Adoption of in vitro systems and zebrafish embryos as alternative models for reducing rodent use in assessments of immunological and oxidative stress responses to nanomaterials

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ABSTRACT
Assessing the safety of engineered nanomaterials (NMs) is paramount to the responsible and sustainable development of nanotechnology, which provides huge societal benefits. Currently, there is no evidence that engineered NMs cause detrimental health effects in humans. However, investigation of NM toxicity using in vivo, in vitro, in chemico, and in silico models has demonstrated that some NMs stimulate oxidative stress and inflammation, which may lead to adverse health effects. Accordingly, investigation of these responses currently dominates NM safety assessments. There is a need to reduce reliance on rodent testing in nanotoxicology for ethical, financial and legislative reasons, and due to evidence that rodent models do not always predict the human response. We advocate that in vitro models and zebrafish embryos should have greater prominence in screening for NM safety, to better align nanotoxicology with the 3Rs principles. Zebrafish are accepted for use by regulatory agencies in chemical safety assessments (e.g. developmental biology) and there is growing acceptance of their use in biomedical research, providing strong foundations for their use in nanotoxicology. We suggest that investigation of the response of phagocytic cells (e.g. neutrophils, macrophages) in vitro should also form a key part of NM safety assessments, due to their prominent role in the first line of defense. The development of a tiered testing strategy for NM hazard assessment that promotes the more widespread adoption of non-rodent, alternative models and focuses on investigation of inflammation and oxidative stress could make nanotoxicology testing more ethical, relevant, and cost and time efficient.

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1. Introduction
Nanotechnology is a fast growing sector and a key enabling technology of the twenty-first century. In particular, the exploitation of engineered nanomaterials (NMs) to develop “nano-enabled” products has already been proven to have many benefits for society and the global economy (Schmidt 2009). Nanomaterials are defined as having at least one dimension that is 1–100 nm in diameter (European Commission 2011). At the nanoscale, novel properties emerge in materials that are not observed in their bulk counterparts, resulting in, for example, changes in reactivity, optical properties, and conductivity. This has been exploited in an array of consumer products, with NMs now used across diverse sectors including pharmaceuticals, cosmetics, textiles, food, electronics, automotive, agriculture, and pigments/inks, to name...
a few. NMs first entered the marketplace in consumer products in the mid-1990s (Fairbrother and Fairbrother 2009) and their exploitation has since grown enormously, and continues to do so today. In 2016, the global market for Nanotechnology was worth $39.2 billion and it is estimated that this will rise to $90.5 billion by 2021 (McWilliams 2016). To illustrate this point further, in 2005 a Nanotechnology Consumer Product Inventory (CPI) identified 54 products that had incorporated nanotechnology, and by 2014 this had increased to 1814 products (Vance et al. 2015). There is now a drive to minimize any risks posed by NMs, to maximize the benefits of this relatively new technology (Maynard et al. 2006). Epidemiological evidence for respirable particles (e.g. particulate air pollution, asbestos, quartz), some of which have a size comparable to that of NMs, demonstrates that they can have detrimental impacts on human health (e.g. Wagner et al. 1960; Dockery et al. 1993; IARC 1997; Borm and Tran 2002; Driscoll et al. 2005), prompting concern regarding the safety of NMs.

Despite the increased prevalence of NMs in the marketplace, there are still uncertainties surrounding their potential impact on humans and the environment. Currently, there is no evidence that engineered NMs are harmful to humans, however, a range of in vivo (e.g. rodent), in vitro, in chemico, and in silico models have demonstrated that some NMs may be toxic. As safety assessments have not kept pace with the desire to exploit NMs, concerns have been raised about their potential to elicit adverse health effects in humans. There is also evidence for toxic effects of certain NMs, most notably metal and metal oxide NMs, from laboratory studies on species that are used to inform environmental protection (e.g. vertebrates, such as the fish species Danio rerio, Pimephales promelas, Oncorhynchus mykiss, Micropterus salmoides, Oryzias latipes; invertebrates, such as Daphnia magna, Lumbriculus variegatus, Lymnaea stagnalis, Mytilus edulis, Eisenia fetida; and a range of other taxa such as algae, bacteria and plants (e.g. reviewed in Selck et al. 2016)). Investigation of impacts on environmental organisms has most commonly encompassed assessment of mortality, reproduction, and accumulation (e.g. body or organ burden), with some studies investigating the mechanism of toxicity, and most prominently oxidative stress. Importantly, existing studies suggest that there are commonalities in the mechanism of NM toxicity across different species (i.e. models which assess impacts on human health and the environment). With few exceptions, however, most of the effects seen for NMs only occur at exposures that far exceed estimated environmental concentrations although it is accepted that hotspots (i.e. areas where concentrations may be higher due to discharges or land application of sludge) do exist and that long-term exposures at low concentrations, which would closely reflect reality, cannot always be replicated in the laboratory (Nowack 2017).

NMs are extremely diverse, and vary with respect to their physicochemical properties including: composition (e.g. metals, metal oxides, carbon, polymers), size (and surface area), morphology, solubility, charge, and surface chemistry. Identification of the properties of NMs that confer toxicity is a core component of toxicity studies, and requires a comprehensive characterization of NM physico-chemical properties in parallel with hazard studies. Human exposure to NMs may occur in occupational, environmental and consumer settings, and it has been established that the properties of NMs may change throughout their life cycle, which can influence their toxicity (Mitrano and Nowack 2017). The great diversity of NMs, and desire to test NM hazard throughout the life cycle/value chain would require a huge number of safety assessments to evaluate their toxicity. For example, for single walled carbon nanotubes (SWCNTs) alone it has been estimated that up to 50 000 varieties could be produced (Schmidt 2007), and as the physico-chemical properties of each SWCNT will be different, they are likely to vary with respect to their toxicity. In 2009, it was estimated that the use of traditional approaches to thoroughly evaluate the toxicity of NMs already on the market (i.e. not including future generations of NMs) would cost up to $1.2 billion, take up to 53 years to complete, and require a very large number of animals (Choi et al. 2009). Many would argue that this is unacceptable, and that while a comprehensive assessment of NM safety is required to ensure their safe use, there is also a need to minimize rodent testing when evaluating NM toxicity. The enhanced used of alternative models has the additional benefit of potentially simultaneously reducing the cost and time of testing, which are major advantages associated with their use.

1.1. Aligning nanomaterial safety assessments with the 3Rs principles

Currently, chemical safety assessments require a systematic evaluation of toxicity using internationally harmonized protocols, which often rely on animal testing (Hartung 2009). There are concerns regarding the numbers of animals required for chemical safety assessments and so there is a desire to minimize animal use (Rovida and Hartung 2009). While rodent testing is often the preferred testing system for human health NM hazard assessment, it is not feasible to rely solely on such rodent testing due to ethical, resource, and legislative considerations (Hartung and Sabbioni 2011; Stone et al. 2014; Burden et al. 2017). In addition, although toxicology testing has remained relatively unchanged for the last 50 years, it is known that rodent models are not always able to accurately predict toxic effects in humans (Olsen et al. 2000; Mestas and Hughes 2004; Hartung 2009). Toxicology for the twenty-first century aspires to make testing more efficient and more ethical, and questions the extent to which the use of rodent models should dominate testing due to their inability to always accurately predict effects in humans (Hartung 2009). Instead, it encourages identification of the mechanism of toxicity to inform evidence-based testing strategies to assess toxicity, and promotes the use of alternative, high-throughput (HTP) systems which use models of human origin (Hartung 2009).

The 3Rs principles of scientific research, which aim to replace, reduce and refine animal testing (Russell and Burch 1959), are widely accepted, and have been implemented internationally. There is a desire to accelerate the development and application of alternative (non-animal) testing methods when assessing chemical toxicity to human health.
and the environment in order to enhance implementation of the 3Rs principles, to reduce the number of vertebrates used for toxicity testing (Törnqvist et al. 2014; Burden et al. 2015, 2016). Recently, the need to better align nanotoxicology testing with the 3Rs principles has been raised (Burden et al. 2017). The EU regulation on the protection of animals used for scientific purposes (Directive 2010/63/EU; European Parliament 2010 (Article 47)) encourages the implementation of alternative approaches when conducting scientific research. Thus, the application of the 3Rs principles is embedded in regulations that are relevant to the safety assessment of NMs (e.g. chemicals, cosmetics, food, pharmaceuticals, medical devices, and biocides) (Rauscher et al. 2017).

The European Chemicals Agency (ECHA) is responsible for implementing REACH, the European chemical safety regulation and came into force in 2007. REACH aims to protect human health and the environment from chemical risks, including NMs. ECHA acknowledges that the safe manufacture and use of chemicals will require significantly more information on potential hazard, and with current testing approaches this is likely to lead to a corresponding increase in animal testing (Rovida and Hartung 2009; ECHA 2016a). However, REACH supports strategies to avoid unnecessary animal testing, such as promoting better data sharing to avoid duplication of research and to support read-across between species, and the use of alternative (non-animal) methods (ECHA 2011, 2014, 2015, 2016b). The European Commission regularly publishes statistics on animal use for experimental procedures, and a reduction in animal use by half a million occurred between 2008 and 2011 (ECHA 2014). Evidence that illustrates registrants actively employ non-animal approaches in fulfillment of the obligations of REACH is illustrated for the 7939 new chemical safety experimental studies logged in the ECHA database for the period between 2009 and 2013, where 62% of the assessments were conducted in vivo, and 38% in vitro (ECHA 2014). Demonstration that legislators and regulators are becoming more receptive to, and confident in, the use of data from alternative testing systems is also exemplified by the EU ban on the use of animals for cosmetic safety testing. Alternative models to assess skin irritation and sensitization have been developed and validated and are used widely. However, for other endpoints alternative models are not yet available. Therefore, while the adoption of alternatives to animal testing is advocated by regulators, currently animal testing still accounts for the majority of research assessing the toxicity of chemicals. Nevertheless, it is expected that the use of alternative models will increase in the future as more alternative approaches are validated.

Transforming the approach for toxicology testing through adoption of alternative systems, provides major opportunities for reducing animal use in chemical safety assessments. This is particularly appealing for NMs, given their diversity and the vast number of these materials that will require safety assessment. This paper explores the potential to enhance the use of zebrafish embryo and cell (in vitro) models as alternatives to rodent testing when assessing NM safety, with a focus placed on investigation of immunological function and oxidative stress; two of the main effector mechanisms for NM toxicity established to date. It is hoped that the identification of alternative (non-rodent) models, which have the potential to be adopted internationally, will support the implementation of an intelligent testing strategy for NM hazard assessment which reduces the reliance placed on rodent testing and better aligns nanotoxicology research with the 3Rs principles. Of interest is that existing reviews have encouraged greater use of zebrafish for assessing NM toxicity (e.g. Lin et al. 2013; Chakraborty et al. 2016). We build on these reviews and extend our analysis on the state of the art to provide recommendations for the experimental design of future studies by, for example, identifying the biological responses that should be prioritized for assessment of NM effects, based on current knowledge on the mechanism of action of NMs. The approach we advocate will help shape future research activities in nanotoxicology to improve establishment of common biological responses across different models (e.g. rodents, zebrafish and cell (in vitro) to facilitate read-across and inform decisions about the suitability of using alternative models).

2. NM mechanism of toxicity

Knowledge of the mechanism of toxicity of NMs is essential when designing intelligent and evidence based testing strategies to screen their safety, and to help understand the potential impact of NMs on human health and the environment (Stone et al. 2014). NMs, depending on their physicochemical properties, have been shown to stimulate a range of biological responses spanning inflammation, fibrosis, oxidative stress, cytotoxicity, mitochondrial perturbations, genotoxicity, and carcinogenicity, at various target sites (e.g. lung, liver, kidneys, cardiovascular system, intestine, CNS). It is established that oxidative stress and inflammation are key drivers of the adverse health effects associated with human exposure to pathogenic particles (e.g. PM10) (reviewed in Stone et al. 2017) and this information has provided the foundations for nanotoxicology research (Oberdörster et al. 2007). More recently, omics technologies have been used in vivo and in vitro to generate hypotheses regarding the molecular mechanism of action of NMs (e.g. van Aerle et al. 2013; Boyles et al. 2016; Labib et al. 2016, reviewed in Costa and Fadeel 2016). The relatively high financial cost associated with these approaches is restrictive for their routine use. Therefore, biochemical assays for detecting oxidative stress and inflammation as indicators of NM toxicity have dominated published studies (see below).

2.1. NMs and inflammation

Inflammation is a normal, protective physiological response to foreign material and injury. However, if inflammatory responses are persistent (chronic) due to an exaggerated or dysregulated response, including a failure of normal resolution mechanisms, a pathological response can emerge (Poon et al. 2014; Robb et al. 2016; Soehnlein et al. 2017) (Figure 1). Therefore, it is essential that inflammation resolves
in a timely manner to prevent the manifestation of detrimental health effects (Poon et al. 2014; Robb et al. 2016; Soehnlein et al. 2017). Disease inducing particles and fibers such as particulate air pollution (PM$_{10}$), silica and asbestos cause a range of adverse health effects in humans (e.g. initiation/exacerbation of respiratory and cardiovascular disease, cancer) via mechanisms involving the stimulation of inflammation (reviewed in e.g. Oberdörster et al. 2007; Donaldson and Seaton 2012). Macrophages and neutrophils play key roles in the body’s first (innate) response to foreign particles and here we first explore knowledge on their roles in the inflammatory response associated with NM toxicity.

2.1.1. Neutrophils
Neutrophils are the most abundant circulating leukocytes in humans, and are recruited to sites of injury or infection to protect the host (e.g. Nathan 2006; Filep and Kebir 2009; Wang and Arase 2014). Neutrophils are a key first line of immune cell defense in the inflammatory process, and possess several strategies to clear and destroy pathogens (Mayadas et al. 2014; Wang and Arase 2014) (Figure 2). The resolution of inflammation requires that neutrophils undergo apoptosis and are cleared by phagocytic cells (e.g. macrophages), a process known as efferocytosis (Poon et al. 2014; Jones et al. 2016; Robb et al. 2016). Failure of neutrophilic inflammation to resolve itself can cause tissue damage and contribute to disease pathogenesis due to the release of toxic mediators (e.g. proteases and reactive oxygen species (ROS)) (Filep and Kebir 2009; Poon et al. 2014; Robb et al. 2016; Soehnlein et al. 2017). Indeed, activation of neutrophil responses has been linked to genotoxicity and carcinogenicity (reviewed in Knaapen et al. 2006). Knowledge of the cellular and molecular events underlying the activation and resolution of neutrophil responses can inform the design of laboratory studies which assess the ability of NMs to stimulate inflammatory responses (Figure 2).

Neutrophil accumulation is recognized as a key indicator of NM toxicity in rodents; with high levels of neutrophil accumulation (neutrophilia) and a lack of inflammation resolution representing high toxicity. Published studies have exposed rodents to NMs via various administration routes (e.g. intratracheal instillation, inhalation, pharyngeal aspiration, intraperitoneal, intrapleural and intravenous injection) and collectively these studies have demonstrated that many NMs induce neutrophilia at target sites including the lungs, liver, peritoneum, and pleural cavity (Brown et al. 2001; Poland et al. 2008; Ma-Hock et al. 2009; Kermanizadeh, Brown et al. 2013; Kermanizadeh, Pojana, et al. 2013; Kermanizadeh, Vranic et al. 2013; Murphy et al. 2013; Kim et al. 2014; Landsiedel, Ma-Hock et al. 2014; Landsiedel, Sauer, et al. 2014; Gosen et al. 2015). Assessment of neutrophil responses, in vitro and in vivo, is therefore recognized as an integral component of NM hazard assessments.

2.1.2. Macrophages
Macrophages, like neutrophils, are key cells in the first line of immune defense against foreign materials (Davies et al. 2013; Davies and Taylor 2015). Macrophages are found in numerous locations throughout the body (e.g. lungs, liver, peritoneal cavity) and are key to the clearance of NMs from various target sites (e.g. Halpern et al. 1953; Ogawara et al. 1999; Sadauskas et al. 2007; Semmler-Behnke et al. 2007, 2008; Geiser et al. 2008; Roberts et al. 2013). The defensive strategies activated by pathogens and particles discussed above for neutrophils (Figure 2) are also relevant to macrophages, although there are also cell specific responses that operate in each type of immune cell.

In vivo, assessment of immune cell accumulation, and cytokine production over time are often prioritized to monitor the inflammatory response to NMs (particularly within the lung, but also other target sites). In vitro, the following responses are commonly assessed in macrophages and neutrophils as indicators of cell activation and toxicity; cytotoxicity (e.g. apoptosis), cytokine production, phagocytic function, NM internalization, and ROS production (intracellular and respiratory burst). Studies which have investigated the response of macrophages and neutrophils to NMs, are discussed in detail below.

Figure 1. The activation of inflammation by NMs: physiological vs pathological responses. NMs can activate acute inflammatory responses that are dominated by neutrophils and macrophages. Apoptosis of immune cells or stimulation of their emigration promotes the resolution of acute inflammatory responses, and activates repair. Failure of inflammation to resolve in a timely manner may stimulate chronic inflammation which can lead to tissue damage and contribute to disease pathogenesis.
2.2. NMs and oxidative stress

Oxidative stress is defined as an imbalance between damaging ROS and protective (ROS scavenging) antioxidants, exemplified by the excessive production of ROS and depletion of antioxidants (Valko et al. 2007). ROS have a dual role in the body, and while they can contribute to important physiological functions, they can also attack biological molecules (lipids, proteins, DNA) and compromise their normal function, leading to cell damage (Valko et al. 2007) (Figure 3). The involvement of oxidative stress in the toxicity of NMs has been extensively investigated both in vivo and in vitro, as the damaging effects of ROS can impair normal cell function, and contribute to disease pathogenesis. NMs may have the inherent ability to produce ROS or generate ROS via cellular sources.

The capacity of NMs to generate ROS is frequently used as an in chemico assay to measure their reactivity (e.g. Wilson et al. 2007; Shi et al. 2012). For the cellular response, it is hypothesized that NMs can elicit three different tiers of oxidative stress, which each dictate a different biological response (e.g. Donaldson et al. 2003; Brown et al. 2004; Nel et al. 2006; Li et al. 2008). At low levels (tier 1), enhanced ROS production can stimulate a protective response in cells via the activation of redox sensitive transcription factors (e.g. nuclear factor erythroid related factor (Nrf2)) which control the expression of cytoprotective genes (e.g. antioxidants). As ROS levels increase (tier 2), and protective antioxidant defenses become overwhelmed, inflammatory responses can be activated (e.g.
via the activation of oxidant and calcium sensitive transcription factors, such as nuclear factor kappa B (NF-κB) and/or activator protein (AP)-1). If ROS levels continue to increase (tier 3) then genotoxicity and cytotoxicity can be stimulated. This hierarchical model of the cellular response to NMs has been used to identify their potential biological effects and can inform the experimental design of studies which investigate NM toxicity.

The respiratory burst is a defensive response that is activated by immune cells (e.g. macrophages, neutrophils), and involves the extracellular release of ROS to destroy invading pathogens. There is evidence that some NMs (e.g. TiO₂, Ag, CNTs, SiO₂) can stimulate a respiratory burst in neutrophils and macrophages (e.g. Brown, Kinloch et al. 2007; Gonçalves et al. 2010; Park and Park 2009; Scherbart et al. 2011; Boyles et al. 2015; Johnston et al. 2015). While this is intended to be a protective response, it can stimulate oxidative stress, and thereby contribute to NM toxicity, and thus should be considered when assessing NM hazard in vitro and in vivo.

For in vitro and in vivo models (e.g. rodent, cell and zebrafish), a wide range of endpoints and assays can be used to probe the involvement of oxidative stress in the toxicity of NMs (Figure 4). Regardless of the experimental approach used, studies have identified that oxidative stress is a key event in the cellular response to many NMs (see below).

Oxidative stress can stimulate inflammation, and vice versa, and thus these processes are intimately linked, and known to contribute to the pathogenesis of many diseases. For example, ROS act as secondary messengers to activate redox responsive signaling pathways and transcription factors (e.g. NFκB) which stimulates the expression of pro-inflammatory proteins (e.g. cytokines, adhesion molecules) that promote inflammation (e.g. reviewed by Torres and Forman 2003; Mittal et al. 2014). Indeed, there is evidence that NMs can stimulate pro-inflammatory cytokine production from macrophages in vitro via an oxidant mechanism (Brown et al. 2004; Lee et al. 2009). In addition, as discussed above, immune cells can generate ROS as a protective response to destroy pathogens, which can promote oxidative stress. A summary of what we know on the relationship between oxidative stress and inflammation in the toxicity of NMs is outlined in Figure 5.

3. Alternative models to investigate NM mediated inflammogenicity and oxidative stress

3.1. Zebrafish

Invertebrate models (e.g. Drosophila melanogaster, Caenorhabditis elegans) have been used as alternatives to rodents for the study of disease pathogenesis, chemical toxicity, and screening new drug candidates for efficacy and toxicity (e.g. Dodd et al. 2000; Lieschke and Currie 2007). Invertebrate models (e.g. Drosophila melanogaster, Caenorhabditis elegans) have been used as alternatives to rodents for the study of disease pathogenesis, chemical
toxicity, and screening new drug candidates for efficacy and toxicity (e.g. Dodd et al. 2000; Lieschke and Currie 2007). However, invertebrates differ in many aspects of their physiology and anatomy compared with humans, thus limiting their ability to accurately model many aspects of human disease.

Zebrafish (*Danio rerio*) are more similar generally to humans in their anatomy, physiology and genome and thus offer many advantages over invertebrates as alternatives to rodent models (Lieschke and Currie 2007; Davis et al. 2014).

Major advantages of using zebrafish include the ability to assess responses in a whole organism, their small size, relative ease of maintenance, genetic similarity to mammals, high fecundity, low cost relative to rodent testing, potential to perform rapid screens of toxicity, availability of genetically manipulated strains, embryo/larval transparency, and the potential to generate high throughput formats (Burns et al. 2005; Renshaw et al. 2006; Meeker and Trede 2008; Strähle et al. 2012; Henry et al. 2013; Howe et al. 2013). From a regulatory perspective, in the EU early life stages of zebrafish prior to exogenous (independent) feeding from 120 h post fertilization (hpf), are not protected and therefore do not require permission from responsible authorities for use in experiments (EU Directive 2010/63/EU, European Parliament 2010; Strähle et al. 2012). Embryo transparency is particularly appealing as it facilitates both the direct visualization of developmental processes including organ/tissue structure and function, and enables evaluation of responses at specific proteins or gene loci within target tissues (e.g. via whole mount *in situ* hybridization and immunohistochemistry). Furthermore, the availability of transgenic zebrafish which express fluorescent proteins in specific cell types enables the visualization and quantification of cellular processes. This is extremely valuable when investigating the cell and molecular basis of disease or the mechanism underlying the toxicity of chemicals (such as NMs). A diverse array of transgenic zebrafish lines are now available (reviewed in Lee et al. 2015), and those particularly relevant to the assessment of NM toxicity are discussed in more detail below. Therefore, zebrafish are arguably relevant as a test species for NM toxicity testing for read across to mammals, including humans with, existing evidence suggesting a good concordance observed between zebrafish and human and rodent studies (see Section 3.3).

Zebrafish are already used widely for biomedical and toxicological research. For example, there are internationally agreed protocols, accepted by regulators, for their use in developmental biology and toxicity studies and as an alternative to rodents (Dodd et al. 2000, OECD Fish Embryo Acute Toxicity Test No. 236, reviewed in He et al. 2014). Zebrafish have been used to unravel the molecular basis of human disease (e.g. cancer, diabetes, cardiovascular diseases, infectious disease (e.g. microbe-host interactions), and neurodegenerative disease), as well as for drug screening (reviewed in Lieschke and Currie 2007; Goldsmith and Jobin 2012; Mathias et al. 2012; MacRae and Peterson 2015). The nanotoxicology community have widely adopted the use of zebrafish embryo and larval stages up to 120 hpf (e.g. Zhu et al. 2012; Massarsky et al. 2013; Osborne et al. 2013, 2016) and adults (e.g. Bilberg et al. 2012; Zhang et al. 2015) to investigate the aquatic toxicity of NMs, typically via assessment of morphological development and mortality (e.g. Lee et al. 2007, 2012; Lin et al. 2011; Duan et al. 2013; Ganesan et al. 2016). Zebrafish embryos have also been used to investigate the bioavailability of NMs when exposed via water (Lee et al. 2007, 2012; Goodhead et al. 2015). Therefore, while zebrafish have received little attention for evaluating possible impacts of NMs on human health, they could be a powerful tool to investigate oxidative effects and inflammogenicity, as an alternative to rodents.

### 3.1.1. Zebrafish and the innate immune response to NMs

The immune system of zebrafish has strong similarities to that of humans with the major cell lineages evident, including cells analogous to monocytes, tissue macrophages, granulocytes (e.g. neutrophils, eosinophils) and lymphocytes (reviewed in Traver et al. 2003; Meeker and Trede 2008; Novoa and Figueras 2012). A functional innate immune system exists in zebrafish within 48 hpf, with the adaptive...
system evident 4 weeks after fertilization (Trede et al. 2004; Renshaw et al. 2006; Novoa and Figueras 2012). Here, we focus on the use of zebrafish embryos to investigate innate, acute immune cell responses, which are dominated by neutrophils and macrophages.

Neutrophils are distributed throughout the zebrafish embryo via the circulation (e.g. Yang et al. 2014; Duan et al. 2016a, 2016b), while macrophages are present in the blood circulation and are resident in tissues (reviewed in Herbomel et al. 1999; Torraca et al. 2014). Several studies have used wildtype zebrafish to investigate inflammatory responses to a range of stimuli (e.g. injury, micro-organisms, chemicals). To quantify the inflammatory response, such studies have identified immune cells based on their morphology, or via staining using immunofluorescence and immunohistochemical techniques. For example, myeloperoxidase (MPO) is a neutrophil-specific enzyme commonly used to identify neutrophils (Herbomel et al. 1999; Renshaw et al. 2006; Mathias et al. 2009; Levraud et al. 2009). Alternatively, the generation of transgenic zebrafish strains with fluorescent, endogenously-labeled immune cells allows inflammatory responses to be visualized and quantified using fluorescent microscopy. The use of such strains presents a major advantage over rodent models – and over traditional histochemical approaches in wildtype fish – owing to the ability to monitor inflammatory responses in real time, in vivo and obtain results more quickly. For example, it is possible to track immune cell movement in zebrafish, which allows monitoring of the recruitment of cells and their reverse migration in order to investigate the activation and resolution of inflammatory responses (Brown, Tucker, et al. 2007; Feng et al. 2010; Ellett et al. 2015). Of further benefit is that automated HTP in vivo screening of inflammatorycy can also be performed in transgenic zebrafish (e.g. Lin et al. 2011; Hall et al. 2014). Transgenic strains that are most commonly used to study inflammation are those with fluorescently labeled (e.g. with green fluorescent protein (GFP) or mCherry) neutrophils (e.g. Tg(mpx:GFP)114, and lyc.C.EGFP)), macrophages (e.g. macrophage expressed gene (mpeg1:GFP)) and double-transgenics with both fluorescently labeled neutrophils and macrophages (e.g. Tg(mpx:GFP)114/Tg (mpeg1:mCherry)923 (Renshaw et al. 2006; Ellett et al. 2011; Jim et al. 2016). To date, transgenic embryos (<120 hpf) have been predominately used to investigate inflammatory responses owing to their transparency, which makes them amenable to microscopic approaches. However, casper strains of zebrafish are now available which lack pigment and the transparency of these strains enables immune cell movement to be tracked in adult zebrafish (e.g. White et al. 2008; Jim et al. 2016).

As leukocyte behavior can be visualized and quantified in transparent zebrafish embryos, they are an extremely useful tool to evaluate fluxes in immune cell accumulation during inflammation (Ellett et al. 2011; Hoodless et al. 2016) (Figure 6). Accordingly, zebrafish have been used to investigate the innate immune response to various stimuli including; lipopolysaccharide (LPS) (Yang et al. 2014), injury (tail fin wound, which involves transection of the zebrafish embryo’s tail; e.g. Renshaw et al. 2006; de Oliveira et al. 2013; Lucas et al. 2013; Hoodless et al. 2016), and micro-organisms (e.g. Renshaw et al. 2006; Li et al. 2012; Nguyen-Chi et al. 2014, reviewed in Meeker and Trede 2008). Existing studies have shown that reproducible and quantifiable neutrophil inflammatory responses are stimulated in zebrafish embryos (e.g. following tailfin injury) and resolve in a time frame similar to mammalian models (e.g. Renshaw et al. 2007; Loynes et al. 2010; Lucas et al. 2013; Hoodless et al. 2016; Robertson et al. 2016). The inflammatory response to injury to the tail fin is well characterized and has been used in drug discovery to assess the efficacy of anti-inflammatory compounds (e.g. Brown, Tucker, et al. 2007; Lucas et al. 2013; Hall et al. 2014; Hoodless et al. 2016; Robertson et al. 2016). We identified only two studies that have used zebrafish embryos to investigate inflammatory responses to NMs (e.g. Duan et al. 2016a, 2016b). In those studies, the neutrophil response induced by benzo[a]pyrene and/or silica NMs in the cardiovascular system was assessed following injection into the caudal vein of transgenic zebrafish embryos.

Mechanisms underlying inflammatory responses to various stimuli in zebrafish embryos (wildtype and transgenic) have typically been investigated using fluorescent probes (e.g. for assessment of apoptosis, ROS production), assessment of changes in gene expression (e.g. cytokines), and the use of knockouts (e.g. deficiency of inflammatory cells (Pase et al. 2012; Shiau et al. 2015) or chemokines such as the neutrophil...
chemoattractant CXCL8, or transcription factors (e.g. nrf2) (Feng et al. 2010; de Oliveira et al. 2013, 2015; de Oliveira et al. 2015; Xu et al. 2015; Osborne et al. 2016)). Reporter (transgenic) zebrafish strains could be used to investigate the role of specific events in NM toxicity, such as calcium signaling (e.g. Beerman et al. 2015), transcription factor activation (e.g. NF-κB; Kanther et al. 2011), or tumor necrosis factor (TNF) expression (e.g. Marjoram et al. 2015) by immune cells, but as yet they have not been adopted. Fluorescent probes can also be used to investigate the cellular and molecular events underlying inflammatory responses such as the role of calcium signaling, and transcription factor activation (e.g. Cheung et al. 2011) in wild type of transgenic zebrafish. Phagocytosis of fluorescent pathogens by (fluorescent) immune cells can be visualized using fluorescent microscopy (e.g. Jim et al. 2016; Meijer 2016), and thus imaging the interaction of fluorescent NMs with phagocytes in zebrafish is possible, although the ability to image individual NMs will depend on microscope resolution.

3.1.2. Zebrafish embryos and oxidative stress

Wildtype and transgenic zebrafish have been used as a test organism to investigate the involvement of oxidative stress in disease pathogenesis, and the toxicity of a variety of chemicals, and including NMs (e.g. Ag, ZnO, TiO2, C60, CuO). A wide range of responses have been used to assess the contribution of oxidative stress to NM toxicity in zebrafish including; depletion of antioxidants (e.g. glutathione, or catalase (e.g. Usenko et al. 2008; Choi et al. 2010; Massarsky et al. 2013; Faria et al. 2014)), activity of antioxidant enzymes (e.g. SOD, CAT and GPx (Xiong et al. 2011; Zhao et al. 2013; Faria et al. 2014; Ganesan et al. 2016), oxidative damage (e.g. malondialdehyde (MDA)) (Choi et al. 2010; Zhao et al. 2013; Massarsky et al. 2013; Zhao et al. 2013; Ganesan et al. 2016), and the ability of antioxidant supplementation to reduce NM mediated toxicity (Usenko et al. 2008). Furthermore, the mechanism of toxicity of Ag NMs has also been investigated via whole mount in situ hybridization for genes forming part of the oxidative response cascade (Osborne et al. 2016). ROS production has also been monitored in zebrafish in vivo, in real time, using fluorescent probes (e.g. Hall et al. 2013; Mugoni et al. 2014). As an example, 2′,7′-dichlorofluorescin diacetate (DCFH) has been used successfully in whole zebrafish to monitor respiratory burst activation in response to phorbol myristate acetate (PMA) (Hermann et al. 2004), and ROS production upon exposure to toxicants (e.g. H2O2), using a HTP technique (Walker et al. 2012). At this time, the DCFH assay has not been used in zebrafish to assess NM mediated ROS production in vivo. Therefore, this assay has the potential to be applied to the study of NM toxicity in future. Of importance, is that existing studies which have investigated the involvement of oxidative stress in the zebrafish response to NMs have focused on assessment of the aquatic toxicity of NMs, however, the results obtained are also relevant to human health. Interestingly, the majority of existing studies have used adult zebrafish, which has the benefit of allowing oxidative stress to be investigated in specific tissues (e.g. gills, liver).

Transgenic zebrafish with fluorescence reporters have been developed for real time monitoring of biological responses to oxidative stressors in whole organisms. Reporter strains developed include those which enable H2O2 production (Niethammer et al. 2009) and lipid peroxidation (Fang et al. 2011) to be investigated. Another transgenic zebrafish line known to be in development includes the electrophile response element (EpRE) reporter for oxidative stress, which shows tissue specificity for different environmental contaminants including NMs (S Mourabit, Personal communication). With the widespread uptake of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) cas, a technique that allows for rapid and specific gene manipulation, it is expected that more transgenic model fish systems will become available in the near future for studying oxidative stress mechanisms.

3.1.3. Zebrafish: recommendations for a testing strategy

As detailed above there are well established zebrafish models and tools that can be immediately applied to study NM toxicity. While the use of zebrafish provides exciting opportunities to investigate the activation and resolution of inflammatory and oxidant driven responses by NMs, careful consideration of the experimental design is required to ensure the models are fit for purpose.

3.1.3.1. Life stage. We recommend the use of non-protected early life stages (<120 hpf) of zebrafish to assess NM toxicity should be prioritized to encourage the more widespread implementation of the 3Rs principles in nanotoxicology. Of benefit is that the innate immune system and many organs (e.g. cardiovascular system, liver, kidney) are functional in zebrafish within this time frame. However, we acknowledge that under certain circumstances it may be more appropriate (and even necessary) to use adult life stages, for example, when (i) investigating impacts on the adaptive immune response, which is not functionally mature until ~4–6 weeks (Trede et al. 2004; Novoa and Figueras 2012), (ii) assessing responses in organs that are not fully developed in early life stages, (iii) assessing the chronic toxicity of NMs, and (iv) investigating impacts following the onset of ingestion beyond 120 hpf at which point the zebrafish are protected.

When investigating the inflammatory response in early, non-protected life stages of zebrafish, embryos have typically been used at three days post fertilization (dpf), which allows inflammatory and oxidative responses to be monitored up to 48 h post exposure (i.e. up until 120 hpf). Existing studies have demonstrated that this time frame is sufficient to capture the activation and resolution of neutrophil and macrophage driven responses following a tail wound injury (e.g. Hoodless et al. 2016). Renshaw et al. (2006, 2007) have demonstrated that the kinetics of the inflammatory response are similar between zebrafish and rodents and typically characterized by an infiltration of neutrophils which peaks between 6 and 24 h, followed by an influx of macrophages. The expression of genes related to oxidative stress, and detoxification following NM exposure have been shown to be dependent on the target site and developmental stage, as well as the
time point under investigation (Osborne et al. 2016). Furthermore, different approaches used to investigate oxidative stress vary in their sensitivity (e.g. biochemical assay assessing protein levels/activity vs gene expression). These findings should be considered when designing experiments to investigate oxidative stress in zebrafish, following exposure to NMs. Of benefit is that zebrafish could potentially be used to assess toxicity following single or repeated NM exposures, and therefore help address a knowledge gap in the area of nanotoxicology (Stone et al. 2017).

3.1.3.2. Route of administration. When investigating NM toxicity, it is advisable to assess both local (i.e. at the exposure site) and systemic effects. In rodents, assessment of toxicity has been assessed following pulmonary exposure, ingestion, dermal application and intravenous injection, and it is relevant to consider what the equivalent exposure routes are in zebrafish. Exposure of zebrafish via water has been the most commonly used technique by studies investigating the aquatic toxicity of NMs, however in order to use zebrafish to assess potential impacts of NMs on human health other administration routes need consideration. The choice of administration route will typically be influenced by the hypotheses under investigation (e.g. the pathway of human NM exposure intended to be represented) and the developmental stage of the zebrafish.

There are a variety of administration routes available for exposure of zebrafish to NMs (reviewed in Benard et al. 2012) (Figure 7). Existing studies have exposed zebrafish via water (e.g. Wang et al. 2014; Xu et al. 2015), or injection into the yolk sac (Yang et al. 2014), caudal vein (Jim et al. 2016), dorsal tail muscle (Lin et al. 2009), hindbrain ventricle (Davis et al. 2002), otic vesicle (ear, Deng et al. 2013), swim bladder (Gratapac et al. 2017), and notochord (Nguyen-Chi et al. 2014) to investigate local or systemic responses to pathogens/toxicants (reviewed in Harvie and Huttenlocher 2015). The majority of rodent studies have assessed the pulmonary response to NMs. Although zebrafish do not have lungs, respiratory tissues on the gills have structural similarities with the gas exchange regions of mammalian lungs, and share common pathways of inflammation upon stimulation (Progatzky et al. 2016). Thus, exposure of zebrafish to NMs via water could be used for investigating effects of substances on human lung tissues (e.g. McLeish et al. 2010; Progatzky et al. 2016). However, the gills are not fully developed and functional until relatively late in the development of a zebrafish (>14 dpf) (Rombough 2002). As an alternative, injection of NMs into other locations (e.g. tail muscle, swim bladder or otic vesicle) could be used to investigate a localized response to NMs as a “surrogate” for the pulmonary response in embryos (or adults). For example, the tail fin wound model, has been used to identify new anti-inflammatory compounds to treat respiratory diseases as this injury model stimulates a neutrophilic response, which if unresolved is the basis of many respiratory diseases (Martin and Renshaw 2009; Robertson et al. 2016). If investigating impacts of NMs following ingestion, injection into the yolk sac of non-protected life stages of zebrafish could be performed. Alternatively, exposure of adult zebrafish to NMs via food or water can be used to investigate impacts following ingestion (e.g. Xiong et al. 2011; Merrifield et al. 2013; Osborne et al. 2015). Exposure to NMs via water could allow for impacts on the skin to be investigated (e.g. McLeish et al. 2010). Injection into the caudal vein could mimic exposure of rodents/humans via intravenous injection, which is particularly relevant to NMs used in biomedical applications (e.g. nanomedicines). Injection of NMs into the hindbrain ventricle, and notochord could be used to investigate impacts on the CNS.

In vivo studies typically investigate NM toxicity at a range of target sites in order to assess their local and systemic toxicity. Some organs are fully functional in non-protected life stages of zebrafish, and thus responses in these organs can be investigated following exposure. For example, the ability of NMs to stimulate inflammation in the heart has been investigated following injection into the caudal vein of zebrafish embryos (e.g. Duan et al. 2016a, 2016b). In rodents, it is known that NMs primarily accumulate in the liver, where they can stimulate toxicity (e.g. Kermanizadeh, Brown, et al. 2013; Kermanizadeh, Pojana, et al. 2013; Kermanizadeh, Vranic, et al. 2013). In zebrafish there are melano-macrophage center sites,
which include spleen, kidney and liver (Agius and Roberts 2003). The liver is identifiable in zebrafish embryos from 22 hpf and its growth is complete by five days dpf (Chu and Sadler 2009), as well as a developed spleen at 14 dpf and pro-nephric kidney by 2 dpf (Drummond and Davidson 2010).

### 3.2. In vitro models

While sometimes criticized for their lack of relevancy to the in vivo situation, in vitro models are extremely useful tools within NM safety assessments, and help reduce the number of intact animals used for toxicology testing (e.g. Farcal et al. 2015; Kermanizadeh et al. 2016; Burden et al. 2016; Stone et al. 2016). The use of in vitro (cell) models is attractive as it provides an opportunity to conduct a rapid, first screen of NM toxicity in order to make testing more time efficient, as tests are typically less than 24 h in duration. Inflammation is challenging to investigate in vitro as inflammatory responses require cellular and humoral interactions, and an intact vascular system (Cho et al. 2010). Investigation of the response of neutrophils and macrophages to NMs in vitro has relied on the use of simple, monocultures of cells. When investigating macrophage responses, the source of cells for in vitro studies is varied and has included cell lines (e.g. mouse J774 macrophage like cells (e.g. Clift et al. 2010; Nguyen et al. 2016), rat NR8383 macrophages (e.g. Wiemann et al. 2016), human MM6 monocytes (e.g. Stone et al. 2000; Foucaud et al. 2007), mouse RAW264.7 macrophages (e.g. Kagan et al. 2006; Kong et al. 2013; Farcal et al. 2015; Pati et al. 2016), and human THP-1 monocytic cells (e.g. Brown, Kinloch, et al. 2007; Lanone et al. 2009; Farcal et al. 2015) and primary cells (e.g. peripheral blood monocytes (PBMCs) isolated from human blood (e.g. Brown, Kinloch, et al. 2007; Lee et al. 2009; Witasp et al. 2009; Tuomela et al. 2013; Farcal et al. 2015), or rat/mouse alveolar or peritoneal macrophages (e.g. Warheit et al. 2009)). For neutrophils, cells are typically isolated from human blood (e.g. Gonc¸alves et al. 2010; Jovanovi´c et al. 2011; Abrikossova et al. 2012; Babin et al. 2013; Couto et al. 2014; Farrera et al. 2014; Haase et al. 2014; Johnston et al. 2015; Liz et al. 2015; Soares et al. 2016). Of interest is that while the uptake of NMs by macrophages has been commonly investigated, there are a lack of studies have investigated the uptake of NMs by neutrophils, despite their phagocytic function, and this knowledge gap needs to be addressed given the key role of neutrophils in the acute inflammatory response to NMs in vivo. More in depth in vitro mechanistic studies are required to assess the molecular mechanism underlying the activation of neutrophils and macrophages by NMs, with existing data suggesting that oxidant and calcium signaling are important (e.g. Brown et al. 2004; Lee et al. 2009; Brown et al. 2010; Johnston et al. 2015).

The contribution of non-immune cells (e.g. epithelial cells) is important to inflammatory responses in vivo and their role in NM toxicity can be investigated in vitro. The release of neutrophil and macrophage chemoattractants (e.g. IL-8, GROα, MCP-1, MIP-1) from a variety of cell types including hepatocytes (e.g. Kermanizadeh, Vranic et al. 2013), alveolar epithelial cells (e.g. Brown et al. 2001; Duffin et al. 2007; Monteiller et al. 2007; Baktur et al. 2011), intestinal epithelial cells (e.g. Gerloff et al. 2013), keratinocytes (e.g. Monteiro-Riviere et al. 2005), and renal cells (Kermanizadeh, Brown et al. 2013) has been observed in vitro. Furthermore, more complex in vitro models which incorporate >2 cell types (e.g. immune and non-immune cells) in static or fluidic conditions, for various target sites such as the lung, and gut (e.g. Lehmann et al. 2011; Kim et al. 2012; Susewind et al. 2016), can be used to better mimic in vivo situations when evaluating NM toxicity, and in order to investigate responses which require communication between different cell types (e.g. immune cell chemotaxis).

Of benefit is that the assays and approaches described previously to investigate oxidative stress (Figure 4) can be applied to both zebrafish and in vitro cell models. The contribution of oxidative stress to the toxicity of NMs is frequently investigated in vitro in immune, and non-immune cells. There is evidence that NMs (e.g. CNTs, Ag, ZnO), depending on their physico-chemical properties, can stimulate; intracellular ROS production, a depletion in antioxidants (e.g. glutathione), changes in antioxidant enzyme activity (e.g. SOD, catalase) and oxidative damage (e.g. lipid peroxidation, DNA damage) and reduction of toxicity with pretreatment of antioxidants in macrophages (e.g. Stone et al. 1998; Wilson et al. 2002; Brown et al. 2004; Kagan et al. 2006; Lee et al. 2009; Nguyen et al. 2016; Pati et al. 2016) and neutrophils (e.g. Liz et al. 2015). As fewer studies have investigated oxidative stress in neutrophils following NM exposure, this area could be prioritized in the future. When investigating the response of neutrophils and macrophages to NM exposure, activation of a
respiratory burst is commonly assessed as a marker of cell activation (e.g. Kagan et al. 2006; Lee et al. 2009; Johnston et al. 2015), with the release of ROS from cells following NM exposure likely to contribute to their toxicity (e.g. van Berlo et al. 2010). Oxidant signaling is important to controlling the function of immune cells, and an increase in intracellular ROS production in macrophages and neutrophils can lead to cell activation (e.g. Brown et al. 2004; Johnston et al. 2015). Increased ROS can also increase intracellular Ca\(^{2+}\) in phagocytes to activate cells (Ermak and Davies 2002). Understanding the mechanism by which immune cells are activated by NMs enables evidence based endpoints to screen their toxicity to be identified.

### 3.3. In vitro to in vivo extrapolation (IVIVE)

A general concern regarding the use of alternative systems (e.g. zebrafish and cell models) is that they cannot always predict the rodent and human response. Therefore, when applying alternative models to assess NM toxicity it is prudent to consider whether they are capable of predicting the findings of in vivo (rodent and human) models. This will support validation of their use so that they may gain regulatory acceptance and are adopted more widely. It is noteworthy that despite their widespread use, it is accepted that rodents will not always correctly predict human responses. Therefore, it is likely that no testing system will provide a perfect prediction of impacts of NMs on human health.

The relevance of using in vitro (macrophage or neutrophil) models to investigate NM toxicity has been explored in the published literature. For example, a good correlation between in vitro (primary human neutrophils) and in vivo (mouse) findings was reported when the response of neutrophils to TiO\(_2\) NMs was assessed (Gonçalves et al. 2010; Gonçalves and Girard 2011). Furthermore, macrophage responses in vitro can predict the pulmonary toxicity of NMs in rodents. For example, Wiemann et al. (2016) demonstrated that macrophage responses in vitro (e.g. LDH release, TNF production) provided a comparable ranking of NM toxicity to that observed in vivo following inhalation (rats; elevation of neutrophils), when particle concentration was quantified on a surface area basis. These findings are supported by several other studies (e.g. Rushton et al. 2010; Han et al. 2012; Kim et al. 2014; Teeguarden et al. 2014), which used a range of in vitro/ex vivo models (e.g. macrophages, lung epithelial cells, mouse lung slices) to demonstrate that a good prediction of the in vivo pulmonary response could be achieved, if NM dose was quantified on a surface area basis. Therefore, while in vitro models are often criticized for not mimicking the in vivo situation, there is evidence that the findings from simple, monocultures of cells which are used to screen the toxicity of NMs in vivo, but this depends on the dose metric used. Conversely, some studies have concluded that in vitro models are not always capable of predicting the in vivo response (Sayes et al. 2007; Warheit et al. 2009; Cho et al. 2010). This may derive from the cell type(s) investigated in the in vitro study, the NMs tested, the dose metric used to quantify exposure, and the lack of compatibility of responses investigated between models (e.g. cytotoxicity in vitro vs inflammation in vivo). Of interest is that the ability of in vitro models to predict the in vivo response has also been explored for a wider range of cell types. For example, as part of the EU funded project ENPRA, a panel of NMs were tested at a range of target sites (e.g. immune system, liver, cardiovascular system, lung, kidney), and a similar ranking of NM toxicity observed across in vitro and in vivo models (Kermanizadeh et al. 2016).

The use of zebrafish has been instrumental to toxicity assessments (and in particular cardiotoxicity, neurotoxicity, teratogenicity and hepatotoxicity), in understanding the pathogenesis of disease, and the development of new therapeutics (i.e. drug discovery) (Hill et al. 2005; Zan and Peterson 2005; Rubinstein 2006; Lieschke and Currie 2007; Goldsmith and Jobin 2012; Mathias et al. 2012; MacRae and Peterson 2015). Of benefit is that the findings from zebrafish studies often are in good concordance with those from human studies, and rodent models (e.g. Hill et al. 2005; Renshaw et al. 2007; Barros et al. 2008; Eimon and Rubinstein 2009; Martin and Renshaw 2009; Vojtech et al. 2009; Sipes et al. 2011; Sukardi et al. 2011; Afrikanova et al. 2013; Mesens et al. 2015). Overall, the good level of predictability of zebrafish models suggests that they could help bridge the gap between in vitro, and in vivo models, when conducting toxicity testing (Sukardi et al. 2011). For example, Driessen et al. (2013, 2015) compared the response of in vivo (zebrafish and mouse) and in vitro (human, mouse and rat hepatocytes) models to known hepatotoxins using transcriptomics and histopathology. Although there were model specific changes in gene expression in each model, overall zebrafish were able to provide similar findings (at a pathway level) to the more traditionally used cell and rodent models, suggesting a good concordance between models. Of note is that there were life stage specific effects observed in zebrafish, which is likely to be related to incomplete development of some systems in embryos. Despite increasing use of zebrafish in biomedical research (e.g. for drug discovery and toxicity testing) the application of zebrafish to assess NM impacts on human health is in its infancy, and few studies have evaluated NM toxicity across rodent, cell and zebrafish models. Rizzo et al. (2013) compared the toxicity of gold, iron oxide and polymeric NMs across zebrafish embryos (via assessment of developmental toxicity) and three cell lines; HeLa (human cervical carcinoma), HUVEC (human umbilical vein endothelial), and SMC (ovine smooth muscle), using cytotoxicity as an indicator of toxicity. In this work, they found there was not always a good correlation in NM toxicity between the zebrafish and in vitro models. However, this may derive from the choice of cells for the in vitro experiments, and the selection of endpoints they compared between models. More work is certainly needed to determine the suitability of using zebrafish to screen for inflammatory and oxidative responses to NMs. We suggest that there are common markers for inflammatory and oxidative responses across rodent, zebrafish and cell models which can be adopted to do this.

Of benefit is that a number of existing in vivo (rodent) studies have assessed the toxicity of NMs from the JRC NM repository (which has a supply of “representative” NMs
available to the scientific community) (e.g. Landsiedel, Mah- 
Hock et al. 2014; Gosens et al. 2015). Therefore, we recom-
mend that these NMs should be prioritized when assessing 
NM toxicity using zebrafish and *in vitro* immune cell models, as 
the existing *in vivo* data can be used to perform *in vitro in 
vivo* extrapolations to determine whether these alternative 
models can predict the *in vivo* response. Indeed, the predict-
itive nature of *in vitro* and *in vivo* models for a selection of 
these NMs has already been performed (Landsiedel, Sauer, et 
al. 2014; Wiemann et al. 2016).

4. Conclusions

Assessment of the toxicity of chemicals has traditionally been 
performed using rodent *in vivo* models. While these models 
have been invaluable in the assessment of chemical safety, 
the use of rodent models is not sustainable in the long term, 
particularly given the number of NMs that will require testing. 
Alternative approaches to investigate the activation and reso-
ution of inflammatory and oxidant responses by NMs could 
 improve implementation of the 3Rs principles and align stud-
ies with the principles of Toxicology for the twenty-first cen-
tury, while simultaneously reducing the cost and time 
associated with testing NM hazard. We suggest that immune 
cell based *in vitro* models, and transgenic zebrafish embryos 
are sensitive and relevant alternative (non-rodent) approaches

that should be prioritized when assessing NM safety in the 
future as part of a tiered testing strategy (Figure 8). More 
specifically, it is suggested that a tiered testing strategy is 
introduced which uses immune cell (macrophage and neutro-
phil) based *in vitro* models (preferably using human cell lines) 
in the first instance to screen NM toxicity, with primary cells 
used for comparative purposes (if deemed appropriate). 
Next, (transgenic) zebrafish embryos should be used to assess 
 inflammatory and oxidant responses in a whole organism 
following single or repeated exposure to NMs. Use of zebrafish 
in biomedical research is well established, and acceptance of 
their use by regulatory agencies (e.g. for developmental bi-
ology) is a strong foundation for development of the zebrafish 
model for assessment of more diverse markers of toxicity 
(e.g. inflammation). Our approach to screening NM toxicity 
would facilitate prioritizing NM selection and dose setting for 
*in vivo* testing, and may inform the need for performing *in 
vivo* studies for NMs and thereby reduce or replace rodent 
testing. Of benefit is that these alternative models are amen-
able to HTP testing. Model systems (e.g. rodents, cell, and 
zebrafish) used for toxicology testing have their own advan-
tages and limitations and these are presented in Table 1. The 
choice of model is influenced by a range of factors including 
access to facilities, biological relevance of model organism, 

cost and speed of testing, ethical implications of performing 
the proposed research, amenability to HTP screening.

![Tiered Testing Strategy for Assessment of NM Toxicity](image)

**Figure 8.** Tiered testing strategy for assessment of NM toxicity. A tiered testing strategy focusing on assessment of immunological and oxidative stress responses as primary effect mechanisms would improve testing efficiency for many NMs, and alignment of testing with the 3Rs principles and toxicology for the twenty-first cen-
tury. In such a strategy, we suggest that toxicity of NMs is first screened *in vitro* using cell (macrophage and neutrophil) lines (preferably of human origin), and 
where possible using HTP systems. Next, focused studies would be performed using primary cells to increase confidence in the data obtained from cell lines. The 
use of zebrafish would then be used to bridge the gap between *in vitro* and the potential for effects in rodents. If significant potential hazard is identified in these 
test systems then this would direct the need for rodent toxicity testing. The use of a tiered testing strategy also helps to inform on dose selection for focused *in vivo* studies.
Oxidative stress and inflammation are common to many diseases, and the mechanism of toxicity of chemicals. Thus, the models discussed are not only relevant to nanotoxicology studies but to other chemicals and pathogens, and thus the models could be widely adopted by toxicologists. This review has focused on assessment of inflammatory and oxidant responses, however, we recognize that NMs may stimulate other biological effects that are not captured in the models identified. Thus, use of the zebrafish and in vitro models described in this review alone are unlikely to be sufficient to provide a comprehensive assessment of NM hazard. What we illustrate, however, is that the use of alternative models can be enhanced in nanotoxicology testing, and suggest how the integration of these alternative models will support the development of a tiered testing strategy that improves the implementation of the 3Rs principles. Adoption of these models will provide an invaluable insight into the toxicity of NMs and have the potential to reduce the burden placed on animal testing, but further analyses are required to assess fully their capacity to reduce and replace rodent testing.

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References

Abrikossova N, Skoglund C, Ahren M, Bengtsson T, Uvdal K. 2012. Effects of gadolinium oxide nanoparticles on the oxidative burst from human neutrophil granulocytes. Nanotechnology. 23:275101.

Afiakanova T, Serruys AS, Buenafe OE, Clinckers R, Smolders I, de Witte PA, Crawford AD, Esguerra CV. 2013. Validation of the zebrafish pentylenetetrazol seizure model: locomotor versus electrographic responses to antiepileptic drugs. PLoS One. 8:e54166.

Agius C, Roberts RJ. 2003. Melano-macrophage centres and their role in fish pathology. J Fish Dis. 26:499–509.

Babin K, Antoine F, Goncalves DM, Girard D. 2013. TiO2, CeO2, and ZnO nanoparticles and modulation of the degranulation process in human neutrophils. Toxicol Lett. 221:57–63.

Baktur R, Patel H, Kwon S. 2011. Effect of exposure conditions on SWCNT-induced inflammatory response in human alveolar epithelial cells. Toxicol In Vitro. 25:1153–1160.

Barros TP, Alderton WK, Reynolds HM, Roach AG, Berghmans S. 2008. Zebrafish: an emerging technology for in vivo pharmacological assessment to identify potential safety liabilities in early drug discovery. Br J Pharmacol. 154:1400–1413.
Beerman RW, Matty MA, Au GG, Looger LL, Choudhury KR, Keller PJ, Tobin DM. 2015. Direct in vivo manipulation and imaging of calcium transients in neutrophils identify a critical role for leading-edge calcium flux. Cell Rep. 13:2107–2117.

Benard EL, van der Sar AM, Eliett F, Lieschke GJ, Spank HP, Meijer AH. 2012. Infection of zebrafish embryos with intracellular bacterial pathogens. J Vis Exp. 15:3781.

Bilberg K, Bruun Hoygaard M, Besenbacher F, Baatrup E. 2012. In vivo toxicity of silver nanoparticles and silver ions in zebrafish (Danio rerio). J Toxicol. 2012:293784.

Borm PJ, Tran L. 2002. From quartz hazard to quartz risk: the coal mines revisited. Ann Occup Hyg. 46:25–32.

Boyles MS, Ranning C, Reischl R, Rurik M, Tessadri R, Kohlbacher O, Duschl A, Huber CG. 2016. Copper oxide nanoparticle toxicity profiling using untargeted metabolomics. Part Fibre Toxicol. 13:49.

Boyles MS, Young L, Brown DM, MacCalman L, Cowie H, Moisala A, Smial F, Smith PJ, Proudfoot L, Windle AH, et al. 2015. Multi-walled carbon nanotube induced frustrated phagocytosis, cytotoxicity and pro-inflammatory conditions in macrophages are length dependent and greater than that of asbestos. Toxicol In Vitro. 29:1513–1528.

Brown DM, Dickson C, Duncan F, Al-Attili F, Stone V. 2010. Interaction between nanoparticles and cytokine proteins: impact on protein and particle functionality. Nanotechnology. 21:215104.

Brown DM, Donaldson K, Borm PJ, Schins RP, Dehnhardt M, Gilmour P, Jimenez LA, Stone V. 2004. Ca2+ and ROS-mediated activation of transcription factors and TNF-cytokine gene expression in macrophages exposed to ultrafine particles. Am J Physiol: Lung Cell Mol Physiol. 286:L344–L353.

Brown DM, Kinloch IA, Bangert U, Windle AH, Walter DM, Walker GS, Scottchord CA, Donaldson K, Stone V. 2007. An in vitro study of the potential of carbon nanotubes and nanofibres to induce inflammatory mediators and frustrated phagocytosis. Carbon. 45:1743–1756.

Brown SB, Tucker CS, Ford C, Lee Y, Dunbar DR, Mullins JJ. 2007. Class Ill antiarrhythmic methanesulfonanilides inhibit leukocyte recruitment in zebrafish. J Leukoc Biol. 82:79–84.

Brown DM, Wilson MR, MacNee W, Stone V, Donaldson K. 2001. Size-dependent proinflammatory effects of ultrafine polystyrene particles: a role for surface area and oxidative stress in the enhanced activity of ultrafines. Toxicol Appl Pharmacol. 175:191–199.

Burden N, Aschberger K, Chaudhry Q, Clift MJ, Doak S, Fowler P, Johnston H, Landsiedel R, Rowland J, Stone V. 2017. The 3Rs as a framework to support a 21st century approach for nanosafety assessment. Nano Today. 12:10–13.

Burden N, Benestrand R, Clok M, Doyle I, Edwards P, Maynard SK, Ryder K, Sheahan D, Whale G, van Emgordon R, et al. 2016. Advancing the 3Rs in regulatory ecotoxicology: a pragmatic cross-sector approach. Integr Environ Assess Manag. 12:417–421.

Burden N, Mahony C, Muller BP, Terry C, Westmoreland C, Kimber I. 2015. Aligning the 3Rs with new paradigms in the safety assessment of chemicals. Toxicology. 330:62–66.

Burns CG, Milan DJ, Grande EJ, Rottbauer W, MacRae CA, Fishman MC. 2005. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. Nat Chem Biol. 1:263–264.

Chakraborty C, Sharma AR, Sharma G, Lee SS. 2016. Zebrafish: a complete animal model to enumerate the nanoparticle toxicity. J Nanobiotechnol. 14:65.

Cheung CY, Webb SE, Love DR, Miller AL. 2011. Visualization, characterization and modulation of calcium signaling during the development of slow muscle cells in intact zebrafish embryos. Int J Dev Biol. 55:153–174.

Cho WS, Duffin R, Poland CA, Howie SE, MacNee W, Bradley M, Megson IL, Donaldson K. 2010. Metal oxide nanoparticles induce unique inflammatory footprints in the lung: important implications for nanoparticle testing. Environ Health Perspect. 118:1699–1706.

Choi JE, Kim S, Ahn JH, Youn P, Kang JS, Park K, Yi J, Ryu DY. 2010. Induction of oxidative stress and apoptosis by silver nanoparticles in the liver of adult zebrafish. Aquat Toxicol. 100:151–159.

Choi JY, Ramachandran G, Kandlikar M. 2009. The impact of toxicity testing costs on nanomaterial regulation. Environ Sci Technol. 43:3030–3034.

Chu J, Sadler KC. 2009. A new school in liver development: lessons from zebrafish. Hepatology. 50:1656–1663.

Clift MJ, Boyles MS, Brown DM, Stone V. 2010. An investigation into the potential for different surface-coated quantum dots to cause oxidative stress and affect macrophage cell signalling in vitro. Nanotoxicology. 4:139–149.

Clift MJ, Rothen-Rutishauser B, Brown DM, Duffin R, Donaldson K, Proudfoot L, Guy K, Stone V. 2008. The impact of different nanoparticle surface chemistry and size on uptake and toxicity in a murine macrophage cell line. Toxicol Appl Pharmacol. 232:418–427.

Costa PM, Fadeel B. 2016. Emerging systems biology approaches in nanotoxicology: towards a mechanism-based understanding of nanomaterial hazard and risk. Toxicol Appl Pharmacol. 299:101–111.

Couto D, Freitas M, Vilas-Boas V, Dias I, Porto G, Lopez-QUINTAL MA, Rivas J, Freitas P, Carvalho F, Fernandes E. 2014. Interaction of polycrylic acid coated and non-coated iron oxide nanoparticles with human neutrophils. Toxicol Lett. 225:57–65.

Davis EE, Frangakis S, Katsanis N. 2014. Interpreting human genetic variation with in vivo zebrafish assays. Biochim Biophys Acta. 1842:1960–1970.

Davies LC, Jenkins SJ, Allen JE, Taylor PR. 2013. Tissue-resident macrophages. Nat Immunol. 14:986–995.

Davies JM, Clay H, Lewis JL, Ghorii N, Herbomel P, Ramakrishnan L. 2002. Real-time visualization of mycobacterium–macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. Immunity. 17:693–702.

Davies LC, Taylor PR. 2015. Tissue-resident macrophages: then and now. Immunology. 144:541–548.

de Oliveira S, Lopez-Munoz A, Martinez-Navarro FJ, Galindo-Villegas J, Mulero V, Callado A. 2015. Cxcl8–11 and Cxcl8–12 are required in the zebrafish defense against Salmonella Typhimurium. Dev Comp Immunol. 49:44–48.

de Oliveira S, Reyes-Aldasoro CC, Candel S, Renshaw SA, Mulero V, Callado A. 2013. Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response. J Immunol. 190:4349–4359.

Deng Q, Sarris M, Bennin DA, Green J, Herbomel P, Huttenlocher A. 2013. Localized bacterial infection induces systemic activation of neutrophils through Cxcr2 signaling in zebrafish. J Leukoc Biol. 93:761–769.

Dockery DW, Pope CA, 3rd, Xu X, Spengler JD, Ware JH, Fay ME, Ferris BG, Speizer FE. 1993. An association between air pollution and mortality in six U.S. cities. N Engl J Med. 329:1753–1759.

Dodd A, Curtis PM, Williams LC, Love DR. 2000. Zebrafish: bridging the gap between development and disease. Hum Mol Genet. 9:2443–2449.

Donaldson K, Seaton A. 2012. A short history of the toxicology of inhaled particles. Part Fibre Toxicol. 9:13.

Donaldson K, Stone V, Donaldson LA. 2015. Localized bacterial infection induces systemic activation of neutrophils through Cxcr2 signaling in zebrafish. J Leukoc Biol. 93:761–769.

Driscoll T, Nelson DJ, Steenland K, Leigh J, Concha-Barrientos M, Fingerhut M, Prüss-Ustün A. 2005. The global burden of non-malignant respiratory disease due to occupational airborne exposures. Am J Ind Med. 48:432–445.

Drummond IA, Davidson AJ. 2010. Zebrafish kidney development. Methods Cell Biol. 100:233–260.

Duffin R, Tran L, Brown D, Stone V, Donaldson K. 2007. Proinflammogenic effects of low-toxicity and metal nanoparticles in vivo and in vitro:
highlighting the role of particle surface area and surface reactivity. Inhal Toxicol. 19:849–856.

Duan J, Yu Y, Li Y, Liu H, Jing L, Yang M, Wang J, Li C, Sun Z. 2016a. Low-dose exposure of silica nanoparticles induces cardiac dysfunction via neutrophil-mediated inflammation and cardiac contraction in zebrafish embryos. Nanotoxicology. 10:575–585.

Duan J, Yu Y, Li Y, Wang Y, Sun Z. 2016b. Inflammatory response and blood hypercoagulable state induced by low level co-exposure with silica nanoparticles and benzo[a]pyrene in zebrafish (Danio rerio) embryos. Chemosphere. 151:152–162.

Duan J, Yu Y, Shi H, Tian L, Guo C, Huang P, Zhou X, Peng S, Sun Z. 2013. Toxic effects of silica nanoparticles on zebrafish embryos and larvae. PLoS One. 8:e74606.

ECHA. 2011. The use of alternatives to testing on animals for the REACH regulation. https://echa.europa.eu/documents/10162/13639/alternatives_test_animals_2011_en.pdf/9b0f7e93-4d61-401d-ba2c-80b369a26e66.

ECHA. 2014. The use of alternatives to testing on animals for the REACH regulation. Second report under Article 117(3) of the REACH Regulation. https://echa.europa.eu/documents/10162/13639/alternatives_test_animals_2014_en.pdf.

ECHA. 2015. Read-across assessment framework (RAAF), https://echa.europa.eu/documents/10162/13628/raaf_en.pdf.

ECHA. 2016a. REACH testing requirements and the ban on animal testing for cosmetics: reply to Peta campaign. http://ec.europa.eu/environment/chemicals/reach/animal_en.htm.

ECHA. 2016b. Practical guide: how to use alternatives to animal testing. https://echa.europa.eu/documents/10162/13655/practical_guide_how_to_use_alternatives_en.pdf.

Eimon PM, Rubinstein AL. 2009. The use of in vivo zebrafish assays in drug toxicity screening. Expert Opin Drug Metab Toxicol. 5:393–401.

Ellett F, Elks PM, Robertson AL, Ogryzko NV, Renshaw SA. 2015. Defining the phenotype of neutrophils following reverse migration in zebrafish. J Leukoc Biol. 98:975–981.

Ellett F, Pase L, Hayman JW, Andrianopoulos A, Lieschke GJ. 2011. mpeg1 promoter transgenes direct macrophage-lineage expression in zebrafish. Blood. 117:e49–e56.

Ermak G, Davies KJ. 2002. Calcium and oxidative stress: from cell signalig to cell death. Mol Immunol. 38:713–721.

European Commission. 2011. Recommendation on the definition of a nanomaterial. http://ec.europa.eu/environment/chemicals/nanotech/pdf/commission_recommendation.pdf.

European Parliament. 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. http://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32010L0063.

Fairbrother A, Fairbrother JR. 2009. Are environmental regulations keeping up with innovation? A case study of the nanotechnology industry. Ecotoxicol Environ Saf. 72:1327–1330.

Fang L, Green SR, Baek JS, Lee SH, Ellett F, Deer E, Lieschke GJ, Wittum JL, Tsimikas S, Miller YI. 2011. In vivo visualization and attenuation of inflammatory response in a zebrafish larval model of endotoxemia. J Leukoc Biol. 98:1046–1057.

Farca l L, Torres Andon F, Di Cristo L, Rotoli BM, Bussolati O, Bergamaschi E, Mech A, Hartmann NB, Rasmussen K, Riego-Santes J, et al. 2015. Comprehensive in vitro toxicity testing of a panel of representative oxide nanomaterials: first steps towards an intelligent testing strategy. PLoS One. 10:e0127174.

Faria M, Navas JM, Raldua D, Soares AM, Barata C. 2014. Oxidative stress effects of titanium dioxide nanoparticle aggregates in zebrafish embryos. Sci Total Environ. 470–471:379–389.

Farrera C, Bhattacharya K, Lazzaretto B, Andon FT, Hultenby K, Kotchev GP, Star A, Fadeel B. 2014. Extracellular entrapment and degradation of single-walled carbon nanotubes. Nanoscale. 6:6974–6983.

Feng Y, Santoriello C, Mione M, Hurlstone A, Martin P. 2010. Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: parallels between tumor initiation and wound inflammation. PLoS Biol. 8:e1000562.

Filip JG, Kebir DE. 2009. Neutrophil apoptosis: a target for enhancing the resolution of inflammation. J Cell Biochem. 108:1039–1046.

Foucauld L, Wilson MR, Brown DM, Stone V. 2007. Measurement of reactive species production by nanoparticles prepared in biologically relevant media. Toxicol Lett. 174:1–9.

Ganesan S, Anaimalai Thirumurthi N, Raghunath A, Vijayakumar S, Perumal E. 2016. Acute and sub-lethal exposure to copper oxide nanoparticles causes oxidative stress and teratogenicity in zebrafish embryos. J Appl Toxicol. 36:554–567.

Geiser M, Casaula M, Kupferschmid B, Schulz H, Semmler-Behnke M, Kreyling W. 2008. The role of macrophages in the clearance of inhaled ultrafine titanium dioxide particles. Am J Respir Cell Mol Biol. 38:371–376.

Gerloff K, Pereira D, Faria N, Boots AW, Kolling J, Förster I, Albrecht C, Powell JJ, Schins RP. 2013. Influence of simulated gastrointestinal conditions on particle-induced cytotoxicity and interleukin-8 regulation in differentiated and undifferentiated Caco-2 cells. Nanotoxicology. 7:353–366.

Goldsmith JR, Jobin C. 2012. Think small: zebrafish as a model system of human pathology. J Biomed Biotechnol. 2012:817341.

Gonzalves DM, Chiasson S, Girard D. 2010. Activation of human neutrophils by titanium dioxide (TiO2) nanoparticles. Toxicol In Vitro. 24:1002–1008.

Gonzalves DM, Girard D. 2011. Titanium dioxide (TiO2) nanoparticles induce neutrophil influx and local production of several pro-inflammatory mediators in vivo. Int Immunopharmacol. 11:1109–1115.

Goodhead RM, Moger J, Galloway TS, Tyler CR. 2015. Tracing engineered nanomaterials in biological tissues using coherent anti- Stokes Raman scattering (CARS) microscopy - a critical review. Nanotoxicology. 9:928–939.

Gosens I, Kermanizadeh A, Jacobsen NR, Lenz AG, Bokkers B, de Jong WH, Krystek P, Tran L, Stone V, Wallin H, et al. 2015. Comparative hazard and identification by a single dose lung exposure of zinc oxide and silver nanomaterials in mice. PLoS One. 10:e126934.

Gratapaci RL, Scherer AK, Seman BG, Wheeler RT. 2017. Control of mucus candidiasis in the zebrafish swim bladder depends on neutrophils that block filament invasion and drive extracellular-trap production. Infect Immun. 85:e00276-17.

Haase H, Fahmi A, Mahltig B. 2014. Impact of silver nanoparticle and silver ions on innate immune cells. J Biomed Nanotechnol. 10:1146–1156.

Hall CJ, Boyle RH, Astin JW, Flores MV, Oehlers SH, Sanderson LE, Ellett F, Lieschke GJ, Crosby KE, Crosby PS. 2013. Immunoresponsive gene 1 augments bacterial activity of macrophage-lineage cells by regulating β-oxidation-dependent mitochondrial ROS production. Cell Metab. 18:265–278.

Hall CJ, Wicker SM, Chien AT, Tropm A, Lawrence LM, Sun X, Krissansen GW, Crosby KE, Crosby PS. 2014. Repositioning drugs for inflammatory disease - fishing for new anti-inflammatory agents. Dis Model Mech. 7:1069–1081.

Halpern BN, Benacerraf B, Bizzi G. 1953. Quantitative study of the granulopoietic activity of the reticuloendothelial system, I: The effect of the ingredients present in india ink and of substances affecting blood clotting in vivo on the fate of carbon particles administered intravenously in rats, mice and rabbits. Br J Exp Pathol. 34:426–440.

Hartung T, Sabbioni E. 2011. Alternative in vitro assays in nanomaterial toxicity. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 3:545–573.

Han X, Corson N, Wade-Mercer P, Gelein R, Jiang J, Sahu M, Biswas P, Finkelstein JN, Elder A, Oberdörster G. 2012. Assessing the relevance of in vitro studies in nanotoxicology by examining correlations between in vitro and in vivo data. Toxicology. 297:1–9.

Hartung T. 2009. Toxicology for the twenty-first century. Nature. 460:208–212.

Harvie EA, Huttenlocher A. 2015. Non-invasive imaging of the innate immune response in a zebrafish larval model of Streptococcus iniae infection. J Vis Exp. 98:e52788.

He JH, Gao JM, Huang CJ, Li CQ. 2014. Zebrafish models for assessing developmental and reproductive toxicity. Neurotoxicol Teratol. 42:35–42.

Henry KM, Loynes CA, Whyte MK, Renshaw SA. 2013. Zebrafish as a model for the study of neutrophil biology. J Lekoc Biol. 94:633–642.
Herbomel P, Thibes B, Thisse C. 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development. 126:3735–3745.

Hermann AC, Millard PJ, Blake SL, Kim CH. 2004. Development of a respiratory burst assay using zebrafish kidneys and embryos. J Immunol Methods. 292:119–129.

Hill AJ, Teraoka H, Heideman W, Peterson RE. 2005. Zebrafish as a model vertebrate for investigating chemical toxicity. Toxicol Sci. 86:6–19.

Hoodless LJ, Lucas CD, Duffin R, Dervir MA, Haslett C, Tucker CS, Rossi AG. 2016. Genetic and pharmacological inhibition of CDK9 drives neutrophil apoptosis to resolve inflammation in zebrafish in vivo. Sci Rep. 5:36980.

Howe K, Clark MD, Torroja CF, Torrance J, Berthelot C, Muffato M, Collins JE, Humphrey S, McLaren K, Matthews L, et al. 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 496:498–503.

International Agency for Research on Cancer (IARC). 1997. Monographs on the evaluation of carcinogenic risks to humans. Vol. 68. Silica, some silicates, coal dust and para-aromatic fibrils. Lyon, France.

Jim KK, Engelen-Lee J, van der Sar AM, Bitter W, Brouwer MC, van der Ende A, Vanering JW, van de Beek D, Vandenbroucke-Grauls CM. 2016. Infection of zebrafish embryos with live fluorescent Streptococcus pneumoniae as a real-time pneumococcal meningitis model. J Neuroinflammation. 13:188.

Johnston H, Brown DM, Kanase N, Euston M, Gaiser BK, Robb CT, Dwyryda E, Rossi AG, Brown ER, Stone V. 2015. Mechanism of neutrophil activation and toxicity elicited by engineered nanomaterials. Toxicol In Vitro. 29:1177–1187.

Jones HR, Robb CT, Perretti M, Rossi AG. 2016. The role of neutrophils in inflammation resolution. Semin Immunol. 28:137–145.

Jovanovic B, Anastasova L, Rowe EW, Zhang Y, Clapp AR, Palić D. 2011. Effects of nanosized titanium dioxide on innate immune system of fat-head minnow (Pimephales promelas Rafinesque, 1820). Ecotoxicol Environ Saf. 74:675–683.

Kagan VE, Tyurina YY, Tyurin VA, Konduru NV, Potapovich AI, Osipov AN, Kisin ER, Schweger-Berry D, Mercer R, Castranova V, et al. 2006. Direct and indirect effects of single walled carbon nanotubes on RAW 264.7 macrophages: role of iron. Toxicol Lett. 165:88–100.

Kanther M, Sun X, Mühlbauer M, Mackey LC, Flynn EJ, III, Bagnat M, Jobin C, Rawls JF. 2011. Microbial colonization induces dynamic temporal and spatial patterns of NF-κB activation in the zebrafish digestive tract. Gastroenterology. 141:197–207.

Kermanizadeh A, Brown DM, Kanase N, Euston M, Gaiser BK, Robb CT, Dwyryda E, Rossi AG, Brown ER, Stone V. 2013. Engineered nanomaterial impact in the liver following exposure via an intravenous route – the role of polymorphonuclear leucocytes and gene expression in the organ. J Nanomed Nanotechnol. 4:157.

Kermanizadeh A, Gosens I, MacCalman L, Johnston H, Danielsen PH, Kermanizadeh A, Brown DM, Hutchison GR, Stone V. 2013. Engineered nanomaterials using a human renal cell line: cytotoxicity, pro-inflammatory cytokines and functional markers. J Nanobiotechnol. 12:47.

Kong H, Zhang Y, Li Y, Cui Z, Xia K, Sun Y, Zhao Q, Zhu Y. 2013. Size-dependent cytotoxicity of nanocarbon blacks. Int J Mol Sci. 14:22529–22543.

Labib S, Williams A, Vauk CL, Nikota JK, Wallin H, Vogel U, Halappanavar S. 2016. Nano-risk Science: application of toxicogenomics in an adverse outcome pathway framework for risk assessment of multi-walled carbon nanotubes. Part Fibre Toxicol. 13:115.

Landsiedel R, Ma-Hock L, Hofmann T, Wiemann M, Strauss V, Treumann S, Wohlenbe W, Gröters S, Wiench K, van Renningswaay B. 2014. Application of short-term inhalation studies to assess the inhalation toxicity of nanomaterials. Part Fibre Toxicol. 11:16.

Landsiedel R, Sauer UG, Ma-Hock L, Schnekenburger J, Wiemann M. 2014. Pulmonary toxicity of nanomaterials: a critical comparison of published in vitro assays and in vivo inhalation or instillation studies. Nanomedicine (Lond). 9:2557–2585.

Lanone S, Rogerieux F, Geys J, Dupont A, Maillot-Marechal E, Boczkowski J, Lacroix G, Hoet P. 2009. Comparative toxicity of 24 manufactured nanoparticles in human alveolar epithelial and macrophage cell lines. Part Fibre Toxicol. 6:14.

Lee HM, Shin DM, Song HM, Yuk JM, Lee ZW, Lee SH, Hwang SM, Kim JM, Lee CS, Jo EK. 2009. Nanoparticles up-regulate tumor necrosis factor-alpha and CXCL8 via reactive oxygen species and mitogen-activated protein kinase activation. Toxicol Appl Pharmcol. 238:160–169.

Lee KJ, Browning LM, Nallathamby PD, Desai T, Cherukuri PK, Xu XH. 2012. In vivo quantitative study of sized-dependent transport and toxicity of single silver nanoparticles using zebrafish embryos. Chem Res Toxicol. 25:1029–1046.

Lee KJ, Nallathamby PD, Browning LM, Osgood CJ, Xu XH. 2007. In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos. ACS Nano. 1:133–143.

Lee D, Green JM, Tyler CR. 2015. Transgenic fish systems and their application in ecotoxicology. Crit Rev Toxicol. 45:124–141.

Lehmann AD, Naum D, Bur M, Lehr CM, Gehr P, Rotten-Rutishauser BM. 2011. An in vitro triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier. Eur J Pharm Biopharm. 77:398–406.

Levraud JP, Disson O, Kissa K, Bonne I, Cossart P, Herbomel P, Lecuit M. 2009. Real-time observation of listeria monocytogenes-phagocyte interactions in living zebrafish larvae. Infect Immun. 77:3651–3660.

Li N, Xie T, Nel AE. 2008. The role of oxidative stress in ambient particulate matter-induced lung diseases and its implications in the toxicity of engineered nanoparticles. Free Radic Biol Med. 44:1689–1699.

Li L, Zhang WQ, Yan B, Shi YQ, Wen ZL. 2012. Live imaging reveals different roles of macrophages and neutrophils during zebrafish tail fin regeneration. J Biol Chem. 287:25353–25360.

Lieschke GJ, Currie PD. 2007. Animal models of human disease: zebrafish swim into view. Nat Rev Genet. 8:353–367.

Lin A, Loughman JA, Zinselmeyer BH, Miller MJ, Caparon MG. 2009. Streptolysin S inhibits neutrophil recruitment during the early stages of Streptococcus pyogenes infection. Infect Immun. 77:5190–5201.

Lin S, Zhao Y, Nel AE, Lin S. 2013. Zebrafish: an in vivo model for nano EHS studies. Small. 9:1608–1618.

Lin S, Zhao Y, Xia T, Meng H, Ji Z, Liu R, George S, Xiong S, Wang X, Zhang H, et al. 2011. High content screening in zebrafish speeds up hazard ranking of transition metal oxide nanoparticles. ACS Nano. 5:7284–7295.

Liz R, Simard JC, LeonardLB, Girard D. 2015. Silver nanoparticles rapidly induce atypical human neutrophil cell death by a process involving inflammatory caspses and reactive oxygen species and induce neutrophil extracellular traps release upon cell adhesion. Int Immunopharmacol. 28:616–625.

Loynes CA, Martin JS, Robertson A, Trushell DM, Ingham PW, Whyte MK, Renshaw SA. 2010. Pivotal advance: pharmacological manipulation of the outcome pathway framework for risk assessment of multi-walled carbon nanotubes. Part Fibre Toxicol. 7:129.

Lucas CD, Allen KC, Dorward DA, Hoodless LJ, Melrose LA, Marwick JA, Tucker CS, Haslett C, Duffin R, Rossi AG. 2013. Flavones induce neutrophil apoptosis by down-regulation of Mcl-1 via a proteasomal-dependent pathway. FASEB J. 27:1084–1094.
MacRae CA, Peterson RT. 2015. Zebrafish as tools for drug discovery. Nat Rev Drug Discov. 14:721–731.

Ma-Hock L, Treumann S, Strauss V, Brill S, Luizi F, Mertler M, Wiencz K, Gamer AO, van Ravenzaaw B, Landsiedel R. 2009. Inhalation toxicity of multiwall carbon nanotubes in rats exposed for 3 months. Toxicol Sci. 112:468–481.

Marjomar J, Alvers A, Deerehke ME, Bagwell J, Mankiewicz JC, Cocchiaro JL, Beerman RW, Miller J, Sumigray KD, Katsanis N, et al. 2015. Epigenetic control of intestinal barrier function and inflammation in zebrafish. Proc Natl Acad Sci USA. 112:2770–2775.

Martin JS, Renshaw SA. 2009. Using in vivo zebrafish models to understand the biochemical basis of neutrophic respiratory disease. Biochem Soc Trans. 37:830–837.

Massarys A, Dupuis L, Taylor J, Eisa-Beygi S, Strek L, Trudeau VL, Moon TW. 2013. Assessment of nanosilver toxicity during zebrafish (Danio rerio) development. Chemosphere. 92:59–66.

Mathias JR, Saxena MT, Mumm JS. 2012. Advances in zebrafish chemical screening technologies. Future Med Chem. 4:1811–1822.

Mathias JR, Walters KB, Huttonlocher A. 2009. Neutrophil motility in vitro using zebrafish. Methods Mol Biol. 571:151–166.

Mayadas TN, Cullere X, Lowell CA. 2014. The multifaceted functions of zebrafish larvae. Thromb Haemost. 103:797–807.

McLeish JA, Chico TJ, Taylor HB, Tucker C, Donaldson K, Brown SB. 2010. Skin exposure to micro- and nano-particles can cause haemostasis in zebrafish larvae. Thromb Haemost. 103:797–807.

Meckers ND, Trede NS. 2010. Immunology and zebrafish: spawning new applications. Wellesley (MA): BCCResearch.

Meeker ND, Trede NS. 2015. Zebrafish as tools for drug discovery. Nat Rev Drug Discov. 14:721–731.

Merrell DL, Shaw BJ, Harper GM, Saoud IP, Davies SJ, Handy RD, Henny TB. 2013. Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (Danio rerio). Environ Pollut. 174:157–163.

Mesens N, Crawford AD, Menke A, Hung PD, Van Goethem F, Nuyts R, Hansen E, Wolterbeek A, Van Gompel J, De Witte P, et al. 2015. Are zebrafish larvae suitable for assessing the hepatotoxicity potential of drug candidates? J Appl Toxicol. 35:1017–1029.

Mestas J, Hughes CC. 2004. Of mice and not men: differences between species in inflammation and tissue injury. Antioxid Redox Signal. 184:18–25.

Meekers ND, Trede NS. 2010. Immunology and zebrafish: spawning new applications. Wellesley (MA): BCCResearch.

Mesers C, Crawford AD, Menke A, Hung PD, Van Goethem F, Nuyts R, Hansen E, Wolterbeek A, Van Gompel J, De Witte P, et al. 2015. Are zebrafish larvae suitable for assessing the hepatotoxicity potential of drug candidates? J Appl Toxicol. 35:1017–1029.

Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. 2014. Reactive oxygen species in inflammation and tissue injury. Antioxid Redox Signal. 20:1126–1167.

Monteiller C, Tran L, MacNee W, Faux S, Jones A, Miller B, Donaldson K. 2007. The pro-inflammatory effects of low-toxicity low-solubility particles, nanoparticles and fine particles, on epithelial cells in vitro: the role of surface area. Occup Environ Med. 64:609–615.

Monteiro-Riviere NA, Nemanich RJ, Inman AO, Wang YY, Riviere JE. 2005. Multi-walled carbon nanotube interactions with human epithelial keratinocytes. Toxicol Lett. 155:377–384.

Mugoni V, Camporeale A, Santoro MM. 2014. Analysis of oxidative stress in zebrafish embryos, JoVE. 89:51328.

Murphy FA, Poon R, Duffin R, Donaldson K. 2013. Length-dependent pleural inflammation and parietal pleural responses after deposition of carbon nanotubes in the pulmonary airspaces of mice. Nanotoxicology. 7:1157–1167.

Nathan C. 2006. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 6:173–182.

Nel A, Xia T, Madler L, Li N. 2006. Toxic potential of materials at the nanolevel. Science. 311:622–627.

Nguyen KC, Richards L, Massarsky A, Moon TW, Tayabali AF. 2016. Toxicological evaluation of representative silver nanoparticles in macrophages and epithelial cells. Toxicol In Vitro. 33:163–173.

Nguyen-Chi M, Phan QT, Gonzalez C, Dubremetz J-F, Levraud J-P, Lutfallah G. 2014. Transient infection of the zebrafish notochord with E. coli induces chronic inflammation. Dis Models Mech. 7:871–882.

Niethammer P, Grabher C, Look AT, Mitchison TJ. 2009. A tissue-scale gradient of hydrogen peroxide mediates rapid wound detection in zebrafish. Nature. 459:996–999.

Novoa B, Figueras A. 2012. Zebrafish: model for the study of inflammation and the innate immune response to infectious diseases. Adv Exp Med Biol. 946:253–275.

Nowack B. 2017. Evaluation of environmental exposure models for engineered nanomaterials in a regulatory context. Nanomultip., 8:38–47.

Oberdörster G, Stone V, Donaldson K. 2007. Toxicology of nanoparticles: a historical perspective. Nanotoxicology. 1:2–25.

Ogawara K, Yoshida M, Higaki K, Kimura T, Shiraiishi K, Nishikawa M, Takakura Y, Hashida M. 1999. Hepatic uptake of polystyrene microspheres in rats: effect of particle size on intrahepatic distribution. J Control Release. 59:15–22.

Olsen H, Betton G, Robinson D, Thomas K, Monro A, Kolaja L, Lilly P, Sanders J, Sipes G, Bracken W, et al. 2000. Concordance of the toxicity of pharmaceuticals in humans and in animals. Regul Toxicol Pharmacol. 32:56–67.

Osborne OJ, Johnston BD, Moger J, Baloumsha M, Lead JR, Kudoh T, Meyer CR. 2013. Effects of particle size and coating on nanoscale Ag and TiO2 exposure in zebrafish (Danio rerio) embryos. Nanotoxicology. 7:1315–1324.

Osborne OJ, Lin S, Chang CH, Ji Z, Yu X, Wang X, Lin S, Xia T, Nel AE. 2015. Organ-specific and size-dependent ag nanoparticle toxicity in gills and intestines of adult zebrafish. ACS Nano. 9:9573–9584.

Osborne OJ, Mukaigasa K, Nakajima H, Stolpe B, Romer I, Philips U, Lynch I, Mourabit S, Hiros S, Lead JR, et al. 2016. Sensory systems and ionocytes are targets for silver nanoparticle effects in fish. Nanotoxicology. 10:1276–1288.

Park EJ, Park K. 2009. Oxidative stress and pro-inflammatory responses induced by silica nanoparticles in vivo and in vitro. Toxicol Lett. 184:18–25.

Pase L, Layton JE, Wittmann C, Ellett F, Nowell CJ, Reyes-Aldasoro CC, Varma S, Rogers KL, Hall CJ, Keightley MC, et al. 2012. Neutrophil-delivered myeloperoxidase dampens the hydrogen peroxide burst after tissue wounding in zebrafish. Curr Biol. 22:1818–1824.

Pati R, Das I, Mehta RK, Sahu R, Sonawane A. 2016. Zinc-oxide nanoparticles exhibit genotoxic, cytotoxic, cytoytic and actin depolymerization effects by inducing oxidative stress responses in macrophages and adult mice. Toxicol Sci. 150:454–472.

Poland CA, Duffin R, Kinloch I, Maynard A, Wallace WA, Seaton A, Stone V, Brown S, Macnee W, Donaldson K. 2008. Carbon nanotubes introduced into the abdominal cavity of mice show asbestos-like pathogenicity in a pilot study. Nat Nanotechnol. 3:423–428.

Poon IK, Lucas CD, Rossi AG, Ravichandran KS. 2014. Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol. 14:166–180.

Progatsky F, Cook HT, Lamb JR, Bugeno L, Dallman MJ. 2016. Mucosal inflammation at the respiratory interface: a zebrafish model. Am J Physiol Lung Cell Mol Physiol. 310:L551–L561.

Rasmussen M, Wiklund R, Olin J, Rasmussen KA, Sjöblom-Pittan M, Forsberg E. 2016. Regulatory aspects of nanomaterials in the EU. Chem Ingenieur Techn. 89:224–231.

Renshaw SA, Lohnes CA, Elworthy S, Ingham PW, Whyte MK. 2007. Modeling inflammation in the zebrafish: how a fish can help us understand lung disease. Exp Lung Res. 33:549–554.

Renshaw SA, Lohnes CA, Thrushell DM, Elworthy S, Ingham PW, Whyte MK. 2006. A transgenic zebrafish model of neutrophilic inflammation. Blood. 108:3976–3978.

Rizzo LY, Golombok SK, Mertens ME, Pan Y, Laaf D, Broda J, Jayapaul J, Möckel D, Subir V, Hennink WE, et al. 2013. In vivo nanotoxicity testing using the zebrafish embryo assay. J Mater Chem B Mater Biol Med. 1:10–23.

Robb CT, Regan KH, Dorward DA, Rossi AG. 2016. Key mechanisms governing resolution of lung inflammation. Semin Immunopathol. 38:425–448.
nanomaterial consumer products inventory. Beilstein J Nanotechnol. 6:1769–1780.

Vojtech LN, Sanders GE, Conway C, Ostland V, Hansen JD. 2009. Host immune response and acute disease in a zebrafish model of Francisella pathogenesis. Infect Immun. 77:914–925.

Wagner JC, Sleggs CA, Marcand P. 1960. Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. Br J Ind Med. 17:260–271.

Walker SL, Ariga J, Mathias JR, Coothankandawamy V, Xie X, Distel M, Köster RW, Parsons MJ, Bhatta KN, Saxena MT, Mummm JS. 2012. Automated reporter quantification in vivo: high-throughput screening method for reporter-based assays in zebrafish. PLoS One. 7:e29916.

Wang J, Arase H. 2014. Regulation of immune responses by neutrophils. Ann N Y Acad Sci. 1319:66–81.

Wang X, Robertson AL, Li J, Chai RJ, Haishan W, Sadiku P, Ogryzko NV, Everett M, Yoganathan K, Luo HR, et al. 2014. Inhibitors of neutrophil recruitment identified using transgenic zebrafish to screen a natural product library. Dis Model Mech. 7:163–169.

Warheit DB, Sayes CM, Reed KL. 2009. Nanoscale and fine zinc oxide particles: can in vitro assays accurately forecast lung hazards following inhalation exposures? Environ Sci Technol. 43:7939–7945.

White RM, Sessa A, Burke C, Bowman T, LeBlanc J, Ceol C, Bourque C, Dovey M, Goessling W, Burns CE, et al. 2008. Transparent adult zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell. 2:183–189.

Wiemann M, Vennemann A, Sauer UG, Wienc K, Ma-Hock L, Landsiedel R. 2016. An in vitro alveolar macrophage assay for predicting the short-term inhalation toxicity of nanomaterials. J Nanobiotechnol. 14:16.

Wilson MR, Foucaud L, Barlow PG, Hutchison GR, Sales J, Simpson RJ, Stone V. 2007. Nanoparticle interactions with zinc and iron: implications for toxicology and inflammation. Toxicol Appl Pharmacol. 225:80–89.

Wilson MR, Lightbody JH, Donaldson K, Sales J, Stone V. 2002. Interactions between ultrafine particles and transition metals in vivo and in vitro. Toxicol Appl Pharmacol. 184:172–179.

Witas E, Shvedova AA, Kagan VE, Fadeel B. 2009. Single-walled carbon nanotubes impair human macrophage engulfment of apoptotic cell corpses. Inhal Toxicol. 21(Suppl 1):131–136.

Xiong D, Fang T, Yu L, Sima X, Zhu W. 2011. Effects of nano-scale TiO2, ZnO and their bulk counterparts on zebrafish: acute toxicity, oxidative stress and oxidative damage. Sci Total Environ. 409:1444–1452.

Xu H, Dong X, Zhang Y, Yang M, Wu X, Liu H, Lao Q, Li C. 2015. Assessment of immunotoxicity of dibutyl phthalate using live zebrafish embryos. Fish Shellfish Immunol. 45:286–292.

Yang LL, Wang GQ, Yang LM, Huang ZB, Zhang WQ, Yu LZ. 2014. Endotoxin molecule lipopolysaccharide-induced zebrafish inflammation model: a novel screening method for anti-inflammatory drugs. Molecules. 19:2390–2409.

Zhang Y, Zhu L, Zhou Y, Chen J. 2015. Accumulation and elimination of iron oxide nanomaterials in zebrafish (Danio rerio) upon chronic aqueous exposure. J Environ Sci (China). 30:223–230.

Zhao X, Wang S, Wu Y, You H, Lv L. 2013. Acute ZnO nanoparticles exposure induces developmental toxicity, oxidative stress and DNA damage in embryo-larval zebrafish. Aquat Toxicol. 136:49–59.

Zhu X, Tian S, Cai Z. 2012. Toxicity assessment of iron oxide nanoparticles in zebrafish (Danio rerio) early life stages. PLoS One. 7:e46286.

Zon LI, Peterson RT. 2005. In vivo drug discovery in zebrafish. Nat Rev Drug Discov. 4:35–44.