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DROSOPHILA MELANOGASTER AS A MODEL SYSTEM TO ASSESS THE EFFECT OF EPSTEIN-BARR VIRUS DNA ON INFLAMMATORY GUT DISEASES

by
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AN ABSTRACT OF THE THESIS OF

Joelle Robert Madi for Master of Science
Major: Microbiology and Immunology

Title: *Drosophila melanogaster* as a model system to assess the effect of Epstein-Barr virus DNA on inflammatory gut diseases

Background: The Epstein-Barr virus (EBV), also referred to as *Human herpesvirus 4* (HHV-4), commonly infects humans and is highly associated with different types of cancer, such as gastric carcinomas, and autoimmune diseases, such as systemic lupus erythematosus. EBV has shown residency in human inflamed gastrointestinal mucosa in patients suffering from inflammation of the digestive tract such as inflammatory bowel disease (IBD); this indicates that this virus potentially plays a role in sustaining inflammation. Our group has previously reported the immune-stimulating capabilities of EBV DNA in *Drosophila melanogaster* as well as in mammalian immune systems. In flies, we observed increased systemic hemocyte counts and enhanced Immune Deficiency (IMD) pathway activation while pro-inflammatory cytokine levels were increased by EBV administration to mice. Hence, we used the fly as a model system to explore the effect of EBV DNA on intestinal damage.

Methods: Adult *D. melanogaster* flies were fed on 5% dextran sodium sulfate (DSS) solubilized in 5% sucrose in the absence or presence of EBV DNA. DSS was used to induce potential damage in fly gut tissues that would then possibly allow EBV DNA to induce alterations. Flies that were fed EBV DNA in the absence of DSS or 5% sucrose as a vehicle were included as well. To assess the impact of EBV DNA administration on the immune status of the gut, Green Fluorescent Protein (GFP)-expressing hemocytes in the gut were examined as well as GFP-expressing systemic hemocytes. GFP-marked intestinal stem cells (ISCs) and enteroblasts were assessed as indicators of fly midgut regeneration while the relative expression of *Diptericin* was determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) as a marker of IMD pathway activation in the gut.

Results: Increased numbers of hemocytes were observed in the hindguts of flies that were administered DSS as compared to the controls; administration of EBV DNA to the DSS-fed flies further increased the levels of hemocytes in fly hindguts. Moreover, the relative gene expression of *Diptericin*, as a marker of the IMD pathway, in the gut was also enhanced upon feeding flies DSS for 24 hours and then EBV DNA for 24 hours compared to controls that were fed DSS for 24 hours then sucrose, the vehicle, for 24 hours.

Conclusions: Observing increased hemocytes and enhanced innate immunity in the fly gut upon feeding EBV DNA is indicative of increased intestinal damage over DSS alone. In light of the immunostimulatory properties of EBV DNA, the study at hand indicates that EBV DNA may trigger proinflammatory processes in human gut diseases. This, however, requires further investigation.

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CHAPTER I

INTRODUCTION

The Epstein-Barr virus (EBV), also referred to as the *Human herpesvirus 4* (HHV-4), belongs to the *Herpesvirinae* family and is capable of establishing life-long latency in the infected host. EBV is associated with infectious mononucleosis in addition to various types of cancers and immune-mediated diseases such as inflammatory bowel diseases (IBDs). IBDs include Crohn's disease and ulcerative colitis, conditions that have the colon inflamed over a long period of time. The reactivation of EBV may result in the consistent shedding of DNA which could trigger innate immune pathways. Our group has previously shown that EBV DNA has immunomodulatory properties in mammalian systems as well as in *Drosophila melanogaster*. In this latter model, EBV DNA led to the activation of the Immune Deficiency (IMD) pathway marked by enhanced *Diptericin* expression in addition to resulting in increased systemic hemocyte numbers. *D. melanogaster*, a simple but efficient model, shares some similarities with mammalian immune responses in addition to digestive tract anatomy and histology. Hence, with the ultimate objective being to establish *D. melanogaster* as a model system to examine the role EBV DNA may play in IBDs, the specific aims of the study at hand were to:

1- Assess the effect of EBV DNA on cellular inflammatory responses and regenerational ones in the *D. melanogaster* gut.
2- Assess the effect of EBV DNA on the IMD humoral innate immune response in the *D. melanogaster* gut.

CHAPTER II

LITERATURE REVIEW

A. Epstein-Barr Virus

The Epstein-Barr virus (EBV), also referred to as the *Human herpesvirus 4* (HHV-4), is a prototype of the *Lymphocryptovirus* genus of the *Gammaherpesvirinae* subfamily belonging to the *Herpesvirinae* family (1). The latter is a large family of viruses divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. The subfamilies have different cellular tropisms whereby the *Alphaherpesvirinae* infect neurons and both *Beta*- and *Gammaherpesvirinae* infect lymphocytes and epithelial cells. Eight members of the *Herpesvirinae* family primarily infect humans, including EBV which infects the majority of the world's population (2). The *Herpesvirinae* family is capable of establishing latency with EBV believed to be persistent due to its ability to commonly undergo reactivation after primary infection and establishment of latency (2-5).

1. *EBV Structure*

Like other herpesviruses, EBV has a lipid bilayer envelope surrounding an icosahedral capsid, comprised of 162 capsomeres, that further encloses the dsDNA viral genome (6). The viral envelope contains virus-encoded glycoproteins that include gp350/220 and gB which are involved in the attachment of the virus to the CD21 receptor on target cell membrane and the fusion of the viral envelope with the target cell membrane, respectively (7-9). The icosahedral capsid consists of major and minor proteins, hence EBV-specific

capsid proteins consist of the following: *Bam*HI-c leftward reading frame number 1 (BcLF1) as the major capsid protein, *Bam*HI-D leftward reading frame number 1 (BDLF 1 (triplex 2)) along with *Bam*HI-O rightward reading frame number 1 (BORF1 (triplex 1)) as the minor capsid proteins and *Bam*HI-F rightward reading frame number 3 (BFRF3) as the small capsid protein (7, 10). EBV capsids range from 80 to 100 μm in diameter and assemble in the nuclei of infected cells (2). A protein tegument or viral matrix is located between the nucleocapsid and the envelope which consists of a number of amorphous proteins including the heat-shock protein 70 (Hsp-70) (11).

2. *EBV Viral Genome*

The EBV genome is linear, double stranded DNA that is approximately 172 kilobase pairs in length and that encodes for more than 85 genes. The viral genome contains terminal direct repeats (TRs), which are implicated in the formation of episomes during latency in addition to four internal repeat sequences (IRs) that divide the genome into short and long sequences, U1 and U2 respectively. The genes coded for by the viral genome are divided into latent and lytic genes. Most of the genes are involved in the lytic phase except for a few latent proteins that include Epstein-Barr nuclear antigens (EBNAs) and three latent membrane proteins (LMPs 1, 2A and 2B) (12).

EBV DNA was shown to have immunomodulatory properties in multiple systems. It was reported to trigger interferon alpha (IFN-∝) and IL-8 secretions from human monocytes and plasmacytoid dendritic cells in a Toll-like receptor 9 (TLR9)-dependent manner. TLR9 is an endosomal pattern recognition receptor (PRR) known to recognize

CpG (cytosine-phosphodiester bond-guanine)-rich unmethylated DNA such as that of the nascent EBV genome (13). In mice, EBV DNA was shown to have immune stimulatory capabilities via enhancing the production of the pro-inflammatory cytokine interleukin 17A (IL-17A) which is associated with autoimmune diseases such as rheumatoid arthritis (RA). This coincided with an increase of IL-23, another pro-autoimmune cytokine required for sustaining the IL-17 response (14). Moreover, another study conducted on mice and mouse peripheral blood mononuclear cells showed that EBV DNA was capable of modulating IL-17A and IL-23 levels by initially altering T helper 17 cells levels with the involvement of TLR9, making this endosomal receptor a possible therapeutic target with autoimmune diseases. The same study assessed a linear correlation between EBV DNA copy numbers and IL-17A levels in RA patients, which was not observed in non-RA patients (15). In *Drosophila melanogaster,* EBV DNA lead to an increase in hemocyte numbers through triggering the immune deficiency (IMD) pathway that is comparable to the tumor necrosis factor receptor signaling pathway in mammals. This was further developed in the same study, by which enhanced RNA expression of the tumor necrosis factor alpha (TNF- $\propto$), a pro-inflammatory cytokine, was observed upon injecting mice with EBV DNA (16).

3. *EBV Strain Variation*

There are two major strains of the EBV virus, EBV1 and EBV2, which are also referred to as type A and type B. The two strains mainly differ in genetic sequences coding for the Epstein-Barr nuclear antigens (EBNAs) (17, 18). Additional variants of EBV have risen likely due to various types of pressure including immunological ones (19-21).

4. *EBV Transmission*

EBV naturally infects humans and its main route of transmission is through saliva (22). Other possible modes of transmission include blood transfusions and organ transplantation (23, 24). Genital contact, transplacental transmission along with breastfeeding as modes of transmission for EBV are limited, although they had been studied due to rare reports (25-31).

5. *EBV Infection*

The EBV virus transmitted through oropharynx secretions (22) has an incubation period of about 6 weeks before its viral symptoms emanate (32). EBV spreads from the saliva to the epithelial cells of the pharynx and salivary glands located in the oropharynx where lytic infection takes place. The EBV viral envelope fuses with the infected cell plasma membrane with the aid of the envelope glycoproteins. Subsequently, the virus infects B-lymphocytes localized in the oropharynx; furthermore infected B-lymphocytes transmit the infection to other B- lymphocytes throughout the body. A long-term latency is then established in memory B cells upon the initiation of the pre-latent phase in which the pre-latent genes are expressed once the viral genome enters the nuclei of the B cells (33, 34).The latent phase takes over the pre-latent phase after a period of 2 weeks due to an epigenetic modification that involves an increase in the level of CpG methylation of the viral genomic DNA. Reactivation involves the reestablishment of the unmethylated genomic state due to an exogenous signal that leads to the reseeding of the virus into lymphoid compartments (34). Although EBV infects epithelial cells, the perception is that

EBV is predominantly a B-lymphotrophic virus. This perception was challenged when multiple lines of evidence indicated that the cellular tropism of EBV is not limited to CD21 lymphocytes based on the detection of the virus in T cells and some other types of cells (35).

6. ***EBV Diseases***

Primary infection with EBV is usually asymptomatic in childhood, but causes infectious mononucleosis syndrome in adulthood (36). EBV was the first human tumor virus to be isolated from a cell line derived from Burkitt lymphoma (37) and it is associated with many types of autoimmune diseases and cancers including Hodgkin's lymphoma (38), Non-Hodgkin's lymphoma (39), post-transplant lymphoproliferative disease (40), nasopharyngeal carcinoma (41) and gastric carcinoma (42, 43). Ten percent of gastric carcinomas contain EBV-carrying tumor cells taking into consideration that the persistence of EBV in gastric epithelium may contribute to the development of the cancer (42, 44-46). EBV infections are also associated with various autoimmune diseases including rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (47).

a. <u>EBV in Gastro-Intestinal Inflammatory Diseases</u>

The detection of EBV in gastric tissue, whether healthy or diseased, dates back to the 1990’s (42, 44-46). Not only has the association of EBV with gastric tumorigenesis been well reported, its detection in inflamed gastrointestinal mucosa indicates an association that is currently under investigation. EBV is present at low levels in healthy gastric and colonic mucosa but the latter tends to carry greater loads of the virus as it conveys a greater

association with chronic inflammatory cells than gastric mucosa. Hence, the viral loads in the different gastro-anatomical regions discussed are related to the number of EBV infected B cells. EBV has been detected in various types of inflammatory gastrointestinal types of conditions including gastritis lesions, Crohn's disease and ulcerative colitis with the following ranges: 55%-63.6% EBV positive Crohn's samples, 64%-76% EBV positive ulcerative colitis samples and 46% EBV positive gastritis samples (48-52). The association of EBV with inflamed tissue in the gut could be due to an infiltration of the virus into inflamed tissue, a gastritis either caused secondarily to an EBV infection (53-56)or due to chronic active Epstein-Barr virus (CAEBV) infection (57). CAEBV infection results from defective EBV-infected natural killer (NK) or T cell activity leading to recurrent or persistent mononucleosis-like symptoms (58, 59). CAEBV patients have persistence in the elevation of the EBV viral load in blood as well as infiltration of tissue by EBV infected NK, T and B cells; EBV is predominantly present in NK cells and T cells among Asian CAEBV patients while it is predominant in T or B cells in patients from the United States (49, 59). The only way to treat CAEBV is for patients to undergo hematopoietic stem cell transplantation. Symptoms associated with CAEBV that differentiate it from other gastro-inflammatory EBV associations include fever, persistent lymphadenopathy, splenomegaly, and EBV hepatitis (59).

Several factors highlight the need to evaluate the role played by EBV in inflammatory diseases of the gastrointestinal tract. On one hand, co-infection with EBV and *Helicobacter pylori* is more severe than infection with either pathogen alone and results in mononuclear (MN) and polymorphonuclear (PMN) cell infiltration (60). On the other

hand, levels of EBV detected in inflammatory bowel disease (IBD) inflamed tissues exceed what is expected based on the number of B-lymphocytes generally present in inflamed tissue; while both the EBV viral load and the number of B lymphocytes are elevated in these inflamed tissues, these elevations are not proportionate to one another (48). Moreover, the detection of EBV viral lytic proteins, *Bam*HI-M rightward reading frame number 1 (BMRF1) and *Bam*HI-Z leftward reading frame number 1 (BZLF1), expressed in some ulcerative colitis lesions raises the possibility that active viral replication plays a role in perpetuating the gastro-intestinal inflammation (48). Therefore, several aspects of the involvement of EBV in these diseases remain to be elucidated.

B. *Drosophila melanogaster*

1. *D. melanogaster – Innate Immunity*

Unlike mammals, flies are devoid of adaptive immunity and rely solely on their innate immunity as a defense mechanism against infections (61). The innate immune system in *D. melanogaster* consists of epithelial barriers, humoral reactions, cellular reactions and the anti-viral RNA interference pathway (62). The three major fly humoral immune pathways include: the Toll pathway, the immune deficiency pathway (IMD) and the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Moreover, humoral reactions also include melanization and coagulation. Cellular reactions include macrophage-like-hemocyte dependent phagocytosis, melanization and fights against parasitic infestation (63-66).

a. Cellular Immunity

The cellular immune response in flies consists of hemocytes which are divided into three groups: plasmatocytes, lamellocytes and crystal cells. They arise from two origins: the embryonic head mesoderm and the lymph gland. Plasmatocytes, represent 95% of total hemocytes, are phagocytic and play a role in clotting responses and are homologs of mammalian monocytes. Crystal cells, represent 5% of total hemocytes and are involved in melanization which leads to the destruction of pathogens. On the other hand, lamellocytes are involved in combatting parasitoid wasps or parasites. (67-69).

b. Humoral Immunity

Signaling pathways known to be activated through distinct activators, mentioned below, have also potential trans-activation and high levels of cross-talk (70).

i. The Toll Pathway

The Toll pathway is involved in innate immune responses against fungal and Gram positive bacterial infections that lead to the production of anti-microbial peptides (AMP) such as drosomycin (71). Nine Toll receptors have been discovered in *D. melanogaster*. The AMP production occurs through Toll-1 and Toll-9 shows the greatest homology to the 10 human Toll-like recepetors (72, 73). Unlike mammalian Toll-like receptors, the *Drosophila* toll pathway is not activated by direct binding to a pathogen but its activation requires the binding of the active form of its ligand spatzle that leads to an activation cascade that results in the transcription of AMPs (74). Mammalian Toll-like receptors play a role solely

in immunity whereas the *Drosophila* Toll pathway plays a role in immunity and development (75, 76). Moreover, the Toll-pathway in *Drosophila* is also involved in controlling the proliferation and differentiation of hemocytes decreased circulating hemocytes were observed in Toll mutants in comparison to wild type flies (77).

ii. The Immune Deficiency Pathway (IMD)

The IMD pathway is activated upon the binding of mono-diaminopimelic acid-type peptidoglycan (DAP-type PGN), that is found in all Gram negative bacteria and in some Gram positive bacteria, to the transmembrane receptor peptidoglycan recognition protein-LC (PGRP-LC) (61, 78-80). It may also play a role in some viral infections (81). The IMD pathway is comparable to the tumor necrosis factor receptor signaling pathway in humans. It is responsible for the expression of the majority of AMPs in *Drosophila* including *Diptericin,* hence it is a vital pathway in immunity (78, 82).

iii. The Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) Pathway

The JAK/STAT pathway plays a role in innate immunity and intestinal stem cell proliferation in *D. melanogaster* (83). It is also shown to be involved in antiviral immune responses in flies and mammals (84). It is activated upon the release of the unpaired proteins Upd1, Upd2 and Upd3 released from damaged cells that attach the transmembrane Domeless receptor. The ligand-receptor binding leads to the phosphorylation of STAT92e, a transcription factor that leads to the expression of genes such as the stress response gene *turnadot A* (78, 85).

2. *Drosophila melanogaster – Digestive Tract*

Drosophila melanogaster serves as a model to study the mammalian gastro-intestinal inflammation. Feeding dextran sodium sulfate (DSS) to flies leads to gut inflammation resembling that observed in inflamed mammalian gastro-intestinal tissue such as that seen in inflammatory bowel disease (IBD) patients (86).

a. *D. melanogaster* – Digestive Tract Anatomy

The modeling of human intestinal diseases is possible in *D. melanogaster* because of high degree of conservation between *D. melanogaster* and mammalian intestinal development, regeneration and the signaling pathways that control them (87). There are structural similarities throughout the mammalian and *D. melanogaster* digestive tracts. As an example, the small intestine- large-intestine-rectum-anus in mammals resemble the midgut-hindgut-rectum-anus in *D. melanogaster*, respectively. The main differences between the two systems include kindey-like malpighian tubules that empty into the *D. melanogaster* gut, along with the red papillae required for water absorption in the *D. melanogaster* rectum and the Fe/Cu cells located in areas of low pH in the *D. melanogaster* midgut (88-90).

b. *D. melanogaster* – Digestive Tract Histology

Tissue similarities are also shared among the fruit fly and human intestines, whereby both the mammalian gut and the Drosophila midgut are of endothelial origin (91, 92). They are both comprised of an epithelial monolayer of columnar or cuboidal enterocytes that contain cytoplasmic extensions on their apical side referred to as microvilli. These microvilli

increase the cellular surface area facing the gut lumen hence creating the brush border (93-95). Above the brush border, a mucus layer protects the intestine from intestinal microbes in addition to the peritrophic matrix, specific for the *Drosophila* gut that also participates in hindering microbial infections in the midgut and the hindgut (96, 97). On the basal side of the enterocytes lies the basement membrane which consists of an extracellular collagenous matrix (98). Underneath the basement membrane is an external innervated and oxygenated muscular layer that drives peristaltic movements of the intestine (99-101). The mammalian gut contains crypts (102) and three layers localized between the external muscular layer and the basement membrane that include: the submucosa, the muscularis mucosae and the lamina propria (103) which are absent in *D. melanogaster* guts.

c. *D. melanogaster* – Digestive Tract Immunity

D. melanogaster gut defenses consist of:

i. Epithelial Barrier/Peritrophic Matrix

The *D. melanogaster* peritrophic matrix, an acellular structure composed of chitin polymers and glycoproteins, is analogous to the mucus layer in the mammalian gut and it mainly functions as an intestinal shield from microbes as it is semi-permeable with its permeability being tightly controlled by protein-protein crosslinking (104-106). The drosocrystallin (dcy) protein that is an important constituent of the *Drosophila* eye lense, is also an important component of the peritrophic matrix. A mutation in the *dcy* gene leads to a reduction in the width of the matrix and increased susceptibility to oral infections as a consequence of increased permeability to larger molecules in the gut. Oral infections

induce remodeling of the peritrophic matrix which includes an up-regulation in the *dcy* expression leading to increased cross-linking in the drosocrystallin protein to further protect the gut from exotoxins (107).

ii. The Production of Reactive Oxygen Species (ROS)

Reactive oxygen species are produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family of proteins which includes the dual oxidase (DUOX) protein that is expressed in *D. melanogaster* plasma membranes (108). The DUOX protein is responsible for gut defenses against yeast and several bacterial infections. Bacterial-derived uracil is the ligand for DUOX-dependent reactive oxygen species formation under the control of the Hedgehog signaling pathway. Uracil-activated Hedgehog signaling leads to the cell adhesion molecule Cadherin 99C (Cad99C) dependent endosome production which results in ROS production (109). The production of ROS eliminates pathogens but also leads to intestinal damage thus ROS production is followed by intestinal stem cell regeneration to maintain gut homeostasis (110).

iii. Antimicrobial Peptide Production

Systemically, antimicrobial peptides (AMPs) are produced by the fat body in *D. melanogaster* and that is controlled by the two major innate immune pathways: The Toll and the IMD pathways. Locally, gut-produced AMPs are solely controlled by the IMD pathway through an inducible manner whereby AMP genes are expressed in the gut epithelia which is also under the control of the IMD pathway (111). While the Toll pathway

is not active in the gut, the JAK/STAT pathway control some gut AMP production in response to intestinal cell damage (112).

iv. Gut-associated Hemocytes

Gut-associated hemocytes are multi-functional, ranging from phagocytosing pathogens, such as bacteria, to taking part in controlling intestinal stem cell regeneration. Hemocytes tend to be found as aggregates in the *Drosophila* intestinal lumen and attached to the visceral muscle located around the midgut. Hemocyte-derived Decapentaplegic (Dpp), a bone morphogenetic protein (BMP) mammalian homolog, is required for the proliferation of intestinal stem cells (67, 113). Mammalian immune cells have also been shown to play a role in the regeneration of intestinal epithelium (114-116).

d. *D. melanogaster* – Intestinal Stem Cell Regeneration

Intestinal stem cells (ISCs) are found in both mammalian and *D. melanogaster* guts (117, 118). The ISC composition, their regeneration and the conserved signaling pathways that control their regeneration show resemblance in both systems (119). Tissue homeostasis requires ISCs to divide and replenish intestinal cells that are lost as a result of any kind of stress imposed on the gut such as inflammation. Mammalian ISCs, located at the bottom of intestinal crypts near Paneth cells self-renew and give rise to the pluripotent transit amplifying (TA) cells (120). TA cells lie directly above the ISCs and undergo divisions to mature into the specialized intestinal stem cells such as secretory Paneth cells as they progress upwards towards the crypt openings (102, 121). *D. melanogaster* ISCs are located basally and divide to give rise to enteroblasts which are transient cells that mature into

either absorptive enterocytes or hormone-secreting enteroendocrine cells which are also present in the mammalian intestine. The *D. melanogaster* midgut is the section that has been the most associated with a rich population of intestinal stem cells; on the other hand, the presence of ISCs in the hindgut has been debatable although some reports have indicated that they may be present in the hindgut proliferative zone (HPZ) (122). There are conserved signaling pathways involved in the control of intestinal stem cell regeneration amongst mammals and the fruit fly. The mammalian Wingless/Integrated (Wnt) and its well conserved *D. melanogaster* Wingless (Wg) pathway is necessary for the maintenance of ISCs in their undifferentiated states (102, 123, 124). The Notch pathway, another well conserved pathway between mammals and the fruit fly, is involved in ISC regeneration and results in the differentiation of transient cells in *D. melanogaster* to specify their fate as either absorptive or secretory cells (102, 124). In mammals, Notch induces the proliferation of ISCs (102). Some other well conserved signaling pathways among mammals and *D. melanogaster* that control intestinal stem cell regeneration include: platelet-derived growth factor (PDGF; PVF in *Drosophila*), c-Jun N-terminal kinases (JNK), insulin receptor (INSR; InR in *Drosophila*) and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways (87). The JAK/STAT pathway is activated in the *D. melanogaster* gut upon the release of the unpaired proteins Upd1, Upd2 and Upd3 cytokines from damaged ISCs (99). The homologous pathway in mammals involves the cytokine interleukin-6 (IL-6) which induces intestinal inflammation (125).

e. The *D. melanogaster* Hindgut

The hindgut begins from the malphigian tubules andis divided into three sections: the pylorus that controls the passage of gut content, the ileum that is responsible for absorption and excretion of water ions and the rectum (126). Although the presence ISCs in the hindgut has not been well determined, previous studies report that stem cells of the hindgut may be confined to an area known as the hindgut proliferative zone (HPZ). The HPZ is confined to the adult pylorus and is controlled by the Wnt and Hedgehog (Hh) signaling pathways (122). Several recent studies refer to these stem cells as "sleeping beauties", as in they remain quiescent but are capable of proliferating in response to tissue injury (127, 128). Moreover, the hindgut responds to tissue damage by stimulating differentiated cells to divide in comparison to the continuously regenerating intestinal stem cells of the midgut (128). The fast turnover of midgut cells and the silent stem cells of the hindgut resemble the mammalian intestinal crypt stem cells, some of which divide rapidly while others are silent (129). Innate immune responses in the hindgut are not well understood; however, a study conducted on a fly cancer model showed that the IMD pathway controls Ras-activated hindgut cell. This innate immune pathway was involved in driving Ras-activated hindgut cell invasion and dissemination upon challenge with a bacterial infection (129, 130). Whether the IMD pathway is involved in innate immune responses in the hindgut of a non-cancer fly model remains to be determined.

CHAPTER III

MATERIALS AND METHODS

A. Flies

1. *Fly Stocks*

Flies were maintained on regular fly food at 25℃. Standard *Drosophila* husbandry procedures were conducted at 25℃. *Hml-Gal4, UAS-GFP* (gift from Dan Hultmark), *esg-Gal4, UAS-GFP/ Cyo* (Bloomington *Drosophila* Stock Center: 67054) and wild type flies (Bloomington *Drosophila* Stock Center: 3605) were used in this study.

2. *Fly Pushing*

Virgin females were identified by their meconium and used to set up crosses. Day 0 males were collected and treated to conduct this study.

B. Treatments

Three-day old adult male flies were transferred from feeding on regular fly food into a vial fitted with a filter paper (Bio-rad, Hercules, CA) at its base. Different treatments were prepared to feed the flies: 5% sucrose was prepared by dissolving 5 g sucrose in 100 ml Millipore water and 5% DSS was prepared by dissolving 0.025 g of DSS (Abcam, Cambridge, UK) in 500 ul of 5% sucrose. For EBV DNA (Amplirun Epstein-Barr Virus DNA Control, Vircell Microbiologists, Granada, Spain) treatment, 288,000 copies of the viral DNA were added to 5% sucrose or 5% DSS as detailed below (Table 1; Figure 1).

Five hundred µl of the sucrose solution were applied to the filter paper for flies in Group 1 which fed on this preparation for the subsequent 48 hours. Five hundred µl of the DSS solution were applied onto the filter paper for flies in Group 2; after 24 hours the filter paper for these flies was switched to one that was wet with 500 µl of sucrose for another 24 hours. For Group 3, flies fed on 500 µl of the DSS solution applied to the filter paper for the subsequent 48 hours. Flies in Group 4 fed on filter paper wet with 500 µl of the sucrose solution that contained 288,000 copies of EBV DNA for 48 hours. Group 5 flies were fed off a filter paper wet with 500 µl of DSS solution containing 288,000 copies of EBV DNA for 48 hours. Five hundred µl of the DSS solution were applied onto the filter paper for flies in Group 6; after 24 hours the filter paper for these flies was switched to one that was wet with 500 µl of sucrose including 288,000 copies of EBV DNA for another 24 hours. To verify that flies were feeding off of the filter paper, 20 adult wild type flies (w^{1118}) were fed on: 500 µl of 5% sucrose that contained 288,000 copies of fluorescein-labelled EBV DNA, or 500 µl of 5% DSS that contained 288,000 copies of fluorescein-labelled EBV DNA or on 500 µl of 5% sucrose only (Advanced Biotechnologies, Colombia, MD). Images to verify the adult wild type fly feeding were taken on a fluorescent stereomicroscope (Olympus SZX 10, Waltham, MA).

All groups were fed at 25°C before gut dissections were performed.

C. Dissections, Immunostaining and Fluorescent Microscopy

For gut dissections, the entire gut was pulled out from the posterior end of the male fly using forceps (Electron Microscopy Sciences, Dumont, Number 5, Hatfield, PA) directly

into 1X phosphate-buffered saline (PBS) on a cold glass plate, by following the listed steps. The fly head was first removed to detach the gut from any attached tissue. Then the posterior end of the gut was pulled out slightly by grasping the male genitals outwards. To prevent the gut from ripping apart, the fly thorax was detached from the fly abdomen before the gut was completely pulled out of the posterior end of the fly. The detaching midway throughout the dissection allows the gut to pass through less tissue on its way out of the posterior end of the fly. Guts were then fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO) for 20 minutes followed by three washes of 10 minutes each using 1X PBS-0.3% Triton X-100 (PBST). Next, guts were placed in blocking solution (5% normal goat serum (Dako, Santa Clara, CA)) for 30 minutes. Guts were subsequently incubated overnight with either or a mixture of the following primary antibodies diluted in blocking solution: mouse anti-P1 IgG (Kind gift from Istvan Ando, 1:100), and rabbit anti-Green Fluorescent Protein (GFP) IgG (Abcam, 1:500). The guts were then washed in PBST three times for 10 minutes and incubated with the following fluorochrome-conjugated secondary antibodies diluted in blocking solution: AlexaFluor-594 anti-mouse IgG (Abcam, 1:500) and AlexaFluor-488 anti-rabbit IgG (Abcam, 1:500). After two hours of incubation with the secondary antibody, the guts were washed with PBST three times for 10 minutes. Samples were then mounted onto microscope slides using Fluoroshield Mounting Medium with DAPI (Abcam). The entire dissection/fixation process was performed on ice. Images were acquired using a laser scanning confocal microscope (Carl Zeiss Laser Scanning Microscopy 710, Jena, Germany).

D. Hemolectin-Positive Cell Count

1. *Gut Hemolectin-Positive Cell Counts*

To count GFP-Hemolectin-positive cells in guts obtained from flies treated as described above (~50 guts per group) using the *Hml-Gal4, UAS-GFP* fly line, the Image-based Tool for Counting Nuclei (ITCN) plugin on ImageJ 1.49v was used. The following parameters were employed: a width of 10 pixels, a minimum distance of 10 pixels and a threshold of 0.8 without detecting any dark peaks.

a. Statistical Analysis

To analyze cell-count statistical significance the chi squared test was used. P-values less than 0.05 were considered statistically significant.

2. *Systemic Hemolectin-Positive Cell Counts*

Five *Hml-Gal4, UAS-GFP* adult flies per group were bled in 40 µl of 8% BSA (Bovine Serum Albumin, Sigma-Aldrich) -1X PBS on a Parafilm strip by pricking the flies on their thorax using a needle. The adult flies were allowed to bleed for 30 seconds before the bleed was removed and suspended in 160 µl of 8%BSA- 1X PBS. The same procedure was done for wild type flies as a negative control. The flies were freshly bled and read by the Guava Millipore machine (Guava EasyCyte8 Flow Cytometer- Milliporesigma, Burlington, MA). The number of events to count the cells was set to 10000. The samples were vortexed before every run. The wild type fly bleed was run first and the GFP fluorescence signal threshold was set so that it does not exceed 10^1. The Gain controls were set to 378 for the

Forward scatter, 159 for the Side scatter and 8 for the green fluorescence. Each sample was repeated 3 times. The protocol was modified from Anderl, et al., 2016 (131).The plots were gated with two gates; GFP and non-GFP populations. Each gate encompassed the cell count, percentage and mean.

a. Statistical Analysis

To analyze statistical significance for GFP positive cells detected by flow cytometry, the unpaired t-test was performed. P-values less than 0.05 were considered statistically significant.

E. Midgut Intestinal Stem Cell Regeneration Analysis

To analyze an increase in midgut intestinal stem cell regeneration, the fly line: *esg-Gal4, UAS-GFP/+* was used. Escargot (Esg) is a zinc-finger transcription factor expressed in ISCs and enteroblasts that also plays a role in maintaining cell identity (117). The cells marked by *esg* are restricted to the midgut region only. The fluorescence intensity measure in the midgut of each group was assessed on ImageJ 1.49v

1. *Statistical Analysis*

To analyze statistical significance for the fluorescence intensity, the unpaired t-test was performed. P-values less than 0.05 were considered statistically significant.

F. *Diptericin* Relative Gene Expression Analysis

To assess the relative expression of *Diptericin* as an indicator of the fly IMD immune pathway, flies were treated as detailed above, their guts were obtained and RNA was then extracted for real time PCR analysis. Duplicates of the experiment were performed.

1. *RNA Extraction*

Two different RNA extraction protocols were followed depending on the sample type: one protocol was used for flies that were administered DSS while the other was employed for all other fly groups. DSS causes DNA polymerase and reverse transcriptase inhibition (132) hence preventing real time assessment unless a particular protocol was employed to remove DSS as described below.

a. RNA Extraction from Non-DSS Treated Flies

Thirty guts per group from flies treated as described in Table 1/Figure 1 were homogenized using a pestle in a volume of 200 µl of TRI reagent (TRI reagent, Sigma-Aldrich). They were then incubated for 5 minutes at room temperature in the lysis medium. Then, 40 µl of chloroform (Sigma-Aldrich) were added to the sample and shaken vigorously for 15 seconds. The sample was left at room temperature for 3 minutes before it was centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was transferred into a new tube and 100 µl of isopropanol was added followed by shaking and a 10-minute incubation period at room temperature. The sample was then centrifuged at 12,000 g for 10 minutes at 4°C and the supernatant was discarded. The pellet was washed with 200 µl of 75% ethanol followed by a centrifugation for 5 minutes at 4°C. The pellet was then air-dried and re-dissolved in 20

µl RNase-free water. The entire procedure was completed on ice. The concentration and purity of the RNA were measured using a microspectrophotometer (Denovix Tc 312, Wilmington, DE).

b. RNA Extraction from DSS Treated-Flies

RNA extraction was also performed using TRIzol (TRI reagent, Sigma-Aldrich) and lithium chloride (Sigma-Aldrich). A volume of 300 µl of TRI reagent was added onto 60 guts from each group that included DSS. The guts were homogenized using a pestle and incubated for 5 minutes at room temperature in the lysis medium. Then, 288 µl of chloroform were added to the sample and shaken vigorously for 15 seconds. The sample was left at room temperature for 3 minutes before 132 µl of isopropanol were added. The sample was shaken vigorously again for 15 seconds before it was centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was then transferred into a new tube containing lithium chloride with a final concentration of 2M and it was incubated overnight at 4°C (133). Subsequently, the sample was centrifuged at 12,000 g for 10 minutes at room temperature. The supernatant was discarded and the pellet was washed with an equal amount of 2M lithium chloride. The sample was centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was discarded again and the pellet was washed twice with 300 µl of 70% ethanol, each wash followed by centrifugation at 12,000 g for 10 minutes at 4°C. The pellet was then re-suspended in 10 µl RNase-free water. The entire procedure was completed on ice. The concentration and purity of the RNA were measured using a microspectrophotometer (Denovix Tc 312, Wilmington, DE).

2. *cDNA Synthesis*

cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-rad) according to the manufacturer's specifications. The entire procedure was completed on ice. Reaction tubes included 1 µl Reverse Transcriptase, 4 µl Reaction Mix and 500 ng of RNA in 20 µl reactions. Samples were then incubated in a thermal cycler (Thermo Electron Corporation, Waltham,MA) undergoing the following steps: priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C, reverse transcription inactivation for 1 minute at 95°C and a hold step at 4°C. The final cDNA products were stored at -20°C for later use.

3. *Real-Time Polymerase Chain Reaction (RT-PCR)*

Real-time PCR was performed to detect the relative gene expression of *Diptericin* which is the downstream product of the IMD pathway. *Ribosomal protein L 11 (RPL 11)* was used as a housekeeping gene. Forward and reverse primers (Macrogen, Seoul, South Korea) used are indicated in Table 2.

The PCR reactions consisted of 5 µl SYBR (Bio-Rad) green, 1µl of the reverse primer at a concentration of 10 pmol/uL, 1µl of the forward primer at a concentration of 10 pmol/uL and 300 ng of cDNA in a total volume of 10 µl per sample tube. Each sample was run in triplicates using the following cycles: initial activation step at 95°C for 5 minutes followed by 40 cycles consisting of 95°C for 15 seconds and 30 seconds at the annealing temperature according to each primer set used. Real time detection was performed using the Bio-Rad CFX96 Real Time System. Relative gene expression analysis was determined using the Livak method (134).

a. Statistical Analysis

To analyze statistical significance of relative expression values by real-time PCR the unpaired t-test was performed. P-values less than 0.05 were considered statistically significant.

CHAPTER IV

RESULTS

A. Validating Fly Treatment Uptake

To examine the effect of EBV DNA on the *D. melanogaster* gut, flies were fed various regimens that included EBV DNA. To validate flies feed on such regimens, fluorescein-labeled EBV DNA was added to either a sucrose or a DSS solution and then applied to a filter paper. Twenty wild type flies were used per feeding regimen and after a period of 48 hours of feeding on the filter paper, fluorescence in the abdominal and proboscis regions of the flies were observed (Figure 2). All flies that were fed EBV DNA or DSS with EBV DNA showed fluorescence hence confirming uptake of the EBV DNA even in the presence of DSS.

B. Number of *Hml-Gal4, UAS-GFP* Cells in Adult Male Fly Hindgut

To examine whether DSS and EBV DNA treatments affect the number of hemocytes in the fly gut, *Hml-Gal4, UAS-GFP* (GFP-Hemolectin) fly line was used. Hemolectin marks hemocytes and is a protein involved in the clotting reaction (135). When observed with confocal microscopy (Figure 3), after 48 hours of feeding, all groups that had DSS included in their treatments showed a higher number of flies with ≥ 20 GFP-Hemolectin positive cells in their hindguts compared to those fed on sucrose (Table 3, Figure 4). Only 9% of analyzed flies feeding on sucrose had ≥ 20 GFP-Hemolectin positive cells in their hindguts. While the percent of flies with ≥ 20 GFP-Hemolectin positive cells in their hindguts was

26% in the group treated with DSS for 48 hours, it was 32% in the group treated with DSS and EBV DNA for 48 hours (p= 0.511). On the other hand, the percent of flies with ≥ 20 GFP-Hemolectin positive cells in their hindguts was 25% in the group of flies treated with DSS for 24 hours followed by sucrose for 24 hours, while it was 55% in the group treated with DSS for 24 hours followed by EBV DNA for 24 hours (p=0.003). This latter group had the highest proportion of flies with ≥ 20 GFP-Hemolectin positive cells in their hindguts. None of the flies that fed on EBV DNA alone for 48 hours showed any GFP-Hemolectin cells in their hindguts; compared to the control group which was fed on sucrose alone hence showing a significant decrease (p=0.023). The 20 GFP-Hemolectin positive cells was selected as a cutoff point since values below 20 were the most recurrently observed in the hindgut of flies feeding on sucrose only for 48 hours.

Since a previous report has indicated that the fly gut harbors hemolectin positive cells that are neuroendocrine rather than hemocytic (67), hindguts were immunostained with a P1 antibody, which recognizes the phagocytic NimC1 receptor expressed on cells with plasmatocytic characteristics (136) (Figure 5). Plasmatocytes comprise 95% of the hemocyte population in flies (68).We observed a co-localization between GFP-Hemolectin and P1 in the gut of flies feeding on DSS then EBV as a representative sample, hence indicating that these cells are plasmatocytic in nature.

C. Number of GFP Positive Cells in Adult Male Fly Bleed

After 48 hours of feeding on the various feeding regimens described above, the number of circulating GFP/Hemolectin positive hemocytes were assessed systemically by collecting

and analyzing the adult fly hemolymph. No significant changes were observed between the different groups (Figure 6) indicating that the feeding regimens employed likely did not induce changes at the systemic level within the timeframe assessed.

D. Intestinal Stem Cell Regeneration in Adult Male Fly Midguts

We further analyzed our phenotype by assessing the regeneration of intestinal stem cells (ISCs) in the midgut of adult male flies subjected to different treatments; the midgut was examined since it is the only gut region with known markers for ISCs (117).Previous reports have indicated that DSS of a high molecular weight, as employed in our experiments, does not induce stem cell regeneration (86). To assess whether inclusion of EBV DNA treatment would affect this, *esg-Gal4, UAS-GFP* (Esg-GFP) flies were used. Escargot (Esg) is a zinc-finger transcription factor expressed in ISCs and enteroblasts of the midgut that also plays a role in maintaining cell identity(117). Laser scanning confocal images of guts from different treatments revealed no significant changes in endogenous GFP expression (Figure 7). This was further confirmed upon quantifying the mean fluorescence intensity in ISCs in different groups, whereby no significant changes were documented (Figure 8).

E. Relative Gene Expression Levels of *Diptericin*

We had previously demonstrated that injecting flies with EBV DNA results in enhanced expression of *Diptericin* as an indicator of the IMD immune pathway (16). To assess whether our DSS and EBV feeding treatments similarly affected the expression of *Diptericin,* the relative gene expression of this mediator was assessed in the gut of adult

male flies subjected to the different treatments by real time PCR. A significant 3-fold increase in *Diptericin* expression levels was observed in the guts of flies feeding on DSS then EBV DNA compared to its expression levels in the control gut of flies feeding on DSS then sucrose ($p=0.043$) (Figure 9). However, no significant increase in *Diptericin* expression levels was documented in other assessed groups.

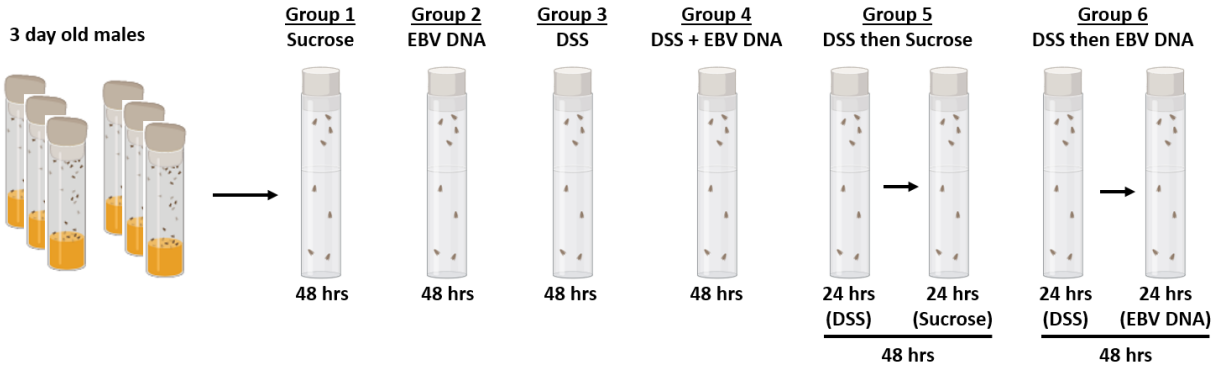

Figure 1: Feeding regimens protocol. Three-day old male flies were fed on different regimens of DSS and/or EBV DNA for 48 hours as indicated.

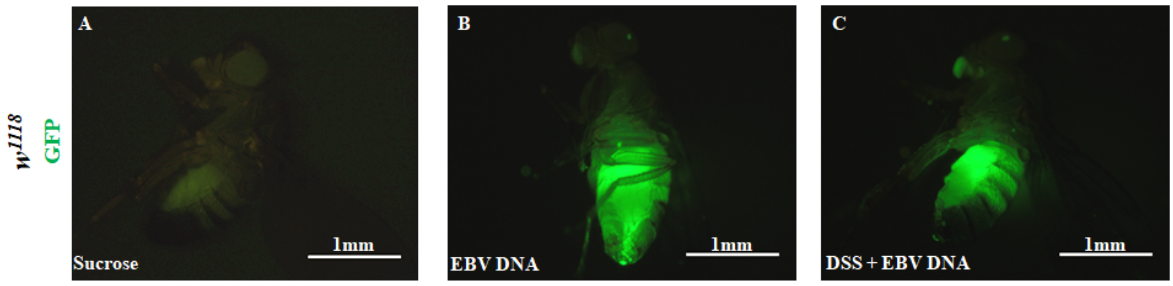

Figure 2: Three day old *w*^{1118} males fed on different regimens of DSS and/or fluorescein-labelled EBV DNA for 48 hours. (A) *w*^{1118} fly fed on sucrose (used as vehicle in the other treatments) for 48 hours. (B) *w*^{1118} fly fed on fluorescein-labelled EBV DNA for 48 hours. (C) *w*^{1118} fly fed on DSS and fluorescein-labelled EBV DNA for 48 hours.

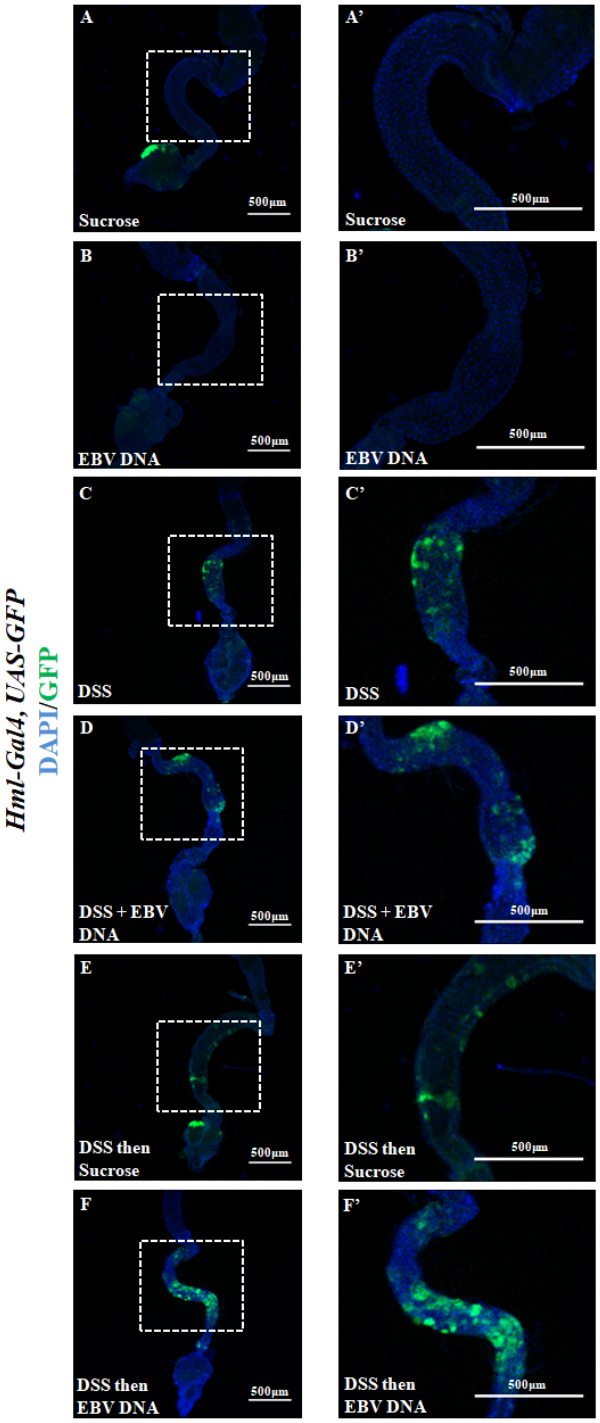

Figure 3: Hindguts of *Hml-Gal4, UAS-GFP* flies fed on different regimens of DSS and/or EBV DNA. (A) Flies fed on sucrose (used as vehicle in the other treatments) for 48 hours. (B) Flies fed on EBV DNA for 48 hours. (C) Flies fed on DSS for 48 hours. (D) Flies fed on DSS and EBV DNA for 48 hours. (E) Flies fed on DSS for 24 hours then sucrose for 24 hours. (F) Flies fed on DSS for 24 hours then EBV DNA for 24 hours. (A'-F') 2X enlargement of area indicated by a box insert denoting gut ilia in A-F respectively. GFP-Hemolectin is in green; DAPI is in blue.

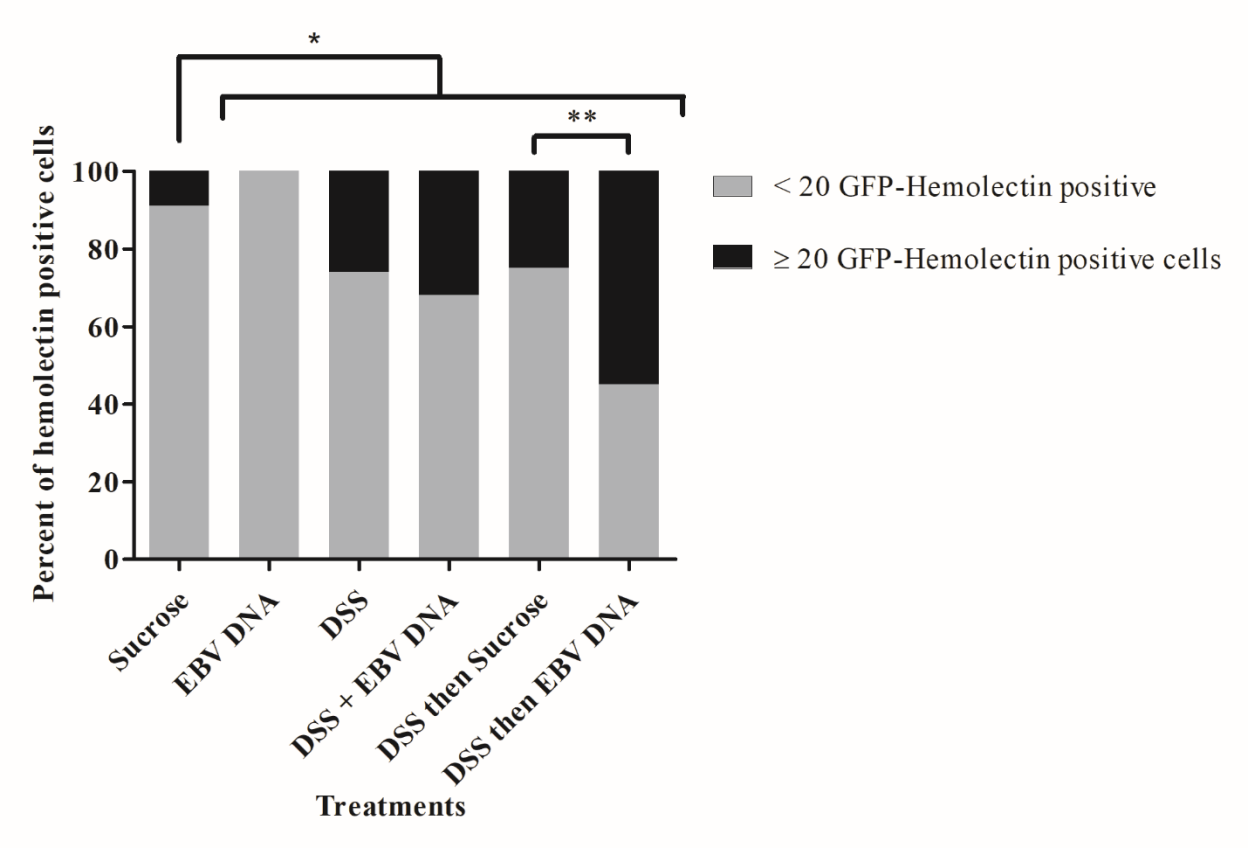

Figure 4: Percent of fly hindguts with GFP-Hemolectin positive cells after feeding on different treatments of DSS and/or EBV. Sucrose was used as vehicle. *p-value<0.05. **p-value<0.01.

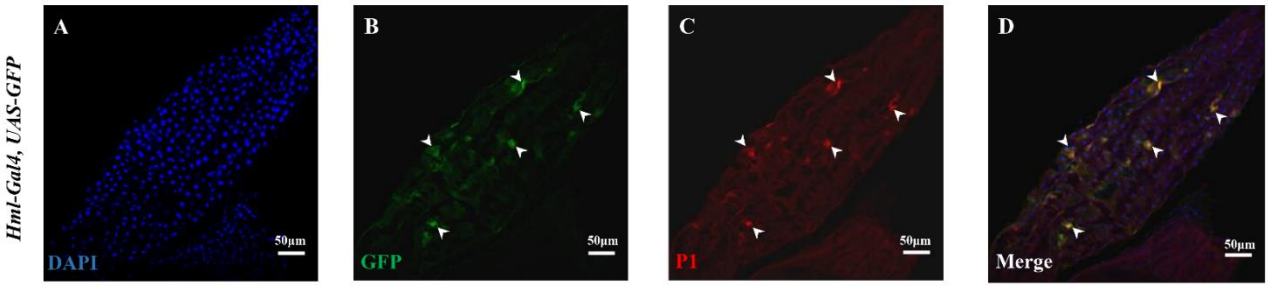

Figure 5: Hindguts of *Hml-Gal4, UAS-GFP* flies after feeding on DSS for 24 hours then EBV DNA for 24 hours. (A) DAPI. (B) GFP. (C) P1. (D) Merged DAPI, GFP and P1 images.

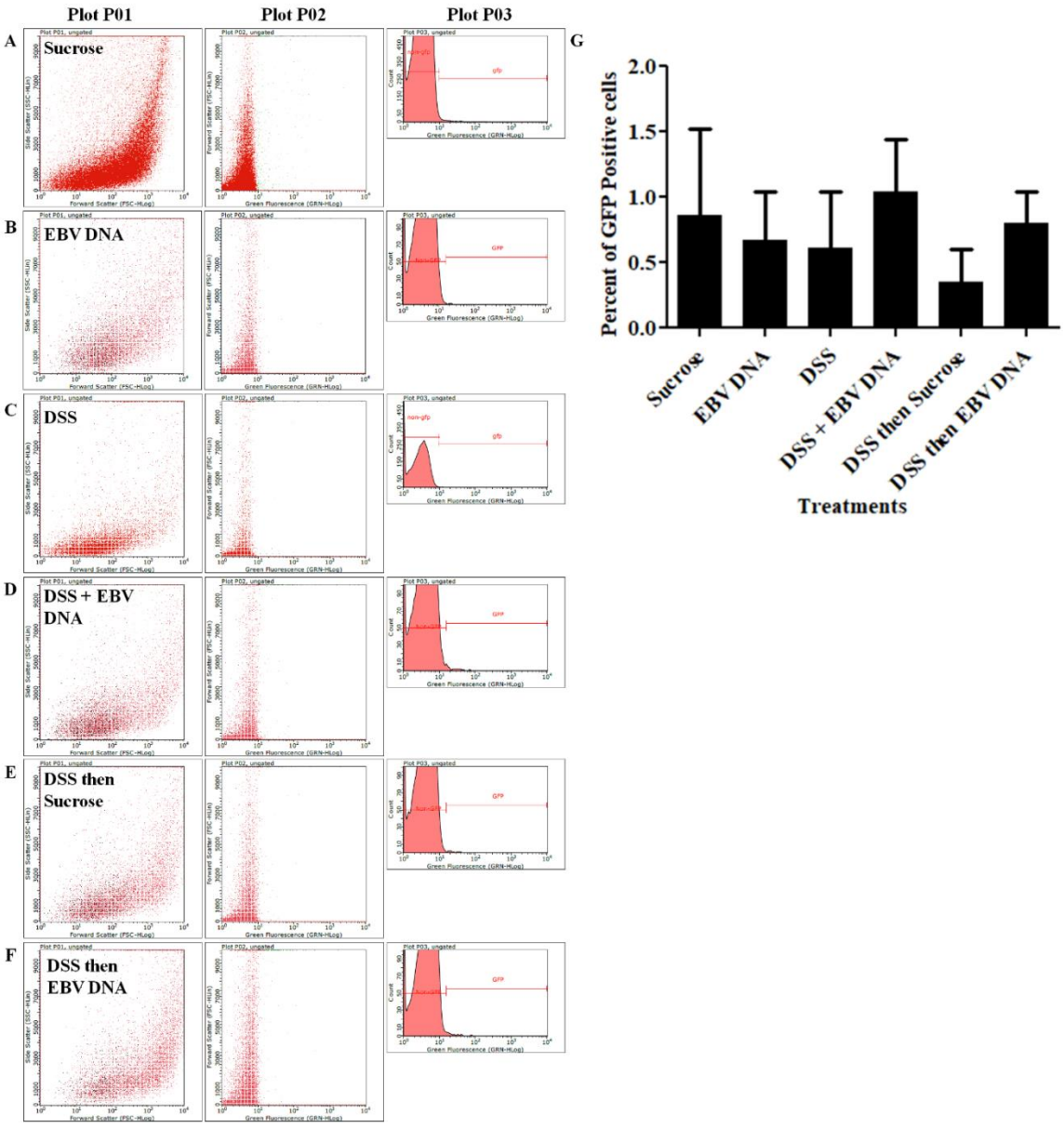

Figure 6: Flow cytometry conducted on *Hml-Gal4, UAS-GFP* hemolymph of adult male flies fed on different regimens of DSS and/or EBV DNA. (A-F) Plot01 represents the forward scatter (X-axis) against the side scatter (Y-axis) which is indicative of size and granularity, respectively. Plot02 represents the forward scatter (Y-axis) against the GFP fluorescence (Y-axis) of the cells. Plot03 represents the GFP intensity of the cells. (A) Flies fed on sucrose (used as vehicle in the other treatments) for 48 hours. (B) Flies fed on EBV DNA for 48 hours. (C) Flies fed on DSS for 48 hours. (D) Flies fed on DSS and EBV DNA for 48 hours. (E) Flies fed on DSS for 24 hours then sucrose for 24 hours. (F) Flies fed on DSS for 24 hours then EBV DNA for 24 hours. (G) Percent of GFP/Hemolectin positive cells in the hemolymph of flies described in A-F.

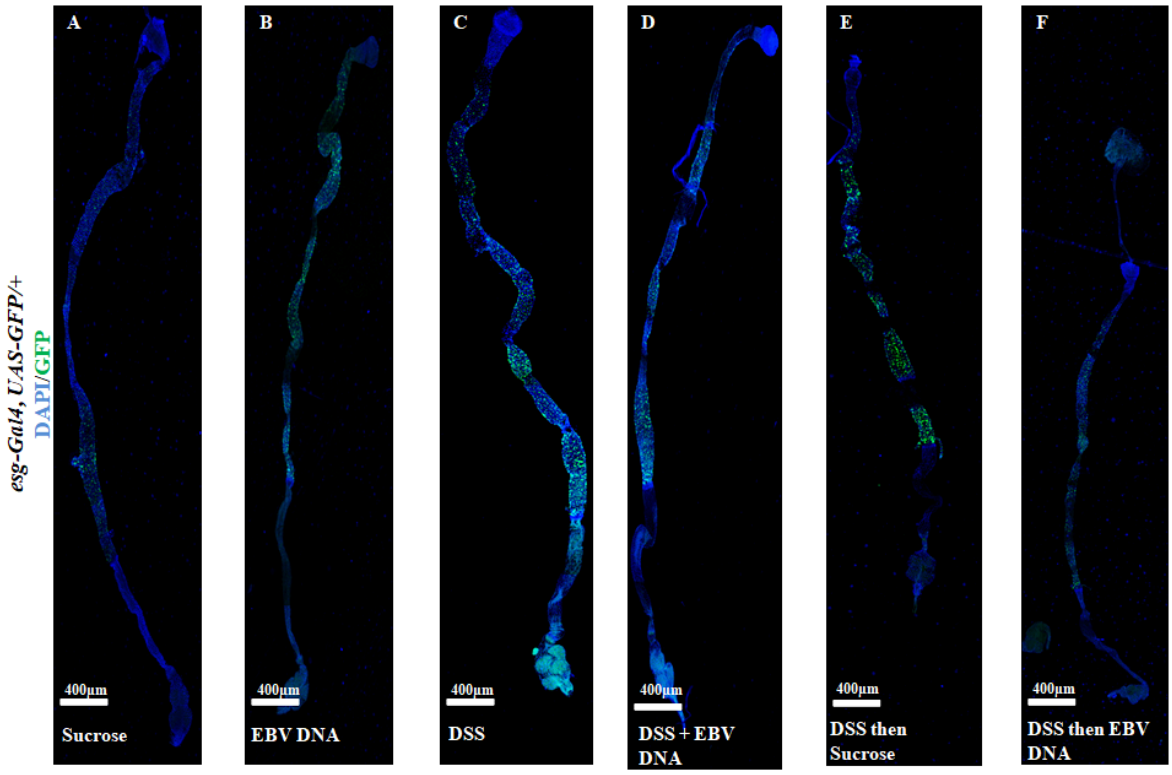

Figure 7: Midguts of *esg-Gal4, UAS-GFP/+* flies fed on different regimens of DSS and/or EBV DNA. (A) Flies fed on sucrose (used as vehicle in the other treatments) for 48 hours. (B) Flies fed on EBV DNA for 48 hours. (C) Flies fed on DSS for 48 hours. (D) Flies fed on DSS and EBV DNA for 48 hours. (E) Flies fed on DSS for 24 hours then sucrose for 24 hours. (F) Flies fed on DSS for 24 hours then EBV DNA for 24 hours. Hemolectin/GFP is in green; DAPI is in blue.

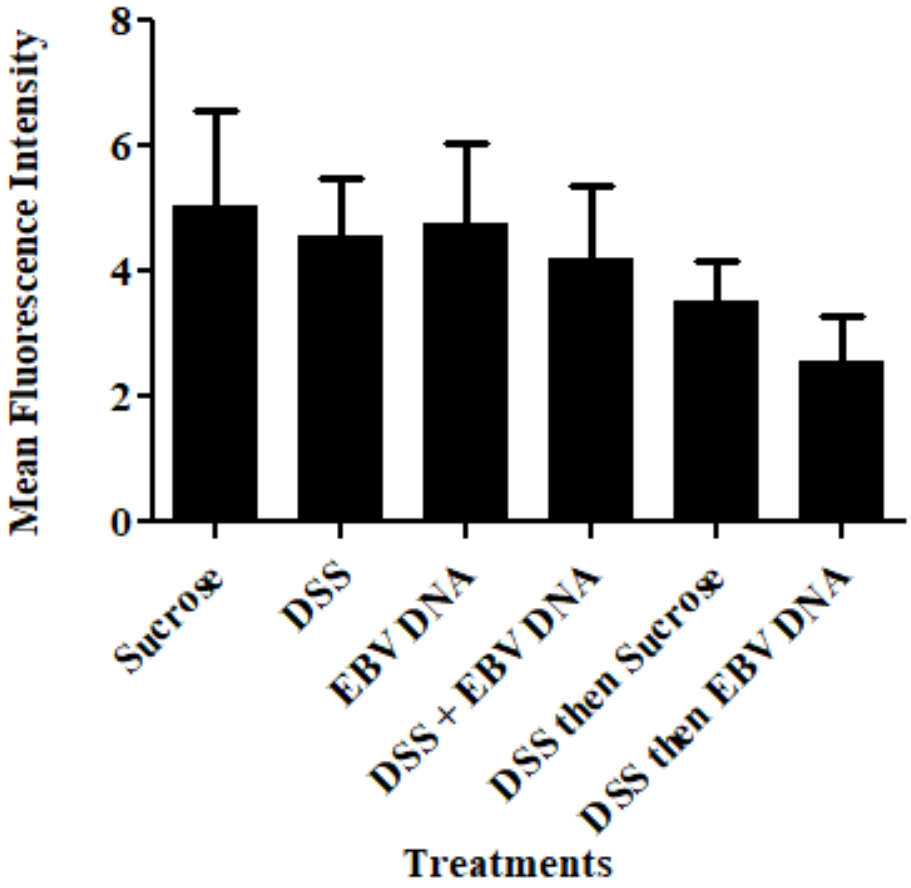

Figure 8: Mean fluorescence intensity of intestinal stem cells (ISCs) and enteroblasts in the midgut of flies after feeding on different regimens of DSS and/or EBV DNA. Sucrose was used as a vehicle. *p-value<0.05

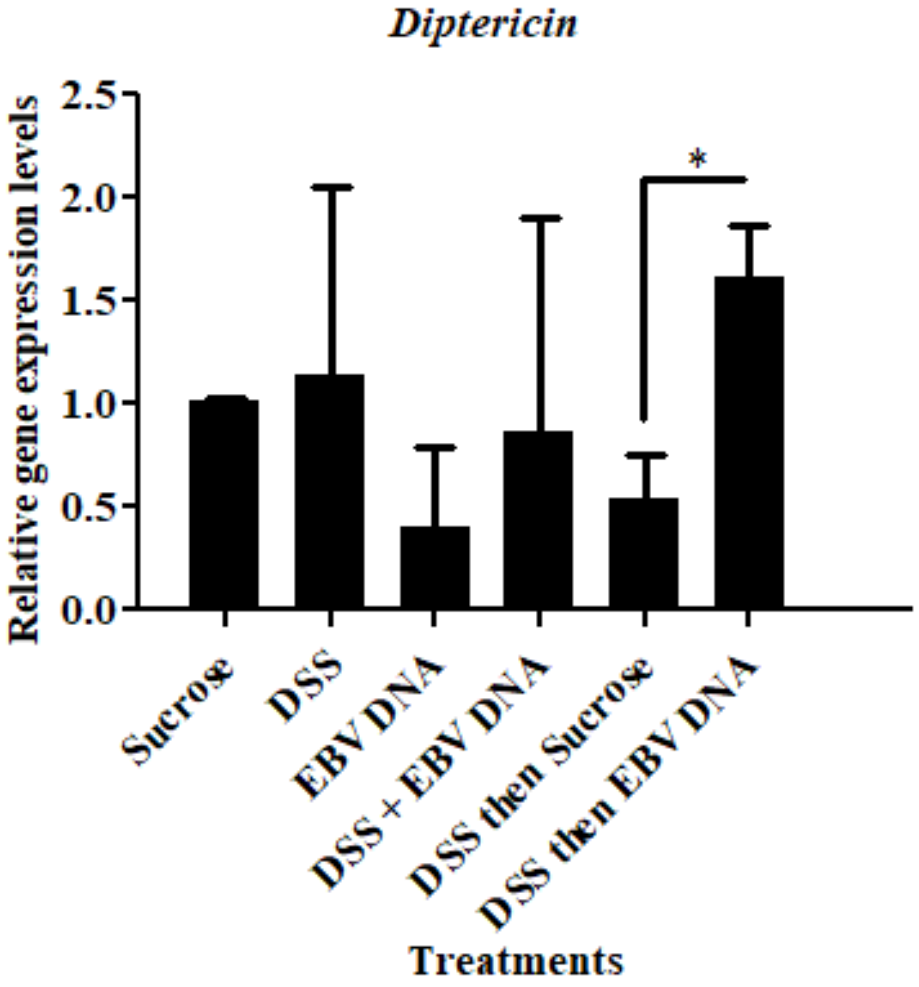

Figure 9: Relative gene expression levels of *Diptericin* in guts from flies fed on different regimens of DSS and/or EBV DNA. Sucrose was used as vehicle. *p-value<0.05.

Table 1: Fly feeding regimens.

	Groups	1	2	3	4	5	6
	Treatments	**Sucrose**	**EBV DNA**	**DSS**	**DSS + EBV DNA**	**DSS then Sucrose**	**DSS then EBV DNA**
48 hours	**24 hours**	500 µl of 5% Sucrose	500 µl of 5% Sucrose + 288,000 copies of EBV DNA	500 µl of 5% DSS	500 µl of 5% DSS +288,000 copies of EBV DNA	500 µl of 5% DSS	500 µl of 5% DSS
	24 hours	500 µl of 5% Sucrose	500 µl of 5% Sucrose + 288,000 copies of EBV DNA	500 µl of 5% DSS	500 µl of 5% DSS +288,000 copies of EBV DNA	500 µl of 5% Sucrose	500 µl of 5% Sucrose + 288,000 copies of EBV DNA

Table 2: Sequences and annealing temperatures for *RPL11* and *Diptericin* primers.

Gene	Direction	Sequence	AT*	Ref.
RPL 11	Forward	5'-CGATCCCTCCATCGGTATCT-3'	57°C	(137)
RPL 11	Reverse	5'-AACCACTTCATGGCATCCTC-3'	57°C	(137)
Diptericin	Forward	5'- CCGCAGTACCCACTCAATCT -3'	57°C	NCBI primer designing tool
Diptericin	Reverse	5'- ACTGCAAAGCCAAAACCATC-3'	57°C	NCBI primer designing tool
***Annealing Temperature (AT)**				

Table 3: Percent of fly hindguts with ≥ 20 GFP-Hemolectin positive cells after feeding on different regimens of DSS and/or EBV.

Treatments	Percent Hindguts with ≥ 20 GFP-Hemolectin positive cells	p-value*
Sucrose	9%	-
EBV DNA	0	0.023
DSS	26%	0.018
DSS+EBV DNA	32%	0.003
DSS then Sucrose	25%	0.030
DSS then EBV DNA	55%	2.706E-07
*Compared to the sucrose-fed group		

CHAPTER V

DISCUSSION

EBV is a prevalent virus that has been associated with different diseases including gastric cancer and inflammatory bowel diseases (IBDs), such as Crohn's disease and ulcerative colitis, conditions that have the colon inflamed over a long period of time (42, 44-46). A significant correlation between IBD and EBV has been shown. Minimal amounts of EBV have been detected in normal gastric tissues but the virus has been highly detectable in gastric lesions from Crohn's disease and ulcerative colitis tissues. One study presented possible perpetuation of inflammation in ulcerative colitis patients due to the active replication of the virus detected in the inflammatory lesions (48). Further research is required to explore the definite role of EBV in IBD. Intriguingly, innate immune responses secondary to EBV DNA have been shown. Our group has previously reported immune stimulation in response to EBV DNA in various models. In mice, pro-inflammatory cytokines were triggered via the involvement of Toll-like receptors (13-15, 138). In *D. melanogaster*, EBV DNA was capable of triggering the activation of the IMD pathway systemically as well as increasing hemocyte levels in the fly hemolymph (16). The fruit fly served as a model to fulfill our aim: assessing the effect of EBV DNA on inflammatory responses in the fly gut by examining markers of the cellular immune response and regeneration, as well as the humoral immune response. The ultimate objective is establishing the fly as a model system to examine the role EBV DNA plays in inflammatory bowel conditions.

Inflammation was established in *D. melanogaster* through the administration of DSS, a sulfated polysaccharide that causes human ulcerative colitis- like pathologies due to its toxicity to intestinal cells (139).

Upon assessing local cellular stimulation in response to different treatments in *hml-GAL4, UAS-GFP*, enhanced levels of GFP-expressing Hemolectin-positive cells were observed in the hindguts of flies administered DSS compared to controls. Administration of EBV DNA to the DSS-fed flies further increased the levels of the Hemolectin-positive cells in fly hindguts. Moreover, the time EBV DNA was administered while feeding on DSS resulted in different outcomes. Feeding DSS on the first day then EBV DNA on the next showed further enhanced Hemolectin positive cell accumulation in the hindgut in comparison to flies feeding on DSS and EBV DNA at the same time. Hence, possibly, establishing inflammation and then allowing the entry of EBV DNA into a damaged gut resulted in a further aggravated cellular response. Conversely, feeding on EBV DNA alone without causing damage to the gut through the administration of DSS led to a decrease in the Hemolectin-positive cells in the hindgut in comparison to flies feeding on sucrose only. It is possible that hindgut injury that may have been caused by DSS allowed EBV DNA access to certain cells that in turn allowed activation of particular pathways that led to an increase in the number of Hemolectin-positive cells; on the other hand, in the absence of possible abrasions caused by DSS, other cell types with a potential suppressive effect may have been triggered by the DNA.

Specifically, the region with a notable cellular response in the gut was the ileum in the hindgut. The innate immune cells of the *D. melanogaster* gut, including those of the

hindgut, have not been well characterized. The ileum is known to consist of polyploid absorptive enterocytes that are quiescent and divide only upon immune challenge (128).

Since Hemolectin positive cells observed accumulating in the hindgut may be endocrine (67, 140) confirming the nature of these GFP-Hemolectin positive cells was of high importance. Upon assessing the nature of these cells through immunofluorescent staining, we were able to confirm that they are plasmatocytes. While fly hindguts have been previously reported to harbor hemocyte-like cells (129), to the best of our knowledge, this is the first report to indicate that these cells are plasmatocytes. On the other hand, the localization of hemocytes in the midgut region of the gut of *D. melanogaster* has been previously reported (67). Hemocytes have been shown to aid in immune responses in the midgut by either taking part in the phagocytosis of pathogens or controlling intestinal stem cell regeneration (67, 109). Hence, possible similar immune contributions may be resulting in the hindgut of the adult flies upon inducing inflammation.

To assess the effect of EBV DNA on systemic cellular components, hemocytes from the adult fly hemolymph were counted after feeding on various treatments for two days. No significant changes in the number of hemocytes were observed and this could be due to a localized damage induced in the gut by DSS and EBV DNA that does not result in systemic changes.

Although it has been previously reported that DSS with a high molecular weight, as employed in our studies, does not show an effect on the regeneration process in the fly midgut, we examined whether inclusion of EBV DNA is capable of triggering regenerational changes (86). Upon assessing the fluorescent intensity of intestinal stem

cells in the midgut, which is indicative of increased division of stem cells, we did not observe any remarkable indication of regeneration. Differences in regeneration and immune responses between midgut and hindgut cells have been reported (128, 129). In a fly oncogenic model, midgut cells were shown to respond to virulent *Pseudomonas aeruginosa* infection by regenerating with a fast turn-over independent of the fly innate immune pathways (129). On the other hand, an avirulent *P. aeruginosa* strain tested did not induce this turn-over; similarly, not all types of DSS induce midgut inflammation and damage resulting in regeneration (86). On the other hand, in flies with Ras-activated hindgut cells, a very low proliferation of ISCs located in an anterior zone in response to immune challenges or damage was observed, but hindgut cells were capable of dissemination, invasion and accumulation in different loci completely under the control of the fly innate immunity (129, 130). Most importantly, both virulent and avirulent bacterial strains that did not cause any alterations in midgut regeneration were capable of inducing dissemination of cells originating from the fly hindgut (129). While these observations were made in a fly cancer model and may not necessarily apply to non-cancer flies, they may indicate differences in immune and regenerational responses between the midgut and the hindgut. This may explain why DSS with a high molecular weight resulted in notable observations in the hindgut in our model, but not in the midgut. Hence, our findings highlight possible differences between responses in the midgut and those in the hindgut.

Regeneration in the hindgut was not examined in our study due to the uncertainty of the presence of ISCs in the hindgut and the lack of specific markers for them (122, 127, 128). Alternatively, general proliferation observations through BrdU labelling and

enhanced mitosis assessment via examining phosphohistone levels (141) may be possible routes of deciphering whether regeneration is triggered in the hindgut in our model. This will be determined in future studies.

Upon assessing the humoral response in the gut of flies feeding on DSS then EBV DNA, we observed that the transcriptional level of *Diptericin* was increased by 3 folds in comparison to flies feeding on DSS then sucrose. These results correlate with our previous studies showing the activation of the IMD pathway systemically in response to EBV DNA (16). Studies have shown the role of the IMD pathway in defense against gut bacterial infections (111, 142). Moreover, studies have demonstrated that the IMD pathway is activated in response to accumulation of chromosomal DNA in flies (143). A previous study suggested that the IMD pathway in *D. melanogaster* larvae induces JNK-dependent PVR ligands such as PVF2 and PVF3 which in turn induce the proliferation of hemocytes (144). Hence, the IMD pathway could be acting upstream of the JNK pathway and inducing hindgut cell responses and plasmatocytic localization/proliferation in the ileum in response to EBV DNA. The involvement of the JNK pathway, along with other innate immune pathways, in our model may be of relevance and could be examined in future studies.

The limitations of our study include the lack of determining the exact amounts of EBV DNA consumed. Hence, these consumed amounts cannot be correlated to the amount of EBV DNA present in human gut tissues in infected subjects. Since EBV stimulates adaptive immune responses in humans, the lack of adaptive immunity in the *D. melanogaster* may pose a potential limitation since these cannot be tested in the fly model.

In conclusion, our results suggest that oral administration of EBV DNA stimulates the proliferation or accumulation of plasmatocytes and the activation of the IMD pathway in the hindgut of *D.melanogaster*. The IMD pathway is comparable to the Tumour Necrosis Factor-α Receptor signaling (TNFR) in mammals. Hence, assessing whether the TNFR pathway and macrophages are stimulated by EBV DNA in mammalian gastric studies can be investigated; such studies may highlight mediators that can serve as potential therapeutic targets to alleviate the inflammatory effects of EBV DNA in IBDs.

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