Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/dci

The zebrafish as a model to study intestinal inflammation

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ARTICLE INFO

ABSTRACT

Starting out as a model for developmental biology, during the last decade, zebrafish have also gained the attention of the immunologists and oncologists. Due to its small size, high fecundity and full annotation of its genome, the zebrafish is an attractive model system. The fact that fish are transparent early in life combined with the growing list of immune cell reporter fish, enables *in vivo* tracking of immune responses in a complete organism. Since zebrafish develop *ex utero* from a fertilized egg, immune development can be monitored from the start of life. Given that several gut functions and immune genes are conserved between zebrafish and mammals, the zebrafish is an interesting model organism to investigate fundamental processes underlying intestinal inflammation and injury. This review will first provide some background on zebrafish intestinal development, bacterial colonization and immunity, showing the similarities and differences compared to mammals. This will be followed by an overview of the existing models for intestinal disease, and concluded by future perspectives in light of the newest technologies and insights.

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1. Introduction

Starting out as a model for developmental biology, during the last decade, zebrafish have also gained the attention of the immunologists and oncologists. Due to its small size, high fecundity and full annotation of its genome, the zebrafish is an attractive model system. From the early nineties until now publications on zebrafish and immunity rose from 4 publications in 1997 to 149 publications in 2014 (Fig. 1). The fact that fish are transparent early in life (<2 weeks) combined with the growing list of immune cell reporter fish, enables *in vivo* tracking of immune responses in a complete organism. Since zebrafish develop *ex utero* from a fertilized egg, immune development can be monitored from the start of life. In zebrafish, cells of the innate immune system (macrophages, neutrophils and eosinophils) arise first followed by adaptive immunity developing from two to three weeks post fertilization (Lam et al., 2004; Page et al., 2013; Zapata et al., 2006). Zebrafish usually hatch from their chorion at 2 days post fertilization although this depends on the temperature. Growing fish at lower temperatures will delay hatching (Villamizar et al., 2012). The zebrafish mouth opens on day 3 and the whole gastrointestinal tube is opened at day 6 post fertilization (Wallace and Pack, 2003). By this time, the yolk sac is consumed and the larvae start to feed on small protozoans

such as paramecia. Over the course of several weeks the intestines develop and simultaneously several bacterial species colonize the gastrointestinal tract.

Given that several gut functions and immune genes are conserved between zebrafish and mammals, the zebrafish is an interesting model organism to investigate fundamental processes underlying intestinal inflammation and injury (Cheesman and Guillemin, 2007; Yang et al., 2014). This review will first provide some background on zebrafish intestinal development, bacterial colonization and immunity, showing the similarities and differences compared to mammals. This will be followed by an overview of the existing models for intestinal disease, and concluded by future perspectives in light of the newest technologies and insights.

2. Zebrafish gastrointestinal tract

Bony fishes (teleosts) are an extremely diverse and abundant (>25.000 species) group of fish. One important feature that needs to be mentioned when the gastrointestinal tract of fish is discussed, is the presence of a stomach (Kwek et al., 2009). Within the teleosts we can find fish that do and those that do not have a stomach. For example, Nile Tilapia and Atlantic Cod have a stomach, while Pufferfish, Platyfish, Common Carp and zebrafish lack a stomach. Gastric glands first appear around 450 million years ago and seem to be a hallmark of most gnathostomic (jawed) vertebrates. A recent study from Castro et al. shows that the loss of gastric glands

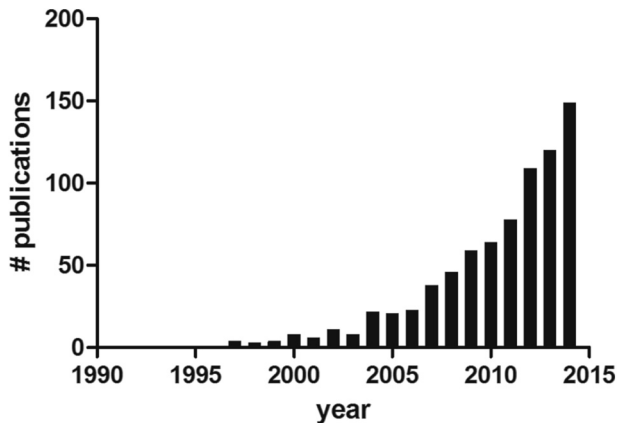E-mail address: Sylvia.brugman@wur.nl.

Fig. 1. Number of publications in PubMed per year using keywords 'zebrafish' and 'immune'.

(and simplification of the vertebrate gut) occurred several times in vertebrate evolution as a result of the loss of genes involved in H+/K+-ATPase (*Atp4A* and *Atp4B*) and those encoding for pepsinogens (*Pga*, *Pgc*, *Cym*); genes at the heart of acid-peptic digestion (Castro et al., 2014). Instead of a stomach, zebrafish have the anterior intestine, called the intestinal bulb, which has a bigger lumen than the posterior part and thus may function as a reservoir comparable to the stomach. However, this intestinal bulb lacks gastric glands, and therefore does not have low pH. According to a study of Nalbant et al. the pH of the zebrafish intestines never reaches below 7.5 under homeostatic conditions (Nalbant et al., 1999).

From the early studies of Wallace and Pack we now know that gut tube formation in zebrafish begins during mid- to late-somite stages (~18 somites), whereas in mammals, the gut begins to form at early-somite stages (1–2 somites). At the 18 somites stage a continuous thin layer of endoderm becomes distinguishable which will give rise to the primitive gut endoderm (Wallace and Pack, 2003). Despite the fact that zebrafish gut formation is later, the temporal sequence of gut tube formation is identical. Like in mammals, the rostral gut of zebrafish forms first, followed by the hindgut, and midgut (Wallace and Pack, 2003).

The zebrafish epithelial layer of the gut lacks intestinal crypts, however, fingerlike protrusions called folds (villi) are present and decrease in size from anterior to posterior (Wallace and Pack, 2003) (Fig. 2). Differentiated epithelial cells such as absorptive enterocytes (anterior and mid-intestine), mucin-producing Goblet cells (entire intestine) and enteroendocrine cells (anterior intestine) are found. To date, Paneth cells are not identified histologically. Also no classical microfold (M) cells are found, although a fourth epithelial cell type is identified within a posterior segment of the mid intestine containing large vacuoles in which luminal contents can be stored (Gebert and Jepson, 1996; Rombout et al., 1985). The absence of Peyer's patches and the lack therefore of follicle associated epithelium suggests zebrafish do not have M cells, however, the aforementioned M-like vacuolated cells might deliver luminal contents to scattered immune cells present underneath the epithelial layer. In contrast to mammals, zebrafish do not have a submucosa. The submucosa in mammals is a layer of loose connective tissue containing blood and lymphatic vessels and nerves, underneath the mucosa (villi), connecting the mucosa with the underlying smooth muscle layer. In zebrafish the smooth muscle layer is less complex and directly attached to the mucosa. The enteric nerve cell bodies in zebrafish are present between the circular and longitudinal smooth muscle layers (Wallace and Pack, 2003).

Like in mammals epithelial cells migrate from the base of the folds to the tip of the fold where they undergo apoptosis. BrdU staining showed that this migration takes 5–7 days in the anterior intestine, and 7–10 days in the mid intestine (Wallace et al., 2005). Like in mammals, epithelial turn-over and differentiation are dependent on microbial colonization (Bates et al., 2006; Rawls et al., 2004). Interestingly, mechanisms controlling differentiation of epithelial cells towards the secretory lineage (i.e. goblet cells) in zebrafish appears to be highly conserved, and like in mammals depends on Delta-Notch signalling. In the absence of Notch activation all epithelial cells differentiate into cells of the secretory lineage (Crosnier et al., 2005). Likewise, zebrafish T cell factor 4 (tcf4) is important in maintaining proliferative self-renewal in the intestine throughout life, just like its murine counterpart (Muncan et al., 2007). In light of this high functional homology, zebrafish are an excellent model to study the mechanisms controlling renewal of gut epithelium.

Although zebrafish do not have 5 intestinal segments like mammals (i.e. jejunum, duodenum, ileum, cecum (appendix) and colon), there is functional homology. In zebrafish, three different gut segments are discriminated on the basis of morphology and gene expression: the anterior gut segment (intestinal bulb), midgut and the posterior gut segment (Wallace et al., 2005; Wallace and Pack, 2003). Digestive enzymes are strongly expressed in the anterior segment where the folds are longest. The presence of these digestive enzymes and solute transporters in the anterior and mid intestines underlines the function of nutrient absorption in these two segments. Concomitantly, expression of intestinal fatty acid binding protein 2 (*ifabp2*) is highest in the anterior and mid-intestinal part (Mudumana et al., 2004), while *ifabp6* is mainly expressed posteriorly (Oehlers et al., 2011a). The last part of the mid intestine contains the aforementioned vacuolated (M-like) cells which might indicate that besides nutrient absorption this region might play a role in mucosal immunity. The epithelial folds of the posterior region are short and this part does not contain absorptive enterocytes, but is likely involved in water absorption (Wallace et al., 2005).

A recent study by Wang et al. performed micro-array analysis the gut of adult zebrafish which they divided in 7 segments of equal lengths (from anterior 1, to posterior 7) (Wang et al., 2010). They could confirm the presence of three distinct regions on the basis of their data on metabolic genes. Sections 1–5 show high expression of well-known human and mouse small intestinal markers fatty acid binding protein 2 (*fabp2*), villin 1 (*vil1*), Apolipoprotein 1 and 4 (*apoa1* and *apoa4*). *Fabp2*, *Apo1* and *Apo4* are all involved in lipid metabolism, while *vil1* is a regulatory gene in small intestinal epithelial cells that has an anti-apoptotic function. The genes *cfl1* (cofilin1) involved in dynamic stabilization of actin filaments and *aqp3* (aquaporin 3) involved in water absorption are both markers of mammalian large intestine and distinguishes segments S5–S7 from S1–S4. The authors conclude that S1–S5 possess molecular features of small intestine, while segments S6 and S7 share features of the large intestine, while S5 forms a transition section (Wang et al., 2010). Section 5 might well be the posterior part of the midgut that might be involved in mucosal immunity. Unfortunately, no immunity related specific probes were taken along in their microarrays. It would be of great interest to perform transcriptomic analysis on the three distinct segments to identify immune differentiation along the zebrafish intestinal tract.

3. Intestinal microbial colonization

All animals on earth, being aquatic or terrestrial, have co-evolved with the microbes in their environment. As we know from advances in the field of microbial ecology, microbes not only
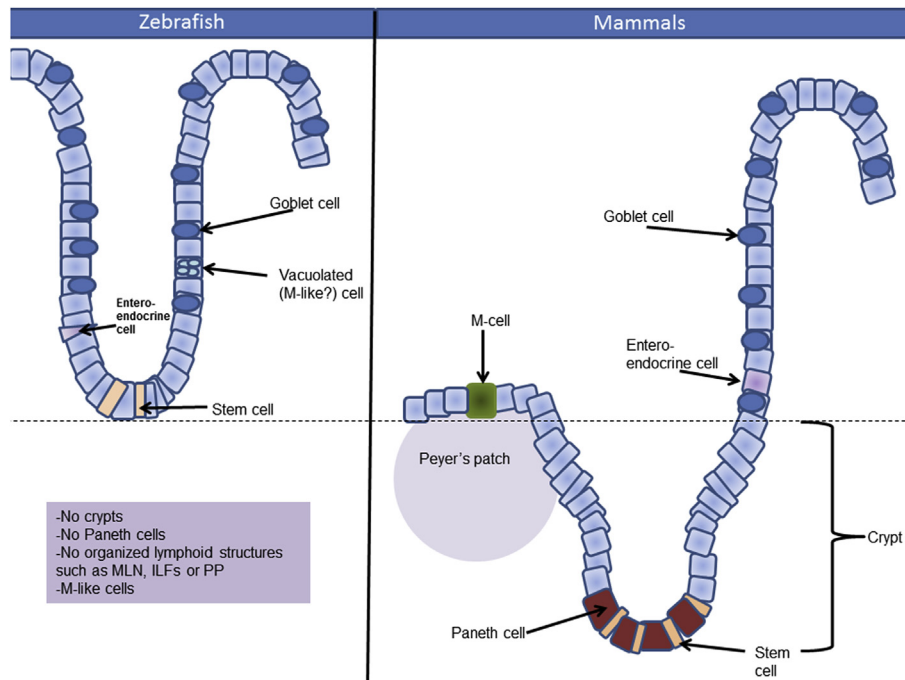
Fig. 2. Difference in cell types and structures between the zebrafish and mammalian (small) intestines. Zebrafish do not have Paneth cells, crypts, and organized lymphoid structures such as mesenteric lymph nodes (MLN), isolated lymphoid follicles (ILFs) or Peyer's patches (PP).

colonize their hosts but also greatly influence the hosts physiology and immunity. Vice versa, the host is able to control which species are able to colonize the intestine, by mounting immune responses to some while tolerating others.

Studies performed in gnotobiotic animals revealed that microbes have an enormous impact on the host (Kanther and Rawls, 2010; Palm et al., 2015). Microbes can affect food processing and make otherwise indigestible food ingredients available for the host. Likewise, microbes can synthesize vitamins and stimulate epithelial renewal (Cheesman et al., 2011). To investigate the effect of microbial colonization on the zebrafish host, Rawls and colleagues reared germfree zebrafish and investigate gene expression profiles (Rawls et al., 2004). They showed that colonization altered the expression of 212 genes. Of these 212 genes, 59 responses were conserved between mice and zebrafish. These conserved genes were mainly involved in epithelial proliferation, promotion of nutrient metabolism, and innate immune responses. This indicates that the response towards microbes is in part highly conserved.

Microbes enhance energy uptake from our food by processing inaccessible food ingredients. In zebrafish it was shown that the presence of a microbiota also results in an increased fat storage in adipose tissue (Camp et al., 2012). The authors found that microbes supress intestinal epithelial expression of Angiopoietin-like 4 (Angptl4/Fiaf). Angptl4/fiaf is a circulating inhibitor of lipoprotein lipase, so by inhibiting the inhibitor colonization results in fat processing and storage. In line with these observations, Semova and colleagues showed that microbes stimulate fatty acid uptake in the intestinal epithelium and liver. Feeding fish a diet of fatty acid analogs increased the abundance of Firmicutes, which in turn can stimulate fatty acid update (Semova et al., 2012). Diet-induced alterations in microbiota composition therefore, like in mice and humans, influence fat absorption and host energy balance.

Microbes also induce intestinal development in zebrafish. Bates and co-workers found that in the absence of the microbiota, the zebrafish gut epithelium was halted in its differentiation, since they observed a lack of brush border intestinal alkaline phosphatase

activity, immature patterns of glycan expression on the surface and lack of goblet and enteroendocrine cells (Bates et al., 2006). Furthermore, in the absence of microbes the intestines of zebrafish fail to take up protein macromolecules in the anterior intestine and show decreased transit time of intestinal content (faster intestinal motility) (Bates et al., 2006). Colonizing these fish with bacteria reversed the effects. Interestingly, exposure of germ-free zebrafish to bacterial lipopolysaccharide (LPS) or heat-killed bacteria could re-establish alkaline phosphatase activity, but not the effects on glycan expression, indicating the presence of different pathways of host-microbe responses (Bates et al., 2006). Cheesman et al. reported that epithelial cell proliferation in the developing zebrafish intestine is stimulated both by the presence of the resident microbiota and by activation of Wnt signaling (Cheesman et al., 2011). Additionally, they showed that homeostatic innate immune responses but not inflammatory signals contribute to the effects on epithelial proliferation since the effect of microbes on epithelial turnover required MyD88 and not TNF receptor. This also relates to the finding that like in mice, bacterial stimulation induces NF-κB (a key regulator of immune gene expression) in zebrafish cells. Bacterial colonization of germ-free zebrafish activated NF-κB and resulted in expression of its target genes in intestinal and extra-intestinal tissues of the gut (Kanther et al., 2011).

Expression of brush border enzyme alkaline phosphatase is induced during establishment of the gut microbiota. Apart from its role in digestion it was shown that zebrafish that lack alkaline phosphatase are sensitive to LPS toxicity and show increased intestinal neutrophil levels. These findings illustrate microbiota shapes the homeostatic level of neutrophils in the zebrafish intestine (Bates et al., 2007). Further investigation into alkaline phosphatase activity in mice revealed indeed it also has a previously unknown immune function (Yang et al., 2015).

Although the response towards microbes appears to be partly conserved, not all organisms are exposed to the same bacteria on the basis of the environment in which they live. Zebrafish, being aquatic animals, obviously encounter different bacteria than mice

or humans. This difference is also reflected in the composition of their microbiota. Zebrafish have a microbiota that is dominated by Proteobacteria, while mice and human have more Bacteroidetes and Firmicutes. Still, zebrafish and mice share six bacterial divisions and the metabolic functions the microbes provide for the host is similar. Rawls and colleagues elegantly showed that exposure is not the only reason the microbiota differs between zebrafish and mammals (Rawls et al., 2006). Host factors also select which species can survive. They showed that transplantation of mouse intestinal microbes (dominated by Firmicutes and Bacteroidetes) into zebrafish, resulted in the outgrowth of the small number of Proteobacteria that were also present in the mouse intestine, so that after two weeks they became dominant in the zebrafish intestine. The opposite occurred in mice colonized with zebrafish bacteria. Here the small number of Bacteroidetes and Firmicutes grew out to become dominant. This illustrates that the microbiota is under selective pressure within the gut habitat of each host (Rawls et al., 2006). Investigation into the gut microbiota of zebrafish in different facilities also confirmed this host selection and observed that although small differences exists from facility to facility, the zebrafish microbiota is still very similar, even to recently caught zebrafish (Roeselers et al., 2011).

In summary, the zebrafish microbiota, although different from the mammalian microbiota in terms of dominant phyla, induces a very conserved host response during colonization and development. We showed that the composition of this microbiota, like in mice, can convey disease susceptibility in a model of intestinal inflammation, emphasizing the usefulness of the zebrafish as a model to study host microbe interactions in health and disease (Brugman et al., 2009).

4. Immune development

4.1. Adaptive immunity

Adaptive immunity first appeared in jawed fish around 500 million years ago (Venkatesh et al., 2014). Since zebrafish develop *ex utero*, immune development can be studied from the beginning of life; from the fertilization of the egg. In this way innate and adaptive immunity development can be studied separately. Early in life zebrafish solely rely on their innate immune system, which enables study of innate immune processes in the absence of adaptive immunity.

It was previously thought that the adaptive immune system did not mature until after 4 weeks of age, since it was shown that the thymus and head kidney (primary immune organs) remain rudimentary throughout the early larval stages. The zebrafish thymus only acquires a more complex shape from 4 wpf onwards and TCR alpha constant region positive cells are seen in the medulla by 2–3 wpf (Lam et al., 2004). Furthermore, Lam et al. detected Rag-1 expression in head kidney by 2 wpf (Lam et al., 2004). However, more and more observations suggest that adaptive immune cells appear earlier in the periphery. For example, intra-epithelial lymphocytes are reported in the zebrafish intestines as early as 8 dpf (Trede et al., 2001; Zapata et al., 2006). Likewise, Danilova and Steiner reported some TCR positive cells in the oesophagus and intestine of 9 day-old zebrafish by in situ hybridisation (Danilova et al., 2000). In contrast, Trede and colleagues were unable to detect T cells outside of the thymus in their lck:GFP T cell reporter zebrafish in the first three weeks of life and we detected lck:GFP cells only from 3 wpf in the intestines (Brugman et al., 2014; Langenau et al., 2004). The recently generated CD4 reporter zebrafish also shows CD4+ cells outside of the thymus from day 7 (personal communication A. Hurlstone). Still, the question remains whether these cells are fully functional at this age.

TCRβ chain expression in zebrafish is detected already at 5 dpf (Schorpp et al., 2006), however, this is not known for TCRα chain expression.

To date, not much is known on the presence and functionality of T cell subsets. Foxp3+ cells have been reported and transcription factors *t*-bet and gata3 have been identified in zebrafish, but extensive functional data on possible T cell subsets is mostly lacking (Mitra et al., 2010; Quintana et al., 2010). This is currently an active area of study, so it is anticipated more discoveries will be made in the near future. For example, recently Yoon and co-workers demonstrated for the first time antigen induced cytokine expression by CD4-1+ lymphocytes in zebrafish (Yoon et al., 2015).

There is also still some discussion on the time of appearance and origin of B cells. Danilova and Steiner observed Rag-1 expression in the zebrafish pancreas by day 4 (whole mount in situ hybridisation) (Danilova et al., 2000). However, Lam and colleagues only detected IgLC-1, 2 and 3 expression in the head kidney and the thymus from 3 wpf (Lam et al., 2004). Furthermore, by using Rag-2:GFP larvae, which exclusively express GFP on B cells in the kidney, showed GFP signal in the kidney (first extrathymic site) at 8 dpf and not in the pancreas (Trede et al., 2004). As for the maturity of B cells, transcripts for membrane and surface IgM were found at 7–13 dpf (Langenau et al., 2004; Trede et al., 2004). Still, positive staining for membrane-IgM was observed from 19 dpf in the head kidney (Danilova et al., 2000). So while transcripts are present during the larval stage, positively stained B cells clearly appear later. In a recent study by Page et al. generation of an IgM1:eGFP transgenic fish showed that the earliest IgM + B cells appear between the dorsal aorta and posterior cardinal vein and in the kidney around 20 dpf by using IgM1:eGFP; rag2:DsRed via their differential expression of *IgM*, *rag2*, and *pax5*. Furthermore, the authors identified pro-B, pre-B, and immature/mature B cells in the adult kidney, indicating that, like in birds and mammals, zebrafish show a shift in location of B-cell development between the embryo and adult (Page et al., 2013). Additionally, in contrast to the earlier observation made by Danilova and colleagues, Page and colleagues did not observe B cells in or around the pancreas at early time points.

Three types of immunoglobulin heavy chain classes have been described for zebrafish. Long it was thought that teleosts only had IgM and IgD, but in 2005 Danilova and colleagues reported on an additional immunoglobulin which they termed IgZ (Danilova et al., 2005). At the same time this class of immunoglobulins was discovered in trout and named IgT (Hansen et al., 2005). Analysis of expression revealed that in contrast to *ighm* expression which is detected from 4 wpf, expression of *ighz* is already prominent at 2 wpf.

Studies performed by Zhang et al. suggested that IgT might be a mucosal antibody in teleost fish, resembling IgA in mammals (Zhang et al., 2010). They detected responses of rainbow trout IgT to an intestinal parasite specifically in the gut. In contrast, IgM responses were only present in the serum. Furthermore, IgT of rainbow trout coated most intestinal bacteria, similar to IgA in the mammalian gut. However, expression of the previously reported IgZ in adult zebrafish was almost entirely localized to the pronephros, mesonephros (kidney) and thymus (primary lymphoid organs), arguing against IgZ being a mucosal antibody in zebrafish (Danilova et al., 2005). This apparent discrepancy seems to be solved by the discovery of a second IgZ-like isotype in zebrafish: IgZ-2, which shares 76.5% nucleotide sequence identity with the previously reported IgZ (Hu et al., 2010). IgZ-2 phylogenetically groups together with IgZ and other known teleost IgT/Z-like sequences. IgZ-2 are expressed in immune-related tissues, and could be up-regulated by *in vivo* stimulation with LPS in various tissues including mucosal tissues such as the intestine, skin, and gills (Hu et al., 2010).

In conclusion, evidence for the age at which functional maturity of the B and T cell compartment is reached is still limited. Lam and colleagues showed that humoral responses (IgM) to formalin-killed *Aeromonas hydrophila* (T cell independent) and human gamma globulin (T cell dependent) could not be observed before 4 wpf, indicating that the IgM response in zebrafish immune system is morphologically and functionally mature by 4–6 wpf (Lam et al., 2004). However, these studies were conducted before identification of IgZ. IgZ-2 transcripts are detected already at 2 wpf which might indicate a faster development of this response (Hu et al., 2010). Based on these data, for now it is relatively safe to state that in the first two weeks of life, zebrafish solely rely on innate immune mechanisms for their defence.

4.2. Innate immunity

Antimicrobial peptides (AMPs), produced by for example the intestinal epithelial cells, form a first line of defence in the larval zebrafish gut. AMPs are potent, broad spectrum antimicrobials that can kill Gram-negative and Gram positive bacteria, enveloped viruses, fungi and even transformed or cancerous cells (Reddy et al., 2004). Multiple defensin-like genes have been discovered in zebrafish. Like vertebrate defensins, they are small, have a cationic charge, and six conserved cysteines. The identified fish defensin-like peptides mostly resemble beta-defensin family members of birds and mammals (Zou et al., 2007). Elevated expression of defensin beta-like is found in the mid-intestine. In this region also most immune cells and the vacuolated (M like) cells reside indicating again its role in immune function (Gebert and Jepson, 1996; Oehlers et al., 2011a; Rombout et al., 1985). Another AMP is hepcidin. Hepcidin is a peptide hormone, which modulates iron absorption and iron delivery to erythrocytes by binding the iron transporter ferroportin (Fraenkel et al., 2009). Zebrafish recombinant hepcidin-2 can very efficiently inhibit the growth of Escherichia coli and Vibrio anguillarum (Gram-negative), and Staphylococcus aureus and Bacillus subtilis (Gram-positive) because these bacteria need iron for their survival (Lin et al., 2014). Another potent mechanism of the innate immune system in zebrafish is ROS mediated killing of pathogens. ROS is generated by the NOX/DUOX family of NADPH oxidases (Niethammer et al., 2009). Zebrafish *Duox* is highly expressed in intestinal epithelial cells. It was shown that knockdown of *Duox* impaired larval capacity to control enteric Salmonella infection (Flores et al., 2010).

That zebrafish research unravels previously unknown mechanisms was nicely illustrated by the finding that alkaline phosphatases (an enzyme abundant in the brush border of the intestinal epithelial cells) can dephosphorylate and detoxify the endotoxin component of LPS (Bates et al., 2007). This immune function of alkaline phosphatase was previously unknown.

Homologues of pattern recognition receptors have been found in zebrafish. Several TLRs have been identified in fish including fish specific TLRs and ten orthologs of mammalian TLR genes (Jault et al., 2004; Meijer et al., 2004). Adaptor protein MyD88 and intracellular pattern recognition receptor Nod2 are present in zebrafish and their function is conserved (Oehlers et al., 2011b; van der Sar et al., 2006; van der Vaart et al., 2013). Several cytokines and chemokines are identified in zebrafish, some appear to be homologous to mammalian cytokines, although some caution is needed. In zebrafish, like in other fish species, several genes are duplicated and diversified which might have resulted in different functionality of these cytokine 'subtypes' (reviewed in (Secombes et al., 2011) and (Alejo and Tafalla, 2011)).

Interestingly, in contrast to mice, zebrafish have Cxcl8 (IL-8) that appears to be homologous also in its function to human CXCL8 (Brugman et al., 2014; de Oliveira et al., 2013; Oehlers et al., 2010).

Cxcl8 is a potent chemokine that is able to attract neutrophils (Deng et al., 2012, 2013; Oehlers et al., 2010). Zebrafish *cxcl8* is expressed in leukocytes and intestinal epithelial cells (Brugman et al., 2014; Oehlers et al., 2010). Zebrafish have four copies of *cxcl8* in their genome: *cxcl8-l1*(*cxcl8a*) and *cxcl8-l2* (*cxcl8b.1, b.2* and *b.3*) (de Oliveira et al., 2013; van der Aa et al., 2010). We have shown that expression of *cxcl8-l1* (*cxcl8a*) in the intestine is dynamic over time (Brugman et al., 2014). Under homeostatic conditions, we detected low expression levels at 1 wpf and at adult age (14 wpf), but increased expression at 5 wpf both in scattered granulocytic cells and in the epithelial cells. Interestingly, this increased expression of *cxcl8a* was not seen in Rag1-deficient zebrafish at 5 wpf and in situ hybridisation only showed staining of some scattered cells, while their epithelial cells were negative. Upon adoptive transfer of T lymphocytes (sorted from Lck:GFP transgenic fish) *cxcl8a* expression was induced in the intestines one week after transfer, and now expression was seen in both scattered cells and the epithelial cells, resembling wildtype animals at 5 wpf (Brugman et al., 2014). These results indicate that the presence of T cells in the intestines influences epithelial *cxcl8a* expression. This might result from a direct interaction of the T cell with the epithelial cell or indirectly by factors released from activated T cells in response to the gut environment upon their first arrival. Subsequent downregulation of *cxcl8a* expression might prevent continuous neutrophil recruitment to the gut, and might be a way to maintain homeostasis.

Just like in mammals, the process of hematopoiesis consists of different phases, gives rise to different myeloid lineages and takes place at different locations (Ellett and Lieschke, 2010; Jing and Zon, 2011). Development of different transgenic reporter zebrafish lines for different innate immune cells (such as the gata2:GFP, mpx:eGFP, and Mpeg1:GFP lines) enabled the study of ontogeny of the immune system (Balla et al., 2010; Ellett et al., 2011; Mathias et al., 2006; Renshaw et al., 2006).

Although a lot is known on hematopoiesis in zebrafish, here I will focus on what is known on innate immune cells in the intestines. Hematopoiesis consists of three phases. The last wave of definitive hematopoiesis, produces hematopoietic stem cells (HSCs). Around 26 hpf these HSCs first migrate from the ventral wall of the dorsal aorta (VDA) to the caudal hematopoietic tissue (CHT) and finally colonize the kidney via the circulation starting from 4 dpf (Ellett and Lieschke, 2010; Xu et al., 2012). At 30 h post fertilisation neutrophils migrate into the circulation from the intermediate cell mass (the region between the somites and the yolk sac). From this time you will also find neutrophils in the intestines, however, most will be present along the major blood vessels or in the surrounding tissues (Xu et al., 2012).

Primitive macrophages also appear before the onset of blood circulation. These primitive macrophages migrate to the brain to form the microglial cells, but are able to phagocytose and kill bacteria that are injected (Herbomel et al., 1999, 2001). The mpeg1 transgenic zebrafish that marks macrophages specifically (no overlap with the mpx-positive neutrophils) shows that macrophages are present, although in low numbers at 28 hpf throughout the embryo including the intestine, and that levels increase with time (Ellett et al., 2011). Recently, by using a transgenic line whose macrophages express tumour necrosis factor alpha (*tnfa*), Nguyen-Chi and coworkers reported the existing of subsets of macrophages (Nguyen-Chi et al., 2015). Using 4D-confocal microscopy, the authors showed that wounding and bacterial exposure triggered macrophage recruitment, and *tnfa* expression in some (but not all) of these macrophages. Fluorescence Activated Cell Sorting of *tnfa*+ and *tnfa*– macrophages showed that expression of *tnfa* correlated with M1 markers, while *tnfa*-negative macrophages expressed alternatively activated (M2) macrophage markers. Interestingly, M1 (*tnfa* expressing) macrophages converted into M2-like phenotype

during resolution of the inflammation (Nguyen-Chi et al., 2015).

Eosinophils are present the intestines, residing just under the epithelial cell layer. At 3 wpf, approximately one eosinophil is found per fold (villus), while at adult age around 2 to 3 cells per fold are observed under homeostatic conditions (Balla et al., 2010; Witte et al., 2014). The number of eosinophils however, do increase upon inflammation and can reach up to 10—20 per fold (Brugman et al., 2009; Witte et al., 2014). In concordance with these observations, the eosinophil reporter gata2:eGFP shows that in adults under homeostatic conditions, eosinophils are relatively scarce in spleen, gills, gut, skin, with the exception of the peritoneal cavity (Balla et al., 2010). The authors suggest that the fact that eosinophils are so abundant in the peritoneal cavity can be attributed to the specific micro-environment, especially since in teleosts the peritoneal cavity is one of the major sites for parasite infection.

Mast cells have also been reported in zebrafish. They rely on gata2 and pu.1 for their development and carboxypeptidase a5 (cpa5) is a specific marker for these cells (Da'as et al., 2011; Dobson et al., 2008). The dependence on gata2 suggests that the eosinophil reporter line might also stain mast cells. Cpa5 expression is seen in blood cells from 24 h post fertilization. Cpa5+ cells are found in the intestines and gills in the adult zebrafish (Dobson et al., 2008).

With the discovery of novel immune-type receptors (NITRs) that resembled ITAM-containing NK receptors which could confer lysis capability to NITR expressing cells, the existence of NK cells in zebrafish was suggested (Yoder et al., 2008, 2004, 2010). NITR expression is highest in lymphocytes (Yoder et al., 2010). Evidence for the existence of NK-like cells came from the analysis of rag1-deficient zebrafish. Petrie-Hanson and co-workers showed that rag1 mutants still have lymphocyte-like cell population (Petrie-Hanson et al., 2009). This lymphocyte-like cell population made up of 7% of total cells in the kidney and expressed mRNA encoding Non-specific Cytotoxic cell receptor protein-1 (NCCRP-1) and Natural Killer (NK) cell lysin, but lacked T cell receptor (TCR) and immunoglobulin (Ig) transcript expression. These cells might well be the evolutionary precursors of the NK cells (or ILCs) we discriminate in mice and humans.

Despite all the major advances in discovery of all these different innate cell types one very important cell involved in intestinal immunity was still missing: the dendritic cell (DC). In 2010, the group of David Traver described an antigen presenting cell (APC) subset greatly resembling DCs (Lugo-Villarino et al., 2010). The frequency of these cells is however very low (0.18 ± 0.1%, within whole kidney marrow). The majority of DC-like cells were found within the myelomonocyte fraction (2.05 ± 0.2% of cells within this population). These DC like cells displayed expression of DC genes like il12, MHC class II invariant chain iclp1, and csf1r. Additionally, activation of T lymphocytes by these cells was achieved in an antigen-dependent manner. Construction of a mhc2dab transgenic fish revealed upon cross with cd45:DsRed transgenics that in peripheral organs these (APC-like) cells were most abundant in the intestine and spleen (3 and 5% DCs, resp.) (Wittamer et al., 2011).

While the presence of APCs has been shown, no gut draining lymph nodes are present in zebrafish. This suggests that APCs and adaptive immune cells meet in the spleen, the only secondary lymphoid organ. In addition, since DCs and other APCs such as B cells are present in the intestine, antigen presentation might take place there independent of organized lymphoid tissue. With the increasing interest in using the zebrafish as a model for disease, new breakthroughs in this area are expected in the near future.

With the sequential development of innate and adaptive immunity, presence of homologues of all innate and adaptive immune cell types and intestines that resemble those of higher vertebrates both functionally and morphologically, the zebrafish offers an interesting novel platform to interrogate cells and pathways involved in intestinal inflammatory diseases.

5. Intestinal inflammation

To make use of the advantage of zebrafish transparency several groups have developed intestinal inflammatory models using zebrafish larvae and adults (Table 1). Together with the development of transgenic reporter fish for the different innate immune cells, for the first time cell recruitment can be monitored in vivo in health and during intestinal inflammation.

Even before most transgenic zebrafish were available, Fleming and colleagues pioneered the field by using the hapten 2,4,6-trinitrobenzenesulfonic acid (TNBS), used in experimental mouse models, to induce intestinal inflammation in zebrafish (Fleming et al., 2010). After immersion of fish in 75 μg/ml of TNBS from 3 to 8 dpf, they observed an expanded gut lumen, disappearance of intestinal folds (villi), increased goblet cell number and positive tumor necrosis factor alpha (TNF-alpha) staining in the epithelium already at 5 dpf. Prednisolone and 5-amino salicylic acid, two drugs prescribed to IBD patients, ameliorated TNBS induced disease in zebrafish larvae. This confirmed that indeed gut physiology and pathology relevant to the human disease state could be modeled in zebrafish larvae.

Around the same time the laboratory of Phil Crosier in New Zealand confirmed the impaired intestinal homeostasis and inflammation in larval zebrafish exposed to TNBS (Oehlers et al., 2011c). Larvae immersed in a solution of TNBS (for 3 days at 50 μg/ml) showed marked induction of pro-inflammatory marker mmp9 and leukocytosis. Since larval immersion could result in damage not only to the intestines but also the skin, they optimized the protocol to induce only intestinal damage without skin damage. Larvae exposed to 75 μg/ml TNBS for three days demonstrated widespread skin damage. Larvae exposed to lower doses of TNBS or to the 75 μg/ml dose for less than three days did not show skin damage. In contrast to the findings of Fleming et al., they did not observe the increase in goblet cells, nor did they observe gross changes to intestinal morphology. However, a significant increase in the number of proliferating cells was observed in the TNBS exposed larvae. Interestingly, like in mice and humans, enterocolitis was dependent on the microbiota and Toll-like receptor signaling, since knockdown of Myd88 resulted in increased mortality upon TNBS exposure. Antibiotic treatment ameliorated the inflammation, since fish co-treated with ampicillin and kanamycin (next to TNBS) did not initiate the transcription of pro-inflammatory cytokines il-1b, tnf-a, ccl20, and cxcl8-l1 (cxcl8a) (Oehlers et al., 2011c).

In a follow-up study Oehlers and colleagues went on to develop another larval model for enterocolitis using DSS and compared the resulting enterocolitis with TNBS-induced enterocolitis (Oehlers et al., 2012, 2013). The DSS model, like the TNBS model showed neutrophilic inflammation that was microbiota dependent. However, the DSS model also showed marked differences from the TNBS induced model: DSS induced colitis showed bacterial overgrowth, since enumeration of bacteria from whole larval homogenates revealed significantly higher counts than either untreated or TNBS treated larvae after 3 days of exposure. The increased production of nitric oxide (NO) seen after 3 days of exposure to TNBS is not seen when larvae are exposed to DSS. An upregulation of pro-inflammatory cytokine and marker gene expression of ccl20, il1b, il23, cxcl8-l1, mmp9 and tnfa is seen as well as reduced proliferation as measured by PCNA transcription. The reduced proliferation was confirmed by BrdU staining and enumeration. whole-mount specimens. A prominent feature of DSS-exposed larvae is the appearance of increased alcian blue staining in the intestinal bulb, however, no difference in the number of mucus-producing goblet cells was reported. Interestingly, antibiotic treatment given

Table 1
Summary larval and adult models for enterocolitis induction in zebrafish.

Treatment	Duration and dose	Characteristics	Ref
TNBS	Larval immersion 3-8 dpf, 75 µg/ml	- expanded gut lumen - disappearance villi (folds) - increase goblet cells - increase TNFα	Fleming et al., 2010
TNBS	Larval immersion 3-6 dpf, 50 µg/ml	- increase intestinal leukocytes - increase proliferating (PCNA+) cells - increased NO production - proinflammatory cytokines	Oehlers et al., 2010
DSS	Larval immersion 3-6 dpf, 0.5%	- increase intestinal neutrophils - bacterial overgrowth - increase pro-inflammatory cytokines - decrease proliferating (PCNA+) cells - increase Alcian Blue (mucus) staining - no alteration goblet cell number	Oehlers, 2011a,b,c, 2012
Oxazolone	Single intra-rectal injection 3 mpf, 0.2% oxazolone in 50% ethanol (w/v)	- increased intestinal neutrophils - inreased intestinal eosinophils - epithelial disruption (loss and thickening villi) - alteration goblet cells (depletion and increased mucus production observed)	Brugman et al., 2009
TNBS	Single intra-rectal injection 3 mpf, 40 mM-320 mM TNBS in 30% Ethanol	- increased intestinal neutrophils - increased pro-inflammatory cytokines - epithelial disruption (thicker and shorter villi) - no change in goblet cells	Geiger et al., 2013

together with DSS prevented this increased alcian blue staining, demonstrating that this mucus-phenotype is a result of the combination of DSS treatment and presence of the microbiota (Oehlers et al., 2012, 2013).

He et al. showed that induction of TNBS colitis in larvae (75 µg/mL TNBS from 3 to 8 dpf) at least partly required the presence of microbes, since in germfree zebrafish the inflammation was less extensive (He et al., 2014). Goblet cell numbers were still increased in germfree zebrafish exposed to TNBS when compared to healthy fish, but less increased compared to conventionally raised (not germ-free) TNBS exposed zebrafish. Furthermore, the authors found a striking reduction in epithelial TNF-α production in germfree zebrafish exposed to TNBS (He et al., 2014). From these studies it is clear that enterocolitis induction is dependent on the microbiota. However, this also suggests that each laboratory has to optimize these models for their specific (microbial) environment.

To further investigate the role of the microbial composition, and model enterocolitis development in the presence of both innate and adaptive immunity, we set-up an enterocolitis using intra-rectal injection of 0.2% oxazolone (Fig. 3) (Brugman et al., 2009). In this study, we exposed adult zebrafish to different antibiotics (colistin or vancomycin) before oxazolone injection, thereby skewing the microbiota either towards a community in which γ-Proteobacteria predominated (colistin) or to one in which Fusobacteria predominated (vancomycin). Pretreatment of vancomycin was able to prevent development of overt inflammation upon oxazolone injection. Colistin pre-treatment, however, while it reduced the number of eosinophils, did not reduce the percentage of neutrophils, and enterocolitis score. The protection from enterocolitis we observed when pretreating our fish with vancomycin associated with a reduction of neutrophil presence in the gut towards levels seen in non-injected animals (Brugman et al., 2009).

Recently, Geiger et al. developed another enterocolitis model in adult zebrafish using TNBS (Geiger et al., 2013). Six hours after rectal injection of TNBS, a significant influx of neutrophils was seen in the intestines as well as upregulation of pro-inflammatory and anti-inflammatory cytokines and disruption of the intestinal villi. In contrast to the oxazolone model, no goblet cell alterations were
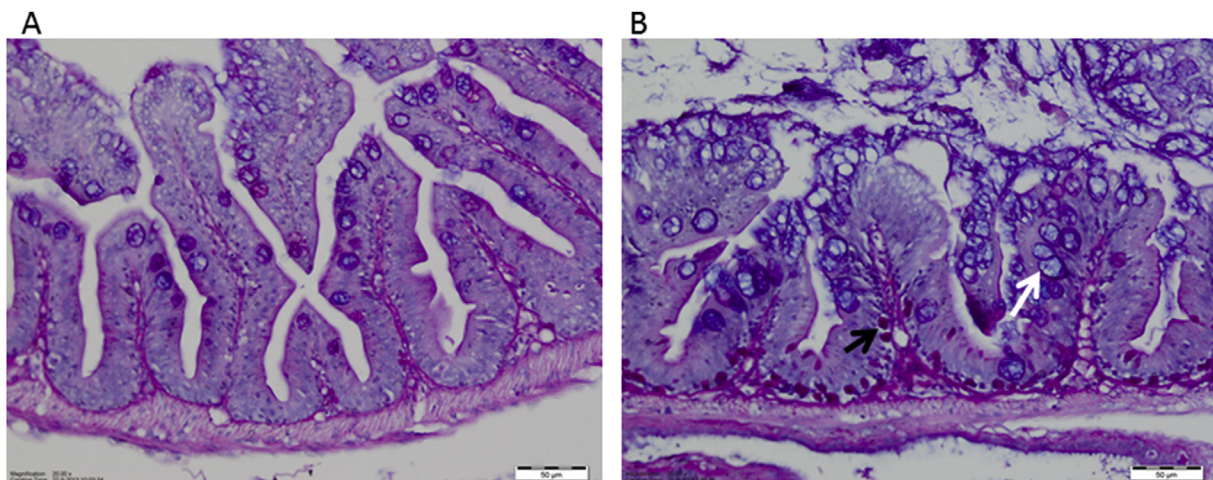

Fig. 3. Example of healthy (A) and inflamed (B) proximal midgut in adult zebrafish intestines. Alcian Blue-Pas staining on paraffin embedded sections. Note the thickening of the villi and infiltrating eosinophils (pink cells, black arrow) as well as increased mucus production (blue staining) and enlarged goblet cells (white arrow) (B).

observed. Interestingly, they went on to show that melanin-concentrating hormone (MCH), an evolutionarily conserved appetite-regulating neuropeptide that is found upregulated in colitis patients, was also upregulated in the TNBS-treated zebrafish (Geiger et al., 2013). Like in the oxazolone-induced adult model, vancomycin pre-treatment resulted in less severe enterocolitis, indicating again that zebrafish enterocolitis is dependent on the microbiota.

Investigating the presence of microbial dysbiosis during zebrafish enterocolitis, He and coworkers showed that the gut bacterial community during TNBS-induced colitis was characterized by an increased abundance of Proteobacteria (especially Burkholderia) and a decrease in Firmicutes (Lactobacillus group) (He et al., 2013). This increased abundance of proteobacteria is also observed in patients with active IBD (Frank et al., 2007; Manichanh et al., 2006). Whether these differences exist in these patients before onset of disease, or whether this is the result of the inflammatory environment favoring these species is yet unknown. The previous studies at least show that the microbial composition at the time of intestinal injury is an important determinant in severity of enterocolitis development.

Overall, these zebrafish models of chemically induced intestinal inflammation resemble inflammation seen in higher vertebrates in terms of morphological changes of intestinal tissue, microbial involvement and response to anti-inflammatory drugs such as prednisolone and 5-amino salicylic acid (5′-ASA). The optical transparency early in life and sequential development of innate and adaptive immunity enables in depth study into inflammatory responses in the gut in great detail. The zebrafish model therefore can be an important tool to elucidate the consequence of disturbances in so-called IBD susceptibility genes or identifying new ones.

6. Genetic susceptibility underlying intestinal inflammation

To date, more than 160 genes have been identified to associated with either Crohn's disease or Ulcerative colitis or both and this list is expanding (Van Limbergen et al., 2014). Now that the zebrafish has proven itself as a novel model to study disease pathogenesis, subsequently we can use the zebrafish to model genetic susceptibility in evolutionary conserved genes.

Marjoram and colleagues performed a forward genetic screen to identify mutants with defects in intestinal epithelial integrity (Marjoram et al., 2015). One of the mutants, named aa51.3[pd1092], showed a significant disruption of the gut epithelium. Analysis of the affected locus revealed a mutation in ubiquitin-like protein containing PHD and RING finger domains 1 (uhrf1), a highly conserved gene involved in methylation regulation, leading to a loss of function. Mutated uhrf1 leads to reduced TNF-α promoter methylation (reduced inhibition) and therefore an increase in TNF-α expression in intestinal epithelial cells. This increased expression led to shedding and apoptosis of epithelial cells, immune cell recruitment, and barrier dysfunction, hallmarks of chronic inflammation, which was microbiota dependent (Marjoram et al., 2015). This study therefore has uncovered a possible susceptibility gene for IBD.

Another forward genetic screen identified a mutant cdipt[hi559] which lacks phosphatidylinositol (PI) synthesis (Thakur et al., 2011). Disturbance of PI signalling has been identified in gastrointestinal disease and inflammation (van Dieren et al., 2011). The cdipt[hi559] mutants showed abnormal villous architecture, depletion of goblet cells (apoptosis), reduced mucus secretion, bacterial overgrowth and neutrophilic influx (Thakur et al., 2011). Additionally, acute phase genes were upregulated as well as endoplasmatic reticulum stress markers such as hspa5 and xbp1. This study, identified a previously unknown link between intracellular PI signaling and ER-stress-mediated mucosal inflammation.

Non-steroidal anti-inflammatory drugs (NSAIDs) can lead to impaired mucosal barrier function (Morteau et al., 2000). Goldsmith and colleagues developed a model for NSAID-induced intestinal injury using zebrafish (Goldsmith et al., 2013). Zebrafish were exposed at 5.5 dpf to the NSAID glafenine (25 μM) for 12 h. Glafenine treatment resulted increased intestinal epithelial apoptosis and ER stress, but did not reduce epithelial barrier function during acute damage. Interestingly, the μ-opioid agonist DALDA protected from glafenine-induced injury by inducing the unfolded protein response (UPR). This UPR usually halts protein translation, degrades misfolded proteins, and activates molecular chaperones involved in protein folding. These last two studies nicely illustrate the usefulness of the zebrafish for the study of (ER-) stress-mediated human gastrointestinal diseases.

Recently, mutations in the genes encoding for Macrophage stimulating protein (Msp) and its receptor Ron (recepteur d'origine nantais) were identified as risk factors in several patient cohorts suffering from IBD (Barrett et al., 2008; Goyette et al., 2008). A forward genetic screen yielded zebrafish with a premature stop mutation in the MSP gene (msp[t34230]) (Huitema et al., 2012). Interestingly, these zebrafish displayed increased numbers of eosinophils in the intestines, increased mmp9 expression, goblet cell alterations and increased intestinal epithelial proliferation spontaneously. Additionally, intra-rectal ethanol administration (20%) results in reduced survival and prolonged inflammatory cytokine responses in some but not all Msp-deficient zebrafish (Witte et al., 2014). Currently, we are investigating innate cell recruitment and barrier dysfunction in the Msp as well as the receptor (Ron) mutant to investigate how disturbance of this pathway leads to chronic inflammation.

7. Future perspectives

A growing amount of studies continues to show the power of the zebrafish model when investigating conserved pathways in gut epithelial homeostasis and inflammation. Currently, we are entering a new era in which the laborious forward genetic screens are supplemented by the highly efficient CRISPR-Cas9 and TALENs genome editing techniques that enable rapid and highly specific gene targeting (Dahlem et al., 2012; Ota et al., 2014). Together with the high fecundity of zebrafish and the availability of transgenic reporter lines this will enable in depth study of several IBD susceptibility genes as well as other pathways involved in mucosal immune homeostasis. Moreover, these zebrafish will provide an excellent platform to develop molecularly targeted therapies by providing a high throughput screening tool in the search for novel compounds that might reverse gene defects associated with intestinal diseases.

Importantly, not only is the zebrafish model useful in the study into underlying pathways of human disease, it can also serve as a great model to study food-induced intestinal inflammation in aquaculture fish species such as Atlantic Salmon. To date, fish represent 16 percent of all animal protein consumed globally and with an increasing human population of an estimated 9 billion by 2050, it is expected to grow further. Production of aquacultured (farmed) fish is rising rapidly and is estimated to equal the production of captured fish in 2030 (FISH TO 2030 Prospects for Fisheries and Aquaculture WORLD BANK REPORT NUMBER 83177-GLB). Fishmeal is the main protein component of many aquaculture diets, however is costly and is dependent on by-catch. Plant-based alternatives to fishmeal, have shown that inclusion of 20% soybean meal in Atlantic salmon feed induced intestinal inflammation (Uran et al., 2008, 2009). Recently, in an initial set of experiments, it was shown that similar to Atlantic salmon, zebrafish also develop

intestinal inflammation as a result of soybean in the diet (Fuentes-Appelgren et al., 2014; Hedrera et al., 2013). Hedrera and colleagues showed that zebrafish larvae that were fed with soybean meal developed a clear intestinal inflammation as early as two days after start of the diet. Understanding the fundamental pathways underlying intestinal inflammation in fish is therefore not only of importance to the medical field but will also be of great importance in maintaining sustainable food chains supplying healthy food to a growing world population.

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