A TRANSGENIC ZEBRAFISH MODEL OF SIGNALING THROUGH TOLL-LIKE RECEPTORS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

Toll-like receptors (TLRs), members of the innate immune system, recognize distinctive components of pathogens to initiate cellular signaling pathways. These pathways lead to the production of inflammatory proteins such as tumor necrosis factor alpha (TNFα), a cytokine that activates immune cells to fight the infection. When infections disseminate into the bloodstream, TLR-signaling is activated systemically and the excessive release of cytokines, chemokines, and other inflammatory mediators produces a life-threatening condition known as septic shock. The majority of drugs recently developed to treat septic shock were beneficial in animal and *in vitro* models, but were ineffective in human clinical trials. One possible solution to resolve this disparity is to use the new animal model *Danio rerio* (zebrafish) whose unique characteristics allow for high-throughput, large-scale screening of chemicals and for exclusive study of the innate immune system.

We undertook three different approaches to establish a functioning transgenic zebrafish model that facilitates visualization of TNFα expression as an indicator of TLR signaling and pathologic immune response. The first expression clone contains the human TNFα (*h*TNFα) enhancer and the enhanced green fluorescent (EGFP) reporter gene under control of the basal carp β-actin promoter. The second expression clone contains all elements of the first clone with the addition of a red fluorescent reporter gene, DsRed, under control of a lens-specific promoter, the *Xenopus laevis* γ-crystallin

promoter (Cry), to facilitate preselection of putative transgenic founders. The third expression clone is a modified BAC clone containing the red fluorescent reporter gene tandem-dimer Tomato (tdTomato) fused to the zebrafish TNFα (*z*TNFα) gene in its genomic context.

We successfully introduced the modified BAC clone into the germline of two male zebrafish. To date, one female offspring from an F_0 founder has been identified as carrying the BAC transgene. In the future, the F_2 embryos from this F_1 female will be analyzed for expression of the fluorescent fusion protein under conditions which induce TNFα production. Additionally, future establishment of a homozygous transgenic generation will allow functional studies of TLR signaling during infections and assessment of chemical and genetic modifiers of TNFα induction.

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1 INTRODUCTION

1.1 Sepsis and Septic Shock

Sepsis is a serious medical condition arising from a localized tissue infection that cannot be contained and then disseminates into the bloodstream. Despite modern advances in medicine, sepsis has continued to be a lethal problem that is increasing in frequency (LEE and SLUTSKY 2010). "Shock due to sepsis is the most common cause of mortality in patients in non-coronary intensive care units" (LOLIS and BUCALA 2003). Sepsis is ranked as the 10th leading cause of death overall in the United States, and kills 20-50% of severely affected patients (WHEELER and BERNARD 1999; MARTIN *et al.* 2003; XU 2010). The resulting sequelae of sepsis are caused by excessive release of cytokines, chemokines, and other inflammatory mediators into the circulation. The repercussion of this "cytokine storm" is the development of systemic inflammatory response syndrome (SIRS). SIRS, in the presence of confirmed or suspected infection, is defined as sepsis. Clinically, systemic inflammatory response syndrome (SIRS) manifests as fever or hypothermia (>38°C or <36°C), tachycardia (>90 beats per minute), tachypnea (>20 breaths per minute), and leukocytosis or leucopenia (<4000 white blood cell per μL or >12,000 white blood cells per μL, or presence of >10 percent immature granulocytes) (JAESCHKE *et al.* 2008). Sustained neutrophil activation and adherence to vascular endothelial cells causes endothelial dysfunction and damage. The permeability of the microvasculature is increased, interstitial edema develops, oxygen diffusion to tissues is

reduced, and blood flow to vital organs is decreased (DE GAUDIO *et al.* 2009). Severe sepsis develops when organ dysfunction, hypoperfusion (decreased blood flow through an organ), and hypotension (low blood pressure: systolic blood pressure less than 90 millimeters of mercury [mm Hg] or diastolic less than 60 mm Hg) are present. If fluid resuscitation fails to correct the hypotension, the body goes into septic shock. All of the tissues and organs now lack sufficient blood flow. Septic shock ultimately leads to multiple organ dysfunction syndrome (MODS) that almost invariably results in death (JAESCHKE *et al.* 2008; DE GAUDIO *et al.* 2009).

1.2 Therapy and Animal Models of Sepsis

Few new therapies have been identified for treating sepsis. The majority of drugs that proved successful in animal and *in vitro* models demonstrated ineffective results in human clinical trials (LEE and SLUTSKY 2010). Lipopolysaccharide (LPS), also referred to as endotoxin, is a cell-wall component of Gram-negative bacteria. Approximately half the cases of sepsis are due to Gram-negative bacterial infections (LOLIS and BUCALA 2003; MARTIN *et al.* 2003). Clinical trials for treating sepsis with a novel human monoclonal antibody against endotoxin, HA-1A, resulted in no decrease of mortality (LUCE 1993). A separate clinical trial for severe sepsis treatment used a chimeric antibody against the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα). The antibody did not alter the pattern of cytokine activation or reduce systemic inflammatory symptoms (CLARK *et al.* 1998). Similar results were observed in a different trial using monoclonal anti-TNFα antibodies (ABRAHAM 1999). Unexpectedly, the anti-TNFα therapies demonstrated beneficial and effective treatment for the chronic inflammatory

diseases such as rheumatoid arthritis, ankylosing spondylitis, and psoriasis arthritis (CHEN *et al.* 2006; NERI *et al.* 2010).

The lack of benefits demonstrated from the anti-endotoxin and anti-TNFα human clinical trials is troubling. Mouse, rat, rabbit, dog, pig, sheep, and nonhuman primate animal models were used in the preclinical experiments of anti-endotoxin and anti-TNFα (POLI-DE-FIGUEIREDO *et al.* 2008; DYSON and SINGER 2009). The disparity between preclinical and clinical data has been observed with other modern drugs; for example, drugs used in chemotherapy of cancer. Starting in 1955, the formal screening of thousands of drugs have shown benefit in either cell or animal models; however, there are only 39 drugs that have gained approval from the U.S. Food and Drug Administration for use as a chemotherapy agent (GURA 1997). Hence, animal models that are not good predictors of human disease and drug responsiveness are not unique to anti-endotoxin and anti-TNFα therapy. One possible solution would be trying a novel animal model with advantageous characteristics that provide different perspectives of the immune system and is amendable to large-scale compound screens. Although animal models will never be optimal for reproducing human disease, the zebrafish, *Danio rerio*, is a promising animal model for drug discovery. While evolutionarily and physiologically more distant to humans than other animal models, the unique features of zebrafish allow for high-throughput, large-scale screening of chemicals for desired effects *in vivo*.

1.3 Advantages of the Zebrafish Animal Model

1.3.1 Inherent Characteristics and Developed Techniques

Several inherent characteristics and developed techniques make zebrafish a promising experimental model for Toll-like receptor (TLR) signaling. A mating pair can produce 200-300 new progeny each week (MEEKER and TREDE 2008). Ova fertilization and embryo development occurs *ex vivo*. Embryos are translucent for several days, allowing for visualization of development, immune cell movement, fluorescent protein reporters such as GFP (green fluorescent protein), and fluorescently-labeled bacteria or viruses (MEEKER and TREDE 2008; ALLEN and NEELY 2010). Fluorescent reporters can be placed under the control of tissue-specific promoters or enhancer elements, allowing specific cell types such as neutrophils to be visualized or tracked *in vivo* using fluorescent microscopy (ALLEN and NEELY 2010; LOYNES *et al.* 2010). Green fluorescent protein (GFP) was originally identified in the *Aequorea victoria* jellyfish (SHIMOMURA *et al.* 1962). Several properties of GFP have been modified for its use in research. Enhanced GFP (EGFP) contains a single-point mutation (S65T) in comparison to wild-type GFP that eliminates one of the bimodal excitation peaks seen in wild-type GFP. EGFP is highly photostable and can be imaged using fluorescein isothiocyanate (FITC) filter sets. DsRed, a red fluorescent protein, was isolated and cloned from a coral belonging to the *Discosoma* genus. Tandem-dimer tomato (tdTomato) fluorescent protein is a derivative of DsRed (SHANER *et al.* 2004; SHANER *et al.* 2007; RIZZO *et al.* 2009).

Other advantages are that in young zebrafish, it is possible to study innate immunity exclusively. A fully functional adaptive immune system does not develop for four-six weeks (SULLIVAN and KIM 2008). Important constituents of the innate immune

system, specifically monocytes, granulocytes, and tissue macrophages, are present in bony fish. Embryos at 23-26 hours postfertilization (hpf) have early macrophages capable of phagocytosis. These early macrophages differentiate in the free space between the yolk cell and epidermis. This locale is easily visible with video-enhanced differential interference contrast (DIC) microscopy allowing cells to be imaged and tracked *in vivo* at high magnification (TRAVER *et al*. 2003). Furthermore, macrophage interaction with other blood cells or with intravenously injected microbes can be imaged in this free space. These interactions are feasible because venous blood flows freely over the surface of the yolk cell without being confined to a blood vessel at the start of circulation and is then collected in the heart (TRAVER *et al.* 2003; LEVRAUD *et al.* 2008).

The entire zebrafish genome has been sequenced multiple times and is continuously being assembled and reassembled (http://uswest.ensembl.org/Danio_rerio/Location/genome). The genetic map of zebrafish reveals highly conserved synteny with the human genome (TRAVER *et al.* 2003; JAULT *et al.* 2004; MEIJER *et al.* 2004). Teleosts or bony fish have a highly developed complement system. Major inflammatory proteins are present in fish, namely, TNFα; nuclear factor-kappaB (NF-κB); cyclooxygenase 2 (COX-2); interleukin 1 (IL-1); IL-8; and other CC and CXC chemokines (TRAVER *et al.* 2003). Homologs of the human IL-1 and IL-18 receptors have been identified in the zebrafish genome.

Techniques for chemical screens in zebrafish have been established. Embryos are small enough to be arrayed in 96-well plates facilitating large-scale *in vivo* chemical screens. Chemical compounds can simply be added to the water, and, using high-throughput *in situ* hybridization or transgenic reporter lines of zebrafish, the read-out of

their functional consequences is straight-forward (TREDE *et al.* 2004). In addition to systemic drug administration, tissue-specific drug delivery can be achieved by electroporation (THUMMEL *et al.* 2006; HENDRICKS and JESUTHASAN 2007). Chemical screens in live zebrafish are unbiased; the molecular target does not have to be pre-determined. Chemicals that restore gene function can be identified. Small molecule screens in zebrafish have already shown potential to translate into clinical benefits. The first human trial of a drug to maximize bone marrow transplants was precipitated by experiments performed in zebrafish that identified prostaglandin E2 (PGE2) as an inducer of stem cell expansion (DURAND and ZON 2010).

Efficient forward and reverse genetic screening techniques have been developed for the zebrafish model including targeting induced local lesions in genome (TILLING), gene knockdown using morpholino oligonucleotides, and transgenesis using the natural fish *Tol2* transposase (TREDE *et al.* 2004; MEEKER and TREDE 2008). The *Tol2* transposition system has been successfully utilized for transgenesis in zebrafish with high efficiency. On average, 50-70% of zebrafish embryos injected with vectors containing the *Tol2* sites produce transgenic F_1 offspring (KAWAKAMI *et al.* 2004b; URASAKI *et al.* 2006). Transgenic fish created with the *Tol2* system have been shown to express GFP beyond the F_5 generation (KAWAKAMI 2005). *Tol2* has also been shown to transpose in frogs, *Xenopus laevis* and *Xenopus tropicalis*, chicken embryos, and mouse embryonic stem cells (KAWAKAMI *et al.* 2004a; KAWAKAMI and NODA 2004; HAMLET *et al.* 2006; KAWAKAMI 2007; SATO *et al.* 2007). *Tol2* can transpose into somatic or germ line cells and can transpose over a range of temperatures, 25°-26°C in zebrafish, and 37°-38°C in chicken embryos. The *Tol2* transposition does not cause gross rearrangements of

surrounding genomic DNA and has no required genome target specificities (KAWAKAMI 2007). A *Tol2* vector can facilitate up to 11 kilobases (kb) of DNA insert without reducing the transpositional activity (URASAKI *et al.* 2006). In addition, the *Tol2* transposon system has been developed for gene trapping and enhancer trapping methods in zebrafish.

A high-throughput automated cell-injection system has been created for introducing materials into zebrafish embryos. The automated system is capable of depositing a precise volume of liquid material into an embryo. Several destinations within the embryo can be chosen for the targeted material. The automated system is capable of injecting 15 embryos per minute with a 99% success rate. Survival rate of the injected embryos is 98% (WANG *et al.* 2007).

1.3.2 Zebrafish as a Tool for Mammalian Infection

Successful infections in zebrafish with both Gram-positive and Gram-negative bacteria have been demonstrated. Infections have been achieved with both natural fish pathogens and genuine human pathogens (O'CALLAGHAN and VERGUNST 2010; VERGUNST *et al.* 2010). For example, extraintestinal pathogenic *Escherichia coli*, *Edwardsiella tarda*, *Burkholderia cenocepacia*, *Aeromonas salmonicida*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Aeromonas hydrophila*, *Streptococcus iniae*, *Streptococcos pyogenes*, *Mycobacterium marinum*, *Salmonella typhimurium*, *Listeria* species, and *Flavobacterium columnare* have all been shown to infect zebrafish (PRESSLEY *et al.* 2005; LIN *et al.* 2007; RODRIGUEZ *et al.* 2008; SULLIVAN and KIM 2008; BRANNON *et al.* 2009; WILES *et al.* 2009; ORDAS *et al.* 2010; VERGUNST *et al.* 2010).

Bacterial infection can be achieved by injection into adults and embryos, or by immersion following dermal abrasion of adults or removal of the chorion from embryos. The ensuing inflammatory reactions can be measured by various methods such as real-time quantitative polymerase chain reaction (PCR). For instance, infection with *Edwardsiella tarda* showed significantly increased levels of TNFα and IL-1β (PRESSLEY *et al.* 2005), while infection with *Aeromonas hydrophila* demonstrated an increase in TNFα, IL-1β, and IFNγ levels as measured by quantitative PCR (RODRIGUEZ *et al.* 2008). Artificial LPS has also been shown to induce inflammatory reactions in zebrafish (WATZKE *et al.* 2007).

In addition to the many similarities, there are also differences between the zebrafish and human response to infection. In humans, it is Toll-like receptor 4 (*h*TLR4) that recognizes endotoxin, the lipopolysaccharides (LPS) of Gram-negative bacteria, and initiates an immune response. In zebrafish, two TLRs have been identified that resemble human TLR4, referred to as *z*TLR4a and *z*TLR4b [Table 1]. However, functional studies in zebrafish have demonstrated that *z*TLR4a and *z*TLR4b do not respond to LPS because of the structural difference in their extracellular domains. Therefore, *z*TLR4a and *z*TLR4b are paralogs to *h*TLR4, not homologs. Other differences exist between zebrafish and mammalian immunology. For example, in adult zebrafish, hematopoiesis occurs in the head kidney, as opposed to the bone marrow in mammals. Teleosts do not have lymph nodes; in comparison, the spleen is the site of antigen presentation and T cell-B cell interactions. Lymphoid aggregates are present in the lamina propria of the gut in teleosts, but the aggregates are not histologically distinguishable as Peyer's patches. Only two immunoglobulin (Ig) heavy chain isotypes are present in teleosts, IgM and IgD

(TRAVER *et al.* 2003). However, despite the differences, the many advantages of zebrafish and the similarities to human infection response make zebrafish an attractive model to study antimicrobial immune responses and to screen for novel compounds that may be used clinically.

1.4 Toll-like Receptor Signaling

Toll-like receptors (TLRs) are an important class of pathogen recognition receptors (PRRs) that are involved in the innate immune response to bacterial infection. Toll-like receptors are expressed by various immune and nonimmune cells such as epithelial cells (AKIRA *et al.* 2006). TLRs recognize biochemical components, referred to as pathogen-associated molecular patterns (PAMPs), that distinguish bacteria, fungi, parasites, and viruses from multicellular animals (CINEL and OPAL 2009). TLRs also recognize endogenous damage/danger-associated molecular patterns (DAMPs), such as heat-shock proteins, neutrophil elastase, or fibrinogen (TSUJIMOTO *et al.* 2008). TLRs recognize their ligands at the plasma membrane and within endosomes, lysosomes, and endolysosomes (KAWAI and AKIRA 2010).

Ten functional Toll-like receptors (TLRs) have been identified in humans, designated TLR1-10 [Appendix A and Appendix B]. Fifteen putative variants of Toll-like receptors have been identified in the zebrafish genome, including 10 homologs to mammalian TLR genes and a fish-specific group of TLRs [Table 1] (JAULT *et al.* 2004; MEIJER *et al.* 2004; SULLIVAN and KIM 2008). At least 15 zebrafish TLR genes, abbreviated *z*TLR, are expressed at one day postfertilization (dpf) when the first macrophages and neutrophils enter the blood circulation (JAULT *et al.* 2004; MEIJER *et al.*

2004; VAN DER SAR *et al.* 2006). Sequencing of cloned *z*TLR2, *z*TLR4b, and *z*MyD88 genes identified the same intron-exon organization present in human TLR2, TLR4, and MyD88 genes (JAULT *et al.* 2004; MEIJER *et al.* 2004; VAN DER SAR *et al.* 2006).

Toll-like receptors (TLRs) are named for their homology to the *Drosophila* Toll receptor. The Toll protein is crucial to dorsal-ventral polarity in *Drosophila* embryos. In the adult *Drosophila*, the Toll protein is essential to the innate immune response against fungal infection (MEDZHITOV *et al.* 1997; AKIRA and TAKEDA 2004). The extracellular domain of Toll-like receptors contains 19-25 tandem copies of leucine-rich repeat (LRR) motifs. The LRR domains form a concave or indented surface important to microbial recognition (JIN and LEE 2008). The cytoplasmic portion of Toll-like receptors have a conserved region of approximately 200 amino acids, termed Toll/interleukin-1 receptor (TIR) domain for its parallel properties to interleukin-1 receptor. The TIR domain has three conserved regions theorized to be important for protein binding and receptor localization (BELVIN and ANDERSON 1996; IMLER and HOFFMANN 2001; WEBER *et al.* 2003; AKIRA and TAKEDA 2004; RALLABHANDI *et al.* 2006; KAWAI and AKIRA 2010).

Subsequent to ligand binding, Toll-like receptors dimerize, either as homodimers or heterodimers, and the TIR domain undergoes conformational changes that recruit a specified adaptor protein to elicit a specific immune response. There are four adaptor proteins: myeloid differentiation primary-response protein 88 (MyD88), TIR-associated protein or MyD88-adaptor-like (TIRAP/MAL), TIR-domain-containing adaptor protein-inducing IFN-β or Toll-IL-1 receptor-containing adaptor molecule 1(TRIF/TICAM-1), and TRIF-related adaptor molecule (TRAM). Zebrafish homologs of the human adaptor proteins and a negative regulator of TRIF-dependent TLR signaling (SARM) have been

identified, suggesting conservation of TLR signaling pathways from teleosts to mammals [Table 2]. In mammals, MyD88 can be recruited by all TLRs except TLR3. The TRIF adaptor is recruited by TLR3 and TLR4 to elicit a type I interferon response to viruses. All of the adaptor proteins have a carboxy-terminal (C-terminal) TIR domain, enabling complementary association with the TLR dimers, and an amino-terminal (N-terminal) death domain (DD) for subsequent recruitment of kinases (IMLER and HOFFMANN 2001; AKIRA and TAKEDA 2004; AKIRA *et al.* 2006; KAWAI and AKIRA 2010).

TLR signaling pathways are categorized as MyD88-dependent or TRIF-dependent. Association of MyD88 with TLRs results in the recruitment of IL-1 receptor-associated kinases, IRAK1 and IRAK4, to MyD88 through complementary death domains. IRAK4 becomes activated and phosphorylates IRAK1. Phosphorylated IRAK1 then associates with TRAF6, tumor-necrosis-factor-associated factor 6, thereby activating the ubiquitin E3-ligase function of TRAF6. In partnership with an ubiquitin E2 complex, TRAF6 catalyzes the synthesis of polyubiquitin chains that provide a temporary structure for recruiting both the TAK1 (transforming-growth-factor-β-activated kinase) and the IκB kinase (IKK) complexes. The large IκB kinase (IKK) complex consists of three subunits, IKK-α, IKK-β, and IKK-γ or NEMO (nuclear factor-κB essential modulator), and is a convergence point for many signaling pathways that lead to activation of the transcription factor nuclear factor-kappaB (NF-κB). It is the IKK-β subunit that subsequently phosphorylates the inhibitor of NF-κB, IκB, marking the IκB protein for rapid degradation. As a result, NF-κB is released from the inhibitor, exposing its nuclear localization signal, allowing translocation of the transcription factor

Table 1: Identified TLR Genes in Zebrafish. Data summarized from (JAULT *et al.* 2004; MEIJER *et al.* 2004).

Zebrafish (*z*) TLR Gene per **Jault** *et al.*	**Zebrafish (*z*) TLR Gene** per **Meijer** *et al.*	**Homolog**
*z*TLR 1.1	*z*TLR 1	*Human* TLR 1 (*h*TLR1); *Fugu rubripes* TLR 1 (*f*TLR 1)
*z*TLR 1.2		*h*TLR 2
*z*TLR 2	*z*TLR 2	*h*TLR 2; *f*TLR 2
*z*TLR 3	*z*TLR 3	*h*TLR 3; *f*TLR 3
*z*TLR 4.1a, 4.1b	*z*TLR 4a, 4b	*h*TLR 4
*z*TLR 5	*z*TLR 5a, 5b	*h*TLR 5; *f*TLR 5
*z*TLR 7a, 7b	*z*TLR7	*h*TLR 7; *f*TLR 7
*z*TLR 8.1	*z*TLR 8a, 8b	*h*TLR 8; *f*TLR 8
*z*TLR 8.2		*h*TLR 8
*z*TLR 9	*z*TLR 9	*h*TLR 9; *f*TLR 9
	*z*TLR 18	*f*TLR 18
	*z*TLR 19	
	*z*TLR 20a, 20b	
*z*TLR 21.1	*z*TLR 21	*f*TLR 21
*z*TLR 21.2		*f*TLR 21
*z*TLR 21.3		*f*TLR 21
*z*TLR 21.4a, 21.4b		*f*TLR 21
*z*TLR 21.5		*f*TLR 21
	*z*TLR 22	*f*TLR 22a, 22b

Table 2: Identified TLR-signaling Genes and IL-Receptor Genes in Zebrafish. Data summarized from (JAULT *et al.* 2004; MEIJER *et al.* 2004; PHELAN *et al.* 2005; SULLIVAN *et al.* 2007; SULLIVAN *et al.* 2009; LIU *et al.* 2010).

Zebrafish (*z*) Gene	**Homolog**
*z*MyD88	*Human* MyD88 (*h*MyD88); *Fugu rubripes* MyD88 (*f*MyD88); *Drosophila* MyD88 (*d*MyD88)
*z*TIRAP	*h*TIRAP; *f*TIRAP
*z*TRIF	*h*TRIF; *f*TRIF
*z*SARM	*h*SARM; *f*SARM
*z*IL-1R	*h*IL-1R
*z*IL-18R	*h*IL-18R; *f*IL-18R
*z*IRAK-4	*h*IRAK-4
*z*TRAF6	*h*TRAF6

across the nuclear membrane. DNA binding of NF-κB recruits RNA polymerase to initiate transcription of several genes including pro-inflammatory cytokines and chemokines, such as tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), IL-6, IL-12, type I interferons (IFNs), IL-8, and macrophage inflammatory protein-1 alpha (MIP-1α), depending on cell type and ligand (BEUTLER and RIETSCHEL 2003; AKIRA and TAKEDA 2004; JIMI and GHOSH 2005; LIU and MALIK 2006; VERSTREPEN *et al.* 2008).

In Gram-negative sepsis, it is Toll-like receptor 4 (TLR4) that recognizes endotoxin or lipopolysaccharide (LPS) and initiates cellular signaling [Table 1]. Monocytes from septic patients were found to have considerable increase in TLR2 and TLR4 expression compared to healthy individuals (TSUJIMOTO *et al.* 2008). Prior to TLR4 recognition, LPS is bound by LPS-binding protein (LBP) and by soluble cluster-of-differentiation-14 (sCD14) protein present in plasma. LBP and sCD14 catalytically transfer LPS to the membrane-bound CD14 (mCD14), a receptor anchored to the surface of macrophages by glycosylphosphatidylinositol (GPI). However, mCD14 does not have an intracellular signaling domain and it must deliver LPS to a TLR4/MD-2 receptor complex. Myeloid differentiation protein-2 (MD-2) is a secreted protein required for optimal TLR4-mediated signaling. MD-2 binds the extracellular domain of TLR4 forming a TLR4/MD-2 receptor complex. (SCHUMANN *et al.* 1990; SCHLETTER *et al.* 1995; YANG *et al.* 1996; AKASHI *et al.* 2000; MIYAKE *et al.* 2000; BEUTLER and RIETSCHEL 2003; RALLABHANDI *et al.* 2006). TLR4/MD-2 dimerization at the plasma membrane preferentially initiates MyD88-dependent signaling via the co-adaptor TIRAP to activate NF-κB. Interestingly, the dimerized TLR4/MD-2 receptor complex is subsequently endocytosed, releasing the TIRAP/MyD88 complex. This allows the co-

adaptor TRAM and the adaptor TRIF to interact with TLR4 and induce intracellular, TRIF-dependent signaling. This signaling pathway specifically leads to IFN-β production (KAGAN *et al.* 2008). Although the zebrafish TLR4 paralogs do not respond to LPS, exposure to the Gram-negative *Edwardsiella tarda* bacteria resulted in increased expression of the homologous signaling proteins *z*TLR3, *z*IRAK-4, and *z*TRAF6. In mammals, TRAF6 is a central intracellular, pro-inflammatory signaling protein used by all known mammalian TLRs, and is involved in antiviral and antibacterial responses (PHELAN *et al.* 2005).

The importance of the MyD88-dependent signaling pathway can be demonstrated by animal models and human patients deficient in key signaling proteins. MyD88-deficient mice infected with a virulent strain of *Mycobacterium avium* or with *Staphylococcus aureus* showed a considerably decreased immune response to both pathogens in comparison to wild-type mice (TAKEUCHI *et al.* 2000; FENG *et al.* 2003). Morpholino knockdowns of MyD88 in zebrafish embryos revealed impaired clearance of *Salmonela enteric* serovar Typhimurium Ra bacteria (VAN DER SAR *et al.* 2006). C3H/HeJ mice with mutated TLR4 were shown to be highly susceptible to infection by *Salmonella typhimurium* or *Neisseria meningitis* (AKIRA *et al.* 2006). In humans, a point mutation within the TLR4 gene causes an amino acid change in the extracellular domain of the receptor and is associated with increased risk of Gram-negative bacterial infection and an increased incidence of systemic inflammatory response syndrome (COOK *et al.* 2004; SCHWARTZ and COOK 2005; RALLABHANDI *et al.* 2006). Experiments using monocytes from humans with inherited IRAK4 deficiency demonstrated an absence of NF-κB activation. The IRAK4-deficient patients were susceptible to Gram-positive

Streptococcus pneumoniae and *Staphylococcus aureus* infections (PICARD *et al.* 2003). Mice lacking p50, a subunit of NF-κB, were unable to sufficiently eliminate infection with *Listeria monocytogenes* and were more susceptible to *Streptococcus pneumonia* infection (SHA *et al.* 1995). Interestingly, newborn mice lacking IκBα were severely runted, had skin defects, and increased granulopoiesis. The levels of nuclear NF-κB in thymocytes and splenocytes from these IκBα deficient mice were extremely elevated. At seven days, the IκBα deficient mice died, suggesting continuously increasing NF-κB activity can be lethal (BEG *et al.* 1995). Indeed, studies demonstrate correlation between NF-κB activity levels and disease severity in septic patients, where significantly higher levels are exhibited in nonsurviving patients compared to patients who survived (ARNALICH *et al.* 2000; LIU and MALIK 2006).

1.5 Tumor Necrosis Factor Alpha

Tumor necrosis factor alpha[1] (TNFα) is one of several pro-inflammatory cytokines activated by NF-κB. TNFα activates and regulates inflammatory reactions and cells of the immune system. TNFα is a major participant in LPS-mediated toxicity during sepsis (VOGL *et al.* 2007; DE GAUDIO *et al.* 2009). Experiments have demonstrated the significant involvement of TNFα in sepsis and septic shock. For example, when BALB/c mice were passively immunized against murine TNF, they were protected against the lethal effects of LPS (BEUTLER *et al.* 1985). TNF by itself is capable of inducing the same pathophysiologic effects provoked by LPS. This has been demonstrated in human

[1] The naming of tumor necrosis factor as TNF-alpha (α) is historical because TNF-beta (β) was subsequently designated as lymphotoxin. TNF, TNFα, and the former term cachectin refer to the same protein.

subjects, rats, goats, and dogs who received intravenous infusions of recombinant TNF (TRACEY *et al.* 1986; TRACEY *et al.* 1987; MICHIE *et al.* 1988). In addition to septic shock, dysregulated TNF levels are associated with numerous diseases ranging from chronic inflammatory disorders to cardiovascular diseases to several forms of cancer (AGGARWAL *et al.* 2006; FALVO *et al.* 2010).

The TNFα gene is an immediate early gene; it is transcribed rapidly in response to pathogens, stress, and other inflammatory proteins (FALVO *et al.* 2010). The TNFα gene is located on chromosome six between class I human leukocyte antigen (HLA)-B and class II HLA-DR loci in a region of the human genome that is highly polymorphic, the major histocompatibility complex (MHC) (FALVO *et al.* 2010). Regulation of TNFα gene expression has been shown to be cell type- and stimulus type-specific (FALVO *et al.* 2000; TSYTSYKOVA *et al.* 2007; FALVO *et al.* 2010). Mononuclear phagocytes, such as macrophages, are the main producers of TNFα. T cells, B cells, NK cells, mast cells, dendritic cells, and nonimmune fibroblasts also produce TNFα (FALVO *et al.* 2010). Receptors for TNFα, TNF-Receptor I and TNF-Receptor II, are present on almost all cell types (VAN DER POLL and LOWRY 1995). These receptors, as well as pattern recognition receptors (PRRs), initiate cellular signaling pathways that lead to TNFα expression. Regulation of TNFα gene expression is achieved by various mechanisms; for example, chromatin remodeling and enhanceosome formation. Repositioning of the nucleosomes proximal to the TNFα promoter regulates the accessibility of the promoter to transcription factors (EL GAZZAR *et al.* 2010). Expression of TNFα can be rapidly upregulated by enhanceosome complexes. These nucleoprotein complexes are composed of transcription factors and coactivators that bind to enhancer and promoter sequences.

This binding manipulates the DNA to form a higher-order structure that helps to increase the rate of transcription (FALVO *et al.* 2010).

In summary, the majority of novel drugs developed in animal and *in vitro* models for the treatment of sepsis proved to be ineffective in human clinical trials, namely anti-endotoxin and anti-TNFα. Although animal models are not optimal for replicating human disease, the zebrafish, *Danio rerio*, has tremendous potential as an animal model for drug discovery. The unique features of zebrafish, such as amendibility to high-throughput, large-scale screening of chemicals, the ability to study the innate immune system exclusively in young zebrafish, and the identification of Toll-like receptor homologs, make zebrafish a promising model for studying signaling events involved in sepsis and other diseases.

2 OBJECTIVE

The aim of this project is to establish a transgenic zebrafish model of signaling events following Toll-like receptor engagement. This transgenic model will provide an unbiased, universal means for studying the functionality of zebrafish TLRs using morpholino knock-down experiments and testing of ligands, and for determining compounds and genes that affect TNFα production through large-scale chemical and genetic screening. These studies could aid in the characterization and understanding of pathways that lead to sepsis and septic shock. Additionally, this transgenic zebrafish model will serve as a versatile research tool for the scientific community to study immune responses in a number of infection-related diseases.

3 METHODS

3.1 Zebrafish Care and Experimental Techniques

3.1.1 Maintenance and Handling

Zebrafish were handled and cared for according to the Institutional Animal Care and Use Committee (IACUC) guidelines. See http://www.iacuc.org/, http://zfin.org, and http://www.nal.usda.gov/awic/pubs/noawicpubs/educ.htm for detailed information.

3.1.2 Strain

**AB*, pronounced star-AB, wild-type line was microinjected with the different expression clones. Refer to http://zfin.org/action/genotype/detail?genotype.zdbID=ZDB-GENO-960809-7 for further information.

3.1.3 Sperm Squeezing, Breeding, and Tail Fin Clipping

Sexually-mature male zebrafish, at least 10-12 weeks old, were anesthetized with tricaine (3-aminobenzoic acid ethyl ester methanesulfonate) and placed belly-side up inside a slit of a dampened sponge. Anal fins were placed to the sides of the fish, and the anal area was dabbed with a Kimwipe to remove excess water that can activate sperm. Sperm were collected into 1.5 μL glass capillary tubes by gently squeezing underneath and somewhat posterior to the anus with forceps. The male fish were then isolated in separate smaller tanks with appropriate water lacking tricaine to ensure restoration of

consciousness, and the fish remained in isolation until their sperm was screened by PCR. Males that were positive by PCR were placed back on the water system and separated to maintain identification.

On the evening before spawning, male and female zebrafish were placed in a small breeding tank and separated by a divider. Spawning and subsequent embryo formation occurred the following morning after removal of the divider. Embryos were incubated at 28.5°C in E3 water supplemented with methylene blue, an antifungal and antibacterial compound. Maintenance of the embryos included daily water changes and removal of dead embryos. Hatched larvae were added to the nursery section of the recirculating water system where larvae received specialized feeding. After 28 days, juvenile zebrafish were moved to an adult section within the recirculating system (LEVRAUD *et al.* 2008).

Prior to clipping the tail fin, zebrafish were anesthetized with tricaine. Anesthetized fish were then placed on plastic lid and a small section of the top of the tail fin was removed with a razor blade. The fish were then isolated in separate smaller tanks with appropriate water lacking tricaine to ensure restoration of consciousness, and the fish remained in isolation until their tail fin DNA was screened by PCR. Zebrafish that were positive by PCR were placed back on the water system and separated to maintain identification.

3.1.4 DNA Extractions and PCR Screening of Zebrafish

DNA from sperm, embryos, and tail fin clippings was extracted using a modified HotSHOT (hot sodium hydroxide and Tris) Protocol (MEEKER *et al.* 2007). Tissues were

placed into a microcentrifuge tube containing 50 mM NaOH: 50 µL for sperm, 75-100 µL per 20-30 embryos, and 100 µL for clipped tails. Specimens in NaOH were vortexed, microcentrifuged, and then heated to 98°C for 10-20 minutes using a thermocycler. After cooling, one-tenth the volume of 1M Tris-HCl at pH of 8.0 was added to each specimen for a final dilution of 1:11 Tris-HCl to NaOH. The Tris-HCl reagent neutralizes the extracted DNA in preparation for PCR. Specimens were microcentrifuged to pellet debris, and 1-5 µL of the supernatant was used for PCR in a total reaction volume of 25 µL. PCR was performed using 10X PCR Buffer, 2.5mM dNTPs, 5µM each of the appropriate forward and reverse primers, molecular grade water, and *Taq* Polymerase diluted to 1:8 with enzyme diluents. The DNA Engine Tetrad thermocycler made by MJ Research was used for cycling the PCR reactions.

3.1.5 Microinjections

The microinjection system used consisted of a Nikon® SMZ645 stereo microscope, a three-dimensional mechanical micromanipulator with micropipette holder made by the Narishige Group, and an automatic pressure regulator, model PLI-100 Basic Pico-Injector by Harvard Apparatus. The pressurized injector permits small liquid volumes to be delivered precisely through the micropipettes. Micropipettes, or needles, were made from fire-polished borosilicate glass capillaries made by Sutter Instrument with an outer diameter (O.D.) of 1.0 mm, an inner diameter (I.D.) of 0.75 mm, and a length of 10 cm. The needle puller instrument used was model P-97 by Sutter Instrument.

Microinjection plate molds were made with Seakem® LE agarose by Lonza at 1.2% in 1X E3 water (deionized water with 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$) using a TU-1 microinjection mold by Adaptive Science Tools. Molds were made in a 100 mm by 20 mm polystyrene petri dish. Injection volumes were calibrated using an eyepiece graticule where five tick marks viewed through a 5X objective corresponds to a diameter of 100 μm and a volume of 520 picoliters (pL). Injection of DNA concentrations higher than 0.1 mg per mL is toxic to the embryo and causes higher lethality (XU 1999; XU *et al.* 2008). 520 pL of mixture containing expression clone DNA and transposase cRNA were co-injected into embryos at a final concentration of 20 ng per μL. Phenol red dye, at a final concentration of 0.0625%, was added to the injection mixture to help visualize the material.

3.1.6 Fluorescent Microscopy

Detection of enhanced green fluorescent protein (EGFP), *Discosoma* red fluorescent protein (DsRed), and tandem-dimer Tomato (tdTomato) fluorescent protein was accomplished using a fluorescent microscope. Excitation of fluorescent molecules is achieved with an ultraviolet (UV) light source. Fluorescent molecules absorb the UV light at a specific wavelength and emit the light at a longer wavelength [Table 3].

3.2 Gateway Cloning Technology

Gateway® Cloning Technology by Invitrogen™ is based on the site-specific recombination system used by the lambda (λ) bacteriophage to integrate into and excise from the *E.coli* chromosome. The λ phage enzyme Integrase (Int) and the *E. coli*

Table 3: Excitation and Emission Wavelengths of Specified Fluorescent Proteins (YARBROUGH *et al.* 2001; SHANER *et al.* 2004; SHANER *et al.* 2007; RIZZO *et al.* 2009).

Fluorescent Protein	Excitation Wavelength (nm)	Emission Wavelength (nm)	Spectral Color
EGFP	488	507	Green
DsRed	558	583	Red/Orange
tdTomato	554	581	Red/Orange

Integration Host Factor (IHF) protein catalyze integration, while an additional λ phage enzyme, Excisionase (Xis), is required for excision. The enzymes bind specifically to recombination sites or attachment sites, denoted as *att* sites: *att*B for bacterial and *att*P for phage. The recombination sites used in Gateway® Cloning are designed with several point mutations in contrast to wild-type sites to confer an even higher specificity (INVITROGEN™ 2006; INVITROGEN™ 2010a; INVITROGEN™ 2010b).

To generate an expression vector that contains a transgene and a reporter gene, the transgene must be amplified by PCR with primers containing appropriate *att*B sites. The PCR product and a donor vector (pDONR) containing *att*P sites and selection genes are then combined with BP Clonase™ II enzyme mix to generate an entry clone or entry plasmid (pENTR) [Figure 1]. BP Clonase™ II enzyme mix contains Integrase and Integration Host Factor proteins to catalyze the recombination between *att*B and *att*P sites to create *att*L and *att*R sites within the entry clone. Two to six entry clones can be made in separate BP recombination reactions depending on the design of the final expression clone.

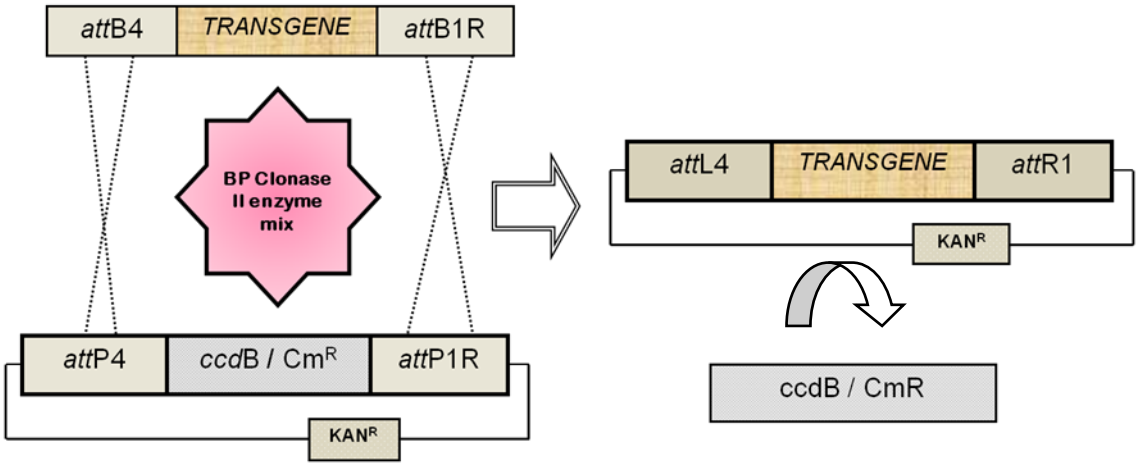

Figure 1. Gateway BP Reaction. KAN^R=kanamycin resistance gene.

The entry clones are then combined with a destination vector (pDEST) and with LR Clonase™ II enzyme mix to produce the expression clone. The LR Clonase™ II enzyme mix contains the Integrase, Integration Host Factor, and Excisionase proteins. The destination vector contains a selective *ccd*B gene and a selective chloramphenicol resistance (Cm^R) gene. The ccdB protein is toxic to most *E. coli* and will be produced in bacteria containing destination vectors that do not successfully undergo the LR recombination reaction. Initially, before the production of the ccdB protein, these bacteria colonies will be capable of growing on media with chloramphenicol and can be excluded. Additionally, the destination vector has been engineered to contain *Tol*2 sites, discussed in Section 3.2, to facilitate transposition into the host genome (INVITROGEN™ 2006; INVITROGEN™ 2010a; INVITROGEN™ 2010b). A basic 2-cassette expression clone has a 5′ element (5E) and a 3′ element (3E) [Figure 2]. The 3-cassette expression clone has a middle element (ME) in addition to the 5′ and 3′ elements [Figure 3].

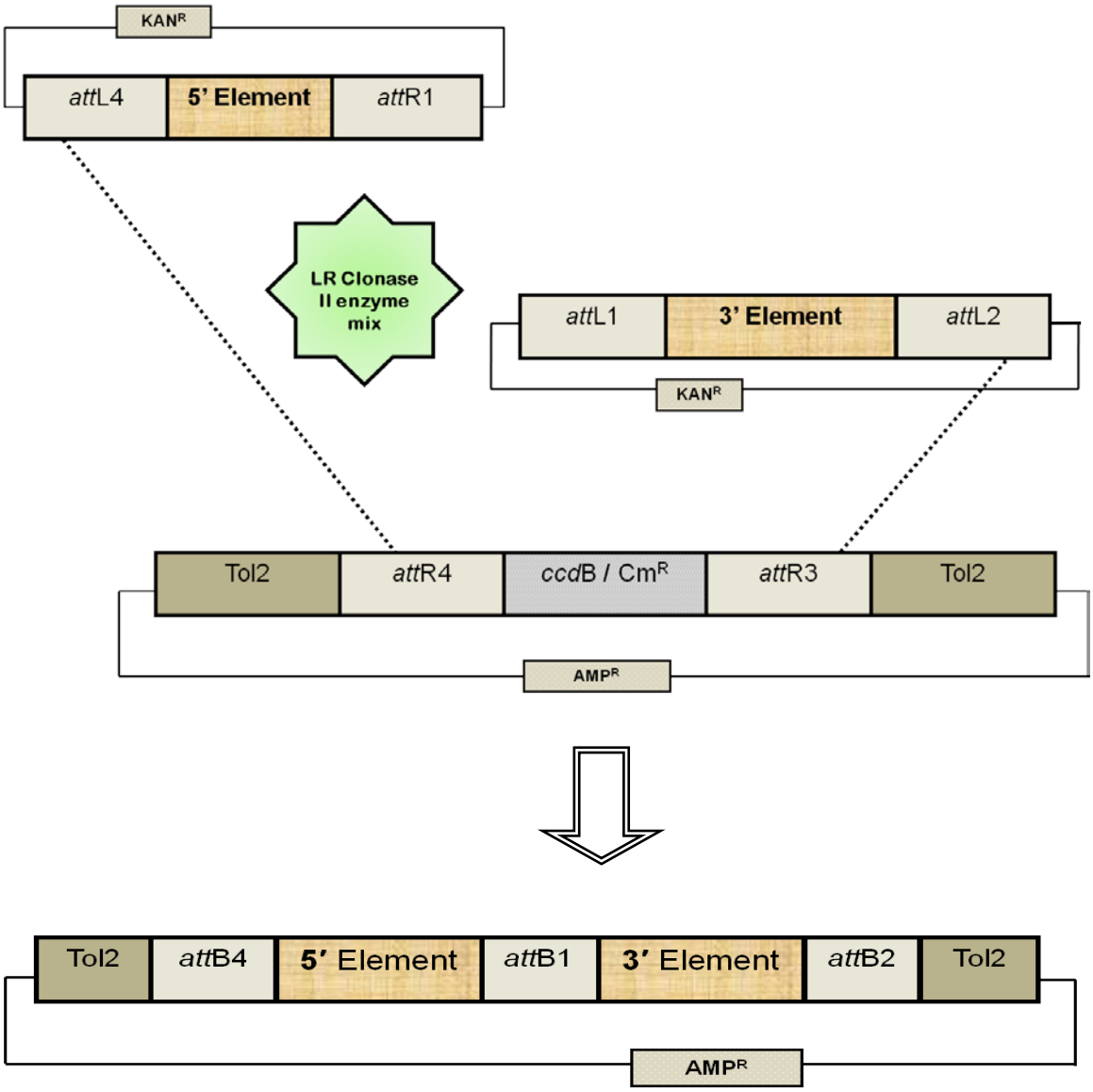

Figure 2. Gateway LR Reaction for Two-cassette Expression Clone. AMP^R=Ampicillin resistance gene.

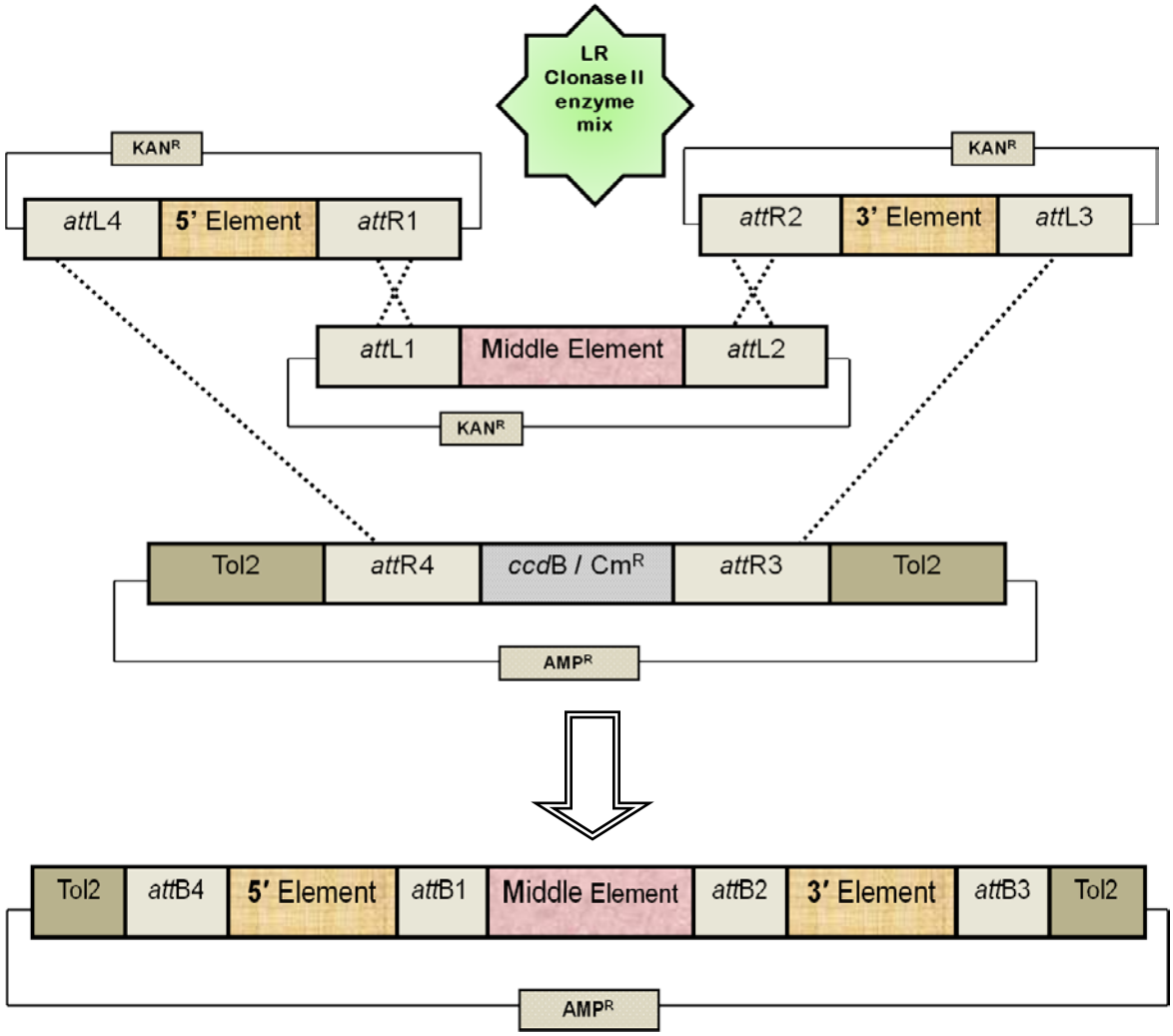

Figure 3. Gateway LR Reaction for Three-cassette Expression Clone.

3.3 *Tol2* Transposition System

The *Tol2* sites present in the destination vector originate from the *Tol*2 gene identified in the genome of the medaka fish, *Oryzias latipes*, a fresh water teleost (KOGA *et al.* 1996). To date, *Tol2* is the only autonomously-active transposable element found in a vertebrate genome. The *Tol2* gene, approximately 4.7 kilobases (kb) in length, encodes a transposase protein that is autonomously active, meaning the protein is capable of catalyzing the excision and integration reaction by itself (KAWAKAMI *et al.* 1998; KAWAKAMI *et al.* 2004a; KAWAKAMI and NODA 2004; HAMLET *et al.* 2006; KAWAKAMI 2007; SATO *et al.* 2007). *Tol2* transgenesis in zebrafish is accomplished by microinjecting a transposon-donor plasmid, such as the final expression clone, along with synthetic transposase messenger RNA (mRNA) into one-cell stage embryos. Synthetic transposase mRNA or complementary RNA (cRNA) must be co-injected with the expression clone because a significant portion of the transposase coding region is not included in the donor plasmid. The transposon-donor plasmid just provides the non-autonomous, *cis*-recognition sequences necessary for transposition. The minimal *cis*-sequences required for transposition are 200 base pairs (bp) from the 5′ end and 150 bp from the 3′ end of the *Tol2* gene (URASAKI *et al.* 2006). The abundance of translation proteins in the embryo will generate transposase protein from the synthetic mRNA. The transposase will then carry out the excision and integration of the transgene.

3.4 Recombineering BAC Clones in *Escherichia coli*

Bacterial artificial chromosomes (BACs) are carriers or vectors of DNA sequences that are capable of entering bacteria for subsequent molecular cloning. BAC

vectors are derived from the fertility (F) plasmid of certain bacteria and possess necessary replication and selection tools. BAC vectors can accommodate long lengths of genomic DNA up to 350 kilobases, a characteristic that makes BAC vector great tools for genetic research. Methods have been developed for modifying the DNA cloned into BAC using homologous and site-specific recombination within *Escherichia coli*. This approach is highly efficient and less time-consuming for introducing linear DNA into BAC clones. Utilizing recombination within *E. coli* to modify DNA cloned into BACs is referred to as recombineering.

The recombineering method employed for this project uses a modified *E. coli* strain, EL250 cells, which possess an intentionally defective lambda (λ) prophage. This λ prophage provides the genes necessary for homologous and site-specific recombination within the EL250 cells. The λ *red*, *exo*, and *bet* genes provide homologous recombination enzymes. A genetically engineered version of the wild-type *flp* gene provides a site-specific recombination enzyme with high recombination efficiency.

Used for homologous recombination, the λ *gam* gene provides a protein that inhibits the *E. coli* RecBCD nuclease from destroying electroporated linear DNA. *Exo*, *bet*, and *gam* genes are under the control of the P_L operon, which is controlled by a temperature-sensitive λ repressor protein, cI857. At 32°C, the repressor protein blocks P_L operon activity. When the EL250 bacteria are heated to 42°C, the repressor no longer inhibits the P_L operon and e*xo*, *bet*, and *gam* recombination proteins are expressed (LEE *et al.* 2001; LIU *et al.* 2003).

The *flpe* gene is derived from a 2 μm plasmid of the *Saccharomyces cerevisiae* yeast. The flippase (Flpe) protein will specifically recombine sequences between Flippase Recognition Target (FRT) sites. The FRT site sequence is: 5′-GAAGTTCCTATTCtctagaaaGTATAGGAACTTC-3′, where lower case indicates the asymmetric core region. The asymmetric core and the placement of the FRT sites dictate whether the intervening DNA will be excised or inverted. Excision will occur when the FRT sites are placed in the same orientation [Figure 4].

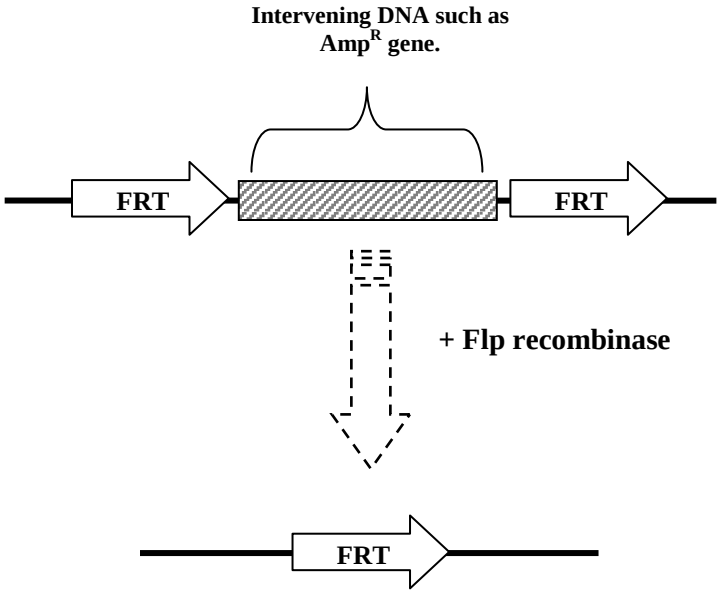

Figure 4. Flp/FRT Recombination.
Adapted from figure 2 of (SCHWEIZER 2003).

3.5 Lipofection

Lipofection or liposomal transfection is a technique for introducing genetic material such as expression clones into cultured cells by means of liposomes. Synthesized cationic lipids form liposomes that interact with anionic DNA. The DNA-containing liposomes fuse with the plasma membrane of cultured cells, resulting in delivery and expression of DNA within the cells (FELGNER *et al.* 1987; MOK and CULLIS 1997). To test the inducibility of the two-cassette expression clone, cultured Jurkat cells, a human leukemia T cell line, were lipofected with the expression clone using the *Trans*IT®-LT1 Transfection Reagent by Mirus Bio LLC. Following lipofection, Jurkat cells were stimulated with PMA (phorbol 12-myristate 13-acetate) and Ionomycin from *Streptomyces conglobatus* (Sigma Aldrich®). The *Trans*IT®-LT1 Transfection Reagent has been used successfully in several studies, including a luciferase reporter assay that helped characterize a novel regulatory pathway involved in an NF-κB-mediated proinflammatory response to respiratory syncytial virus infection (YOBOUA *et al.* 2010).

4 RESULTS

4.1 Approach 1: Two-Cassette Design

4.1.1 Two-Cassette Expression Clone

The Gateway® cloning system by Invitrogen was used to generate an expression clone containing the basal carp beta-actin (β-actin) promoter, the enhanced green fluorescent protein (EGFP) gene, and the human TNFα (*h*TNFα) enhancer donated by Dr. Anne Goldfeld, MD (GOLDFELD *et al.* 1990) [Figure 5]. The *h*TNFα enhancer sequence is: 5′-ATGCTTGTGTGTCCCCAACTTTCCAAATCCCCGCCCCCGCGATGGAGAA GAAACCGAGACAGAAGGTGCAGGGCCCACTACCGCTTCCTCCAGATGAGCTC ATGGGTTTCTCCACCAAGGAAGTTTTCCGCTGGTTGAATGATTCTTTCCCCGC CCTCCTCTCGCCCCAGGGACATATAAAGGCAGTTGTTGGCACACCCAGCCAG CAGACGCTCCCTCAGCAAGGACAGCAGAGGACCAGCTAAGAGGGAGAGAAG CAACTACAGACCCCCCCTGAAAACAACCCTCAGACG-3′

A human enhancer was chosen because the zebrafish TNFα (*z*TNFα) promoter has not been characterized functionally. Thus, it is unclear where exactly the important regulatory elements are located. A previous attempt, using a 5.0 kilobase sequence located upstream of the zebrafish TNFα gene, did not produce an inducible expression model; data not published (REDD 2007) [Figure 6].

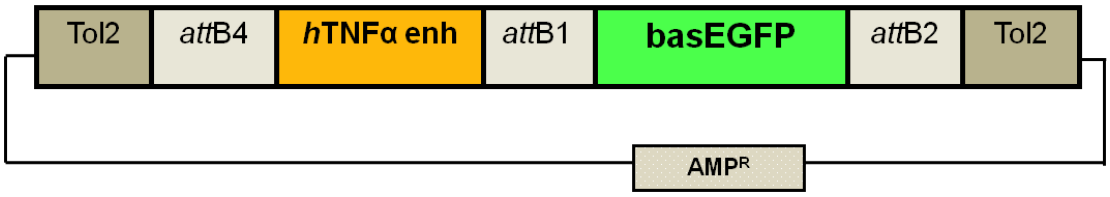

Figure 5. Two-cassette Expression Clone for First Approach

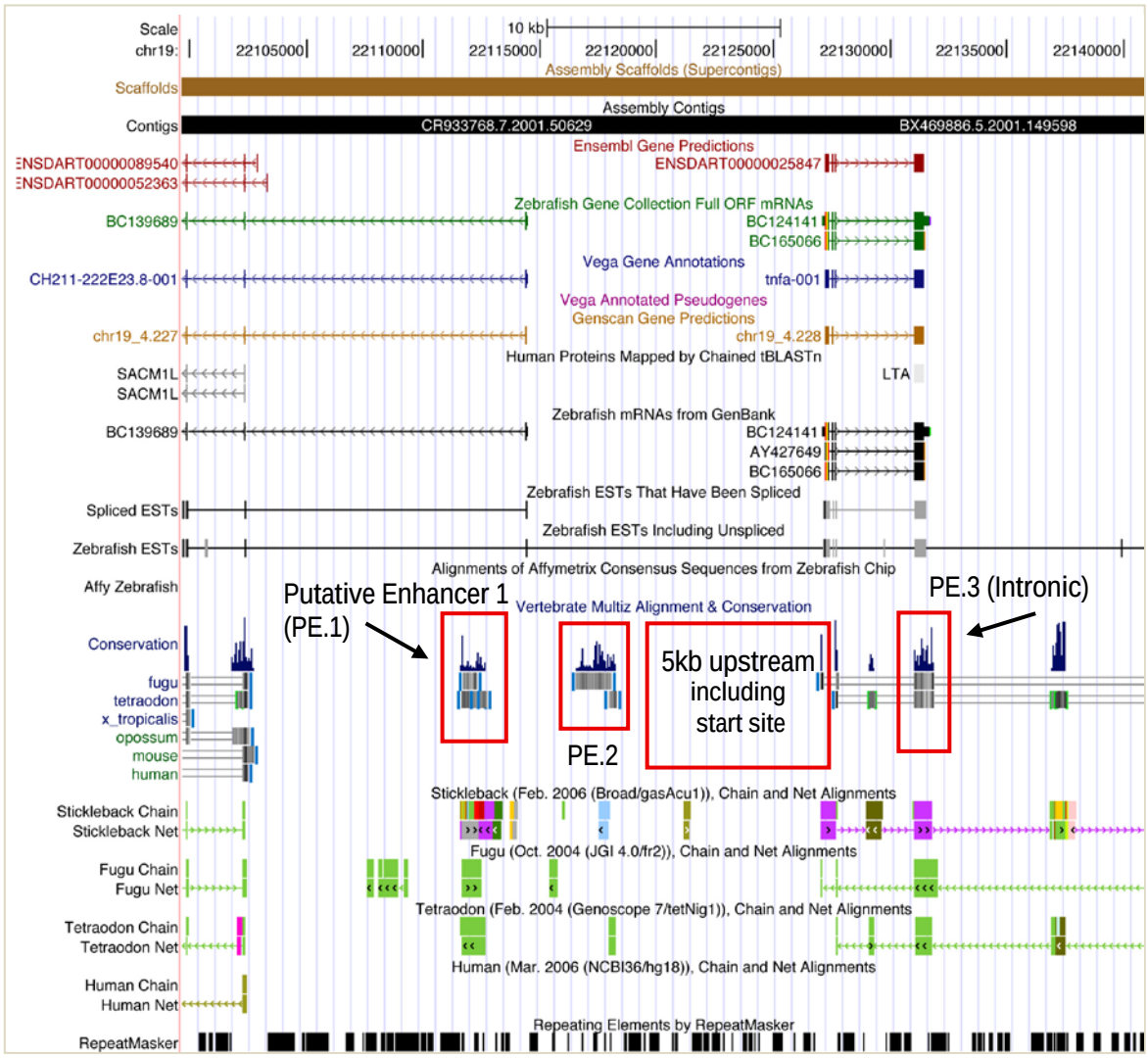

Figure 6. Putative Enhancers for *z*TNFα. UCSC Genome Browser: Zebrafish March 2006 Assembly (Zv6/danRer4). The image shows a view of chromosome 19 at position 22,099,500-22,142,000 approximately. http://www.genome.ucsc.edu (KENT *et al.* 2002).

Additionally, although genome database searches reveal conserved putative enhancers of the TNFα gene between sequenced teleosts, such sequence conservation is not seen between mammals and teleosts. In this scenario, the options are to use either regulatory elements from a different species that drives TNFα expression or to use a bacterial artificial chromosome (BAC) containing the entire zebrafish TNFα gene that should provide the majority of *cis*-regulatory elements for expression of a transgene.

Established mammalian enhancers have been used successfully in zebrafish to induce expression of reporter genes in a tissue-specific manner (FISHER *et al.* 2006). Therefore, we first chose the well-characterized human TNFα (*h*TNFα) enhancer in conjunction with the EGFP reporter gene under control of basal carp β-actin promoter, abbreviated as bas or bactin, to drive expression of the EGFP reporter gene in zebrafish cells that are expressing *z*TNFα (CALDOVIC and HACKETT 1995).

The *h*TNFα enhancer was provided in the pOCAT vector that contains an ampicillin resistance gene (GOLDFELD *et al.* 1990). Plasmid DNA was extracted using the Wizard® *Plus* Miniprep DNA Purification System by Promega. PCR amplification of the *h*TNFα enhancer was accomplished using Advantage® *Taq* polymerase, the CA55S thermocycler protocol [Appendix D], and primers designed to contain the appropriate *att*B sites as well as a few sequences from the 5′- and 3′-end of the *h*TNFα enhancer (*att*B *h*TNFα enhancer forward and reverse primers) [Appendix C]. PCR gel bands were excised, extracted using Qiagen's QIAquick® Gel Extraction Kit, and sequenced using a *h*TNFα enhancer sequencing primer [Appendix C]. The *att*B-flanked *h*TNFα enhancer cDNA was cloned into the pDONR P4-P1R vector by Invitrogen using BP Clonase™ II enzyme mix to generate a 5′ Entry clone. The 5′ Entry clone was transformed into DH5α competent *E. coli* cells which were subsequently grown in S.O.C. media; *E. coli* cells and media are made by Invitrogen™ by Life Technologies™. Selection of transformed bacteria was performed using Lysogeny broth (LB) media with 50 μg per mL of Kanamycin. Twenty colonies were chosen for PCR screening using M13 forward and reverse primers that amplify the region where *h*TNFα enhancer incorporates via *att* recombination sites [Appendix C]. The CA55S thermocycler protocol was used for this

PCR amplification [Appendix D]. Two of the twenty colonies were positive by PCR and were subsequently subcultured, followed by DNA extraction using Qiagen's QIAprep® Spin Miniprep Kit. Extracted DNA was sequenced using the same M13 forward and reverse primers. Contig analysis of the sequencing results demonstrated the presence of the *h*TNFα enhancer within the pDONR P4-P1R vector flanked by appropriate *att*R1 and *att*L4 sites.

The pENTRbasEGFP vector provided by Dr. N.D. Lawson's laboratory at the University of Massachusetts was used as the 3′ Entry clone in this two-cassette design. The 3′ and 5′ ENTRY clones were combined with the destination vector pTolR4R2pA, also provided by Dr. Lawson's laboratory, in an LR Clonase™ II enzyme reaction mix to produce the final expression clone (VILLEFRANC *et al.* 2007). The expression clone was transformed into DH5α competent *E. coli* cells which were subsequently grown in S.O.C. media. Positive selection of transformed bacteria was performed using LB media with 50 μg per mL of ampicillin. Fourteen colonies grew on the selection media and were by screened by PCR for the presence of *Tol2* and EGFP. DNA was amplified using a modified CA55S thermocycler protocol [Appendix D] and by using the forward primer Tol2_F, designed to anneal at the *Tol2* site adjacent to the 5′ entry cassette, and the reverse primer EGFP_R, designed to anneal at the 3′-end of the EGFP gene [Appendix C]. All 14 colonies were positive by PCR. All of the colonies were subcultured, followed by DNA extraction using Qiagen's QIAprep® Spin Miniprep Kit. Three positive colonies were chosen for sequencing confirmation using primers that anneal to the *h*TNFα enhancer (Expr_F), the EGFP gene (Expr_R), and the flanking *Tol2* sites (Tol2_F; Tol2_R_b) [Appendix C]. Contig analysis of the sequencing results

demonstrated the presence of a correct expression clone containing the *h*TNFα enhancer element, the basal carp beta-actin promoter, and the EGFP gene within the pTolR4R2pA vector.

4.1.2 Lipofection and Drug Induction of Jurkat Cells

Prior to microinjection, the inducibility of the two-cassette expression clone was tested by lipofection into the human leukemia T cell line Jurkat using the *Trans*IT®-LT1 Transfection Reagent by Mirus Bio LLC followed by stimulation with PMA (phorbol 12-myristate 13-acetate) and Ionomycin from *Streptomyces conglobatus*, supplied by Sigma Aldrich®. PMA and Ionomycin are known to activate protein kinase C which targets the transcription factors NF-κB and AP-1 (SAITOH and DOBKINS 1986; CHATILA *et al.* 1989; ISAKOV and ALTMAN 2002). Also, PMA induction of TNFα expression has been shown in T and B cell lines; specifically, HUT78, P30, HPB-ALL, and Jurkat T cell lines and the Raji B cell line (GOLDFELD *et al.* 1991). Jurkat cells were cultured in RMPI 1640 medium with 10X Fetal Bovine Serum and 1X Penicillin and Streptomycin (Invitrogen™ by Life Technologies™). Jurkat cells were grown overnight in a T75 culture flask at 37°C and 5% CO_2 incubator. Cells were quantitated with a hemocytometer and then replated into a 6-well cell-culture plate at a concentration of $1x10^6$ cells per mL.

The Jurkat cells in wells 4-6 were lipofected for five hours. Jurkat cells in wells 1-3 were not lipofected [Figure7]. Additional culture medium was added to bring the total volume of each well to 3 mL. Subsequently, the lipofected cells in wells 5 and 6 were stimulated with PMA and Ionomycin. Concentrations of PMA and Ionomycin were chosen based on previous experiments using Jurkat cells (BALLESTER *et al.* 1998).

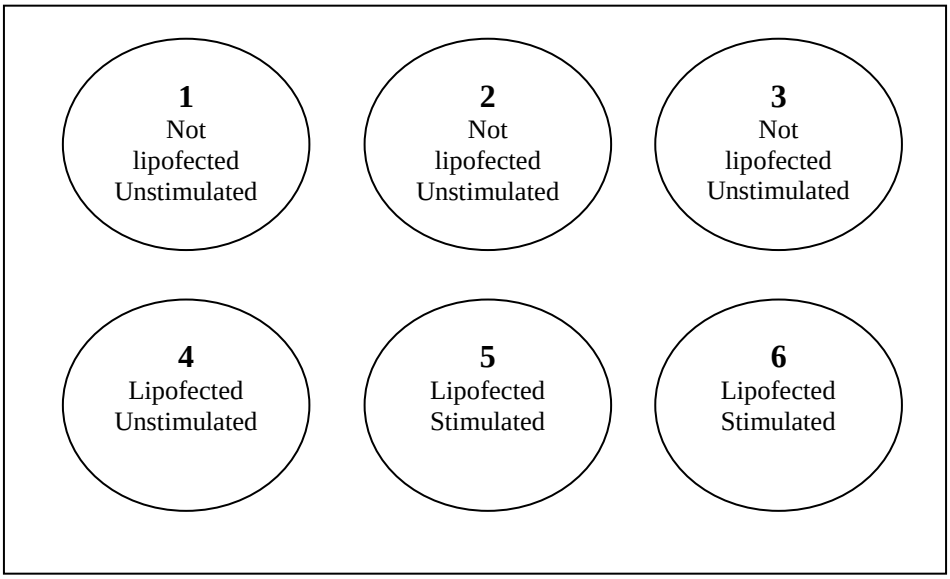

Figure 7. Lipofection and Drug Stimulation Assay. 6-well plate with Jurkat cells at a concentration of $1x10^6$ cells per mL in each well.

The final concentrations of PMA and Ionomycin in well 5 were 50ng per mL and 0.25μM, respectively. The final concentrations of PMA and Ionomycin in well 6 were 250.9 ng per mL and 0.125μM, respectively.

Cells were examined for EGFP fluorescence prior to stimulation, at 7.5 hours poststimulation, 24 hours poststimulation, and 48 hours poststimulation. "Leakiness," basal transgene expression in the absence of stimulation, was assessed in well 4. Fluorescent cells were quantitated between 49 and 52 hours poststimulation. Only cells showing bright green fluorescence were counted; cells showing faint green fluorescence were not counted [Figure 8]. Jurkat cells lipofected with the two-cassette expression clone demonstrated dose-dependent fluorescence [Figure 9]. Bright fluorescent cells were attributed to successful enhancer-driven expression of EGFP. The faint fluorescence was attributed to background fluorescence produced by the basal promoter.

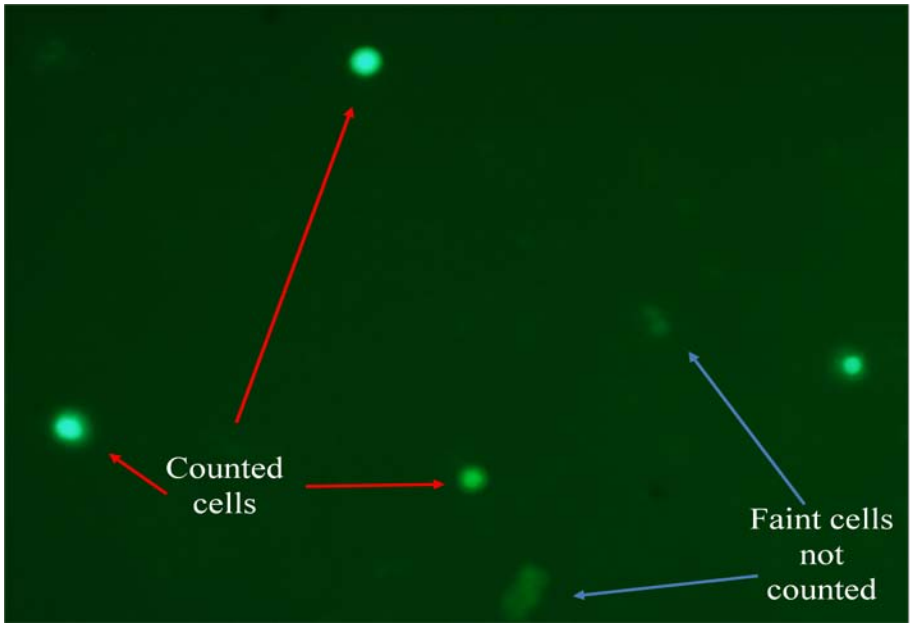

Figure 8. Well 6 at 24 Hours Poststimulation.

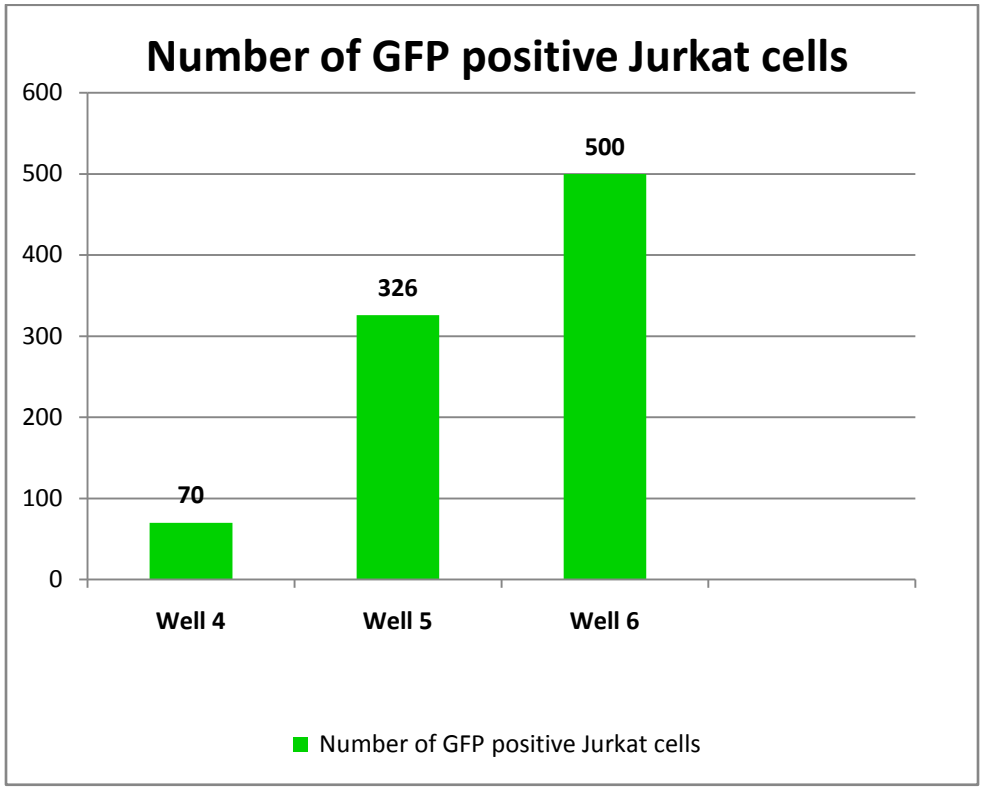

Figure 9. Graphical Chart Representation of Quantitated Fluorescent Cells per Well.

4.1.3 Microinjection and PCR Screening

Between November 2008 and February 2009, 382 zebrafish embryos obtained from the **AB* wild-type strain were injected at the one-cell stage with of the two-cassette expression clone containing the *h*TNFα enhancer, the carp beta-actin basal promoter, and the enhanced green fluorescent protein gene, along with transposase cRNA and phenol red dye. DNA and transposase cRNA were at a final concentration of 20 ng per μL, while phenol red dye was at a final concentration of 0.0625%. Approximately 271 of the 382 injected embryos survived to adulthood. Sperm were collected from 74 males and fertilized eggs from 17 females. Females were bred with **AB* wild-type males to obtain embryos at 24 hours postfertilization (hpf) which have a greater amount of DNA compared to a single-celled egg. DNA was extracted from the sperm and embryos using the modified HotSHOT protocol and screened by PCR for the presence of the enhanced green fluorescent protein (EGFP) gene using EGFP_F and EGFP_R primers and the EGFP thermocycler protocol [Appendix C and D]. Genomic DNA from the tail fin of a wild-type zebrafish, most often **AB* wild-type, was used as a negative control. The pENTRbasEGFP vector was used as the positive control. A tube of PCR reagents without any DNA template served as a negative reagent control. A separate PCR amplification was performed simultaneously to provide a quality control check of the DNA extraction and of *Taq* polymerase performance. This PCR reaction using the EGFP thermocycler protocol confirms the presence of DNA using primers that target a marker on zebrafish chromosome eight, named Z60737 [Appendix C]. Unfortunately, the germ-line cells from all 91 zebrafish were negative for the EGFP gene. This prompted the design of a new three-cassette expression clone.

4.2 Approach 2: Three-Cassette Design

4.2.1 Three-Cassette Expression Clone

A three-cassette expression clone was created to introduce a second fluorescent reporter protein gene, *Discosoma* red fluorescent protein (DsRed), under control of the *Xenopus laevis* γ-crystallin promoter, Cry (SMOLICH *et al.* 1993; OFFIELD *et al.* 2000; YARBROUGH *et al.* 2001). Crystallins are expressed at high levels in the lens of the eye. As a result, the γ-crystallin promoter makes DsRed expression lens-specific. Addition of this lens-specific fluorescent protein facilitates preselection of putative transgenic founders. The presence of red fluorescence in the lens should be associated with a higher frequency of expression clone transgenesis into germ-line cells (VOPALENSKY *et al.* 2010). Therefore, only zebrafish with red fluorescent lenses will be further screened for germ-line transgenesis.

An ENTRY vector containing the CryDsRed insert was obtained from Dr. H. J. Yost's laboratory at the University of Utah. The CryDsRed vector is the 3′ ENTRY clone in this design. The 5′ ENTRY clone is the same vector used in the two-cassette design. The pENTRbasEGFP vector becomes the middle entry clone for this three-cassette expression clone design [Figure 10].

The 3′, Middle, and 5′ ENTRY clones were combined in an LR Clonase™ II

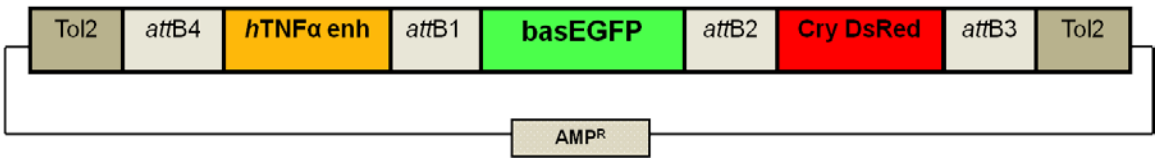

Figure 10. Three-cassette Expression Clone for Second Approach.

enzyme reaction with the destination vector pDEST Tol2pA, which contains the appropriate *att* recombination sites for generating a three-cassette expression clone (KWAN *et al.* 2007). The destination vector pDEST Tol2pA is provided by Dr. N.D. Lawson's laboratory at the University of Massachusetts. The final expression clone was transformed into Library Efficiency® DH5α™ Competent *E. coli* cells which were subsequently grown in S.O.C. media. Positive selection of transformed bacteria was performed using LB media with 50 μg per mL of ampicillin. Thirty colonies were chosen for additional concurrent selection using LB media with 50 μg per mL of ampicillin for positive selection, and LB media with 12.5 μg per mL of chloramphenicol for negative selection. The 30 colonies were screened by two separate PCR amplifications using different sets of primers. The first amplification was accomplished with expression clone primers and the EXPR thermocycler protocol; Expr_F anneals to the *h*TNFα enhancer and Expr_R anneals to the EGFP gene. The second amplification was performed using the DSRED thermocycler protocol and using primers designed to anneal to the DsRed gene (DsRed_F; DsRed_R) [Appendix C and D]. Twenty-nine of thirty colonies were positive for the DsRed gene, the *h*TNFα enhancer, and the EGFP gene. Three colonies were chosen for subculturing and DNA extraction using Qiagen's QIAprep® Spin Miniprep Kit. DNA from the three colonies was sequenced to confirm the presence of the expression clone using a reverse primer (ME_Rev) designed to anneal to the middle entry cassette and amplify the middle and 5′ entry cassettes, and a reverse primer (3E_Rev) designed to anneal to the crystalline promoter of the 3′ entry cassette and amplify the 3′ and middle entry cassettes [Appendix C]. The three colonies contained all three cassettes.

4.2.2 Microinjection and PCR Screening of Three-Cassette Expression Clone

Between November and December of 2009, approximately 1,040 zebrafish embryos obtained from the **AB* wild-type strain were injected at the one-cell stage with 520 pL of the three-cassette expression clone along with transposase cRNA and phenol red dye. DNA and transposase cRNA were at a final concentration of 20 ng per μL, and phenol red dye at a final concentration of 0.0625%. Approximately 938 of 1,040 injected embryos survived to adulthood and were screened by fluorescent microscopy for the presence of red fluorescent lenses. Fourteen fish were positive for lens-specific red fluorescence: five fish with both eyes, and nine fish with only one eye, a partial eye(s), or a possible gill location [Figure 11].

Six of the fourteen DsRed-positive fish died before they were sexually mature for germ-line PCR screening. Sperm and fertilized eggs from seven of the eight remaining DsRed-positive fish, three males and four females, were screened by PCR for the presence of the EGFP gene using EGFP_F and EGFP_R primers and the EGFP thermocycler protocol [Appendix C and D]. Genomic DNA from the tail fin of a wild-type zebrafish served as a negative control, the pENTRbasEGFP vector served as the positive control, and a tube of PCR reagents without any DNA template served as a negative reagent control. A separate PCR amplification was performed simultaneously to provide a quality control check of the DNA extraction and of *Taq* polymerase performance. This PCR reaction using the EGFP thermocycler protocol confirms the presence of DNA using primers that target a marker on zebrafish chromosome eight, named Z60737 [Appendix C].

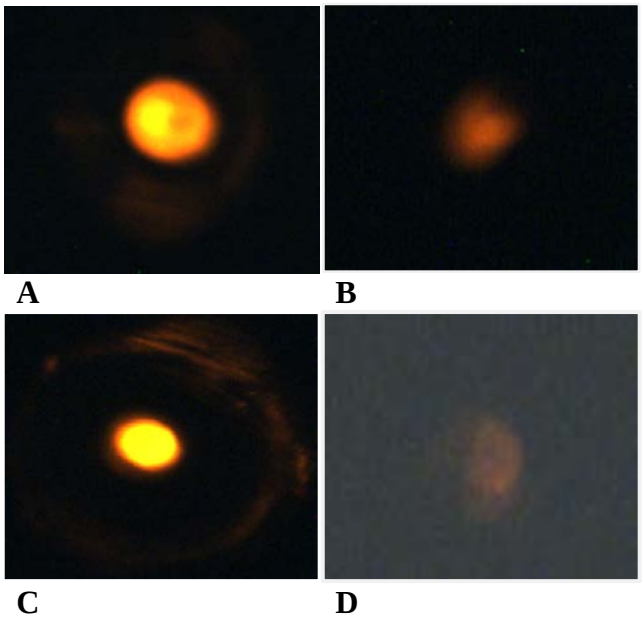

Figure 11. DsRed Fluorescent Eye Lenses.

A,B: Right and Left eye lens showing DsRed fluorescence from a putative male founder.
C: Left eye lens showing DsRed fluorescence from another putative male founder.
D: Faint DsRed fluorescence from eye lens of a putative female founder.

All seven were negative for the EGFP gene. Embryos from the four DsRed-positive females were subsequently screened further by PCR for the presence of the *h*TNFα enhancer using hTNFαEnh_F and hTNFαEnh_R primers and the EGFP thermocycler protocol and for the presence of the DsRed gene using DsRed_F and DsRed_R primers and the DSRED thermocycler protocol [Appendix C and D]. In these PCR assays, the 3-cassette expression clone and the entry clone containing the CryDsRed cassette were used as positive controls. The fertilized eggs from all four of the females were negative for the *h*TNFα enhancer and for the DsRed gene.

4.3 Approach 3: BAC Expression Clone

4.3.1 Recombineering in *E. coli*

A bacterial artificial chromosome (BAC) clone (locus: BX469886; clone CH211-127D20) was obtained from http://bacpac.chori.org. This BAC clone contains the entire zebrafish TNFα (*z*TNFα) gene, ~6.0 kb of additional upstream sequence, ~140 kb downstream sequence, and a chloramphenicol (Cm) resistance gene. Dr. Jindong Wang, MD, PhD performed all of the steps necessary to generate the retro-fitted BAC expression clone. For further details regarding the construction of the BAC expression clone that are not provided in this thesis, direct questions to Dr. Jindong Wang, MD, PhD. Microinjection of the BAC expression clone and subsequent screening were performed by Danielle Elsberry.

The BAC clone was received in DH10 *Escherichia coli* cells which were cultured and BAC DNA was extracted. BAC DNA was then electroporated into EL250 *E. coli* followed by selection with chloramphenicol (Cm). A targeting cassette was created for electroporation into the EL250 bacteria containing the CH211-127D20 BAC DNA. This initial targeting cassette was cloned into a pCR-XL-TOPO® vector by Invitrogen™ and PCR amplified to produce the linear DNA product depicted in Figure 12.

L. ARM	Linker	tdTomato	FRT	Amp^R	FRT	R. ARM

Figure 12. First Targeting Cassette for Recombineering.

Amplification primers were designed to add 50 bp flanking sequences onto the targeting cassette which are homologous to the BAC vector, specifically, 50 bp upstream and 50 bp downstream of the *z*TNFα stop codon, referred to as the left arm (L. ARM) and the right arm (R. ARM). Additionally, the left primer was designed to add a 21 bp linker sequence following the left arm which replaces the *z*TNFα stop codon and links the *z*TNFα and tdTomato genes creating a *z*TNFα:tdTomato transgene. With this design, translation of the fluorescent tdTomato protein will be in-frame with the *z*TNFα protein, thereby providing a visual indicator of *z*TNFα expression. The linker sequence also contributes to independent folding of the two proteins to preserve their function (OOI *et al.* 2006).

EL250 *E. coli* with the CH211-127D20 BAC DNA were heated to 42°C for 15 minutes to induce expression of the recombination genes. Then the linear DNA or targeting cassette was electroporated into the EL250 cells. The linear DNA undergoes homologous recombination to be added into the CH211-127D20 BAC DNA [Figure 13] (LEE *et al.* 2001; LIU *et al.* 2003). Bacteria were then grown on double selection media containing ampicillin and chloramphenicol.

The selection marker, ampicillin resistance gene (Amp^R), introduced by the first targeting cassette into the CH211-127D20 BAC DNA is located in the 3′ untranslated region of the fusion protein, and has the potential to interfere with transcription and translation. Site-specific recombination by the Flp protein will excise the Amp^R gene. The FRT sites in the initial targeting cassette were placed in the same orientation to facilitate the excision. Expression of the flpe enzyme within EL250 *E. coli* is inducible with the addition of 0.1% $_L$-arabinose.

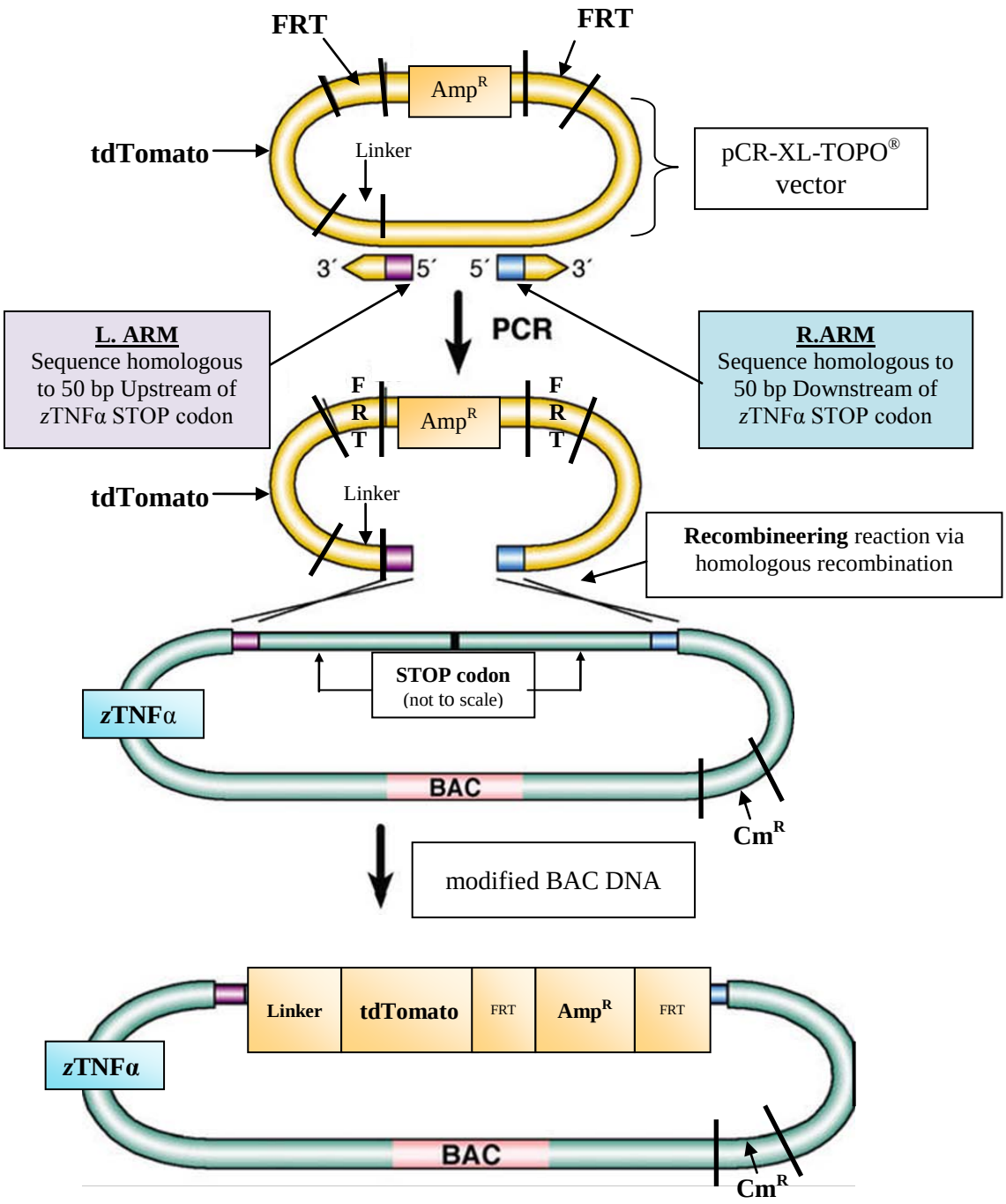

Figure 13. Recombineering Reaction of First Targeting Cassette. Adapted from figure 1 of (LIU *et al.* 2003).

The bacterial culture was incubated with the arabinose for one hour to ensure removal of the Amp^R gene. Colonies were then selected by positive growth on chloramphenicol media and by negative growth on ampicillin media.

The final modification made to the CH211-127D20 BAC vector was the addition of *Tol2* sites to facilitate the upcoming transposase-mediated integration into zebrafish genome. A second targeting cassette of linear DNA was generated using primers designed to add 50 bp flanking sequences onto the targeting cassette which are homologous to the BAC clone, specifically, 50 bp upstream and 50 bp downstream of Cm^R gene. The linear DNA is also designed to introduce a selection marker, Amp^R gene, and the minimal *Tol2 cis*-sequences in an inverted orientation, referred to as *iTol* [Figure 14] (SUSTER *et al.* 2009).

The EL250 *E. coli* were heated to 42°C to induce expression of the recombination genes e*xo*, *bet*, and *gam*. The second targeting cassette containing the *Tol2* sites adjacent to an Amp^R selection marker is electroporated in the EL250 *E. coli* cells and integrates by homologous recombination. Correctly modified colonies are selected by growth on ampicillin media. The final BAC vector includes the *z*TNFα:tdTomato transgene as well as the necessary *Tol2* sites [Figure 15]. The final BAC expression clone is referred to as retro-fitted because the modifications were made to an existing BAC clone.

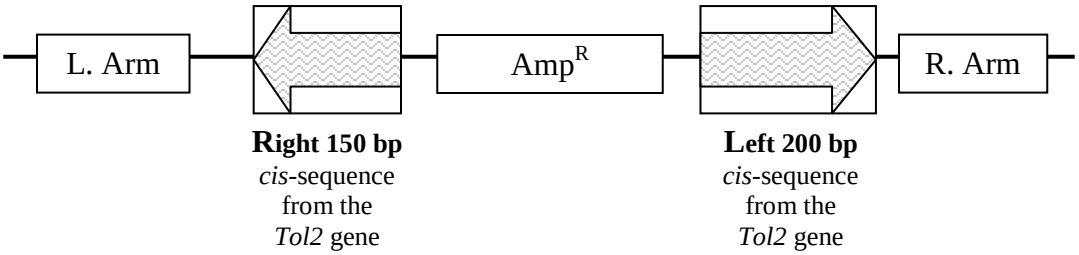

Figure 14. Second Targeting Cassette to Introduce *Tol2* Sites. Adapted from (SUSTER *et al.* 2009).

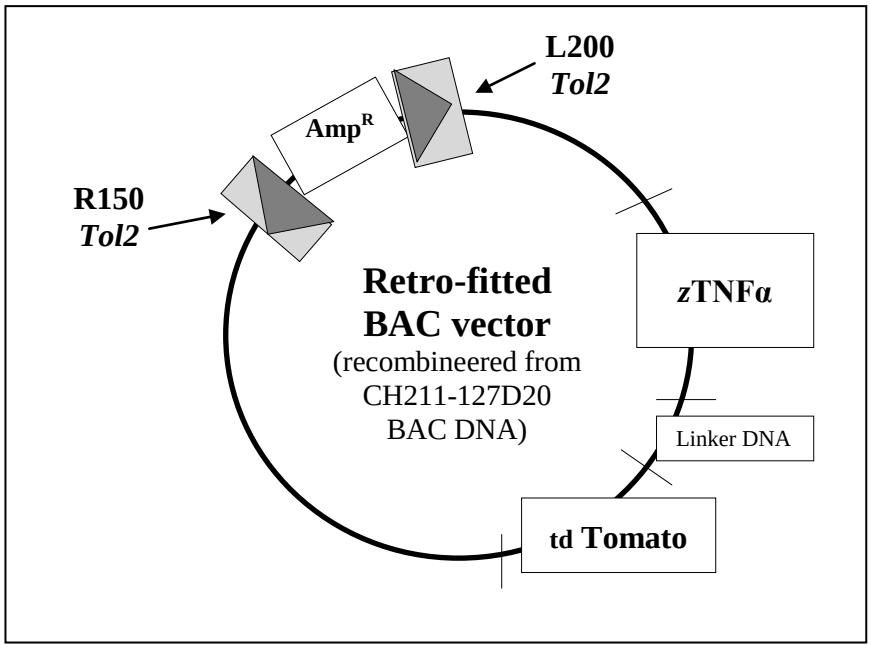

Figure 15. Retro-fitted BAC Expression Clone for Third Approach.

4.3.2 Microinjection and PCR Screening of BAC Expression Clone

During December 2009 and March 2010, one-cell stage zebrafish embryos obtained from the **AB* wild-type strain were injected with 520 pL of the retro-fitted BAC expression clone and the CryDsRed vector. The combined final DNA concentration of BAC expression clone and CryDsRed vector was 20 ng per μL. The injection mixture also contained transposase cRNA at a final concentration of 20 ng per μL and phenol red dye at 0.0625%. Transgenesis of the co-injected CryDsRed vector provides a means for preselecting putative transgenic founders; successful incorporation and expression of DsRed in the lens should be associated with a higher frequency of BAC clone transgenesis into germ-line cells (VOPALENSKY *et al.* 2010). Approximately 722 zebrafish embryos were injected with the retro-fitted BAC clone and the CryDsRed vector. Approximately 596 to 599 embryos survived to the juvenile stage and were screened by fluorescent microscopy for the presence of red fluorescent lenses. Sixty-two juveniles from the 722 injected embryos had DsRed-positive lenses, a transgenesis rate of 8.6%. Varying degrees of red fluorescence was observed. For example, red fluorescence could be seen emitting from 80% of the lens or emitting only as pinpoints within the lens. Some fish had higher fluorescence in one eye compared to their other eye or fluorescence in one eye only. Two fish that had pinpoint red fluorescence in the head region, not in the lens, were also kept for further screening. Fifty-eight of the sixty-two DsRed-positive fish survived into adulthood for further screening.

To date, sperm and fertilized eggs from 45 DsRed-positive zebrafish, 36 males and 9 females, have been screened by PCR for the presence of the *z*TNFα:tdTomato transgene. Primers were designed to amplify from the 3′-end of the *z*TNFα gene into the

linker DNA sequence (zTNFaTom_F) and to amplify from the 5′-end of the tdTomato gene (zTNFaTom_R) using the ZTNF thermocycler protocol [Appendix C and D]. Therefore, the PCR product is unique to the BAC vector transgene and not indicative of native *z*TNFα. Genomic DNA from the tail fin of wild-type zebrafish served as a negative control, BAC clone DNA served as the positive control, and a tube of PCR reagents without any DNA template served as a negative reagent control. A separate PCR amplification was performed simultaneously to provide a quality control check of the DNA extraction and of *Taq* polymerase performance. This PCR reaction using the EGFP thermocycler protocol confirms the presence of DNA using primers that target a marker on zebrafish chromosome eight, named Z60737 [Appendix C]. Two males were sperm-positive for the BAC vector transgene [Figure 16]. Two positives out of 45 fish is a germ-line integration rate of 4.4%. Seventeen DsRed-positive zebrafish remain that need their germ-line cells screened by PCR.

4.3.3 Stimulation Assays and PCR Screening of F_1 Embryos

The two sperm-positive F_0 males for the BAC vector transgene were bred with **AB* wild-type females as well as with DsRed-positive females not yet confirmed to be germ-line positive. Due to mosaicism within the sperm, only a small percentage of the F_1 offspring will be positive for the BAC transgene. The positive F_1 offspring will be heterozygous, and a minimum of one positive F_1 male and one positive F_1 female must be isolated for subsequent in-crossing to produce homozygous F_2 offspring. Breeding of the two sperm-positive F_0 males produced approximately 15 tanks of F_1 larvae to be screened by PCR for the presence of the *z*TNFα:tdTomato transgene.

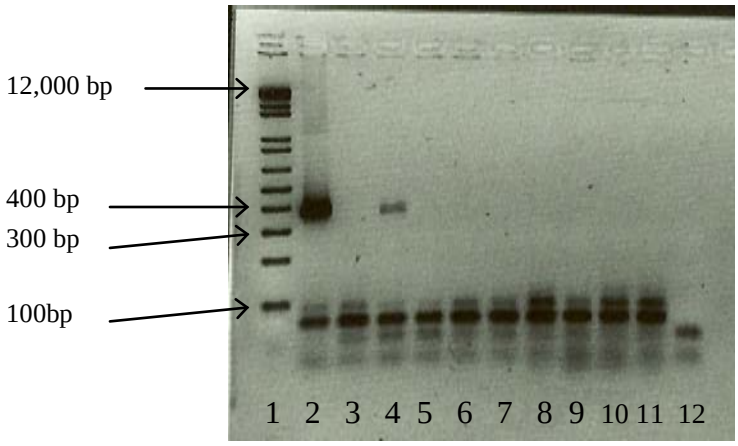

Figure 16. Gel Electrophoresis of Sperm DNA. DNA was amplified by PCR using primers that detect the *z*TNFα:tdTomato transgene. *Lane 1*: 1Kb Plus DNA Ladder by Invitrogen used for sizing linear double-stranded DNA fragments. The ladder contains 20 reference bands ranging in size from 100 bp to 12,000 bp. *Lane 2*: Positive control; BAC expression clone DNA with expected base pair size of 376. *Lane 3*: Negative control; genomic DNA from the tail fin clipping of a wild-type zebrafish. *Lane 4*: F_0 sperm DNA that is positive for the *z*TNFα:tdTomato transgene. *Lane 5-11*: F_0 sperm DNA that is negative for the transgene. *Lane 12*: Reagent control. Please note that the bands seen below the 100 bp reference band are primer dimers.

To date, DNA has been extracted from the tail fin clippings of 101 F_1 zebrafish using the modified HotSHOT protocol. The same primer set, zTNFaTom_F and zTNFaTom_R, and ZTNF thermocycler protocol that was used to detect the presence of the BAC vector transgene in F_0 germ-line cells was employed for screening the tail fin DNA of the F_1 larvae [Appendix C and D]. Genomic DNA from the tail fin of wild-type zebrafish served as a negative control, BAC clone DNA served as the positive control, and a tube of PCR reagents without any DNA template served as a negative reagent control. A separate PCR amplification was performed simultaneously to provide a quality control check of the DNA extraction and of *Taq* polymerase performance. This PCR reaction using the EGFP thermocycler protocol confirms the presence of DNA using primers that target a marker on zebrafish chromosome eight, named Z60737 [Appendix

C]. To date, DNA from one F_1 female is positive for the BAC vector transgene [Figure 17]. One positive heterozygote in 101 F_1 zebrafish indicates that a low percentage of the F_0 sperm is transgenic positive. PCR screening of the remaining F_1 zebrafish will continue with the aim of identifying a positive F_1 male to in-cross with the positive F_1 female. In the meantime, the positive heterozygous F_1 female will be bred with a wild-type male to produce F_2 embryos. Fifty percent of these F_2 embryos will be positive for the transgene and can be tested for inducibility of fluorescence by drug and infection stimulation.

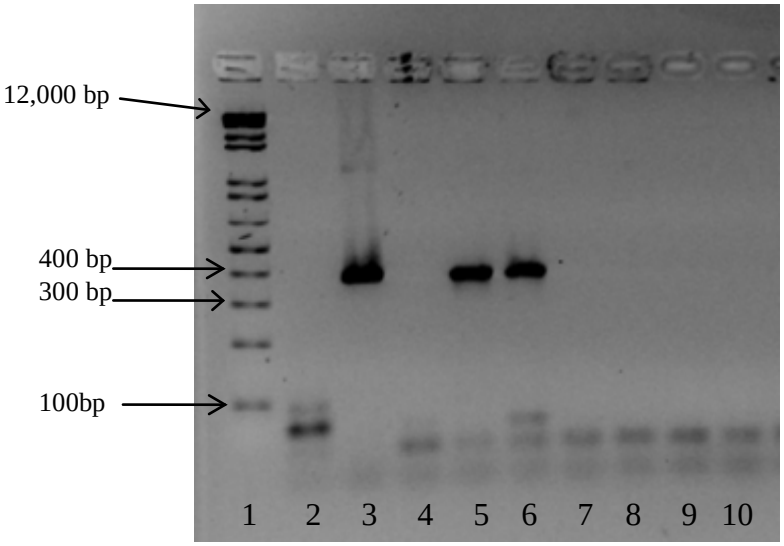

Figure 17. Gel Electrophoresis of Tail Fin DNA. DNA was amplified by PCR using primers that detect the *z*TNFα:tdTomato transgene. *Lane 1*: 1Kb Plus DNA Ladder by Invitrogen used for sizing linear double-stranded DNA fragments. The ladder contains 20 reference bands ranging in size from 100 bp to 12,000 bp. *Lane 2*: Negative control; genomic DNA from the tail fin clipping of a wild-type zebrafish. *Lane 3*: Positive control; BAC expression clone DNA. Expected base pair size of 376. *Lane 5-6*: F_1 tail DNA, in duplicate from the same zebrafish, that is positive for the *z*TNFα:tdTomato transgene. *Lane 4 and 7-10*: F_1 tail DNA that is negative for the transgene. *Lane 38 (not shown)*: Reagent control. Please note that the bands seen below the 100 bp reference band are primer dimers.

In addition to creating F_1 larvae for PCR screening, separate F_1 embryo populations were generated for experiment purposes only. These F_1 embryo populations were stimulated either with PMA and Ionomycin or injected with *E. coli* to see if any fluorescence could be observed. F_1 embryo populations were stimulated with 100 ng per mL of PMA and 0.10 μM of Ionomycin. These optimal concentrations of PMA and Ionomycin were determined by a separate toxicity experiment performed by Dr. Nikolaus Trede, MD, PhD, on wild-type embryos at two days postfertilization (dpf). PMA and Ionomycin were added to the water without de-chorionating the embryos. The 100 ng per mL of PMA and 0.10 μM of Ionomycin caused death in only 10% of the embryos at 24 hours poststimulation, whereas 250 ng per mL of PMA and 0.25 μM of Ionomycin caused death in 50% of embryos, and 500 ng per mL of PMA and 0.50 μM of Ionomycin caused death in 90% of the embryos.

One-hundred and thirty-five F_1 embryos from male founder A1 and ninety F_1 embryos from male founder A2 were stimulated with PMA and Ionomycin. In addition, 42 F_1 embryos from male founder A1 and 25 F_1 embryos from male founder A2 were not stimulated to serve as a negative control. The F_1 embryos were divided into three groups to be stimulated with 100 ng per mL of PMA and 0.10 μM of Ionomycin at three different time points: the first group at 24 hours postfertilization (hpf), the second group at 48 hpf, and the third group at 72 hpf. Embryos at 24 and 48 hpf were not de-chorionated. The majority of embryos at 72 hpf had hatched from their chorion. Stimulated embryos were screened for tdTomato fluorescence at 1, 3, 6, 8, 24, 48, and 72 hours poststimulation; no fluorescence was observed in any population at any designated time-point. We concluded that the percentage of sperm that are positive for the BAC

vector must be low in both male founders. Another possibility is that the chorion could have hindered diffusion of the drugs.

In addition to the PMA and Ionomycin stimulation assays, a population of F_1 embryos was injected with standard laboratory *E. coli* bacteria by Dr. Michael Redd, PhD. Forty embryos at 24 hpf, twenty from each male founder, were anesthetized with tricaine and mounted in 1% low-melt agarose made with 1X E3 water. Glass capillary needles that were attached to an automatic pressure-regulated injector were loaded with the bacteria. Mounted embryos were injected with the bacteria at the end of the yolk sac just above the anus. Embryos were observed for tdTomato fluorescence prior to injection, one hour postinjection, and six hours postinjection. No fluorescence signal was visualized at any time-point. Again, it was concluded that the percentage of sperm that are positive for the BAC vector must be low in both male founders.

5 DISCUSSION

5.1 Utility of the *h*TNFα Enhancer in Zebrafish

The utility of the *h*TNFα enhancer as an inducer of a fluorescent reporter protein in zebrafish has not yet been determined due to the lack of germ-line-positive founders (F_0) from microinjections of either the two- or three-cassette design. Failure to achieve germ-line positive zebrafish was due to technical difficulties with the microinjections and possibly because the human enhancer does not incorporate or function in zebrafish. The injection volume of the two-cassette expression clone was calibrated incorrectly and likely caused the lack of transgenesis. Injecting volumes greater than 1nL can cause the nucleus to burst or result in nonspecific deformities (XU 1999). Although the injection volumes were calibrated correctly for the three-cassette expression clone and DsRed expression was observed in the lenses of some of the injected fish, this three-cassette clone also failed to incorporate into germ-line cells. We reasoned that the single putative male founder with both eyes positive for lens-specific DsRed fluorescence would be germ-line positive for the expression clone. However, this was not the case with this male or any other DsRed-positive zebrafish. Incorporation of the expression clone into the genomes of the embryos must have occurred too late for transmission to germ-line cells. It is also possible that the human source of the TNFα enhancer caused problems with transgenesis. DsRed expression in only 14 of ~1,040 injected zebrafish translates to a transgenesis rate of only 1.3%; this low rate of transgenesis for the three-cassette clone

is much lower than reported transgenesis rates using the *Tol2* transposition system (KAWAKAMI *et al.* 2004b; URASAKI *et al.* 2006). Again, this low transgenesis rate is probably attributed to technical difficulties with the microinjections.

5.2 Utility of the BAC Expression Clone

Until offspring with reliable transgenesis are obtained from positive F_1 or F_2 zebrafish, the utility of the BAC expression clone cannot be assessed. However, we are hopeful that the BAC expression clone will work and become a useful research tool for the following reasons. 1) The BAC expression clone introduces a gene that is native to the zebrafish, an advantage compared to artificially-designed or nonnative genes that can be recognized as foreign and become methylated. 2) TNFα is known to be expressed in several diseases; therefore, our transgenic model will be a useful tool to the scientific community for studying multiple diseases, including infection and autoimmune disorders. It is known that not all septic patients have detectable TNFα levels. However, we will choose bacteria that do induce TNFα expression in infection studies using our transgenic model. Also, baseline TNFα expression in humans has not been defined (VAN DER POLL and LOWRY 1995); therefore, only transgenics that demonstrate a low baseline level of TNFα expression will be chosen for TLR signaling assays. 3) Our transgenic BAC model contains 31 potential transcription factor or transcription coactivator binding sites for TNFα activation that have been characterized in other species. An informatic search of sequence from 5.0 kilobases upstream, from the first exon, and from the first intron of the native zebrafish TNFα gene, identified these 31 potential binding sites including two NF-κB sites, a single AP-1 and CRE-BP1 site, and several NF-1, Sp1, Oct-1 and TBP

sites[2]. The search was performed using TRANSFAC version 4.0 software at http://www.gene-regulation.com/pub/programs/alibaba2 and by applying the following settings: pair similarity to known sites-50%, matrix width in bp-12, minimum number of sites-5, minimum matrix conservation-75. The Sp1 transcription factor is known to be involved in TNFα gene activation within mammalian T cell lines stimulated with virus or within B cells stimulated with LPS or *Mycobacterium tuberculosis* (FALVO *et al.* 2000; FALVO *et al.* 2010). The NF-κB and Oct-1 binding sites located within the first intron of the *z*TNFα gene may be important sites where transcription factors can interact and cooperate with other factors bound to sites upstream and downstream of the transcription start site. In mammals, the induction of TNF gene transcription by virus or LPS does not depend on NF-κB binding to the proximal promoter. However, attainment of maximal TNF mRNA levels does involve an NF-κB postinduction mechanism that is not fully characterized (TSYTSYKOVA *et al.* 2007). 4) The BAC expression clone includes ~6.0 kb of additional upstream sequence and ~140 kb of downstream sequence to the zebrafish TNFα gene that may contain further regulatory elements, such as enhancer sequences, to possibly aid in TLR signaling and disease model studies.

5.3 Future Objectives and Proposed Alternatives

The main objective for future experiments is to finish the PCR screening of the remaining F_1 offspring derived from the sperm-positive BAC clone males. Identifying a heterozygous F_1 female and F_1 male will facilitate the generation of homozygous F_2

[2] NF-κB, nuclear factor-kappa B; AP-1, activator protein-1; CRE-BP1, cAMP-response element-binding protein-1; NF-1, nuclear factor-1; Sp1, specificity protein 1; Oct-1, octamer-binding protein 1; TBP, TATA-box binding protein.

offspring. One hundred percent of the offspring from a homozygous zebrafish will have the transgene. This facilitates the interpretation of experiments and obviates the need for genotyping. These offspring are best-suited for the ultimate goal of studying TLR signaling and for determining compounds and genes that affect TNFα production through large-scale chemical and genetic screening.

We decided to abandon further embryo injections of the expression clones containing the *h*TNFα enhancer because the advantages of the BAC expression clone outweigh the possible advantages of the *h*TNFα enhancer clone. The functionality of the *h*TNFα enhancer in zebrafish could be tested by transiently injecting embryos with the expression clone and with LPS to see if any green fluorescence is produced. Furthermore, it is possible that the fluorescence observed in drug-stimulated, lipofected Jurkat cells was not dose-dependent. The number of Jurkat cells that produced EGFP fluorescence via the *h*TNFα enhancer could be attributed to better-quality lipofection isolated to a particular well. Perhaps cells in one well received slightly more lipofection reagent than another well which resulted in the greater number of fluorescent cells. The assay could be repeated with lipofection occurring in a larger, single culture dish before splitting the cells into a 6-well plate for drug stimulation. This would increase the confidence of our conclusion that the fluorescence was dose-dependent.

One alternative to our approach would be using the genome of pufferfish, *Fugu rubripes*. Although conservation of putative TNFα enhancers is not seen between mammals and teleosts, genome database searches comparing the pufferfish and zebrafish genomes do show conserved putative enhancers for the TNFα gene; data not shown. Characteristics of the *Fugu* genome offer several advantages for research. The complete

genome of *Fugu* is only 400Megabases (Mb) as compared to 1700Mb in the zebrafish genome and 3000Mb in the human genome. The smaller *Fugu* genome is attributed to highly-compressed intergenic and intronic sequences and a low amount of repeats. The result is a gene density of 17% coding sequence in the *Fugu* genome compared to just 3% in the human genome. The *Fugu* genome still contains necessary noncoding sequences, and the evolutionary distance between mammals and fish provides a high stringency for identifying conserved noncoding sequences (BRENNER *et al.* 1993; VENKATESH *et al.* 2000; VENKATESH and YAP 2005). All of these characteristics make the *Fugu* genome a good reference for more complex genomes and an easier genome to use in research. A second alternative to our approach would be designing a fluorescent reporter system inducible by the expression of different cytokines such as interleukin-1 (IL-1), IL-12, or type I interferons (IFNs).

APPENDIX A

HUMAN TLRs EXPRESSED ON THE CELL SURFACE

Table 4: Human TLRs (*h*TLRs) Expressed on the Cell Surface. Data summarized from (AKIRA *et al.* 2006; KAWAI and AKIRA 2010).

***h*TLRs Expressed** on the **Cell Surface**	**Ligand(s)**	**Source**
TLR**2**-TLR**1** heterodimer	Triacylated lipopeptides	Gram-negative bacteria & Mycoplasma
TLR**2**-TLR**6** heterodimer	Diacylated lipopeptides	Gram-positive bacteria & Mycoplasma
TLR**2**	Lipopeptides	Bacteria
	Peptidoglycan (PG) & Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Zymosan	Fungi
	tGPI-mucin	*Trypanosoma cruzi*
	Hemagglutinin protein	Measles virus
TLR**4**	Lipopolysaccharide (LPS)	Gram-negative bacteria
	Fusion (F) protein	Respiratory syncytial virus (RSV)
	Envelope (Env) protein	Mouse mammary tumor virus (MMTV)
	Pneumolysin	*Streptococcus pneumonia* (Gram-positive)
	Paclitaxel, a cytostatic drug	Plant-derived
TLR**5**	Flagellin	Flagellated bacteria
TLR**10**	Unknown	Unknown
TLR**2**, TLR**4**, or TLR**2**-TLR**4** heterodimer	Extracellular Matrix (ECM) degradation products: biglycan, hyaluronic acid, versican, extradomain A of fibronectin, and surfactant protein A.	Endogenous host molecule
	High-mobility group box 1 (HMGB1) protein	Endogenous host molecule
	Heat-shock proteins: Hsp60, Hsp70, Hsp22, and gp96	Endogenous host molecule

APPENDIX B

HUMAN TLRs EXPRESSED IN INTRACELLULAR VESICLES

Table 5: Human TLRs (*h*TLRs) Expressed in Intracellular Vesicles. Data summarized from (AKIRA *et al.* 2006; KAWAI and AKIRA 2010).

***h*TLRs Expressed** in **Intracellular Vesicles**	**Ligand(s)**	**Source**
TLR**3**	dsRNA	Produced during replication of ssRNA viruses
	Genomic RNA	Reoviruses
	Polyinosinic-polycytidylic acid (poly(I:C))	Synthetic analog of dsRNA
TLR**7**	ssRNA	RNA viruses
	Imidazoquinoline derivatives & guanine analogs	Synthetic
TLR**8**	ssRNA	RNA viruses
TLR**9**	Unmethylated 2′-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs	Bacteria & DNA viruses
	Insoluble crystal hemozoin	*Plasmodium falciparum*
	CpG oligodeoxynucleotides (CpG ODNs)	Synthetic

APPENDIX C

PCR PRIMER SEQUENCES

Table 6: PCR Primer Sequences. The sequence of *att*B sites are indicated in lower case.

Primer Designation	Sequence
*att*B *h*TNFα enhancer Forward primer	5′- gggacaactttgtatagaaaagttgATG CTTGTGTGTCCCCAACTTTCCA -3′
*att*B *h*TNFα enhancer Reverse primer	5′- ggggactgctttttgtacaaacttgCGT CTGAGGGTTGTTTTCAGG -3′
*h*TNFα enhancer sequencing primer	5′- ATGCTTGTGTGTCCCCAACTT -3′
M13 Forward primer	5′- GTAAAACGACGGCCAG -3′
M13 Reverse primer	5′- CAGGAAACAGCTATGAC -3′
Tol2 Forward primer (Tol2_F)	5′- TCAAGTGAAAGTACAAGTACTTAGG -3′
Tol2 Reverse primer (Tol2_R_b)	5′- CGTGCAGTTCATTATTAGTT -3′
EGFP Forward primer (EGFP_F)	5′- ATGGTGAGCAAGGGCGAGGA -3′
EGFP Reverse primer (EGFP_R)	5′- CGTCCTTGAAGAAGATGGTGCG -3′
Expression Clone Forward primer (Expr_F)	5′- CAGTTGTTGGCACACCCAGC -3′
Expression Clone Reverse primer (Expr_R)	5′- TTTACGTCGCCGTCCAGCTC -3′
DsRed Forward primer (DsRed_F)	5′- TCTAGGTTCATCGCATGCAG -3′
DsRed Reverse primer (DsRed_R)	5′- GCCGTCCTCGAAGTTCATC -3′
Z60737 Forward primer	5′- AGGGCATTAGGATCTCCGTT -3′
Z60737 Reverse primer	5′- TGTGACTGACAGTCAAACCTGA -3′
Middle Entry Reverse primer (ME_Rev)	5′- AAAGTGAGGCTGAGACGC -3′
3′ Entry Reverse primer (3E_Rev)	5′- TATTTGTGAGCCAGGGCATT -3′
*h*TNFα Enhancer Forward primer	5′- CCCGCGATGGAGAAGAAACC -3′
*h*TNFα Enhancer Reverse primer	5′- TGTGCCAACAACTGCCTTTA -3′
*z*TNFαTomato Forward primer (zTNFaTom_F)	5′- TTTCAGTGCAATCCGCTCAATCTG -3′
*z*TNFαTomato Reverse primer (zTNFaTom_R)	5′- GCCGCCCTTGGTCACCTTCAGCTT -3′

APPENDIX D

THERMOCYCLER PROTOCOLS FOR PCR AMPLIFICATION

Table 7: Thermocycler Protocols for PCR Amplification

Thermocycler Protocol Designation	Protocol
CA55S	Initial denaturation at 94°C (3′); 40 cycles of denaturing, annealing, and elongation: 94°C (15″); 55°C (30″); 72°C (30″); an additional elongation at 72°C (10′); and cool down of 10°C (60′)
modified CA55S	Initial denaturation at 94°C (3′); 40 cycles of denaturing, annealing, and elongation: 94°C (15″); 55°C (30″); 72°C (75″); an additional elongation at 72°C (10′); and cool down of 10°C (60′)
EGFP	Initial denaturation at 95°C (2′); 30 cycles of denaturing, annealing, and elongation: 95°C (30″); 55°C (30″); 72°C (60″); an additional elongation at 72°C (2′); and cool down of 10°C (60′)
EXPR	Initial denaturation at 95°C (2′); 35 cycles of denaturing, annealing, and elongation: 95°C (20″); 60°C (20″); 72°C (30″); an additional elongation at 72°C (5′); and cool down of 10°C (60′)
DSRED	Initial denaturation at 95°C (2′); 35 cycles of denaturing, annealing, and elongation: 95°C (20″); 55°C (20″); 72°C (30″); an additional elongation at 72°C (5′); and cool down of 10°C (60′)
ZTNF	Initial denaturation at 95°C (3′); 35 cycles of denaturing, annealing, and elongation: 95°C (15″); 60°C (30″); 72°C (60″); an additional elongation at 72°C (7′); and cool down of 10°C (60′)

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