Glucocorticoid modulation of the immune response: Studies in zebrafish

Xie, Y

Chapter 2

Modeling inflammation in zebrafish for the development of anti-inflammatory drugs

Yufei Xie, Annemarie H. Meijer, Marcel J.M. Schaaf

Abstract

Dysregulation of the inflammatory response in humans can lead to various inflammatory diseases, like asthma and rheumatoid arthritis. The innate branch of the immune system, including macrophage and neutrophil functions, plays a critical role in all inflammatory diseases. This part of the immune system is well conserved between humans and the zebrafish, which has emerged as a powerful animal model for inflammation, because it offers the possibility to image and study inflammatory responses *in vivo* at the early life stages. This review focuses on different inflammation models established in zebrafish, and how they are being used for the development of novel anti-inflammatory drugs. The most commonly used model is the tail fin amputation model, in which part of the tail fin of a zebrafish larva is clipped. This model has been used to study fundamental aspects of the inflammatory response, like the role of specific signaling pathways, the migration of leukocytes, and the interaction between different immune cells, and has also been used to screen libraries of natural compounds, approved drugs, and well-characterized pathway inhibitors. In other models the inflammation is induced by chemical treatment, such as lipopolysaccharide (LPS), leukotriene B4 (LTB4) and copper, and some chemical-induced models, such as treatment with trinitrobenzene sulfonic acid (TNBS), specifically model inflammation in the gastro-intestinal tract. Two mutant zebrafish lines, carrying a mutation in the hepatocyte growth factor activator inhibitor 1a gene (*hai1a*) and the cdp-diacylglycerolinositol 3-phosphatidyltransferase (*cdipt*) gene, show an inflammatory phenotype, and they provide interesting model systems for studying inflammation. These zebrafish inflammation models are often used to study the anti-inflammatory effects of glucocorticoids, to increase our understanding of the mechanism of action of this class of drugs and to develop novel glucocorticoid drugs. In this review, an overview is provided of the available inflammation models in zebrafish, and how they are used to unravel molecular mechanisms underlying the inflammatory response and to screen for novel anti-inflammatory drugs.

1. Introduction

1.1. Inflammation and inflammatory diseases

When the body encounters harmful stimuli, such as invading pathogens, wounding or damaged cells, the immune system will be activated and an inflammatory response is triggered [1, 2]. This response is induced by Pattern Recognition Receptors (PRRs) such as Toll-Like Receptors (TLRs) recognizing patterns in molecules characteristic for microbes (Pathogen-Associated Molecular Patterns (PAMPs)), or molecules released by damaged cells (Damage-Associated Molecular Patterns (DAMPs)). Subsequently, immune cells release pro-inflammatory cytokines, such as IL-1β and TNF-α, which in

turn stimulate the synthesis and release of inflammatory mediators, including chemokines and prostaglandins [1, 3]. Directed by the chemokine gradients, leukocytes migrate towards the inflamed site to deal with the damaged tissue or invading microbes [4, 5]. These changes at the molecular level will lead to the five classical symptoms of inflammation: heat, pain, redness, swelling and eventually loss of function. Normally, the inflammatory processes are actively terminated through functional reprogramming of involved cells, which results in restored homeostasis [1].

A dysregulated inflammatory response is observed in various diseases. Abnormally and excessively activated inflammation plays an essential role in the pathogenesis of inflammatory disorders such as asthma, rheumatoid arthritis and allergic and autoimmune diseases [6, 7]. Chronic inflammation in the gastrointestinal tract can lead to inflammatory bowel disease (IBD), which may even cause non-digestive tract complications [8]. In addition, it has become apparent that chronic inflammation is involved in some diseases that were previously not considered to be inflammation-related, including cancer, type 2 diabetes, neurodegenerative diseases and atherosclerosis [9-12]. Finally, although inflammation serves primarily as a beneficial defence response against infections, acute or chronic overactivation of the inflammatory response is well known to exacerbate infectious disease pathologies, for example in COVID-19 and tuberculosis [13, 14].

Traditionally, the therapeutic regimen for inflammation includes the use of steroidal (glucocorticoid (GC)) and non-steroidal anti-inflammatory drugs [15]. However, the use of these drugs may provoke multiple side effects including osteoporosis, gastrointestinal disorders, cardiovascular or cerebrovascular events and infection [16, 17]. Moreover, drug resistance may occur in a subpopulation of patients [17]. In the past decades, successful monoclonal antibody therapies have dramatically improved the prognosis of patients with inflammatory disorders, for example rheumatoid arthritis [18] and novel inhibitors of critical components of inflammatory signaling pathways have been discovered [15, 19]. Despite this notable progress, there is still an unmet need for more effective and safer anti-inflammatory drugs. In this review, we discuss the usefulness of the zebrafish as an animal model for studying the mechanisms of inflammation and as a screening system to accelerate research aimed at the discovery of novel anti-inflammatory drugs (an overview is presented in Tables 1 and 2).

1.2. The zebrafish as an animal model for biomedical research

The use of zebrafish (*Danio rerio*) as a research model started in the 1950s and it was initially applied for studying embryonic development [20]. The zebrafish is a tropical fish that grows healthily in freshwater at temperatures around 24.6°C to 38.6°C [21]. When zebrafish find a shore of shallow water, they tend to spawn in the morning, which can be easily simulated in the laboratory with sliding

bottom inserts and lamp light at 28°C [22, 23]. The transparent embryonic and larval stages, the relatively short generation time, the small size and strong reproduction ability of zebrafish make it a highly versatile animal model. Over the years, more and more genetic tools and experimental methods have been applied, leading to the successful sequencing of the zebrafish genome, enabling rapid screening of gene function, and the generation of various transgenic or mutant fish lines and models for studying human diseases [24-26]. Due to the accumulation of knowledge and available tools for zebrafish, we are currently able to optimally exploit the advantages of this model.

Although initially used to study embryonic development, the zebrafish has emerged as a versatile animal model in diverse areas of biomedical research, including immunology, toxicology, cancer, and behavioral biology [27, 28]. In recent years, there have been many successful attempts modelling human diseases using zebrafish. For example, the characteristics of benign and malignant tumors that develop in zebrafish are similar to the histological symptoms of human tumors [29], zebrafish infected with *Mycobacterium marinum* adequately simulate hallmarks of human tuberculosis [30], and the phenotype of zebrafish carrying a mutation in the gene *sauternes* closely resembles the pathology of human X-linked congenital sideroblastic anemia [31]. In this review, we will discuss how the zebrafish is used as an animal model for inflammatory diseases and how the available models have been used for research on anti-inflammatory drugs.

An important advantage of the model is that the small size of zebrafish embryos and the development of automated techniques facilitate high-throughput screening [32-34]. Although *C. elegans* and *Drosophila* are also frequently used for high-throughput screening, their cuticles may act as a barrier for diffusion [35, 36]. Zebrafish embryos do not have cuticles, and most drugs can therefore be delivered by simply adding them to the culture medium at a relatively low dose. As a vertebrate, zebrafish are evolutionarily more closely related to mammals compared to worms and flies, so results can more easily be extrapolated to humans. Therefore, zebrafish models have a strong potential to serve as whole animal models to be used in preclinical bioassays during drug development.

The immune system of zebrafish is highly similar to that of humans. Important components of the innate immune system, macrophages, can be observed from 15 hours post fertilization (hpf) [37]. By the onset of blood circulation at 26 hpf, embryonic macrophages are already capable of phagocytosing particles, producing reactive oxygen species (ROS), and killing pathogens [37, 38]. The zebrafish neutrophils, which develop by 18 hpf and mature between 24-48 hpf, resemble human neutrophils regarding the segmented nuclei, granules, and expression of myeloperoxidase [39, 40]. Additionally, zebrafish show conserved critical parts of the adaptive immune system, including thymus development, thymocyte development and the function of T-cells and B-cells [41]. The adaptive

immune system matures after three to four weeks [42, 43], which means that the innate immune system can be studied separately during early embryonic and larval stages. The inflammatory response has also been found to be well conserved in zebrafish and this has been successfully exploited to increase our mechanistic understanding of the role of neutrophils in inflammatory diseases [44, 45]. The inflammatory response in zebrafish larvae can be induced using a variety of approaches. In this review we provide an overview of different methods to trigger inflammation (see Figure 2 for a schematic overview of these different methods), and we discuss how they are used for studies on the molecular mechanisms underlying the inflammatory response as well as for research aiming at the development of novel anti-inflammatory drugs, in particular novel GC drugs.

2. Inflammatory disease models in zebrafish

2.1. Wounding-induced inflammation

2.1.1. Introduction

Acute inflammation induced by tail wounding is a well-established model for inflammation and regeneration studies in zebrafish (Fig. 1, Fig. 2A). Tail wounding can be performed by amputation of part of the tail fin, or incision of the fin with a sterile scalpel or needle under a stereo microscope, which can be performed in zebrafish embryos, larvae and adults [46-48]. In embryos (stages up to 72 hpf) and larvae (72 hpf onwards), the amputation may include a distal part of the notochord, to induce a stronger response (Fig. 1A). Subsequently, an acute local inflammatory response can be observed, inducing accumulation of macrophages and neutrophils near the wounded area [46]. The visualization of leukocytes is possible through the use of transgenic fish in which the expression of autofluorescent proteins, such as GFP and mCherry, is driven by promoters which are specifically active in neutrophils (such as the myeloperoxidase (*mpx*) [46] and lysozyme (*lyz*) promoter [49]), or in macrophages (such as the macrophage-expressed gene-1 (*mpeg-1*) [50, 51] and *mfap4* promoter [52]), or by a promoter that marks both these cell types (*coro1a* [53]). Besides direct transection, the wounding can also be inflicted by laser irradiation of the epidermis on the trunk [54], the yolk sac [55], skeletal muscle tissue [56], or melanocytes over the yolk sac [57] and in the caudal hematopoietic tissue (CHT) [58]. Recently, thermal damage inflicted to the tail fin by a cautery pen has been shown to result in a dramatic loss of collagen fibers in the wound region (unlike tail fin transection), which was accompanied by a stronger inflammatory response and a delayed regeneration than observed after tail transection [59, 60].

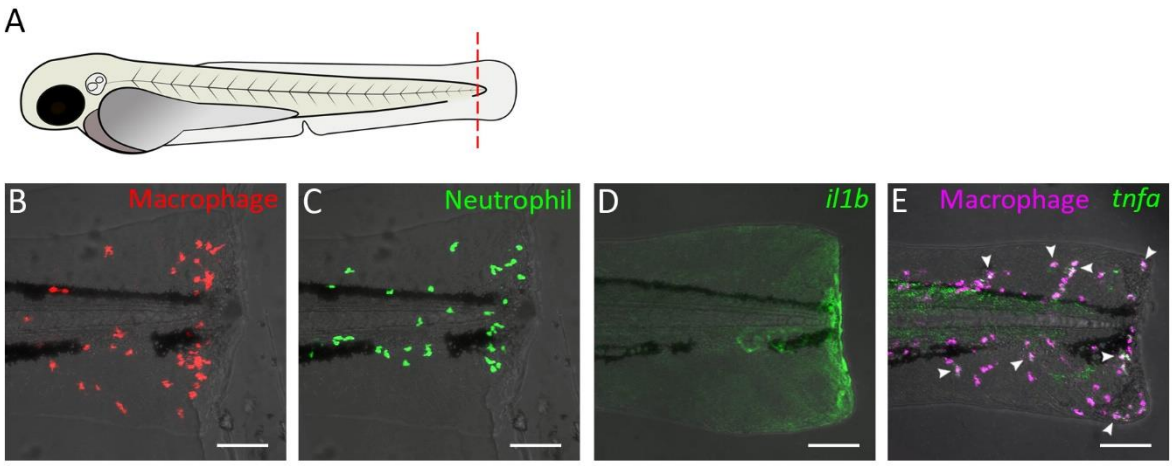

Figure 1. Tail transection in zebrafish larvae as a model for inflammation. A. Schematic drawing of a zebrafish larva at 3 dpf. The dashed red line shows a site of transection (in some studies, the transection site may not include a part of the notochord). B-E. Confocal microscopy images of tail from amputated larvae of the following transgenic lines: *Tg(mpeg1:mcherry-F)* (B), *Tg(mpx:GFP)* (C), *Tg(il1b:GFP)* (D), *Tg(mpeg1:mCherry-F/tnfa:eGFP-F)* (E). Images were taken at 4 hours post amputation using a Nikon Eclipse Ti-E microscope with a Plan Apo 20X/0.75 NA objective. Images show accumulation of macrophage (B) and neutrophils (C), activation of the *il1b* gene (D) and *tnfa* expression in macrophage (E) near the wound. In panel E, arrow heads indicate macrophages in which *tnfa* was activated.

2.1.2. Studies on molecular mechanisms underlying the inflammatory response

Using the zebrafish tail wounding model for inflammation, different molecular pathways of the inflammatory response have been unraveled. As a first response to wounding, the damaged epithelium generates a sustained hydrogen peroxide (H_2O_2, a major reactive oxygen species (ROS)) gradient from the wounded site, through local activation of the epithelial NADPH oxidase Duox [61, 62]. This gradient initiates the recruitment of leukocytes to the wounded area, in particular neutrophils, which use the Src family kinase Lyn as a redox sensor to detect the H_2O_2 gradient [63]. In addition, epithelial cells have been shown to use fatty acid β-oxidation to increase their mitochondrial ROS production in response to wounding. This process requires the activity of a zebrafish homologue of the mammalian mitochondrial enzyme, Immunoresponsive gene 1 (IRG1), and was shown to contribute to neutrophil recruitment [64, 65].

In neutrophils, phosphoinositide 3-kinase (PI3K) was found to mediate migration by inducing actin polymerization and generating membrane protrusions at the leading edge through Rac activation and polarization of F-actin dynamics (in a Rac-independent way), which is required for actomyosin-mediated tail contraction [58]. Treatment with the microtubule-destabilizing drug nocadozole impairs neutrophil migration towards wounds, even though this process enhances the polarity of F-actin dynamics [66]. SHIP phosphatases limit neutrophil mobility and their migration towards a wound,

probably by inhibiting PI3K activity [67]. Neutrophil migration also appears to require the Wiskott-Aldrich syndrome protein (WASp) for their proper migratory behavior [68].

Macrophages migrate to a wounded area by extension of pseudopods and they are capable of phagocytosing tissue debris [57]. Microtubule disassembly by nocadozole inhibited macrophage migration towards a wound, through global activation of Rho kinase (ROK) and thus a loss of the polarity of ROK activity [55]. Two distinct subsets of zebrafish macrophages were identified using a *Tg(tnfa:GFP)* reporter line, similar to the differentiation processes that are observed in mammalian macrophages [69, 70]. GFP-positive macrophages could already be observed at 1 hour post-wounding, and they are characterized by a flattened and lobulated morphology, and expression of markers characteristic of classically activated, pro-inflammatory (M1) macrophages. Those GFP-positive macrophages could convert to negative ones, which dominate the population at later stages, showing features of alternatively activated, anti-inflammatory (M2) macrophages.

The migration of leukocytes upon tail wounding is dependent on *de novo* protein synthesis, since treatment with the protein synthesis inhibitor cycloheximide was shown to inhibit the migration of neutrophils and macrophages [71]. Both AP-1- and NF-κB-induced transcription have been shown to be involved and the action of these transcription factor complexes is highly regulated by MAP kinase (MAPK) activity. One class of MAPKs, the c-Jun N-terminal Kinases (JNKs), are involved in the regulation of the AP-1-induced transcription, , whereas another class, the p38 MAPKs, appeared not to be alter the function of AP-1. Upon wounding, active JNKs were shown to activate c-Jun, which in turn induces the transcription of *mmp13* in neutrophils, which is required for the migration of these cells [72]. This JNK/c-Jun/Mmp13 pathway can be inhibited by Mkp-1 [72]. In addition, JNK-mediated c-Jun activation results in an increased expression of the *alox5* gene, encoding the 5-lipoxigenase Alox5, a key enzyme involved in the biosynthesis of leukotrienes, including LTB4 [73]. This pathway was also shown to be required for neutrophil migration upon tail wounding and could be inhibited by activation of the cannabinoid receptor type 2 (Cnr2) [73]. NF-κB activation, characterized by p65 phosphorylation, was shown to be dependent on the phosphorylation of another class of MAPKs, the Extracellular signal-regulated kinases (ERKs) [74]. The activation of this pathway was shown to be dependent on the circadian gene *period1b* (*per1b*), and results in an increased expression of pro-inflammatory molecules like *tnfa*, *il1b*, *il6*, and *il8* [74].

The cytokine IL-8 (or CXCL8) is known to be a potent chemoattractant for neutrophils in mammalian systems [75, 76]. The zebrafish homologs Cxcl8a (Cxcl8-l1) and Cxcl8b.1 (Cxcl8-l2) have been shown to be upregulated upon tail wounding, mediating neutrophil recruitment through Cxcr2 [77, 78]. The chemokines Ccl2 and Cxcl11aa were demonstrated to be required for the wound-induced migration of

macrophages by knocking down the expression of the genes encoding their respective receptors, Ccr2 and Cxcr3.2 [79]. Suppressing the activation of the cytokine IL-1β (by caspase-1 inhibitors and P2X7 antagonists) resulted in attenuated migration of neutrophils and macrophages [80]. In addition, knockdown of the gene encoding Il-1β by morpholino treatment was shown to decrease the migration of neutrophils towards the wounded area in two studies (although Il-1β appeared to be dispensable for random basal motility) [80, 81]. The migration of macrophages was not affected upon by *il1b* morpholino knockdown in one study [80], and decreased in another [81]. The Il-1β pathway (also involving the adaptor protein Myd88) was shown to act independently of NADPH oxidase-mediated ROS production, since treatment with the NADPH oxidase inhibitor DPI did not affect *il1b* expression levels (and vice versa: *il1b* and *myd88* knockdown did not affect ROS production upon tail wounding) [81].

Several hours after the wounding, the response enters the resolution phase, and active Wnt/β-catenin signaling has been suggested to play a role in this transition [82]. In the resolution phase of the inflammatory response, neutrophils leave the wounded area (a process called reverse migration) or undergo apoptosis [83]. The survival of neutrophils is regulated by Serum and Glucocorticoid Regulated Kinase 1 (SGK1), which is an anti-apoptotic protein downstream of the neutrophil survival factor GM-CSF [84]. The hypoxia-inducible factor-1α (HIF-1α) has been proven to be a critical factor for the regulation of myeloid cell function in mammals, and the activation of Hif-1α delays the resolution of inflammation in zebrafish by inhibiting neutrophil apoptosis and reverse migration [85]. The reverse-migrating neutrophils were found to exhibit an activated morphology and to respond normally to a secondary challenge [86]. The Cxcl12/Cxcr4 signaling axis plays a role in neutrophil retention and the knockdown of *cxcr4b* and *cxcl12a* or the pharmacological inhibition of this signaling increased the movement of neutrophils away from the wounded area [87].

For regeneration studies, the amputation is usually performed on 2 days post fertilization (dpf) zebrafish, after which tissue repair can be observed gradually and complete regeneration can be established 3 days later, at 5 dpf, which is within the time frame in which regulations of animal experimentation do not apply [88, 89]. It was demonstrated that the tissue regeneration of zebrafish embryos is dependent on ROS-induced vimentin production at the wound edge, and that the Stat3 and Tgfβ signaling pathways are involved in this process [59, 60]. Furthermore, It was shown that regeneration was not affected after ablation of macrophages and neutrophils using morpholino knockdown of the *pu.1/spi1b* gene, which encodes a transcription factor required to permit myeloid cell development [88]. However in later studies, macrophages were shown to be crucial for cell proliferation and tissue regeneration, since ablation of macrophages by an *irf8* morpholino (which

drives myeloid cell fate toward neutrophil development) resulted in impairment of the fin regeneration, and the presence of large vacuoles in the regenerated tissue [53]. A specific subset of macrophages, peripheral tissue-resident macrophages, were shown to contribute to tail fin regeneration by ROS production and downregulation of inflammatory mediators such as Il-1β at the damaged site [90]. In the adult zebrafish tail fin amputation model, macrophages have also been shown to enhance tail fin regeneration, by regulating tissue growth and bone ray patterning, which was demonstrated by depletion of macrophages in transgenic fish using the nitroreductase (NTR)/metronidazole(MTZ) cell ablation technology [82]. Mutation of the *runx1* gene reduced neutrophil numbers, but did not affect tail fin regeneration [53]. These findings suggest that the inflammatory response induced by wounding, in particular the recruitment of macrophages, is critical for tissue repair and regeneration.

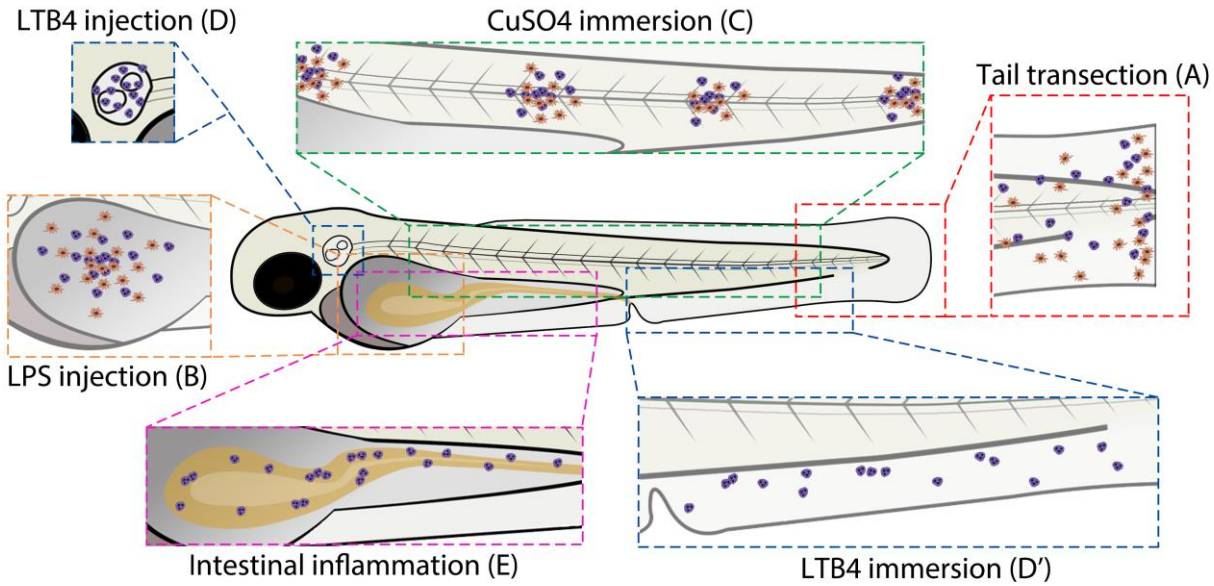

Figure 2. Schematic overview of commonly used zebrafish larval inflammation models. A. Transection of the tail fin. B. LPS injection in the yolk sac. C. CuSO4 immersion causing damage to the neuromasts. D. LTB4 injection in the otic vesicle. D'. LTB4 immersion. E. Chemical-induced intestinal inflammation. All presented models induce leukocyte recruitment. Red cells represent macrophages and purple cells represent neutrophils (in some models, the macrophage infiltration is not shown because it has not been investigated in the studies cited in this review). Alternative zebrafish larval inflammation models, discussed in the text but not presented in this figure, include laser wounding-, tail fin incision-, LPS immersion- and mutation-induced inflammation. For the tail fin transection/incision, CuSO4 immersion and intestinal inflammation models, adult zebrafish have been used as well.

2.1.3. 2Drug discovery studies

Tail wounding-induced inflammation in zebrafish has been used for anti-inflammatory drug testing and screening in numerous studies. Natural compounds, well-characterized drugs and defined pathway inhibitors have been tested and several libraries of such compounds have been screened in this model system. These studies contributed to the validation of this inflammation model and resulted in the identification of a number of novel anti-inflammatory compounds, requiring validation in other models and further optimization and testing.

In order to find new anti-inflammatory drugs from collections of existing or clinically approved drugs (drug repositioning [91]), a library of approved drugs that had not previously been characterized as anti-inflammatory compounds, were screened for their ability of suppressing neutrophil recruitment in the zebrafish tail wounding assay [92]. Interestingly, the ten most potent repositioned drugs from this zebrafish screen (including amodiaquin dihydrochloride, alfuzosin hydrochloride and clonidine hydrochloride) also displayed anti-inflammatory activity in a mouse model of skin inflammation. To discover novel analogs of an existing drug with reduced side effects, several analogs of thalidomide were screened using the zebrafish tail wounding model. A number of these analogs were shown to cause a reduction in neutrophil recruitment, without displaying the infamous teratogenic side effects of the original drug [93]. Important drug targets for accelerating the resolution of inflammation, ErbBs, were identified by screening kinase inhibitors in the zebrafish tail fin wounding model [94]. ErbB inhibitors and simultaneous gene knockdown of two genes that encode ErbB kinases (*egfra* and *erbb2*) resulted in suppression of neutrophil apoptosis and reduced the level of inflammation in zebrafish larvae.

In addition, structure-function studies have been performed using this model. For example, meisoindigo, which is a derivative of indirubin, a chemical constitute of the traditional Chinese herbal medicine Qing Dai was found to inhibit leukocyte migration induced by tail wounding without affecting reverse migration or Akt and Erk activity, whereas indirubin (which represents the core structure of meisoindigo) did not show an effect [95]. Moreover, a particular chemical group, consisting of fused benzene and pyran rings with an attached carbonyl group (1,4-benzopyrone) or its isomer ‘coumarin’ (1-benzopyran-2-one), was found to be present in four of the nine most-active pro-resolution compounds identified in a large screen of 2000 well-characterized and approved drugs. All four of these drugs accelerated the resolution of the inflammation and three of them also inhibited neutrophil migration towards the wound [96]. Other compounds containing this benzopyrone structure were shown to have similar effects and the most active one, isopimpinellin, was found to inhibit the

recruitment of leukocytes (by inhibiting PI3K), and to promote the resolution phase (by inducing neutrophil apoptosis) [97].

Natural compound libraries are rich sources for drug discovery. Various natural products have been demonstrated to have an inhibitory effect on the infiltration of leukocytes near the wounded area, including extracts from the medicinal herb ginseng (roots of plants in the genus *Panax, such as Panax ginseng*) [98]. One of the bioactive compounds in these extracts was shown to be the ginsenoside Rg1, a glycosylated steroid that exerts its anti-inflammatory activity through the glucocorticoid receptor (GR) [99]. Similar effects on leukocyte migration have been observed for an essential oil from *Thymus vulgaris* [100], for the coumarin-derivative bergapten isolated from *Ficus hirta* roots [101], for an extract from the green seaweed *Cymopolia barbata* and its major active component, cymopol [102], and for the compound *micrometam C* isolated from *Micromelum falcatum* trees, which are mangrove associates [103]. Downregulation of the expression of various pro-inflammatory genes and upregulation of the anti-inflammatory gene *il10* in the tail-wounding assay was found for an extract from *Clerodendrum cyrthophyllum* Turcz leaves [104]. Inhibition on tail wound-induced ROS generation was shown for a metabolite isolated from the red seaweed *Laurencia snackeyi*, 5β-Hydroxypalisadin B [105], for bergapten [101] and for the polysaccharide fucoidan, extracted from the brown seaweed *Ecklonia cava* [106]. The latter two compounds also attenuated the inflammatory response by inhibiting the synthesis of Nitric Oxide (NO), which is an important inflammatory mediator. Enhancement of the resolution of the inflammation, by promoting neutrophil apoptosis and reverse migration, was demonstrated for tanshinone IIA, a compound extracted from the root of the plant species *Salvia miltiorrhiza* [96].

Table 1. Overview of zebrafish models for inflammation.

Inflammatory models	Age	Treatment	Inflammatory responses	References
Tail wounding-induced inflammation				
Transection	2-5 dpf	Amputation	Accumulation of macrophages and neutrophils; increased ROS production; upregulated inflammatory genes	[46, 61-63]
	2-4 dpf	Incision	Accumulation of macrophages and neutrophils; increased ROS production;	[47, 57, 62]
Laser	4 dpf	Epidermis	Accumulation of neutrophils	[54]
	22 hpf	Yolk sac	Accumulation of macrophages	[55, 57]
	1-2 dpf	Skeletal muscle	Myofibril damage	[56]
	2-3 dpf	Tail fin	Accumulation of macrophages and neutrophils; ROS signaling; upregulated inflammatory genes (*tnfa*)	[59, 60]
Chemical-induced inflammation				
LPS	1-3 dpf	Immersion	Increased ROS and NO production; upregulated inflammatory genes (*il1b, tnfa, il10, p65, nfkbia*)	[107, 108]
	3 dpf	Yolk injeciton	Accumulation of macrophages and neutrophils; upregulated inflammatory genes (*il1b, tnfa, il6*)	[109]
$CuSO_4$	2-7 dpf	Immersion	Hair cell death; infiltration of macrophages and neutrophils; oxidative stress	[110-114]
	Adult	Immersion	Oxidative damage and apoptosis in the gills; upregulated inflammatory genes (*tnfa, mmp9, myd88, il6, il8*)	[115-117]
LTB4	3 dpf	Otic vesicle injection	Neutrophil recruitment	[77, 118, 119]
	2 dpf	Hindbrain injection	Macrophage recruitment	[120]
	3 dpf	Immersion	Neutrophil accumulation in the fin	[33, 63]
Enterocolitis	3-8 dpf	TNBS immersion	Gut dilation; loss of villi and clefts; infiltration of neutrophils; increased number of goblet cells; upregulation of inflammatory genes (*il1b, tnfa, mmp9, ccl20, il8*)	[121, 122]
	3-6 dpf	DSS immersion	Mucus accumulation; infiltration of neutrophils; reduced proliferation; upregulation of inflammatory genes (*il1b, tnfa, mmp9, ccl20, il8, il23*)	[123]
	3-6 dpf	Glafenine immersion	Intestinal epithelial cell apoptosis and shedding; ER stress	[124, 125]
	Adult	TNBS intrarectal injection	Epithelial disruption; neutrophil infiltration; upregulation of inflammatory genes (*il1b, tnfa,il8, il10*)	[126]

	Adult	Oxazolone intrarectal injection	Epithelial damage; infiltration of granulocytes; goblet cell depletion; upregulation of inflammatory genes (*il1b*, *tnfa*, *il10*)	[127]
Mutation-induced inflammation				
hai1a	1-3 dpf		Epidermal defects (skin); leukocyte accumulation; enhanced keratinocytes apoptosis; upregulation of inflammatory genes (*mmp9*)	[128-130]
cdipt	5-6 dpf		Intestinal damage; reduced mucos ecretion; infiltration of macrophages and neutrophils; globlet cell apoptosis; impaired proliferation; ER stress; upregulation of inflammatory genes	[131, 132]

Table 2. Overview of drugs showing anti-inflammatory effects in zebrafish inflammation models

Inflammatory models	Drugs showing anti-inflammatory effect	References
Tail amputation	Extract from *Clerodendrum cyrthophyllum* Turcz	[104]
	Extract from *Cymopolia barbata* and its major active component cymopol	[102]
	Ginsenoside Rg1 from ginseng; beclomethasone	[99]
	ErbB kinase inhibitors	[94]
	Extracts from ginseng	[98]
	Bergapten from *Ficus hirta*	[101]
	Meisoindigo, a derivative of indirubin	[95]
	Analogs of thalidomide	[93]
	Mircometam C from *Micromelum falcatum*	[103]
	Isopimpinellin from the Apiaceae family, and other compounds containing a benzopyrone structure	[96, 97]
	Tanshinone IIA from *Salvia miltiorrhiza*; dexamethasone	[96]
	Approved drugs (eg: glipizide, tetracycline HCl, dexamethasone)	[92]
	5β-Hydroxypalisadin B from *Laurencia snackeyi*; dexamethasone	[105]
	Fucoidan from *Ecklonia cava*	[106]
Tail incision	Essential oil from Thymus vulgaris	[100]
LPS immersion	Polyphyllin VII from *Paris polyphylla*	[133]
	Caffeine	[134]
	Oleuropein from *Olea europaea*	[135]
	Polyphenol-rich extract from *Ecklonia cava*	[136]
	5β-Hydroxypalisadin B from *Laurencia snackeyi*; dexamethasone	[105]
	Fucoidan from *Ecklonia cava*	[106]
LPS injection	Phillyrin from *Forsythia suspensa Vahl*; dexamethasone	[137]
	Extracts from *Chimonanthus nitens* Oliv.; dexamethasone	[138]
	Chlorogenic acid	[109]
$CuSO_4$ immersion	Extract from *Clerodendrum cyrtophyllum* Turcz	[104]
	Enzymatic peptide from skipjack (*Katsuwonus pelamis*); indometacin	[139]
	Terpene glycoside from *Sanguisorba officinalis*	[140]
	Polyphyllin VII from *Paris polyphylla*	[133]
	Pituitary adenylate cyclase-activating polypeptide(PACAP)-38)	[141]
	Extract from Quzhou Fructus Aurantii; indometacin	[142]
	Clinically approved drugs (eg: tenatoprazole, candesartan)	[143]
TNBS immersion	Cholecystokinin; dopamine receptor agonists; dexamethasone	[144]
	5-aminosalicylic acid; prednisolone	[121, 122]
	NOS inhibitors; thalidomide; parthenolide;	[121]
DSS immersion	Cholecystokinin; dopamine receptor agonists	[144]
	Retinoic acid; dexamethasone	[123]

2.2. Chemical-induced inflammation

2.2.1. LPS-induced inflammation

Lipopolysaccharide (LPS) is an endotoxin in the cell walls of Gram-negative bacteria which acts as a PAMP that induces the innate immune response upon recognition by TLRs [145]. LPS-induced inflammation in zebrafish is generally established by non-invasive immersion of embryos in egg medium containing LPS [107, 146] or injection into the yolk [109] (Fig. 2B). In mammals, the immune response to LPS is characterized by TLR4-mediated induction of NF-κB and the expression of various inflammatory mediators, including TNFα and IL-1 [147, 148]. However, the TLR4 paralogs identified in zebrafish do not recognize LPS, due to the differences in extracellular structures for recognition and the lack of essential costimulatory molecules [149-151].

Despite the poorly characterized recognition mechanism for LPS, a response similar to that observed in mammalians has been observed, indicating a high degree of conservation between the zebrafish and mammalian transcription factors and signaling pathways in response to LPS stimulation [152, 153]. LPS stimulation enhanced the production of NO and ROS, increased the levels of iNos and Cox2 proteins, and the mRNA levels for *p65*, *nfkbiaa* and other genes encoding key pro-inflammatory cytokines including *tnfa* and *il1b* [107, 108]. Pre-treatment of zebrafish with a sublethal dose of LPS was shown to prevent mortality as a result of a subsequent lethal dose of LPS, which demonstrates that tolerance, generally observed in mammals, can be reproduced in zebrafish. Cxcr4 signaling appeared to play an important role in the occurrence of LPS tolerance [146, 154].

LPS-induced inflammation in zebrafish has been used as a model for research on anti-inflammatory drugs. A lot of compounds and extracts from traditional medicinal or non-medicinal herbs were tested using this model, and several of these showed inhibition on LPS injection-induced inflammation. Chlorogenic acid, a polyphenolic compound which occurs in coffee and phillyrin (the main ingredient in *Forsythia suspensa* Vahl fruits) inhibited macrophage and neutrophil recruitment to the site where LPS was injected, and improved the survival rate [109, 137]. The latter compound inhibited the MyD88/NF-κB signalling pathway by decreasing expression levels of genes encoding IκBα, Il-1β, Il-6, and Tnf-α [137]. Extracts from *Chimonanthus nitens* Oliv. leaves also inhibited recruitment of neutrophils (and not macrophages), reduced the LPS-induced upregulation of *il1b*, *il6* and *tnfa* expression [138].

In many studies, the ROS and/or NO production have been used as a readout for the anti-inflammatory effect. Polyphyllin VII (PP7) from *Paris polyphylla* inhibited NO generation, and also deceased the heartbeat and attenuated the yolk sac edema after LPS injection into the yolk sac [133]. Fucoidan and

a polyphenol-rich fraction extracted from *Ecklonia cava* inhibited both NO and ROS formation [106, 136], just like the compound 5β-Hydroxypalisadin B, which was also shown to be anti-inflammatory in the tail-wounding model [105]. The polyphenol-rich fraction extracted from *Ecklonia cava* also decreased cell death and improved survival [136]. In some reports, only the NO generation was used as an indicator for the anti-inflammatory effect of drugs, and this has been used to demonstrate the effects of caffeine [134] and oleuropein, a phenolic compound present in olives and leaves of the olive tree (*Olea europaea*) [135].

Apolipoprotein(apo)A-I is one of the major constituents of high-density lipoproteins (HDLs) which has been shown to have anti-inflammatory effects [155]. The role of apoA-I modification was tested in zebrafish embryos by co-injecting LPS and HDLs containing either native or glycated apoA-I. The results demonstrated a reduced mortality upon injection of HDLs with native apoA-I, probably due to its anti-inflammatory effect [156].

LPS treatment has also been used in combination with tail wounding to enhance leukocyte accumulation near the wound. This model was utilized to evaluate the bioactivity of structurally diverse natural products of an East African medicinal plant, *Rhynchosia viscosa*, resulting in the identification of both known and novel isoflavone derivatives with anti-inflammatory activity [157, 158].

2.2.2. Copper-induced inflammation

Copper is a trace element acting as a catalytic cofactor for various enzymes involved in energy and antioxidant metabolism [159]. Excessive inorganic copper from the environment could disturb the copper balance in zebrafish and lead to an inflammatory response mediated by damage from the oxidative stress [160]. In adults, soluble copper was reported to induce oxidative damage and apoptosis in the gills and showed dose-dependent lethality [115, 116]. Upon copper sulfate ($CuSO_4$) treatment, the neutrophils in the kidney marrow were found to be activated, and analysis of the proteome of neutrophils revealed regulation of proteins involved in cell cycle, NO signaling, regulation of cytoskeleton and immune-related processes [117].

Exposure of zebrafish embryos to $CuSO_4$ was reported to inhibit the survival and development of embryos [161, 162]. It induces an inflammatory status, which is related to exacerbated damage and oxidative stress, and the endogenous signaling molecule adenosine was shown to be involved [113]. Importantly, within two hours this treatment induces death of hair cells in the neuromasts of the lateral line, which regenerate and reach full functionality one day later [110, 111]. This damage to the neuromasts results in a localized robust inflammatory response in the neuromasts, including the

infiltration of macrophages and neutrophils [112] (Fig. 2C). The recruited macrophages play a critical role in the regeneration of damaged hair cells since ablation of macrophages significantly delays this process, while neutrophils are not required [114].

The accumulation of neutrophils in the neuromasts is one of the most frequently used indicators for the level of inflammation in this model and has been applied to assess the effect of known anti-inflammatory drugs [112]. Since the induction of inflammation by $CuSO_4$ can be established by just adding the compound into the culture medium, an automated high-throughput drug screening assay could be developed with this model based on leukocyte accumulation around neuromasts, using a double transgenic line with the neutrophils labelled in red and the neuromasts in green (using the claudin b (*cldnb*) promoter driving GFP expression) [112, 163]. Using this automated system, various drugs from a library of clinically approved drugs were identified to have an anti-inflammatory effect, among which the NOS1 inhibitor 3-Bromo-7-nitroindazole. Further investigation revealed a novel pro-inflammatory role of NO signaling via soluble guanylate cyclase and in a soluble guanylate cyclase - independent manner through protein S-nitrosylation [143].

Furthermore, a neuropeptide, pituitary adenylate cyclase-activating polypeptide(PACAP)-38, known to be an anti-apoptotic and anti-inflammatory factor, was reported to inhibit neutrophil migration towards the neuromasts and expression of pro-inflammatory genes (*il8, il1b, il6* and *atf3*) [141]. Several natural products were reported to exert an inhibitory effect on the $CuSO_4$-induced neutrophil accumulation, including a new terpene glycoside extracted from the root of *Sanguisorba officinalis* [140], an enzymatic peptide (SEP) from skipjack (*Katsuwonus pelamis*) [139] and an extract from Quzhou Fructus Aurantii, an unripe fruit from the bitter orange tree (*Rutaceae Citrus changshan-huyou Y.B. Chang)* [142]. The compound PP7 (from *Paris polyphylla)* also showed an inhibition of the neutrophil migration upon $CuSO_4$ stimulation, similar to what was observed for this compound in the LPS-induced inflammation model [133]. An extract from leaves of *Clerodendrum cyrtophyllum* Turcz decreased the oxidative stress induced by $CuSO_4$ and inhibited inflammation by downregulating genes related to inflammatory processes (*cox2, pla2, c3a, mpx*) and cytokines (*il1b*, *il]8*, *tnfa* and *il10*) [104].

2.2.3. LTB4-induced inflammation

Leukotriene B4 (LTB4) is an eicosanoid released by leukocytes, acting as a pro-inflammatory mediator and enhancing leukocyte accumulation at sites of inflammation [164, 165]. In zebrafish, LTB4 was demonstrated to attract both neutrophils and macrophages [119, 120]. Upon injection of LTB4 into the otic vesicle of 3 dpf zebrafish larvae, neutrophil recruitment to the ear was observed at one hour after the injection, and this recruitment was not dependent on Cxcl8/Cxcr2 signaling [77, 118] (Fig. 2D). In addition, injection of LTB4 into the hindbrain at 30 hpf recruited macrophages independent of

Cxcl11aa/Cxcr3.2 signaling [120]. Bath application of LTB4 induced dissemination of neutrophils into fins, which can be quantitated by counting cells in the ventral fin (Fig. 2D'). This LTB4-induced migration of neutrophils was not prevented by inhibition of the Cxcl8/Cxcr2 signaling pathway either [118], or by DPI or Lyn knockdown [63]. A Zebrafish Entrapment by Restriction Array (ZEBRA) microfluidic device was designed to quickly position zebrafish embryos and larvae in a predictable array, suitable for automated imaging. The effectiveness of this device was demonstrated by assessing the inhibitory effect of the PI3K inhibitor LY294002 on LTB4-induced neutrophil migration [33]. The device can be designed with access ports to enable the administration of treatments, and it could potentially be used for other inflammation assays like tail wounding as well [33].

2.2.4. Chemical-induced intestinal inflammation

Inflammatory bowel disease (IBD) represents a group of intestinal disorders that are characterized by inflammation of the digestive tract [8]. IBD is modeled in zebrafish by treatment of fish with chemicals that induce an IBD-like enterocolitis [166] (Fig. 2E). In adult zebrafish, intrarectal administration of the hapten oxazolone was reported to induce enterocolitis, characterized by infiltration of granulocytes, epithelial damage, goblet cell depletion and upregulated expression of genes encoding cytokines (*il1b*, *tnfa*, *il-10*) [127]. Similar results were obtained upon intrarectal administration in adult zebrafish of another hapten, trinitrobenzene sulfonic acid (TNBS), which was also shown to increase the intestinal mRNA and peptide levels of melanin-concentrating hormone (Mch) and the mRNA levels encoding its receptor [126].

Immersion of larvae in egg water containing TNBS between 3 and 8 dpf induced an inflammatory response in the gut [121]. Using a fluorescent dye, which was swallowed by the fish, the gut architecture and motility could be assessed, showing TNBS-induced dilation of the gut, reduction in villus length, expansion of crypts, and a loss of peristalsis. Throughout the length of the gut, histological analysis showed an expansion of the lumen, a smoothening of the gut lining which was devoid of villi and clefts, and an increase in the number of goblet cells [121]. The reported changes in intestinal cell morphology were not observed in a different study in which different doses and durations of TNBS exposure were used [122]. In this study an increased number of neutrophils in the inflamed intestine and increased expression of *il1b*, *tnfa, mmp9, ccl20* and *il8* was observed upon TNBS treatment [122].

Exposure of zebrafish larvae to the detergent dextran sodium sulfate (DSS) has also been utilized to induce intestinal inflammation. This treatment recapitulates several aspects of TNBS-induced enterocolitis, inducing symptoms such as elevated expression of pro-inflammatory genes and neutrophil recruitment around the intestine [123]. However, the inflammatory phenotype was not identical to that induced by TNBS, and DSS treatment actually protects against TNBS-induced

enterocolitis [123]. The non-steroidal anti-inflammatory drug glafenine has also been used in zebrafish larvae to induce intestinal injury after 12 hours of exposure, which is characterized by profound intestine-specific pathological changes. Glafenine was shown to induce intestinal epithelial cell apoptosis and shedding, which resulted from ER stress and the induction of the unfolded protein response [124]. The inhibition of multidrug resistance (MDR) efflux pumps by glafenine appeared to play an important role in the intestinal epithelial cell shedding. This shedding plays a protective role by restricting inflammation and promoting survival [125].

Just like in humans suffering from IBD and in mouse models of IBD [167], the variable composition of the gut microbiota was demonstrated to be an important determinant of intestinal inflammation in zebrafish. Treatment of adult zebrafish with vancomycin or colistin sulphate differentially affected the components of the intestinal microbiota, which influenced the severity of the oxazolone-induced enterocolitis and the composition of the intestinal leukocyte infiltration [127]. In larvae, treatment with the broad-spectrum antibiotics kanamycin and ampicillin, which resulted in a severe loss of microbiota, decreased mortality after TNBS exposure, and inhibited the induction of pro-inflammatory gene expression and leukocyte migration to the intestine [122]. Using a protocol to generate germ-free zebrafish larvae, it was confirmed that the TNBS-induced pathology, including histological changes and an increased expression of genes encoding pro-inflammatory cytokines, entirely depended on the presence of resident microbiota [168]. TNBS-induced enterocolitis in larvae increased the proportion of *Proteobacteria* (especially *Burkholderia*) and decreased the relative number of Firmicutes (*Lactobacillus* group) in the composition of the larval microbiota, and these changes correlated with the severity of the enterocolitis [169]. Cotreatment with excretory-secretory products from the nematode *Anisakis* showed a suppression on TNBS-induced mortality and pro-inflammatory gene expression in adult zebrafish, suggesting that the exposure to the immunomodulatory effects of parasitic helminths could be protective against IBD [170].

Validation of the larval TNBS-induced enterocolitis model was further performed using known (steroidal and non-steroidal) anti-inflammatory and antibiotic drug treatments which ameliorated the response to TNBS [121, 122]. A small drug screen was performed using this model as well, in which NOS inhibitors and thalidomide and parthenolide were tested. Whereas all compounds showed a reduction of *tnfa* expression, only the NOS inhibitors rescued the *in vivo* disease phenotype, assessed by histological analysis [121]. Similarly, the DSS-induced model was validated by demonstrating the role of retinoic acid (RA) in suppressing the pathological intestinal mucin production [123]. In addition, the DSS- and TNBS-induced larval enterocolitis models have been used for screening small molecules from a large clinical compound library using the neutrophil accumulation in the intestine as a readout [144]. Most of the hits were known antibiotics or anti-inflammatory agents, confirming the validity of

the screening assay. Novel drug hits were also identified using this assay, such as cholecystokinin (CCK) and dopamine receptor agonists, and the involvement of these receptors was confirmed by using CCK and dopamine receptor antagonists, which were shown to exacerbate inflammation in these models [144].

2.3. Mutation-induced inflammation

2.3.1. The *hai1a* mutant

To identify genes with essential functions during zebrafish skin development, a screen of mutants generated by insertional mutagenesis was performed [171], and a mutant line was identified carrying an insertion in the hepatocyte growth factor activator inhibitor 1a gene (*hai1a*, also known as *spint1lb*) [128, 129]. Hai1a is known to be an inhibitor of serine proteases, in particular of Matriptase 1a. The *hai1a* mutant zebrafish larvae display a phenotype reminiscent of the human condition psoriasis: the basal keratinocytes in the epidermis lose their regular polygonal shape and the tight contact to adjacent cells, form aggregates and display enhanced apoptosis. These epidermal defects induce an inflammatory response in the skin, which is illustrated by leukocytes strongly accumulating near aggregates of keratinocytes with apoptotic cells at 1 dpf [128, 129]. The mutant neutrophils display a more random motility, but retain their ability to respond to directional signals [129]. A microarray transcriptome analysis showed that the expression of pro-inflammatory genes was increased in the mutant fish [130]. Among those genes, matrix metalloproteinase 9 gene (*mmp9*) played a critical role. Morpholino knockdown of *mmp9* partially rescued the abnormal epithelial phenotype as well as the neutrophilic infiltration of the epithelium, and restored the organization of collagen fibers.

2.3.2. The *cdipt* mutant

Screening the same collection of insertional mutants, in which the *hai1a* mutant was found [171], for liver defects, a mutant with an insertion in the cdp-diacylglycerolinositol 3-phosphatidyltransferase (*cdipt)* gene was identified [131]. Cdipt, also known as Phosphatidylinositol synthase, has an indispensable role in the synthesis of a critical phospholipid, phosphatidylinositol (PtdIns). The mutant larvae displayed chronic endoplasmic reticulum (ER) stress which contributes to hepatic steatosis around 5 dpf, resembling features of nonalcoholic fatty liver disease in humans [131]. A mild inflammatory response was observed, reflected by the presence of macrophages adjacent to necrotic hepatocytes and increased expression of inflammatory genes. More recently, it was reported that the *cdipt* mutant shows a pathological phenotype in the gastro-intestinal tract reminiscent of IBD [132]. The PtdIns deficiency led to an ER stress-mediated cytopathology in intestinal epithelial cells, including vacuolation, microvillus atrophy and impaired proliferation, subsequently resulting in reduced mucus

secretion, goblet cell apoptosis, autophagy, and bacterial overgrowth. Eventually, this results in an inflammatory response, reflected by the infiltration of macrophages and neutrophils into the intestines. The inflammation could be suppressed by antibiotics and anti-inflammatory drugs, but these treatments failed to suppress the ER stress phenotype. Treatment of mutant larvae with phenylbutyric acid (PBA), a small chemical chaperone and a well-established drug proven to reduce ER stress, was shown to alleviate the mutant phenotype [132].

3. The use of zebrafish inflammation models for research on glucocorticoid drugs

Steroidal anti-inflammatory drugs, also referred to as GCs, have been studied extensively using zebrafish inflammation models. This research has focused on the molecular mechanisms underlying the anti-inflammatory action of these compounds and aims at the development of novel GC drugs. In addition, due to their well-characterized anti-inflammatory effects, GCs are frequently used as a positive control in anti-inflammatory drug screens and the golden standard for anti-inflammatory drugs, and therefore provide a useful method for validation of novel animal models for inflammation.

GCs are a class of steroid hormones secreted by the adrenal gland, regulating a wide variety of systems in the body, like the immune, metabolic, reproductive, cardiovascular and central nervous system [172-174]. In humans, the secretion of the main endogenous GC, cortisol, shows a diurnal pattern, is greatly enhanced upon stress, and is mainly regulated by the hypothalamic-pituitary-adrenal (HPA) axis [175, 176]. The immune-suppressive effects of GCs were first reported by Hench et al. (1949), who demonstrated that adrenocorticotropic hormone (ACTH) and cortisone improved clinical features of rheumatoid arthritis patients [177]. Subsequently, GCs were soon applied in eye inflammation [178, 179], and currently GCs are frequently prescribed worldwide to treat various immune-related diseases, including asthma, rheumatoid arthritis, dermatitis, leukemia, several autoimmune diseases and even some cancers, due to their potent and well-established anti-inflammatory and immune-suppressive effects [180, 181]. These effects of GCs are mediated by an intracellular receptor, the glucocorticoid receptor (GR). GCs activate the translocation of this receptor from the cytoplasm to the nucleus, where it acts as a transcription factor, inducing the expression of anti-inflammatory genes and inhibiting the transcriptional activity of pro-inflammatory genes [180, 182].

Like in humans, the main endogenous GC hormone in fish is cortisol and its secretion is regulated by the hypothalamus-pituitary-interrenal (HPI) axis, the fish equivalent of the HPA axis [183, 184]. Zebrafish, similarly to humans, have a Gr that is encoded by a single *gr* gene [185, 186]. In addition, both zebrafish and humans express an alternative splice variant, Grβ, which is notably absent in mice

[186]. The zebrafish Gris structurally and functionally highly similar to its mammalian equivalent, which includes the immune-suppressive action that is observed upon Gr activation in zebrafish [186, 187].

Upon tail amputation in embryos, treatment with several synthetic GCs has been shown to inhibit the migration of neutrophils towards the wounded site in a Gr-dependent manner. However, GCs leave the migration of macrophages unaffected [65, 71, 72, 79, 88]. The Gr-induced upregulation of the expression of the gene encoding MAPK phosphatase-1 (Mkp-1) was suggested to be involved in the inhibition of neutrophil migration, by inactivation of JNK, resulting in a reduced AP-1-induced transcriptional activation of pro-inflammatory genes [72]. Indeed, studying the transcriptome by microarray analysis showed that almost all wounding-induced changes in transcription were attenuated by GC treatment [71]. Although the chemotactic migration of macrophages is not affected by GCs, their differentiation towards a pro-inflammatory (M1) phenotype is inhibited upon GC treatment [79]. In a combined infection/tail wounding model, GCs were shown to inhibit the infection-induced expression in epidermal and/or epidermal cells of *irg1l*, thereby inhibiting the ROS production which is important for leukocyte migration [65]. In adult zebrafish, no effect of GC treatment on neutrophil recruitment upon tail wounding was detected [188]. In adult zebrafish models for brain and heart injuries, GCs were shown to inhibit the expression of pro-inflammatory genes like *il8*, *tnfa* and *il1b* and reduced the recruitment of leukocytes towards the wounded area [189, 190].

In the LPS-induced inflammation model, GC administration was reported to inhibit the production of ROS and NO, the expression of pro-inflammatory genes, the recruitment of leukocytes, and the mortality [105, 109, 137, 138]. In the Copper-induced inflammation model using $CuSO_4$ immersion of larvae, GCs also caused inhibition of neutrophil accumulation [112]. Similarly, utilizing the DSS-induced enterocolitis model, GCs were observed to inhibit the expression of pro-inflammatory genes and neutrophil infiltration [123]. Interestingly, in larvae from a CRISP/Cas9-generated Gr mutant line, the DSS-induced increase in pro-inflammatory gene expression was abolished due to the deficiency in Gr signaling, suggesting a dual action, both pro- and anti-inflammatory, of GC signaling in the immune system [191].

The clinical use of GCs is severely limited by the severity of their side effects, which include diabetes and obesity, osteoporosis and impaired wound healing. Interestingly, these effects have been modeled in zebrafish as well, opening up the possibility to evaluate both the therapeutic anti-inflammatory effect and the adverse effects. GC effects on metabolism, including increased glucose concentrations, were observed in zebrafish embryos and the global transcriptional changes underlying these effects have been characterized [192]. GC-induced osteoporosis was modeled by treating larvae with GCs between 5 and 10 dpf and performing staining with alizarin red (which binds to calcified matrix) [193],

and studying extracellular matrix (ECM)-, osteoblast-, and osteoclast-related genes [194, 195]. Alternatively, regenerating scales that were removed from GC-treated adult fish have been used to model GC-induced osteoporosis [196]. Finally, inhibitory effects on tissue regeneration and wound healing have been shown in many zebrafish injury models. Inhibition of regeneration by GCs was observed after spinal motor neuron lesions in larvae [197], and in adult zebrafish after tail fin amputation, brain lesion, and cardiac injury, GCs were demonstrated to inhibit tissue regeneration [188-190, 198]. GC treatment of zebrafish embryos blocks the regeneration of the tail fin upon amputation through inhibition on blastemal formation and cell proliferation [88, 199]. Interestingly, the ginsenoside Rg1 was shown to inhibit neutrophil migration in a Gr-dependent manner, but did not show any effect on tissue regeneration. These data suggest that this compound may be provide an interesting lead for the development of novel anti-inflammatory drugs with reduced side effects [99].

4. Concluding remarks

The use of animal models is a critical part of biomedical research and crucial for the development of novel drugs. A wide range of human disease models have been established in mammalian models such as rats and mice, which have largely contributed to the remarkable progress in our understanding of the mechanisms underlying these diseases and the development of novel therapies. However, the rodent systems have limitations such as the high cost of housing and breeding and they are not suited for large-scale automated screening. The development of the zebrafish animal model in the past decades has added a complementary system, which allows the performance of automated high through-put screening *in vivo*, mainly due to the small size and transparency of zebrafish larvae. The similarities of the immune system and inflammatory responses between zebrafish and mammals guarantee good translational value.

In order to model inflammatory diseases, three types of inflammation models have been developed in zebrafish: wounding-, chemical- and mutation-induced inflammation. These models have enabled a detailed investigation of the cellular and molecular mechanisms underlying the inflammatory response, adding to our knowledge of the mechanisms of leukocyte behavior and the identification of potential drug targets. For example, using the zebrafish model, it was observed for the first time that a tissue-scale H_2O_2 gradient is created during the onset of an inflammatory response which signals to leukocytes in the tissues [61], and that Lyn acts as a redox sensor to mediate the migration of leukocyte [63]. In addition, the described models have been used for the screening of compound libraries. This has led to the discovery of important novel targets for anti-inflammatory drugs, such as ErbBs [94]. Moreover, various drug candidates were tested or identified, such as natural extracts (e.g. fucoidan [106], tashinone IIA [96] and cymopol [102]), thalidomide analogs [93], and the PI3K inhibitor LY294002

[33]). In summary, these zebrafish inflammation models have been shown to be very useful to unravel the molecular and cellular aspects of the inflammatory response and for the discovery of novel drug targets. Besides, these models have proven to be effective screening tools for candidate drugs, providing an intermediate between in vitro assays and rodent experiments with great potential to accelerate the preclinical phase of anti-inflammatory drug development.

References

1. Netea, M.G., et al., *A guiding map for inflammation.* Nature immunology, 2017. **18**(8): p. 826.
2. Chen, L., et al., *Inflammatory responses and inflammation-associated diseases in organs.* Oncotarget, 2018. **9**(6): p. 7204.
3. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010. **140**(6): p. 805-820.
4. MacLeod, A.S. and J.N. Mansbridge, *The Innate Immune System in Acute and Chronic Wounds.* Adv Wound Care (New Rochelle), 2016. **5**(2): p. 65-78.
5. Bonecchi, R., et al., *Chemokines and chemokine receptors: an overview.* Frontiers in bioscience (Landmark edition), 2009. **14**: p. 540.
6. Marrack, P., J. Kappler, and B.L. Kotzin, *Autoimmune disease: why and where it occurs.* Nature medicine, 2001. **7**(8): p. 899-905.
7. Ngoc, L.P., et al., *Cytokines, allergy, and asthma.* Current opinion in allergy and clinical immunology, 2005. **5**(2): p. 161-166.
8. Hanauer, S.B., *Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities.* Inflammatory bowel diseases, 2006. **12**(suppl_1): p. S3-S9.
9. Mathis, D. and S.E. Shoelson, *Immunometabolism: an emerging frontier*. 2011, Nature Publishing Group.
10. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-899.
11. DeLegge, M.H. and A. Smoke, *Neurodegeneration and inflammation.* Nutrition in clinical practice, 2008. **23**(1): p. 35-41.
12. Geovanini, G.R. and P. Libby, *Atherosclerosis and inflammation: overview and updates.* Clinical Science, 2018. **132**(12): p. 1243-1252.
13. Merad, M. and J.C. Martin, *Pathological inflammation in patients with COVID-19: a key role for monocytes and macrophages.* Nature Reviews Immunology, 2020: p. 1-8.
14. Kaufmann, S.H. and A. Dorhoi, *Inflammation in tuberculosis: interactions, imbalances and interventions.* Current opinion in immunology, 2013. **25**(4): p. 441-449.
15. Li, P., Y. Zheng, and X. Chen, *Drugs for Autoimmune Inflammatory Diseases: From Small Molecule Compounds to Anti-TNF Biologics.* Frontiers in Pharmacology, 2017. **8**(460).
16. Antman, E.M., et al., *Use of nonsteroidal antiinflammatory drugs: an update for clinicians: a scientific statement from the American Heart Association.* Circulation, 2007. **115**(12): p. 1634-1642.
17. Moghadam-Kia, S. and V.P. Werth, *Prevention and treatment of systemic glucocorticoid side effects.* International journal of dermatology, 2010. **49**(3): p. 239-248.
18. Senolt, L., *Emerging therapies in rheumatoid arthritis: focus on monoclonal antibodies.* F1000Research, 2019. **8**.
19. O'Neill, L.A., *Targeting signal transduction as a strategy to treat inflammatory diseases.* Nature reviews Drug discovery, 2006. **5**(7): p. 549-563.
20. Streisinger, G., et al., *Production of clones of homozygous diploid zebra fish (Brachydanio rerio).* Nature, 1981. **291**(5813): p. 293-296.

21. Engeszer, R.E., et al., *Zebrafish in the wild: a review of natural history and new notes from the field.* Zebrafish, 2007. **4**(1): p. 21-40.
22. Eaton, R.C. and R.D. Farley, *Spawning Cycle and Egg Production of Zebrafish, Brachydanio rerio, in the Laboratory.* Copeia, 1974. **1974**(1): p. 195-204.
23. Avdesh, A., et al., *Regular care and maintenance of a zebrafish (Danio rerio) laboratory: an introduction.* JoVE (Journal of Visualized Experiments), 2012(69): p. e4196.
24. Lieschke, G.J. and P.D. Currie, *Animal models of human disease: zebrafish swim into view.* Nat Rev Genet, 2007. **8**(5): p. 353-67.
25. Barut, B.A. and L.I. Zon, *Realizing the potential of zebrafish as a model for human disease.* Physiological Genomics, 2000. **2**(2): p. 49.
26. Vogel, G., *Sanger Will Sequence Zebrafish Genome.* Science, 2000. **290**(5497): p. 1671.
27. Tavares, B. and S. Santos Lopes, *The Importance of Zebrafish in Biomedical Research.* Acta Medica Portuguesa, 2013. **26**(5): p. 583-592.
28. Patton, E.E. and D.M. Tobin, *Spotlight on zebrafish: the next wave of translational research*. 2019, The Company of Biologists Ltd.
29. Amatruda, J.F., et al., *Zebrafish as a cancer model system.* Cancer Cell, 2002. **1**(3): p. 229-231.
30. Prouty, M.G., et al., *Zebrafish-Mycobacterium marinummodel for mycobacterial pathogenesis.* FEMS Microbiology Letters, 2003. **225**(2): p. 177-182.
31. Brownlie, A., et al., *Positional cloning of the zebrafish sauternes gene: a model for congenital sideroblastic anaemia.* Nat Genet, 1998. **20**(3): p. 244-50.
32. Carvalho, R., et al., *A high-throughput screen for tuberculosis progression.* PloS one, 2011. **6**(2).
33. Bischel, L.L., et al., *Zebrafish Entrapment By Restriction Array (ZEBRA) device: a low-cost, agarose-free zebrafish mounting technique for automated imaging.* Lab Chip, 2013. **13**(9): p. 1732-6.
34. Meijer, A. and H. Spaink, *Host-pathogen interactions made transparent with the zebrafish model.* Current drug targets, 2011. **12**(7): p. 1000-1017.
35. Strecker, T.R., et al., *The effects of the glucocorticoid, dexamethasone, on the development of the Drosophila embryo.* Roux's archives of developmental biology, 1995. **204**(6): p. 359-368.
36. Burns, A.R., et al., *A predictive model for drug bioaccumulation and bioactivity in Caenorhabditis elegans.* Nature chemical biology, 2010. **6**(7): p. 549.
37. Herbomel, P., B. Thisse, and C. Thisse, *Ontogeny and behaviour of early macrophages in the zebrafish embryo.* Development, 1999. **126**(17): p. 3735-3745.
38. Hermann, A.C., et al., *Development of a respiratory burst assay using zebrafish kidneys and embryos.* Journal of immunological methods, 2004. **292**(1-2): p. 119-129.
39. Bennett, C.M., et al., *Myelopoiesis in the zebrafish, Danio rerio.* Blood, The Journal of the American Society of Hematology, 2001. **98**(3): p. 643-651.
40. Lieschke, G.J., et al., *Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish.* Blood, 2001. **98**(10): p. 3087-3096.
41. Langenau, D.M. and L.I. Zon, *The zebrafish: a new model of T-cell and thymic development.* Nature Reviews Immunology, 2005. **5**(4): p. 307-317.
42. Lam, S.H., et al., *Development and maturation of the immune system in zebrafish, Danio rerio: a gene expression profiling, in situ hybridization and immunological study.* Developmental & Comparative Immunology, 2004. **28**(1): p. 9-28.
43. Page, D.M., et al., *An evolutionarily conserved program of B-cell development and activation in zebrafish.* Blood, 2013. **122**(8): p. e1-11.
44. Henry, K.M., et al., *Zebrafish as a model for the study of neutrophil biology.* Journal of leukocyte biology, 2013. **94**(4): p. 633-642.
45. Shelef, M.A., S. Tauzin, and A. Huttenlocher, *Neutrophil migration: moving from zebrafish models to human autoimmunity.* Immunological reviews, 2013. **256**(1): p. 269-281.
46. Renshaw, S.A., et al., *A transgenic zebrafish model of neutrophilic inflammation.* Blood, 2006. **108**(13): p. 3976-8.

47. Mathias, J.R., et al., *Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish.* Journal of leukocyte biology, 2006. **80**(6): p. 1281-1288.
48. Lee, Y., et al., *Fgf signaling instructs position-dependent growth rate during zebrafish fin regeneration.* Development, 2005. **132**(23): p. 5173-5183.
49. Hall, C., et al., *The zebrafish lysozyme C promoter drives myeloid-specific expression in transgenic fish.* BMC developmental biology, 2007. **7**(1): p. 42.
50. Bernut, A., et al., *Mycobacterium abscessus cording prevents phagocytosis and promotes abscess formation.* Proc Natl Acad Sci U S A, 2014. **111**(10): p. E943-52.
51. Ellett, F., et al., *mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish.* Blood, The Journal of the American Society of Hematology, 2011. **117**(4): p. e49-e56.
52. Walton, E.M., et al., *The macrophage-specific promoter mfap4 allows live, long-term analysis of macrophage behavior during mycobacterial infection in zebrafish.* PloS one, 2015. **10**(10): p. e0138949.
53. Li, L., et al., *Live imaging reveals differing roles of macrophages and neutrophils during zebrafish tail fin regeneration.* Journal of Biological Chemistry, 2012. **287**(30): p. 25353-25360.
54. Feng, Y., et al., *Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation.* PLoS biology, 2010. **8**(12).
55. Redd, M.J., et al., *Imaging macrophage chemotaxis in vivo: studies of microtubule function in zebrafish wound inflammation.* Cell motility and the cytoskeleton, 2006. **63**(7): p. 415-422.
56. Otten, C. and S. Abdelilah-Seyfried, *Laser-inflicted injury of zebrafish embryonic skeletal muscle.* JoVE (Journal of Visualized Experiments), 2013(71): p. e4351.
57. Mathias, J.R., et al., *Characterization of zebrafish larval inflammatory macrophages.* Developmental & Comparative Immunology, 2009. **33**(11): p. 1212-1217.
58. Yoo, S.K., et al., *Differential regulation of protrusion and polarity by PI (3) K during neutrophil motility in live zebrafish.* Developmental cell, 2010. **18**(2): p. 226-236.
59. Miskolci, V., et al., *Distinct inflammatory and wound healing responses to complex caudal fin injuries of larval zebrafish.* Elife, 2019. **8**.
60. LeBert, D., et al., *Damage-induced reactive oxygen species regulate vimentin and dynamic collagen-based projections to mediate wound repair.* Elife, 2018. **7**: p. e30703.
61. Niethammer, P., et al., *A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish.* Nature, 2009. **459**: p. 996.
62. Enyedi, B. and P. Niethammer, *H2O2: a chemoattractant?*, in *Methods in enzymology*. 2013, Elsevier. p. 237-255.
63. Yoo, S.K., et al., *Lyn is a redox sensor that mediates leukocyte wound attraction in vivo.* Nature, 2011. **480**: p. 109.
64. Hall, C.J., et al., *Immunoresponsive gene 1 augments bactericidal activity of macrophage-lineage cells by regulating β-oxidation-dependent mitochondrial ROS production.* Cell Metab, 2013. **18**(2): p. 265-78.
65. Hall, C.J., et al., *Epidermal cells help coordinate leukocyte migration during inflammation through fatty acid-fuelled matrix metalloproteinase production.* Nat Commun, 2014. **5**: p. 3880.
66. Yoo, S.K., et al., *The role of microtubules in neutrophil polarity and migration in live zebrafish.* Journal of cell science, 2012. **125**(23): p. 5702-5710.
67. Lam, P.-y., et al., *The SH2-domain-containing inositol 5-phosphatase (SHIP) limits the motility of neutrophils and their recruitment to wounds in zebrafish.* Journal of cell science, 2012. **125**(21): p. 4973-4978.
68. Cvejic, A., et al., *Analysis of WASp function during the wound inflammatory response–live-imaging studies in zebrafish larvae.* Journal of cell science, 2008. **121**(19): p. 3196-3206.
69. Nguyen-Chi, M., et al., *Identification of polarized macrophage subsets in zebrafish.* Elife, 2015. **4**: p. e07288.
70. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nat Rev Immunol, 2008. **8**(12): p. 958-69.

71. Chatzopoulou, A., et al., *Glucocorticoid-Induced Attenuation of the Inflammatory Response in Zebrafish.* Endocrinology, 2016. **157**(7): p. 2772-84.
72. Zhang, Y., et al., *In Vivo Interstitial Migration of Primitive Macrophages Mediated by JNK-Matrix Metalloproteinase 13 Signaling in Response to Acute Injury.* The Journal of Immunology, 2008. **181**(3): p. 2155-2164.
73. Liu, Y.-J., et al., *Cannabinoid receptor 2 suppresses leukocyte inflammatory migration by modulating the JNK/c-Jun/Alox5 pathway.* Journal of Biological Chemistry, 2013. **288**(19): p. 13551-13562.
74. Ren, D.-l., X.-b. Wang, and B. Hu, *Circadian gene period1b regulates proinflammatory cytokine expression through NF–κB signalling in zebrafish.* Fish & shellfish immunology, 2018. **80**: p. 528-533.
75. Baggiolini, M. and I. Clark-Lewis, *Interleukin‐8, a chemotactic and inflammatory cytokine.* FEBS letters, 1992. **307**(1): p. 97-101.
76. Huber, A.R., et al., *Regulation of transendothelial neutrophil migration by endogenous interleukin-8.* Science, 1991. **254**(5028): p. 99-102.
77. de Oliveira, S., et al., *Cxcl8 (IL-8) Mediates Neutrophil Recruitment and Behavior in the Zebrafish Inflammatory Response.* The Journal of Immunology, 2013.
78. Sarris, M., et al., *Inflammatory chemokines direct and restrict leukocyte migration within live tissues as glycan-bound gradients.* Current Biology, 2012. **22**(24): p. 2375-2382.
79. Xie, Y., et al., *Glucocorticoids inhibit macrophage differentiation towards a pro-inflammatory phenotype upon wounding without affecting their migration.* Dis Model Mech, 2019. **12**(5).
80. Ogryzko, N.V., et al., *Zebrafish tissue injury causes upregulation of interleukin-1 and caspase-dependent amplification of the inflammatory response.* Dis Model Mech, 2014. **7**(2): p. 259-64.
81. Yan, B., et al., *Il-1β and Reactive Oxygen Species Differentially Regulate Neutrophil Directional Migration and Basal Random Motility in a Zebrafish Injury–Induced Inflammation Model.* The Journal of Immunology, 2014. **192**(12): p. 5998-6008.
82. Petrie, T.A., et al., *Macrophages modulate adult zebrafish tail fin regeneration.* Development, 2014. **141**(13): p. 2581-2591.
83. de Oliveira, S., E.E. Rosowski, and A. Huttenlocher, *Neutrophil migration in infection and wound repair: going forward in reverse.* Nature Reviews Immunology, 2016. **16**(6): p. 378.
84. Burgon, J., et al., *Serum and glucocorticoid–regulated kinase 1 regulates neutrophil clearance during inflammation resolution.* The Journal of Immunology, 2014. **192**(4): p. 1796-1805.
85. Elks, P.M., et al., *Activation of hypoxia-inducible factor-1α (Hif-1α) delays inflammation resolution by reducing neutrophil apoptosis and reverse migration in a zebrafish inflammation model.* Blood, 2011. **118**(3): p. 712-722.
86. Ellett, F., et al., *Defining the phenotype of neutrophils following reverse migration in zebrafish.* Journal of leukocyte biology, 2015. **98**(6): p. 975-981.
87. Isles, H.M., et al., *The CXCL12/CXCR4 signaling Axis retains neutrophils at inflammatory sites in zebrafish.* Frontiers in immunology, 2019. **10**: p. 1784.
88. Mathew, L.K., et al., *Unraveling tissue regeneration pathways using chemical genetics.* J Biol Chem, 2007. **282**(48): p. 35202-10.
89. Kawakami, A., T. Fukazawa, and H. Takeda, *Early fin primordia of zebrafish larvae regenerate by a similar growth control mechanism with adult regeneration.* Developmental dynamics: an official publication of the American Association of Anatomists, 2004. **231**(4): p. 693-699.
90. Morales, R.A. and M.L. Allende, *Peripheral macrophages promote tissue regeneration in zebrafish by fine-tuning the inflammatory response.* Frontiers in immunology, 2019. **10**: p. 253.
91. Ashburn, T.T. and K.B. Thor, *Drug repositioning: identifying and developing new uses for existing drugs.* Nature reviews Drug discovery, 2004. **3**(8): p. 673-683.
92. Hall, C.J., et al., *Repositioning drugs for inflammatory disease–fishing for new anti-inflammatory agents.* Disease models & mechanisms, 2014. **7**(9): p. 1069-1081.

93. Beedie, S.L., et al., *In vivo screening and discovery of novel candidate thalidomide analogs in the zebrafish embryo and chicken embryo model systems.* Oncotarget, 2016. **7**(22): p. 33237.
94. Rahman, A., et al., *Inhibition of ErbB kinase signalling promotes resolution of neutrophilic inflammation.* Elife, 2019. **8**: p. e50990.
95. Ye, B., et al., *Meisoindigo, but not its core chemical structure indirubin, inhibits zebrafish interstitial leukocyte chemotactic migration.* Pharm Biol, 2017. **55**(1): p. 673-679.
96. Robertson, A.L., et al., *A zebrafish compound screen reveals modulation of neutrophil reverse migration as an anti-inflammatory mechanism.* Sci Transl Med, 2014. **6**(225): p. 225ra29.
97. Robertson, A.L., et al., *Identification of benzopyrone as a common structural feature in compounds with anti-inflammatory activity in a zebrafish phenotypic screen.* Disease models & mechanisms, 2016. **9**(6): p. 621-632.
98. Sun, M., et al., *Characterization of ginsenoside extracts by delayed luminescence, high-performance liquid chromatography, and bioactivity tests.* Photochemical & Photobiological Sciences, 2019. **18**(5): p. 1138-1146.
99. He, M., et al., *Ginsenoside Rg1 Acts as a Selective Glucocorticoid Receptor Agonist with Anti-Inflammatory Action without Affecting Tissue Regeneration in Zebrafish Larvae.* Cells, 2020. **9**(5).
100. Polednik, K.M., A.C. Koch, and L.K. Felzien, *Effects of essential oil from Thymus vulgaris on viability and inflammation in zebrafish embryos.* Zebrafish, 2018. **15**(4): p. 361-371.
101. Yang, Y., et al., *Anti-inflammatory and proresolution activities of bergapten isolated from the roots of Ficus hirta in an in vivo zebrafish model.* Biochemical and biophysical research communications, 2018. **496**(2): p. 763-769.
102. Bousquet, M.S., et al., *Seaweed natural products modify the host inflammatory response via Nrf2 signaling and alter colon microbiota composition and gene expression.* Free Radical Biology and Medicine, 2020. **146**: p. 306-323.
103. Tang, H., et al., *Micrometam C protects against oxidative stress in inflammation models in zebrafish and RAW264. 7 macrophages.* Marine drugs, 2015. **13**(9): p. 5593-5605.
104. Nguyen, T.H., et al., *Anti–Inflammatory and Antioxidant Properties of the Ethanol Extract of Clerodendrum Cyrtophyllum Turcz in Copper Sulfate-Induced Inflammation in Zebrafish.* Antioxidants, 2020. **9**(3): p. 192.
105. Wijesinghe, W., et al., *Assessment of anti-inflammatory effect of 5β-hydroxypalisadin B isolated from red seaweed Laurencia snackeyi in zebrafish embryo in vivo model.* Environmental Toxicology and Pharmacology, 2014. **37**(1): p. 110-117.
106. Lee, S.-H., et al., *Anti-inflammatory effect of fucoidan extracted from Ecklonia cava in zebrafish model.* Carbohydrate polymers, 2013. **92**(1): p. 84-89.
107. Watzke, J., K. Schirmer, and S. Scholz, *Bacterial lipopolysaccharides induce genes involved in the innate immune response in embryos of the zebrafish (Danio rerio).* Fish & shellfish immunology, 2007. **23**(4): p. 901-905.
108. Ko, E.-Y., et al., *The roles of NF-κB and ROS in regulation of pro-inflammatory mediators of inflammation induction in LPS-stimulated zebrafish embryos.* Fish & shellfish immunology, 2017. **68**: p. 525-529.
109. Yang, L.-L., et al., *Endotoxin molecule lipopolysaccharide-induced zebrafish inflammation model: a novel screening method for anti-inflammatory drugs.* Molecules, 2014. **19**(2): p. 2390-2409.
110. Hernández, P.P., et al., *Sub-lethal concentrations of waterborne copper are toxic to lateral line neuromasts in zebrafish (Danio rerio).* Hearing research, 2006. **213**(1-2): p. 1-10.
111. Olivari, F.A., P.P. Hernández, and M.L. Allende, *Acute copper exposure induces oxidative stress and cell death in lateral line hair cells of zebrafish larvae.* Brain research, 2008. **1244**: p. 1-12.
112. d'Alençon, C.A., et al., *A high-throughput chemically induced inflammation assay in zebrafish.* BMC biology, 2010. **8**(1): p. 151.
113. Leite, C.E., et al., *Involvement of purinergic system in inflammation and toxicity induced by copper in zebrafish larvae.* Toxicology and applied pharmacology, 2013. **272**(3): p. 681-689.

114. Carrillo, S.A., et al., *Macrophage recruitment contributes to regeneration of mechanosensory hair cells in the zebrafish lateral line.* Journal of cellular biochemistry, 2016. **117**(8): p. 1880-1889.
115. Craig, P.M., C.M. Wood, and G.B. McClelland, *Oxidative stress response and gene expression with acute copper exposure in zebrafish (Danio rerio).* American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 2007. **293**(5): p. R1882-R1892.
116. Griffitt, R.J., et al., *Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (Danio rerio).* Environmental science & technology, 2007. **41**(23): p. 8178-8186.
117. Singh, S.K., et al., *Proteome dynamics in neutrophils of adult zebrafish upon chemically-induced inflammation.* Fish & shellfish immunology, 2014. **40**(1): p. 217-224.
118. Deng, Q., et al., *Localized bacterial infection induces systemic activation of neutrophils through Cxcr2 signaling in zebrafish.* J Leukoc Biol, 2013. **93**(5): p. 761-9.
119. Tobin, D.M., et al., *The lta4h locus modulates susceptibility to mycobacterial infection in zebrafish and humans.* Cell, 2010. **140**(5): p. 717-730.
120. Torraca, V., et al., *The CXCR3-CXCL11 signaling axis mediates macrophage recruitment and dissemination of mycobacterial infection.* Dis Model Mech, 2015. **8**(3): p. 253-69.
121. Fleming, A., J. Jankowski, and P. Goldsmith, *In vivo analysis of gut function and disease changes in a zebrafish larvae model of inflammatory bowel disease: a feasibility study.* Inflammatory bowel diseases, 2010. **16**(7): p. 1162-1172.
122. Oehlers, S.H., et al., *A chemical enterocolitis model in zebrafish larvae that is dependent on microbiota and responsive to pharmacological agents.* Developmental Dynamics, 2011. **240**(1): p. 288-298.
123. Oehlers, S.H., et al., *Retinoic acid suppresses intestinal mucus production and exacerbates experimental enterocolitis.* Disease models & mechanisms, 2012. **5**(4): p. 457-467.
124. Goldsmith, J.R., et al., *Glafenine-induced intestinal injury in zebrafish is ameliorated by μ-opioid signaling via enhancement of Atf6-dependent cellular stress responses.* Disease models & mechanisms, 2013. **6**(1): p. 146-159.
125. Espenschied, S.T., et al., *Epithelial delamination is protective during pharmaceutical-induced enteropathy.* Proceedings of the National Academy of Sciences, 2019. **116**(34): p. 16961-16970.
126. Geiger, B.M., et al., *Intestinal upregulation of melanin-concentrating hormone in TNBS-induced enterocolitis in adult zebrafish.* PloS one, 2013. **8**(12).
127. Brugman, S., et al., *Oxazolone-induced enterocolitis in zebrafish depends on the composition of the intestinal microbiota.* Gastroenterology, 2009. **137**(5): p. 1757-1767. e1.
128. Carney, T.J., et al., *Inactivation of serine protease Matriptase1a by its inhibitor Hai1 is required for epithelial integrity of the zebrafish epidermis.* Development, 2007. **134**(19): p. 3461-3471.
129. Mathias, J.R., et al., *Live imaging of chronic inflammation caused by mutation of zebrafish Hai1.* Journal of Cell Science, 2007. **120**(19): p. 3372-3383.
130. LeBert, D.C., et al., *Matrix metalloproteinase 9 modulates collagen matrices and wound repair.* Development, 2015. **142**(12): p. 2136-2146.
131. Thakur, P.C., et al., *Lack of de novo phosphatidylinositol synthesis leads to endoplasmic reticulum stress and hepatic steatosis in cdipt‐deficient zebrafish.* Hepatology, 2011. **54**(2): p. 452-462.
132. Thakur, P.C., et al., *Dysregulated phosphatidylinositol signaling promotes endoplasmic-reticulum-stress-mediated intestinal mucosal injury and inflammation in zebrafish.* Disease models & mechanisms, 2014. **7**(1): p. 93-106.
133. Zhang, C., et al., *In vitro and in vivo anti-inflammatory effects of polyphyllin VII through downregulating MAPK and NF-κB pathways.* Molecules, 2019. **24**(5): p. 875.
134. Hwang, J.-H., et al., *Caffeine prevents LPS-induced inflammatory responses in RAW264. 7 cells and zebrafish.* Chemico-biological interactions, 2016. **248**: p. 1-7.
135. Ryu, S.-J., et al., *Oleuropein suppresses LPS-induced inflammatory responses in RAW 264.7 cell and zebrafish.* Journal of agricultural and food chemistry, 2015. **63**(7): p. 2098-2105.

136. Kim, S.-Y., et al., *Polyphenol-rich fraction from Ecklonia cava (a brown alga) processing by-product reduces LPS-induced inflammation in vitro and in vivo in a zebrafish model.* Algae, 2014. **29**(2): p. 165.
137. Yang, L., et al., *Protective effect of phillyrin on lethal LPS-induced neutrophil inflammation in zebrafish.* Cellular Physiology and Biochemistry, 2017. **43**(5): p. 2074-2087.
138. Sun, Q., et al., *Anti-inflammatory properties of extracts from Chimonanthus nitens Oliv. leaf.* PloS one, 2017. **12**(7).
139. Wang, Z.-g., et al., *Anti-Inflammatory Activity of a Peptide from Skipjack (Katsuwonus pelamis).* Marine drugs, 2019. **17**(10): p. 582.
140. Guo, D.-L., et al., *Terpene Glycosides from Sanguisorba officinalis and Their Anti-Inflammatory Effects.* Molecules, 2019. **24**(16): p. 2906.
141. Kasica-Jarosz, N., P. Podlasz, and J. Kaleczyc, *Pituitary adenylate cyclase–activating polypeptide (PACAP-38) plays an inhibitory role against inflammation induced by chemical damage to zebrafish hair cells.* PloS one, 2018. **13**(6).
142. Li, L., et al., *Role of Quzhou Fructus Aurantii extract in preventing and treating acute lung injury and inflammation.* Scientific Reports, 2018. **8**(1): p. 1-11.
143. Wittmann, C., et al., *A zebrafish drug-repurposing screen reveals sGC-dependent and sGC-independent pro-inflammatory activities of nitric oxide.* PloS one, 2015. **10**(10).
144. Oehlers, S.H., et al., *A whole animal chemical screen approach to identify modifiers of intestinal neutrophilic inflammation.* Febs j, 2017. **284**(3): p. 402-413.
145. Beutler, B. and E.T. Rietschel, *Innate immune sensing and its roots: the story of endotoxin.* Nature Reviews Immunology, 2003. **3**(2): p. 169-176.
146. Novoa, B., et al., *LPS response and tolerance in the zebrafish (Danio rerio).* Fish & shellfish immunology, 2009. **26**(2): p. 326-331.
147. Chow, J.C., et al., *Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction.* Journal of Biological Chemistry, 1999. **274**(16): p. 10689-10692.
148. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nature reviews immunology, 2004. **4**(7): p. 499-511.
149. Sullivan, C., et al., *The gene history of zebrafish tlr4a and tlr4b is predictive of their divergent functions.* The Journal of Immunology, 2009. **183**(9): p. 5896-5908.
150. Sepulcre, M.P., et al., *Evolution of lipopolysaccharide (LPS) recognition and signaling: fish TLR4 does not recognize LPS and negatively regulates NF-κB activation.* The Journal of Immunology, 2009. **182**(4): p. 1836-1845.
151. Iliev, D.B., et al., *Endotoxin recognition: in fish or not in fish?* FEBS letters, 2005. **579**(29): p. 6519-6528.
152. Forn-Cuní, G., et al., *Conserved gene regulation during acute inflammation between zebrafish and mammals.* Scientific reports, 2017. **7**: p. 41905.
153. Copeland, S., et al., *Acute inflammatory response to endotoxin in mice and humans.* Clin. Diagn. Lab. Immunol., 2005. **12**(1): p. 60-67.
154. Dios, S., et al., *The involvement of cholesterol in sepsis and tolerance to lipopolysaccharide highlighted by the transcriptome analysis of zebrafish (Danio rerio).* Zebrafish, 2014. **11**(5): p. 421-433.
155. McDonald, M.C., et al., *Reconstituted high-density lipoprotein attenuates organ injury and adhesion molecule expression in a rodent model of endotoxic shock.* Shock, 2003. **20**(6): p. 551-7.
156. Park, K.-H. and K.-H. Cho, *A zebrafish model for the rapid evaluation of pro-oxidative and inflammatory death by lipopolysaccharide, oxidized low-density lipoproteins, and glycated high-density lipoproteins.* Fish & shellfish immunology, 2011. **31**(6): p. 904-910.
157. Cordero-Maldonado, M.L., et al., *Optimization and pharmacological validation of a leukocyte migration assay in zebrafish larvae for the rapid in vivo bioactivity analysis of anti-inflammatory secondary metabolites.* PloS one, 2013. **8**(10).

158. Bohni, N., et al., *Integration of microfractionation, qNMR and zebrafish screening for the in vivo bioassay-guided isolation and quantitative bioactivity analysis of natural products.* PLoS One, 2013. **8**(5).
159. Linder, M.C. and M. Hazegh-Azam, *Copper biochemistry and molecular biology.* The American journal of clinical nutrition, 1996. **63**(5): p. 797S-811S.
160. Pereira, T.C.B., M.M. Campos, and M.R. Bogo, *Copper toxicology, oxidative stress and inflammation using zebrafish as experimental model.* Journal of Applied Toxicology, 2016. **36**(7): p. 876-885.
161. Dave, G. and R. Xiu, *Toxicity of mercury, copper, nickel, lead, and cobalt to embryos and larvae of zebrafish, Brachydanio rerio.* Archives of Environmental Contamination and Toxicology, 1991. **21**(1): p. 126-134.
162. Johnson, A., E. Carew, and K. Sloman, *The effects of copper on the morphological and functional development of zebrafish embryos.* Aquatic Toxicology, 2007. **84**(4): p. 431-438.
163. Wittmann, C., et al., *Facilitating drug discovery: an automated high-content inflammation assay in zebrafish.* JoVE (Journal of Visualized Experiments), 2012(65): p. e4203.
164. Yokomizo, T., T. Izumi, and T. Shimizu, *Leukotriene B4: metabolism and signal transduction.* Archives of biochemistry and biophysics, 2001. **385**(2): p. 231-241.
165. Peters-Golden, M., et al., *Leukotrienes: underappreciated mediators of innate immune responses.* The Journal of Immunology, 2005. **174**(2): p. 589-594.
166. Lee, J.A. and S.A. Renshaw, *Zebrafish screens for new colitis treatments – a bottom‐up approach.* The FEBS Journal, 2017. **284**(3): p. 399-401.
167. Packey, C. and R. Sartor, *Interplay of commensal and pathogenic bacteria, genetic mutations, and immunoregulatory defects in the pathogenesis of inflammatory bowel diseases.* Journal of internal medicine, 2008. **263**(6): p. 597-606.
168. He, Q., et al., *Role of gut microbiota in a zebrafish model with chemically induced enterocolitis involving toll-like receptor signaling pathways.* Zebrafish, 2014. **11**(3): p. 255-264.
169. He, Q., et al., *Microbial fingerprinting detects intestinal microbiota dysbiosis in Zebrafish models with chemically-induced enterocolitis.* BMC microbiology, 2013. **13**(1): p. 289.
170. Haarder, S., et al., *Effect of ES products from Anisakis (Nematoda: Anisakidae) on experimentally induced colitis in adult zebrafish.* Parasite Immunology, 2017. **39**(10): p. e12456.
171. Amsterdam, A., et al., *A large-scale insertional mutagenesis screen in zebrafish.* Genes & development, 1999. **13**(20): p. 2713-2724.
172. Oakley, R.H. and J.A. Cidlowski, *The biology of the glucocorticoid receptor: New signaling mechanisms in health and disease.* Journal of Allergy and Clinical Immunology, 2013. **132**(5): p. 1033-1044.
173. Chrousos, G.P. and T. Kino, *Intracellular Glucocorticoid Signaling: A Formerly Simple System Turns Stochastic.* Science's STKE, 2005. **2005**(304): p. pe48-pe48.
174. Revollo, J.R. and J.A. Cidlowski, *Mechanisms generating diversity in glucocorticoid receptor signaling.* Ann N Y Acad Sci, 2009. **1179**: p. 167-78.
175. Wei, Y., et al., *Regulation of hypothalamic-pituitary-adrenal axis activity and immunologic function contributed to the anti-inflammatory effect of acupuncture in the OVA-induced murine asthma model.* Neuroscience Letters.
176. Tsigos, C. and G.P. Chrousos, *Hypothalamic–pituitary–adrenal axis, neuroendocrine factors and stress.* Journal of Psychosomatic Research, 2002. **53**(4): p. 865-871.
177. Hench, P.S., et al., *The effect of a hormone of the adrenal cortex (17-hydroxy-11-dehydrocorticosterone: compound E) and of pituitary adrenocortical hormone in arthritis: preliminary report.* Ann Rheum Dis, 1949. **8**(2): p. 97-104.
178. Duke-Elder, S. and N. Ashton, *Action of cortisone on tissue reactions of inflammation and repair with special reference to the eye.* Br J Ophthalmol, 1951. **35**(11): p. 695-707.
179. O'Rourke, J.F., G. Iser, and R.W. Ryan, *An initial evaluation of prednisone therapy in ocular inflammation.* AMA Arch Ophthalmol, 1956. **55**(3): p. 323-32.

180. Busillo, J.M. and J.A. Cidlowski, *The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore.* Trends in Endocrinology & Metabolism, 2013. **24**(3): p. 109-119.
181. Barnes, P.J., *Glucocorticosteroids: current and future directions.* British Journal of Pharmacology, 2011. **163**(1): p. 29-43.
182. Baschant, U. and J. Tuckermann, *The role of the glucocorticoid receptor in inflammation and immunity.* J Steroid Biochem Mol Biol, 2010. **120**(2-3): p. 69-75.
183. Schreck, C.B., et al., *Biology of stress in fish*. 2016: Academic Press.
184. Wendelaar Bonga, S.E., *The stress response in fish.* Physiological reviews, 1997. **77**(3): p. 591-625.
185. Alsop, D. and M.M. Vijayan, *Development of the corticosteroid stress axis and receptor expression in zebrafish.* American Journal of Physiology - Regulatory, Integrative and Comparative Physiology, 2008. **294**(3): p. R711-R719.
186. Schaaf, M.J., et al., *Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish.* Endocrinology, 2008. **149**(4): p. 1591-9.
187. Schaaf, M.J., A. Chatzopoulou, and H.P. Spaink, *The zebrafish as a model system for glucocorticoid receptor research.* Comp Biochem Physiol A Mol Integr Physiol, 2009. **153**(1): p. 75-82.
188. Geurtzen, K., et al., *Immune suppressive and bone inhibitory effects of prednisolone in growing and regenerating zebrafish tissues.* Journal of Bone and Mineral Research, 2017. **32**(12): p. 2476-2488.
189. Huang, W.C., et al., *Treatment of Glucocorticoids Inhibited Early Immune Responses and Impaired Cardiac Repair in Adult Zebrafish.* PLoS One, 2013. **8**(6): p. e66613.
190. Kyritsis, N., et al., *Acute inflammation initiates the regenerative response in the adult zebrafish brain.* Science, 2012. **338**(6112): p. 1353-1356.
191. Facchinello, N., et al., *nr3c1 null mutant zebrafish are viable and reveal DNA-binding-independent activities of the glucocorticoid receptor.* Scientific reports, 2017. **7**(1): p. 1-13.
192. Chatzopoulou, A., et al., *Transcriptional and metabolic effects of glucocorticoid receptor alpha and beta signaling in zebrafish.* Endocrinology, 2015. **156**(5): p. 1757-69.
193. Barrett, R., et al., *A rapid, high content, in vivo model of glucocorticoid‐induced osteoporosis.* Biotechnology Journal: Healthcare Nutrition Technology, 2006. **1**(6): p. 651-655.
194. He, H., et al., *Possible mechanisms of prednisolone-induced osteoporosis in zebrafish larva.* Biomedicine & Pharmacotherapy, 2018. **101**: p. 981-987.
195. Huo, L., et al., *Prednisolone induces osteoporosis-like phenotypes via focal adhesion signaling pathway in zebrafish larvae.* Biology open, 2018. **7**(7): p. bio029405.
196. De Vrieze, E., et al., *Prednisolone induces osteoporosis-like phenotype in regenerating zebrafish scales.* Osteoporosis International, 2014. **25**(2): p. 567-578.
197. Ohnmacht, J., et al., *Spinal motor neurons are regenerated after mechanical lesion and genetic ablation in larval zebrafish.* Development, 2016. **143**(9): p. 1464-1474.
198. Geurtzen, K. and F. Knopf, *Adult zebrafish injury models to study the effects of prednisolone in regenerating bone tissue.* JoVE (Journal of Visualized Experiments), 2018(140): p. e58429.
199. Sengupta, S., et al., *Alternate glucocorticoid receptor ligand binding structures influence outcomes in an in vivo tissue regeneration model.* Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 2012. **156**(2): p. 121-129.