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LEUKOCYTE RECRUITMENT TO THE PERITONEUM OF CULTURED SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) DURING BACTERIAL

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LEUKOCYTE RECRUITMENT TO THE PERITONEUM OF CULTURED SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) DURING BACTERIAL INFECTION

BY

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Abstract

Summer flounder (*Paralichthys dentatus*) constitutes a major fishery in the coastal northeastern United States. Spurred by population declines, cultivation of summer flounder began in 1996, but expansion of culture efforts has been constrained by disease outbreaks. Flounder Infectious Necrotizing Enteritis (FINE) causes significant mortality of juvenile flounder. The disease is characterized by ascites, intestinal prolapse, and severe inflammation of the mesentery and posterior intestinal serosa, often resulting in intestinal rupture. FINE is caused by *Vibrio harveyi,* a bacterial pathogen of cultured marine fish and invertebrates. To better understand the immune response to FINE, recruitment and fluctuation of leukocytes within the summer flounder peritoneum were investigated during intraperitoneal challenge with *V. harveyi*. Consistent with the pathology of FINE, massive infiltration of large granular leukocytes was observed in coelom of flounder 24 hours after intra-peritoneal injection of live bacteria. In these fish, the number of lymphocytes doubled, though the number of B-cells was not significantly different from saline-injected animals. The large influx of granular cells resulted in a 15-fold increase in that population, decreasing the lymphocyte population from ~65% to ~20% of the total coelomic leukocyte pool. It is hypothesized that the influx of large granular leukocytes into the coelom of challenged animals may contribute significantly to the pathology of the disease.

Acknowledgements

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For Bee

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Introduction

The summer flounder (*Paralichthys dentatus*) commercial and recreational fishery has traditionally been a significant one in the northeastern United States (Bengtson 1999). Precipitous declines in wild stocks in the late 1980's and the resulting restrictions placed on the fishery fueled interest in developing the species for aquaculture (Waters 1996) and in 1996 commercial cultivation of flounder began in New Hampshire (Bengtson 1999). Typical conditions of intensive culture can result in infectious disease outbreaks and in the summer of 1998, a severe epizootic in newly transported juvenile flounder occurred at a Rhode Island grow-out facility resulting in significant mortality and severe economic losses to the facility (Soffientino et al. 1999). The etiological agent was identified as *Vibrio carchariae*, later found to be synonymous with *Vibrio harveyi* (Gauger and Gomez-Chiarri 2002), a significant opportunistic bacterial pathogen of a number of cultured marine fish and invertebrates, including grouper, sea bream, red drum, penaeid shrimp, and abalone (Lee et al. 2002; Austin and Zhang 2006).

The disease, named Flounder Infectious Necrotizing Enteritis (FINE) is a severe bacterial disease characterized by profuse inflammation in the peritoneum. Gross clinical signs of FINE include ascites formation resulting in a distended abdomen, cloacal petechiae, and intestinal prolapse that often results in rupture and separation of the posterior intestine from the body wall. Histologically, inflammation is evident on the peritoneal mesentery and serosa of the posterior intestine of susceptible fish. Survivors of FINE are often found to possess a blind sac intestine, presumably a result from the healing over of the intestine ruptured during active

infection (Soffientino et al. 1999, Gauger et al. 2006). While these fish do survive, they do not grow and thrive as intact fish do (Specker et al. 2000), thereby negatively impacting their future market value and potentially causing additional losses to producers.

Early stages of the disease have not yet been well characterized, though transport and temperature stress have been identified as triggers of infection (Soffientino et al. 1999; Gauger 2004). Much of the pathology associated with FINE is in the peritoneum (Soffientino et al. 1999, Gauger et al. 2006), yet the mechanisms leading to intestinal rupture remain unknown. In the peritoneum of mammals, three main cell systems are present; macrophages, lymphocytes, and mesothelium, and all are involved in the early immune response in that body cavity (Sammour et al. 2010). The mesothelium lines the peritoneum and lies between the cells of the cavity and the underlying microvasculature and plays a major role in the recruitment of leukocytes. Typically, primary peritoneal infections originate from the bloodstream during immunocompromise or may be secondarily derived from pathology in a visceral organ or originate from infection of the digestive organs (Sammour et al. 2010). The first phase of the inflammatory response in the peritoneum is characterized by the recruitment of granulocytes which provide machinery for effective elimination of bacteria. During the second phase of inflammation, the peritoneal infiltrate is dominated by macrophages and lymphocytes and exemplifies the transition from the innate to the acquired immune response. At this point in successful clearance of infection, peritoneal damage is repaired and foreign antigens are presented to T lymphocytes (Rapoport et al. 1999; Glik and Douvdevani 2006).

In order to develop effective strategies to prevent and combat future outbreaks of FINE, it is critical to understand what perturbations of the normal response in summer flounder may be occurring and resulting in the pathologies associated with the disease, as well as the elements involved in a successful immune response and clearance of the pathogen. The aim of the present work is to develop an effective tool to study the immune response of summer flounder and illustrate the utility of this tool by characterizing aspects of the immune response in the peritoneum to intraperitoneal challenge by *Vibrio harveyi*. The development of a monoclonal antibody (mAb) against summer flounder immunoglobulin (SFIg) provides a marker for B lymphocyte populations that will help to shed light on basic host immune responses, as well as responses relevant to this disease. A mAb specific for SFIg was generated from mice immunized with immunoglobulin purified from captive summer flounder, as shown by SDS-PAGE, Western blot, protein A co-precipitation, and flow cytometry. We describe how summer flounder peritoneal cell populations fluctuate during challenge with *V. harveyi* and how B lymphocytes contribute to the peritoneal response. These investigations will provide some insight on the nature of critical host responses in the summer flounder peritoneum which will be important for developing strategies, such as vaccines, to manage bacterial infections of summer flounder.

Materials & Methods

Fish

Juvenile summer flounder (*Paralichthys dentatus*) for both mAb production and bacterial challenge were obtained from GreatBay Aquaculture (Portsmouth, NH) and held at the University of Rhode Island's (URI) Blount Aquaculture Laboratory in

Narragansett, RI for several months. All fish were maintained in a flow-through system supplied with filtered seawater pumped in from Narragansett Bay, kept at ambient temperature (14-19°C) and salinity (30-32‰), and fed a commercial diet (Skretting Gemma Diamond 0.8mm, Stavanger, Norway) twice daily. Wild-caught summer flounder, four-spot flounder (*Paralichthys oblongus*), windowpane flounder (*Scophthalmus aquosus*), winter flounder (*Pseudopleuronectes americanus*), yellowtail flounder (*Limanda ferruginea*), all ranging in size between 700-1500g, were sampled during commercial trawling operations off of southeastern Massachusetts and Rhode Island. Blood collection was done as follows: fish (except those sampled aboard fishing vessels) were sedated with 90ppm MS-222 (tricaine methanesulfonate, Sigma) by bath immersion and 0.5-3.0mL blood was drawn from the caudal tail vein with a 3mL syringe and 23ga needle. All fish were maintained and sampled in accordance with approved URI IACUC protocols (AN 10-10-008).

Purification of Summer Flounder Immunoglobulin & Monoclonal Antibody Production

Plasma immunoglobulins from wild-caught summer flounder were purified using size-exclusion chromatography as previously described (Bromage et al. 2004). In brief, 1ml fractions were collected from a sephracryl S300 column (16mm × 1m) after 500µl of whole plasma was added. Fractions containing purified antibody were determined by the presence of putative antibody heavy and light chains during reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Three Balb/c mice were immunized by intraperitoneal injection with 100µg of purified summer flounder Ig (SFIg) emulsified in Freund's complete adjuvant in 100µL PBS on day one. An additional injection using Freund's incomplete adjuvant was administered to the mice 14 days later with booster injections given twice more at one-week intervals. At 90 days, mice were euthanized and a standard 50% polyethylene glycol fusion was performed between mouse splenic plasma cells and SP2/0 myeloma cells. The resulting hybridomas were distributed and cultured in 96-well plates and culture supernatant was screened for production of anti-SFIg by enzyme-linked immunosorbent assay (ELISA). Positive hybridomas were selected and diluted into 24-well plates where they were re-cultured. This selection process was repeated until only single clones were contained in the wells. Cell culture of specific hybridomas was expanded and the supernatant was collected and kept frozen at -20°C. The cultured cells were kept frozen at -80^{o}C for storage.

Binding specificity of mAbs against summer flounder IgM

Binding specificity of the mAbs against summer flounder IgM were analyzed using reducing SDS-PAGE and Western blot. Purified SFIg (1µg), whole summer flounder plasma (1:100, 5µl), and trout immunoglobulin (positive control, 5µg) were run on a 12% SDS-PAGE gel under reducing conditions (Harlow and Lane 1999) and transferred to a nitrocellulose membrane to be probed by candidate mAbs 6C11 and 10C11. Binding specificity to SFIg was further investigated by Protein A coprecipitation assay. The mAb was incubated in a 10mL chromatography column containing 6mL Protein A beads and MAPS® binding buffer (BioRad). 1mL whole

biotinylated summer flounder plasma was later added with 4mls phosphate buffered saline (PBS) + 0.02% azide and incubated for 1hr. After washing with PBS azide, the column was eluted by the addition of 0.1M Glycine pH2.5 and 1ml aliquots containing eluted protein complexes were collected and analyzed by SDS-PAGE.

In order to determine whether the anti-SFIg mAb generated for summer flounder cross-reacted with closely related fish species, plasma from winter flounder, yellowtail flounder, windowpane flounder, and four-spot flounder was collected as described above for assay by Western blot using summer flounder plasma as a positive control. Plasma was run on SDS-PAGE as described previously and transferred to a membrane. Blots were then probed with 10C11 anti-SFIg mAb.

Ig+ lymphocytes were identified in peripheral blood leukocyte samples from healthy summer flounder as follows. Whole blood samples (1-3mL) were centrifuged at 500g x 5min and the buffy coat containing leukocytes was drawn off and put into 5mL heparinized HyClone. Leukocytes were then separated using a Histopaque 1077 (Sigma-Aldrich) gradient. Cell suspensions were underlaid with 2mL Histopaque 1077 and centrifuged at 500g x 40min. The leukocytes were then put into 4ml non-sterile RPMI-1640 and centrifuged at 500g for 10min. The supernatant was disposed of and the remaining cells were washed twice with 3mL non-sterile RPMI at 500g at 5min, once with 3mL FACS buffer (Hank's Balanced salt solution + 3% fetal bovine serum + 0.02% sodium azide) at 500g x 5min and then resuspended with 2mL FACS buffer. Leukocyte preparations were then incubated with 5mg/mL anti-SFIg mAb (10C11) diluted in FACS buffer, washed three times, then subsequently incubated with 1:2000

goat anti-mouse RPE, washed three times, resuspended and analyzed by flow cytometry in order to identify Ig+ lymphocytes.

Flow Cytometry

The flow cytometry analyses were done on a FACSArray flow cytometer (Becton Dickinson). Cell preparations for analysis were as described above for leukocyte isolation. Cyflogic software (CyFlo Ltd.) was used for further analysis. Ratios are presented as the means of the percentages of the total cells analyzed ±SD from four fish per treatment per time point.

Vibrio harveyi Challenge

Thirty-six fish averaging 27.3±2.49cm were randomly distributed to each of 6 20L aquaria, comprising duplicate aquaria for each of the experimental treatments (n = 4 fish per treatment per time point) and control fish were maintained under the same conditions, but sampled separately. All fish were fasted during the challenge. Bacterial solutions of *Vibrio harveyi* strain DN01 (Soffientino et al. 1999) were prepared for challenge by growing cultures in Luria Bertani 20 (LB20) media overnight at room temperature, collecting the cells by centrifugation (3000×g, 5min), and washing 3× in filtered sterile seawater (FSSW). Concentrations of all bacterial suspensions were determined by measuring optical density (OD) at 490nm and comparing values to a standard curve showing the relationship between OD_{490} and colony forming units (CFU) of *V. harveyi* (Gauger et al. 2006). Heat-killed bacteria preparations were obtained by the additional step of placing bacterial cell suspensions in a 70°C water bath for 45min. Fish were anesthetized in MS-222 (Sigma) and inoculated by intraperitoneal (IP) injection with $5x10^7$ CFU of live or killed *V. harveyi* per fish in

100µL FSSW (challenged groups) or FSSW alone (negative control). Fish were sampled for isolation of peritoneal leukocytes prior to challenge, as well as at 1, 4, and 24hr post-challenge.

Isolation of Peritoneal Leukocytes

Sample collection. Summer flounder were euthanized in MS-222 (Sigma) and the abdomen was disinfected with 70% ethanol. The posterior aspect of the peritoneal cavity was then injected with 4mL of cold RPMI-1640 (Sigma). After gently massaging the abdominal surface for several minutes, the medium containing leukocytes was harvested from the peritoneum with a syringe. To obtain the maximum number of cells, the peritoneal lavage was repeated twice and then a plastic Pasteur pipette was inserted into a peritoneal incision aseptically to collect any remaining fluid from the cavity. Peritoneal leukocytes were then centrifuged at 1500rpm for 8min, washed and resuspended with RPMI-1640, and kept cold until separation.

Leukocyte isolation. Peritoneal leukocytes were obtained using a Histopaque 1077 (Sigma-Aldrich) gradient and were subsequently prepared for analysis by flow cytometry as described above for leukocytes from peripheral blood. Cell counts were obtained by adding 2% Trypan Blue to resuspended peritoneal leukocyte samples and then counting the cells on a hemacytometer.

Results

α-SFIg 10C11 binds to summer flounder Ig Heavy Chain

Purity of isolated summer flounder immunoglobulin was confirmed by the presence of distinct bands on SDS-PAGE corresponding to expected weights of immunoglobulin heavy- and light-chain subunits, approximately 70kDa and 20kDa (Fig. 1). Western blotting using the 10C11 clone as a probe confirms the specificity of this mAb to the heavy-chain subunit of SFIg as indicated by the presence of 70kDa bands in the summer flounder samples, but not in the trout control (Fig. 1). Furthermore, anti-SFIg 10C11 bound to protein A captured immunoglobulins in the summer flounder plasma, as demonstrated by the presence of proteins with molecular weights corresponding to both the heavy (70kDa) and light (20kDa) chain subunits when the eluted proteins from the protein A were run under reducing SDS-PAGE. In an effort to determine if the mAb would cross-react with immunoglobulins of closely related fish species, additional Western blots were performed against the plasma of winter flounder, yellowtail flounder, windowpane flounder, and four-spot flounder plasma, using summer flounder plasma as a positive control. The mAb 10C11 reacted solely with the summer flounder plasma, further indicating its' specificity (Fig. 2).

Summer flounder peripheral blood leukocyte samples incubated with the 10C11 SFIg mAb were analyzed by flow cytometry. The resulting scatter plot was consistent with typical plots of leukocyte populations (granulocytes and lymphocytes, Fig. 3). Fine analysis of lymphocyte subpopulations using the anti-SFIg mAb enabled the separation of Ig+ and Ig-lymphocytes, that is, presumptive B cells and T cells, respectively (Fig. 3).

Leukocyte recruitment to the peritoneum during V. harveyi challenge

At the conclusion of the bacterial challenge, there was 100% survival of flounder in all treatment groups and no gross pathology associated with FINE was evident in any of the challenged fish. However, analysis of peritoneal leukocyte samples by flow cytometry allowed the visualization and characterization of shifts in the ratios of the cell types during intraperitoneal challenge by both live and killed *V. harveyi*. The ratio of lymphocytes to total cells present in the peritoneum decreased significantly in fish injected with either live or killed Vh (Table 1, Fig. 3). For granular cells, the ratio to total cells present increased significantly for the same groups. For Ig+ cells, the ratio of Ig+ B lymphocytes to total cells present also decreased significantly in fish injected with either live or killed Vh. However, the ratio of large Ig+ cells to total cells for all groups did not differ significantly from one another (Table 1, Fig. 3).

Cell count data showed a significant increase in the number of total peritoneal leukocytes at 24hr post-challenge of 4.5-fold and 9-fold in killed Vh-injected fish and live Vh-injected fish, respectively, over control fish ($p \leq 0.0005$, Table 1, Fig. 3), while FSSW-injected showed a slight, non-significant increase. Total numbers of lymphocytes in the three injected treatment groups all increased, with killed Vh-injected fish and live Vh-injected fish lymphocyte numbers doubling over the control fish ($p \leq 0.0005$, Fig. 4). The number of lymphocytes in killed and live bacteria-injected fish did not differ significantly from one another. Granular cells in FSSW-injected fish, killed Vh-injected fish and live Vh-injected fish all increased as well, but while

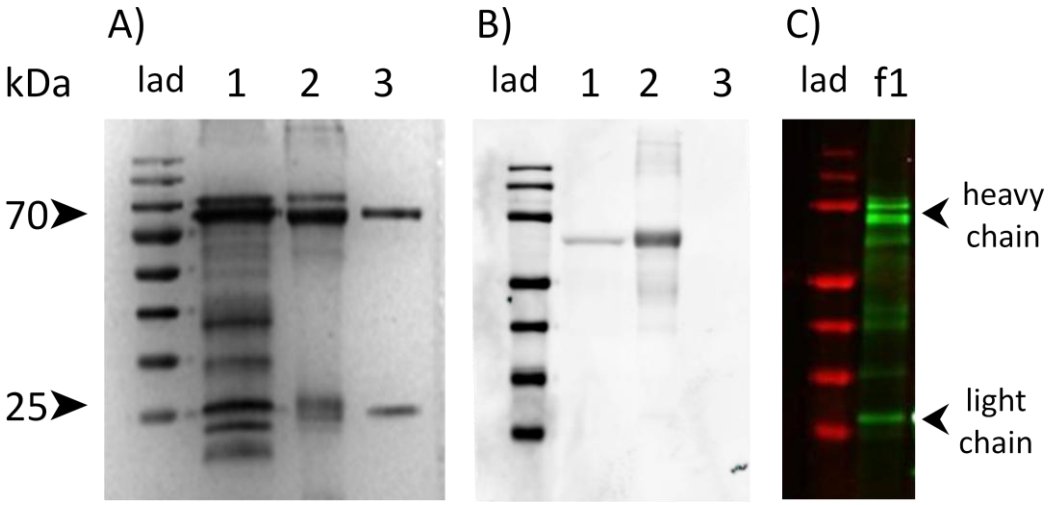

Figure 1. ***Mab 10C11 is specific to heavy chain of summer flounder Ig.*** A) 12% SDS-PAGE reducing gel, total protein visualized by silver staining, B) Western blot, C) Protein A co-precipitation gel. Lane 1, summer flounder (*P. dentatus*) plasma, lane 2, *P. dentatus* purified Ig, lane 3, positive control, rainbow trout (*O. mykiss*), lane f1 – eluted fraction of biotinylated *P. dentatus* plasma.

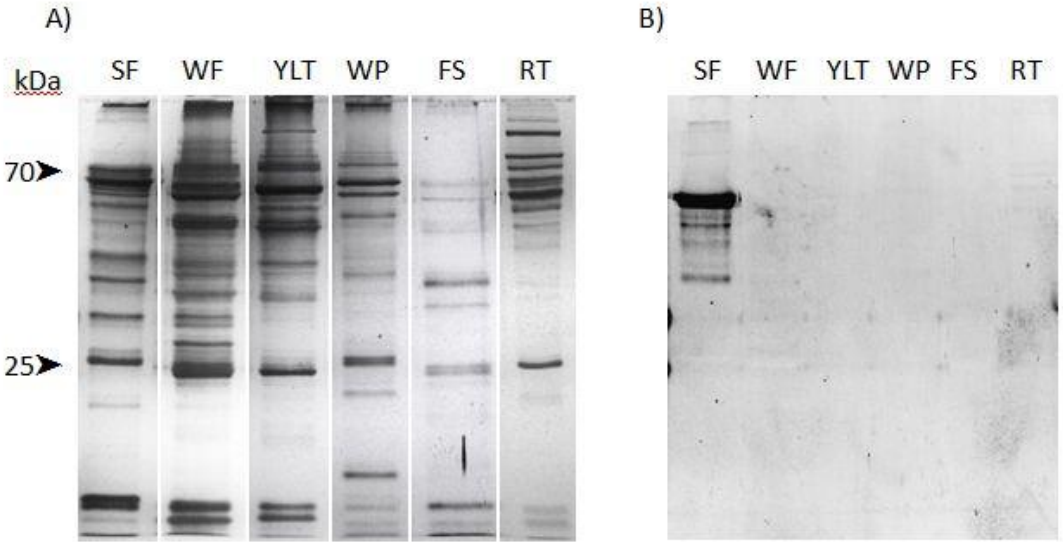

Figure 2. *Mab 10C11 is specific to summer flounder.* A) 12% SDS-PAGE reducing gel of plasma proteins showing strong bands corresponding to Ig, B) Western blot with 10C11 reveals binding only to the heavy chain subunit of summer flounder Ig. SF, summer flounder (*P. dentatus*), WF, winter flounder (*P. americanus*), YLT, yellowtail flounder (*L. ferruginea*), WP, windowpane flounder (*S. aquosus*), FS, fourspot flounder (*H. oblonga*), RT, negative control, rainbow trout (*O. mykiss*).

the FSSW-injected fish cells doubled over the control fish, the killed Vh-injected fish and live Vh-injected fish increased by 16-fold and 30-fold, respectively (Fig. 4). For the Ig+ B cell population, numbers of cells in the three injected treatment groups all increased to nearly double the control fish, but none of them differed significantly from one another (Fig. 5). Lastly, the population of large Ig+ cells increased in FSSW-injected fish 1.5-fold over control fish, but these cells in killed Vh-injected fish and live Vh-injected fish increased by 5-fold and 8-fold, respectively (Fig. 5).

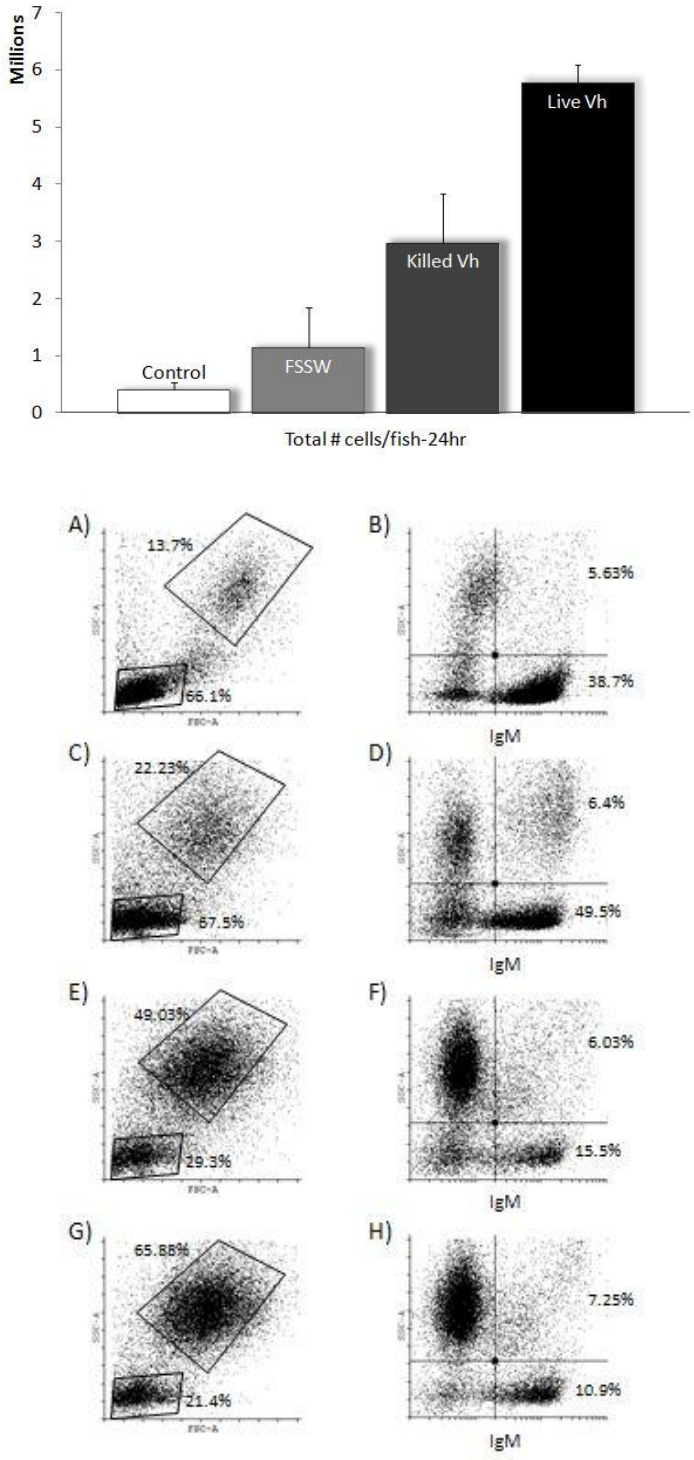

Figure 3. *Effect of bacterial challenge on leukocyte cell populations in the peritoneum of summer flounder*. Plotting cell size (FSC) against cell complexity (SSC) (A, C, E, G) and fluorescence (IgM+/-) against SSC (B, D, F, H) illustrated changes in the ratio of IgM+ B cells (B cells, R bottom quadrant) and large granular IgM+ cells (R upper quadrant) to total cells. (A, B) control, (C, D) FSSW, (E, F) heat-killed *V. harveyi*, (G, H) live *V. harveyi.* Percentages represent mean ratios of subpopulation to total cells.

Treatment	Hour	% Cells of Total (±SD, n=4)				Cell Counts @ 0 and 24hr (cells/fish, n=4)				
		Lymphocytes	Granular cells	IgM+ B cells	Large IgM+ cells	**Total Cells**	Lymphocytes	Granular cells	IgM+ B cells	Large IgM+ cells
Control	0	**66.10 ± 2.74**	**13.73 ± 3.2**	**38.73 ± 2.65**	**5.63 ± 0.67**	377,025 ±115,117	249,214 ±76,092	51,766 ±15,806	146,022 ±44,585	21,227 ±6,481
	1	61.50±11.38	14.7 ± 3.47	37.08±14.06	3.03 ± 1.32					
	4	66.33 ± 9.12	17.38 ± 8.35	41.9 ± 14.35	3.55 ± 3.63					
FSSW	**24**	**67.53±15.01**	**22.23 ± 11.26**	**49.5 ± 12.31**	**6.4 ± 5.17**	1,046,681 ±839,221	706,824 ±566,726	232,677 ±186,559	518,107 ±415,414	66,988 ±53,710
	1	67.73±19.27	12.53 ± 6.47	42.23±21.67	3.33 ± 1.96					
	4	67.80 ± 10.2	19.83 ± 6.1	52.83±16.15	3.93 ± 3.31					
Heat-killed *V. harveyi*	**24**	**29.28±9.68***	**49.03±20.08***	**15.53 ± 6.1***	**6.03±2.98****	2,712,150 ±941,213	794,118 ±275,587	1,329,767 ± 461,477	421,197 ±146,170	163,543 ±56,755
	1	70.05 ± 7.89	16.25 ± 6.63	58.05 ± 7.91	7.65 ± 4.42					
	4	60.58 ± 8.93	17.23 ± 3.26	40.05±12.47	2.9 ± 1.67					
Live *V. harveyi*	**24**	**21.43±9.66***	**65.88±12.08***	**10.9 ± 5.12***	**7.25±1.97****	5,062,500 ±618,718	1,084,894 ±132,591	3,335,175 ±407,612	551,813 ±67,440	367,031 ±44,857

Table 1. ***Leukocytes in the peritoneum of summer flounder after challenge with V. harveyi.*** Data displayed as percentages of each subpopulation to total number of cells in the sample +/- SD, as determined by flow cytometry (Fig. 3). Cell counts (cells/mL ± SD) at 0 and 24hr were derived from the percentages of the total cell counts. *=significant difference from hour 1, **=significant difference from hour 4.

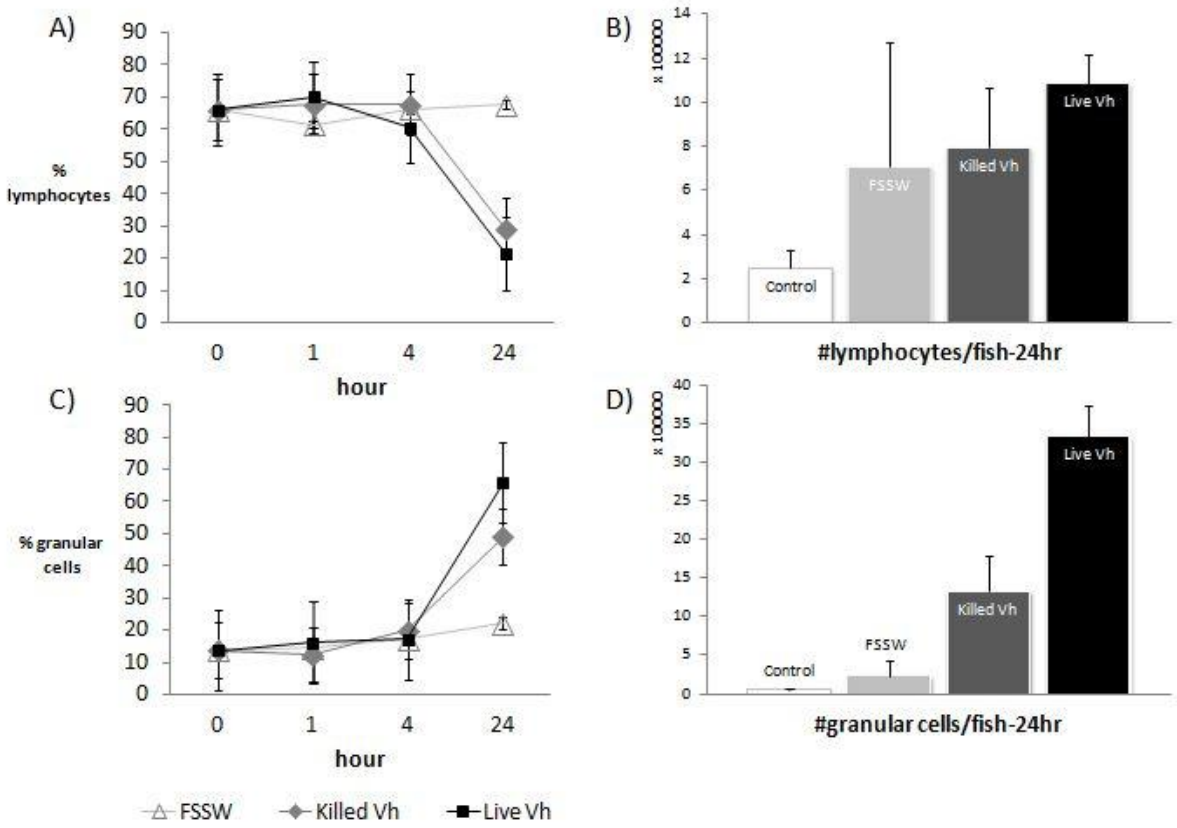

Figure 4. *Effect of bacterial challenge on lymphocytes & granular cell populations in the peritoneum of summer flounder*. A, C) Percentages (± SD) of peritoneal leukocytes (A. lymphocytes, C. granular cells) to total cell samples in bacteria-challenged summer flounder as determined by flow cytometry at 0, 1, 4, and 24hr post-challenge. B, D) Numbers of peritoneal leukocytes at 24hr (B. lymphocytes, D. granular cells) in those samples as calculated from above percentages and total cell counts. While the ratio of lymphocytes to total cells decreased significantly in Killed Vh-injected and Live Vh-injected flounder at 24hr, the absolute numbers of cells actually increased. This can be explained by the massive increase in the ratio of granular cells to total cells in those treatment groups, as well as the corresponding increase in number of granular cells (16-fold and 30-fold, respectively).

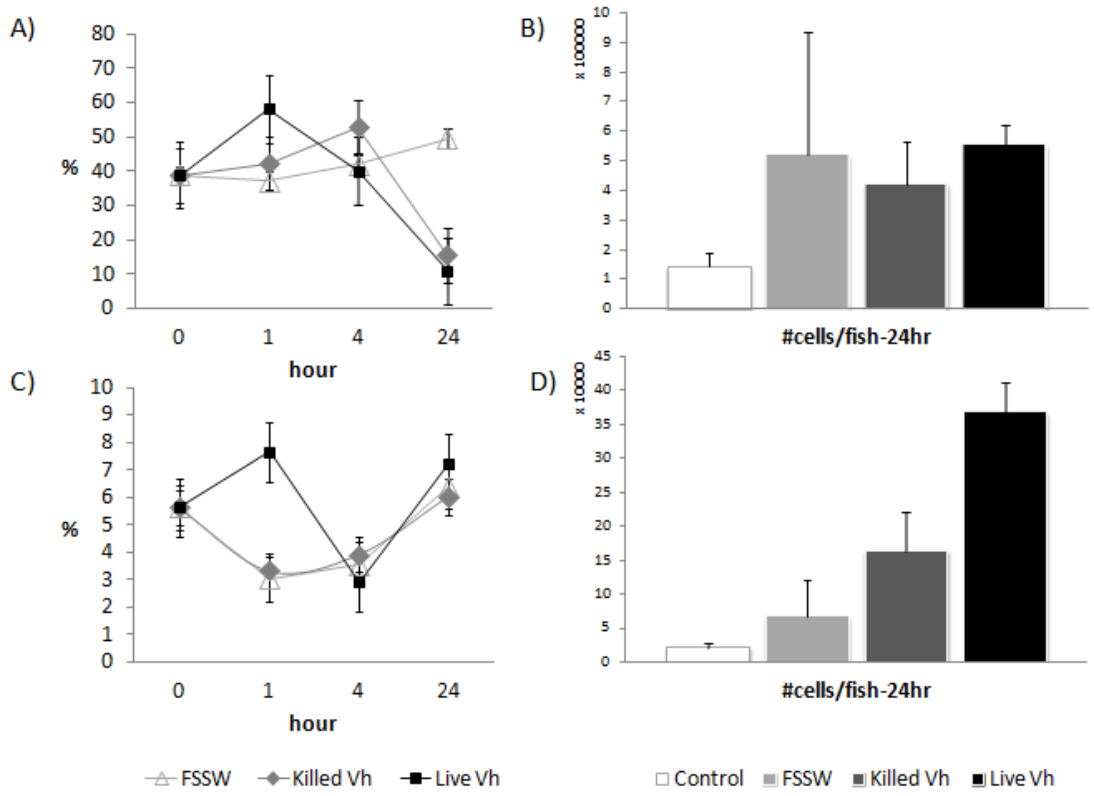

Figure 5. *Effect of bacterial challenge on Ig+ peritoneal lymphocytes & granular cell populations in the peritoneum of summer flounder*. A, C) Percentage of peritoneal leukocytes expressing Ig (A. Ig+ B cells, C. large, granular Ig+ cells) relative to total cell samples in bacteria-challenged summer flounder at 0, 1, 4, and 24hr post-injection. Cell samples were incubated with α-summer flounder Ig Mab 10C11 prior to analysis by flow cytometry. B, D) Numbers of peritoneal leukocytes at 24hr (B. Ig+ B cells, D. large, granular Ig+ cells) in those samples as calculated from above percentages and total cell counts

Discussion

The dearth of specific markers for the analysis of immune function in teleost fish places some limitations on the characterization of immunological processes for this vertebrate group (Randelli et al. 2008), particularly for non-model species such as summer flounder. In this study, we successfully developed a marker for summer flounder immunoglobulin to evaluate immune responses in this species and utilized this tool to characterize impact of intraperitoneal challenge with an important bacterial pathogen, *V. harveyi*. We found that intraperitoneal challenge with *V. harveyi* stimulated a large influx of granulocytes to the peritoneum, as well as a significant increase in the presence of large, granular Ig+ cells within the peritoneal cavity. To our knowledge, this is one of the first studies characterizing the peritoneal leukocyte population in response to a bacterial challenge and the disease FINE, characterized by acute peritonitis, provides a relevant model in which to examine this process in fish. Peritoneal cells have been sourced extensively for the study of leukocytes, particularly macrophages, and the study of inflammation in many vertebrate species (Meseguer et al. 1993; Afonso et al. 1997; Watanabe et al. 1997; Moss et al. 2009; Tumbol et al. 2009). The composition of resident peritoneal cells has been studied in several teleosts, but the classification of leukocytes has been hampered by the lack of specific markers to identify cell subsets, and variable methodologies make comparison between studies challenging. Previous work has focused generally on assaying phagocytic activity of peritoneal macrophages in response to immune challenge (Afonso et al. 1998; Moss et al. 2009) or on evaluating differential counts of resident peritoneal cells in unstimulated fish (Meseguer et al. 1993; Watanabe et al. 1997; Afonso et al. 1998; Tumbol et al. 2009). These studies reveal differences in the

resident peritoneal cell populations between fish species. For example, in sea bass, the most abundant cell type in the peritoneum of unstimulated fish was found to be macrophages, in contrast to sea bream (Meseguer et al. 1993) and zebrafish (Moss et al. 2009), where granulocytes dominate. In unstimulated rainbow trout, lymphocytes were found to be the dominant peritoneal cell type (Afonso et al. 1997). In the present study, we also found lymphocytes to be the dominant cell type in unstimulated, control fish. Understanding the composition of cell types present in healthy, unstimulated fish helps to provide a baseline from which to both examine what deviations occur during an active immune response to specific disease states as well as provide a basis for comparison among and between fish species and other vertebrates.

In our study, intraperitoneal injection of killed and live *V. harveyi* cells led to a significant change in the composition of peritoneal leukocyte populations and a dominance of granulocytes. As expected during a bacterial invasion, there was a massive increase in the number of cells to the site of insult. The ratio of lymphocytes present drastically decreased at 24hr as a result of the massive infiltration of granular cells, shifting the ratio to favor the granulocyte population. Even though the ratio of lymphocytes to total cells present in the peritoneum decreased significantly, there was still an increase in absolute numbers of lymphocytes, indicating that these cells were being recruited to the peritoneum in challenged fish. An alternative explanation for the total increase in leukocytes in the peritoneum during bacterial challenge is an increase in lymphocyte proliferation. However, this is unlikely, considering the timing of the increase (4 – 24 h).

The mechanism of cell recruitment to the peritoneum is not very well described for teleost fish. Much of what is currently understood about trafficking and recruitment of cells to the peritoneal cavity is described in numerous studies in the human and murine literature (Rapoport et al. 1999; Fagarasan et al. 2000; Thiriot et al. 2008; Sammour et al. 2010). This remains a significant gap in our understanding of teleost peritoneal immunology, considering that there are several major diseases in fish, such as bacterial septicemias caused by Gram-negative organisms like *Aeromonas* sp., which result in peritoneal inflammation, making investigations into these processes relevant. The fact that peritoneal inflammation, the hallmark of FINE, can be consistently replicated by intraperitoneal challenge with *V. harveyi* in summer flounder, indicates that this bacterial challenge is a useful and relevant model for examining immune cell recruitment to the peritoneum of teleosts. In our experiment, no animals succumbed to the infection, so our results may represent leukocyte population fluctuations that are associated with successful clearance of infection from the peritoneum.

Differences in the magnitude of change of leukocyte populations in response to the different treatments (FSSW, killed, and live *V. harveyi*) suggested differences in recruitment dynamics between the different cell populations. The slight, but not significant, increase in lymphocytes in the FSSW-injected fish, suggested a mild response to injection, likely triggered by tissue injury and/or introduction of microbes. There was no difference between lymphocyte numbers in the peritoneum between killed and live bacteria-injected fish, suggesting that lymphocyte recruitment was stimulated in response to antigens derived from both killed and live bacteria. In

contrast, granular cells, while significantly increased in killed Vh-injected fish, increased even more significantly in live Vh-injected fish, suggesting an active response to factors present in live, not dead, bacteria, secreted virulence factors and/or signals of cell death. This makes inherent sense because granular cells are important for a fast initial response to counter active microbial infection, so a quick, robust response to live pathogenic organisms would be critical, whereas lymphocytes are responsible for a slower, adaptive immune response and recognition and processing of microbial antigens, whether derived from live or dead organisms, would be more essential in order to develop long-term memory for future immune protection.

The marker developed in this study, an anti-SFIg mAb, allowed the identification of Ig+ B cells and the characterization of their population fluctuations in the summer flounder peritoneum. We were particularly interested in exploring the role B cells play in the peritoneum during early response to bacterial intraperitoneal challenge. Recent research has demonstrated functional aspects of IgM^+ B lymphocytes in teleost fish, amphibians, and reptiles that are important for the innate immune response, whereby subsets of B cells in rainbow trout, Atlantic cod, Atlantic salmon, *Xenopus laevis*, and red-eared sliders have been shown to behave as professional phagocytes possessing the ability to phagocytose particles and kill internalized bacteria (Li et al. 2006; Overland et al. 2010; Zimmerman et al. 2010). Other B cell functions relevant for the innate immune response have been identified in mice. IFN-α-producing murine B cells were found to be capable of triggering IFN-γ response by NK cells, and by doing so, were participating in the early innate response (Bao et al. 2011) and an additional function has been demonstrated where B cell

subsets in mice identified as Innate Response Activator (IRA) B cells produce granulocyte-macrophage colony-stimulating factor (GM-CSF), which possesses a known role in the activation of innate leukocytes (Rauch et al. 2012). It is clear that B cells may possess similar functions in teleosts, as well as functions yet undescribed for teleost fish that are relevant for the innate immune response.

Summer flounder Ig+ B cells in the peritoneum, not surprisingly, followed closely the pattern of lymphocytes in all of the experimental groups except that the numbers did not rise as sharply as granulocytes and there was no real difference between numbers of lymphocytes between fish injected with either killed or live bacteria. More interesting is the finding that the absolute numbers of large Ig+ cells increases dramatically in response to bacterial challenge. The overall percentage of these cells in the peritoneum was small in all groups studied and there was some variability in the data in early time points. However, though the earliest time points were unresolved, there was a significant difference observed in this population for all injected groups between 4 and 24hr, indicating the response was real. When extrapolating cell population numbers from those ratios, we observe a large increase of large, granular Ig+ cells in the bacteria-injected fish, with those of the live Vh-injected fish nearly doubling those of the killed Vh-injected fish. This suggests a real response of large Ig+ cells to the presence of bacteria in the peritoneum and may signify any number of conclusions. One, it is possible that these cells represent a subset of B cells undergoing activation; two, they may represent a separate B cell subset recruited to the peritoneum as a result of the presence of bacteria; or three, they may be granulocytes coated with IgM. Based on their large size and granularity profile, as well as how their

population numbers fluctuated in a pattern very similar to that of the granulocytes in this challenge, it is likely that these cells fall in the third category. In any instance, this novel cell subset deserves further attention in the characterization of the relevant cellular responses for successful clearance of microbial invasion of the peritoneum in flounder.

The anti-SFIg mAb 10C11, by specifically binding Ig from summer flounder in both serum or on the surface of antibody producing cells, provides a very useful tool for the analysis of a range of innate and acquired immune functions in summer flounder. Our future goal is to use this antibody to assay phagocytic activity of summer flounder peritoneal cells, particularly Ig+ B cells, during *V. harveyi* infection, investigate potential peritoneal immune factors responsible for tissue destruction of posterior intestine, as well as examine further aspects of the ecology and pathology of FINE. It is critical to analyze the response in the peritoneum, the major site where pathology is seen. This report describes the first description of peritoneal cell populations in summer flounder and by further exploring peritoneal immunology, we hope to identify parameters critical for immune defenses against bacterial infections of the gut and peritoneum, thereby providing key information for the development of effective fish health management strategies, such vaccines and immunostimulants.

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