PEARLS

Nucleated red blood cells: Immune cell mediators of the antiviral response
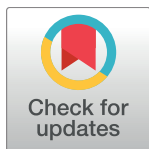
Ivan Nombela, Maria del Mar Ortega-Villaizan*

IBMC, Miguel Hernández University, Elche, Spain

* mortega-villaizan@umh.es

Introduction

The involvement of nucleated red blood cells (RBCs) as immune response cell mediators is a novel topic of research. RBCs are the most abundant cell type in the bloodstream and are best known for their roles in gas exchange and respiration. In mammals, mature RBCs are flexible, oval, biconcave disks that lack cell nuclei, organelles, and ribosomes (reviewed in Moras et al. 2017 [1]). In nonmammalian vertebrates, RBCs are oval, flattened, biconvex disks with a cytoskeleton composed of a marginal band of microtubules and a cell nucleus and organelles in their cytoplasm [2], which allow them to de novo synthesize proteins and molecules in response to stress and stimuli. In the recent past, a set of biological processes related to immunity–such as phagocytosis [3], antigen presentation [3], and interleukin-like production [4–7]–have been reported in nucleated RBCs from different species. However, elucidating the role of RBCs during viral infections is an emergent research topic of great interest. Here, we provide a brief overview of the novel role of nucleated RBCs against viral infections.

Viral pathogen-associated molecular patterns (PAMPs) induce pattern-recognition receptor (PRR) signaling in nucleated RBCs

Nucleated RBCs are implicated in the immune response to viral infections based on their response to viral PAMPs through various PRR signaling pathways. Among these receptors, the expression of Toll-like receptor 3 (TLR3) and TLR9–which are endosomal TLRs that recognize viral double-stranded RNA (dsRNA) and nonmethylated viral 5'-C-phosphate-G-3' (CpG)-containing DNA, respectively–and retinoic acid-inducible gene I (RIG-I)–a member of the RIG-I-like receptor (RLR) family that interacts intracellularly with viral dsRNA–have been reported in rainbow trout RBCs [5, 8] and Atlantic salmon [9], respectively. Chicken RBCs constitutively express *tlr3* and *tlr21*, which is a homolog of mammalian TLR9 [5, 10]. Stimulation of these receptors with their corresponding PAMPs leads to the activation of signaling networks that induce the transcription of a set of genes, resulting in a characteristic immune response.

The activation of these receptors by viral pathogens induces expression of the interferon system [11, 12]. Stimulation of rainbow trout RBCs with polyinosinic:polycytidylic acid (poly I:C, a molecule structurally similar to dsRNA) induces the de novo synthesis of mRNAs from immune genes such as chemokine (C-C motif) ligand 4 (*ccl4*), interferon-α (*ifn-α*), and myxovirus resistance gene *(mx)* [5]; and in chickens, RBCs respond to poly I:C by upregulating type I IFN (*ifn1*) and interleukin-8 (*il-8*) genes [10]. Moreover, the infectious pancreatic necrosis virus (IPNV)–a dsRNA virus–has been reported to stimulate the expression of *tlr3*, *ifn1* and *mx* genes [13]. The piscine orthoreovirus (PRV) also increases the expression of *rig-I*, *mx*, and *ifn-α* genes in Atlantic salmon RBCs [6].

The roles that other members of the RLR family, such as melanoma differentiation-associated protein 5 (MDA5) or probable ATP-dependent RNA helicase DExH-box helicase 58 (LGP2), assume in RBCs are still unknown. In addition, we still do not know if RBCs express other PRRs that recognize viral genomic RNA, such as TLR7 or TLR8. While IFN1 is thought to play a similar role in mammalian and nonmammalian species and induce similar sets of genes [14], the extent of nucleated RBCs' involvement in the global organism IFN1 response and how RBCs' involvement influences defense against viral infections remain to be defined.

Nucleated RBCs may be capable of inducing an adaptive immune response

Nucleated RBCs are linked to the adaptive immune response. Major histocompatibility complex I (MHCI) plays a key role in the antigen presentation of intracellular pathogens, which initiates adaptive immunity mechanisms. MHCI is expressed on the surface of RBCs from rainbow trout [15], Atlantic salmon [6], African clawed frogs [16], and chickens [17]. However, to date, it has only been reported that PRV infection induces genes involved in antigen presentation via MHCI in salmon RBCs [6] and that poly I:C upregulates gene ontology (GO) categories related to antigen processing, antigen presentation, and MHCI receptor activity in rainbow trout RBCs [18].

Molecules bearing the immunoreceptor tyrosine-based activation motif (ITAM), which is contained in certain transmembrane proteins of the immune system and is important for signal transduction in immune cells, are known markers of hematopoietic and immune cells [19]. ITAM-bearing molecules are expressed on rainbow trout RBCs [20]. Further, Epstein–Barr virus G-protein-coupled receptor 2 (EBI2) 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 [21]. EBI2 is highly expressed in rainbow trout young RBCs [22]. The presence of these molecules in nucleated RBCs may indicate a role of these cells in the adaptive immune response. However, the function of these molecules on RBCs and their effect on the antiviral adaptive immune response remain to be studied.

Nucleated RBCs trigger diverse immune responses against viral aggression

Three viruses from different families that infect or replicate inside nucleated RBCs have been identified: (i) infectious salmonid anemia virus (ISAV) from the Orthomyxoviridae family with single-stranded RNA (ssRNA) [7], (ii) PRV from the Reoviridae family with dsRNA [6, 23], and (iii) erythrocytic viral infections, reviewed in Paperna and Alves de Matos [24]. Fig 1 schematically summarizes the response of nucleated RBCs to these viruses. Unfortunately, information on the immune response of RBCs to erythrocytic viral infections is not available.

A study of nucleated RBCs from ISAV-infected Atlantic salmon first demonstrated the ability of RBCs to induce an immunological response against a viral pathogen. This response was characterized by the induction of *ifn-α* in hemagglutinated RBCs [7]. Recently, it has been shown that PRV also can induce the expression of *ifn-α*–in addition to *mx*, protein kinase RNA-activated (*pkr*) [6], *viperin*, and interferon-stimulated gene 15 (*isg15*) [25] antiviral genes–in PRV-challenged Atlantic salmon RBCs.

Recently, Nombela and colleagues demonstrated that nucleated RBCs can generate immune responses to viruses despite not being infected. Rainbow trout RBCs are nonpermissive to viral hemorrhagic septicemia virus (VHSV) [26] and infectious pancreatic necrosis virus (IPNV) infections [13], likely due to the inability of VHSV and IPNV to replicate in ex vivo purified rainbow trout RBCs. This phenomenon is known as nonproductive or abortive infection in nonpermissive cells and occurs when a virus enters a host cell and some or all viral components are synthesized but nonproductive or defective viruses are ultimately released because
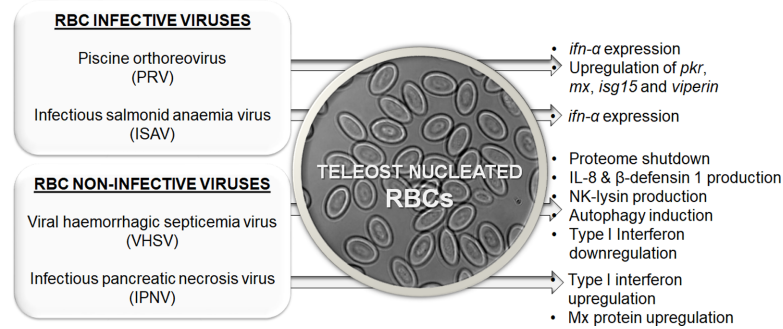
Fig 1. Schematic representation of teleost nucleated RBC immune responses against different infective (target: RBCs) or noninfective (target: other cell types) viral pathogens. *ifn-α*, interferon-α; IL-8, interleukin-8; *isg15*, interferon-stimulated gene 15; *mx*, myxovirus resistance gene; *pkr*, protein kinase RNA-activated; RBC, red blood cell.

the host cell is nonpermissive or inhibits the replication of the virus. Previously, abortive infection in a macrophage cell line was linked with the constitutive expression of the antiviral Mx protein by macrophages [27]. Similarly, high levels of constitutive Mx transcripts and protein have been identified in rainbow trout RBCs (Fig 2), suggesting a possible mechanism for aborted or halted infections in RBCs [13, 26]. Nevertheless, rainbow trout RBCs can develop diverse immune responses to VHSV halted replication, a process characterized by global proteome downregulation–mainly of proteins from the proteasome and RNA stability processes–increased expression of IL-8 and β-defensin 1, decreased expression of genes related to the IFN1 pathway, and an antioxidant response [13]. In the case of IPNV aborted infection in rainbow trout RBCs, there was an increase in the expression of *ifn1*, *mx*, interferon regulatory factor 7 (*irf7*), and *pkr* genes, followed by upregulation of Mx protein expression [13] (summarized in Fig 1).

Considering their ability to produce immune proteins related to interferon, pro-inflammatory cytokines, antimicrobial peptides, proteasome [26], and autophagy [28] pathways, nucleated RBCs likely are able to trigger an immune response similar to that of their leukocyte counterparts by activating diverse immune mechanisms to complement the protection against infection conferred to the host organism.
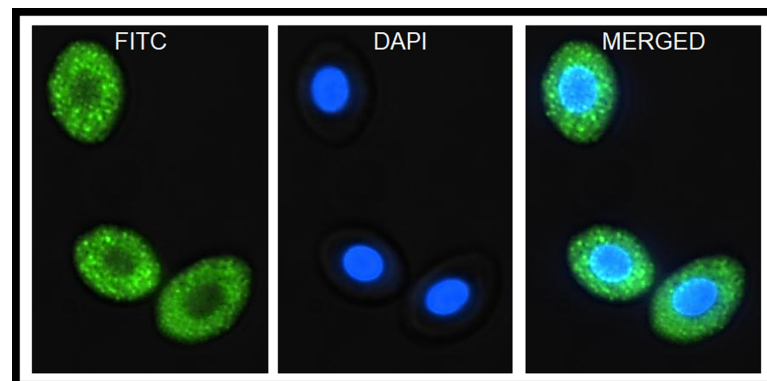
Fig 2. Constitutive expression of Mx antiviral protein in rainbow trout nucleated RBCs. Immunofluorescence images of Mx protein expression in nucleated RBCs. FITC: Mx protein expression; DAPI: nuclei. Images were obtained using an INCell Analyzer 6000 Cell imaging system (GE Healthcare, Little Chalfont, United Kingdom). DAPI, 4',6-diamidino-2-phenylindole; FITC, Fluorescein-5-isothiocyanate; Mx, myxovirus resistance gene; RBC, red blood cells.

Nucleated RBCs can mount immune responses against nonviral pathogens

The RBCs of mammalian and nonmammalian vertebrates are hosts for approximately 40 genera, including protists, prokaryotes, and viruses [18, 29]. Few blood infections of fish, amphibians, reptiles, and birds have proven pathogenicity, in contrast to the many known intraerythrocytic mammalian pathogens [29]. To date, few studies have reported diverse immune responses of nucleated RBCs to this broad spectrum of pathogens.

As previously described, the immune response against viruses is generally associated with the expression of IFN1 and ISGs. In response to bacterial lipopolysaccharide (LPS), rainbow trout RBCs upregulate the expression of tumor necrosis factor receptor-like (*tnfr-like*), oxidative-stress response 1 (*oxsr1*), *irf1*, and *mhcI* genes. Several reports have shown that hemoglobin, the most abundant protein of RBCs, has antibacterial activity and can elicit antimicrobial activity through reactive oxygen species production when under pathogen attack [30]. In rainbow trout, acid-soluble extracts from RBCs showed antibacterial activity against a variety of bacteria, including *Planococcus citreus* and *Escherichia coli* [31]. In the presence of the fungus *Candida albicans*, rainbow trout [3], and chickens [4], RBCs performed innate immunity functions, using phagocytosis to bind and engulf *C. albicans* and present to macrophages. Ultimately, little is known regarding the immune response triggered by nucleated RBCs against the broad range of pathogens that infect them.

Nucleated RBCs are future targets for vaccines

Human non-nucleated RBCs have long been investigated for the transportation of drugs or antigens through the blood [32, 33]. Proteomic studies of human [34] and nonhuman primate species [35] aim to further characterize the biology of human RBCs and identify future targets for newer-generation vaccines, especially against malaria. Because of the ability of nucleated RBCs to generate and modulate immune responses, development of a new generation of vaccines targeting membrane receptors or intracellular molecules of nucleated RBCs capable of triggering and stimulating the antiviral immune response is a promising and exciting field. Such vaccines may contribute greatly to organism survival, given the large volume of RBCs and their fast distribution through the organism. However, additional proteomic studies of nucleated RBCs are needed to identify potential therapeutic targets.

Acknowledgments

References

1. Moras M., Lefevre S.D., and Ostuni M.A., *From Erythroblasts to Mature Red Blood Cells: Organelle Clearance in Mammals*. Front Physiol, 2017. 8: p. 1076. https://doi.org/10.3389/fphys.2017.01076 PMID: 29311991

2. Glomski C.A., Tamburlin J., and Chainani M., *The phylogenetic odyssey of the erythrocyte. III. Fish, the lower vertebrate experience*. Histol Histopathol, 1992. 7(3): p. 501–28. PMID: 1504472

3. Passantino L, et al., *Fish immunology. I. Binding and engulfment of Candida albicans by erythrocytes of rainbow trout (Salmo gairdneri Richardson)*. Immunopharmacol Immunotoxicol, 2002. 24(4): p. 665–78. https://doi.org/10.1081/IPH-120016050 PMID: 12510797

4. Passantino L, et al., *Antigenically activated avian erythrocytes release cytokine-like factors: a conserved phylogenetic function discovered in fish*. Immunopharmacol Immunotoxicol, 2007. 29(1): p. 141–52. https://doi.org/10.1080/08923970701284664 PMID: 17464774

5. Morera D, et al., *RNA-Seq reveals an integrated immune response in nucleated erythrocytes*. PLoS ONE, 2011. 6(10): p. e26998. https://doi.org/10.1371/journal.pone.0026998 PMID: 22046430

6. Dahle M.K, et al., *Transcriptome analyses of Atlantic salmon (Salmo salar L.) erythrocytes infected with piscine orthoreovirus (PRV)*. Fish Shellfish Immunol, 2015. 45(2): p. 780–90. https://doi.org/10.1016/j.fsi.2015.05.049 PMID: 26057463

7. Workenhe S.T, et al., *Infectious salmon anaemia virus replication and induction of alpha interferon in Atlantic salmon erythrocytes*. Virol J, 2008. 5: p. 36. https://doi.org/10.1186/1743-422X-5-36 PMID: 18307775

8. Rodriguez M.F, et al., *Characterization of Toll-like receptor 3 gene in rainbow trout (Oncorhynchus mykiss)*. Immunogenetics, 2005. 57(7): p. 510–9. https://doi.org/10.1007/s00251-005-0013-1 PMID: 16086174

9. Wessel O, et al., *Piscine orthoreovirus (PRV) replicates in Atlantic salmon (Salmo salar L.) erythrocytes ex vivo*. Vet Res, 2015. 46: p. 26. https://doi.org/10.1186/s13567-015-0154-7 PMID: 25888832

10. St Paul M, et al., *Chicken erythrocytes respond to Toll-like receptor ligands by up-regulating cytokine transcripts*. Res Vet Sci, 2013. 95(1): p. 87–91. https://doi.org/10.1016/j.rvsc.2013.01.024 PMID: 23433682

11. Robertsen B, *The interferon system of teleost fish*. Fish Shellfish Immunol, 2006. 20(2): p. 172–91. https://doi.org/10.1016/j.fsi.2005.01.010 PMID: 15939626

12. Zou J, Bird S, and Secombes C, *Antiviral sensing in teleost fish*. Curr Pharm Des, 2010. 16(38): p. 4185–93. PMID: 21184657

13. Nombela I, et al., *Infectious pancreatic necrosis virus triggers antiviral immune response in rainbow trout red blood cells, despite not being infective*. F1000Research, 2017. 6: p. 1968. https://doi.org/10.12688/f1000research.12994.2 PMID: 29333244

14. Schultz U, Kaspers B, and Staeheli P, *The interferon system of non-mammalian vertebrates*. Dev Comp Immunol, 2004. 28(5): p. 499–508. https://doi.org/10.1016/j.dci.2003.09.009 PMID: 15062646

15. Sarder M.R, et al., *The MHC class I linkage group is a major determinant in the in vivo rejection of allogeneic erythrocytes in rainbow trout (Oncorhynchus mykiss)*. Immunogenetics, 2003. 55(5): p. 315–24. https://doi.org/10.1007/s00251-003-0587-4 PMID: 12879308

16. Nedelkovska H, et al., *Effective RNAi-mediated beta2-microglobulin loss of function by transgenesis in Xenopus laevis*. Biol Open, 2013. 2(3): p. 335–42. https://doi.org/10.1242/bio.20133483 PMID: 23519478

17. Delany M.E, et al., *Cellular expression of MHC glycoproteins on erythrocytes from normal and aneuploid chickens*. Dev Comp Immunol, 1987. 11(3): p. 613–25. PMID: 3678561

18. Morera D and MacKenzie S.A, *Is there a direct role for erythrocytes in the immune response?* Vet Res, 2011. 42(1): p. 89.

19. Humphrey M.B, Lanier L.L, and Nakamura M.C, *Role of ITAM-containing adapter proteins and their receptors in the immune system and bone*. Immunol Rev, 2005. 208: p. 50–65. https://doi.org/10.1111/j.0105-2896.2005.00325.x PMID: 16313340

20. Ohashi K, et al., *A molecule in teleost fish, related with human MHC-encoded G6F, has a cytoplasmic tail with ITAM and marks the surface of thrombocytes and in some fishes also of erythrocytes*. Immunogenetics, 2010. 62(8): p. 543–59. https://doi.org/10.1007/s00251-010-0460-1 PMID: 20614118

21. Gatto D, et al., *Guidance of B cells by the orphan G protein-coupled receptor EBI2 shapes humoral immune responses*. Immunity, 2009. 31(2): p. 259–69. https://doi.org/10.1016/j.immuni.2009.06.016 PMID: 19615922

22. Gotting M and Nikinmaa M.J, *Transcriptomic Analysis of Young and Old Erythrocytes of Fish*. Front Physiol, 2017. 8: p. 1046. https://doi.org/10.3389/fphys.2017.01046 PMID: 29311976

23. Finstad O.W, et al., *Piscine orthoreovirus (PRV) infects Atlantic salmon erythrocytes*. Vet Res, 2014. 45: p. 35. https://doi.org/10.1186/1297-9716-45-35 PMID: 24694042

24. Paperna I and Alves de Matos A.P, *Erythrocytic viral infections of lizards and frogs: new hosts, geographical locations and description of the infection process*. Annales de Parasitologie Humaine et Comparée, 2016. 68(1): p. 11–23.

25. Haatveit H.M, et al., *Viral Protein Kinetics of Piscine Orthoreovirus Infection in Atlantic Salmon Blood Cells*. Viruses, 2017. 9(3): p. 49.

26. Nombela I, et al., *Identification of diverse defense mechanisms in rainbow trout red blood cells in response to halted replication of VHS virus*. F1000Research, 2017. 6: p. 1958. https://doi.org/10.12688/f1000research.12985.2 PMID: 29527292

27. Pham P.H, et al., *Differential effects of viral hemorrhagic septicaemia virus (VHSV) genotypes IVa and IVb on gill epithelial and spleen macrophage cell lines from rainbow trout (Oncorhynchus mykiss)*. Fish Shellfish Immunol, 2013. 34(2): p. 632–40. https://doi.org/10.1016/j.fsi.2012.12.004 PMID: 23257204

28. Pereiro P, et al., *Nucleated Teleost Erythrocytes Play an Nk-Lysin- and Autophagy-Dependent Role in Antiviral Immunity*. Frontiers in Immunology, 2017. 8: p. 1458. https://doi.org/10.3389/fimmu.2017.01458 PMID: 29163526

29. Davies A.J and Johnston M.R, *The biology of some intraerythrocytic parasites of fishes, amphibia and reptiles*. Adv Parasitol, 2000. 45: p. 1–107. PMID: 10751939

30. Jiang N, et al., *Respiratory protein-generated reactive oxygen species as an antimicrobial strategy*. Nat Immunol, 2007. 8(10): p. 1114–22. https://doi.org/10.1038/ni1501 PMID: 17721536

31. Fernandes J.M and Smith V.J, *Partial purification of antibacterial proteinaceous factors from erythrocytes of Oncorhynchus mykiss*. Fish Shellfish Immunol, 2004. 16(1): p. 1–9. PMID: 14675829

32. Cremel M, et al., *Red blood cells as innovative antigen carrier to induce specific immune tolerance*. Int J Pharm, 2013. 443(1–2): p. 39–49. https://doi.org/10.1016/j.ijpharm.2012.12.044 PMID: 23305866

33. Muzykantov V.R, *Drug delivery by red blood cells: vascular carriers designed by mother nature*. Expert Opin Drug Deliv, 2010. 7(4): p. 403–27. https://doi.org/10.1517/17425241003610633 PMID: 20192900

34. D'Alessandro A and Zolla L, *Proteomic analysis of red blood cells and the potential for the clinic: what have we learned so far*? Expert Rev Proteomics, 2017. 14(3): p. 243–252. https://doi.org/10.1080/14789450.2017.1291347 PMID: 28162022

35. Moreno-Perez D.A, et al., *The Aotus nancymaae erythrocyte proteome and its importance for biomedical research*. J Proteomics, 2017. 152: p. 131–137. https://doi.org/10.1016/j.jprot.2016.10.018 PMID: 27989940