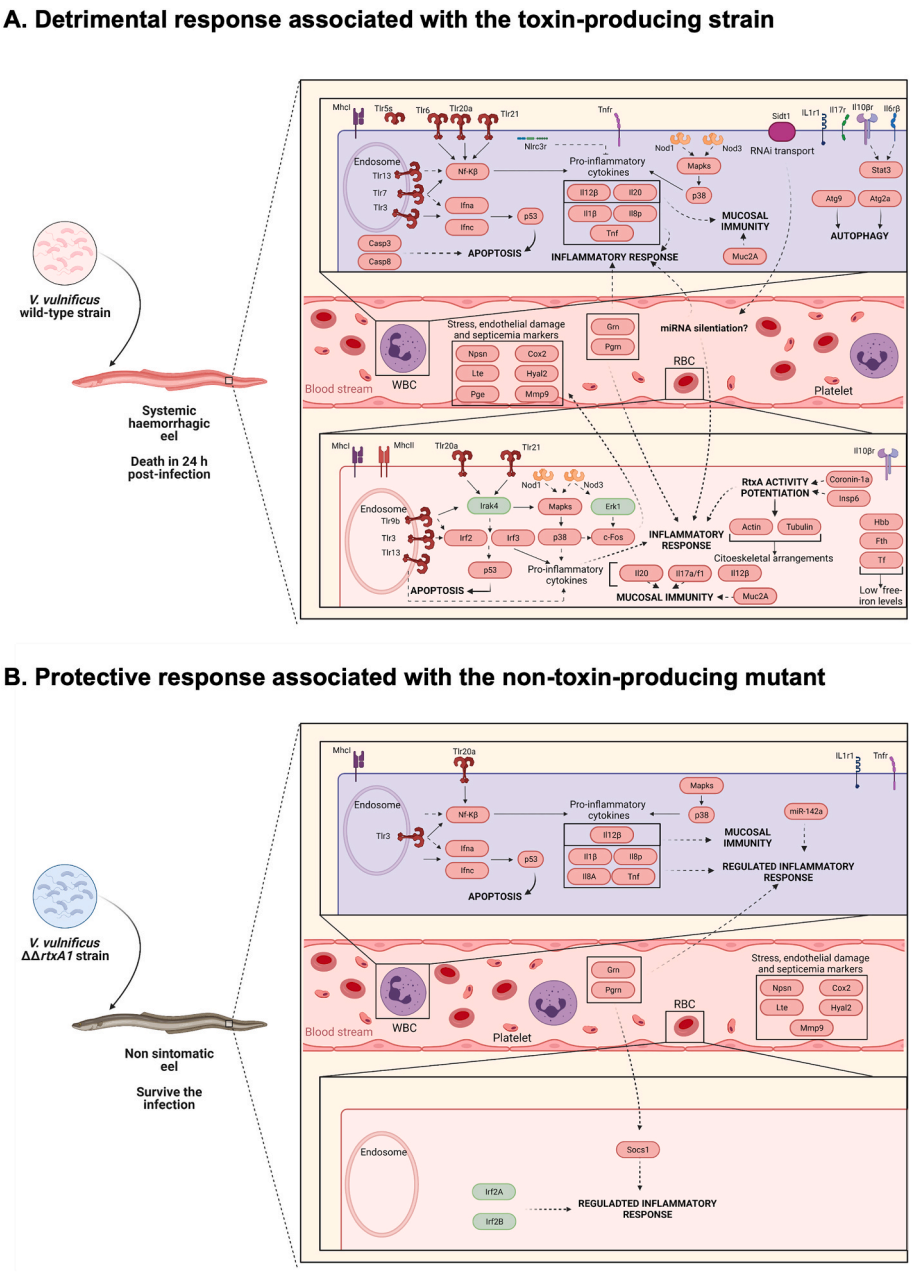

Fig. 5. Eel immune response as a function of RtxA1 toxin production by *V. vulnificus*: A. Detrimental response associated with the toxin-producing strain (R99); B: Protective response associated with the non-toxin-producing mutant (*ΔΔrtxA1*). The model shows the resultant proteins produced by the main transcripts differentially expressed by eels during *V. vulnificus* infection. The putative translated proteins are represented in a code color depending if it is encoded by a gene detected as: upregulated (red), downregulated (green). Figure A modified from Hernández-Cabanyero et al., [7]. Created with BioRender.com.

other fish species infected with other pathogens although it has never been demonstrated what the molecular/cellular basis of this relationship would be [35].

3.2. Functional assays

Complementary to the transcriptomic assays we performed a series of *in vivo* functional assays. First, we assessed the early eel colonization/invasion by monitoring bacterial counts in gills and blood from animals infected either with the toxin-producing strain or the non-toxin-producing mutant at 0, 3 and 12 hpi. Both strains colonized the gills giving counts between 10^5-10^6 CFU/gr, with no significant differences between strains or times. In addition, both strains were also able to invade internal organs and were detected in blood at 3 (around 10^2 CFU/ml) and 12 hpi (around 10^3 CFU/ml) without significant differences in bacterial counts between strains per sampling time, confirming that both were equally septicemic. However, the mutant strain was unable to kill the eels, while the parental strain produced 50 % mortality at the tested infective dose (2×10^6 CFU/ml), a result in accordance with that obtained by Lee et al. [9]. Therefore, we conclude that i) the immune response associated to the non-toxin-producing mutant could be considered as representative of a fish protective response against bacteria, and ii) the differences in the immune response developed against the parental and the mutant strains are related to the production of the RtxA1 toxin *in vivo*.

Then the levels of the pro-inflammatory cytokines Tnfα and Il1β were determined by indirect ELISA in eel serum samples. The results showed that the production of both cytokines increased at 12 hpi compared to

those found in serum samples at 0 hpi, with a higher increase in samples from animals infected with the toxin-producing strain compared to the those infected with the non-toxin-producing mutant and those found in serum samples from non-infected animals (Table 3). This result is in accordance with those obtained in the transcriptomic analysis and again relates the production of the RtxA1 toxin *in vivo* with a higher inflammatory response.

Finally, a series of enzymatic and lytic activities were determined in the serum from infected and non-infected eels. We found that serum from eels infected with the non-toxin-producing mutant was less proteolytic, hemolytic and bacteriolytic than serum from eels infected with the toxin-producing strains, pointing out a lower tissue damage in the absence of the toxin. This result is in accordance with those obtained in the transcriptomic analysis which relates the production of the RtxA1 toxin *in vivo* with death by a hemorrhagic septicemia (Table 4).

4. Concluding remarks

V. vulnificus is a zoonotic pathogen of concern in aquaculture and public health, since fish farms are the hot spot for outbreaks and evolution of this species [2]. For those reasons is especially relevant to understand how this pathogen cause disease in fish in order to develop strategies to prevent outbreaks of vibriosis in fish farms. In the present study, we aimed to unravel the role of the RtxA1 toxin (major *V. vulnificus* virulent factor) in triggering an unbalanced immune response that leads to the eel death by sepsis.

Fig. 5 summarizes the results obtained and propose two models of eel response to septicemic infection with *V. vulnificus*, one would lead to a cytokine storm and death by sepsis and would be linked to toxin production, similarly as previously described in mice [10] (Fig. 5A). The other would lead to eel survival and would occur if the infectious *Vibrio* did not express the toxin gene or produce the toxin (Fig. 5B).

In this model, the role of RBCs stands out because they are the most responsive blood cells when the bacterium produces the toxin *in vivo*. This increased susceptibility could be related to the overexpression of pathways that lead to calcium mobilization and InsP formation, processes that increase toxin release and intracellular activation, in this way the toxin would be feeding back its own release and activation. In early events after infection, the toxin would activate in the RBCs both cell death mechanisms and a mixed-type of pro-inflammatory response, which would have anti-viral response characteristics probably related to the presence of the toxin inside the host's cells. This early pro-inflammatory response would also involve WBCs, but as RBCs are 1000 times more abundant than WBCs in eel's blood [6,7,47], the role of RBCs in vibriosis would be far superior. This early response would lead to a global inflammatory response that would be maintained in the blood of the eel simultaneously with the production of a set of sepsis markers of protease type that would damage the endothelium causing the death of the animals by the hemorrhagic septicemia typical of vibriosis.

But more interestingly, there is evidence of an RNA-based response that underlies the inflammatory response and is also toxin-dependent. Prominent in this response are transcriptional modulators such as a systemic RNAi and miR-142a [34,35]. RNAi is part of the innate defenses against viruses that are common to all vertebrates. Its exact function is unknown. In our study we found it linked to toxin and the deregulated inflammatory response while we found that miR-142a would be linked to a balanced and protective response of the eel against bacteria (Fig. 5). We hypothesize that the toxin would indirectly activate an anti-RNA response leading to the overproduction of a systemic RNAi that would silence the miR-142a regulator responsible for the balanced response. The role of the systemic RNAi in silencing miR-142a and triggering an RNA-related response that induces an atypical and non-effective immune response, along with the likely role of miR-142a in proper modulation of host immunity and survival deserves further investigation. Specifically, miR-142a is emerging as a useful avenue to develop new therapeutic tools, based on the use of silencing/anti-silencing RNAs, to prevent infectious diseases in fish farms. We are currently working to demonstrate this hypothesis.

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CRediT authorship contribution statement

Carmen Amaro (CA) and Carla Hernéndez-Cabanyero (CH-C) conceived and designed the study. CH-C and Eva Sanjuán performed the laboratory experiments. Luis Mercado designed the antibodies and the ELISA experiments. CH-C analyzed the data. CH-C wrote the first draft of the manuscript that was corrected and improved by CA. CA and CH-C built the final version taking into account all the corrections and suggestions of the other authors. All authors read and approved the submitted version.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2023.109131.

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