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Evolutionary conserved pathway of the innate immune response after a viral insult in *Paracentrotus lividus* sea urchin

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Abstract

Despite the apparent simplicity of the body organization of echinoderms, their immune system is competent to perform a complex innate immune response, which is far from being well understood. The echinoderms represent the most advanced invertebrates that form a bridge with the primitive chordates. In fact, they possess numerous receptors and effectors that are used to obtain a fast immune response. After an infection, the humoral and cellular immune response determines a network in which the main protagonists are membrane and endosomal receptors. The recognition of nonself molecules by specific membrane receptors triggers the immune response, stimulating consecutive intracellular events. We have previously shown how the polyinosinic–polycytidylic acid (polyI:C) that mimics a viral infection is able to induce an immune response in the sea urchin *Paracentrotus lividus* immune cells. It activates a specific membrane receptor belonging to the Toll-like receptor (TLR) family. Here, we show the activated expression pattern of some genes involved in the downstream cascade of TLR signalling pathway, such as *Pl-Tbk* and *Pl-Irf*, whose partial sequence was isolated from *P. lividus* immune cells. Their mRNA expression increases consequentially to the polyI:C stimulation and in a temporal way. In addition, we analysed the expression of *Pl-NF-kB and we found that its upregulation was time-dependent, preceding Pl-Tbk* and *Pl-Irf* increase. Protein analysis showed that also some cytokine (TNF-α and IL-1α) expression increased after polyI:C insult. Therefore, the purpose of this study was to discover the molecular mechanisms of the innate defence strategies, similarly to vertebrates, implemented by the sea urchins in order to cope with viral infection challenge.

KEYWORDS
evolution, innate immunity, invertebrate animals, model organism

1 | INTRODUCTION

Invertebrates such as echinoderms show an innate immune response similar to that of vertebrates as it is a low-specificity cellular defence used to prevent and combat infectious agents (Satake & Sekiguchi, 2012). In addition, vertebrates have the adaptive immune response.

Echinoderms perform this innate immune response through the varied populations of immune cells present in the coelomic cavity and well described by diverse authors in two species of sea urchin, the purple *Strongylocentrotus purpuratus* and the Mediterranean *Paracentrotus lividus* (Arizza, Giaramita, Parrinello, Cammarata, & Parrinello, 2007; Branco, Figueiredo, & Silva, 2014; Matranga, Pinsino, Celi, Di Bella, & Natoli, 2006; Smith, 2012).

The innate immune response is activated by pathogen-associated molecular pattern (PAMP) molecules identified as nonself that are recognized by immune cells via specific cell pattern recognition receptors. The most common PAMPs include components of the bacterial cell wall, such as lipopolysaccharide (LPS) and peptidoglycan, or viral double-stranded RNA (dsRNA) (Kawai & Akira, 2010). Diverse families of proteins are upregulated in the sea urchins after immune activation; among these, Sp185/333 genes represent a highly diversified family of genes expressed in the phagocyte class of immune cells in *S. purpuratus* (Smith, 2012). Moreover, different immune membrane receptors have been found both in invertebrates and in vertebrates, such as the C-type lectins, the Nod-like receptors, the scavenger cysteine-rich receptors (Franchi, Warner, Viani, & Nuñez, 2009; Ospelt & Gay, 2010; Smith et al., 2006) and the Toll-like receptor (TLR) family (Kawai & Akira, 2010). TLRs have been identified in all metazoans, endorsing their significant role in immunity of vertebrates (Kawai & Akira, 2010), and invertebrates (Rauta, Samanta, Dash, Nayak, & Das, 2014). TLRs are type I membrane receptors whose signalling domain is known as the Toll/IL-1 receptor (TIR). TIR performs the first step of the signalling cascade, which culminates in the induction of specific transcription factors (TFs) that in turn activate pro-inflammatory cytokines, interleukins, interferons and tumour necrosis factors (Kaisho & Akira, 2006; Ospelt & Gay, 2010).

Sixteen vertebrate TLRs have been classified into six subfamilies based on sequence homology (Kawai & Akira, 2010). Differently from vertebrates or insects, TLR receptors in the sea urchins are encoded by a large multigene family: 68 and 253 *TLR* genes were isolated from *L. variegatus* and *S. purpuratus* sea urchins, respectively, representing a great expansion (equal to fivefold to 10-fold) compared to vertebrates (Buckley & Rast, 2012; Smith et al., 2006). They can perform different functions; for instance, it is known that the TLR4 receptors are specifically responsive to bacterial LPS stimuli while the TLR3 receptors, which are found exclusively in the endosome membrane, respond specifically to dsRNA resulting from viral infections (Satake & Sekiguchi, 2012).

The signalling pathway cascade of vertebrate TLR family is quite known: TLRs bind to adapter molecules such as the protein myeloid differentiation primary response 88 (MyD88) and the protein TIR-domain-containing adapter-inducing interferon-β (TRIF). They are connecting proteins that receive signals from outside the cell and transmit signals inside the cell. They activate specific kinases such as the serine/threonine protein kinase (TBK1) or the inhibitor of kappa NF-κB (IKK), which in turn phosphorylates TFs such as the interferon regulatory factor (IRF). Finally, other TFs, the nuclear factor-kappa B (NF-kB) and the activator protein 1, enter the nucleus in order to express effector genes important for the immune response, such as interferons and pro-inflammatory cytokines and chemokines (Iwanaszko & Kimmel, 2015; Turner, Nedjai, Hurst, & Pennington, 2014; Zhao, Jiang, & Li, 2015). TBK1 kinase has a central role in innate immunity, as a nexus of multiple signals induced by pathogen organisms and as a modulator of IFN levels, via IRF (Ma et al., 2012). IRF family is TFs important in the regulation of expression of interferons, especially in response to virus infections (Escalante, Yie, Thanos, & Aggarwal, 1998). The main innate immune signalling pathways are conserved in all living organisms, in invertebrates including sponges, worms, cnidarians, molluscs, crustaceans, insects and echinoderms and in all vertebrates. A diversification is known in the components that interact with pathogens, while the components that act downstream of the receptors are highly conserved (Buchmann, 2014; Stein, Caccamo, Laird, & Leptin, 2007).

Although many studies on echinoderms have contributed to the knowledge of the immune system, the complex mechanisms of the innate immune response are not yet clear. Echinoderms are phylogenetically related to vertebrates, as they are deuterostomes like chordates, to such an extent that they share many genes of receptor families and effectors involved in the immune response.

Many echinoderms have been used as model systems for studies on immunology as well as cell and developmental biology (Chiaramonte & Russo, 2015; Smith et al., 2018).

One TLR family member in *P. lividus* sea urchin immune cells that we refer to as *Pl-Tlr* was previously isolated (Russo, Chiaramonte, Matranga, & Arizza, 2015). Its expression in response to challenge with LPS and polyinosinic–polycytidylic acid (polyI:C) was characterized, in that *Pl-Tlr* was specifically upregulated by viral infection and not by bacterial infection.

Here, we report the isolation, from *P. lividus* sea urchin immune cells, of the partial cDNAs corresponding to TBK1 and IRF3 genes involved in the cascade signalling pathway downstream a TLR gene, which is specifically stimulated by dsRNA during a viral infection. We determined their increased expression after a viral infection produced by a chemical agent (polyI:C), in addition to another TF, NF-kB. Finally, we demonstrated that they are sequentially expressed during the time and that they can activate cytokines important for the innate immune response such as TNF-α and IL-1α.

2 | MATERIALS AND METHODS

2.1 | Animal sampling and coelomic fluid extraction

Adult individuals of *P. lividus* were collected from the Palermo gulf and maintained at 15°C in an aerated aquarium with filtered sea water and a 10/14-hour light/dark cycle. Sea water was prepared using Instant Ocean Sea Salt (Mentor, OH) dissolved in deionized water corrected for salinity and pH. A small volume of water was changed weekly, and the animals were fed once a week with commercial invertebrate food (Azoo, Taikong Corp., Taiwan). Coelomic fluid (CF) was withdrawn by inserting the needle of a syringe preloaded with isosmotic anticoagulant solution (ISO-EDTA; 0.5 M NaCl, 20 mM Tris-HCl and 30 mM EDTA; pH 7.4) into the peristomial membrane. After centrifugation (900 *g* for 10 min at 4°C), the pellet containing the sea urchin immune cells was washed twice in ISO–EDTA and resuspended at a density of 1×10^5 cells per ml in ISO–EDTA.

2.2 | Treatment with polyinosinic–polycytidylic acid (polyI:C)

Different adult individuals of *P. lividus* received injections, into the coelomic cavity through the peristomial membrane, of polyinosinic–polycytidylic acid (polyI:C) (Sigma Chemical Co., St. Louis, MO) at doses of 4 µg/ml of CF. The reagent was dissolved in artificial CF (aCF) as described by Terwilliger (Terwilliger, Buckley, Brockton, Ritter, & Smith, 2007). Control individuals were injected with aCF alone. Subsequently, the CF (4 ml) was withdrawn by syringe preloaded with isosmotic anticoagulant solution at 3, 9, 15, 18, 24 and 48 hr post-polyI:C treatment. Cells were washed by centrifugation, suspended in ISO–EDTA and counted using a Burker chamber. The immune cells were then aliquoted in tubes at a density of 1×10^7 cells per ml and stored at 4°C until further use.

2.3 | Differential immune cell count

Differential immune cell count was performed using a Burker chamber under a light microscope at 1,000 × magnifications (Leica DMLB equipped with a digital camera Leica DC 200, Germany). The count values were the average number of immune cells observed in 30 microscopic fields for each animal (in total ~1,000 coelomocytes). The dead cells were evaluated by using the eosin-y exclusion test (0.5% in ISO–EDTA). The number of individuals utilized in this study was 18 untreated (control) and 18 treated specimens (three animals for each time point).

2.4 | Extraction of RNA, cDNA synthesis, cloning and sequencing

Total RNA from control and polyI:C treated immune cells was extracted using the GenElute Mammalian Total RNA Miniprep Kit according to the manufacturer's instructions (Sigma Chemical Co.) and quantified using a bio-photometer (Eppendorf S.r.l., Hamburg, Germany). An average quantity of mRNA equal to 3 µg was obtained from the immune cell pellet of about 20 µl. Before synthesizing cDNA, we tested the obtained RNAs by PCR with primers for the *Pl-Z12-1* reference/housekeeping gene to ensure that no genomic DNA contaminant was present. Total RNA (1 µg) from immune cells was reverse transcribed according to the manufacturer's instructions (Applied Biosystems, Life Technologies, Carlsbad, CA). An aliquot of each cDNA (20 ng) was used to perform the polymerase chain reaction (PCR) using the specific oligonucleotides. The amplicons obtained from the PCR were cloned into the pGEM-T Easy Vector following the manufacturer's instructions (Promega, Madison, WI) and sequenced by a service company (Bio-Fab research srl, Rome, Italy).

2.5 | Real-time quantitative PCR

Quantification of gene expression was performed using the StepOnePlus Real-Time PCR (a comparative threshold cycle method) as described in the manufacturer's manual (Applied Biosystems) with SYBR Green Chemistry (Livak & Schmittgen, 2001). The *Pl-Z12-1* was used as the internal reference (Costa, Karakostis, Zito, & Matranga, 2012). Primer sequences for *Pl-Tbk* and *Pl-Irf* used in the quantitative PCR (QPCR) analysis were as follows: *Pl-Tbk* Forward (F): 5'ACACAAGGTATTAGAAGCACCAA3' and Reverse (R): 5'AACTGCA ACAACATCTCCAGT3', *Pl-IRF* F: 5' TGACAAGGAAAGGCAGATTG TGA3' and R: 5'TCGGTCGCGGGGTCGTATTTCTTT3'. The amplicon lengths were 120 and 126 nt, respectively. The NCBI accession numbers of the sequences are summarized in Tables 1 and 2.

Primer sequences for *Pl-NF-kB* are F: 5'TCCCATGGAGGACTGC CGTGTCA3' and R: 5'TCGTTGGTTACCAAGGAGACCACA3'; the amplicon length was 116nt; and the accession number was HE574572. The primers were manually designed following the indications of Applied Biosystems QPCR manual, and the amplified fragment of the target gene was sent to be sequenced.

The QPCR was run as follows: 1x cycle: denaturing 95°C for 10′ for DNA polymerase activation; 38x cycles: melting 95°C for 15″, annealing/extension 60°C for 60″. Statistical analysis was performed on QPCR values obtained from at least two independent experiments using the one-way ANOVA test followed by Tukey's multiple comparison test using R statistical software (http://www.r-project.org).

TABLE 1 Name of species, databank accession numbers, protein name and similarity percentage with Pl-Tbk, compared to the number of amino acids

Species	Accession number	Protein name	% similarity/ number of aa	Class/phylum
Paracentrotus lividus	MK229194	Pl-Tbk		Echinoidea/Echinodermata
Strongylocentrotus purpuratus	XP_011670140	Serine/threonine protein kinase TBK1 isoform X1	80%/40	Echinoidea/Echinodermata
Strongylocentrotus purpuratus	XP_784300.3	TBK1 isoform X2	80%/40	Echinoidea/Echinodermata
Acanthaster planci	XP_022080976	TBK1-like	77%/36	Asteroidea/Echinodermata
Nematostella vectensis	XP_001636449	Predicted TBK1-like	69%/36	Anthozoa/Cnidaria
Branchiostoma belcheri	XP_019616006	TBK1-like	70%/37	Leptocardii/Chordata
Branchiostoma floridae	XP_002607727	Hypothetical TBK1-like	70%/37	Leptocardii/Chordata
Pan troglodytes	PNI28490.1	TBK1 isoform 8	78%/37	Mammalia/Chordata

TABLE 2 Name of species, databank accession numbers, protein name and similarity percentage with Pl-Irf, compared to the number of amino acids

Species	Accession number	Protein name	% similarity /number of aa	Phylum class/phylum
Paracentrotus lividus	MK229195	Pl-Irf		Echinoidea/Echinodermata
Strongylocentrotus purpuratus	XP_011666057	Interferon regulatory factor IRF-like isoform X1	88%/42	Echinoidea/Echinodermata
Strongylocentrotus purpuratus	XP_003723620	IRF-like isoform X2	88%/42	Echinoidea/Echinodermata
Acanthaster planci	XP_022106007	IRF-like isoform X1	80%/41	Asteroidea/Echinodermata
Austrofundulus limnaeus	XP_013881638	IRF1-like isoform X1	66%/42	Fish/Chordata
Xenopus laevis	XP_018093096	IRF2 isoform X1	71%/42	Amphibia/Chordata

2.6 | Extraction of proteins from immune cells

The pellet rich of the immune cells, obtained from the CF extracted from adult sea urchins control and polyI:C treated collected after 24 and 48 hr, was lysed in RIPA buffer (50 mM TRIS-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) in the presence of an anti-protease cocktail (pepstatin A, E-64, bestatin, leupeptin, aprotinin and AEBSF 0.1% final concentration) (Sigma Chemical Co.), incubated on ice for 20 min, then sonicated (Branson, Model B15, Danbury, CT) at 4°C for 20 s (1 pulse/s, 70% duty cycle) and finally centrifuged at 27,000 *g* for 20 min at 4°C to remove any debris. Protein concentrations were determined by Qubit fluorimetry (Qubit 2.0 Fluorometer), and sample aliquots were stored at −80°C until further use.

2.7 | Electrophoresis on SDS-polyacrylamide gel

Procedures for 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions were performed according to Laemmli (1970). After determining the total protein contents of CLS, 10 μg of proteins from each sample was added to the sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol 10% SDS, 0.05% bromophenol blue, SB) containing 5% β-mercaptoethanol topped up to a total volume of 20 μl. The mixed samples were then denatured for 10 min at 100°C. The obtained protein pattern was compared with those of standard proteins (SDS-PAGE Color Prestained Protein Standard, Broad range, EuroClone) in order to calculate the molecular weight of each band.

2.8 | Immunoblotting

Proteins were transferred to nitrocellulose paper (Hybond ECL Amersham Pharmacia Biotech) according to Towbin, Staehelin, and Gordon (1979), using the Bio-Rad Trans-Blot SD Semi Dry Transfer Cell appliance. Immunoblots were performed according to Celi, Vazzana, Sanfratello, and Parrinello (2012). The nitrocellulose sheets were soaked for 1 hr at 20°C with 5% bovine serum albumin (BSA) in 10 mM Tris-base, 150 mM NaCl, pH 7.4, 0.1% Tween-20 (TBS-T) and then were incubated overnight at 4°C in a humid chamber with gentle stirring, in a solution of various anti-HSP antibodies: anti-IL-1α monoclonal antibody produced in mice (Sigma Chemical Co.) 1:1,000 in 3% BSA in TBS-T; anti-TNF-α monoclonal antibody produced in mice

(Sigma Chemical Co.) 1:1,000 in 3% BSA in TBS-T; after, the membranes were incubated for 1 hr at 20°C with a diluted solution of the secondary antibody anti-mouse IgG conjugated with alkaline phosphatase (1:10,000 anti-mouse IgG in TBS-T) to highlight the presence of specific proteins. Immunoreactivity was then demonstrated using a mixture of BCIP-NBT (Roche, Germany). The anti-tubulin monoclonal antibody (Sigma, 181 T5168; 1:1000) was used as a control to normalize protein expression, for 1 hr at room temperature.

2.9 | Statistical analysis

For immune cell counts, a total of 18 untreated (control) and 18 treated specimens (three animals for each time point) of sea urchins were used. The significance of the results obtained was assessed through the Student's *t* test, with considered significance at $p < 0.05$. Statistical analysis was performed on QPCR values obtained from at least three independent experiments using the one-way ANOVA test followed by Tukey's multiple comparison test using R statistical software (http://www.r-project.org).

3 | RESULTS

3.1 | Analysis of the immune cell percentages after polyI:C insult

In order to evaluate the differences in the relative composition of the circulating immune cell populations after polyI:C stimulus (at 3, 9, 15, 18, 24 and 48 hr post-injection), we collected the immune cells of the coelomic cavity from 18 controls and 18 treated individuals (three individuals for each time), as described in Materials and Methods section. Figure 1 shows the images of different types of immune cells found in a control sample, classified as follows: phagocytic cells (PC) (double black arrow), colourless/white spherule cells (CSC) (black arrow), red spherule cells (RSC) (black asterisk) and vibratile cells (VC) (black arrowhead), according to the classification of Matranga et al. (2006) and Arizza et al. (2007).

In Figure 2 are shown the histograms of the statistically significant variations in immune cell populations after polyI:C treatment, compared to the control, assumed as 1. The number of PCs increased significantly ($p < 0.001$), reaching its highest value, at 9 hr, when the value increased to 1.7-fold. The trend remained such until 24 hr and
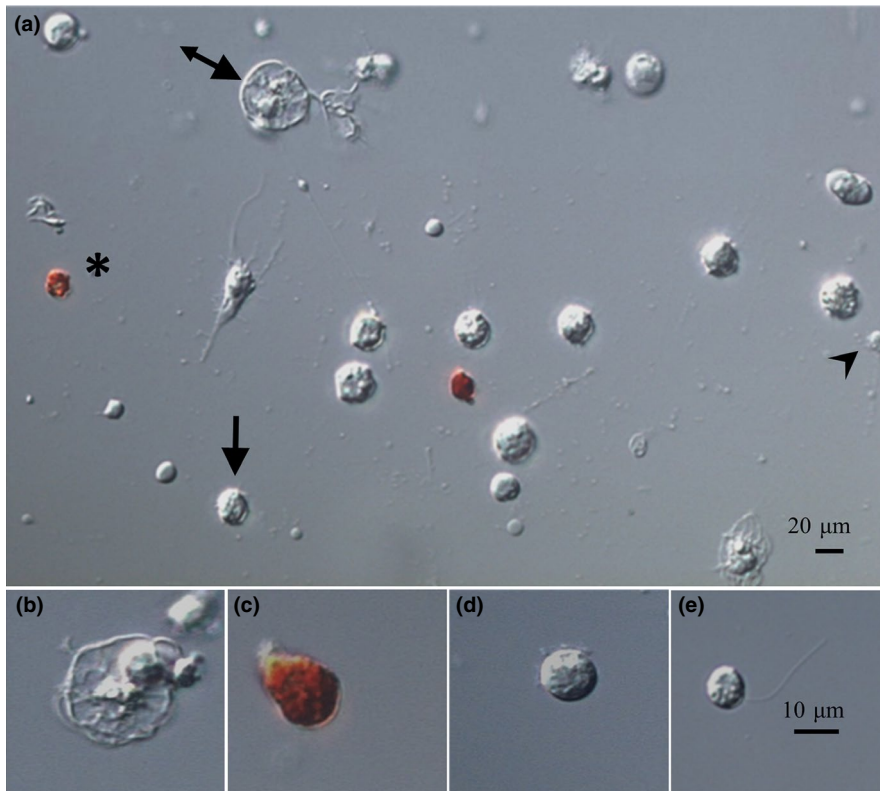
FIGURE 1 Immune cell types in the sea urchin, *Paracentrotus lividus*. Specifically: phagocytes (double arrow), red spherule cells (asterisk), colourless spherule cells (arrow) and vibratile cell (arrowhead). All images were collected Zeiss Axioskop 2 plus microscope (Zeiss). Scale bar in (a) is 20 μm. Scale bar in b–e is 10 μm [Colour figure can be viewed at wileyonlinelibrary.com]
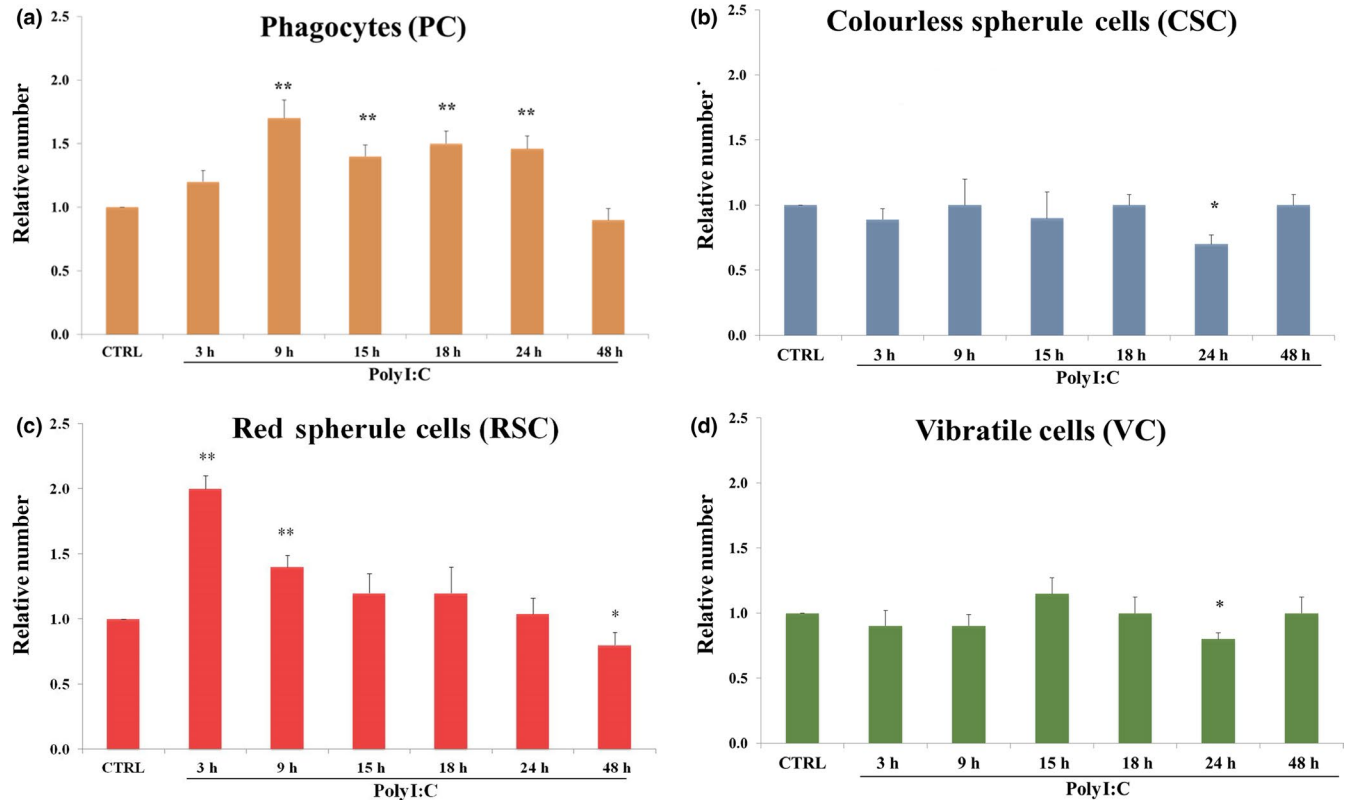

FIGURE 2 Effect of polyI:C treatment on the relative number of phagocytic cells (a), colourless spherule cells (b), red spherule cells (c) and vibratile cells (d) of *Paracentrotus lividus* immune cell populations compared to their relative untreated control group (aCF alone without polyI:C). Mean values were significantly different according to R statistical method (**$p < 0.001$; *$p < 0.01$) [Colour figure can be viewed at wileyonlinelibrary.com]
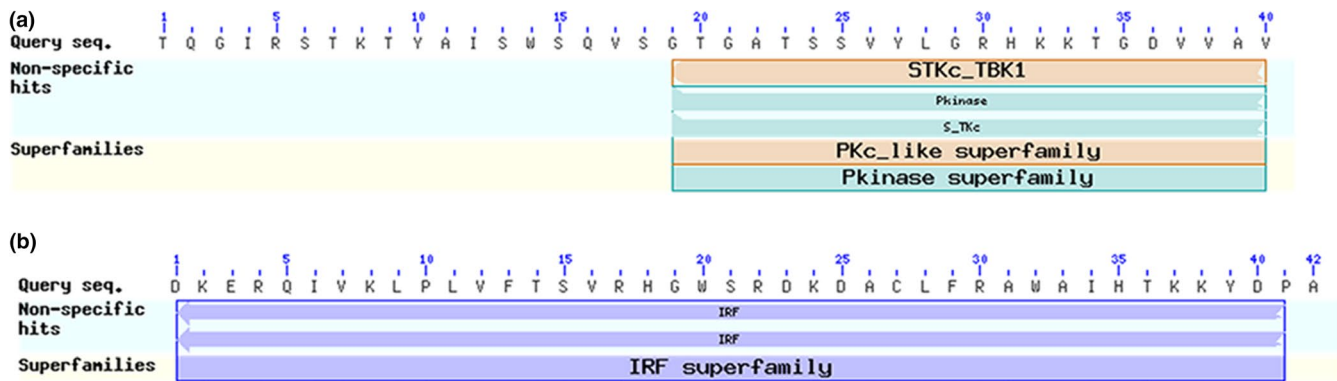
FIGURE 3 Schematic drawing of the partial sequence of Pl-Tbk (a) and Pl-Irf (b) proteins. It was exported from BLAST software at NCBI website and indicates the functional domains of the isolated partial proteins. STKc_TBK1 = catalytic domain of the serine/threonine kinase TANK binding kinase 1; S_TKc = serine/threonine protein kinases, catalytic domain; Pkinase = protein kinase domain [Colour figure can be viewed at wileyonlinelibrary.com]

decreased at 48 hr (Figure 2a). Moreover, the RSC responded to polyI:C stimulus by significantly increasing after 3 and 9 hr (twofold and 1.3-fold, respectively) (Figure 2c).

Ratio values of CSC and VC populations at 3, 9, 15, 18 and 48 hr did not differ significantly when compared to the control untreated individuals, at the same time. Their value differed significantly ($p < 0.001$) from the control set only at 24 hr when they decreased at the value of 0.65 and 0.7, respectively (Figure 2b,d).

3.2 | Isolation of the partial gene Tbk and Irf from *P. lividus* immune cells

This study aimed to discover the expression pattern of proteins belonging to the TLR pathway linked to the viral (ds-RNA) response. Therefore, we isolated from *P. lividus* adult immune cells the partial cDNAs corresponding to *Tbk* and *Irf* genes, 120 and 126 nt long, respectively, using specific oligonucleotides designed on the base of sea urchin *S. purpuratus* published sequences (Sodergren et al., 2006). The nucleotidic sequence of the cDNA fragment was blasted at GenBank NCBI (https://www.ncbi.nlm.nih.gov) to confirm its correctness. The isolated cDNA, named *Pl-Tbk* and *Pl-Irf*, showed a high homology with *S. purpuratus* serine/threonine kinase TANK binding kinase 1 (*TBK*) and *IRF* genes. In particular, the deduced protein of Pl-Tbk, 40 aa long, was blasted at NCBI website in order to catch information of the protein functional domains and the obtained scheme is showed in Figure 3a. The isolated mRNA belongs to TBK1 superfamily, and the functional domain is located between the amino acids 19–40 of our partial sequence. Pl-Tbk protein had the following amino acid sequence percentages of similarity: 80% with two isoforms of *S. purpuratus* TBK (X1 and X2), 77% with the sea star *Acanthaster planci*, 69% with the sea anemone *Nematostella vectensis* (phylum: Cnidaria), 70% with the *Branchiostoma belcheri* and *floridae* (phylum: Chordata; subphylum: Cephalochordata) and unexpectedly 78% with the mammals *Pan troglodytes* (phylum: Chordata; subphylum: Vertebrata) (see Table 1).

Moreover, we isolated the partial sequence corresponding to *IRF* gene, and the deduced protein of Pl-Irf, 42 aa long (scheme in Figure 3b), showed a percentage of similarity equal to the following:

88% with two isoforms of *S. purpuratus* IRF (X1 and X2), 80% with the sea star *A. planci* (phylum: Echinodermata) and 66%–71% with the fish *Austrofundulus limnaeus*, the amphibian *Xenopus laevis* (phylum: Chordata; subphylum: Vertebrata) (see Table 2). These results indicated that both genes possess a high degree of conservation in the evolutionary scale.

3.3 | Expression analysis of *Pl-Tbk* and *Pl-Irf* mRNAs in polyI:C treated immune cells

We determined the effects on the transcriptional activity of *Pl-Tbk* and *Pl-Irf* in *P. lividus* immune cells, after injection of polyI:C into the coelomic cavity of adult sea urchins at the concentrations suggested by Terwilliger et al. (2007). Immune cells were withdrawn 3, 9, 15, 18, 24 and 48 hr post-treatment from the treated and control sea urchins. The quantitative expression was analysed by QPCR. Immune cells injected with artificial CF were used as the reference control sample and assigned an arbitrary value of 1. In particular, the *Pl-Tbk* mRNA expression (Figure 4a) increased following the polyI:C treatment at 9 hr (1.54-fold) and reached the maximum value at 15 hr (1.91-fold). At 3, 18, 24 and 48 hr, the *Pl-Tbk* gene expression modulation was found comparable to the control values. Instead, after the polyI:C treatment, *Pl-Irf* mRNA (Figure 4b) began to increase, significantly, at 15 hr (2.13-fold) and reached a consistent expression at 24 hr (5.72-fold). *Pl-NF-kB* expression was investigated at 3, 9, 15, 18, 24 and 48 hr post-treatment, and an increased activity was found at 3 and 9 hr (of 9.67- and 11.54-fold, respectively), while a significant decrease was shown at 15, 18 and 48 hr (up to a value of 0.41, 0.22 and 0.48, respectively) (Figure 4c). The relative quantitative values expressed as fold increases with respect to the control were the average of two/three independent QPCR analyses using at least two cDNAs obtained from different experiments.

3.4 | Protein expression analysis of TNF-α and IL-1α, in polyI:C treated immune cells

In order to evaluate other molecules involved in the *P. lividus* pathway of the immune response after polyI:C insult, total proteins were
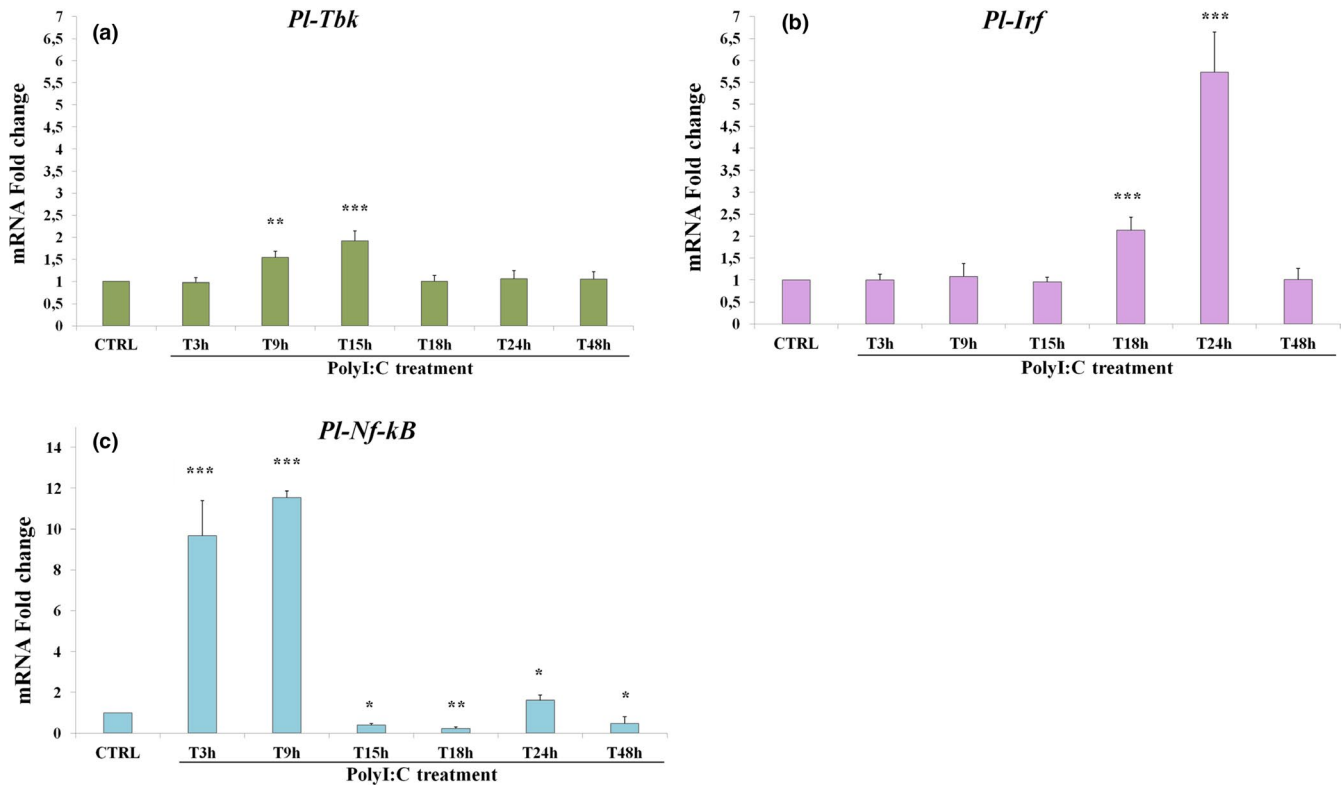
FIGURE 4 Quantitative analysis of the expression of *Pl-Tbk* (a) and *Pl-Irf* (b), *Pl-NF-kB* (c) performed by QPCR. *Paracentrotus lividus* immune cells treated with polyI:C and collected after 3, 9, 15, 18, 24 and 48 hr. CTRL = control that consisted of immune cells injected with only artificial coelomic fluid whose was assigned an arbitrary value of 1 in the histogram. It was representative of all the controls collected after 3, 9, 15, 18, 24 and 48 hr. Each bar represents the mean of two independent experiments and two different QPCR analysis ± *SD*. Mean values were significantly different according to R statistical method (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$) [Colour figure can be viewed at wileyonlinelibrary.com]
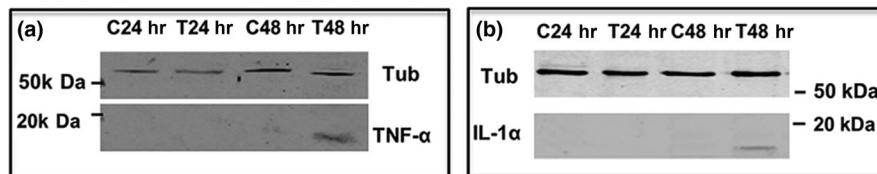

FIGURE 5 Protein analysis by Western blot in the sea urchin immune cells control (C) and treated (T) with polyI:C collected at 24 and 48 hr. (a) Total lysates from immune cells reacted with an antibody against the human protein TNF-α. (b) Total lysates from immune cells reacted with an antibody against the human IL-1α

examined from controls and treated samples, 24 and 48 hr after treatment. We used two commercial polyclonal antibodies to investigate the expression of cytokines: one against a highly conserved region of human IL-1α, and one against the human TNF-α. The Western blot analysis conducted with the TNF-α human antibody showed a single band having a molecular weight of about 17/18 kDa (exactly corresponding to what reported in the antibody datasheet), only present at 48 hr from polyI:C treatment, and not present in the control (Figure 5a).

Similarly, the time course of IL-1α protein expression showed a band only present at 48 hr post-polyI:C treatment, having a molecular weight of about 17/18 kDa (corresponding to what reported in

the antibody datasheet) (Figure 5b). The anti-tubulin monoclonal antibody was used as a control to normalize protein expression.

4 | DISCUSSION

In the present study, we attempt to contribute to the analysis of innate immune response mechanisms that occur in the Mediterranean species of *P. lividus* sea urchin, following a viral infection. In fact, numerous microorganisms can cause diseases in echinoderms and can hold pathogenic or symbiotic relationship with hosts. These agents include bacteria, fungi, viruses and parasites (Gudenkauf, Eaglesham,

Aragundi, & Hewson, 2013; Kiselev, Ageenko, & Kurilenko, 2013; Schmid-Hempel, 2003, 2011; Stein et al., 2007; Turton & Wardlaw, 1987). We further wished to demonstrate the conservation during evolution of the signalling pathway implemented by an invertebrate such as the sea urchin in the immune response. In the last years, several studies were performed on the sea urchin innate immune system that reinforced the notion of its use as a model for immunological purpose, due to its evolutionary proximity to chordates and vertebrates (Branco et al., 2014; Rast, Smith, Loza-Coll, Hibino, & Litman, 2006). Moreover, *P. lividus* was extensively used as a biological indicator of local pollution and of stress (Bonaventura, Zito, Chiaramonte, Costa, & Russo, 2018; Russo et al., 2018; Scanu et al., 2015).

We have treated the *P. lividus* sea urchin with polyI:C, a chemical compound that mimics a viral infections, at the aim to induce an immunological response. In a precedent study (Russo et al., 2015), we have isolated one member of TLR membrane receptor family from *P. lividus*, named *Pl-Tlr*, and we have found that *Pl-Tlr* gene responded to polyI:C treatment by increasing its mRNA expression at 9 hours post-treatment.

Here, we showed that after polyI:C insult, a multifaceted phenomenon happens, including: (a) an increase in the relative number of two cellular subpopulations, the PCs and the RSCs; (b) an increase in the gene expression of *Pl-Tbk*, *Pl-NF-kB* and *Pl-Irf* mRNAs, at different time points; and (c) an increase in the protein expression of IL-1α and TNF-α.

Invertebrates such as sea urchins have a complex defence system able to protect the animals from pathogen attacks. They possess an innate immune system that acts through humoral and cellular responses. The cellular response is mediated by a particular class of cells, the immune cells, that circulate in the coelomic cavity and can infiltrate tissues and organs; they act through different activating pathways in response to host invasion, insult and cytotoxic agents (Chiaramonte & Russo, 2015; Pinsino & Matranga, 2015; Smith et al., 2018). Since the appearance of the first eukaryotic cells on Earth, many defence mechanisms have evolved to ensure cell integrity, homeostasis and organ survival. For instance, invertebrates have increased the number of cell receptors able to bind to nonself molecules. This ability is associated with the presence of phagocytic immune cells such as amoebocytes and hemocytes. These cells have similar appearance and functions to those of vertebrates macrophages (Dzik, 2010).

In our study, we investigated the changes in immune cell subpopulations after treatment with polyI:C and we have found an increasing number of PC and RSC. The first cells are responsible of many immune functions including the phagocytic activity of possible pathogens, while the second ones are cells that migrate to the site of infection during the first phase of response, at the aim to encapsulate the invasive particles and degrade them (Smith et al., 2018). Moreover, the RSC contains the echinochrome A with antimicrobic activity (Service & Wardlaw, 1985) suggesting that it has a protective role of the sea urchin animals. Instead, the VC and the CSC population seem not to be influenced by the polyI:C treatment. Phagocytic

cells are the best evolutionary conserved immune cells, able to solve immune response throughout amoeba-like motility and phagocyting activity. They were first discovered in the invertebrate starfish by Metchnikoff in 1893 and reported by Tauber (2003). The PCs correspond to the macrophage cells present in vertebrates (Ovchinnikov, 2008) and were found in all other organisms of the animal kingdom, from advanced invertebrates (echinoderms) to tunicates (ascidians), where both granulocyte-like cells and macrophages occur (Buchmann, 2014).

These results indicate that a first important step of response is the changed composition in the subpopulations of immune cells. Indeed, a different number of immune cells was shown in the arctic sea urchin *S. neumayeri*, with an increase in RSC population after LPS treatment (Gonzalez-Aravena et al., 2015). Moreover, an increase in the immune cell number was found in other marine invertebrates in response to different stimuli. For example, hemocytes increased in the mollusc *Ruditapes philippinarum* after treatment with zinc oxide nanoparticles (Marisa et al., 2016).

After polyI:C treatment of the sea urchin immune cells, we assist to an increased expression of *Pl-Tbk* and *Pl-Irf* mRNAs in a temporal manner, indicating a consequentiality of expression of the signalling pathway actors.

In the kidney human epithelial HEK293 cell line, after a viral infection, *TBK1* transcription is activated by TRIF adapter protein activated by a TLR receptor present in the endosomal membrane. In turn, cytoplasmatic TBK1 kinase phosphorylates the TF IRF3, which leads to the translocation of protein IRF3 from the cytoplasm to the nucleus (Fitzgerald et al., 2003). This indicates a key role of the TBK1 protein in the immune response signalling pathway, mainly related to viral infections. Moreover, the statistically significant increased expression of *Pl-Irf* mRNA after 18 hr post-polyI:C stimulation, reaching a peak after 24 hr, suggests a late temporal transcriptional activation. IRF are TFs composed by a unique helix-turn-helix DNA-binding domain involved in host immune response, haemotopoietic differentiation and immunomodulation. IRF-like genes were detected in the invertebrate deuterostomes, and they showed similarity to those of IRF family members in vertebrates, as revealed by phylogenetic analysis (Huang, Qi, Xu, & Nie, 2010).

Really, the presence of TKB1 and IRF proteins also in marine organisms, such as sea urchins, and the high percentage of protein sequence similarity with vertebrates' orthologous genes, demonstrates the importance of the role of these proteins in the immune response, which has been well preserved during evolution from invertebrates to vertebrates. TBK-like protein sequences were found in the genomes of the cephalochordates *Branchiostoma* sp. and in the sea anemones *N. vectensis* (Wolenski et al., 2011). An IRF-like protein was found in the genome of the invertebrate echinoderm *A. planci* sea star (Hall et al., 2017).

Instead, *Pl-NF-kB* increased from 3 to 9 hr being activated rapidly and transiently. *Pl-NF-kB* was previously isolated from *P. lividus* and used as it is one of the most sensitive TF markers associated with inflammation in a study investigating the pathway signalling

triggered by nanoparticles (Pinsino et al., 2015). It is known that NF-κB/RelA is not important for regulating IFN but is specifically required during an early phase after virus infection (Wang et al., 2010). A very interesting work, based on in silico and experimental analyses in mammals, reported that there is a crosstalk in the pathway of the immune response between NF-kB and IRF. NF-kB may be a negative regulator of IRF3 and vice versa (Iwanaszko & Kimmel, 2015). In our system, it is likely that at 3–9 hr post-viral infection, *Pl-NF-kB* is highly expressed and IRF3 is repressed. After, at 18–24 hr when *Pl-NF-kB* expression is reduced, *Pl-Irf* mRNA is expressed and can be phosphorylated by *Pl-Tbk*, and enters the nucleus.

The effect of polyI:C treatment was further investigated at the protein level, and we found that it induced the over-expression of the TNF-α and IL-1α at 48 hr post-treatment. In contrast to the receptors for interleukins, present in the sea urchin *S. purpuratus* (Hibino et al., 2006) and isolated in the starfish *Asteria forbesi* (Beck & Habicht, 1986), interleukins have so far neither been isolated nor characterized in echinoderms. However, in the GenBank database is reported the presence of different isoforms of IL-17 in *S. purpuratus*, whose molecular weight is equal to 17.5 kDa, which perfectly corresponds to the weight of our band obtained for WB, using a human IL-1α antibody. Moreover, Hibino et al. (2006) reported the presence, in *S. purpuratus*, of 25 IL-17 isoforms, one Sp-IL-17 receptor-like and one IL-1R/CD121 receptor. Recently, Buckley and Rast (Buckley & Rast, 2017) reported the presence of 30 genes and 5 pseudogenes coding for IL-17-like. Therefore, the protein band found by WB in *P. lividus* immune cells stimulated with polyI:C could correspond to one of these isoforms present in the *S. purpuratus* genome. These isoforms result differentially expressed during the sea urchin immune response. For example, the subfamily IL-17-9 is upregulated in adult immune cells (Buckley & Rast, 2017).

The IL-17 family in vertebrates has six members (IL-17 A to F) and represents a distinct signalling system with a well-documented pro-inflammatory function (Kawaguchi, Adachi, Oda, Kokubu, & Huang, 2004).

The GenBank database also reports other cytokines-like proteins present in *S. purpuratus* genome such as TNF-α. Furthermore, Hibino et al. (2006) report that four isoforms of TNF-α-like and 8 isoforms of TNF-α receptors are present in the *S. purpuratus* genome.

Our data highlight that TLR signalling pathway proteins in *P. lividus* are similar to their vertebrate homologs, and provide a framework for understanding their evolutionary origins.

Thus, based on this similarity it was possible to draft the hypothetical temporal regulation pathway of gene expression of the immune response after a viral insult, summarized in Figure 6. We propose a working model where polyI:C (a potential virus) initially activates, through a transmembrane endosome receptor (maybe TLR3), an adapter cytoplasmatic protein such as TRAF, TRIF and MyD88. As a consequence, the adapter protein induced an increased expression of the serine/threonine kinase *Pl-Tbk* which in turn activates the TF *Pl-Irf* that entering the nucleus directly activates cytokine gene transcription, affecting the balance between pro- and anti-inflammatory
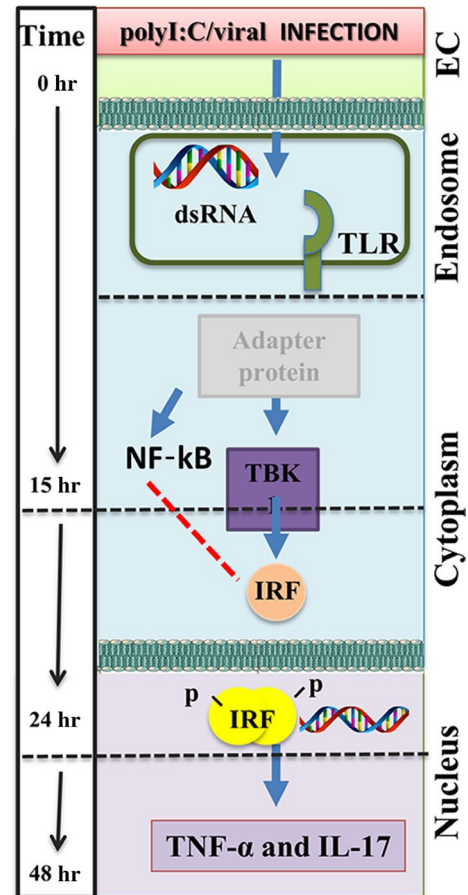

FIGURE 6 *Paracentrotus lividus* signalling pathway of TLR downstream genes, after polyI:C/viral infection. Dashed red arrow means a negative regulation (repression of expression). Blue arrows indicate a positive regulation (stimulation of activity/expression). EC = extracellular [Colour figure can be viewed at wileyonlinelibrary.com]

immune response. To this process probably participate other TFs such as NF-kB, as a negative regulator of IRF.

5 | CONCLUSIONS

The discovery of some genes involved in the pathways activated by TLR family, belonging to TBK and IRF families, provides new perspectives of knowledge of the immune response in invertebrates and vertebrates. The evolutionary study, starting from marine invertebrates such as the sea urchin *P. lividus*, can offer interesting indications for the understanding of more complex systems, present in higher vertebrates. Effectively, the study of echinoderms is particularly remarkable because they possess typical characteristics of protostomes and deuterostomes, suggesting a key passage in evolution up to the higher vertebrates (Smith et al., 2018).

As in this report we described only some of the different actors involved in the sea urchin immune response at the transcriptional and translational level, further investigations are needed in

order to increase our knowledge on invertebrate innate immunity response.

DISCLOSURES

The authors declare no conflict of interest.

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