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ACTIVATION OF INNATE IMMUNE PATHWAYS BY EPSTEIN-BARR VIRUS DNA USING DROSOPHILA MELANOGASTER AS A MODEL SYSTEM

by
NOUR ALI SHERRI

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AN ABSTRACT OF THE THESIS OF

Nour Ali Sherri for Master of Science
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Title: Activation of innate immune pathways by Epstein-Barr virus DNA using *Drosophila melanogaster* as a model system

Introduction: The mammalian Epstein-Barr virus (EBV) is associated with several malignancies and autoimmune diseases. Following EBV infection and establishment of latency, recurrences frequently occur resulting in potential viral DNA shedding, which may then trigger the activation of immune pathways. A previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology indicated that levels of IL-17, a pro-inflammatory cytokine associated with several autoimmune diseases, is increased in response to EBV DNA injection in mice. Whether other pro-inflammatory pathways are induced in EBV DNA pathobiology remains to be investigated. The molecular complexity of mammalian immune systems makes it challenging to study differential activities of specific immune pathways in response to a particular challenge. In this study we used *Drosophila melanogaster* to identify innate immune pathways that are activated in response to EBV DNA.

Methods: To assess the activation of the three major *Drosophila* immune pathways Toll, Immune deficiency (IMD), and Janus kinase/signal transducers and activators of transcription (JAK-STAT) in response to EBV DNA, wild type adult flies were injected with 70, 140 or 280 EBV DNA copies. As a non-viral DNA control, wild type flies were injected with 0.01, 0.02 or 0.05 pg of *Staphyloccocus epidermidis* DNA. In addition, flies that overexpress Toll, IMD and JAK-STAT pathway components were used as positive controls. Transcriptional levels of downstream targets of each pathway were measured by real time reverse transcriptase PCR. We also investigated the effect of EBV DNA on activating the cellular arm of the fly innate immunity. Wild type flies that were treated as described above were bled and hemocytes were counted using a Neubauer chamber.

Results: Upon injection of 70 copies of EBV DNA into flies, the transcriptional level of diptericin, indicative of the IMD pathway, was increased by 115 folds on day 1 but not day 3 post-injection compared to flies injected with sterile water. Higher copy numbers of EBV DNA did not result in a similar increase in the expression of diptericin. On the other hand, the transcriptional levels of drosomycin and TotA, which are indicative of Toll and JAK/SAT pathway activation respectively, were not affected compared to flies injected with sterile water on days 1 and 3 post-injection. A 7-fold increase in hemocyte number was only observed on day 1 post-injection of 70 copies of EBV DNA compared to flies injected with sterile water.

Conclusions: Our results suggest that EBV DNA triggers the activation of the IMD pathway and stimulates hemocyte proliferation in flies. Whether the activation of the mammalian tumor necrosis factor receptor (TNFR) pathway, which is often compared to IMD in flies, is triggered by EBV DNA remains to be investigated.

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CHAPTER I

INTRODUCTION

Epstein Bar virus (EBV) belongs to the herpes family of viruses. Like other herpes viruses, EBV is capable of establishing latency. During latency only a small portion of EBV genes are expressed. In addition to causing infectious mononucleosis, this virus is associated with several autoimmune diseases as well as malignancies such as Burkitt and Hodgkin lymphomas, nasopharyngeal carcinoma, and post-transplant lymphoproliferative disorders (PTLD). EBV reactivation may result in consistent shedding of DNA which could then trigger innate immune pathways. Previous studies have shown that EBV DNA is capable of triggering the production of pro-inflammatory cytokines in mammalian systems. The mechanisms by which these cytokines are triggered remain to be fully understood. Such cytokines would then play a role in disease processes such as autoimmunity. We hence sought to identify innate immunological pathways that are activated in response to EBV DNA. Owing to the complexity of mammalian systems we initiated our studies in a relatively simple but efficient model, *Drosophila melanogaster,* whose immune system is equivalent to that of mammals. Innate immunity in flies involves humoral and cellular responses. The humoral immune response is characterized by the secretion of antimicrobial agents through the activation of Toll and immune deficiency (IMD) pathways, which result in the expression of the antimicrobial peptides (AMPs) drosomycin and diptericin, respectively. Additionally, the JAK-STAT pathway can induce the expression of genes, such as Turandot (TotA), that play a role in the humoral immune response

The specific aims of this study were to:

1- Assess the activation of humoral innate immune responses in *Drosophila melanogaster* injected with EBV DNA.

2- Assess hemocyte proliferation in *Drosophila melanogaster* injected with EBV DNA.

CHAPTER II

LITERATURE REVIEW

A. Epstein-Barr virus (EBV)

M. A. Epstein and Y. M. Barr were the first to discover the Epstein Bar virus (EBV*)* in a Burkitt's lymphoma derived cell line in 1964 (1). EBV is classified within the *Lymphocryptovirus* genus of the gamma subfamily which belongs to the *Herpesviridae* family (2). The *Herpesviridae* is a family of viruses consisting of more than 100 herpesviruses, 8 of them routinely infect humans: *Herpes simplex virus* types 1 and 2 (HSV-1 and 2), the Epstein-Barr virus (EBV), the cytomegalovirus (CMV), the varicella zoster virus (VZV) and the *Human herpes virus* types 6, 7 and 8 (HHV-6, 7 and 8) (3, 4). Following infection, viruses of this family are capable of establishing latency with possible recurrent reactivation (3). The herperviruses are divided into three subfamilies. 1) The *Alphaherpesvirinae* subfamily comprises both HSV and VZV; these viruses primarily establish latency in sensory and cranial nerve gangalia. 2) The *Betaherpesvirinae* subfamily comprises CMV, HHV-6 and HHV-7, these viruses primarily establish latency in lymphocytes and 3) the *Gammaherpesvirinae* subfamily comprises both EBV and HHV-8 these viruses primarily establish latency in B lymphocytes (3, 4).

1. EBV Structure

Similar to other herpesviruses, EBV is composed of four major components: 1) The envelope which is a lipid bilayer acquired from the cell membrane of the host; the envelope contains several types of glycoproteins (also known as spikes) such as glycoprotein B (gB), glycoprotein (gH), glycoprotein (gL) and glycoprotein 350 gp350 (5). 2) The viral matrix or tegument is composed of about seventeen amorphous proteins. It lies between the envelope and the nucleocapsid. The proteins constituting the viral matrix include host proteins such as heat-shock protein70 (Hsp 70) and actin (6). 3) An icosadeltahedral nucleocapsid made up of 162 capsomeres. 4) Enclosed in the nucleocapsid, the genetic material consists of double-stranded DNA that encodes for approximately 80 proteins (7) .

2. Viral Genome

The EBV genome is formed of linear-double stranded DNA (dsDNA) with a length of approximately 172 kilobase pairs (kb) (8). The EBV virus typically has 0.5 kb terminal direct repeats (TRs) that are found on both ends of the linear dsDNA. TRs are involved in the formation of the episome during the latent phase (9). The EBV genome also contains the major internal repeat sequences (IRs), which consists of 5 to 10 copies of a sequence about 3 kilo base pair (kbp) long. IRs are considered to divide the genome into the unique short (U_S) sequence, which is 12 kbp in length, and the unique long (U_L) sequence which is 134 kbp. Moreover, the promoter of the EBV nuclear antigens (EBNAs) is located in IRs (10).

Most of the EBV genes play a role in the lytic phase (or productive phase). On the other hand, only 12 genes, the Epstein-Bar virus encoded small RNAs (EBERs) and micro RNA (miRNA) are expressed during the latent (or nonproductive phase) (11).

3. EBV Strain Variation

Despite the fact that the two existing types of EBV (EBV-1 and EBV-2) are identical for the most part of the whole viral genome, they show allelic polymorphism in a series of latency genes such as EBNA-LP, EBNA2, EBNA3A, EBNA3B and EBNA3C (12-14). EBV-1 isolates are prevalent in numerous Western countries, while the two types are predominant in New Guinea and African equatorial countries (15). Moreover, studies show that EBV-2 is less effective than type 1 in causing the transformation of B-cells *in vitro* (16).

4. EBV Epidemiology

Based on the detection of antibodies, the International Agency for Research on Cancer (IARC) reported that the percentage of adults infected with EBV exceeds 90% worldwide (17). In developing countries, EBV infection is usually acquired at the age of 3 to 4 and most cases are subclinical. On the other hand, EBV infection is acquired at adolescence in developed countries and can manifest as infectious mononucleosis (17, 18).

5. Transmission

Transmission of EBV occurs most frequently through oropharyngeal secretion (19). Infection may occur via blood transfusion, genital transmission, and following organ transplantation, which may lead to post transplantation lymphoproliferative disease (PTLD)

(20-22). Moreover, some studies suggested that EBV may be transmitted transplacentally and through breast milk; however, low rate of transmission is observed through these modes (23-25).

6. EBV Infection

In oral transmission cases, the virus initially enters the differentiated oropharyngeal epithelial cells,(26); where it replicates, and then it is disseminated into salivary glands, oropharyngeal lymphoid tissues, and the B-lymphocytes of the tonsils (27). The DNA within a virion upon release from an infected cell lacks histones and contains unmethylated CpG dinucleotides (28). Hence, the DNA delivered into a cell upon infection is in this form. The "pre-latent" phase is initiated when the viral genome enters the nucleus of the B-lymphocytes and shapes into a circular plasmid (29). After 1 to 2 weeks post-infection, the latent phase takes over the pre-latent phase, and a steady association between the virus and the host is established (30). In this phase epigenetic modifications results in a significant level of CpG methylation of the genomic DNA leading to the transcriptional repression of lytic genes (29). Reactivation may then be induced in the latently infected cells upon exogenous signals. The viral progeny then released from the lytically induced cells enclose viral DNA in its naïve unmethylated state (29).

7. Diseases

EBV is linked to many diseases. They can be classified into infectious mononucleosis, epithelial diseases, and other associated diseases.

EBV manifests as infectious mononucleosis in more than fifty percent of infected individuals. It is a self-limiting infection common in adolescents and young adults. Its symptoms typically include: fever, lymphadenopathy and pharyngitis (31). EBV also plays a role in several epithelial diseases including: nasopharyngeal carcinoma and oral hairy leukoplakia (OHL). Moreover, EBV is carcinogenic in humans due to its association with Burkitt's lymphoma (BL), Hodgkin's disease (HD),T -cell non-Hodgkin lymphomas (NHL), post-transplant lymphoproliferative disorders (PTLD), and nasopharyngeal carcinoma (NPC) (32).

EBV is correlated with many autoimmune diseases including: systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS) (33, 34) It was previously shown that SLE patients have high EBV viral load in the peripheral blood mononuclear cells (PBMCs), which indicates that SLE is associated with EBV infections (35, 36). Similar observations were seen in RA patients, where high EBV viral load was detected in the blood, synovial fluid cells, and synovial membranes in RA subjects (37). Moreover, various studies suggested that the risk of developing MS increased in EBV-infected subjects (38, 39). In addition, a previous study conducted at the Department of Experimental Pathology, Immunology and Microbiology suggested that EBV DNA triggers the expression of IL-17, a proinflammatory cytokine associated with autoimmune processes, following the injection of EBV DNA into mice *in vivo* (40). Follow up studies indicated that Toll-like receptor 9 (TLR9) activation may be involved in triggering IL-17 synthesis in response to EBV DNA injection in mice (41). Whether other pro-inflammatory

pathways are triggered by EBV DNA and play a role in its pathogenic associations remain to be investigated.

B. *Drosophila melanogaster*

Drosophila melanogaster, the fruit fly, is a genetic model system used for exploring molecular mechanisms of human diseases, such as Parkinson's disease (42), heritable cancer syndromes such as multiple endocrine neoplasia (43), and metabolic disorders like diabetes(44). During the past few decades, *D. melanogaster* has been extensively used to study innate immune responses elicited against a variety of pathogens including human viruses (45).The following factors make these insects an attractive model: Short generation time, inexpensive, easy to maintain , availability of numerous genetic tools such as RNAi-mediated gene silencing, UAS-GAL4 system, and P transposable elements (46).. Although flies and vertebrates share a common ancestor that is tracked back 700 million years at the Protostome-Deuterostome split (47), numerous genes in *D. melanogaster* have homologues in humans(48) .

1. Innate immunity in Drosophila melanogaster

Drosophila are devoid of an adaptive immune system, hence it solely relies on innate immune responses for its defense (49). The innate immunity in flies involves humoral and cellular responses in addition to the RNA interference pathway (49). The RNA interference (RNAi) pathway is also involved in the antiviral responses in flies. The viral dsRNA is detected and cleaved by dicer into short interfering RNAs (siRNA) (50). These are then

loaded into the RNA-induced silencing complex (RISC), which will finally degrade the viral RNA (51).

a. The cellular immune response

The cellular immune response comprises three groups of hemocytes. The first group, the plasmatocytes, represents 95% of the total hemocytes and is responsible for phagocytosis. Receptors expressed on the plasmatocytes include the vascular endothelial growth factor (VEGF) and the platelet-derived growth factor (PDGF) receptor (PVR) (52, 53). PVR has known functions in the guidance of cell migration (53). Moreover, PVR has three potential ligands, the PDGF/VEGF related factors (PVF1, PVF2, and PV3) (52). A previous study suggested that PVF2 induces hemocyte proliferation in *Drosophil*a larvae (52). Crystal cells, the second group, comprise 5% of total hemocytes and contribute to melanization (54). Melanization is a prominent immune response that leads to the sequestration and destruction of the pathogen. Melanin is formed when phenols are oxidized to quinines. Moreover, melanization acts together with diverse immune reactions including: blood clotting, wound healing, phagocytosis and antimicrobial peptide (AMP) secretion (55). The third type of cells, which is the lamellocyte, is produced in response to parasitic infections; for example, the prasitization by the wasp *Leptopilina boulardi* results in a dramatic increase in the number of lamellocyte (56). These cells are also induced in response to mutations that lead to the production of melanotic tumors. For instance, a mutation in the allele hop^{Tuml} leads to the formation of melanotic tumor and extensive lamellocyte differentiation (57).

b. The humoral immune response

The humoral arm of the innate immune system in *Drosophila* includes several immune responses such as melanization and hemolymph coagulation (49). Hemolymph coagulation is one of the immediate reactions that seals wounds to prevent hemolymph loss and to avoid the entrance of pathogens (58).The hallmark of humoral immune responses is thesecretion of antimicrobial pepetides (AMPs) by the fat body, the functional equivalent of the mammalian liver and adipose tissue, through nuclear factor-kB (NF-κB) signaling pathways (49). The transcription of about half of the immune-inducible genes is regulated by the NF-κB/Rel proteins (49). Despite the fact that the fat body is the site where the bulk of AMP production occurs, AMPs may also be produced by epithelial tissues including the trachea and the gut (59, 60). Hemocytes can produce AMPs to a minimal extent; therefore, their contribution to overall AMP production is limited. The hemocytes rather play a crucial role in inducing AMP secretion from the fat body by producing ligands required to trigger their production from this organ (61). The secretion of AMPs is regulated by two NF-kB signaling pathways, Toll and the immune deficiency (IMD) (49). Each pathway is activated by different microbial component and triggers the transcription of overlapping subsets of antimicrobial peptides as well as other immune inducible genes. For instance, AMPs such as metchnikowin and drosomycin are strictly triggered through the Toll pathway, while diptericin is induced through the IMD pathway (62, 63). On the contrary, certain AMPs, in particular attacin and cecropin are co-operatively regulated by Toll and IMD pathways(64).

i. Toll pathway

Toll was originally discovered in flies as a type I trans-membrane receptor needed for determining the dorsal–ventral axis at the time of embryonic development (65)Lethal abnormalities in the pattern of gastrulation and the differentiation of cuticular structures are observed in *D. melanogaster* Toll mutants (66). Later on, it was shown that the Toll signaling pathway is involved in host responses to Gram-positive bacterial and fungal infections (67). In the genome of the *D. melanogaster*, nine Toll receptors have been uncovered so far (68). AMP induction through the Toll pathway occurs via Toll-1, which is the first identified Toll receptor in Drosophila (68).As opposed to the other Toll receptors, Toll-9 has a structure that resembles mammalian Toll-like receptors (TLRs) to a high extent, whereby it is the only Toll receptor that is devoid of N-flanking cysteine-rich motifs (69). Moreover, Toll/IL-1R (TIR) domain is common between *Drosophila* Tolls and the (Interleukin -1 Receptor) IL-1R in humans. This domain interacts with adaptor molecules thus activating downstream events.(70)

Unlike mammalian Toll-like receptors, *Drosophila* Toll receptors do not bind directly to pathogens (71, 72). The Toll pathway is instead activated in development or immunity via the binding of the active (split) form of spatzle, which leads to the activation of intracellular components such as the myeloid differentiation primary response protein 88 (MyD88), Tube and the Pelle kinase. This event results in the destabilization of IκB-family protein Cactus, and the localization of the NF-kB-like transcription factors (Dorsal and Dif) to the nucleus (49, 72). Dorsal and Dif will further induce the transcription of AMPs, such as drosomycin, metchnikowin, and defensin (49, 72, 73).

In humans, 10 TLRs have been identified . Despite the fact that Toll receptors in *Drosophila* share similarities to mammalian TLR domain the latter do not play a prominent role in development, while the Toll pathway in *Drosophila* is involved in development as well as immunity (74, 75).

ii. IMD pathway

In addition to the Toll pathway, fruit flies utilize the IMD pathway to regulate the expression of many anti-microbial peptides. Gram-negative bacteria are preferentially detected through the IMD pathway (67). The IMD pathway may also play a role in the immune reaction to viral infections in flies (76). This pathway controls the activation of Relish, a member of the NF-κB family found in *Drosophila* (77). This pathway regulates the expression of the majority of AMPs and hence, plays a pivotal role in normal immunity (78). Unlike the Toll pathway, IMD is activated via the binding of the mono-diaminopimelic acid-type peptidoglycans (DAP-type PGN) to the transmembrane receptor: peptidoglycan recognition protein-LC (PGRP-LC) (46, 71, 79). Other components of this pathway include: Transforming growth factor beta-activated kinase 1 (TAK1), IκB kinase-γ (IKKγ), the Fas Associated protein with Death Domain (FADD) adaptor, the death-relatedCED-3/NEDD2-like protein (Dredd) caspase, and Relish (46, 54, 79). Relish is a transcription factor, activated by proteolytic cleavage allowing its N-terminal to translocate to the nucleus and stimulates the expression of AMPs such as attacin, cecropin and diptericin (49, 54, 71). Moreover, the IMD pathway branches into the c-Jun N-terminal kinases (JNK) pathway at the level of Tak1(80). The JNK pathway can be activated in

response to infection, lipopolysaccharide, and inflammatory cytokines such as Eiger (Egr) in *Drosophila* and tumor necrosis factor (TNF) and mammals (81-83).

The IMD pathway is comparable to the tumor necrosis factor receptor (TNFR) signaling pathway in humans (54, 71). TNFR, a transmembrane protein, is not only expressed by activated mammalian immune cells, but also by non-immune cells including fibroblasts and endothelial cells (84). Activation of TNFR triggers the expression of several cytokines involved in inflammation such as IL-6 and IL-8 in mammalian systems (85).

iii. JAK–STAT pathway

In addition to the Toll and IMD pathways, the Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway is involved in innate immunity. Moreover, it is involved in the antiviral immune response in flies and mammals (86). In addition to its role in immunity, the JAK-STAT pathway plays a vital role in several processes such as development and cellular proliferation in *D. melanogaster* (87) .

In flies, the JAK-STAT pathway was initially detected by its function in embryonic segmentation (87). This pathway is activated through the attachment of the unpaired (Upd) proteins to the Domeless receptor, this results in the activation of a signaling cascade that eventually leads to the phosphorylation of STAT92e (46). This transcription factor plays a role in the expression of immune inducible genes including the stress response gene turandot A (TotA) (88).

It was previously shown that the JAK-STAT pathway possesses an antiviral activity in *D. melanogaster* (89, 90), whereby the Drosophila C virus (DCV) and Feline

Herpesvirus (FHV) in flies both trigger a transcriptional program that depends in part on the JAK-STAT pathway (91). Although the mammalian JAK-STAT pathway is broadly antiviral, several studies conducted using flies suggested that the response induced by JAK-STAT depends on the type of the virus. For example, the JAK-STAT pathway is involved in the host defense against Drosophila C virus (DCV) and Cricket Paralysis Virus (CrPV) infections. However, it is not triggered in response to infections by Sindbis Virus (SINV) and Vesicular Stomatitis Virus (VSV) (90, 92).

CHAPTER III

MATERIALS AND METHODS

A. Flies

1. Fly stocks

To identify the innate immune components that may be involved in the response to EBV DNA in *Drosophila melanogaster*, 1-day-old adult flies were injected with various treatments of EBV and *Staphylococcus epidermidis* DNA as indicated in Section 5 below.

Flies were raised and crossed at 25˚C; standard *Drosophila* husbandry procedures were followed. Wild type flies (Bloomington Drosophila Stock Center #3605, Bloomington, IN,) were used; in addition, the UAS-STAT92e(93), UAS-Relish (94)and UAS-Toll10b (95) flies were employed. RNAi lines used included IMD-RNAi (Vienna Stock center #9253) obtained from the Vienna Drosophila Stock Center (Vienna, Austria). Moreover, the hemocyte driver Cg25C-GAL4 (Bloomington stock center #7011) was employed (Table 1).

2. *Fly Pushing*

Virgin females were used in order to set up crosses. Virgin females were physically identified by the presence of a dark spot on the ventral abdominal wall, the meconium.

3. Overexpression of Reslish, STAT92e and Toll10b using the UAS-GAL4 system

As a positive control for gene expression studies described below, overexpression of Toll10b, Relish, and STAT92e in the Toll, immune deficiency (IMD) and Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathways respectively was performed using the UAS-GAL4 system adapted from the yeast *Saccharomyces cerevisiae*. Briefly, GAL4 regulates the transcription of its targets genes by binding an Upstream Activating Sequences (UAS) element (96). UAS and GAL4 are inserted in separate fly lines. The GAL4 is inserted in the driver lines along with a genomic enhancer, which provides tissue-specific GAL4 expression. On the other hand, UAS is inserted in the responder line along with the coding sequence of the target gene (97). Hence, the expression of a target gene can be obtained by crossing the appropriate driver lines with the responder lines that carry UAS and the target genes.

Overexpression of Relish, Toll10b and STAT92e in hemocyte and fat body was performed using the following crosses: UAS-Relish > CG25C-Gal4, UAS-Toll10b > CG25C-Gal4, UAS-STAT92e > CG25C-Gal4

4. RNAi-mediated gene silencing of IMD using the UAS-GAL4 system

IMD was conditionally silenced using the UAS/GAL4 system described above. Briefly, hairpin RNA (hpRNAs) downstream the UAS sequence will target the IMD gene. Dicer cleaves the expressed hpRNA into small fragments of small interfering RNA molecules (siRNAs). The anticoding strand of the siRNA acts as a template for the RNA-induced

silencing complex (RISC) to recognize and process complementary messenger RNA (mRNA), which will then be degraded (98).The following cross was set up to knock down IMD gene expression:

UAS-IMD RNAi > CG25C-Gal4

5. Injections and specimen procurement

EBV DNA was obtained from Advanced Biotechnologies (Columbia, MD) while *S. epidermidis* DNA was prepared from an isolate collected by the Department of Experimental Pathology, Immunology and Microbiology.

To assess the effect of EBV DNA on the expression of drosomycin, diptericn, and turnadot (TotA), three groups of wild type flies were injected with 70, 140 or 280 copies of EBV DNA. As non-viral DNA control, three different groups of wild type flies received 0.01, 0.02 or 0.05 pg of *S. epidermidis* DNA; the indicated weight of bacterial DNA injected is equivalent to the weight of 70, 140, 280 copies of EBV DNA respectively. In addition, a group of wild type flies received no injections while another received sterile water, the DNA solvent, thus both groups served as negative controls. Groups of 1-day-old flies that overexpressed Toll10b, STAT92e and Relish were included as positive controls for Toll, JAK-STAT and IMD pathway activation as well. All injections were administered into the thorax of CO_2-anesthetized flies using a Nano-injector and glass capillary needles. A total volume of 55.2 nl was injected into each fly (Table 2). Ten flies were collected per group per time point for gene expression studies on days 1 and 3 post-injection. Collected

flies were placed at -20˚C for RNA extraction performed as described in Section 6 below. Hence a total of 190 flies were utilized for this experiment.

The copy numbers of EBV DNA injected into flies were extrapolated from our previous studies in mice (40). *Drosophila* equivalent copy numbers were calculated using the following formula:

$$Drosophila\ equivalent\ dose = Mouse\ dose \times \frac{Mouse\ Km}{Drosophila\ Km}$$

Where $Km = \frac{Weight\ (kg)}{Body\ surface\ area(m^2)}$

The used formula is extrapolated from the Food and Drug Administration (FDA) recommended formula for dose conversion (99):

$$Human\ equivalent\ dose = Animal\ (mg/kg) \times \frac{Animal\ Km}{Human\ Km}$$

The FDA approach is based on the body surface area (BSA); hence the BSA of *D. melanogaster* was calculated using Mosteller's formula (100):

$$BSA = \sqrt{\frac{height\ (cm) \times weight\ (kg)}{3600}}$$

For further assessment of diptericin gene expression in response to EBV DNA, three groups of wild type flies received 10, 35 or 70 copies of EBV DNA. A group of wild type flies was left un-injected while another received sterile water, both serving as negative controls. Injections were administered as described above. In addition, flies that overexpress Relish were included as positive control for IMD pathway activation as well. Ten flies were collected after 6, 12 and 24-hours post-injection.

To examine the effect of abrogating the IMD pathway, IMD-RNAi flies were injected with 70 copies of EBV DNA or were left un-injected. As controls, groups of wild type flies were injected with 70 copies of EBV DNA, sterile water or left un-injected. Injections were administered as above. In addition, flies that overexpressed Relish were included as positive controls for IMD pathway activation. Ten flies were collected per group on day 1 post-injection for diptericin gene expression assessment. These studies thus utilized a total of 60 flies.

For cell proliferation studies, wild type flies were injected with 70, 140 or 280 copies of EBV DNA or with 0.01, 0.02, 0.05 pg of *S.epidermidis* DNA. Wild type flies that received sterile water or were left un-injected were included as negative controls. Three flies were collected per group on days 1 and 3 post-injection and examined for cell proliferation as described in Section 9 below. Hence, a total of 72 flies were used for the proliferation studies.

B. RNA Extraction

RNA extraction was performed using TRIzol (TRI reagent) (Sigma-Aldrich, St. Louis , MO) according to the manufacturer's specifications. One hundred and fifty μl of lysis reagent were added to the flies from each group and the homogenized using a Pestle. The homogenate was incubated at room temperature for 5 minutes and then centrifuged at 15,000g for 5 minutes at 4°C. Thirty μl of chloroform was added to the homogenate and the tube was vigorously vortexed for 1 minute, incubated for 1 minute at room temperature and centrifuged at 15,000 g for 10 minutes at 4°C. The upper aqueous phase was transferred to a new tube and then isopropanol was added. The volume of isopropanol used was 0.7 x the volume of the sample. Samples were centrifuged at 15,000g for 10 minutes at 4°C. Two hundred and fifty μl of 70% ethanol was added to the RNA pellet before centrifuging at 15,000g for 10 minutes at 4°C. The supernatant was removed, and the RNA pellet was air-dried for 2 minutes. The RNA was then resuspended in 25 μl RNase-free water. The concentration and purity was assessed using a nanodrop spectrophotometer (ds11 Denovix Tc 312, Wilmington, DE).

C. Reverse Transcriptase Real-Time PCR

Reverse transcriptase real-time PCR was performed to detect the relative gene expression of drosomycin, diptericn, and TotA which are downstream products of the Toll, IMD, and JAK-STAT pathways respectively.

1. cDNA synthesis

cDNA synthesis was performed using the QuantiTect® Reverse Transcription Kit (QIAGEN, Hilden, Germany) according to the manufacturer's specification. All the reagents were thawed and kept on ice throughout the preparation and procedure.

1µg of RNA in a volume of 2 µl were placed in a sterile, nuclease-free, thin-walled PCR tube. Elimination of genomic DNA was achieved by adding 2 µl of the gDNA Wipeout Buffer and incubating for 2 minutes at 42ºC on a heat block, and then placing the tube immediately on ice.

For cDNA synthesis, 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer, and 1 µl of RT Primer Mix were added to each sample tube. These were gently mixed and then incubated for 15 minutes at 42ºC. To inactivate the Quantiscript Reverse Transcriptase, samples were incubated at 95ºC for 3 minutes using a thermal cycler (PCR SPRINT, Thermo Electron Corporation, Waltham, MA) with a heated lid. The final cDNA products were stored at -20 ºC for later use.

2. Real-time polymerase chain reaction

Real-time PCR was performed to detect the relative gene expression of drosomycin, diptericn, and TotA which are downstream products of the Toll, IMD, and JAK-STAT pathways respectively.

Primers used were obtained from Thermo Scientific (Ulm, Germany) and were blasted to ensure specificity using the NCBI primer BLAST tool .The forward and reverse primers for Drosomycin had the following respective sequences: 5′-

TACTTGTTCGCCCTCTTCG-3′ and 5′-GTATCTTCCGGACAGGCAGT-3′ (101); the expected product length was 84 base pairs. The forward and reverse primers for Diptericin had the following respective sequence: 5'- AAGTGGGAAGCACCTACACCTACA -3' and 5'-GTATCTTCCGGACAGGCAGT-3' (91); the expected product length was 247 base pairs. The forward and reverse primers for TotA had the following respective sequence: 5′-CCCAGTTTGACCCCTGAG -3′ and 5′-GCCCTTCACACCTGGAGA -3′ (102); the expected product length was 144 base pairs. Finally, the forward and reverse primers for RPL32 had the following respective sequences: 5'- GACGCTTCAAGGGACAGTATCTG -3' and 5'-AGGGCCACAGCATGGGTCTGT-3' (103); the expected product length was 144 base pairs (Table 3).

Each real-time PCR reaction consisted of 10 µl and contained 5 µl of SYBR green, 150 pmoles of the forward primer, 150 pmoles of the reverse primer and 150 ng of cDNA. Samples were run in triplicates. Real time detection was then performed in a BioRad CFX96 Real Time System employing a C1000 Thermal Cycler (Munich, Germany). The cycling conditions were as follows: PCR initial activation step took place at 95ºC for 5 minutes followed by 40 cycles of 95ºC for 15 seconds and 30 seconds at the annealing temperature for each primer (Table 3).

3. Relative Gene Expression Analysis

The threshold cycle (C_T) for each target gene was normalized to that of the reference (ref) gene (RPL32), for both the test sample and the calibrator sample (water injection):

$\rightarrow$ $\Delta C_T(\text{test}) = C_T(\text{target, test}) - C_T(\text{ref, test})$

→ ΔC_T(calibrator) = C_T (target, calibrator) – C_T (ref, calibrator)

Then, the ΔC_T of the test sample was normalized to the ΔC_T of the calibrator:

→ $\Delta\Delta C_T = \Delta C_T$ (test) – ΔC_T (calibrator)

Finally, relative gene expression normalized to the water-injected group was calculated using the Livak method (104)

→ Normalized relative expression = $2^{-\Delta\Delta CT}$

D. Hemocyte count

To analyze fly immune cell proliferation, hemocytes were counted as previously described (105). Three female flies per time point from each assessed group were anesthetized with CO_2 and the wings were removed using fine scissors. The three flies were then placed in 10 µl of phosphate buffered saline (PBS). Without disturbing the adult abdomen, the thorax was exposed by a fine incision using dissecting forceps with superfine tips. The flies were kept in the aforementioned PBS for 20 seconds in order to collect the adult bleed. The total bleed volume was then transferred to a hemocytometer and the hemocytes were counted under a light microscope using a 40X magnification. The experiment was performed at two time points (days 1 and 3 post-injection) and repeated three times.

E. Statistical analysis

To analyze statistical significance unpaired t-tests were performed using the Graphpad software; p-values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Relative gene expression of drosomycin

Intrathoracic injection of 70, 140, and 280 copies of EBV DNA into *Drosophila melanogaster* did not result in any significant increase in the relative gene expression of drosomycin compared to flies injected with distilled water on days 1 and 3 post-injection (Table 4, Figure 1).

The injection of flies with *Staphylococcus epidermidis* DNA did not result in notable changes in the gene expression of drosomycin compared to the water-injected group on days 1 and 3 post-injection either (Table 4, Figure 1). On the other hand, the normalized expression level of drosomycin in the Toll10b flies used as positive controls for the expression of this gene was 9.39 (p=0.0001).

B. Relative gene expression of TotA

Intrathoracic injection of 70, 140, and 280 copies of EBV DNA into *D. melanogaster* did not result in any significant increase in the relative gene expression of TotA compared to flies injected with sterile water on days 1 and 3 post-injection (Table 5, Figure 2). Similarly, no notable effects on TotA expression were observed in flies injected with *S. epidermidis* DNA. (Table 5, Figure 2). On the other hand, the normalized expression level

of TotA in the STAT92e flies used as positive controls for the expression of this gene was 14.46 (p=0.0178).

C. Relative gene expression of diptericin

The injection of 70 copies of EBV DNA into *D. melanogaster* resulted in a 115-fold increase (p=0.0002) in the transcriptional levels of diptericin compared to its expression on day 1 post-injection with sterile water (Table 6, Figure 3). This level then decreased to 5.62 on day 3. No notable changes were observed upon injecting flies with 140 or 280 copies of EBV DNA or with *S. epidermidis* DNA on days 1 and 3 post-injection.

To examine whether lower copy numbers of EBV DNA induce the expression of diptericin and if its expression is affected prior to day 1 post injection, flies were injected with various copy numbers of EBV DNA and assessed 6, 12 and 24 hours post-injection. Intrathoracic injection of 10 and 35 copies of EBV DNA into *Drosophila melanogaster* resulted in a significant increase in the relative gene expression of diptericin by 13 folds (p=0.0109) and 16 folds (p= 0.0199) respectively at 24 hours but not at 6 or 12 hours post-injection (Table 7, Figure 4). On the other hand, the injection of 70 copies of EBV DNA resulted in a significant increase in the relative gene expression of diptericin by 16 folds (p=0.0163) at 12 hours but not at 6 hours post-injection.

In contrast to the results observed for wild-type flies, the injection of 70 copies of EBV DNA into IMD-knockdown flies did not result in any increase in the gene expression of diptericin relative to the water injected group on day 1 post-injection (Table 8, Figure 5).

D. Number of hemocytes in adult female fly bleed

On day 1 post-injection, a significant 7-fold increase ($p=0.0009$) in the number of circulating hemocytes was observed in flies administered 70 copies of EBV DNA compared to flies injected with distilled water. In comparison, an injection of *S. epidermidis* DNA consisting of 0.01 pg, which is equivalent to the weight of 70 copies of EBV DNA, only resulted in a 24% increase ($p=0.0493$). On the other hand, a 46% decrease ($p=0.0009$) in the hemocyte number was seen upon injecting flies with 140 copies of EBV DNA on day 1 post-injection compared to the number of hemocytes in control flies injected with distilled water. Injection with 280 copies of EBV DNA did not yield any notable changes on day 1 post-injection compared to the water-injected group.

On day 3 post-injection, administration of 70 or 140 copies of EBV DNA resulted in a significant decrease in the number of hemocytes. A 42 % decrease ($p=0.0016$) was noted in the group injected with 70 copies of EBV DNA while a 50% decrease ($p=0.0013$) was seen in the group injected with 140 copies compared to the water-treated group on day 3 post-injection. The flies injected with 280 copies, on the other hand, did not display a statistically significant decrease in the number of circulating hemocytes compared to the water-treated group on day 3 post-injection.

Table 1: *Drosophila melanogaster* strains used

Flies	**Property**
W1118	Wild type flies
UAS-STAT92e	Activation of the JAK-STAT Pathway
UAS-Relish	Activation of the IMD Pathway
UAS-Toll10b	Activation of the Toll Pathway
IMD-RNAi	Knock-down of the IMD gene
Cg25c-GAL4	Hemocyte and fat body driver

Table 2: *Drosophila melanogaster* groups and injections used

Group	Number of flies	Injection
1-Assessement of drosomycin, diptericin and TotA on days 1 and 3 post-injection		
G1	20	-
G2	20	Sterile water
G3	20	EBV DNA(70 copies)
G4	20	EBV DNA(140 copies)
G5	20	EBV DNA(280 copies)
G6	20	*S. epidermidis* DNA(0.01 pg)
G7	20	*S. epidermidis* DNA(0.02 pg)
G8	20	*S. epidermidis* DNA(0.05 pg)
2- Assessment of diptericin at 6, 12 and 24 hrs post-injection		
G9	30	-
G10	30	Sterile water
G11	30	EBV DNA (10 copies)
G12	30	EBV DNA (35 copies)
G13	30	EBV DNA (70 copies)
3-Assessement of circulating hemocyte counts on days 1 and 3 post-injection		
G14	9	-
G15	9	Sterile water
G16	9	EBV DNA (70 copies)
G17	9	EBV DNA (140 copies)
G18	9	EBV DNA (280 copies)
G19	9	*S. epidermidis* DNA (0.01 pg)
G20	9	*S. epidermidis* DNA (0.02 pg)
G21	9	*S. epidermidis* DNA (0.05 pg)

Table 3: Primer sequences and annealing temperatures for drosomycin, diptericin, TotA and RPL32 primers

Primer	Sequences	Annealing temperature (ºC)
Drosomycin	F: 5′-TACTTGTTCGCCCTCTTCG-3 R: 5′-GTATCTTCCGGACAGGCAGT-3′	63
Diptericin	F: 5'- AAGTGGGAAGCACCTACACCTACA-3' R: 5'-GTATCTTCCGGACAGGCAGT-3'	63
TotA	F: 5′- CCCAGTTTGACCCCTGAG-3′ R: 5′-GCCCTTCACACCTGGAGA-3′	58.7
RPL32	F: 5'- GACGCTTCAAGGGACAGTATCTG-3' R: 5'-AGGGCCACAGCATGGGTCTGT-3'	59.6

Table 4: Relative gene expression of drosomycin in *Drosophila melanogaster* on days 1 and 3 post-injection with microbial DNA

	Flies	Injections	Drosomycin relative gene expression	SD	*P-value
Day 1	Wild type	Water	1.015678	0.234049	
	Wild type	Not injected	0.02704	0.00353	0.0019
	Wild type	EBV DNA (70 copies)	0.112123322	0.194203	0.0068
	Wild type	EBV DNA (140 copies)	0	0	0.0017
	Wild type	EBV DNA (280 copies)	0	0	0.0017
	Wild type	*S. epidermidis* DNA (0.01pg)	1.00	0.60602	0.9687
	Wild type	*S. epidermidis* DNA (0.02pg)	1.42435	0.279617	0.1242
	Wild type	*S. epidermidis* DNA (0.05pg)	0	0	0.0017
Day 3	Wild type	Water	0.296439	0.039395	0.0063
	Wild type	Not injected	0.019106	0.000682	0.0018
	Wild type	EBV DNA (70 copies)	0.131035	0.090048	0.0036
	Wild type	EBV DNA (140 copies)	0.045893	0.010149	0.0020
	Wild type	EBV DNA (280 copies)	0	0	0.0017
	Wild type	*S. epidermidis* DNA (0.01pg)	0.015525	0.015949	0.0019
	Wild type	*S. epidermidis* DNA (0.02pg)	0.094545	0.040579	0.0027
	Wild type	*S. epidermidis* DNA (0.05pg)	0.307154	0.463597	0.0820
	Toll 10b (positive control)	Not injected	9.397963	0.678948692	0.0001

** compared to the water-injected group on day 1.*

Table 5: Relative gene expression of TotA in *Drosophila melanogaster* on days 1 and 3 post-injection with microbial DNA

	Flies	Injections	TotA relative gene expression	SD	P-value*
Day 1	Wild type	Water	1.009903071	0.16715	
	Wild type	Not injected	0.007666	0.001506	0.0005
	Wild type	EBV DNA (70 copies)	0.911405	0.295529	0.6417
	Wild type	EBV DNA (140 copies)	0.016648	0.027871	0.0005
	Wild type	EBV DNA (280 copies)	0	0	0.0005
	Wild type	*S. epidermidis* DNA (0.01pg)	1.099646	0.738482	0.8474
	Wild type	*S. epidermidis* DNA (0.02pg)	0.869258	0.197277	0.3995
	Wild type	*S. epidermidis* DNA (0.05pg)	0	0	0.0005
Day 3	Wild type	Water	0.157543	0.080042	0.0013
	Wild type	Not injected	0.009214	0.00133	0.0005
	Wild type	EBV DNA (70 copies)	0.055049	0.005332	0.0006
	Wild type	EBV DNA (140 copies)	0.034854	0.006013	0.0005
	Wild type	EBV DNA (280 copies)	0	0	0.0005
	Wild type	*S. epidermidis* DNA (0.01pg)	0.090843	0.063157	0.0009
	Wild type	*S. epidermidis* DNA (0.02pg)	0.022224	0.002707	0.0005
	Wild type	*S. epidermidis* DNA (0.05pg)	0.01754	0.015163	0.0005
	STAT92e (positive control)	Not injected	14.46203	5.99617	0.0178

** compared to the water-injected group on day 1.*

Table 6: Relative gene expression of diptericin in *Drosophila melanogaster* on days 1 and 3 post-injection with microbial DNA

	Flies	Injections	Diptericin relative gene expression	SD	P-value*
Day 1	Wild type	Water	1	4.48549196	
	Wild type	Not injected	0.02485022	0.003836782	0.7256
	Wild type	EBV DNA (70 copies)	115.1918518	15.59413263	0.0003
	Wild type	EBV DNA (140 copies)	0.03	0.046497328	0.7270
	Wild type	EBV DNA (280 copies)	0	0	0.7190
	Wild type	*S. epidermidis* DNA (0.01pg)	0.83	0.120385149	0.9508
	Wild type	*S. epidermidis* DNA (0.02pg)	6.03	3.254745571	0.1910
	Wild type	*S. epidermidis* DNA (0.05pg)	0	0	0.7190
Day 3	Wild type	Water	11.41	3.64	0.0355
	Wild type	Not injected	0.022449302	0.00334363	0.7250
	Wild type	EBV DNA (70 copies)	5.62	2.907825503	0.2087
	Wild type	EBV DNA (140 copies)	0.25	0.226914338	0.7868
	Wild type	EBV DNA (280 copies)	0	0	0.7190
	Wild type	*S. epidermidis* DNA (0.01pg)	5.48	3.243729676	0.2336
	Wild type	*S. epidermidis* DNA (0.02pg)	14.96	8.388829083	0.0639
	Wild type	*S. epidermidis* DNA (0.05pg)	0.45	0.771496652	0.8444
	Relish (positive control)	Not injected	113.1635903	51.42564133	0.0197

**Compared to the water-injected group on day 1*

Table 7: Relative gene expression of diptericin in *Drosophila melanogaster* at 6, 12 and 24 hours post-injection with EBV DNA

	Flies	Injections	Diptericin relative gene expression	SD	P-value*
6 hours	Wild type	Water	1	1.672	
	Wild type	Not injected	0.74	0.391	0.8094
	Wild type	EBV DNA(10 copies)	3.86	0.69	0.0519
	Wild type	EBV DNA (35 copies)	1.824462358	0.9635	0.5005
	Wild type	EBV DNA (70 copies)	1.712615879	0.458	0.5160
12 hours	Wild type	Water	1.680086774	0.861	0.5652
	Wild type	Not injected	3.095263056	1.37	0.1687
	Wild type	EBV DNA (10 copies)	3.151259152	0.58	0.1033
	Wild type	EBV DNA (35 copies)	3.649628973	0.60	0.0612
	Wild type	EBV DNA (70 copies)	16.35476914	6.454	0.0163
24 hours	Wild type	Water	0.622844503	0.20	0.7180
	Wild type	Not injected	0.018513851	0.002	0.3669
	Wild type	EBV DNA (10 copies)	13.13868895	4.36	0.0109
	Wild type	EBV DNA (35 copies)	16.50020737	6.95	0.0199
	Wild type	EBV DNA (70 copies)	115.1918518	15.5	0.0002
	Relish (positive control)	Not injected Relish	113.1635903	51.4	0.0195

**Compared to the water-injected group at 6 hours*

Table 8: Relative gene expression of diptericin in IMD-knocked down flies on day 1 post-injection with EBV DNA

Flies	Injections	Drosomycin relative gene expression	SD	P-value*
Wild type	Water	1.026551	0.33594	
Wild type	Not injected	0.02485022	0.003836782	0.0067
Wild type	EBV DNA (70)	115.1918518	15.59413263	0.0002
IMD-RNAi	EBV DNA (70)	0.055273171	0.012274771	0.0075
IMD-RNAi	Not injected	0.28389224	0.097148873	0.0212
Relish (positive control)	Not injected	113.1635903	51.42564133	0.0212

**Compared to the water-injected group on day 1*

Table 9: Number of circulating hemocytes in adult female fly bleed on days 1 and 3 post-injection with microbial DNA.

	Flies	Injections	Number of hemocytes	SD	P-value*
Day 1	Wild type	Water	187.33	18.14	
	Wild type	Not injected	177.33	11.93035345	0.4698
	Wild type	EBV DNA (70 copies)	1304.33	215.5025135	0.0009
	Wild type	EBV DNA (140 copies)	95	2	0.0009
	Wild type	EBV DNA (280 copies)	159.66	21.07921567	0.1600
	Wild type	*S. epidermidis* DNA (0.01pg)	232.66	21.5019379	0.0493
	Wild type	*S. epidermidis* DNA (0.02pg)	94.16	9.358596761	0.0014
	Wild type	*S. epidermidis* DNA (0.05pg)	176.66	3.214550254	0.3729
Day 3	Wild type	Water	324.33	19.13983629	0.0008
	Wild type	Not injected	355.66	30.98924545	0.2105
	Wild type	EBV DNA (70 copies)	187.66	24.78574859	0.0016
	Wild type	EBV DNA (140 copies)	160.66	29.39954648	0.0013
	Wild type	EBV DNA (280 copies)	262	23.64318084	0.0238
	Wild type	*S. epidermidis* DNA (0.01pg)	380	27.18455444	0.0441
	Wild type	*S. epidermidis* DNA (0.02pg)	313	11.13552873	0.4254
	Wild type	*S. epidermidis* DNA (0.05pg)	240	39.03844259	0.0283

** compared to the water injected group on respective day.*

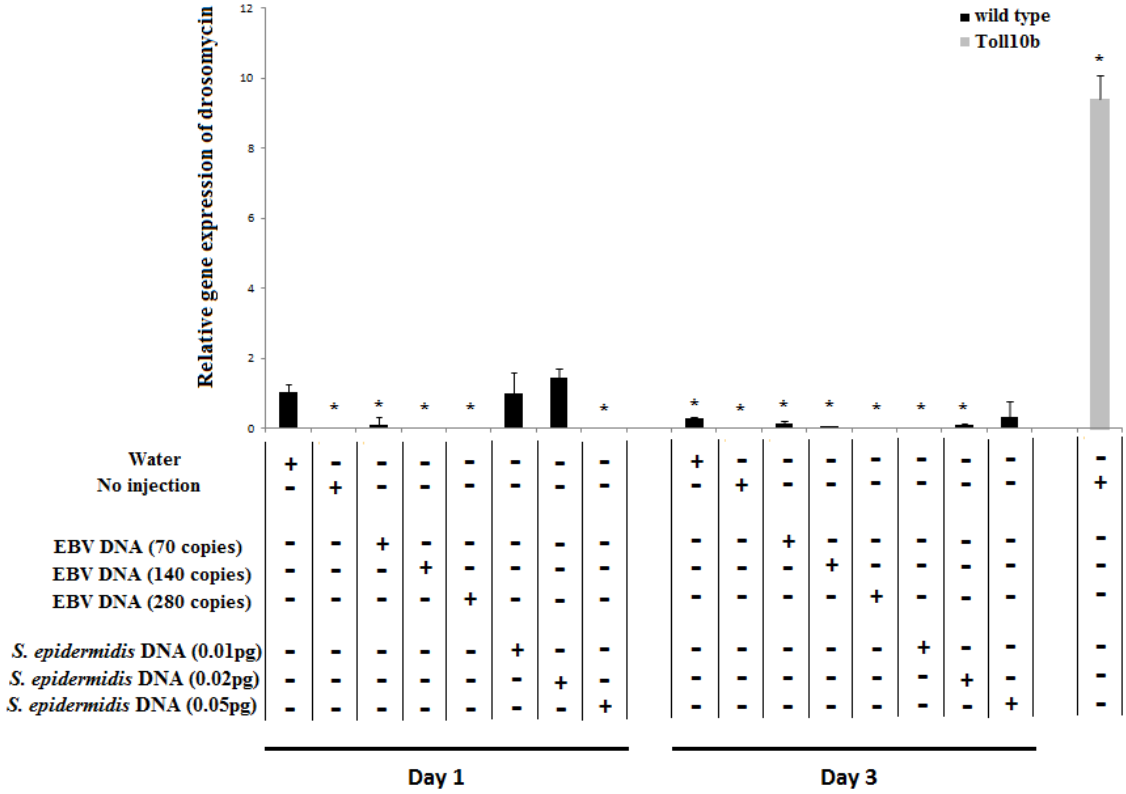

Figure.1: Drosomycin relative gene expression in wild-type flies that received no injection and wild-type flies injected with sterile water, EBV DNA (70, 140, or 280 copies) and *S. epidermidis* DNA (0.0.1pg, 0.02pg, or 0.05pg) on days 1 and 3 post-injection. Flies overexpressing toll10b were included as positive control for drosomycin expression. Data is normalized to expression in water-injected flies on day 1 post-injection. * indicates $p<0.005$.

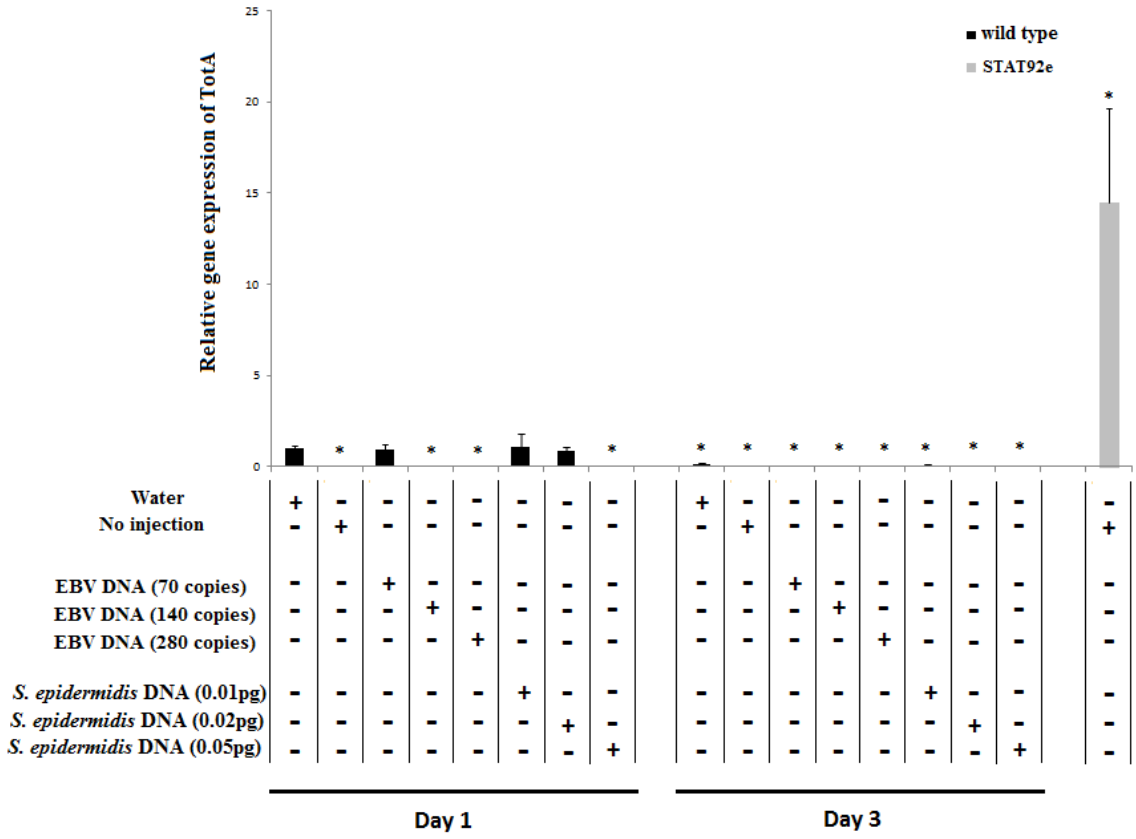

Figure.2: TotA relative gene expression in wild-type flies that received no injection and wild-type flies injected with sterile water, EBV DNA (70, 140, or 280 copies) and *S. epidermidis* DNA (0.0.1pg, 0.02pg, or 0.05pg) on days 1 and 3 post-injection. Flies overexpressing STAT92e were included as positive control for TotA expression. Data is normalized to expression in water-injected flies on day 1 post-injection. * indicates $p<0.005$.

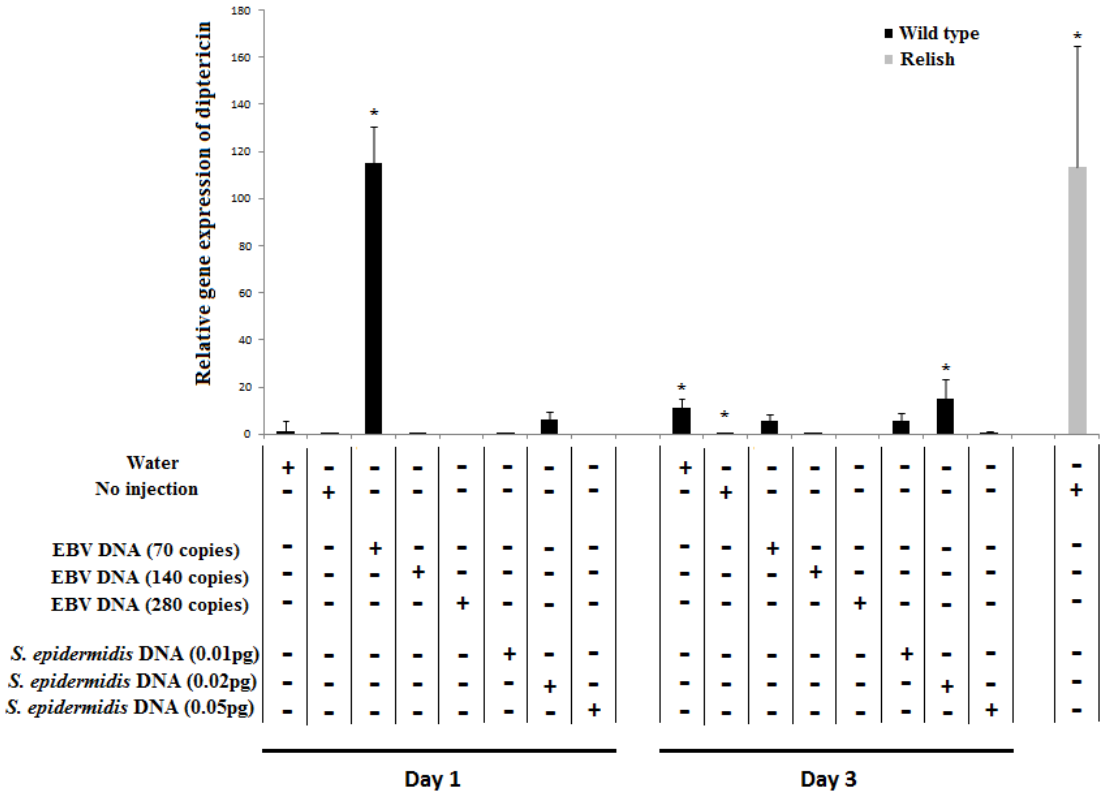

Figure.3: Diptericin relative gene expression in wild-type flies that received no injection and wild-type flies injected with sterile water, EBV DNA (70, 140, or 280 copies) and *S. epidermidis* DNA (0.0.1pg, 0.02pg, or 0.05pg) on days 1 and 3 post-injection. Flies overexpressing Relish were included as positive control for diptericin expression. Data is normalized to expression in water-injected flies on day 1 post-injection. * indicates $p<0.005$.

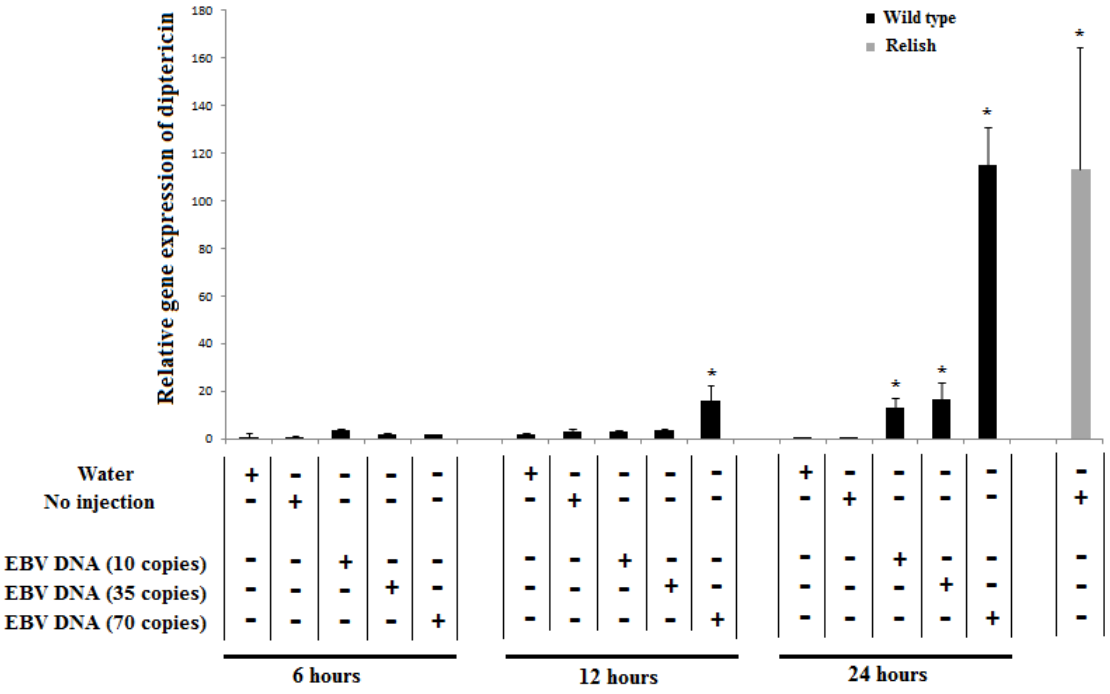

Figure.4: Diptericin relative gene expression in wild-type flies that received no injection and wild-type flies injected with sterile water, EBV DNA (70, 140, or 280 copies) and *S. epidermidis* DNA (0.0.1pg, 0.02pg, or 0.05pg) at 6, 12 and 24 hours post-injection. Flies overexpressing Relish were included as positive control for diptericin expression. Data is normalized to expression in water-injected flies at 6 hours post-injection. * indicates $p<0.005$.

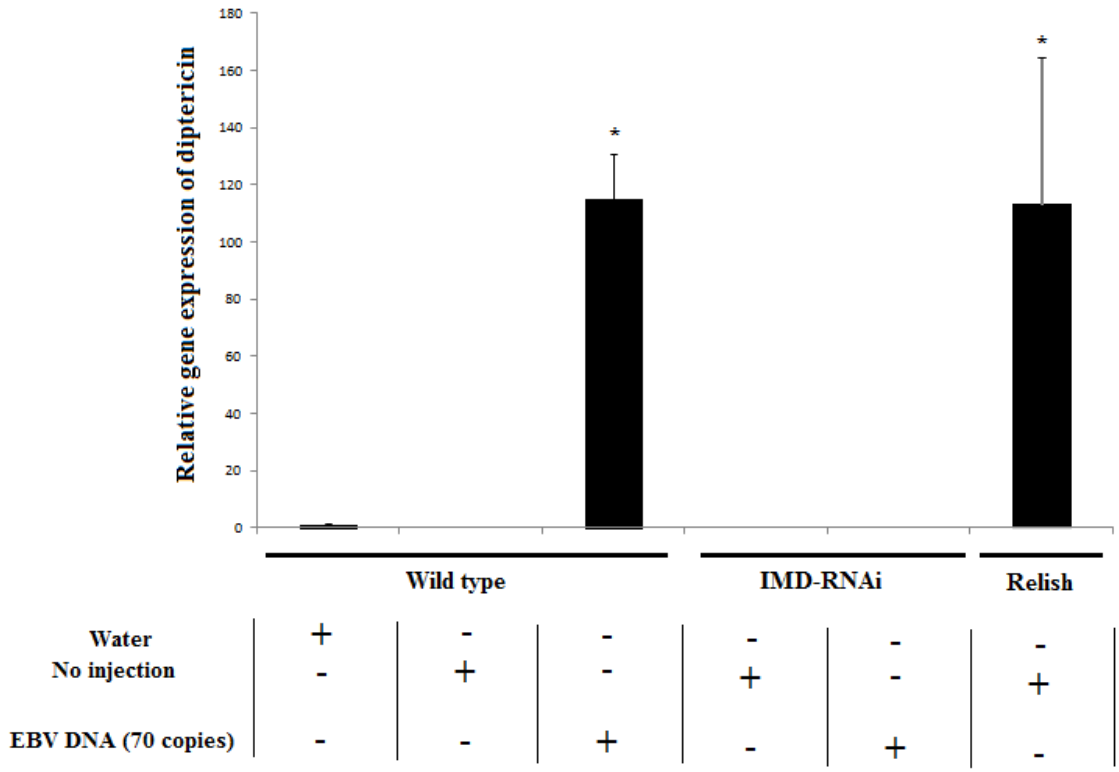

Figure.5: Diptericin relative gene expression in wild-type flies that received no injection, wild-type flies injected with sterile water and wild-type flies injected with EBV DNA (70 copies) in addition to IMD-knocked down flies that received no injection and IMD-knocked down flies injected with EBV DNA (70 copies). Expression was assessed on day 1 post-injection. Flies overexpressing Relish were included as positive control for diptericin expression. * indicates $p<0.05$

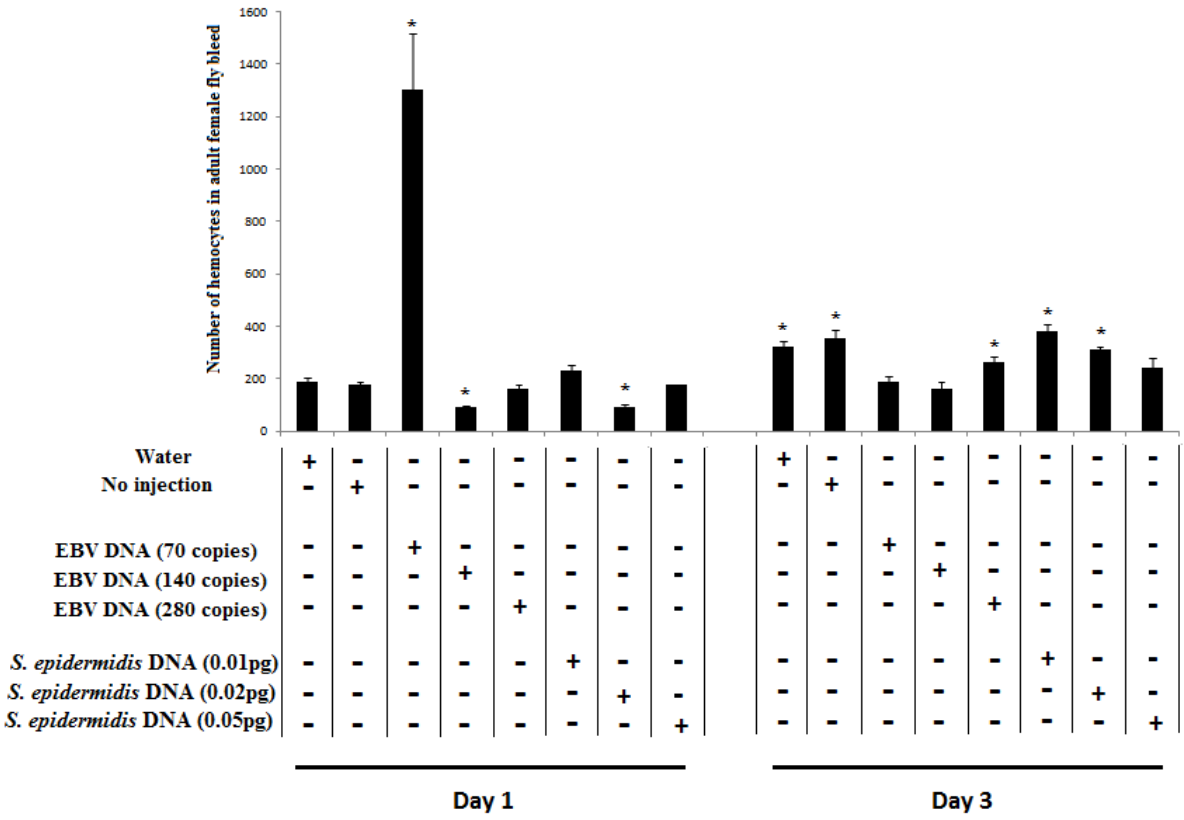

Figure.6: Number of hemocytes in adult female fly bleed in wild-type flies that received no injection and wild-type flies injected with sterile water, EBV DNA (70, 140, or 280 copies) and *S. epidermidis* DNA (0.0.1pg, 0.02pg, or 0.05pg) on days 1 and 3 post-injection. Data is normalized to the number of hemocytes in water-injected flies on day 1 post-injection.
* indicates $p<0.05$.

CHAPTER V

DISCUSSION

Innate immune responses secondary to EBV DNA presence, have been shown to be activated potentially contributing to autoimmune disease manifestation (40). Involvement of Toll-like receptor 9 (TLR9) has been previously shown at the Department of Experimental Pathology, Immunology and Microbiology to potentially trigger proinflammatory cytokine IL-17 production in response to EBV DNA in mice (41). Production of other pro-inflammatory cytokines upon treatment with EBV DNA in mammalian systems was also seen by our group, among others (40, 106), raising the possibility of involvement of other immune regulators in response to EBV DNA. The molecular complexity of mammalian immune pathways as well as the tight interaction between adaptive and immune pathway components makes it challenging to study additional immune pathways involved in response to EBV DNA. Hence, the use of a simple model organism such as *Drosophila melanogaster* with well-conserved cellular and humoral innate immune pathways in addition to the absence of adaptive immune pathways facilitates the identification of innate pathways involved in =response to EBV DNA. Therefore, the aim of this study was to examine the effect of EBV DNA on cellular and humoral immune pathways in flies.

Upon injection of 70 copies of EBV DNA into flies, the transcriptional level of diptericin was increased by 115 folds on day 1 but not day 3 post-injection compared to

flies injected with sterile water. Its levels following injection of higher copies of EBV DNA (140 and 280 copies) were not affected at these time points. This may indicate dose and time-dependent activation or repression of IMD pathway-dependent AMP (diptericin) expression. On the other hand, the transcriptional levels of drosomycin and TotA, which are indicative of Toll and JAK/SAT pathway activation, respectively, were not remarkably affected compared to flies injected with sterile water on days 1 and 3. This may indicate that EBV DNA does not activate these pathways or that their activation occurs at other time points or upon using EBV copy numbers that were not assessed. Previous studies have indicated that unmethylated CpG DNA motifs, which are abundant in the EBV genome in a nascent viral particle (107), activate innate immunity through TLR9 in mammals (41, 106). Our observations that the Toll pathway is not involved in the response to EBV DNA in flies may highlight the differences in responses of Toll and Toll-like receptors in flies and mammals. Alternatively, Toll activation in flies may occur at EBV DNA copy numbers and/or time points that were not assessed in the current study. On the other hand, it has been shown that in *D. melanogaster*, endogenous accumulation of chromosomal DNA triggered an immune response through the production of dipterican (108). Although the nature of DNA used is not microbial in this study, it may indicate that responses to various types of DNA occur via activation of the IMD pathway in flies.

To assess the effect of EBV DNA on the cellular compartment, hemocytes were counted following the injection of 70, 140, and 280 copies of EBV DNA at days 1 and 3 post-injection. A 7-fold increase in hemocyte number was only observed on day 1 post-injection of 70 copies of EBV DNA compared to flies injected with sterile water. These

results suggest that cross-talk between humoral and cellular pathways may occur in response to EBV DNA injection. A previous study suggested that the IMD pathway induces (JNK)-dependent expression of *Drosophila* PVR ligands, PVF2 and PVF3 (109), which in turn induce hemocyte proliferation in Drosophila larvae. (52). Hence, the increase in hemocyte number and diptericin expression levels upon EBV DNA injection might be regulated through IMD/JNK pathways. However, this needs to be validated in future experiments. Uncovering mediators that may be involved in the cross-talk between the IMD pathway and hemocyte proliferation may be examined by investigating whether the observed hemocyte proliferation persists in EBV DNA-injected IMD-deficient flies.

In conclusion, our results suggest that EBV DNA triggers the activation of the IMD pathway and stimulates hemocyte proliferation in flies. The IMD pathway is often compared to Tumor Necrosis Factor-α Receptor signaling (TNFR) in mammals (71); whether activation of TNFR signaling is triggered by EBV DNA in a mammalian system remains to be investigated and may provide possible therapeutic targets to control pro-inflammatory processes induced by EBV DNA.

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