Characterization of a Rhodanese Homologue from Haemonchus Contortus and its Immune-Modulatory Effects on Goat Immune Cells in Vitro

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Research

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Abstract

Background: Suppression and modulation of the immune response of the host by nematode parasites have been reported widely. Rhodaneses or thiosulfate: cyanide sulfurtransferases are present in a wide range of organisms, such as archea, bacteria, fungi, plants and animals. Previously, it was reported that a rhodanese homology could bind by goat peripheral blood mononuclear cells (PBMCs) *in vivo*.

Results: In the present study, we cloned and produced recombinant rhodanese protein originated from *Haemonchus contortus* (rHCRD), which was one of the parasitic nematodes of small ruminants. The effect of this protein on modulating the immunity of goat PBMC and monocyte was studied in the current work. The predominant localization of the natural HCRD protein was verified as the bowel wall and body surface of worms, according to the immunohistochemical tests. It was proved in this study that the serum produced by artificially infecting goats with *H. contortus* successfully recognized rHCRD which conjugated goat PBMCs. The rHCRD was co-incubated with goat PBMCs to observe the immunomodulatory effect on proliferation, apoptosis and secretion of cytokines exerted by HCRD. The results showed that the interaction of rHCRD suppressed proliferation of goat PBMCs stimulated by ConA but did not induce the apoptosis of goat PBMCs. After rHCRD exposure, the production of TNF-α and IFN-γ were significantly decreased, however, it significantly increased the secretion of IL-10 and TGF-β1 in goat PBMCs. Phagocytotic assay by FITC-dextran internalization showed that rHCRD inhibited the phagocytosis of goat monocytes. Moreover, rHCRD could down-regulate the expression of MHC-II on goat monocytes in a dose-dependent manner.

Conclusions: These discoveries proposed a possible target as immunomodulator, which was potentially beneficial to illuminate the interaction between parasites and hosts in the molecular level and hunt for innovative protein species as candidate targets of drug and vaccine.

Background

The parasitic nematode *Haemonchus contortus* occurs generally in small ruminants, and it is an especially severe threat to the production and health of goats and sheep in warm and tropical temperate zones [1]. Specialized and highly complex interaction between hosts and parasites is associated with parasitic nematodes escaping from immunological response of hosts which supports the parasitic life history within hosts [2, 3]. With the aim of surviving under the immune system of hosts, a variety of molecules are produced by worm parasites, released as excretory secretory proteins and/or located on the cuticle surface, during host-parasite interface [4, 5].

Rhodaneses or thiosulfate: cyanide sulfurtransferases are present in a wide range of organisms, such as archea, bacteria, fungi, plants and animals [6, 7]. Rhodanese homology domains (RHDs) which the C-terminal domain possessing the active-site cysteine residue are ubiquitous structural modules [8]. There is a superfamily of variant rhodaneses composed of single or tandem RHDs, or combined with other protein domains of different functions [9]. Rhodanese catalyzes the irreversible transfer of a sulfane

sulfur atom from a suitable donor (i.e., thiosulfate) to cyanide *in vitro*, leading to formation of less toxic sulfite and thiocyanate [10]. Generally, during catalysis two separate sulfur-transfer steps are believed to occur. In the first step, the thiosulfate ($S_2O_3^{2-}$) anion is attacked by the sulfhydryl (-SH) group of the conserved cysteine residue, forming a covalent persulfide intermediate; in the second step, a cyanide (CN^-) ion attacks the persulfide intermediate, the latter released the thiocyanate (SCN^-) product and regenerated the cysteine sulfhydryl group [6, 11]. Rhodanese is thought to play a key role in cyanide detoxification [12-16]. It is very important for herbivorous and omnivorous mammals, due to some plants which they eaten containing cyanogenetic glucoside. It was reported that the level of rhodanese in different tissues and organs of mammalian animals is correlated with levels of exposure to cyanide [12, 15, 17, 18]. Except cyanide detoxification, growing body of evidences support that rhodaneses and RHD contatining proteins are involved in many other physiological processes including iron-sulfur cluster assembly and regulation of oxidative phosphorylation [19-21], maintenance of the sulfane pool [7], maintenance of redox homeostasis [22], selenium metabolism [23, 24], thiamin biosynthesis [25], assisted protein refolding [26, 27], regulation of cell cycle [9], aging-related regulations [28], molybdenum cofactor biosynthesis [29], protection of Fe-S enzymes against oxidative damage [30], xenobiotic-induced oxidative-stress and detoxification [31, 32].

Baghshani et al. reported that at least some rhodanese activity was present in all the seven investigated parasitic helminths including *Haemonchus longistipes*, although its activity was much lower than the values previously reported for some tissues of their vertebrate hosts [33]. The role of rhodanese in parasitic helminth is fascinating given it's widely distribution. Previously, Gadahi et al. reported that the excretory and secretory products from *H. contortus* could exert immunomodulatory effects on goat peripheral blood mononuclear cells (PBMCs) *in vitro* [34], and subsequently identified a series of excretory and secretory proteins capable of binding to goat PBMCs *in vivo* [35], including a rhodanese (HCRD).

On the basis of the current work, a rhodanese gene of *H. contortus* was cloned and the recombined protein of which was expressed to analyze the effect on modulating host immunity. The modulation effect on goat PBMC and monocyte function exerted by the recombined *H. contortus* rhodanese protein (rHCRD) was revealed variously.

Methods

Parasites, animals, and cells

The *H. contortus* strain Nanjing 2005 was acquired from Nanjing in Jiangsu province, China. Helminth-free goats aged 3–6 months were used to maintain the strain through continuous passage [36]. Experimental animals were challenged with stage 3 larvae (L3), which were isolated from the excrement of goats given monospecific infection, cultured at 26 °C and preserved in water at 4 °C at a larval concentration of 2500 per ml.

Native mongrel male goats aged 3–6 months were acquired from a herd maintained for study and education at Nanjing Agricultural University and kept in indoor pens containing six goats per pen. The goats were provided with cured hay and whole grain maize and allowed to drink water freely. Levamisole at a dose of 8 mg per kg body weight was given P.O. every 14 days to expel naturally-occurring strongylids. After 14 days, helminth eggs were collected from goat feces under a light microscope using standard parasitology techniques. Goats confirmed to be free from nematode infections were utilized to conduct the following experiments. Goats were observed every day throughout the study to ensure their health.

Sprague-Dawley rats weighing approximately 150 g were bought from the Experimental Animal Center of Jiangsu, China (qualified certificate: SCXK 2008-0004), maintained in a sterile environment and supplied with sterile food and water.

The standard Ficoll-Hypaque (GE Healthcare, Little Chalfont, UK) gradient centrifugation method was used to separate PBMCs from whole blood with added heparin, and the obtained cells were washed twice with phosphate buffered saline (PBS) [37]. The cell density was then adjusted to 1×10^6 cells/ml and cells were cultured in RPMI 1640 (GIBCO, Grand Island, New York, USA) containing 100 mg/ml streptomycin (GIBCO), 100 U/ml penicillin (GIBCO) and 10% heat-inactivated fetal calf serum (GIBCO) in a humidified cell chamber at 37 °C and 5% CO_2. Trypan blue dye was used to determine cell viability, which was >95% for all relevant experiments.

To obtain goat monocytes, a six welled flat-bottom tissue culture plate (Corning, New York, USA) was used to culture goat PBMCs, cells were incubated with culture medium RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum (GIBCO), 100 U/ml penicillin and 100 mg/ml streptomycin (GIBCO). Plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 1 h. Non-adherent cells were drain out by twice washing with PBS. The cells (monocytes) stick to the bottom of plate were collected and adjusted to a density of 1×10^6 cells/ml, and viability was confirmed by trypan blue exclusion test, which was >95% in all preparations.

Cloning of HCRD and bioinformatics analyses

Based on the sequence of the open reading frame (ORF) of the rhodanese-like gene (GenBank accession number: CDJ82729.1) retrieved from the online database, the following primers were specifically designed to amplify the gene using reverse transcription-polymerase chain reaction (RT-PCR): forward primer, 5'-ACGGATCCATGATGTGTCCACCTCCA-3'; and reverse primer, 5'-GCAAGCTTGGAGAACTGTAACTGCCT-3'. The underlined sequences refer to *BamH*I and *Hind*III restriction endonuclease sites. The RT-PCR products were ligated with pMD19-T vector (Takara, Dalian, China) to produce pMD-rhodanese. Fragments of rhodanese were then cleaved from the pMD-rhodanese plasmid using *BamH*I and *Hind*III before being subcloned into the relevant location of the pET32a vector (Invitrogen, Carlsbad, CA, USA). Sequencing analysis was used to confirm proper plasmid construction.

Expression and purification of rHCRD in *Escherichia coli*

To express the recombinant fusion protein, isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM was used to induce *Escherichia coli* BL-21 cells (DE3) cultured in Luria-Bertini (LB) medium containing 100 μg/ml ampicillin at 37 °C for 6 h. A His•Bind® 128 Resin Chromatography kit (Novagen, Madison, WI, USA) was used to purify the His-tagged fusion protein from the precipitated bacterial lysate according to the manufacturer's instructions. The refolded protein was purified in renaturation buffer (20 mM Tris-Cl, 500 mM NaCl, 1 mM reduced glutathione, 0.1 mM oxidized glutathione, pH 8.0) containing different concentrations of urea (0, 2, 4, 6 or 8 M) and dialyzed using PBS (pH 7.4). Subsequently, 12% SDS-PAGE and Coomassie bright blue staining were used to assess the purity of rHCRD after purification, and the Bradford method was used to quantify protein samples. Detoxi-Gel Affinity Pak prepacked columns (Thermo Fisher Scientific, Waltham, MA, USA) were used to deplete lipopolysaccharide from the obtained rHCRD protein, and the concentration of the recombinant protein samples was adjusted to 1 mg/ml before performing limulus amebocyte lysate (LAL) assays. A Pyrosate® Kit (Cape Cod Inc., East Falmouth, MA, USA) was used for LAL gel clot assays to measure endotoxin units (EU) in protein samples. Only samples with endotoxin levels lower than 1 EU/mg recombinant protein were used in further experiments.

Generation of polyclonal antibodies

Five goats were used to produce antiserum against *H. contortus*, which was used for western blots. A total of 5000 L3 larvae with infective activity was administered P.O. to goats who were maintained in a helminth-free environment. After 30 days, antiserum samples were collected and preserved at -70 °C for further use.

In order to generate polyclonal antibodies against rHCRD, a mixture of Freund's complete adjuvant and 0.3 mg purified rHCRD was used to inoculate Sprague-Dawley rats by subcutaneous injection at a series of sites, based on the protocol proposed by Wang et al. [38]. The rats received an initial immunization followed by four booster immunizations of the same dose at intervals of 14 days. Ten days after the final immunization, antiserum, which contained specific antibodies against rHCRD, was collected, and its reactivity was determined using an enzyme-linked immunosorbent assay (ELISA).

Western blot analysis

Protein samples containing 20 μg purified rHCRD were isolated by 12% SDS-PAGE, and the protein bands in the gel were transferred onto Hybond-C Extra nitrocellulose membranes (Amersham Biosciences, London, UK). The membranes were immersed in blocking buffer containing 5% skim milk and Tris-buffered saline (TBS) for 1 h under ambient conditions to block non-specific binding sites. Subsequently, TBS containing 0.1% Tween-20 (TBST) was used to wash the membranes five times for 5 min each. The primary antibody (i.e., antiserum obtained from goats experimentally infected with *H. contortus*) was diluted 1:100 in TBST and used to incubate the membranes for 1 h at 37 °C. The membranes were then washed five times with TBST and treated with horseradish peroxidase (HRP)-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO, USA) diluted 1:2000 in TBST for 1 h at 37 °C. Finally, a fresh preparation of

diaminobenzidine (DAB, Sigma), which acted as a chromogenic reagent, was added for 5 min to visualize the immune reaction.

Localization of HCRD by immunohistochemical study

Adult nematodes were immersed in TISSUE-TeK® O.C.T. compound (SAKURA, Torrance, CA, USA), washed with PBS and immersed in PBS containing 0.2% glutaraldehyde and 4% formaldehyde for 1.5 h. The nematodes were then flash frozen in liquid nitrogen and stored at -20 °C for further use. Cryostat sections with a thickness of 10 μm were washed with PBS and immersed in PBS containing 10% normal goat serum in order to block non-specific binding sites. Subsequently, sera of rats immunized with rHCRD diluted to 1:100 was used to incubate separate sections for 1 h at 37 °C. Serum from normal rats was used as the control. The sections were then washed three times with PBS for 15 min and treated with Cy3 goat anti-rat IgG (ab6953, Abcam, Cambridge, MA, USA) for 1 h. Finally, DAPI (Beyotime, Haimen, Jiangsu, China) and PBS were used to stain and wash the sections, respectively, and Anti-Fade Fluoromount solution (Beyotime) was applied to prevent fading as the samples were observed under a fluorescent microscope.

Binding of rHCRD to goat PBMCs

Goat PBMCs were isolated as described above immediately prior to experiments, inoculated into 24-well plates and cultured with 40 μg/ml rHCRD on glass cover slides for 1 h at 37 °C. Untreated cells were used as controls. Then, 0.1 M PBS was used to wash the slides, and 4% paraformaldehyde was used to fix the slides under ambient conditions for 30 min. Subsequently, PBS containing 5% normal goat serum was used to incubate the slides to block non-specific binding sites, and rat polyclonal antibody against rHCRD diluted 1:100 in PBS containing 5% normal goat serum was used to incubate the slides overnight at 4 °C. Cy3 goat anti-rat IgG (ab6953, Abcam) diluted 1:400 in PBS containing 5% normal goat serum was used to incubate the slides at 37 °C for 1 h before counterstaining with DAPI (Beyotime). The nucleus and rHCRD were indicated by blue and red, respectively, under scanning confocal laser microscopy (LSM710, Zeiss, Jena, Germany) with an oil immersion lens. Images were acquired by selecting the blue and red color channels corresponding to DAPI and Cy3, respectively. The fluorescent microscope settings used for observing control samples were identical to those used for rHCRD-treated cells. Unstained controls were used to detect background staining and auto-fluorescence of the protein and cells. ZEN software (Zeiss) was used to perform synergistic combinations and for photo acquisition. Observations were independently collected from three individual samples.

Cell proliferation assays

Concanavalin A (ConA, 10 μg/ml) was used to activate goat PBMCs at the same time as they were incubated with a range of concentrations of rHCRD at 37 °C and 5% CO_2 for 72 h. Cell Counting Kit-8 assay reagent (Beyotime) was added into each well of a 96-well plate and the OD450 was measured using a microplate reader (Thermo Fisher Scientific) after incubation for 4 h. The OD450 of control cells

in the blank group was set as 100%. The following equation was used to calculate the proliferation index of the cells: OD450 sample/OD450 control.

Apoptosis assay

Flow cytometry was used to analyze cell apoptosis, as previously described [39]. Briefly, a range of concentrations of rHCRD was used to culture goat PBMCs. Control cells received no rHCRD treatment. Subsequently, the cells were stained with annexin V and propidium iodide (PI, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Detection of cytokine secretion

To assess cytokine secretion, 10 μg/ml of ConA with or without rHCRD was used to stimulate goat PBMCs for 72 h. After collecting the supernatant, cytokines were measured using ELISAs. Commercial goat ELISA kits (Mlbio, Shanghai, China) were used to determine the concentrations of IL-2, IL-4, IL-10, IL-17A, TNF-α, IFN-γ and TGF-β1 in the supernatant of samples. Data obtained from three individual experiments were used to perform the analyses.

FITC-dextran internalization

The phagocytic ability of goat monocytes in response to rHCRD was determined by FITC-dextran internalization followed by flow cytometry analysis (BD Biosciences). Monocytes were treated with rHCRD for two days and then incubated with FITC-dextran (1 mg/ml in RPMI1640) for 1 h at 37 °C. Cells incubated with equal concentration of FITC-Dextran were used as the baseline of monocyte phagocytosis. Finally, cells were twice washed to eliminate excess of FITC-dextran and results were analyzed using FlowJo 7.6 software (Tree Star, Ashland, OR, USA) by considering the statistical data of median fluorescence intensity (MFI) values in the control as 100%.

Analysis of MHC molecules expression

The purified monocytes (0.5×10^6 cells/ml) were poured into 24-well culture plates with different concentrations of rHCRD or equal volumes of control buffer for 24 h in complete RPMI 1640 at 37 °C. Afterward, monocytes were marked with the monoclonal MHC-I (MCA2189A647) and MHC-II (MCA2226F) antibodies (AbD serotec, BioRad Laboratories, CA, USA). The results were expressed as the percentage of mean fluorescence intensity (MFI) followed by analyzed on a FACS Calibur cytometer (BD Biosciences).

Statistical analysis

The data are represented as average ± standard deviation. Analysis of variance was used to calculate significant differences and Student's *t*-tests were used to evaluate parametric samples (GraphPad Prism, San Diego, CA, USA).

Results

Cloning and sequencing HCRD gene

In a search for the rhodanese gene in online databases, a homologous *H. contortus* HCRD gene was identified. The HCRD protein is composed of 443 amino acid residues and has an isoionic point of 8.26 and a predicted molecular weight of 50.6 kDa. A conserved rhodanese homology domain was detected in the putative amino acid sequence (position 220–336). No signal peptide was identified by analysis of amino acids using the SignalP program.

Expressing and purifying HCRD

By ligating the HCRD gene into the pET32a plasmid, the recombinant protein with two His 6 tags was successfully expressed in *E. coli*, resulting in a protein with a predicted molecular weight of 70.6 kDa (Fig. 1A). A His•Bind® 128 Resin Chromatography kit (Novagen) was used to purify rHCRD using affinity chromatography according to the manufacturer's protocol. SDS-PAGE results showed that the purity of the rHCRD was >90%.

Western blots

To determine whether HCRD evokes an immune reaction in the host, serum of goats subjected to experimental *H. contortus* infection was used as a primary antibody to detect rHCRD. The results showed that the serum recognized rHCRD (Fig. 1B), indicating that HCRD was exposed to host immunity in the process of infection.

Immunolocalization of HCRD

Fig. 2 shows a longitudinal section of a female imago in which blue and red fluorescence indicate DNA and HCRD, respectively. The predominant binding sites of antibodies eluted from rHCRD included the inner surface of the intestinal wall and the body surface, as shown in Fig. 2. Control sections exhibited no fluorescent signal. According to these results, HCRD acts as a secretory/excretory antigen.

Binding of rHCRD to PBMCs of goats

Immunofluorescence assays in which cells were incubated with rHCRD protein were used to investigate the binding of PBMCs to rHCRD. As shown in Fig. 3, blue and red fluorescence indicate the nucleus stained with DAPI and rHCRD labelled with Cy3, respectively. The unstained background control exhibited no fluorescence in any color channel (data not shown). As shown in the lower panel of Fig. 3, no red fluorescence signal was observed in controls, whereas a strong red fluorescence signal was observed in cells that were treated with rHCRD, as shown in the upper panel of Fig. 3.

Cell proliferation assay

Cell counting kits (CCK8) were used to measure the effect of rHCRD on goat PBMC proliferation. It was revealed that rHCRD remarkably inhibited ConA-induced proliferation of goat PBMCs in a dose-dependent

manner (10 μg/ml: $t_{(4)}$=0.7383, p =0.5013; 20 μg/ml: $t_{(4)}$=5.006, p =0.0075; 40 μg/ml: $t_{(4)}$=9.939, p =0.0006) (Fig. 4).

rHCRD did not induce apoptosis of PBMCs of goats

rHCRD-induced apoptosis of goat PBMCs was measured using annexin V-fluorescein-5-isothiocyanate (FITC)/PI double staining apoptosis assays. The results showed that rHCRD has no significantly effect on the apoptosis of goat PBMCs (10 μg/ml: $t_{(4)}$=2.045, p =0.1103; 20 μg/ml: $t_{(4)}$=1.422, p =0.2280; 40 μg/ml: $t_{(4)}$=2.697, p =0.0543) (Fig. 5).

Altered quantities of secretory cytokines

According to the results of ELISA assays, compared with PBMCs treated with ConA only, rHCRD inhibited the expression of TNF-α (10 μg/ml: $t_{(4)}$=0.5788, p =0.5938; 20 μg/ml: $t_{(4)}$=10.04, p =0.0006; 40 μg/ml: $t_{(4)}$=9.302, p =0.0007) and IFN-γ (10 μg/ml: $t_{(4)}$=5.920, p =0.0041; 20 μg/ml: $t_{(4)}$=9.255, p =0.0008; 40 μg/ml: $t_{(4)}$=10.88, p =0.0004) and remarkably enhanced the secretion of IL-10 (10 μg/ml: $t_{(4)}$=4.268, p =0.0130; 20 μg/ml: $t_{(4)}$=5.488, p =0.0054; 40 μg/ml: $t_{(4)}$=8.155, p =0.0012) and TGF-β1 (10 μg/ml: $t_{(4)}$=1.469, p =0.2158; 20 μg/ml: $t_{(4)}$=1.287, p =0.2675; 40 μg/ml: $t_{(4)}$=7.577, p =0.0016) in goat PBMCs induced with ConA (Fig. 6).

Phagocytosis ability of goat monocytes

The goat monocytes were treated with different concentrations of rHCRD for 48 as shown in Fig. 7. The results of flow cytometry analysis showed that, protein concentration of 20 μg/ml and 40 μg/ml significantly decreased the FITC-dextran uptake ability of goat monocytes (10 μg/ml: $t_{(4)}$=1.625, p =0.1794; 20 μg/ml: $t_{(4)}$=13.04, p =0.0002; 40 μg/ml: $t_{(4)}$=16.86, p <0.0001), whereas, no significant change was observed in His-tagged protein treated group. The results suggested that native HCRD protein can restrain phagocytic capacity of goat monocytes *in vivo*.

rHCRD inhibited MHC-II expression on goat monocytes

The results illustrated in Fig. 8, showed that, rHCRD significantly decreased MHC-II expression in a dose-dependent manner as compared to the baseline expression of MHC-II in the control buffer (10 μg/ml: $t_{(4)}$=5.373, p =0.0058; 20 μg/ml: $t_{(4)}$=6.367, p =0.0031; 40 μg/ml: $t_{(4)}$=7.691, p =0.0015), whereas, no significant change was observed in control section (His-tagged protein). However, goat monocytes exposure to rHCRD at different concentrations did not show any change in MHC-I molecule expression (10 μg/ml: $t_{(4)}$=0.1773, p =0.8679; 20 μg/ml: $t_{(4)}$=0.6904, p =0.5279; 40 μg/ml: $t_{(4)}$=0.09299, p =0.9304) (Fig. 8).

Discussion

Excretory and secretory products are those which may diffuse or leak from the parasite body or actively exported through secretory pathways [40]. Modulation of the immune response of host by helminths involves the excretory and secretory products released by these parasites [41-43]. In the present study a rhodanese homologue was described from the parasitic nematode *H. contortus* for the first time. We found that rHCRD could be recognized by the antiserum from goats experimentally infected with *H. contortus* and the native HCRD protein was predominantly localized at the body surface and internal surface of the parasite's gut. Furthermore, the immunofluorescence assay determined that rHCRD could bind on the surface of goat PBMCs *in vitro*.

The naive T cell activated by the antigen-presenting cells triggers adaptive immune response and promotes the secretion of the corresponding cytokines, resulting in both T-cell differentiation and the proliferation of further T cells [44]. The phenomenon of diminished proliferation of peripheral T cells to filarial-specific antigens was called lymphocyte hypoproliferation, was already shown in filarial infected humans [45]. Diliani et al. demonstrated that draining lymph node cells and splenocytes from mice injected with *Ancylostoma ceylanicum* excretory/secretory (ES) products showed decreased proliferation in response to both species-specific antigens and mitogen [46]. In present study, rHCRD significantly suppress Con A-stimulated goat PBMCs proliferation in a dose-dependent manner. Furthermore, two *Onchocerca volvulus* excreted/secreted proteins, OvALT-2 and OvNLT-1, suppress antigen-specific T cell proliferation via induction cell apoptosis [47]. However, rHCRD has no significantly effect on the apoptosis of goat PBMCs.

IL-2, TNF-α, and IFN-γ belong to Th1 cytokine involved in cell-mediated immune response, such as inflammatory response. IL-4 plays an important role in the activation, differentiation and proliferation of B lymphocytes and participates in antibody class switch to IgG and IgE. Currently, IL-17A has been considered as a key role in driving inflammation and protective immunity at both mucosal and non-mucosal sites [48-50]. It was reported that intestinal nematode infection levels correlate with both the production of IL-10 and TGF-β of host [51]. In the model of mouse infected *Trichuris muris*, IL-10 plays a key role in controlling the inflammation caused by parasite infected and the establishing of long-term infection [52-54]. T cell TGF-β signaling plays an essential role in the modulation of mouse intestinal immune response to *Heligmosomoides polygyrus* infection by limiting mucosal Th1 and Th2 cytokines production and increasing IL-10 production [55]. Furthermore, the inhibition of filarial-specific T-cell proliferation can be reversed *in vitro* by antibodies against IL-10 and/or TGF-β [56, 57]. In present study, after incubated with rHCRD, the production of IL-10 and TNF-β1 of ConA-stimulated goat PBMCs were significantly increased. However, rHCRD could significantly decrease the secretion of TNF-α and IFN-γ and no significantly effect on IL-2, IL-4 and IL-17A production. The cytokines profile modulated by rHCRD is responsible for the induction of an anti-inflammatory responses which favorable for the survival of worms.

Peripheral blood monocytes which represent one of the major classes of APCs play a crucial role in the innate response of vertebrate hosts to viral, fungal, bacterial, and parasitic infections [58, 59]. Phagocytosis is the process by which unwanted cells or invading pathogens are efficiently removed from

tissues and organs by professional phagocytes, predominantly macrophages [60]. In the present study, phagocytic capacity of goat monocytes was significantly decreased after treatment with different concentrations of rHCRD. MHC-II molecules are constantly expressed on the surface of APCs, permitting them to present extracellular antigens and initiate the adaptive immune response [61]. Activation of APCs increases MHC-II expression [62]. In the present study, we observed that rHCRD was able to inhibit MHC-II expression on goat monocytes in a dose dependent manner.

Conclusion

In conclusion, our results showed that rHCRD could bind by goat PBMCs and exerts its immunomodulatory effects on multiple aspects to facilitate the immune evasion of *H. contortus*. These findings shed new light on the molecular mechanisms of helminthic immune evasion.

Abbreviations

HCRD: *Haemonchus contortus* rhodanese; rHCRD: recombinant proteins of *Haemonchus contortus* rhodanese; PBMC: peripheral blood mononuclear cell; FITC: fluorescein-5-isothiocyanate; RHD: Rhodanese homology domain; L3: third-stage larvae; ORF: open reading frame; RT-PCR: reverse transcription-polymerase chain reaction; IPTG: isopropyl-β-D-thiogalactopyranoside; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; LAL: *Limulus* amoebocyte lysate; EU: Endotoxin Unit; ELISA: enzyme-linked immunosorbent assay; TBS: Tris-buffered saline; TBST: Tris-buffered saline containing 0.1% Tween-20; PBS: phosphate buffered saline; HRP: horseradish peroxidase; DAB: diaminobenzidine; LPS: Lipophosphoglycan; MFI: median fluorescence intensity; DAPI: 2-(4-amidinophenyl)-6-indole carbamidinedihydrochloride; ConA: Concanavalin A; DMEM: Dulbecco's Modified Eagle Medium; PI: propidium iodide; IL-2: interleukin-2; IL-4: interleukin-4; IL-10: interleukin-10; IL-17A: interleukin-17A; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; TGF-β1: transforming growth factor-β1.

Declaration

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Authors' contributions

Prof. LXR directed the project and participated in the coordination and management of the study. WYJ performed the laboratory tests and the data analysis and wrote the manuscript. ME, HJM, KA and YRF helped with various aspects of the experiments and manuscript revising. XLX and SXK provided new analytical reagents and tools. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article.

Ethics approval and consent to participate

All experiments described in the current work were performed in strict accordance with the guidelines of the Animal Ethics Committee of Nanjing Agricultural University, China. Approval from the Science and Technology Agency of Jiangsu Province was obtained for all protocols (ID: SYXK [SU] 2010-0005).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

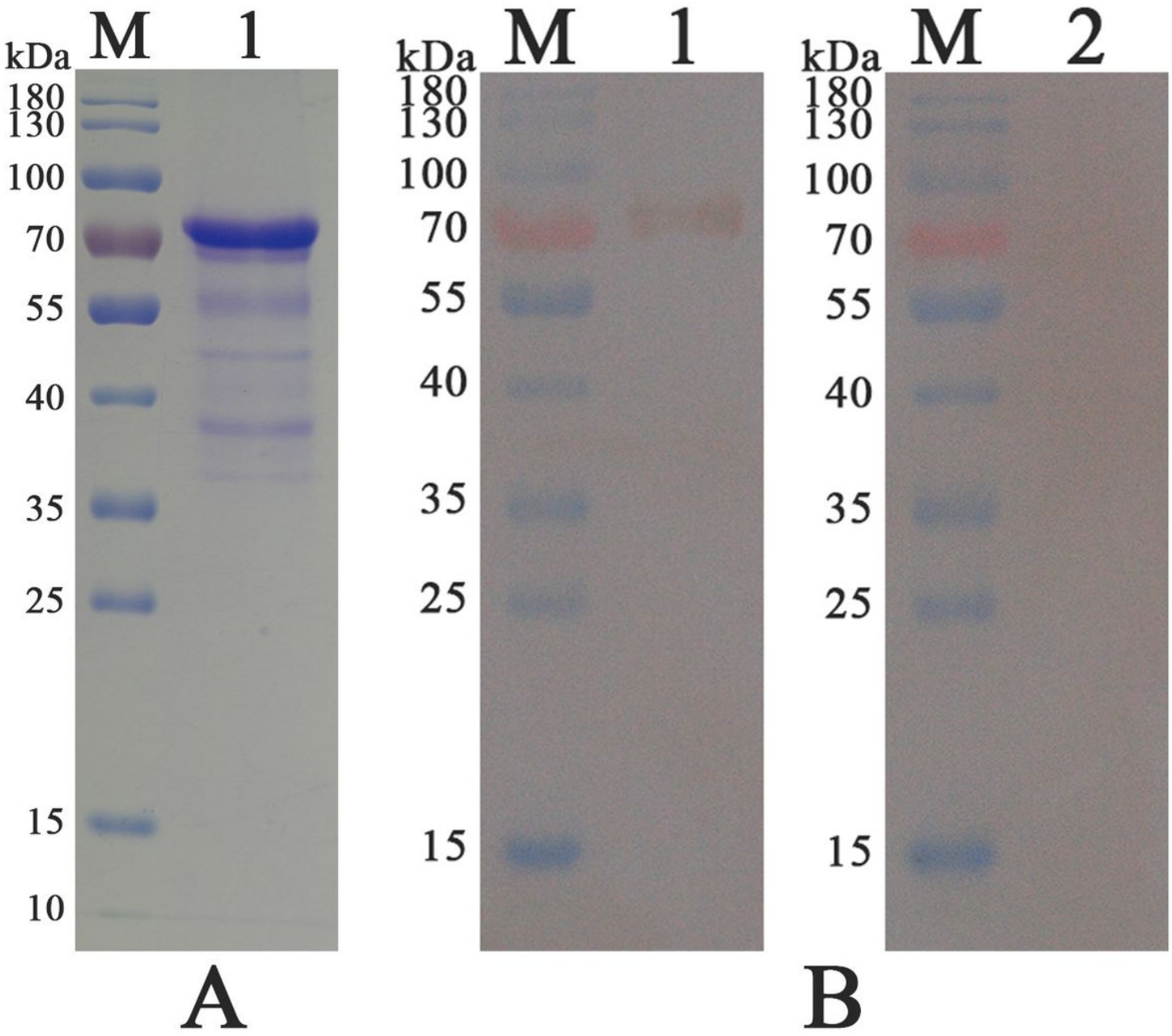

Figure 1

Purification of recombinant rhodanese protein from Haemonchus contortus (rHCRD) and western blots. A: Separation of purified rHCRD by SDS-PAGE using a 12% polyacrylamide gel and Coomassie brilliant blue R250 staining. B: Western blots of rHCRD after purification. Antisera obtained from goats experimentally infected with H. contortus was used as the primary antibody to recognize the protein (lane 1), whereas sera from uninfected goats was used as the control (lane 2).

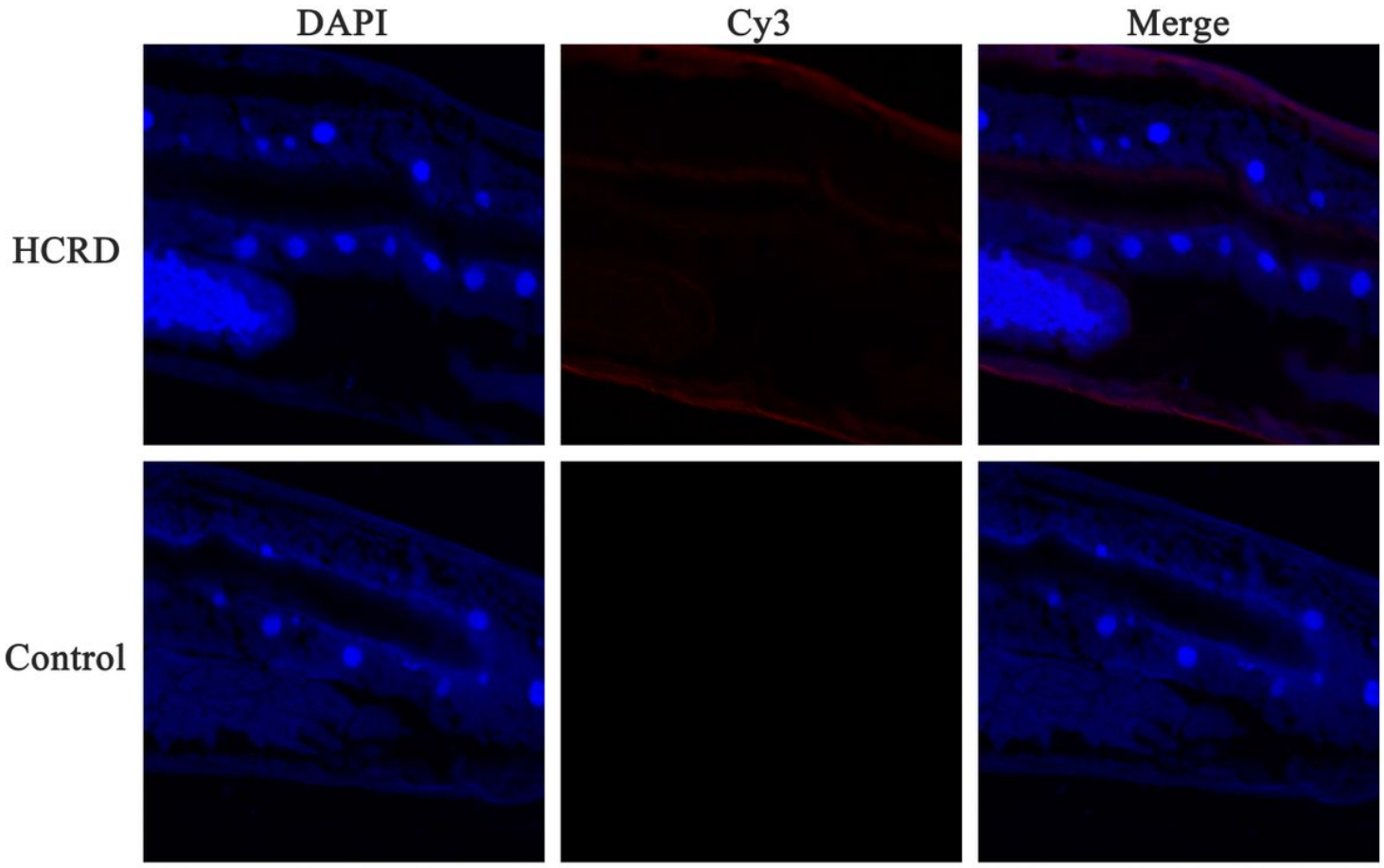

Figure 2

Immunohistochemical localization of recombinant rhodanese protein from Haemonchus contortus (rHCRD) in frozen sections of H. contortus. Cy3-labelled goat anti-rat IgG (ab6953, Abcam) was used as the secondary antibody to detect HCRD protein by indirect immunofluorescence. DAPI was used to counter-stain sections in order to observe DNA.

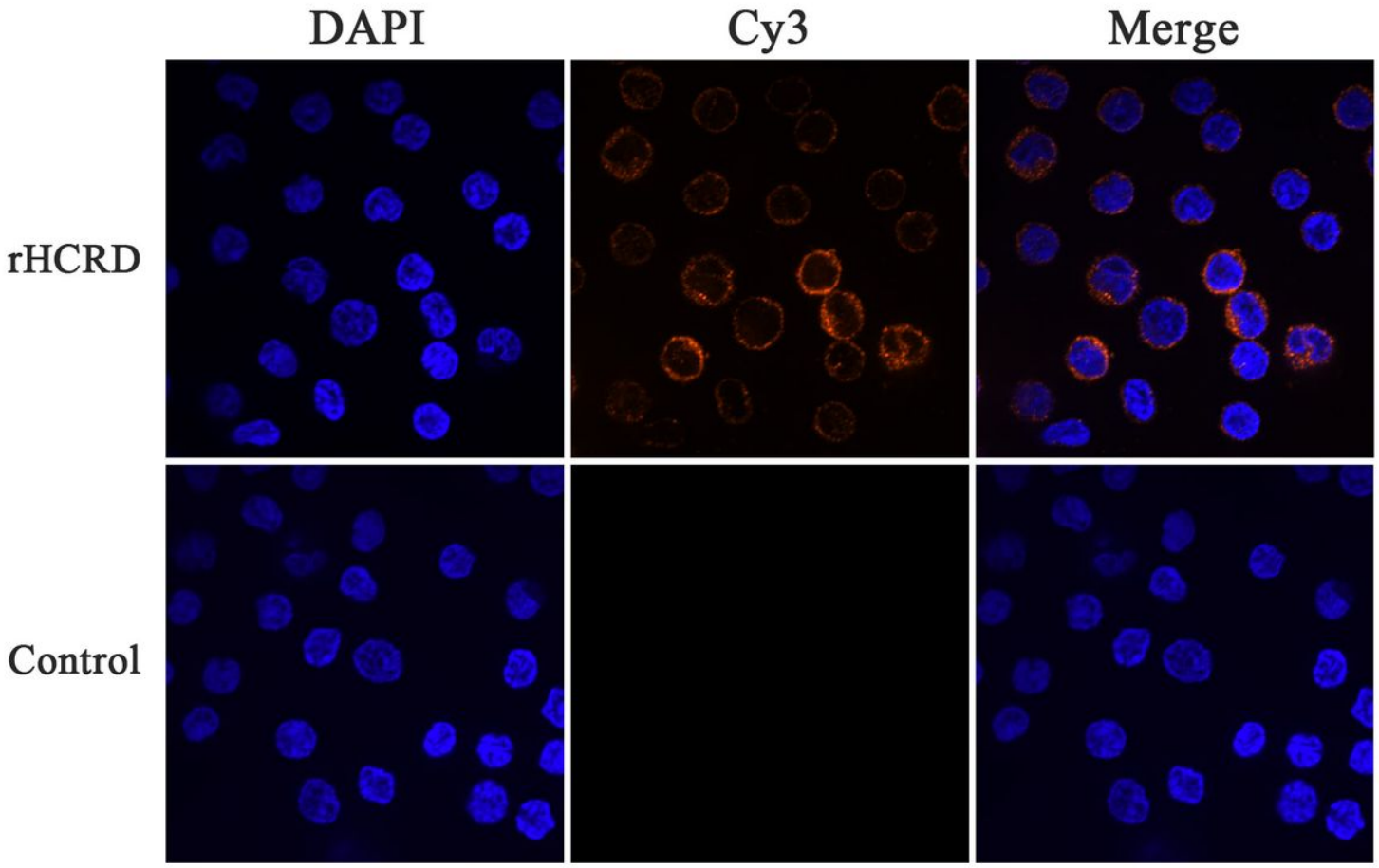

Figure 3

Conjugation of recombinant rhodanese protein from Haemonchus contortus (rHCRD) and goat peripheral blood mononuclear cells (PBMCs). Goat PBMCs were treated with rHCRD (40 μg/ml) or untreated at 37 °C for 1 h. After fixing, the cells were incubated with rat anti-rHCRD antibody followed by Cy3-labelled goat anti-rat IgG (red), and DAPI (blue) staining was used to visualize the nuclei. A confocal laser scanning microscope was used to visualize the binding of rHCRD and goat PBMCs. The overlapping blue and red channels were merged. Tests were independently performed in triplicate.

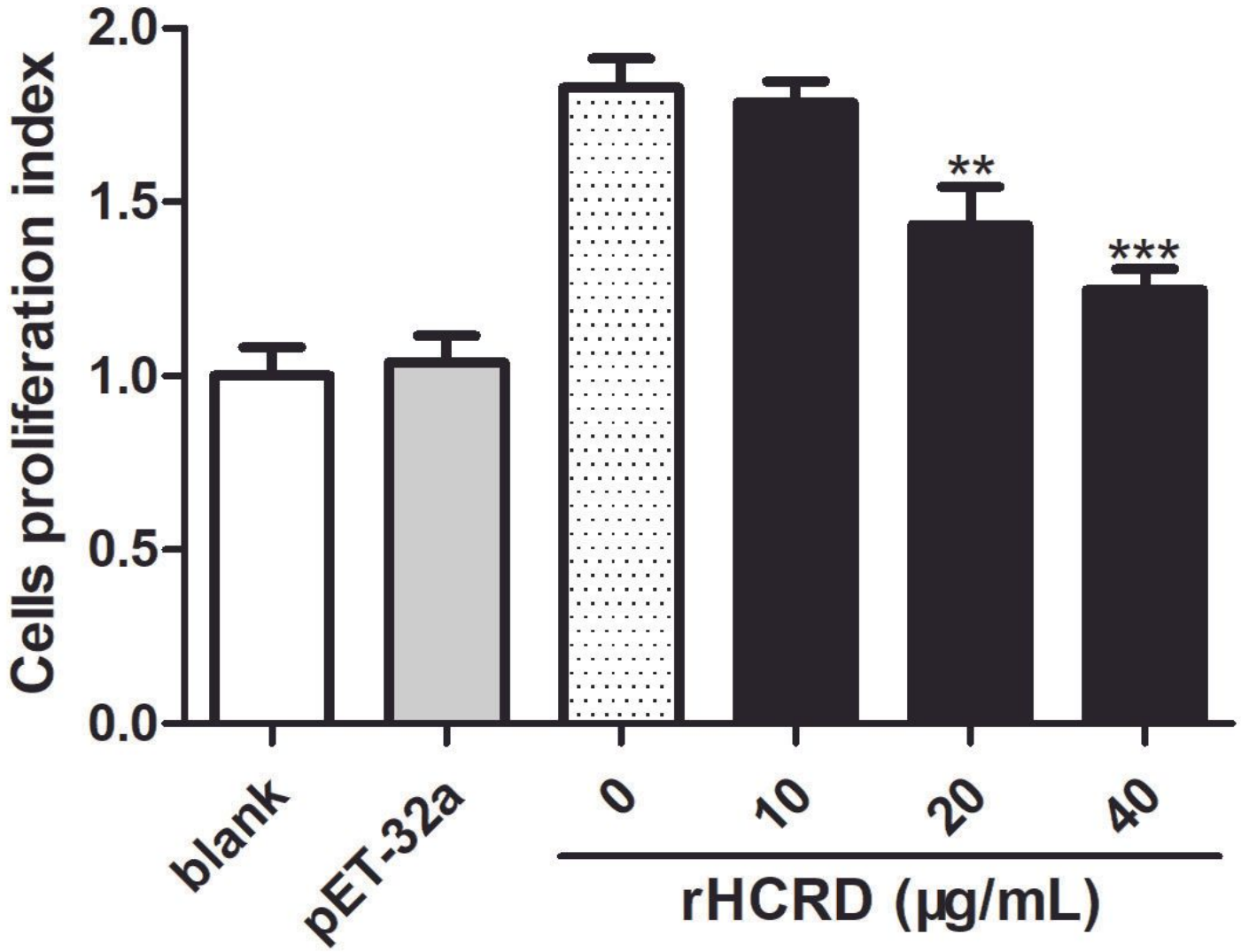

Figure 4

Inhibitory effect of recombinant rhodanese protein from Haemonchus contortus (rHCRD) on goat peripheral blood mononuclear cell (PBMC) proliferation. ConA (10 μg/mL) was used to stimulate goat PBMCs for 72 h with or without a range of concentrations of rHCRD and His-tagged protein. CCK-8 incorporation was used to measure proliferation and the cell proliferation index was calculated based on the assumption that the absorbance at 450 nm of the blank group was 100%. Tests were independently performed in triplicate. (*P< 0.05, **P< 0.01, ***P< 0.001).

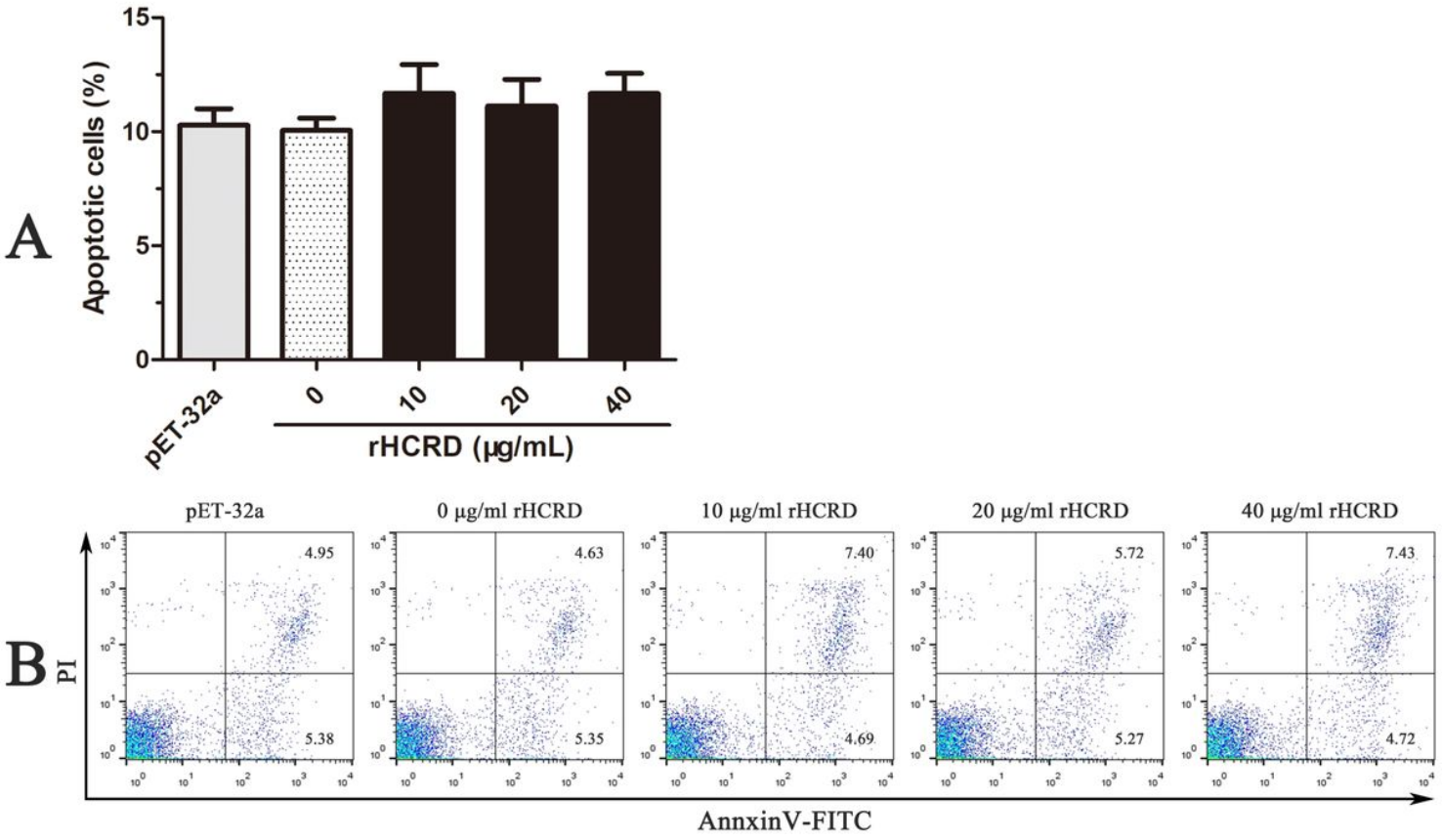

Figure 5

Recombinant rhodanese protein from Haemonchus contortus (rHCRD) did not induce apoptosis in goat peripheral blood mononuclear cells (PBMCs). PBMCs were cultured for 24 h with or without a range of concentrations of rHCRD and His-tagged protein. Propidium iodide (PI) and annexin V were used to stain the cells, which were subsequently analyzed by flow cytometry to quantify apoptotic cells. A: Apoptotic cells (annexin V+/PI-) were plotted as a percentage of the total cell population. B: Death of goat PBMCs after exposure to rHCRD is shown by a dot plot. Tests were independently performed in triplicate. (*P< 0.05, **P< 0.01, ***P< 0.001).

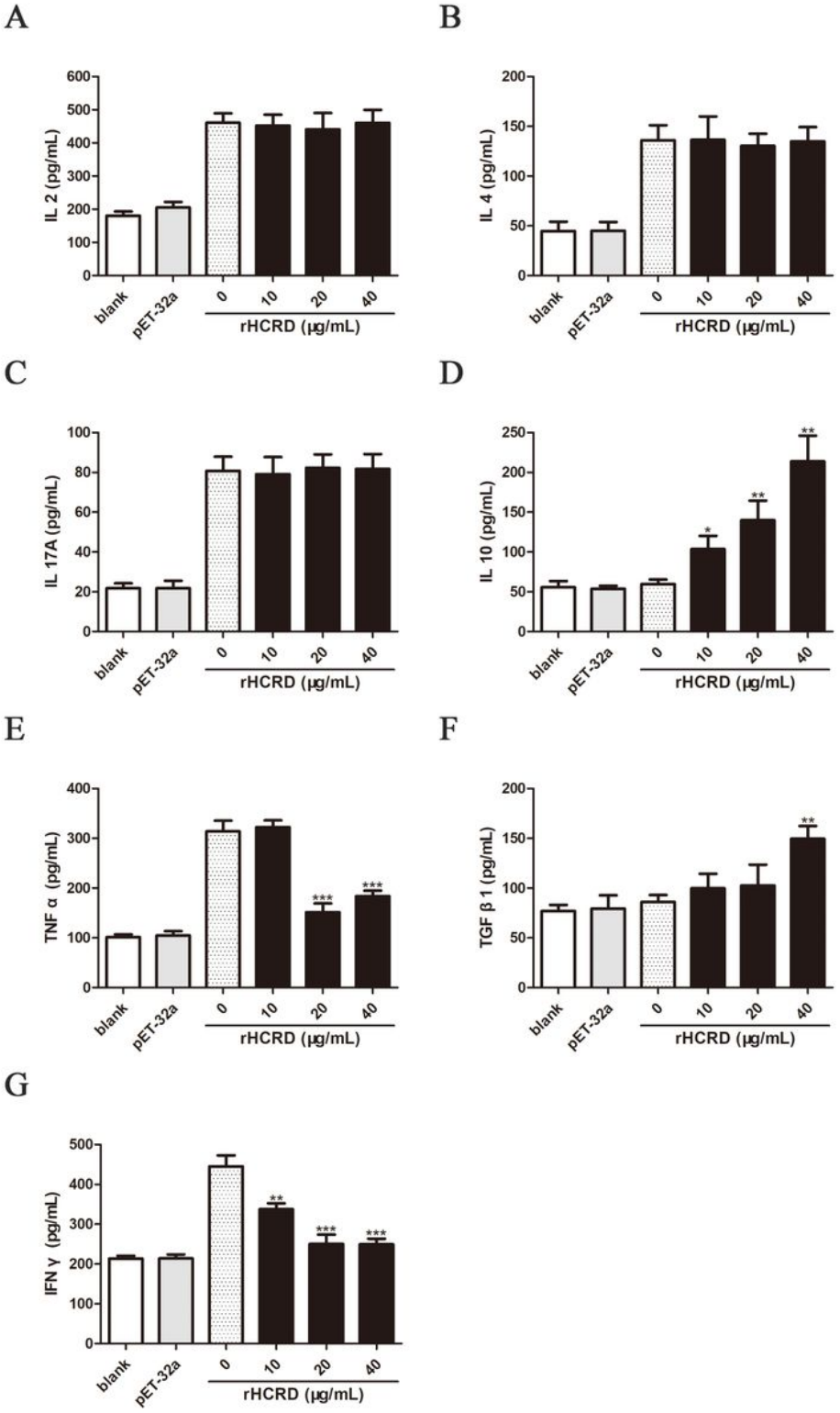

Figure 6

The cytokine profile of goat peripheral blood mononuclear cells (PBMCs) is modulated by recombinant rhodanese protein from Haemonchus contortus (rHCRD). Concanavalin A (ConA, 10 μg/mL) was used to stimulate goat PBMCs for 72 h with or without a series of concentrations of rHCRD and His-tagged protein. Enzyme-linked immunosorbent assays (ELISAs) were used to quantify cytokine secretion in the

cell culture supernatant. A: IL-2. B: IL-4. C: IL-17A. D: IL-10. E: TNF-α. F: TGF-β1. G: IFN-γ. Tests were independently performed in triplicate. (*P< 0.05, **P< 0.01, ***P< 0.001).

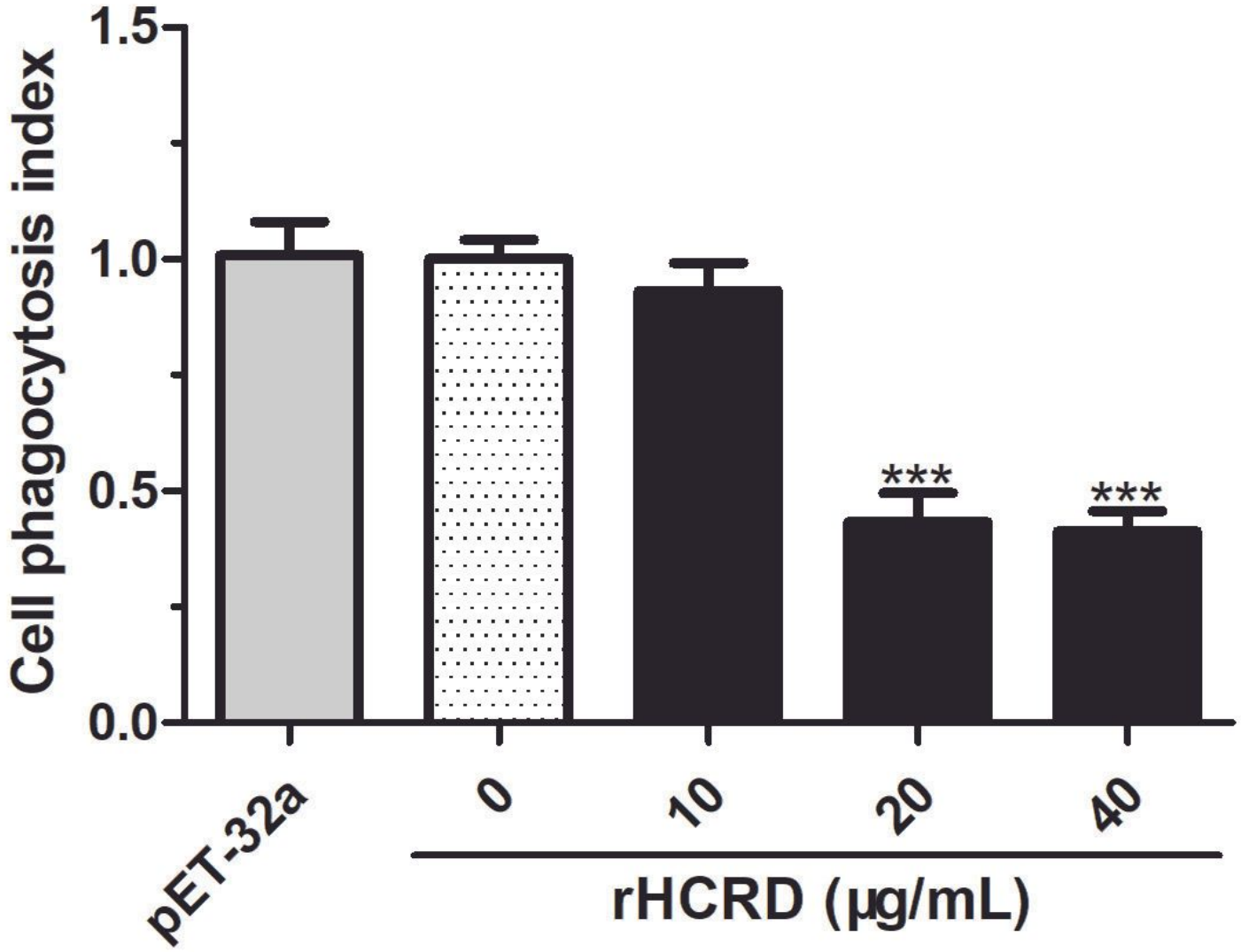

Figure 7

Inhibitory effect of recombinant rhodanese protein from Haemonchus contortus (rHCRD) on phagocytosis of goat monocytes. Cells were collected after rHCRD or his-tagged protein treatment for 48 h and incubated with FITC-dextran (1 mg/ml) for 1 h at 37 °C. The phagocytic activity of cells was analyzed on flow cytometry and calculated as mean fluorescence intensity (MFI). The data presented are results of three independent experiments (*P< 0.05, **P< 0.01, ***P< 0.001).

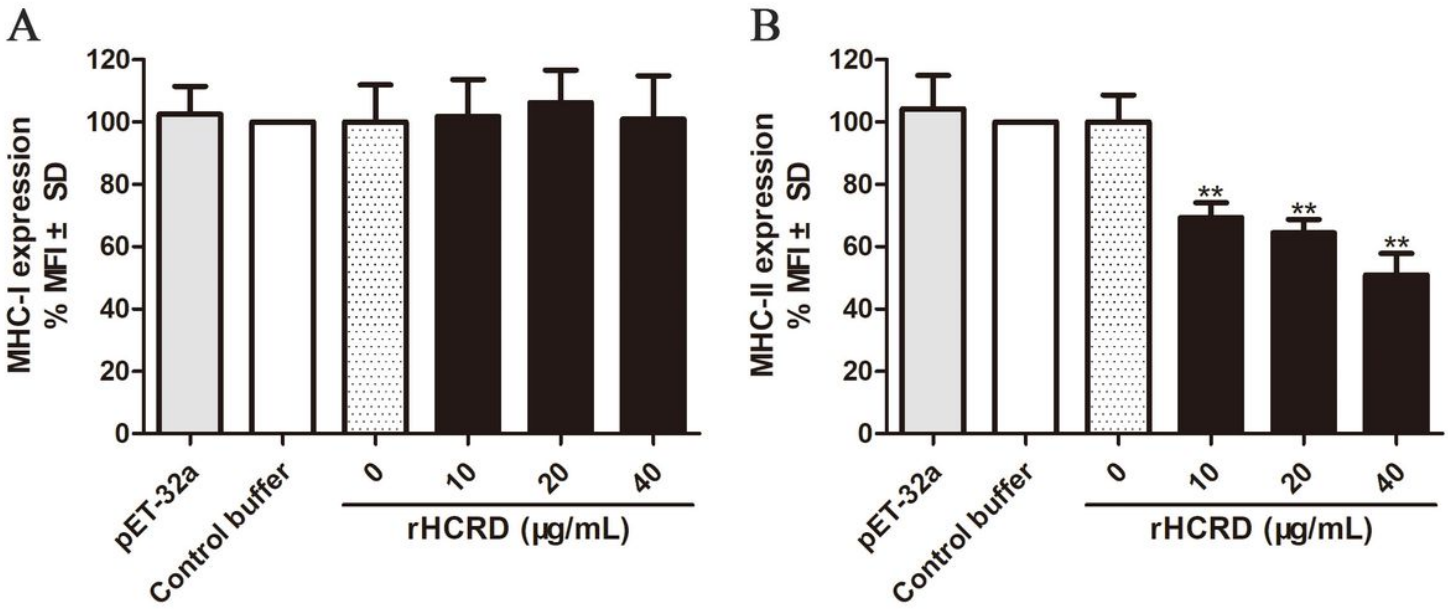

Figure 8

Inhibitory effect of recombinant rhodanese protein from Haemonchus contortus (rHCRD) on the expression of MHC-II by goat monocytes. Cells were cultured in the presence of varies rHCRD concentrations and his-tagged protein or control buffer (PBS/DTT) for 24 h. The cells treated with LPS were used as positive control. MHC-II expression was analysed on flow cytometric analysis and calculated as the percentage of mean fluorescence intensity (MFI) of controls. Bars represent the MFI ± SD of controls. The data presented are results of three independent experiments (*P< 0.05, **P< 0.01, ***P< 0.001). A: MHC-I; B: MHC-II.

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