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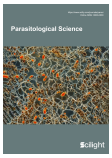

Article

Cloning and Expression of a Molecule with Immunomodulatory Potential, a Cysteine Protease Inhibitor from *Toxocara canis*

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Abstract: Parasites are known for their ability to escape from the immune system of their hosts. One of the strategies is the release of specific molecules with high immunomodulatory activity. Cysteine protease inhibitors (cystatins) have been shown to play an essential role in suppressing the host's immune response. Cystatins have been found in many species of parasites from different taxa. Herein, we report cloning and expression of the cysteine protease inhibitor, *Tc*-CPI-2, from dog roundworm (*Toxocara canis*) infective L3 larvae, a causative agent of human toxocarosis. Sequence analysis revealed two specific cystatin-like domains, the Q-x-V-x-G motif and the S-N-D motif. Phylogenetic analysis indicated that *Tc*-CPI-2 belongs to type 2 cystatin subfamily. The recombinant cystatin was expressed in *Escherichia coli*, purified, and used to stimulate human THP-1 macrophages in vitro and mouse splenocytes ex vivo. Cell treatment with the recombinant molecule induced significant cytokine secretion changes, suggesting that it may be responsible for the Th2/Th1 immune response switch in *T. canis*-infected mice. Altogether, our results prove that *Tc*-CPI-2 is a molecule with promising immunoregulatory and therapeutic potential.

Keywords: cystatins; immunomodulation; toxocarosis; recombinant molecules

1. Introduction

The cystatins are a family of cysteine protease inhibitors. These proteins are widely distributed and found in the plant and animal kingdoms, including mammals, nematodes, and arthropods. They participate in various biological processes by regulating proper proteolysis and are also involved in the development of autoimmune diseases, cerebral amyloid angiopathy and hereditary brain haemorrhage, atherosclerosis and aortic aneurysms multiple sclerosis, and liver dysfunction [1]. Cystatins are primarily located in endosomes and lysosomes, but they can also be found in the nucleus, cytosol, cell membrane, or secreted from cells [2].

According to the MEROPS database of peptidases and their inhibitors [3], cystatins belong to the I25 family, which is divided into three subfamilies. Type 1 cystatins (also termed stefins) are low molecular weight cytoplasmic proteins that do not contain disulfide bonds or glycosylation sites. Stefins inhibit papain-like peptidases of the C1 family, and human cystatin A is a representative prototype for this family [4,5]. Type 2 cystatins are secretory proteins of about 13–15 kDa, and most cystatins, also those produced by parasites, belong to this subfamily. Chicken ovocystatin is a representative molecule for this group of proteins [4]. These cystatins have several characteristic elements such as an N-terminal signal peptide, 2 conserved disulfide bonds, and the cysteine protease interaction site formed by a conserved glycine residue at the N-terminal region, central Q-X-V-X-G motif, and a C-terminal PW hairpin loop [5]. Type 2 cystatins act as inhibitors of family C1 peptidases, but

some of these molecules can inhibit the legumain-like peptidases of C13 family. This activity is due to the presence of a distinct conserved S-N-D/V motif [6]. Type 3 cystatins are divergent, high molecular mass proteins (>60 kDa) and include kininogens, fetuins, and histidine-rich proteins, and apart from kininogens, the proteins from this subfamily appear to lack cystatin activity [7].

The first nematode parasite cystatin identified in 1992 was onchocystatin (*Ov*-17), a highly-antigenic protein from *Onchocerca volvulus* [8]. Since then, cystatins were discovered in many species of parasitic protozoans, trematodes, nematodes, and arthropods [9–14]. The key role of parasite cystatins is the regulation of oogenesis, moulting, and migration through host tissues, thereby promoting parasite development [5]. Apart from their role in physiological processes, the cystatins have been recognized as one of the major molecules responsible for immunoevasion mechanisms [15,16].

First studies describing such mechanisms concern a cystatin from a model filarial species, *Acanthocheilonema viteae* (*Av*-17), which directly inhibits the proliferation of T cells and stimulates the production of IL-10 [17]. A similar effect on T cells is caused by *Ov*-17 from. *O. volvulus*, which also inhibits the expression of MHC class II and co-stimulatory molecule CD86 on monocytes [18]. A cystatin from another filarial species, *Brugia malayi Bm*-CPI-2, was shown to inhibit the activity of asparaginyl endopeptidase (AEP), an antigen processing enzyme in B lymphocytes [12], which proved that parasite cystatins can directly interfere with antigen presentation. Later studies confirmed that cystatins modulate the production of cytokines by regulation of ERK, MAPK, and p38-dependent pathways in macrophages, which results in IL-10 upregulation and induction of regulatory macrophages [19]. Finally, Acevedo et al. showed that a cystatin from *Ascaris lumbricoides* roundworm (*Al*-CPI) induces differential expression of over four hundred transcripts in monocyte-derived dendritic cells (mo-DC) [20]. Some of these transcripts encoded members of the TGF-β and the IL-10 families, others were involved in the TNF-α signaling pathway, and the regulation of the mevalonate pathway and cholesterol biosynthesis.

The first prediction of cystatin presence in *T. canis* secretome was reported in 2015 by Zhu et al., who published the genome and transcriptome sequences [21]. *Toxocara canis*, the dog roundworm, is a nematode present worldwide and is characterized by high zoonotic potential. In humans serving as paratenic hosts, parasites from *Toxocara* species cause the toxocarosis disease, with an estimated global seroprevalence rate of 19.0% [22]. After ingestion of the infective stages (embryonated eggs or larvae encapsulated in improperly cooked animal tissues), larvae hatch in the small intestine, migrate through the intestinal wall, and enter the circulatory system and migrate throughout the body [23]. Their presence in various organs leads to a marked inflammatory response, causing four clinical symptoms: visceral larva migrans (VLM), ocular larva migrans (OLM), neurotoxocarosis, and covert toxocarosis [24]. Although many aspects of host-parasite molecular interactions have been described [25], there are still many gaps in our understanding of the pathogenesis of toxocarosis and the immunomodulatory mechanisms induced by the parasite. The possible role of cystatins during *T. canis* infection is one of these insufficiently explored lines of research.

Here we describe cloning of a novel *T. canis* cysteine protease inhibitor (*Tc*-CPI-2) and its expression in *E. coli* cells. We have also analysed the immunomodulatory potential of the recombinant molecule. The in vitro and ex vivo experiments proved that *Tc*-CPI-2 can affect the production of cytokines by human macrophages and splenocytes isolated from naïve and *T. canis*-infected mice.

2. Materials and Methods

2.1. Toxocara canis L3 Larvae Collection

Adult *T. canis* worms were collected from the feces of dewormed dogs treated in veterinary clinics. Eggs were obtained from dissected female worms and incubated in 1% paraformaldehyde at room temperature for approximately 3–4 weeks. The embryonation process was monitored microscopically. Fully embryonated eggs were hatched in vitro using the protocol described by Oaks and Kayes [26] with some modifications. Eggs were washed three times in sterile 0.85% NaCl, followed by centrifugation (500× *g*, 5 min.). The egg pellet was resuspended in a 10% commercial bleach solution in 0.85% NaCl and incubated for 15 min on a roller shaker at room temperature. After centrifugation, eggs were washed two times in 0.85% NaCl and two washes in Minimal Essential Medium (Sigma-Aldrich, St. Louis, MI, USA) supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2.5 µg/mL) (Biowest, Nuaillé, France) (MEM/A). After washing, eggs were suspended in MEM/A, placed on a 40 µm nylon mesh strainer in a 6-well tissue culture plate, and incubated (37 °C, 5% CO_2). After three days, hatched larvae were collected from the bottom of the plate, centrifuged, and used for RNA isolation.

2.2. cDNA Cloning and Bioinformatic Analysis

Total RNA was isolated from *T. canis* L3 larvae using the Total RNA Mini purification kit according to the manufacturer's protocol (A&A Biotechnology, Poland). One microgram of RNA was then reverse transcribed into cDNA with RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania) and used as a template for coding sequence amplification. PCR primers (F: ATGCATACAGCAACGAAGGGACTGT and R: TTACGACGCTTCCTTGATCGCCTTG) were designed based on the *T. canis* whole genome shotgun sequence (GenBank acc no JPKZ01003225) locus tag Tcan_07240. The amplified cDNA fragment was cloned into pGEM-T-Easy vector (Promega, USA) and sequenced in a commercial service (Genomed, Poland).

The DNA and corresponding amino acid sequence were analysed by Basic Local Alignment Search Tool (BLAST) using sequences from the National Center for Biotechnology Information (NCBI) database [27,28]. Protein sequence alignments were conducted using Clustal Omega [29,30]. SignalP was used to predict the signal sequence length [31]. Net-O-glyc was used to analyse potential glycosylation sites [32,33]. The three-dimensional model was generated using Phyre2 [34,35].

2.3. Protein Production and Purification

The sequence encoding the mature protein lacking the signal peptide was amplified with the following linker primers: F: GGATCCGGAAGGGATGGTCGGTGGT and R: CTCGAGCGACGCTTCCTTGATCGC and cloned in frame into pET28b expression vector using BamHI and XhoI restriction enzymes. *Escherichia coli* SoluBL21 strain was used to express the recombinant protein. The presence of the recombinant protein in the soluble and insoluble fractions was analysed with SDS-PAGE and western blotting with His-tag specific antibodies (Sigma-Aldrich). The protein was purified using HisTrap FF columns (Cytiva, Uppsala, Sweeden) according to the protocol. The purity of elution fractions was monitored with SDS-PAGE.

After purification, the recombinant protein was dialysed into PBS buffer using the Zeba™ Spin Desalting Columns (7 K MWCO, 2 mL, Thermo Scientific, Waltham, MA, USA). After that, endotoxins were removed using Triton X-114 according to the method described by Teodorowicz et al. [36]. The remains of Triton X-114 were removed from the solution with Bio-Beads SM-2 nonpolar polystyrene adsorbents (BioRad, Hercules, MA, USA) according to the manufacturer's protocol. The effect of endotoxin removal was monitored with Pierce™ Chromogenic Endotoxin Quant Kit (Thermo Scientific, USA). The protein concentration was measured with Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA).

2.4. In Vitro Cell Culture

The THP-1 human monocyte cell line was purchased from Merck (Germany). The culture protocol was adapted from previous studies [37,38]. Cells were maintained in RPMI 1640 culture medium supplemented with 10% foetal bovine serum (FBS), 2-mM glutamine, 100-U/mL penicillin, 100 µg/mL streptomycin, and amphotericin B (2.5 µg/mL) (all from Biowest, France) at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were seeded into 24-well plates at a concentration of 5×10^5/mL in a 1 mL/well volume. The cells were differentiated into macrophages by the addition of 100 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, USA) for 48 h. After differentiation, the cells were washed three times with sterile PBS and stimulated with three different concentrations of *Tc*-CPI-2 (0.01, 0.1 and 1 µg/mL). An equal volume of PBS buffer was added to control cells. Additionally, cells were pre-stimulated for 2h with lipopolysaccharide (LPS) (Sigma Aldrich) (100 ng/mL) and then treated with *Tc*-CPI-2 or pre-stimulated with *Tc*-CPI-2 and, after 2h, treated with LPS. Cells treated only with LPS served as controls. Four culture replicates were performed. The culture media were collected after 24 h and stored at −20 °C until use.

2.5. Ex Vivo Splenocyte Culture

Splenocytes used in this study originated from an experimental study described elsewhere (Lekki-Jóźwiak et al., 2025, under submission) [39]. Briefly, C57BL6/J female mice were infected with 1000 *T. canis* eggs and sacrificed at 7 and 28 days post-infection, which represent the early and established infection stages, respectively. Uninfected mice served as controls. Each group consisted of five animals. The study was conducted with the approval of the Second Local Ethical Committee at Warsaw University of Life Sciences (Approval no. WAW2/094/2022) and following the local governmental guidelines.

After isolation from mouse spleens, splenocytes were suspended at a concentration of 5×10^6/mL in RPMI 1640 medium containing 10% FBS, 100 µg/mL gentamycin, and 0.5 mM β-mercaptoethanol. The suspension was transferred to a 96-well culture plate (200 µL per well) and stimulated with *Tc*-CPI-2 (0.1, 1, and 10 µg/mL).

Unstimulated cells were used as controls. Splenocytes were incubated at 37 °C with 5% CO_2 for 72 h. The culture medium was collected and stored at −20 °C.

2.6. Cytokine Measurements

The concentration of CXCL-8, IL-1β, IL-6, IL-10, and TNF-α was measured in THP-1 culture supernatants using Human DuoSet ELISA kits (R&D Systems, Minneapolis, MI, USA). Levels of IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-17, and TNF-α were measured in splenocyte supernatants using Mouse DuoSet ELISA kits (R&D Systems, USA). The absorbance was measured with a Synergy H1 ELISA microplate reader (BioTek Instruments, Charlotte, VT, USA) at 450 nm, with corrections made at 570 nm. A four-parameter logistic (4-PL) curve was generated, and cytokine concentrations were calculated using BioTek Gen5 software version 3.12 (Agilent Technologies, Santa Clara, CA, USA).

2.7. Statistical Analysis

The statistical analysis was performed as follows: the normality of the data distribution was confirmed using the Shapiro-Wilk test, followed by an evaluation of variance equality using the Brown-Forsythe test. Then, one-way ANOVA was performed, followed by Dunnett's post-hoc multiple comparisons test. All analyses were conducted using GraphPad Prism 9.5.1, and results were considered statistically significant at $p < 0.05$.

3. Results

3.1. Cloning, Bioinformatical Analysis and Expression of Tc-CPI-2

The amplified *Tc*-CPI-2 cDNA sequence was 423 nucleotides long and was identical to the corresponding genome sequence. The protein sequence consisted of 140 amino acids. The analysis in SignalP 5.0 bioinformatic tool revealed the presence of a signal peptide with a cleavage site between positions 23 and 24. The molecular mass of the mature protein, calculated with the Expasy PeptideMass tool, was 13.48 kDa. The protein sequence contains 15 O-glycosylation sites as predicted by NetOGlyc tool from DTU Health Tech service.

The BLAST alignment showed that *Tc*-CPI-2 shared the highest homology with a cysteine protease inhibitor from *Baylisascaris schroederi* (GenBank: UOT91678.1) and an unnamed protein product from *Enterobius vermicularis* (GenBank: VDD95074.1) (Supplementary Table S1). According to a distance tree of these pairwise comparisons, the second closest group consists of filarial cystatins (Supplementary Figure S1). The coding sequence was aligned with selected homologous cystatins from different nematode species (Figure 1).

As in other cystatins, a conserved cysteine protease interaction site was recognized within the sequence formed by the N-terminal glycine, central Q-X-V-X-G motif, and a C-terminal PW hairpin loop. The S-N-D motif responsible for the inhibition of legumain-like peptidases was also identified. Only one disulfide bond was recognized (Figure 1). The predicted three-dimensional structure is presented in Figure 2A. Interestingly, a phylogenetic tree generated with Clustal Omega tool (Figure 2B) shows that the cystatin from *T. canis* is more closely related to cystatins from *B. schroderi* and *Anisakis simplex* than to the one originating from *Ascaris lumbricoides*.

Construction of a recombinant pET28b expression vector encoding *Tc*-CPI-2 protein and its transformation into *E. coli* SoluBL21 enabled the production of significant amounts of the recombinant protein. The protein of an apparent 18–19 kDa size was expressed in both soluble and insoluble forms (Figure 3A,B). Western blot analysis with His-tag-specific antibodies revealed the presence of the *Tc*-CPI-2 dimer in the soluble fraction (Figure 3B).

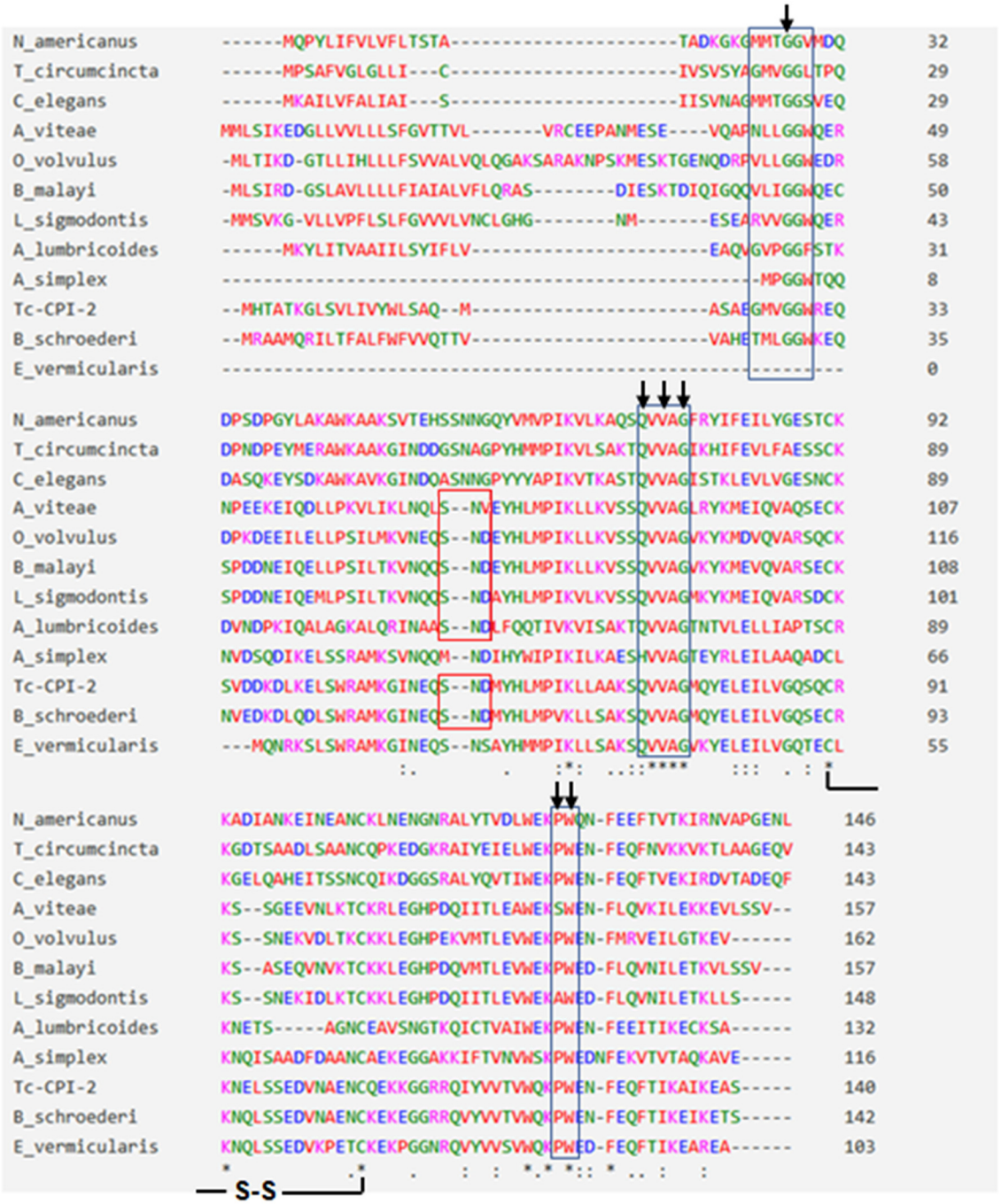

Figure 1. Nematode cystatin amino acid sequences alignment generated with Clustal Omega tool. A conserved N-terminal glycine, central Q-X-V-X-G motif, and a C-terminal PW hairpin loop are marked with arrows. The S-N-D/V motif is marked with red boxes. Two conservative cysteines forming a disulfide bond are also marked. Identical residues are indicated by (*), conserved amino acid substitutions with (:) and semi-conservative substitutions with (.). The following protein sequences were aligned with *Tc*-CPI-2; GenBank accession numbers are given in brackets: *Baylisascaris schroederi* cysteine protease inhibitor (UOT91678.1); *Enterobius vermicularis* unnamed protein product (VDD95074.1); *Anisakis simplex* unnamed protein product (VDK63755.1); *Teladorsagia circumcincta* cystatin domain protein (PIO70811.1); *Caenorhabditis elegans* cystatin CPI-2 (NP504565.1); *Brugia malayi* cystatin-type cysteine proteinase inhibitor CPI-2 (XP042938176.1); *Necator americanus* hypothetical protein (XP_064063042.1); *Litomosoides sigmodontis* Ls-cystatin precursor (AAF35896.1); *Onchocerca volvulus* Onchocystatin OV17 (P22085.2); *Acanthocheilonema viteae* cystatin (AAA87228.1); *Ascaris lumbricoides* CPI (ADR51550.1).

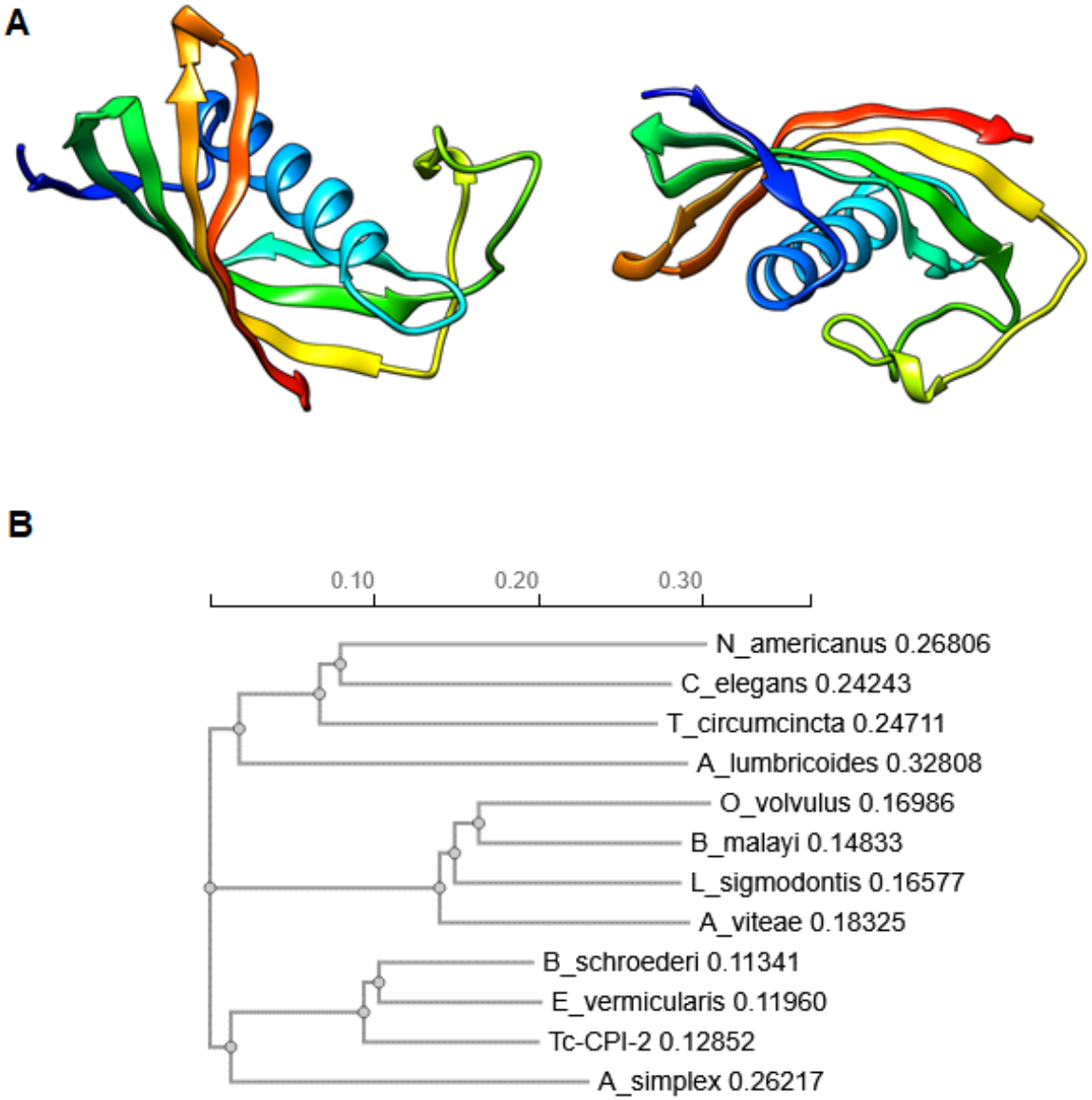

Figure 2. *Tc*-CPI-2 potential three-dimensional structure predicted using Phyre2 (**A**) and a phylogenetic tree constructed using Clustal Omega (**B**). The evolutionary distance is shown next to each branch. The following protein sequences were used to generate the phylogenetic tree; GenBank accession numbers are given in brackets: *Baylisascaris schroederi* cysteine protease inhibitor (UOT91678.1); *Enterobius vermicularis* unnamed protein product (VDD95074.1); *Anisakis simplex* unnamed protein product (VDK63755.1); *Teladorsagia circumcincta* cystatin domain protein (PIO70811.1); *Caenorhabditis elegans* cystatin CPI-2 (NP504565.1); *Brugia malayi* cystatin-type cysteine proteinase inhibitor CPI-2 (XP042938176.1); *Necator americanus* hypothetical protein (XP_064063042.1); *Litomosoides sigmodontis* Ls-cystatin precursor (AAF35896.1); *Onchocerca volvulus* Onchocystatin OV17 (P22085.2); *Acanthocheilonema viteae* cystatin (AAA87228.1); *Ascaris lumbricoides* CPI (ADR51550.1).

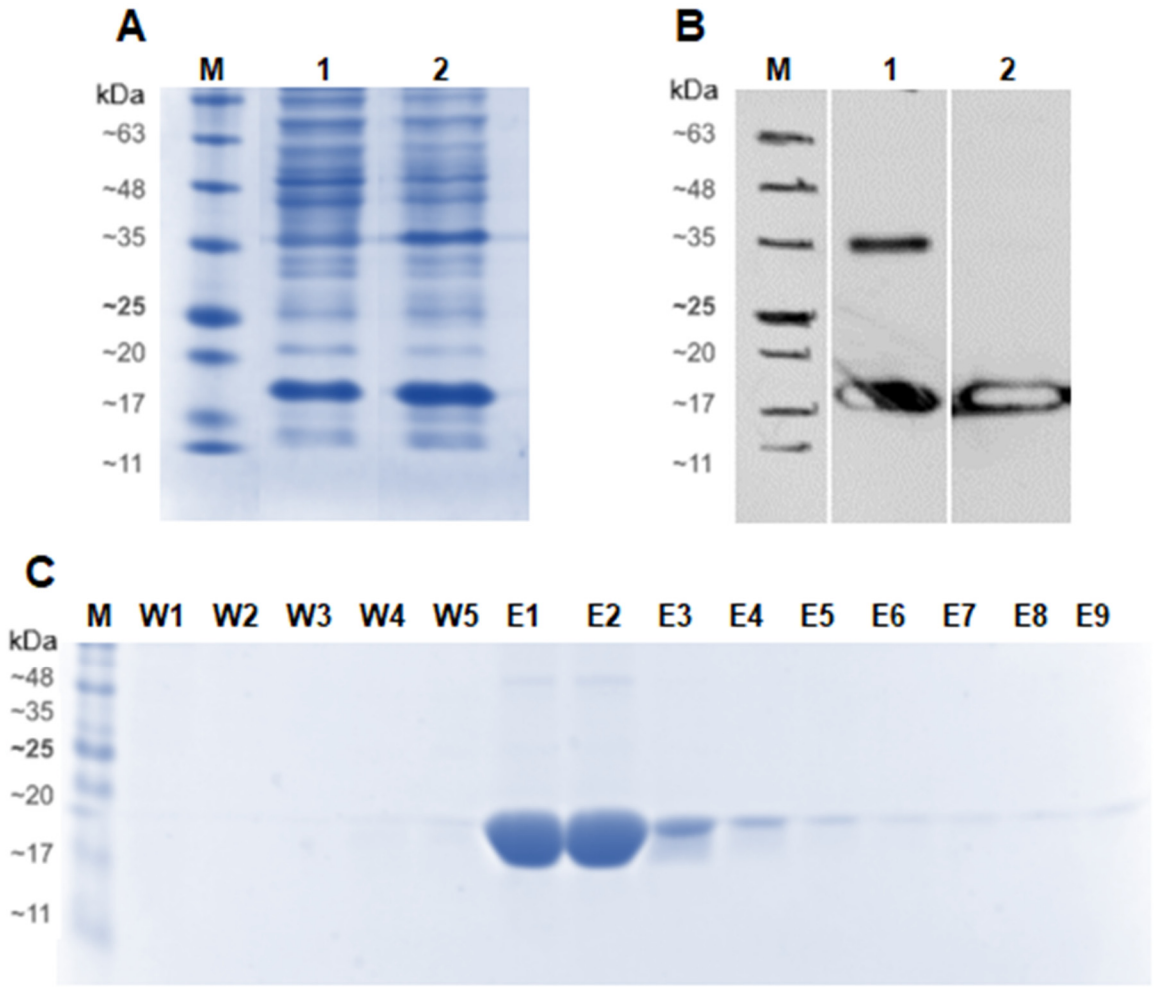

Figure 3. *Tc*-CPI-2 expression and purification. Soluble (lane 1) and insoluble (lane 2) protein fractions isolated from *E. coli* SoluBl21 were resolved in a 15% polyacrylamide gel and stained with Coomassie blue (**A**) or probed with anti-His-tag antibodies (**B**). The recombinant protein was purified using affinity chromatography. Wash (W1-W5) and elution (E1–E9) fractions were analysed by SDS-PAGE (**C**). M–Perfect Tricolor Protein Ladder (Eurx, Poland).

3.2. Tc-CPI-2 Effect on Cytokine Release by THP-1 Cells

The immunomodulatory potential of *Tc*-CPI-2 was then investigated in vitro. Stimulation of THP-1 macrophages with different concentrations of the recombinant protein resulted in significant upregulation of CXCL-8 and downregulation of TNF-α and IL-10 (Figure 4). No IL-6 was detected in cell culture media. In the second experiment, the effect of the recombinant cystatin in combination with LPS was examined. The addition of LPS alone resulted in a substantial increase in all examined cytokines in culture media compared to unstimulated cultures. Pre-treatment of macrophages with *Tc*-CPI-2 for 2 h significantly increased TNF-α secretion and decreased IL-6 release by LPS-treated macrophages (Figure 5A). No changes were observed in cytokine production when cells were first incubated with LPS then with the cystatin (Figure 5B).

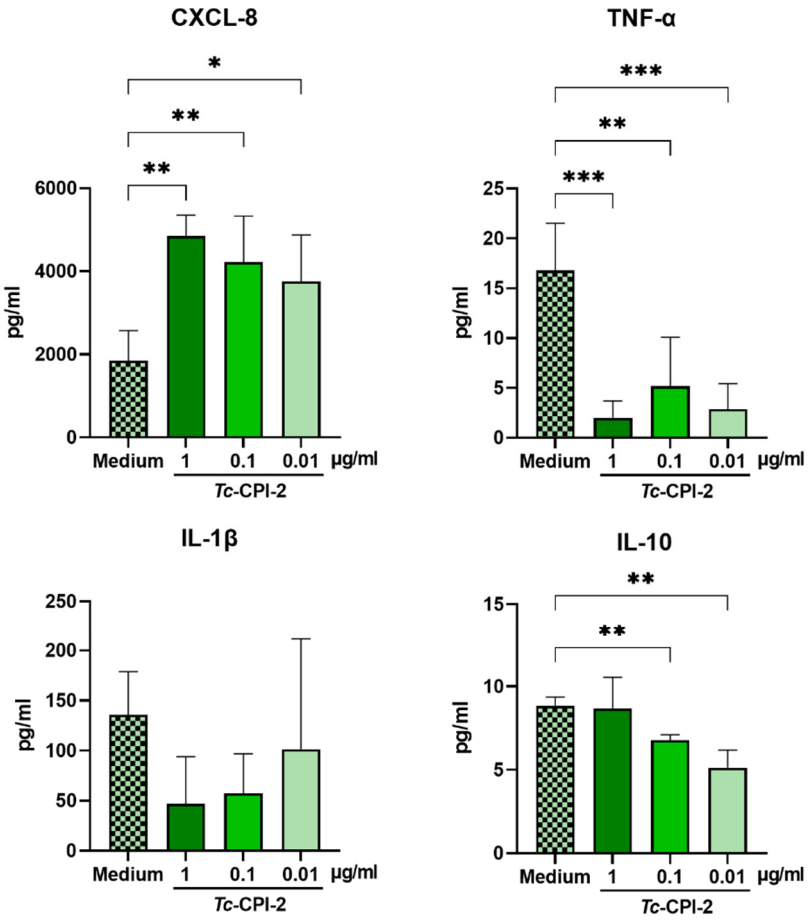

Figure 4. In vitro cytokine production by THP-1 macrophages after stimulation with *Tc*-CPI-2 recombinant protein (1, 0.1, and 0.01 µg/mL). Data is presented as mean ± SD from four technical replicates. Significant differences in cytokine concentrations compared to unstimulated controls are marked: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

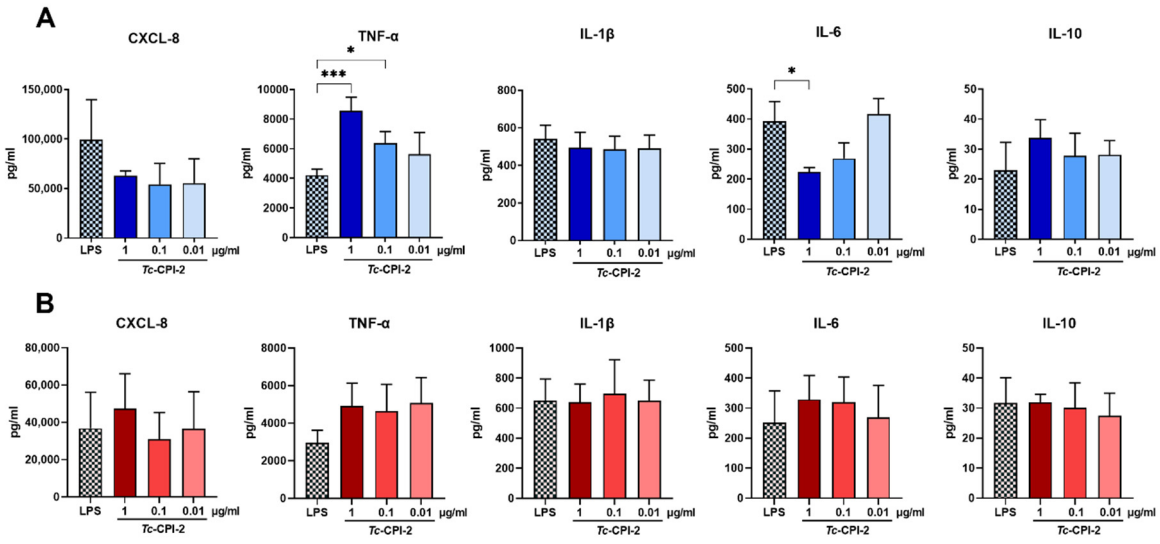

Figure 5. In vitro cytokine production by THP-1 macrophages after stimulation with *Tc*-CPI-2 (1, 0.1 and 0.01 µg/mL) in combination with LPS treatment (100 ng/mL). (**A**) Cells were pre-treated with *Tc*-CPI-2 for 2 h and then stimulated with LPS for additional 24 h. (**B**) Cells were pre-treated with LPS for 2 h and then stimulated with *Tc*-CPI-2 for 24 h. Data is presented as mean ± SD from four technical replicates. Significant differences in cytokine concentrations compared to cells treated only with LPS are marked: * $p < 0.05$, *** $p < 0.001$.

3.3. Tc-CPI-2 Effect on Cytokine Release by Splenocytes Isolated from T. canis-Infected Mice

Spleen cells were isolated from *T. canis*-infected C57BL6/j mice at 7 and 28 days post-infection (dpi). Cytokine release by unstimulated and *Tc*-CPI-2-stimulated cells was analysed compared to uninfected controls (0 dpi) (Figure 6). Cells isolated from uninfected mice responded to *Tc*-CPI-2 treatment with upregulation of TNF-α, IL-6, and IL-10. Seven days post-infection, the production of IFN-γ dropped markedly compared to uninfected mice, however cystatin addition to splenocyte cultures increased IFN-γ release. There was no effect on IFN-γ secretion at 28 dpi. In contrast, the cystatin affected the production of TNF-α at 28 dpi, resulting in significant upregulation, which was dose-dependent. At the same time, *Tc*-CPI-2 significantly downregulated the secretion of IL-5 (at 10 µg/mL) and IL-6 (at 0.1 and 1 µg/mL) by spleen cells. The production of IL-10 after cystatin treatment increased at 7 dpi and decreased at 28 dpi. Neither IL-4 nor IL-17 was noted at any time point of the experiment.

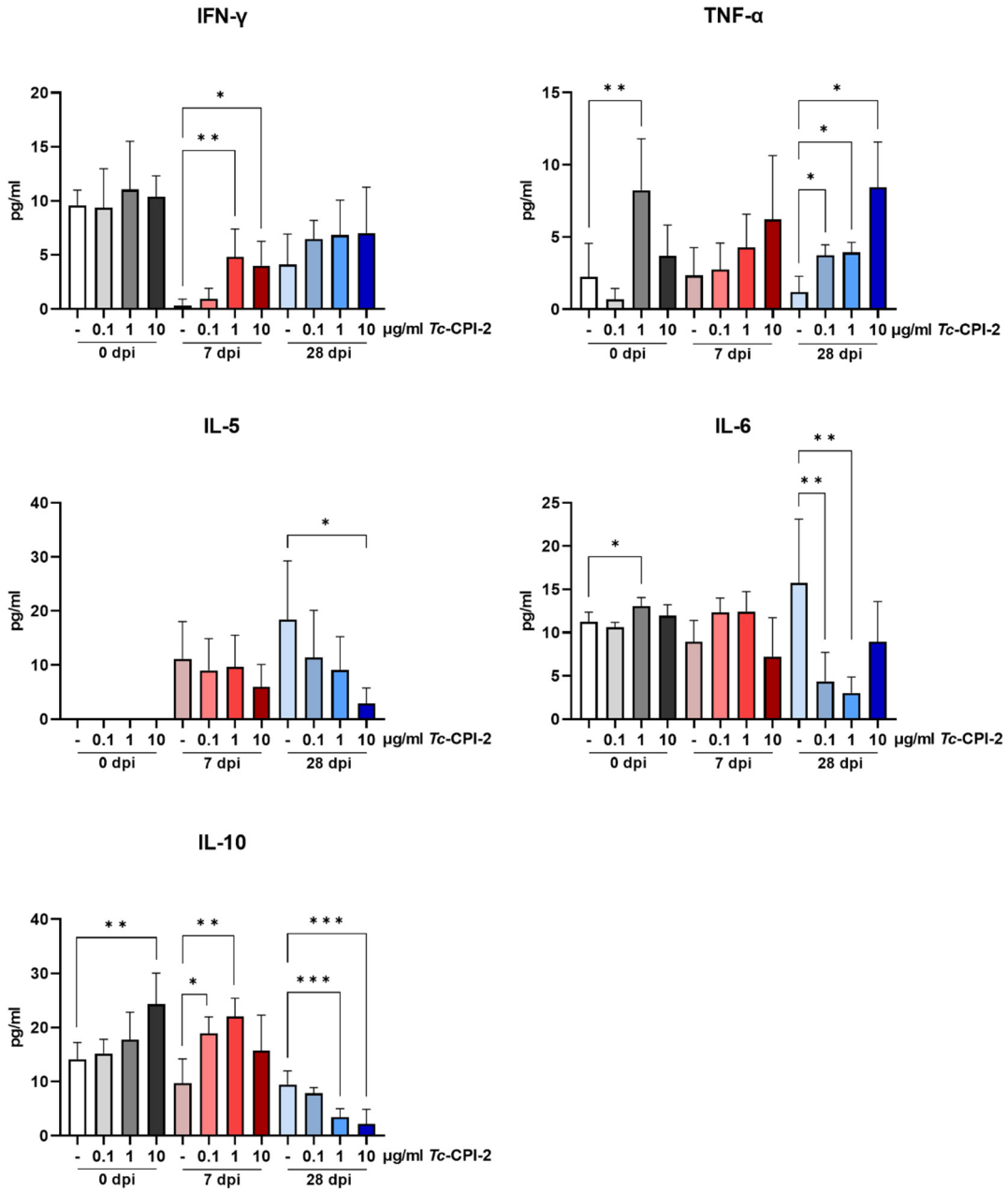

Figure 6. Ex vivo cytokine production by spleen cells isolated from *T. canis*-infected and control mice after stimulation with *Tc*-CPI-2 (0.1, 1, and 10 µg/mL) for 72 h. Mice were sacrificed before infection, 7, and 28 days post-infection (0, 7, and 28 dpi, respectively). Data is presented as mean ± SD from 5 experimental animals. Significant differences in cytokine concentrations between untreated and *Tc*-CPI-2-treated splenocytes at particular timepoints are marked: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

The cysteine protease inhibitor originating from *T. canis* larvae, *Tc*-CPI-2, contains motifs typical for other parasitic type 2 cystatins such as the cysteine protease interaction site, the S-N-D motif, and single disulfide bond. This suggests that it shows the same inhibitory activity towards peptidases from C1 and C13 family [5]. The recombinant protein was expressed in *E. coli* system. One major drawback of our study is that we did not confirm the biological activity of the protein using experimental inhibition assays. However, most of the recombinant parasite cystatins described so far have been produced in *E. coli* [17,18,40,41], some using the same pET28 expression vector [13,42] and they were all biologically active molecules. A different study shows that recombinant cystatins from a parasitic protozoan *Giardia intestinalis* produced in *E. coli* or in *Pichia pastoris* yeast show a similar inhibitory activity [11]. We can therefore assume that the recombinant *Tc*-CPI-2 is also a biologically active molecule.

Another limitation of the study is that we did not analyse the effect of cystatin on lymphocyte proliferation activity. All cystatins analysed so far were shown to effectively inhibit the proliferatory response. A study published by Junginger and colleagues shows that *T. canis* larval excretory-secretory (TES) antigens are able to inhibit polyclonal proliferation of canine T cells [43]. Moreover, TES antigens induced IL-10 production and impaired LPS-induced maturation of monocyte-derived dendritic cells by downmodulating MHCII and CD80 expression. These effects clearly resemble the effect of parasite cystatins on antigen presenting cells [16]. TES antigens most probably contain cystatins [21], but whether the abovementioned effects may be attributed only to cystatins or other molecules in the secretome remains to be investigated.

Our results confirm *Tc*-CPI-2 immunomodulatory activity on human macrophages and murine splenocytes. We have used THP-1 macrophages to analyse *Tc*-CPI-2 effect on cytokine release by these cells and noted that the cystatin alone upregulated CXCL-8 and downregulated TNF-α, but also IL-10 secretion. However, when LPS treatment was followed by stimulation with the cystatin, TNF-α production was significantly higher compared to cells treated only with LPS.

The only study analysing parasitic cystatins with the use of THP-1 cells concerns the molecule originating from the *Fasciola gigantica* trematode [44]. The authors show that the cystatin, without additional stimulation with LPS, had no effect on the signalling pathways and production of cytokines by THP-1 macrophages. However, the ex vivo stimulation of $CD14^+$ monocytes and $CD14^+$-derived macrophages isolated from human peripheral blood mononuclear cells (PBMCs) with recombinant *Bm*-CPI-2 resulted in an increase of CXCL-8, IL-6, and IL-10 secretion, and there was no effect on TNF-α [45]. The *Ov*-17 cystatin increased TNF-α production in human PBMCs, which preceded the upregulation of IL-10 [18]. Another example is the *Di*-CPI from the dog heartworm, *Dirofilaria immitis*, which decreased TNF-α and increased IL-10 release by dog PBMCs [10].

The abovementioned studies are not unanimous, especially in documenting cystatins' effect on TNF-α synthesis. This may result from differences in experimental design, type of immune cells used, the concentration of the cystatin stimulant and the species of parasite from which the cystatin originates. Monocytes are the target cells for cystatins [18], this may be one of the reasons why our results obtained with use of THP-1 macrophages differed from those noted using monocytes.

A common feature of parasitic cystatins is their ability to stimulate IL-10 production. At first glance, our results from in vitro experiments are not in line with this statement. This may be due to the fact that the concentration of *Tc*-CPI-2 used was relatively low (1 μg/mL) compared to other studies, where *Di*-CPI was effective at 5 μg/mL and *Bm*-CPI-2 was effective starting from 10 μg/mL [10,45]. Also, our ex vivo stimulation results support this assumption. *Tc*-CPI-2 stimulated a substantial increase in IL-10 secretion by splenocytes from naïve mice only at the highest of applied concentrations. Interestingly, splenocytes from mice infected with *T. canis* for 7 days responded with IL-10 upregulation even at lower cystatin concentrations.

Because of their potent ability to upregulate immunoregulatory cytokines, IL-10, but also TGF-β [41,46,47], parasite cystatins are recognized as molecules with therapeutic activity against immune-mediated diseases, which is widely reviewed by Khatri et al. [16]. For example, allergic responses in the murine ovalbumin model can be ameliorated by *A. vitae* cystatin, *Av*-17, in an IL-10-dependent manner [48]. The same molecule is also able to suppress grass pollen-specific allergic response [49]. A cystatin from *A. lumbricoides* prevents the development of an allergic inflammation induced by the house dust mite [46]. These studies report that cystatins modulate the disease severity by inducing IL-10 and TGF-β production and decreasing pro-inflammatory cytokines such as IL-4, IL-5, IL-6 and TNF-α.

Cystatins were also shown to modify the course of parasitic infections. A weekly pre-treatment of mice with *Litomosoides sigmodontis* cystatin resulted in the reduction of nitric oxide production and antigen-specific proliferative responses of splenocytes and upregulation of TNF-α mRNA expression upon infection with

microfilariae [50]. Chen and colleagues applied *Schistosoma japonicum* cystatin after the infection had already been established, and such treatment resulted in increased proliferation of regulatory T cells and upregulation of IL-4 and TGF- β compared to infected but untreated mice [51]. Our results prove that *Tc*-CPI-2 also affects the cytokine response in *T. canis*-infected mice, and the stimulation effect depends on infection duration. At 7 dpi, cystatin significantly enhanced IL-10 but also IFN-γ production by spleen cells. Mouse splenocytes responded to *T. canis* infection with upregulation of IL-10 and downmodulation of IFN-γ [52,53].

IFN-γ is especially decreased in the early phase of infection [39], and here we show that ex vivo stimulation of cells with cystatin at 7 dpi restored the cytokine production, but its level was still significantly lower compared to uninfected controls. A similar observation was made for TNF-α, which production by splenocytes is downmodulated by TES antigens [52,53]. *Tc*-CPI-2 alone increased the TNF-α secretion, and the differences were significant at 0 and 28 dpi. This is consistent with other findings, where parasite cystatins were shown to increase TNF-α secretion [18,50,54].

Interestingly, the cystatin-stimulated upregulation of IFN-γ and TNF-α, which are Th1 cytokines at 28 dpi, was accompanied by a decrease in Th2 cytokines, IL-5 and IL-10, and also in IL-6 production. Generally, *T. canis* infection causes a severe inflammatory reaction in host tissues, with dominant CD4+ Th2 activity, eosinophilia, and production of specific antibodies [24]. Moreover, the infection may exacerbate other diseases, such as allergic airway inflammation [55]. Our results suggest that *Tc*-CPI-2 could be responsible for switching from Th2 to Th1 immune response during *T. canis* infection in mice, and this shows that this particular molecule could be used to limit excessive inflammation. More studies are needed to explore this issue.

Unfortunately, there are no studies describing the cystatin expression profile in parasites at different infection timepoints. We do not know whether cystatins are equally secreted throughout the infection or if the expression changes with time. And although we know a lot from in vitro experiments about the immunomodulatory mechanisms induced by cystatins, we are not able to confirm their actual importance in real-time infection process. This might be achieved by generating cystatin knock-out parasites and performing experimental infections.

The idea that the neutralisation of cystatins could contribute to the generation of protective immune responses was proposed some time ago [50]. However, vaccination of gerbils with *B. malayi* cystatin did not result in significant protection and reduction of parasite numbers [56]. Since cystatins, as already mentioned, are effective in the amelioration of severe inflammatory reactions in the course of many immune-mediated diseases, their therapeutic potential for parasitic infections should also be analysed. Perhaps, instead of cystatin neutralisation, their exogenous application to parasite-infected hosts would be beneficial.

In conclusion, our study shows that *Tc*-CPI-2 exerts a significant immunomodulatory effect on the immune response of the paratenic host during *T. canis* infection. The ability of the cystatin to influence cytokine production by immune cells, including the up-regulation of Th1 (IFN-γ and TNF-α) and Th2 (IL-10) cytokines, suggests that it plays a key role in the immune response modulation. Although further studies are needed to fully understand the mechanism by which cystatins act during infection, our results highlight that *Tc*-CPI-2 is a molecule with promising immunoregulatory and therapeutic potential.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/250626105015 8793/ParSci-1158-accept-SI.zip. Supplementary Table S1 contains BLAST results, Supplementary Figure S1 presents a phylogenetic distance tree generated by BLAST tool.

Author Contributions

J.K.: methodology, investigation, writing—reviewing and editing; O.Ś.: investigation; J.W.: investigation; E.D.: conceptualization, supervision, data curation, writing—original draft preparation. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

Data will be available at a request from the corresponding author.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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