Development of an enzyme linked immunosorbent assay (ELISA) for the detection of Zebrafish Interleukin 1 beta upregulation

by
Samuel Cook

A THESIS

submitted to
Oregon State University
Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Biology
(Honors Scholar)

Presented November 28, 2017
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AN ABSTRACT OF THE THESIS OF

Samuel Cook for the degree of Honors Baccalaureate of Science in Biology presented on November 28, 2017. Title: Development of an enzyme linked immunosorbent assay (ELISA) for the detection of Zebrafish Interleukin 1 beta upregulation

Abstract approved:_____________________________________________________

Justin Sanders

The study and utilization of Danio rerio (Zebrafish) as a model organism has increased greatly in recent decades. They are used in many areas of biological research, but one field that has used them extensively is immunology. Since zebrafish have been found to possess many orthologs to human genes, study of the relationship between gene transcription and protein production has been a key area of interest. One gene and protein of particular interest to the field of immunology is interleukin-1β (IL1β), a pro-inflammatory cytokine that is produced in an inactive pro-form before being post-translationally modified and excreted into its active form. While IL1β is a well-studied cytokine, research into its expression and regulation at the protein level is still growing. One reason for this is a lack of reagents and straightforward assays that can be used to detect changes in protein production with precision. The body of zebrafish research would benefit greatly from a sensitive assay based on monoclonal antibodies. In this paper, the generation and characterization of two monoclonal antibodies reactive against zebrafish IL-1β is reported. These were used in conjunction to develop an IL1β-specific sandwich ELISA assay
system to detect native IL-1β produced by bacteria-stimulated zebrafish leucocytes and individually to develop a competitive ELISA for the same purpose.

Key Words: Zebrafish, cytokine, ELISA, Interleukin, IL1β, qPCR

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

____________________________________
Samuel Cook, Author
Introduction

The study and utilization of *Danio rerio* (Zebrafish) as a model organism has increased greatly in recent decades. They have proven to be an indispensable model for the study of neurobiology, developmental biology, and genetics in humans. Their use as a model for human disease is also growing in the fields of behavioral sciences, toxicology, and immunology (Sullivan & Kim, 2008). In 2016 the Zebrafish Information Network recorded that there were over 7500 researchers working with zebrafish and that there were over 28,000 academic publications using zebrafish between 1998 and 2016 (ZFIN, 2017). Nearly a third of those were published after 2013.

Zebrafish have become a key biological model because of their many distinct characteristics. These include their resilience, high number of offspring, ease of husbandry, and our extensive knowledge of their genome (Sanders, 2014). While most of these characteristics are also true of other model organisms, such as mice and fruit flies, zebrafish can be easier to work with because of their size, about an inch long. At this size, they are large enough to easily be dissected under a normal dissecting scope, individual organs are easy to visualize and extract, and entire cross-sections of the animal can be prepped and fixed onto a microscope slide, something that is not possible with mice. Also, unlike fruit flies, zebrafish are vertebrates, making them much closer to humans evolutionarily and physiologically.

While knowledge of the zebrafish genome is still growing, there is already an expansive database of known zebrafish genes and well-annotated genetic data. The Zebrafish Information Network (ZFIN) maintains a database with much of this information and in 2016 had over 36,000 recorded gene records (ZFIN, 2017). Many genes for immune proteins, or at least many components of them, are conserved among various vertebrates, even ones as different as humans...
and zebrafish (Zou and Secombes, 2016). The ZFIN has records of over 16,000 genes with recorded human orthologs, many of which are related to the immune system (ZIFN, 2017). These human orthologs are what make zebrafish so useful; They allow researchers to run tests with zebrafish and examine how various bacteria and viruses effect the regulation of these genes that have human orthologs. If we can reliably predict how a gene is regulated in zebrafish, we should be able to predict how its human ortholog will be regulated under similar conditions. Researchers are also able to observe how these changes in gene regulation correlate with changes in protein production and pathology of infections.

All vertebrates have an immune system, a way of preventing illness and responding to toxins and pathogens. This response is accomplished using a network of specific proteins that are expressed or activated in response to foreign compounds. They can identify the foreign compounds and either destroy them or signal the body to mount some sort of response, such as inflammation. These foreign compounds can be viruses, bacteria, or chemicals created by other organisms. A common inflammatory response in humans that many people are familiar with is nasal congestion due to pollen. The pollen activates receptors that trigger a chain reaction of proteins to mount an immune response, in this case, inflammation in the sinuses. These reactions can be measured at both the genetic (transcription) level and the protein (translation) level, since the genes that encode the proteins must first be upregulated so that more copies of the protein can be produced by the lymphocytes.

To observe immune responses at the level of transcription, a technique called quantitative polymerase chain reaction, or qPCR can be used to detect increases or decreases in the number of copies of a gene of interest relative to a gene that is known to be produced at a constant rate under varying conditions. This control gene is often called a housekeeping gene. To measure
protein expression, there are two common techniques used, western blotting and enzyme-linked immunosorbent assays, or ELISAs. Both techniques require antibodies that specifically recognize and bind to the protein of interest. Researchers will often develop what is called a monoclonal antibody, one that is produced by chimeric cells called hybridomas that are all clonal, or identical (Milstein & Köhler, 1975). They are produced by repeatedly injecting one animal (often a mouse or guinea pig) with an antigen of interest (ex: zebrafish IL1β), allowing the host animal to produce antibodies to that antigen, then harvesting the spleen of the host. The B cells from the spleen are then fused with myeloma cells so that they will be able to divide and survive continuously in cell culture media. These cells can then be diluted and plated so that colonies of individual clones can be grown and screened for sensitivity and specificity to the desired antigen. Once purified, the antibodies can be used for the assays named above.

One common class of immune proteins are small, secreted proteins called cytokines. Cytokines are a large group that can be either pro- or anti-inflammatory, and can be part of adaptive or innate immune responses (Zou and Secombes, 2016). Cytokines are generated by certain leukocytes and lymphocytes, which drive particular types of immune responses. For example, interleukins are made by one leukocyte and act on another leukocyte (Zhang and An, 2007). One important interleukin is interleukin-1β, or IL1β, it is produced mainly by macrophages and monocytes and is involved in the upregulation of inflammatory responses. In humans, IL1β has also been shown to be involved in pathological pain, such as upregulation in response to damage to peripheral nerves or cells in the central nervous system (Zhang and An, 2007). In in vivo tests with zebrafish, tissue injury has been shown to illicit IL1β upregulation as well (Ogryzko et al., 2014) as determined by measuring changes in the transcript levels of mRNA encoding IL-1B. Similar to mammals, zebrafish have been shown to produce a pro-form
of IL1β that is ~30.5 kDa and is activated by an inflammatory caspase resulting in an 18kDa active form (Vojtech et al 2012).

While IL1β is a well-studied cytokine, research into its expression and regulation at the protein level is still growing. One reason for this is a lack of reagents and established assays that can be used to detect changes in protein production with precision. The body of zebrafish research would benefit greatly from a sensitive assay based on monoclonal antibodies. In this paper, the generation and characterization of two monoclonal antibodies reactive against zebrafish IL-1β is reported. These were used in conjunction to develop an IL-1β-specific sandwich ELISA assay system to detect native IL-1β produced by bacteria-stimulated zebrafish leucocytes and individually to develop a competitive ELISA for the same purpose.

**Materials and methods**

**Zebrafish living conditions**

Zebrafish (casper-line) were housed in a flow-through system which used municipal water that was dechlorinated and filtered via charcoal filtration. Fish were kept at 27°C and subjected to a 14/10 hour light/dark photoperiod. Water was maintained at a pH of 7.0-7.4, alkalinity of 80 ppm, hardness of 75 ppm, and a conductivity of 135 ls. Fish were fed once daily. All animal handling procedures were approved by the Oregon State University Institutional Animal Care and Use Committee.
**Zebrafish tissue extraction**

Zebrafish were euthanized via submersion in ice water to induce fatal hypothermic shock in accordance with Oregon State University Institutional Animal Care and Use Committee approved procedure. They remained in ice water for at least 10 minutes after last movement was seen. Once euthanized, the kidney, intestines, and liver were removed and placed in microfuge tubes with 500 μL of RPMI cell culture medium with antibiotics. For this experiment, liver and kidney samples were placed in the same tube. Once enough tissue samples had been collected, 20-30 fish, the tissue/ RPMI mix was homogenized using vigorous pipetting with gradually smaller pipettes to dissociate the tissues and release the lymphocytes.

**Fish tissue sample cell density quantification**

The cell density of each homogenized sample was determined by counting with a hemocytometer. Briefly, once homogenized and dissociated enough to be pipetted, 10 μL of each tissue mixture was removed, mixed with 10 μL of trypan blue to determine cell viability. Ten μL of this mixture was placed on a hemocytometer and under a phase-contrast microscope. Once a known area of the hemocytometer was counted, the total cells/ml were calculated.

**Incubation of cells with bacteria**

To determine the effect of bacterial exposure on immune cells, 90 μL of the intestine cell mixture, and 75 μL of liver/kidney cell mixture was placed into a 24 well plate. Added to each was 10 μL of one of three treatments: Physiological saline (negative control), *Edwardsiella ictaluri* suspension (160.33 CFU/mL), or *Mycobacterium chelonae* suspension (391 CFU/mL). The bacterial inoculum consisted of a 0.5 McFarland dilution made from an overnight (*E. ictaluri*) or a one-week (*M. chelonae*) broth culture. The wells were then filled to a final volume
of 500 μL with RPMI cell culture medium and allowed to incubate at room temperature for 2, 4, or 6-hour collection times. Cells were pelleted and both the supernatant (containing secreted proteins) and pelleted cells were frozen. The intracellular components of the samples are labeled as ‘pellet’ and the extracellular ones as ‘supe’ in future sections.

**Bacterial cell density quantification**

In order to determine quantity of the bacterial inocula, serial dilutions from cultures of *Edwardsiella ictaluri* (*E. ictaluri*) and *Mycobacterium chelonae* (*M. chelonae*) were made and 100 μL of each dilution was spread onto petri dishes of tryptic soy agar (TSA) and 7H10 media respectively. The plates were allowed to incubate at 25 °C until the most diluted plate had enough colonies to count, 7 days for the *E. ictaluri* and 14 days for *M. chelonae*.

**RNA isolation and cDNA synthesis**

RNA was extracted from zebrafish cells that had been treated as described above. The NucleoSpin RNA Extraction kit (Macherey-Nagel, Bethlehem, PA) was used, following the manufacturer’s instructions. The extracted RNA was then used to make cDNA, using a RNA to cDNA EcoDry premix kit (Clontech Laboratories, Mountain View, CA) following the manufacturers recommendations. Briefly, 20 μL of RNA was added to each well of EcoDry Premix in the cDNA strips. These strips were then placed in a Veriti 96 Well Thermal Cycler (Applied Biosystems, Foster City, CA) at 42°C for 60 minutes, and then 70°C for 10 minutes. Upon completion, the cDNA was refrigerated at 4°C.

**Quantitative Polymerase Chain Reaction (qPCR)**

Quantitative PCR reactions were performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and intron-spanning PrimePCR SYBR Green Assays
(Bio-Rad, Hercules, CA) for GapDH (assay ID qDreCED0021000) and IL1β (qDreCID0003801), following the manufacturer’s recommended guidelines for both DNA and primer concentrations. 20 µL reaction volumes were used for each well of a Fast Optical 96-Well qPCR plate (Applied Biosystems, Foster City, CA). Negative controls were included on each plate and replaced cDNA with molecular grade water. The plate was covered and spun in a centrifuge at 1000 rpm for 1 minute. The plate was then placed in Applied Biosystems StepOnePlus Real Time PCR System for qPCR. The qPCR was run on the Quantitative setting with a reaction volume of 20 µl. The qPCR took measurements in real time of the amount of DNA at each step in a PCR. Fluorescence directly related to the amount of DNA present was measured by the StepOnePlus PCR System. Relative gene expression was determined using the 2⁻ΔΔCt method (Livak & Schmittgen 2001). Briefly, the CT of the housekeeping gene, GapDH, was subtracted from the CT of the gene of interest, IL1β to create the delta CT value(ΔCT). Next, the ΔCT of the negative control for a sample was subtracted from the ΔCT of a bacteria-treated sample to obtain the delta delta CT value (ΔΔCT). Lastly, the formula $2^{-ΔΔCT}$ was used to calculate the relative quantity (RQ), or fold increase. This is number represents the fold increase of IL1β gene copies in the sample of interest when compared to the housekeeping gene, GapDH, and normalized to the negative control for that sample.

*Indirect enzyme-linked immunosorbent assay (ELISA) procedure*

Indirect enzyme-linked immunosorbent assays (ELISAs) were performed to determine the specificity and sensitivity of monoclonal anti-zebrafish IL1β antibodies being produced by hybridomas. First, a 96-well plate was coated with zebrafish IL1β antigen diluted to 1 µg/mL in 100 mM bicarbonate buffer, ph 9.6. After overnight incubation at 4°C, the plate was washed with PBS containing 0.05% Tween 20 (Amresco Inc., Solon, OH). The plate was then coated in a
blocking buffer (1% bovine serum albumin in PBS) and incubated overnight again at 4°C. The plate was then washed again and 50 μL of cell supernatant or serial dilutions of purified antibody were added to each well. After two hours of incubation at room temperature on a 60 RPM rocker, the plate was washed again and 50 μL of the detection antibody, anti-mouse IgG HRP conjugate (Enzo Life Sciences, Farmingdale, NY) diluted 1:5000 in blocking buffer, was added. After another hour of incubation at room temperature on a 60 RPM rocker, the detection antibody was discarded, and the plate was washed again. 50 μL of pre-made TMB substrate (Amresco, Inc., Solon, OH) was added to each well and incubated for 10-30 minutes, until a blue color developed. Next, 50 μL of a stop solution of 0.28M sulfuric acid was added and the plate absorbance was read at 450 nm on a plate reader.

Development and screening of monoclonal antibodies

Anti-IL1β antibody producing hybridomas were developed using the method described in Antibodies: A laboratory manual Second ed. (Greenfield, 2014). Briefly, two BALB/C mice were vaccinated IP with a 1:1 solution of a synthesized peptide (Biosynthesis, Lewisville, TX) based on an 8 kDa fragment of zebrafish IL1β and TiterMax Gold adjuvant (Sigma-Aldrich). After 3 rounds of IP injection, mouse serum was screened for the presence of antibodies against the IL1β peptide by indirect ELISA. Two days after a final IV injection of peptide without adjuvant, the mice were sacrificed, and spleens removed. Splenocytes were fused to SP 2.0 cells to produce hybridomas and grown in selective medium (HAT Medium). Confluent wells of hybridomas were screened for their sensitivity to IL1β via enzyme-linked immunosorbent assay (ELISA). The colonies that were reactive were then sub-cloned onto new 96-well plates, at a dilution of a single cell for every 3 wells, with the goal of growing cultures of only one single clone in each well that showed growth. These clones were then incubated and allowed to grow. They were then screened for IL1β
reactivity again via ELISA, and the reactive clones were subbed again onto a new 96-well plate, with the goal of one cell per three wells. The most reactive clones from this last set of sub-clones were then grown up in larger flasks in RPMI cell culture media with pen-strep (Gibco, Grand Island, NY), gentamicin (Gibco, Grand Island, NY) and fetal bovine serum. Once the cells were growing steadily, they were transferred to a flask containing hybridoma serum-free media (Gibco, Grand Island, NY) with the same antibiotics. After growing for 2-3 days in this media, the serum-free media was collected and stored at 4 °C with 0.03%μL sodium azide to prevent bacterial growth.

*Purification of monoclonal antibodies via protein G antibody affinity chromatography*

Protein G antibody affinity chromatography was used to purify anti-zebrafish IL1β antibodies from serum-free cell culture medium. The cell culture media was first diluted 1:1 with filtered PBS and filtered through a .2μm vacuum bottle filter top (Nalgene, Rochester, NY) to remove cellular debris. A column with 5mL of protein G agarose resin was prepared and allowed to compact upright overnight. The column was set up in a 4°C refrigerator and equilibrated by running ten column volumes of filtered PBS through the system followed by previously collected hybridoma supernatants allowing the antibodies to bind to the protein G. Flow-through was collected for later testing to ensure complete binding. Next the column was washed with ten column volumes of filtered PBS which also was saved for later analysis. Seven microfuge tubes were then prepared for elution by adding 50 μL of 1M dibasic Na₂HPO₄. Elution buffer (0.2M glycine) was added to the column, and fractions were collected in 1mL increments. The fractions were then tested for protein concentration using a NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA) against a blank of 1mL filtered PBS with 50 μL of 1M dibasic Na₂HPO₄.
Testing antibodies and tissue samples via western blot

To test the specificity and sensitivity of the developed monoclonal antibodies, western blotting was done examining antibody binding to known concentrations of IL1β peptide or proteins extracted from fish tissues. First, 80µL of processed fish tissue or IL1β peptide of known concentrations (1mg/mL and 250 µg/mL) were combined with 20 µL of Bolt LDS sample buffer (Life Technologies, Carlsbad, CA) and 2 µL of Bolt sample reducing agent (Life Technologies, Carlsbad, CA) and boiled at 95°C for 5 minutes to denature the proteins. Bolt 4–12% Bis-Tris Plus Gels (Thermo Fisher Scientific Waltham, MA) were set up to run with Bolt MES running buffer (Life Technologies, Carlsbad, CA) in the iBlot gel tank. 15 µL of each sample mix was pipetted into the wells and the tank lid was attached to a Bio-Rad Powerpac HC (Bio-Rad Laboratories, Hercules, CA). The gel ran at 165 volts until the loading dye began to run out of the bottom of the gel. It was then removed from its mold and placed in a bath of 20% ethanol for five minutes. Next the gel was carefully placed on an iBlot transfer stack (Thermo Fisher Scientific, Waltham, MA) and manufacturer instructions were followed to complete the transfer from the gel to the nitrocellulose in the stack using the iBlot 2 gel transfer device. After the transfer was complete, the blot was placed in a LI-COR incubation box (LI-COR biosciences, Lincoln, NE) and blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline containing 0.25% Tween-20) for at least 30 minutes on a room temperature rocker at 60 RPM. Next, the milk was poured off and the blot was rinsed with TBS-t for 5 minutes on a room temperature rocker at 60 RPM. For cell culture screening, blots were coated in cell supernatant of interest along with 0.03% sodium azide as the primary antibody. They were left on a room temperature rocker @ 60 RPM overnight. For testing purified monoclonal antibodies, both with known
concentrations of IL1β peptide and with fish tissues, the blots were covered with 1: 10,000 dilutions of the antibody with .5% nonfat dry milk in TBS-t and left on a room temperature rocker @ 60 RPM overnight. Next, the contents of the (box) were poured off and the blots were rinsed with TBS-t. The blots were then covered with LI-COR IR Dye 800CW goat anti mouse or goat anti mouse IgG at 1: 10,000 with .5% milk in TBS-t and set on a room temperature rocker at 60 rpm for 60 minutes. The previous step was repeated with LI-COR IR dye 800CW streptavidin if the secondary antibody was not fluorescent, but instead labeled with biotin. Blots were scanned at 800 nm with an Odyssey Sa imaging system (LI-COR biosciences, Lincoln, NE) to visualize the blot.

**Biotin labeling of monoclonal antibodies**

After purification, one fraction of each antibody was selected to be labeled with biotin. The EZ-Link Sulfo-NHS Biotinylation Kit by Thermo Fisher Scientific was used to biotin-label the antibody. Manufacturer instructions were followed. The HABA assay included in the kit was used on the labeled samples to test level of biotinylation, following manufacturer instructions.

**Testing for IL1β in zebrafish samples with a sandwich ELISA**

![Figure 1: Sandwich ELISA diagram](image)
Sandwich ELISAs were created using a combination of biotin-labeled and non-biotin labeled monoclonal antibodies that recognize the protein of interest, IL1β, at different epitopes. As seen in figure 1, the process is very similar to the indirect ELISA described above, and all buffers used are the same. The biggest difference is the variable of interest. Sandwich ELISAs test for differences in levels of protein (IL1β) expression, while the indirect ELISA described above tested the affinity of the antibodies to a known amount of protein. To develop the sandwich ELISA, the capture antibody was diluted in blocking buffer and used to coat the plate, incubating overnight at 4 °C. The plate was then blocked with blocking buffer just as in the indirect ELISA. Next, samples of intra- and extra- cellular proteins from bacteria-treated zebrafish tissues were added to the wells and allowed to incubate for one hour at room temperature on a 60 RPM rocker. After washing, a biotin-labeled monoclonal antibody was added to the wells to act as a detection antibody. Again, the plate was incubated on the 60 RPM rocker at room temperature for one hour. After another wash, streptavidin-HRP conjugate (Enzo Life Sciences, Farmingdale, NY) was added to the wells to attach to all bound biotin-labeled antibodies. After another hour of incubation at room temperature on a 60 RPM rocker, the unbound streptavidin-HRP was discarded, and the plate was washed again. 50 μL of pre-made TMB substrate (Amresco, Inc., Solon, OH) was added to each well and incubated for 10-30 minutes, until a blue color developed. Next, 50 μL of a stop solution of .28M sulfuric acid was added and the plate was read at 450 nm on a plate reader.
A competitive ELISA (figure 2) was created to test the levels of IL1β in various intra- and extra-cellular samples from zebrafish treated with bacteria. First, a 96-well plate was coated with zebrafish IL1β antigen diluted to 1 µg/mL in 100 mM bicarbonate buffer, pH 9.6. After overnight incubation at 4 °C, the plate was washed with a buffer made of .05% Tween 20 (Amresco Inc., Solon, OH) in PBS. The plate was then coated in a blocking buffer made of 1% bovine serum albumin in PBS and incubated overnight again at 4 °C. Next, the samples, as well as some known concentrations of purified IL1β (1µg/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.25 ng/mL, 15.625 ng/mL) were mixed 1:1 with the monoclonal antibody in a 96 well plate and incubated at room temperature for one hour. Next, the coated and blocked plate was washed with wash buffer and 50 µl of the sample-antibody mix was added to each well. After one hour of incubation at room temperature on a 60 RPM rocker, the plate was washed again and 50 µL of the detection antibody, goat anti-mouse IgG HRP conjugate (Enzo Life Sciences, Farmingdale, NY) diluted 1:5000 in blocking buffer, was added. After another hour of incubation at room temperature on a 60 RPM rocker, the detection antibody was discarded, and the plate was washed again. 50 µL of pre-made TMB substrate (Amresco, Inc., Solon, OH) was added to each well and incubated for 10-30 minutes, until a blue color developed. Next, 50 µL of
a stop solution of .28M sulfuric acid was added and the plate was read at 450 nm on a plate reader.

Results

Screening of sub-clones:

<table>
<thead>
<tr>
<th>Cell supernatant line</th>
<th>Absorbance 1</th>
<th>Absorbance 2</th>
<th>Average Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E2 H11H1 w/ serum</td>
<td>1.191</td>
<td>0.774</td>
<td>0.9825</td>
</tr>
<tr>
<td>5D10 F7H12 w/ serum</td>
<td>0.315</td>
<td>0.261</td>
<td>0.288</td>
</tr>
<tr>
<td>4E2 H11H1 SFM</td>
<td>1.477</td>
<td>0.83</td>
<td>1.1535</td>
</tr>
<tr>
<td>5D10 F7H12 SFM</td>
<td>0.121</td>
<td>0.138</td>
<td>0.1295</td>
</tr>
<tr>
<td>6E5 SFM</td>
<td>0.156</td>
<td>0.176</td>
<td>0.166</td>
</tr>
<tr>
<td>4F2 SFM</td>
<td>0.977</td>
<td>0.771</td>
<td>0.874</td>
</tr>
<tr>
<td>5D10 F7H12 SFM + trypsin</td>
<td>0.133</td>
<td>0.119</td>
<td>0.126</td>
</tr>
<tr>
<td>4E2 H11H1 SFM + trypsin</td>
<td>0.859</td>
<td>0.756</td>
<td>0.8075</td>
</tr>
<tr>
<td>6E5 SFM + trypsin</td>
<td>0.144</td>
<td>0.774</td>
<td>0.459</td>
</tr>
<tr>
<td>4F2 SFM + trypsin</td>
<td>0.469</td>
<td>0.261</td>
<td>0.365</td>
</tr>
</tbody>
</table>

Table 1: Hybridoma sub-clone supernatant screening results

After expanding four different sub-clones that had initially tested positive for IL1β reactivity, cell culture media, both with and without serum, was collected from each line. The different cell supernatants were screened to test which
ones were the most reactive and if growing in the serum free media (SFM) reduced their ability to produce and excrete effective anti-IL1β antibodies (Table 1). As seen in table 1, the 4E2 H11H1 line was the most reactive, even when grown in serum free media. The next most reactive cell line was 4F2. These two lines were selected to be expanded in higher volumes of media, so that the media could be purified to collect the secreted antibodies. The 4E2H11H1 line was also tested via western blot (Figure 3) and was shown to be reactive with IL1β peptide. The 4F2 line was not reactive via western (not shown).

*Concentration of purified antibodies:*

<table>
<thead>
<tr>
<th>Purified antibody</th>
<th>Concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4E2H11H1 (non-biotinylated)</td>
<td>2.1</td>
</tr>
<tr>
<td>4E2H11H1 (biotinylated)</td>
<td>2.42</td>
</tr>
<tr>
<td>4F2 (non-biotinylated)</td>
<td>6.8</td>
</tr>
<tr>
<td>4F2 (biotinylated)</td>
<td>2.9</td>
</tr>
</tbody>
</table>

Table 2: Purified antibody concentrations

Once the antibodies were purified via protein G antibody affinity chromatography, their concentrations were measured via microvolume spectrophotometry, using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA). The highest concentrated fraction was chosen to be biotinylated. The next most concentrated fraction would serve as the stock of non-biotinylated antibody for each line. The final concentrations are seen in table 2.
**Bacterial cell density:**

The *Edwardsiella ictaluri* suspension used was found to have a cell density of 160 CFU/mL and the *Mycobacterium chelonae* suspension was found to have a density of 391 CFU/mL.

**Sensitivity of purified antibodies to IL1β:**

An indirect ELISA was run with serial dilutions of each purified antibody to determine their levels of sensitivity. The data in figure 4 shows that there was a decrease in reactivity as concentration decreased, which is to be expected.
Sandwich ELISA results:

Sandwich ELISAs were done with the purified antibodies, using both known concentrations of IL1β peptide and cell samples from zebrafish tissues that had been incubated with either *Edwardsiella ictaluri* (Ei) or *Mycobacterium chelonae* (Mch). The results shown in figure 4 are from a test that used the nonbiotinylated 4E2H11H1 antibody as the capture antibody and the biotinylated 4F2 antibody as the detection antibody. IL1β was undetectable in the sandwich ELISA, but did open the door for another type of ELISA, a competitive ELISA (figure 6).
Competitive ELISA results:

Competitive ELISAs were completed with the purified antibodies, using both known concentrations of IL1β peptide as controls and cell samples from zebrafish tissues that had been incubated with either *Edwardsiella ictaluri* (Ei) or *Mycobacterium chelonae* (Mch). The results shown in figure 6 are from a test that used the non-biotinylated 4F2 antibody.
Quantitative PCR Results:

Quantitative PCR was performed with the cDNA that was synthesized from the RNA isolated from the zebrafish tissues that had been treated with the different bacteria (figure 6). The intestine upregulated IL1β transcription in response to Edwardsiella ictaluri (12.51 fold) at time 1 (two hours), then decreased in the next two time points (four and six hours). The intestinal cells were not very reactive to Mycobacterium chelonae, though did downregulate IL1β transcription at four hours (0.48). The result for liver and kidney at six hours in response to Edwardsiella ictaluri (19.92-fold increase) is notable, since it seems to have increased at some point between time 2 (four hours) and time 3 (six hours).
Discussion

This paper describes the development of a pair of monoclonal antibodies that recognize zebrafish IL1β and their use in the construction of sensitive sandwich and competitive ELISAs. Newly developed monoclonal antibodies were used in conjunction with quantitative PCR to attempt to detect a change in IL1β gene expression as well as functional cytokine production. Both assays test for the presence of IL1β, but achieve it in different ways. In sandwich ELISAs, if there is more antigen present in the sample, more will attach to the capture antibody. With more antigen attached to the capture antibody, more detection antibody can bind, resulting in a higher absorbance. With competitive ELISAs, if there is more antigen present in the sample, then less antibody will be available to bind to the antigen in the well, since it is already bound to the antigen in the sample. This means that lower absorbance levels equate with higher concentrations of the antigen.

While the sandwich ELISAs failed to detect IL1β in zebrafish tissues competitive ELISAs proved successful in detecting changes in explanted tissues infected with different bacteria. When comparing the results of the competitive ELISA and the quantitative PCR for the same samples, an interesting pattern emerges. When treated with Edwardsiella ictaluri, the intestines increased the copy number of the IL1β gene 12.51-fold in the first 2 hours. Increasing the number of copies of a gene does not necessarily mean that all of those will be translated into functional proteins. Additionally, IL1β must be post-translationally cleaved to release an active cytokine, so a disruption in this post-translational processing could result in increased transcription without seeing an increase in functional protein secretion. The absorbance in the competitive ELISA stayed stable throughout the first four hours, with a slight decrease from .382
to .247. However, by time point 3 (six hours), the absorbance in the intracellular intestine sample had dropped to .09, indicating a large increase in amount of actual IL1β protein.

Similar patterns were seen in the liver/kidney samples in response to *Edwardsiella ictaluri*, however the increase in gene copy number happened at the later time points, and consequently, the absorbance in the competitive ELISA did not drop appreciably until the six hour point. We suspect that if a later time point, at eight hours for example, had been collected, it would make sense that the absorbance on the competitive ELISA would be even lower, correlating with a higher number of active IL1β proteins and a greater inflammatory response.

These patterns are likely explained by the fact that *Edwardsiella ictaluri* is an aggressive gram-negative rod that causes clinical symptoms in zebrafish that include tissue necrosis, skin ulceration, nasal, brain, liver, and kidney inflammation (Hawke et al., 2013). Knowing this, it is unlikely that zebrafish cells would be equipped to prevent a massive inflammatory reaction unless they had been exposed previously and developed some adaptive immunity to the bacteria. If the IL1β upregulation seen in these samples occurred *in vivo*, the affected fish would experience inflammation of the examined organs (intestine, liver, kidney). This would likely result in intestinal symptoms such as gastroenteritis, colitis, and other complications of inflamed intestines. Upregulation of IL1β in the liver and kidney could lead to hepatitis and nephritis, leading to a decrease in function of these organs and decreased blood filtration.

Increased knowledge of the zebrafish immune response to *Edwardsiella ictaluri* could lead to improved treatments for *Edwardsiella tarda* infections in humans, or even other infections by gram-negative bacteria in the family Enterobacteriaceae, which include *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella*, and *Shigella*. 
Another interesting pattern emerges when the intestinal cell responses to *Mycobacterium chelonae* are examined. The relative quantity of gene copies stays close to 1 initially (1.38), with a decrease to .48 at four hours, and a small jump back to 1.14 at six hours. The absorbance values in the quantitative PCR data stayed very flat as well, ranging between .181 and .203 for all samples except the intracellular sample at time 3 (6 hours). At that point it dropped to .061, which could be an outlier or a sign that IL1β was starting to be produced at a higher level within the cell at the six hour point.

The pattern is slightly different in the liver and kidney samples, especially at the genetic level. The relative quantity of IL1β increased 4.76-fold at four hours and 15.58-fold at six hours. The two hour data point (51.29 fold) for the liver and kidney samples in response to *Mycobacterium chelonae* seems to be an outlier, considering the other two time points, as well as the corresponding competitive ELISA data. Interestingly, the absorbance values in the competitive ELISA for these samples stayed relatively high (.245-.714), indicating that there was not a lot of active IL1β produced, despite the large increase in relative gene copies. It is very possible, however, that at longer time periods, an increase in protein production would be seen.

These results make sense, considering that *Mycobacterium chelonae* tends to cause a low-level, chronic infection in zebrafish that can be clinically insignificant for a long time (Whipps, Lieggi, & Wagner, 2012). It would be very detrimental for zebrafish to have a strong IL1β response to *Mycobacterium chelonae*, since it is a pro-inflammatory cytokine and *Mycobacterium chelonae* is a fairly common bacterium. It would also be disadvantageous for *Mycobacterium chelonae* to cause severe intestinal symptoms, since this would likely cause the fish to expel any bacteria that had been able to grow inside it. An improved understanding of zebrafish immune regulation in response to *Mycobacterium chelonae* may allow for increased
understanding of human immune responses to similar mycobacteria, such as the ones that cause tuberculosis (Mycobacterium tuberculosis) and leprosy (Mycobacterium leprae). It is important to note that these bacteria cause a long-lasting, chronic infection in humans, similar to the chronic infection Mycobacterium chelonae causes in fish.

While the patterns seen in the competitive ELISA are promising, the absorbance values of the antibodies developed were quite low (see figure 1 and figure 3), considering the stock concentration of the antibody used was high (6.2 mg/mL). Most commercially available monoclonal antibodies are at a stock concentration of 1mg/mL and can be diluted to 1:10,000 and be much more reactive than the data seen here. The low reactivity could be due to several reasons, but there are two likely causes, low affinity and low avidity. Low affinity describes weak binding between the antibody and the epitope of the antigen (IL1β). Given that binding is reversible, a weak affinity will cause the binding to not last as long and take longer to occur. Avidity is a similar concept, but it describes the stability of the interaction between the antibody and antigen. While it is influenced by affinity, it is also determined by the number of available binding sites. A low avidity is a likely explanation of the low absorbance values seen even at the highest concentrations of antibody in figure 1. These deficits are also likely the cause of the failure of the sandwich ELISAs attempted.

While the findings discussed here are limited, they shine a light on an interesting phenomenon in the immune response of zebrafish at both the transcription and translation levels to different bacteria. The ability to easily compare these different levels of regulation is a tool that the field could benefit from greatly. While sandwich ELISAs can be highly sensitive, they require a lot of work to optimize, and cross-reactivity can occur between the two antibodies used. On the other hand, competitive ELISAs are less sensitive to sample dilutions, and can be used
with samples that have undergone minimal processing (Bio-Rad, 2017). These differences may also account for the differences in IL1β detection between the ELISAs developed. Future steps to build on this research should include developing monoclonal antibodies with higher avidity and affinity to expand the number of commercially available tools. These would be able to be used in sandwich ELISAs that yield reliable results, as well as competition ELISAs that yield much stronger results. It would also be beneficial to replicate this study with a different pro-inflammatory cytokine, such as Interleukin-6 (IL-6) or Tumor necrosis factor alpha (TNF-α) to see if the regulation of the transcription and translation of these proteins is comparable to that of IL1β. It would also be beneficial to test IL1β and other cytokine regulation in response to other bacteria that share a genus with problematic bacteria in human medicine.
Works Cited


