Understanding the impact of High-Sugar Diet on the innate immunity of *Drosophila melanogaster*

Sreelekshmi S R
MS15109

A dissertation submitted for the partial fulfilment of BS-MS dual degree in science

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

June 2020

Dedicated to all those people who enlightened my path with wisdom and knowledge

Acknowledgement

I am grateful to each and everyone who has supported and guided me in this life. I am beholden to my parents and grandparents. If it were not for them, I would never have lived on this glorious earth, which has so many reasons to live for. My sister Rajalekshmi is my most prominent pillar of strength. She is my Boss Baby. From gathering crumbled pieces of my broken heart to crumbling the hearts of my naysayers, she has done everything that it takes to be an amazing sister. I would like to express my heartfelt gratitude to all my teachers back in school who had unveiled a whole new world for me.

I would like to sincerely thank Dr Lolitika Mandal, my thesis advisor, for allowing me to work in her lab. She has taught me to be passionate about my work, which raises my spirit to work up to my fullest potential. She has inspired me to explore Science further. She has been an admirable teacher and has directed me not only along the academic lines but also has been a mentor. She is a mentor who sees more talent and capability within you than you see in yourself and helps bring it out of you. I learned from her that confidence is something I can create within me by believing in myself. She is a supporter who walks alongside you to show you what you can do. My life itself, and the best heart of it thank her for everything she has done for me and her care and support. I am grateful to Dr Sudip Mandal for all the support he has given me through my stay in the lab. His teaching, enthusiasm, and dedication to work have motivated me to pursue more in the field of research. I learned the importance of discipline from him. I sincerely acknowledge him for the motivation and encouragement that he has offered throughout my stay in the lab.

I am indebted to Parvathy Ramesh for her constant advice, support, and encouragement that she has shown towards me. She has taught me lots of things from the time I started working with her in the lab and has made me a better scholar in the long-run. The knowledge she has imparted upon me has been a great asset throughout my project work. I am grateful for her patience towards me. She is an exemplar in the field of scientific research. I can nothing render but be allegiant and thankful.

I am obliged to Aiswarya, Ramsi and Farzana for their support and help. They have always been there for me from weakness to strength, from grass to grace and from nothing to something. They are the critic who rips me apart not to let me down, but to aid me to sail longer.

I would like to convey my deepest gratitude to all the members of Drosophila Genetics Laboratory and Molecular Cell and Developmental Laboratory namely Kaustuv Ghosh, Sushmit Ghosh, Satish Tiwari, Aditya Kanwal, Jayati Gera, Prerna Budakoti, Gunjan Chauhan, Shiv Kumar Sharma, Harleen Kaur, Ramsi Nilopher, Farzana N, Aiswarya A S, Pranav Joshy, kaustav Ray and Saswata for the constant support and a pleasant lab environment.

I would like to thank the former Directors Prof. N. Sathyamurthy and Prof.Debi Prasad Sarkar and Director Dr Gowrishankar of IISER Mohali, for giving me the opportunity to work at this premier research institute. I sincerely acknowledge the library facility in IISER Mohali for giving access to textbooks, journals. I express most respectful regards to my thesis committee members and other faculties for their valuable suggestions for improving my project work.

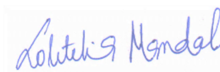
I am thankful to all my friends in the IISER Mohali for their love and care. They filled my life with incredible memories.

Last but not least, I would like to thank the INSPIRE for supporting me through the entire duration of my course and IISER Mohali for giving me the best education and the best five years of my life.

Certificate of Examination

This is to certify that the dissertation titled "Understanding the impact of High-Sugar Diet on the innate immunity of Drosophila melanogaster" submitted by Sreelekshmi S R (Reg. No. MS15109) for the partial fulfillment of B.S.-M.S. dual degree programme of the Institute, has been examined by the thesis committee duly appointed by Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr Rhitoban Ray Choudhury **Dr Sudip Mandal** **Dr Lolitika Mandal**

(Supervisor) 

Signature

Date: 12-06-2020

Declaration

The work presented in this dissertation has been carried out by me under the supervision of Dr Lolitika Mandal at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institution. Whenever contributions of others are involved, every effort has made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me, and all sources listed within have been detailed in the bibliography.

Sreelekshmi S R (Candidate)

(Signature)

Date: 12-06-2020

In my capacity as a supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr Lolitika Mandal (Supervisor)

(Signature)

" These woods are lovely, dark and deep,
But I have promises to keep,
And miles to go before I sleep,
And miles to go before I sleep."

— Robert Frost, Stopping by Woods on a Snowy Evening

List of figures

Figure 1: **Life Cycle of *Drosophila melanogaster*** 6
Figure 2: **The Gal4-UAS system**. 7

Figure 3: **Toll and Imd pathway in *Drosophila melanogaster*** 10

Figure 4: **Comparison of *Drosophila melanogaster* Imd pathway and human TNFR Signalling**. 11

Figure 5: **Schematic representation of experimental strategy** 13

Figure 6: ***Drosophila melanogaster* adult hematopoietic hub and enlarged first cluster** 14

Figure 7: **Infection procedure for adult *Drosophila melanogaster*** 19

Figure 8: **Infection response after 2 hours of infection in *Drosophila*** 27

Figure 9: **Infection response after 8 hours of infection in *Drosophila*** 28

Figure10: **Infection response after 24 hours of infection in *Drosophila*** 28

Figure 11: **Response of Extracellular matrix (ECM) upon infection** 30

Figure 12: **Intensity analysis of Extracellular matrix (ECM) upon infection** 30

Figure 13: **Increased uptake of glucose in High Sugar fed adult flies** 31

Figure 14: **Response of blood cells in Normal Diet and High Sugar Diet flies in pre-diabetic condition (Day 5)** 32

Figure 15: **Response of Extracellular matrix (ECM) Normal Diet and High Sugar Diet flies in pre-diabetic condition** 33

Figure 16: **Response of blood cells in Normal Diet and High Sugar Diet flies in Day15** 34

Figure 17: **Expression of Dpt in Normal Diet fed flies (Day 5)** 36

Figure 18: **Expression of Dpt in Normal Diet fed flies after infection (Day 5)** 36

Figure 19: **Expression of Dpt in High Sugar Diet flies (Day 5)** 37

Figure 20: **Expression of Dpt in High Sugar Diet flies after infection (Day 5)** 37

Graph 1: **Level of Dpt in adult *Drosophila melanogaster* in different conditions (Day 5)** 38

Contents

Abstract 1

Chapter 1: Introduction

1.1 High Sugar Diet and its consequences 4

1.2 Drosophila as a model system 4

1.2.1 The lifecycle of *Drosophila melanogaster* 5

1.2.2 Gal4-UAS system 6

1.3 Drosophila as a model for studying the immune system 8

1.3.1 Signalling pathways involved in immune responses 9

1.3.2 Drosophila Immune pathways show striking similarity with vertebrates 11

1.4 Drosophila hematopoiesis and immunity 12

1.5 Experimental Model: A powerful system to study the effect of High Sugar Diet on Immunity 12

Chapter 2: Objectives 16

Chapter 3: Materials and Methods

1. Materials and Methods

1.1 Rearing of flies and maintenance 18

1.2 Fly stocks and genotypes 18

1.3 Experimental setup 18

1.3.1 Infection of flies 19

1.3.2 Bacterial culturing and plating 19

1.4 Immunohistochemistry 21

1.4.1 Dissection of adult flies 21

1.4.2 Reagents and Buffers 21

1.4.3 Procedure for immunostaining of adult hematopoietic hub 23

1.4.4 Imaging and Image processing 24

1.4.5 Unfixed tissue labelling with 2-NBDG 24

Chapter 4: Results **25**

Chapter 5: Discussions & Future direction **40**

Bibliography

Abstract

The diet of an organism has a significant influence on its health and homeostasis. In globally, Diabetes and related metabolic disorders are emerging health complications. Few reports suggest that diabetic patients have a compromised immune system. Our goal was to understand the impact of High Sugar Diet on the immune system using model organism *Drosophila melanogaster*. In this study, we provided experimental fly batches 1M sugar to create a high sugar condition compared to Normal Diet concentration which is 0.15M. Our results showed a variation in the blood cell population in the High sugar diet compared to the Normal Diet. On pre-diabetic conditions, flies reared in the High Sugar Diet could not mount an immune response upon infection.

Chapter 1: Introduction

1.INTRODUCTION

1.1 High Sugar Diet and its consequences

No one can deny the relation between health and diet. It has been reported by the World Health Organization (WHO) that dietary and lifestyle changes can trigger the spread of chronic diseases all over the world [1]. Some of the nutritional components can step up the feasible occurrences of such conditions. Because of the lifestyle changes over the past few decades, which lead us into the fast-food culture, all these eventually caused a rapid surge in chronic diseases such as Diabetes Mellitus(T2DM), Obesity, Cardiovascular diseases(CVD) and some type of cancer, etc.

A good percentage of the population across the world consumes sugar as a significant dietary component [2][3]. Excess amount of sugar in the blood causes hyperglycemia and which in turn lead to the resistance to the hormone insulin – blood sugar-regulating hormone. Persisting problems in distinction to high blood sugar include chronic heart diseases, strokes, diabetic retinopathy, tooth mineralisation, kidney failure, and poor blood flow in the limbs, which under severe conditions may lead to amputations [4]. It is a compelling hardship for the healthcare system worldwide. From an estimation, 425 million people had diabetes worldwide as of 2017 [5] with type 2 diabetes making more than 80% of the cases with identical proportions in men and women [6][7] [8]. From the studies, it was evident that amid headway of T2DM, the anomaly of either innate immunity, adaptive immunity, or together they intricate and collaborated [9]. Patients suffering from obesity and T2DM, are more vulnerable to infections, and significant delay in wound healing is also observed [10].

1.2 *Drosophila* as a model system

For more than a hundred years, *Drosophila melanogaster,* universally recognised as fruit fly or Vinegar fly, has been serving as an excellent model system in biomedical research. Native to Africa, these fly species belong to order Diptera and family Drosophilidae. William Ernest Castle (1867-1962) introduced flies to the workbenches. However, as a model organism, these flies rose prominently by the works of Thomas Hunt Morgan (1866-1945) and his students at

Columbia University. Thomas Hunt Morgan and his lab members deciphered various elementary principles of heredity, including epistasis, sex-linked inheritance, multiple alleles, and gene mapping [10].

There are so many reasons such as easy maintenance with little space, human resources, and equipment even it comes to large culturing, high fecundity, and short generation time with a clear cut distinction between male and female flies, which makes flies one of the most widely used model organism. Completely sequenced genome spreads over four chromosomes. By the year 2000, complete sequencing and mapping of the entire *Drosophila* genome have completed. They concluded that there is a sixty per cent homology between the human genome and *Drosophila* genome [11]. The size of the Drosophila genome is nearby 1/20th of the size of a regular mammalian genome, which simplifies molecular genetic analysis [12]. Seventy-five Percentage of genes accountable for human diseases have homologs in Drosophila [13].

It is easy to manipulate the *Drosophila* genome. It ranges from the early mutagenesis tools, which counted on ethyl methane sulfonate (EMS) and X-rays, respectively, to alter DNA at a nucleotide level randomly. Recurrently using genome editing tools in *Drosophila* include P-elements, CRISPR/Cas9 mutagenesis, FLP/FRT recombination, transcription activator-like effector nucleases (TALENs), phiC31 integrase-mediated target insertion, zinc-finger nucleases (ZFNs) [14]. Most phenotypes in *Drosophila* are easily perceptible with the naked eye or with the assistance of a microscope.

1.2.1Life Cycle of *Drosophila melanogaster*

Drosophila melanogaster is a holometabolous insect with four distinct stages of development -egg, larvae, pupae, and adult. (**Figure-1**) Depending on the temperature at which they are growing, Drosophila growth and development can change. At 25℃, it will take about ten days to become a fully developed adult fly. Drosophila has internal fertilisation. Females lay approximately 50-80 embryos soon after fertilisation. After 24 hours, the larval stage begins, and there are three distinct phases in the larval stage, namely, first instar, second instar, and third instar. The embryos hatch out to the first instar larvae, smallest among the different forms. After 24 hours, it changes to second instar, and within the next 24 hours' time span, it becomes third instar larvae. After their maturation, they leave the culture medium and crawl up on the walls of the culturing bottle. After that, they will go into a sedentary mode and prepare for the

pupariation event. During this period, metamorphosis takes place. Adult structures replace larval structures through this time. Within 3-4 days' flies will emerge out. The average lifespan of flies at 25 ºC will be around six weeks [16].

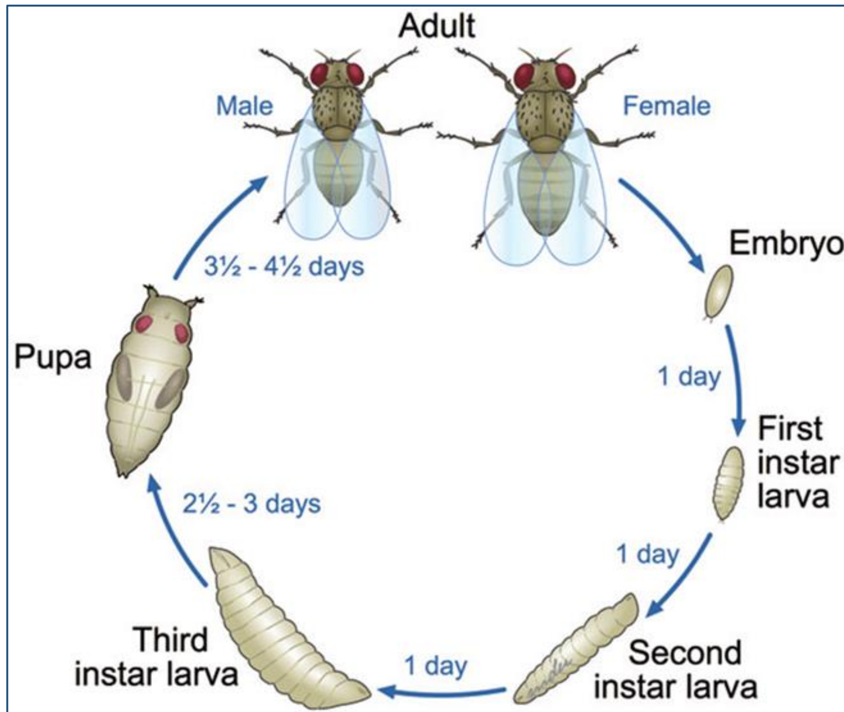


Figure 1: The life cycle of *Drosophila melanogaster*
Drosophila has four distinct developmental stages-embryos, larvae, pupae, and the adult fly. Larval stages are significantly different from adult stages. After growing and undergoing several moults, larvae become pupae. Pupae undergo metamorphosis, and after 3-4 days, fly emerges out of the pupal case.

Image copyright @ Ong C, Yung LY, Cai Y, Bay BH, Baeg GH. Drosophila melanogaster as a model organism to study nontoxicity. *Nanotoxicology*. 2015;9(3):396-403. DOI:10.3109/17435390.2014.940405

1.2.2 Gal4-UAS System

The Gal4 UAS system is an outstanding potent genetic tool which allows us targeted gene expression in *Drosophila* in a spatiotemporal manner. In 1993, Andrea Brand and Norbert Perrimon discovered the Gal4-UAS System [15]. GAL4 is a regulator protein of genes first identified in yeast *Saccharomyces cerevisiae*. GAL4 binds to an Upstream Activating Sequences (UAS) element sites, equivalent to an enhancer element demarcated in multicellular

eukaryotes. The binding of GAL4 to UAS is essential for the transcriptional activation of these GAL4-regulated genes. Gal4-UAS system is a bipartite system in which the driver line and responder line are retained in separate parental lines. Here GAL4 with the tissue-specific promoter is called the driver line, and UAS with the specific gene of interest is called the responder line. By crossing both the transgenic lines, we will get our desired gene expression in F1 progeny in which both GAL4 and UAS elements are present. As a result, GAL4 protein will go and bind to UAS and activates transcription of the target gene which downstream of UAS element (**Figure 2**)

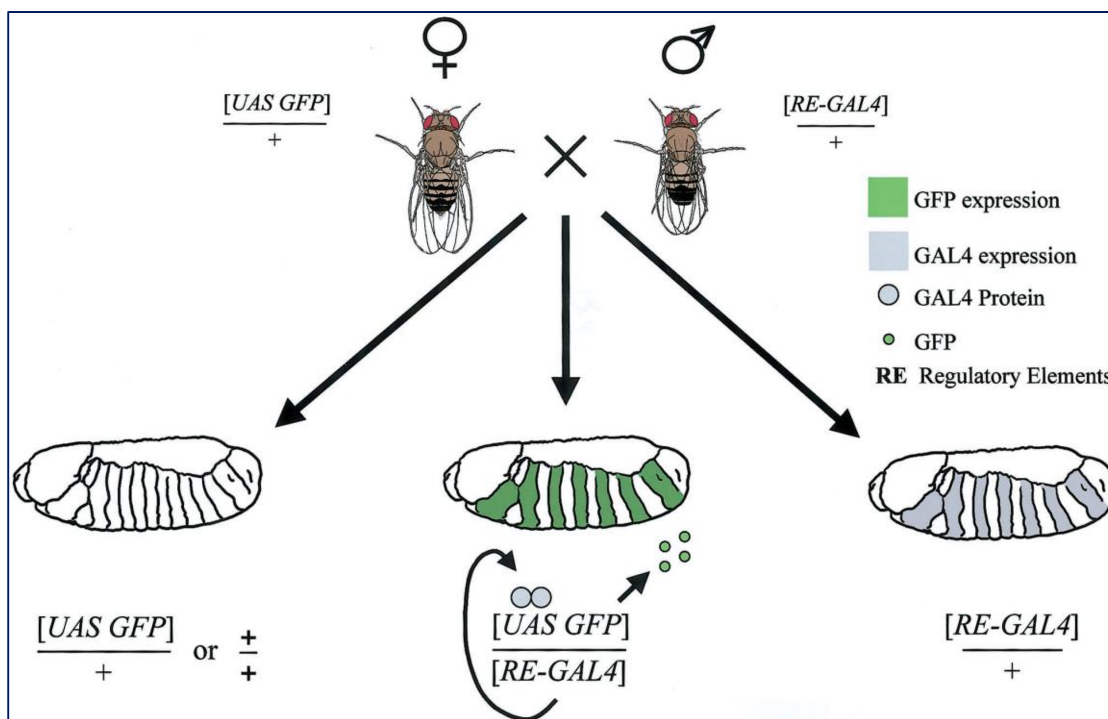


Figure 2: The Gal4 UAS System The UAS-GAL4 system in *Drosophila*. When females with a UAS responder (*UAS-GFP*) are mated to males with a GAL4 driver, the progeny will have both the elements in their system. The presence of GAL4 in an alternating segmental pattern in the illustrated embryos then drives the expression of the UAS responder gene analogously.

Image copyright @ Duffy JB. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis*. 2002;34(1-2):1-15. DOI:10.1002/gene.10150

1.3 *Drosophila* as a model for studying the immune system

The immune system is encompassing many biological structures and processes within an organism to fight against diseases and invading pathogens. The protection is accomplished through multiple layers of defence mechanisms to increase the specificity within the system. Based on the responses, in the majority of the higher-level species, the immune system is divided into two, namely innate immune response and adaptive immune response. Innate immune response provides the first line of immediate, but non-specific defence against pathogens that breaches the physical barriers such as skin, membrane linings, etc. Innate immune systems are found in all plants and animals [17]. The adaptive immune response, only present in higher vertebrates, which enable them to keep exquisite specificity and strong immunological memory. Both the immune responses act side by side to prevent pathogen activities in the body [18].

Unlike mammals, *Drosophila* has no adaptive immunity; they solely rely upon the innate immune system. Insects do have many of the features of vertebrate immunity; some of them are their ability to discriminate between self-versus non-self, extension and distribution of defences all over the body [19].

The major two categories of the *Drosophila* immune system are cellular mediated immunity and humoral immunity. Apart from these two, there are epithelial barriers such as cuticle, trachea, and gut; in the gut, there is commensal microbiota, which can mount an immune response when needed [20]. There is melanisation reaction in which the blackening of the wound site associated with septic injuries or pathogen attacks [21]. *Drosophila* protects a wound by clotting of the hemolymph; the clot often contains different proteins that could cross-link the bacteria and prevent them [22].

In cellular mediated immunity, a group of hemocytes called plasmatocytes - the mammalian analogue for macrophages, they can phagocyte pathogens. Comparable to mammals, *D. melanogaster* consumes numerous mechanisms of bacterial sensings, such as complement-like opsonisation and scavenger receptors. Most of the machinery downstream of the cell-surface receptors are mutual among flies and humans. It has been demonstrated by RNA-interference-based screens [23] [24].

Drosophila can make a humoral immune reaction in contrast to invaders by the production of antimicrobial peptides (AMPs) into the hemolymph [25]. Antimicrobial peptides (AMPs) are oligopeptides with five to hundreds of amino acids. From viruses to bacteria, these molecules can target a wide range of pathogens [26]. Both prokaryotes and eukaryotes have AMPs [27] [28]. Because of their rapid killing effect and targeted lipopolysaccharide activities make them a better defender. Eukaryotic tissues have a reduced level of anionic charges and an increased level of cholesterol. This property enabled them to get away from the target range of many AMPs [29]. AMPs like Diptericin, Drosomycin, Attacin, Drosocin, Cecropins, and defensin are some of the chief proteins used by *Drosophila.*

1.3.1 Signalling pathways involved in immune responses

The Toll pathway and Immune deficiency (Imd) pathway are primarily controlling the Drosophila immune system. Mammalian TLR signalling mechanisms share resemblances with the Drosophila Toll pathway.

Infection by a Gram-positive bacteria or fungi prompts the Toll pathway and which leads to the activation of both cellular and humoral immunity in flies. It can control the haemocyte proliferation and density along with other signalling pathways. This pathway controls the melanisation reactions in the larval stage [30] [31] [32]. The primary readout from the Toll pathway is AMP Drosomycin. Extracellular recognition factors initiate protease cascades leading to the activation of the Toll receptor ligand Spätzle. Once the Toll pathway gets initiated, it settles in the destruction of the IκB-family protein Cactus and localisation of Dorsal and Dif to the nucleus. Dorsal/Dif activate Drosomycin expression [33]. In **Figure 3**, is illustrating both the pathways.

Imd pathway gets activated when there is an infection from Gram-negative bacteria. Peptidoglycan(PGN), component in the bacterial cell wall, recognised by the PGN- recognition protein receptors(PGRPs), which is conserved among insects and mammals. In the case of Gram-negative bacteria, the receptor is PGRP-LC. The reaction events cause the activation of transcription factor Relish, an NF-κB factor in flies. Once relish makes its entry into the nucleus, it can trigger activation of AMPs like Diptericin [34]. The epithelial and mucosal surfaces of the tracheal and digestive system can initiate localised activation of Imd signalling.

These areas are always engaged in encounters with pathogenic bacteria, fungi, and yeast [35] [36].

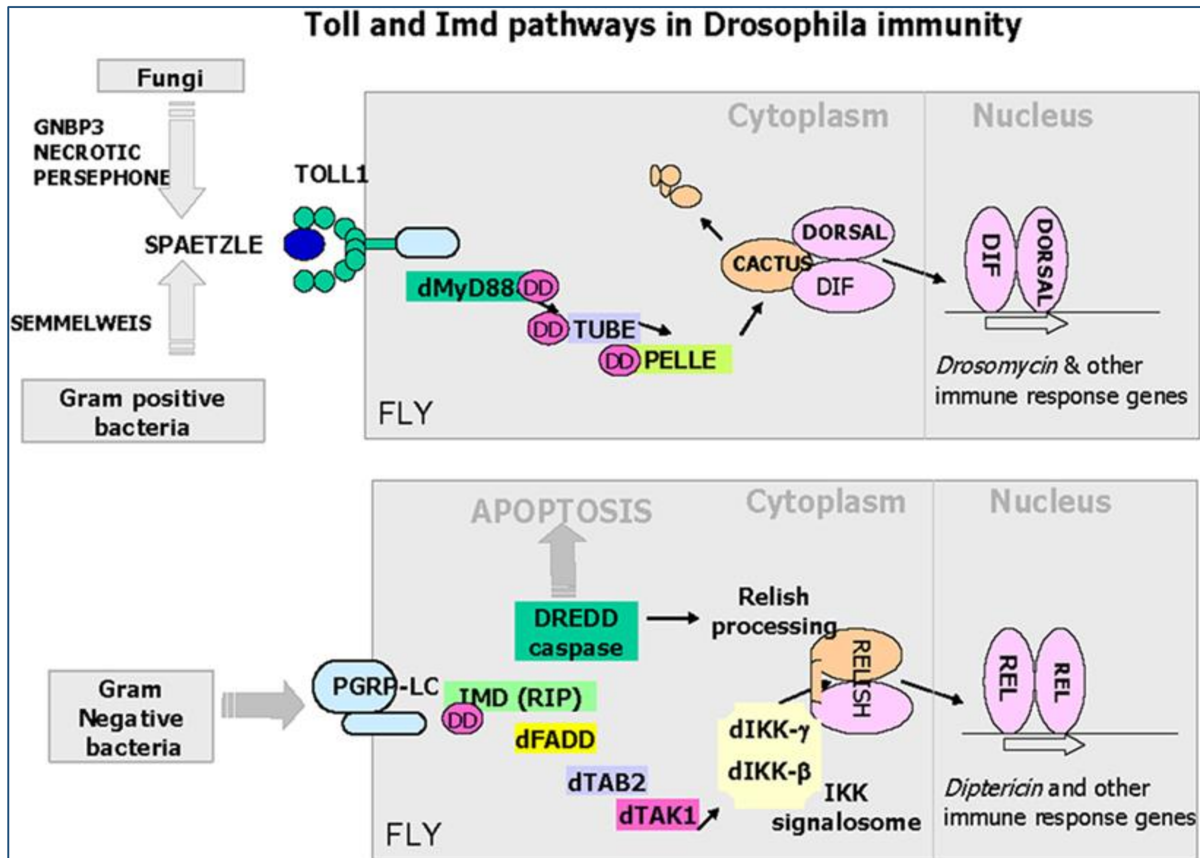


Figure: 3 The Toll and Imd pathways (i) fungal or Gram-positive bacterial infection activates the Toll pathway by the wakening of Toll receptor ligand spatzle. Destruction of cactus leads to the activation Dorsal and Dif, which translocate into the nucleus and activating the production of AMPs. (ii) The IMD pathway triggered by the infection of Gram-negative bacteria and which leads to the production of AMPs like Diptericin, attacin, drosocin, cecropins, and defensin

Image copyright @ Govind S. Innate immunity in Drosophila: Pathogens and pathways. Insect Science. 2008 Feb;15(1):29-43. DOI: 10.1111/j.1744-7917.2008.00185.x.

1.3.2 *Drosophila* Immune pathways show striking similarity with vertebrates

The Imd pathway of Drosophila shares similarities with mammalian TNFR signalling and TLR signalling pathways. Activation of the Imd pathway requires the activation of relish transcription factor while in TNFR signalling, it must need the NF-Kb transcription factor [37 [38]. In **Figure 4,** conserved constituents in both pathways are shown by identical colour and shape. The Toll/IL-1R (TIR) domain in mammals intermingles with adaptor molecules and activates the downstream cascade. This domain has a cytosolic homology with Drosophila Tolls [39].

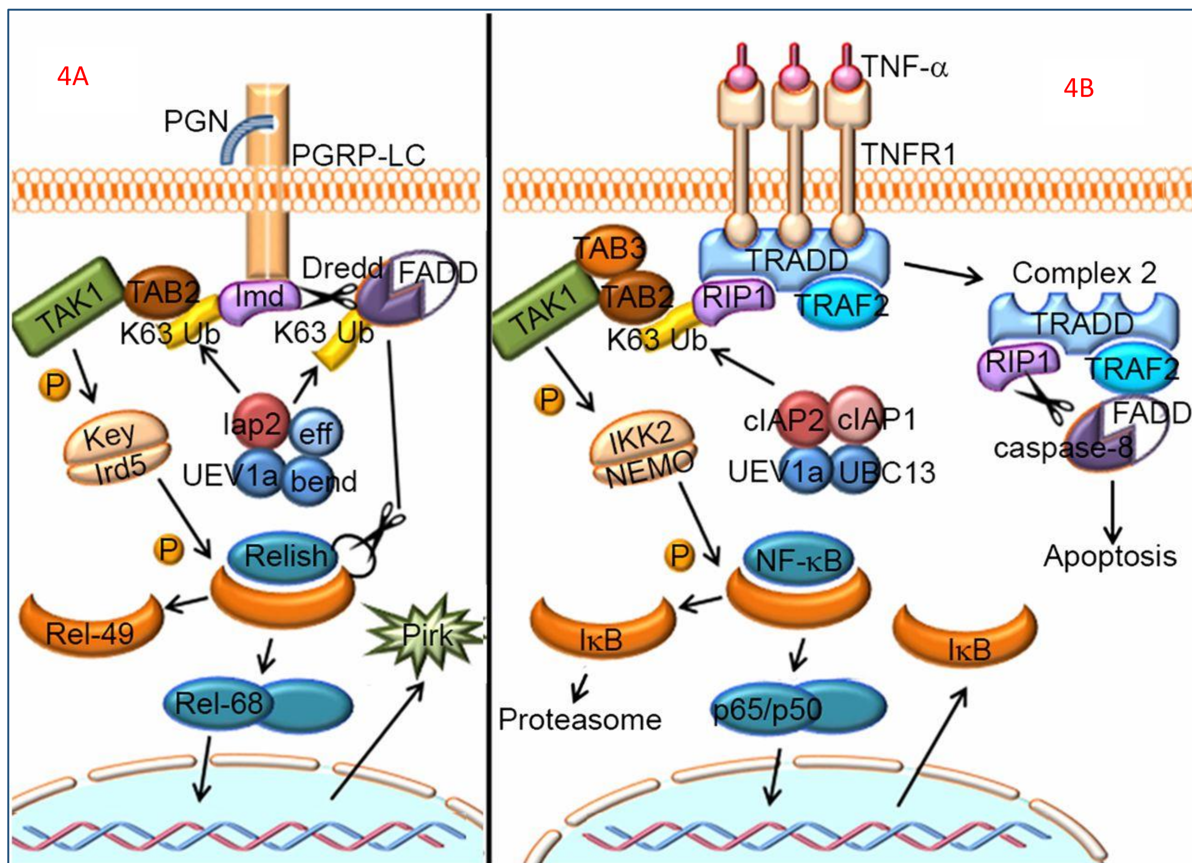


Figure 4: *Drosophila* Imd pathway and human TNFR signalling. Figure 4A is the Drosophila Imd pathway and 4B is the human TNFR signalling. Similar colours and shapes are used to articulate the conserved components.

Image copyright @ The Drosophila Imd Signaling Pathway Henna Myllymäki, Susanna Valanne and Mika Rämet

J Immunol April 15, 2014, 192 (8) 3455-3462; DOI: https://doi.org/10.4049/jimmunol.1303309

1.4 Drosophila hematopoiesis and immunity

Hematopoiesis is the process through which new blood cells are formed and specified. *Drosophila* hematopoiesis shows striking similarity with vertebrate hematopoiesis. It extends from the biphasic mode of blood cell formation to the signalling molecules essential for hematopoiesis. The primitive or first wave of hematopoiesis that takes place in the head mesoderm contributes to the generation of hemocytes for all the stages of the lifecycle. The second wave, the definitive phase, includes the lymph gland (larval hematopoietic organ) and adult hematopoietic clusters. The differentiated hemocytes are mainly of two types: Plasmatocytes and Crystal cells. When prone to immune challenge, the third type of differentiated haemocyte appears, the Lamellocytes [40] [41].

Along the dorsal midline in the abdominal segments, A1–A4 of adult flies, four hematopoietic blood cell clusters are present. Extracellular matrix proteins such as laminin A, pericardin form an elaborate mesh-like structure, adjoining the heart and the pericardial cells in which these hemocytes are entrenched [42].

1.5 Experimental Model

A powerful system to study the effect of High Sugar Diet in Immunity

Decades of studies established *Drosophil*a as an excellent model system for various biological aspects. Very recently, researchers have been using the *Drosophila* model system to study various lifestyle diseases, including diabetes mellitus. (both type 1 and type 2)

An earlier study has reported that there is a defect in phagocytosis in both high sugar-fed larvae and adult flies. There was an induction of lamellocytes in the lymph gland (larval hematopoietic organ) and also in circulating hemolymph by the activation of JNK signalling. The fat bodies - functional analogue of vertebrate adipocytes, has shown to activate the Toll and JNK signalling pathways, which, in general, occurs when there is an immune challenge [43] [44].

Musselman et al. forged a useful Drosophila Type 2 Diabetes Mellitus (T2DM) model in which a High Sugar Diet can induce hyperglycemia, insulin resistance and increased levels of triglycerides [45]. We came up with an experimental strategy based on this. According to our experimental strategy, flies are fed (experimental one) on the High Sugar Diet (HSD), which contains one molar sugar. When the fly emerges from the pupal case, for two days, we feed it on a Normal diet (ND), which has a 0.15 molar sugar in it. After two days, on 2:1 ratio of females to male flies were transferred into a High Sugar Diet bottle and fed for 13 days. From 13 Day onwards (threshold), they show human T2DM characteristics such as increased glucose level in hemolymph, increased Trehalose (sugar consisting of 2 molecules of glucose, the primary source of energy in Fruit fly), and insulin resistance with unaffected insulin production.

For analysing the effect of sugar feeding on immune response, we infected the flies with DH5α RFP (red fluorescent protein) positive *Escherichia coli* bacteria. We monitored the phagocytosis at different time windows to find out the maximum activity of phagocytosis during infection; The time windows were 0.5, 2,8,24,36, and 48 hours' post-infection. The flies were reared on normal food and control flies kept without infection. Infection experiments were done on the sixth day.

For my analysis, I checked the effect of high sugar feeding on fly immunity in a pre-diabetic condition (5-day feeding) as well. There will be a high glucose level in the model system. Still, they do not have diabetes. We infected the flies with bacteria on the seventh day, which were reared on HSD for five days. (dissections was performed after 8 hours of infection) The same set of experiments repeated on the 15th day when flies are diabetic.

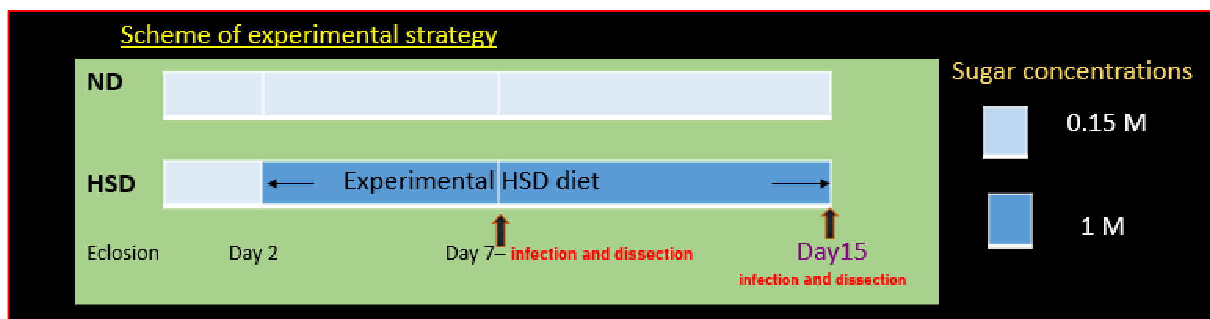


Figure 5: Schematic Representation of experimental strategy

High Sugar Diet (HSD) and Normal Diet (ND) batches were made on Day 2 after eclosion. Infection and Dissections were done on Day 7 and Day 15. In this diagram **Light blue** boxes represent Normal Diet and **Dark blue** boxes represent High Sugar Diet.

For my experiments, the region of interest was the first cluster of an adult hematopoietic hub and fat body cells next to it. The reason behind this choice is the first cluster is the largest cluster in the adult hematopoietic hub and therefore will be easy to score a phenotype.

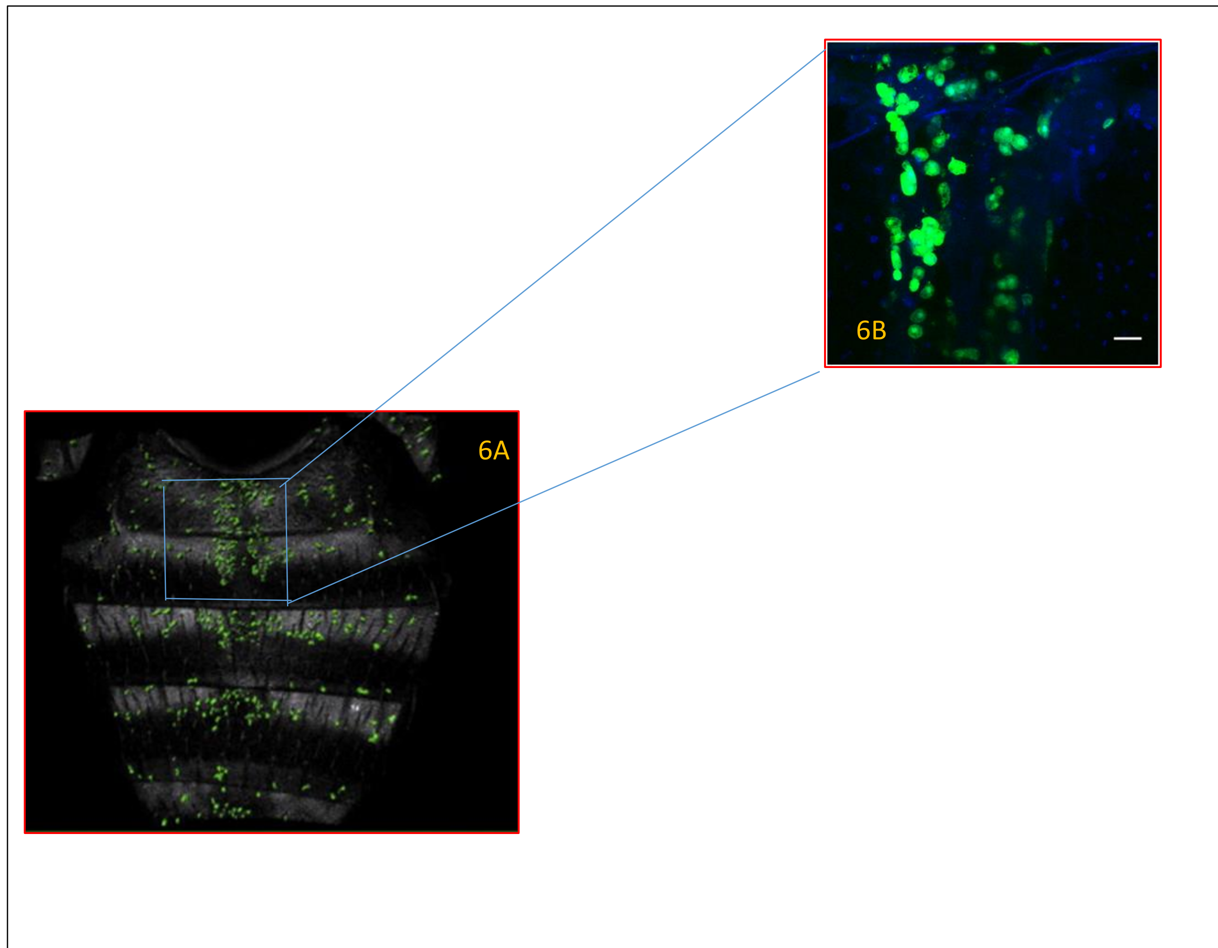


Figure 6: Enlarged view of first cluster of hematopoietic hub.

In 6A the adult hematopoietic hub of Drosophila is shown (The green color indicates the plasmatocytes). In 6B, (Scale bar 20 µm) enlarged view of the first cluster. The blue colour is DAPI and green is plasmatocytes.

Image copyright@ Ghosh S, Singh A, Mandal S, Mandal L. Active hematopoietic hubs in Drosophila adults generate hemocytes and contribute to immune response. *Dev Cell*. 2015;33(4):478-488. doi: 10.1016/j.devcel.2015.03.014

Chapter 2: Objectives

1. Objectives

Through my studies, I am trying to understand the following:

1. To study the changes in blood cells (morphology, number of cells) in *Drosophila* upon bacterial infection.
2. To study the difference in blood cells (morphology, number of cells) on normal food and high sugar food.
3. To study the changes in immune pathways if any in flies reared on high sugar food and thereafter infected with bacteria.

Chapter 3: Materials and Methods

1. Materials and methods

1.1 Rearing of flies and maintenance

Standard food bottles and food vials are used for rearing the flies in the lab. The fly food consists of cornmeal, agar, yeast, and fungicides. The consistency of the food is essential. It must be in medium condition. If the bottle is too much dry, then flies cannot eat the food.

Furthermore, if the water content is high, flies will stick on to the food. For maintaining a healthy culture, we need to flip into a new bottle every alternate day. In the case of the High Sugar Diet, we need to flip the flies every day. To prevent the flies from escaping the food bottles are plugged with clean cotton. Since temperature can alter the *Drosophila* development, The culture bottles were maintained at 25°C incubators, if not mentioned otherwise.

1.2 Fly stocks and genotypes

- Oregon R: Wild-type laboratory stock of *Drosophila.*

Transgenic lines used:

- **P{ry[+t7.2] =*Dipt*2.2-lacZ}1, P{w[+mC] =*Drs*-GFP**: This transgenic line component P{Drs-GFP.JM804}1 expresses GFP-tagged Drosomycin under the control of its native promoter, and component P{Dipt2.2-lacZ}1 Expresses lacZ in the fat body under the control of the Dpt on the X chromosome. It brought from Bloomington Drosophila stock center, Bloomington number - 55707
- **w; hml-Gal4Δ-UAS-GFP; +/+:** This transgenic line component {hml-gal4Δ} expresses GFP tagged hemolectin in blood cells. This line was obtained from Dr Utpal Banerjee.

1.3 Experimental setup

Freshly eclosed flies of the desired genotype were collected and transferred to normal diet bottle for two days. After two days, the experimental flies transferred to the High

Sugar Diet (HSD) and control flies on a normal diet on 2:1 female to male's ratio. In a standard food bottle, added 40–50 female flies and 20–25 males respectively. The number of flies in each bottle was kept equal. The fly bottles were flipped on every alternate day to maintain a healthy culture. As per the experimental strategy, female flies were infected and dissected on the 7th day and 15th day.

1.3.1 Infection of flies

A sharp and thin glass capillary with a pointy tip is used to infect the flies with RFP positive *E. coli* bacteria. For that anesthetise, the adult female flies with ether. Then take a single colony from the bacterial plate with the needle and then inject at the lateral side of the thoracic region (**Figure 7**)

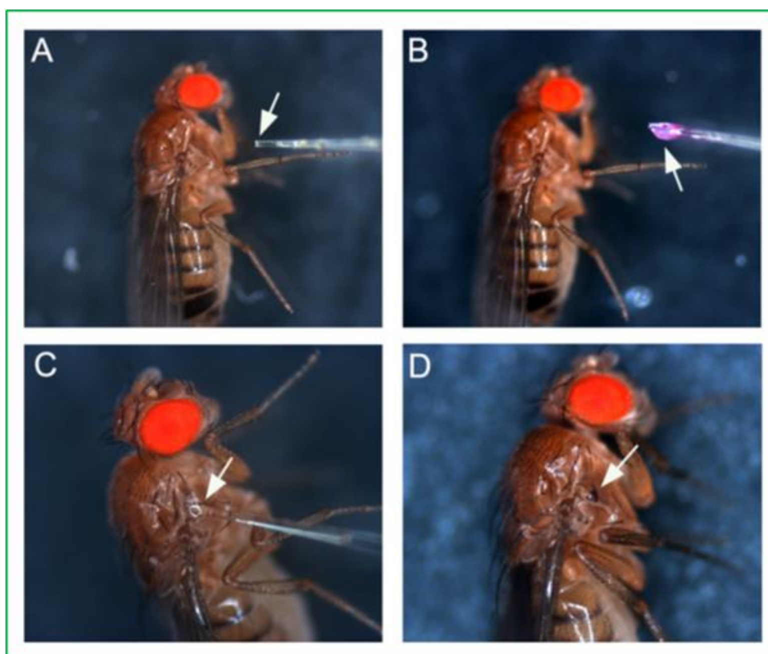


Figure 7: Different stages in infecting a fly.(A) Glass capillary with a pointed tip (arrow) is used to infect an anesthetized adult female fly. (B) A single colony of RFP expressing *E. coli* (DH5-Alpha) picked up at the tip of the capillary (arrow). (C) With the capillary, pricking the lateral side of the thoracic region (D) On post infection; a black melanised spot can be seen at the thoracic region because of the wound. *Image copyright @ Ghosh et al,2015*

1.3.2 Bacterial culturing and plating

For our analysis, we used RFP (red fluorescent protein) positive *E. coli*(DH5α) Bacteria. The bacteria will be in red due to the expression of reporter plasmid RFP.

So that we can visualise and locate the bacteria in flies while observing under a fluorescence microscope. The given strain is resistant to ampicillin antibiotic. We initially inoculated the bacteria in a liquid culture prepared from nutrient-rich LB (Luria Broth) powder. Liquid culturing will enable us to grow bacteria in higher densities, and it will have a sufficient number of bacteria. We needed a single colony of bacteria for our experiments hence made the Ampicillin resistant LB-Agar plates [46].

- Liquid culturing
  2.5 gm of LB (Luria-Bertani) powder was added in a 500 ml autoclavable reagent bottle. We dissolved it using 100 ml of distilled water. Once it wholly dissolved then loosely closed the lid and covered the top of the bottle with aluminium foil paper and subject it to autoclave. Pst autoclaving, it was transferred into a laminar hood, and we poured 10 ml of media into falcon tube and added 10µl from 100 µg/ml concentrated Ampicillin (only after the media come into room temperature). Then using a sterile toothpick, a single colony was collected from the LB Agar plate and dropped it into the falcon tube and gently swirled. The tube was loosely covered with its cap and incubated at 37°C for 16-18 hours in a shaking incubator.
- Culturing of bacteria in LB Agar plate
  We used disposable LB Agar plate of 10 ml capacity to culture the bacteria. For the making of LB Agar media, we need 2.5 gm of LB powder and 1.5 gm of agar powder in a 500 ml autoclavable reagent bottle and dissolved it in 100 ml of distilled water. This was then set for autoclaving. Post autoclaving, it is transferred into the laminar hood. Its neck region gently heated over the spirit lamp. Once the media is sufficiently cooled to room temperature, 10µl of Ampicillin from a stock of 100 µg/ml concentrated was next added. The plates were placed in front of the spirit lamp, and the media was poured into each plate (10 ml each). Care should be taken to ensure that the media is distributed equally over the plates and

allowed to solidify. Bacteria from the liquid culture was used to streak the plates. A sterilise wire loop was dipped in liquid culture, and the surface area of the plate was streaked with it. Plates were then incubated overnight at 37°C for 16-18 hours.

- Precautions while using the laminar hood
  Before using the laminar hood, the UV light was turned on for 20 minutes. UV light can kill the microbes and sterilises the hood. Make sure to turn off UV before working in the hood. Sterilise hands using 70% ethanol.

1.4 Immunohistochemistry

1.4.1 Dissection of adult flies

- ❖ Anesthetise the flies using ether and transferred to a drop of PBS solution on a glass slide.
- ❖ The flies are placed dorsal down and wholly immersed in PBS.
- ❖ Fly wings are stretched apart using the dissection needles and place a surgical scissor was placed above the ventral side in a parallel manner. Then an incision was made from the posterior tip of the abdomen ventrally and continued up to the head.
- ❖ Utilising this first ventral cut, removed the ventral side of the abdomen, legs and head region.
- ❖ Using a scissor, removed both the wings from the fly. Care was taken to remove the fat bodies, gut, Malpighian tubules and ovaries present in the abdominal cavity.
- ❖ Thereafter, the dissected sample will only have the dorsal side of the abdomen and the thorax.

1.4.2 Reagents and Buffers

1. 1X PBS (phosphate buffer saline)

PBS is a buffer solution that helps to sustain a constant pH in the cell. The solvent for the PBS solution is water.

Salts and required amount: NaCl, - 8 g

KCl - 0.2 g

Na_2HPO_4 - 1.44 g

KH_2PO_4 - 0.24 g

Weigh the above mentioned chemical and transfer them into a 1000 ml reagent bottle and dissolve with distilled water and adjust the pH of the solution to 7.20-7.40. It can be adjusted using an acid such as HCl or a base like NaOH. Autoclave it for 45 minutes at 121 °C and 22 psi

2. Paraformaldehyde (PFA)

Paraformaldehyde is a polymerised form of formaldehyde, and it helps fix the tissues. Take 0.2 g of PFA powder in a 2ml Eppendorf and add 2ml of 1X PBS and mix it well. It will take time to dissolve so keep it in 65°C water bath and shake it in time interval until it gets dissolved. PFA is a potential carcinogen. Need to take the necessary precautions while handling it.

3. **PBT (PBS- TritonX100)**
   PBT is a detergent which helps in solubilising the membrane proteins and thereby allow the molecules such as antibodies to get inside the cell.

<u>0.3% PBT preparation</u>

• Add 120 µl of TritonX100 (Detergent) in 40 ml of 1XPBS.

• Keep it on a rotator for 30 - 45 minutes to dissolve properly

4. **Bovine Serum Albumin (BSA)**
   It functions as a blocking agent. It binds to the non-specific sites in the cell surfaces and thereby decreases the chances of non-specific antigen-antibody interaction.
   <u>5% BSA preparation</u>
   - Weigh 0.1 g of BSA and dissolve it in 2 ml of 0.3% PBT and mix it well.

5. Antibodies used

Antibody	Dilution factor	Raised in
Beta galactosidase	1:200	Mouse
Pericardin	1:5	Mouse
Secondary antibody cy3	1:500	Mouse

6. DAPI (2-(4-Amidinophenyl)-1*H*-indole-6-carboxamidine)

DAPI is a light-sensitive fluorescent stain used to mark the nuclei. It binds to the AT-rich regions in DNA can pass through the cell membrane. It is stable at a pH level 4 – 11

7. VECTASHEILD

It is an antifade mounting medium which inhibits rapid photobleaching of fluorescent proteins and fluorescent dyes.

1.4.3 Procedure for immunostaining of adult hematopoietic hub

Dissected the adult hematopoietic hub after 8 hours of infection

- The dissection must be completed within 20 minutes and put the tissues in a cavity block containing PBS
- Once done with the dissection, the PBS was removed, and 5% PFA was added. Fixation was done at room temperature for 1 hour.
- After fixation PFA was replaced by PBS and gave two five minute PBS wash at room temperature (RT) was done.
- After removing PBS, two washes in 0.3% PBT of 15-minute duration on a shaker (at RT, speed- 60 rpm) was done.
- 5% BSA (made in 0.3% of PBT) was next added, and the tissues were incubated in the block for 1 hour on a shaker. (at RT, speed- 60 rpm)
- A Nunc plate was taken, 7µl of the primary antibody was added in each well, using a dissection needle, each tissue was gently transferred in wells for incubation at 4°C for 48 hours.
- After 48 hours, the tissues from the well were transferred to cavity blocks for three washes in 0.3% PBT for 15 minutes on a shaker. (at RT, speed- 60 rpm)
- 5% BSA (made in 0.3% of PBT) was next added, and the tissues were left on a shaker for 1 hour (at RT, speed- 60 rpm)
- BSA was replaced with secondary antibody and incubated at 4°C for 48 hours (Secondary antibodies can be added in the cavity block, and they are light-sensitive).
- Post incubation, the tissues from the well were transferred to a cavity bock and washed twice in 0.3% PBT. (15 minutes at RT, speed- 60 rpm)

- The tissues were washed briefly in 1X PBS for five minutes and incubated in DAPI for overnight (at 4°C).
- Two washes followed DAPI treatment in PBS, and the samples were ready to mount in VECTASHIELD.

1.4.4 Imaging and Image processing

Images of the mounted samples were taken by using the confocal microscope (Zeiss LSM 780 and Leica SP8).

The processing is done by using Fiji or Image J 9 software; it is an image processing program functioning with Java created by the scientists at the National Institutes of Health(NIH) in the USA and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin).

1.4.5 Unfixed tissue labelling with 2-NBDG

2- NBDG is a fluorescent analogue for glucose. The flies were dissected in Schneider's media at room temperature. The media was replaced with 2- NBDG – 1Mm concentration made in 1X PBS and incubated at room temperature for 45 minutes. This was followed by washing the tissues with pre-cooled 1X PBS. Post washing, the tissue was fixed with 5% PFA for 1 hour. Post washing washes the samples were incubated with DAPI (1:1000 in 1X PBS) for 10 minutes to stain the nucleus. The samples were then mounted in VECTASHIELD medium.

Chapter4: Results

1. Results

1.1 Infection response at different time windows

To verify the maximum phagocytic activity after infection, we observed the population of blood cell – plasmatocytes in the adult hematopoietic hub cluster 1 at different time intervals after infection on the sixth day.

After 2 hours of infection, we could see that phagocytosis was initiated, but still, there were many bacteria freely in the system. (**Figure: 8**)

When looked into the 8 hours of the post-infection time window, there was an increase in phagocytosis and individual cells formed a lawn like structure, and most of the bacteria were inside the plasmatocytes. (**Figure: 9**)

In the case of 24 hr post-infection, there was lawn formation in cells, and the number of bacteria in the system, i.e. bacterial load, was also less. Blood cell response towards infection has reached its plateau at this stage. (**Figure: 10**)

Among the different time windows, 8 hours' post-infection period has the maximum phagocytic activity. Towards later time windows clearance of bacteria in the system observed. Hence for my further studies, I adopted 8 hours of the post-infection time window.

2 Hours post-infection

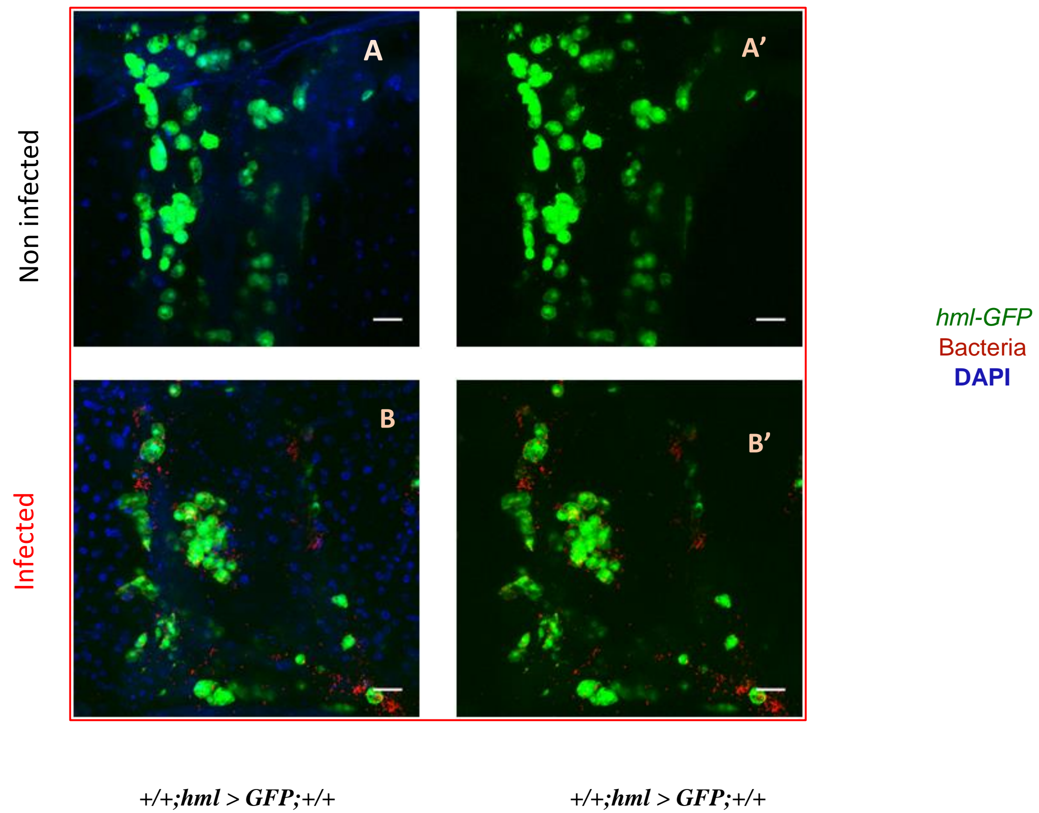


Figure: 8 Infection response after 2 hours of infection in adult flies. (Scale bar 20 µm)

In infected system, we can see *E.coli* bacteria (in red) some of them are within the blood cells(in green). Engulfing of bacteria just started.

8 Hours post infection

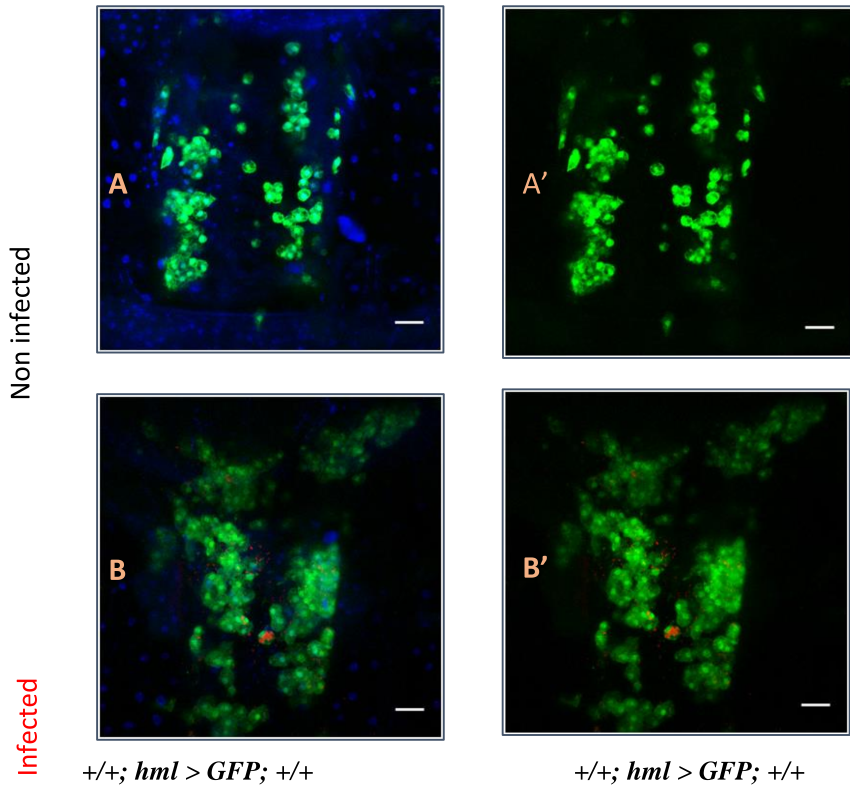


Figure: 9 Infection response after 8 hours of infection in adult flies. (Scale bar 20 µm) Compared to non infected, (A) the infected one's (B) cells are come together and formed a lawn like structures. Number of bacteria reduced and there is bacteria within the green colored cells - plasmatocytes

24 hours post infection

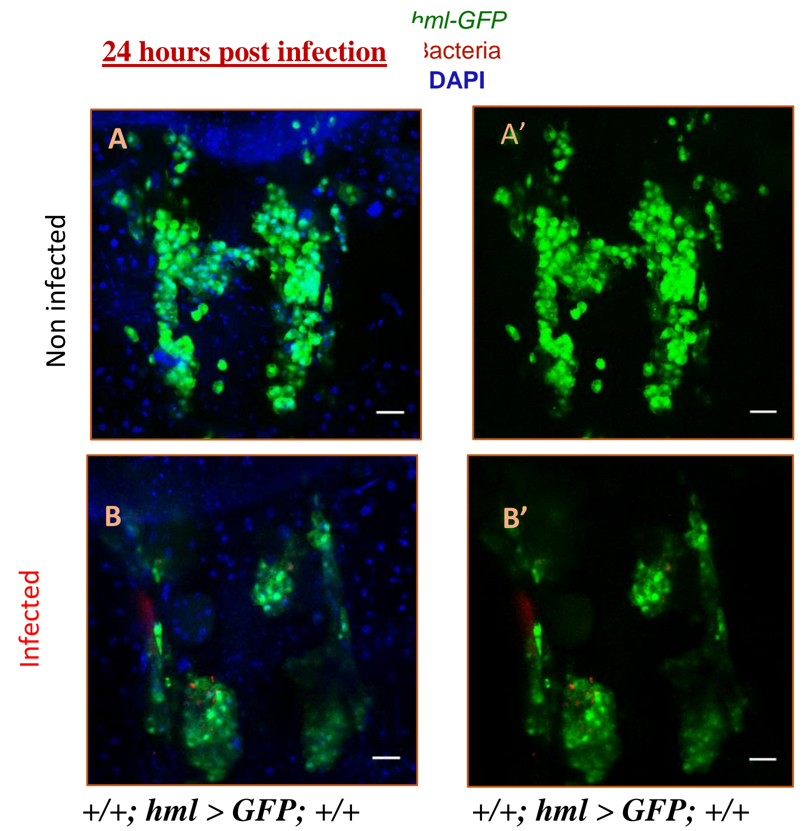


Figure: 10 Infection response after 24 hours of infection in adult flies. (Scale bar 20 µm)In B, phagocytosis is not completed but there is a rapid decrease in number of bacteria in the system

1.4 Infection response of Extracellular matrix(ECM)

Extracellular matrix (ECM) is a web of secreted proteins and carbohydrates which heaps the intercellular space. It facilitates several cellular functions like migration, proliferation, adhesion, and differentiation. The hemocytes within the clusters are inlayed in an elaborated network of ECM proteins. Some of the essential proteins are collagen IV like Pericardin and Laminin A. They maintain the adhesive interaction with the hemocytes and also helping in the formation of clusters. We checked the status of Pericardin upon infection. From our infection analysis, since we found that maximum phagocytic activity occurs after 8 hours of infection, we assayed the status of Pericardin in the same hour.

Interestingly, we could not identify any significant difference between non- infected and infected individuals. The clusters were intact in both cases, and from the intensity analysis, there is no significant difference in the expression level of Pericardin as well (**Figure: 11** and **12**).

Response of Extracellular matrix upon infection

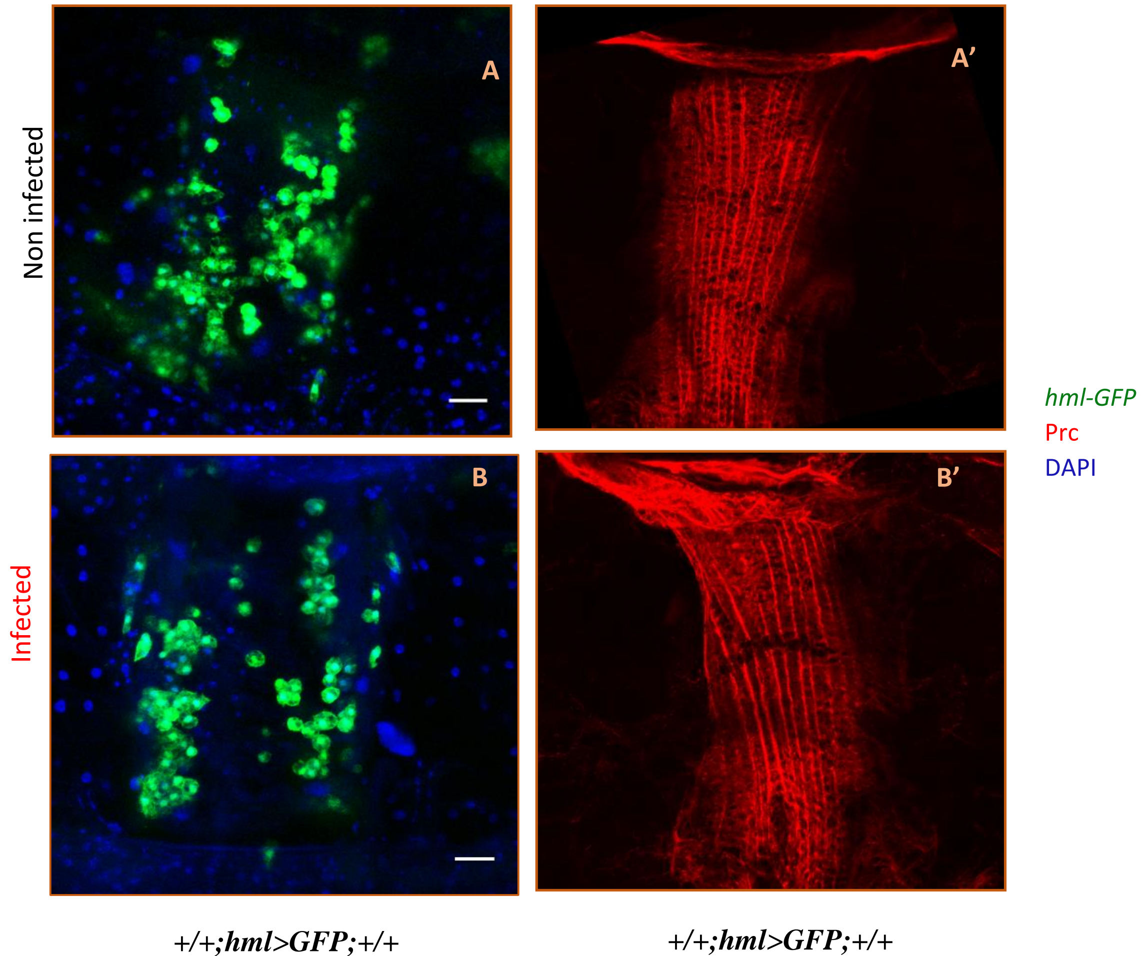


Figure: 11 Response of ECM upon infection. (Scale bar is 20 µm)Expression of *hml GFP* in non infected(A) and expression of *pericardin*, ECM component(A'). In the infected sample, expression of *hml GFP* (B) and *pericardin*(B')

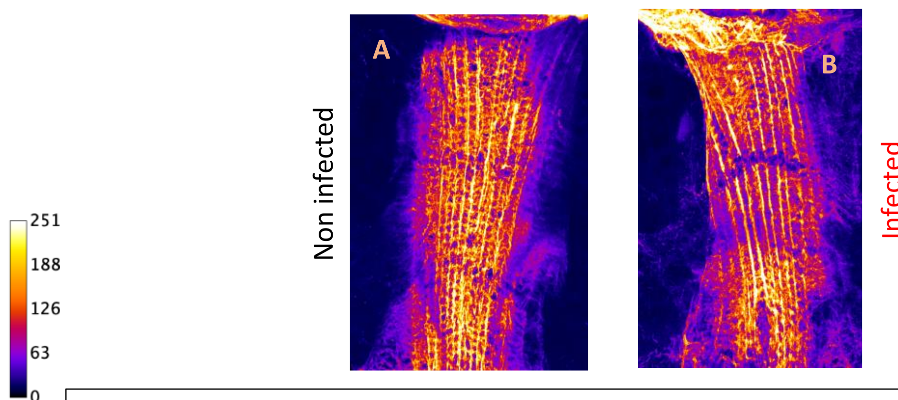


Figure: 12 Intensity analysis: Intensity analysis of non infected(A) and infected (B)ECM, there are no variations in them.

1.3 Overall glucose uptake increased in High Sugar fed flies

When flies fed with High Sugar for five days from the second day after eclosion onwards (a pre-diabetic condition), we could see an increase in glucose uptake by cells in the flies compared to the Normal Diet. We used glucose fluorescent analogue 2 – NBDG for glucose monitoring validating the accuracy of our experimental setup.

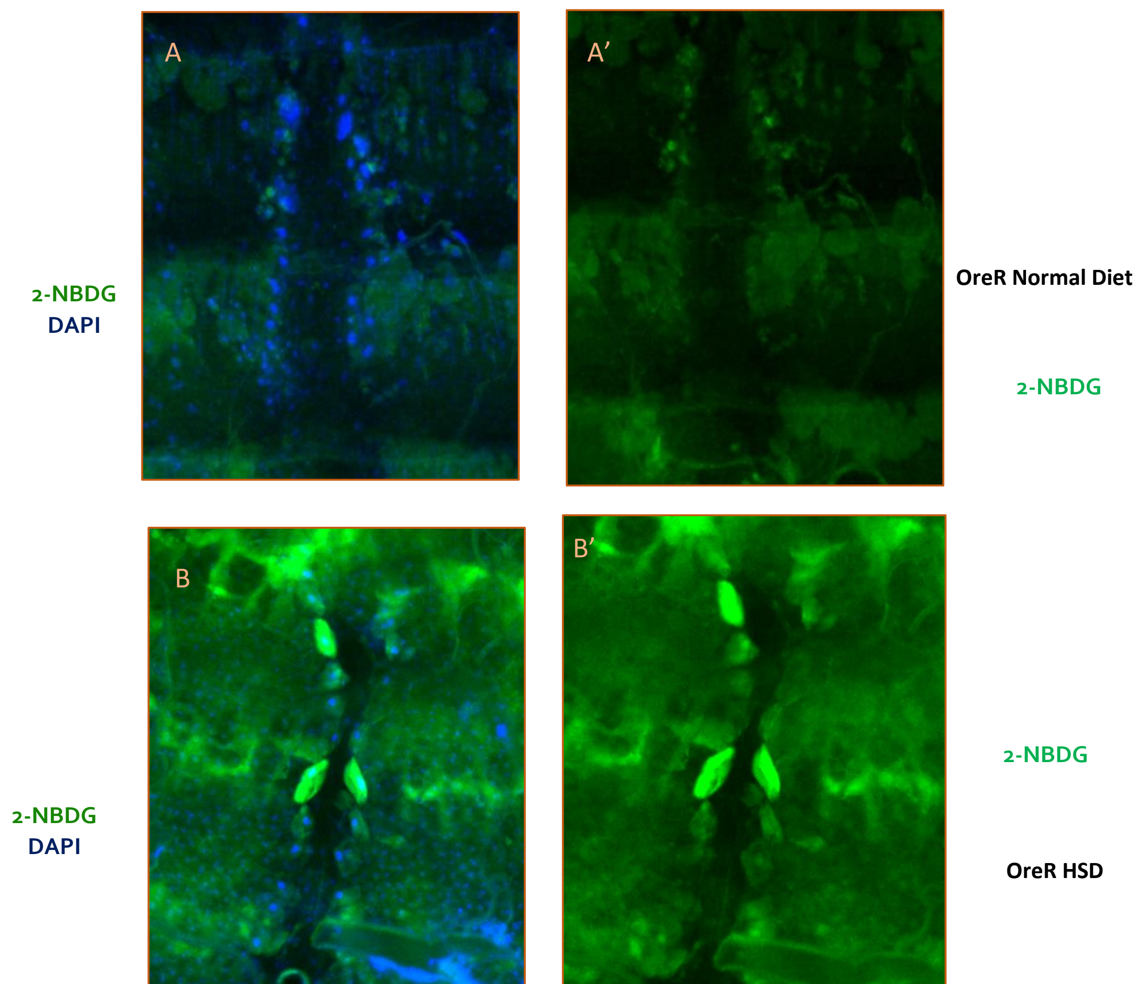


Figure: 13 Increased uptake of glucose in High Sugar fed adult flies. (Scale bar 20 µm) Flies fed in High Sugar diet showed an increase in glucose (B, B’) compared to normal diet (A,A’). Expression of 2-NBDG in green and the blue (DAPI) marks the nucleus.

1.4 Response of blood cells on High Sugar diet and Normal food on High glucose condition

As per our experimental scheme, the flies become type 2 diabetic from 15th day (post eclosion) only. Before that, there is a high glucose level in the hemolymph. We looked into the blood cells after five days of High sugar food treatment. At high glucose level, there is no significant difference in blood cell morphology and number on normal food and high sugar food.

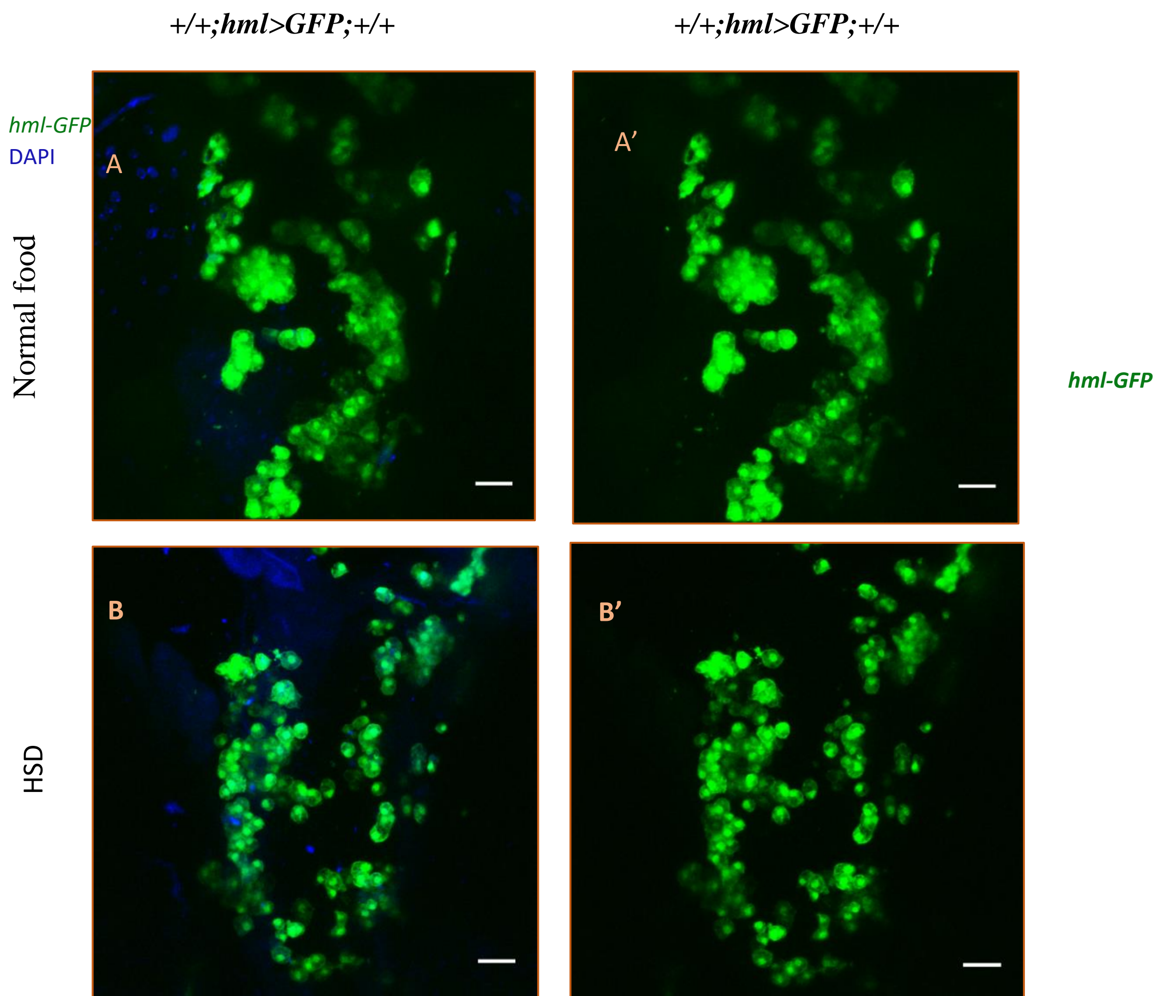


Figure: 14 Response of blood cells in normal diet and HSD in pre diabetic condtion. (Scale bar 20 µm)**Expression of *hml-GFP* in blood cells of flies fed in both normal diet(A') and HSD(B') has no significant difference.A and B are DAPI stained images of ND and HSD along with *hml-GFP* expression**

So far, we look into the time series of infection response, analysed the effect of infection on the extracellular matrix, and observed the blood cells when there is high glucose in the fly system. After 13 days of feeding in the High Sugar diet, the flies become diabetic. The next step requires repeating the entire analyses in the diabetic flies, i.e. post 13 days on High Sugar Diet.

1.5 The response of blood cells upon infection in Normal Diet and High Sugar Diet (At type 2 diabetic condition)

In diabetic conditions, there was a significant change in the number of blood cells in comparison to the control. We found a substantial drop in blood cell number in High Sugar fed flies. Compared to Normal and High Sugar fed (without infection), the survival of the infected flies in the High Sugar Diet was much less. Survival of flies in the Normal Diet and Normal Diet & infected were comparatively the same. We can observe in **Figure 15** that blood cell numbers are very less in infected flies in High Sugar compared to the rest.

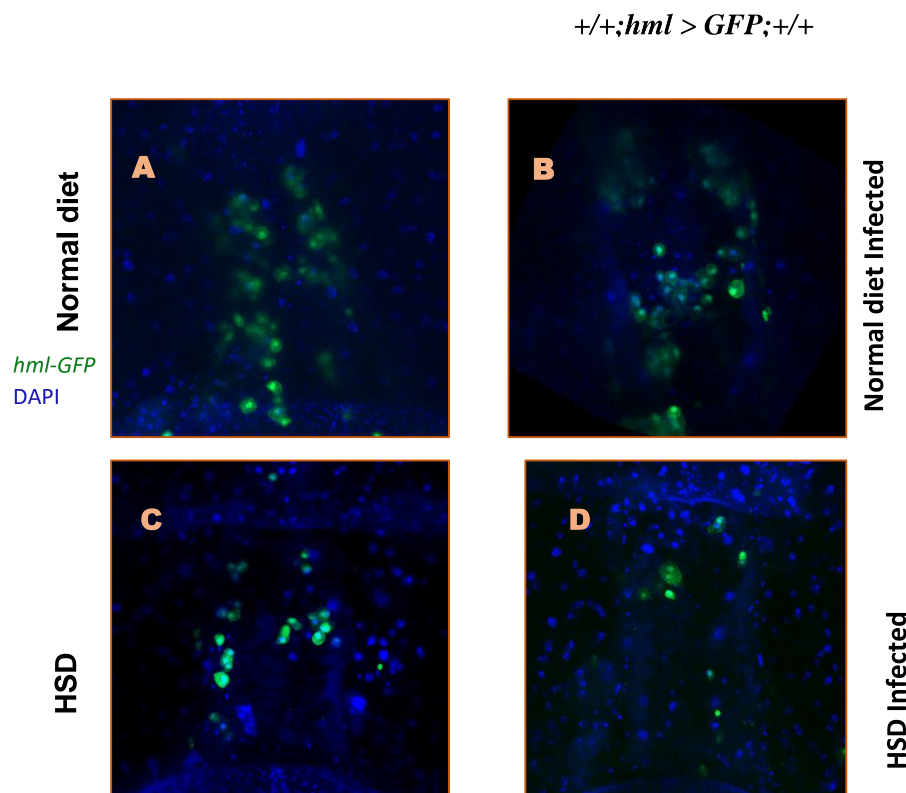


Figure: 15 Response of blood cells upon infection in Normal Diet and HSD: (Scale bar 20 µm) Expression of blood cell marker *hml-gfp* in ND (A),ND infected(B), HSD(C)and HSD infected(D). (DAPI is in blue colour.) There is a variation in HSD(C) and HSD infected(D) compared to ND and ND infected.

1.5 The response of the Extracellular matrix in Normal Diet and High Sugar Diet (At type 2 diabetic condition)

When the diabetic condition established in the High Sugar Diet, we analysed the status of Pericardin, which is one of the major components in the extracellular matrix. Fascinatingly, we see that there is an increase in the amount of Pericardin in High Sugar fed flies compared to Normal Diet fed flies. Surprisingly, this increase in Pericardin did not support the maintenance of the blood cell cluster in the High Sugar flies indicating that the decrease in hemocyte number (Figure:15) is not due to the problem with ECM.

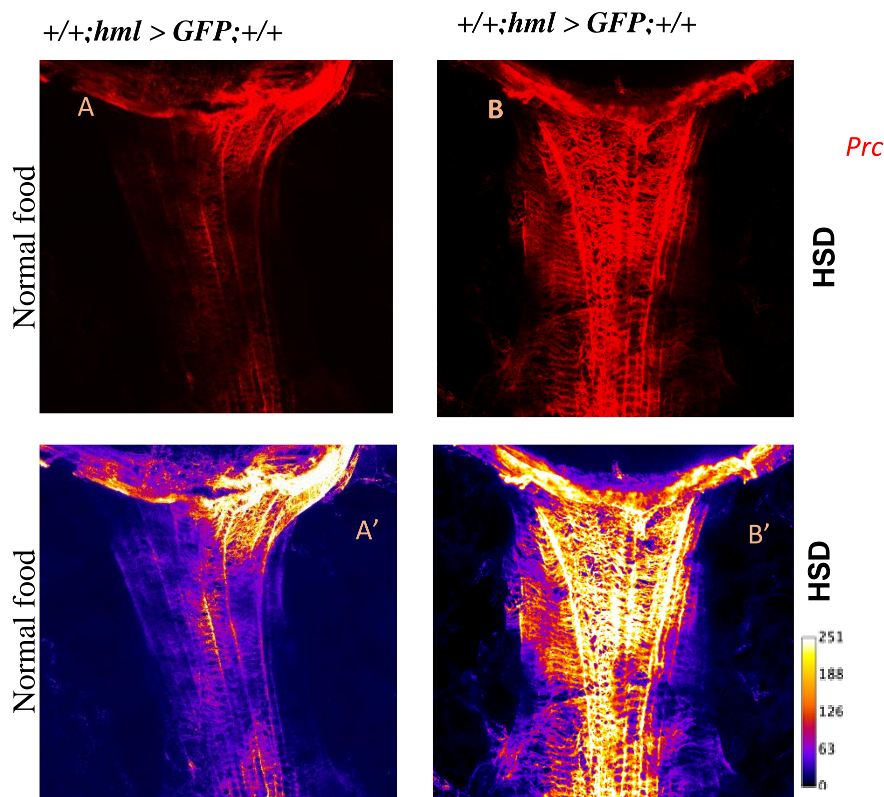


Figure: 16 Response of ECM in Normal diet and High Sugar Diet: (Scale bar 20 µm) In HSD, compared to Normal Diet pericardin intensity is high which means there is an increase in the amount of ECM.(A is normal diet and B is HSD,A' and B' are their intensity analysis respectively)

1.6 The response of Immune pathways in High Sugar Diet upon infection

Drosophila immune system is under the control of two major signalling pathways – Toll and Imd. The primary function of these two cascades is to produce Antimicrobial peptides(AMPs), which are host defence oligopeptides and they can induce permeation of large molecules and cell membrane damage and cell death of the pathogen. For understanding whether diabetes has any effect on immune response, we infected the flies and checked the level of Diptericin(Dpt) in High Sugar fed flies. Diptericin is majorly produced in the fat body cells in adult and released into the hemolymph. The control of the experiment was flies reared in a normal diet.

For achieving high glucose condition flies were, reared on High Sugar for five days and infected on the sixth day with RFP positive *E. coli* bacteria. Post-infection flies subjected to dissection after 8 hours, and the expression of Diptericin in the fat bodies was analysed. For imaging and quantitation, we mainly focused on the fat body population near the first cluster of the adult hematopoietic hub.

There was a basal level expression of Diptericin in control (Normal Diet) flies **(Figure:17)**. The flies on Normal Diet infected with Bacteria, as expected, exhibited a surge in the expression of Diptericin. **(Figure:18)** In High Sugar fed flies, the expression level of Diptericin was slightly less in comparison with control (Normal Diet) **(Figure:19)**. However, in the significant population of High Sugar infected fly's expression of Diptericin was very weak. We found that about 70% of the population were unable to mount an immune response. (**Figure:20**). We could see the RFP positive *E. coli* bacteria in the system (a white arrow in **Figure:20)**

In **Figure: 21**, the graph showing the variation of Diptericin in infected and non-infected flies in both Normal and High Sugar Diet.

w; drosomycin -GFP,diptericin-lacz ;+/+

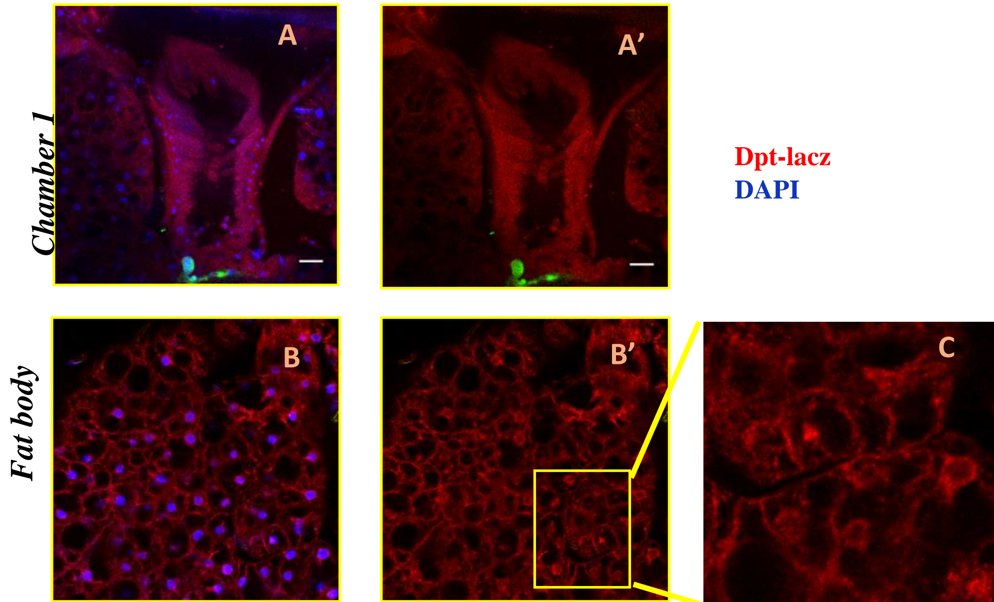


Figure: 17 Expression of Dpt in Normal Diet fed flies. Expression of Dpt in red colour. (Scale bar 20 µm)_First chamber of hematopoietic hub and fat body cells next to it.(A') Basal level of expression of Dpt in fatbody cells(B- expressing DAPI and Dpt and in B' only the Dpt expression is there. In A, the first chamber have both DAPI and Dpt having a basal level expression

w; drosomycin -GFP,diptericin-lacz ;+/+

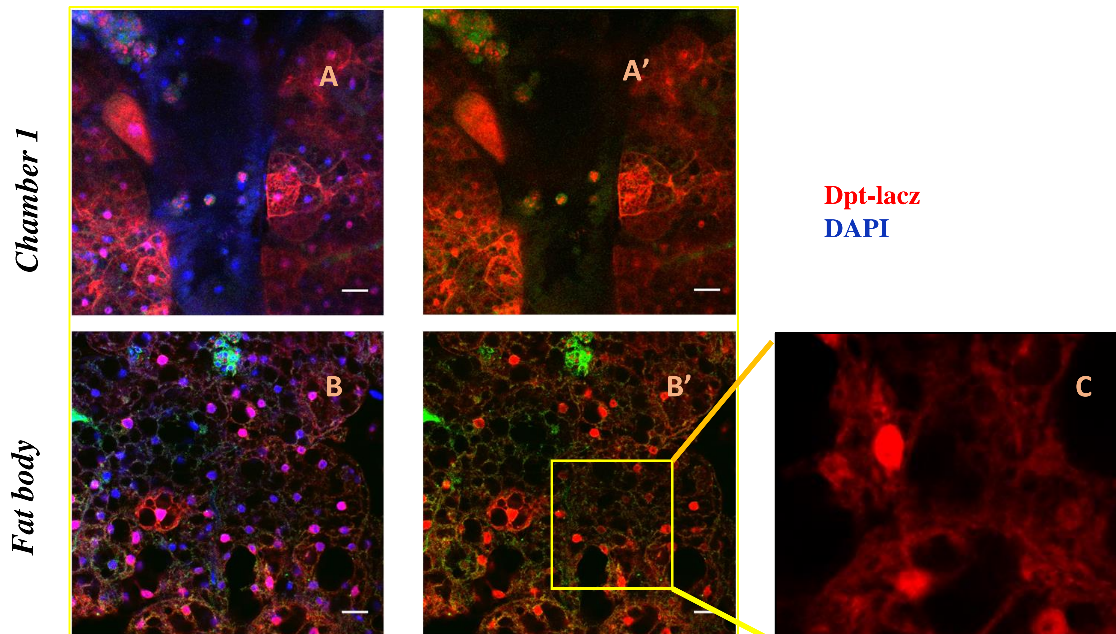


Figure: 18 Expression of Dpt in Normal Diet flies when infected. (Scale bar 20 µm) Expression of Dpt_is strong in infected flies(B' expression of Dpt in fat bodies and enlarged portion of it in C

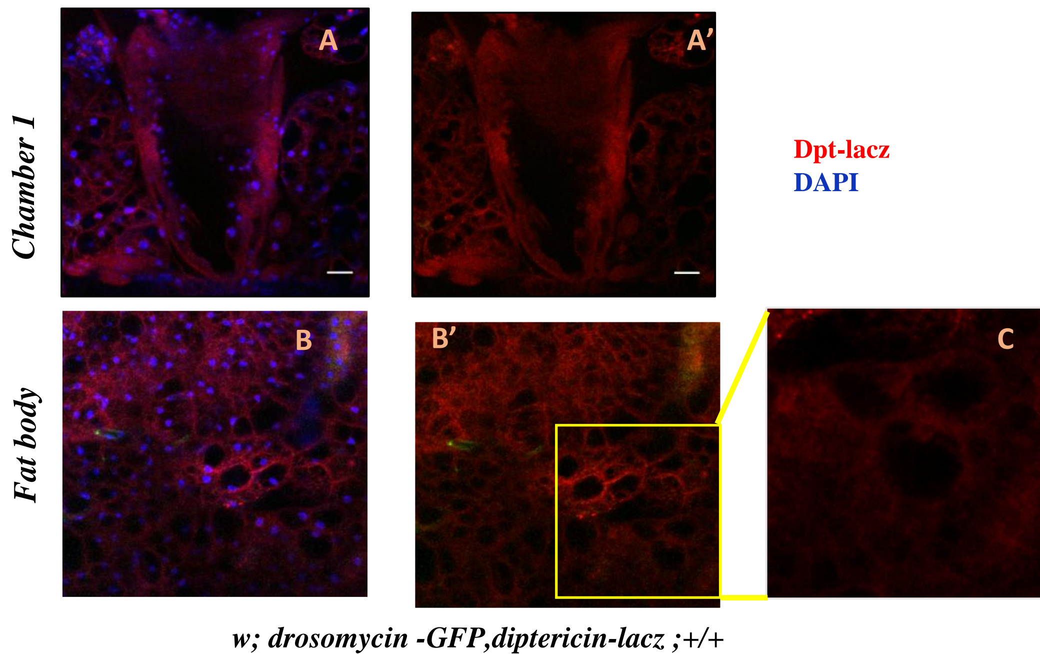


Figure: 19 Expression of Dpt in High Sugar Diet flies . (Scale bar 20 µm) A' represents the expression of Dpt in the first chamber in A there is both DAPI and Dpt. C is the enlarged portion of the fat bodies and expression of Dpt is slightly less than that of ND. B' is the fat body's expressing Dpt and B is with both DAPI and Dpt.

w; drosomycin -GFP,diptericin-lacz ;+/+

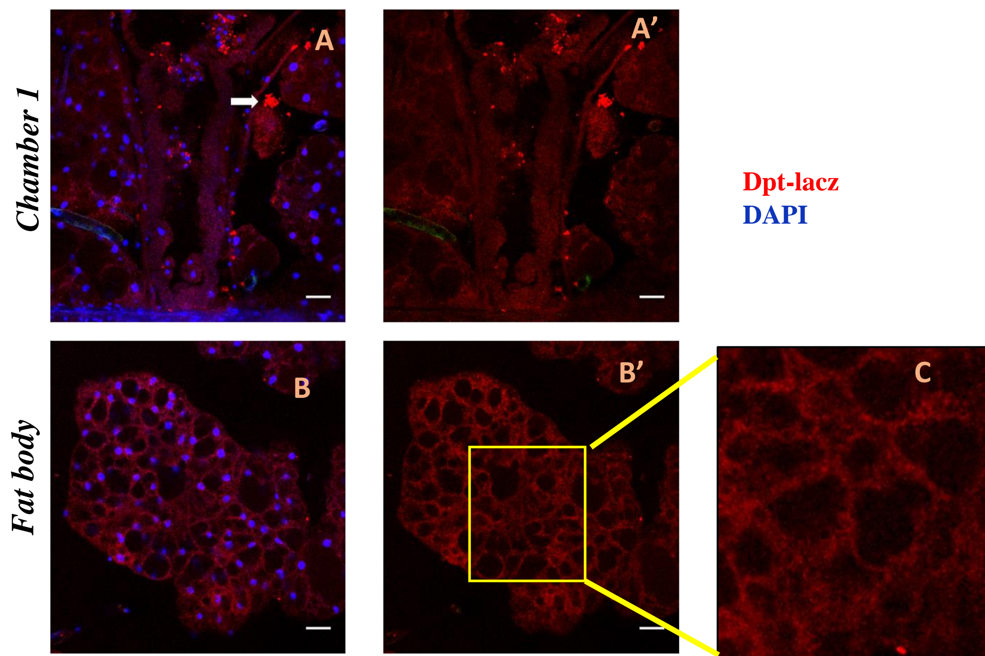
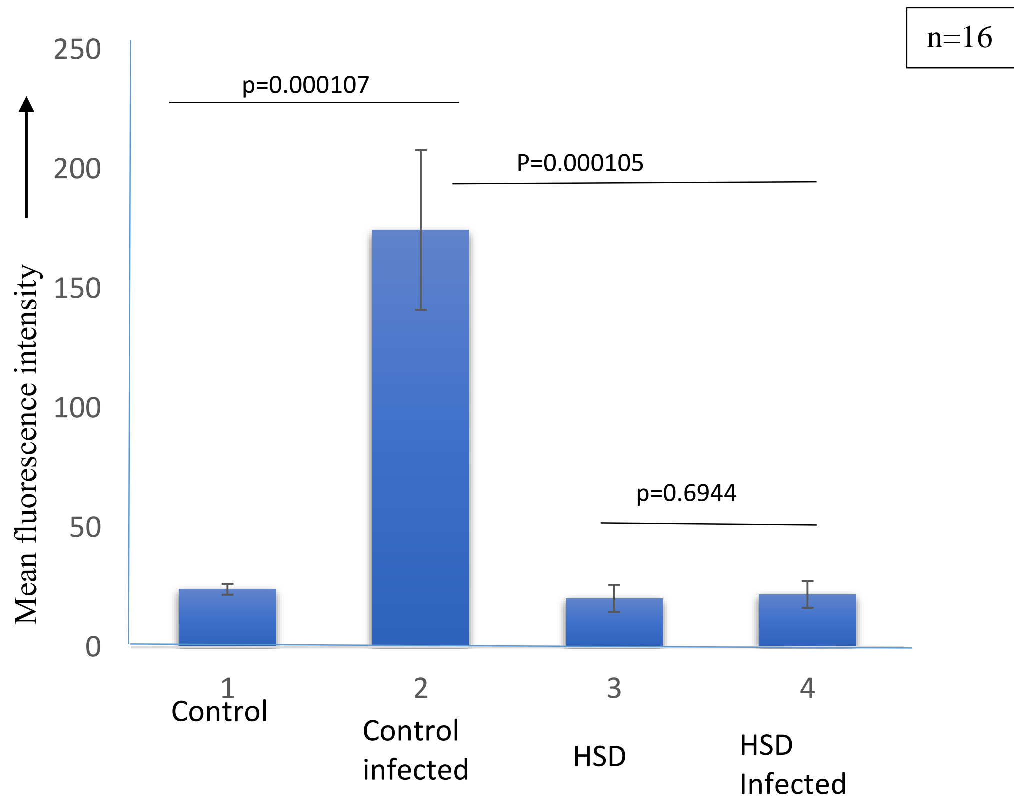


Figure: 20 Expression of Dpt in High Sugar Diet flies when infected(Scale bar 20 µm). There was a reduction in Dpt expression. In A we can see bacteria freely in the system. (white arrow mark) In the enlarged portion of fat body(C) is also showing less Dpt expression. A and B represent the first chamber and fat body 's expressing DAPI and Dpt.

Level of Diptericin in the adult fat body



Graph 1 : Level of Diptericin in adult fat body: Compared to normal diet infected (control infected) HSD fed infected flies have much less amount of Diptericin in them. n= 16

Chapter 5

Discussions & Future direction

Discussions& Future direction

Erratic changes in the lifestyle and diet invited metabolic disorders such as Diabetes mellitus into our life. Patients with diabetes mellitus have a higher chance to get an infection compared to a healthy individual. Also, there is a delay in wound healing in patients with diabetes. Glitches in immunity can be one of the possible reason for this kind of variations. The immune system itself is very complex and efficient, but the fluctuation in the level of glucose can affect the whole system. From the very elemental layer such as the innate immune system to miscellaneous adaptive immune system will turn away from its roles. It is evident that diabetes has a noteworthy part in the formation of macrovascular complications. Such complications are exceedingly swayed through inflammatory cytokines allied with the innate immunity. The convenience of adopting the *Drosophila* model system is because of the availability of only one type of immune system – the innate immune system in them, and we could dodge the influence of the adaptive immune system. Thus through our study, we addressed the issue of how High Sugar Diet can induce the changes immune system. Our investigations resulted in the finding that phagocytosis is significantly altering in the adult hematopoietic system in both sessile and hub population. From Day 5 to Day 15, we could see a descending trend in the blood cell number in High Sugar fed flies compared to Normal Diet. When they infected, compared to Normal Diet, there was a further reduction of blood cell population which is very intriguing. We observed a lawn like the organisation of blood cells when the healthy flies are infected, and it was less intense in High Sugar infected flies. It may be because of the cell-cell interaction in healthy flies will be higher than flies in High Sugar Diet.

We also observed an enhancement in the Extracellular matrix of infected flies in High Sugar Diet on diabetic conditions. The function of the Extracellular matrix is to adhere to the blood cells together in the animal body (Ghosh et al., 2015). The variation we have seen in infected High Sugar Condition may be for balancing the reduced blood cell count, and also the functionality of the extracellular matrix needs to be rechecked with other components such as Laminin A. Further we checked the level of antimicrobial peptide Diptericin associated with the Imd signalling pathway. After five days of High Sugar feeding, a significant number of the infected flies could not mount an immune response against the pathogen. (RFP positive *E. coli)* There was a basal level of expression in control flies; even then there was a slight decrease in the basal level expression in High Sugar control flies. The absence of nuclear localisation of

Diptericin in High Sugar infected flies strongly indicate that their immune system is compromised in the given condition of High Sugar Diet. It needs further validation with Day 15 analysis (onset of Type2 Diabetes).

Imd signalling pathway activates different components of the nuclear factor-κB (NF-κB) family in *Drosophila*. The pathway is evolutionary primaeval, and it can evoke the tumour-necrosis factor (TNF) signalling pathways which have a fundamental part in the innate immunity of mammals. We have studies indicated that numerous diabetic problems are often concomitant with TNF-alpha. Escalated level of TNF-alpha can observe in type 2 diabetic model in immune challenges such as bacterial infection and other complications related to diabetes such as impaired wound healing, diabetic retinopathy etc. [43]. Because of the decreased production of Dpt and inefficient phagocytosis, we can interpret that the system is immunocompromised. We have other studies using *Drosophila* for studying the progression of T2DM; they have shown that the Toll pathway, the evolutionary ancient of the TLR signalling pathway, is affected by High Sugar. In the future, we can check whether any cross-talk between these pathways exists or by how the immune system will respond. In mammal's regulation of NF-κB by TNF, Family Cytokines is a well-entrenched fact, and so is in its *Drosophila* counterpart. In the subsequent studies using *Drosophila,* we can alter the transcription factor Relish and see its effects on the High Sugar Diet and also when the immune response gets activated post-infection.

Initiation and regulation of normal wound healing in mammals are linked with Toll-like receptors which are part of the innate immune system. [44] Since TLR and toll pathways are evolutionary conserved. We know that one of the major problems faced by Diabetes mellitus patients is the delay in wound healing. Employing the experimental setup standardised in this study, we can further look into the involvement of Toll pathway if any.

We also need to look into the rate of cell death in the High Sugar Diet and also upon infection.

The lawn like aggregation of the blood cells was much higher in control infected condition in contrast with its High Sugar counterpart. Further analysis should also aim in understanding its contribution towards immunity of the fly. Along with that, we need to look into the proliferation of cells in a much broader way. A cell cycle monitoring in High Sugar Diet and Normal Diet in both normal condition and infected scenario will be helpful in our further understanding.

Bibliography

[1] Diet, nutrition and the prevention of chronic diseases., ‖ Switzerland, 2003.

[2] V. Azais-Braesco, D. Sluik, M. Maillot, F. Kok, and L. A. Moreno, ―A review of total& added sugar intakes and dietary sources in Europe., ‖ Nutr. J. vol. 16, no. 1, p. 6, Jan. 2017.

[3] Q. Yang, Z. Zhang, E. W. Gregg, W. D. Flanders, R. Merritt, and F. B. Hu, Added sugar intake and cardiovascular diseases mortality among US adults., ‖ JAMA Intern. Med., vol.174, no. 4, pp. 516–524, Apr. 2014.

[4] Kitabchi AE, Umpierrez GE, Miles JM, Fisher JN (July 2009), Hyperglycaemic crises in adult patients with diabetes. Diabetes Care. 32 (7): 1335–43

[5] I DF DIABETES ATLAS Ninth Edition 2019(PDF). www.diabetesatlas.org. Retrieved May 18 2020

[6] Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. (December 2012). "Years lived with disability (YLDs) for 1160 sequelae of 289 diseases and injuries 1990–2010: a systematic analysis for the Global Burden of Disease Study 2010". Lancet. 380, (9859): 2163–96.

[7] "What is Diabetes?". Centers for Disease Control and Prevention. March 11 2020.

[8] "About diabetes". World Health Organization. Archived from the original on March 31 2014. It was retrieved April 4 2014.

[9] Zhou T, Hu Z, Yang S, Sun L, Yu Z, Wang G. "Role of Adaptive and Innate Immunity in Type 2 Diabetes Mellitus". J Diabetes Res. 2018; 2018:7457269. Published 2018 November 8

[10] B. A. Pierce, Genetics A conceptual approach, 4th ed.

[11] Gilbert SF (2006). "9: Fertilisation in Drosophila". In eighth (ed.). Developmental Biology. Sinauer Associates.

[12] G. M. Rubin, "Drosophila melanogaster as an experimental Organism," no. 7, 1988.

[13] Fernández-Moreno, Miguel Angel; Farr, Carol L.; Kaguni, Laurie S.; Garesse, Rafael (2007). "Drosophila melanogaster as a Model System to Study Mitochondrial Biology". Mitochondria. Methods in Molecular Biology (Clifton, N.J.). 372. pp. 33–49.

[14] Bohni R, Riesgo-Escovar J, Oldham S, Brogiolo W, Stocker H, Andruss BF Beckingham K, Hafen E. 1999. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1– 4. Cell 97:865– 875.

[15]. Duffy, Joseph. (2002). GAL4 System in Drosophila: A Fly Geneticist's Swiss Army Knife. Genesis. 34. 1-15. 10.1002/gene.10150.

[16] Ashburner M, Golic KG, Hawley RS (2005). Drosophila: A Laboratory Handbook (2nd ed.). Cold Spring Harbor Laboratory Press. Pp. 162–4. ISBN 978-0-87969-706-8.

[17] Nürnberger T, Brunner F, Kemmerling B, Piater L. Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev. 2004;198:249-266. DOI:10.1111/j.0105-2896.2004.0119.x

[18] Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001. Immunological memory. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27158/

[19] Sackton, T., Lazzaro, B., Schlenke, T. et al. Dynamic evolution of the innate immune system in Drosophila. Nat Genet 39, 1461–1468 (2007). https://doi.org/10.1038/ng.2007.60

[20] Davis MM, Engström Y. Immune response in the barrier epithelia: lessons from the fruit fly Drosophila melanogaster. J Innate Immun. 2012;4(3):273-283. DOI:10.1159/000332947

[21] Tang H. Regulation and function of the melanisation reaction in Drosophila. Fly (Austin). 2009;3(1):105-111. DOI:10.4161/fly.3.1.7747

[22] Wang L, Kounatidis I, Ligoxygakis P. Drosophila as a model to study the role of blood cells in inflammation, innate immunity and cancer. Front Cell Infect Microbiol. 2014;3:113. Published 2014 January 9. doi:10.3389/fcimb.2013.00113

[23] Stuart, L., Ezekowitz, R. Phagocytosis and comparative innate immunity: learning on the fly. Nat Rev Immunol 8, 131–141 (2008). https://doi.org/10.1038/nri2240

[24] Stroschein-Stevenson SL, Foley E, O'Farrell PH, Johnson AD. Identification of Drosophila gene products required for phagocytosis of Candida albicans. PLoS Biol. 2006;4(1):e4. doi:10.1371/journal.pbio.0040004

[25] Elrod-Erickson M, Mishra S, Schneider D. Interactions between the cellular and humoral immune responses in Drosophila. Curr Biol. 2000;10(13):781-784. doi:10.1016/s0960-9822(00)00569-8

[26] Bahar, Ali Adem, and Dacheng Ren. "Antimicrobial peptides." Pharmaceuticals (Basel, Switzerland) vol. 6,12 1543-75. 28 Nov. 2013, doi:10.3390/ph6121543

[27] Radek K, Gallo R. Antimicrobial peptides: natural effectors of the innate immune system. Semin Immunopathol. 2007;29(1):27-43. doi:10.1007/s00281-007-0064-5

[28] Leippe M. Antimicrobial and cytolytic polypeptides of amoeboid protozoa—Effector molecules of primitive phagocytes. Dev. Comp. Immunol. 1999;23:267–279. doi: 10.1016/S0145-305X(99)00010-5.

[29] Jenssen H, Hamill P, Hancock RE. Peptide antimicrobial agents. Clin Microbiol Rev. 2006;19(3):491-511. doi:10.1128/CMR.00056-05

[30] V Susanna, H- Jing, R.Mika. The Drosophila Toll Signaling Pathway.The Journal of Immunology January 15, 2011, 186 (2) 649-656; DOI: 10.4049/jimmunol.1002302

[31] Rutschmann S., A. Kilinc, D. Ferrandon. 2002. Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in Drosophila. J. Immunol. 168: 1542–1546

[32] Bettencourt R., H. Asha, C. Dearolf, Y. T. Ip. 2004. Hemolymph-dependent and -independent responses in Drosophila immune tissue. J. Cell. Biochem. 92: 849–863

[33] Haghayeghi A., A. Sarac, S. Czerniecki, J. Grosshans, F. Schöck. 2010. Pellino enhances innate immunity in Drosophila. Mech. Dev. 127: 301–307.

[34] M.Henna, Valanne S, R. Mika. The Drosophila Imd Signaling Pathway. The Journal of Immunology April 15, 2014, 192 (8) 3455-3462; DOI: 10.4049/jimmunol.1303309

[35] Tzou, P., S. Ohresser, D. Ferrandon, M. Capovilla, J. M. Reichhart, B. Lemaitre, J. A. Hoffmann, J. L. Imler. 2000. Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity 13: 737–748.

[36] Ferrandon D.1998. A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO J. 17: 1217–1227

[37] Valanne S., A. Kleino, H. Myllymäki, J. Vuoristo, M. Rämet. 2007. Iap2 is required for a sustained response in the Drosophila Imd pathway. Dev. Comp. Immunol. 31: 991–1001.

[38] Dushay M. S., B. Asling, D. Hultmark. 1996. Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defence of Drosophila. Proc. Natl. Acad. Sci. USA 93: 10343–10347.

[39] Mler J. L., J. A. Hoffmann. 2001. Toll receptors in innate immunity. Trends Cell Biol. 11: 304–311

[40] Jung, S. H., Evans, C. J., Uemura, C., & Banerjee, U. (2005). The Drosophila lymph gland as a developmental model of hematopoiesis. Development, 132(11), 2521-2533.

[41] Letourneau M, Lapraz F, Sharma A, Vanzo N, Waltzer L, Crozatier M. Drosophila hematopoiesis under normal conditions and in response to immune stress. FEBS Lett. 2016;590(22):4034-4051. doi:10.1002/1873-3468.12327

[42] Ghosh, S., Singh, A., Mandal, S., & Mandal, L. (2015). Active hematopoietic hubs in Drosophila adults generate hemocytes and contribute to immune response. Developmental cell, 33(4), 478-488.

[43] Musselman LP, Fink JL, Ramachandran PV, Patterson BW, Okunade AL, Maier E, Brent MR, Turk J, Baranski TJ. 2013. Role of fat body lipogenesis in protection against the effects of caloric overload in *Drosophila*. J Biol Chem https://doi.org/10.1074/jbc.M112.371047.

[44] Ganeshan, Kirthana, and Ajay Chawla. "Metabolic regulation of immune responses." Annual review of immunology vol. 32 (2014): 609-34. doi:10.1146/annurev-Immunol-032713-120236

[45] Palanker Musselman, Laura (2011). "A high-sugar diet produces obesity and insulin resistance in wild-type Drosophila." Disease Models & Mechanisms 2011 4: 842-849; doi: 10.1242/dmm.007948

[46] Elbing, Karen L, and Roger Brent. "Recipes and Tools for Culture of Escherichia coli." Current protocols in molecular biology vol. 125,1 (2019): e83. doi:10.1002/cpmb.83