Danio rerio: A Past, Present, and Future Hallmark for Immunological Studies

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Abstract

An emerging species that is slowly gaining reputation as a common model organism is *Danio rerio*. Due to its similarities to our own immune response and its general ease of use, hundreds of studies have been conducted which have increased our understanding of how chemicals or pathogens can affect both our own health, and the health of the environment.

This study aims to briefly describe a brief history of *D. rerio*, as well as discuss key experiments that have been performed using *D. rerio*. A comparison will also be made between the human and zebrafish immune responses, and discuss how future experiments can exploit this similarity to benefit future studies.

***Danio rerio*: A Past, Present, and Future Hallmark for Immunological Studies**

Model organisms are important for the overall benefit of humankind through immunological studies. A model organism can be defined as widely studied and having a genetic makeup that is relatively well-documented and well-understood by scientists (Alleyne, 2013). Model organisms can provide insight into human diseases and the subsequent treatment of said diseases through careful testing and observation. For this reason, they are frequently used to study the effects of pathogens on the immune response. More importantly, what researchers have learned from these creatures has had huge impacts on the field of medicine; more specifically, they demonstrate how we can understand the relationship between immunity and the disease state of the body. Their importance to the study of immunology, and nearly anything modern scientists know about modern biology and medicine as a whole, is due to the research that has been done on these types of organisms (Fields & Johnston, 2005). Mice serve as good model organisms, and thus have been used extensively to test novel therapeutic strategies and drug screenings; these have and could eventually lead to expanded humanized models (Schinnerling et al., 2019).

While classic vertebrate model organisms must remain incredibly important to the study of immunology, including classics such as rats, mice, or guinea pigs, there exists a newer model. What makes them useful to immunologists is their relative ease of use when compared to more traditional model organisms, while still retaining the immunological similarities with humans which is essential. *Danio rerio*, more commonly known to the layman as the zebrafish, is a still-emerging model organism that will most likely continue to see extensive use years into the future, and has an enormous potential that is still waiting to be tapped. *D. rerio* is a small, shoaling cyprinid species of fish that is native to southern Asia, with some artificially planted

populations springing up in both North and South America (Markowski, 2011; Spence et al., 2007). Their development cycle is characterized by rapid growth; most zebrafish are fully grown and differentiate into their respective genders less than four months after hatching (Spence et al., 2007). When fully grown, the genders are distinguishable from each other; males tend to be slimmer and golden in color, while females tend to be wider (due to the retention of eggs) and bluish in color.

Figure 1. *The zebrafish* (*Danio rerio). Credit: Live Science*

The first recorded mention of zebrafish possessing traits characteristic of a model organism comes from Dr. CW Creaser in 1934, where he described them as easy to raise and breed (Creaser, 1934). Along with the American Society of Zoologists, he was one of the first to pioneer controlled breeding and caring for zebrafish in a laboratory environment. Alas, he was also one of the first scientific pioneers to recognize that *D. rerio's* contributions in research programs could integrate valuable and novel perspectives into experimental and student

laboratories (Creaser, 1934). His foresight regarding both the zebrafish's ease of use and its potential to be utilized in future research over 80 years ago holds true to this day.

Zebrafish have proven themselves a cost-effective and oftentimes faster solution as testers for experimental drugs and other chemicals, giving them an advantage over traditional rodent model organisms. The zebrafish retains these advantages over the latter species, while also maintaining factors such as genetic similarity that make rodents the great model organisms that they are. For example, zebrafish, like humans, have a fully developed immune response. *Danio rerio* shares many similarities with humans in regards to the innate side of immunity, which extends to cells such as macrophages, neutrophils, eosinophils, and dendritic cells (Goody et al., 2014; Nik et al., 2017). Innate immunity serves as the first line of defense, and is necessary for the activation of the adaptive response through effector molecules called cytokines and chemokines (Nayak et al., 2007; Vasta et al., 2004). Antibodies, an integral part of adaptive immunity, can undergo class switching to better adapt to pathogenic response (Trede et al., 2004). Zebrafish also contain secondary lymphoid tissues (including lymph nodes, the spleen, Peyer's patches, etc.) which provide a bridge between the innate and adaptive branches of the immune system by serving as the primary location for antigen presentation and activation (Trede et al., 2004). Specifically, naïve B and T lymphocytes of the adaptive response mature in these secondary lymphoid tissues, where they can await their activation (Page et al., 2013; Langenau & Zon, 2005). *Danio rerio*'s fully developed adaptive side of immunity also allows them to participate in memory response, which is essential to mounting more efficient defenses against secondary infections by a pathogen (Zhang & Cui, 2014).

As the use of *Danio rerio* in microbiological and genetic applications continues to increase, the previously mentioned similarities across both the innate and adaptive portions of the

immune response have already yielded positive results in immunological research. For example, multiple studies have been carried out on embryonic zebrafish hematopoietic stem cells (HSCs) within the aorta-gonad-mesonephros (AGM) regions in order to better understand hematopoiesis (Kissa et al., 2008; Burns et al., 2009). North et al. (2007) discovered that chemicals which induced the expression of prostaglandin (PGE2) could cause an elevation of HSC numbers downstream of embryonic development. This study led to future discoveries involving prostaglandin's connection to umbilical cord HSC transplantation, and also a downstream potential treatment option for type I diabetes through hematopoietic stem and progenitor cell (HSPC) infusion (Cutler et al., 2013; Nasr et al., 2018). Furthermore, North et al.'s (2007) research coincided with contemporary findings at the time, which was focused on discovering prostaglandin's role in immune development. Prostaglandin was also discovered to play a role in *RAG-1* expression and subsequent T-lymphocyte maturation by signaling through the PGE2-EP4 mechanism, a pathway which is also conserved in humans (Villablanca et al., 2007; Yokoyama et al., 2013). HSCs are the primary method of blood cell synthesis of the body in vertebrates, including humans, mice, and zebrafish (Gunsilius et al., 2001). In both humans and zebrafish, HSCs undergo differentiation in the AGM region during development; similarly, there is also a common myeloid and lymphoid lineage, through which both organisms can develop the different types of leukocytes that are commonly present after development (Iwasaki & Akashi, 2007). The common lymphoid progenitors in particular include all adaptive-sided cells such as T and B lymphocytes, along with natural killer (NK) cells (Akashi et al., 1999). Meanwhile, myeloid progenitors generally differentiate into innate-sided cells such as macrophages, granulocytes, dendritic cells, and even erythrocytes (Iwasaki & Akashi, 2007). In the similarities therein lies the importance of classifying zebrafish hematopoiesis and factors that

can manipulate it, which allows researchers to gain a greater understanding of how the early immune development in *Homo sapiens* functions. See Figure 2.

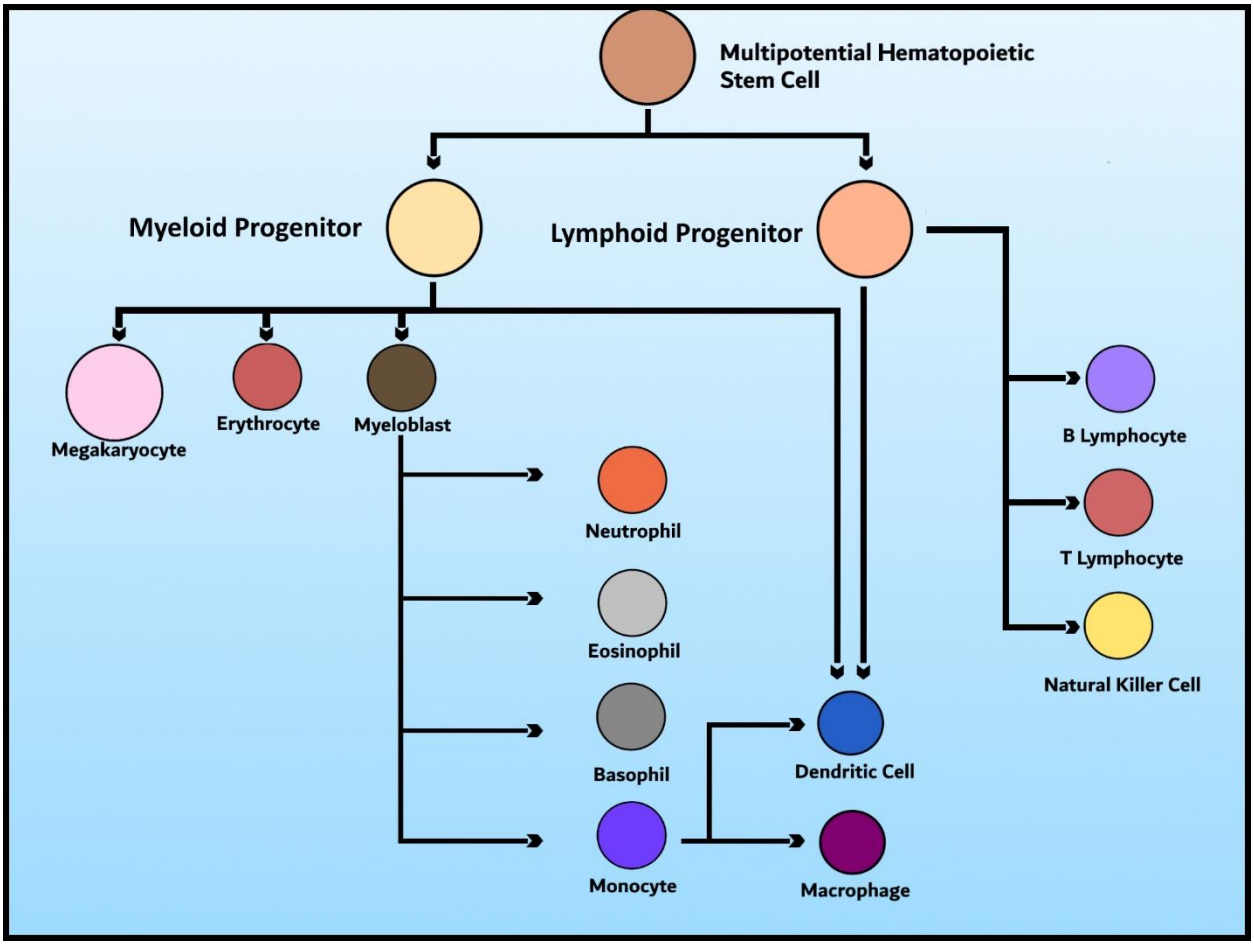

Figure 2. *Hematopoietic Stem Cells (HSCs) differentiate into one of two progenitors, the lymphoid or myeloid. From the lymphoid progenitor come B lymphocytes, T lymphocytes, Natural Killer Cells, and some dendritic cells. From the myeloid progenitor come the majority of innate immune cells, from neutrophils, eosinophils, and basophils, to monocytes which can further differentiate into dendritic cells and macrophages. Note that dendritic cells can differentiate from either lymphoid or myeloid progenitors, hence their label as the 'bridge' between immunity branches. Original illustration by Michael S. Chembars.*

Because the zebrafish's DNA has been fully sequenced, thanks to indispensable work by the Zebrafish Genome Project, scientists have also discovered that zebrafish can produce B lymphocyte variable genes that are very similar to ours (Wellcome Sanger Institute, n.d.).

Additionally, several separate studies discovered new perspectives regarding V(D)J segments in the adaptive immune response development (Jiang et al., 2011). Naïve B lymphocytes from zebrafish, however, experience strikingly similar maturation processes as they do in humans. Due to the zebrafish genome sequencing, immunologists have also gained a better understanding of how we can use this information further study human genetic diseases, such as those concerning hematopoietic stem cell development (Meeker & Trede, 2008; Wellcome Sanger Institute, n.d.; Ransom et al., 1996).

Adaptive Immunity

Similarities exist between human and zebrafish T lymphocytes as well, which has aided our understanding of autoreactivity and dysregulation mechanisms in the immune system. An effective experimental treatment method was devised for reducing clinical symptoms of encephalomyelitis in humans, through a zebrafish model which involved testing a relatively new chemical called Lenaldekar (LDK) (Cusick et al., 2012). This chemical can inhibit leukemic T lymphocytes in both species, and was proven to be effective in modulating T lymphocyte expansion through disrupting the PI3K/mTOR pathway (Ridges et al., 2012). Ridges et al. (2012) determined that LDK mechanism of action involved the dephosphorylation of both mTOR and p70S6, the latter of which is a target molecule of mTOR. LDK has seen use in several zebrafish cancer research models, and may be a viable method of treatment for leukemia in the future. Additionally, several studies are present on anti-cancer drug screening in the treatment of leukemia through zebrafish models (Mizgirev & Revskoy; Deveau et al., 2017).

Another study involving zebrafish and T lymphocyte research concerned a mutation in the ARPC1B (standing for actin-related protein 2/3 complex subunit 1B) gene, which is crucial for the development of T lymphocytes and thrombocytes (Papadatou et al., 2021). The function

of the gene was previously known, but the mechanism through which it caused disease was not (Kahr et al., 2017). Somech et al. (2017) discovered that mutating and/or knocking out this gene induced severe immunodeficiencies, which expressed themselves as pathologies in the fish. One of these pathologies included symptoms of Wiskott-Aldrich Syndrome, which in humans is characterized by chronic infections resulting from said immunodeficiencies and also thrombocytopenia (Ochs & Thrasher, 2006). A deficiency in this gene can also lead to a heightened type I hypersensitivity response in the body, which can lead to more severe allergic and asthmatic pathologies. (Papadatou et al., 2021). Now that the mechanism for disease is more known, future research could focus on how ARPC1B regulates lymphocyte development, in order to effectively treat patients with Wiskott-Aldrich Syndrome.

A greater understanding of adaptive immunity through the context of zebrafish models has also been explored through studying *Mycobacterium marinum,* a close relative of *Mycobacterium tuberculosis,* which is the bacteria that causes human tuberculosis (Banuls et al., 2015). Swaim et al. (2006) discovered that a RAG1 gene deficiency caused hyper-susceptibility in mutant zebrafish to *M. marinum*, which demonstrated that an adaptive response was crucial for effectively defending the host from this pathogen. The study's goals were focused on contributing to the lesser-known mechanisms and importance of caseous necrosis, which is apt to occur in victims of tuberculosis, whether that be in vertebrate fish or mammals (Swaim et al., 2006). RAG1 is in the family of recombinase activating genes (RAG), which are tasked with ensuring proper V(D)J recombination occurs (Schatz et al., 1989). RAG1 in particular forms a complex with recombination signal sequences (RSS) along with RAG2, which then splice DNA at the appropriate sites (Murphy, 2012). V(D)J recombination as a whole ensures that T and B lymphocytes gain antigen receptor variability, which aids in the identification of millions of

combinations of different foreign substances (Nishana & Raghavan, 2012). A downregulation or deletion of this gene or its activity can lead to decreased V(D)J recombination, resulting in a more compromised and less effective adaptive branch of immunity as a result.

Another study in zebrafish that concerned *M. marinum* led to a further understanding of granuloma formation post-infection (Davis et al., 2002). Davis et al. (2002) discovered two novel mechanisms through which macrophages can be recruited and cause eventual granuloma formation, which occurs in humans as a result of *M. tuberculosis* infection; this can result in failure to completely clear the bacterium and leads to a chronic infection as a result (Russell et al., 2009; Novoa & Figueras, 2011).

SARS-CoV-2 Response

Several researchers have also expressed interest in using zebrafish in SARS-CoV-2 related studies. Interestingly, there have also been novel discoveries in efferocytosis, or the apoptotic cell removal process, in zebrafish (Hosseini et al., 2016). There are several prevalent viruses such as SARS-CoV-2, which causes Covid-19, that exploit this process to help cause disease (Dos-Santos et al., 2021). Hosseini et al.'s (2016) discoveries led to the conclusion that macrophages (and neutrophils to a lesser extent) play a major role in efferocytosis. A major component of SARS-CoV-2's mortality was later discovered to be a result of impaired macrophage efficiency in the clearance of apoptotic cells, leading to impaired efferocytosis (Erol, 2022; Dutta et al., 2022). This primarily impedes the recovery process through reduced or completely retarded immune retraction, but also has negative effects on the adaptive side of immunity (Doran et al., 2020). Few studies are present on Covid-19 efferocytosis impediment,

and future research would benefit by utilizing zebrafish models to determine these mechanisms in greater depth (Amaral et al., 2021).

New antiviral therapeutics, which could alleviate symptoms of disease caused by Covid-19, have been proposed for further study in zebrafish due to our current understanding of viral glycoprotein crosstalk (Galindo-Villegas, 2020). Additionally, proposals through which a novel vaccine against this virus could be developed and deployed have been discussed (Kraus et al., 2020). In relation to this topic, Fernandes et al. (2020) has already laid out the groundwork involving preliminary trials for a vaccine and other prophylactic medications by infecting female zebrafish with SARS-CoV-2 spike proteins, and harvesting specific antibodies from them.

The previously mentioned study by Kraus et al. (2020) also determined that infection with a specific SARS-CoV-2 receptor binding domain causes an upregulation of proinflammatory cytokines such as CCL20a.3, resulting in the fish exhibiting tachycardia-like symptoms. In several studies, tachycardia has also been identified as a post-Covid infection phenomenon in humans (Stahlberg et al., 2021; Blitshteyn & Whitelaw, 2021). In order to gain better understanding into the SARS-CoV-2 mechanism of action, Costa et al. (2021) proposes that *in vivo* models may be used to study the effects of cytokine storms, which are caused by a mass release of proinflammatory cytokines in the body which can lead to organ failure and death. On this topic, novel research has discovered new insights into cytokine storms associated with Covid-19, which includes a Tlr2/Myd88 independent signaling pathway, as well as cementing the theory of TLR4 and TLR2's importance to said cytokine storms (Tyrkalska et al., 2022).

Zebrafish have also been used to study flavonoids extracted from jackfruit that can reduce ACE-2 receptor-mediated infiltration (Kusumaningtyas & Retnoaji, 2021). ACE-2 is an

enzyme that functions as a receptor in cardiac and pulmonary tissue for the Covid-19 virus, and for several years, research has been focused on finding ways to hinder or otherwise entirely prevent ACE-2 infiltration from occurring, while simultaneously boosting immune system effectiveness (Samavati & Uhal, 2020; Kusumaningtyas & Retnoaji, 2021). There have been several successful attempts using zebrafish models to create a therapeutic agent to combat this virus as well. Zar'ah & Retnoaji (2021) also extracted flavonoids from plant matter, and tested their effectiveness by infecting zebrafish with SARS-CoV-2, treating them with flavonoids, and then monitoring pulmonary, cardiac, and general physiological activity; this led them to the conclusion that the chemical was effective as an ACE-2 inhibitor, and subsequently as a therapeutic agent against Covid-19.

The use of other therapeutic agents for treating Covid-19 have also been performed successfully in zebrafish through the use of Ayurvedic medicines (Ali et al., 2022). This was done through modulating inflammatory damage by downregulating IL-6 and TNFα cytokines; the fish treated with these medicines recovered from infection faster than their control counterparts, and the overall survival rate was also enhanced (Balkrishna et al., 2021; Balkrishna et al., 2020). Balkrishna et al. (2021) also discovered that Patanjali Special Chyawanprash (PSCP) was successful as a therapeutic agent through its inhibition of the nuclear factor kappa B (NF-κB) signaling pathway in an *in vitro* study that used first a zebrafish model, then a modified human THP-1 macrophage model. Ayurvedics have been approved by several countries for use as a therapeutic agent against Covid-19, notably in part due to the information gleaned from zebrafish laboratories (Ali et al., 2022).

Estrogen on Immune System Development

Estrogen can have a variety of complex effects on the immune system, and have been studied in depth using the zebrafish model. However, these effects can occur chiefly due to the presence of estrogen receptors in immune cells, or immune related organs such as the spleen. As mentioned previously, zebrafish contain several subtypes of estrogen receptors. These receptors, sometimes referred to as ERa, ERb, and GPER in humans, are similar structurally to those found in the zebrafish and conserve many of the same functions and response patterns (Lam et al., 2011; Eyster, 2016).

Estrogen (one of its major forms being 17 β-estradiol, or estradiol) can have many effects on the immune system through binding with the aforementioned receptors, one of which is through complement activation. The complement system is an innate-sided immune process that utilizes a myriad of proteins through either a classical, lectin, or alternative pathway (Mariscalco, 2011; Noris & Remuzzi, 2013). Complement is responsible for neutralizing pathogens through opsonization, eventually lysing the pathogens through the formation and activation of a membrane attack complex (MAC), or by recruiting phagocytic cells to the site of infection (Murphy, 2012).

Estradiol can modulate complement effectiveness by inducing Factor H in zebrafish; an accumulation of Factor H, which is a complement control protein, can cause certain pathogens to escape from the complement system (Kumwenda et al., 2022). This is due to Factor H's known inactivation of C3b; C3b's primary function is to spearhead MAC formation (Kopp et al., 2012). This particular study by Kumwenda et al. (2022) was used to further ascertain the reasoning behind higher female susceptibility to contract urogenital tract infections during pregnancy.

Estradiol can also be involved with regulating TLRs, as was the case with BPA. A study by Sun et al. (2019) highlighted the hormone's modulatory effect on both TLR and NF-κB expression, through which exposure led to higher transcription levels. This is important as NF-κB activation and subsequent nuclear translocation is crucial for the release of pro-inflammatory cytokines and adhesion molecules into the microenvironment (Serasanambati & Chilakapati, 2016). If left unchecked, this can lead to low-level chronic inflammation, resulting in the pathogenesis of several diseases in humans, such as cardiovascular and neurodegenerative diseases, gastric disorders, or even metastatic cancer (Biswas & Bagchi, 2016; Haffner, 2006; Schottelius & Baldwin, 1999). This research on estrogen's role in NF-κB regulation in zebrafish is fairly novel and unique, and could be pursued further to determine its specific signaling pathway.

Estradiol can also modulate one of the Notch pathways, demonstrating its ability to control hematopoietic stem cells and ultimately immune cell differentiation in zebrafish (Nik et al, 2017; Carroll et al., 2014). There were several approaches that were attempted in order to determine estradiol's effect on this process, and several pathways of modulation were discovered. Carroll et al. (2014) discovered that vascular endothelial growth factor (VEGF), a glycoprotein that has a significant role in hematopoiesis and works in one of the Notch pathways, can be modulated by estradiol, with two different respective outcomes (Gerber & Ferrara, 2003). If embryos were exposed to estradiol without antagonizing estrogen receptors, there was a significant decrease in HSC differentiation and vasculature development; this was quantified by analyzing conserved HSC markers such as *runx1* and *cmyb* (Carroll et al., 2014). However, if the treatment occurred while also antagonizing estrogen receptor signaling, then HSC levels and vasculature development were elevated (Carroll et al., 2014). Previous research has determined

that VEGF typically has immunosuppressive tendencies, especially as it pertains to the inhibition of T lymphocyte development through modulating thymic maturation (Ohm et al., 2003; Lapeyre-Prost et al., 2017). This research is joined by similar experiments in different model organisms to determine what effect estrogen (or synthetic estrogens that may be classified as EDCs) may have on the developing immune systems of mammalian fetuses, as well as VEGF/Notch's role in antitumor therapy and treatment (Nik et al., 2017; Yang et al., 2018).

Estradiol can also have marked effects on microRNA regulation, according to several studies. These microRNAs have notably been linked to several important aspects of the immune system, especially in studies that involve type I hypersensitivity reactions (Wienholds et al., 2005; Lu & Rothenberg, 2017). Estradiol exposure has been linked to increased miR-17-92 expression, which is a specific microRNA that is crucial for B lymphocyte development from the pre-B to immature B lymphocyte stage (Spierings et al., 2011). It can also cause inflammation through a dual mechanism of simultaneously promoting helper T lymphocyte response, as well as inducing regulatory T lymphocyte production (Kuo et al., 2019). A failure of miR-17-92 expression to recede after its initial use in early development can cause cancers in zebrafish, such as B-lymphocyte lymphoma and lymphocytic leukemia, which results in higher B lymphocyte mortality rates (Kuo et al., 2019). It is also apparent that this elevated expression mechanism can lead to several autoimmune and lymphoproliferative diseases such as lupus and multiple sclerosis, the former of which can be caused through targeting the apoptotic protein *Bim*; elevated miR-17-92 can additionally decrease the expression of tumor suppressors such as *PTEN*. (Lindberg et al., 2010; Dai et al., 2010; Jiang et al., 2011). Apoptotic proteins such as Bim are very important for modulating immune contraction and preventing autoimmunity, as they can work together with and independently from the Fas ligands (FasL) and Fas, which have

similar roles in transducing apoptotic signals through an extrinsic pathway (Hughes et al., 2008; Nagata, 1994). Interestingly, this particular microRNA miR-17-92 (along with the proteins it modulates, *Bim* and *PTEN*), is also found in humans, and is currently being studied in part due to its carcinogenic and autoimmune-inducing activity when overstimulated (Gomez-Bougie et al., 2004; Liu et al., 2017; Bonneau & Longy, 2000). While the functional conservation of these genes between zebrafish and humans has been noted for the past 20 years, its contributions to the study of human cancers have increased in the last five years (Jette et al., 2008). However, miR-17-92 appears to have both angiogenic and antiangiogenic activities depending on the cell it is expressed in; Kuhnert & Kuo (2010) have highlighted the need for cell specific targeting strategies to fully ascertain miR-17-92's (along with other potentially carcinogenic microRNAs') capabilities (Kaluza et al., 2013).

Estrogen Disrupting Chemicals on Immune System Development

Research models that use *D. rerio* are not just beneficial for humankind's future health studies. It has also been used to expand our knowledge on how we could be impacting the aquatic ecosystem, through studying the environmental pollutant sinks and its correlated sediment toxicity; in particular, extensive research has been done on polycyclic aromatic hydrocarbons, such as bisphenol A (BPA) (Saiki et al., 2021). For example, many studies over the years have tried to expose the toxicity of BPA and its homologs (Eladak et al., 2015). Bisphenols are a common chemical that are used primarily in the polycarbonate plastic industry, which are used to manufacture many household and commercial items (Rochester, 2013). Because a large amount of BPA is released into the atmosphere and oceans every year, studies have been conducted on how it can impact wildlife and human health (Molina et al., 2018). In another study, it was discovered that low doses of BPA could act as an endocrine disrupting

chemical (EDC) in both humans and zebrafish, specifically affecting reproductive fidelity (Hachfi et al., 2012; Molina et al., 2018).

The effect of estrogen disrupting chemicals (EDCs) and estrogen on immune system development is a newer branch of study that utilizes *Danio rerio* as a model organism, especially as it pertains to the impact of human and ecological health. Zebrafish possess several estrogen receptors, such as EsrA, GPER, and EsrB, the latter of which is additionally subdivided into receptor subtypes Esr2a and Esr2b (Menuet et al., 2004; Deroo & Korach, 2006). While GPER, or the G protein-coupled estrogen receptor, was recently discovered in zebrafish (and thus their functions are still largely unknown in the species), EsrA and EsrB appear to have similar immunomodulatory abilities in both humans and zebrafish through their binding of estrogen and estrogen-like compounds (Lopez-Munoz et al., 2015; Prossnitz & Barton, 2011). Estrogen and EDCs are major environmental pollutants, and have been proven to cause severe side effects in zebrafish embryonic development (Bambino & Chu, 2017). As mentioned earlier, the bisphenols (BPA, BPC, etc.) can have profound effects not only on other body systems, but also immune development. Bisphenol derivatives can affect not only embryos directly, but can also affect offspring if the parents were exposed to the EDC (Dong et al., 2018).

In a transgenerational experiment, Dong et al. (2018) treated adult zebrafish with BPA as well as BPS and BPF, and afterwards monitored genes that are important for immune development, such as those coding for TLR, MyD88, TNFα, RAG, and IFN. They discovered that exposure to bisphenols caused the embryos to exhibit a downregulation of all these genes, and thus the offspring were more susceptible to infection and had a higher mortality rate. Toll-like receptors (TLRs) are part of the innate branch of immunity, and are tasked with recognizing pathogen-associated molecular patterns (PAMPs) from foreign substances, which can include

lipopolysaccharide (LPS), flagellin, and even nucleic acids such as RNA (Lu et al., 2008; Hayashi et al., 2001; Alexopoulou et al., 2001). Upon detection, they can then activate a signaling cascade through a canonical MyD88 pathway, or through a noncanonical TRIF pathway; this ends in the release of pro-inflammatory cytokines such as TNF-a, or antiviral interferons like Type 1 IFN, into the microenvironment of the cell (Serasanambati & Chilakapati, 2016). Therefore, the inhibition of TLR seems to cause a downstream downregulation of MyD88, which will reduce the efficiency of innate pro-inflammatory cytokine release. See Figure 3 for a summary of both canonical and noncanonical TLR-4 signaling.

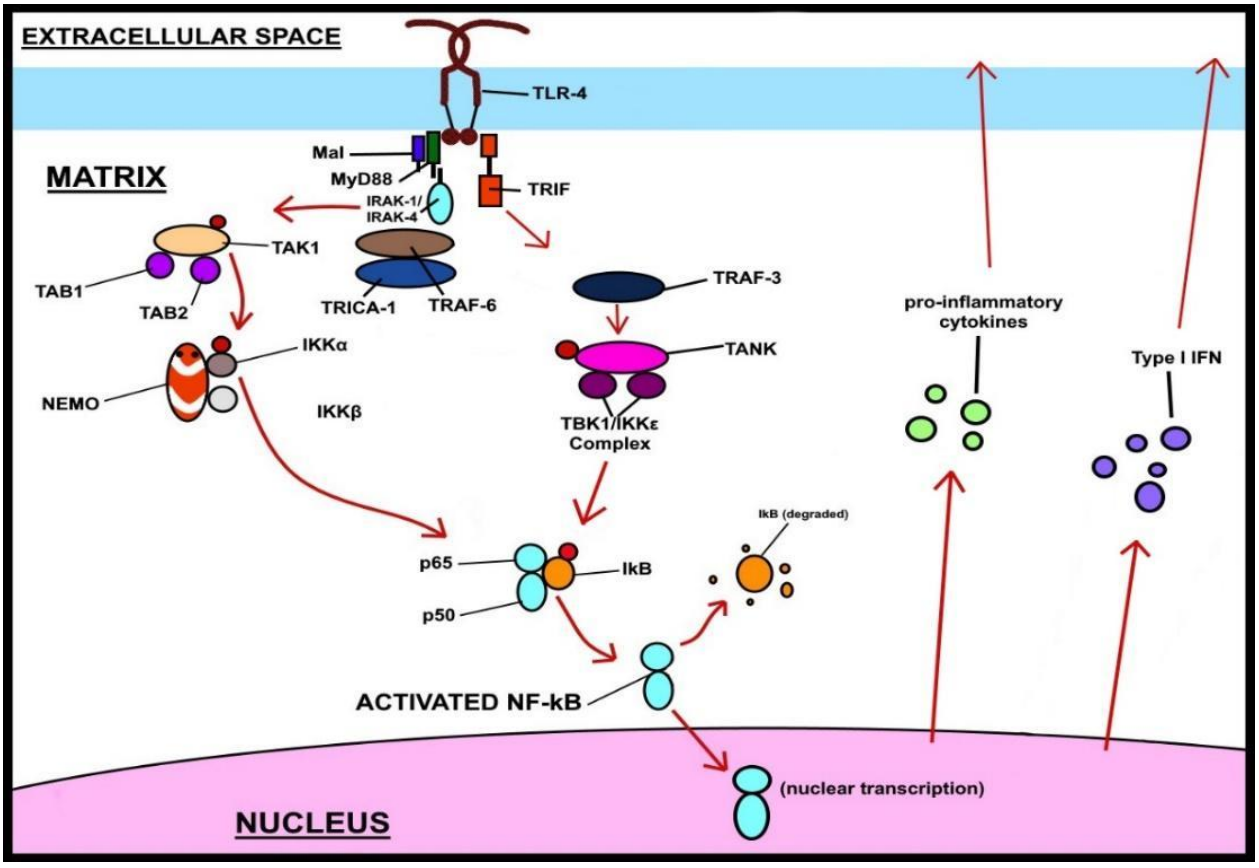

Figure 3. *Canonical and noncanonical pathways of TLR-4 signaling. In the canonical pathway, MyD88-dependent signaling works in conjunction with IRAK to activate NF-κB through degradation of IκB, and eventually results in the release of pro-inflammatory cytokines such as TNF-a, IL-1β, and IL-6 (van der Sar et al., 2006; Adachi et al.,*

1998). The noncanonical pathway, which utilizes TRIF to cause a downstream activation of NF-κB as well, causes the release of Type I IFN, which modulates the induction of the antiviral state (Durbin et al., 2000). Figure adapted from Chembars & Stevenson (in review).

In another experiment, Qiu et al. (2018) treated zebrafish embryos with two analogs of BPA (BPS and BPF) in response to public health concerns from EDC exposure; conversely to Dong et al.'s (2018) research, however, their results concluded that embryos treated with these two bisphenols tended to have higher concentrations of TNFα and IL-6 expression. These contrasting results may differ due to the concentration of BPS and BPF used in the experiments. Whereas the Dong et al. (2018) used 10 μg/mL of bisphenol, Qiu et al. (2018) used 100 μg/mL and 1000 μg/mL. Lee et al. (2018) determined that BPS could additionally alter microRNA expression in zebrafish adults by increasing hematopoiesis and overall immune development as a whole. These microRNAs are small individual segments of regulatory RNA that are involved in post-transcriptional regulation of protein expression, and can play a key part in immune development (Tanase, et al., 2012). It is likely that this increase in hematopoiesis due to BPS exposure can cause toxic effects, due to several other studies using BPA, a very structurally similar analog of BPS; in the experiment that utilized BPA, the treated zebrafish ultimately built up an accumulation of enlarged, immature lymphocytes, which may be indicative of acute myeloid leukemia, among other serious conditions (Sundarraj et al., 2021).

Additionally, heightened TNFα levels, if not corrected and released in large amounts systemically, may cause endotoxin-mediated septic shock as a result of lowered blood pressure (Murphy, 2012). This is primarily due to TNFα's pro-inflammatory nature, through its ability to activate NF-κB and other pro-inflammatory transcription factors such as AP-1 (Sethi et al.,

2008). Chronic inflammation due to TNFα levels ceasing to recede is also linked to tumorigenesis and metastatic promotion (Aggarwal et al., 2002).

Interleukin 6 (IL-6) is crucial for the differentiation and activation of T lymphocytes, and also provides T lymphocytes with apoptotic resistance through the induction of IL-2 (Neurath & Finnoto, 2011). Elevated IL-6 has also been linked to several inflammatory-related cancers in humans, as well as several autoimmune diseases including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus (Ireland et al., 2015). In summary, there is a respectable amount of noteworthy research on the effects of bisphenol on immune system development, and as such, a lot of groundwork has been cleared for further studies on this topic.

There are, of course, many other EDCs that have been identified and studied in the zebrafish model, in order to ascertain what their impact might be on human immune competency. Nonylphenol (NP), an organic compound used in manufacturing, caused several immune-related genes such as IFNγ, IL-1β, TNFα, and IL-10, to be significantly upregulated in zebrafish embryos; additionally, exposure also led to an upregulation of TLR and MyD88 genes (Soares et al., 2008; Xu et al., 2013).

Interleukin 10 (IL-10) is an anti-inflammatory cytokine primarily expressed by regulatory T lymphocytes, which in humans reduces the actions of pro-inflammatory cytokines such as TNFα, IL-1, and IL-6 (Glocker et al., 2012). It also has modulatory effects on both the adaptive and innate branches of immunity through its ability to control the differentiation process in immune cells such as macrophages, T lymphocytes, and B lymphocytes (Moore et al., 2001). As can be expected, if IL-10 is circulating in higher than appreciable quantities, this can lead to conditions associated with immune suppression such as the disruption of delayed

hypersensitivity, decreased eosinophil and basophil activity, or even decreased monocyte proinflammatory cytokine production (Wu et al., 2007; Peyron et al., 2008; Malefyt et al., 1991).

Additionally, the study by Xu et al. (2013) found that reactive oxygen species (ROS) were elevated in zebrafish embryos who were exposed to NP. ROS can modulate several functions within the immune system, one of which aids the innate branch through its activation of a TLR-4 pathway; in T lymphocytes, they can control the rates of activation and differentiation (Matsuzawa et al., 2005; Moro-Garcia et al., 2018). ROS are classified as a biocidal agent, and in large quantities, can cause a host of diseases in humans and zebrafish (Kohchi et al., 2009). This can range from chronic inflammation, accelerated aging, as well as certain cancers (Yang et al., 2012).

There is also evidence to suggest that certain EDCs like NP can cause the upregulation of CXCL chemokines, which are known to be a key factor in certain cancers and autoimmune diseases (Xu et al., 2013; Antonelli et al., 2014). In a healthy individual, CXCL family chemokines are secreted by natural killer and natural killer T lymphocytes (in response to IFN-y and TNFα signaling pathways), and can mediate inflammation, along with tumor regulation and metastasis (Chen et al., 2020; Roy et al., 2017). Medical conditions caused by an overexpression of this chemokine may include the development of type I diabetes, gastric cancer, or even systemic sclerosis (Antonelli et al., 2014; Chen et al., 2020).

An interesting point that may be observed is the upregulation of both pro-inflammatory and anti-inflammatory cytokines as a result of NP exposure. It is unclear at this time whether this mutual upregulation causes a balance between the two to allow homeostatic function in the organism, or whether an imbalance is created leading to pathogenesis.

Fulvic acid has been proposed as a water-soluble immune stimulant for use as an alternative of antibiotics to keep eco-systems healthy, and to increase the survival rate of aquatic species; its mechanism of action involves a stimulation of growth hormone, and through this an increase in lysozyme and myeloperoxidase activity (Lieke et al., 2021). Previous to this study, fulvic acid has been experimented with to a minimal degree, and was proposed as a potential medication for the prevention of chronic inflammatory diseases such as diabetes; however, there is still a toxicity factor to consider, as it can actually increase inflammatory activity in high doses, through increasing ROS concentrations (Winkler & Ghosh, 2018; Lieke et al., 2022). Although its exact structure and purification methods are still in its infancy, it has potential as a helpful drug, and is seeing increasing use in zebrafish models in order to ascertain its usefulness in healthcare.

Di-2-(ethylhexyl) phthalate (DEHP), an EDC used in the polymer manufacturing process, has also been associated with the disruption of immune function as well as other toxic effects in human and animal species (Yuan et al., 2022). Adamovsky et al. (2020) determined that DEHP-exposed zebrafish developed stronger adaptive responses in the gut through an increase of helper T lymphocyte expression. This specifically led to dysregulation-like levels of Th1 and Th2 subsets, which have been associated with an onset of Crohn's disease and/or ulcerative colitis (Adamovsky et al., 2020; Zhu et al., 2018; Clough et al., 2020). DEHP can also cause a downregulation of TLR-5 and IL-1β in female zebrafish, which could also be a factor in promoting gut dysbiosis (Jia et al., 2021). There seem to be some contradictions with an earlier report that IL-1β levels were actually upregulated by a factor of approximately 1.5 times after DEHP exposure; this may be due to treatment time parameters between a chronic versus acute response, where Jia et al. (2021) tested embryos well past 3 months post fertilization, while Mu

et al. (2018) treated embryos only to the 96 hours post fertilization mark. Mu et al. (2018) also reports that TNFα and IL-8 levels were upregulated after acute DEHP exposure.

Cancer

The extrinsic pathway of apoptosis is mostly conserved between zebrafish and humans, as zebrafish possess FasL homologs, FADD, and caspases 8, 10, and 3 (Eimon et al., 2006). As a result, these Fas ligand interactions and downstream effectors have been studied using a zebrafish model, and have helped researchers gain a better perspective of the mammalian extrinsic pathway of apoptosis, along with cell death mechanisms in general (Pyati et al., 2007; Yamashita, 2003). These studies have given insight into autoimmune diseases and cancers, as FasL (a member of the TNF superfamily of cytokines) is usually secreted by cytotoxic T lymphocytes or some helper T lymphocyte subtypes (National Library of Medicine, 2022; Janeway et al., 2001). CD8 and some CD4 positive cytotoxic T lymphocytes modulate anti-tumor activity and terminate autoreactive thymocytes through the recognition of abnormal surface markers (Janeway et al., 2001).

For example, Ferrari et al. (2014) discovered a novel mechanism of evasion for chordoma, a rare bone cancer that usually presents itself in the sacrum, through studying Fas/FasL interactions in zebrafish (Williams et al., 2013). This research contributed to the treatment of this specific cancer by analyzing notochord development, its dependency on Fas/FasL, and its potential in chordoma formation. There has also been some work focused on inhibiting P13K/mTOR pathways in zebrafish, to determine if there was any correlation between these pathways and chordoma formation (Burger et al., 2014). This particular study by Burger et al. (2014) instead discovered a potential chordoma model through the expression of HRASV12,

a Ras-family member that has been shown to be critical for helper T lymphocyte response (Iborra et al., 2011). HRAS genes are found in both humans and zebrafish alike, and the discovery that oncogenic HRAS can also cause melanoma through ignoring tumor suppressing agents from the immune system was pioneered by Santoriello et al. (2010). See Figure 4 for a summary on Fas and extrinsic apoptotic signaling.

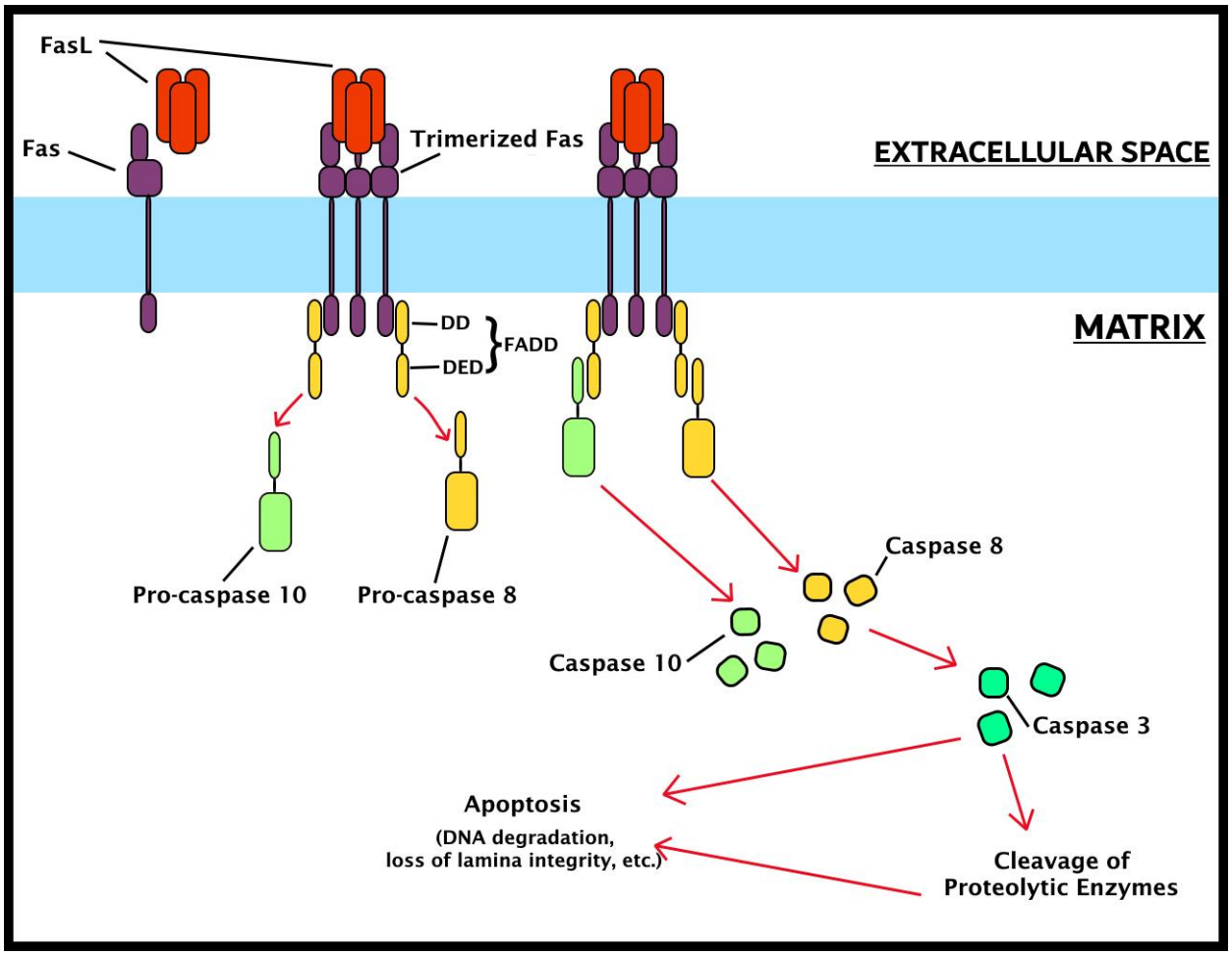

Figure 4. *The canonical pathway of FasL-induced apoptosis through the extrinsic pathway. A signaling cell such as a cytotoxic T lymphocyte will express FasL, which binds and trimerizes Fas receptors on the target cell. FADD adapter molecules are then recruited with a death effector domain (DED), which recruits pro-caspase 8 and pro-caspase 10. Caspase 8 can activate itself from pro-caspase 8 when recruited in this manner, and the Death Inducing Signaling Complex (DISC) is formed (Holler et al., 2003). Caspase 8 then cleaves Procaspase 3 into Caspase 3,*

which subsequently activates proteolytic enzymes such as caspase-activated endonuclease in zebrafish (Porter & Janicke, 1999; Sakahira et al., 1998). These then have a lethal effect on the cell from DNAase activity and degradation of the nuclear lamina. Original illustration by Michael S. Chembars.

Dysbiosis

Recently, many researchers have adopted zebrafish as their model organism to learn more about the mechanisms that govern our own gut microbiota, especially as it pertains to immunological interactions associated therein. For example, recent studies led to the discovery of neutrophil recruitment and response in the gut in response to specific dual combinations of bacteria such as *Aeromonas* or *Vibrio* (Rolig et al., 2015). This induced higher neutrophil counts and subsequently increased the inflammatory potential in the gut (Rolig et al., 2015). Research utilizing zebrafish also contributed to the discovery of an immunomodulatory protein that is secreted by bacteria belonging to the *Aeromonas* genus, which can prevent a toxic buildup of neutrophils and contributes to the mutualistic tendencies of the gut microbiota (Rolig et al., 2018). This and similar research could assist medical professionals in diagnosing dysbiosis in patients, as well as providing researchers with newfound insight on the mutualism between the innate branch of immunity and the gut microbiota, much of which is hard to decipher and requires further research, given the many species of bacteria that reside in the gut.

In regards to the environmental impact, research on effectors of the gut microbiota in zebrafish has also led to key discoveries. Qiao et al. (2019) determined that microplastics, which are known aquatic ecosystem contaminants, can cause a reduction in key gut bacterial genera such as *Proteobacteria*, *Pseudomonas, Actinobacteria,* and *Aeromonas*. This could lead to deficiencies in secondary metabolite secretion and reduced epithelial cell regeneration, both of which are crucial for a healthy gut in zebrafish (Li et al., 1998; Fernandes et al., 2014).

Conclusion

In summary, *Danio rerio* has proven itself as a stellar model organism in regards to immunological studies due to several factors. They are cost-effective, easy to breed/raise, and are easy to maintain compared to more classic model organisms. They also exhibit many similarities with our own immune response, ranging from hematopoietic stem cell development, all the way to lymphocyte maturation and subsequent memory after a primary infection. In the past, many studies ranging from cancer to gut microbiota research have uplifted zebrafish as a prime model organism, through which this data may eventually be used to gain a better understanding of how the immune system works in our own bodies (or the lack thereof).

Research that has been performed on EDCs and estrogen in regards to their effect on the immune system and overall organismal health are also important topics of study. As many of these chemicals have already been shown to be polluting global waterways that may eventually be traced back to human consumption, it is crucial that we understand the mechanisms through which these chemicals can manipulate our body systems. In the future, perhaps safer chemicals can be derived from the research done on zebrafish, for a safer ecosystem and a healthier community. For these punctuated reasons, *D. rerio* will continue to serve future researchers as a stellar model organism in immunological studies.

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