Chapter

Nucleated Red Blood Cells Contribute to the Host Immune Response Against Pathogens

Verónica Chico, Ivan Nombela, Sara Puente-Marín and María del Mar Ortega-Villaizan

Abstract

It has recently come to light that nucleated red blood cells (RBCs) of fish, amphibians, reptiles and birds are multifunctional cells, because in addition to being involved in gas exchange and transport, it has also been reported that they respond to pathogens by means of (i) phagocytosis, (ii) antigen presentation, (iii) production of cytokines and antimicrobial peptides, (iv) regulation of complement system, and (v) exerting paracrine molecular communication with other immune cells and modulating their functions. Similarly, human cord blood nucleated RBCs have been shown to exert a regulatory function in the innate immune response, by means of the suppression of the production of inflammatory cytokines. This chapter comprises the study of the implications of nucleated RBCs as mediators of both branches of immune system (innate and adaptive immune responses).

Keywords: nucleated red blood cells, erythrocytes, immune response, cytokines, antimicrobial peptides, virus, antigen presentation

1. Introduction

Red blood cells (RBCs) are the most abundant cell type in the bloodstream, and their life span has been estimated to be 120 and 50 days in human and murine species, respectively [1]. In mammals, mature RBCs are biconcave disks that lack cell nucleus, organelles, and ribosomes [2], and their best known function is gas exchange and respiration. However, the most characteristic feature of nonmammalian RBCs is the presence of a nucleus which allows them to transcribe and translate proteins and therefore intervene in additional functions different from delivery of oxygen to tissues (**Figure 1**) [3]. The nucleated RBCs are able to respond against pathogens by employing various mechanisms. This chapter review encompasses the up-to-date studies about the involvement of nucleated red blood cells (RBCs) as immune response cell mediators against microbes.

2. The role of nucleated RBCs in innate immune system

The innate immune system is an evolutionarily older defense strategy found in many organisms such as animals, plants, fungi, insects, and primitive multicellular

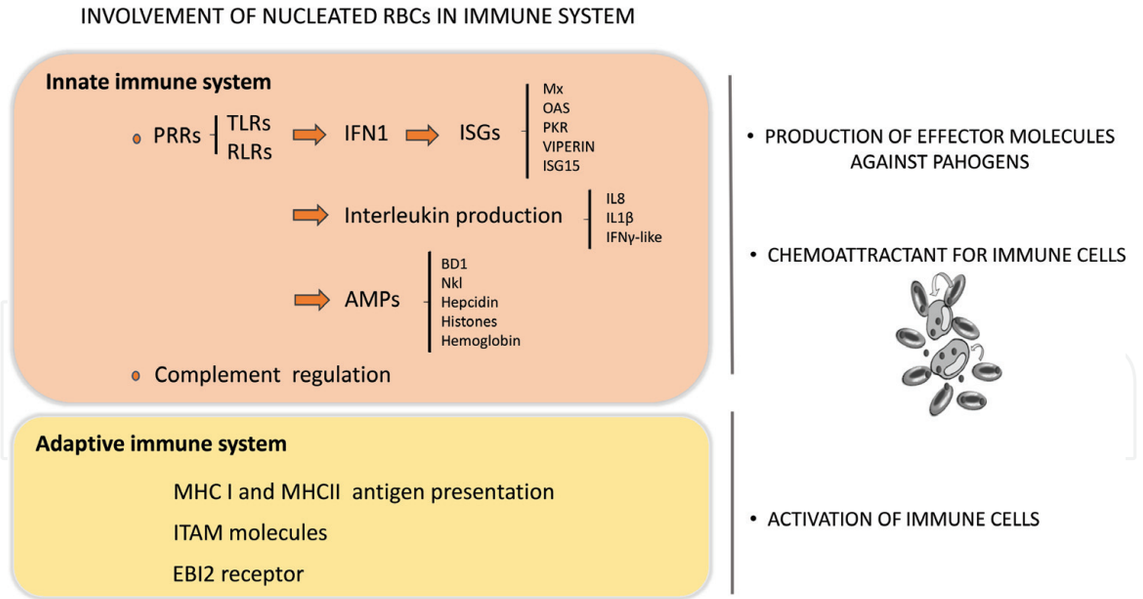

Figure 1.
Schematic representation of the suggested roles of nucleated RBCs in the immune response. *PRRs, pattern recognition receptors; TLRs, Toll-like receptor; RLR, RIG-I like receptor; IFN1, interferon type 1; ISGs, interferon-stimulated genes; Mx, myxovirus resistance gene; OAS, oligoadenylate synthetase; PKR, protein kinase RNA-activated; ISG15, interferon-stimulated gene 15; IL8, interleukin 8; IL1β, interleukin 1β; IFNγ-like, interferon γ-like; AMPs, antimicrobial peptides; BD1, β-defensin; Nkl, Nk-lysin; MHC, major histocompatibility complex; ITAM, immunoreceptor tyrosine-based activation motif; and EBI2, Epstein–Barr virus G-protein-coupled receptor 2.*

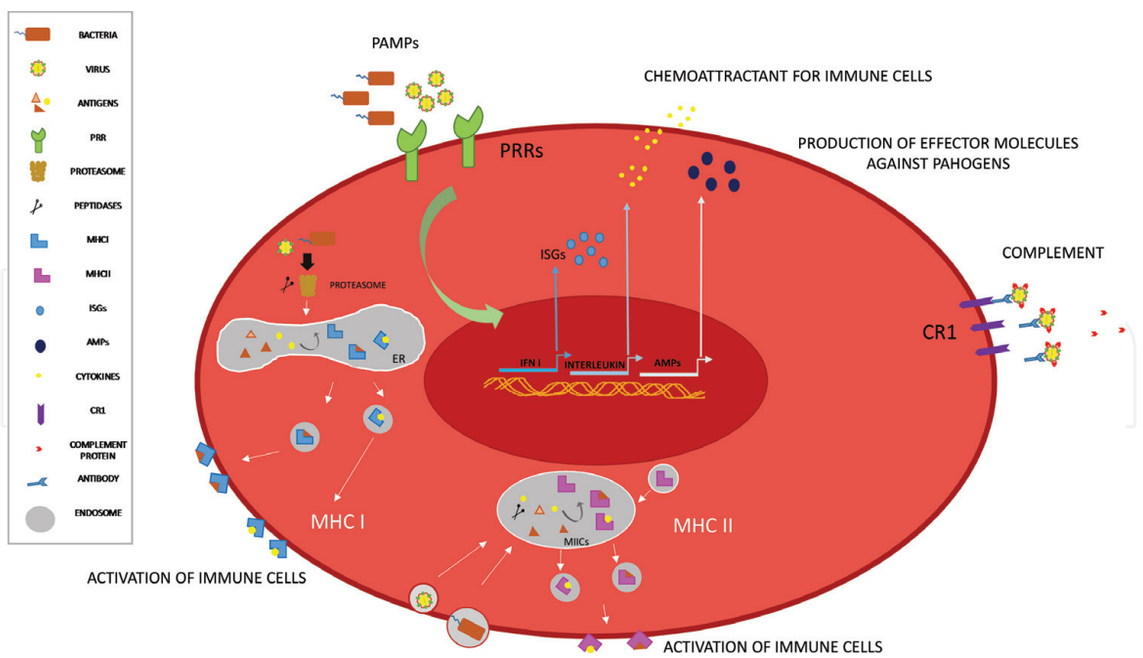

Figure 2.
Nucleated RBCs immune response suggested signaling involved in production of effector molecules against pathogens, chemoattractant proteins, and activation of immune cells. *PAMPs from microbes are recognized by specific PRRs. Activation of these receptors triggers the signaling pathways that induce the transcription of a set of genes such as IFN1, ISGs, interleukins, and AMPs. On the other hand, pathogens can be recognized by proteasome proteins and digested by peptidases. In the endoplasmic reticulum (ER), digested antigens from proteasome are bound to MHCI and exposed on the surface of RBCs. In addition, pathogens can enter inside the cell by endocytosis and delivered to MHC class II-loading compartments (MIICs) where they are digested and finally exposed on the surface of the cells by MHC II complex. Another pathway is the recognition of complement-opsonized immune complexes by CR1.*

organisms. This system is the first line of defense against pathogen infections. It is known as non-specific immune system and does not provide long-lasting immunity to the host [4, 5]. The innate immune system includes many types of molecules (receptors and effectors) to sense and eliminate pathogens. Moreover, nucleated RBCs release signaling molecules that trigger the activation of adaptive immune system. The implication of these cells in the innate immune response described to date is shown in **Figures 1** and **2**.

2.1 Nucleated RBCs trigger IFN type I response

It has been reported that nucleated RBCs do express pattern recognition receptors (PRRs) for pathogen-associated molecular patterns (PAMPs) [6]. PAMPs are small molecular motifs conserved in evolution and characteristic from pathogens. There are a vast variety of PAMPs, for example, bacterial lipopolysaccharides (LPS), bacterial flagellin, lipoteichoic acid from Gram-positive bacteria, peptidoglycan, and nucleic acid variants from viruses—such as double-stranded RNA (dsRNA) or nonmethylated viral 5'-C-phosphate-G-3′ (CpG)-containing DNA [7, 8]. PAMPs are recognized by the host cells through their PRRs such as Toll-like receptors (TLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs), AIM2-like receptors (ALRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [8, 9].

Among these receptors, a wide repertoire of TLRs have been described in nucleated RBCs, which allow them to respond to both bacterial and viral pathogens [10]. Chicken RBCs constitutively express gene transcripts of *tlr3* (which recognize viral patterns like viral double-stranded RNA (dsRNA)), *tlr21* (a homolog of mammalian TLR9 [3, 10]), and *tlr2*, *tlr4*, and *tlr5* (which recognize bacterial patterns [10]). In addition, rainbow trout RBCs [3, 11] and Atlantic salmon RBCs [12] constitutively express genes *tlr3* and *tlr9*, which recognizes CpG motifs present in microbial genome. Besides, *tlr3* upregulation with polyinosinic:polycytidylic acid (polyI:C, a molecule structurally similar to dsRNA) was found in tilapia RBCs [13]. It is noteworthy to highlight that it has been reported that the type of TLRs found in chicken nucleated RBCs is equivalent to that of many types of leukocytes [14]. This could be due to the fact that chicken RBCs and myeloid cells arise from a common progenitor cell [15].

Among RLRs, a family of receptors which interact intracellularly with viral dsRNA [3, 11, 12], RIG-I has also been reported in salmon RBCs. However, the expression of other members of RLR family, such as melanoma differentiation-associated protein 5 (MDA5) or probable ATP-dependent RNA helicase DExH-box helicase 58 (LGP2), in nucleated RBCs is still unknown [16].

Activation of these receptors with their corresponding PAMPs triggers the signaling networks that induce the transcription of a set of genes characteristic of the innate immune response such as the expression of interferon type I (IFN1) [17, 18]. The IFN1 is reportedly known to play a similar role in mammalian and nonmammalian species [19]. The binding of IFN1 to their cellular receptors induces different cell signaling pathways leading to the transcription of interferon-stimulated genes (ISGs), including important mediators of antiviral response such as myxovirus resistance protein (Mx), 2′–5′oligoadenylate synthetase (OAS), protein kinase RNA-activated (PKR), viperin, interferon-stimulated gene 15 (ISG15), IFN-induced protein with tetratricopeptide repeats (IFIT), and tripartite motif (TRIM) family, tetherin, among others [20].

In this regard, chicken and rainbow trout RBCs treated with polyI:C have been reported to induce upregulation of type I IFNs [3, 10] and also interferon-inducible genes Mx [3] and OAS [10], a protein responsible for initiating the RNAse L pathway in order to cleave viral RNA [21]. In addition, it has been described that

nucleated RBCs when infected with a virus increase the expression of IFN1 and their ISGs. A study of Atlantic salmon-infected individuals with salmon anemia virus (ISAV) first demonstrated the ability of RBCs to induce *ifnα* expression in hemagglutinated RBCs [22]. In another example, Atlantic salmon challenged with piscine orthoreovirus (PRV), PRV-infected RBCs, induced the expression of *ifn1*, *mx*, *pkr* [23], *viperin*, and *isg15* [24] antiviral genes. Recently, Nombela and colleagues demonstrated that rainbow trout RBCs could generate IFN1-related responses to viruses despite not being infected. In response to infectious pancreatic necrosis virus (IPNV), authors observed that ex vivo purified RBCs exposed to the virus showed an increment in the expression of *ifn1*, *mx*, interferon regulatory factor7 (*irf7*), and *pkr* genes followed by upregulation of Mx protein expression [25]. Likely to IPNV, viral hemorrhagic septicemia virus (VHSV) was unable to replicate in ex vivo purified rainbow trout RBCs [26]. However, rainbow trout RBCs exposed to this virus showed a decrease in the expression of genes related to IFN1 pathway. The possible explanation that the authors found for this phenomenon was a process characterized by global proteome downregulation or shutoff in order to inhibit viral protein synthesis [26]. In addition, high levels of constitutive Mx transcripts and protein were also identified in rainbow trout RBCs (**Figure 3**) suggesting that the expression of this ISG could be a possible mechanism for aborted or halted infections in rainbow trout RBCs [25, 26].

Nevertheless, the involvement of IFN 1 response in nucleated RBCs and how does this response influence the global defense against viral infections remain to be demonstrated.

2.2 Nucleated RBCs induce interleukin response

TLR signaling culminates in cellular activation and production of cytokines [27]. Cytokines are secreted proteins involved in cell recruitment and regulation of both innate and adaptive immune responses. They are essential for an effective host immune response to pathogens [28]. The nucleated RBCs apart from type I IFN expression have been reported to produce other cytokines, at gene or protein level, in response to several PAMPs.

Chicken RBCs stimulated with polyI:C have shown an increase in interleukin 8 (*il8*) transcripts of approximately 4 log, which was at least two to three orders of

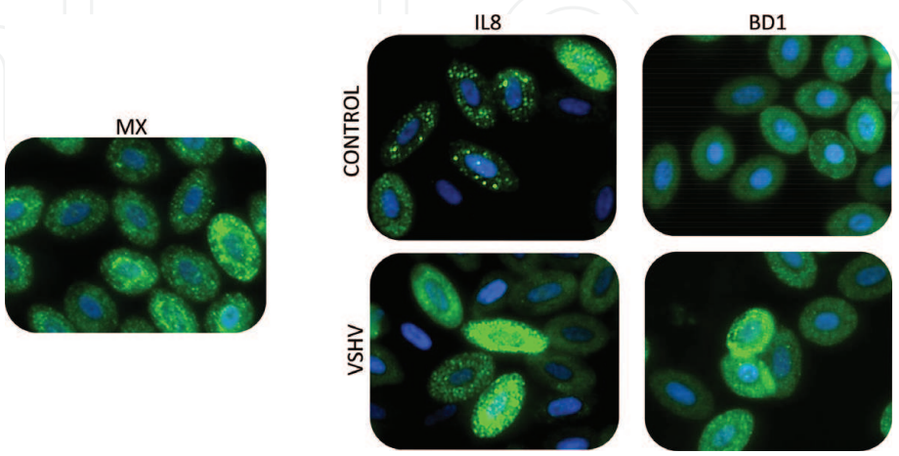

Figure 3.
Representative innate immune response in rainbow trout RBCs. *Representative immunofluorescence of Mx constitutive expression in rainbow trout RBCs. Representative immunofluorescence of IL8 and BD1 protein expression in rainbow trout RBCs control and exposed to VHSV. Images were taken using INCell Analyzer 6000 Cell imaging system (GE Healthcare, Little Chalfont, United Kingdom). FITC, fluorescein-5-isothiocyanate—for protein stain; DAPI, 4′,6-diamidino-2-phenylindole—for nuclei stain; Mx, myxovirus resistance protein; IL8, interleukin 8; BD1, β-defensin 1; and RBC, red blood cells.*

magnitude higher than those observed in monocytes, thrombocytes, and heterophils [10]. Besides, stimulation of rainbow trout RBCs with polyI:C was reported to induce de novo synthesis of mRNAs from chemokine (C-C motif) ligand 4 (*ccl4*) [3], which is a chemoattractant for natural killer cells, monocytes, and a variety of other immune cells [29].

Rainbow trout RBCs exposed to VHSV have shown augmented IL-8 (**Figure 3**) and interleukin 1β (IL-1β) protein levels [26]. IL-8 acts as a chemotactic factor for heterophils and other leukocytes such as monocytes [30]. Further studies are needed to consider the chemotactic properties of nucleated RBCs, however. On the other hand, rainbow trout RBCs exposed to IPNV showed a downregulation trend of IL-8, IL-1β, and tumor necrosis factor-α (TNF-α) protein expression [25]. Similarly, human cord blood nucleated RBCs have been shown to exert a regulatory function in the innate immune response, by means of the suppression of the production of inflammatory cytokines such as TNF-α and IL-1β from monocytes in lipopolysaccharide (LPS)-mimicked system to suppress a vigorous innate immune reaction, which can be harmful to fetuses [31].

Interferon gamma (IFNγ), a highly pleiotropic pro-inflammatory and antiviral cytokine exclusively produced in immune-related cells, has been detected in murine nucleated erythroid cells and may exert a regulatory influence on development and functionality of cells pertaining to monocyte/ macrophage lineage [32]. In addition, it has been shown that chicken RBCs stimulated with fungal species *Candida albicans* release cytokine-like factors with IFNγ-like activity [33]. The conditioned medium of shape-shifted RBCs (shRBCs), a new cell stage of rainbow trout RBCs, induced communication with rainbow trout stromal pronephros cells (TPS-2 cells) and resulted in the upregulation of IFNγ-activated genes in this cell line [34].

Taken altogether, these evidences indicate that nucleated RBCs exert paracrine molecular communication with other cells by means of cytokine production. Therefore, nucleated RBCs could play an important role in the recruitment and/or activation of immune cells.

2.3 Nucleated RBCs produce antimicrobial peptide and protein expression

Antimicrobial peptides (AMPs) exist in all living creatures in nature and present the first line of host defense against infectious pathogens [35] by means of molecular mechanisms of cellular disruption [36] and multifaceted immunomodulatory functions [35]. Fish nucleated RBCs have been reported to produce antimicrobial peptides in response to the viral infection.

Rainbow trout RBCs expose to VHSV induced an upregulation of β-defensin 1 (BD1) protein levels (**Figure 3**). Defensins belong to a family of small cysteine-rich peptides that have amphiphilic and cationic properties [37]. BD is produced and stored in epithelial cells, neutrophils, and phagocytes [38]. During infection by pathogens, BD stored in granular bodies is released into the phagosomes or the extracellular system [38]. Additionally, they are known as chemotactic attractants for immune cells and participate in immune regulation [39].

Another AMP recently found in fish RBCs is Nk-lysin (Nkl) [40]. Nkl is orthologous to human cytolytic protein granulysin, produced by natural killer cells and cytotoxic T lymphocytes [41, 42], and involved in the destruction of bacteria, fungi, and parasites [43]. Nkl is stored in cytolytic granules together with perforin and granzymes [41, 42]. However, Nkl in turbot RBCs was found in autophagolysosomes. This is reportedly known to link mechanism to VHSV defense in turbot RBCs [40].

Hepcidins, another family of cysteine-rich antimicrobial peptides, have also been found to be produced by fish RBCs [26]. They were first identified in the human liver [44] and also in some fish species [45]. But these peptides have also

been reported to be expressed in other organs such as cardiac stomach, esophagus [46], heart, gill, spleen, kidney, and peripheral blood leucocytes [47] dependent upon the species. They have been shown to respond to bacterial and viral infections [48]. Regarding RBCs, Nombela and colleagues found that rainbow trout RBCs exposed to VHSV did not vary hepcidin protein levels [26]. Therefore, the possible role of hepcidin in nucleated RBCs against infectious pathogens is not known yet.

Histone proteins share all of the essential traits of cationic AMPs (CAMPs); they are hydrophobic and cationic and can form amphipathic alpha-helical structures [49]. Recently, it has been demonstrated that a histone mixture (H1, H2A, H2B, H3, H4, and H5) extracted and purified from chicken RBCs had antimicrobial activity against a variety of Gram-negative and Gram-positive planktonic bacteria [50], as well as eradication activity against Gram-positive bacterial biofilms [51]. It has also been reported that histone H5 from chicken RBCs has a broad-spectrum antimicrobial activity [52].

In addition to AMPs, another protein with antimicrobial activity found in RBCs is hemoglobin [53], which is the most abundant protein of RBCs. It has been described that hemoglobin can elicit antimicrobial activity through reactive oxygen species production under pathogen attack [53]. The pathogen clearance from the bloodstream is also carried out by the hemoglobin oxygen [54].

In brief, nucleated RBCs can produce antimicrobial molecules in response to pathogens. It therefore supports the important contribution of RBCs in the regulation of host defense against pathogens.

2.4 Complement regulation on RBCs

The complement system is a component of the innate immune system which is involved in the clearance of pathogens, dying cells and immune complexes through opsonization, induction of an inflammatory response, and formation of a lytic pore. This system is composed by a group of 30 different plasma and membrane proteins, which are involved in three distinct pathways of complement activation: the classical, lectin, and alternative pathway. The classical pathway is activated by immune complexes, by pattern recognition molecules such as C-reactive protein (CRP), or directly by apoptotic cells and microbial surfaces. The lectin pathway is triggered by carbohydrate structures from pathogen, and the alternative pathway is activated by the spontaneous hydrolysis of the protein C3 (reviewed in [55]).

Autologous cells are protected from complement activation and posterior lysis by regulatory proteins [56]. RBCs are continuously in contact with complement proteins in the blood plasma; therefore, they have complement regulatory proteins on their cell membrane to prevent this activation [55]. It has been reported that human and rainbow trout RBCs highly express the regulatory protein complement receptor 1 (CR1) or CD35 [56, 57]. An important function of RBC CR1 is to eliminate complement-opsonized immune complexes from the circulation. A failure in this receptor can end up in inflammation and damage to healthy tissues [58]. In addition, it has been described that human RBCs can sequester typ. 5 adenovirus (Ad5) through CR1 and Coxsackie virus-adenovirus receptor (CAR), in the presence and absence of anti-Ad5 antibodies and complement, respectively. In this context, human RBCs may act as circulating viral traps or clarifiers and prevent systemic virus infection [59]. The studies of immune complex clearance in rainbow trout showed a similar complement-dependent way to eliminate immune complex as found in humans, suggesting that rainbow trout CR1 has a similar function to human CR1 [60].

3. The role of nucleated RBCs in adaptive immune response activation

The adaptive immune system consists of a specialized group of cells responsible of a specific immune response which eliminates and prevents reoccurrence of pathogens by immunological memory [61]. The cells that carry out adaptive immune response are B and T lymphocytes [62]. All nucleated cells are capable of presenting an antigen, through major histocompatibility complex (MHC) molecules [62]. MHCI plays a key role in antigen presentation of intracellular pathogens. Nucleated RBCs can express MHCI, and this molecule has been found on the surface of RBCs from rainbow trout [63], African clawed frogs [64], and chickens [65]. In addition, it has been reported that PRV infection induces genes involved in antigen presentation via MHCI in salmon RBCs [23], and incubation with polyI:C upregulates gene ontology (GO) categories related to antigen processing, antigen presentation, and MHCI receptor activity in rainbow trout RBCs [6]. Unlike MHCI, MHCII molecules are generally restricted to some endothelial cells and a subset of antigen-presenting cells (APCs), such as macrophages, dendritic cells, and B cells [66]. However, MHCII transcripts have been detected in chicken [10] and rainbow trout RBCs [67]. Moreover, in rainbow trout RBCs, a combination of transcriptome- and proteome-sequencing data identified functional pathways related to antigen presentation via major histocompatibility complex class II. The set of genes/proteins identified were ARP1 actin-related protein 1 homolog B (ACTR1B), adaptor-related protein complex 1 beta 1 subunit (AP1B1), adaptor-related protein complex 2 alpha 1 subunit (AP2A1), adaptor-related protein complex 2 alpha 2 subunit (AP2A2), ADP ribosylation factor 1 (ARF1), calnexin (CANX), capping actin protein of muscle Z-line alpha subunit 1 (CAPZA1), clathrin light chain A (CLTA), clathrin heavy chain (CLTC), cathepsin D (CTSD), dynamin 2 (DNM2), dynein cytoplasmic 1 heavy chain 1 (DYNC1H1), dynein light chain LC8 typ. 2 (DYNLL2), and member RAS oncogene family (RAB7A) (**Figure 4**) [67].

Taking the above-said observations into account, these facts indicate that nucleated RBCs might participate in antigen presentation through MHCI and MHCII and suggest that RBCs may be participants in the immunological synapse with T and NK cells. Besides, it has been published that human RBCs could play a biological role in the modulation of T-cell differentiation and survival in the active cell division [68]. Also, natural killer enhancing factor (NKEF) protein in human RBC cytosol mediates enhancement of NK cell activity [69]. In addition, in rainbow trout RBCs, functional pathways related to regulation of leukocyte activation were identified by a combination of transcriptome- and proteome-sequencing data [67]. Separately, rainbow trout RBCs have been reported to use phagocytosis to bind and engulf *Candida albicans* and present it to macrophages [70]. In fact, the identification of clusters of cells composed by RBCs and immune cells, commonly termed rosettes, leads to a crosstalk between RBCs and immune cells [70]. These evidences broaden the horizon of nucleated RBC immune functions as they open a novel topic of investigation where nucleated RBCs may act as professional APCs.

Separately, other molecules related to adaptive immune response have been identified in nucleated RBCs. An example of these molecules is the immunoreceptor tyrosine-based activation motif (ITAM) which is contained in certain transmembrane proteins of the immune system and is important for the signal transduction in immune cells [71]. ITAM-bearing molecules are expressed on rainbow trout RBCs [72]. Another molecule, Epstein–Barr virus G-protein-coupled receptor 2 (EBI2), which plays a critical role in the regulation of T-cell-dependent antibody responses and provides a mechanism to balance short- versus long-term antibody responses [73], has also been reported to be highly expressed in rainbow trout young RBCs

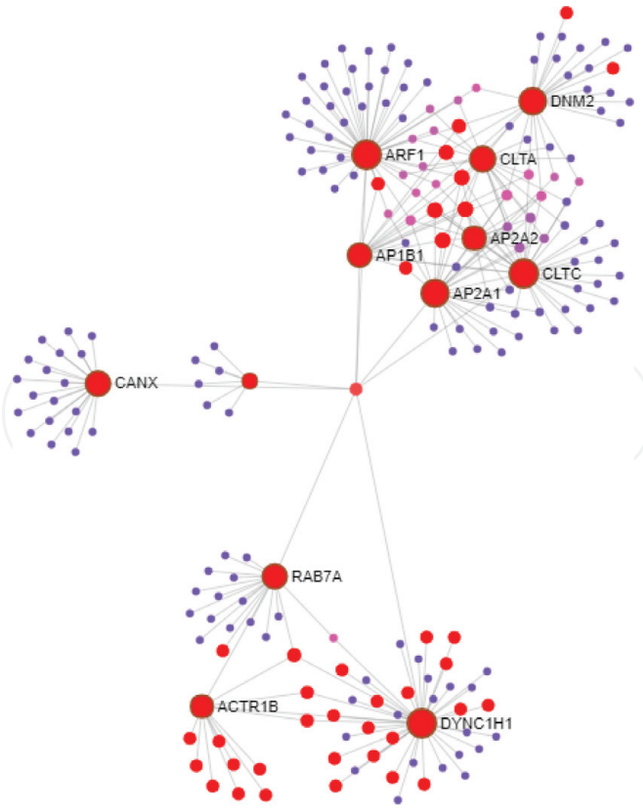

Figure 4.
An overview of protein–protein interaction network of a set of proteins, identified in rainbow trout proteome profiling, related to antigen processing and presentation of exogenous peptide antigen via MHCII. *Protein–protein interaction network was constructed using NetworkAnalyst software [75]. Highlighted red nodes represent the input protein-related antigen processing and presentation of exogenous peptide antigen via MHCII pathway (Reactome database). Other nodes represent other protein interactions within the same pathway (red nodes) or related to other pathways (other colors).*

[74]. Based on these facts, a role for RBCs in the adaptive immune response may be established. However, the function of these molecules and their effect on the antiviral adaptive immune response of nucleated RBCs remain to be studied.

4. Conclusion

Nucleated red blood cells (RBCs) of fish, amphibians, reptiles, and birds contain the transcriptional and translational machinery necessary to produce characteristic molecules of the immune system to respond against pathogen attacks. The mechanisms by which nucleated RBCs may contribute to the clearance of the pathogens are (i) phagocytosis, (ii) antigen presentation, (iii) producing cytokines and antimicrobial peptides, (iv) regulation of complement system, and (v) exerting paracrine molecular communication with other immune cells and modulate their functions. The nucleated RBCs seem to be involved in regulation of both innate and adaptive immune responses, and these findings highlight the important contribution of RBCs in the host defense against pathogens. However, more studies are needed to elucidate the role of RBCs in the immune response and the molecular mechanisms involved in these processes. And, the RBCs could be considered as potential targets for new prophylactic or therapeutic strategies against viral infections.

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Conflict of interest

The authors declare no conflict of interest.

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