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Translocation of nanoparticles and *Mycobacterium marinum* across the intestinal epithelium in zebrafish and the role of the mucosal immune system

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ABSTRACT

Nano- and microparticles are promising carrier systems for oral delivery of drugs or vaccines, particularly in fish aquaculture. However, the mechanisms of uptake, *trans*-epithelial transport and immune response to nano/micrometer sized particles, or microorganisms such as bacteria are poorly understood in fish. Here, adult zebrafish were used to study the uptake of different nano- and microparticles and the pathogenic bacteria *Mycobacterium marinum* in the intestine, and their interactions with epithelial cells and the mucosal immune system. Fluorescent particles or bacteria were delivered directly into the adult zebrafish intestine by oral intubation and their localization was imaged in intestine, liver and spleen sections. Zebrafish do not appear to have M-cells, but both nanoparticles and bacteria were rapidly taken up in the intestine and transported to the liver and spleen. In each tissue, both bacteria and particles largely localized to leukocytes, presumably macrophages.

1. Introduction

The mucosal surface of the gastro-intestinal tract forms a vast interface between the body and the external environment and functions as a physical and immunological barrier in the first-line of defence against infection by pathogenic bacteria and viruses. Moreover, intestinal mucosal surfaces are the primary site of nutrient uptake and waste secretion (Kim and Jang, 2014). Intestinal epithelia are constantly exposed to both environmental antigens and luminal bacteria and have to balance the activation of the immune system in response to both beneficial commensal and pathogenic microorganisms.

In mammals, the gut-associated lymphoid tissue (GALT), comprising follicular structures such as Peyer's patches and mesenteric lymph nodes, is the major induction site of the gut mucosal immune system, where antigens are sampled from the gut lumen and presented to naïve T- and B-cells by antigen-presenting cells (APCs). Specialized enterocytes, called Microfold (M) - cells, line the surface of Peyer's patches and actively transport antigens and whole microorganisms across the epithelium to closely associated APCs, which then migrate to mesenteric lymph nodes (Corr et al., 2008; Owen, 1999). More diffuse lymphoid tissue in the lamina propria and the intraepithelial lymphocyte (IEL) compartment act as effector sites harbouring plasma B cells, which secrete neutralizing IgA into the intestinal lumen (Neutra and Kozlowski, 2006).

In contrast, teleost fish essentially lack organized GALT structures, but lymphoid cells are abundantly present in a scattered manner throughout the intestinal epithelium. Both T cells and Ig-positive B cells were identified in the lamina propria of several teleost fish species, including sea bass (Abelli et al., 1997; Picchietti et al., 1997), carp (Rombout et al., 1998), salmonids (Zhang et al., 2010) and zebrafish (Danilova and Steiner, 2002; Langenau et al., 2004). Macrophages have been described in the lamina propria and between intestinal epithelial cells in a number of teleost fish

Abbreviations: GALT, gut-associated lymphoid tissue; PLGA, poly(lactic-*co*-glycolic acid); PS, polystyrene; M-cells, microfold cells; TEM, transmission electron microscopy.

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species (Georgopoulou and Vernier, 1986; Rombout et al., 1985, 1986). So far, M-cells have not been shown in teleost fish; however, recent studies in salmonid fish identified specialized enterocytes with M cell-like characteristics, including the overall cellular and microvillar morphology and surface carbohydrate composition (Fuglem et al., 2010). These M cell-like cells are restricted to the posterior part, also called the second segment, of the mid-intestine, which is the major site of antigen uptake in fish, evident by uptake of macromolecules such as ferritin or horseradish peroxide (HRP) and the bacterium *Vibrio anguillarum* (Georgopoulou et al., 1988; Joosten et al., 1996; Rombout et al., 1989b). In carp, enterocytes in this segment were shown to be able to take up macromolecular antigens from the intestinal lumen and transfer them to closely associated intra-epithelial macrophages (Rombout et al., 2011).

The physical and immunologic barrier function of the intestinal epithelium not only prevents intrusion of pathogenic microorganisms from the intestinal lumen but, importantly also limits the ability of oral vaccines to reach the inductive sites of the GALT and consequently to induce a protective immune response. Ensuring sufficient uptake of vaccine formulations by the intestinal epithelium is a major challenge in oral vaccination, particularly since low levels of absorbed antigens might induce immunologic tolerance. Encapsulation of vaccines in nano-/microparticles promises to help overcome many of the obstacles seen with oral vaccines by providing increased protection against the hostile environment of the gastro-intestinal tract and by enhancing uptake by the intestinal epithelium. Although there is no uniform definition, nanoparticles are often defined as dispersed particles with a size of 1–1000 nm and microparticles as micron-sized particles (>1 μm) (Couvreur, 2013; Zhao et al., 2014). Antigens and immune-stimulatory agents can be incorporated into particles by covalent linking or physical entrapment and are then released as the particle degrades intracellularly, which provides a depot of vaccine antigens (Sinyakov et al., 2006). Moreover, nanoparticles have been shown to possess adjuvant capabilities by directly activating immune cells (Gamucci et al., 2014). Compounds like alginate, poly (lactic-*co*-glycolic acid) (PLGA), chitosan or liposomes have been successfully tested for use in oral vaccines for mammals and fish, but until now no nano-/microparticle based vaccine has been licensed for vaccination in fish (Chadwick et al., 2010; Plant and LaPatra, 2011).

The cyprinid zebrafish (*Danio rerio*) is a well-characterized and increasingly used model organism in biology, including for human intestinal inflammatory diseases, such as Crohn's disease and inflammatory bowel disease (Brugman, 2016, in press; Marjoram and Bagnat, 2015). As a well-established model to study host-pathogen interactions, zebrafish have further provided key insights into human tuberculosis (TB), which is caused by *Mycobacterium tuberculosis (M.tb)* (Meijer, 2016; Ramakrishnan, 2013), using the closely related fish pathogen *Mycobacterium marinum*. These pathogenic mycobacteria reside and multiply within host macrophages, evading the immune system by interfering with host macrophage functions, such as phagosome maturation and antigen presentation (Tobin and Ramakrishnan, 2008). However, the natural infection route of *M. marinum* in fish is not clear, although there are strong indications that the primary route of infection is via the oral-intestinal pathway (Harriff et al., 2007).

Studies indicate that in the zebrafish - as in the carp and other teleost species - a specialized type of enterocytes located in the posterior part of the mid-intestine segment is involved in sampling of antigens from the intestinal lumen. These potential antigen-sampling cells can be identified by the presence of large, supra-nuclear vacuoles, in which macromolecule antigens accumulate after uptake (Rawls et al., 2004; Wallace et al., 2005). However, it is not clear if this cell type resembles mammalian M-cells or if they have similar ability to sample or even to deliver particles such as nano-/microparticles or whole microorganisms to associated APCs.

The study presented here aimed to investigate the uptake and transport of nano-/microparticles and a pathogenic bacterium *M. marinum* via the teleost intestine. First, antigen-sampling cells in the zebrafish intestine were characterized with respect to key characteristics of mammalian M-cells, such as morphology, macromolecule uptake and selective binding of lectins. Then, adult zebrafish were intubated with different fluorescent particles or *M. marinum* and their uptake and transport within the intestinal epithelium and to other organs was analyzed by histochemistry, immunohistochemistry and electron microscopy.

2. Materials and methods

2.1. Zebrafish care and husbandry

Adult zebrafish used in this study were kept at a constant 28 °C, with continuous water circulation. All experiments were done using wild type zebrafish, aged between one and three years. Experiments were approved of and conducted in accordance with the Norwegian national animal research authority (FOTS permission ID: 7276) ethical standards.

2.2. Oral intubation of adult zebrafish

Adult zebrafish were orally intubated as described in (Collymore et al., 2013). Prior to intubation fish were starved for 24 h to ensure an empty intestine. Fish were sedated in 150 mg/L Tricane (Sigma-Aldrich, St. Louis, MO, USA) and placed in a sponge soaked in water with the head protruding. A rubber tube (Perifix® Soft B. Braun, Melsungen, Germany) attached to a 50 μl Hamilton Microliter™ syringe (Hamilton Company, Nevada, USA) was inserted approximately 1 cm into the mouth, ensuring that the tube extended past the gills and the oesophagus. Then, 5 μl of PBS, nano-/microparticles or bacteria in PBS was directly injected in the anterior segment of the gastro-intestinal tract and the tube was gently retracted from the zebrafish. After the procedure, fish were immediately transferred into fresh system water for recovery. Fish that showed any signs of injuries (e.g. bleeding, slow swimming, inability to keep upright) during recovery were eliminated from the analysis. Fish were euthanized after defined time points (5 min, 1 h, 3 h, 5 h and 24 h) with an overdose of Tricaine (300 mg/ml). In all experiments, at least three fish were analyzed at each time point.

2.3. Nanoparticles used for oral intubation

2.3.1. BSA-coated gold particles

BSA-coated 10 nm colloidal gold particles were prepared as described previously (Slot and Geuze, 1985).

2.3.2. Carboxylated polystyrene (PS) particles

For oral intubation, red fluorescent carboxylate-modified polystyrene particles were used with a diameter of 500 nm (Sigma-Aldrich) or 1 μm (FluoSpheres®, Thermo Scientific, Waltham, MA, USA). Approximately 5×10^7 polystyrene particles in a volume of 5 μl were injected per fish by oral intubation.

2.3.3. PLGA nanoparticles

Red fluorescent PLGA nanoparticles were prepared by oil in water emulsion technique as previously reported (Kalluru et al., 2013). Briefly, PLGA (50:50 RESOMER® RG 500, Evonik Röhm Gmbh, Essen, Germany) and Nile red (Sigma-Aldrich) were

dissolved in dichloromethane (DCM) and mixed with 0.001% (w/v) polyvinyl alcohol (PVA) in water. After sonication, the primary emulsion was kept stirring overnight to evaporate the DCM. The resulting particles were then washed twice with water and collected via ultracentrifugation. For oral intubation, PLGA particles were diluted in PBS and used at a concentration of 1 μg/ml. Particles were between 50 nm and 400 nm in size, as determined by electron microscopy.

2.4. Culturing of Mycobacterium marinum and preparation for oral infection

M.marinum was cultured and prepared for oral intubation of zebrafish as previously described (Cosma et al., 2005; Gao and Manoranjan, 2005). A *M. marinum* strain with a plasmid coding for a red fluorescent protein, dsRed, (msp 12:deRed2, kind gift from Lalita Ramakrishnan, University of Washington, Seattle, WA, USA) was used. *M. marinum* was grown in 7H9 medium supplemented with 0.02% glycerol, 10% ADC (Albumin dextrose catalase), 0.05% tween 80 and 50 μg/ml kanamycin (Sigma-Aldrich) and incubated at 30 °C on an orbital shaker in the dark.

For oral infection, *M. marinum* was harvested in the exponential growth phase with an OD_{600} between 0.5 and 0.9 by centrifugation at 3000 × *g* for 10 min. After two washes in PBS, bacteria were re-suspended in 1 ml of PBS and passed through a 27-gauge needle 10 times to disrupt any bacterial aggregates before oral intubation. Fish were finally intubated with 5 μl of *M. marinum* suspension containing approximately 5×10^6 colony forming units (CFUs).

2.5. Histology

2.5.1. Preparation for tissue sections

Zebrafish were sacrificed and organs were quickly dissected as previously outlined (Gupta and Mullins, 2010). The organ 'package' (intestine, liver, spleen, pancreas and gallbladder) was taken out as a whole to avoid damage to individual tissues. Samples were then prepared for different applications as described below. Samples of salmon intestine were kindly provided by Alf Seljenes Dalum at NMBU and mice were obtained from the animal facility at IBV, University of Oslo.

Samples for paraffin sectioning were fixed in 10% neutral buffered formalin overnight and processed for sectioning using standard paraffin infiltration protocol as described earlier (Løkka et al., 2014). Samples were finally sectioned to a thickness of 5 μm using a Sliding Microtome (Ticrom HM 450, Thermo Scientific).

For cryostat sectioning, samples were fixed in 4% paraformaldehyde for 2 h at 4 °C. After fixing, the tissue was washed three times in PBS for 5 min, organs were separated, and connective tissue removed. The intestine was kept either whole or divided into three segments (anterior, mid and posterior segments) and embedded in Tissue-Tek® O.C.T. Compound (Sakura® Finetek, Tokyo, Japan) and snap frozen in liquid nitrogen. Cryostat sections (7 μm) were collected on Superfrost™ plus slides and either stained immediately or stored at −20 °C for later use.

2.5.2. Hematoxylin and eosin staining (H&E)

Paraffin sections were stained with H&E on an automatic multiple slide stainer, and cryostat sections were stained manually according to standard protocols. Slides were finally dried and mounted with PVA.

2.5.3. Lectin histochemistry

Cryostat sections of zebrafish intestine were stained with FITC-conjugated UEA-1 lectin (Sigma-Aldrich) or rhodamine-conjugated WGA (Vector laboratories, CA, USA) as described before (Fuglem et al., 2010). Briefly, slides with cryostat sections were incubated in a blocking solution consisting of 2% BSA in PBS Tween-20 (PBST) for 1 h at room temperature. Thereafter, sections were stained with 10 μg/ml UEA-1 or 2 μg/ml WGA in blocking solution for 1 h, washed three times in PBS and counterstained with nuclear counterstain (Hoechst, 1 μg/ml). After 2 washes in PBS, slides were mounted with PVA under a cover glass.

2.5.4. Immunohistochemistry

To identify the brush border of the intestinal epithelium, the antibody 4E8 was used.

This antibody recognizes an unknown epitope at the microvilli apices of absorptive cells bordering to the intestinal lumen of zebrafish (Crosnier et al., 2005). Cryostat sections of the zebrafish intestine were stained with the 4E8 as previously described (Crosnier et al., 2005). Briefly, cryostat sections were incubated with 10 μg/ml primary 4E8 antibody in a blocking solution containing 10% goat serum, 2% BSA, 0.1% Triton-X PBS for 2 h at room temperature or overnight at 4 °C. After three washes in PBS, sections were incubated with a green fluorescent secondary antibody (cy2-conjugated goat anti-mouse, Jackson ImmunoResearch, PA, USA), in blocking solution for 2 h. Finally, sections were washed, stained with nuclear counter stain and mounted as described for lectin stained sections.

To identify leukocytes in the intestine, liver and spleen, antibody against L-plastin was used (kindly provided by Paul Martin, University of Bristol, United Kingdom). In zebrafish, L-plastin is a pan-leukocyte marker staining both neutrophils, macrophages, eosinophil, and T- and B-lymphocytes (Morley, 2012). Cryostat sections of zebrafish intestine and spleen were stained with *anti*-L-plastin according a previous published protocol (Cui et al., 2011). Briefly, sections were incubated with primary antibody (1:500) in 2% BSA in PBST overnight at 4 °C. After three washes in PBS, sections were stained with a secondary antibody (1:200, Cy2-conjugated goat anti-rabbit, Jackson ImmunoResearch, PA, USA) for 2 h. Sections were then washed, stained with nuclear counter stain and mounted as described above.

2.5.5. Transmission electron microscopy (TEM)

For the ultrastructural analysis zebrafish intestine and spleen were embedded in epoxy resin as described previously (Repnik et al., 2015). The organs were dissected and fixed in 1% glutaraldehyde in 0.2 M HEPES buffer, pH 7.4, overnight. Thereafter they were post-fixed with 2% osmium tetroxide and contrasted with 2% uranyl acetate, both for 2 h. Samples were dehydrated with a graded ethanol series (70–80–90–95–100%), progressively infiltrated with epoxy resin and polymerized overnight at 60 °C. 70 nm thin sections were prepared using an ultramicrotome (Ultracut EM UCT-Leica Microsystems) and a diamond knife (Diatome) and contrasted with 0.2% lead citrate for 15 s. Samples were analyzed with JEM1400 transmission electron microscope (JEOL). Images were recorded with TemCam-F216 (Tvips).

2.6. Correlative confocal laser and correlative light microscopy

Antibody or lectin-labelled sections were analyzed with an Olympus FluoView 1000 inverted confocal microscope. Correlative confocal laser and light microscopy of zebrafish spleen cryosections was performed as follows: after fluorescence imaging, glass slides with sections were soaked in PBS for 2 h to remove cover slips. Sections were then stained with H&E, as described in section 2.5.2. and imaged with an Axio Imager. M2 (Zeiss, Oberkochen, Germany).

3. Results

3.1. Characterization of antigen-sampling cells in the zebrafish intestine

Previous studies in zebrafish and carp have identified a special phenotype of enterocytes, characterized by large supranuclear vacuoles, to be responsible for the prominent role of the posterior mid-segment in intestinal uptake and transepithelial transport of macromolecular antigens from the lumen. These specialized enterocytes are therefore considered to have important antigen-sampling functions in the fish intestine (Rombout et al., 1985, 2011; Wallace et al., 2005).

To identify regions of the adult zebrafish intestine containing these antigen-sampling cells, histological analysis of H&E stained paraffin sections was performed (Fig. 1A).

Antigen-sampling cells, as identified by their distinct supranuclear vacuoles, localized mainly to the posterior part of the mid segment of the zebrafish intestine, where they represented the predominant phenotype of epithelial cells (Fig. 1C and Fig. S1). In the anterior and posterior segments, antigen-sampling cells were absent (Fig. 1B, D).

The most important antigen-sampling cell type in the mammalian intestine, M-cells, are characterized by short, irregular and poorly organized microvilli, which sometimes lack completely (Mabbott et al., 2013). In salmon, the microvilli of antigen-sampling cells similarly differ from regular enterocytes (Fuglem et al., 2010). In contrast, in our ultrastructural analysis, no major morphological differences in microvillar morphology were observed in zebrafish antigen-sampling cells compared to regular enterocytes, except a moderately shorter length. However, in contrast to regular enterocytes, antigen-sampling cells were characterized by invaginations at the base of the microvilli and apical cytoplasm containing multiple cisternal channels and vesicles (Fig. 1E, F).

In fish intubated with 10 nm gold particles, the majority of antigen-sampling cells contained small vesicles at the apical side with internalized gold particles, suggesting fluid-phase uptake (Fig. 1H). Gold particles accumulated in large supranuclear vacuoles, which were typically localized between the apical plasma membrane and the cell nucleus (Fig. 1G, I). However, no particles were detected in cells beneath the epithelial layer by 1 h after the intubation.

The presence of M-cells was investigated by lectin histochemistry. The fucose binding lectin UEA-I, a positive marker of M-cells in mammals, showed no labelling in any part of the zebrafish intestine, including the posterior part of the mid segment (Fig. 2A). In contrast, using identical sample preparation and labelling conditions, a positive labelling was present in salmon (Fig. 2C) and mouse (Fig. 2E) intestine. In all three species, WGA lectin strongly stained goblet cells and less intensely the brush border of the intestine (Fig. 2D, F and B).

3.2. Uptake of differently sized polystyrene nanoparticle in the intestine and spleen after oral intubation

To study the ability of the zebrafish intestinal epithelium to take up particles in a micrometer range, fish were orally intubated with 500 nm or 1 μm red fluorescent polystyrene particles and their localization was investigated in cryosections. Polystyrene particles are inert and non-degradable and are therefore widely used for studying endocytosis and phagocytosis *in vitro* and *in vivo*. The smaller polystyrene particles (500 nm) attached to the epithelial brush border in the posterior part of the mid-intestine as early as 5 min after intubation and were localized to the lamina propria after 1 h (Fig. 3A, B). Uptake of particles was not limited to this segment, but was likewise observed in the anterior intestine. No apparent difference in particle uptake was observed between the anterior and the mid segments; however, occasionally, larger accumulations of 500 nm sized particles were seen in epithelial cells of the mid segment (Fig. 3C). In the lamina propria, a significant majority of the particles co-localized with leukocytes, as identified by L-plastin immunohistochemistry (Fig. 3D).

To investigate transport of particles to other organs, spleens from orally intubated fish were isolated and analyzed for particles. Already 3 h after oral intubation, particles accumulated in distinct areas of the spleen (Fig. 3E). Correlative light microscopy with H&E staining on the same sections confirmed that particle accumulations were located in the white pulp area of the spleen (Fig. 3F). Furthermore, particles in the spleen were primarily localized to leukocytes, identified as L-plastin-positive cells (Fig. 3G).

Larger polystyrene particles (1 μm) were likewise taken up by the intestinal epithelia and were found in the lamina propria of the mid segment after 1 h (Fig. 3H). However, in contrast to the 500 nm particles, no particles could be found in the spleen at any time-point (data not shown).

3.3. Uptake of PLGA nanoparticles in the intestine and spleen after oral intubation

PLGA nanoparticles are considered to be one of the most promising approaches for oral delivery of therapeutic agents or vaccines. Therefore, uptake of fluorescent PLGA-nanoparticles was analyzed in intestine and spleen cryosections of orally intubated zebrafish. PLGA nanoparticles were rapidly taken up in the intestine and accumulated in cells located in the lamina propria of the mid-intestine after only 5 min (Fig. 4A). This rapid uptake was not limited to the mid-intestine and was also seen in the anterior, but not in the posterior segment of the intestine (data not shown). PLGA particles were partially associated with leukocytes in the lamina propria, but not in the epithelial layer (Fig. 4C, D). Similarly to 500 nm polystyrene particles, PLGA particles accumulated massively in the white pulp areas of the spleen after a few hours (Fig. 4B).

3.4. Mycobacterium marinum infection via the intestine

The gastro-intestinal tract is believed to be a natural port of entry for the fish pathogen *M. marinum* (Harriff et al., 2007). To study the ability of this pathogen to infect via the intestinal epithelium, red fluorescent *M. marinum* was introduced into the zebrafish intestine by oral intubation. At an early time-point (5 min after intubation), fluorescent bacteria were mainly found in the intestinal lumen and attached to the epithelial brush border (data not shown). After 1 h, bacteria were detected in leukocytes located at the basal side of the intestinal wall (Fig. 5A, B). In general, uptake of *M. marinum* by the intestinal epithelium was observed mainly in the mid segment and in particular in the posterior part of the mid segment. At later time-points (5 h after intubation), aggregates of bacteria appeared within leukocytes in the white pulp of the spleen (Fig. 5D) and in liver tissue directly adjacent to the intestine (Fig. 5C). To study the interaction of bacteria with enterocytes in more detail, intestines of orally intubated zebrafish were sampled and processed for TEM at different time points. Accumulations of *M. marinum* were observed in supranuclear vacuoles of antigen-sampling cells as soon as 20 min after intubation (Fig. 6A). Neither goblet cells nor regular enterocytes were found to contain bacteria at any time point. Intracellular bacteria localized not only to supranuclear vacuoles but also to small vesicular structures that were in many cases closely associated with the endoplasmic reticulum (ER) (Fig. 6B). In a few instances, *M. marinum* bacteria were

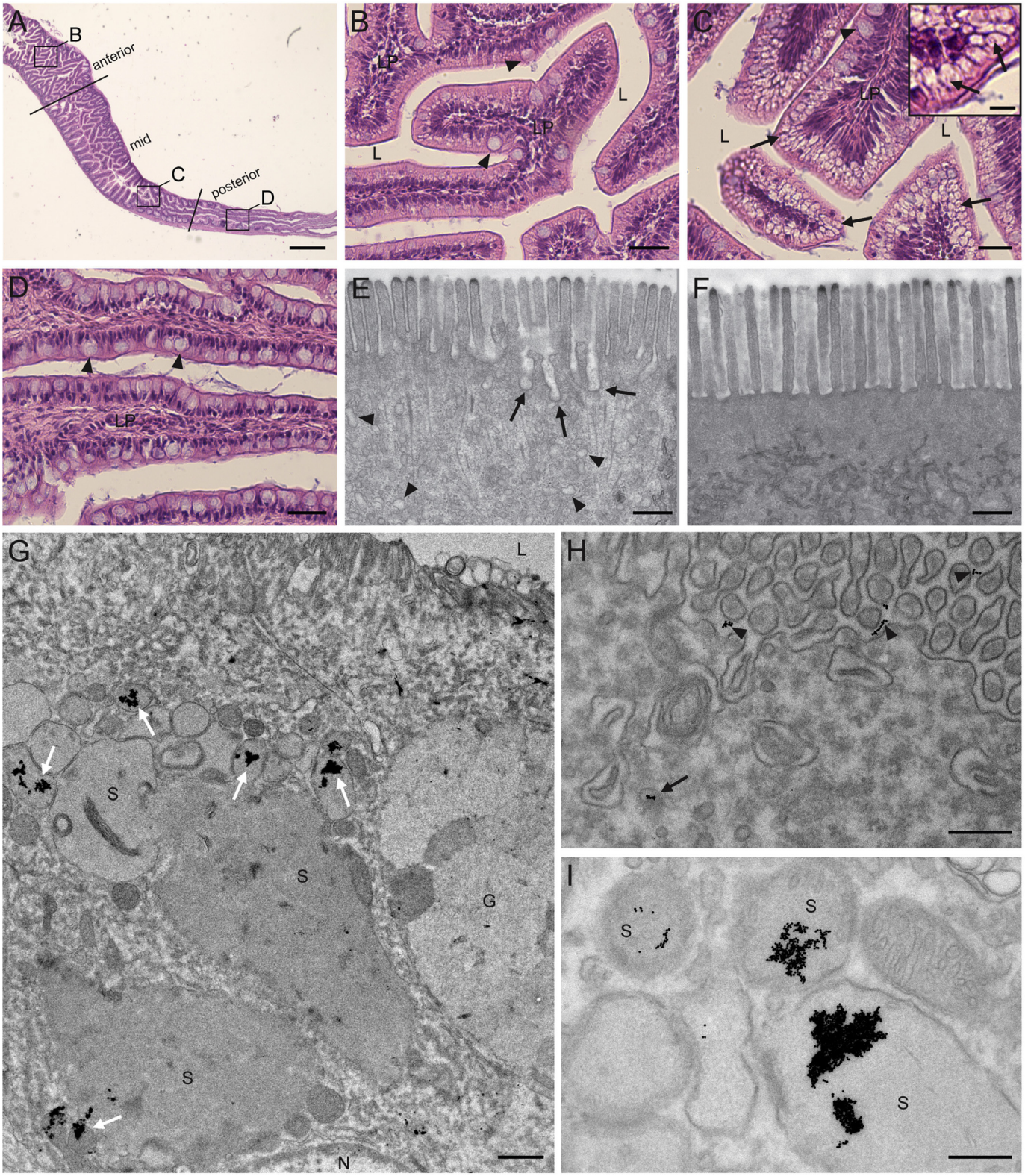

Fig. 1. Characterization of antigen-sampling cells in the zebrafish intestine. (A–D) Longitudinal H&E stained paraffin sections of the zebrafish intestine, indicating the regions where samples were taken: (A) sampling site for anterior (B), posterior part of the mid (C) and posterior segment (D) of the intestine. (C–D) Antigen-sampling cells are characterized by large supranuclear vacuoles in their cytosol (arrows) and make up the majority of the epithelium of the posterior mid segment (C, inset shows a higher magnification view), but are absent in the anterior (B) or posterior segment (D). Goblet cells are indicated by arrowheads; LP: lamina propria; L: intestinal lumen. (E, F) Ultrastructural analysis by TEM of the apical part and microvillar morphology of antigen-sampling cells and of regular enterocytes. In contrast to regular enterocytes (F), antigen-sampling cells display cisternal channels (arrows) intruding from the base of the brush border into the highly vacuolated apical cytoplasm (arrowheads) (E). (G–I) Uptake of 10 nm BSA-gold particles by antigen-sampling cells in the mid segment 1 h after intubation analyzed by TEM. (H) BSA-gold particles can be seen in the extracellular space between microvilli and at the base of the brush border (arrowheads) being taken up by antigen-sampling cells into small vesicles (arrows). (G, I) Accumulations of BSA-gold particles in supranuclear vacuoles located in the apical cytoplasm. G: goblet cell; N: nucleus; S: supranuclear vacuoles; scale bars: (A) = 500 μm, (B, C, D) = 30 μm, (E,F) = 500 nm, (G) = 1 μm, (H, I) = 300 nm.

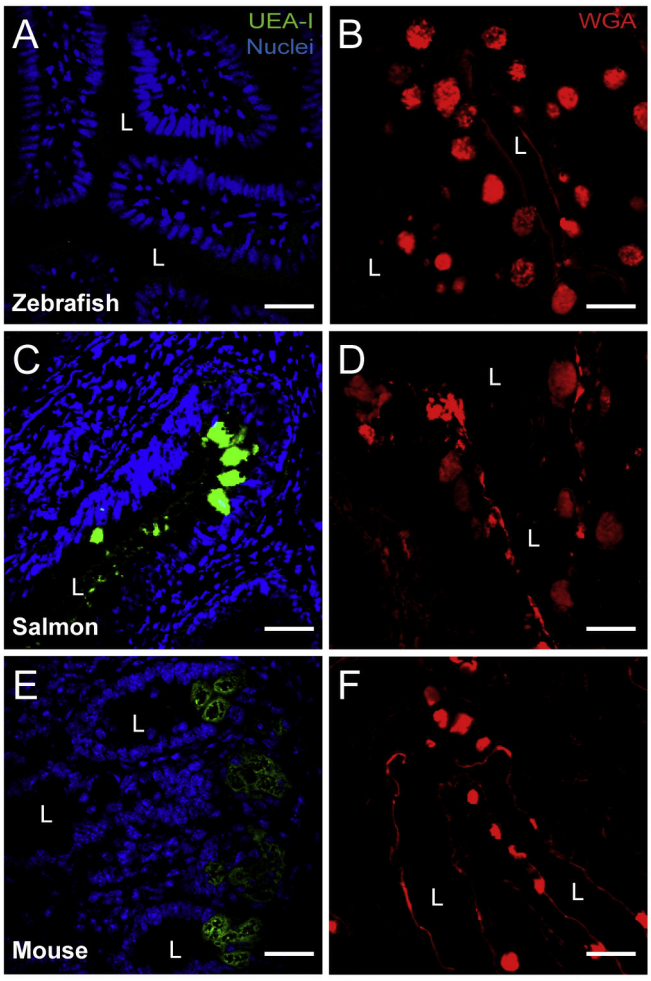

Fig. 2. Comparative lectin histochemistry on sections of the intestine from zebrafish (A–B), salmon (C–D) and mouse (E–F). Zebrafish, salmon and mouse intestine cryosections were labelled with the positive M-cell marker UEA-I (A, C, E) and the negative M-cell marker WGA (B, D, F). WGA labels goblet cells and the brush border in all three species. UEA-I positive cells are frequent in salmon (C) and mouse intestine (E), but are entirely absent in the zebrafish intestine (A). L: intestinal lumen; scale bars: (A–F) = 50 μm.

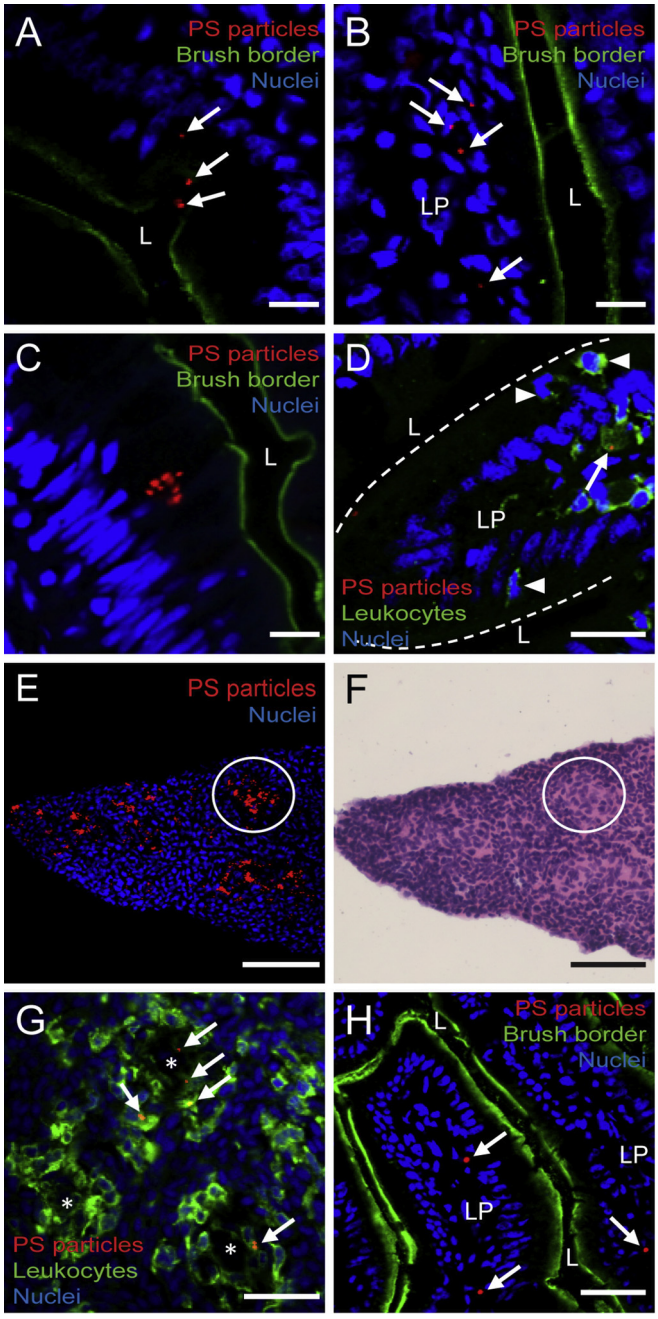

Fig. 3. Uptake of polystyrene (PS) particles in the zebrafish intestine and spleen after oral intubation. (A–C) Cryosections of the posterior mid-intestine of zebrafish intubated with 500 nm sized polystyrene particles (red). (A) Five min after oral intubation, polystyrene particles (arrows) were mostly found in the lumen or adjacent to the intestinal brush border, visualised with the antibody 4E8. (B) Particles located within the lamina propria were seen after 1 h. (C) On rare occasions, larger accumulations of particles in epithelial cells could be observed. (D) Polystyrene particles (500 nm) co-localized with L-plastin-positive leucokytes (arrow) in the lamina propria but not with intra-epithelial leukocytes (arrowheads) 1 h after oral intubation; the stippled line indicates the brush border. (E, F) Correlative light microscopy of spleen tissue 1 h after oral intubation with 500 nm polystyrene particles. Confocal laser scanning pictures (E) show accumulation of particles in the spleen, located in the white pulp areas surrounding arterioles (white circles); these are characterized by areas with significantly lower cell density as evident in H&E staining of the same section (F). (G) Polystyrene particles in spleen tissue 3 h after oral intubation were nearly exclusively located within L-plastin positive leukocytes surrounding arterioles (*). (H) Uptake of 1 μm sized polystyrene particles (arrows) across the intestinal epithelium into the lamina propria 1 h after oral intubation. LP: lamina propria; L: lumen; scale bars: (A, B, C) = 10 μm, (D) = 15 μm, (E, F) = 50 μm, (G) = 20 μm and (H) = 30 μm.

found in non-epithelial cells at the basal side of antigen-sampling cells (Fig. 6C, D). These cells were characterized by electron-pale cytoplasm with vacuoles containing various material or organelles, thus resembling macrophage morphology (Lieschke et al., 2001).

4. Discussion

In the mammalian intestine, M-cells have crucial functions in the homeostasis of the mucosal immune system by continuously sampling and transporting antigens or whole microorganisms from the intestinal lumen to the underlying lymphoid tissue of the Peyer's patches. So far, no cell type equivalent to M-cells has been identified in the intestine of teleost fish, but uptake of macromolecular antigens and particles by enterocytes has been shown in several fish species.

The current study is consistent with earlier reports showing that specialized enterocytes in the posterior part of the mid segment of the zebrafish intestine, (and other teleost species), function as antigen-sampling cells. These cells are characterized by large supranuclear vacuoles and are able to sample and transport

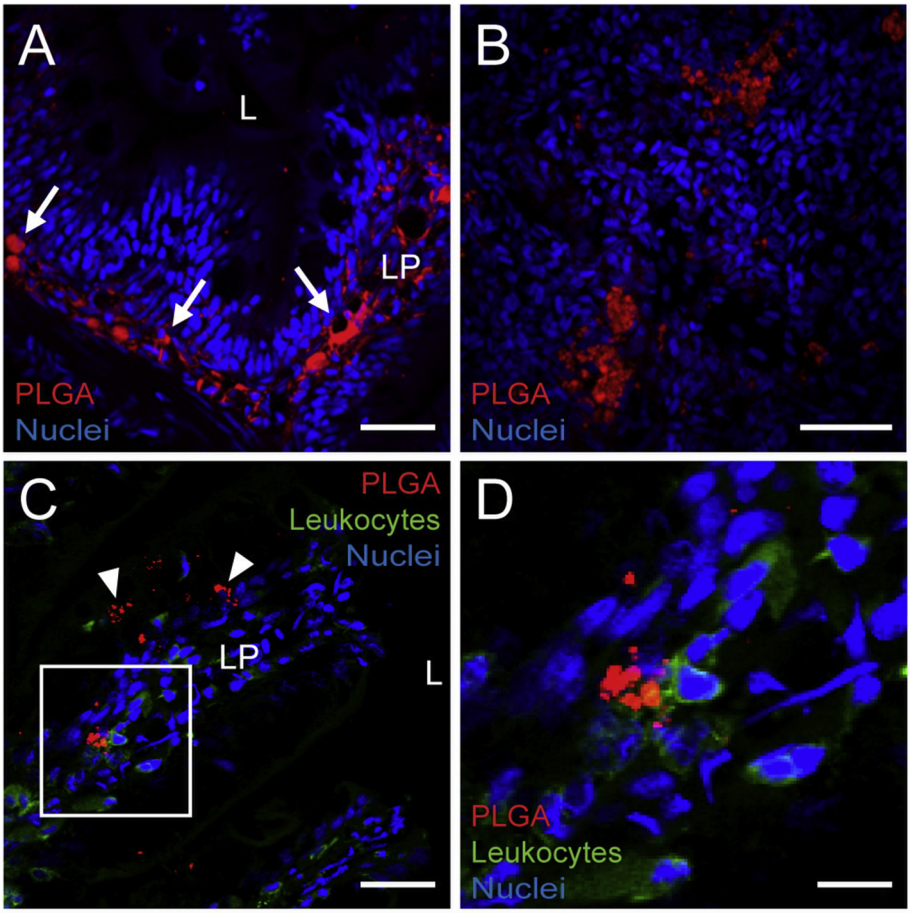

Fig. 4. Uptake of PLGA nanoparticles in the zebrafish intestine and spleen after oral intubation. (A) Cryosection of the posterior part of the mid-intestine shows rapid uptake of PLGA nanoparticles (red) by the intestinal epithelium and significant accumulation inside the lamina propria (arrows) only 5 min after oral intubation. (B) Strong accumulations of PLGA particles were seen in cryosections of the spleen 3 h after oral intubation. (C, D) One hour after oral intubation, PLGA particles partially co-localized with L-plastin positive leukocytes in the lamina propria but not in the epithelial layer (arrowheads). Image D shows the area indicated by the white box in C at larger magnification. L: intestinal lumen; LP: lamina propria; scale bars: (A, B, C) = 30 μm and (D) = 10 μm.

macromolecular antigens such as horseradish peroxidase and ferritin to leukocytes, mainly macrophages, in the lamina propria (Rombout et al., 1985; Rombout and van den Berg, 1989; Wallace et al., 2005). Areas containing antigen-sampling cells are associated with high densities of leukocytes and high expression of antimicrobial peptides and cytokines, suggesting a close interaction between antigen-sampling cells and the mucosal immune system (Oehlers et al., 2011; Rombout et al., 1989a, 1989b). However, it is not clear if these cells can also transcytose micrometer sized particles, such as bacteria or yeast. For this reason, we investigated antigen-sampling cells in the zebrafish intestine for key characteristics of mammalian M-cells.

In the present study, ultrastructural analysis of zebrafish antigen-sampling cells showed cisternal channels intruding from the brush border base into the cytoplasm, indicating high endocytic activity in these cells. Similarly to salmon M-cell like cells, antigen-sampling cells in zebrafish are able to take up and accumulate 10 nm gold particles, however they lacked key M-cell characteristics such as short, irregular microvilli and UEA-I positive staining of the glycocalyx. This raises a question whether or not the antigen-sampling cells we identified in the zebrafish intestine are directly homologous to mammalian M-cells. This concern is strengthened by our observation that uptake of micrometer-sized polystyrene and PLGA particles was not limited to the posterior mid segment but also occurred throughout the anterior segment and the anterior part of the mid segment, where no or few antigen-sampling cells are present. Both polystyrene and PLGA particles used in this study are in a size range that predominantly triggers internalization by phagocytosis. Neither particle type seemed to be located in L-plastin positive cells (leukocytes) in the epithelial layer but they did co-localize with leukocytes in the lamina propria. Together, this indicates that particles are directly taken up by enterocytes and that phagocytic activity is not limited to antigen-sampling cells in the mid-segment but is most likely also found in regular enterocytes in other segments.

A prerequisite for oral vaccination is that a sufficient amount of antigen and adjuvant crosses the intestinal epithelium and reaches components of intestinal and systemic immune system. Encapsulation of antigen and adjuvants in nano-/microparticles has been studied as a potential approach to develop oral vaccines in fish by many groups (Plant and LaPatra, 2011). Particularly, PLGA-nanoparticles have been suggested as a suitable vector for oral vaccines, showing promising results in multiple studies (Altun et al., 2010; Behera et al., 2010; Lavelle et al., 1997; Rombout et al., 2014). PLGA is a hydrophobic, biodegradable and non-toxic polymer that is easy and cheap to produce; it facilitates cellular uptake and enables sustained release of encapsulated antigen or adjuvant (O'Donnell et al., 1996). Other studies have in addition reported that PLGA nanoparticles facilitate uptake and expression of antigen-encoding plasmid DNA by the intestinal epithelium and

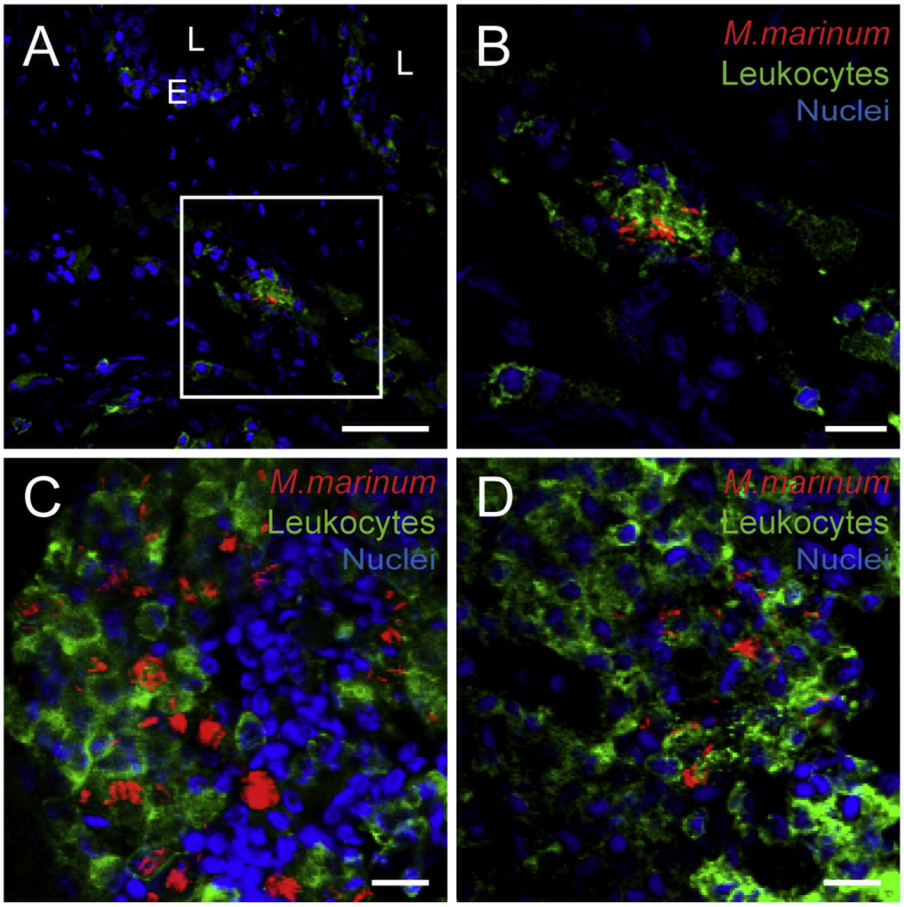

Fig. 5. *Mycobacterium marinum* infection via the zebrafish gastro-intestinal tract after oral intubation. (A, B) Accumulations of fluorescent *M. marinum* at the basal side of the intestinal wall 1 h after oral intubation, co-localizing with L-plastin positive leukocytes. Image B shows the boxed area in A at larger magnification. (C, D) *M. marinum* accumulated in the liver (C) and spleen (D) 5 h after oral intubation. In both tissues, bacteria are within or closely associated with leukocytes. L: intestinal lumen, E: epithelial layer; scale bars: (A) = 30 μm and (B, C, D) = 10 μm.

other organs and enhance antigen-specific antibody responses after oral vaccination (Adomako et al., 2012; Tian et al., 2008).

An additional challenge for oral vaccination is not only to deliver sufficient amounts of the antigen but also to elicit a sufficiently strong specific immune response at the same time. This is also highlighted by this study. Zebrafish given an intra-peritoneal injection of *M. marinum* showed strong stimulation of immune regulatory genes in the intestine or spleen as measured by qPCR. In contrast, oral intubation of the same dose of bacteria failed to induce any measurable response (Fig. S2). This was likely due to more defined dosing since i. p injection delivers stimulatory compounds directly to the fish without the need for selective uptake by the intestine. PLGA has been suggested to have adjuvant-like effects, but we did not see a strong immune response to our PLGA particles by either intubation or i. p. injection. This would suggest that additional adjuvants or immune-stimulatory compounds would need to be incorporated in the particles to provide a sufficient response and subsequent protection.

In this study, we show directly that fluorescent PLGA nanoparticles are not only rapidly taken up in the zebrafish intestine but also quickly reach sub-epithelial layers, where they partly co-localize with leukocytes, presumably macrophages. Only a few hours after intubation, significant accumulation of PLGA-particles can be found in the spleen, in particular in ellipsoids within the white pulp area. This rapid transport to splenic tissue seems to be a general feature of particle uptake in the zebrafish intestine as we saw the same pattern with inert 0.5 μm polystyrene particles. Furthermore, others have reported transport of polystyrene nano-/microparticles from the intestinal lumen to central organs of the fish immune system, such as spleen and head kidney (Dalmo et al., 1995; Glenney and Petrie-Hanson, 2006).

The size, shape and surface-properties of nano-/microparticles can have a significant impact on cellular uptake, complement activation, immune response and biodistribution *in vivo* (Oh and Park, 2014; Zolnik et al., 2010). Polystyrene particles with sizes between 100 nm and 10 μm were shown to be taken up in salmon intestine to different degrees, but only 1 μm sized particles were rapidly transported to spleen and kidney (Petrie and Ellis, 2006). The lack of transport of 1 μm sized polystyrene particles to the spleen in our study may therefore be explained by possible different size-dependent uptake dynamics. However, it was not determined if this is due to a lack of transport from the intestine or if the larger particles accumulated at other sites or organs.

The spleen in teleosts is considered to be a secondary lymphoid organ with important functions in screening and trapping antigens from the blood, and in B-cell activation and differentiation. Blood-borne antigens or particles are retained in structures called ellipsoids, where they are taken up by surrounding macrophages. These macrophages were shown to be able to retain antigens for long periods, which may indicate their involvement in activation of B-cells (Press and Evensen, 1999; Salinas et al., 2011; Solem and Stenvik, 2006). As particles in this study exclusively co-localized

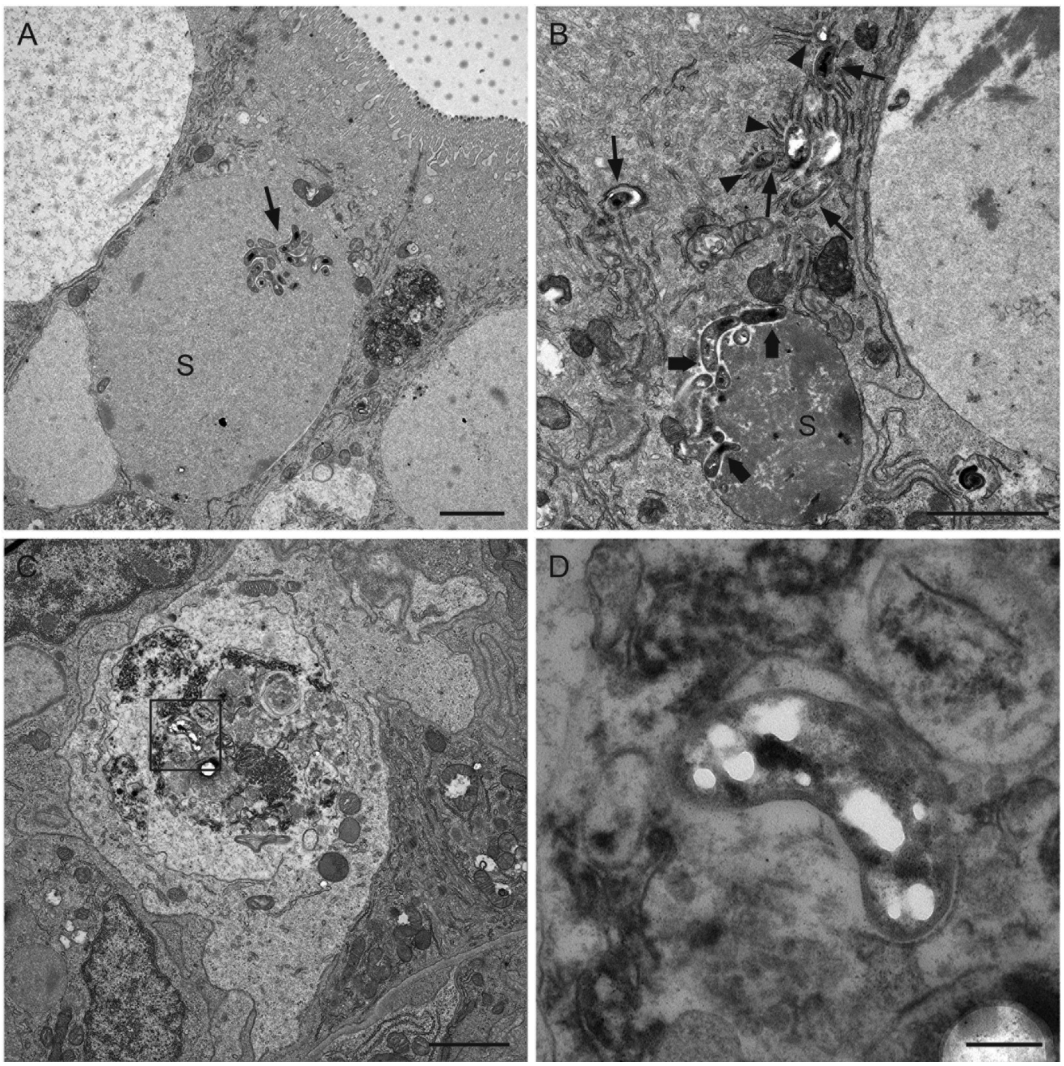

Fig. 6. TEM micrographs of the zebrafish intestine after oral intubation with *M. marinum*. (A) Accumulations of *M. marinum* (arrow) in supranuclear vacuoles of antigen-sampling cells 20 min after intubation. (B) Antigen-sampling cell with intracellular *M. marinum* inside the supranuclear vacuole (thick arrows) or in small vesicular structures (thin arrows), which are associated with endoplasmic reticulum cisternae (arrowheads). (C) A single *M. marinum* bacterium within a cell with electron pale cytoplasm, located at the basal side of the epithelial layer. (D) The area indicated by the white box in C, showing the bacterium at increased magnification. S: supranuclear vacuole; scale bars: (A, B, C) = 2 μm and (D) = 250 nm.

with leukocytes in the spleen but only partially in sub-epithelial layers, it is unclear if they are transported to the spleen already within leukocytes originating from the lamina propria, or whether they travel freely in the blood, from where they are then taken up by splenic leukocytes. In carp, antigen-loaded macrophages were shown to migrate from the intestine to the spleen after anal intubation with ferritin (Rombout and van den Berg, 1989). Furthermore, PLGA-particles injected directly into the blood circulatory system of zebrafish embryos, are cleared within minutes from the bloodstream by macrophages located along the endothelium (Fenaroli et al., 2014). This suggests that particles can also travel within leukocytes, presumably macrophages, rather than freely in the blood to the spleen.

The ability of the fish intestine to take up micro-sized particulates might also be exploited by pathogenic bacteria to invade the host during infection. The fish pathogen *M. marinum* was shown to infect zebrafish via the gastro-intestinal tract and induce granuloma formation in different organs, such as liver, spleen and kidney (Harriff et al., 2007; Peterson et al., 2013). Similarly, *M. marinum* infection in Japanese medaka (*Oryzias latipes*) can be transmitted via the oral route (Mutoji, 2011). This strongly suggests that this is a natural route of infection for *M. marinum* in fish. Here, we show direct evidence that *M. marinum* can cross the intestinal epithelium via transcytosis by antigen sampling cells and that bacteria disseminate to spleen and liver after only few hours. *M. marinum* bacteria were-contrary to polystyrene particles - exclusively taken up by antigen sampling cells and accumulated in supranuclear vacuoles after a short time. In the mammalian intestine, receptors for pathogen-associated molecular patterns (PAMPs) were shown to regulate phagocytosis and transcytosis of bacteria by M-cells (Chabot et al., 2006; Tyrer et al., 2006). However, if similar mechanisms are involved in the selective uptake of *M. marinum* and inert polystyrene particles by antigen sampling cells remains unclear.

In antigen sampling cells, vesicles containing *M. marinum* bacteria were closely associated with ER membranes, a feature strongly resembling the recruitment of ER during ER-mediated phagocytosis of bacterial pathogens (Gagnon et al., 2002). At the base of the epithelial layer, bacteria were found in macrophage-like cells with characteristic electron-pale cytoplasm, which were located between antigen sampling cells. In spleen and liver, aggregations of *M.*

marinum were consistently located within L-plastin positive leukocytes. Uptake of intact non-pathogenic and pathogenic bacteria by enterocytes has been shown in other fish species such as spotted wolf fish, arctic charr and salmon (Ringø et al., 2001, 2006, 2007; Salinas et al., 2008). Furthermore, dissemination of bacteria and bacterial antigens from the intestine to the spleen and liver was observed in rainbow trout after oral challenge with live *Yersinia ruckeri*, the cause of enteric redmouth disease in salmonids, or with *Vibrio anguillarum* in turbot (Khimmakthong et al., 2013; Grisez et al., 1996). *M. marinum*, and possibly other pathogenic bacteria, may cross the intestinal barrier by transcytosis by enterocytes and spread - possibly within leukocytes - to other organs. The rapid transport of bacteria (and particles) to the spleen may represent a mechanism for the early sensing of invading pathogens and may also reflect the important role of the spleen in initiating systemic immune responses to bacterial and viral infections in teleosts (Castro et al., 2013; Hadidi et al., 2008).

In conclusion, we show that zebrafish have a broad and non-M-cell like antigen sampling activity in the intestine. Nano-/microparticles are frequently taken up in the intestine, presumably by transcytosis, which is not limited to specialized antigen-sampling cells. Although we show that designated antigen-sampling cells in zebrafish lack key-characteristics of mammalian M-cells, we provide for the first time clear evidence for the ability of this cell type to take up and transcytose whole bacteria from the intestinal lumen. This uptake mechanism is likely exploited by the fish-pathogen *M. marinum* as port of entry during infection. A more detailed study of the cell types and mechanisms involved in intestinal particle uptake would further give valuable information for more systematic and targeted development of nanoparticle-based oral vaccines. In addition, the differences in bio-distribution between different particles observed in this study, emphasizes the need to study the effect of nano-/microparticle properties on uptake and transport dynamics *in vivo* more systematically. The zebrafish offers a valuable tool for this task, especially taken into consideration the availability of transgenic lines with fluorescently labelled immune cell types such as macrophages and dendritic cells, neutrophils, T- and B-cells (Langenau et al., 2004; Wittamer et al., 2011). Furthermore, we show that using the zebrafish oral intubation model, infection routes of fish pathogens in the gastrointestinal tract can be studied in *vivo* as shown for *M. marinum*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.dci.2016.06.016.

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