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A model 450 million years in the making: zebrafish and vertebrate immunity

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Since its first splash 30 years ago, the use of the zebrafish model has been extended from a tool for genetic dissection of early vertebrate development to the functional interrogation of organogenesis and disease processes such as infection and cancer. In particular, there is recent and growing attention in the scientific community directed at the immune systems of zebrafish. This development is based on the ability to image cell movements and organogenesis in an entire vertebrate organism, complemented by increasing recognition that zebrafish and vertebrate immunity have many aspects in common. Here, we review zebrafish immunity with a particular focus on recent studies that exploit the unique genetic and in vivo imaging advantages available for this organism. These unique advantages are driving forward our study of vertebrate immunity in general, with important consequences for the understanding of mammalian immune function and its role in disease pathogenesis.

Introduction

Similar to all other multicellular organisms, we spend our lives continuously interacting with a broad range of unicellular organisms: some beneficial, many potentially deadly. To survive in the midst of this continual competition, vertebrates have evolved a highly complex immune system that can recognise many components of foreign organisms, and remove those that are threatening. This system can 'learn' at two levels to better recognise and neutralise threats: the adaptive arm of the immune system can learn to respond better to threats it has seen before at the level of an individual organism, whereas, at a population level, natural selection shapes the arm of the immune system that learns to recognise pathogens over evolutionary time – the innate immune system.

Even in the relatively sterile environment of a modern laboratory or operating room, tissue damage would be invariably followed by infection were it not for the robust action of the immune system. Because pathogen numbers can increase rapidly, vertebrate immunity must be able to detect tiny numbers of pathogens, but also to recognise environments in which pathogens might be trying to establish a foothold – including damaged tissues. Thus, recognising tissue damage and recognising pathogens are part of the same process. The machinery used for identifying the 'danger signals' produced in these two contexts is shared, and is conserved over large stretches of evolutionary time. Recognition of these danger signals needs to be translated into an effective response, and a broad range of mechanisms exists to do this efficiently. The immune response is a complex and flexible series of cellular and signalling events that alerts the organism to the presence of infection and sends cellular or humoral effectors to combat the pathogen. The cells of the adaptive and innate immune systems are distinct (granulocytes and macrophages are traditionally considered innate immune cells, and lymphocytes considered adaptive immune cells), but there is important crosstalk between these systems. For example, neutrophils have receptors for antibodies produced by B cells, and dendritic cells (DCs) recognise pathogens innately and then present their antigens to cells of the adaptive immune system.

Thus, our day-to-day survival depends on a robust immune response, but this response must be under strict control to ensure that unwanted host damage does not accompany pathogen clearance. Infectious diseases are still a threat, with the spectre of untreatable diseases such as extreme drug-resistant tuberculosis (TB) ever closer, but we have also witnessed the emergence of diseases associated with overactive immunity, such as asthma, inflammatory bowel disease, atherosclerosis, chronic obstructive pulmonary disease and rheumatoid arthritis; these are just select examples of the many autoimmune and inflammatory diseases we now face. Treatment of these diseases often demands toxic immunosuppressive therapies, and we remain unable to balance this toxicity against the certainty of cure. There is therefore an urgent need to better understand the molecular processes of host-pathogen interactions, and of immune system regulation, in order to develop new approaches to treat infectious and autoimmune and/or inflammatory diseases. As such, there is a pressing demand to extend the range of models available to study immunity to infections and their treatment.

Our knowledge of the molecular controls of immune processes has been acquired by the study of a range of vertebrate models and in vitro systems, predominantly mouse models and in vitro primary immune cell culture. To avoid some of the limitations of these

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models, researchers have begun to study immunity in non-mammalian models. Given the relatively recent evolutionary increase in complexity of the immune system, there are clear benefits to the zebrafish over other non-mammalian models. Of these, two stand out for us: the capacity to carry out in vivo imaging and the availability of powerful genetic tools. Previous authors have reviewed zebrafish immunity in depth (Ward and Lieschke, 2002; Traver et al., 2003; Trede et al., 2004; Lieschke and Currie, 2007; Lesley and Ramakrishnan, 2008; Meeker and Trede, 2008; Sullivan and Kim, 2008; Meijer and Spaink, 2011); in this review, we aim to illustrate how these two main advantages, in concert, allow new experimental approaches using this model system that can advance our understanding of the components of vertebrate immunity. In many areas, these new approaches are already delivering new advances to our understanding of immunity more generally; in others, the potential for rapid advances is clear.

Granulocytes: the first wave

Recognition of tissue damage and infection

Immune cells with a granular cytoplasmic pattern on traditional histochemical stains are termed granulocytes, and include neutrophils and eosinophils. The neutrophil is the most numerous innate immune cell, and is the dominant leukocyte in zebrafish larvae from 2 days post fertilisation (dpf) (Ellett et al., 2010) and in adult mammals. The neutrophil is a key cellular effector of the inflammatory response. Of the cardinal features of inflammation, heat, swelling and redness arise from the vasodilation and increased vascular permeability of the capillary bed, caused by the release of local inflammatory mediators. One of the key functions of this inflammatory response is the delivery of neutrophils into damaged or infected tissues. A robust response to tissue injury or infection must include the recruitment of cells of the innate immune system to the affected site through chemotactic stimuli. Neutrophils are recruited to sites of infection or tissue injury by responding to gradients of chemoattractant molecules, which include the contents of damaged cells, bacterial products and chemokines produced by the host in response to injurious stimuli. Recently, experiments in zebrafish have yielded new insights into this process, specifically the discovery of hydrogen peroxide (H_2O_2) release by injured tissues. By unknown mechanisms, cellular damage leads rapidly to activation of dual oxidase (Duox), which in turn establishes a tissue gradient of H_2O_2. This gradient was known previously and was assumed to have a direct antibacterial function. However, studies in transparent zebrafish larvae using in vivo genetic reporters have clearly demonstrated that this H_2O_2 forms a transient gradient that recruits neutrophils in the first minutes of the immune response (Niethammer et al., 2009). This transient but important phenomenon is likely to be conserved over evolution, and might represent an evolutionarily ancient 'danger signal' (Matzinger, 2002). This experiment is a revealing illustration of how the transparency and genetic tractability of the zebrafish combine to make a substantial advance in the understanding of immunity – one that might not have been made in such a timely fashion had we not been able to transcend the limitations of mammalian models. This observation in the zebrafish might have broad biological significance, but will need new mammalian models to confirm its applicability in higher vertebrates.

Following on from the initial H_2O_2 gradient, tissue injury causes the release of intracellular components, which further signal tissue damage. Some of these components are known to trigger the activation of Toll-like receptors (TLRs) or activate intracellular signalling molecules, including the nucleotide-binding-domain- and leucine-rich-repeat-containing family proteins (NLRs). The importance of these pathways is being defined in the zebrafish, and their contribution to host defence against infectious agents in vertebrates is being dissected in detail (Meijer et al., 2004; van der Sar et al., 2006; Hall et al., 2009; Sepulcre et al., 2009; Stockhammer et al., 2009; Sullivan et al., 2009; Liu et al., 2010; Palti, 2011). Zebrafish genetic knockouts of components of these pathways are eagerly awaited. Finally, chemokine signalling is currently being defined in the zebrafish model (Oehlers et al., 2009), and the rapid pace of research in this area suggests that major advances are not far away.

Inflammation resolution and immune cell removal

The fate of neutrophils following completion of their role in inflammation is an area of considerable importance in the clinical setting, where failure of normal inflammation resolution is considered a key part of the pathogenesis of a range of inflammatory diseases (Serhan et al., 2007). In mammalian systems, neutrophil apoptosis has been identified as a key part of inflammation resolution, although emerging evidence is suggesting that not all neutrophils die at the site of inflammation (Buckley et al., 2006; Uller et al., 2006). It is obvious from inspecting time-lapse datasets generated using transgenically labelled zebrafish neutrophils that these cells migrate away from the inflammatory site (Mathias et al., 2006); the importance of this phenomenon is under exploration. Work from several groups has described this 'reverse migration' in zebrafish, and transgenic lines expressing new photoconvertable pigments in myeloid cells are helping to define the role of this process in inflammation resolution (Elks et al., 2011; Yoo and Huttenlocher, 2011). Importantly, activation of genetic pathways involved in in vivo inflammation [specifically the hypoxia-inducible factor (HIF) signalling pathway] has recently been shown to alter the reverse migration of neutrophils (Elks et al., 2011). A picture is beginning to emerge that neutrophils might receive signals that influence both their ability to move away from inflammatory sites and simultaneously prolong their life span. Although it might be true that some of these features are specific to teleosts, current advances in mammalian in vivo imaging are confirming the importance of these observations in mammalian systems (Woodfin et al., 2011).

Eosinophils and other immune cell types

Eosinophils are important in host defence against parasites, and also have a central role in the aetiology of human asthma and other allergic diseases. In the zebrafish, the eosinophil has only been extensively characterised in the last year with the generation of a *gata2* transgenic line that marks this population (Balla et al., 2010). This opens up a new area for the in vivo visualisation and study of eosinophil function, which has been notoriously difficult in mammalian systems. Cells sharing histochemical and biochemical characteristics with human mast cells have also been identified in the zebrafish (Dobson et al., 2008). The characterisation of these markers in the zebrafish has not yet led to the development of a

Table 1. Zebrafish transgenic lines for studying the immune system: approaches and examples

Approach	Examples	Advantages	Disadvantages
Small promoter	*Tg(rag1:EGFP)la5*[a] *Tg(rag2:EGFP)la6*[b] *Tg(rag2:EGFP-Bcl2)zdf9*[c] *Tg(mpx:GFP)uwm1*[d] *Tg(lyz:EGFP)nz117*[e] *Tg(NFkB:GFP)nc*[f] *Tg(Cμ:EGFP)*[g]	Flexible, relatively quick and easy; [*rag1* and *rag2* lines mark all immature lymphocytes; *Bcl2* transgene prevents apoptosis in immature lymphocytes; *Cμ:* expressed only by immature and mature B cells[g]]	[*rag* promoters: unpredictable specificity; also expressed in testes and olfactory bulb, but not expressed in mature lymphocytes]
Large PCR-generated promoters	*Tg(lck:EGFP)*[h]	[*lck* promoter is specifically expressed in immature and mature T cells]	[*lck* line shows some expression in muscle]
BAC	*Tg(mpx:GFP)i114*[i] *Tg(Gata2:eGFP)la3*[j] *Tg(fms:Gal4)i186*[k]	High specificity	Technically demanding; time consuming; low efficiency of transgenesis; [*fms* expressed in xanthophores]
iTol2 BAC	None published in immune system to date	Markedly increased efficiency of transgenesis; other advantages of BACs retained	Requires second targeting; new technology
Tol2kit/Gateway	*Tg(mpeg1:EGFP)*[l] *Tg(mpeg1:mCherry)*[l]	Highly flexible; high efficiency of transgenesis	Expensive reagents
Gal4/UAS	*Tg(fms:Gal4)i186*[k] *Tg(mpeg1:Gal4)*[l]	Strong expression of transgene; flexibility: one good *Gal4* line can be crossed to many different *UAS* lines	*UAS* silencing; may be overcome by new non-repeating *UAS*
Cre driver lines	Double *Tg(rag2:lox-dsRED2-lox-EGFP-mMyc);Tg(hsp70:Cre)*[m]	Allows tissue-specific expression after elimination of sequences flanked by *loxP* sites	Specific genes cannot yet be excised by this method

Specific advantages and disadvantages of individual lines are shown in square brackets. [a]Jessen et al., 1999; [b]Jessen et al., 2001; [c]Langenau et al., 2005; [d]Mathias et al., 2006; [e]Hall et al., 2007; [f]Kanther et al., 2011; [g]David Traver, personal communication; [h]Langenau et al., 2004; [i]Renshaw et al., 2006; [j]Balla et al., 2010; [k]Gray et al., 2011; [l]Ellett et al., 2010; [m]Feng et al., 2007.

transgenic in which to study these cells, but such a development is eagerly awaited. Meanwhile, more detailed characterisation is revealing the extent to which these cells are similar to mammalian mast cells (Da'as et al., 2011).

Advances in imaging drive new discoveries in innate immunity

Neutrophil granulocytes are known to be important for host defence against bacterial and fungal pathogens, and our own clinical experience regularly brings home to us how dependent we are on adequate neutrophil function[†]. In zebrafish models of host defence, there has been a lack of clarity on the phagocytic role of neutrophils (Le Guyader et al., 2008; Prajsnar et al., 2008; Brannon et al., 2009), and it has been suggested that, in some circumstances, macrophages are the predominant larval phagocyte. The reason for this lack of clarity has recently been identified following a serendipitous discovery by Herbomel and colleagues that showed how the context of the encounter between host and pathogen can influence the outcome of the encounter. They found that bacteria injected into fluid environments, such as the blood or the developing ear vesicle, were almost exclusively phagocytosed by macrophages, but that when bacteria were presented on tissue surfaces, they were efficiently taken up by neutrophils (Colucci-Guyon et al., 2011). These findings had been previously identified in human and mammalian systems, but had been forgotten, and are clearly important for understanding neutrophil responses to abscess formation, biofilms and in designing indwelling or prosthetic devices for which infection is such an important problem. This example demonstrates how the superb imaging potential of the zebrafish can enable suitably prepared researchers to make crucial advances in understanding a fundamental biological process such as phagocytosis.

The ability to visualise inflammatory processes at the level of individual cells has enabled important advances in neutrophil biology, and the Huttenlocher laboratory has taken this a step further and looked at the subcellular level at activation of important intracellular signalling systems during inflammation in vivo. In elegant studies investigating phosphatidylinositol 3-kinase (PI3K) pathways (Yoo et al., 2010), the activation of PI3K in individual cells was visualised in vivo by expressing the Pleckstrin homology (PH) domain of AKT fused to EGFP in neutrophils. Using ratiometric comparisons to an mCherry construct, activation of PI3K was visualised at the leading edge during neutrophil migration. This was true for forward and reverse migration, as well as for the random movement of unstimulated leukocytes in the head mesenchyme. Furthermore, the dynamics of F-actin were visualised in vivo, revealing for the first time the real-time kinetics of actin polymerisation and its relation to neutrophil migration in vivo. The novel observation that PI3K can regulate F-actin in a manner that is dissociable from Rac-induced membrane protrusion is another example of how the unique properties of the zebrafish system are driving forward our understanding of vertebrate immunity.

Advances in genetic techniques reveal new inflammatory pathways

The advances in our understanding of neutrophil biology described above have been driven by the ability to visualise inflammatory cells in vivo, in combination with the ability to generate tissue-specific

[†]N.T. is a paediatric haematologist/oncologist and regularly renders his patients neutropenic as a side effect of treatment. S.A.R. is a chest physician and is called on to see such patients in his own hospital.

Table 2. Approaches to mutagenesis screens for immune system genes in zebrafish

Approach	Examples	Advantages	Disadvantages
ENU mutagenesis	Innate immunity: persisting neutrophilic inflammation[a]; altered myeloid cell development[b] Adaptive immunity: lymphocyte-specific mutant (*Ikaros*)[c]; collection of mutants with thymus defects in zebrafish[d] and medaka[e]	High mutation rate; well-established and validated technique	Technique can be labour-intensive; identification of mutation can be time-consuming [Screens have so far mainly identified housekeeping genes affecting thymus organogenesis]
Retroviral insertional mutagenesis	Innate immunity: early mitotic inhibitor 1 (*Emi1*)[f] Adaptive immunity: *grechetto*[g]	Ease of cloning affected gene	Lower mutation rates than ENU [Screen designed to select for embryonic lethality, so resulting phenotypes are pleotropic[h]]
Transposable element insertional mutagenesis	No published screens in the field of immunity	Easier identification of mutation	Use in immune screens yet to be established

Specific disadvantages of particular approaches for studying adaptive immunity are shown in square brackets. [a]Martin and Renshaw, 2009; [b]Hogan et al., 2006; [c]Schorpp et al., 2006; [d]Trede, 2007; Trede et al., 2008; [e]Iwanami et al., 2004; Iwanami et al., 2008; [f]Rhodes et al., 2009; [g]Bolli et al., 2011; [h]Amsterdam et al., 1999.

transgenics. Generating transgenics in zebrafish is now becoming commonplace; the various approaches and their relative merits are set out in Table 1.

In addition to advances made using transgenic constructs, the zebrafish model is well suited to unbiased screening to identify the pathways involved in important biological pathways. This includes both genetic and so-called 'chemical-genetic' approaches. These approaches are often combined with in vivo cell labelling using transgenic lines to visualise individual cell populations in mutant or chemically altered populations. To date, we are aware of four genetic screens for neutrophil specification and function, two of which have been formally reported (Table 2). An insertional mutagenesis screen performed in the laboratory of Nancy Hopkins (Amsterdam et al., 1999) has to date revealed three distinct proinflammatory phenotypes (Carney et al., 2007; Mathias et al., 2007; Dodd et al., 2009; Walters et al., 2009) with chronic inflammation in either the tailfin, the muscle or the epidermis. In addition, ENU mutagenesis screens have been performed in a number of laboratories. The first reported was the Melbourne Myeloid screen, which screened haploid ENU mutants for altered expression of myeloid markers and identified a number of interesting mutants, including a new allele of *lost-a-fin* (*laf*), mutants of which had defective myeloid cell development (Hogan et al., 2006). The results of other screens are awaited, including screens of altered Sudan Black staining (Michael J. Redd, personal communication) and of persisting neutrophilic inflammation (Martin and Renshaw, 2009). Such mutants can be combined with transgenic lines to enable detailed phenotyping of the immune defect. Identification of interesting genotypes is expected to bring significant advances to our understanding of the key regulators of myeloid cell production and function.

Genetic screens carried out in the context of infection have also recently delivered substantial advances. The Ramakrishnan group performed a gynecogenic diploid ENU screen for zebrafish mutants with defective host defence against *Mycobacterium marinum* (Mm; a close relative of the human pathogen *Mycobacterium tuberculosis*) and identified a range of mutant phenotypes (Tobin et al., 2010). The first mutant from this screen to be described in detail has a defect in LTA4 hydrolase, an enzyme that is responsible for the production of proinflammatory leukotrienes. The absence of leukotriene B4 (the product of LTA4 hydrolase) in this mutant does not, however, explain the phenotype; it is the conversion of LTA4 to lipoxin A4 (a pro-resolution compound) that prevents adequate host defence to Mm infection. This work provides a better understanding of the ways in which host defence to mycobacteria is mediated, and highlights the important potential infectious complications of pro-resolution pharmacologicals. Combined with other advances in Mm infection in zebrafish (see below), it seems likely that treatment strategies for human TB will change as a direct consequence of work performed in zebrafish. Other translational advances in immunity made using zebrafish are discussed in Box 1.

Macrophages and DCs: bridging innate and adaptive immunity

Macrophages are central in directing the host immune response, acting both as key phagocytic cells and as regulators of cytokine-mediated immunity (Mosser and Edwards, 2008). They are important for host defence against pathogens and are responsible for phagocytosis of apoptotic cells during development and inflammation. Recently, new macrophage-specific genes suitable for driving macrophage-specific transgenics have been identified (Zakrzewska et al., 2010), and two transgenic lines have now been established that allow detailed study of this key immune cell type in vivo. A novel *mpeg1*:Gal4 line from the Lieschke group (Ellett et al., 2010) was generated using a small promoter fragment in the multisite Gateway construct Tol2kit system (Kwan et al., 2007). An *fms*:Gal4 line generated in Sheffield, UK, used a bacterial artificial chromosome (BAC) containing the *fms* promoter, targeted with the Gal4 gene (Gray et al., 2011). Both lines can drive expression of constructs in macrophages using the UAS promoter (Fig. 1; supplementary material Movies 1, 2). The *fms* gene is also expressed in xanthophores, but in the immune system its expression seems to be exclusively restricted to cells of the macrophage lineage. Already, these lines have identified a role for macrophages in the clearance of apoptotic neutrophils during inflammation in vivo – the first such observation in zebrafish (Ellett et al., 2010) – and a previously unsuspected preference for migratory paths along the abluminal surface of vessels (Gray et al., 2011). The use of the nitroreductase ablation system (Davison et al., 2007) has been shown to successfully ablate macrophages in this system, and this has the potential to be of huge importance in determining the relative roles of these cells

Box 1. Disease modelling and drug screening

Much work in the zebrafish has used simple tissue injury as the initiating stimulus: it is simple, relatively well understood and reproducible. However, in order to model human disease, organ-specific inflammation has been induced in several organ systems. This enables disease pathogenesis to be studied and potential therapies screened. To date, the most successful models in the zebrafish have been of colitis, in both adult (Brugman et al., 2009) and larval zebrafish (Fleming et al., 2010; Oehlers et al., 2010), and skin inflammation (Dodd et al., 2009). Furthermore, human diseases resulting in innate immune dysfunction have been modelled recently in the zebrafish. For example, WHIM syndrome is a disorder characterised by warts, hypo-gammaglobulinaemia, infections and myelokathexis caused by gain-of-function mutations in the CXCR4 chemokine receptor. Expression of mutated CXCR4 proteins in zebrafish neutrophils leads to neutrophil retention at their sites of production, and an inability to recruit neutrophils to sites of inflammation (Walters et al., 2010). This model recapitulates some of the features observed in human WHIM syndrome, and allows in vivo observations to be made that are not possible in other systems. In another important advance, the cystic fibrosis transmembrane conductance regulator (CFTR) has been identified and knocked down in zebrafish, revealing a specific sensitivity to infection with *Pseudomonas aeruginosa* (Phennicie et al., 2010), in line with the susceptibility of cystic fibrosis patients to this bacterium. The generation of a *CFTR* knockout from the Zinc Finger Consortium is eagerly awaited.

In addition to organ-specific inflammation to model human disease, a new method of initiating an immune response has been identified by the group of Miguel Allende: the chemically induced neutrophilic inflammation (or ChIN) assay. In this assay, cell damage to the fish-specific lateral line caused by copper exposure causes abrupt and spontaneously resolving neutrophilic inflammation (d'Alençon et al., 2010). This offers the possibility to carry out automated compound screens for anti-inflammatory compounds and complements manual methods of inducing inflammation (e.g. by sterile tissue injury) (Loynes et al., 2010). These screens can be used to identify anti-inflammatory or pro-resolution compounds, and might have an important place in the translation of zebrafish biology into the clinic.

in host defence and inflammatory disease. Furthermore, in vivo imaging has for the first time allowed the capture of the earliest interactions of macrophage with transformed cells (Feng et al., 2010), which consist of cytoplasmic tethering and engulfment. This study revealed activation of the innate immune response almost immediately following the transforming event. The cue for recruitment of innate immune cells was H_2O_2 secreted by HRAS-G12V transformed cells, similarly to neutrophil recruitment to wounds by H_2O_2 (discussed earlier).

Macrophages have been shown to provide an intracellular niche for the replication of several pathogens. The new models that allow the detailed study of zebrafish macrophages will enable the real-time visualisation of host-pathogen interactions in vivo. *Burkholderia cepacia* is an important respiratory pathogen in immunocompromised hosts, particularly in individuals with cystic fibrosis. The in vivo life cycle of this pathogen has been difficult to study, and new data suggest that there is an intra-phagocyte stage of its life cycle and that this might be important for evading the host response (Vergunst et al., 2010). Similarly, the intracellular life cycle of mycobacteria has been further elucidated by elegant studies in the zebrafish, which have demonstrated a role for macrophages in mycobacterial dissemination and in granuloma formation (Volkman et al., 2004; Clay et al., 2007; Davis and Ramakrishnan, 2009; Volkman et al., 2010). These findings overturn existing hypotheses regarding the role of the granuloma: rather than protecting against mycobacterial dissemination, as previously thought, granulomata seem to be involved in dissemination and expansion of mycobacteria. Furthermore, the Ramakrishnan group has further identified circumstances in which mycobacteria can evade both the host response and antibiotic-mediated killing by assuming a tolerant phenotype within macrophages. This phenomenon was previously thought to reflect a quiescent state of the mycobacteria, but recent groundbreaking work in the zebrafish has shown that these intracellular mycobacteria are actively dividing (Adams et al., 2011). The tolerance of mycobacteria to antibiotics is a result of an induction of a bacterial efflux pump, which is induced when mycobacteria take up residence inside macrophages. The observation that verapamil, a blocker of these efflux pumps, can shorten the duration of antibiotic therapy that is needed to clear a mycobacterial infection would have profound implications for the global TB epidemic if the findings were replicated in human populations.

Innate and adaptive immunity must be integrated for successful acute defence against microorganisms, and for immunological memory to protect against future severe infections. Macrophages and DCs are professional antigen presenting cells (APCs) that are endowed with both phagocytic functions and cell-surface receptors that allow them to interact with and activate lymphocytes. Among these cell-surface receptors, the co-stimulatory molecules B7-1 (also known as CD80) and B7-2 (CD86) are required for antigen-specific stimulation of naive T cells (Lanier et al., 1995). Expression of these co-stimulatory molecules, which represent an important part of the bridge between the innate and adaptive arms of the immune response, is governed by the adapter TRAF6 (Kobayashi et al., 2003), and also involves CD40 ligation, antigen presentation and co-stimulation (Fujii et al., 2004). The counter-receptors for B7 molecules are the activating molecule CD28 and the inhibitory receptor CTLA-4.

Research in mammals has shown that DCs are pivotal to integrating the innate and adaptive arms of the immune system: once stimulated to mature by an infection, they orchestrate the activation of T and B cells. But do DCs exist in fish? DCs have been demonstrated in Atlantic salmon (Pettersen et al., 2008), and the survival of zebrafish in the notoriously microbe-infested waters of their native habitat in the tributaries of the Ganges river and in the laboratory setting strongly suggests their existence in *Danio rerio*. However, DCs have been the most elusive of the haematopoietic lineages in zebrafish. The first evidence of DCs in zebrafish was reported by Ai-Fu and colleagues, who elegantly showed co-expression of DC-SIGN (also known as CD209) with other DC markers (CD80, CD83), as well as the functionality of CD209, in vaccination studies (Lin et al., 2009). A recent paper by Lugo-Villarino et al. provided morphological data and further functional evidence for the existence of the DC lineage in zebrafish (Lugo-Villarino et al., 2010). Similarly, the pivotal stimulatory receptors CD40-CD40 ligand (Gong et al., 2009) and CD28, as well as the inhibitory receptor CTLA-4 (Bernard et al., 2007) and the adaptor molecule TRAF6, which is required for DC maturation (Kobayashi et al., 2003), have been identified in zebrafish and other jawed vertebrates. Therefore, there is ample evidence for the existence of DCs and other types of APCs in zebrafish. Now that the tools have been generated, the zebrafish is poised to give unprecedented access to scrutinise APC-lymphocyte interactions by in vivo imaging.

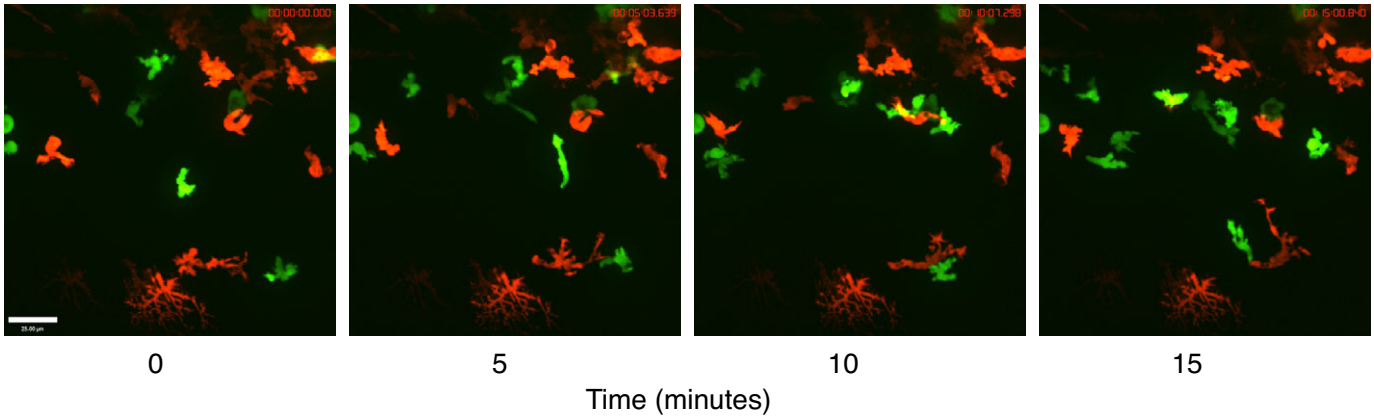

Fig. 1. In vivo imaging of zebrafish immunity. Neutrophils (green) and macrophages (red) can be visualised during inflammation in vivo, showing clear differences in cell morphology and behaviour. Dynamic interaction between these cell types can also be visualised as they participate in the inflammatory process. Time-course images show a day-3 *Tg(mpx:GFP)i114;Tg(fms:gal4)i186;Tg(UAS:nfsB.mCherry)i149* larva. A single immotile xanthophore can be seen at the bottom of the image. Images were acquired on a Perkin Elmer UltraVOX spinning disc confocal mounted on an Olympus IX81 microscope using a 40× oil immersion lens, NA 1.3. The movie from which these stills were acquired is available as supplementary material Movie 1, and a 3D reconstruction of the data is available as supplementary material Movie 2. Scale bar: 25 µm.

Lymphocytes: the adaptive immune response

T cells

T cells make up a multifunctional subset of immune cells. $CD8^+$ killer T cells are involved in controlling cancer cells and eliminating virus-infected cells, whereas $CD4^+$ helper T cells assist B cells in antibody production and $CD4^+CD25^+$ regulatory T cells prevent autoimmune responses. Although there is genetic evidence for the existence of all three types of T cells in zebrafish, functional assays are still lacking. Several zebrafish mutagenesis screens have been conducted, which were designed to identify new or unsuspected players in the development of this haematopoietic lineage (Table 2). Surprisingly, apart from two known transcription factors (Schorpp et al., 2006; Soza-Ried et al., 2010), most genes identified in these studies and a similar screen conducted in medaka are involved in housekeeping functions, several of them affecting thymus development (Trede et al., 2007; Iwanami et al., 2008; Iwanami et al., 2009; Monnich et al., 2010).

Transient knock down of genes in zebrafish has helped to validate newly identified human immunodeficiency genes in an animal model (Pannicke et al., 2009). The T-cell-specific *p56^{lck}*:EGFP transgenic line (Langenau et al., 2004) was instrumental in uncovering the presence of mature lymphocytes in extra-thymic sites such as the epithelium of the gut and nose, and to track engraftment of transplanted cells in the thymus. This transgenic line also labels immature lymphocytes and was employed for identification of mutants with immunodeficiency (N.S.T., unpublished data) and heritable T-cell acute lymphoblastic leukaemia (T-ALL) (Frazer et al., 2009). Finally, the *lck*:EGFP line can also be used in chemical screens to identify immunomodulatory compounds (Langenau et al., 2004). This concept has been successful in identifying compounds that can kill zebrafish and human T-ALL cells (N.S.T., unpublished data). Recently, screens of chemical libraries have identified compounds that expand human haematopoietic stem cells (North et al., 2007) and are active in an embryonic zebrafish model of acute myelogenous leukaemia (Yeh et al., 2009). Together, these studies demonstrate the power of high-content small-molecule screens to identify chemical modifiers of normal and malignant haematopoiesis in an entire living vertebrate.

B cells

Because B cells develop in the zebrafish kidney, they are far more difficult to visualise than T cells, which mature in the superficial thymus. For this reason, B cell development has not been addressed in mutagenesis screens. Most studies have focused on the origin of B cells and the structure of the B cell antigen receptors, and their respective peculiarities in zebrafish compared with mammals (Danilova et al., 2005; Hsu and Criscitiello, 2006; Hu et al., 2010; Danilova et al., 2011; Marianes and Zimmerman, 2011). Recently, the first functional studies of the B lineage in zebrafish have been reported (Wakae et al., 2006; Gong et al., 2009; Liang et al., 2010). The recent generation of the first B-cell-specific transgenic zebrafish line (*Cμ*:EGFP) (David Traver, personal communication) will probably lead to increased interest in studying this lymphoid lineage in zebrafish.

NITR cluster and NK cells

Natural killer (NK) cells act as part of the innate immune system, but are derived from the common lymphoid progenitor and share expression of some surface receptors (e.g. CD8) with T cells. Their main role is to recognise non-self cells (such as virus-infected cells, tumour cells and foreign cells), meaning that NK cells have important functions in defence against infection and malignancy. There is a complex array of receptors on NK cells that recognise self-molecules on the cells that they interact with. The outcome of such interactions – either tolerance or apoptosis induction in the opposing cell – is governed by receptors with inhibitory (ITIM) or activating (ITAM) motifs. The discovery of a new gene family known as novel immune-type receptors (NITRs) in zebrafish (Yoder et al., 2001; Yoder et al., 2004; Yoder et al., 2010) suggests the existence of NK cells in this species. In particular, the NITR9 family member shares structural similarities with ITAM-containing NK receptors expressed in mammals and has been shown to confer

lysis capability to cells in which it is expressed (Yoder et al., 2001; Wei et al., 2007; Cannon et al., 2008). Expression analyses have shown that NITR transcripts are detected at highest levels in zebrafish lymphocytes, compared with the myeloid population (Yoder et al., 2010). Although the specific ligands of NITRs remain to be identified, their activating and inhibitory forms, their expression in lymphocytes and the ability of at least one NITR to mediate allogeneic recognition has led to the hypothesis that the NITRs are functional orthologues of mammalian NK receptors (Yoder, 2009).

Lymphatic tissues in the zebrafish

Lymphatic vessels

The adaptive immune response to infection initiates with APCs presenting processed peptides derived from invading microbes to lymphocytes. This interaction occurs in secondary lymphoid organs: in mammals, these are primarily the spleen and lymph nodes, and also the Peyer's patches (the lymphatic tissues of the gut). At this juncture, we must ask: where do APCs and lymphocytes interact in zebrafish? This question is particularly significant because, during evolution, lymph nodes first appear in aves (Hofmann et al., 2010). Therefore, it was long held that lymphatics in general were absent in teleosts. However, the first evidence for teleost lymphangiogenesis was recently provided by in vivo imaging in zebrafish (Kuchler et al., 2006; Yaniv et al., 2006). Since that seminal discovery, the power of zebrafish as a tool for revealing developmental processes through genetics and in vivo imaging has contributed to our understanding of the molecular mechanisms of lymphangiogenesis in health (Isogai et al., 2009) and disease (Hogan et al., 2009). For example, morpholino knockdown of the *collagen and calcium-binding EGF domain-1* (*ccbe1*) gene led to a phenocopy of the zebrafish mutant *full of fluid* (*fof*) (Hogan et al., 2009) and was instrumental in the identification of *CCBE1* mutations in patients with Hennekam syndrome, which is characterised by lymphedema, lymphangiectasias, mental retardation and unusual facial characteristics (Alders et al., 2009). Using the double transgenic zebrafish line *Tg(flt1:YFP);Tg(kdrl:RFP)*, and morpholinos to knock down *vascular endothelial growth factor c* (*vegfc*) and *phospholipase cγ1* (*plcγ*), the authors then dissected angiogenic sprouting and lymphangiogenic budding from the posterior cardinal vein by in vivo imaging. These studies showed that both angiogenic sprouting and lymphangiogenic sprouting require *ccbe1* and *vegfc*, but that only angiogenic sprouting requires *plcγ*, genetically separating the two processes (Hogan et al., 2009).

However, lymph nodes are truly absent in zebrafish. In their absence, APCs and lymphocytes could interact in the only truly secondary lymphoid organ of zebrafish – the spleen, a site where DCs reside (Lugo-Villarino et al., 2010). DCs have also been isolated from the gut, where large numbers of lymphocytes are found (Danilova and Steiner, 2002; Langenau et al., 2004), albeit scattered and not in organised lymphoid tissues, as in mammalian Peyer's patches. The liver can serve as a surrogate lymphoid organ (Hofmann et al., 2010) and, finally, T and B cells, as well as DCs, are found in the kidney, the primary lymphoid organ and zebrafish equivalent of mammalian bone marrow. Once transgenically labelled lines for all of these cell types are established, the issue of where and when APCs and lymphocytes interact can be addressed by in vivo imaging in developing zebrafish.

Thymus

It was long held that jawed fish were the first vertebrates with a thymus, the specialised primary lymphoid organ where developing T cells undergo fate decisions, maturation and antigen-driven selection. However, the presence of discrete thymus-like lympho-epithelial structures, termed thymoids, in the gill structures of the cyclostome lamprey has recently been reported (Bajoghli et al., 2011). Similarly, the two thymi in zebrafish are in close proximity of the gills and remain bilateral, in continuity with pharyngeal endoderm (Willett et al., 1997). During zebrafish development, the thymus is first detectable at 48 hours post fertilisation (hpf). Population of the mammalian thymus with T cells occurs in waves (Foss et al., 2001; Porritt et al., 2003), and there is evidence that the first T cell progenitors arise in the aorta-gonad-mesonephros (AGM) region of the mammalian embryo (Cumano et al., 1996). However, studies in mammals required cell culture assays to deduce lineage potential of progenitors derived from known sites of haematopoiesis. Exploiting the transparency of fish larvae, the groups of Philippe Herbomel (in zebrafish) and Yosuke Takahama (in medaka) provided the first in vivo evidence of migration of T cell progenitors to the thymus. In the former case, a zebrafish line carrying the *cd41* transgene allowed imaging of in vivo migration of T cell progenitors from the AGM equivalent in zebrafish to the thymic anlage (Kissa et al., 2008). In the latter case, similar in vivo imaging experiments showed migration of T cell progenitors to the thymus anlage in *rag1* transgenic medaka (Li et al., 2007). The use of different transgenic lines has allowed imaging of cell trafficking from shifting hematopoietic sites during development of the thymus, clarifying the functional equivalence of these sites and their mammalian counterparts.

Where next?

In recent years, the combination of in vivo imaging and powerful genetics in the zebrafish has driven significant advances in our understanding of vertebrate immunity in general. These latest advances show how these advantages combine to drive innovation and knowledge gain. There is still much we do not know about the immune system, and the re-emergence of infectious diseases as a serious health threat in the developed world drives a continuing need for new and better understanding. Work on zebrafish infection models, particularly of mycobacterial disease, has shown how novel insights into infectious disease can come from zebrafish research. Inflammatory diseases caused by unresolved innate immune responses are also proving tractable in zebrafish models.

Although it is clear that zebrafish can make an important contribution to this field, many challenges remain. The rapid progress in recent years in understanding zebrafish innate immunity and inflammation have paved the way for studies that address adaptive immunity and autoimmunity. A wish list for the future also includes: development and completion of well-designed compound screens to identify pharmacological agents for the therapeutic manipulation of immunity; generation of new transgenic lines to label immune cell populations that have not yet been studied in zebrafish; execution of new genetic screens targeting immune responses; and complete characterisation of the differences and similarities between zebrafish and mammalian immune systems, enabling fine-tuning of zebrafish models so as to model human pathology as closely as possible.

In our view, the translational potential of the zebrafish model is clear, and to realise it we must unite clinical researchers with researchers working on all relevant model systems (including zebrafish) to develop the translational potential of these models into clinically important advances. In parallel, we would like to see integration of different model systems and communities working together to push forward the boundaries of knowledge. Zebrafish provide an excellent opportunity to address questions that are difficult to solve in mammalian systems. In return, advances in zebrafish must be confirmed in mammalian systems to maximise their translational impact.

Although it may have taken 450 million years for zebrafish to evolve as a model, the next few are set to be the most exciting of them all.

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COMPETING INTERESTS

S.A.R. has filed a patent for the use of zebrafish in screens for inflammatory response modifiers. N.S.T. has no financial or competing interests to declare.

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SUPPLEMENTARY MATERIAL

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