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Teaser This article presents an overview of the potential advantages of zebrafish use for the discovery of new anti-inflammatory drugs.

Zebrafish as a model for inflammation and drug discovery

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Zebrafish is a small teleost (bony) fish used in many areas of pharmacology and toxicology. This animal model has advantages for the discovery of anti-inflammatory drugs, such as the potential for real-time assessment of cell migration mechanisms. Additionally, zebrafish display a repertoire of inflammatory cells, mediators, and receptors that are similar to those in mammals, including humans. Inflammatory disease modeling in either larvae or adult zebrafish represents a promising tool for the screening of new anti-inflammatory compounds, contributing to our understanding of the mechanisms involved in chronic inflammatory conditions. In this review, we provide an overview of the characterization of inflammatory responses in zebrafish, emphasizing its relevance for drug discovery in this research area.

Rodrigo Zanandrea is pursuing an MSc in pharmacology on disease modeling in zebrafish, having previous publications in zebrafish and neuropharmacology.

Carla Bonan has recognized expertise in zebrafish research, with a special focus on the purinergic system and disease modeling.

Maria Campos has been working on inflammatory pathways and animal models of inflammation for almost 20 years.

Introduction

Chronic inflammation involves dysregulated and maladaptive responses, encompassed by unremitting inflammation, tissue destruction, and failed attempts at tissue repair, lasting for weeks to years. The mechanisms implicated in acute inflammation are well defined. However, the exact processes involved in chronic inflammation remain unknown. Human diseases, such as asthma, allergy, atherosclerosis, cancer, arthritis, and autoimmune diseases, are examples of chronic inflammatory conditions that have no cure. This group of diseases is the current focus of research and development worldwide, aiming to discover new anti-inflammatory drugs, with increased efficacy and fewer adverse effects [1,2]. Validated preclinical experimental models of inflammation are crucial to gain further insights into chronic inflammatory diseases, from their pathophysiology to innovative treatment options.

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https://doi.org/10.1016/j.drudis.2020.09.036

Zebrafish is a small teleost that has gained attention in inflammation research during the past few decades. Its popularity in biomedical research is associated with genetic and physiological homology with humans as well as the possibility to assess real-time behavioral and cellular alterations. *In vivo* models have additional translational validity over *in vitro* models, accounting for the use of zebrafish in inflammation research [3]. The use of zebrafish as an animal model to study inflammation started during the 2000s, with a substantial increase in the number of publications from 2011 onwards. A search of the Scopus database returned 1441 results, with an expressive number of publications in 2019 (Fig. 1). Given the involvement of inflammation in many chronic diseases, preclinical inflammatory disease modeling in zebrafish is relevant to gain advances in this area.

In this review, we discuss the zebrafish inflammatory repertoire, and the main studies using zebrafish as a model of inflammation. We also highlight pharmacological approaches using zebrafish as an experimental organism.

Inflammatory cells in zebrafish

The hematopoiesis system of zebrafish is similar to that in other vertebrates, with several phases of cell differentiation. Hematopoietic stem cells (HSCs) originate from the hemogenic endothelium of the ventral wall of the dorsal aorta, and a subset of these HSCs migrate to the caudal hematopoietic tissue (CHT), where several cell lineages are produced. In the thymus, HSCs generate T lymphocytes, whereas in the kidney, HSCs produce erythroid, myeloid, and B lymphocytes [4]. However, the adaptive immune system of zebrafish is not fully functional until the fish develop from the larval to the adult stage; the first B cells arise only at 20–21 days post fertilization (dpf). The transparency of the zebrafish larvae allows real-time visualization of the inflammatory cell migration (Fig. 2) [5,6]. Thus, the use of larval zebrafish up to 20 dpf is a valuable tool for evaluating the innate immune responses, which is an advantage of zebrafish over rodent models.

For microbial infections, the inflammatory response is triggered by recognition molecules, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain (NOD)-like receptors. Currently, drugs targeting TLR and NOD signaling are attractive alternatives for treating chronic inflammation [7,8]. TLR genes have already been characterized in zebrafish, with a core set of orthologous genes highly conserved with human TLRs. Overall, 14 distinct TLR types have been identified in zebrafish [9]. The NOD like-receptors have also been characterized, with five distinct member orthologous to those in humans [10]. However, even orthologous receptors might have different functions in humans and zebrafish. Therefore, in-depth studies of the specific functions of microbial recognition receptors in zebrafish are still required to gain further insights into their role in this teleost in relation to mammals.

After the detection of infection, inflammatory cells are recruited, driving the production of a variety of inflammatory mediators. The main leukocytes recruited in zebrafish are neutrophils, which are the most abundant and the first cells to reach the injured sites. In addition, the apoptosis or exit of neutrophils has an important role in the resolution of inflammation and can be a strategy to treat chronic inflammatory diseases, which can be screened in zebrafish [6]. Macrophages are present in zebrafish helping to control inflammation and are involved in organogenesis, tissue regeneration, and remodeling. The identification of

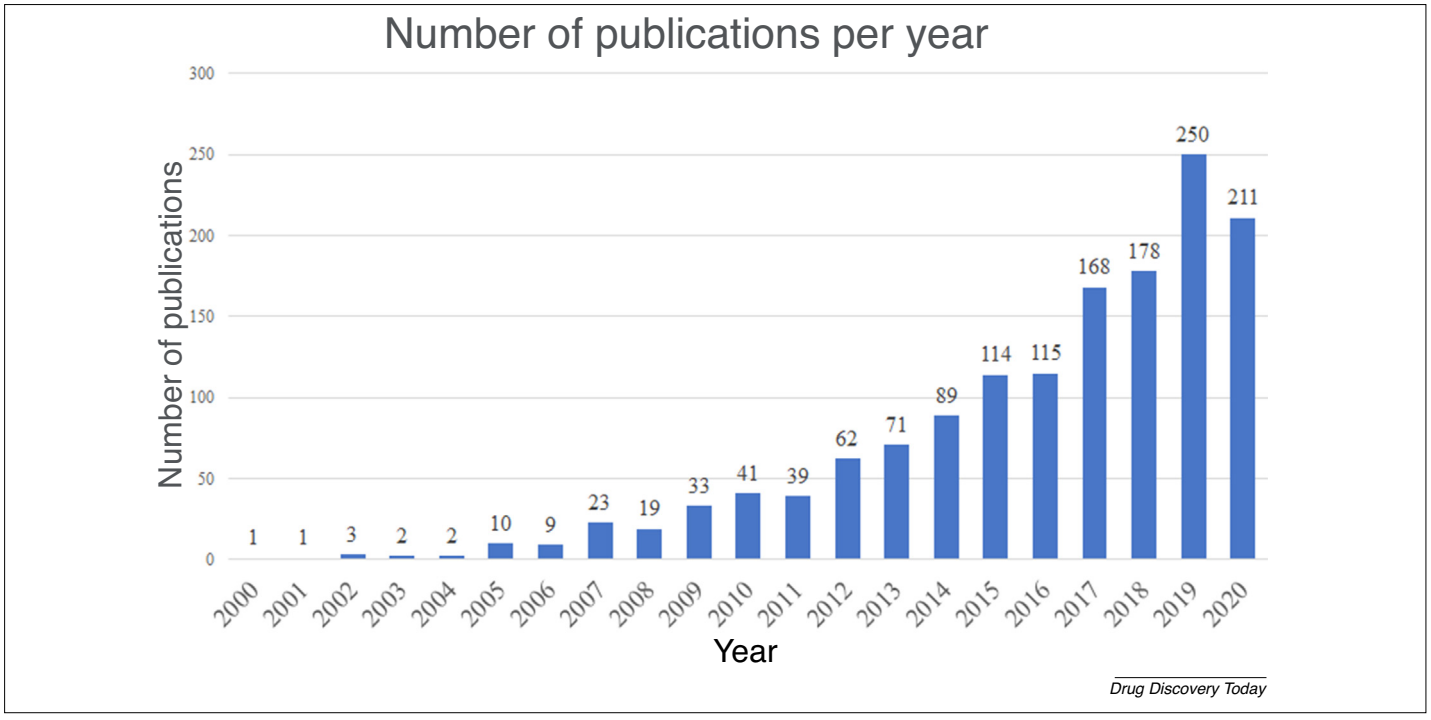

FIGURE 1

Number of publications per year (1999–2019) based on a search of Scopus with the settings: TITLE-ABS (zebrafish OR 'zebra fish' OR 'danio rerio' AND inflammation OR inflammatory) AND PUBYEAR > 1999 AND (LIMIT-TO (DOCTYPE, 'ar') OR LIMIT-TO (DOCTYPE, 're')) AND (LIMIT-TO (LANGUAGE, 'English')). Data obtained on August 16 2020, based on articles or reviews published in English only).

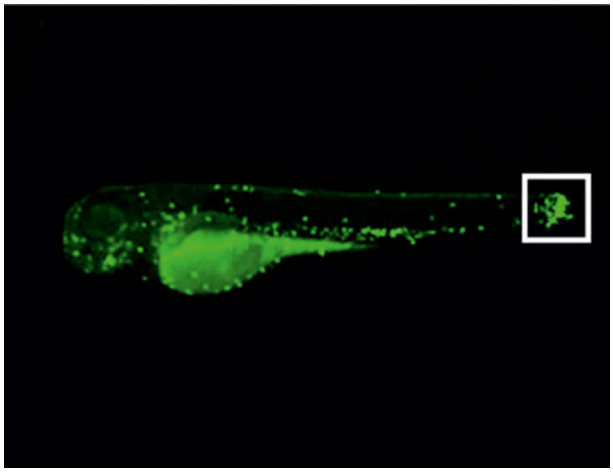

FIGURE 2

Green fluorescence emitted by neutrophils in an inflammation induced by cutting off the apical region of the tail in zebrafish [6].

subtypes of macrophage in zebrafish highlights the evolutionary conservation of these cells from fish to mammals [11,12].

Mast cells are a sentinel group that act against infection; they coordinate the balance between pro- and anti-inflammatory responses, releasing mediators that activate effector neutrophils [13]. They were identified in the gill and intestine of zebrafish, showing structural and functional similarity to mammalian cells [14]. Eosinophils are granulocytic leukocytes with a conserved role in the response to helminth antigens and allergens. In zebrafish, eosinophil granules display peroxidase activity, being negative for myeloperoxidase without a segmented nucleus [15]. Alternatively, mammal eosinophils undergo nuclear segmentation during maturation [15,16]. However, zebrafish can be a good model to evaluate the role of these cells in chronic inflammation, because they are relatively conserved over evolution [15]. In addition, antiallergic therapies targeting eosinophils are promising strategies to suppress excessive inflammation in a series of unmet medical conditions, such as asthma and atopic dermatitis, for which zebrafish could be useful as a screening model [17].

Although platelets are known for their role in thrombosis and wound repair, they recruit other immune cells and have a key role in inflammation [18]. Zebrafish blood contains mature erythrocytes and thrombocytes, which are nucleated, differing from enucleated human platelets. Nevertheless, the characterization of platelets suggests that zebrafish thrombocytes are the hemostatic homologs of mammalian platelets [19]. Therefore, with staining techniques and genetic tools, zebrafish becomes an appropriate model for evaluating the thrombocyte function under inflammation.

The two lineages of lymphocytes are present in zebrafish: B and T cells. B cell development is dynamic and shows sites of change during development; in addition, their phagocytic activity is low compared with other teleost species [5]. Some T lymphocyte subgroups have been characterized in teleosts, but the lack of selective monoclonal antibodies (mAbs) against zebrafish CD4+ and CD8α + T cells imposes an obstacle to greater advances in this area. Nonetheless, with the application of cross-reactivity of monoclonal antibodies, there is evidence for the differentiation and subspecialization of these cells in zebrafish [20].

The cells of the immune system are pivotal in the inflammatory response. Their tissue distribution and phenotypes completely modify inflammatory outcomes. Despite not having a complete description of immune cells in zebrafish, the major effector cells have been characterized in this teleost, displaying morphology and hematopoiesis similarities with both rodents and humans (Fig. 3). Although further studies are still needed, research focusing on inflammatory cells can be made using zebrafish, with a relevant translational value. In this context, research focused on neutrophil migration in zebrafish revealed novel evidence for cell migration mechanisms. In addition, advances in imaging tools and the application of gene-editing technologies (e.g., TALENS and CRISPR/Cas9) provide an interesting platform for *in vivo* studies using zebrafish as an animal model of inflammation.

Mediators and effectors of inflammation in zebrafish

The inflammatory response is regulated by a variety of chemical mediators derived from plasma, inflammatory cells, and/or injured tissue. Generally, these mediators can be classified into seven groups according to their biochemical properties: (i) vasoactive amines; (ii) vasoactive peptides; (iii) complement components;

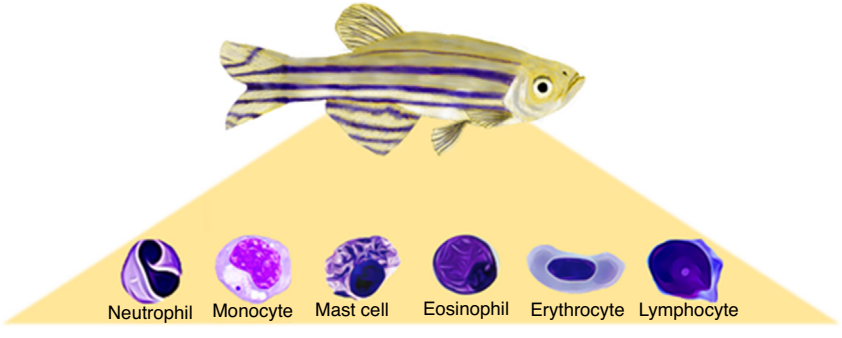

FIGURE 3

Morphological representation of different hematologic cells in zebrafish.

(iv) lipid mediators; (v) cytokines; (vi) chemokines; and (vii) proteolytic enzymes [21]. Zebrafish is an animal model with potential for the discovery of mechanisms involving inflammatory mediators, including the inflammasome activation [22].

Vasoactive amines

Many biological effects of mast cells, basophils, and platelets are mediated by biogenic amines that are released from cytoplasmic granules, which in turn act on blood vessels and smooth muscle, causing increased vascular permeability, and vasodilation or vasoconstriction, depending on the context. Members of this class of mediators include histamine and serotonin (5-HT), which act by binding to distinct receptors on target cells. Given that 5-HT receptors are ubiquitously expressed, it is difficult to associate a unique receptor with inflammation. 5-HT exerts its immunomodulatory effects by inducing chemoattraction and cytokine secretion [23]. Two receptors of this family, 5-HT1 and 5-HT2, were identified in zebrafish [24,25]. Other 5-HT receptors, such as 5-HT4 and 5-HT7, have an important role in inflammation in mammals [23], but they have not been characterized in zebrafish. Recent evidence suggests that 5-HT displays an important role in the peripheral immune system. Platelets, mast cells, antigen-presenting cells, and T cells are likely to synthesize, transport, store, and/or respond to 5-HT, as indicated by a series of studies in mammals [26]. To the best of our knowledge, there is no study relating and/or identifying 5-HT and its receptors in zebrafish immune cells. However, a role for 5-HT in the regulation of immune cells has been reported in another teleost model [27]. Moreover, 5-HT immunoreactive paraneuronal cells were identified during caudal fin regeneration in zebrafish. This is an indicative of the relevance of 5-HT in zebrafish inflammation, opening new possibilities to the use of this teleost in preclinical inflammation research [28].

Histamine mediates physiological and pathological processes associated with neuroinflammation and neurodegenerative diseases [29]. Histamine induces immunomodulatory effects via the activation of four G-protein-coupled receptors, being the H_1 the main subtype associated with allergic responses [30]. In zebrafish, H_1, H_2, and H_3 receptors are expressed by different cells, but histamine is not expressed in peripheral tissues of zebrafish. By contrast, the histamine concentration in the brain is in the same range as in other vertebrates [31,32]. This feature in zebrafish can be particularly useful for exploring the role of histamine in the brain and the action of drugs in this system, without any influence of peripheral mechanisms. However, Hrh4, a promising receptor in the development of anti-inflammatory drugs, does not appear to have an orthologous gene in zebrafish [32], generating some limitations in this model. It might be that the functions that overlap between the H_1 and H_4 receptors in humans are only performed by the H_1 receptor in zebrafish. Therefore, an evaluation of anti-H_1 receptor strategies in this animal model could provide initial insights into their therapeutic and neurotoxicological actions.

Vasoactive peptides

Vasoactive peptides are autacoids with actions in various tissues, especially on vascular smooth muscle cells. This class includes vasoconstrictors, vasodilators, and peptides with mixed effects. Substance P is released from sensory neurons and can itself cause mast cell degranulation; in zebrafish; it is encoded by *tac1* [21,33]. Vasoactive peptides can be generated by proteolytic processing of inactive precursors in the extracellular fluid, such as fibrin degradation products. The presence of highly conserved orthologs of the fibrinogen chains in zebrafish is related to the coagulation cascade and inflammation process, the function of which is similar to that in mammals [21,34,35]. In an inflammatory situation, pain sensation has an important physiological role by alerting the organism to the abnormal state of the damaged tissue. In this case, bradykinin affects the vasculature and has potent proalgesic effects. Single-copy genes exist for each of the bradykinin receptors, namely B_1 and B_2, in zebrafish [21,36]. With vasoactive peptides similar to mammals, zebrafish represents a good animal model for testing fibrin/fibrinogen inhibitors and bradykinin receptor antagonists, which are potential alternatives for treating neuroinflammation and inflammatory pain, respectively.

Complement components

The complement system mediates several major effector functions and modulates adaptive immune responses. Many components of the complement system and the signaling pathways known from mammals are highly conserved in zebrafish [37]. The mammalian anaphylatoxins C3a, C4a, and C5a display relevant effects on inflammatory responses. They promote granulocyte and monocyte recruitment, inducing mast cell degranulation, thereby affecting the vasculature [21]. The complement receptors C5aR1 and C3aR1 are highly upregulated in zebrafish during early cardiac regeneration, as observed in other species [38]. The presence of eight genes encoding C3 in the zebrafish genome expressed in different tissues was reported, unlike mammals, in which complement factors are secreted predominantly in the liver. Research into endotoxin-induced expression also revealed the differential regulation of C3 in distinct organs [39]. However, little is known of the role of anaphylatoxins in zebrafish. Thus, further research exploring complement members and their physiological roles in zebrafish is required.

Lipidic mediators

Lipidic mediators are derived from phospholipids and can be released when damage occurs in the cell membrane, thus generating arachidonic acid from phospholipids, via phospholipase A2. Subsequently, by the action of the lipoxygenases and cyclooxygenases, leukotrienes, thromboxanes, and prostaglandins are produced, causing vasodilation, hyperalgesia, and fever. Prostaglandin E_2 (PGE_2) leads to the resolution of trauma-elicited inflammation in zebrafish via the activation of its receptor EP4, with the subsequent stimulation of 15-lipooxygenase and lipoxin A_4 release [40]. This latter pathway is part of the lipid resolution machinery, involving the removal of neutrophils from the inflammatory site. Most lipid mediators are produced by zebrafish cells and are widely involved in inflammatory changes observed in this teleost [41,42].

Cytokines

Cytokines are key modulators of inflammation, acting via complex signaling pathways, and participate in acute and chronic inflammation. Their effects can be autocrine, paracrine, or endocrine and their roles in cellular communication go beyond the immune

system [43]. Interleukins (IL) bind to specific membrane receptors and have a pivotal role in intercellular communication. Tumor necrosis factor (TNF) is a multifunctional cytokine produced by immune cells that induces proinflammatory responses during infection, acting in diverse cellular events, such as cell survival, proliferation, and differentiation. Interferons (IFNs) are released in response to pathogens, mainly viruses, besides having antitumor actions. Colony-stimulating factors (CSF) have essential functions in the differentiation and proliferation of hematopoietic cells. Finally, transforming growth factors (TGFs) are mainly involved in cell proliferation [12,13,43–46]. Comparing zebrafish cytokines with human cytokines, we observe a low amino acid sequence identity. However, the identification and characterization of cytokines in zebrafish revealed both similar function and structure to that in mammals, supporting the use of zebrafish inflammation models. Table 1 summarizes the similarities between 31 zebrafish cytokines from different families compared with those in humans. This is relevant given the growing number of molecules targeting cytokines as new strategies to treat chronic inflammatory diseases. Thus, zebrafish might be useful for the initial screening of cytokine-based molecules and for testing either the potential efficacy or toxicity of those compounds.

Chemokines

Indispensable for immune cell migration, the chemokines are a group of inflammatory mediators that act under both inflammatory and normal physiological conditions. The four groups in this class are the CXC, CC, C and CX3C chemokines [47]. For zebrafish, 33 chemokine receptors and 89 chemokine genes have been identified so far. However, functional characterizations were performed only for Cxcl12a, Cxcl12b, Ccl19, and Cxcl8 (Il8), for which there are conserved counterparts in mammals [48]. The importance of these inflammatory mediators opens the possibility of using zebrafish as an organism model to test chemokine-based therapeutic strategies [49]. Genetic and biochemical characterization of the remaining zebrafish chemokines is needed to further explore all the advantages that this model can offer.

Proteolytic enzymes

The last inflammatory group of mediators discussed here are the proteolytic enzymes, also termed peptidases, proteases or proteinases, which are molecules that break the peptide bond between protein amino acids. They have important roles in various processes, including host defence, tissue remodeling, and leukocyte migration. Given their intrinsic biological activities, this group of

TABLE 1

Amino acid sequence identity between the zebrafish cytokine and the corresponding human cytokine[a]

Cytokine	Percentage of identity amino acid sequence corresponding to human cytokine	Sequence number reference (Genbank; Entry Uniprot)		Refs
		Zebrafish	Human	
IL-1β	24.24%	NP_998009.2; E6N152	AAM88883.1; P01584	[122,123]
IL-4	19.62%	CAL48253.2; D1YSM1	AAH70123.1; P05112	[124]
IL-6	18.44%	NP_001248378.1; H9A0J9	AAK48987.1; P05231	[125]
IL-8/Cxcl8	33%	XP_009305130.1; A0A0G2KYH9	AAH13615.1; P10145	[126]
IL-10	29.12%	AAI63038.1; Q5EFQ8	AAA80104.1; P22301	[127]
IL-11α	21.55%	CAI61346.1; Q494Q5	AAH12506.1; P20809	[128]
IL-11β	19.09%	CAI61347.1; Q494Q4	AAH12506.1; P20809	[128]
IL-12α (IL-12 p35)	17.84%	BAD26596.1; Q6F3R0	AAK84425.1; P29459	[129]
IL-12β (IL-12 p40)	24%	AAI64577.1; Q0V941	AAG32620.1; P29460;	[129]
IL-13	17.58%	BAG50536.1; B3IWZ9	AAH96139.1; P35225	[130]
IL-15	22%	AAI62843.1; Q15KG7	AAI00964.1; P40933	[131]
IL-17_5	31.36%	AAI15082.1; Q5TKT0	AAH67505.1; Q16552	[132]
IL-17_3	25.24%	BAD72788.1; Q5TKT2	AAH69152.1; Q9P0M4	[132]
IL-17_4	52.53%	AAI62897.1; Q5TKT1	AAH36243.1; Q8TAD2	[132]
IL-21	16.46%	ABM46913.1; A1YYP5	AAH69124.1; Q9HBE4	[133]
IL-22	15.35%	BAD72867.1; Q5TLE4	AAK62468.1; Q9GZX6	[134]
IL-23 p19	18.90%	CBM41294.1; L0N860	AAQ89442.1; Q9NPF7	[129]
IL-26	17.03%	AAI63119.1; Q5TLE5	AAH66270.1; Q9NPH9	[134]
IL-34	24.23%	BAM75187.1; L8AZT5	AAH29804.1; Q6ZMJ4	[135]
IFN type I	22.63%	AAM95448.1; Q8AY12	EAW58611.1; P01563	[136]
IFN-β	22.63%	BAD20663.1; Q75S22	EAW97180.1; P01579	[137]
TNF-α	25.93%	AAR06286.1; Q6T9C7	CAA26669.1; P01375	[12]
TNF-β	25.40%	AAR06286.1; Q1JQ40	CAA26670.1; P01374	[138]
CSF1a	17.29%	CAP58787.1; A9JRD6	AAH21117.1; P09603	[139]
CSF1b	12.06%	CAP58788.1; B0UYR0	AAH21117.1; P09603	[139]
CSF3a	19.01%	CAQ64749.1; B8ZHI7	AAK62469.1; P09919	[45]
CSF3b	22.58%	ABX57823.1; B5L332	AAK62469.1; P09919	[45]
TGF-β1	42.21%	AAI62366.1; Q7ZZU7	AAH22242.1; P01137	[140]
TGF-β2	78.50%	AAQ18012.1; Q7SZV4	AAH99635.1; P61812	[141]
TGF-β3	73.36%	AAW66727.1; Q66I23	AAC79727.1; P10600	[142].

[a] Percentages obtained by alignment using UniProt Proteome [121].

enzymes has a multitude of biotechnological applications [21,50]. Proteases can be divided into four groups, according to the essential catalytic component: (i) cysteine proteases; (ii) serine proteases; (iii) aspartic proteases; and (iv) metalloproteinases.

The cathepsins belong to the cysteine protease family, leading to tissue damage and triggering chronic inflammation, which could represent an attractive therapeutic strategy [51]. Cathepsin L gene (*ctsl*), later named *ctslb*, was reported in zebrafish as *ctsla* and *ctslc* genes. Both genes have a highly conserved structure compared with humans, but they present a distinct exon organization related to earlier evolution [52]. Lepage and Bruce characterized the zebrafish calpain system genes in embryos, and compared the expression with mammals, observing that *capns1a* and *capns1b* have high sequence identity to human *capsn* [53]. The cysteine protease family also encompasses caspases, which cleave protein substrates into aspartate residues and are involved in a range of cell signaling processes implicated in health and disease [54]. Although 12 caspase genes have been identified in humans, 19 distinct caspase genes are present in zebrafish, with *casp2*, *casp3a*, *casp8a*, *casp9*, and *casp20* being strongly expressed in the developing nervous system. Notably, *casp6a* and *casp19a* have a dynamic expression pattern that changes during zebrafish larval development [55].

Serine proteases are also involved in the production of proinflammatory cytokines, leading to the activation of immune cells. Several diseases are related to the dysregulation of these enzymes, including skin and lung inflammation, neuroinflammation, and arthritis [56]. The trypsin gene was cloned in zebrafish, and demonstrated homology to sequences seen in other vertebrate species [57]. Four proteinase-activated receptors (PARs) genes are present in zebrafish, which are homologs of mammalian PAR1–3. The zebrafish PAR-1 is a thrombin receptor and PAR-2b is a trypsin receptor [58]. Thrombin has a pivotal role in hemostasis and is generated from prothrombin, for which zebrafish share 53% amino acid identity with humans [19]. Other serine protease families expressed in zebrafish include kallikreins [59], elastases [60], matriptases [61], and subtilisins [62]. Members of the aspartic proteases also occur in this animal model, such as renin and cathepsin D [63,64], as well as a series of metalloproteinases [65,66]. There are a few drugs targeting proteases currently available in clinics. The development of new drugs using this therapeutic target has adverse effects caused by the nonspecificity of the compound. Although zebrafish has orthologous proteases, they do not always have the same specificity as the human enzyme, as observed in other animal models. By contrast, studies using a combination of cell models and/or cells from patients, with zebrafish as an animal model, could be useful in early screening, mainly for the identification and functional characterization of proteases that are currently unknown.

Zebrafish and mechanisms of inflammation resolution over the past few years, the modulation of pro-resolution pathways has become a promising strategy for treating inflammatory diseases in the place of traditional anti-inflammatory drugs. This premise is supported by the primary physiological role of the inflammatory response [67]. The resolution of inflammation encompasses the cessation of leukocyte infiltration, accompanied by a switch of chemical mediators, and uptake of apoptotic neutrophils and cellular debris. Specific pools of lipid mediators coordinate the resolution process, including prostanoids, leukotrienes, lipoxins, and resolvins [68]. So far, few studies have addressed the characterization of resolution mediators and their receptors in zebrafish. Five prostanoid receptors, (EP_{2a} e EP_{2b}, EP_{4a}, EP_{4b}, and EP_{4c}) were identified in zebrafish, with EP_{4a} and EP_{4b} being very close genetically to, and with drugs acting similarly as on, human receptors [69]. Resolvin E1 binds to the BLT_1 receptor and mediates the resolution of inflammation [67]. The biochemical characterization of three Blt receptors was carried out in zebrafish, including a Blt1 ortholog of human BLT_1, with low homologies to human and mouse BLT_1 (~40%), but with similar functions [70]. Nevertheless, the visualization of the cellular components of inflammation and the possibility of genetic manipulation offered by zebrafish makes this animal model suitable for studies involving the resolution of inflammation [71]. For this reason, different groups have used this model for screening drug libraries, with a special focus on neutrophil-targeting compounds [72–74]. Both transporter proteins [the solute carrier (SLC) and ATP-binding cassette (ABC)] have been identified in zebrafish neutrophils, providing an ideal model to identify novel strategies to modulate neutrophil fates and inflammation solving [75].

Zebrafish inflammation modeling

Compelling evidence indicates the importance of zebrafish for inflammation research, considering the cellular and molecular pathways sharing similarity to humans. Therefore, several models of inflammation using zebrafish have been developed over the past few years. An advantage of zebrafish is the option to use the larval and/or the adult phase of this teleost. As described for rodents, zebrafish inflammation can be induced by physical, chemical, or biological stimuli.

During the larval stage, it is usual to induce physical injury via a tail fin amputation; this can be performed using a 24-gauge needle cutting, or more accurately with a cryostat, commonly at 72 h post fertilization. The procedure must be conducted under aseptic conditions, because infection-induced inflammatory responses can be a confounding factor in this experimental paradigm. After anesthesia with 0.02% tricaine, the larvae are aligned in agarose-coated Petri dishes for partial amputation of the tail fin. The transection is performed by using the posterior section of the ventral pigmentation gap in the tail fin, as an anatomical reference, under a stereomicroscope. Other physical stimuli used to induce inflammation in zebrafish include ultraviolet exposure and electroablation. Either model induces a massive inflammatory response, being valuable for preclinical analysis of inflammation in zebrafish larvae [76,77].

In adult zebrafish (~ 6 months), similar protocols of physical damage are adopted. Before fin amputations, the fish must be anesthetized with 0.1% tricaine and placed on a soft and humid surface. For the cut, a razor blade can be used and ~50% of the caudal fin of zebrafish is amputated. After the surgery, the fish is allowed to recover in tanks placed in an incubator set at 33 °C, to facilitate regeneration [78]. Fin regeneration and the expression of inflammatory markers can be examined at different time points after amputation, and the effects of pharmacological treatments can be assessed. In this case, adult zebrafish have even more benefits, permitting the simultaneous analysis of several lesions. This animal model has an extraordinary regenerative capacity,

especially in the case of neuronal lesions. Brain injuries can be carried out after anesthesia with a needle pushed through a nostril [79], directly inserted into the brain in the area of interest, such as the telencephalon [80]. Alternatively, a nonpenetrating diffuse injury to the brain can be induced by using the weight drop model [81]. This injury leads to the activation of microglia and leukocytes, leading to acute neuroinflammation [82]. Of interest, the model of spinal cord injury secondary to surgical procedures allows the evaluation of many processes involved in regeneration, such as inflammation, cell death, cell migration, cell proliferation, and neurogenesis [83]. Other protocols of physical injury-induced inflammation in zebrafish have been described, such as optic nerve crush [84], ventricular resection [85], hypoxia/reoxygenation cardiac injury [86], and traumatic bone injury [87].

Despite the wide applicability of physical injury models of inflammation in zebrafish, these approaches are timeless, requiring individual manipulation and special equipment, precluding their use in large-scale screening protocols. In addition, physical injuries can compromise the evaluation of some behavioral tasks. Thus, a series of chemical zebrafish models of inflammation has been validated. D'Alençon *et.al.* [88] proposed a model of chemical inflammation induced by copper sulfate that induces infiltration of leukocytes to neuromasts within 20 min, according to the evaluation of zebrafish in the larval stage. The intraperitoneal injection of the algae-derived product carrageenan leads to abdominal edema in zebrafish, accompanied by an increase in TNF levels and inducible nitric oxide synthase (iNOS) expression [89]. A model of enterocolitis was proposed based on the rectal administration of oxazolone in adult zebrafish [90]. Tris(1,3-dichloro-2-propyl) phosphanovote (TDCIPP) is a chemical compound usually used in flame retardants, pesticides, and plasticizers, but can also be used to induce hepatic inflammation in zebrafish [91].

Given the number and variety of inflammatory diseases caused by pathogens, specific protocols of infection-related inflammation in zebrafish have been used for preclinical assays. In most cases, inflammation is induced by lipopolysaccharide (LPS), a wall component of all Gram-negative bacteria. LPS causes a series of immunological responses in zebrafish, and can be used in larvae by exposure in water or injection [6,92]. When used for immersion or injection, LPS can also be applied to adult zebrafish [93]. Additional models of infection using different bacteria strains shed new light on the mechanisms involved in host–pathogen interaction, accounting for the growing use of zebrafish in preclinical infection studies [94].

The development of methods that mimic chronic inflammation in zebrafish is important because most chronic inflammatory diseases still lack effective pharmacological therapies. Adult zebrafish knocked out for *Sirt1* exhibit an upregulation of the genes encoding IL-1b, IL-6, and TNF, correlating with chronic inflammation and intestinal atrophy, thereby increasing proapoptotic events [95]. The first zebrafish embryo chronic inflammation mutant was obtained through the insertion of the hepatocyte growth factor activator inhibitor 1 (*hai1*) gene, resulting in a phenotype with typical alterations featuring chronic inflammation [96]. By using a different approach, it was proposed that notochord infection of zebrafish larvae causes prolonged inflammation, and this could be a new model to study cellular and molecular mechanisms related in cartilage and/or bone chronic inflammation [97]. Zebrafish have also been proposed for screening new strategies to manage chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, by exposing embryos to dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS). Both chemical agents are also used to induce chronic intestinal inflammation in rodents for screening new anti-inflammatory drugs [98]. Interestingly, Kulkarni *et al.* validated a model of multiple sclerosis by inoculating the myelin oligodendrocyte protein (MOG) in adult zebrafish, which resulted in the fish displaying the mains signs and symptoms of this neuroimmune disease [99].

Effects of anti-inflammatory drugs on zebrafish inflammation

Zebrafish models of inflammation are responsive to treatment with classical steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs), emphasizing the applicability of this organism in inflammation research. For instance, in a model of chronic alcohol intake in adult zebrafish, the NSAID mefenamic acid was able to prevent cognitive deficits by regulating acetylcholinesterase brain activity, confirming the effects of mefenamic acid against neuroinflammation [100]. Another NSAID, indomethacin, displayed neuroprotective effects in the model of pentylenetetrazole-induced seizures in zebrafish larvae, likely by modulating the proinflammatory genes *il1b* and *cox2b* [101]. The benefits of NSAIDs, such as aspirin, were demonstrated in a nociception model induced by acetic acid exposure using zebrafish larvae [102]. Aspirin also displayed analgesic effects in a model of painful-like behavior induced by tail fin clipping in adult zebrafish [103]. Additionally, pretreatment with nonsteroidal agents, namely indomethacin or diclofenac sodium, ameliorated the nociception behavior elicited by formalin or acetic acid in adult zebrafish, respectively [104,105]. Recently, an automated method named the 'Fish Behaviour Index' was developed to detect nociceptive changes in adult zebrafish, based on general activity and distance swum after mechanical or chemical noxious stimuli [106]. In this experimental paradigm, the NSAID flunixin displayed dose-related analgesic effects in zebrafish submitted to tail fin clipping. These pieces of evidence indicate that zebrafish can also be a reliable model for the assessment of new potential analgesic drugs.

Although this is not a focus of this review, over-the-counter anti-inflammatory drugs have been detected in the environment, indicating the relevance of ecotoxicological studies for this group of drugs. Indeed, zebrafish is a reliable model to assess the potential aquatic ecotoxicity of pharmaceutical products. For instance, the acute exposure of zebrafish embryos to the NSAID diclofenac elicited a reduction in lipid peroxidation, an effect that was significant only at a low concentration of 0.03 mg/l [107]. There were no adverse effects of chronic exposure of zebrafish larvae to diclofenac in concentrations up to 320 mg/l, despite a slight reduction in growth at doses as low as 10 mg/l [108]. Diclofenac and ibuprofen (5–500 mg/ml) led to a decrease in hatching and motor activity, according to an evaluation of zebrafish embryos from 6 to 120 hpf. However, neither morphological defects nor increased mortality rates were observed for either drug in this study [109]. Alternatively, an investigation testing sublethal concentrations of diclofenac (from 0.4–7 mg/l) revealed distinct toxic effects for diclofenac in embryo and early-life stages of zebrafish

development [110]. In addition, studies demonstrated that non-steroidal agents alter sex differentiation in zebrafish, primarily through inhibition of COX-2 [111]. Altogether, these studies suggest that zebrafish is also a useful experimental model to predict the environmental effects of NSAIDs.

The exposure of 3-day-old zebrafish larvae to the glucocorticoid beclomethasone prevented neutrophil migration induced by tail fin amputation. This effect involved the modulation of *Il8* and *Cxcl18b* genes, which are implicated in the chemoattraction of neutrophils. Alternatively, beclomethasone failed to alter the macrophage influx or the expression of macrophage recruitment-related genes, namely *Ccl2* and *Cxcl11aa* [112]. In this model of trauma-induced inflammation, the administration of beclomethasone inhibited the differentiation of macrophages into the M1 proinflammatory phenotype and reduced the upregulation of several inflammatory genes elicited by tail amputation [112,113]. The steroidal drug dexamethasone was able to lessen both cell migration and mortality rate in a lethal inflammation model induced by LPS injection into the yolk of 3-dpf zebrafish larvae [114]. Dexamethasone also alleviated leukocyte migration and apoptosis taxes in the hearts of *breakdance* mutant zebrafish submitted to heart cryoinjury inflammation [115]. Therefore, zebrafish models recapitulate the main signs of inflammation, including pain, and these responses are sensitive to marketed anti-inflammatory drugs. In this regard, inflammation models in zebrafish have been used for the screening of innovative and repurposed agents with anti-inflammatory potential, presenting predictive validity [2,116].

Concluding remarks and perspectives

Zebrafish presents a complex immune system featuring mammalian characteristics [117]. The machinery involved in inflammation is also greatly developed in this organism, with the identification of a series of inflammatory mediators and receptors underlying inflammatory responses [118,119]. Zebrafish has also been demonstrated to be useful to study the mechanisms of resolution, mainly by characterization of real-time neutrophil migration patterns. Modeling inflammatory diseases in zebrafish is an interesting way to increase our understanding of the intricate mechanisms implicated in chronic inflammatory diseases, opening new avenues for the identification of efficacious and safe pharmacological strategies to treat inflammation-related unmet clinical needs. Acute models of inflammation in zebrafish are well established, but additional models of chronic inflammation need further validation, which continues to be a challenge in this area. In this regard, additional studies characterizing the mediators and receptors switching inflammation to resolution in zebrafish are required. Furthermore, the development of selective antibodies would enable an in-depth characterization of inflammatory responses in zebrafish at the protein level. Finally, the possibility to evaluate the effects of biologicals, such as anti-TNF strategies, in zebrafish could be addressed by generating new mutant strains of zebrafish, via new genetic tools, such as TALENS and CRISPR-Cas9 technologies. Considering the evidence presented in this review, it is tempting to propose this animal model as a relatively lower cost and potentially higher throughput screening strategy. Additionally, using zebrafish for testing inflammation-targeted compound libraries provides clear advantages over cell culture strategies, enabling the detection of adverse effects in a whole organism. Nonetheless, it will be imperative to adopt the principles of 3Rs when using zebrafish as an organism model in inflammation research [120]. Accordingly, refinement procedures must be adopted to maximally reduce the stress, and to improve animal well-being, regardless of whether working with larvae or adult zebrafish. It will also be relevant to perform sample size calculation *a priori*, to keep the number of animals per group as low as possible. Finally, replacement of zebrafish by using *in vitro* techniques or bioinformatics is also desirable, depending on the stage of drug development.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was funded in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Proc. 409485/2017-9), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS; Proc. 17/2551-0000977-0), and Instituto Nacional de Ciências e Tecnologia para Doenças Cerebrais, Excitotoxicidade e Neuroproteção. C.D.B. and M.M.C. are Research Career Awardees of the CNPq/Brazil. R.Z. was a recipient of a fellowship from CNPq. We thank Everton Paz for assistance with Fig. 3.

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