

# Innate immunity in vertebrates: an overview

Mario Riera Romo,<sup>1</sup> Dayana  
Pérez-Martínez<sup>2</sup> and Camila  
Castillo Ferrer<sup>3</sup>

<sup>1</sup>Pharmacology Department, Centre of Marine  
Bioproducts, Havana, <sup>2</sup>Tumour Immunology  
Direction, Centre of Molecular Immunology,  
Havana, and <sup>3</sup>Biochemistry Department,  
Faculty of Biology, University of Havana,  
Havana, Cuba

doi:10.1111/imm.12597

Received 3 December 2015; revised 10

February 2016; accepted 11 February 2016.

Correspondence: Mario Riera Romo, Centre  
of Marine Bioproducts, Loma and 37 St,  
Alturas del Vedado, PA 10600, Havana,  
Cuba. Email: rieraromo@gmail.com  
Senior author: Mario Riera Romo

## Summary

Innate immunity is a semi-specific and widely distributed form of immunity, which represents the first line of defence against pathogens. This type of immunity is critical to maintain homeostasis and prevent microbe invasion, eliminating a great variety of pathogens and contributing with the activation of the adaptive immune response. The components of innate immunity include physical and chemical barriers, humoral and cell-mediated components, which are present in all jawed vertebrates. The understanding of innate defence mechanisms in non-mammalian vertebrates is the key to comprehend the general picture of vertebrate innate immunity and its evolutionary history. This is also essential for the identification of new molecules with applications in immunopharmacology and immunotherapy. In this review, we describe and discuss the main elements of vertebrate innate immunity, presenting core findings in this field and identifying areas that need further investigation.

**Keywords:** defence mechanisms; evolutionary history; infection; innate immunity; vertebrates.

## Introduction

Innate immunity is defined as the first line of defence against pathogens, representing a crucial systemic response to prevent infection and maintain homeostasis.<sup>1–3</sup> This type of immunity also has a critical role in the activation and regulation of adaptive immunity.<sup>4</sup> Despite its constitutive elements, innate defence mechanisms have the capacity to develop an induced response during primary infection and create inflammatory conditions.<sup>5,6</sup> This response is specific due to the cell surface expression of pattern recognition receptors, which are capable of recognizing complex polysaccharides, glycolipids, lipoproteins, nucleotides and nucleic acids.<sup>4,7</sup>

The elements of innate immunity include external physical barriers, humoral and cellular effector mechanisms, components that are conserved among jawed vertebrates (Table 1) with certain variations and specific characteristics.<sup>2,3,8</sup> Innate immunity has been extensively studied in mammals in comparison to other vertebrates, because of its significance for human health and veterinary sciences.<sup>3,9–11</sup> However, a major exploration of these

innate mechanisms in non-mammalian species is essential for comprehension of the complex events involved in human innate immunity and is also crucial for the discovery of new antimicrobial, antitumour and immunomodulatory molecules with therapeutic applications.

Recent studies have evaluated the innate immune mechanisms in pathogen–host interactions and their phylogenetic conservation across animal species, revealing new evidence that is changing the concept of innate immunity. The former view of a non-inducible and pre-existing innate immune response is now evolving to the concept of a more specific immunity based on pattern recognition receptor molecular recognition and even memory development, leading to increased responses to secondary infections.<sup>6,7,12–14</sup> This innate immunological memory depends mainly on natural killer (NK) cells and macrophages, which provide protection against re-infection in a T/B-cell-independent manner.<sup>13</sup> The development of memory responses in innate cells, involves epigenetic changes based on methylation and acetylation, a functional reprogramming that induces cell reactivation upon secondary stimulation.<sup>14</sup> The memory in innate

Abbreviations: AMP, antimicrobial peptide; APP, acute phase protein; CRP, C-reactive protein; FV3, frog virus 3; IFN, interferon; IL, interleukin; ITL, intraepithelial lymphocyte; NAb, natural antibody; NK, natural killer; TGF, transforming growth factor; TNF, tumour necrosis factor

**Table 1.** General overview of vertebrate innate immunity

	Fish	Amphibians	Reptiles	Birds	Mammals	References
Physical/chemical barriers						
External protective structures	+	—	++	++	—	15,16,18,19
External mucous secretions	++	++	+	+	+	15,17,16,18
Specialized skin and mucosa	±	+	+	+	++	2,16,20,24
Humoral innate immunity						
Lysozyme	+	+	+	+	+	20,21,33,34,35
Antimicrobial peptides	+	++	+	+	++	15,20,22,37,40
Acute phase proteins	++	+	+	+	++	9,10,47,49,51
Complement	++	+	+	+	++	49,52,53,56,60
Cytokines	+	+	+	+	++	65,66,68,70,71,73
Natural antibodies	+	+	+	+	+	60,93,96,97,100
Cell-mediated innate immunity						
Intraepithelial T lymphocytes	+	+	+	+	+	11,25,26,27,28
Myeloid phagocytic cells	+	+	+	+	+	73,75,104,108,112
Nuocytes	—	—	—	—	+	85
Innate lymphoid cells	—	—	—	—	+	113
Phagocytic B cells	+	+	+	?	—	94,114,110
Non-specific cytotoxic cells	+	+	?	+	+	116,118,121,123
Mucosa-associated invariant T cells	—	—	—	—	+	84

immunity has been described as a defence mechanism present not only in vertebrate organisms, but also in invertebrates such as plants and insects.<sup>12</sup> In this context, the study of innate immunity in non-mammalian vertebrates could provide integrative evidence for the understanding of this changing type of immunity and its evolutionary history.

In the current review, we examine the main defence systems of vertebrate innate immunity, describing its components, functions and properties. We also discuss the evolutionary conservation and diversification of these mechanisms, comparing them with their counterpart in mammals and providing a background of what is known and what needs further investigation in vertebrate innate immunity.

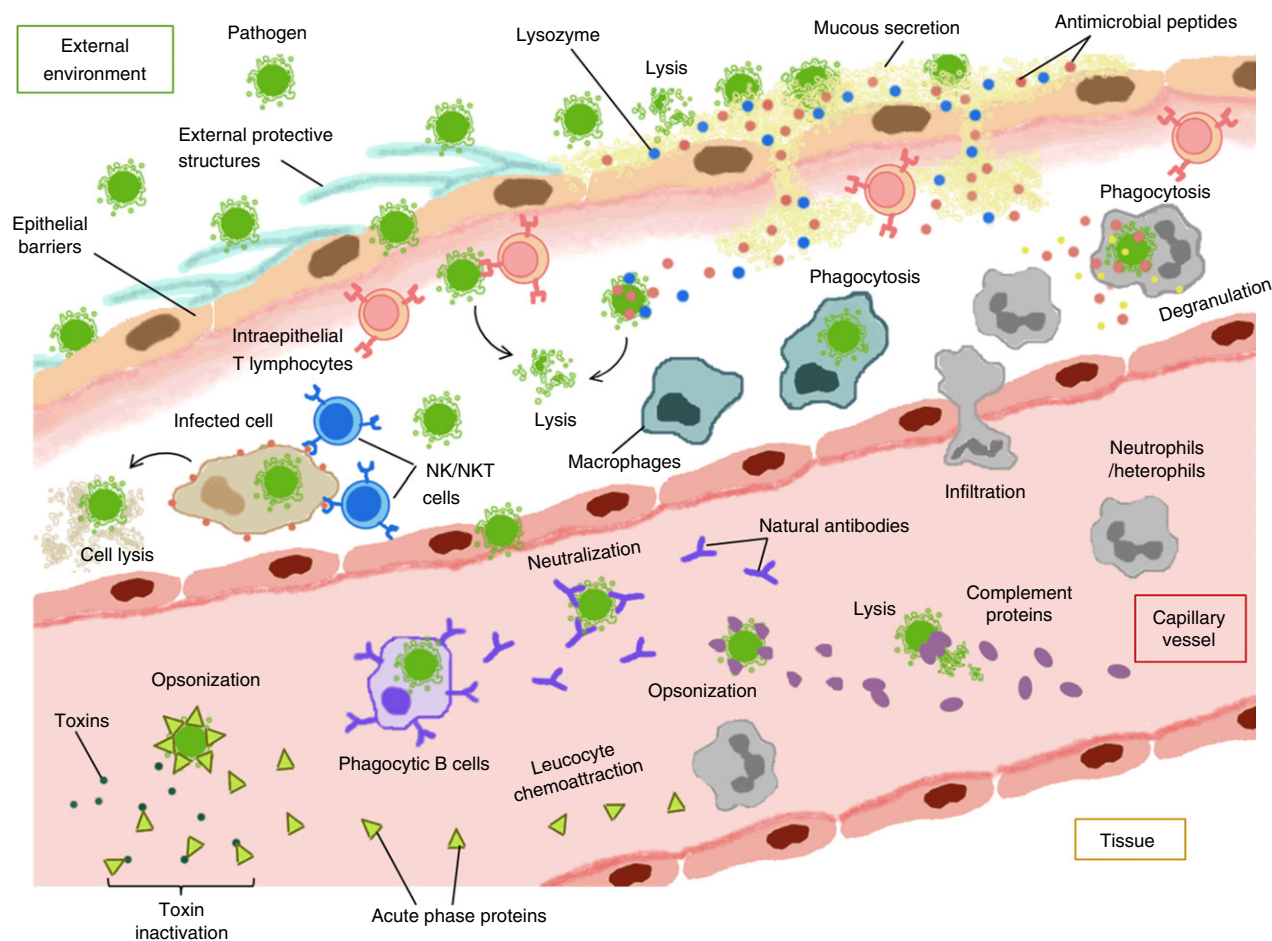
### Physical and chemical barriers

External structures as a protection strategy against pathogens are the primary mechanism of innate immunity, which prevents microbe spreading and infection.<sup>1,2,15,16</sup> Among different species these barriers have morphological and anatomical variations, but they preserve the essential role of isolating the internal environment from external factors, such as harmful substances and pathogenic microorganisms<sup>2,15,17</sup> (Fig. 1).

The skin is the main protective barrier in all living vertebrates and consists of stratified epithelial layers with tight junctions and structural proteins that provide mechanical protection, preventing water loss and pathogen infiltration, in addition to other sensory and excretory functions.<sup>16,18</sup> Vertebrate integument contains two multilayered zones or

regions: the dermis, which is the most internal and the epidermis, which is the most external and is in direct contact with environmental factors and pathogens.<sup>16,18,19</sup> This basic morphology is shared by the five classes of vertebrates, but the adaptation to their respective habitats has led to the evolution of certain functional and structural differences. For example, fish epidermis is protected by ossified scales, actin-rich filaments called microridges and localized keratin filaments, which are adequate mechanical defences for aquatic environments and their associated pathogens.<sup>15,16</sup> The subsequent colonization of terrestrial habitats by tetrapods led to the evolution of a more reinforced and specialized epidermis with an active keratinization process and a resistant outer keratinized layer, the stratum corneum, which is made of dead cells that are continually desquamated.<sup>16,18</sup>

In amphibians this corneous envelope contains a few cell layers with  $\alpha$ -keratin and does not form scales, whereas in reptiles it is improved by a hard cornification process that allows the formation of a thicker epidermis with multiple layers of  $\alpha$ - and  $\beta$ -keratin and rigid corneal scales that confer additional protection.<sup>2,16,18,19</sup> Avian epidermis is similar to its reptilian predecessor and the multiple  $\alpha$ - and  $\beta$ -keratin layers, with a predominant  $\beta$ -keratinized stratum corneum, is conserved.<sup>16,18,19</sup> Birds do not possess scales but instead they have feathers, which are chemically and structurally resistant to pathogens and are also essential for aerial locomotion.<sup>18</sup> Interestingly, mammalian epidermis and its main appendages, the hair, only contain  $\alpha$ -keratin and keratin-associated proteins, consequently they are mechanically less resistant in comparison with scales or feathers.<sup>16,18,19</sup> However,



**Figure 1.** Integrative view of vertebrate innate immunity. Schematic illustration of the main innate defence systems in jawed vertebrates, which represents its physical, humoral and cell-mediated components and the interactions between them, in a physiological context.

this loss of rigidity is compensated with a soft, flexible and moistened stratum corneum, which is necessary for mammalian locomotion and sensory functions. This cornuous layer has a specialized desquamation process and a weakly acidic pH condition that prevent infections with high efficiency.<sup>16,19</sup>

In addition, the skin of all vertebrates is constantly lubricated with external secretions that maintain the epidermal integrity and moisture and also exhibit important antimicrobial properties.<sup>16,18</sup> These secretions in aquatic and semi-aquatic anamniotic vertebrates (fish and amphibians) form a mucous cuticle that contains abundant mucopolysaccharides and glycoproteins, whereas in terrestrial amniotes (reptilians, birds and mammals) they form a sebaceous fluid with glycolipids and lipids.<sup>15,17,18</sup> Despite these differences in chemical composition, the external secretions of all vertebrates are enriched in microbicidal molecules, such as lysozyme and antimicrobial peptides (AMPs), which are an important innate defence mechanism.<sup>2,3,15,20–22</sup> The phylogenetic conservation and functional properties of these molecules are discussed in a separate section.

The vertebrate skin also possesses specific cellular responses to pathogen invasion. For instance, epithelial cells can react to microbe contact by thickening and cellular hyperplasia.<sup>8,18</sup> Likewise, immature dendritic cells, called Langerhans cells, infiltrate into the stratified layers of epidermis where they extend dendritic prolongations, capturing antigens and preventing local infection.<sup>2,16,20,23</sup> Langerhans cells have been extensively characterized in mammals but they are present in other vertebrates with similar phenotypic and functional characteristics.<sup>16,23</sup> Other innate cells associated with these epithelial barriers are the specific intraepithelial lymphocytes and macrophages that destroy pathogens as well as transformed or infected cells.<sup>1,2,23</sup> The role of these innate immune cells is analysed in detail in the Cell-mediated innate immunity section.

Mucosal membranes form another important epithelial barrier conserved in vertebrate evolution that protects the body cavities from invading pathogens.<sup>2,20,24</sup> In fish, the mucosal epithelium of the gills and the alimentary tract protects the areas that are most vulnerable to microbe penetration in aquatic environments.<sup>8</sup> On the other hand, more specialized mucosa of respiratory and gastrointestinal

tracts in terrestrial amniotes, prevent the infiltration of foreign particles and air-associated or ingested pathogens.<sup>16,20</sup> In general, the mucosa of vertebrates exhibit basic biochemical defences against microbes and parasites such as the low pH, hydrolytic enzymes, the internal mucus layer and the secretion of bioactive molecules that include lysozyme, AMPs and pro-inflammatory cytokines.<sup>1,2,20,22,25</sup> Other immune-related tissues, like the gut-associated lymphoid tissue, are also present in all jawed vertebrates and contain high levels of infiltrating intraepithelial lymphocytes that destroy pathogens and preserve epithelial integrity.<sup>3,11,26–29</sup> In addition, the presence of symbiont microbes, organized in communities called microbiomes, have been reported in the majority of vertebrates; where they are associated with the mucosal surfaces and the skin.<sup>17,20</sup> They inhibit the colonization by pathogenic microorganisms, competing with them for attachment sites and producing antimicrobial metabolites.<sup>17,20,24</sup>

## Humoral innate immunity

### Lysozyme

The mucous secretions and the plasma of all vertebrates are enriched in proteins with antimicrobial activity

(Table 2). One of the most active is the lysozyme (muramidase or N-acetyl muramide glycanohydrolase), an enzyme that lyses bacteria.<sup>1,3,21,30</sup> This protein has been found in fish,<sup>30</sup> generally associated with the mucous cuticle,<sup>8,15,31</sup> whereas in amphibians like *Rana pipiens* eight lysozyme isoforms were identified by chromatographic and electrophoretic methods in the skin, serum, liver, kidney, ovary and spleen, demonstrating lytic capacity on a *Micrococcus lysodeikticus*-agar suspension.<sup>32</sup> This enzyme has also been identified in anurans like *Xenopus laevis*<sup>33</sup> and *Xenopus tropicalis*.<sup>31</sup> In addition, different lysozyme homologues have been isolated from reptiles such as the Chinese soft-shelled turtle (*Pelodiscus sinensis*), Asian soft-shelled turtle (*Amyda cartilaginea*) and green sea turtle (*Cheloni amydas*), demonstrating lytic ability against several strains of Gram-positive and Gram-negative bacteria.<sup>34</sup> In the same way, using an improved lytic assay on *Micrococcus lysodeikticus*-agar plates, lysozyme has been detected and quantified in the plasma of captive birds, such as chickens (*Gallus gallus*), American kestrels (*Falco sparverius*), cockatiels (*Nymphicus hollandicus*) and zebra finches (*Taeniopygia guttata*) and free-living birds, such as Clay-coloured thrushes (*Turdus grayi*), blue-grey tanagers (*Thraupis episcopus*), crimson-backed tanagers (*Ramphocelus dimidiatus*), variable seedeaters

Table 2. Detailed humoral immunity in jawed vertebrates

Molecule	Function	Organism <sup>1</sup>	References
$\alpha$ -Defensins	Antimicrobial peptides	B,M	20,22,37
$\beta$ -Defensins	Antimicrobial peptides	R,B,M	20,22,37,38
$\theta$ -Defensins	Antimicrobial peptides	M	22,37
Cathelicidins	Antimicrobial peptides	F, R, M	15,20,37,39
Hepcidin	Antimicrobial peptide	F, A, R, M	40
Cationic peptides	Antimicrobial peptides	F, A, R, M	15,22,37,43,44
C-reactive protein	Acute phase protein	F, A, M	9,10,48,49
Serum amyloid P	Acute phase protein	F, M	10,48,49
Serum amyloid A	Acute phase protein	F, R, B, M	9,10,49,51
Haptoglobin	Acute phase protein	F, B, M	10,46,52
$\alpha$ -1 acid glycoprotein	Acute phase protein	B, M	9,10
$\alpha$ -2 macroglobulin	Acute phase protein	F, M	9,10,52
Fibrinogen	Acute phase protein	F, R, B, M	9,10,49,51,52
C3	Complement protein	F, A, R, B, M	23,49,51,52,53, 55
B factor	C3 convertase	F, A, B, M	49,51,52,61
C4	Complement protein	F, B, M	49,51,52,53
C5	Complement protein	F, A, B, M	23,49,51,52
C6	Complement proteins	M	52,53,55
C1, C2, C7, C8, C9	Complement proteins	F, M	52,53,55
Type I interferons	Antiviral cytokines	F, A, B, M	65,66,67,70
Type II interferons	Phagocytosis stimulators	F, A, R, B, M	65,70,73,74
Type III interferons	Antiviral cytokines	A, M	71
Interleukins	Immunomodulatory cytokines	F, A, R, B, M	64,70,75,77,80
Tumour necrosis factor- $\alpha$	Pro-inflammatory cytokine	F, A, B, M	49,64,70,75
Transforming growth factor- $\beta$	Immunomodulatory cytokine	F, A, R, B, M	25,80,89,90
IgM	Natural antibody	F, A, R, B, M	98,93,96,97,100

<sup>1</sup>F, fish; A, amphibians; R, reptiles; B, birds; M, mammals.



(*Sporophila americana*) and white-tipped doves (*Leptotila verreauxi*).<sup>35</sup> The bactericidal activity of these avian plasma samples against *Staphylococcus aureus* and *Escherichia coli* was evaluated, demonstrating lytic capacity against both bacterial strains with higher levels for *Escherichia coli* in all avian species.<sup>35</sup> This enzyme is also expressed in different mouse, rat and human tissues and is secreted by glandular serous cells, surface epithelial cells, and macrophages into the airway lumen.<sup>20,21</sup> Mammalian lysozyme has been characterized at gene and protein levels in rodents, where different isoforms have been identified.<sup>21,36</sup> The bacteriolytic capacity of rat lysozyme has been evaluated *in vitro* and *in vivo*, in transgenic mice, demonstrating a major activity against Gram-positive bacteria like *Pseudomonas aeruginosa* and *Staphylococcus aureus*.<sup>21</sup> In humans, high concentrations of lysozyme can be found in the airway surface liquid, where it contributes to the microbicidal properties of nasal secretions.<sup>20</sup> This protein is also produced by neutrophils, macrophages and specialized intestinal secretory cells (Paneth cells), but can be secreted by gastrointestinal epithelial cells during inflammatory and infectious conditions, such as ulcerative colitis and Crohn's disease.<sup>20</sup>

Recent phylogenetic analyses demonstrate the wide distribution of this enzyme across the whole animal kingdom.<sup>33</sup> The amino acid sequences of different lysozyme types have been identified in vertebrates such as humans, mice (*Mus musculus*), chickens (*Gallus gallus*), turtles (*Pelodiscus sinensis*), frogs (*Xenopus laevis*), fish (*Epinephelus coioides* and *Paralichthys olivaceus*), in cephalochordates like amphioxus (*Branchiostoma japonicum*) and in several invertebrates and plants.<sup>33</sup> The high conservation of this protein in multicellular organisms is strong evidence of its crucial role in innate immunity.

### Antimicrobial peptides

The AMPs are another innate humoral defence that is highly conserved in vertebrates. These molecules exhibit antibacterial, antifungal and antiparasitic activity through a wide variety of mechanisms including destabilization and disruption of microbial membrane, pore formation, protein aggregation, inhibition of intracellular targets, interference in DNA transcription and blockade of protein synthesis and folding.<sup>22,37</sup> The most conserved peptides include defensins, cathelicidins and hepcidin. Defensins and cathelicidins are present in the mucous secretions of fish<sup>15</sup> and reptiles like the European pond turtle (*Emys orbicularis*)<sup>38</sup> and the banded krait (*Bungarus fasciatus*).<sup>39</sup> The  $\beta$ -defensin and cathelicidin-BF, isolated from reptiles exhibit bactericidal capacity against *Salmonella typhimurium*, *Listeria monocytogenes*, *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* and antifungal activity against *Candida albicans*.<sup>38,39</sup> Likewise, the defensin-like peptides and cathelicidins

isolated from fish have potent antimicrobial activity against different pathogens, such as bacteria, fungi, viruses or parasites.<sup>15</sup> The  $\alpha$ - and  $\beta$ -defensins are also found in birds and different mammals, such as cows, goats, pigs, rats and mice.<sup>22,37</sup> In humans, specific  $\beta$ -defensins such as the human  $\beta$ -defensins 1, 2 and 3, produced by epithelial cells and neutrophils, have been identified.<sup>20,37</sup> Other neutrophil-derived antimicrobial peptides include azurocidins and  $\alpha$ -defensins like the human neutrophil peptides 1, 2 and 3.<sup>20</sup> On the other hand, in some mammals specific cathelicidins have been found, such as PR-39 in pigs and LL-37 in humans<sup>20,37</sup> and a unique type of defensin, the  $\theta$ -defensins, has been isolated from the rhesus macaque (*Macaca mulatta*).<sup>22,37</sup> The mammalian defensins and cathelicidins have been shown to be an effective mechanism against bacterial infections with *Salmonella typhimurium*, group A streptococcus, some *Staphylococcus* species and *Pseudomonas aeruginosa*.<sup>20</sup> Besides, they display multiple immunomodulatory functions that include the stimulation of chemokine production, inhibition of pro-inflammatory cytokines, direct leucocyte chemotaxis and modulation of host cell gene expression.<sup>20</sup>

Hepcidin, is possibly the most conserved AMP across vertebrates. This small peptide enriched in cysteine residues is present in > 51 vertebrate species, where it acts as an acute phase reactant synthesized by the liver (reviewed in ref. 40). The presence of hepcidin in birds is debatable, but experiments based on mRNA expression and searches in the EST and HTGS databases, did not reveal any evidence that confirms the presence of this gene in birds.<sup>40</sup>

The EST database (<http://www.ncbi.nlm.nih.gov/nucleotide/>) is a collection of short single-read transcript sequences from GenBank, whereas the HTGS database (<http://www.ncbi.nlm.nih.gov/genbank/htgs>) contains unfinished DNA sequences generated by the high-throughput sequencing centers using traditional clone-based sequencing.

Other cationic peptides, with linear or helical structure, have been found in different vertebrates. For example, piscidin and pleurocidin have been isolated from fish,<sup>15,37</sup> while pelovaterins are present in the eggs of the Chinese soft-shelled turtle (*Pelodiscus sinensis*).<sup>41</sup> Several AMPs have been identified in amphibians, such as ascaphine-1 and ascaphine-8, isolated from *Ascaphus truei* and *Ascaphus montanus*,<sup>42,43</sup> bombinin H6, isolated from *Bombina bombina*, *Bombina orientalis* and *Bombina variegata*<sup>44</sup> and alyteserin-1 and alyteserin-2, obtained from *Alytes obstetricans*.<sup>45</sup> Other examples are magainin 2, dermaseptin I, brevinin IT, esculentin I, ranalexin and ranateurin I, which were isolated from different amphibians.<sup>22,37</sup> In mammals, cationic peptides have also been found, such as profenins in pigs and histatins in the saliva of humans and some primates.<sup>37</sup>

In the same way, AMPs derived from domains of other proteins have also been described in vertebrates. Some examples include hipposin, a histone-derived peptide from Atlantic halibut (*Hippoglossus hippoglossus* L),<sup>30</sup> lactoferricin from mammalian lactoferrin I, casodigin I from human casein, buforin II, derived from the histone 2A, which is highly conserved among vertebrates, and soluble antimicrobial domains of bovine lactalbumin, human hemoglobin, lysozyme and ovalbumin.<sup>22,37</sup>

### Acute phase proteins

The vertebrate plasma and epithelial secretions contain several proteins that are synthesized during infection and inflammation, playing a crucial role in innate immunity and host defence<sup>9,10,46</sup> (Fig. 1). The main acute phase proteins (APPs) described in mammals are C-reactive protein (CRP), serum amyloid A and P, haptoglobin,  $\alpha$ 1-acid glycoprotein,  $\alpha$ 2-macroglobulin, ceruloplasmin, fibrinogen and transferrin.<sup>10</sup> Serum amyloid P and CRP are pentraxins implicated in pathogen recognition, agglutination, opsonization and complement activation.<sup>10,47</sup> CRP is a major and moderate APP in many mammals, such as sheep, cows, rabbits, dogs, mice, rats, humans and other primates<sup>9,10</sup> and different CRP homologues have been identified in teleost fish,<sup>47,48</sup> but not in reptiles or birds. On the other hand, serum amyloid P seems to be a CRP counterpart in some species.<sup>10,49</sup> This lectin is a major APP in mice, but has not been further identified in other mammals.<sup>10</sup> More recently, both CRP and serum amyloid P were purified from the serum of the iridescent shark (*Pangasianodon hypophthalmus*), demonstrating the capacity to agglutinate rabbit erythrocytes and pathogenic bacteria like *Edwardsiella ictaluri* and *Aeromonas hydrophila*.<sup>48</sup> On the other hand, different pentraxins with CRP domains have been described in amphibians of *Xenopus* genus.<sup>47</sup> Serum amyloid A is a more conserved APP that has been identified in fish,<sup>49,50</sup> reptiles,<sup>51</sup> birds and mammals,<sup>9,10</sup> but not in amphibians. The expression of the gene encoding the serum amyloid A protein is increased in zebrafish (*Danio rerio*), rainbow trout (*Onchorhynchus mykiss*) and Chinese soft-shelled turtle (*Pelodiscus sinensis*) during bacterial infections.<sup>49–51</sup> Serum amyloid A is a major APP in mammals such as sheep, goats, cows, horses, cats, dogs, pigs, mice and humans, but is a moderate APP in chickens and non-human primates.<sup>10</sup> This protein is implicated in leucocyte chemoattraction and regulation of the inflammatory response<sup>10</sup> and is classified as the major APP due to its high plasma levels during inflammation.<sup>9,10</sup> Interestingly, a trypsin inhibitor only present in pigs is known as major acute phase protein and is increased > 10-fold during infections.<sup>10</sup>

Haptoglobin and  $\alpha$ 1-acid glycoprotein are other important acute phase reactants that are not present in all vertebrates. Haptoglobin binds to free haemoglobin to

prevent oxidative damage and also displays antimicrobial and immunomodulatory effects.<sup>10</sup> This protein has only been detected in fish and mammals,<sup>9,46,50,52</sup> but recently an avian counterpart of mammalian haptoglobin, called PIT 54, was identified, which is induced in *Escherichia coli*-infected chickens.<sup>46</sup> Likewise, the  $\alpha$ 1-acid glycoprotein has only been described in birds and mammals.<sup>9,10</sup> Its main function is the inactivation of harmful molecules like lipopolysaccharide or different types of drugs.<sup>10</sup>

Other plasma proteins such as  $\alpha$ 2-macroglobulin, ceruloplasmin, fibrinogen and transferrin, in addition to their normal activity, can act as APPs and increase their plasma levels during inflammation and infection.<sup>9,10</sup> The protease inhibitor  $\alpha$ 2-macroglobulin inactivates toxins and proteolytic enzymes<sup>10</sup> and has been identified in teleost fish like *Ictalurus punctatus*<sup>52</sup> and mammals such as rats and primates,<sup>9,10</sup> whereas ceruloplasmin is a copper-binding protein that scavenges free radicals,<sup>10</sup> and has been identified as a positive APP in fish,<sup>52</sup> birds<sup>46</sup> and mammals.<sup>10</sup> Fibrinogen is involved in tissue repair and is up-regulated during acute infections in fish,<sup>52</sup> reptiles,<sup>51</sup> birds<sup>46</sup> and mammals,<sup>9,10</sup> while transferrin acts as a negative APP in mammals,<sup>10</sup> but is a positive acute phase reactant in other vertebrates like fish<sup>52</sup> and birds.<sup>10</sup> In contrast, albumin decreases its plasma levels during acute infections or inflammatory conditions, representing a negative APP among domestic animals like chickens and mammals.<sup>10</sup> The loss of albumin is caused by renal and gastrointestinal changes during infection and its hepatic synthesis decreases, representing a strategy to preserve the unused amino acids for the production of positive APPs.<sup>10</sup>

### Complement system

The complement system is composed of serum proteins that react against pathogens through a molecular cascade, resulting in opsonization, enhanced inflammatory response and formation of a membrane-penetrating macromolecular pore, known as the membrane attack complex, which produces microbial lysis<sup>1,3,53</sup> (Fig. 1). This system is better characterized in mammals, where three activation pathways have been identified: the classical, the alternative and the lectin pathways.<sup>1,53–55</sup> In the classical pathway the proteolytic cascade is activated by antigen–antibody immune complexes (generally with IgG or IgM), whereas in the lectin pathway the recognition of microbial surface carbohydrates by mannose-binding lectin triggers the activation of mannose-binding-lectin-associated serine proteases that initiate the complement proteolytic cascade.<sup>53</sup> The alternative pathway, on the other hand, is self-activated by spontaneous hydrolysis of the complement protein C3 at the microbial membrane.<sup>1,53</sup> Different studies *in vitro* on immobilized proteins and *in vivo* in mouse models have revealed a novel

role of properdin in the activation of the alternative pathway.<sup>53</sup> Other evidence demonstrates the involvement of coagulation-associated proteases like kallikrein and thrombin in the complement proteolytic cascade, indicating a functional connection between these molecular pathways.<sup>53</sup> In immunodeficient patients and specific knockout mice, the relevance of the complement system for mammalian immunity has been evaluated, demonstrating that several complement proteins or their proteolytic fragments are capable of activating B- or T-cell function and are also crucial for the innate immune response against bacterial and viral pathogens, such as *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *Haemophilus influenzae*, herpes simplex virus and West Nile virus (reviewed in ref. 53). The reactivity and antimicrobial properties of the serum complement has also been evaluated in different vertebrates. For instance, complement-mediated opsonization and antibody-mediated complement activation have been described in teleost fish, such as common carp (*Cyprinus carpio*), catfish (*Ictalurus punctatus*) and salmonids.<sup>8</sup> Likewise, the serum haemolytic capacity has been studied in different amphibians like *Rana pipiens* and *Lithobates berlandieri*.<sup>56</sup> This study demonstrates that lytic activity against heterologous cells is stimulated by antibodies, reaches the levels reported for mammals and is influenced by heat, EDTA, hydrazine, carrageenan and antigen-antibody precipitate to a similar extent as the complement of some mammals, like pigs.<sup>56</sup>

The serum of reptiles also exhibits antimicrobial properties. Some comparative studies between the human serum complement and the complement of the American alligator (*Alligator mississippiensis*) demonstrate a major range of action against Gram-negative bacteria for the alligator serum.<sup>57</sup> The complement of *Alligator mississippiensis* displays antiviral activity against human immunodeficiency virus type 1, West Nile virus and herpes simplex virus,<sup>58</sup> and additionally, amoebicidal properties against various species of *Naegleria* and *Acanthamoeba* that have been reported as resistant to human complement lysis.<sup>59</sup> In birds, evidence shows that the complement system is also responsible for the lysis of heterologous cells. Matson *et al.* in 2005<sup>60</sup> evaluated the haemolytic capacity on rabbit erythrocyte suspension of the sera from several bird species, including American kestrel (*Falco sparverius*), Japanese quail (*Coturnix coturnix japonica*), zebra finch (*Taeniopygia guttata*), mourning dove (*Zenaidura macroura*), waved albatross (*Phoebastria irrorata*), grey catbird (*Dumetella carolinensis*) and common grackle (*Quiscalus quiscula*). This work demonstrates that haemolysis decreases with heat, which indicates that it is mediated by the complement system. A comparison between subjects of different age and species also indicates that complement-mediated lysis is affected by age and varies among species.<sup>60</sup>

The mammalian complement system has been extensively studied from a molecular perspective and nine complement proteins have been identified: C1, C2, C3, C4, C5, C6, C7, C8 and C9.<sup>1,53,55</sup> Several complement inhibitors detected in mammals impair the complement cascade activation on host cell membranes and ensure the specificity of this innate mechanism.<sup>53</sup> The identified mammalian inhibitors include Factor I, involved in C3b and C4b proteolysis, the decay-accelerating factor, complement receptor 1 (CD35), Factor H and C4-binding protein, which are enzymatic inhibitors of C3 convertase activity.<sup>53</sup> Other inhibitors that impair the assembly of the membrane attack complex, preventing C9 binding and polymerization are CD59, vitronectin and S protein.<sup>53</sup> In other vertebrates the presence of complement proteins has been described, confirming that this mechanism is preserved among vertebrates. Key components of the complement system, such as C3 and C5, have been isolated from some anurans of the genus *Xenopus*<sup>23</sup> and have been detected as down-regulated genes in teleosts like *Onchorhynchus mykiss* during infection with *Yersinia ruckeri* O1.<sup>49</sup> In other osteichthyes like catfish (*Ictalurus punctatus*), several complement proteins such as C1, C3, C4, C5, C7, C8 and C9 have been identified.<sup>52</sup> In addition, the B factor that is a crucial element of the C3 convertase cascade implicated in alternative C3 activation has been identified in *Onchorhynchus mykiss*,<sup>49</sup> *Ictalurus punctatus*,<sup>52</sup> *Silurana (Xenopus) tropicalis* and *Xenopus laevis*.<sup>61</sup>

Similarly, different proteins of this enzymatic cascade like C3, factor B, C4 and C5, have been identified in domestic birds like chickens.<sup>62</sup> In contrast, the molecular composition of the complement cascade of reptiles remains elusive and has only been identified the complement protein C3.<sup>51</sup>

## Cytokines

Cytokines are proteins that mediate the communication between cells and are essential for the immune response.<sup>63</sup> These soluble factors are one of the most conserved elements of vertebrate innate immunity. Cytokines are subdivided into different classes according to their genomic encoding regions, target receptors, associated signalling pathways and biological functions; these classes include interferons (IFNs), interleukins (ILs), tumour necrosis factors (TNFs) and transforming growth factors (TGFs).<sup>1,63,64</sup> Although the cytokines characterized to date have been cloned and extensively studied in mice, many of them are present in other vertebrates with similar functions.

Interferons are mainly involved in antiviral defence, although they display additional immunomodulatory function.<sup>63–65</sup> In mammals, three types of IFNs have been identified so far: type I IFNs, which include IFN- $\alpha$ , IFN- $\beta$ , IFN- $\omega$ , IFN- $\kappa$  and IFN- $\epsilon$  (reported in humans), type II IFNs (IFN- $\gamma$ ) and type III IFNs (IFN- $\lambda$ ).<sup>65</sup> Type I IFNs

represent a conserved antiviral system in vertebrates but they have a different genomic organization in lower and higher vertebrates.<sup>66</sup> Fish and amphibian type I IFNs are encoded by characteristic five-exon/four-intron transcripts, in contrast to reptilian, avian and mammalian type I IFNs, which are encoded by intronless transcripts.<sup>66,67</sup> The type I IFNs in lower vertebrates could not be phylogenetically classified as  $\alpha$  or  $\beta$ , but a detailed structural analysis in teleost fish has subdivided it into groups I and II, based on the amount and position of cysteine residues.<sup>67</sup> The role of type I IFNs in antiviral immunity has been corroborated in different vertebrates. In teleost fish like the crucian carp (*Carassius auratus* L.) these cytokines provide strong antiviral protection against grass carp haemorrhagic virus infection and mediate the Poly I:C-induced antiviral response, acting through the signal transducer and activator of transcription 1 pathway, as occurs in mammals.<sup>68</sup> Likewise, type I IFNs have been identified as repressors of viral protein synthesis and stimulators of NK cells in amphibians<sup>69</sup> and have been recently evaluated in frog virus 3 (FV3)-infected frogs (*Xenopus laevis*), demonstrating its capacity to inhibit the replication of FV3 and expand the mean survival time of infected tadpoles.<sup>66</sup> In reptilians, IFN-like activity have been reported in virus-infected animals and cell lines,<sup>3</sup> but a detailed evaluation of type I IFNs in these vertebrates is still missing. On the other hand, this type of IFN has been characterized in birds, at genetic and molecular levels (reviewed in ref 70). The presence of an IFN- $\alpha$  homologue and other closely related genes was reported in chickens, where they are encoded by a single intronless transcript, like in mammals.<sup>70</sup> These IFN-like molecules showed significant antiviral properties in virus-infected chicken embryos and cell lines and were also identified in ducks and turkeys.<sup>25,70</sup> A second type I IFN, homologue to mammalian IFN- $\beta$  and encoded by an independent intronless transcript, was further identified in chickens demonstrating antiviral activity in cell cultures.<sup>71,72</sup>

Type II IFNs are also conserved among vertebrates and their main functions include antiviral defence, regulation of MHC expression, phagocytosis stimulation, inhibition of cell growth and apoptosis.<sup>63–65,70</sup> Interferon- $\gamma$  is the only type II IFN identified in mammals, whereas in lower vertebrates like teleost fish multiple type II IFNs have been found.<sup>71</sup> In *Takifugu rubripes* and other teleosts, IFN- $\gamma$  homologues have been identified and they share the antiviral properties and genomic organization of mammalian IFN- $\gamma$ .<sup>72,73</sup> Likewise, in the amphibian *Xenopus tropicalis* the expression of the IFN- $\gamma$  gene has been corroborated.<sup>74</sup> This result was confirmed by Guanchun and Jacques in *Xenopus laevis*, demonstrating that the IFN- $\gamma$  gene is up-regulated by FV3 infection in tadpoles and adult frogs.<sup>75</sup> New evidence demonstrates the presence of an IFN- $\gamma$  gene in the North American green anole

lizard (*Anolis carolinensis*) and its expression in different tissues.<sup>65</sup> On the other hand, in different birds such as chickens, ducks, turkeys, pheasants, quails and Guinea fowls the presence of IFN- $\gamma$  genes with high sequence homology to mammalian IFN- $\gamma$  and strong antiviral activity as well as macrophage-stimulating capacity has been reported.<sup>70,76</sup> An increased expression of IFN- $\gamma$  gene has also been detected in chickens during coccidial infections.<sup>25</sup> In contrast, type III IFNs (IFN- $\lambda$ ) have only been identified in mammals and recently in the amphibian *Xenopus laevis*.<sup>71</sup> In both species IFN- $\lambda$  seems to be involved in antiviral defence and displays common functions with type I IFNs.<sup>65,71</sup> Although type III IFNs are not present in teleost fish,<sup>71</sup> the existence of IFN- $\lambda$  genes in reptilians or birds has not been properly evaluated. Hence, a genomic screening for IFN- $\lambda$  in amniotes is crucial to determine the evolution of type III IFNs in jawed vertebrates.

Interleukins are another class of cytokines conserved in vertebrate immunity, but they are not equally represented in all classes of vertebrates. Interleukin-1, IL-2 and IL-8 are among the most preserved ILs in amphibians, reptiles, birds and mammals.<sup>3,61,63,64,70,77</sup> Additionally, IL-1 has been identified in teleost fish with the same functions that it exhibits in other vertebrate species.<sup>8,49</sup> The mammalian IL-1 cytokine family has three characterized members: IL-1 $\alpha$  and IL-1 $\beta$ , which mediate inflammatory responses and leucocyte stimulation and a third member, the IL-1 receptor antagonist (IL-1Ra) that inhibits the biological activity of IL-1 $\alpha$  and IL-1 $\beta$ .<sup>1,64</sup> Of these three genes, only the IL-1 $\beta$  has been identified in other vertebrates. This gene has been detected in 13 teleost species,<sup>8,50</sup> in amphibians<sup>75</sup> and in chickens,<sup>25,70</sup> where it preserves the pro-inflammatory and cell-stimulating capacities that have been observed in mammals. In reptiles an IL-1-like molecule was detected but it could not be classified as  $\alpha$  or  $\beta$ .<sup>78</sup> This cytokine is produced by splenic macrophages of the wall lizard (*Hemidactylus flaviviridis*) in response to lipopolysaccharide stimulation.<sup>78</sup> On the other hand, IL-2 that is involved in lymphoid and myeloid cell differentiation in mammals<sup>63</sup> has been identified in reptilians like diadem snake (*Spalerosophis diadema*) as a thymocyte proliferating factor<sup>3</sup> and in chickens associated to the nuclear factor- $\kappa$ B to regulate cell differentiation.<sup>70</sup> Furthermore, the IL-2 gene is over-expressed in amphibians like *Xenopus tropicalis*, during *Batrachochytrium dendrobatidis* infection.<sup>61</sup>

Interleukin-8 is another cytokine conserved in vertebrate immunity. This molecule acts as a chemokine that participates in macrophage and leucocyte chemoattraction.<sup>1,70,75,79</sup> It has been identified in elasmobranchs like dogfish (*Triakis scyllia*)<sup>80</sup> and teleosts like *Onchorhynchus mykiss*,<sup>81</sup> reptiles like *Pelodiscus sinensis*,<sup>77</sup> domestic birds<sup>71</sup> and mammals.<sup>1,79,80</sup> However, no IL-8 homologue gene has been reported in amphibians. Interleukin-6 is



another important cytokine in the immune response that stimulates the proliferation of B lymphocytes and the production of fibronectin and APPs in hepatocytes.<sup>64,82</sup> It has been found in fish,<sup>8,50</sup> amphibians like *Rana esculenta*,<sup>80</sup> birds and mammals,<sup>70,79,80,82</sup> but not in reptiles. Other ILs such as IL-15, IL-16, IL-17 and IL-18 seem to be restricted to birds and mammals and their functions include cell differentiation, chemoattraction of T lymphocytes, induction of cytokine expression and stimulation of IFN- $\gamma$  secretion.<sup>25,70,79,80,83,84</sup> Additional ILs that display a variety of functions in innate and adaptive immunity have been identified in mammals. For example, IL-4, IL-5, IL-10 and IL-13, which are secreted during T helper type 2 response and regulate lymphoid cell activation and cytokine secretion.<sup>64,79,85</sup> Likewise, IL-12, IL-20,<sup>64</sup> IL-21,<sup>86</sup> IL-22, IL-26,<sup>73,87</sup> IL-25 and IL-33<sup>85</sup> have been characterized in mice or human tissues, where they exhibit specific biological properties related with inflammation, leucocyte proliferation and lymphocyte differentiation. Interestingly, IL-12, IL-22 and IL-26 were also discovered in teleost fish,<sup>73,80</sup> suggesting that some of these cytokines could be present in other vertebrates but remain unidentified.

Among pro-inflammatory cytokines, TNF- $\alpha$  is the second most conserved across vertebrate species after IL-1 $\beta$  and is implicated in leucocyte chemoattraction and macrophage stimulation.<sup>8,25,63,64,79</sup> This cytokine has been detected in teleosts such as rainbow trout (*Onchorhynchus mykiss*), sea bream (*Sparus aurata*), goldfish (*Carassius auratus*) and catfish (*Ictalurus punctatus*),<sup>8,49</sup> in amphibians like *Xenopus laevis*<sup>75</sup> and mammals.<sup>63,64,79</sup> A recent work identified the *TNFSF13* gene (a member of the TNF superfamily) in *Xenopus laevis* and demonstrated that its encoding protein promotes the proliferation of *Xenopus* B lymphocytes and mouse splenic B cells.<sup>88</sup> The similar structure, tissue expression and bioactivity of *TNFSF13* between mammals and *Xenopus laevis*, suggest that this anuran could be an interesting model to evaluate different immunological disorders caused by this cytokine.<sup>88</sup> On the other hand, although TNF- $\alpha$ -like activity was reported in chickens, the sequence of a chicken *TNF- $\alpha$*  homologue gene has not been identified or described in any database.<sup>25,70</sup> Similarly, the presence of this molecule or its encoding gene has not been reported in reptiles.

Other immunoregulatory cytokines like TGF- $\beta$ <sup>80</sup> are also well-preserved across vertebrate species and are present in early metazoans and protozoans.<sup>89</sup> The mammalian TGF- $\beta$  family have been extensively characterized and include three related isoforms encoded by independent genes, that regulate cell growth and differentiation and are involved in oocyte maturation, wound repair, modulation of inflammatory response, immunosuppression and tolerance.<sup>90</sup> In the same way, the presence of TGF- $\beta$  in other vertebrates has been reported and the ubiquitous expression of this pleiotropic cytokine among animals could be more related to its function in repro-

duction and embryonic development than to its role in immunity.<sup>80</sup> In 2009, a genomic screening of 33 animal species of different taxa identified the *TGF- $\beta$*  gene in humans, primates such as *Pan troglodytes* and *Macaca mulatta*, rodents like *Mus musculus* and *Rattus norvegicus*, in dogs (*Canis domesticus*), birds like roosters (*Gallus gallus*), osteichthyes (bony fish) such as *Oryzias latipes*, *Danio rerio*, *Tetraodon nigroviridis* and *Takifugu rubripes*.<sup>89</sup> The TGF- $\beta$  is also secreted by the placenta of viviparous squamate reptiles like *Chalcides chalcides*.<sup>80</sup> Additionally, the three mammalian isoforms of TGF- $\beta$  were identified in chickens as regulators of embryonic development and haematopoiesis.<sup>70</sup> Interestingly, the mRNA expression of a fourth isoform, TGF- $\beta_4$ , is induced by coccidial infection in the intestinal epithelia of chickens.<sup>25,70</sup>

### Natural antibodies

In the plasma of vertebrates there are circling antibodies known as natural antibodies (NABs) (Fig. 1), which are mainly of IgM isotype, possess a limited genetic repertoire and are synthesized by B cells in absence of pathogens.<sup>91</sup> These pre-existent immunoglobulins are essential components of humoral innate immunity; they react against foreign antigens and microbe-derived substances and can activate the complement cascade by the classical pathway.<sup>60,91</sup>

The NABs have been described as part of humoral immunity in fish, reacting with proteins exposed on the microbial surface and providing protection against pathogens like *Aeromonas salmonicida*.<sup>92</sup> This natural IgM is found in chondrichthyes (sturgeons and sharks) and some osteichthyes such as *Onchorhynchus mykiss* and *Pagrus auratus*.<sup>93</sup> Such IgM isotype NABs have also been identified in amphibians,<sup>94</sup> where they are implicated in the inactivation of modified bacteriophages and other viral particles,<sup>95</sup> as well as haemoagglutination of foreign red blood cells from chicken, sheep, rat and human.<sup>96</sup> High levels of NABs have been detected in anurans like *Bufo regularis*, *Bufo arenarum* and *Xenopus laevis*.<sup>96</sup>

The production of NABs is also present in reptiles, as an essential element of the humoral immune response that has been found in some species, e.g. water python (*Liasis fuscus*).<sup>97</sup> This study demonstrates that NABs increase with age for tropical pythons, possibly to compensate for the immunosenescence and loss of effectiveness in the adaptive humoral immune response.<sup>97</sup> Natural immunoglobulins like IgM and IgG, are also produced by the gut-associated lymphoid tissue of birds, due to stimulation by the intestinal microbiota.<sup>98</sup> These NABs eliminate bacteria and spirochaetes *in vivo* and promote the clearance of bacterial substances like lipopolysaccharides.<sup>60</sup> They represent an important humoral defence mechanism that correlates with resistance to pathogens,

like Amblyceran lice in some bird species such as Galápagos hawks (*Buteo galapagoensis*).<sup>99</sup> In addition, NAb against parasites like *Plasmodium lophurae* have been identified in White Leghorn chickens (*Gallus domesticus*).<sup>60</sup>

The NAb are present in mammals as well, representing a conserved element from lower vertebrates involved in humoral innate immunity; they neutralize pathogens and helminth parasites<sup>2</sup> and confer resistance against influenza virus.<sup>91</sup> These antibodies also display new modulatory functions in complex vertebrates like mammals. For example, the binding to oxidized proteins like oxidized low-density lipoprotein, in a mechanism that reduces its uptake by macrophages and attenuates the atherogenic process.<sup>100</sup> Additionally, some reports demonstrate that these natural IgMs participate in immune surveillance, recognizing tumour-associated or tumour-specific antigens such as CFR-1, expressed in malignant cells of different epithelial cancers, and even inducing apoptosis.<sup>101</sup>

## Cell-mediated innate immunity

### Intraepithelial T lymphocytes

The mucosa and epithelial barriers of vertebrates are infiltrated by a specific type of lymphocyte – intraepithelial T lymphocytes (ITLs) (Fig. 1), which recognize and destroy pathogens.<sup>102</sup> These cells have been better characterized in mammals and are phenotypically classified as  $\gamma\delta$  T lymphocytes.<sup>11,102</sup> However, they have more characteristics in common with the effector cells of innate immunity. For example, its limited antigenic repertoire and the low variability of its T-cell receptors make them different from the typical lymphocytes present in the adaptive immune response.<sup>11,102</sup>

Intraepithelial lymphocytes have been isolated from the intestinal epithelial tissue of fish, like *Onchorhynchus mykiss*, and its cytotoxic capacity against EL4 mouse thymoma cells has been demonstrated.<sup>27</sup> Likewise, in some specialized epithelia of amphibians, such as the gut-associated lymphoid tissue, the presence of intraepithelial lymphocytes has been reported; these are similar to those found in fish and share many phenotypic characteristics with the ITLs of mammals.<sup>26</sup>

These lymphocytes have also been identified in reptiles, as an element of protective immunity, in intestinal epithelium and gut-associated lymphoid tissue,<sup>28</sup> a characteristic that is preserved from fish and amphibians. They are present in some species, such as platypus (*Ornithorhynchus anatinus*), in the lamina propria of intestinal mucosa, and are phenotypically similar to mammalian ITLs.<sup>28</sup> The gut-associated lymphoid tissue of birds is also infiltrated by intraepithelial lymphocytes, which diversify into various subsets of T lymphocytes that include CD8<sup>+</sup> T cells (CD8 $\alpha\alpha$ ), and  $\gamma\delta$  T CD8 $\alpha\beta$  cells.<sup>29</sup> These cells represent a

primary active barrier against pathogen dissemination, and constitute the main component of protective immunity to avian coccidiosis caused by the intestinal parasitic pathogen *Eimeria*.<sup>25</sup> Some investigations indicate that this cellular component of epithelium increases in number after infection with intestinal parasites such as *Eimeria acervulina* and *Eimeria tenella*.<sup>103</sup>

In humans, two subsets of  $\gamma\delta$  T lymphocytes have been identified: V $\delta$ 1 and V $\delta$ 2  $\gamma\delta$  T cells. V $\delta$ 1 T cells reside mainly in the epithelial mucosa, whereas V $\delta$ 2 T cells are abundant in the lymphatic system and peripheral blood.<sup>11</sup> These cells can recognize non-peptidic antigens, soluble proteins and MHC molecules, displaying multiple functions, such as cytokine secretion, activation of dendritic cell maturation, macrophage recruitment, cytotoxic activity against transformed cells, maintenance of epidermal integrity and B- or T-cell cooperation, among others.<sup>11</sup>

### Myeloid phagocytic cells

The main cellular components of vertebrate innate immunity are myeloid phagocytic cells, also called ‘professional’ phagocytes<sup>3,94</sup> (Fig. 1). This type of leucocyte includes monocyte–macrophages, neutrophils/heterophils, basophils and eosinophils.<sup>1–3,94</sup> These cells phagocytose and destroy pathogens, secrete cytokines and release soluble mediators like histamine, reactive oxygen species, reactive nitrogen species, lysozyme and AMPs.<sup>2,3,79,104</sup> They recognize senescent cells and microbes by pattern recognizing receptors and present phagocytosed antigens to B or T cells, acting as the first line of defence against infection and contributing with the activation of adaptive immune response.<sup>1,79,104</sup> The main pattern recognizing receptors expressed in vertebrate phagocytes are the Toll-like receptors, C-type lectin receptors, NOD-like receptors and Rig I-helicases, which recognize chemical structures that are present in all microbes, such as lipopolysaccharides, lipoproteins, complex glycans, formylmethionine and even nucleotides and nucleic acids.<sup>5–7,12</sup> These receptors are able to distinguish between chemical isomers and can also recognize a variety of polysaccharides, lipids or nucleotides, cooperating with each other to selectively activate different signalling pathways that induce specific pro-inflammatory and antimicrobial innate responses.<sup>5,6</sup>

Myeloid phagocytes represent a potent defence mechanism in mammals, essential against infections with Gram-negative (*Escherichia coli*, *Salmonella typhimurium*, *Yersinia enterocolitica*) and Gram-positive (*Listeria monocytogenes*, *Staphylococcus aureus*) bacteria.<sup>104,105</sup> The immune functions of mammalian innate myeloid cells have been evaluated in mouse models and they include phagocytosis, antigen presentation, releasing of histamine, pro-inflammatory cytokines, AMPs and granule-derived proteins that destroy microorganisms.<sup>1,104,105</sup> These cells are also involved in wound healing and normal tissue

functions.<sup>106</sup> Among myeloid phagocytic cells, neutrophils represent the earliest innate response in humans, phagocytosing microbes, cooperating with other immune cells and releasing AMPs, proteases, hydrolytic enzymes and phospholipases.<sup>105,107</sup>

In other vertebrates, leucocytes such as macrophages, heterophils (homologues of mammalian neutrophils), basophils and eosinophils are crucial for the effectiveness of innate immunity.<sup>3,94,108</sup> These cells have been described in fish,<sup>8,108,109</sup> amphibians<sup>23,75</sup> and reptiles,<sup>3,78,110</sup> preserving their functions in phagocytosis, inflammation and the release of soluble mediators. In these ectothermic vertebrates, the phagocytosis plays an important role in immune response, because it is less influenced by temperature than adaptive immune mechanisms.<sup>111</sup> Recent imaging techniques in fish model organisms like *Danio rerio*, reveal new insights into the phagocytotic process and immune cell recruitment during inflammation, from a unique *in vivo* perspective.<sup>108</sup> These approaches combined with genetic manipulation have allowed the discovery of novel inflammatory pathways, danger signals and myeloid development-related genes, which are integrative findings for the comprehension of the mammalian immune system.<sup>108</sup>

Innate myeloid cells have also been characterized in birds. They include macrophages, heterophils, eosinophils and basophils, which are preserved from the early vertebrates and whose main function is the recognition and phagocytosis of pathogens.<sup>112</sup> Its pattern recognizing receptor-mediated recognition stimulates phagocytosis and activates destruction of pathogens, antigen presentation, stimulation of the adaptive immune response and production of pro-inflammatory cytokines.<sup>112</sup>

On the other hand, the lymphoid lineage of vertebrate leucocytes has recently been a focus of research in innate immunity to comprehend the developmental relationships between the various lineages that have been identified to date as well as their functionality. A new type of leucocyte, known as a nuocyte, has been identified in mammals, implicated in type 2 immunity against helminth parasites.<sup>85</sup> These cells were characterized in the *IL13-eGFP* reporter mice, demonstrating an IL-25/IL-33-dependent proliferation and a capacity to secrete IL-13 during infection with the helminth *Nippostrongylus brasiliensis*.<sup>85</sup> In addition, recent reports describe the existence of lineage-marker-negative innate lymphoid cell subsets that do not express cell surface markers associated with other immune cell lineages. These newly identified members of the lymphoid lineage, which include nuocytes, have emerging roles in mediating immune responses and in regulating tissue homeostasis and inflammation.<sup>113</sup>

### Phagocytic B cells

The B lymphocytes are considered another type of cell involved in vertebrate innate immunity (Fig. 1). In lower

vertebrates these cells display phagocytic capacity and activation of degradative pathways that result in phagolysosome fusion and killing of internalized bacteria.<sup>94,114</sup> B cells seem to act like leucocytes; however, they are able to produce antibodies of IgG and IgA isotype, involved in classical adaptive immune response, and also NAb of IgM isotype.<sup>92,110</sup>

Phagocytic B cells have been identified in teleost fish like *Onchorhynchus mykiss* and *Ictalurus punctatus*, in amphibians like *Xenopus laevis* and in reptiles like the red-eared slider (*Trachemys scripta*).<sup>110,114</sup> In mammals, the B cells do not exhibit phagocytic capacity and their immune functions seem to be limited to adaptive immunity.<sup>94,110,114</sup> However, a mammalian subpopulation of B lymphocytes known as B-1 lymphocytes, retains the phagocytic capacity and antimicrobial properties of the B cells from lower vertebrates.<sup>114</sup> These cells reside mainly in the peritoneal and pleural cavities<sup>91</sup> where they produce NAb and present phagocytosed antigens to CD4<sup>+</sup> T cells, playing an important role in innate and adaptive immune responses.<sup>114</sup> The phagocytic ability of non-mammalian B cells which is preserved in B-1 cells, suggests an evolutionary connection between both cell types. In fact, the discovery of a bipotent B macrophage precursor in fetal liver and adult bone marrow of mammals, supports the idea of a common origin for B cells and macrophages, from a phagocytic ancestor.<sup>94,114</sup> In contrast, the phagocytic capacity of B cells in birds has not been evaluated, making it hard to determine the evolution of this function into the higher amniotes.<sup>110</sup> However, subsets of B cells, similar to B-1 lymphocytes of mammals, are found in birds, in the gut-associated lymphoid tissue where they produce NAb.<sup>98</sup>

### Non-specific cytotoxic cells

Non-specific cytotoxicity is one of the most important cellular mechanisms in vertebrate innate immunity (Fig. 1), playing an essential role in antiviral defence and immune surveillance.<sup>115,116</sup> This function is carried out in mammals mainly by NK lymphocytes, which are involved in the cytotoxic response against cells that are transformed or infected by virus or intracellular microbes.<sup>115</sup> In addition, NK cells secrete cytokines with antiviral activity<sup>117</sup> and are also involved in hepatic defence and tissue regeneration and remodelling.<sup>87</sup>

The presence of non-specific cytotoxic cells similar to NK cells has been described in lower vertebrates. These cells have been identified in fish like *Ictalurus punctatus* where they are distinguishable by their small, non-granular morphology and their abundance in lymphoid tissues, such as kidney and spleen.<sup>8,118</sup> In addition, the identification of a new gene family known as novel immune-type receptors in zebrafish (*Danio rerio*) is strong evidence for the presence of NK-like cells in teleosts.<sup>108</sup> These proteins

are structurally similar to mammalian NK receptors, are differentially expressed in fish lymphoid cells but not in myeloid cells, and confer lytic capacity on the cells in which they are expressed.<sup>108</sup>

Natural killer-like lymphocytes have also been reported in amphibians, with an essential role in cytotoxic response against virus-infected cells.<sup>69,75</sup> Many NK candidates have been identified in the intraepithelial layer and the spleen tissue of *Xenopus laevis*, using a staining method with specific anti-NK monoclonal antibodies 1F8, 1G5 and 4D4.<sup>119</sup> This study demonstrates the existence of at least three subsets of NK lymphocytes in *Xenopus laevis*: 1F8<sup>+</sup> CD5<sup>-</sup> splenocytes, 1F8<sup>+</sup> CD5<sup>+</sup> gut cells and 1F8<sup>+</sup> F17<sup>-</sup> lymphocytes, which exhibit strong *in vitro* cytotoxic activity, against the *Xenopus* thymus lymphoid tumour cell line B3B7.<sup>119</sup> These results have been confirmed by Goyos and Robert in 2009,<sup>116</sup> through the identification of 1F8<sup>+</sup> NK cells and NKT-like cells (with NK- and T-cell-associated markers), which are similar to those found in mammals and are involved in *in vivo* anti-tumour immune responses in *Xenopus laevis*. Different studies in this anuran species have revealed the connection between tumorigenesis and embryonic development and are also providing evidence for the innate cellular responses to oncogenic processes.<sup>116</sup> In comparison to murine models, anurans like *Xenopus laevis* and *Xenopus tropicalis* provide advantages in the genetic manipulation and constitute a powerful alternative for the identification of new genes involved in tumorigenesis and anti-tumour immune responses.<sup>116</sup>

In birds, non-specific cytotoxic activity against infected or transformed cells has also been reported, carried out by NK-like lymphocytes called TRC0 cells.<sup>120</sup> These cells are homologues to mammalian NK lymphocytes and express their typical surface antigens, such as CD3.<sup>120</sup> This kind of lymphocyte has been identified in the intestinal mucosa of chickens, where they are subdivided into two distinct populations: 28-4<sup>+</sup> CD3<sup>-</sup> NK cells (detected with 28-4 monoclonal antibody) and 28-4<sup>+</sup> CD3<sup>+</sup> NK cells, which are homologues to the NKT cells of mammals.<sup>121</sup> They represent a conserved cellular element from the lower vertebrates that plays an important role in anti-parasitic immune response and mucosal immunity.<sup>25</sup>

In contrast with other vertebrates, non-specific cytotoxic cells or NK cells are not found in reptiles, or at least have not been identified and studied thoroughly. They are present in the other classes of vertebrates, suggesting that they should exist in reptiles as well. This theory can be supported by the finding of NK-mediated cytotoxicity against tumour cells in the turtle *Mauremys caspica*.<sup>122</sup> This activity was identified in thymic cells, but was not defined in a specific subset.<sup>122</sup>

In addition, a particular type of NK cell, with many phenotypic characteristics of a T lymphocyte, known as NKT lymphocyte, has been described in mammals.<sup>117,123</sup>

These cells recognize glycolipid antigens in the context of the CD1 molecule and release cytokines implicated in the innate immune response.<sup>123</sup> The presence of NKT functional analogues has been reported in amphibians<sup>116</sup> and birds,<sup>25,121</sup> suggesting that this subset of NK lymphocytes is a conserved component among vertebrates, which represents an evolutionary link between innate and adaptive immunity. The main functions of these cells include cytotoxic response, antitumour activity<sup>123</sup> and the secretion of antiviral cytokines like type I IFNs<sup>117</sup> and IL-21.<sup>86</sup> Other types of invariant T cell, similar to NKT lymphocytes, are known as mucosal-associated invariant T cells and are also implicated in the mammalian innate immune response by the release of IL-17, mainly in the gastrointestinal tract<sup>84</sup> and the liver.<sup>124</sup>

Recent comparative studies across animal species have revealed new functional properties for NK lymphocytes and other innate cells, like monocyte-macrophages.<sup>13,14</sup> These novel findings indicate that innate immune cells display enhanced responses to secondary infections in a T/B-cell-independent manner, through epigenetic mechanisms and a biochemical reprogramming that predisposes cells to a rapid activation by secondary stimulation.<sup>13,14</sup> This innate immunological memory termed as 'trained immunity' is an interesting new feature of innate cells that is changing the concept of innate immunity.<sup>13,14</sup>

## Conclusions

Across jawed vertebrates, innate immunity displays a diversity of mechanisms that adapt to the anatomy and behaviour of different species, while preserving its core elements and main functions. Physical barriers are conserved across all vertebrates, nevertheless they have anatomical and chemical variations that exhibit a tendency to specialization of the skin and mucosa. Besides, among humoral components lysozyme, AMPs, complement system and NAb are the most conserved, showing the same basic functions and sharing their main properties. On the other hand, the acute phase response and cytokine release are conserved mechanisms, triggered in the presence of pathogens, even though the involved molecules differ among vertebrate species. In general, humoral components are poorly characterized in amphibians and reptiles, making it difficult to understand the evolution of humoral innate immunity in vertebrates.

The current evidence reveals non-mammalian vertebrates as a source of potent antimicrobial and antiviral proteins and peptides, which could also have antitumour or immunomodulatory activity. There are several examples of antimicrobial molecules isolated from invertebrates and plants with potential applications in biomedicine; however, vertebrates are an interesting alternative for the development of new therapeutic agents to deal with antibiotic resistance and chemotherapy toxicity.



Cell-mediated components of innate immunity are preserved across vertebrates and they include ITLs, myeloid phagocytes and non-specific cytotoxic cells. Nevertheless, the existence of reptilian non-specific cytotoxicity remains unclear. Further investigation and characterization of cell-mediated immunity in reptiles is required to elucidate the transition of non-specific cytotoxic cells into the amniotes. On the other hand, the B lymphocytes are also described as innate effector cells, a recent concept in vertebrate immunity. B cells produce NABs and display phagocytic capacity and antimicrobial properties in lower vertebrates, a characteristic that remains in mammalian B-1 lymphocytes. These findings suggest an evolutionary connection between macrophages and B cells, which could have evolved from a common phagocytic ancestor. However, the phagocytic capacity of B cells from birds has not been examined. A detailed evaluation of phagocytic and antimicrobial characteristics of avian B cells is the key to trace the evolution of B-cell-mediated phagocytosis into the higher amniotes.

In spite of the evident physiological and morphological differences among the five classes of jawed vertebrates, that determine the kinetics and overall effectiveness of immune responses, the molecules and cells involved in the innate response are conserved and maintain their functionality throughout vertebrates. Hence, even if the immunity in higher vertebrates is more complex than in lower vertebrates, they share some of the basic mechanisms involved in cutaneous protection, serum antimicrobial activity, acute phase response, opsonization, pathogen recognition, phagocytosis and cytotoxicity, among others. From this point of view, non-mammalian vertebrates can be considered as models for the study of the immune system. In this sense, different zebrafish mutants have been used to examine key aspects of immune system ontogeny, phagocytosis, cell recruitment and inflammatory pathways, while the frog *Xenopus laevis* is a potent model of tumorigenesis, tumour immunity and other immune-related pathophysiological conditions. Likewise, chickens and other domestic birds constitute models for the study of the gut-associated lymphoid tissue and the pathogen–host interactions at the intestinal epithelium.

Further investigation is required to achieve the complete characterization of innate immune mechanisms and their evolution across vertebrates. This could lead to a better understanding of the cellular and molecular processes that determine the dynamic functionality of innate immunity.

## Disclosures

The authors declare that there are no financial or commercial conflicts of interest associated with this work.

## References

- Abbas AK, Litchman AH, Pillai S. Cellular and molecular immunology. Philadelphia: Elsevier/Saunders, 2012.
- de Veer MJ, Kemp JM, Meeusen EN. The innate host defence against nematode parasites. *Parasite Immunol* 2007; **29**:1–9.
- Zimmerman LM, Vogel LA, Bowden RM. Understanding the vertebrate immune system: insights from the reptilian perspective. *J Exp Biol* 2010; **213**:661–71.
- Iwasaki A, Medzhitov R. Control of adaptive immunity by the innate immune system. *Nat Immunol* 2015; **16**:343–53.
- Newton K, Dixit VM. Signaling in innate immunity and inflammation. *Cold Spring Harb Perspect Biol* 2012; **4**: pii:006049.
- Ip WK, Takahashi K, Ezekowitz RA, Stuart LM. Mannose-binding lectin and innate immunity. *Immunol Rev* 2009; **230**:9–21.
- Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; **140**:805–20.
- Uribe C, Folch H, Enriquez R, Moran G. Innate and adaptive immunity in teleost fish: a review. *Vet Med* 2011; **56**:486–503.
- Petersen HH, Nielsen JP, Heegaard PM. Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res* 2004; **35**:163–87.
- Cray C, Ziaias J, Altman NH. Acute phase response in animals: a review. *Comp Med* 2009; **59**:517–26.
- Wu Y, Ding Y, Tanaka Y, Shen L, Wei C, Minato N *et al.*  $\gamma\delta$  T cells and their potential for immunotherapy. *Int J Biol Sci* 2014; **10**:119–35.
- Brehélin M, Roch P. Specificity, learning and memory in the innate immune response. *ISJ Vision Perspect* 2008; **5**:103–9.
- Netea MG. Training innate immunity: the changing concept of immunological memory in innate host defence. *Eur J Clin Invest* 2013; **43**:881–4.
- Netea MG, Latz E, Mills KH, O'Neill LA. Innate immune memory: a paradigm shift in understanding host defense. *Nat Immunol* 2015; **16**:675–9.
- Rakers S, Niklasson L, Steinhagen D, Kruse C, Schaubert J, Sundell K *et al.* Antimicrobial peptides (AMPs) from fish epidermis: perspectives for investigative dermatology. *J Invest Dermatol* 2013; **133**:1140–9.
- Matsui T, Amagai M. Dissecting the formation, structure and barrier function of the stratum corneum. *Int Immunol* 2015; **27**:269–80.
- Kueneman JG, Parfrey LW, Woodhams DC, Archer HM, Knight R, McKenzie VJ. The amphibian skin-associated microbiome across species, space and life history stages. *Mol Ecol* 2014; **23**:1238–50.
- Lillywhite HB. Water relations of tetrapod integument. *J Exp Biol* 2005; **209**:202–26.
- Alibardi L, Toni M, Valle LD. Hard cornification in reptilian epidermis in comparison to cornification in mammalian epidermis. *Exp Dermatol* 2007; **16**:961–76.
- Bowditch DM, Davidson DJ, Hancock RE. A re-evaluation of the role of host defence peptides in mammalian immunity. *Curr Protein Pept Sci* 2005; **6**:35–51.
- Akinbi HT, Epaul R, Bhatt H, Weaver TE. Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice. *J Immunol* 2000; **165**:5760–6.
- Zaslouf M. Antimicrobial peptides of multicellular organism. *Nature* 2002; **415**:389–95.
- Mescher A, Neff A. Limb regeneration in amphibians: immunological considerations. *Sci World J* 2006; **6**:1–11.
- Van Immerseel F, Methner U, Rychlik I, Nagy B, Velge P, Martin G *et al.* Vaccination and early protection against non-host-specific *Salmonella* serotypes in poultry: exploitation of innate immunity and microbial activity. *Epidemiol Infect* 2005; **133**:959–78.
- Lillehoj HS, Min W, Dalloul RA. Recent progress on the cytokine regulation of intestinal immune responses to *Eimeria*. *Poult Sci* 2004; **83**:611–23.
- Chin KN, Wong WC. Some ultrastructural observations on the intestinal mucosa of the toad (*Bufo melanostictus*). *J Anat* 1977; **123**:331–9.
- McMillan DN, Secobes CJ. Isolation of rainbow trout (*Oncorhynchus mykiss*) intestinal intraepithelial lymphocytes (IEL) and measurement of their cytotoxic activity. *Fish Shellfish Immunol* 1997; **7**:527–41.
- Osogoe B, Tsujii T, Liszczynsky HR, Naora H, Seno S. Intestinal mucosa and intra-abdominal lymphoid tissues of the platypus, *Ornithorhynchus anatinus*. *Okajimas Folia Anat Jpn* 1991; **67**:457–66.
- Tregaskes CA, Kong FK, Paramithiotis E, Chen CL, Ratcliffe MJ, Davison TF *et al.* Identification and analysis of the expression of CD8  $\alpha\beta$  and CD8  $\alpha\alpha$  isoforms in chickens reveals a major TCR- $\gamma\delta$  CD8  $\alpha\beta$  subset of intestinal intraepithelial lymphocytes. *J Immunol* 1995; **154**:4485–94.
- Birkemo GA, Luders T, Andersen O, Nes IF, Nissen-Meyer J. Hippocin, a histone-derived antimicrobial peptide in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Biochim Biophys Acta* 2003; **1646**:207–15.
- Irwin DM, Biegel JM, Stewart CB. Evolution of the mammalian lysozyme gene family. *BMC Evol Biol* 2011; **11**:166.

- 32 Ostrovsky D, Snyder JA, Iwata T, Izaka K, Maglott DS, Nace GW. Frog lysozyme. I. Its identification, occurrence as isozymes, and quantitative distribution in tissues of the leopard frog, *Rana pipiens*. *J Exp Zool* 1976; **195**:279–90.
- 33 Xu N, Pan J, Liu S, Xue Q, Zhang S. Three in one: identification, expression and enzymatic activity of lysozymes in amphioxus. *Dev Comp Immunol* 2014; **46**:508–17.
- 34 Thammasiriak S, Ponkham P, Preecharram S, Khanchuan R, Phonyothee P, Dadaung S *et al.* Purification, characterization and comparison of reptile lysozymes. *Comp Biochem Physiol* 2006; **143**:209–17.
- 35 Mille S, Bennetta J, Leeb K, Haub M, Klasing K. Quantifying and comparing constitutive immunity across avian species. *Dev Comp Immunol* 2007; **31**:188–201.
- 36 White TJ, Moss GA, Osseman EF, Wilson AC. Primary structure of rat lysozyme. *Biochemistry* 1977; **16**:1430–6.
- 37 Tellez GA, Castaño JC. Péptidos antimicrobianos. *Infectio* 2010; **14**:55–67.
- 38 Stegemann C, Kolobov A Jr, Leonova YF, Knappe D, Shamova O, Ovchinnikova TV *et al.* Isolation, purification and de novo sequencing of TBD-1, the first  $\beta$ -defensin from leukocytes of reptiles. *Proteomics* 2009; **9**:1364–73.
- 39 Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J *et al.* Snake cathelicidin from *Bungarus fasciatus* is a potent peptide antibiotic. *PLoS ONE* 2008; **3**:e3217.
- 40 Hilton KB, Lambert LA. Molecular evolution and characterization of hepcidin gene products in vertebrates. *Gene* 2008; **415**:40–8.
- 41 Lakshminarayanan R, Vivekanandan S, Samy RP, Banerjee Y, Chi-Jin EO, Teo KW *et al.* Structure, self-assembly, and dual role of a  $\beta$ -defensin-like peptide from the Chinese soft-shelled turtle eggshell matrix. *J Am Chem Soc* 2008; **130**:4660–8.
- 42 Colon JM, Sonnevend A, Davidson C, Smith DD, Nielsen PF. The ascapins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, *Ascapus truei*. *Biochem Biophys Res Commun* 2004; **320**:170–5.
- 43 Colon JM, Bevier CR, Coquet L, Leprince J, Jouenne T, Vandry H. Peptidomic analysis of skin secretions supports separate species status for the tailed frogs, *Ascapus truei* and *Ascapus montanus*. *Comp Biochem Physiol* 2007; **2**:121–5.
- 44 Mangoni ML, Marcellini HG, Simmaco M. Biological characterization and modes of action of temporins and bombinins H, multiple forms of short and mildly cationic anti-microbial peptides from amphibian skin. *J Pept Sci* 2007; **13**:603–13.
- 45 Colon JM, Demandt A, Nielsen F, Leprince J, Vaundry H, Woodhams D. The alyte-serins: two families of antimicrobial peptides from the skin secretions of the midwife toad *Alytes obstetricans* (Alytidae). *Peptides* 2009; **30**:1069–73.
- 46 Georgieva TM, Koinarski VN, Urumova VS, Marutsov PD, Christov TT, Nikolov J *et al.* Effects of *Escherichia coli* infection and *Eimeria tenella* invasion on blood concentrations of some positive acute phase proteins (haptoglobin (PIT 54), fibrinogen and ceruloplasmin) in chickens. *Revue Méd Vét* 2010; **161**:84–9.
- 47 Vasta GR, Ahmed H, Odom EW. Structural and functional diversity of lectin repertoires in invertebrates, protochordates and ectothermic vertebrates. *Curr Opin Struct Biol* 2004; **14**:617–30.
- 48 Huang Giang DT, Van Driessche E, Vandenbergh I, Devreese B, Beeckmans S. Isolation and characterization of SAP and CRP, two pentraxins from *Pangasianodon (Pangasius) hypophthalmus*. *Fish Shellfish Immunol* 2010; **28**:743–53.
- 49 Raida MK, Buchmann K. Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1. *Dev Comp Immunol* 2009; **33**:35–45.
- 50 Lin B, Chen S, Cao Z, Lin Y, Mo D, Zhang H *et al.* Acute phase response in zebrafish upon *Aeromonas salmonicida* and *Staphylococcus aureus* infection: striking similarities and obvious differences with mammals. *Mol Immunol* 2007; **44**:295–301.
- 51 Zhou X, Wang L, Feng H, Guo Q, Dai H. Acute phase response in Chinese soft-shelled turtle (*Trionyx sinensis*) with *Aeromonas hydrophila* infection. *Dev Comp Immunol* 2011; **35**:441–51.
- 52 Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H *et al.* Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium. *Dev Comp Immunol* 2007; **31**:1183–96.
- 53 Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res* 2010; **20**:34–50.
- 54 Zhang S, Wang C, Wang Y, Wei R, Jiang G, Ju H. Presence and characterization of complement-like activity in the amphioxus *Branchiostoma belcheri tsingtauense*. *Zoolog Sci* 2003; **20**:1207–14.
- 55 Kang YH, Tan LA, Carroll MV, Gentle ME, Sim RB. Target pattern recognition by complement proteins of the classical and alternative pathways. *Adv Exp Med Biol* 2009; **653**:117–28.
- 56 Legler D, Evans E. Comparative Immunology. Haemolytic Complement in Amphibia. *Proc Soc Exp Biol Med* 1966; **121**:1158–62.
- 57 Merchant ME, Roche C, Elsey RM, Prudhomme J. Antibacterial properties of serum from the American alligator (*Alligator mississippiensis*). *Comp Biochem Physiol B Biochem Mol Biol* 2003; **136**:505–13.
- 58 Merchant ME, Pallansch M, Paulman RL, Wells JB, Nalca A, Ptak R. Antiviral activity of serum from the American alligator (*Alligator mississippiensis*). *Antiviral Res* 2005; **66**:35–8.
- 59 Merchant M, Thibodeaux D, Loubser K, Elsey RM. Amoebicidal effects of serum from the American alligator (*Alligator mississippiensis*). *J Parasitol* 2004; **90**:1480–3.
- 60 Matson KD, Ricklefs R, Klasing KC. A hemolysis–hemagglutination assay for characterizing constitutive innate humoral immunity in wild and domestic birds. *Dev Comp Immunol* 2005; **29**:275–86.
- 61 Ribas L, Li MS, Doddington BJ, Robert J, Seidel JA, Kroll JS *et al.* Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. *PLoS ONE* 2009; **4**:e8408.
- 62 Parmentier HK, Baelmans R, Nieuwland MG, Dorny P, Demy F. Haemolytic complement activity, C3 and Factor B consumption in serum from chickens divergently selected for antibody responses to sheep red blood cells. *Vet Immunol Immunopathol* 2002; **90**:91–100.
- 63 O'Shea JJ, Ma A, Lipsky P. Cytokines and autoimmunity. *Nat Rev Immunol* 2002; **2**:37–45.
- 64 Kleemann R, Zadelar S, Kooistra T. Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovasc Res* 2008; **79**:360–76.
- 65 Chen SN, Huang B, Zhang XW, Li Y, Zhao LJ, Li N *et al.* IFN- $\gamma$  and its receptors in a reptile reveal the evolutionary conservation of type II IFNs in vertebrates. *Dev Comp Immunol* 2013; **41**:587–96.
- 66 Grayfer L, De Jesus Andino F, Robert J. The amphibian (*Xenopus laevis*) type I interferon response to frog virus 3: new insight into ranavirus pathogenicity. *J Virol* 2014; **88**:5766–77.
- 67 Zou J, Tafalla C, Truckle J, Secombes CJ. Identification of a second group of type I IFNs in fish sheds light on IFN evolution in vertebrates. *J Immunol* 2007; **179**:3859–71.
- 68 Yu FF, Zhang YB, Liu TK, Liu Y, Sun F, Jiang J *et al.* Fish virus-induced interferon exerts antiviral function through Stat1 pathway. *Mol Immunol* 2010; **47**:2330–41.
- 69 Goodbourn S, Didcock L, Randall E. Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J Gen Virol* 2000; **81**:2341–64.
- 70 Staeheli P, Puehler F, Schneider K, Gobel TW, Kaspers B. Cytokines of birds: conserved functions – a largely different look. *J Interferon Cytokine Res* 2001; **21**:993–1010.
- 71 Grayfer L, De Jesus Andino F, Robert J. Prominent amphibian (*Xenopus laevis*) tadpole type III interferon response to the frog virus 3 ranavirus. *J Virol* 2015; **89**:5072–82.
- 72 Zou J, Yoshiura Y, Dijkstra JM, Sakai M, Ototake M, Secombes C. Identification of an interferon  $\gamma$  homologue in Fugu, *Takifugu rubripes*. *Fish Shellfish Immunol* 2004; **17**:403–9.
- 73 Igawa D, Sakai M, Savan R. An unexpected discovery of two interferon  $\gamma$ -like genes along with interleukin (IL)-22 and 26 from teleost: IL-22 and 26 genes have been described for the first time outside mammals. *Mol Immunol* 2006; **43**:999–1009.
- 74 Qi ZT, Nie P. Comparative study and expression analysis of the interferon  $\gamma$  gene locus cytokines in *Xenopus tropicalis*. *Immunogenetics* 2008; **60**:699–710.
- 75 Guanchun C, Jacques R. Antiviral Immunity in Amphibians. *Viruses* 2011; **3**:2065–86.
- 76 Lowenthal JW, Staeheli P, Schultz U, Sekellick MJ, Marcus PI. Nomenclature of avian interferon proteins. *J Interferon Cytokine Res* 2001; **21**:547–9.
- 77 Zhou X, Guo Q, Dai H. Molecular characterization and expression profiles in response to bacterial infection of Chinese soft-shelled turtle interleukin-8 (IL-8), the first reptilian chemokine gene. *Dev Comp Immunol* 2009; **33**:838–47.
- 78 Mondal S, Rai U. *In vitro* effect of temperature on phagocytic and cytotoxic activities of splenic phagocytes of the wall lizard, *Hemidactylus flaviviridis*. *Comp Biochem Physiol A Mol Integr Physiol* 2001; **129**:391–8.
- 79 Laskin L, Weinberger B, Laskin J. Functional heterogeneity in liver and lung macrophages. *J Leukoc Biol* 2001; **70**:163–70.
- 80 Bubanovic I, Najman S. Diversification of Cytokines Across Vertebrate Immune System Evolution, Reproductive Efficacy and Tumor Escape. 12th International Congress of Immunology and 4th annual Conference of FOCIS. Montreal, Canada, 2004:369–74.
- 81 Laing KJ, Zou JJ, Wang T, Bols N, Hirono I, Aoki T *et al.* Identification and analysis of an interleukin 8-like molecule in rainbow trout *Oncorhynchus mykiss*. *Dev Comp Immunol* 2002; **26**:433–44.
- 82 Lynagh GR, Bailey M, Kaiser P. Interleukin 6 is produced during both murine and avian *Eimeria* infections. *Vet Immunol Immunopathol* 2000; **76**:89–102.
- 83 Schneider K, Puehler F, Baeuerle D, Elvers S, Staeheli P, Kaspers B *et al.* cDNA cloning of biologically active chicken interleukin-18. *J Interferon Cytokine Res* 2000; **20**:879–83.
- 84 Dusseaux M, Martin E, Serriari N, Peguillet I, Premel V, Louis D *et al.* Human MAIT cells are xenobiotic-resistant, tissue-targeted, CD161hi IL-17-secreting T cells. *Blood* 2011; **117**:1250–9.
- 85 Neil DR, Heng S, Bellosi A, Flynn RJ, Daly M, Langford TK *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 2010; **464**:1367–70.
- 86 Coquet JM, Kyparissoudis K, Pellicci DG, Besra G, Berzins SP, Synth MJ *et al.* IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J Immunol* 2007; **178**:2827–34.

- 87 Vivier E, Spits H, Cupedo T. Interleukin-22 producing innate immune cells: new players in mucosal immunity and tissue repair. *Nat Rev Immunol* 2009; **9**:229–34.
- 88 Liu X, Ji XM, Du XN, Zong XC, Liang DF, Ma L *et al*. Molecular cloning, expression, bioinformatics analysis, and bioactivity of TNFSF13 (APRIL) in the South African clawed frog (*Xenopus laevis*): a new model to study immunological diseases. *OMICS* 2013; **17**:384–92.
- 89 Huminiacki L, Goldovsky L, Freilich S, Moustakas A, Ouzounis C, Heldin CH. Emergence, development and diversification of the TGF- $\beta$  signalling pathway within the animal kingdom. *BMC Evol Biol* 2009; **9**:28.
- 90 Taylor AW. Review of the activation of TGF- $\beta$  in immunity. *J Leukoc Biol* 2009; **85**:29–33.
- 91 Baumgarth N, Tung JW, Herzenberg LA. Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Semin Immunopathol* 2005; **26**:347–62.
- 92 Sinyakov MS, Dror M, Zhelev HM, Margel S, Avatation R. Natural antibodies and their significance in active immunization and protection against a defined pathogen in fish. *Vaccine* 2002; **20**:3668–74.
- 93 Morrison RN, Bruce Lyons A, Nowak BF, Hayball JD. Assessment of snapper (*Pagrus auratus*) natural IgM binding to bromelain treated sheep erythrocytes. *Fish Shellfish Immunol* 2005; **18**:91–9.
- 94 Li J, Barreda DR, Zhang Y, Boshira H, Gelman AE, LaPatra S *et al*. B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nat Immunol* 2006; **7**:1116–24.
- 95 Haimovich J, Du Pasquier L. Specificity of antibodies in amphibian larvae possessing a small number of lymphocytes. *Proc Natl Acad Sci U S A* 1973; **70**:1898–902.
- 96 Rosenberg CE, Salibian A, Fink NE. An enzyme-linked immunosorbent assay for measuring anti-sheep red blood cells antibodies in lead-exposed toads. *J Pharmacol Toxicol Methods* 2002; **47**:121–8.
- 97 Ujvari B, Madsen T. Do natural antibodies compensate for humoral immunosenescence in tropical pythons? *Funct Ecol* 2011; **25**:813–7.
- 98 Haghighi HR, Gong J, Gyles CL, Hayes MA, Zhou H, Sanei B *et al*. Probiotics stimulate production of natural antibodies in chickens. *Clin Vaccine Immunol* 2006; **13**:975–80.
- 99 Whitman NK, Matson KD, Bollmer JL, Parker PG. Disease ecology in the Galapagos Hawk (*Buteo galapagoensis*): host genetic diversity, parasite load and natural antibodies. *Proc Biol Sci* 2006; **273**:797–804.
- 100 Shaw PX, Horkko S, Chang M, Curtiss L, Palinski W, Silverman GJ *et al*. Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity. *J Clin Invest* 2000; **105**:1731–40.
- 101 Vollmers HP, Brandlein S. The, “early birds”: natural IgM antibodies and immune surveillance. *Histol Histopathol* 2005; **20**:927–37.
- 102 Hayday A, Theodoridis E, Ramsburg E, Shires J. Intraepithelial lymphocytes: exploring the Third Way in immunology. *Nat Immunol* 2001; **2**:997–1003.
- 103 Klasing KC, Adler KL, Remus JC, Calvert CC. Dietary betaine increases intraepithelial lymphocytes in the duodenum of coccidia-infected chicks and increases functional properties of phagocytes. *J Nutr* 2002; **132**:2274–82.
- 104 Zenewicz LA, Shen H. Innate and adaptive immune responses to *Listeria monocytogenes*: a short overview. *Microbes Infect* 2007; **9**:1208–15.
- 105 Levy O. Antimicrobial proteins and peptides of blood: templates for novel antimicrobial agents. *Blood* 2000; **96**:2664–72.
- 106 Bielecki T, Dohan Ehrenfest DM, Everts PA, Wiczowski A. The role of leukocytes from L-PRP/L-PRF in wound healing and immune defense: new perspectives. *Curr Pharm Biotechnol* 2012; **13**:1153–62.
- 107 Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev* 2007; **219**:88–102.
- 108 Renshaw SA, Trede NS. A model 450 million years in the making: zebrafish and vertebrate immunity. *Dis Model Mech* 2012; **5**:38–47.
- 109 Neumann NF, Stafford JL, Barreda D, Ainsworth AJ, Belosevic M. Antimicrobial mechanisms of fish phagocytes and their role in host defense. *Dev Comp Immunol* 2001; **25**:807–25.
- 110 Zimmerman LM, Vogel LA, Edwards KA, Bowden RM. Phagocytic B cells in a reptile. *Biol Lett* 2010; **6**:270–3.
- 111 Ellis AE. Innate host defense mechanism of fish against viruses and bacteria. *Dev Comp Immunol* 2001; **25**:827–39.
- 112 Buehler DM, Encinas-Viso F, Petit M, Vezina F, Tielemans BI, Piersma T. Limited access to food and physiological trade-offs in a long-distance migrant shorebird. II. Constitutive immune function and the acute-phase response. *Physiol Biochem Zool* 2009; **82**:567–71.
- 113 Walker JA, Barlow JL, McKenzie AN. Innate lymphoid cells – how did we miss them? *Nat Rev Immunol* 2013; **13**:75–87.
- 114 Parra D, Rieger A, Li J, Zhang Y, Randall L, Hunter C *et al*. Pivotal Advance: peritoneal cavity B-1 B cells have phagocytic and microbicidal capacities and present phagocytosed antigen to CD4<sup>+</sup> T cells. *J Leukoc Biol* 2012; **91**:525–36.
- 115 Caligiuri MA. Human natural killer cells. *Blood* 2008; **112**:461–9.
- 116 Goyos A, Robert J. Tumorigenesis and anti-tumor immune responses in *Xenopus*. *Front Biosci* 2009; **14**:167–76.
- 117 Kakimia K, Guidottia L, Koezuka Y, Chisaria F. Natural killer T cell activation inhibits hepatitis B virus replication *in vivo*. *J Exp Med* 2000; **192**:921–30.
- 118 Utke K, Bergmann S, Lorenzen N, Kollner B, Ototake M, Fischer U. Cell-mediated cytotoxicity in rainbow trout, *Oncorhynchus mykiss*, infected with viral haemorrhagic septicaemia virus. *Fish Shellfish Immunol* 2007; **22**:182–96.
- 119 Horton TL, Minter R, Stewart R, Ritchie P, Watson MD, Horton JD. *Xenopus* NK cells identified by novel monoclonal antibodies. *Eur J Immunol* 2000; **30**:604–13.
- 120 Gobel T, Chen C, Shrimpflo J, Grossin C, Bernoto A, Bucy P *et al*. Characterization of avian natural killer cells and their intracellular CD3 protein complex. *J Immunol* 1994; **24**:1685–91.
- 121 Gobel TW, Kaspers B, Stangassinger M. NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. *Int Immunol* 2001; **24**:757–62.
- 122 Muñoz F, De La Fuente M. The immune response of thymic cells from the turtle *Mauremys caspica*. *J Comp Physiol* 2001; **171**:195–200.
- 123 Bendelac A, Savage P, Teyton L. The biology of NKT cells. *Annu Rev Immunol* 2007; **25**:297–336.
- 124 Billerbeck E, Kang YH, Walker L, Lockstone H, Grafmueller S, Fleming V *et al*. Analysis of CD161 expression on human CD8<sup>+</sup> T cells defines a distinct functional subset with tissue-homing properties. *Proc Natl Acad Sci U S A* 2010; **107**:3006–11.