

Zebrafish mast cells possess an FcεRI-like receptor and participate in innate and adaptive immune responses

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ABSTRACT

We previously identified a zebrafish mast cell (MC) lineage and now aim to determine if these cells function analogously in innate and adaptive immunity like their mammalian counterparts. Intraperitoneal (IP) injection of compound 48/80 or live *Aeromonas salmonicida* resulted in significant MC degranulation evident histologically and by increased plasma tryptase compared with saline-injected controls ($p = 0.0006$, 0.005 , respectively). Pre-treatment with ketotifen abrogated these responses ($p = 0.0004$, 0.005 , respectively). Cross-reactivity was observed in zebrafish to anti-human high-affinity IgE receptor gamma (FcεRIγ) and IgE heavy chain-directed antibodies. Whole mount *in situ* hybridization on 7-day embryos demonstrated co-localization of *cpa5*, a MC-specific marker, with *myd88*, a toll-like receptor adaptor, and zebrafish FcεRI subunit homologs. Zebrafish injected IP with matched dinitrophenyl-sensitized mouse (anti-DNP) IgE and DNP-BSA or trinitrophenyl-sensitized mouse (anti-TNP) IgE and TNP-BSA demonstrated increased plasma tryptase compared with mismatched controls ($p = 0.03$, 0.010 , respectively). These results confirm functional conservation and validate the zebrafish model as an *in vivo* screening tool for novel MC modulating agents.

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1. Introduction

The zebrafish, a robust model for studying vertebrate hematopoiesis (Berman et al., 2005; Carradice and Lieschke, 2008), has recently been exploited as a versatile organism for better understanding the complexity of both innate and adaptive immune responses (Meeker and Trede, 2008; Traver et al., 2003; Trede et al., 2004). Putative orthologs of eight of ten human toll-like receptors (TLRs), key regulators of innate immune signaling, have been identified in the zebrafish, as have downstream adaptor molecules, including Toll/interleukin-1 receptor (TIR)-domain containing adaptor inducing INF-β (TRIF) and myeloid differentiation primary response gene (88) (MyD88) (Jault et al., 2004; Meijer et al., 2004). Similarly, conserved elements required for adaptive immune responses have been described in zebrafish and other

teleosts (Fujiki et al., 2000; Stafford et al., 2006; Yoder et al., 2001, 2004), such as FcRγ and FcRγ-like receptors, found to be syntenic to the gamma subunit of the human high-affinity IgE receptor located at 1q23 (Yoder et al., 2007).

Mast cells (MCs) serve a critical role as sentinels of the immune system, initiating many of the events involved in immune responsiveness, in addition to functioning as the key effector cell in allergic reactions. Our recent discovery of a zebrafish MC equivalent possessing structural and functional similarities to its mammalian counterpart (Dobson et al., 2008) has expanded the repertoire of immune cells in this organism and provided the opportunity to further investigate the conservation of functional genomics as it applies to immunity in general, and MCs in particular.

In this study, we reconcile the conservation of immune cell receptor genes with MC responses *in vivo*. We demonstrate that zebrafish MC degranulation stimulated by compound 48/80 (c48/80) or *Aeromonas salmonicida* can be abrogated by the MC stabilizing agent, ketotifen. *Myd88* is expressed in a proportion of these mature MCs, suggesting conservation of innate immune responses mediated through TLRs. Most significantly, we present structure–function evidence that zebrafish MCs possess an analogous high-affinity immunoglobulin E (IgE)-like receptor (FcεRI),

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which following stimulation, results in reproducible classic passive systemic anaphylactic (PSA) responses.

2. Materials and methods

2.1. Zebrafish maintenance

Zebrafish husbandry was carried out according to Westerfield (Westerfield, 1995). The use of zebrafish in these studies has been approved by the Dalhousie University Animal Care Committee.

2.2. Histochemical stains

Standard protocols were used for staining 5 μ m sections of zebrafish intestine for Periodic Acid Schiff (PAS) and toluidine blue (TB). Slides were visualized using Zeiss Z1 microscope and Axiocam Rev 3.0 camera, Wetzlar, Germany.

2.3. Electron microscopy (EM)

Zebrafish intestine was collected immediately following cardiac puncture and fixed with 2.5% glutaraldehyde with 0.1 M sodium cacodylate buffer at 4 °C overnight. Samples were further fixed in 1% osmium tetroxide and 0.25% uranyl acetate and embedded in 100% epon araldite resin. Thin sections of 100 nm were cut and placed on 300 mesh copper grids and stained with 2% aqueous uranyl acetate followed by lead citrate. The samples were then viewed using a JEOL JEM 1230 Transmission Electron Microscope (Tokyo, Japan) at 80 kV, and images captured using a Hamamatsu ORCA-HR digital camera (Bridgewater, NJ).

2.4. Functional assays

Intraperitoneal injections (IP) were performed as described previously (Dobson et al., 2008; Klein et al., 2009) with drug or infectious agent of interest diluted using 0.07% Bromophenol blue to ensure correct anatomical location (Dobson et al.,

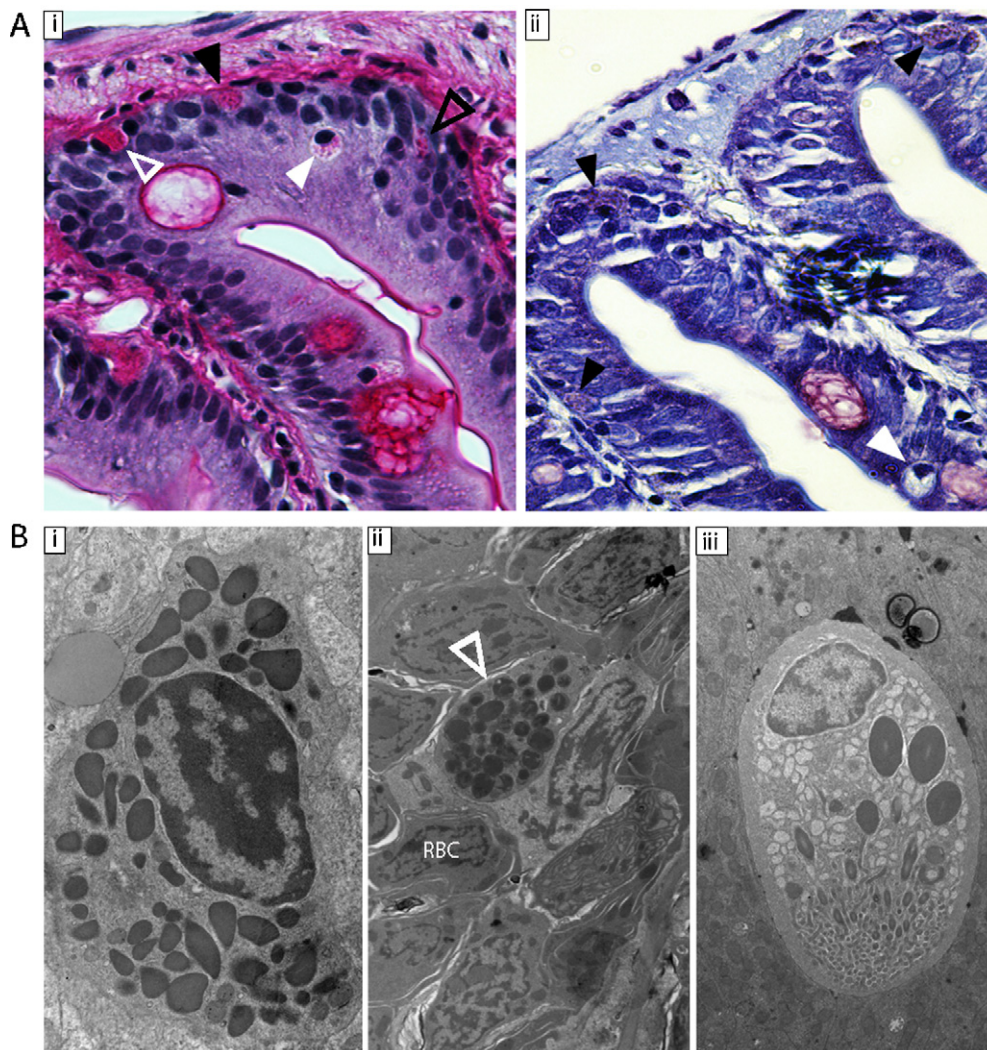


Fig. 1. Zebrafish intestinal immune cells can be distinguished by light and electron microscopy. (A) Light microscopic images of wild type zebrafish intestine (magnification 100 $\times$) under resting conditions. (i) Periodic Acid Schiff (PAS) stain. Mast cells (black arrowhead) and eosinophils (open white arrowhead), which have more compact eosinophilic granules. A degranulating mast cell is indicated by an open black arrowhead. Rodlet cells display fewer distinct coarse round amphophilic granules (white arrowhead). (ii) Toluidine blue stain. Mast cells demonstrate metachromatic granules (black arrowheads). Rodlet cell granules do not stain giving the cytoplasm a clear appearance (white arrowhead). (B) Transmission electron microscopic (TEM) images of wild type zebrafish intestine. (i) Unstimulated mast cell, displays dense, coarse and oblong granules (magnification 15,000 $\times$). (ii) Eosinophil located in the vasculature as indicated by the adjacent red blood cells (RBC) (magnification 8000 $\times$). (iii) Rodlet cell (magnification 8000 $\times$).

2008). c48/80 10 μ g, live *A. salmonicida* in a final concentration of 3×10^7 cfu/adult fish, or saline was used for IP injections. Fish recovered for 5 min to 1 h prior to blood sampling by cardiac puncture. Ketotifen fumarate salt (Sigma–Aldrich, St. Louis, MO) was added directly to the water at 400 μ g/mL final concentration, 1 h prior to c48/80 or *A. salmonicida*. Tryptase activity was measured in plasma spectrophotometrically at 415 nm by the release of p-nitroanilide from N-benzoyl-DL-arginine p-nitroanilide (BAPNA), a tryptase substrate (Lavens et al., 1993), using a 96-well plate spectrophotometer (BIORAD 680 Microplate reader, Japan). Relative levels of tryptase activity in zebrafish samples were measured and normalized by using a standard curve generated from a human LAD2 mast cell line.

2.5. Immunohistochemistry

Tissue mounted on silanized slides was de-paraffinized and antigen retrieval was performed in 10 mM citric acid, pH 6.0 in a steamer for 30 min. Slides were immersed in 3% hydrogen peroxide in methanol to quench endogenous peroxidase activity for 30 min. Slides were then incubated with normal goat serum diluted at 1:20 in $1 \times$ PBS for 30 min on a shaker. Polyclonal rabbit immunoglobulin G (IgG), anti-human Fc ϵ RI γ (Millipore, Bellerica, MA) antibody diluted at 1:400 in $1 \times$ PBS was applied and incubated overnight at 4 °C. Slides were rinsed in $1 \times$ PBS and a secondary antibody, biotinylated goat anti-rabbit IgG diluted in 1:600 in $1 \times$ PBS was applied and incubated for 30 min. The avidin–biotin complex (ABC) method was utilized, and the peroxidase reaction with diaminobenzidine (DAB) was employed for visualization (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin. Negative controls consisted of tissue sections in which the primary antibody was replaced with an isotype control consisting of non-immunized rabbit IgG (Cedarlane Laboratories, Hornby, ON), but otherwise treated as above.

2.6. Western blot

Protein was extracted from zebrafish embryos 7 days post-fertilization (Sidi et al., 2008). Protein extract was treated with or without 5% β -mercaptoethanol. A total of 50 μ g of protein were loaded for Western blot. The detection of the zebrafish Ig heavy chain was determined with a 1:1000 dilution of the polyclonal rabbit anti-human IgE that reacts with the epsilon-chain of human IgE (Dako, Denmark). Chemiluminescence detection after adding the secondary antibody was done by using Image Kodak.

2.7. Identification of zebrafish homologs of Fc ϵ RI subunits

cDNA clones were identified using human protein sequences of the high-affinity Fc ϵ RI α (FCER1, GenBank: NP.001992) and β subunits (MS4A.2, GenBank: AAF17243) as queries in tBLASTn searches against the zebrafish nucleotide collection. A putative zebrafish *fc ϵ 1a* sequence (GenBank: BC095877), encoded by MGC 113199, and *ms4a.17a.4* sequence (GenBank: BC150377) encoded by MGC 171691 were retrieved. cDNAs were purchased from Thermo Scientific Open Biosystems (Huntsville, AL). cDNAs were amplified using *fc ϵ 1a* (Fc ϵ RI alpha) forward primer 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GAT TTA CAT GAT CAT ATT CC-3'/reverse primer 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TTT AGT CGT TGT TAT AAT-3', *ms4a.17a.4* (Fc ϵ RI beta) forward primer 5'-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT AAT GAA TCC TTC AAC GAT T-3'/reverse primer 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA TCA TGT TGA TTC ACA TTT-3'. cDNAs coding for zebrafish homologs of human Fc ϵ RI γ , *fc ϵ 1g* and *fc ϵ 1gl*, were generously provided by Dr. Jeffrey Yoder (Yoder et al., 2007).

2.8. Whole mount in situ hybridization (WISH) and fluorescence-activated cell sorting (FACS)

Digoxigenin- and fluorescein-labeled RNA probes were transcribed from linearized cDNA according to manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis, IN). WISH assays were performed on wild type embryos at 7 days post-fertilization (dpf) as described previously (Dobson et al., 2008). For double WISH, *cpa5* (Dobson et al., 2008) was used in conjunction with probes to *fc ϵ 1* subunits on wild type embryos. Similarly, fluorescein-labeled *cpa5* and digoxigenin-labeled *EGFP* probes were used in combination on *myd88::EGFP* embryos. FACS and cytopspins of dissociated wild type embryos at 7 dpf WISH-labeled with *fc ϵ 1a*, were carried out as described previously (Dobson et al., 2009).

2.9. Induction of IgE-dependent systemic reaction

Adult zebrafish were sensitized by IP injection with 5 μ L containing 20 ng of mouse monoclonal anti-DNP IgE (dinitrophenyl; Sigma–Aldrich, St. Louis, MO), anti-TNP IgE (trinitrophenyl; Biosearch Technologies, USA) or saline and allowed to recover for 24 h prior to being challenged with 20 μ g of DNP-BSA (bovine serum albumin; Sigma–Aldrich, St. Louis, MO) or 40 μ g of TNP-BSA (Biosearch Technologies, USA). Plasma was isolated for tryptase assay as described above and for measurement of leukotriene C₄ (LTC₄) by ELISA as outlined in Olynych et al. (2006).

2.10. Statistical analysis

Tryptase assays performed on individual fish were pooled across identical experiments and analyzed using an analysis of variance (ANOVA) model to account for experiment-to-experiment variability. Factors for treatment and experiment were included in each model. *p*-Values were adjusted for multiple comparisons using Tukey's least significant difference. A Student's *t*-test was used to analyze differences between anti-DNP IgE/DNP-BSA, anti-TNP IgE/TNP-BSA, and anti-TNP IgE/DNP-BSA as there was no significant experiment-to-experiment variability. A *p*-value < 0.05 was deemed statistically significant.

3. Results

3.1. Staining properties of zebrafish intestinal immune cells

MCs are a component of a repertoire of immune cells in the zebrafish intestine. We have previously shown that MC granules are

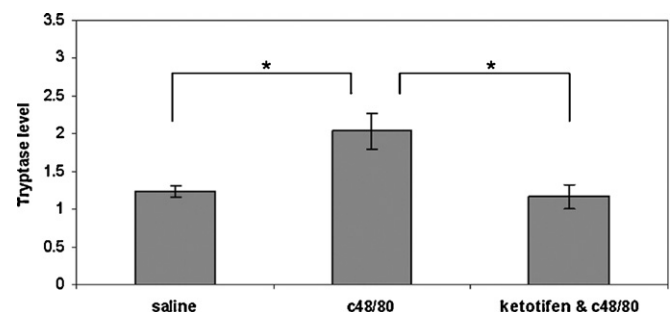


Fig. 2. Zebrafish intestinal mast cells are activated by c48/80, which can be abrogated following treatment with ketotifen. Individual adult wild type zebrafish injected IP with 10 μ g of c48/80 demonstrate increased plasma tryptase levels compared to saline controls (**p* = 0.0006). Ketotifen added to the fish water (400 μ g/mL) 1 h prior to injection abrogated this effect (*p* = 0.0004) to control levels observed with saline (*p* = 0.92). Presented as mean tryptase levels $\pm$ SEM. Tryptase levels are equivalent to a 1×10^6 human mast cell line (LAD2) used to generate a standard curve.

eosinophilic following Periodic Acid Schiff (PAS) staining (Dobson et al., 2008). However, an additional population of cells that we have labeled eosinophils, stain similarly (albeit with more compact and more intensely eosinophilic granules), making the distinction between these two populations extremely challenging under PAS staining (Fig. 1Ai). Conversely, following staining with toluidine blue, zebrafish MC granules demonstrate metachromatic staining like their mammalian counterparts, while eosinophils are not visualized (Fig. 1Aii). A third immune granulocyte identified is the rodlet cell, a cell type unique to teleosts (Reite, 2005; Reite and Evensen, 2006). Rodlet cells have been identified as components of the immune system in the gastrointestinal tract of a number of teleost fish (Reite, 2005). However, they had previously only been identified in zebrafish for their role as sensory cells of the olfactory system (Dezfuli et al., 2007). Rodlet cells are easily

discernible due to their characteristic large ordered eosinophilic granules observed under PAS and granular pallor under toluidine blue staining (Fig. 1Ai and ii). MCs at rest (Fig. 1Bi) and eosinophils (Fig. 1Bii) share similar morphology under transmission electron microscopy (TEM) with a single-lobed eccentric nucleus and homogeneous dense round granules. This appearance of eosinophils (Fig. 1Bii) in adult intestine is consistent with the morphology of these cells previously described in zebrafish embryos (Lieschke et al., 2001). However, following stimulation, MC granules become more oblong to cuboidal and frequently, a void may be apparent within the cytoplasm, from a granule that has released its contents. Cytoplasmic blebs and extracellular granular contents can also be observed following MC activation (Fig. 7C and F). By contrast, TEM reveals that rodlet cells have large ordered pale appearing granules (Fig. 1Biii).

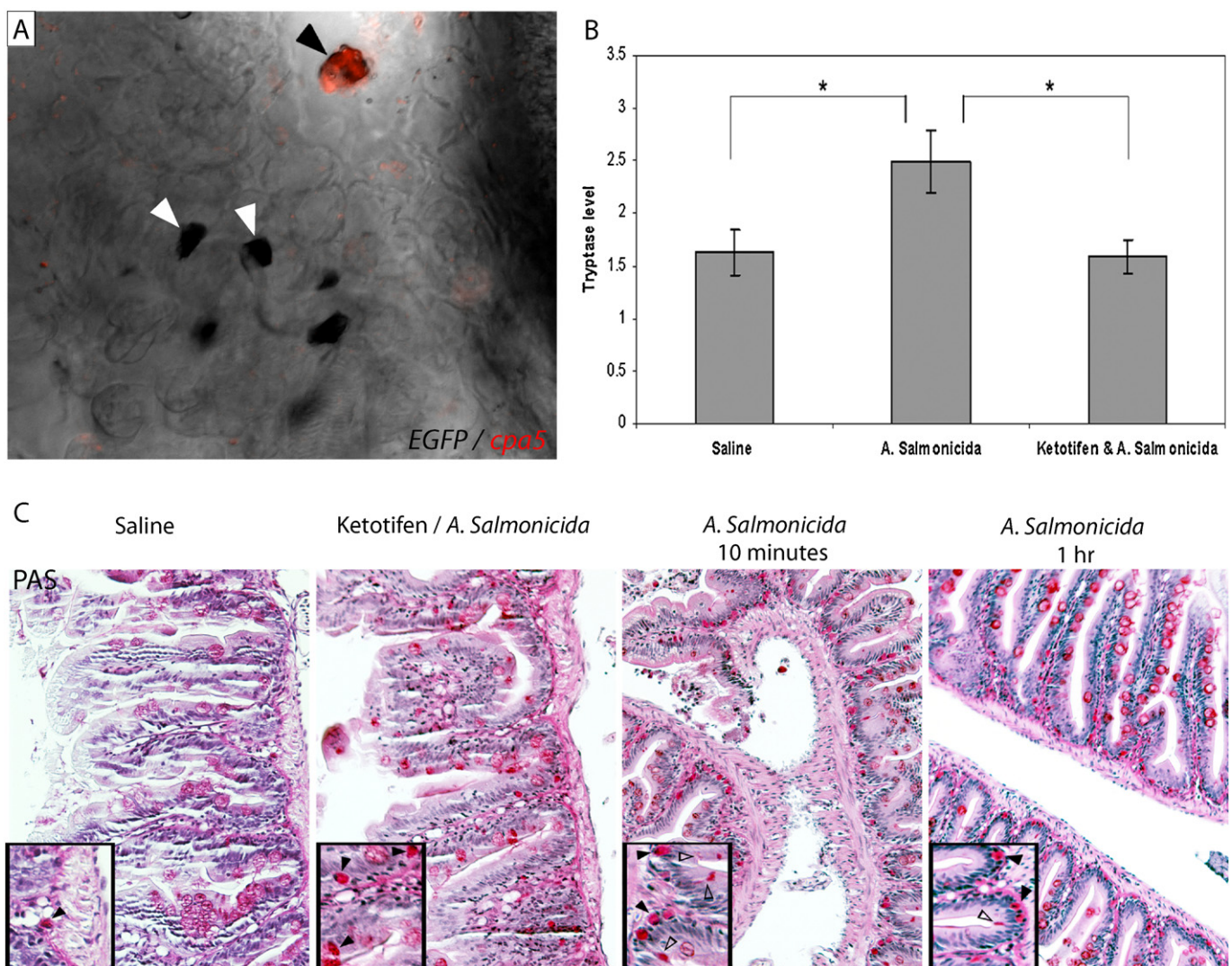


Fig. 3. Zebrafish mast cells participate in innate immune responses. (A) Double WISH performed on *myd88::EGFP* transgenic embryos at 7 dpf using a dig-labeled RNA anti-sense probe to *EGFP* (black) and FITC-labeled RNA anti-sense probe to zebrafish *cpas* (red), demonstrate co-localization in a proportion of cells (black arrowhead). Other leukocytes that express *myd88* alone are identified by white arrowheads. (B) Individual adult wild type zebrafish injected IP with live *A. salmonicida* 3×10^7 cfu/adult fish demonstrate elevated plasma tryptase levels compared to saline-injected controls ($*p = 0.005$). Treatment with ketotifen (400 μ g/mL) 1 h prior to injection prevented mast cell degranulation as evident by significantly lower tryptase levels following live *A. salmonicida* injection ($*p = 0.005$) that were comparable to saline controls ($p = 0.98$). Presented as mean tryptase levels $\pm$ SEM. Tryptase levels are equivalent to a 1×10^6 human mast cell line (LAD2) used to generate a standard curve. (C) PAS staining (insets to highlight cells of interest) of histological sections of adult zebrafish intestine demonstrate an accumulation of mast cells in ketotifen pre-treated and *A. salmonicida*-infected fish compared with saline-injected controls that is greatest in the *A. salmonicida*-infected cohort at 10 min post-injection. Degranulating mast cells are observed predominantly in the *A. salmonicida*-infected cohort, peaking at 10 min post-injection (magnification for panel A 40 $\times$; C PAS 20 $\times$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Zebrafish mast cell degranulation following treatment with compound 48/80 is abrogated following exposure to ketotifen

Individual adult wild type zebrafish underwent IP injection with 10 µg of c48/80. Despite this relatively high dose, c48/80 injected fish showed no evidence of distress following IP injection. Plasma was obtained via cardiac puncture for measurement of tryptase level and examined individually for each fish. IP injection of c48/80 resulted in a significant treatment effect as measured by increased tryptase levels ($p=0.0002$) in keeping with our previously reported data on pooled samples (Dobson et al., 2008). Tryptase levels following c48/80 were consistently elevated compared to saline-injected controls ($p=0.0006$). Ketotifen is an H1 histamine antagonist and MC stabilizing agent used in the treatment of asthma and other allergic diseases (Schwarzer et al., 2004). Ketotifen significantly abrogated MC degranulation induced by c48/80 ($p=0.0004$) to control levels observed with saline ($p=0.92$, Fig. 2).

3.3. Zebrafish mast cells demonstrate conservation of innate immune pathways

Responses to live *A. salmonicida* are rapid following initial exposure (Lin et al., 2007), thus reasoned to be mediated through innate immune pathways, initiated through the stimulation of TLRs. MyD88 has been found to be necessary for the majority of TLR-mediated signaling in mammals (Kawai et al., 1999). Endogenous *myd88* expression in zebrafish embryonic leukocytes has been demonstrated by whole mount *in situ* hybridization (WISH) (Jault et al., 2004; van der Sar et al., 2006) and further characterized with the recent development of a *myd88::EGFP* transgenic line (Hall et al., 2009). We previously identified carboxypeptidase a5 (*cpa5*) as a specific marker for zebrafish MCs. At 7 days post-fertilization (dpf), *cpa5*-positive cells represent a mature MC population as determined by morphology (Dobson et al., 2008). WISH employing probes to *EGFP* and *cpa5* was performed on 7-day *myd88::EGFP* transgenic embryos and co-localization observed in a proportion of cells (Fig. 3A and Supplemental Fig. 1). These results reveal that zebrafish MCs express *myd88* and suggest a role in TLR-mediated signaling.

Individual adult wild type zebrafish treated by IP injection with live *A. salmonicida* exhibited no differences in swimming behavior, but demonstrated a significant treatment effect ($p=0.002$) with increased levels of plasma tryptase compared to saline-injected controls ($p=0.005$, Fig. 3B). Pre-treatment with ketotifen prevented MC degranulation as evident by significantly lower tryptase levels following live *A. salmonicida* injection ($p=0.005$) that were comparable to saline controls ($p=0.98$, Fig. 3B). PAS and toluidine blue staining of adult zebrafish intestine comparing saline injection, ketotifen pre-treatment and *A. salmonicida* infection revealed an accumulation of MCs in the latter two groups compared to the saline-injected control group at 10 min (Fig. 3C and Supplemental Fig. 2). However, the most dramatic MC accumulation occurred in the *A. salmonicida*-infected cohort not pre-treated with ketotifen, which was also the only group to demonstrate MC degranulation, a feature in keeping with the peak tryptase level observed under these conditions. MC accumulation and degranulation persisted to 1 h post-infection with *A. salmonicida* (Fig. 3C).

3.4. Zebrafish mast cells express an FcεRI-like receptor and mediate adaptive immune responses

Immunohistochemical staining of adult zebrafish intestinal tissue was performed employing a polyclonal antibody directed to the gamma subunit of the human high-affinity IgE receptor (FcεRIγ), and revealed specific staining of zebrafish intestinal MCs (Fig. 4A).

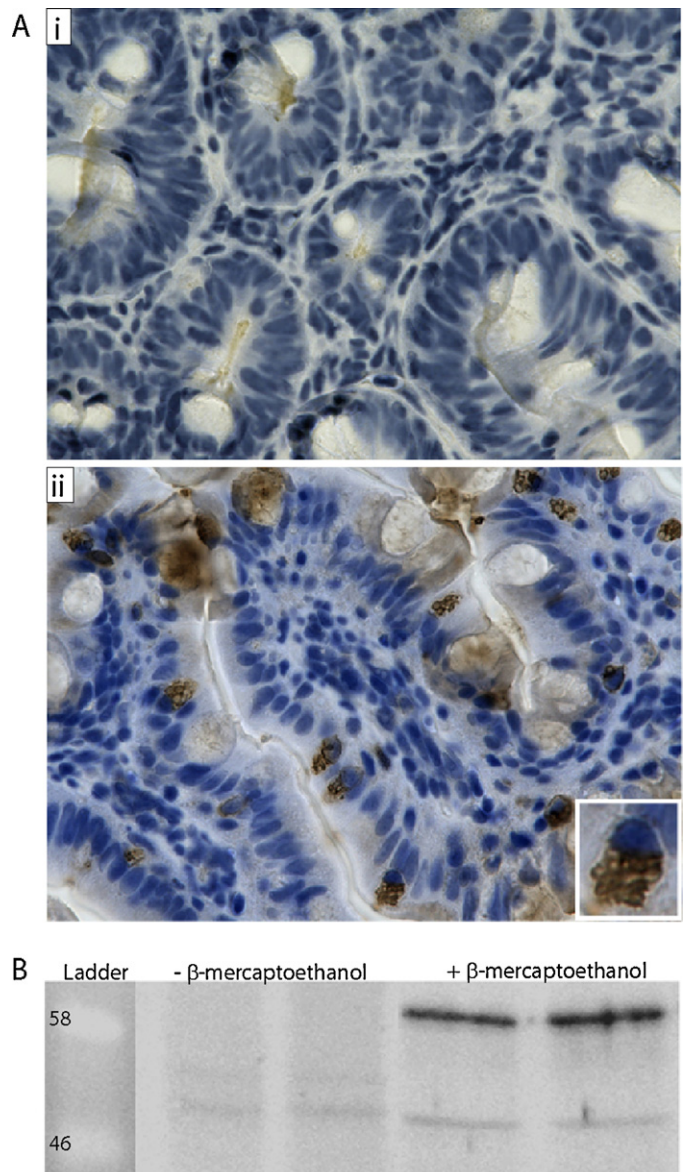


Fig. 4. Zebrafish mast cells express an FcεRI-like receptor and antibody. (A) Immunohistochemistry of wild type adult zebrafish intestine using anti-human FcεRIγ demonstrates specific staining in intestinal mast cells (ii, and inset) compared with human IgG isotype control (i) (magnification 63×). (B) Western blot of protein with and without treatment with β-mercaptoethanol extracted from zebrafish embryos at 7 days post-fertilization demonstrates a specific 57.7 kD protein band. This band corresponds to zebrafish Ig heavy chain (NCBI AAK96442.1).

Seeing evidence for a conserved IgE receptor, we looked for an indication of a conserved IgE-like antibody. A Western blot employing a polyclonal rabbit antibody directed to human IgE, revealed a specific 58 kD band (Fig. 4B) corresponding to a 57.7 kD zebrafish Ig heavy chain (NCBI AAK96442.1). Given these positive results, we subsequently performed WISH analysis on 7-day embryos using probes generated from the following cDNAs: a predicted sequence called *fcεr1a* (corresponding to FcεRIα), *ms4a.17a.4* (corresponding to FcεRIβ), which were found to have conserved protein domains and shared phylogeny to their mammalian counterparts (Fig. 5 and Supplemental Fig. 3), and gamma and gamma-like orthologs, *fcεr1g* and *fcεr1gl* (corresponding to FcεRIγ, Yoder et al., 2007). For all probes examined, we observed expression in individual cells in anterior regions where *cpa5*-positive MCs were previously identified (Dobson et al., 2008 and Supplemental Fig. 4). Co-localization studies employing anti-sense probes to both *cpa5* and the various

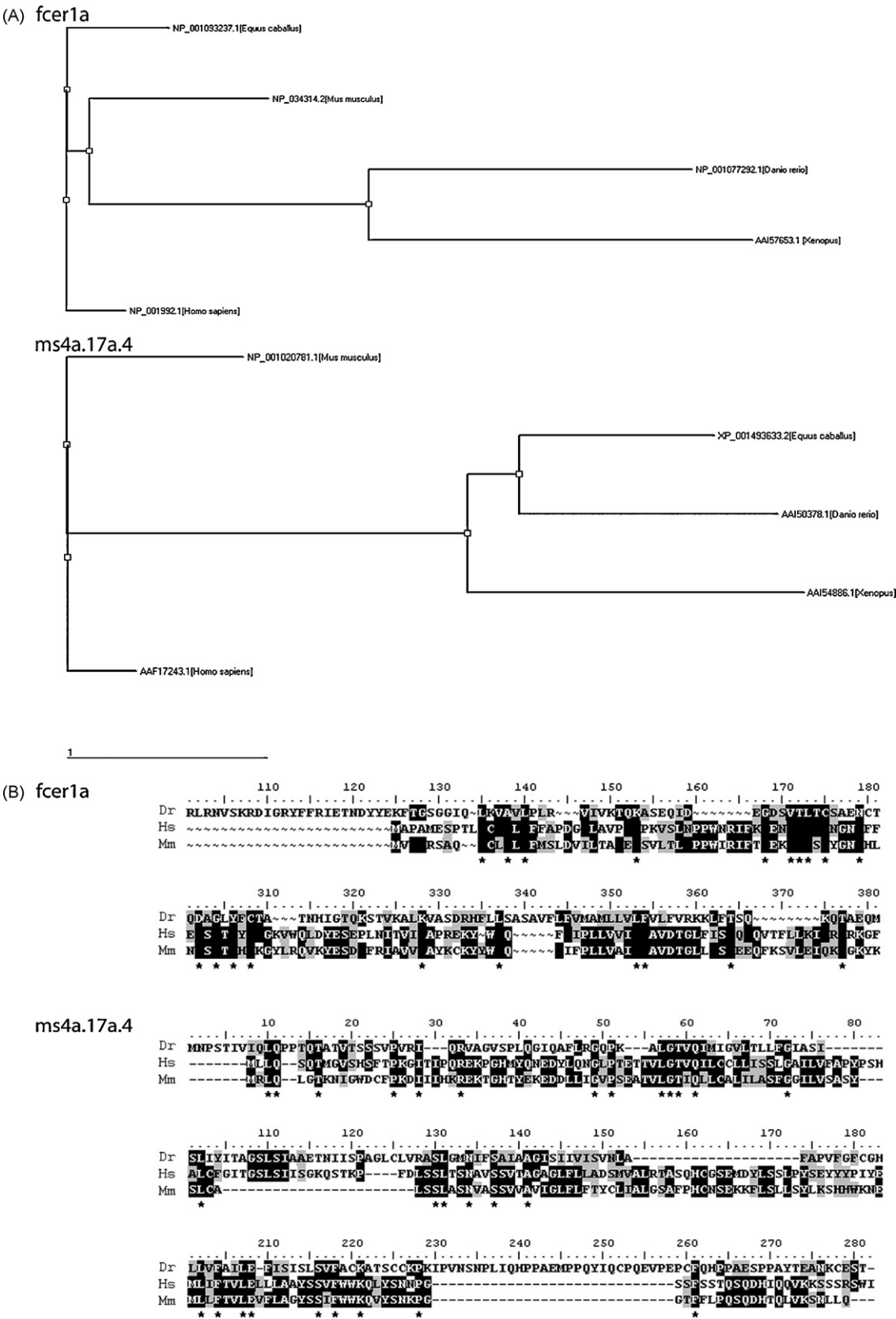


Fig. 5. Zebrafish FcεRI-like receptor predicted subunits fcer1a and ms4a.17a.4 demonstrate conservation with their mammalian counterparts. (A) Phylogenetic analysis. Neighbor phylogenetic trees were constructed based on the ClustalW multiple alignment of the amino acid sequences of the zebrafish (*Danio rerio*) fcer1a and ms4a.17a.4 proteins compared with human (*Homo sapiens*), mouse (*Mus musculus*), horse (*Equus caballus*) and frog (*Xenopus* (*Silurana*) *tropicalis*). (B) Zebrafish fcer1a (NP.001077292.1) shared identities with its mammalian orthologs: human NP.001992.1 (26%) and mouse NP.034314.2 (34%). Zebrafish ms4a.17a.4 (AAI50378) shared identities with its mammalian orthologs: human AAF17243 (23%) and mouse NP.001020781 (36%). Black shading indicates identical amino acids and gray shading indicates functionally similar amino acids.

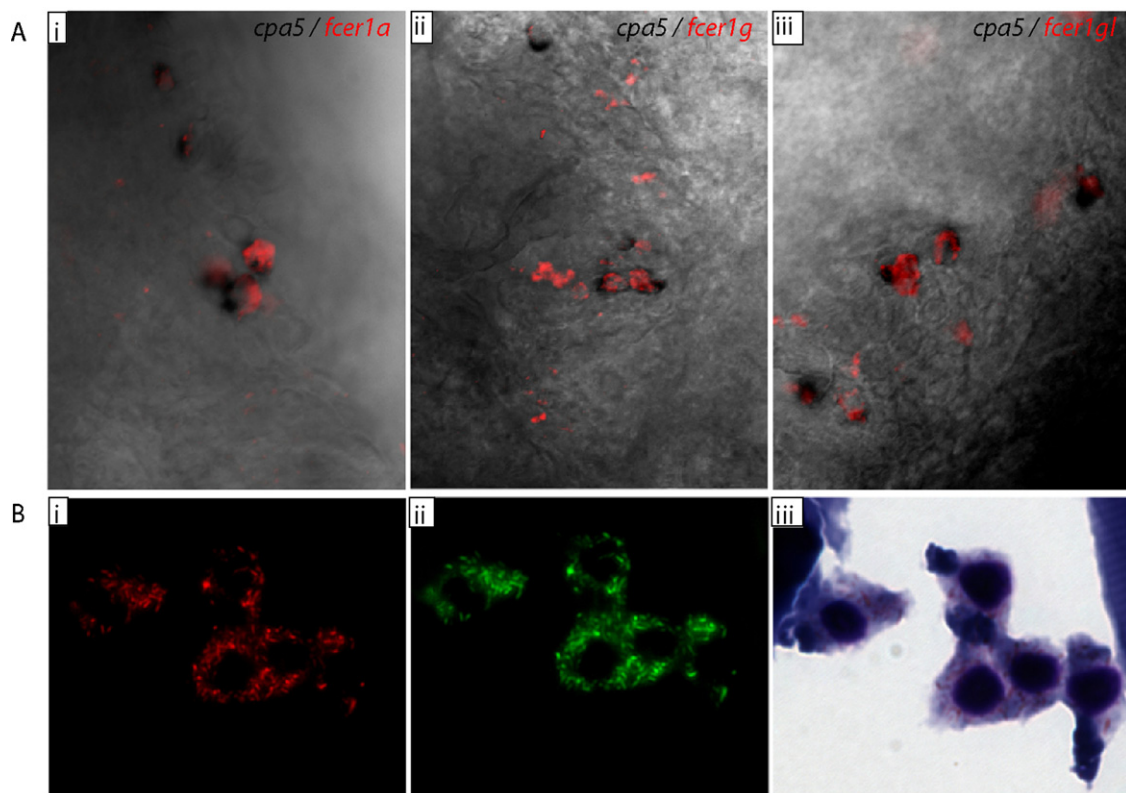


Fig. 6. The zebrafish *cpa5* mast cell-specific marker colocalizes with FcεRI-like receptor subunits. (A) Double WISH performed on wild type zebrafish embryos at 7 dpf using a dig-labeled RNA anti-sense probe to zebrafish *cpa5* (black) and FITC-labeled RNA anti-sense probe to *fcer1a*, *fcer1g* and *fcer1gl* (red), demonstrates co-localization in a proportion of cells. (B) FACS sorted FITC+/Fast Red+ *fcer1a* positive cells demonstrate a mast cell phenotype at 7 dpf. Cellular labeling is confirmed by fluorescence under both Fast Red (i) and FITC (ii) fluorescent filters and Wright–Giemsa staining (iii) (magnification for panel A 40×; B 100×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

FcεRI subunit homologs were undertaken in order to determine if these positively expressing cells were in fact MCs. *fcer1a* demonstrated complete co-localization with *cpa5* (Fig. 6Ai), while *fcer1g* and *fcer1gl* demonstrated co-localization in a proportion of cells (Fig. 6Aii and iii). No co-localization was observed for *cpa5* and *ms4a.17a.4*. While the alpha and gamma subunits are the most essential components for MC signaling (Kinet et al., 1991; Riske et al., 1991; Takai, 1996; Taube et al., 2004), the beta subunit may confer MC specificity (MacGlashan, 2008). Thus, we employed a novel technique we developed that provides morphological confirmation of RNA expression by combining WISH with fluorescence-activated cell sorting (FACS) (Dobson et al., 2009). Cells exclusively expressing *fcer1a* (FcεRIα) were isolated and a cytospin corroborated a mature MC phenotype (Fig. 6B).

With evidence for an FcεRI-like receptor, we wanted to evaluate if zebrafish MCs participated in IgE-mediated allergic responses following sensitization with an antigen-primed antibody and subsequent antigen exposure. We observed no behavioral changes, but a significant treatment effect in adult zebrafish sensitized with anti-DNP IgE followed by DNP-BSA ($p=0.03$). Fish treated with anti-DNP IgE/DNP-BSA demonstrated a suggestive increase in plasma tryptase compared to fish treated with saline and DNP-BSA ($p=0.07$), while a significant difference was observed compared to fish treated with anti-DNP IgE and saline ($p=0.04$, Fig. 7A). Preferentially in the anti-DNP IgE/DNP-BSA fish, TEM demonstrated MC degranulation with cytoplasmic blebbing, agranular cytoplasmic areas and extracellular granules (Fig. 7B and C). Finally, to demonstrate that this response was antigen specific, we compared treatment with anti-DNP IgE/DNP-BSA to the response of treatment with anti-TNP IgE to DNP-BSA, an antibody which does not cross-react with DNP. We found significant increases in

plasma tryptase for anti-DNP IgE/DNP-BSA ($p=0.03$) as well as anti-TNP IgE/TNP-BSA ($p=0.01$) compared with anti-TNP IgE mismatched with DNP-BSA and corresponding morphologic changes by TEM (Fig. 7D–F). These results suggest that degranulation and increased plasma tryptase levels resulted from sensitized direct antibody-antigen recognition, in keeping with what is observed in mammalian models.

4. Discussion

We previously identified the gastrointestinal submucosa and gills as prominent anatomic sites of zebrafish MC activity (Dobson et al., 2008). These locations correspond to the respective mammalian tissues, where MCs predominate in order to easily function as early effector cells in response to noxious or infectious environmental stimuli. Similarly, mast cell/eosinophilic granule cells (MC/EGCs) were previously described in gastrointestinal tissue sections of a number of other teleost species (Reite, 1997, 1998, 2005; Reite and Evensen, 2006). In zebrafish, we demonstrate that toluidine blue staining can effectively distinguish between MCs and eosinophils. However, the nomenclature MC/EGCs has persisted in the literature in reference to these cells in other teleosts, likely due to a failure of certain fixation techniques to consistently demonstrate metachromatic staining in a subpopulation of these cells stained with toluidine blue (Reite and Evensen, 2006). This distinction cannot be easily made using PAS or hematoxylin and eosin due to similar cell size and staining characteristics. However, we found that eosinophils were decidedly rare inhabitants of the G.I. tract and when present, more localized in and around the vasculature. Others have similarly observed a lack of eosinophils in the intestine (G. Lugo and D. Traver, unpublished data).

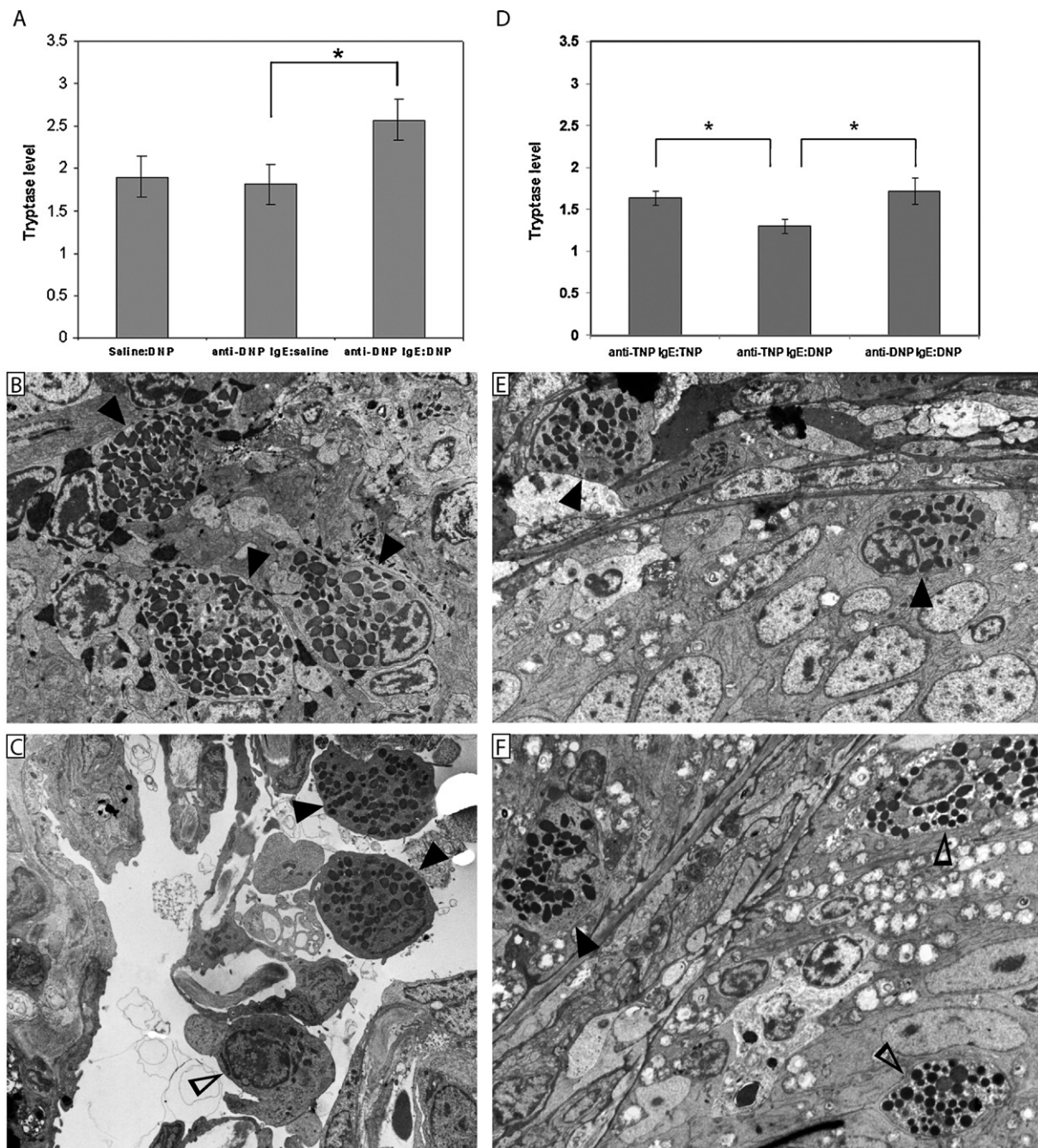


Fig. 7. Zebrafish mast cells participate in passive systemic anaphylaxis. (A) Individual adult wild type zebrafish injected IP with anti-DNP IgE 20 ng and then exposed to DNP-BSA 20 μ g demonstrate an increase in plasma tryptase level compared with saline and DNP-BSA 20 μ g ($p = 0.07$) or anti-DNP IgE 20 ng and saline ($*p = 0.04$). (B) TEM of adult wild type zebrafish intestine treated with anti-DNP IgE and saline demonstrate persistence of intact mast cells (black arrowheads). (C) TEM of adult wild type zebrafish intestine treated with anti-DNP IgE and DNP-BSA demonstrate mast cell activation with cytoplasmic blebbing and areas of agranular cytoplasm post-degranulation. Extracellular granules are also visible (open black arrowhead). Intact mast cells are indicated by black arrowheads. (D) Individual adult wild type zebrafish injected IP with matched anti-TNP IgE 20 ng and then exposed to TNP-BSA 40 μ g or anti-DNP IgE 20 ng and then exposed to DNP-BSA 20 μ g demonstrate an increase in plasma tryptase level compared with mismatched anti-TNP IgE 20 ng and DNP-BSA 20 μ g ($p = 0.01, 0.03$, respectively). (E) TEM of adult wild type zebrafish intestine treated with mismatched anti-TNP IgE and DNP-BSA demonstrate persistence of intact mast cells (black arrowheads). (F) TEM of adult wild type zebrafish intestine treated with anti-TNP IgE and TNP-BSA demonstrate mast cell activation with cytoplasmic blebbing and different granular morphology, and areas of agranular cytoplasm post-degranulation (open black arrowhead). Intact mast cells are indicated by black arrowheads. (A) and (D) presented as mean tryptase levels $\pm$ SEM. Tryptase levels are equivalent to a 1×10^6 human mast cell line (LAD2) used to generate a standard curve. Magnification: B and C, 5000 $\times$; E and F, 4000 $\times$.

Tryptase is the major type of serine protease stored in MC granules and seemingly well conserved among vertebrate species (McNeil et al., 2007). Elevated plasma tryptase levels accurately reflect MC burden or severity of reaction, providing a robust assay for detection of MC stimulation (Hallgren and Pejler, 2006). c48/80 functions through the direct stimulation of intracellular G proteins,

activation of phospholipase D, increased cell membrane permeability, influx of intracellular calcium and subsequent release of MC granular mediators (Chahdi et al., 2000). We previously determined the chromogenic tryptase assay was the most reliable assay for measuring MC degranulation in plasma pooled from adult zebrafish (Dobson et al., 2008). Individual fish likewise demonstrate

increased tryptase release following 10 µg of c48/80, in keeping with MC degranulation without any compromise to fish health or behavior (Dobson et al., 2008). This effect was effectively blocked with prior administration of the MC stabilizing agent, ketotifen.

Conservation of innate immune responses has been demonstrated in zebrafish and other teleosts through evidence of TLR expression and activity. *In vitro* studies using rainbow trout leukocyte culture demonstrated comparable functional responses to those in mammals following exposure to bacterially derived TLR agonists (Purcell et al., 2006). Infection of adult zebrafish with *Mycobacterium marinum* resulted in upregulation of TLRs (Meijer et al., 2004); while a prior study infecting zebrafish with *A. salmonicida* found increased levels of TNFα and IL-1, which lay downstream of the TLRs (Lin et al., 2007). We have added to this literature, by demonstrating evidence suggesting TLR-mediated signaling may occur in zebrafish MCs. Utilizing the *myd88::EGFP* transgenic line generated by Hall et al. (2009), we showed that in keeping with the other leukocyte lineages presented in their study, a proportion of mature MCs express *myd88*, a downstream adaptor protein frequently required for TLR signaling.

Moreover, we utilized the tryptase assay to examine the role of MCs in initiating an innate immune response to infection by *A. salmonicida*. The robust rapid response following active infection suggests innate immune mechanisms and the successful inhibition of degranulation by prior exposure to ketotifen, may implicate MCs. A time course was conducted from 5 min to 1 h following infection. MC accumulation observed clearly under both PAS and toluidine blue staining and degranulation, seen best under PAS staining, was most striking histologically at 10 min, which corresponded to peak tryptase levels (Fig. 3B and C, and Supplemental Fig. 2). Taken together with evidence of *myd88* expression in zebrafish MCs, our data suggest that zebrafish MCs participate in innate immune responses to pathogens through a conserved TLR-initiated pathway.

In mammals, MCs classically participate in adaptive immune responses through the binding of IgE to a high-affinity IgE surface receptor (FcεRI). While a specific IgE counterpart has not been identified in zebrafish, we have demonstrated cross-reactivity of antibodies directed both against human FcεRIγ and human IgE. A unique IgZ antibody has been described (Danilova et al., 2000), which may serve a broad effector purpose given the more restrictive immunoglobulin profile in teleost fish (Flajnik, 2005). Moreover, stimulation of the FcεRI receptor by IgG and other immune mediators is well-described in mammals (Katz et al., 1992; Mehlhop et al., 1997; Vaz et al., 1970). In support of this contention, we identified the 57.7 kD zebrafish Ig heavy chain band by Western blot using an antibody directed against the human IgE heavy chain (Fig. 4B). Taken together, these data suggest conserved function of MC antibody-receptor responses in zebrafish.

The mammalian FcεRI receptor is heteromeric, composed of alpha, beta and a pair of gamma subunits. The alpha subunit is considered the most critical component, necessary for IgE binding and MC function (Kinet et al., 1991; Riske et al., 1991; Taube et al., 2004). The beta subunit is specific to MCs, but not necessary, rather serving to enhance receptor-mediated signals resulting in the amplified response typical of MC activation (Kraft et al., 2004). Phosphorylation of the gamma subunits by Lyn, a Src family kinase, initiates downstream signaling and ultimately degranulation (Holowka et al., 2007). In the absence of gamma subunits, bound IgE fails to activate MCs effectively (Takai, 1996). We examined 7-day zebrafish embryos and demonstrated co-localization of zebrafish *fcεr1a*, *fcεr1g* and *fcεr1gl* with *cpa5*. *Cpa5* is a protease which shares significant identity with the human carboxypeptidases and which we had identified as specifically labeling zebrafish MCs throughout embryonic development (Dobson et al., 2008). These studies confirmed conservation of FcεRI subunit expression

on zebrafish MCs (Fig. 6A). These findings were further substantiated using FACS to demonstrate that cells expressing *fcεr1a* all displayed mature MC morphology (Fig. 6B).

Following this structural characterization, functional studies invoking classic PSA assays displayed activation of zebrafish intestinal MCs, both by tryptase assay and TEM imaging (Fig. 7). A significant increase in tryptase level was observed for fish treated with anti-DNP IgE/DNP-BSA compared to anti-DNP IgE without BSA antigen ($p=0.04$). The difference in tryptase release between the combined injection of antibody and antigen versus saline and DNP-BSA was less dramatic ($p=0.07$), but may be accounted for by stimulation and cross-reactivity of endogenous fish antibodies to DNP-BSA injection. Confirmation of an antigen specific response was accomplished by performing similar experiments with mismatched anti-TNP IgE and DNP-BSA and observing significantly less tryptase produced compared to either matched anti-DNP and DNP-BSA or anti-TNP IgE and TNP-BSA. Leukotrienes are another mediator frequently released in response to IgE-activated adaptive MC responses (Castells, 2006; Ogawa and Grant, 2007). We attempted to measure the leukotriene, LTC₄, by ELISA following PSA experiments (Olynych et al., 2006). While leukotriene levels were measurable, increases following anti-DNP IgE/DNP exposure were inconsistent (Supplemental Table 1). Histamine is another classic component of MC granules, frequently measured as a marker of MC response (Castells, 2006; Ogawa and Grant, 2007). We also attempted to measure histamine levels both using chromogenic assay and ELISA, but were unsuccessful. In keeping with these findings, to date histamine receptors have only been identified in the CNS of the zebrafish (Choich et al., 2004; Peitsaro et al., 2000) and a recent paper demonstrated that while other teleost fish contain histamine in their MC granules, it is decidedly absent from the MC granules of the *Cyprinidae* family, which includes zebrafish (Mulero et al., 2007).

In summary, we have expanded upon our initial identification and characterization of zebrafish MCs to demonstrate that they share some similar functions in innate and adaptive immune responses to their mammalian counterparts. Structurally, zebrafish MCs possess the prerequisite “molecular apparatus” and functionally, degranulate releasing tryptase following infectious, noxious and allergic stimuli. MC stabilizers effectively abrogate these responses. These results extend the utility of this versatile model system for interrogating vertebrate immune pathways with the potential to serve as an *in vivo* screening tool to identify novel MC modulating agents.

Authorship

S.D. performed research, analyzed data and helped write the paper. E.M.T. performed research and analyzed data. J.T.D. and G.K.N. performed research. E.R.M. analyzed data and provided pathology expertise. H.W. and D.S.N. analyzed data and provided statistical expertise. J.S.M. and T.-J.L. designed research and analyzed data. J.N.B. designed research, analyzed data, and wrote the paper.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2010.09.001.

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