The requirement of *Unpaired2* (*upd2*), the ligand for JAK-STAT signaling pathway for the homeostasis of *Drosophila* larval hematopoietic organ

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A dissertation submitted for the partial fulfillment of BS-MS dual degree in Science

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Indian Institute of Science Education and Research

Mohali

June 2020

Dedicated to my family

Acknowledgement

I am thankful to each and every person who has supported and guided me in my life. I am thankful to my family members – my mother, father and sister for all their support and understanding. Without them, I wouldn't have been able to complete my thesis work successfully. They have always been there in my ups and downs.

I am grateful to Dr. Lolitika Mandal, my project supervisor and mentor, for her love and support throughout my degree. She is a very good listener, and she has patiently guided me throughout my project. She is an excellent teacher, and I am sincerely thankful to her for introducing me to the basics of developmental biology. She has taught me how to tackle things in academics as well in my life. She also gave me insights about my future. I am thankful to her for introducing me to the amazing world of *Drosophila melanogaster* – the "Cinderella of Genetics".

I am sincerely grateful to Dr. Sudip Mandal for his guidance and support. He taught me how to be methodical, careful and punctual about my work.

I would like to convey my deepest gratitude to Parvathy Ramesh for her support, encouragement and valuable advice. She has helped me a lot with my labwork.

I thank all the members of *Drosophila* Genetics Laboratory and Molecular Cell and Developmental Laboratory, Satish Tiwari, Ashish Toshniwal, Shiv Kumar Sharma, Harleen Kaur, Jayati Gera, Aditya Kanwal, Sushmit Ghosh, Prerna Budakoti, Gunjan Chauhan, Greeshma P. Bose, Pranav Joshy, Sreelekshmi S R, Farzana N, Ramsi Nilopher, Susovan, Kaustav Ray, Saswata and Kaustuv Ghosh for a lively lab environment.

I would also like to thank my batchmates and friends who have helped me throughout my life in IISER Mohali. I will always cherish the beautiful memories I have with my friends. They have always been there for me.

I would like to thank the former Directors Prof. N. Sathyamurthy and Prof. Debi Prasad Sarkar, the current director Prof. J Gowrishankar of IISER Mohali, for giving me the opportunity to study and work at this premier research institute. I express most respectful

regards to my thesis committee members and other faculties for their valuable suggestions. Finally, I would like to thank IISER Mohali for the best five years in my life – academically and non-academically.

Aiswarya A S.

Certificate of Examination

This is to certify that the dissertation titled "The requirement of *Unpaired2 (upd2)*, the ligand for JAK-STAT signaling pathway for the homeostasis of *Drosophila* larval hematopoietic organ" submitted by Aiswarya A S (Reg. No. MS15028) for the partial fulfilment 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)

Dated: June 12, 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 is made to indicate this clearly, with due acknowledgment 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.

Aiswarya A S

(Candidate)

Dated: 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)

Abstract

Hematopoiesis is the process by which blood cells are formed. There are studies going on across the globe on hematopoiesis by using various model systems. Amongst its diverse roles in vertebrates, most important roles of blood are the transport of oxygen, nutrients and rendering of immunity for the organism. The WBCs or white blood corpuscles are responsible for the immunity of the organism, which is crucial for its protection from pathogens. There is conservation of the overall hematopoietic process from invertebrates to vertebrates. The invertebrate in which the process of hematopoiesis is widely studied is *Drosophila melanogaster*. The *Drosophila* immune system has three types of cells – (1) the plasmatocytes, which functions like the macrophages or monocytes in vertebrates by phagocytosing the foreign bodies, (2) crystal cells that are insect specific and involved in wound healing, (3) lamellocytes which also performs phagocytosis of pathogens that are too large for the plasmatocytes to deal with. What makes this model system favourite in the areas of hematopoietic research is that the similarity of the transcription factors and signaling pathways involved in both *Drosophila* and vertebrate hematopoiesis [2] [5].

One of the major signaling pathways involved in *Drosophila* hematopoiesis is the JAK-STAT signaling pathway. In general, the JAK-STAT pathway in *Drosophila* is turned on by the binding of the ligand, either cytokines Upd1, 2 or 3. The role of Upd3 in the lymph gland hematopoiesis is known. But the role of Upd2 is still a mystery. We were able to show that the knockdown of *upd2* from the differentiated hemocytes (cortical zone) of the lymph gland severely imbalances the homeostasis of the three zones of the lymph gland. This includes the early differentiation of progenitors, complete absence of crystal cells and the abnormal increase in niche cells. Our results suggests that Upd2 plays an important role in the maintenance of lymph gland homeostasis. Further studies on this aspect will throw further insights to the role of JAK-STAT signaling in blood disorders and cancers.

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CHAPTER I

INTRODUCTION

I.I *Drosophila melanogaster* as a model system

Drosophila melanogaster, commonly known as fruit fly, is a model organism which is widely used for biological research in genetics, physiology, microbial pathogenesis and life history evolution. It belongs to the kingdom Animalia, phylum Arthropoda, class Insecta, order Diptera, family Drosophilidae and genus *Drosophila*.

The main reasons for using fruit fly as a model organism include – (1) short life cycle, (2) ease and robustness of culturing, (3) low maintenance cost, (4) sexual dimorphism, (5) small size, (6) high reproductive rate, (7) easily observable phenotype.

The male and female fruit flies are morphologically different (Fig. 1). Males are smaller as compared to the female. Males are dark in colour, have rounded genitalia at the tip of their abdomen whereas females have a light coloured, pointed genitalia. In males, a sex comb (a small patch of bristles) is present on the fourth segment of the first pair of legs. The male fly uses this to grab the female while mating.

Figure 1: Male and female *Drosophila melanogaster*
Image courtesy: Floris van Breugel

I.I.I Life cycle of *Drosophila melanogaster*

The fruit fly is a holometabolous insect, that is, its life cycle has four stages – fertilized egg, larva, pupa and imago or adult (Fig. 2). A female fly will lay eggs in ripe or rotten fruits. After 24 hours, a first instar larva will hatch out from this egg. After the next 24 hours, the first molting happens, and the second instar larval stage begins. And after the next 24 hours, the second molting happens, and the third instar larval stage begins. After the next 60-72 hours, puparium forms and after the next 96 hours (4 days), the adult fly ecloses from the pupal case [45] (Fig. 2).

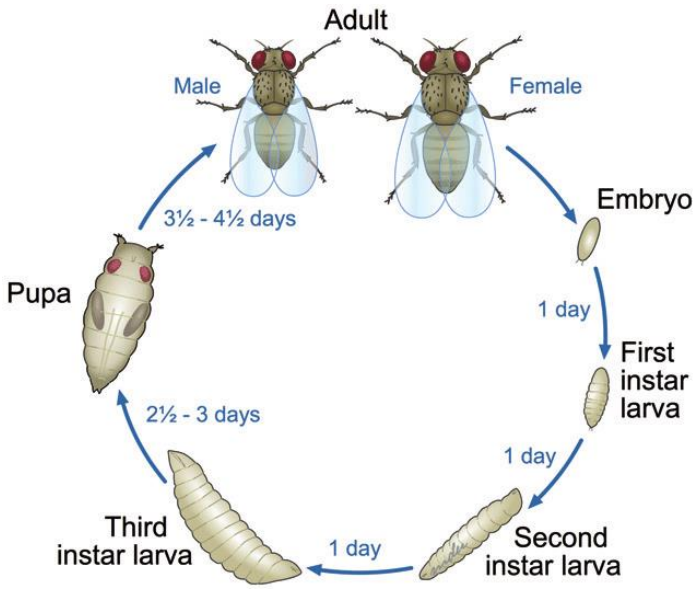


Figure 2: Life Cycle of *Drosophila melanogaster*

Schematic representation of male and female fly and different stages of the life cycle (at 25^0C) of *Drosophila melanogaster* [26].

The metabolic rate of fly changes with the surrounding temperature. 25^0C is the optimum temperature for fruit fly growth. At 18^0C the enzymatic reactions become slower, and thus

the metabolism becomes slower resulting in an increase in the duration of life cycle. At 29^0C the enzymatic reactions becomes faster and thus the metabolism become faster leading to shortening of the duration the life cycle. At temperatures lower than 16^0C the fly collapses and at temperatures higher than 31^0C the fly becomes sterile.

I.II The GAL4-UAS System

The GAL4-UAS system is used to overexpress or knockdown a gene in a tissue-specific manner. Thus, we can study the role played by a particular protein (or gene) in the tissue of our interest by observing the phenotypic changes that happened as a result of overexpression or knockdown of the gene/protein [21] [30].

The GAL4-UAS system has two parts – the *GAL4* gene, encoding the yeast transcription activator protein GAL4, and the UAS (Upstream Activation Sequence), an enhancer to which GAL4 specifically binds to activate gene transcription [21] [30].

Two fly lines are required for this system – the driver and the responder. The driver line will drive the expression of the protein of interest. This line will have the *GAL4* gene under tissue-specific promoter. So GAL4 protein will be produced in a tissue specific-manner. *GAL4* gene is not endogenous to *Drosophila*. In fact, yeast has this *GAL4* gene for their galactose metabolism. So the *GAL4* driver line flies are transgenic flies. The *GAL4* gene is cloned in a tissue-specific manner and put inside these flies by a P-element vector. In the responder line, the gene that has to be overexpressed or the RNAi construct will be under the control of UAS (Upstream Activation Sequence). The responder line will respond to the driver thereby expressing a knockdown or an overexpression construct of the desired gene.

Upon mating the driver and the responder flies, the F1 progeny obtained will have both the *GAL4* driver and the UAS with knockdown or overexpression construct downstream to it. Since *GAL4* is only expressed in cells where the driver gene is active, the responder will also only be activated in those cells. In the cells that are expressing *GAL4*, the GAL4 protein binds with the UAS region and thus the UAS is activated, the reporter gene is transcribed and this activates the transcription of the reporter or drives the expression of the RNAi (Fig. 3).

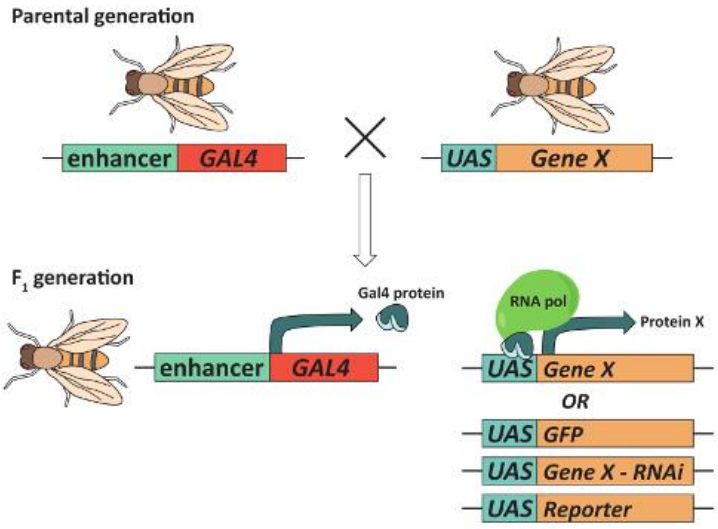


Figure 3: The GAL4-UAS System

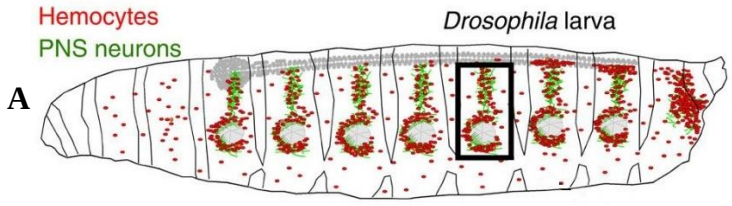
Schematic representation of the GAL4-UAS system which is used for targeted gene expression in *Drosophila melanogaster* [25].

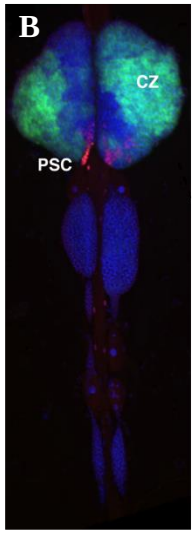
I.III *Drosophila melanogaster* as a model for studying hematopoiesis

I.III.I Lymph gland

As in vertebrates, *Drosophila* also has two phases of hematopoiesis – primitive phase and definitive phase. Primitive phase of hematopoiesis takes place in the embryonic head mesoderm [44] (Fig. 4). Definitive phase of hematopoiesis takes place at three sites – in a larval hematopoietic organ called lymph gland [3] [44], sessile patches in larva [1] [8] and hematopoietic hubs in adults [7] (Fig. 5).

Figure 4: Site of primitive phase of hematopoiesis in *Drosophila* - Embryonic head mesoderm [6].





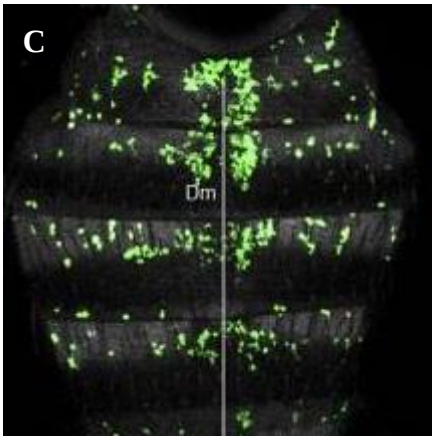


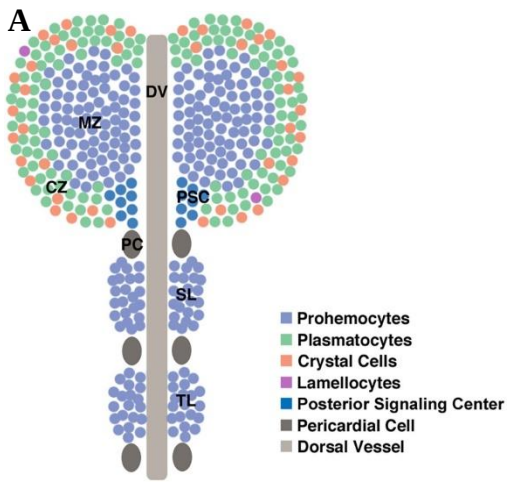
Figure 5: Sites of definitive phase of hematopoiesis in *Drosophila*

(A) Sessile patches in larva [9], **(B)** Lymph gland in larva [10], **(C)** Hematopoietic hubs in adult fly [7]

The process of hematopoiesis (the production of blood cells) in *Drosophila* larva is performed by the dorsally placed lymph gland and the sessile patches, which are seen on the lateral sides of each segment. The *Drosophila* lymph gland is used as a developmental model for studying hematopoiesis. The lymph gland is a set of 4-6 lobe pairs located at the anterior end of the dorsal vessel [62]. The primary lobe of lymph gland has three regions – the medullary zone (MZ), the cortical zone (CZ), and the niche or the posterior signaling centre (PSC) (Fig. 6B).

The medullary zone contains the blood progenitors which has stem-cell like properties. These are multipotent cells (cells that can develop into more than one cell type) that can give rise to the three kinds of hemocytes or the blood cells. The cortical zone has differentiated hemocytes. The niche consists of a small cluster of cells at the posterior tip of each of the primary lobes of the lymph gland. The niche sends certain signals to the progenitors in the MZ as well as to the differentiated hemocytes of CZ. In the cortical zone, there are three types of differentiated hemocytes [13] – the plasmatocytes (more than 90% of the total hemocyte population), the crystal cells (6-7%) [12] and the lamellocytes (0-3%) [11] (Fig. 6A). The plamatocytes removes dead cells and microbial pathogens by phagocytosis. A protein called Hemolectin (Hml) is expressed by the plasmatocytes [3] [61]. Crystal cells are hexagonal shaped non-phagocytic cells that facilitate innate immune and wound-healing responses by mediating the process of melanization. Crystal cells express the insect specific enzyme phenoloxidase (Pro-phenoloxidase A1), responsible for the initiation of the melanogenesis cascade [65]. Lamellocytes are relatively large, flat cells that encapsulate and neutralize objects that are too large to be engulfed by plasmatocytes and can only be seen upon wasp infection. In infected larvae, lamellocytes are engaged in phagocytosis of the wasp eggs [35] [36].

It is the niche or the PSC that maintains the critical balance between progenitor and differentiated cell populations of the lymph gland [8] [58]. In spite of the phylogenetic differences between mammals and invertebrates, the strategies and the mechanisms utilized are similar and are conserved between vertebrate and *Drosophila* hematopoietic niche-stem cell interactions [58].



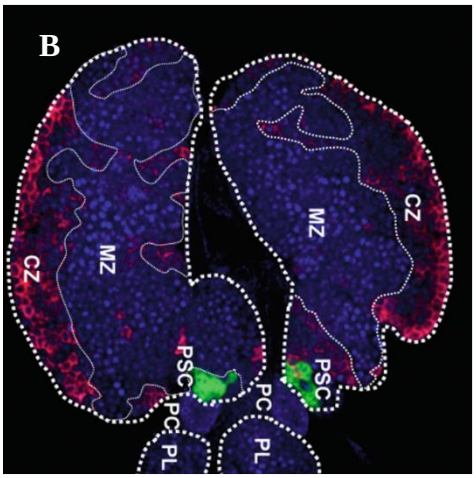


Figure 6: Drosophila lymph gland

(A) Schematic representation of *Drosophila* lymph gland showing different types of cells [27]

(B) Confocal image of *Drosophila* lymph gland showing different zones [28]. CZ – Cortical zone, MZ – Medullary zone, PSC – Posterior signaling center, SL – Secondary lobe, TL – Tertiary lobe, PL – Posterior lobe, DV - Dorsal vessel, PC – Pericardial cell

During pupation, the lymph gland ruptures and releases its cells into the circulation [43]. Thus, adult that emerges out of the pupal case will not have a blood forming organ instead they have active hematopoietic hubs throughout the dorsal part of their body which are capable of blood cell production [7]. These sites in adult fly can be considered equivalent to bone marrow [46]. The molecular signaling processes involved in hematopoiesis in fruit fly is similar to that in human body. Thus, *Drosophila melanogaster* can be used as an *in vivo* model to study the formation of blood stem cells, their migration, their roles in immunity, wound healing, ageing, etc.

I.III.II JAK-STAT Signaling Pathway

One of the major signaling pathways of *Drosophila* hematopoiesis is the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway. As mentioned earlier, the signaling molecules and transcription factors involved in hematopoiesis in both *Drosophila* and mammals are similar [2] [5] (Table 1).

Conserved Transcription Factors	
Drosophila	**Mammals**
Serpent	***GATA***
U-Shaped	***Friend of GAT (FOG)***
Lozenge	***Runx***
Cut	***Cux***
Dorsal/Cactus	***NFkB/IkB***
Collier	***Early B Factor***
Notch/ Serrate	***Notch/ Jagged***
Hop/ STAT 92E	***JAK/ STAT***
Toll	***Toll-like Receptor***
Dpp	***BMP***

Table 1: Conservation of Transcription factors during hematopoiesis between *Drosophila* and mammals (*Table courtesy: Dr. Lolitika Mandal*)

JAKs and STATs mediate intracellular signaling in response to secreted type I cytokines. In mammals, four JAKs, seven STATs, and more than thirty different cytokines and growth factors have been identified [22]. In *Drosophila* there is only one active type I cytokine receptor (Domeless, Dome), one JAK (Hopscotch/Hop), one STAT (Stat92E/Marelle), and three cytokines called Unpaired (Upd, Upd2, and Upd3) [22].

In *Drosophila*, binding of either cytokine, Unpaired (Upd1, 2, and 3), to the type I cytokine receptor Domeless activates trans-phosphorylation of the JAK kinase Hopscotch (Hop) and Dome phosphorylation, creating a docking site for STAT (Stat92E) [22]. Hop

phosphorylated STAT forms dimers which translocate into the nucleus and activate target genes [22] [23] (Fig. 7A).

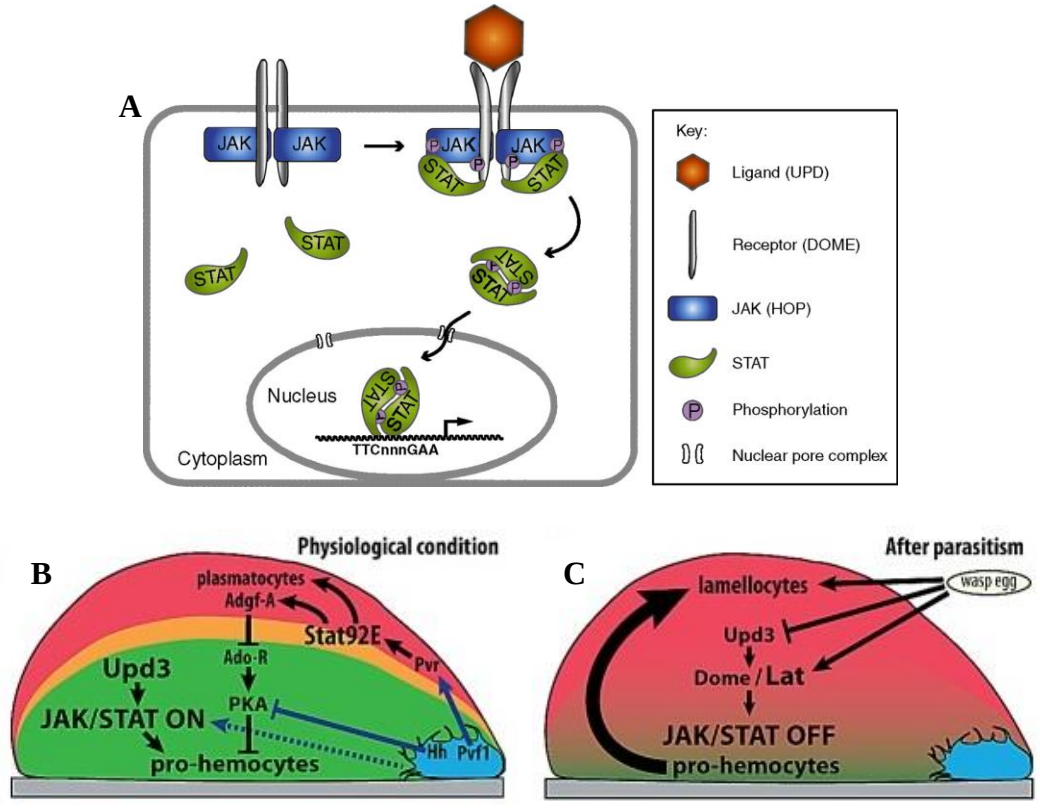


Figure 7: The JAK-STAT Signaling Pathway in *Drosophila melanogaster*

(A) Schematic representation of the JAK-STAT signaling pathway in *Drosophila* [24]

(B) Schematic diagram showing the role of Upd3 in maintaining a pool of pro-hemocytes in the larval lymph gland [22]

(C) Schematic diagram showing the decrease of *Upd3* and switching off of the JAK-STAT pathway upon wasp parasitism. This leads to the massive differentiation of lamellocytes [22]

Studies show that Upd3 has functions in hematopoiesis, immunity, and tissue homeostasis in *Drosophila* [59]. We also know that Upd2 can act as a potent activator of JAK/STAT signaling in *Drosophila* [60]. In *Drosophila* lymph gland, role of the ligands Upd2 and Upd3 is not well understood. It is known that, in the lymph gland, under normal conditions, Upd3 is required for the activation of JAK-STAT signaling in the progenitor for their maintenance [22] (Fig. 7B). PSC/niche is known to produce Pvf1 (REF). Its binding to its receptor Pvr which is located in the differentiating cells activates Stat92E. Stat92E, in turn, activates Adgf-A expression, leading to downregulation of Ado-R (Adenosine Receptor), which upregulates the PKA (protein kinase A), activity in the medullary zone. PKA activity, which contributes to the progenitor to hemocyte transition, is negatively regulated by Hh signaling from the PSC [22] [29].

When there is a wasp parasitism, *Upd3* and *Dome* simultaneously decrease and the expression of *lat* (Latran, acts as a dominant negative Dome co-receptor) increases. This leads to complete switching off of the JAK-STAT pathway in the medullary zone (Fig. 7C). This is a prerequisite to the massive differentiation of lamellocytes [22].

I.III.III Similarities between hematopoiesis in *Drosophila* and vertebrates

- The primitive and definitive stages of hematopoiesis in *Drosophila* is similar to that in vertebrates. [14]
- Both the lymph gland in *Drosophila* larvae and definitive hematopoiesis in vertebrates produce large populations of cells important for immunity. [14]
- Primitive macrophages in vertebrates are similar to *Drosophila* plasmatocytes, both functionally and developmentally. [14]
- Lymph gland development in *Drosophila* and definitive hematopoiesis that occurs in the AGM (aorta-gonad-mesonephros) region of mammals show high degree of similarity. [14]
- The signaling pathways involved in hematopoiesis in both *Drosophila* and vertebrates are similar. There are apparent similarities in developmental strategies in vertebrates and *Drosophila*. [14]

- *Drosophila* hemocyte-specific protein, Hemolectin is similar to human Von Willebrand factor. It is a blood glycoprotein involved in hemostasis (a process to prevent and stop bleeding). [15]

I.IV Objectives

Upd2 and Upd3 are ligands of the JAK-STAT signaling pathway in *Drosophila melanogaster*. JAK-STAT pathway regulates the maintenance of a pool of pro-hemocytes in the MZ and plasmatocyte differentiation [22]. The role of the ligands Upd2 and Upd3 in the JAK-STAT signaling pathway in the lymph gland is not well understood. The activation of JAK-STAT pathway by Upd3 in the medullary zone is required for the maintenance of the progenitor population [22] [56]. When there is a wasp parasitism, the reduction in Upd3 level leads to the complete switching off of the JAK-STAT pathway in the medullary zone and this results in the massive differentiation of lamellocytes. It has been reported that high Upd3 induced by ablation of Hml positive hemocytes is required for the increase in JAK/STAT signaling [57].

The role of both these ligands in the differentiating cells in the cortical zone is not known, and in addition to that, the role of Upd2 in the lymph gland is yet to be explored. Therefore, this project aims to know if these ligands are present in the cortical zone, which houses the differentiated hemocytes. If they are present in the cortical zone, we want to know if they have any role in the lymph gland hematopoiesis.

Our objectives are,

1) To knockdown *upd2* and *upd3* genes from differentiating cells of the lymph gland and to see its effects on the morphology of the lymph gland.
2) To see the effects of the knockdown of these genes in progenitor cell and niche cell maintenance and functionality.
3) To see if it has any effect in the different types of differentiated cell population of the lymph gland – plasmatocytes, crystal cells and lamellocytes.
4) To see if it has any effect on the circulating hemocyte population in the larvae.

CHAPTER II

MATERIALS AND METHODS

II.I Rearing of flies in laboratory

II.I.I Virgin Selection

All female flies used in control and genetic crosses must be virgins. The female flies are capable of mating as soon as they emerge out of the pupal case and are capable of mating with multiple males (polyandry). Once mated, they can retain viable sperm for several days. This will confuse the results of a subsequent controlled mating. Therefore, females are selected and separated from the males at the pupal stage itself. For this:

1. All brown pupae from the culture bottle were collected.
2. Ventral side of each pupa was observed under the microscope, for the presence or absence of sex comb. If sex combs is seen, it is a male pupa. Otherwise, it is a female.
3. Collected males and females in separate vials containing food.

II.I.II Setup of Genetic Cross

1. A fresh vial containing food is taken.
2. The surface of food is scratched with a needle (this creates rough edges that facilitates the female to dig in her ovipositor for laying eggs).
3. Place a paper cone made of Whatman filter paper in the surface of food.
4. Put 4-5 yeast grains on the surface of the food.
5. Etherized both the male and virgin female sets of flies required to set up the cross and transferred them into this vial. (Usually, number of males = half of the number of females) (Ether should be used in scanty amount, over-etherization kills flies)
6. Flip the flies every day in a fresh vial, the vials containing the eggs are reared to larval stages for analysis.

II.I.III Rearing of flies in cornmeal yeast fly food

Egg collection in vials

1) Flip the cross that was set up into an empty vial (without food). To flip the flies, the vial is tapped gently on the workbench. Once the flies settle down to the bottom of the vial, cotton plug can be taken off and the vial can be inverted to the empty vial. Care is to be taken that there is no gap between the two edges of the vials. Once the flies are transferred to the empty vial, a cotton plug is immediately used to close the mouth of the vial. While flipping, maximum care should be taken to prevent flies from entering or escaping the vials. The flies are kept in the empty vial for an hour.
2) After one hour, the crosses are flipped into a food vial with paper cone. The flies will lay eggs in the food, but they will not be synchronized. So it will be discarded.
3) After one hour, flies are flipped into a fresh food vial.
4) After 4 hours, the flies were transferred to another food vial. The vials in which the flies were kept for four hours will have eggs in it. If the number of eggs laid is large, excess eggs can be removed using a spatula cleaned with 70% ethanol. There shouldn't be more than 30 eggs/vial.
5) These egg vials were kept at 29^0C for further processing.

II.I.IV Rearing of flies in fruit plates

Preparation of fruit plates

1) Autoclaved Petri plates with lids are wiped with a tissue paper dipped in 70% ethanol.
2) Once wiped can be air dried or kept in hot air oven for about 10 minutes.
3) 10g agar powder and 8g sugar are taken in a beaker, and mixed well in distilled water. The volume is adjusted to 100 ml.
4) The mixture is heated in a microwave oven. As soon as it starts boiling, add fruit juice with constant stirring. Continue adding juice till the solution achieves the required consistency.
5) The solution is then ready for pouring into plates. Plate pouring should be done in a clean place, away from any chemicals.

6) The mixture solidifies within 10 minutes. Once solidifies, the plates can be covered with lids and stored at 4^0C.

Egg collection in fruit plates

1) The fruit plates stored at 4^0C are kept at room temperature for few minutes.
2) The surface of fruit plates is scratched.
3) The plate is then fitted at the bottom of the autoclaved cages (food bottles without bottom). The gap between the plate and the cage is tightly sealed with a labeling tape.
4) The crosses are flipped into these cages and properly labeled.
5) The cages with flies are kept at 25^0C for 4 hours for habituation.
6) After 4 hours, the flies are flipped into a fresh cage with a fruit plate that contains a tiny drop of yeast paste for egg collection.
7) Post 4 hours, the flies were transferred to food bottles and the plate (labeled the bottom with the genotype and date) was kept for 24 hr at 25^0C for larval collection.
8) The first batch of hatched larvae was removed and, thereafter, larvae were collected with an interval of 1hr in a food plate and reared till the desired age.
9) Each plate should have no more than 30 larvae.

II.II Experimental setup and calculation of the phenocritical time point

As control, we have used a fly line which had GAL4 gene expressed under the promoter Hemolectin which is expressed in the differentiated hemocytes only. It was already recombined with GFP. So we were successful in visualizing the differentiated cell population through fluorescence. For our experiment, we crossed *Hml-GAL4* with both *UAS-upd2RNAi* and *UAS-upd3RNAi* and analyzed the phenotype in F1 larval progeny. This line will have *upd2* and *upd3* genes specifically knocked down respectively, from the Hemolectin positive cell population (Fig. 8).

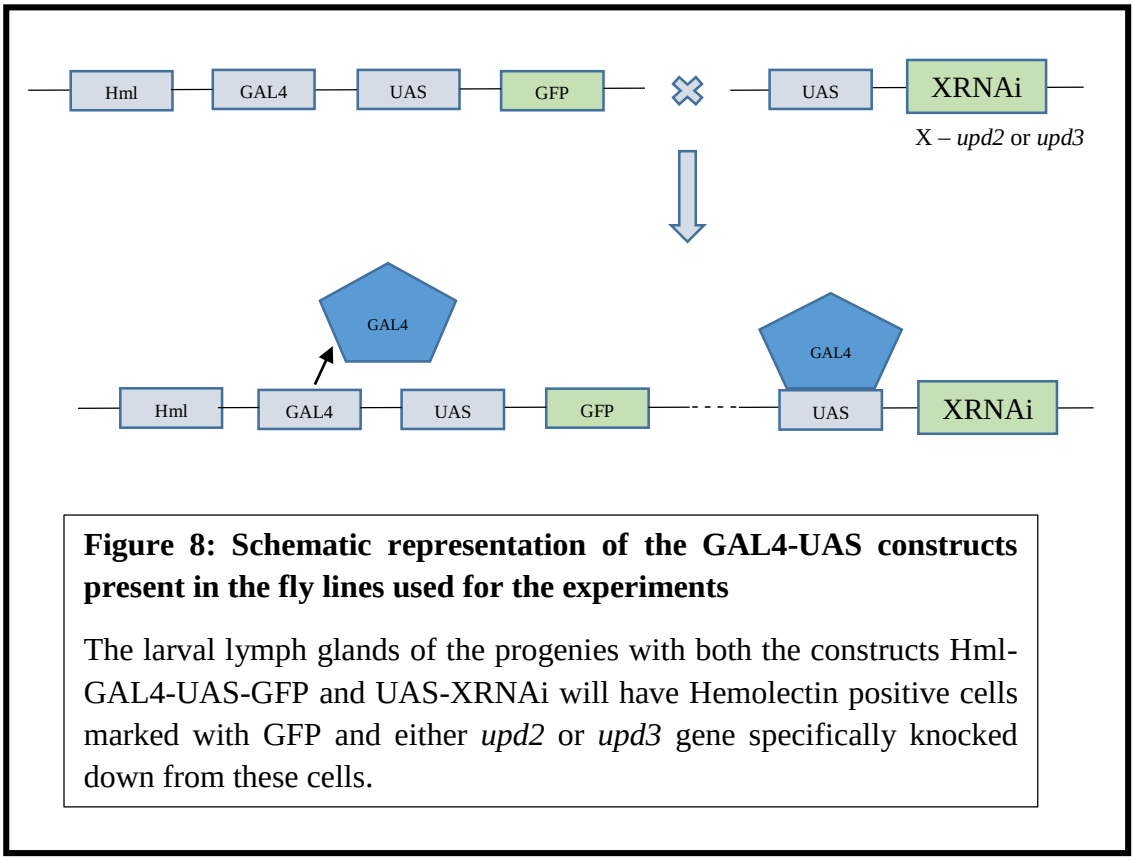


Figure 8: Schematic representation of the GAL4-UAS constructs present in the fly lines used for the experiments

The larval lymph glands of the progenies with both the constructs Hml-GAL4-UAS-GFP and UAS-XRNAi will have Hemolectin positive cells marked with GFP and either *upd2* or *upd3* gene specifically knocked down from these cells.

Lymph glands of third late instar larvae in which either *upd2* or *upd3* was down regulated were dissected out and observed. There was no differentiation defect in *upd3* knockdown lymph gland. But *upd2* knockdown lymph gland showed a differentiation defect.

To find out in which stage the phenotype sets in in the case of *upd2* knockdown from the cortical zone, we did time series experiment. We dissected both control (Oregon-R) and experimental (*upd2* knocked down from Hemolectin positive cells) larvae at different larval stages – 2nd instar (37 hrs post egg laying at 29^{0}C), 3rd early instar (55 hrs post egg laying at 29^{0}C), 3rd mid instar (74 hrs post egg laying at 29^{0}C), 3rd late instar (92 hrs post egg laying at 29^{0}C) and observed their lymph glands under fluorescence microscope. For maximum GAL4 activity, experiments were done at 29^{0}C.

There was no differentiation defect in 2nd instar lymph gland. We observed that the phenotype starts to manifest only after the second instar stage. By the 3rd mid-late stage i.e.,

around 84 hrs post egg laying at 29^0C, the phenotype completely sets in. Beyond this stage, the lymph gland peels off precociously except the niche that can still be detected. So this is the phenocritical time point for our experiment. Time series experiments were done in larvae which were grown in fruit plates.

II.III Fly stocks and genotypes

Flies were reared at 25°C, and eggs were kept at 29°C for maximum GAL4 activity.

1. Oregon-R - Wild-type laboratory stock of *Drosophila*.
2. W^{1118} – Wild-type stock of *Drosophila*, from BDSC (Bloomington *Drosophila* Stock Center, Indiana University), Stock number – 3605
3. *+ ; HmlGal4*Δ*-UASGFP ; +* - In this transgenic fly line, Hml protein will be marked with GFP. This stock was a gift from Dr. Utpal Banerjee, UCLA.
4. *+ ; + ; UAS-Upd2RNAi* – In this transgenic fly line, mRNA of *Upd2* gene is silenced. BDSC, Stock No. – 33949
5. *+ ; + ; UAS-Upd3RNAi* – In this transgenic fly line, mRNA of *Upd3* gene is silenced. BDSC, Stock No. - 32859
6. *zcl1973, HmldsRed, HmlGal4/cyo* – In this transgenic fly line, Hml protein will be marked with dsRed and Trol (Terribly Reduced Optic Lobes) protein will be marked with GFP.
7. *+ ; HmlGal4,HmldsRed/+ ; +* - In this transgenic fly line, Hml protein will be marked with dsRed. This stock was a gift from Katja Brückner, UCSF.

II.IV Larval dissection to take out the lymph gland

1) Took out the larvae using a brush and kept them briefly in water in a cavity block to remove food particles attached to their cuticles.
2) Took a few drops of 1X PBS in a glass slide and placed a larva in it with the help of a brush.
3) Using one needle grabbed the mouth hook. Placed the other needle gently at 2/3rd body length to hold the animal still. Held steady the needle near the mouth hooks while quickly pulling the rest of the body away with the second needle. Released the larva from the needles and allowed for its gut to spill out.
4) Using one needle, grasped the mouth hook again to hold the front end of the larva in place. Removed the lower 2/3rd part of the larva using the other needle.
5) Transferred the pullouts in a cavity block containing 1X PBS kept in ice.
6) Replaced this PBS with 2 ml of freshly prepared 5% Paraformaldehyde (PFA) solution. Made sure that all the pullouts are submerged in the fixative. Kept the cavity blocks in a moist container. Incubated tissues in PFA for 1.5 hours at room temperature by keeping them on the shaker.
7) After 1.5 hours, the fixative was replaced with 1 ml 1X PBS with two washes of 5 minutes each.

II.V Larval bleed assay

1) The hemocytometer and coverslip are cleaned with ethanol.
2) The larvae was cleaned using distilled water in a cavity block.
3) Five larvae were transferred to a drop of 1X PBS on a cover slip
4) 20 µl of 1X PBS was placed near this drop and a larva was transferred into it using needles.
5) It was then pricked near the mouth hook to release hemocytes. Took the next larva and repeated the same procedure.
6) The entire collection should be done within 30 seconds.
7) Post bleeding the larvae were removed from the PBS.

8) With help of a pipette, the PBS was then mixed with hemolymph properly. .
9) Put the coverslip over the hemocytometer.
10) Took 10 µl of this solution (PBS + hemolymph) and released it through the edge of the coverslip so that the solution goes inside the gap and spreads over the grids in the hemocytometer.
11) Observed the hemocytometer under fluorescence microscope. Brightfield and fluorescence was used for observing the grids and hemocytes, respectively.
12) Took images of both and merged the images to make the composite.
13) Counted the number of blood cells in each grid.

II.V.I Calculation factors

Number of hemocytes per ml of hemolymph = (Total number of hemocytes in the grids that are being considered for counting / Number of grids that are being considered) * (Dilution factor / Number of larvae bled) * (1 / Volume of liquid each grid of the hemocytometer can hold) = (x / 5) * (2 / 5) * (1 / (10^{-4} mL)) = (x * 800) ml^{-1}

We made 20 µl of 1XPBS+hemolymph solution, but took only 10 µl for counting.

∴ the dilution factor = (20 / 10) = 2

Volume of liquid each grid of the hemocytometer can hold = 100 µl = 10^{-4} ml

II.VI Immunohistochemistry

Immunostaining is a method used to detect a specific protein in a tissue by staining it with fluorophore-tagged antibodies. In this technique, a primary antibody is allowed to bind to the protein, the expression of which we want to detect. Then a fluorophore-tagged secondary antibody is allowed to bind to the constant region of this primary antibody. When observed under a fluorescence microscope, the protein with this 1^0 antibody-2^0 antibody-fluorophore complex attached to it will show fluorescence. More than one 2^0 antibody can bind with a 1^0 antibody.

II.VI.I Immunostaining Protocol for Lymph Gland

1. Larval pullouts were fixed for 1.5 hours in 5% Paraformaldehyde (PFA) in 1X PBS solution. This was followed by two washes of 1X PBS for 2 minutes each.
2. Post these brief washes, three washes of 0.3% PBT Phosphate Buffer Saline in Triton X made in 1X PBS (PBT is a detergent which is capable of removing the proteins from the cell membranes and thus allowing the entry of the reagent which is used next into the cell) for 15 minutes each was carried out. During this time removal of the excess cuticle, fat bodies, gut and other unwanted tissues from the pullouts using needles can be carried out, still keeping them in the solution.
3. Incubated the pullouts for 45 minutes in 500 µl of 10% BSA (Bovine Serum Albumen – It is a blocking agent which prevents the non-specific binding of the antibody with proteins. It non-specifically binds to all the proteins in the cell and blocks majority of the epitopes of proteins) in 0.1% PBT.
4. Filled each well of a NUNC plate with 10 µl of the primary antibody. Transferred three pullouts into each well with the help of needles and incubated for 18-20 hours at 4^0C. Kept the NUNC plate in a moist chamber.
5. The next day, transferred these pullouts into a cavity block containing 0.3% PBT with the help of needles and gave three washes of PBT for 15 minutes each.
6. Incubated the pullouts for 45 minutes in 500 µl of 10% BSA.
7. Added secondary antibody (1:500 dilution in 10% BSA) and incubated for 18-20 hours at 4^0C. Since the secondary antibody is light sensitive, this was kept in a container that will not allow light to enter.
8. Post secondary antibody incubation, three washes of PBT for 15 minutes each was done followed by one wash of PBS for 5 minutes.
9. Tissues were then incubated with 500 µl of 10% DAPI (4′,6-diamidino-2-phenylindole) nuclear dye solution in 1X PBS for 1 hr (DAPI is a fluorescent stain that binds strongly to the adenine-thymine rich regions in DNA. Thus, it marks all the nuclei in blue).
10. Removed excess DAPI with two PBS washes for 5 minutes each.
11. The lymph glands were then mounted in VECTASHIELD Mounting Medium.

II.VI.II Imaging of tissues

Images of the mounted samples were taken by using confocal microscope (Zeiss LSM 780 and Leica SP8) and processed using Fiji or ImageJ (NIH) software.

II.VI.III Antibodies used

	Antibody	Raised in	Source	Dilution used
Primary antibody	Nimrod (P1)	Mouse	Gift from Prof. Istavan Ando	1:60
	Enabled (Ena)	Mouse	DSHB, Iowa	1:50
	β-PS	Mouse	DSHB, Iowa	1:5
	Hindsight (Hnt)	Mouse	DSHB, Iowa	1:3
	Antennapedia (Antp)	Mouse	DSHB, Iowa	1:10
Secondary antibody	mouseCy3	Goat	Jackson ImmunoResearch Laboratories	1:500

II.VI.IV Stains used

DAPI (4′,6-diamidino-2-phenylindole)

DAPI is a blue fluorescent dye that binds to A-T rich region in double-stranded DNA. It can pass through an intact cell membrane. Its absorption maxima/emission maxima are 351nm/461nm.

II.VII RNA isolation and PCR

II.VII.I Protocol

1) Washed the larvae properly in distilled water to remove the food particles.
2) Dissected the larvae in SCHNIDER media and separated the lymph gland. Then transferred it into 1.5 ml microcentrifuge tube containing 1X PBS using low retention pipette tip (around 50 lymph glands).
3) Spin the microcentrifuge tube for 5 minutes, 5000 rpm (revolutions per minute) at 4^0C. Checked for the pellet.
4) Removed the PBS and added 500 µl of TRIZOL.
5) Crushed it properly for around 2-3 minutes at regular intervals keeping it in ice. Continuous crushing will cause the temperature to rise, so kept the microcentrifuge tube in ice at regular intervals.
6) Made up the volume of TRIZOL up to 1 ml.
7) Kept it at room temperature for 5 minutes.
8) Phase separation step – added 500 µl chloroform and mixed vigorously.
9) Kept it at room temperature for 5 minutes.
10) Centrifuged for 15 minutes, 12000 rcf (relative centrifugal force), at 4^0C.
11) Transferred supernatant (~500 µl) into a new microcentrifuge tube and added equal amount of 2-Propanol (~500 µl).
12) Added 1 µl of glycogen and stored it at -80^0C.
13) The next day, took out the microcentrifuge tubes kept at -80^0C and kept it in ice at room temperature for 10 minutes for the ice to thaw.
14) Spin at 12000 rcf at 4^0C for 10 minutes.
15) Removed 2-Propanol without using a pipette.
16) Added 75% pre-chilled ethanol.
17) Spin at 7500 rcf for 5 minutes at 4^0C.
18) Removed the ethanol carefully without moving the pellet.
19) Dry spin the microcentrifuge tubes at 5000 rcf for 5 minutes at 4^0C.
20) Air dried the pellet by keeping it in laminar hood.

21) Once the rest of the ethanol evaporated, added molecular grade water and kept it at -20^{0}C.
22) The next day, took out microcentrifuge tubes kept at -20^{0}C and kept it in ice at room temperature for the ice to thaw.
23) Estimated the concentration of RNA in both control and experimental samples using NanoDrop (Thermofisher).

cDNA preparation

1) Made master mix in microcentrifuge tubes.

	1 X Reaction	3 X Reaction
cDNA mix	7 µl	21 µl
Primer mix	2 µl	6 µl
Enzyme	1 µl	1 µl

2) Calculate the amount of RNA required to make final concentration to be 1 microgram. Add molecular grade water and make the volume up to 10 µl.
3) Add 10 µl of reaction mixture to this to make a total volume of 20 µl.
4) Mixed it well and ran cDNA protocol in thermocycler,
   at 42^{0}C for 45 minutes for elongation step
   at 92^{0}C for 2 minutes for termination step
5) Then stored at -20^{0}C.

qPCR

1) The next day, took out microcentrifuge tubes kept at -20^{0}C and kept it in ice at room temperature for the ice to thaw.
2) Did qPCR.
3) Ran PCR according to the following protocol,

at 95^0C for 3 minutes

Denaturation → at 95^0C for 30 seconds

Annealing → at 55^0C for 30 seconds

Extension → at 72^0C for 1 minute

} 40X

at 72^0C for 10 minutes

at 4^0C for ∞ time

4) Stored it at -20^0C.
5) The next day, took out microcentrifuge tubes kept at -20^0C and kept it in ice at room temperature for the ice to thaw.

II.VII.II Gel Electrophoresis

1) Made 1X TAE buffer.
2) Made 50 ml of 1.5% agarose gel by mixing 0.6g agarose powder in 50 ml 1X TAE buffer.
3) Levelled the gel holder in a levelling table using a spirit level and put a gel comb.
4) Heated agarose powder and buffer in an oven until the solution was clear without any particles.
5) Kept this solution outside for some time. Once when the fumes stopped coming, added 2.5 µl ethidium bromide and swirled the conical flask.
6) Poured this mixture into the gel holder. Removed bubbles to the corners (if any). Once the gel solidified, removed the comb carefully and transferred it to the gel electrophoresis apparatus along with the casting tray.
7) For loading the PCR products, took a piece of parafilm and sticked it on the table. Then took four drops of 1.66 µl loading dye in it. (Kept enough distance between the drops.)
8) Took 8.34 µl of one PCR product sample and added it to one drop of loading dye. Mixed it well and loaded it into the well in the gel using a pipette. Loaded each sample like this.

   1.66 µl 6X loading dye + 8.34 µl PCR product
9) Finally, loaded 10 µl ladder (100bp ladder of 10ng/µl concentration).

10) Closed the apparatus with the lid and connected the electrodes.
11) Once the samples ran up to the required distance, took the gel out and took the gel image.

II.VII.III Forward and reverse primers used

mRNA – *Upd2*

- Forward primer sequence → 5'- ACC TTA AAC GCC AGC CAA CA -3'
  Tm: 57.5^{0}C
  GC Content: 50.0%
- Reverse primer sequence → 5'- AGA CCG CGA TAC GGA TTG AC -3'
  Tm: 57.0^{0}C
  GC Content: 55.0%

(The housekeeping gene RP49 is the positive control used for the experiment)

II.VIII Buffers and Reagents

1X PBS (Phosphate Buffer Saline) – A buffer

1X PBS is isotonic and non-toxic to cells. It prevents exosmosis of cells.

To make 1 L of 1X PBS,

1) Weighed,
   NaCl = 8 gm
   KCl = 0.2 gm
   Na_2HPO_4= 1.44 gm
   KH_2PO_4 = 0.24 gm
2) Added these into a 1 L glass bottle and added 1000 ml of distilled water into it and shook well till the components dissolved.
3) Autoclaved the bottle by keeping the lid loosely closed at 121^{0}C, 100 kPa for 30 minutes using saturated steam.

4) After autoclaving, checked pH. pH should be in the range of 7.2-7.4.

0.3% PBTX (Triton-X 100 made in PBS) – A detergent

Triton-X 100 inserts a detergent monomer into the lipid membrane ultimately permeabilizing the membrane for the antibody to bind to its specific antigen.

1) Took 40 ml 1X PBS in a falcon tube.
2) Took a 200 µl microtip and cut its tip. Using this tip, pipetted out 120 µl Triton X 100 and added it into the falcon tube.
3) Shook the mixture well and kept it in a roller shaker for proper mixing.

5% Paraformaldehyde (PFA) solution – A fixative

PFA is a crosslinking fixative that acts by creating covalent chemical bonds between proteins in tissue. It has long-term storage and good tissue penetration properties.

1) Weighed 0.1 gm of PFA powder in 2 ml microcentrifuge tube.
2) Added 1 ml of 1X PBS into it and mixed it well.
3) Sealed the lid of the tube with parafilm.
4) The tube is kept in a hot water bath which was set up at 65^0C for the powder to dissolve.
5) Checked after every 20 minutes interval to see if the powder has dissolved or not.
6) Once the powder dissolved, the solution was made up to 2 ml by adding 1X PBS.
7) The tubes can be stored at -20^0C.

5% BSA (Bovine Serum Albumen) – A blocking agent

Before using specific antibodies to detect antigens by immunohistochemistry, all potential non-specific binding sites in the tissue sample must be blocked by treating the tissues with a blocking agent to prevent nonspecific antibody binding.

1) Weighed 0.1 gm of crystalline BSA powder in 2 ml microcentrifuge tube.
2) Added 2 ml of 0.3% PBTX into it and dissolved the crystals using a vortex.
3) Then kept it in rotator shaker for proper mixing.

CHAPTER III

RESULTS AND DISCUSSION

III.I Loss of Upd3 specifically from the differentiated cell population does not affect differentiation

To begin with, we have dissected out lymph glands from third instar larvae in which either *upd2* or *upd3* was knocked down specifically from the differentiated cell population (Hemolectin positive, by *hmlΔ-Gal4*). It was observed that loss of Upd3 from the cortical zone cell population does not affect differentiation (Fig. 9).

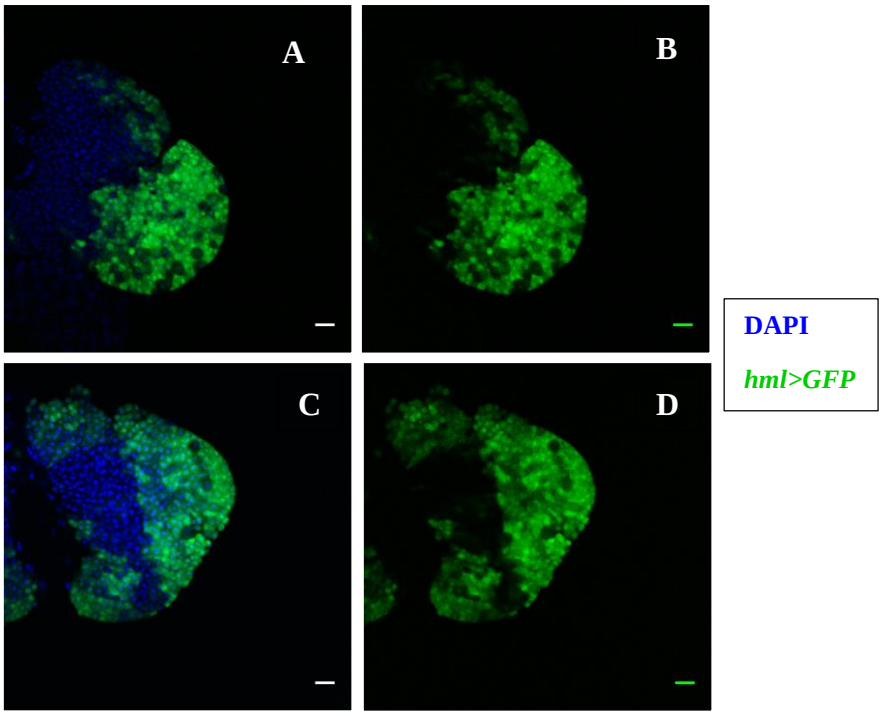


Figure 9: Confocal images of wild type and *upd3* knockdown lymph glands

A, B – Primary lobe of lymph gland of wild type larva

Genotype - +/+ *; hml>gfp/+ ;* +/+

C, D – Primary lobe of lymph gland of *upd3* knocked down larva

Genotype - +/+ *; hml>gfp/+ ; upd3RNAi/+*

GFP marks the differentiated cells.

III.II Upd2 loss specifically from the differentiated cell population results in a differentiation defect

It was observed that lymph glands from third instar larvae in which *upd2* was knocked down specifically from their differentiated cell population shows a differentiation defect. It is the cortical zone that houses the differentiated cell population. The control lymph glands had intact cortical and medullary zones (Fig. 10A and B). But the cortical zones of *upd2* knocked down lymph glands were enlarged and the medullary zones were highly reduced (Fig. 10C and D). There was not an intact boundary between the two zones. Since both the control and experimental lymph glands were from larvae of the same age, we can also conclude from the images that there is precocious differentiation in *upd2* knockdown lymph gland compared to the control lymph glands.

To further confirm this result, we analysed lymph glands in which Hemolectin positive cells were marked with an independent marker *hmldsRed*, an enhancer trap construct [50]. DsRed is a basic red fluorescent protein derived from *Discosoma sp.* [47], in the above genotype will mark the differentiated hemocytes. The lymph glands of larvae of this fly line also showed the same phenotype (Fig. 11). Thus, it was confirmed that the loss of *upd2* specifically from the differentiated cell population results in the differentiation defect.

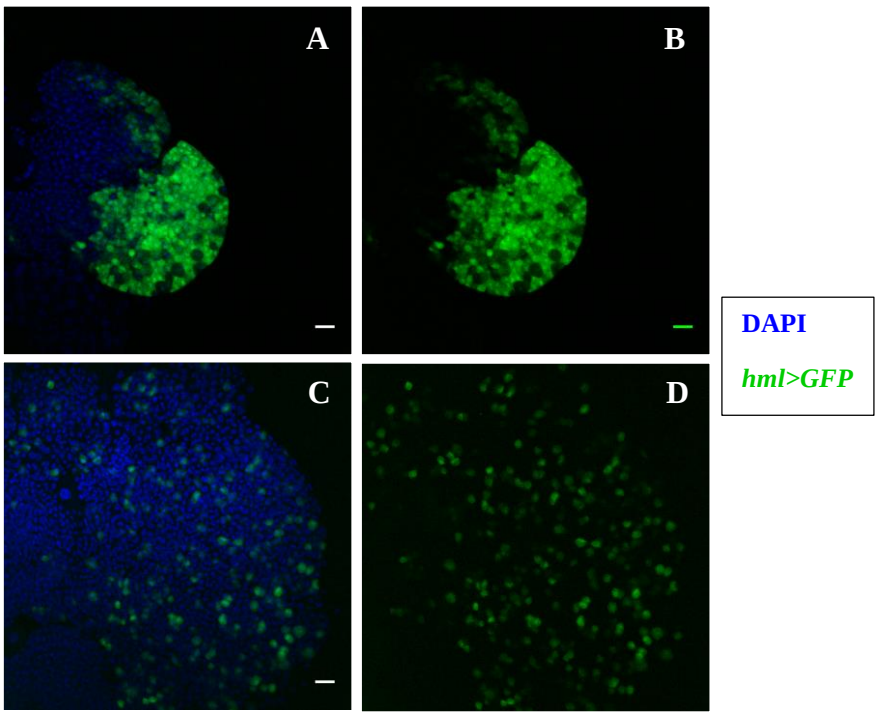


Figure 10: Control and *upd2* knockdown lymph glands – differentiated cells marked with GFP

A, B – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

C, D – *upd2* knockdown lymph gland (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

GFP marks the differentiated cells.

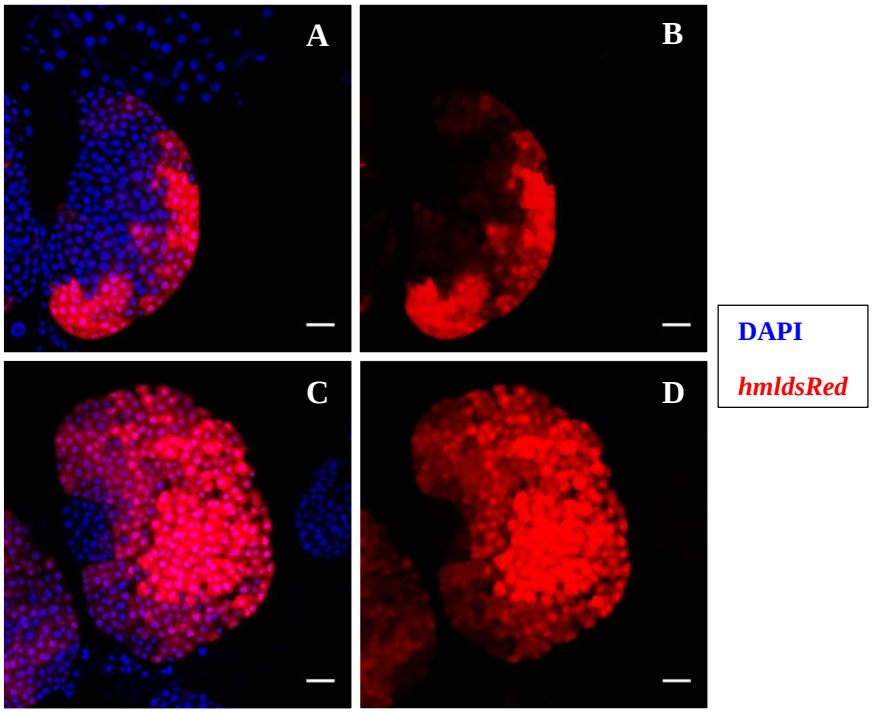


Figure 11: Control and *upd2* knockdown lymph glands – differentiated cells marked with DsRed

A, B - (+/+ *; hmlGal4, hmldsred/+ ; +/+*)

C, D - (+/+ *; hmlGal4, hmldsred/+ ; upd2RNAi/+*).

Age of the lymph glands - 84 hours post egg laying (3rd mid-late stage)

DsRed marks the differentiated cells.

III.III The phenocritical time point of the differentiation defect due to *upd2* knockdown is 84 hours post egg laying or third mid-late stage

To find at which stage the phenotype due to *upd2* knockdown specifically from the differentiated cell population sets in, we have done a time series experiment. For this, we have dissected larvae at different larval stages – 2nd instar (37 hrs post egg laying at 29^{0}C), 3rd early instar (55 hrs post egg laying at 29^{0}C), 3rd mid instar (74 hrs post egg laying at 29^{0}C), 3rd late instar (92 hrs post egg laying at 29^{0}C) – and observed their lymph glands.

In both the control and experimental lymph glands, differentiation just starts to manifest by the 2nd late-3rd early instar stage (Fig. 12). The differentiation defect in *upd2* knockdown lymph gland is evident by the 3rd early-mid stage (Fig. 13). And, the phenotype is full blown by the 3rd mid-late stage, i.e., 84 hours post egg laying at 29^{0}C (Fig. 14). After this stage, the lymph gland ruptures and releases its cells into the circulation.

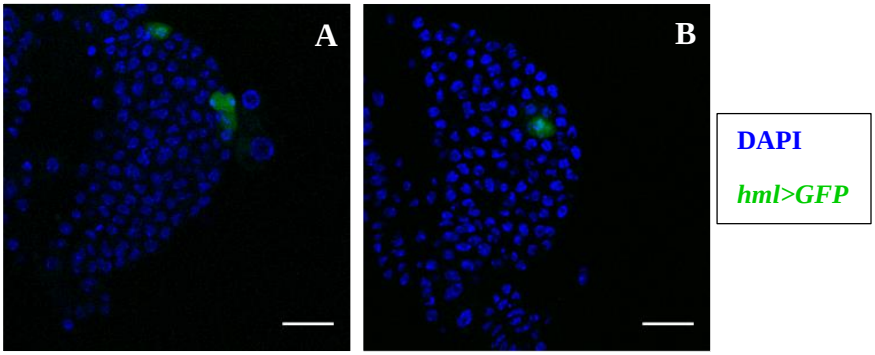


Figure 12: Control and *upd2* knockdown lymph glands of 2nd late-3rd early instar larval stage

A – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

B – *upd2* knocked down lymph gland (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

GFP marks the differentiated cells.

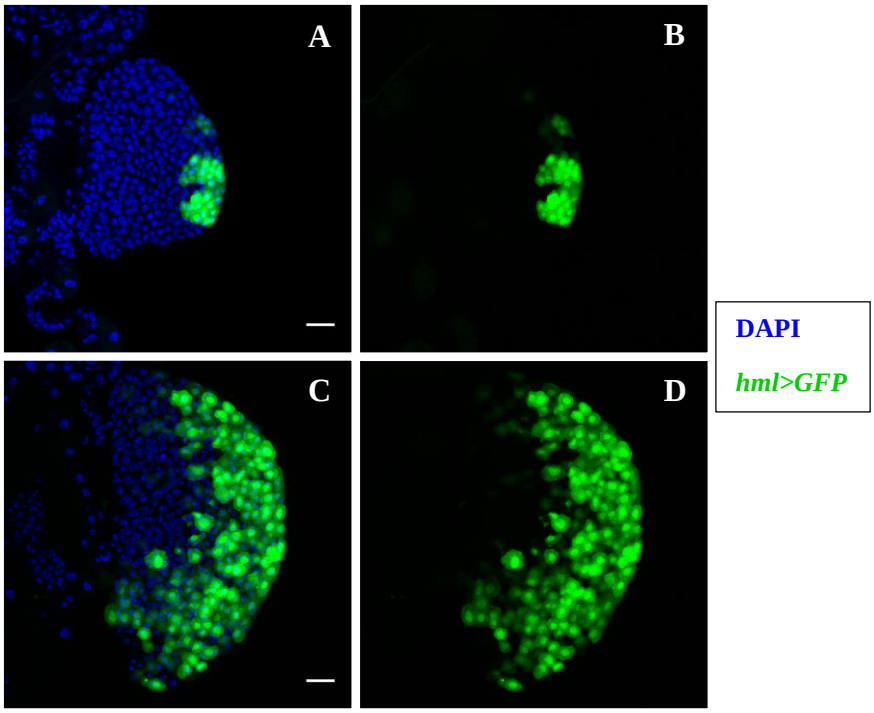


Figure 13: Control and *upd2* knockdown lymph glands of 3rd early-mid instar larval stage

A, B – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

C, D – *upd2* knocked down lymph gland (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

GFP marks the differentiated cells.

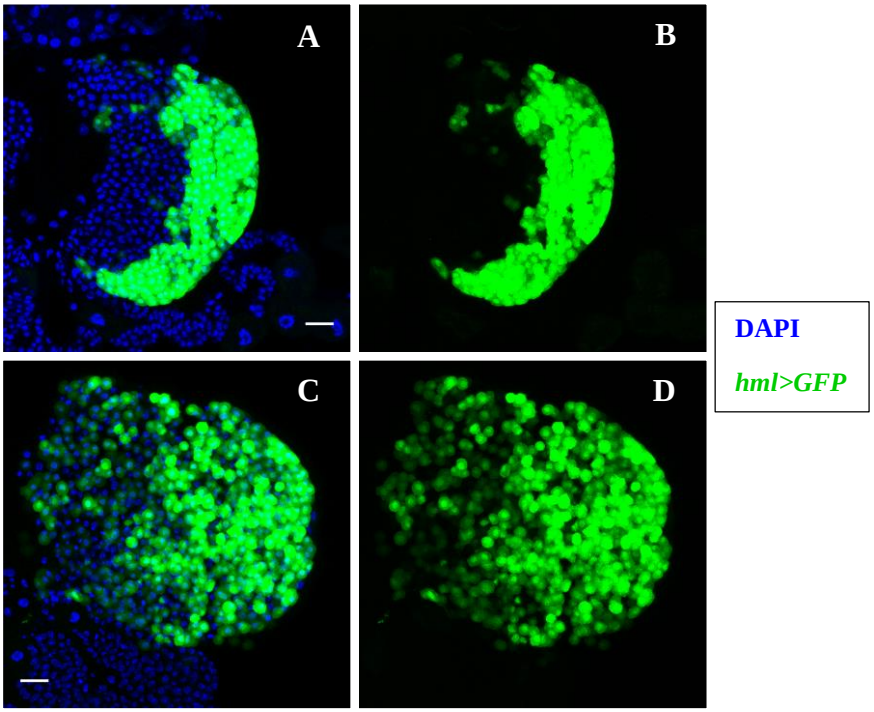


Figure 14: Control and *upd2* knockdown lymph glands of 3rd mid-late (84 hrs post egg laying at 29^{0}C) instar larval stage

A, B – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

C, D – *upd2* knocked down lymph gland (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

In *upd2* knockdown lymph gland, we can see a reduction in progenitor population and an increase in differentiated cell population as compared to the control.

GFP marks the differentiated cells.

III.IV *Upd2* transcripts are present in the *Drosophila* larval lymph gland

Next, we wanted to know if *upd2* is present in the lymph gland. Unfortunately, there are no antibodies available to check the expression of Upd2 gene. Also, there are no GAL4-UAS constructs available to mark the *upd2* expressing cells. So in order to see if *upd2* is present in the lymph gland, we have checked the *upd2* transcript level. For this, we have isolated the RNA from the lymph gland, prepared its cDNA, amplified it, ran the gel and analyzed the gel image. We have observed that *upd2* is present in the control lymph gland and absent in the *upd2* knockdown lymph gland (Fig. 15). This shows that *upd2* knockdown specifically from the differentiated cell population is responsible for the differentiation defect. This experiment also validates the efficiency of the *upd2RNAi* construct in the fly line, which we are using for the experiments.

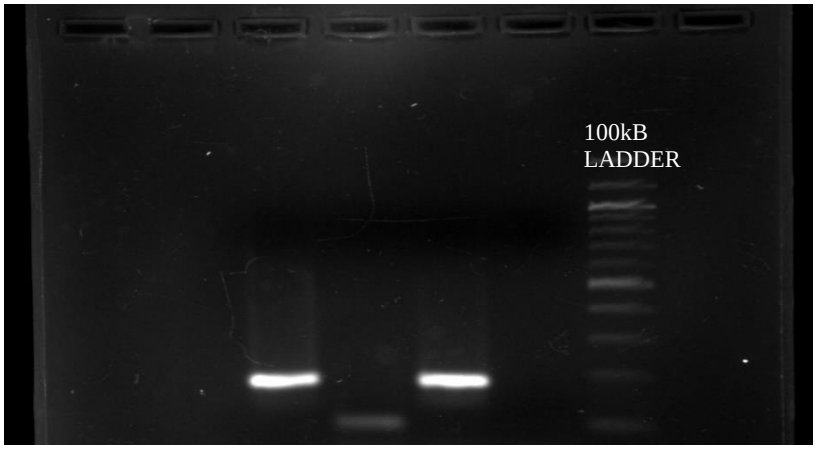


Figure 15: Upd2 transcript is present in the *Drosophila* larval lymph gland

Gel image showing the presence of *Upd2* transcripts in wild type lymph gland and absence of *Upd2* in *upd2* knocked down lymph gland. The constitutive gene RP49 is the positive control used for the experiment.

III.V Progenitor population is highly compromised in Upd2 loss condition

Plasmatocytes function by removing microorganisms and apoptotic cells by phagocytosis. Plasmatocytes are also known to promote the humoral immune response by secreting cytokine-like proteins and antimicrobial peptides [4] [41]. Thus they play an important role in immunity and development. On the surface of both circulating and sessile plasmatocytes, a protein called Nimrod C1 (NimC1) is present. It is also known as the P1 protein and is involved in the phagocytosis of bacteria [16]. It has been reported that NimC1-deficient hemocytes show adhesion defects *in* vitro [48]. We wanted to see the number of cells that are expressing P1 both in the control lymph gland and in the *upd2* knockdown lymph gland.

The areas of the primary lobe that do not show the expression of Hemolectin is the medullary zone which houses the blood cell progenitors and the niche. A proper medullary zone is nearly absent in the *upd2* knockdown lymph gland whereas we can distinctly see a medullary zone and a cortical zone in the control lymph gland. As compared to the control (Fig. 16A, B), the differentiated cell population in the experimental lymph gland showed strong expression of P1 (Fig. 16C, D), i.e. the number of plasmatocytes in the experimental lymph gland is more than that in a control lymph gland from larvae of the same age. Also, cortical zone of the experimental lymph gland is enlarged and bulged as evident from the confocal images. The images also reveal the early maturation of the progenitor population.

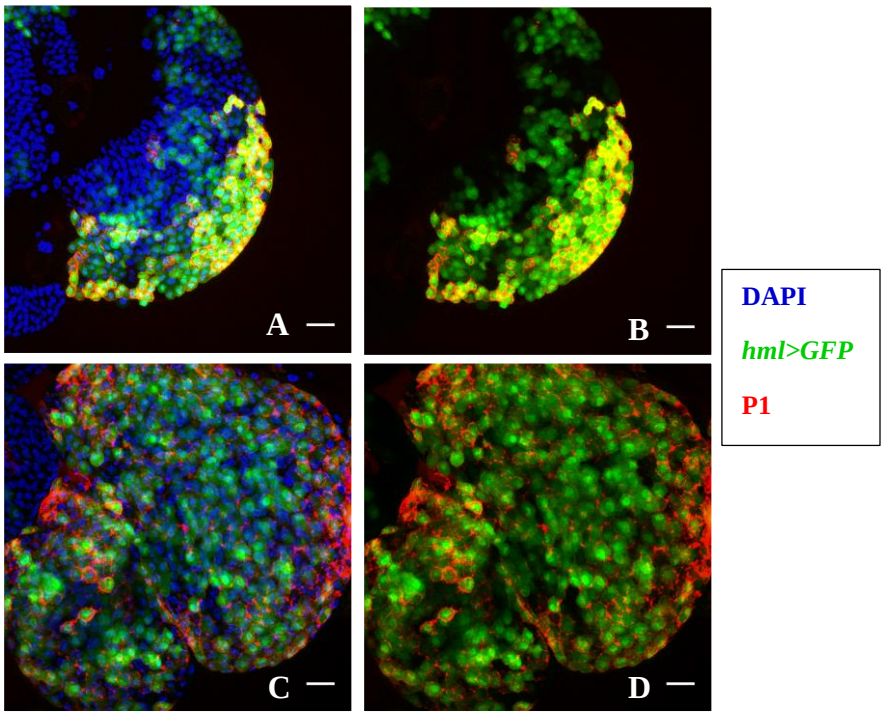


Figure 16: Control and experimental lymph glands labeled with P1 (Nimrod)

A, B – Wild type lymph gland (+/+ *; hml>gfp/+ ;* +/+)

C, D – *upd2* knockdown lymph gland (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

In *upd2* knockdown lymph gland, we can see a reduction in progenitor population and an increase in differentiated cell population as compared to the control.

GFP marks the differentiated cells and P1 marks the mature plasmatocytes [43].

III.VI Ectopic differentiation, early peeling off and reduction in progenitor number are observed in Upd2 loss condition

To confirm the findings stated in result number III.V we have used another antibody, Ena or Enabled. Enabled is a cytoskeletal regulator that facilitates continued actin polymerization at the barbed ends of actin filaments [17] [42]. Ena is expressed only in the mature plasmatocytes. Mature plasmatocytes have filopodial extensions that help in their migration. Filopodia are used for attachment of the cell to various surfaces and also for its movement through the hemolymph. So if the plasmatocytes of the lymph gland start expressing Ena, it suggests that they are ready to migrate and join the circulation.

The confocal images of the control and experimental lymph gland show that the expression of Ena among the plasmatocyte population of the *upd2* knockdown lymph gland (Fig. 17C, D) is stronger than that in control (Fig. 17A, B). This says that these plasmatocytes will soon peel off and leave the lymph gland. The basal level of Ena expression in the control lymph gland denotes that its plasmatocytes will peel off at a later stage in development. In the experimental lymph gland, reduced medullary zone and enriched Ena in the cortical zone cells suggests ectopic differentiation of the progenitors and precocious maturation of the hemocytes.

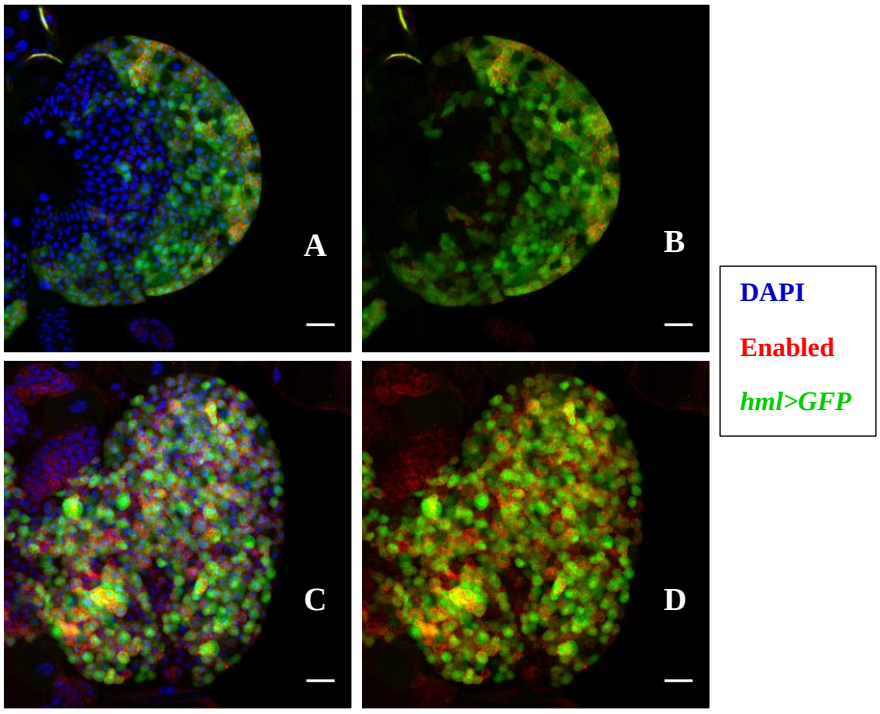


Figure 17: Control and experimental lymph glands labeled with Enabled (Ena)

A, B – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

C, D – *upd2* knockdown lymph gland (+/+ *; hml>gfp/+ ; UAS upd2 RNAi/+*)

In the experimental lymph gland, we can see a reduction in the progenitor population and an increase in the differentiated cell population as compared to the control.

GFP marks the differentiated cell population and Ena marks the mature plasmatocytes that show phagocytic activity.

III.VII Medullary zone is highly compromised and lamellocytes are absent in Upd2 loss condition

The status of progenitor population was next checked by β-PS, a marker for integrin, a cell adhesion receptor [49]. In order to visualize the progenitor cells that are tightly packed cells, we have marked it with β-PS. Its expression is strong in tightly packed cell population and lamellocytes in the cortical zone [34].

As mentioned earlier, lamellocytes are formed only when there is a wasp parasitism. There is a regulatory network where co-activation of Toll/NF-κB and EGFR signaling by ROS levels in the niche controls lymph gland hematopoiesis under parasitism [51] [52] [53].

We can see a strong expression of β-PS in the medullary zone of the control lymph gland (Fig. 18A, B) whereas no proper medullary zone can be seen in *upd2* knockdown lymph gland (Fig. C, D). The cortical zone of the control lymph gland shows faded expression of β-PS as it consists of loosely packed differentiated cells, which are ready to leave the lymph gland. Interestingly, we can see the expression of β-PS in the differentiated hemocytes of the experimental lymph gland, which is not the case in control lymph gland. This is because, when the plasmatocytes mature and get ready to leave the lymph gland they starts expressing β-PS. Lamellocytes are large cells and have elevated β-PS expression. In the experimental lymph gland, as there are no such strong expressions, we can conclude that lamellocytes are absent in it. But further confirmations with independent markers for lamellocytes are required.

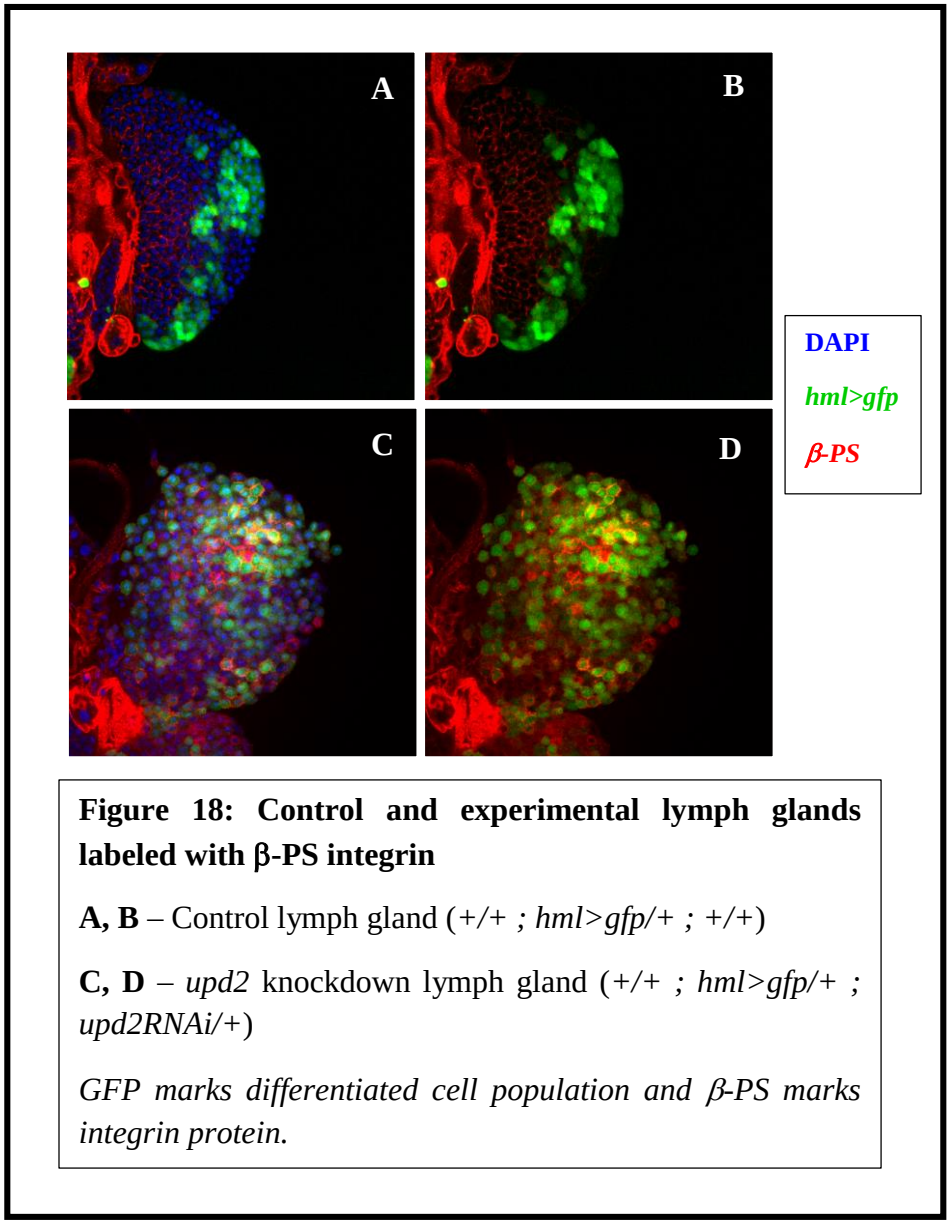


Figure 18: Control and experimental lymph glands labeled with β-PS integrin

A, B – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

C, D – *upd2* knockdown lymph gland (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

GFP marks differentiated cell population and β-PS marks integrin protein.

III.VIII ECM component is disturbed and loosely packed in Upd2 loss condition

The heparin sulfate proteoglycan Trol (Terribly Reduced Optic Lobes) is the *Drosophila melanogaster* homolog of the vertebrate protein Perlecan. Perlecans are the secreted components of the extracellular matrix [40]. Trol is expressed as part of the extracellular matrix (ECM) in the cells of the lymph gland [37]. In a normal lymph gland, the ECM forms

thin basement membranes around individual or small groups of blood progenitors [54]. The medullary zone has many closely spaced membranes, whereas the cortical zone has fewer basement membranes [54]. Therefore, Trol is enriched in the progenitor population than that in the differentiated cell population. We can see the medullary zone clearly in the control lymph gland (visualized by *zcl1973*: enhancer trap line for Trol, Fig. 19A, B, C), whereas there is no defined medullary zone in *upd2* knockdown lymph gland (Fig. 19D, E, F). The experimental lymph gland is filled with differentiated cells and its cell integrity and zonation is disturbed [54].

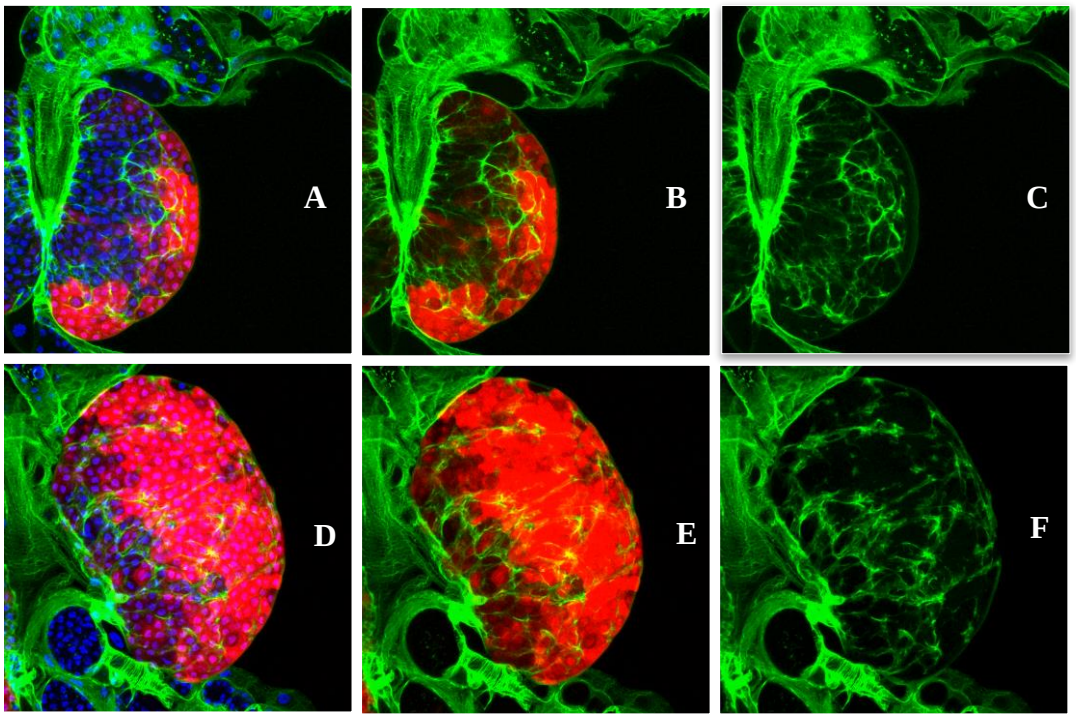


Figure 19: Upd2 loss condition affects the ECM of the lymph gland

A, B, C – Control lymph gland (*zcl1973/+; hmldsred, hmlGal4/+ ; +/+*)

D, E, F – Experimental lymph gland (*zcl1973/+; hmldsred, hmlGal4/+ ; +/upd2RNAi*)

DsRed marks differentiated cell population and zcl1973 marks the ECM component Trol.

DAPI

hmldsRed

zcl1973

III.IX Crystal cells are absent in Upd2 loss condition

Crystal cells are insect specific and involved in wound healing [33]. Crystal cells perform this by the process of melanization, the darkening and hardening of tissue due to the local deposition of melanin. Phenoloxidase (PO) enzymes mediate the oxidation of phenols into quinones that then polymerize into melanin [3] [39]. In order to visualize crystal cells through fluorescence, they were marked with the crystal cell specific antibody, Hindsight or Hnt [38]. As compared to a control lymph gland (Fig. 20A, B), crystal cells are completely absent in *upd2* knockdown lymph gland (Fig. 20C, D). In a normal lymph gland, a progenitor cell either differentiates into a plasmatocyte or a crystal cell (or during wasp parasitism a progenitor can also differentiate into a lamellocyte). But in *upd2* loss condition the differentiation is completely biased towards plasmatocytes.

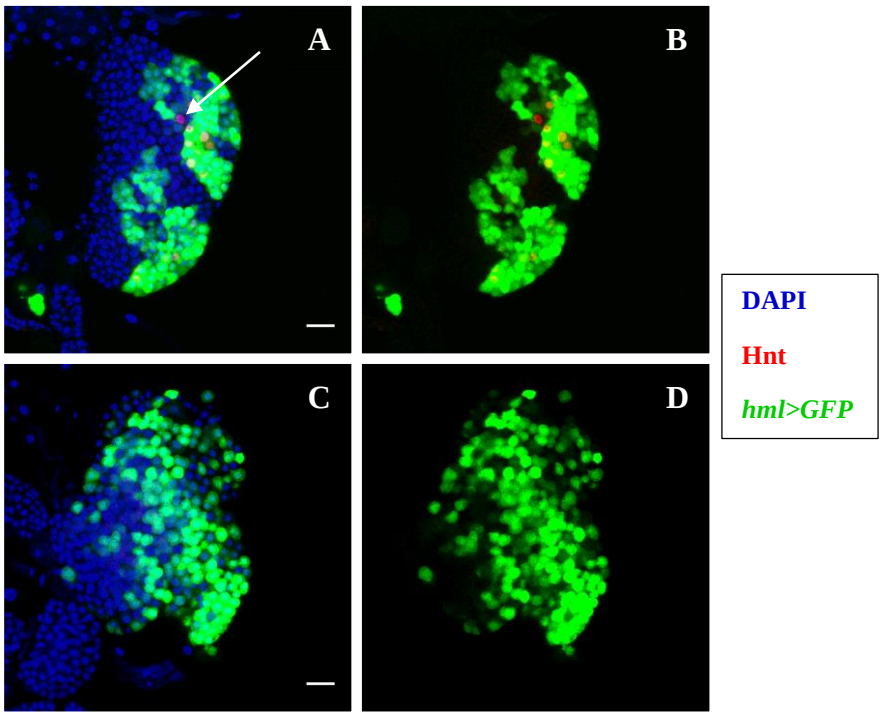


Figure 20: Control and experimental lymph glands labeled with Hindsight (Hnt)

A, B – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

C, D – *upd2* knockdown lymph gland (+/+ *; hml>gfp/+ ; UAS upd2 RNAi/*+)

GFP marks the differentiated cells and Hnt marks the crystal cells.

The white arrow marks the crystal cells.

III.X Niche cell number is higher in Upd2 loss condition compared to the wild type

The fine balance between the number of progenitors in the medullary zone and differentiating cells in the cortical zone is maintained by the niche [19] [31]. We next wanted to assay the niche cells. Using an antibody Antp or Antennapedia which is a bonafide marker for the niche cells [31], we labeled the control as well as the *upd2* knockdown lymph glands. Antennapedia is a homeobox gene which provides cells with specific positional identities on the anterior-posterior axis and is shown to be essential for specifying the hematopoietic niche. It was observed that the number of niche cells in *upd2* knockdown lymph gland (Fig. 21B) is more than that in the control lymph gland (Fig. 21A). As evident from the quantitation (Graph 1), this increase in the niche cell number in the experimental lymph gland is more than double the number of niche cells of the control lymph gland.

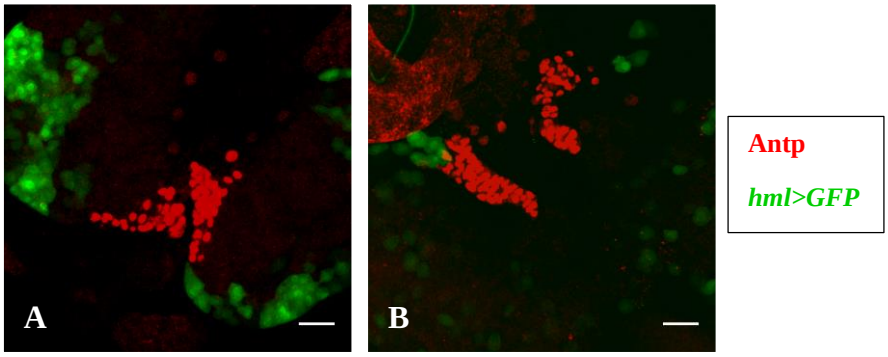
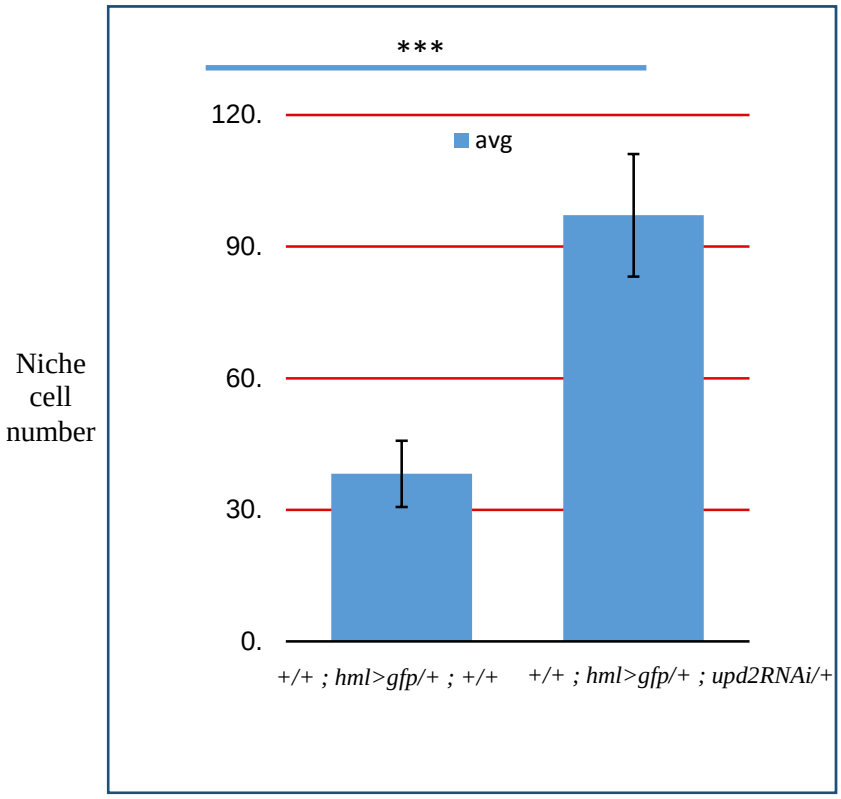


Figure 21: Control and *upd2* knockdown lymph glands labeled with Antennapedia (Antp) antibody

A – Control lymph gland (+/+ *; hml>gfp/+ ;* +/+)

B – *upd2* knocked down lymph gland (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

GFP marks differentiated cells and Antp (red) marks the cells of the PSC.



Graph 1: Graphical representation of niche cell count in control and *upd2* knockdown lymph glands

n = 10

It is known that in the niche cells, Col (Collier) promotes the expression of Dlp (Dally like protein), which in turn promotes the expression of Dpp (Decapentaplegic), a bone morphogenetic protein (BMP). Dpp then acts through two ways to maintain the niche cell number – either it directly represses dMyc or it represses Wg (Wingless), Wingless can also activate dMyc [18] (Fig. 22). This regulation described in literature defines an autonomous regulation on niche cell number. Our current findings describes a non-autonomous control on niche proliferation through *upd2* from the cortical zone (Fig. 22). It will be interesting to further look at the status of the autonomous circuit in a condition in which *upd2* is lost from the Hemolectin positive cells.

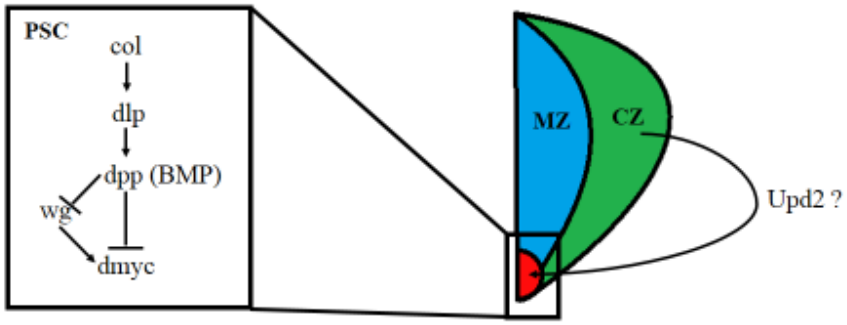


Figure 22: Upd2 might have a non-autonomous role in maintaining the niche cell number

Schematic representation showing the known autonomous circuit and the proposed non-autonomous role of Upd2 in maintaining the niche cell number. This conclusion is based on the results we have got.

III.XI Circulating hemocytes in *upd2* knocked down larvae are less than that in wild type larvae

We have observed the early differentiation of progenitors and early maturation and peeling off of the lymph gland in the *upd2* knocked down *Drosophila* larvae. Now we wanted to see if the differentiated cells are present in the hemolymph of these larvae. For this, we did a bleed assay to check if any circulating Hemolectin positive cells are present in the larvae [32] (Fig. 23). The result we got was confusing - the bleed count of *upd2* knockdown larvae was just about 1/3rd of that of the control lymph gland (Graph 2). But the larval lymph gland of the *upd2* knockdown fly line was enlarged. If this lymph gland has more number of differentiated cells than the control lymph gland and the bleed count of these larvae are less compared to the control, then where do these cells go? The quantitation of differentiated cells of these lymph glands is necessary for the further analysis of this. Subsequent experiments aimed in cell death assay should be done to evaluate if the hemocytes are dying in the *upd2* knockdown larvae.

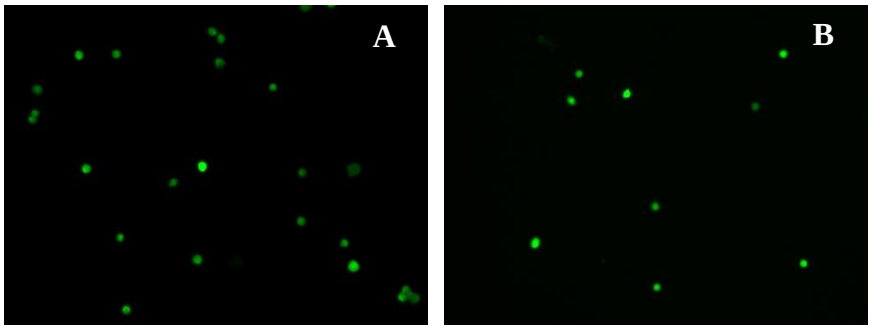


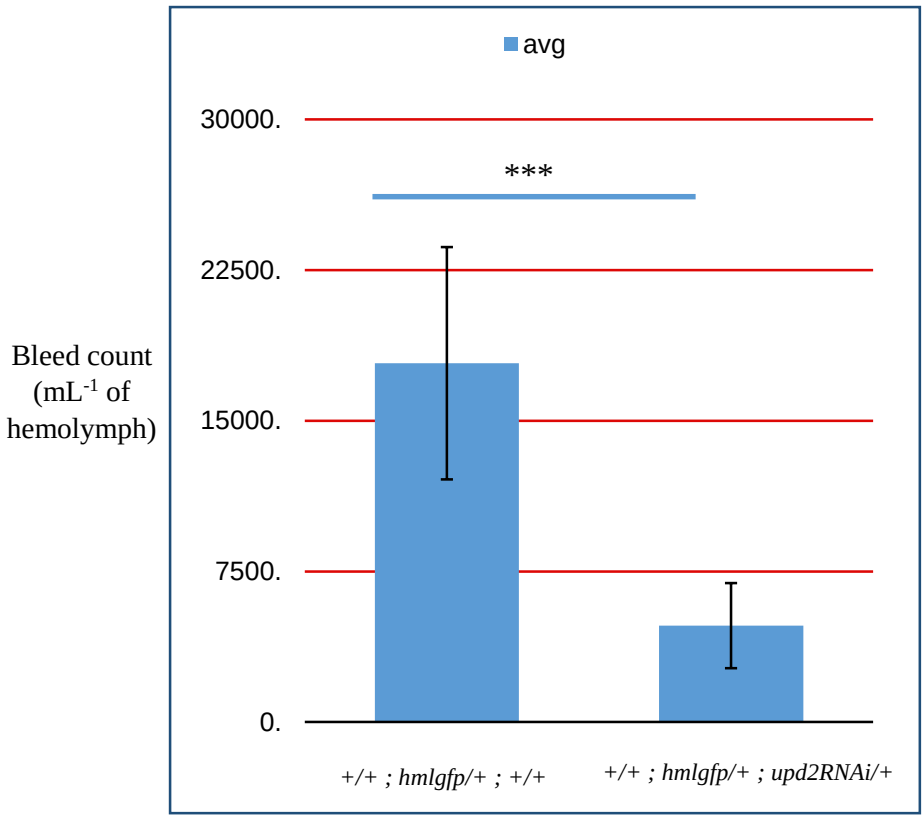
Figure 23: Fluorescence image of differentiated cells in control and *upd2* knockdown larval hemolymph

A – Control larval hemolymph (+/+ *; hml>gfp/+ ;* +/+)

B – *upd2* knocked down larval hemolymph (+/+ *; hml>gfp/+ ; upd2RNAi/+*)

Age of larvae – 84 hours post egg laying at 29^{0}C

GFP (green) marks the differentiated cells.



Graph 2: Graphical representation of Hemolectin positive cell count in control and *upd2* knockdown larval hemolymph

n = 3

CHAPTER IV

FUTURE DIRECTIONS AND SIGNIFICANCE

The JAK-STAT pathway and its components are evolutionarily conserved from small fruit flies to the most complex vertebrate life forms. JAK-STAT signaling pathway is involved in processes such as immunity, cell division, cell death, and tumor formation. *Drosophila melanogaster* is used as a model system in studying the signaling pathways involved in hematopoiesis – especially the JAK-STAT pathway. In animals, this pathway promotes blood cell division and its differentiation. In *Drosophila*, some mutations in JAK genes causes overproliferation of blood cells and this results in leukemia [66]. Cytokines are the ligands that activate JAK-STAT signaling pathway. In *Drosophila*, the role of the ligand upd3 in JAK-STAT signaling in hematopoiesis is known. Our results show that upd2 is also involved in maintaining the homeostasis of hematopoiesis in *Drosophila* – suggesting that upd2 might also be acting as a ligand in the activation of JAK-STAT signaling in *Drosophila* larval lymph gland. The complete knockdown of *upd2* from the lymph gland resulted in early differentiation of the progenitors, increase in niche cell number, absence of crystal cells, and disturbed ECM component in the lymph gland.

In humans, the mutations in different components of the JAK-STAT signaling pathway leads to various immunological defects. The mutations in STAT3 results in defective responses to the cytokines IL-17 (Interleukin-17) and IL-22 by various blood cells [67]. The defective response by the cells to IL-17 causes Hyperimmunoglobulin E syndrome (HIES, or Job's syndrome). The inability of the blood cells to respond to IL-22, which is important for epithelial barrier function, leads to impaired barrier function in HIES [67]. Gain-of-function STAT1 mutations causes increase in IFN-γ signaling. This inhibits IL-17 transcription and this results in susceptibility to fungal infections like Chronic mucocutaneous candidiasis (CMC) [67]. This also increases the risk for autoimmune disease. The mutations in TYK2 (Tyrosine kinase 2) leads to different clinical disorders [67]. This might be because of its association with the cytoplasmic domains of receptors for several cytokines including Type I interferon (IFN), IL-6, and IL-12. Deficiency of STAT2 affects Type 1 IFN signaling and this increases the susceptibility to viral infections [67]. INFs are required for the destruction of transformed malignant cells [67].

Activating mutations of *JAK2* causes myeloproliferative diseases (MPD) in humans - like, polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF)

[67]. Mutations in *JAK1* have been associated with the development of acute myeloid leukemia (AML) and mutations in *JAK3* have been associated with juvenile myelomonocytic leukemia [67]. Constitutively activated STAT3 leads to the formation of tumors and hematological cancers [67]. Constitutively active tyrosine kinase causes Chronic myeloid leukemia (CML) [67]. Polymorphisms of *STAT1* increases the risk of malignancy, of STAT3 are associated with Crohn's disease and psoriasis, of *STAT4* are associated with rheumatoid arthritis and systemic lupus erythematosus (SLE), and of *STAT6* are associated with asthma and allergy [67].

In *Drosophila*, a mutant Jak kinase can cause leukemia like abnormalities. A dominant temperature sensitive mutation in *Drosophila* Jak kinase, *hopscotch*$^{Tumorous\text{-}lethal}$ (*hop*$^{Tum\text{-}l}$) causes overproliferation of plasmatocytes and the formation of melanotic tumors [62] [63] [64] [65]. In *hop*$^{Tum\text{-}l}$ flies, the number of plasmatocytes and lamellocytes in circulation is dramatically increased. *T42* is another dominant mutation in *hop* and it is functionally identical to Tum-l [66]. Both these hop mutations result in a hyperactive kinase which hyperphosphorylates Stat92E. This leads to increased association of Stat92E with DNA [64]. Sustained JAK/STAT signaling leads to the loss of several distinct tumor suppressors and a mutation of the tumor suppressor results in ectopic expression of Upd [64]. The link between JAK-STAT activity and oncogenesis is clear in both flies and mammals. But the targets that mediate this transformation has to be identified. The JAK-STAT pathway in *Drosophila* is less complex and some genetic relationships in tumorigenesis in both flies and mammals are conserved. So research in *Drosophila* will help in understanding tumor formation.

All these studies suggests that defects and dysfunctions in the cytokines in JAK-STAT signaling pathway can lead to various immunological and hematological diseases. The mutations in JAKs and STATs can cause defective responses of blood cells to cytokines. Our study also shows that the absence of upd2 in plasmatocytes in *Drosophila* larvae causes hematological defects. This will give new information about the unknown components of the signaling pathways acting in the *Drosophila* immune system. Further studies on this may be helpful in understanding better the processes behind blood disorders in *Drosophila* as well as in humans.

CHAPTER V

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