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Diverse Transformers (Trf) protein family in the sea urchin *Paracentrotus lividus* act through collaboration between cellular and humoral immune effector arms

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Abstract

Sea urchins are long-living invertebrates with a complex immune system which includes extended families of immune receptors. A central immune gene family in the sea urchins encodes for the Transformer (Trf) proteins. The Trf family was so far studied mainly in the purple sea urchin *Strongylocentrotus purpuratus*. In this study, we explored this protein family in the Mediterranean Sea urchin *Paracentrotus lividus*. The *PlTrf* genes and predicted proteins were found to be highly diverse and showed a typical Trf size range and structure. We found that *P. lividus* coelomocytes and hemolymph contain different PlTrf protein repertoires with a shared subset which specifically bind *E. coli* bacteria. Using FACS, we identified five different *P. lividus* coelomocyte sub-populations with cell surface Trf protein expression. The relative abundance of the Trf-positive cells sharply increased following immune challenge with *E. coli* bacteria, but not following challenge with LPS or sea urchin pathogen *V. penaeicida*. Finally, we demonstrated that the phagocytosis of *E. coli* bacteria by *P. lividus* phagocytes is mediated through the hemolymph and is inhibited by blocking Trf activity with anti-Trf antibodies. Together, our results suggest collaboration between cellular and humoral Trf-mediated effector arms in the *P. lividus* specific immune response to pathogens.

Keywords: *Paracentrotus lividus*, Transformers, Trf, 185/333, invertebrate immunity, phagocytosis, model organism

Introduction

Sea urchins are long-living invertebrates that are constantly being exposed in nature to the marine environment and its pathogens through direct contact with marine substrates, seawater, and consumed food. For protection, they have evolved a very sophisticated and robust immune system with multiple effector arms and a wide genome representation of immune genes [1]. The echinoid immune gene repertoire includes several extended immune gene families that encode for receptors and hemolymph proteins that participate in either cellular responses (e.g., Toll-like receptors, NOD-like receptors, scavenger receptor) or humoral responses (e.g., C-type lectins, complement such as factor B) [2, 3]. In spite of the absence of vertebrate-like adaptive immunity, the large number of the germline-encoded immune proteins in the sea urchins suggest that they may specifically recognize and act against a variety of pathogen-associated molecular patterns (PAMPs). One of the central immune gene families in sea urchin's immune response encodes for the Transformer (Trf) proteins.

The Trf protein family (formerly termed 185/333) was first characterized in the California purple sea urchin *Strongylocentrotus Purpuratus* [4]. Since then, more than 30 studies about the family in this species were published and only one study in a different sea urchin species - *Heliocidaris erythrogramma* [5]. To date, no *Trf* genes or transcript sequences have been identified outside of the echinoid lineage, suggesting that Trf-based immunity is unique to sea urchins. In *S. purpuratus*, a robust Trf-mediated response was documented upon challenge with bacteria including *Escherichia coli* and *Vibrio diazotrophicus* [6, 7] and with PAMPs including lipopolysaccharide (LPS), β-1,3-glucan and double-stranded RNA [8]. The response was characterized by a significant increase in *SpTrf* transcript

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and protein quantities [4, 6, 8-10] as well as in increasing Trf-expressing cell ratios [11-13]. The *SpTrf* proteins are capable to selectively bind to different antigens [14], augment phagocytosis and retard bacterial growth [15]. It was also shown that a recombinant SpTrf protein is transforming its shape upon binding to antigens. Consequently, the family name was recently changed from 185/333 to Transformers [16].

The *SpTrf* genes are 1.2 - 2 kb long and consist of two exons; a short leader and a second exon which encodes for the functional protein [4]. The structure of the second exon is made of a mosaic of blocks of conserved sequences, termed elements, that are either present or absent in different members of the family [4, 9, 17]. The defined combinations of the gene elements of a second exon are the primary source of diversity in gene sequence and size. However, additional diversification processes occur downstream, post-transcription [18] and post-translation, resulting in a diversity of up to 260 different SpTrf proteins per individual as was deduced from 2DE Western blot analysis [6, 10]. Fifteen *SpTrf* genes were identified within two to three gene clusters in the genome of a single sea urchin [19]. However, the *SpTrf* loci might be highly unstable due to gene clustering and tandem sequence repeats [20] and the *SpTrf* gene repertoire show high variability within the sea urchin population [19]. Furthermore, it was shown that *SpTrf* genes may be subjected to regulated genomic diversification processes leading to gene deletions, duplications and single point mutations (SNPs) in single cells [12] and that each cell may express a unique single *SpTrf* message [7].

The Trf proteins are intrinsically unstructured in their native form and are predicted to adopt an α helical structure upon binding to a target [16]. Their predicted molecular weight based on the size of the messages ranged from 4 to 55 kDa in *S. purpuratus* [8] and from 8 to 39 kDa in *Heliocidaris erythrogramma* [5], while the actual protein range as observed on western blots is between 30 kDa to >200 kDa in both sea urchin species [5, 10, 13]. This difference in size is attributed to the multimerization of the Trf proteins [13]. The primary structure of the Trf proteins consists of a glycine-rich region, a histidine-rich region and a C-terminal region [4]. The Gly-rich region contains the protein multimerization motif in its C-terminus, which mediates the Trf protein multimerization [14]. Recombinant peptides of the Gly-rich and His-rich Trf regions were found to have both different and overlapping antigen-binding specificities [14].

The Trf proteins were so far found to be expressed in a phagocytic subset of sea urchin coelomocytes in at least two different expression patterns: in small phagocytes, Trf proteins were localized to the cell surface and in cytoplasmatic vesicles in filopodia, whereas in large polygonal and discoidal phagocytes the proteins were localized to perinuclear vesicles [5, 13]. On the other hand, no Trf proteins were so far localized in other cell types, including red spherule cells and the vibratile cells [21]. Immunolocalization of Trf proteins in *S. purpuratus* histological sections identified cellular Trf protein expression in all major sea urchin organs, including axial organ, gut, esophagus, gonad, and pharynx [11], which is attributed to infiltrated coelomocytes [21].

Based on the multiple studies in *S. purpuratus*, a rough model for Trf-mediated immune response was recently proposed [15, 21]. According to the model, the Trf protein isoforms that are stored within the perinuclear vesicles of phagocytes are secreted to the coelomic fluid following the detection of bacterial challenge. The secreted Trf protein isoforms multimerize with the same/other isoforms upon binding to bacteria and may adapt an α helical shape. The proteins are also predicted to be involved in bacterial growth retardation [15]. It is not known how Trf-bound bacteria are recognized by the phagocytes; however, it was suggested that it may be facilitated through either an unknown receptor, multimerization with surface Trf proteins or through phosphatidic acid (PA) within the cell membranes. It was shown that Trf protein binding to PA may cause membrane deformation and therefore may participate in phagocytosis [22].

To date, the *Trf* gene/protein family was characterized in only two sea urchin species - *S. purpuratus* and *H. erythrogramma.* In this study, we characterize the *Trf* genes and proteins and the Trf-mediated immune response in a third sea urchin species - the Mediterranean Sea urchin *Paracentrotus lividus*. *P. lividus* is abundant in shallow subtidal areas and in tidal pools on rocks or seagrass meadows in the Mediterranean sea and in the northeastern Atlantic ocean [23]. It is widely used in the food industry (e.g. [24]) as well as a model in scientific research, including in the fields of developmental biology (e.g. [25]) and comparative immunology (e.g. [26]).

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Our results suggest that similarly to *S. purpuratus* and *H. erythrogramma*, *P. lividus* utilize a diverse Trf protein repertoire as part of its immune response to pathogens. We found that the *PlTrf* genes and the translated protein sequences, although phylogenetically distinct from *S. purpuratus* and *H. erythrogramma*, bear the same basic structure of *SpTrf* and *HeTrf* genes and proteins. In this study, we used three anti-SpTrf antibodies previously raised against the peptides within the N terminus, the central part and the C terminus of the Trf proteins [13]. Using three different approaches, we first verified the cross-reactivity of these antibodies with PlTrf proteins. Western blot analyses depicted high PlTrf protein diversity among different *P. lividus* individuals. Furthermore, each of the sea urchins tested expressed a different Trf protein profile in the coelomocytes vs. the cell-free coelomic fluid (CF). Using FACS, we identified five different coelomocyte sub-populations with detectable membranal PlTrf expression. We also showed that a challenge with heat-killed *E. coli* bacteria is followed by a sharp increase in the ratios of TRF-positive coelomocytes and that the *E. coli*-specific response is likely being mediated by a specific set of Trf proteins. Lastly, we demonstrated that *P. lividus* hemolymph plays a role in the phagocytosis of *E. coli* bacteria, a process which is inhibited by blocking membranal PlTrf activity using the anti-Trf antibodies.

Results

The Trf family in the Mediterenean sea urchin *Paracentrotus lividus*

For the initial characterization of the Trf family in *P. lividus,* we obtained a total of 94 *PlTrf* transcript and DNA sequences from the Transcriptome Shotgun Assembly (TSA) databases HACU01, GCZS01, GEDS01 and GFRN01 (NCBI) and Octopus database (http://octopus.obs-vlfr.fr/blast/oursin/blast_oursin.php), of which 70 sequences were unique after UTRs removal (Fig. S1). To understand the phylogenetic position of the PlTrf sequences, twenty-five *PlTrf* CDS sequences of different sizes were chosen and aligned to an equal number of randomly picked full *SpTrf* and *HeTrf* CDS sequences. The alignment-based phylogenetic tree showed clustering of the Trf sequences according to species, from which they were originated with similar evolutionary distance among the clusters (Fig 1A). In contrast to the other two species, of which sequences showed homogeneous distribution, the *P. lividus* Trf cluster was sub-divided into three clades. This subdivision of *PlTrf* sequences may reflect segregation in function or may be caused by the limited sequence repertoire of the currently available databases from which the sequences were obtained. So far, no *Trf* sequences have been identified in non-echinoid echinoderm classes such as sea stars (class: Asterozoa) and sea cucumbers (class: Holothuroidea) nor in basal sea urchins such as pencil sea urchin *Eucidaris tribuloides* (Fig. 1A), suggesting the Trf family is unique to higher sea urchins [21]. Within the echinoid lineage, in addition to Trf sequences from *S. purpuratus*, *H. erythrogramma* and *P. lividus,* Trf sequences were also identified in *Strongylocentrotus franciscanus* and *Alocentrotus fragilus* [27], but are not publicly available. A single *Trf*-like sequence from *S. intermedius* (genebank accession: ADO13494.1) was also identified.

To characterize the *P. lividus* Trf primary protein sequences, we selected twelve full-length translated *PlTrf* CDS with unique sequence element content. The translated PlTrf coding sequences were between 220-308 amino acids (aa) in length, which is similar to the size range of the HeTrf protein sequences (257-349 aa long) and the SpTrf sequences (276-354 aa long). All PlTrf aa sequences had the typical signatures of Trf sequences, including the leader sequence, glycine-rich (Gly-rich) and histidine-rich (His-rich) regions and conserved sequence elements. However, the RGD motif (Arg-Gly-Asp cell-binding peptide motif), previously characterized in *S. purpuratus* [4], was absent from both PlTrf and HeTrf protein sequences suggesting that it is not a canonical Trf motif. The translated protein sequences had a variable number of glycine residues in the Gly-rich region. The His-rich region also varied in the number of histidines, where shorter versions contained 9 histidines (element 20 in sequences PL001, PL005, PL006, PL009, PL023) and longer versions had up to 19 histidines (elements 19-20 in sequences PL10, PL011, PL015, PL018, PL021 and PL022) (Fig. 1B). An alignment of all unique translated *PlTrf* CDS identified 22 sequence elements determined based on the alignment gaps. The PlTrf elements contained between 3 and 64 amino acids (aa). The PlTrf hydrophobic leader sequences contained 21 aa, and the most common leader sequence was MELKLILIIAIVAAITISA with variations at positions 5, 7, 9 and 16 (Fig. 1B, 12 chosen sequences shown).

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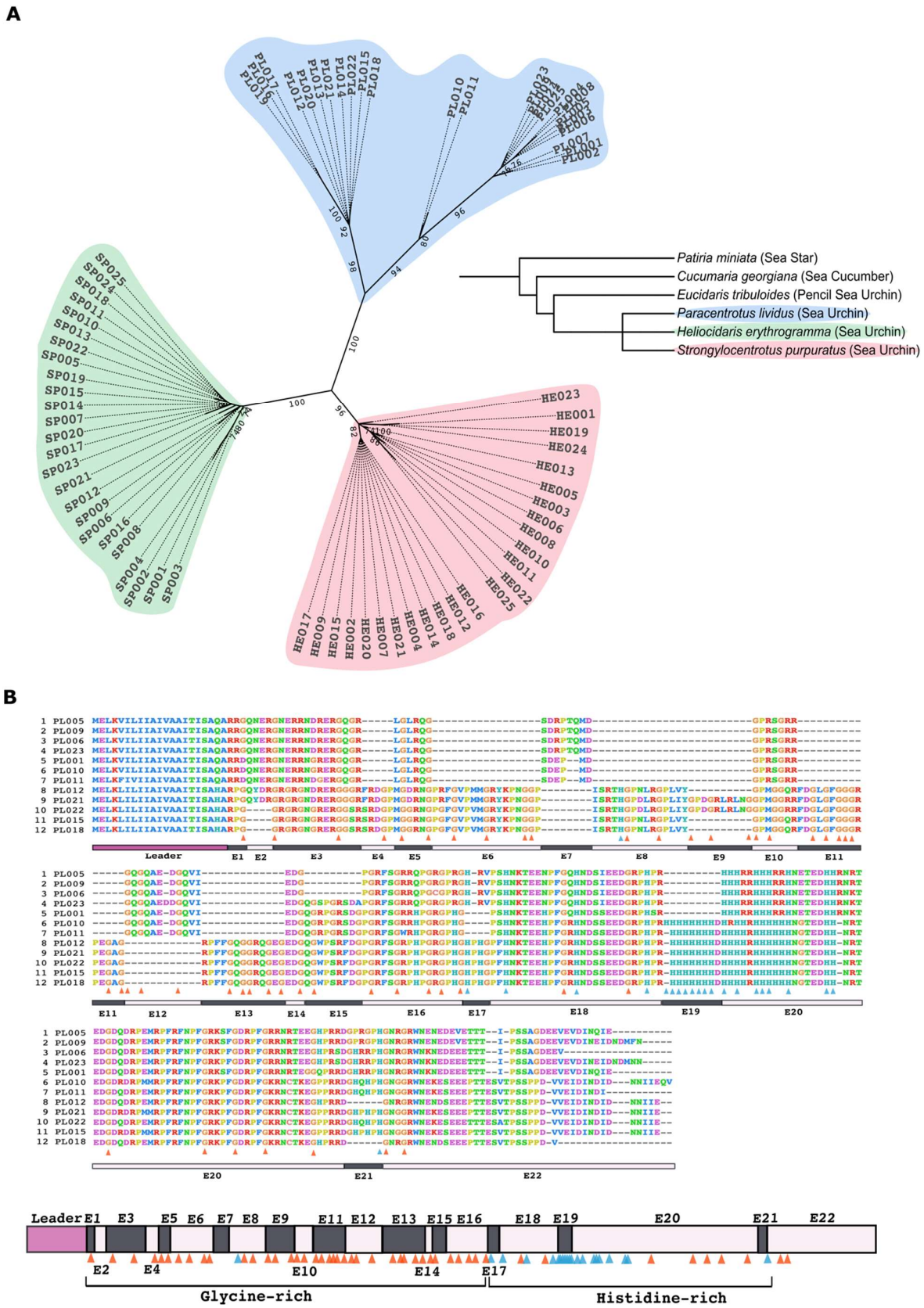

Fig. 1. The *PlTrf* phylogeny and the structure of the translated protein sequences. A. An unrooted phylogenetic tree of unique *PlTrf, HeTrf* and *SpTrf* CDS. Maximum likelihood (ML), Neighbour-Joining (NJ), and Minimum Evolution (ME) analyses resulted in similar phylogenetic trees. Only the ML-based tree (Hasegawa-Kishino-Yano model [28], highest log likelihood (-1640.98) (+*G*, parameter =

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2.7099)) is presented. The *Trf* sequences were clustered according to the three sea urchin species (*P. lividus*, *S. purpuratus* and *H. erythrogramma*). In *P. lividus*, sequences were subdivided into 3 clades according to the element patterns. The whole genome-based phylogenetic tree (phyloT, NCBI taxonomy) shows evolutionary relationships among echinoderms species. The pencil sea urchin- *Eucidaris tribuloides*, *Paritia miniata* (a sea star) and *Cucumaria georgiana* (a sea cucumber) do not contain *Trf*-like sequences in their genomes. **B. Alignment of 12 full translated PlTrf aa sequences**. The sequences were aligned in MEGAX Muscle [29] with manual correction considering three or more aa surrounded by gaps as an element. The 12 sequences were chosen from all publicly available *PlTrf* sequences so they will cover all available sequence elements. Sequence alignment is shown. Bars underneath the alignment representing the leader (In purple) and the 22 sequence elements (in dark and light shades of gray and light pink). PlTrf protein structure scheme summarizing the alignment is shown at the bottom. Orange triangles indicate glycine residues. Blue triangles indicate histidine residues. Sequence elements are marked as E1-E22.

Anti-SpTrf polyclonal antibodies cross-bind PlTrf proteins

Polyclonal SpTrf antibodies, provided by Prof. L. Courtney Smith from the George Washington University, were originally raised against three synthetic peptides corresponding to conserved sequences of the N-terminus, central part and C-terminus of the SpTrf proteins [13]. The three peptides for which the antibodies were raised are termed "66" (AHAQRDFNERRGKENDTER), "68" (GGRRGDGEEDTDAAQQIGDGLG), and "71" (GTEEGSPRRDGQRRPYGNR) and are part of SpTrf sequence elements 1, 7 and 25 accordingly. Similar sequences (41-66% aa identity to the 66 region, 30-40% to the 68 region and 70-88% to the 71 region) were identified in the *P. lividus* translated sequences within elements 1-3, 12-15 and 20-22 (Fig. 2A). Since their development, the SpTrf antibodies have been extensively used and became an essential tool in studying the Trf protein family. In order to use these antibodies to study the Trf family in *P. lividus*, as was done for *H. erythrograma* [5], we began with verification of its cross-reactivity with PlTrf proteins by three different approaches:

We first used a Nickel (Ni)-column to enrich the PlTrf proteins from whole coelomic fluid protein extract based on the binding of Ni to the multiple histidine residues of the Trf proteins. The resulting WB showed different patterns of Ni-bound vs. unbound proteins. Two major Trf protein sizes of ~120 and ~50 kDa for sea urchin #1 vs. ~250 and ~30 kDa in sea urchin #2 were identified in the Ni-bound fraction. In contrary, proteins mainly of ~12 and ~20 kDa in size were identified in the unbound fraction of Sea urchin #1 and were absent from Sea urchin #2, which had only >250 and several weak bands of smaller sizes in its unbound fraction (Fig. 2B). These results are similar to previous results from Ni-column enrichment of SpTrf proteins that identified different Trf protein sizes in the Ni-bound and unbound fraction of the Ni-column assay [6, 15]. The unbound PlTrf proteins may include family members with shorter His-rich regions, such as the proteins that would be encoded based on sequences PL001, PL005, Pl006, PL009 (Fig. 1B).

To test whether each of three SpTrf polyclonal antibodies (66, 68 and 71) in the mixture have a similar binding pattern, we used each of the antibodies separately in WB of coelomocytes and CF protein extract from the same individual. Except for two differences in the coelomocyte fractions of the 68 and 71 anti-SpTrf blots, the resulted WB band patterns were identical for the three antibodies, suggesting that the Trf-binding is specific. The two differences were 1) a ~30 kDa band that was present in the coelomocyte fraction of the 68 anti-SpTrf blot, which replaced the ~20 kDa band that appeared in the coelomocyte fractions identified by the two other antibodies and 2) an additional, weak ~17 kDa band that was found in the coelomocyte fraction of the 71 anti-SpTrf blot, which was not present in any of the other blots (Fig 2C). We speculated that these differences are due to the truncation and multimerization processes of specific membrane attached PlTrf proteins. It was also noticed that the WB band patterns received for the three anti-SpTrf antibodies were significantly different between the coelomocytes and the CF (Fig 2C). Since this difference between the cellular and humoral Trf fractions was so far not characterized, we followed these results with further investigation using other sea urchins as described in the next section.

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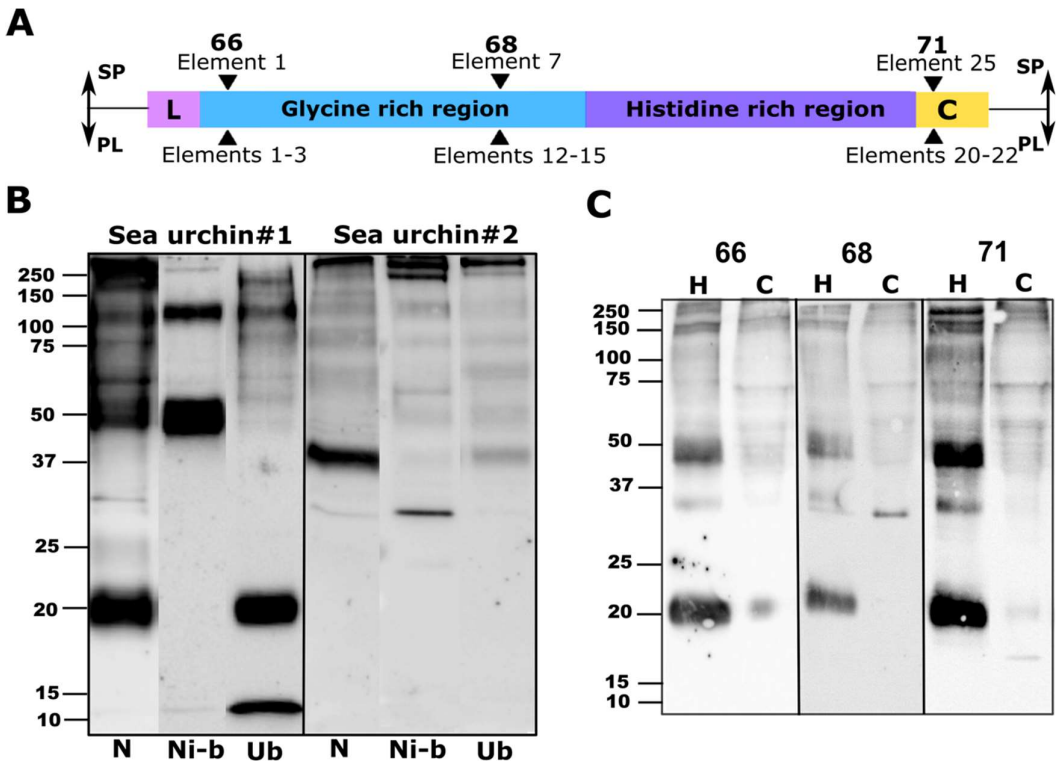

Fig. 2. Specific binding of anti-SpTrf antibodies to PlTrf proteins A. Schematic structure of a Trf protein with the locations to which the three SpTrf antibodies bind. The locations for 66, 68, and 71 Trf antibodies binding are marked with black triangles for *S. purpuratus* (SP) and their equivalents in *P. lividus* (PL). **B. WB of native, nickel-bound and unbound PlTrf proteins with anti-SpTrf antibody mixture.** Ni-Column Trf protein enrichment was performed according to [6], based on the binding of the histidine residues to the Nickel. N - native protein extract from the CF. Ni-b-Nickel bound proteins. Ub-Nickel column washed flow-through fraction (unbound proteins). **C. WB of PlTrf proteins from hemolymph and coelomocytes with each of the three anti-SpTRF antibodies seperately.** The western blot was made based on a whole protein extract from CF (hemolymph (H)) and coelomocytes (C) of the same sea urchin. Each blot was incubated with either 66, 68 or 71 SpTrf antibodies.

In a third anti-SpTrf cross-binding verification approach, we implemented a targeted mass-spectrometry (MS) analysis on anti-SpTrf bound proteins from native *P. lividus* protein extracts and from the Ni-enriched protein fractions. Using the relevant WBs as a reference, SDS gel slices, an equivalent of anti-SpTrf positive high-molecular weight bands, were sent for MS. The MS analysis identified PlTrf peptide sequences from both native and Ni-enriched fractions. As expected, a higher number of PlTrf matches were observed in the Ni-enriched fraction compared to the whole native extract (6 vs. 4). While matches of numerous conserved proteins from different sea urchins were obtained, Trf protein matches were strictly of *P. lividus* (Table 1). The size range of the full MS-identified PlTrf proteins was 239-307 aa with a predicted molecular weight of 27.2 to 34.3 kDa and predicted uniform isoelectric points (Pi) of pH 7.5 to 8.8. The presence of multiple Trf isoforms with high number of matches in the analyzed samples points with a high confidence level to the presence of the PlTrf proteins in the samples from *P. lividus*.

Table 1. Mass spectrometry PlTrf hits

	Accession	Name	Coverage [%]	Unique Peptides**	Length aa	MW [kD]	calc. pI
Elution*	GEDS01012456.1	Trf [P_lividus]	27	14	301	33.7	7.64
	GCZS01069164.1	Trf [P_lividus]	16	4	243	27.9	8.34
	GCZS01069162.1	Trf [P_lividus]	4	2	241	27.6	8.46
	HACU01465648.1	Trf [P_lividus]	6	2	244	27.7	6.87
	GFRN01311978.1	Trf [P_lividus]	2	1	239	27.3	8.09
	GEDS01012454.1	Trf [P_lividus]	2	1	307	34.3	8.82

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Native*	GEDS01012454.1	Trf [P_lividus]	18	8	307	34.3	8.82
	GFRN01311979.1	Trf [P_lividus]	13	7	239	27.3	8.09
	HACU01465651.1	Trf [P_lividus]	6	2	239	27.2	7.52
	GCZS01069162.1	Trf [P_lividus]	4	2	241	27.6	8.46

* Western blot bands corresponding to the gel slices used for MS analyses are presented in Fig. S1.
**Unique peptides: the number of peptides uniquely mapping to the protein.

P. lividus coelomocytes and hemolymph have different subsets of TRF proteins

Following our initial results showing different Trf protein profiles in coelomocytes vs. cell-free CF (hemolymph), we tested whether this pattern is consistent for other *P. lividus* individuals. WB with SpTrf antibody mixture resulted in different Trf profiles of the coelomocytes and hemolymph fractions of 7 sea urchins both in Trf band content and relative band intensity (given that the general protein concentrations of the coelomocyte and cell-free CF fractions samples for each animal were equalized). No consistent pattern was identified for the coelomocytes and hemolymph fractions, except for a short band of ~20 kDa that was present in the hemolymph fractions of all tested individuals but was absent or very weak in the coelomocyte samples (Fig. 3). A strong ~35 kDa band was present in five of the seven individuals in either the coelomocytes (sea urchins 3, 4, 5) or hemolymph (sea urchin 2, 7) fractions (Fig. 3). Although the PlTrf proteins sampled for MS were of high molecular mass of 200-300 kDa (Fig. S2), the highest PlTrf protein mass according to the MS analysis was ~34 kDa (Table 1). We, therefore, assume that all PlTrf bands of larger sizes represent multimerized proteins in similar to what has been established for *S. purpuratus* and *H. erythrograma.*

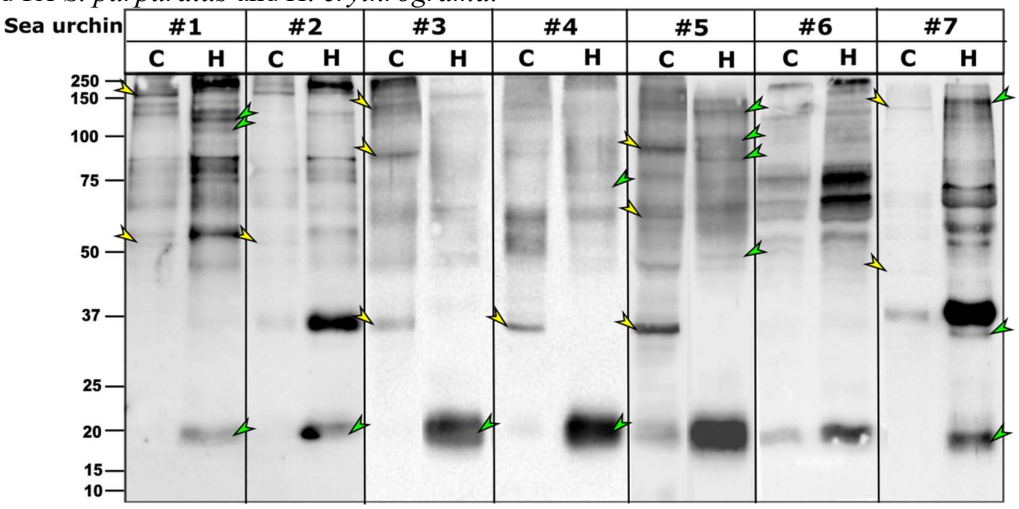

Fig. 3. Trf protein repertoires in coelomocytes and hemolymph of 7 *P. lividus* individuals. C – coelomocytes fraction, H – cell-free CF (hemolymph) fraction. Multiple differences were identified between the two fractions in each of the tested sea urchins, including unique bands in the coelomocyte fractions (yellow arrowheads) and unique bands in the cell-free fractions (green arrowheads).

Trf-positive cell types in *P. lividus*

P. lividus coelomocytes are morphologically divided into phagocytes (filopodial and small), vibratile cells, red and colorless spherule cells [30], also termed amoebocytes [26]. We used FACS to sort Trf-positive coelomocytes based on their binding to the anti-SpTrf antibody mixture according to [12](Fig S4). Using fluorescent microscopy, we identified cell surface Trf proteins in five immune coelomocyte sub-populations (Fig. 4A). This is in contrary to results from *S .purpurtus* that detected cell surface Trf proteins only in small phagocytes, whereas red spherule cells were specifically reported to lack Trf receptors [21]. Based on their distinct autofluorescence, red cells could be separately analyzed (as performed in [12, 30, 31]), showing the presence of the Trf-positive red spherule cells (double-positive for APC and FITC channels) compared to secondary antibody-only control (APC-positive only, Fig. 4B).

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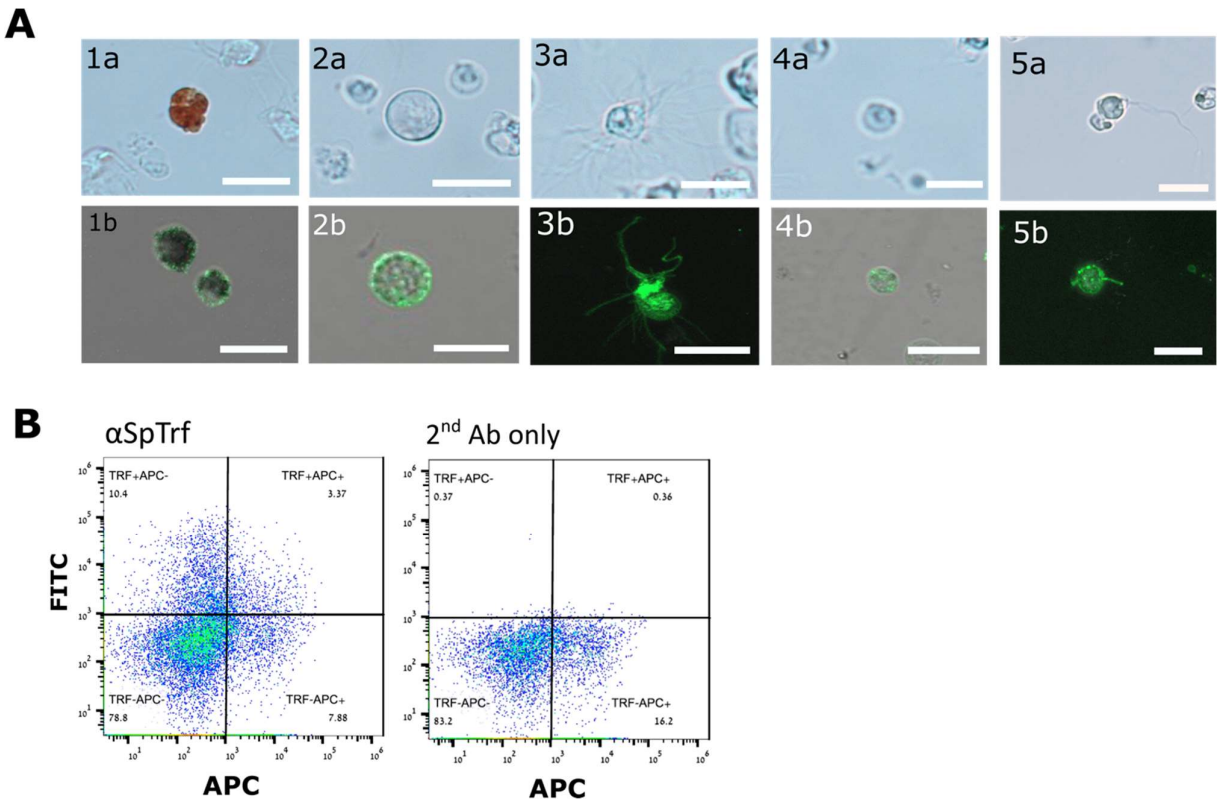

Fig. 4. *P.lividus* Trf-positive coelomocytes. A. Live Trf-positive *P. lividus* cells of five coelomocyte sub-populations. a –bright field, b – confocal microscope imaging. Trf proteins were detected by rabbit anti-SpTrf antibodies and Alexa Fluor 488 secondary anti-rabbit antibody. Trf-positive coelomocytes include: 1. red spherule cells, 2. petaloid or small phagocytes, 3. filopodial phagocytes, 4. white spherule cells and 5. vibratile cells. Scale bars = 10 µm **B. Trf-positive red spherule cells sorting.** The APC-positive cells are the Red spherule cells. In the αSpTrf treated sample (left) - The APC/FITC double-positive cells (upper right corner) are the Trf-expressing Red spherule cells. The FITC-positive/APC-negative cells (Upper left corner) represent all other SpTrf-positive cells. In the 2nd Ab only control (right) coelomocytes were incubated with the Alexa Fluor 488 secondary anti-rabbit antibody only. Almost no FITC-positive cells were observed.

P. lividus Trf-expressing coelomocytes ratios increase in response to *E.coli* but not to LPS or sea urchin pathogen *V. penaeicida* challenge

We previously showed that the *S. purpuratus* Trf-positive coelomocytes ratios increase following the challenge with heat-killed *V. diazitrophicus* bacteria [12]. In this study, we characterised the changes in the *P. lividus* Trf-positive coelomocyte ratios following challenge with heat-killed *E. coli* bacteria, LPS, and heat-killed *P. lividus* pathogenic bacteria *V. penaeicida* in comparison to sterile artificial CF (aCF) injections. Although sea urchins were kept in isolated aquaria for more than 6 months before the experiment, their basal levels of Trf-positive coelomocyte ratios were highly variable (13-65% of the total coelomocyte counts). In the first experiment, 10^6 heat-killed *E. coli* bacteria or 1 µg of LPS per ml of CF, diluted in 100µl of sterile aCF or equivalent volumes of aCF as control, were injected to corresponding sea urchin groups of three individuals each. Coelomocytes were collected for FACS analysis at day 0, just before the injection, and at days 1, 2, 13 and 43 post-injection. A significant (p value < 0.05) sharp increase in the Trf-positive coelomocyte ratios was observed in response to the *E. coli* challenge after the first day, which lasted up to day 43 post-challenge. On the contrary, the aCF-injected sea urchin controls and LPS-injected sea urchins showed a decrease in the Trf-positive coelomocyte ratios one and two days post-challenge followed by an increase in ratios toward the basal levels in day 13 and 43 post-injection (Fig. 5A). We assumed that the decrease in the Trf-positive coelomocyte ratios compared to the basal levels was possibly due to the insufficient time for the coelomocyte to differentiate into Trf-expressing cells after removal of substantial 0.2-0.4 ml of sea urchin CF from their coelomic cavity. We therefore adopted a different experimental design, which

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included CF sampling starting from day 2 to allow enough time for Trf-expressing coelomocytes to accumulate. Indeed, when we followed this design, we did not observe a decrease in the Trf-expressing coelomocytes population in the unchallenged sea urchin controls. On the contrary, the injection of aCF in the control group resulted in a mild increase the Trf-expressing coelomocytes ratios (Fig. 5B). This suggests that the decrease in Trf-positive cells in the first experiment was not related to the injections but rather to the timing of the sampling. We further assume that the time needed for Trf-expressing cell progenitors to differentiate after depletion due to sampling was between one and two days. In the second experiment, we tested the Trf-mediated cellular response to *V. penaeicida*, which is considered a possible *P. lividus* pathogen [32]. 10^6 heat-killed *V. penaeicida* cells for 1 ml of sea urchin coelomic fluid and equivalent volumes of aCF as a control were injected in the two corresponding groups of five sea urchins each. No significant difference was observed between the challenged sea urchins and the control group over the time course of the experiment (Fig. 5B). In both groups, a moderate increase in Trf-expressing coelomocytes ratios was observed on day 2 followed by a significant (p value < 0.05) decrease to the basal levels in day 14 and another unexplained increase on day 30. Additionally, a distinct, highly Trf-positive cell population appeared in the flow cytometry analysis of day 2 in most of the experiment and control samples (Fig. S3), which was not present in days 14 and 30, suggesting Trf-based short-time response to both bacteria and aCF injections.

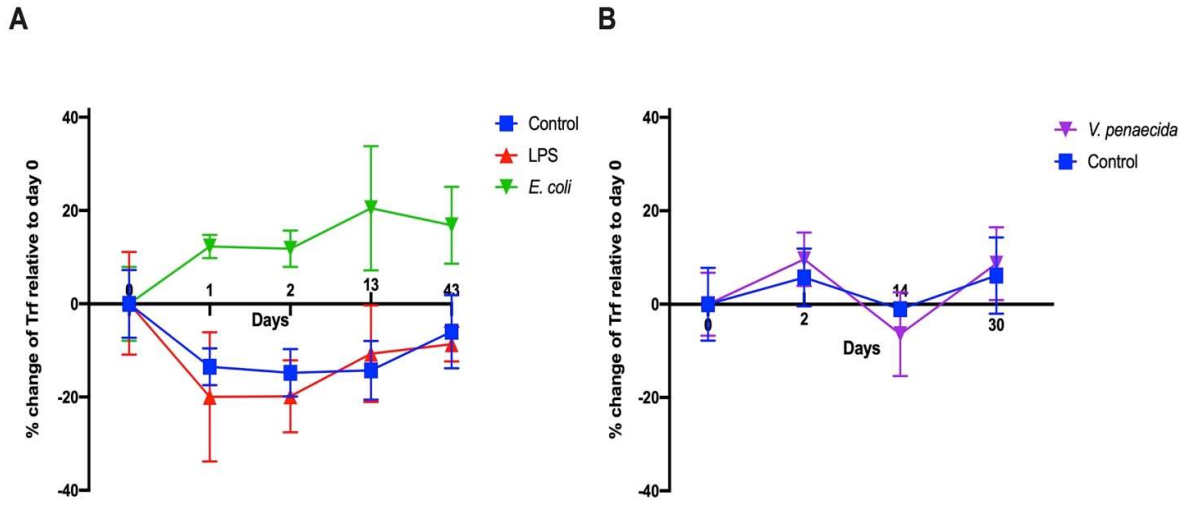

Fig. 5. Percent change of Trf-positive cells in response to a challenge with different immune elicitors. Due to the high variability in the basal levels of the Trf-positive coelomocytes within the sea urchin population, only the changes in the ratios relative to day 0 are presented. **A. Trf-positive coelomocytes percent change in response to injections of heat-killed *E. coli*, LPS and aCF (control)**. Sampling and measurements were subsequently done on days 1, 2, 13 and 43 post-injection. A significant increase in the percentage of Trf-positive coelomocytes was observed following the challenge with heat-killed *E. coli* over the course of the experiment. **B. Trf-positive coelomocytes percent change in response to injections of heat-killed *V. penaeicida* and aCF (control)**. Experiment and control groups did not show significant differences.

Specific subset of Trf proteins bind live *E. coli* bacteria

Quite a few *S. purpuratus* studies demonstrated specific Trf-mediated responses to different types of immune elicitors, including the expression and binding of specific subsets of Trf proteins [4, 7, 8, 15, 33]. Following the strong PlTrf cellular effector arm reaction to the *E. coli* challenge, we tested the profiles of the Trf proteins that specifically bind to live *E. coli*. We used the cellular and cell-free CF (hemolymph) fractions of coelomic fluid extracted from three sea urchins. WB analysis of the *E. coli*-bound proteins showed a conserved band pattern in both coelomocytes and hemolymph fractions of the tested sea urchins after 30 minutes incubation with live *E. coli*. Most of the *E. coli*-bound Trf proteins were confined within 5-9 bands of ~ 30 to ~60 kDa (Fig.6). The *E. coli*-bound protein band sizes were absent from the untreated protein extract taken from the same three sea urchin individuals, suggesting they represent diverse Trf monomers or dimers that may be bound to *E.coli* molecules.

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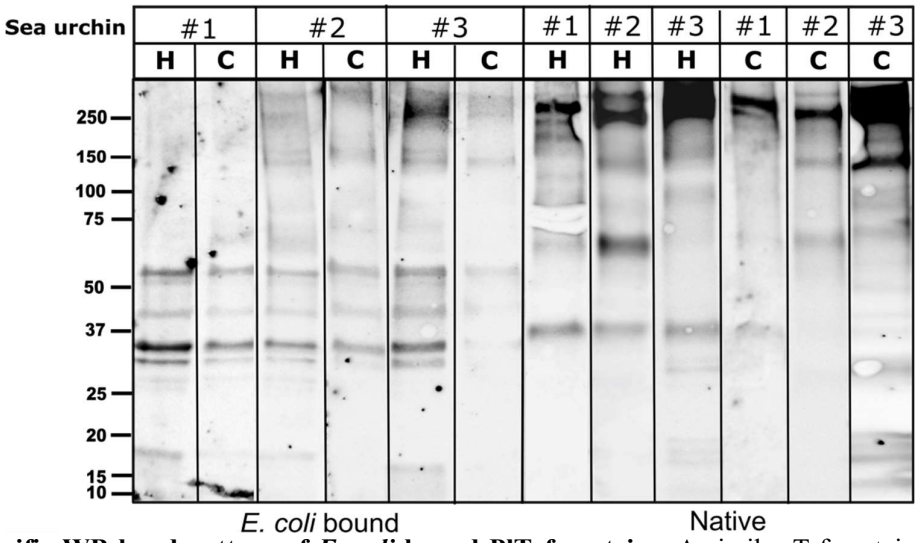

Fig. 6. Specific WB band pattern of *E. coli* bound PlTrf proteins. A similar Trf protein band pattern was observed in both coelomocytes (C) and hemolymph (H) from three sea urchins that were incubated with live *E. coli* bacteria. The band pattern of the untreated coelomocytes and hemolymph protein extract counterparts are shown on the right side of the figure.

Phagocytosis in *P. lividus* is mediated through the hemolymph and is partially inhibited by blocking surface PlTrf protein activity

Two morphological types of phagocytes were described in *P. lividus* –filopodial and petaloid phagocytes [26, 34]. The two phagocyte types are similar in size and contain long microfilamental cytoplasmatic projections of different morphology. We performed phagocytosis assays using *E.coli* in similar to [15]. Fluorescent bacteria were identified inside both *P. lividus* phagocyte types, the petaloid phagocytes were observed mostly with a single internalized bacterial cell, whereas filopodial cells mostly included multiple (up to 15) bacteria (Fig. 7A). The ratios of the phagocytes with internalized *E. coli* within the general coelomocyte population were between 1 and 3%. The hemolymph in many invertebrates plays a major role in augmenting phagocytosis. Accordingly, our results show almost two-fold higher phagocytosis rates by *P. lividus* phagocytes of bacteria that were pre-incubated with hemolymph compared to non-incubated ones (Fig. 7B, treatment A vs. B). It was demonstrated that the Trf proteins augment the phagocytosis of bacteria in *S. purpuratus* [15]. To test whether the membrane-bound Trf proteins participate in mediating phagocytosis in *P. lividus,* we used the anti-SpTrf antibody mixture to block the cell surface Trf activity. Indeed, phagocytosed bacteria were found in significantly ($p < 0.05$) lower ratios in the anti-Trf pre-treated coelomocytes (Fig. 7B, treatment C) compared with the equivalent untreated coelomocytes of the same sea urchins (n=3) (Fig. 7B, treatment A). This is in spite of the pre-incubation of the bacteria with the hemolymph in both treatments.

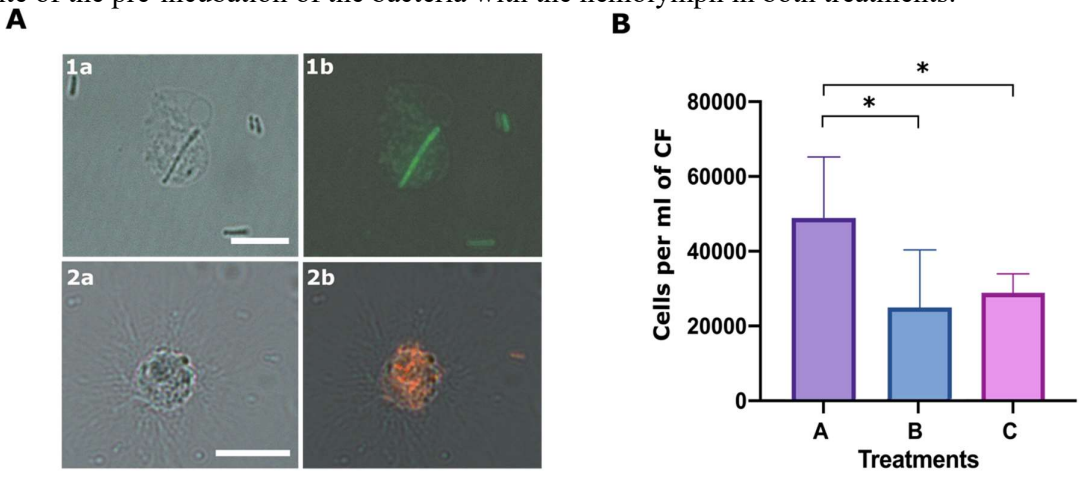

Fig. 7. Phagocytosis of *E. coli* bacteria by *P. lividus* phagocytes and its inhibition with anti-SpTrf antibody. A. *P. lividus* sea urchin phagocytes with internalized *E. coli* bacteria. 1a-b. petaloid phagocyte in the process

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with internalized FITC-stained *E. coli* bacteria. 2a-b. filopodial phagocyte with multiple mCherry-expressing *E. coli* bacteria in its cytoplasm. Scale bars = 10 µm. **B. Concentrations of *P. lividus* coelomocytes containing *E. coli* bacteria under different treatments.** A. phagocytosis ratioes in coelomocytes that were incubated with bacteria that were pre-incubated with hemolymph, B. phagocytosis ratios in coelomocytes that were incubated with untreated bacteria C. phagocytosis ratios in coelomocytes that were pre-incubated with anti-SpTrf antibodies and incubated with bacteria that were pre-incubated with hemolymph. Vertical bars indicate standard errors. Differences in phagocytosis ratios in different treatments were significant by Anova two-factor analysis. Anova single factor analysis indicated that treatment A resulted in significantly higher phagocytosis ratios compared with treatments B and C. * indicates a significant difference ($p < 0.05$).

Discussion

Immune systems are probably the most diverse systems in multicellular organisms, both among species as well as within a specific population, and even among different cells of the same individual. This is especially true for invertebrates that use many different ways to achieve protection against a great variety of pathogens in their surrounding (e.g. [35]). The Trfs are an example of unique proteins that participate in a specialized immune response in sea urchins. To date, this family was studied in *S. purpuratus* as well as in *H. erythrogramma*. In this study, we characterize, for the first time, a family of Trf genes and proteins in the Mediterranean Sea urchin *P. lividus*, a known echinoid model organism. Our results suggest that despite the differences among PlTrf, SpTrf and HeTrf gene and protein sequences, the basic features and the mechanism of action seem to be conserved. The PlTrf protein sequences included specific features like size, typical regions including the characteristic hydrophobic leader, glycine-rich and histidine-rich regions, distinguished element patterns, and repeats that were characterized for the two other species. On the other hand, the RGD cell-binding motif seems to be absent from both *P. lividus* and *H. erythrogramma* and therefore may have a specific role only in *S. purpuratus*. To be able to study the Trf-mediated response in *P. lividus*, we had to establish a strong molecular tool, which is sometimes hard to find for such a non-classical invertebrate immunity model. We, therefore, invested resources to validate the cross-reactivity of a potent anti-Trf antibodies mix that was developed by the Smith group against conserved areas of the SpTrf proteins [13]. We used the validated antibodies mix to characterize the PlTrf protein repertoire in cells and hemolymph of different individuals, for detection of PlTrf-expressing coelomocytes, FACS assays, and for the blocking of the cell surface PlTrf activity in functional phagocytosis assays.

As in other sea urchin species, the PlTrf proteins vary significantly among individuals, although they often share subsets of proteins of the same molecular weight. It was previously established that some SpTrf proteins multimerize through the glycine-rich Trf protein region in order to function [14]. The PlTrf protein multimers vary greatly in size, indicating several levels of multimerization. The Trf protein multimerization is irreversible and stable in high temperature, 2-Mercaptoethanol, and SDS treatments and that is why multimers are observed in WB. We identified two size categories of PlTrf protein monomers of approximately 20 and 35 kDa that are similar to the sizes of the SpTrf and HeTrf protein monomers. While the ~34 kDa monomer size was predicted based on the CDS sequences, the ~20 kDa monomer could not be predicted by the available full primary sequences and may represent truncated proteins. In *S. purpuratus*, truncated Trf sequences that were lacking the histidine-rich region, were referred to as potential surveillance proteins [6]. We think that this is the case with the PlTrf as it is also supported by the Ni-column isolation, in which the ~20 kDa proteins were found in the unbound fraction of one of the tested sea urchins (Fig. 2A).

Recent works about the function of the SpTrf proteins suggested that they work through both cellular and humoral effector arms in augmenting phagocytosis [15, 22]. In this study, we identified significant differences between the PlTrf protein repertoires of the coelomocytes (cellular) and the cell-free CF or hemolymph (humoral) fractions of the coelomic fluid in each of the tested sea urchins. The differences were observed in the monomers and the multimers content of the two fractions. The smaller PlTrf monomers of ~20kDa, that were consistently present in the hemolymph, were either fully or almost absent from coelomocytes. On the other hand, the ~35 kDa monomers were present in either coelomocytes, hemolymph or in both, depending on the genotype. Significant variability was observed in the PlTrf multimers content in all tested sea urchins. To better understand the functionality of each of these Trf-mediated effector routes and the possible interplay between them, we studied each of the two

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fractions separately and tested how the absence or inhibition of each of the fractions is effecting the phagocytosis process.

In contrary to the cell surface localization of Trf proteins which was restricted to small phagocytes (petaloid coelomocytes) in *S. purpuratus* [21], we identified at least five morphological sub-populations of coelomocyte with cell surface-bound PlTrf proteins. These differences between *S. purpuratus* and *P. lividus* may be due to differences in Trf activity between the species. However, they may also be due to the use of FACS to sort live coelomocytes, which enabled sensitive detection prior to imaging. An increase in membranal Trf-expressing coelomocyte ratios was recorded in *S. purpuratus* in response to LPS [13] and *V. diazotrophicus* [7, 12]. We followed a similar experimental design to first test the effect of LPS and heat-killed *E. coli* bacteria. We were surprised to see that differently than the results obtained from *S. purpurtus,* LPS did not influence the ratio of PlTrf-expressing coelomocytes. On the other hand, a sharp increase was recorded from day 1 and up to day 13 *E. coli* post-challenge, suggesting that LPS might not be the main targets of the PlTrf proteins but rather different molecules on *E. coli* surface. We also used heat-killed *V. penaeicida*, a *P. lividus* pathogen, in similar to *V. diazotrophicus,* which was used for the *S. purpurtus* immune challenge. Unexpectedly, *V. penaeicida* did not have an effect on the PlTrf expressing coelomocytes, suggesting it may escape the surveillance of the PlTrf proteins.

Specificity is a key feature in immune systems since it enables optimal recognition of pathogens within a variable environment and prevents self-distraction and autoimmunity [36]. Although this attribute is classically linked with adaptive immunity, a high degree of specificity may characterize certain types of innate immunity and innate immune receptors. The great protein diversity of the Trf family within the sea urchin population [10] and the unique single-gene expression in individual coelomocytes [7] allow a high degree of specificity. Indeed, SpTrf specificity was demonstrated in the specific gene-expression response to different elicitors [6-8, 15], the specific binding of a recombinant SpTrf protein [14] and the selective PlTrf-mediated cellular response to different types of immune challenges, as is shown here. By incubating live *E. coli* with coelomocytes or hemolymph, we profiled the specific *E. coli* bound PlTrf proteins. The *E. coli* bound PlTrf protein profiles included the same specific sets of PlTrf proteins, regardless of the genotype or the coelomic fluid fraction they were identified in.

In the last experiment, we studied the role of the Trf-mediated cellular and humoral fractions in the phagocytosis of *E. coli*. Although it was demonstrated that SpTrf proteins augment phagocytosis [15], it was still not clear what is the function of the membranal Trf proteins in this process. We found that although the coelomocytes alone (without hemolymph) are sufficient for phagocytosis, the absence of hemolymph (humoral fraction) reduces phagocytosis efficiency. We also used the anti-Trf antibody mix to block the cell surface PlTrf proteins and test whether this will affect phagocytosis efficiency. Our results demonstrate that the inhibition of membranal PlTrf activity results in significant inhibition of phagocytosis.

Taken together, this study characterizes for the first time, a diverse Trf gene/protein family in the Mediterranean Sea urchin *P. lividus*. It demonstrates the collaboration of distinct cellular and humoral Trf-mediated effector arms in mounting specific *P. lividus* immune responses, opsonization of bacteria and phagocytosis.

Materials and methods

Alignment and phylogenetic analyses of Trf sequences

The search for *PlTrf* sequences was performed in 4 TSA databases: HACU01, GCZS01, GEDS01, GFRN01 and in the Octopus database (http://octopus.obs-vlfr.fr/blast/oursin/blast_oursin.php). *S. purpuratus* and *H. erythrogramma Trf* sequences were compared to the databases using local BLAST algorithm. For the initial characterization of the Trf family in *P. lividus,* we obtained a total of 94 *PlTrf* transcript and DNA sequences, of which 70 were sequences with unique full open reading frames after the removal of UTR sequence edges. 25 sequences from each of the three species were aligned using ClustalW multiple alignments with default parameters and further manual refinement using MEGAX [29]. Phylogenetic analyses of PlTrf sequences were performed using Maximum Parsimony, Neighbor-joining and Maximum Likelihood analyses with bootstrapping of 100 repetitions. The best algorithm

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combination was inferred by MEGAX as Maximum Likelihood with Hasegawa-Kishino-Yano model [28]. Initial trees for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites (3 categories (+*G*, parameter = 2.7099)). The tree was visualized as unrooted with bootstrap value displayed in ITOL program [37]. Twelve translated *PlTrf* sequences were chosen based on their unique aa sequence to generate alignments and to represent all available element patterns.

Sea urchins

Sea urchins were obtained from the Israeli National Center for Mariculture in Eilat. Urchins were kept in artificial seawater (Red Sea Fish Pharm Ltd.) in a 165-liter aquarium in the Molecular Ecology Laboratory at the Ariel University, of which 1/10 was replaced every week. The water salinity was kept around 40 ppt and the temperature between 20 and 22°C. The animals were fed once a week with either fresh or frozen Ulva algae. Unhealthy-looking or dying sea urchins are isolated to a different aquarium to avoid contamination of healthy individuals. Experiment animals were isolated in individual floating plastic cages in a separate compartment in the aquarium.

Bacteria culturing

Escherichia coli (25922 strain) and mCherry-expressing *E. coli* were kindly provided by prof. Shiri Navon-Venezia and Ms. Helena Tuchinsky, *Vibrio penaeicida* (51842 strain) were obtained from ATCC bioresource center (USA), Commercial lipopolysaccharide (LPS) from *E. coli* was purchased from Sigma-Aldrich (Israel). *E. coli* bacteria were cultured in liquid Luria Bertani (LB) medium overnight at 37°C. *V. penaeicida* bacteria were cultured in Marine Broth media (MB) from Sigma-Aldrich (Israel) overnight at 26°C. The concentration of bacteria was either calculated based on OD values measured in a Nanodrop NP80 spectrophotometer (Implen) or manually counted with a hemocytometer. All bacteria were heat-killed for 5 min at 95°C and then washed and resuspended in aCF (10 mM CaCl2, 14 mM KCl, 50 mM MgCl2, 398 mM NaCl, 1.7 mM Na2HCO3, 25 mM Na2SO4) and used shortly after in the different experiments.

Preparation of coelomocytes and cell free CF samples

To study the *P. lividus* Trf-mediated immune response, ~300 µl of coelomic fluid was collected from the coelomic cavity of the sea urchin with a 23 G syringe filled with the same volume of cold calcium-magnesium free seawater CMFSW-EH (460 mM NaCl, 10.73 mM KCl, 7.06 mM Na2SO4, 2.38 mM NaHCO3, 70 mM EDTA, 20 mM HEPES; pH 7.4) and kept on ice until processing. The CF was then centrifuged at 500 g at 4℃ for 5 min. The supernatant was carefully collected without disturbing the cell pellet and transferred to a separate tube. The supernatant was centrifuged again at the higher speed of 2000 g at 4℃ for 5 min, to obtain the cell-free CF. The cells (the pellet of the first centrifugation) were carefully resuspended and washed in CMFSW-EH. The cell-free CF and the hemolymph-free coelomocytes were separately used for WB and phagocytosis assays.

Protein extraction

The whole coelomic fluid, the cell-free CF and the coelomocytes were separately lysed using RIPA buffer (Thermo Fisher Scientific, USA). The lysate was incubated for 30 min at room temperature with protease inhibitor cocktail III (Calbiochem) with constant vortexing. Alternatively, cells were sonicated for 5 min. Cell debris was pelleted by centrifugation for 10 min at 12,000 rpm at 4℃. The supernatant was stored as sample aliquots at −80°C until use. To equalize the protein quantities before loading on the WB gels, protein concentrations were measured using Bradford assay and with an NP80 spectrophotometer (Implen).

His60 Ni-column binding assay

The His60 Ni-column binding assay was performed according to [6]. The whole coelomic fluid was mixed with 2 mL of lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole, adjusted pH to 8.0 with NaOH, supplemented with 1 mg/ml lysozyme). The mixture was sonicated for 5 minutes and incubated at room temperature for 10 min. The lysate was centrifuged for 30 min at 10,000 g at 4˚C, and the supernatant was collected. 2 mL of His60 Ni Superflow Resin (Takara Bio, Japan) was added to a gravity column, followed by 10 min incubation with 4 mL equilibration buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM Imidazole in water, adjusted pH to 8.0 with NaOH). The lysate supernatant was added to the column and incubated with resin for 1h at 4˚C with gentle shaking after which it was drained and

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discarded. Unbound proteins were collected with 4 volumes of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 30 mM Imidazole in water, Adjusted pH to 8.0 with NaOH) that ran through the column. Ni-bound proteins were then eluted with 5 volumes of elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM Imidazole, water, Adjusted pH to 8.0 with NaOH). The washed (unbound) and eluted proteins were further used for WB and MS.

Western blots

Samples with equal quantities of whole protein extracts were mixed with X4 Laemmli sample buffer (Bio-Rad). The samples were then incubated at 90˚C for 5 min, and 10-30 µg of protein was loaded on 10% or 12% SDS-PAGE gels. Gel electrophoresis was performed in a fresh running buffer (10 x 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Gels were subjected to semi-dry transfer with Trans-Blot Turbo™ System (Bio-Rad) onto commercial PVDF membranes (Bio-Rad) with the preprogrammed Bio-Rad 1.5 mm gel transfer program. The membranes were blocked in BSA-TBST blocking solution (5% BSA in 20 mM Tris, 150 mM NaCl, 0.1% Tween20) for 1h. Blocking solution was then replaced with 2.5% BSA-TBST solution containing 1:10000 of primary Rabbit anti-SpTrf antibodies and kept overnight at 4˚C. The membranes were washed 5 times for 5 min with TBST and then incubated in TBST containing 1:20000 HRP Goat anti-Rabbit antibody (Abcam) for 1h. Pictures were obtained by Quantity One or with ChemiDoc imaging systems (Bio-Rad) and processed with ImageJ (FIJI) or the Image Lab (Bio-Rad) softwares.

Mass spectrometry

The nickel-bound (eluted) and washed protein fractions from the Nickel column were run in two identical batches on an SDS-PAGE gel. The gel was cut according to the batches. The first gel half was used for the identification of the band sizes by WB. The second gel half was used to cut gel slices that corresponded to high molecular Trf-positive bands that were identified in the WB. An empty gel slice from the gel corner was used as a background control. Gel slices were further processed for MS as follow: The bands were subjected to in-gel tryptic digestion, followed by a desalting step. The resulting peptides were analyzed using Waters HSS-T3 column on nanoflow liquid chromatography (nanoAcquity) coupled to high resolution, high mass accuracy mass spectrometry (Q Exactive Plus). The samples were analyzed on the instrument in a discovery mode. The data was processed using Proteome Discoverer version 2.4.1.15, searched against custom databases, to which a list of common lab contaminants was added. The search was done with the Byonic search algorithms using the following modifications: fixed modification of cysteine carbamidomethylation, and variable modification of methionine oxidation, asparagine- or glutamine-deamidation, and protein N-terminal acetylation. One combined database was generated from the custom tailored subjected databases: Euechinoidea_proteins_collective (53221 protein entries of all identified to the date proteins for Euechinoidea subclass uploaded from NCBI), P_lividus_translated_EMBOSS (raw translated by all 6 frames peptides untreated while removing all ‘X’ and ‘*’ signs of the stop codons) (468 entries), Trf_P_lividus_cleaned (53 entries of cleaned manually confirmed sequences).

Bacterial binding essay

For characterizing the cell-free CF and coelomocyte Trf protein repertoire that binds live *E. coli* bacteria we performed a binding assay. 10^9 live *E. coli* was incubated with either live *P. lividus* coelomocytes or cell-free CF for 30 min at room temp with gentle shaking. After the incubation, coelomocytes were washed 3 times for 5 min with CMFSW-EH. Bacteria that were incubated with cell-free CF were pelleted at 10000 g for 5 min and washed 2 times for 5 min. Both coelomocytes and bacteria were lysed and used for Trf protein profiling by Western blot as described above.

Immune challenge

For the immune challenge, we injected 100µl aCF containing 10^6 heat-killed (95˚C for 5 min) *E. coli* or *V. penaeicida* bacteria or 1 µg of LPS per ml of sea urchin CF. The CF volume was calculated according to (weight of animal (g) x 0.22 = ml whole coelomic fluid (wCF)) [38]. Equivalent volumes of aCF were injected in the control animals. Before the injection untreated animals CF was taken for measuring the base level of Trf expression.

FCM and FACS

For flow cytometry (FCM) analysis, we used challenged and unchallenged animals at day 0, 1, 2, 14, 30, 43 post-challenge. The coelomic fluid was collected as described above. All steps were

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performed on ice. Cells were counted and adjusted to 10^6 cells/ml. Coelomocytes were washed as described above and pelleted by centrifugation at 500 g for 5 min at 4°C. The pellet was gently resuspended in 100µl of a staining medium (3.3X PBS with 20 mM HEPES, 2% fetal calf serum and 0.09% Sodium Azid-NaN3, pH 7.4) containing 1:100 concentration of three polyclonal SpTrf antibodies mixture provided by prof. L. Courtney Smith (1:300 of each polyclonal antibody). Control samples were resuspended in a staining medium without the antibody. Samples were incubated for 30 min on ice in the darkness to allow antibody binding. Then cells were pelleted again, resuspended, and washed in 500µl staining medium. The washed cells were resuspended in 30 µl of staining medium with the 1:250 goat anti-rabbit secondary antibody conjugated to Alexa Fluor 488 (Thermo Fisher Pierce) and incubated on ice for 30 min in the darkness. Cells were pelleted and resuspended in 500 µl staining medium containing 1µg/ml DAPI for the exclusion of dead cells and subjected to the FCM evaluation or FACS sorting. The evaluation was done on the Calibur flow cytometer (Becton Dickinson, USA) and analyzed by FlowJo software (FlowJo LLC, USA). Sorting was done using MA900 cell sorter (Sony, Japan). Sorted live Trf-positive cells were imaged with Nikon Eclipse fluorescent microscope and with ZEISS LSM900 confocal microscope.

Phagocytosis essay

Cells and cell-free CF from three sea urchins were used in the phagocytosis assay. The cells were washed two times with cold staining medium (3.3X PBS with 20 mM HEPES, 2% fetal calf serum and 0.09% Sodium Azid-NaN3, pH7.4) and pelleted at 500g and 4°C. Washed cells of each animal were divided into three aliquots and kept in the staining medium on ice until use. For detection of phagocytosed bacteria, we used either FITC-stained or mCherry expressing *E. coli* bacteria. Bacteria were diluted to a concentration of 10^7 per ml and divided into three groups (A, B and C). Group A and C were incubated for 40 min with sea urchin hemolymph at 16°C in the dark. Groups B bacteria remained untreated. Group C bacteria was additionally incubated with antiSpTrf polyclonal antibodies mixture in 1:100 ration in 100 µl for 30 min in the dark. All bacteria samples (A, B, C) were then mixed with the corresponding coelomocyte aliquots of each of three sea urchins and incubated in the dark for 70 min. After the incubation, cells were shortly pelleted and loaded to the hemocytometer for the visual evaluation of phagocytosis using fluorescent microscope Nikon Eclipse with standard fluorescent lamp and company filters, Olympus (model IX81, with company filters). Phagocytosis events were blindly counted (without knowing the type of treatment), using 16 middle squares of the hemocytometer (n=3) for A, B and C phagocytosis treatments. Differences in the number of phagocyted cells in different treatments were assessed by Anova two-factor and one-factor analyses.

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Supplementary material

Fig. S1 Unique Trf sequences obtained from publicly available databases.

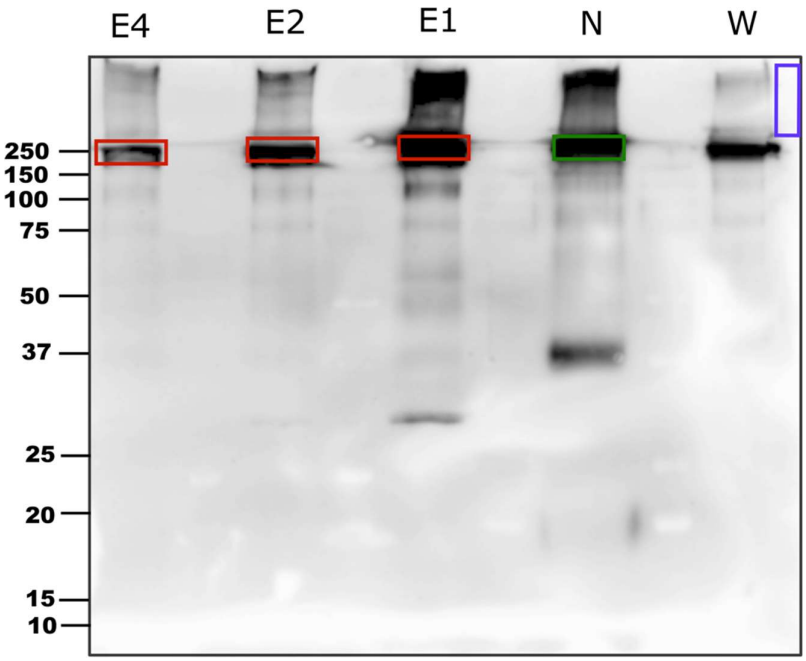

Fig. S2 Western blot of the gel that was used for the mass spectrometry. E1, E2, E4 – elution fractions, N – untreated native whole coelomic fluid protein, W – wash fraction. Inside boxes are the bands that were cut from PAGE gel. In red – elution fractions, in green – native protein from the same animal, in blue – the background control that was cut from the side of the gel.

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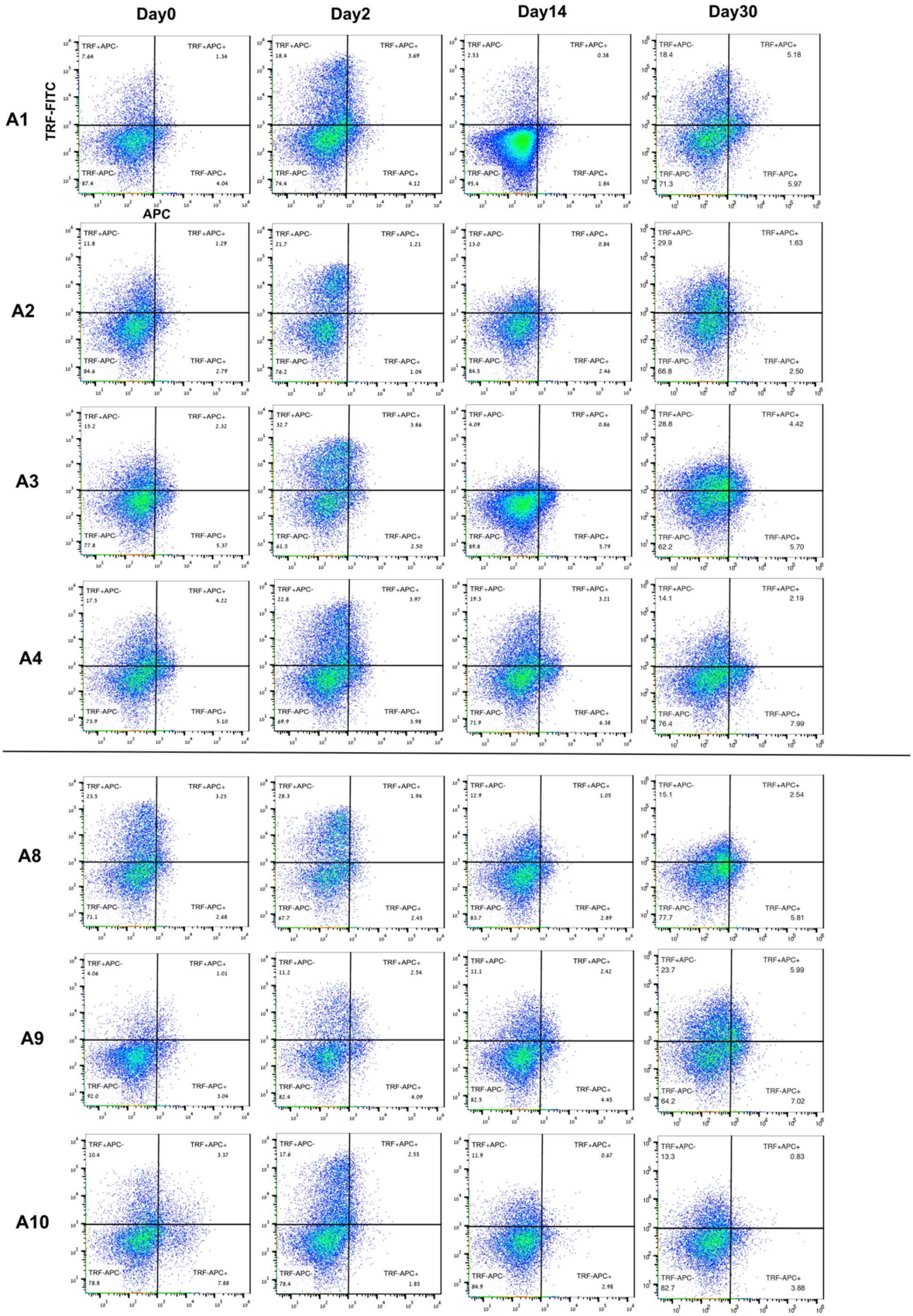

Fig. S3 Trf-positive cell population change over time after injection of either *V. penaeicida* or Acf. A1-A4 – animals challenged with heat-killed *V. penaeicida*, A8-A10 - control animals injected with sterile aCF. Y-axis Trf labeled with AF488, X-axis autofluorescence in APC channel indicates red spherule cells.

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Table S1 accessions of the Trf sequences used to build the phylogenetic tree.

*H. erythrogramma**		*S. purpuratus*s*		*P. lividus***	
JX245053.1	HE001	KJ408453.1	SP001	HACU01465650.1	PL001
JQ780274.1	HE002	KJ408451.1	SP002	HACU01465648.1	PL002
JX245045.1	HE003	KJ408450.1	SP003	HACU01465646.1	PL003
JQ780273.1	HE004	KJ408449.1	SP004	HACU01465645.1	PL004
JX245024.1	HE005	EF607775.1	SP005	HACU01465644.1	PL005
JX245021.1	HE006	EF607759.1	SP006	HACU01465643.1	PL006
JQ780304.1	HE007	EF607755.1	SP007	GFRN01311981.1	PL007
JX245020.1	HE008	EF607753.1	SP008	GFRN01311979.1	PL008
JQ780312.1	HE009	EF607742.1	SP009	GFRN01311974.1	PL009
JQ780311.1	HE010	EF607719.1	SP010	GFRN01311972.1	PL010
JQ780305.1	HE011	EF607717.1	SP011	GEDS01012457.1	PL011
JQ780303.1	HE012	EF607713.1	SP012	GEDS01012456.1	PL012
JQ780287.1	HE013	EF066272.1	SP013	GEDS01012454.1	PL013
JQ780284.1	HE014	EF066267.1	SP014	GEDS01012453.1	PL014
JQ780276.1	HE015	EF066259.1	SP015	GEDS01012452.1	PL015
JQ780275.1	HE016	EF066253.1	SP016	GEDS01012451.1	PL016
JQ780271.1	HE017	EF066219.1	SP017	GEDS01012450.1	PL017
JQ780270.1	HE018	EF066159.1	SP018	GEDS01012448.1	PL018
JQ780269.1	HE019	EF065743.1	SP019	GEDS01012447.1	PL019
JQ780268.1	HE020	EF065742.1	SP020	GEDS01012446.1	PL020
JQ780266.1	HE021	EF065733.1	SP021	GEDS01012445.1	PL021
JQ780265.1	HE022	DQ183180.1	SP022	GEDS01012443.1	PL022
JQ780262.1	HE023	DQ183182.1	SP023	GCZS01069164.1	PL023
JQ780244.1	HE024	DQ183168.1	SP024	GCZS01069162.1	PL024
JQ780233.1	HE025	DQ183167.1	SP025	GCZS01069159.1	PL025

* *P. lividus*, *S. purpuratus* and *H. erythrogramma* Trf sequence accession numbers from NCBI protein database
**P. lividus* sequences from TSA NCBI databases (HACU01, GCZS01, GEDS01, GFRN01)

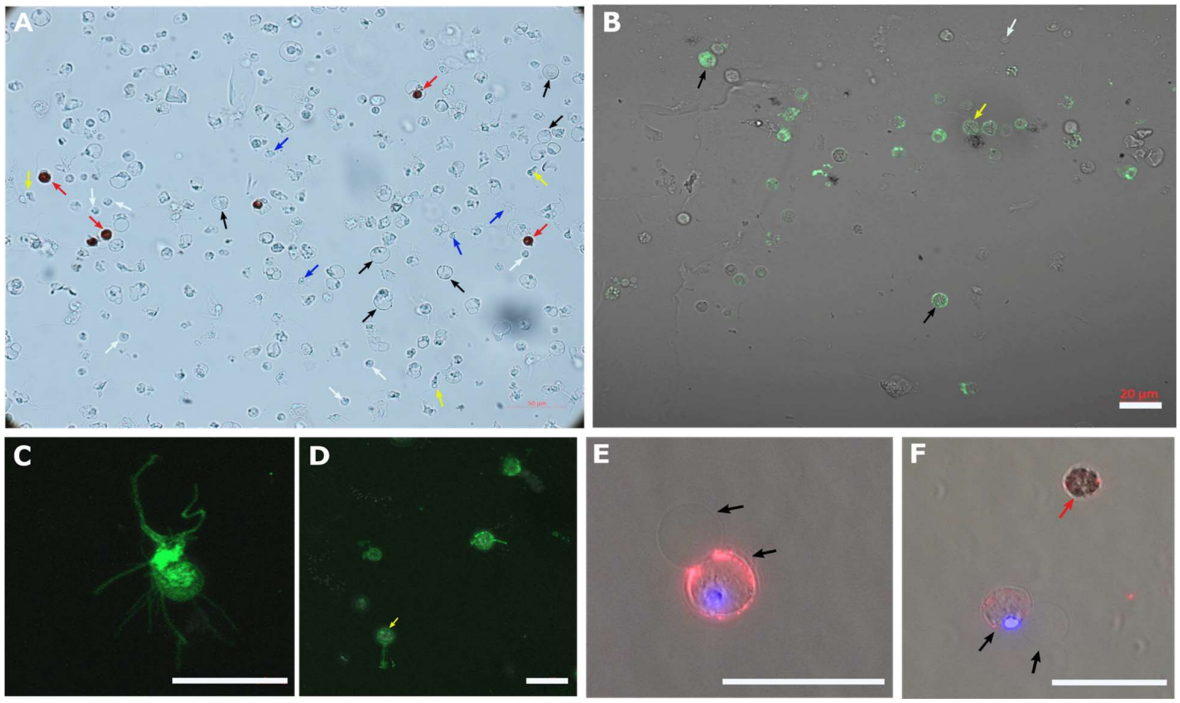

Fig S4. *P. lividus* coelomocyte types and sorted Trf-positive coelomocytes. A. *P. lividus* unsorted coelomocytes in whole CF shown in a bright field. **B.** Sorted Trf-positive cells - petaloid phagocytes and a vibratile cell labeling is clearly seen, other coelomocyte types have a weaker signal. **C.** Trf protein expression on

the cell surface and filopodia of a filopodial phagocyte (confocal microscopy) **D.** Trf-positive *P. lividus* vibratile cell (yellow arrow) (confocal microscopy). **E and F.** Membranal interactions of Trf-positive and Trf-negative petaloid phagocytes (black arrows). Scale bars = 20 μm. Blue arrows - filopodial phagocytes. Red arrows- red spherule cells. Yellow arrows - vibratile cells. Green fluorophore — 2nd Ab AlexaFluor 488. Red fluorophore - 2nd Ab AlexaFluor 567. Blue is DAPI.

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