TITLE OF THE THESIS

Methotrexate (MTX) restores hematopoietic equilibrium by affecting the nutritional signaling in the fat body via Toll/NF-κB Pathway

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY (Ph.D.)

By

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DECLARATION

I, Dushyant Kumar Gautam, hereby declare that this thesis entitled "Methotrexate (MTX) restores hematopoietic equilibrium by affecting the nutritional signaling in the fat body via Toll/NF-κB Pathway" submitted by me under the guidance of Dr. Indira Paddibhatla and supervision of Prof. Ravi Kumar Gutti is a bonafide work.

I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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Further, the student has the following publications(s) before submission of the thesis/monograph for adjudication and has produced evidence for the same in the form of reprint in the relevant area of his research.

1. Methotrexate negatively acts on inflammatory responses triggered in Drosophila larva with hyperactive JAK/STAT pathway. Yadav RK, **Gautam DK**, Muj C, Gajula Balija MB, Paddibhatla I. *Dev Comp Immunol*. 2021 Oct; 123:104161. doi: 10.1016/j.dci.2021.104161.

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BC803	Scientific Writing and Research Proposal	4.00	Passed

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DEDICATION

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LIST OF ABBREVIATIONS

A

Aorta- Gonad-Mesonephros (AGM)

Anti-Microbial Peptides (AMPs)

Acute Lymphoblastic Leukemia (ALL)

B

Bone Marrow (BM)

Bone Morphogenetic Protein (BMP)

Black cells (Bc)

Beta-Human Chorion Gonadotropin (β-HCG)

C

Carnegie Stage (CS)

Collagen (Cg)

Chemokine (C-X-C motif) ligand 12 (CXCL12)

Common Lymphoid Progenitors (CLPs)

Common Myeloid Progenitors (CMPs)

Cluster of Differentiation (CD)

Cortical Zone (CZ)

Croquemort (Crq)

D

Dihydro Folate Reductase (DHFR)

Developmental Studies Hybridoma Bank (DSHB)

Drosophila Fork Head Box Transcription factors (dFOXO)

Drosophila Insulin Like Peptides (dILPS)

E

Erythromyeloid Progenitors (EMPs)

Extra-cellular Matrix (ECM)

F

Fetal Liver (FL)

Food and Drug Administration (FDA)

G

Gestational Carcinoma (GC)

Gain of Function (GOF)

H

Hematopoietic Stem Cell (HSC)

Hematopoietic Stem cell Progenitors (HSPCs)

Hematopoietic Precursors (HSs)

Human Epithelial Cells (HeLa)

Hemolectin (Hml)

I

Insulin Like Peptides (ILPs)

Immunoglobulins (Ig)

Insulin Receptor (InR)

Insulin Receptor Substrate (IRS)

L

Lymph Gland (LG)

Long term (LT)

Loss of Function (LOF)

Leptopilina boulardi- 17 (*Lb-17*)

M

Methotrexate (MTX)

Medullary Zone (MZ)

N

Nimrod C1 (NIMC1)

P

Paraaortic Splanchnopleures (P-Sp)

Posterior Signaling Centre (PSC)

Peroxidasin (Pxn)

Prophenoloxidase (PPO)

Phosphate Buffer Saline (PBS)

Paraformaldehyde (PFA)

R

Rheumatoid Arthritis (RA)

S

Stem Cell Factor (SCF)

Short time (ST)

Serpin (Srp)

Spätzle Processing Enzyme (SPE)

Spätzle (Spz)

U

Ubiquitin Conjugating 9 (Ubc9)

Y

Yolk Sac (YS)

1.1 Hematopoiesis: An overview

The creation of a diverse set of blood cells with a variety of functions and morphologies is known as hematopoiesis [1, 2]. Published and prevailing research extensively contributed to our understanding about the blood cell development and hematopoietic signaling in different model organisms [3]. The heterogeneous blood cell populations that are present within the various anatomical regions that display chronological changes were used in both *in vivo* and *in vitro* research to draw these observations and conclusions. HSCs, blood cell progenitors, blood cell precursors, red blood cells, white blood cells, and platelets are among the variety of blood cells found in the bone marrow [4]. Each of these hematopoietic progenitors, precursors and matured cells have a significant and unique role in maintaining hematopoietic homeostasis and blood cell development.

1.1.1 Multipotent Progenitors (MPPs), or multipotent progenitors are stem cells that possess intrinsic self-renewal capacity to evolve into any type of functional cell in an organism [5]. Their differentiation into distinct types of stem cells, such as totipotent stem cells, pluripotent stem cells, multipotent stem cells, and unipotent stem cells, aids in their classification [6]. The cells generated from the zygote up to the 8-cell division stage are regarded as totipotent after successive fertilization events (Figure 1.1). These cells are dynamic in generating every call type of the body as well as an extraembryonic tissue (placenta), which leads to the formation of an entire organism [7, 8]. They maintain their pool up until the embryonic stage not only by differentiation but also by creating their clones [9]. Totipotent cells have the potency to develop into pluripotent cells, and pro-generate all the three germ layers, [10] although these pluripotent cells do not exhibit the capacity to create entire organisms [10]. Compared to pluripotent stem cells, multipotent stem cells can differentiate into more than one type of cell, but they are unable to produce the three germ layers. Skin and muscle stem cells, which can only create cell types from one particular lineage are examples of unipotent stem cells that have the least differentiation potential [11]. These oligopotent MPPs display myeloid and erythroid cell. Lineages along with a build-up of uncommitted cells. MPPs develop into CMPs and CLPS (Figure 1), which further differentiate into cells with the cellular markers of the lymphoid and myeloid lineages, respectively [12].

Long-term (LT) and short-term (ST) HSCs are two subpopulations of the HSC pool that are distinguished by their capacity for self-renewal. In comparison to ST-HSCs, LT-HSCs are known to have a longer self-renewal capacity and the potential to re-establish the hematopoietic lineage [13]. Sub-structuring of the hematopoietic lineage requires ST-HSC self-renewal for around 6 weeks. MPPs belong to closest progenitor of ST-HSCI, but their lack of capacity for self-renewal allows them to sustain the hematopoietic system temporarily [14].

1.1.2 Common Myeloid Progenitors (CMPs) are the source of all myeloid cells, including erythrocytes, megakaryocytes, platelets, macrophages, dendritic cells, and all granulocytes (Figure 1.1) [15]. These are bone marrow derived terminally differentiated cells exhibiting unipotent features. During embryonic development, erythrocytes aid in the process of tissue oxygenation [16]. Platelets are released by megakaryocytes into the circulatory system [17]. While recent studies have highlighted their significance in immunological response as well [18, 19]. these platelets function in wound healing and homeostasis. Macrophages are designed to play a specific role in phagocytosis of foreign elements. Myeloid and lymphoid lineages are combined with dendritic cells since both can produce it [20, 21] and these dendritic cells are regarded as a specific type of antigen-presenting cell [22]. Granulocytes aid in the eradication of the infection [23, 24]. They amplify in number and get released in the blood stream at the sites of infection to detoxify the pathogen causing serious infection, thereby imparts healing.

1.1.3 Common Lymphoid Progenitors (CLPs) originates from the lymphatic tissue and generates multiple types of lymphocytes. CLPs are constrained from a variety of lineage commitments, equivalent to CMPs. They produce unipotent immune cells such as NK-cell, B-cell and T-cell [25]. NK cells and are regarded as unique cells that counteracts with the infectious pathogens. They are also responsible for inhibiting tumor growth [26, 27]. CLPs develop from the bone marrow and propagate T-cells whose maturation occurs in the thymus. T cells are one of the major types of lymphocytes that depict a central role in the adaptive immune response [28, 29]. Specific functions delivered by T cells is immune mediated cell death via CD8+ and CD4+ subtypes of T cells [30]. B cells are antibody producing white blood cells and one of the broad classes of adaptive humoral immune system. They are known to generate antigen specific

immunoglobulins (Ig), recognized as antibodies that abrogate the invasive effects of microbial pathogens. They aid in T cell immune response and other immune related homeostasis. Dendritic cells are named as accessory cells functioning in immune surveillance by antigen presentation generated by endogenous and exogenous process (Figure 1.1). Understanding the mechanisms of hematopoiesis is crucial for developing therapies in case of blood disorders such as leukemia and anaemia. Research on hematopoiesis is continued intending to understand blood cell production is regulated. Hematopoiesis research is in the evolutionary phase to acquire latest knowledge on the regulation of hemocyte development.

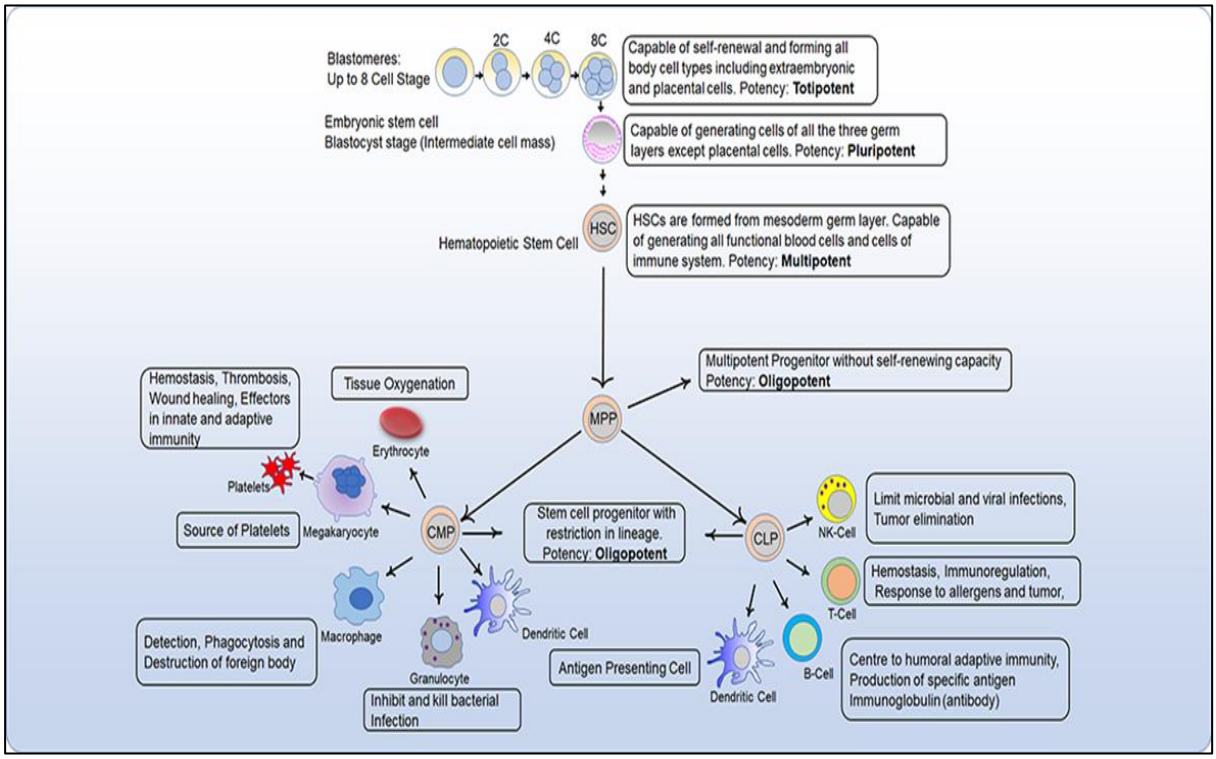


Figure 1.1 Hematopoietic lineage with its downstream hierarchy, *Gautam et al., 2023*: Adapted from: Modern trends in Molecular Biology, Book: *Hematopoiesis, Cellular, Molecular and Genomic Perspectives.* Chapter 2 (Accepted for publication) CRC Press Taylor & Francis Group

1.2 Hallmarks of HSCs

The standard illustration of multipotent stem cells is HSCs. They generate the myeloid and lymphoid lineage progenitors, which are the two distinct hematopoietic branches. These lineages are what continue to differentiate to produce all the many types of blood cells [31]. HSCs exhibit a number of distinctive characteristics that set them apart from other hematopoietic progenitors [32]. Self-renewal, differentiation, migration, quiescence, and apoptosis are characteristics of HSCs (Figure 1.2). One of the key traits of HSCs is thought to be **self-renewal**. This capability limits HSCs' ability to differentiate while assisting them in making identical duplicates of themselves with comparable potential [33]. Through the development of HSCs, mature blood cells are continuously replenished [34]. Asymmetric and symmetric division, which allows for a balance between their capacity for self-renewal and differentiation, determines the fate of HSCs [35]. A sophisticated network of cytokines and growth factors powers HSC differentiation. These variables are controlled by external stimulation or according to the requirement of the cells [36]. Blood cell differentiation is thought to be a dynamic, irreversible process that, once initiated, cannot be undone to return blood cells to their progenitor condition [37]. Therefore, in order to generate hierarchical progenitors and differentiate into mature cells, HSCs require a set of specialised signals. The growth of hematopoietic tissues depends on the HSCs' migration. The HSCs' regeneration capacity is conferred by this characteristic [38]. At various developmental stages, HSC migration is seen to both embryonic and extraembryonic regions, including the foetal liver, spleen, and ultimately bone marrow. HSCs are kept in these anatomical places until they are required to develop into adult cells [39]**.** Circulating HSCs "home" in the designated "niche" region of the bone marrow microenvironment [40, 41]. A well-controlled and coordinated mechanism is required for the HSC to travel from the bone marrow to anatomical areas via the circulating blood. The generation of hematopoietic cells is a lifelong process that the HSCs support with little stem cell fatigue [12]. HSCs strategically pursue this during the latent period. The intrinsic and extrinsic signals created in response to stress and blood loss greatly influence the fate of HSCs' quiescence [42].

By preventing the HSC from entering the cell cycle, these signals aid in preserving HSC quiescence. Proliferating cells are more susceptible to genetic alterations than quiescent cells are, and when they reach the Hayflick limit, they undergo apoptosis to prevent faulty hematopoiesis. As a result, blood cell quiescence is a crucial characteristic that guards HSCs against the emergence of haematological cancers. **Apoptosis** is a biological process that requires energy and self-destructs in order to maintain the hemostasis mechanism throughout development, ageing, and immunological responses [43]. The delicate balance between self-renewal and apoptosis is directly related to the size of the hematopoietic stem cell bulk [12]. Hematopoietic deficiencies and cancers are often the outcome of any imbalance between apoptosis and self-renewal of HSCs [44]. Similar to other cells, the number of HSCs is controlled by a process known as apoptosis.

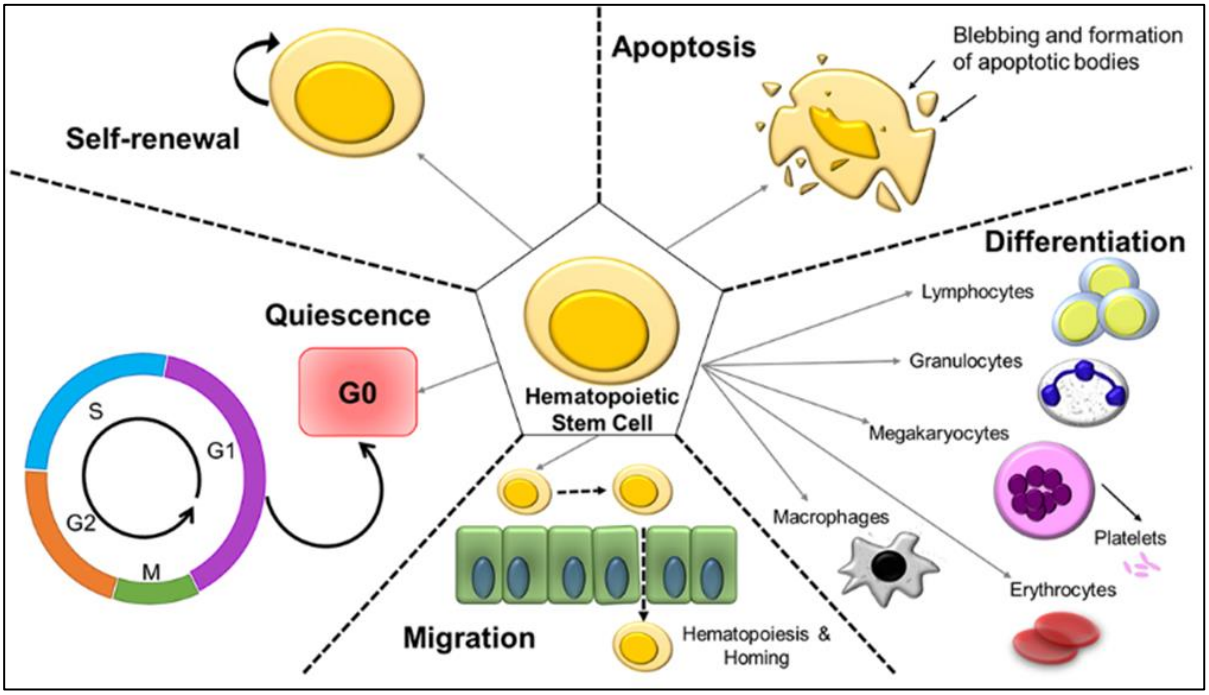


Figure 1.2 Hallmarks of HSCs, *Gautam et al., 2023*

Adapted from**:** Modern trends in Molecular Biology, Book: *Hematopoiesis, Cellular, Molecular and Genomic Perspectives.* Chapter 2 (Accepted for publication) CRC Press Taylor & Francis Group

1.3 Hematopoietic Theories

There exist three theories behind development of human blood cells. To date, the most accepted theory for hematopoiesis is Unitary or Monophyletic theory proposed by A. A. Maximow [45, 46]. Monophyletic theory highlights presence of a common pluripotent stem cell for all the mature blood cells [47]. However, dualistic and trialistic/ polyphyletic theories did not gain significant scientific attention. Dualistic theory proposed by Ehrlich, Schridde, and Naegeli states that all the blood cells are generated from two sources of hematopoietic progenitor cells i.e., the myeloid and the lymphoid. In contrast Aschoff gave the polyphyletic theory suggesting that separate stem cells exist for the formation of each type of blood cells.

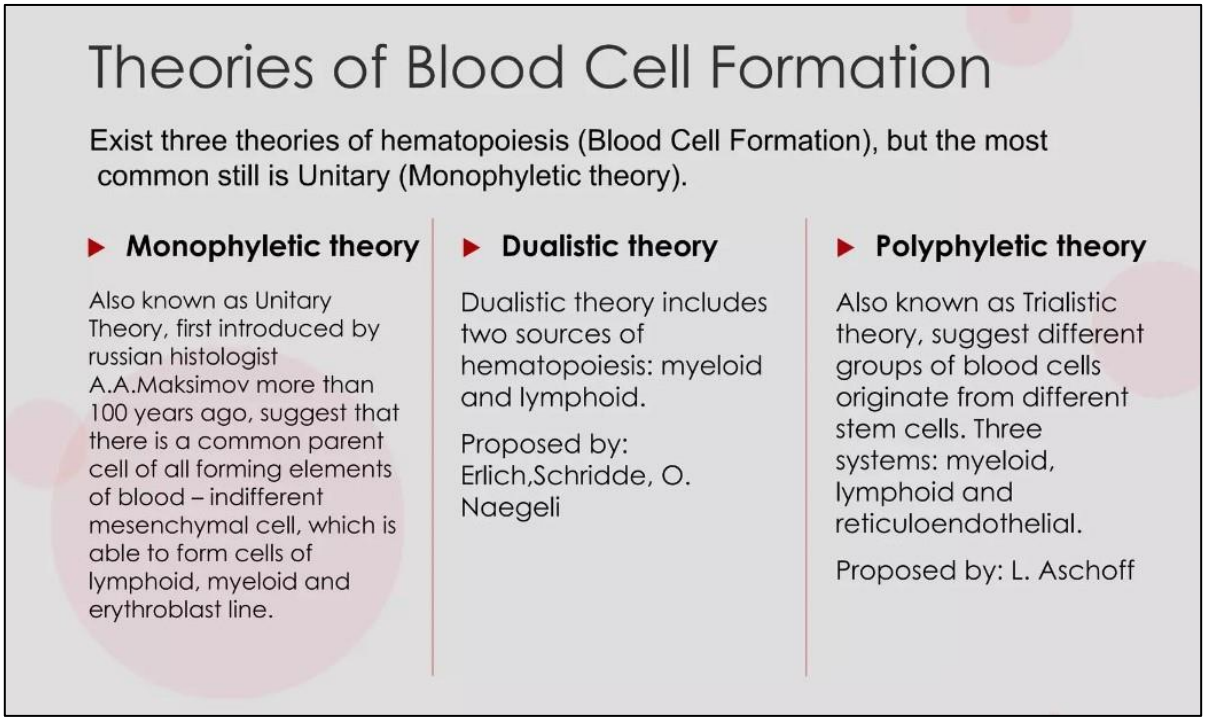


Figure 1.3 Theories of Blood Cell Formation

Adapted from SlideShare: *https://www.slideshare.net/TereshchenkoO/monophyletic-theory-of-erythropoiesis-stem-cells*

1.4 Hematopoietic niche, signal transduction in HSCs and hematopoietic regulation

The hematopoietic niche refers to the specialized microenvironment within the bone marrow where hematopoietic stem cells (HSCs) reside and undergo the process of hematopoiesis, i.e., the formation of blood cells [48]. The niche provides crucial support and regulation for HSCs, ensuring their self-renewal, differentiation, and maintenance [49]. Cell-cell signaling is an essential event during these cellular processes. This enables cells to perceive and transmit signals for development, growth and immunity. Stem cells' self-renewal, maturation, differentiation and regulation properties are fundamentally dependent on their niche microenvironment [50, 51]. Crosstalk between the HSCs and the hematopoietic niche establishes homeostasis and steady state of HSCs [52]. Hematopoietic equilibrium is observed by asymmetrical cell division through which one daughter cell maintains the stem cell identity and the other becomes differentiated [53-55]. This asymmetry can be achieved by environmental signals, which create pro-differentiation or pro-renewal environments. HSC's self-renewal and differentiation properties are critically regulated by cellular signals produced from HSCs' niche as well as systemic environment [12, 50]. CXCL12 [chemokine (C-X-C motif) ligand 12], a chemokine and Stem Cell Factor (SCF), a cytokine are factors which are implicated in HSC maintenance and retention. Recent studies also shed light on different effects of CXCL12 on blood cells. CXCL12 expressing perivascular region stromal cells are required for HSCs and this was elucidated by deleting CXCL12 function from specific subset of cells [56].

Similarly, function of SCF in perivascular cells and endothelial cells is also crucial for HSC maintenance [57]. Past few research publications have established that murine bone marrow niche's cellular constituents interact with the HSCs leading to its self-renewal or differentiation [58, 59]. The HSCs localization is often witnessed adjacent to the blood vessels (but not limited to) hinting that HSCs could be maintained in a perivascular niche by endothelial cells [60, 61]. Recent study about transcriptomic profiling of murine bone marrow niche at a single cell resolution has precisely determined the understanding of HSCs self-renewal and differentiation [62]. Studies on functional significance of E-selectin, a niche factor expressed in the murine

bone marrow established its requirement in quiescence, proliferation and self-renewal of HSCs [63]. Loss of vascular endothelial expressed Notch ligand (Dll4) in bone marrow narrowed down the HSPCs differentiation towards myeloid commitment [62]. Bone marrow osteoblast cells interact with HSCs and in contrast mice deficient in bone morphogenetic protein (BMP) signaling show increased number of osteoblast and HSCs [59, 64]. Adult bone marrow provides sufficient nourishment through the niche predominantly from two cellular compartments i.e., the osteoblast and the vascular endothelial cells [59-61, 64-67].

1.5 Ontogeny of blood in mammals (Vertebrates)

During ontogeny, in most of the vertebrates, the prime sites for blood cell production are changed along with the course of development [68]. In vertebrates, particularly in birds and mammals, bone marrow is regarded as the primary site for new blood cell production [69]. In mammals, the hematopoietic niche is primarily located in the yolk sac and with the course of development the bone marrow niche is responsible for hematopoiesis. Hematopoietic niche maintains the heterogeneity of HSCs and other committed hematopoietic progenitors [70]. Vertebrate blood cells are of multiple types forming an array of intricate networks, destined for a specific function [71]. For seven weeks, early hematopoietic cells can be seen in the yolk sac. The liver, spleen, and thymus then temporarily share hematopoiesis. Primitive hematopoietic cells enter the circulation and develop once blood circulation begins. Hematopoietic progenitor cells begin to slowly move from the aorta-mesonephros-gonad (AGM) area to colonise the liver, which thereafter becomes the primary hematopoietic organ, at the age of 10 weeks. At 16 weeks of gestation, the foetal liver gives way to bone marrow as the site of hematopoiesis [72].

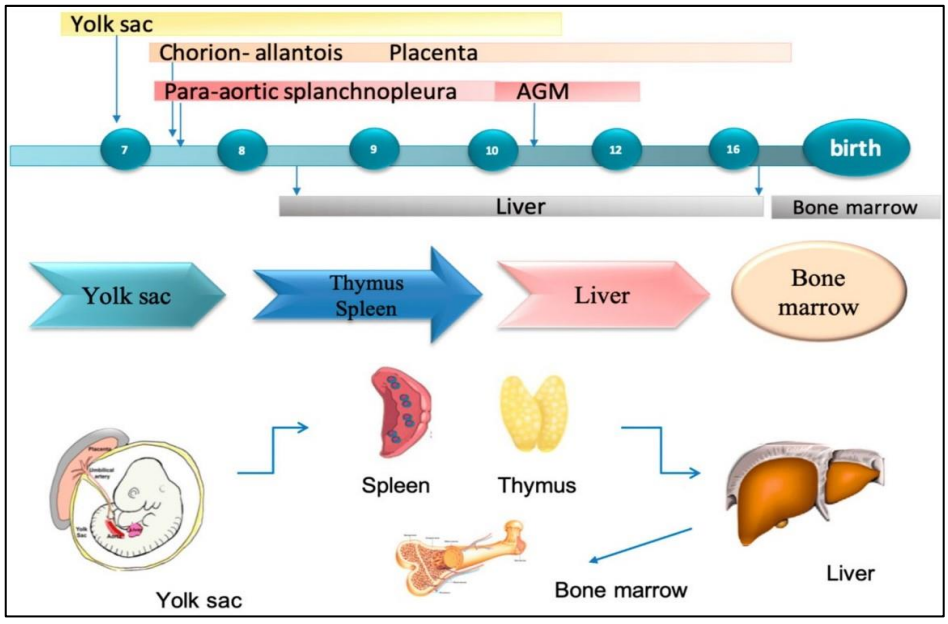


Figure 1.5 Foetal hematopoiesis time scale
Adapted from *Giancotti, A., et al.*, 2019

1.5.1 Primitive and Definitive hematopoiesis in *Homo sapiens*

The purpose of primitive hematopoiesis in humans is to produce red blood cells, which are essential for tissue oxygenation by the early developing embryo [39]. **Primitive wave** is of short period during which erythrocytes are derived from the erythroid progenitors (figure 4). In mammals the embryonic YS, FL, spleen and adult BM are the sites of sequential emergence of stem cells [68]. In spleen hematopoiesis occurs only till the fifth month of the fetus after which spleen does not participate in the development of blood cells. The differentiation of endodermal cells give rise to mesodermal precursors known as intermediate cell mass. After 12-15 days of fertilization, YS is collapsed into small vesicles forming secondary YS from the remnants. Primitive hematopoiesis is initiated in the secondary YS 16-19 days post-conception [73, 74]. Along with the switching of primitive hematopoiesis to definitive hematopoiesis the anatomical site is also changed i.e., from YS to FL (Figure 1.5.1). In case of mice this switch occurs between the embryonic days 10 to day 11 whereas in case of humans it occurs during 8^{th} week of embryonic development. Primitive hematopoiesis is distinguished by the generation of large nucleated erythrocytes that express embryonic globin. **Definitive hematopoiesis** shows initially nucleated erythrocytes that transform into the enucleated adult-type erythrocytes in humans and mouse, that produce fetal globin and adult globin respectively [2]. From the published literature it is quite evident that definitive hematopoiesis in humans has two distinct sites for the production of differentiated blood cells similar to mouse model [75-77]. These two sites are referred as P-Sp and AGM. AGM is thought to evolve from P-Sp identified in early embryos of mammals such as human, mice and non-vertebrates. Definitive HSCs produce all the mature blood cells during the adult life span [78]. Definitive hematopoiesis in humans is further divided into two waves [75]. One wave has the multipotent progenitors known as the erythromyeloid progenitors (EMPs). The differentiation potential of EMPs is limited to erythrocytes, mega-karyocytes and myeloid cells [79, 80]. In the second wave HSCs attain capabilities such as self-renewal and multipotency all through the life of an individual. These HSCs are also further capable of producing complete adult lineages, including lymphocytes (Figure 1.5.1).

They are the B- and T-lymphocytes that form in the bone marrow (CS23) and thymus (CS22) respectively [81, 82]. Murine EMPs produce erythrocytes expressing adult globins and this knowledge created confusion, especially in the *in-vitro* assays seeking to generate HSCs. Also, it is impossible to distinguish EMPs and HSCs on the basis of assays with only an erythro-myeloid readout [83]. HSCs in the placenta are found in week 6 of the gestation [84]. Furthermore, EMPs and HSCs cannot be distinguished by cell surface markers [83]. These issues highlight the need to test hematopoietic precursors for their long-term reconstitution and lymphoid potential. Furthermore, they can help decipher the success of *in vitro* HSC specification. Several unknown factors and aberrant signaling pathways deregulate the normal process of blood cells' development in hematopoietic diseases.

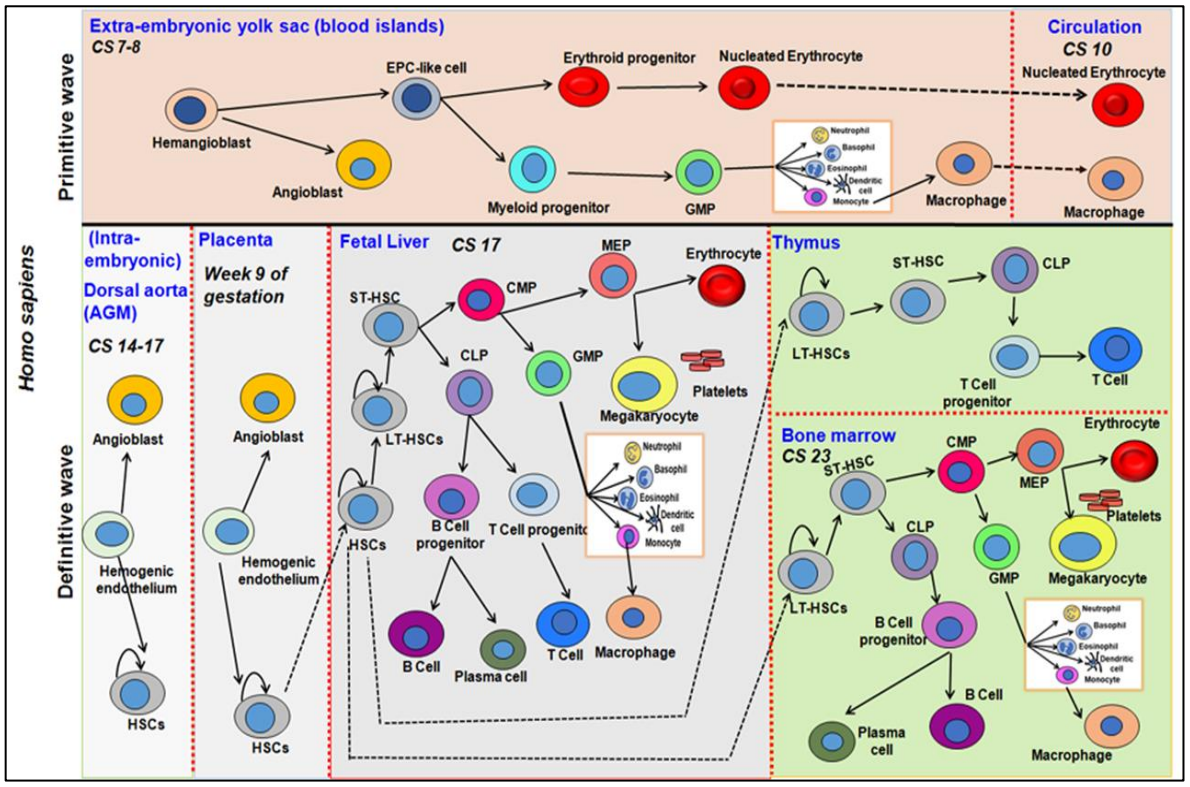


Figure 1.5.1 The schematic overview of hematopoiesis in *Homo sapiens* with the anatomical sites, blood cells, and time points, *Gautam et al., 2021*

1.6 Ontogeny of blood in *Drosophila melanogaster* (Invertebrate)

Drosophila melanogaster is a versatile model organism with simple hematopoietic system. Unlike vertebrates, *Drosophila* lacks vasculature with no physical barrier between blood cells and other tissues and organs [85]. Blood in the open circulatory system of *Drosophila* is referred as hemolymph. It comprises of three morphologically different types of mature blood cells [Plasmatocytes (>95%), Crystal cells (~5%) and lamellocytes (<1%)] differentiating from a common progenitor [71, 86-89]. Along with the hematopoietic precursors and progenitors, all the matured blood cells are actively found in the hemolymph and in the lymph gland. Plasmatocytes are functionally equivalent to mammalian monocytes, macrophages and neutrophils [90]. Plasmatocytes serve in surveillance of damaged tissue, repair and defense. Crystal cells are larger in size compared to plasmatocytes and functionally equivalent to mammalian platelet-like cells. These cells have para-crystalline inclusions that are involved in melanization reaction and wound healing [91, 92]. Lamellocytes are large flat adhesive cells [86]. They appear least in healthy larvae whereas they are absent in the embryonic and adult stages of the fly [86, 93].

1.6.1 Primitive and Definitive hematopoiesis in *Drosophila melanogaster*

Hematopoietic development in an invertebrate model *Drosophila*, that is a metamorphosizing organism, can be studied at different developmental stages i.e., the embryo, the larva and the adult. Fruit flies have two different anatomical sites for hematopoietic development in larval stages that are not present in the adults. Whereas, in vertebrate model system, the anatomical sites for hematopoietic development remains the same during the fetal and the adult stages. Published literature separates the development of *Drosophila* blood cells into two phases: primitive and definitive. During the primitive phase of hematopoiesis, in the head (procephalic) mesoderm, hemocyte precursors (HSs) arise from an identical set of progenitors known as multipotent hematopoietic progenitors/prohemocytes [86]. Further, these HSs give rise to two types of mature blood cells, plasmatocytes and crystal cells, in the early embryo (Figure 1.6) [89, 94, 95].

As the embryo develops, plasmatocytes populate it totally, found in circulation, whereas crystal cells limit themselves specifically to the proventriculus region [89]. The second phase occurs in the larval stages known as "Definitive phase of hematopoiesis". The formation of lymph gland (LG), a small hematopoietic organ begins in the 16 celled stage of the embryo, but the definitive stages occur only during larval stages marking the difference between the two stages. During definitive phase, the LG gives rise to all types of hemocytes [86, 96, 97]. LG is a bilobed structure composed of a single pair of anterior lobes, followed by 2-3 pairs of smaller posteriors lobes [98] along the dorsal vessel [86, 99, 100]. The core of the anterior lobe is known as the medullary zone (MZ) where the undifferentiated hematopoietic progenitor stem cells reside. The cortical zone (CZ) i.e., the periphery of the anterior lobe is mostly occupied with differentiated hemocytes and few intermediate progenitors. Framework of the base of the anterior lobe has the posterior signaling centre (PSC) [99-101]. PSC, has a key role in regulating the balance between undifferentiated hematopoietic progenitors (prohemocytes) and their differentiated lineages [99]. The existence of stem cells in the hematopoietic organ, lymph gland was initially determined by clonal studies i.e., by inducing mitotic clones in the hematopoietic cells of the embryos and first instar larvae of *Drosophila* [102]. One of the GATA family of transcription factors, serpent (srp) in *Drosophila*, is known to be involved in blood formation apart from gut and fat body development. Srp also regulates the hemocyte precursor formation and is essentially involved for the plasmatocytes and crystal cell specification during larval development [103-105]. Like many metazoans, *Drosophila* lacks both the lymphoid lineages and the adaptive immune responses. Nevertheless, *Drosophila* blood cells (plasmatocytes and crystal cells) are considered functional equivalents to mammalian lymphoid lineage for their participation in repair of damaged tissue, macrophagy, wound healing, and innate immune defense mechanisms against pathogens such as microbes and parasitoid wasps [94, 106].

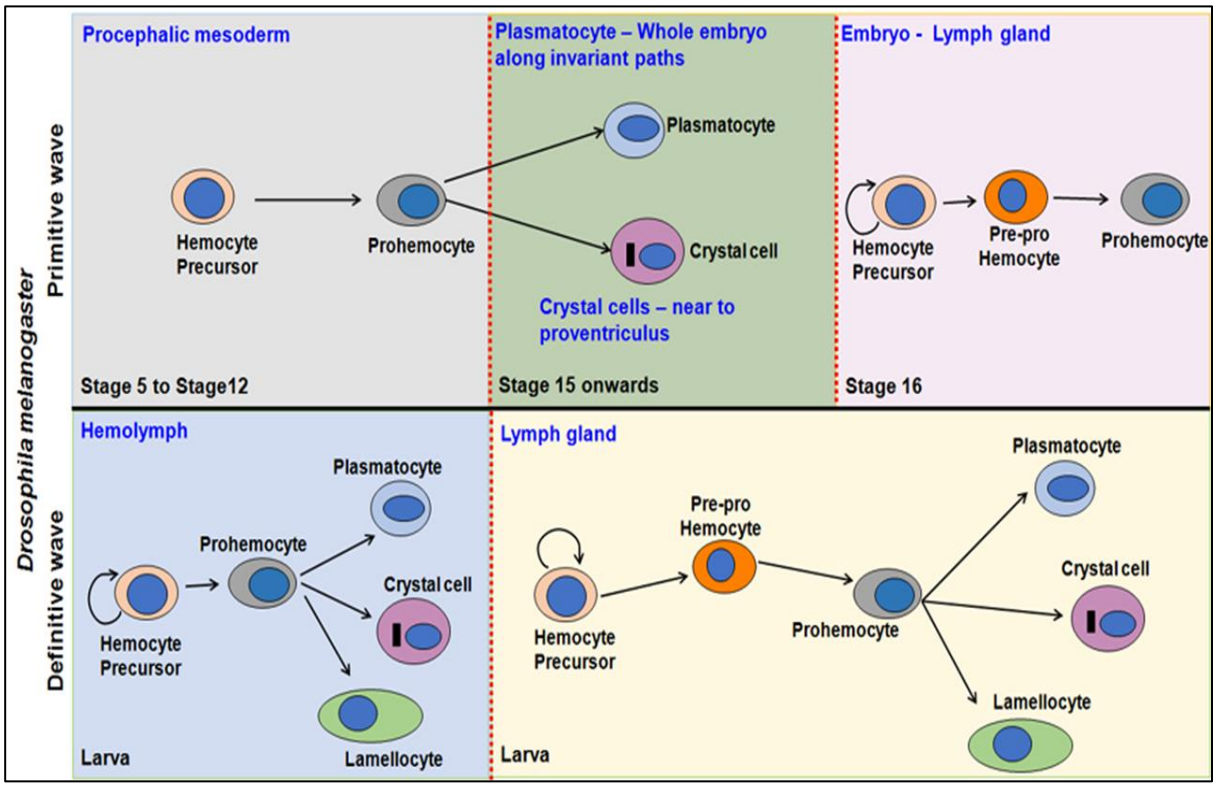


Figure 1.6 Diagrammatic illustration of hematopoiesis in *Drosophila* specifying the stages and tissues of development, *Gautam et al., 2021*

1.7 *Drosophila* as a model system

Drosophila melanogaster, commonly known as the fruit fly, has long been recognized as an exceptional model organism in biological research. It offers several advantages that have contributed significantly to our understanding of various biological processes. The following are key advantages of using *Drosophila* as a model system:

Genetic tractability: *Drosophila* has a well-annotated and sequenced genome, with a relatively small number of chromosomes (four pairs) [107]. This genetic simplicity makes it easier to identify and study specific genes and their functions. The fruit fly also possesses a wide array of genetic tools, such as transgenic techniques, gene knockouts, and RNA interference (RNAi), allowing precise manipulation of gene expression and investigation of gene function [108].

Conserved genetic and developmental pathways: Many fundamental genetic and developmental processes are highly conserved between *Drosophila* and higher organisms, including humans. Genes and pathways identified in fruit flies often have counterparts in humans, making *Drosophila* a valuable model for studying human biology and diseases[109]. Insights gained from *Drosophila* studies have provided valuable information on topics such as cell cycle regulation, cell signaling, and organ development.

Short generation time: *Drosophila* has a rapid life cycle, with a generation time of around 10-14 days. This short generation time allows for the study of multiple generations within a short period, facilitating genetic crosses and experiments. It enables researchers to observe and analyse the effects of genetic manipulations and environmental factors across multiple generations in a relatively short timeframe [110].

Large number of offspring: Fruit flies produce a large number of offspring from a single mating pair. A female fruit fly can lay hundreds of eggs in her lifetime. This high fecundity provides researchers with a large sample size for statistical analyses and allows for the efficient screening of genetic variations, mutations, or phenotypic traits [111, 112].

Easy and cost-effective maintenance: *Drosophila* adults are small and can be easily housed in laboratory vials or culture bottles. They require minimal space, food, and care

compared to larger animal models. This ease of maintenance allows for the establishment of large populations in a relatively small space. Additionally, the cost of maintaining *Drosophila* colonies is relatively low, making it a cost-effective model organism for research purposes[113].

Well-characterized developmental processes: The development of *Drosophila* is highly organized and well-understood. The embryos undergo a series of precisely regulated cell divisions and differentiation, resulting in a predictable pattern of tissue formation and organ development [114]. The transparency of *Drosophila* embryos allows for the direct observation and manipulation of developmental processes, making it an excellent model for studying tissue patterning, organogenesis, and morphogenesis [115].

Extensive resources and community support: The *Drosophila* research community has established a vast array of resources and tools to facilitate research. These include a comprehensive collection of mutant strains, genomic libraries, transgenic techniques, and databases that catalogue gene functions and interactions [116]. The availability of these resources, along with the collaborative nature of the research community, promotes knowledge sharing and accelerates discoveries.

In conclusion, *Drosophila* melanogaster offers numerous advantages as a model system, including its genetic tractability, conservation of genetic pathways, short generation time, large offspring numbers, easy maintenance, well-characterized development, and extensive resources. These advantages have positioned *Drosophila* as a powerful model organism for addressing a wide range of biological questions and have played a pivotal role in advancing our understanding of genetics, development, behaviour, and human biology

Although hematopoiesis in *Drosophila* larvae is well understood, little is known about adult blood cells and their developmental signals. Recently, four scattered "hematopoietic hubs" (hemocyte clusters) were discovered by *Drosophila* biologists in the fly's dorsal abdomen. According to *Ghosh et al., 2015*, these hematopoietic hubs house progenitors that have the capacity to mature into functioning plasmatocytes and crystal cells, revealing the real nature of the hematopoietic organ in adult *Drosophila* [117]. As a result, *Drosophila* makes a great model for studying how hemocytes drive immunological responses.

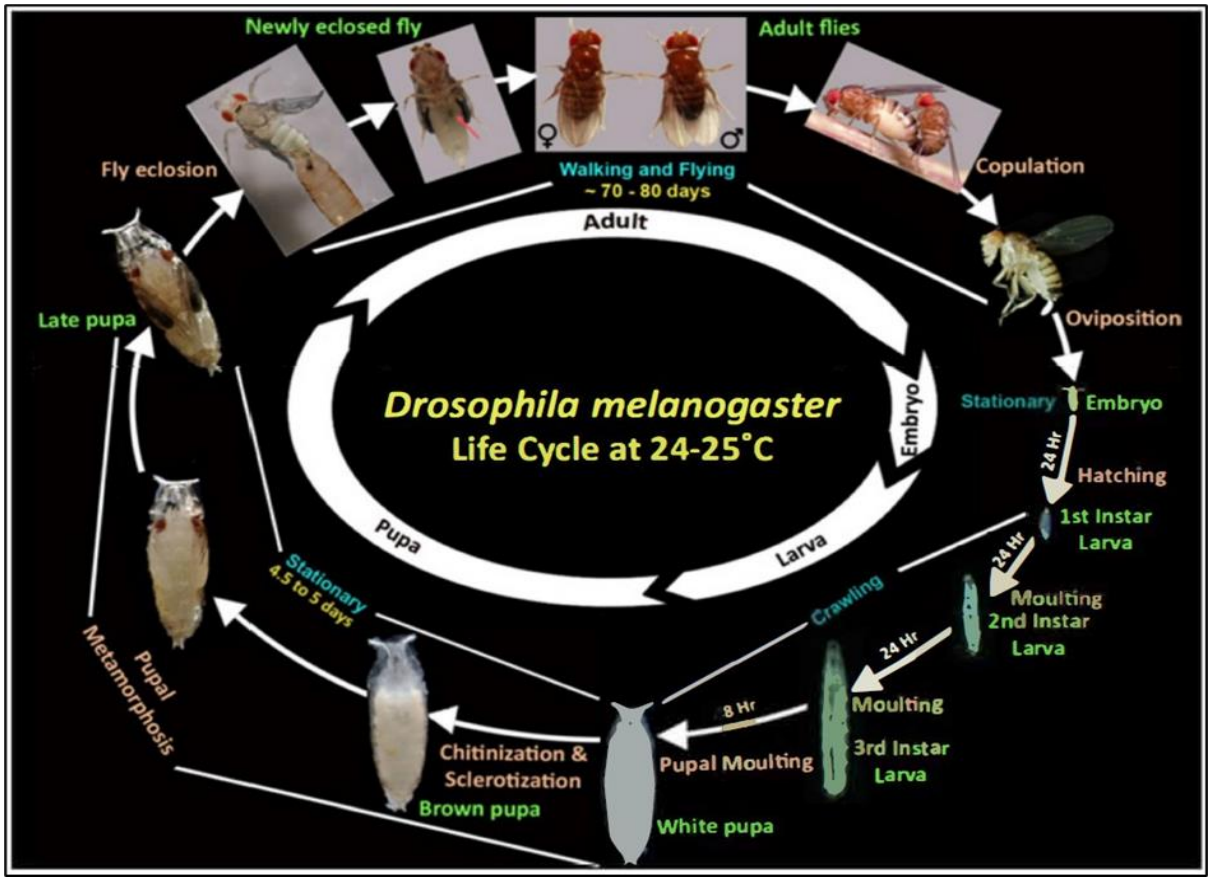


Figure 1.7 *Drosophila melanogaster* life cycle, projecting major events, motility and average durations (at 24° - 25°C) of different stages. Red arrow points to the 'meconium' in newly emerged fly. Adapted from *Hamid and Mishra 2021***,** Book "Experiments with *Drosophila* for Biology Courses" Chapter 1, Figure 2 **[113]** Author: S.C. Lakhotia.

1.8 Host-pathogen interaction

The interaction between *Drosophila* (fruit fly) and *Leptopilina boulardi* (parasitoid wasp) is a well-studied model system in evolutionary biology and host-parasite interactions. The parasitoid species strategically attack and utilize their hosts' machinery to pursue their own offspring survival and development, often resulting into death of the host. To circumvent this endoparasite strategy, the host triggers immune responses that include the formation of lamellocytes capable of inducing the encapsulation response. Encapsulation, as a complex cellular immune response against parasitism, resulting from primarily the increased differentiation of progenitors into lamellocytes (specialized hemocyte produced during wasp infestation) along with the involvement of crystal cells necessary for the melanisation process

(Prophenoloxidase cascade) [87, 93, 118, 119]

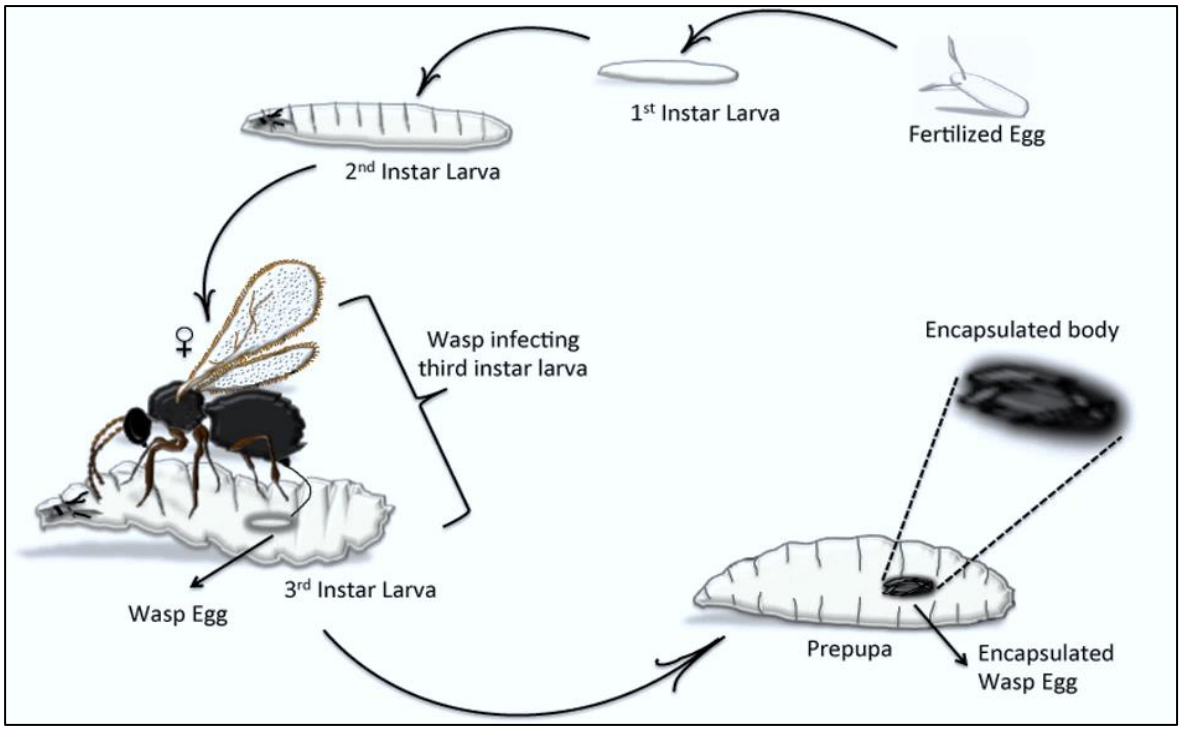


Figure 1.8 Immune response of third instar *Drosophila* larva to *Leptopilina boulardi-17* wasp infection. Adapted from: *Hamid and Mishra 2021***,** Book "Experiments with *Drosophila* for Biology Courses" Chapter 18, Figure 2 **[113]** Author: Indira Paddibhatla

1.9 Aberrant hematopoiesis in *Drosophila*

The blood homeostasis is disturbed in two scenarios: in wild type upon wasp/microbial/viral infection and due to hematopoietic deregulation [118, 120, 121]. Flies respond to infection by coordinating complex defense responses. Wasp infection activates both the humoral (gene expression changes affecting systemic concentrations of antimicrobial peptides) and cellular (differentiation, melanisation and aggregation of blood cells) components of the host's immune system [122]. Hematopoietic homeostasis is crucial for maintaining a balance in precursor, progenitor and matured blood cell population. This stable equilibrium in the hematopoietic population is disturbed in hematopoietic mutants. These mutants include *hop*$^{Tum\text{-}l}$ (JAK/STAT), *Toll10*^{b} (Toll/NF-κB), *Ubc9*$^{4\text{-}3/5}$ (Toll/NF-κB), *RAS*^{v12} (RAS) [123-127].

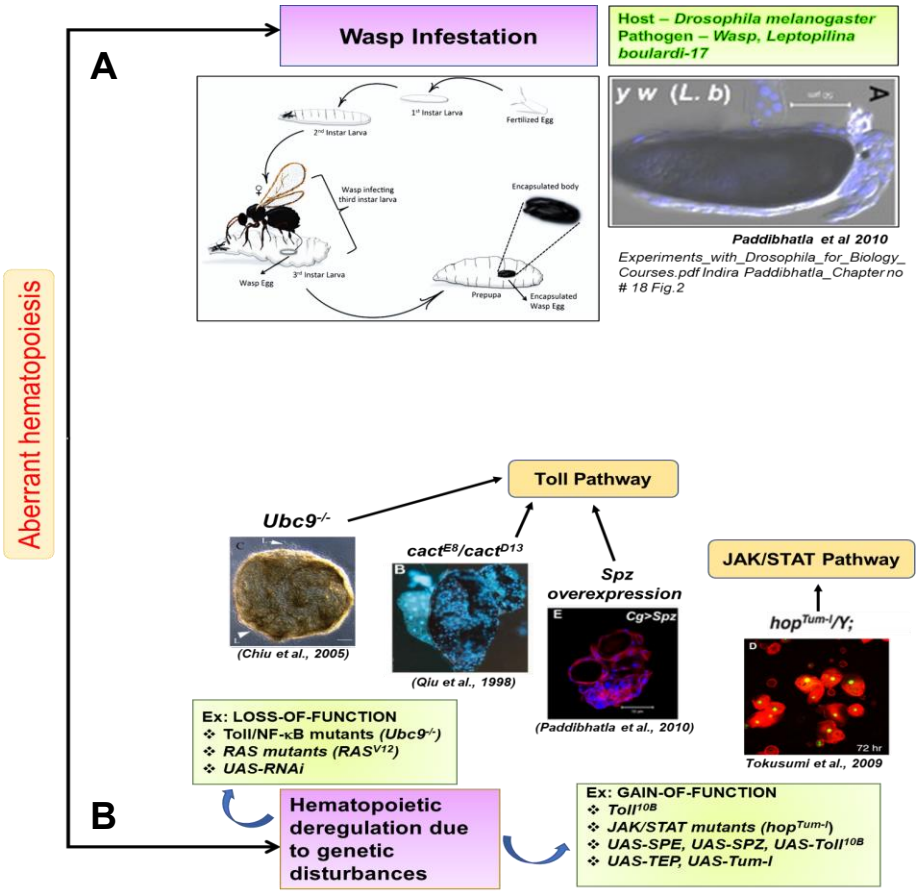


Figure 1.9 Schematic showing the disturbances in blood homeostasis via **A**: Wasp attack and **B**: Hematopoietic mutants

Modified from *Hamid and Mishra 2021***,** Book "Experiments with *Drosophila* for Biology Courses" Chapter 18, Figure 2 **[**113**],** Author: Indira Paddibhatla and adapted from **[118]** *Paddibhatla et al., 2010*.

1.10 Immune tissues in *Drosophila* (Blood cells)

The internal organs of the fruit fly are covered in hemolymph because they lack a vascular network to separate the blood cells from other tissues and organs [85]. Three distinct types of blood cells, or hemocytes, circulate in the hemolymph throughout a fly's lifespan in the primitive open blood circulation system of *Drosophila* [85]. Haemocytes are responsible for a number of immune functions in *Drosophila*, among which phagocytosis, encapsulation and melanization have been documented [128]. Hemocyte deprivation in *Drosophila* causes infection sensitization, which is obvious. The domino mutation, which causes drastically lower haemocyte counts, was used to demonstrate this in larvae. When this mutation is coupled with mutations that alter humoral immunity (Imd) or melanization, the resistance of larvae to infection is significantly reduced compared to single mutants [129]. Plasmatocytes and crystal cells are produced in *Drosophila* embryos. Although they do so in different ways, both protect against dangerous germs. Invaders are engulfed and destroyed by plasmatocytes, whereas crystal cells produce substances that encapsulate bacteria in a solid gel. Once the *Drosophila* fly enters its larval stages, the blood cells that were created there are still there. During this stage of development, the majority of the blood cells are found in clusters attached to the cuticle that covers the larva's surface, but a small number also move freely within the body of the larva [130]. The most frequent hemocyte type is the plasmatocyte, which is relatively small, 8–10 μm in diameter, accounting for ~90–95% of hemocytes. They generally have phagocytic and antimicrobial capabilities, analogous to human macrophages. Additionally, the plasmatocytes secret extracellular matrix (ECM) proteins to promote tissue formation and remodelling [131-133].

Plasmatocytes are derived from ancient forms of metazoans, reminiscent of the cells of mammalian macrophage lineage, from which they acquired the function of phagocytosis. They are the predominant hemocyte population that differentiate or fuse to transform into large, flattened cells. *Drosophila* plasmatocytes differentiation give rise to a special type of cells called lamellocytes. The markers responsible for the fate of the plasmatocytes are Hemolectin (Hml), Peroxidasin (Pxn), NimC1 (P1 antigen), Croquemort (Crq), Collagen and Eater but none of them is completely successful in identifying every plasmatocyte [89, 99, 127, 134, 135]. However, they are all very representative. Mature plasmatocytes are recognized by an

antibody against P1 antigen in larval and adult flies but not in embryonic plasmatocytes [134]. They create segmentally repeating and terminal segment patterns in microenvironments in the larval body wall (Hematopoietic Pockets) and static arrays in certain regions of the digestive tract (proventriculus) [136, 137]. High rates of plasmatocyte self-renewal cause the macrophage pool to grow by more than 30 times, from about 300 cells in the 1st instar to around 10,000 in the late 3rd instar [138, 139]. Majority of the hemocytes in the 1st larval instar are resident, but there is high rise in the number of hemocytes from the late 2nd larval instar onward observed during circulation, thereby forming a steady state with the resident hemocyte population. In addition, *Drosophila* resident plasmatocytes can produce two additional blood cell subtypes: crystal cells [130] and lamellocytes (due to immune trigger)[140] a subtype of hemocyte designed specifically to encapsulate big foreign entities like parasitoid wasp eggs. This highlights additional similarities between plasmatocyte EMPs and vertebrate EMPs by indicating that at least some plasmatocytes, if not all, have lineage-restricted progenitor potential [79].

Crystal cells are a broader group of blood cells, ~10-12 um in size as compared to plasmatocytes, and accounts for 2-5% of the total hemocyte population in circulation [86, 134]. They are named so because they possess the key enzyme for melanin biosynthesis, prophenoloxidase (PPO) in the form of crystalline inclusion [141]. They are a form of non-phagocytic blood cells that help circulate melanin proteases to induce innate immunity and wound healing. Black cell (Bc) mutation reveals the visibility of crystal cells as a result of abortive / premature melanization of the crystalline inclusions [142].

Lamellocytes is large, flat, irregular-shaped (diameter of 15–40 μm) and are a group of hemocytes with asymmetrical giant appearance. They are flat macrophage- like cells with adhesive properties that engulf invading pathogens, that escape the machinery of phagocytosis, leading to the degradation of pathogens and apoptotic cells. They are specialized in mediating the encapsulation and killing of pathogens too large to be phagocytosed. They rarely exist in healthy larvae and their mass production is due to a trigger of immune stimulus such as wasp colonization, injury or mechanical stress. Alike, plasmatocytes and crystal cells, they prevail only in the larval and not in embryonic or adult stage, even after post wasp invasion [86, 143, 144].

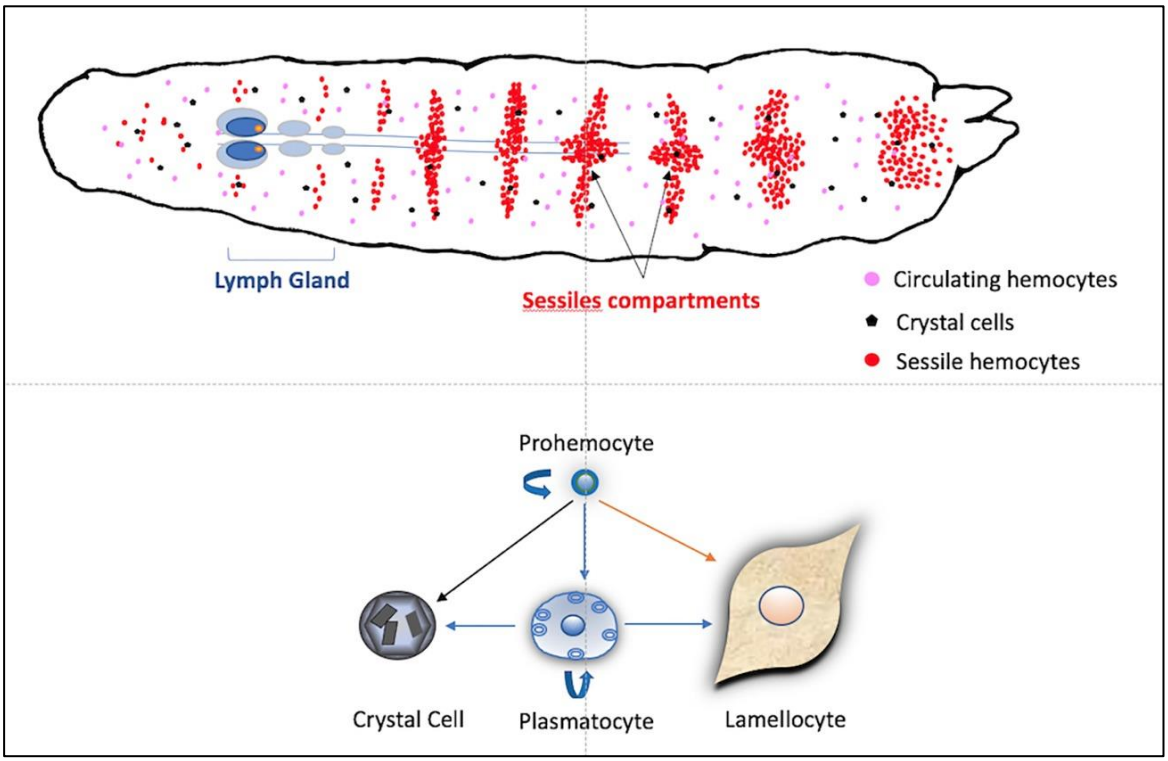


Figure 1.10 Schematic of third instar *Drosophila* larva showing hemocytes mainly crystal cells, plasmatocytes and lamellocytes.

Adapted from *C. Kim-Jo, J.-L. Gatti and M. Poirié* (2019)

1.11 Immune tissues in *Drosophila* (Fat body)

Similar to other arthropods, *Drosophila* fat body is also derived from the mesoderm germ layer [145]. The term "fat body" is in fact used to describe a tissue in *Drosophila* that functions similarly to adipose tissue in mammals [145]. Unlike, other tissues fat body undergoes endoreplication (256C DNA content) and are basically large cells of monolayer [146]. It is covered with an outer layer of extracellular matrix separating it from the other larval parts including hemolymph [147]. An important organ involved in energy storage, metabolism, immunity, and nutrition sensing is the fat body of the *Drosophila* [148, 149]. Triglycerides are stored by the body as a source of energy that can be accessed during fasting or times when there is a greater need for energy. In order to control energy metabolism, the fat body stores and releases lipids and glycogen [148]. It contributes to the production, breakdown, and transportation of lipids. It also helps with nutrition sensing and glucose metabolism. The fat body recognises the availability of nutrients and signals the body's other tissues about its nutritional condition. In response to signals from nutrients, it creates and secretes metabolic hormones including insulin-like peptides (ILPs) that control growth, development, and metabolism [150]. Although energy metabolism is the fat body's main function in *Drosophila*, it also plays a role in the immune system's defences [151]. As a component of the innate immune system's defence against infections, it creates antimicrobial peptides (AMPs) [151]. In *Drosophila*, the fat body is essential for growth, development, and reproduction also. It offers the nutrients and signalling compounds required for the healthy growth and maturation of different tissues and organs. The fat body contributes to the detoxification and elimination of toxins and waste products in *Drosophila* [152]. Since *Drosophila* fat body, similar to mammalian adipose tissue, serves as a versatile metabolic organ with roles in energy storage, nutrient sensing, metabolism, immunity, and development, its functions are essential for maintaining homeostasis and supporting the overall physiology of the organism.

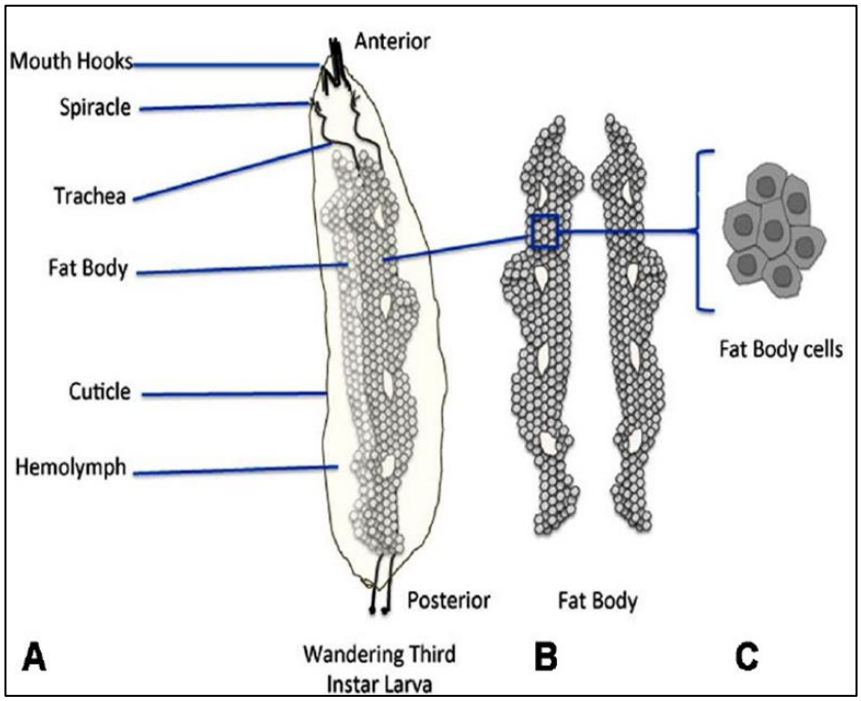


Figure 1.11 Schematic of the fat body tissue of third instar *Drosophila* larva

A. Distribution of the fat body tissue in larval body cavity;

B. Schematic of the dissected-out fat body.

C. Schematic of hexagonal and large fat body cells.

Adapted from Experiments with *Drosophila* for Biology Courses" Chapter 16, Figure 1A,B,C **[**113**]** Author: Indira Paddibhatla

1.12 History of Methotrexate (MTX)

Yellapragada Subba Rao, an eminent biochemist, who unravelled anti-cancer activity of MTX for the treatment of cancer which revolutionized that era. Prior 1950, the majority of the cancers were treated either by surgery or radiation most of which was not successful at times [153-156]. In the course of 1949, Sidney Farber conjectured that cancerous cells proliferate rapidly and so require folate for their continuous maintenance of cell growth [157]. Folate, being one of the nutrients of the B group vitamins, mark a vital role in the production and maintenance of early developing cells particularly in infants and in pregnant women [158]. This made him think that if an anti-folate drug, such as MTX, administered to the suffering patients probably would have some inhibitory effect on the growth rate of these cancerous cells. Furthermore, children with acute lymphoblastic leukemia (ALL), rapid developing blood cancer, fed with MTX showed remarkable improvement in their symptomatic behaviour [157]. Nevertheless, the success of improvement was of short term, due to relapse. Roy Hertz, another scientist working in the field of medical oncology, studied the effects of folic acid on the female urogenital system (reproductive plus urinary system) specifically urogenital tract. His early research work sheds light on the effect of the anti-folate treatments, such as MTX, on actively proliferating cells of the female urogenital tract [153].

Meanwhile, Min C. Li, endeavoured anti-folate treatment in patients with metastatic melanoma leading to an intangible success. However, it had an intense reducing effect in urinary human chorionic gonadotropin (β-HCG) which went unnoticeable at that time. Since healthy pregnant women are (β-HCG) positive and (β-HCG) is also associated with multiple tumors, carcinomas, and melanomas [155].

Li's result intrigued Hertz and he speculated that MTX could be a beneficial tool to treat cancer specific to the female reproductive tract called gestational carcinoma (GC). GC occurs due to the abnormal growth of cells which are specific to the placental origin. Hertz and Li together showed that metastatic GC tumors were inhibited drastically against MTX use. The case was remarkable as for the first time the solid tumor has ever responded to the chemotherapy treatment. The treatment was breakthrough since its perseverance was for the first time. In 1958, Hertz group came up with another publication, Hertz et al. showing how 27 patients who suffered from

choriocarcinoma and related diseases responded to treatment. Five patients were reported to achieve a complete cure [153-156]. Alan Rabson, M.D., Deputy Director of NCI, praised Dr. Hertz for his outstanding work which had had an immense impact on cancer therapy and was not only limited to leukemia. New challenges were being the scientist in cancer biology which paved the new pathway in the field of medical oncology (treatment of cancer with chemotherapy). Previous perceptions about cancer being fatal were getting remoulded and revised. Early results published by [157]Farber et al, showed the success of an antifolate (Aminopterin) treatment to childhood leukemia. This attracted the scientist for the use of an anti-metabolite in the treatment of childhood leukemia [157]. Aminopterin, an anti-metabolite compound structurally show resemblance to MTX, interferes with the connective tissue and this observation was extrapolated, by Gubner research group in 1951 in, rheumatoid arthritis (RA) [159]. Several patients with RA, psoriasis and psoriatic arthritis were treated with aminopterin routinely. They showed a rapid recovery in RA signs and symptoms but drug discontinuation led to the emergence of RA back. Due to struggle in manufacturing aminopterin, the compound was synthetically modified in the structure for the easier production [160]. The modified version was MTX. Over past 25 years, MTX has become an emerging standard and popular drug in the treatment of adult rheumatoid arthritis.

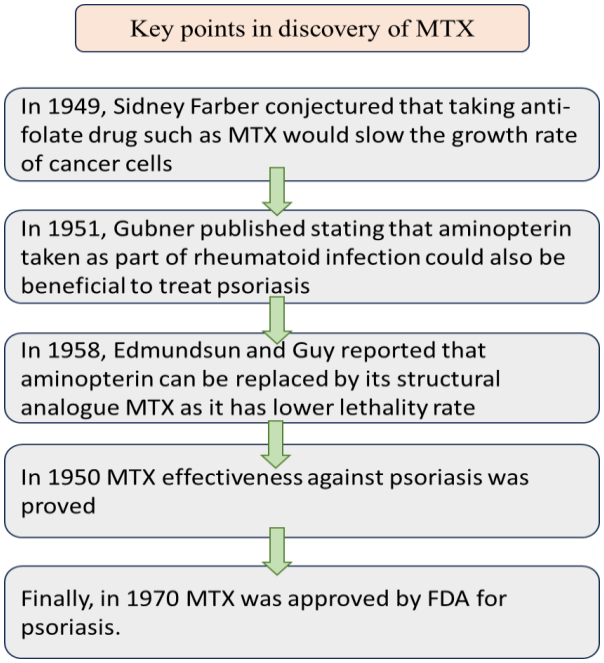


Figure 1.12 Flowchart of MTX hierarchical discovery

1.13 MTX Physiological properties

MTX is a solid odourless, yellow or orange-brown crystallite compound. Chemically MTX is 4-amino-4-deoxy-N-methyl- Pteroylglutamic acid. It shows the molecular structural difference from folic acid only in that folic acid has a hydroxyl group (OH-) in place of the 4-amino group on the pteridine ring and there is no methyl group at the N10 position [161]. Methotrexate, (previously amethopterin), differs from aminopterin in that the latter is also not methylated at the N10 position. Therefore, the active site of the molecule appears to involve the pteridine ring portion [162]. MTX is a weak organic acid in nature and lipid insoluble at physiological pH. Its solubility in human urine is directly proportional to its increasing pH. It is soluble in dilute solutions of alkali hydroxides and carbonates, slightly soluble in dilute hydrochloric acid whereas practically insoluble in water and alcohol. (Lewis, R.J., Sr (Ed.). Hawley's Condensed Chemical Dictionary. 13th ed. New York, NY: John Wiley & Sons, Inc. 1997., p. 722). Molecular weight is 454.44 g/mol and empirical formula is C20H22N8O5 with biological half-life 3-10 hours (lower doses, <20mg/m2) and 8-15 hours (higher doses, >30mg/m2). The bioavailability at lower doses is approximately 60% and comparatively less at higher doses.

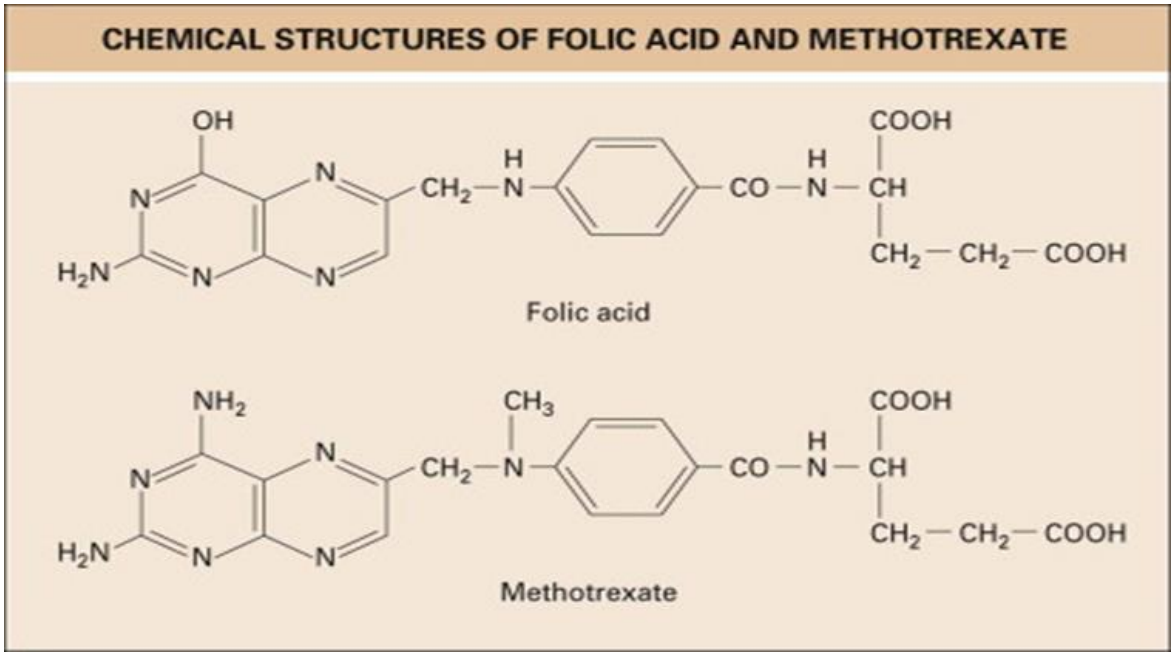


Figure 1.13 Molecular Chemical structure of Methotrexate and Folic acid

1.14 MTX mode of action

Methotrexate (MTX) is a Food and Drug Administration (FDA) approved chemotherapeutic agent and widely used anti-cancer drug formerly known as amethopterin [163]. Methotrexate is known to inhibit Dihydrofolate reductase (DHFR) through competitive inhibition having 1000-fold more affinity than folate. DHFR is a key enzyme functionally associated to convert dihydrofolate to active tetrahydrofolate (reduced folate factors) [164]. Tetrahydrofolate plays an important role in transferring one carbon unit in biochemical reactions specific for the synthesis of thymidylic acid and ionosinic acid. The former is an important component of DNA, whereas latter is the precursor of purines involved in the synthesis of both DNA and RNA [161]. MTX is widely used in different types of cancers and in autoimmune diseases. In spite of the fact that it is considered as an immunosuppressant, it has severe side effects, which could be mild to severe [165, 166]. These include nausea, vomiting, liver disease, lung disease and even low blood cell count synthesized by the bone marrow. MTX is recommended in low doses as high dose often leads to severe side effects. Existing literature has shown the effect of MTX on the survival of adult flies, female fecundity, egg morphology, larval tumors, and wing, eye, and leg defects [167].

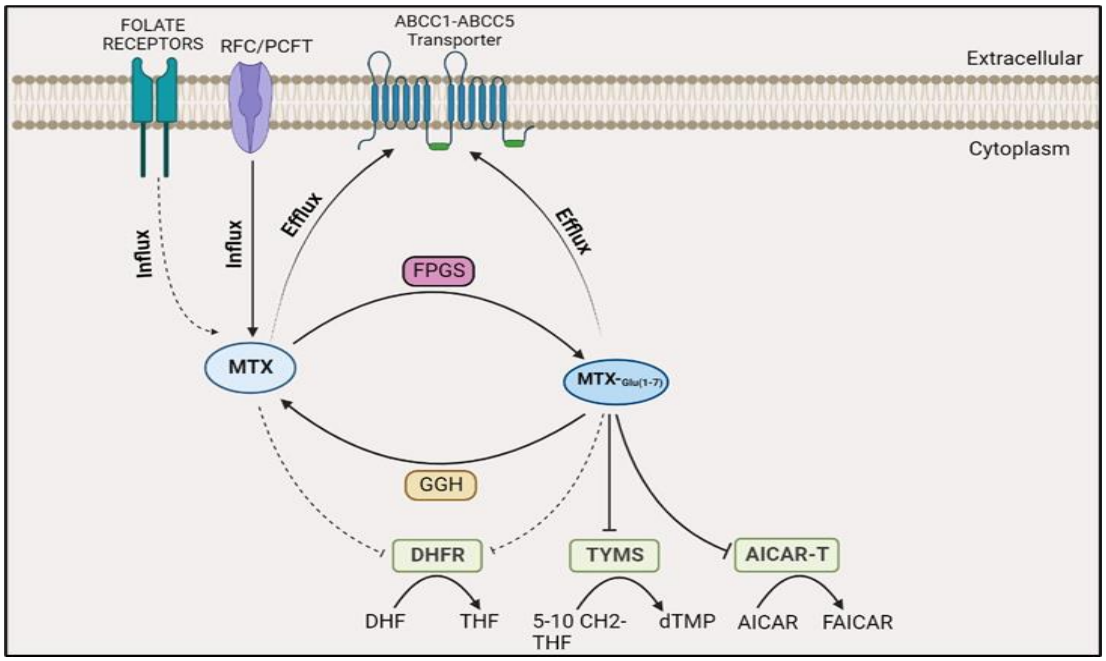


Figure 1.14 Schematic diagram showing the mechanism of action of MTX in DNA and RNA Created in Biorender

1.15 Objectives of the Study

Recent publications have established the fact that MTX inhibits the JAK-STAT pathway through dephosphorylation of STAT1 and STAT5 [168]. In most of the higher animals, MTX is a known teratogen but its effect on invertebrate's reveals very limited information. Previous literature provides some studies showing that MTX treated flies have an effect on their survival, oviposition, and egg morphologies. In spite of MTX treatment rare survived flies showed some developmental aberrations such as larval tumors, bristle, wing, and leg and eye defects. Developmental abnormalities due to toxic effects of MTX are also studied in another vertebrate model system such as rats, rabbits, and mice [169-171].

Also, MTX activity interferes with the JAK/STAT pathway leading to the deregulation of the hemocytes (blood cells in *Drosophila*) [168]. The action of a mechanism still remains enigmatic. In humans, MTX inhibits DNA synthesis to a greater degree as compared to RNA synthesis signifying that thymidylate synthesis is a most crucial mechanism for MTX cytotoxicity. This makes it cell cycle-dependent thereby acting primarily on DNA synthesis (S-phase) [172]. Hence the cells undergoing rapid proliferation in the cell cycle are more liable to experience the cytotoxic effect of MTX. Due to the anti-leukaemia effects of MTX it is important to determine if MTX can affect hematopoiesis. The MTX treatment of *Drosophila* blood cells will be a new and interesting work figuring out the how it is targeting the blood cells at the individual level. By which the knowledge can be predicted (numbers, based on what's known) to human cancer cells treated with MTX. This drug is widely used for treatment of several types of cancer including leukemia [163]. It is important to comprehend MTX's non-canonical effect on signaling pathways that are essential for immune cell responses against growing cancerous cells. Recent research articles have also provided evidences that MTX can function non-canonically through JAK/STAT pathway in myeloproliferative neoplasms [168]. Another *in-vitro* study using cell lines like T-Jurkat (T cells), HeLa (human epithelial cells), and U937 (human histiocytic lymphoma) reported that MTX specifically inhibits the phosphorylation status of IκBα, thereby hindering the NF-κB activation [173].

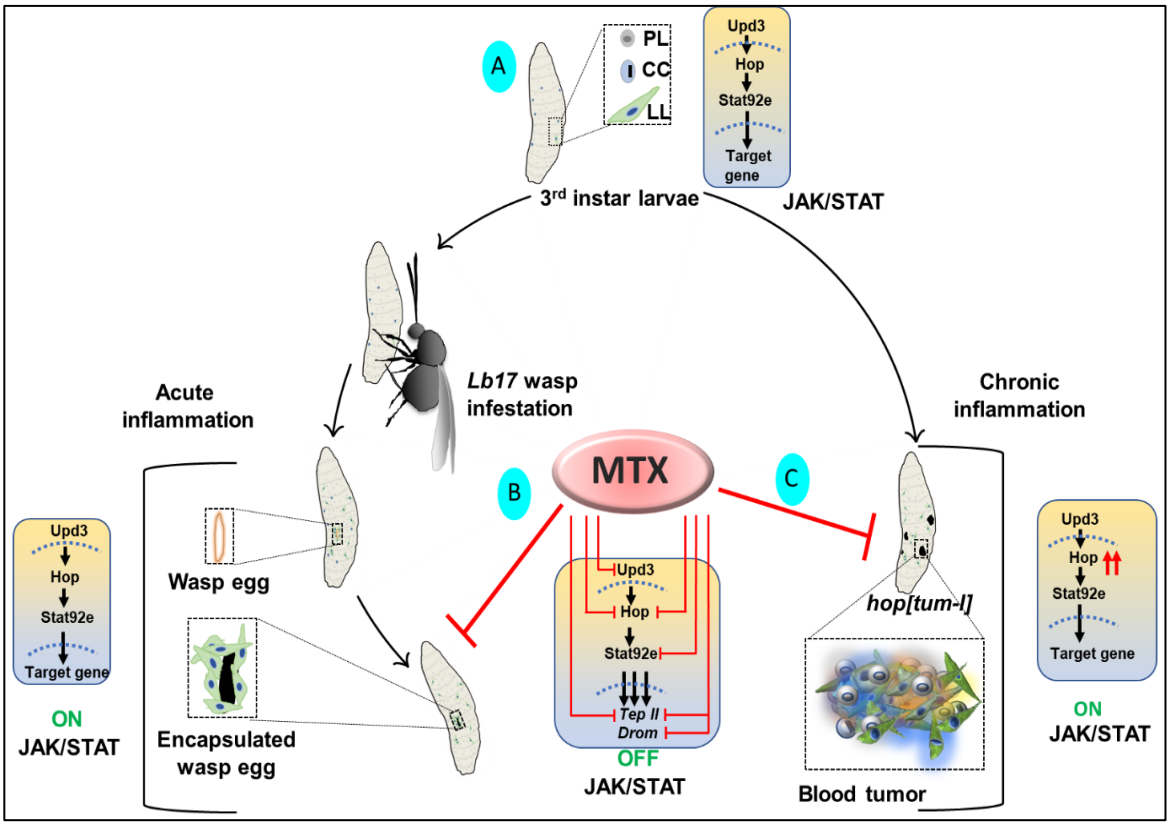


Figure 1.15 Schematic representation of MTX's role in both acute (wasp induced) inflammation and chronic inflammation (*hop*$^{Tum\text{-}l}$) in 3rd instar *Drosophila* larvae. Adapted from *Yadav et al., 2021*

Based on the rationale [168] and our own published research [174] it was evident that MTX inhibits JAK/STAT pathway *in vitro* and *in vivo*. So, in this study we hypothesised that *MTX can also exhibit an inhibitory effect on Toll/NF-κB pathway and if it does whether it could relieve or restore the defects associated with hyperactive (parasitoid infected/hematopoietic mutant) Toll pathway*?

In order to test this hypothesis, we designed specific objectives to carry out the study

specific objectives:

1. To study the effect of Methotrexate (MTX) on parasitoid wasp (*Lb-17*) infestation triggered Toll/NF-κB Pathway, in the immune tissues of wild type third instar larvae

2. To study the effect of MTX in larvae with hyperactive Toll/NF-κB pathway (melanotic tumors associated with hematopoietic deregulation) in both the gain of function (*SPE* overexpression) loss of function ($Ubc9^{-/-}$) background

3. To study effect of MTX on the nutritional metabolism (insulin signaling) in the larvae with hyperactive Toll/NF-κB pathway in the $Ubc9^{-/-}$ mutant model

2. MATERIALS AND METHODS

2.1 *Drosophila* stocks and genetics

A standard protocol of cornmeal-malt-agar mixed media was implemented for breeding of all the fly stocks and same were maintained at 25°C under 12hr: 12hr light-dark cycles respectively. Fly stock, *Canton S*, *y w*, *hop*$^{Tum\text{-}l}$ (gain of function mutants of JAK/STAT pathway), *Ubc9* stocks were a gift from Dr. Shubha Govind's laboratory, City College, CUNY.

2.2 Wasp Infestation strains and breeding

A standard protocol [93] was followed for rearing *Leptopilina boulardi* (strain 17) wasps [175] on the wild type fly *y w* strain. Infestation was performed on 3-day-old larvae (after 72 hours of egg-laying).

2.3 Wasp Infestation setup

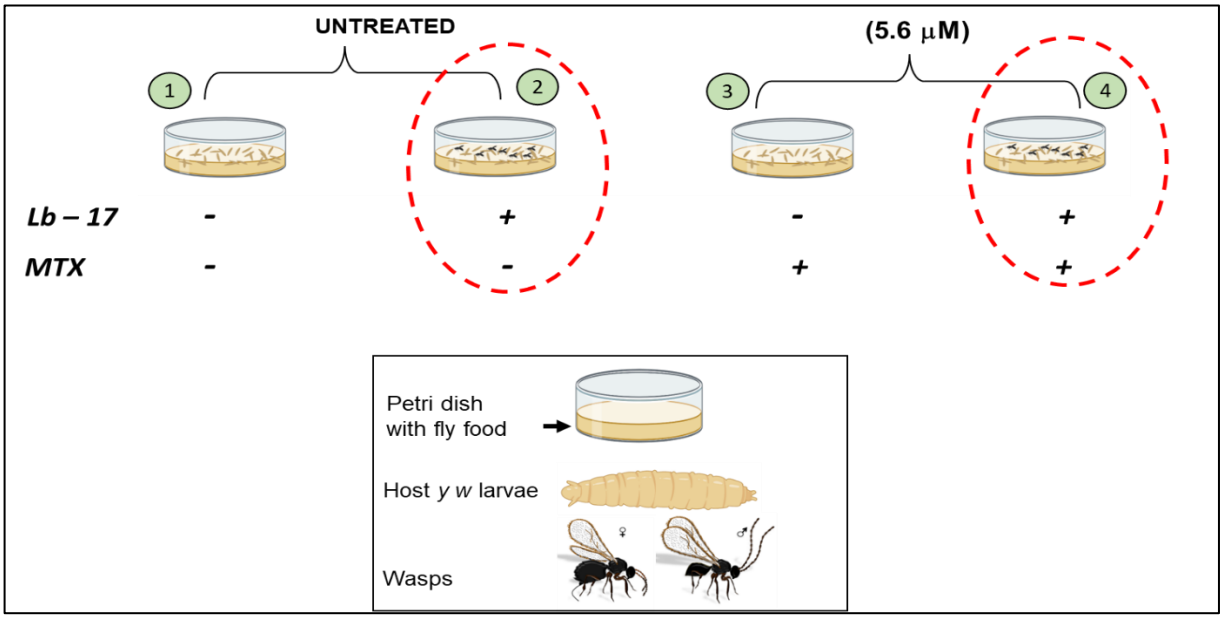


Figure 2.1 Schematic showing wasp infestation - *Leptopilina boulardi 17*

Host - *Drosophila melanogaster (y w)*

Created in Biorender

2.4 Egg lay treatment by MTX in wasp infestation background synonymous to acute inflammation

Drosophila wild type (*y w*) flies were kept in an egg laying chamber for 12 h at room temperature for egg lay. After 48 h, the 1st instar larvae, were transferred to a petri dish containing fly food mixed with MTX drug (1ml of drug + 3ml of fly food). This was referred as First MTX treatment. After 12 h, late 2nd instar larvae were exposed to their parasitoid wasps, *Lb17*, for 12 h of duration and then wasps were removed. After 36 h, early 3rd instar larvae were transferred to another petri dish containing fly food mixed with MTX drug (1ml of drug+ 3ml of drug). This was referred as Second MTX Treatment. Then, after 24 h, 3rd instar host larvae were dissected for fat body and immune tissues to study the subcellular localization of Toll pathway components. The procedure is adapted and modified from our published article [174]. All the data were analysed using Graph Pad Prism software (version 8.0.2).

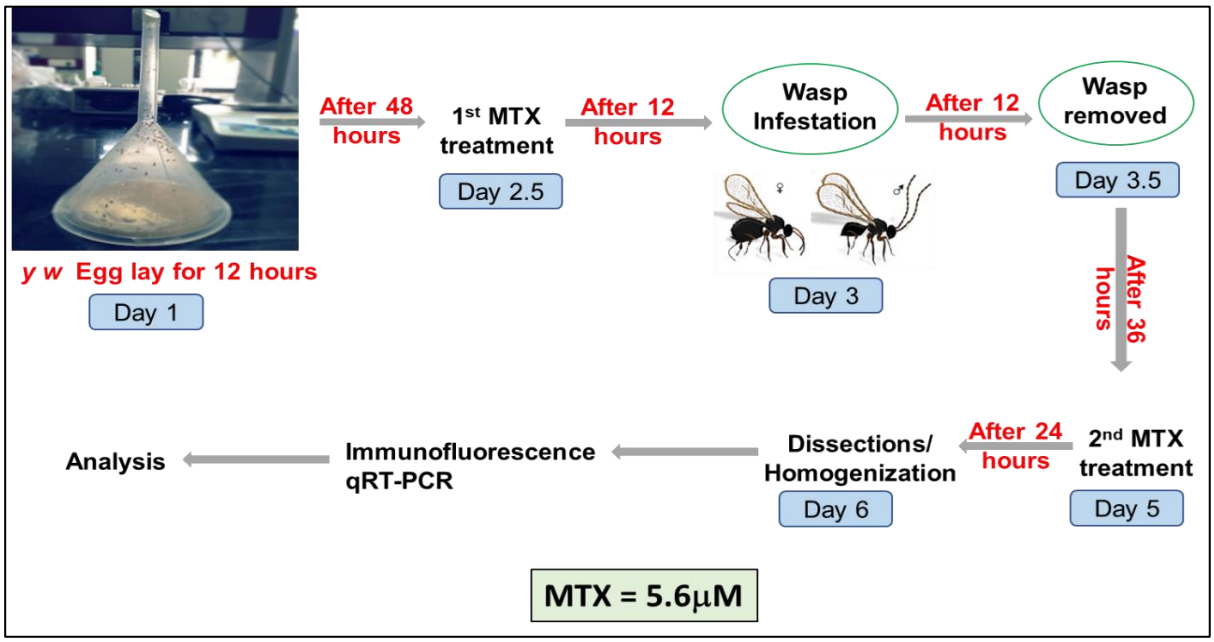


Figure 2.2 Methodology flow chart for MTX treatment in wasp infestation background

2.5 *Ubc9* stocks *Drosophila Ubc9* is encoded by an enzyme also known as lesswright (lwr) or semushi [125, 126] *y w; Ubc9^{5} FRT40/CyO y+* and *y w; Ubc9$^{4-3}$/CyO y+* (gift from Dr. Shubha Govind's laboratory, City College, CUNY) [125]. *Ubc9$^{4-3}$/Ubc9^{5}* trans heterozygote

mutants showed extended larval development where most of them died by day 10 as larvae [118].

2.6 *Ubc9$^{4-3}$/CyOTb, Ubc9^{5} FRT40/CyOTb* and *UAS-SPE Activated/CyOTb* stock preparation

For the ease of selection between homozygous lethal mutants (*Ubc9$^{4-3}$/Ubc9^{5} FRT40*) and heterozygote siblings (*Ubc9^{5} FRT40/CyO y+*, *Ubc9$^{4-3}$/CyO y+*) at larval stages, *Ubc9$^{4-3}$/CyOTb* and *Ubc9^{5} FRT40/CyOTb* strain was prepared by crossbreeding with the stocks carrying CyOTb marker (*CyOTb* marker stocks was a gift from Dr. Rakesh Kumar Mishra's lab, CCMB, Hyderabad). *CyOTb* marker carrying larvae are short and stout at larval stages compared to *Non-CyOTb* larvae. The homozygous mutant stock (*Ubc9$^{4-3}$/Ubc9^{5} FRT40*) showed both melanized and non-melanized tumor. Melanized tumor was visible due to black pigmentation while the non-melanized were transparent. Whereas *Ubc9$^{4-3}$/CyOTb* and *Ubc9^{5} FRT40/CyOTb* which did not show any defects were served as control larvae.

2.7 UAS line

UAS-SPE-Activated (amino acid 135–400 from B. Lemaitre [176], Bloomington line 7011 (obtained from Dr. Shubha Govind's laboratory). The *Ubc9* alleles were recombined or crossed into the Cg-Gal4 to produce *Ubc9^{5} Cg-Gal4, UAS-Cact-RFP//Ubc9$^{4-3}$*.

2.8 GAL4 lines

Cg-Gal4 (Cg is ubiquitously expressed in fat body, circulating hemocytes and in some cells of the lymph gland) [127] (gift from Dr. Shubha Govind's laboratory, City College, CUNY). Hemese-Gal4 (Hemese is expressed exclusively by blood cells and hematopoietic tissue) [177]. The UAS-SPE-Act alleles were crossed into the Cg-Gal4 or Hemese-Gal4 backgrounds to produce: *UAS-SPE-Act, Cg-Gal4/CyOTb* and *UAS-SPE-Act, Hemese-Gal4/CyOTb* respectively

2.9 Genetic rescue cross setup

we performed a genetic rescue of *Ubc9*$^{-/-}$ mutants by overexpressing Cactus, the inhibitor of the transcription factor, Dorsal. In *Ubc9* mutant background we overexpressed a wild type cactus protein (*UAS-Cactus-RFP*) using *Cg-Gal4* promoter. We setup the genetic rescue crosses and screened those larvae that were genetically rescued by comparing with their control siblings.

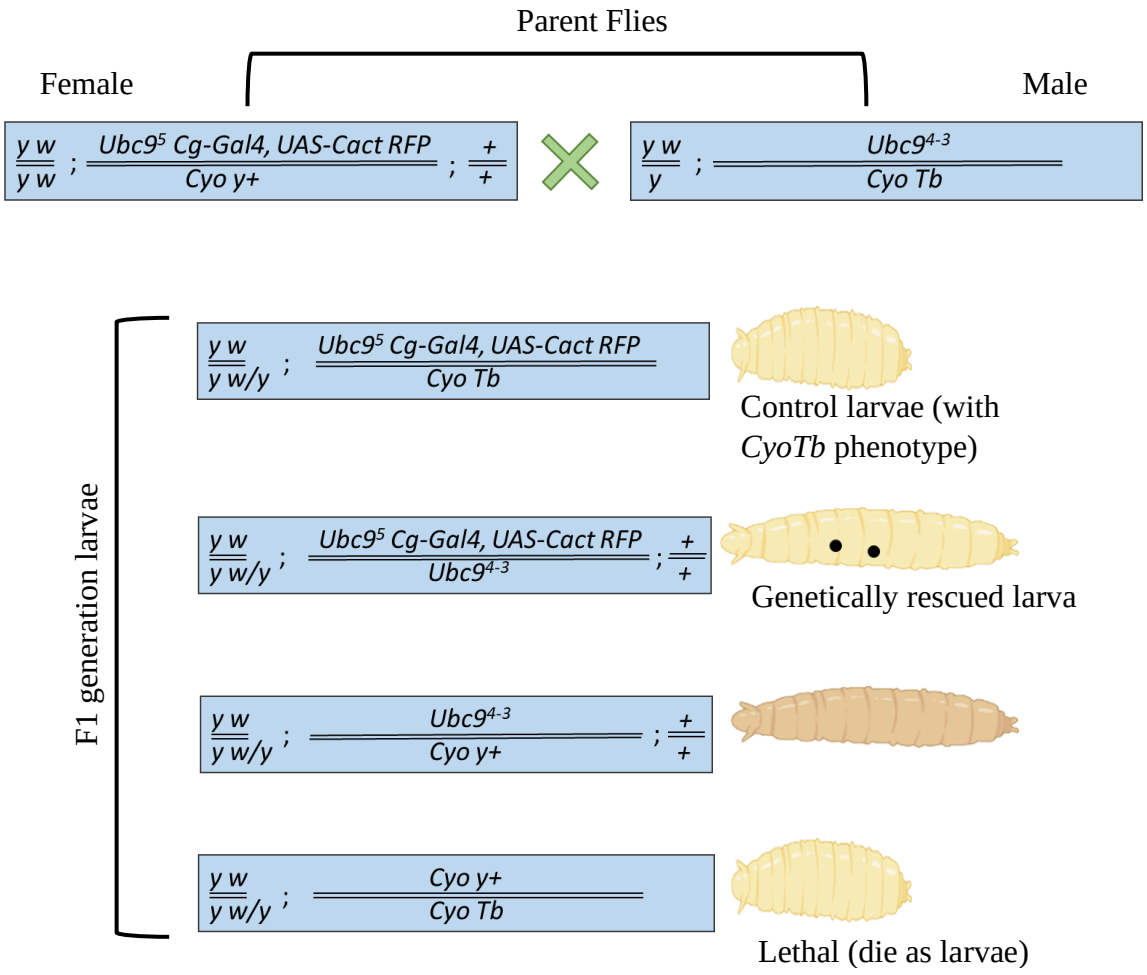


Figure 2.3 Set up of genetic rescue crosses.

Out of four probable progenies in the first generation, the genetically rescued larvae are picked up upon comparing with its control heterozygote siblings. The chosen rescued larvae are documented for further experimental analysis. Created in Biorender

***Ubc9*$^{-/-}$ experimental setup**

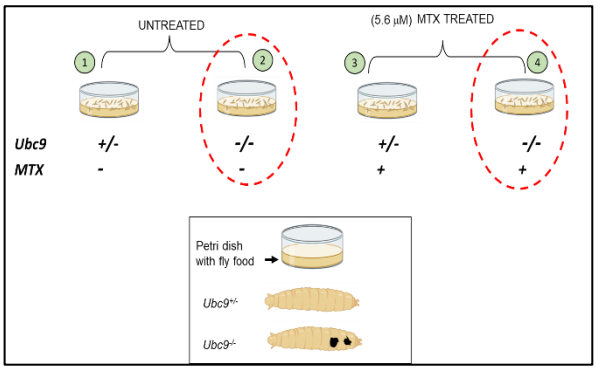


Figure 2.4 Schematic showing *Ubc9*$^{+/-}$ & *Ubc9*$^{-/-}$ in untreated and MTX treated Created in Biorender

***SPE* overexpression experimental setup**

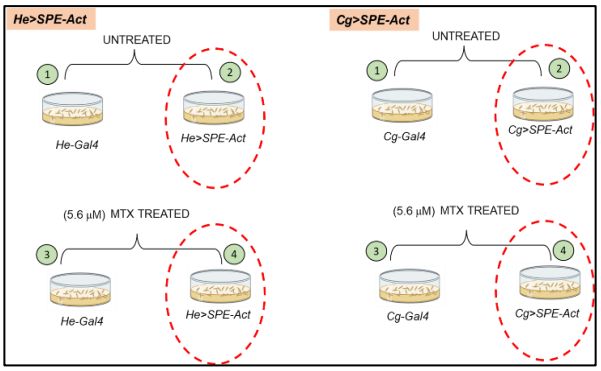


Figure 2.5 Schematic showing, *He>SPE-Act* & *Cg>SPE-Act* untreated and MTX treated background. Created in Biorender

2.10 Egg lay treatment by MTX in loss of function (*Ubc9*$^{-/-}$ mutant) and in overexpression (*Cg>UAS-SPE Act*; *He>UAS SPE Act*) background synonymous to chronic inflammation

Ubc9$^{4-3}$/*Ubc9*^{5} mutant flies, *He>SPE-Act* and *Cg>SPE-Act* flies were kept separately in an egg laying chamber for 24 hours (12h:12h dark and night cycle) to lay eggs (*Their respective control genotype larvae were also kept for egg lay in separate egg laying chamber for the same conditions as shown in the experimental setup*). After 36 hours, larvae were transferred to the petri dish containing fly food mixed with MTX drug (1ml of drug + 3ml of fly food). This was referred as First MTX treatment. After 60 hours, early 3rd instar larvae were transferred to petri dish containing fly food mixed with MTX drug (1ml of drug + 3ml of fly food). This was referred as Second MTX Treatment). The procedure is adapted and modified from our published article [174].

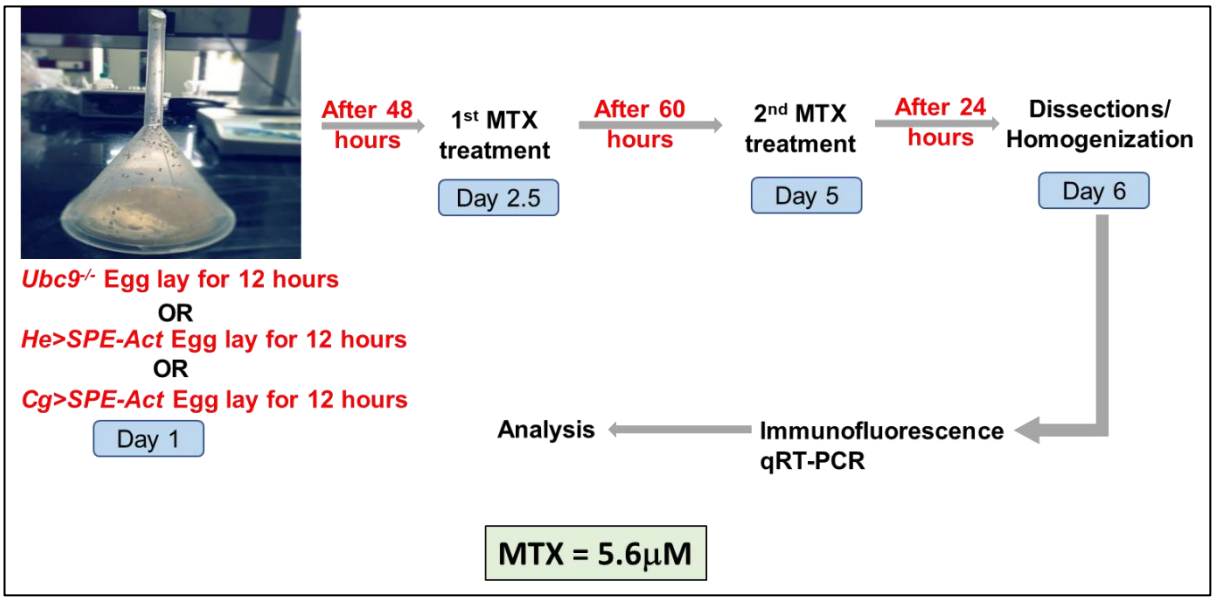


Figure 2.6 Methodology flow chart for MTX treatment in *Ubc9*$^{-/-}$, *He>SPE-Act* and *Cg>SPE-Act* background.

2.11 Tumor penetrance, tumor expressivity, tumor encapsulation and infiltration index

After second MTX treatment of larvae in loss of function and in overexpression background, the 3rd instar host larvae were dissected to score the total number of melanized tumor, visible under the cuticle, along with the non-melanized tumor. Fat body of the control and mutant larvae were dissected out as per the experimental setup. Dissected fat body was fixed, blocked and stained with the Alexa Fluor® 555 phalloidin along with the nuclear counter stain DAPI followed by mounting in 50% glycerol. The number of single blood cells adhered/fat body cell were documented with the help axial fluorescence microscope. Aggregates of blood cells on the fat body were omitted from documentation. Data was analysed by the GraphPad prism 8.0.2 tool and student t-test (unpaired) was utilized for statistical significance.

2.12 Immunostaining, microscopy, data collection and analysis

12-15 3rd instar developmentally synchronised larvae were dissected for fat bodies, hemolymph and lymph gland after cleaning with 1X PBS, double distilled water, 70% EtOH and again with 1XPBS to avoid contamination. Samples were fixed with 4% paraformaldehyde (PFA) at room temperature for 15 min. Fixed samples were given quick washes with 1X PBS followed by 30 min of incubation with 3% Bovine Albumin Serum with 1XPBS and incubated overnight (at 4°C)

with primary antibodies in 1X PBS+ 3%BSA+ 0.2% Triton X-100 (mouse monoclonal anti-dorsal (74A, 1:10; Developmental Studies Hybridoma Bank (DSHB), Biogenuix Medsystem Pvt Ltd; [9]), mouse monoclonal anti-cactus (3H12, 1:10, DHSB, Biogenuix Medsystem Pvt Ltd; [9]), lamellocyte-specific mouse monoclonal anti-L1/Atilla (1:100, gift of Dr. Istvan Ando, [10]), plasmatocyte-specific mouse monoclonal anti-Nimrod C (1:100, gift of Dr. Istvan Ando, [10]), rabbit polyclonal anti-relish (sc-26912, were kindly provided by Dr. Rajendra Chilukuri ,1:10, SCBT), and rabbit polyclonal anti-Phospho-Histone H3 ((ser10), #9701, 1: 600, Cell Signalling Technology (CST). Washed samples were incubated for 3 hrs 45 min at room temperature with commercially available secondary antibody conjugated with Alexa Fluor®488-anti mouse IgG (#4408, 1:200, CST), and/or Alexa Fluor®488-anti rabbit IgG (#4412, 1:200, CST), and /or Alexa Fluor®555-anti-mouse IgG (#4409, 1:200, CST) and/or Alexa Fluor®555-anti-rabbit IgG (#4413, 1:200, CST). Samples were washed twice with 1XPBS and once with 1XPBS+ 0.2% Triton X-100 (1XPBST). Samples were incubated overnight (at 4°C) with Alexa Flour® 488 Phalloidin (#8878, 1:20, CST) and Alexa Flour® 555 Phalloidin (#8953, 1:20, CST) for polymerized F-actin staining. Following day after three washes the samples were counterstained for 15 min at room temperature with the nuclear dye DAPI (#4083, 2μg/ml, CST) or Hoechst 33342 (#4082, 2.5 μg/ml, CST) as the final step for staining procedure. Final slides were mounted in 50% glycerol. Imaging was performed using Carl Zeiss Laser scanning confocal microscope (LSM710) in 20X and 40X (with immersion oil) objectives. Raw images (CZI files) were taken and processed into final images (TIF files) by using ZEN 3.1(Zen lite) blue edition software. Identical settings were used for obtaining the control and experimental images.

2.13 Pixel Quantification

The intensity of the fluorescent signal (pixels) was quantified after anti-Cactus antibody staining was done in ZEN 3.1(Zen lite) blue edition software. More than 300 cells from 12 larvae were analysed for each experimental setup. A student-t test (unpaired) analysis was performed for statistical significance by using GraphPad Prism 8.0.2. Controls were normalized and only two experimental setups were compared for better understanding in both acute and chronic inflammation.

2.14 Oil red staining for triglycerides

8-10 larvae were thoroughly washed with 1XPBS. Entire fat body was gently taken out. Following treatments as indicated, the fat body tissues were washed twice with (1XPBS) and fixed in 3.7% formaldehyde for 1 h at room temperature. After aspiration of the formaldehyde, the cells were stained with Oil Red O (#23576, SRL Pvt Ltd.) for 1 h. Oil Red O was prepared by dissolving 0.5 g Oil Red O in 100 ml 2-propanol and diluting it with water (6:4), followed by filtration. Stained tissue was washed gently in PBS. Later the slides were imaged using the 20X and 40X lens of Carl Zeiss Laser scanning confocal microscope (LSM710).

2.15 Sample preparations, RNA isolation and real time-PCR

More than 50 3rd instar developmentally synchronised larvae were washed and proceeded for RNA Isolation (TRIzol method, Invitrogen, Carlsbad, CA). RNA concentration was quantified by Nanodrop 2000 spectrophotometer (Thermo Scientific). Approximately 2.5µg RNA was taken as template for kit-based cDNA synthesis ((Iscript TM; Bio-Rad Laboratories, Hercules, CA). Real-time qPCR was performed running the standard step-one plus PCR program: 1.0µl of the cDNA sample was mixed KAPA SYBRR FAST Universal (KAPA Biosystems, Lot # 006255-8-1) and primers to set up a 10µl reaction mix. Transcript levels detected were normalized to Rp49 mRNA values. Primer's sequence used for real-time PCR.

2.16 Statistics

All the documented data from the control and experimental samples were analysed for statistical significance. To compare the characteristics between two groups of cohorts, we utilized Student's t-test (unpaired, two-tailed). Graphs representing the error bars show ± SEM. In all cases $p > 0.05$ (shows no significance), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). Biological repeats (N), sample size (n) and student's t-test results are mentioned in the figure legends section. All the graphical data was analysed using GraphPad Prism software (version 8.0.2).

Table 2.1. Primers used in current study

QRT-PCR primers **Primer sequence (5'-3')**

Gene	**Forward primer sequence**	**Reverse Primer sequence**
Rp49	GACGCTTCAAGGGACAGTATCTG	AAACGCGGTTCTGCATGAG
Cyclin A	TCAGCGTGGGCACTGAAACGG	GGGCGATGTTTCTTCTCGCTC TCC
Cyclin B	GCCGAGGACGAGCACCATACG	GTGAGGCAGCTGCAATCTCCGA
Cyclin D	CAGCTTGCCTCTTACTGGCT	ACACTGCTCCCTTGCCATAC
Dacapo	CCCGAGTCCTGAATCCTGTG	TGGAGCTACCGAAGAGGTCA
Spätzle	GGAGCGGATCAACCCTGTG	TTGGATTATAGCTCTGCGGAAAG
SPE	CTTTTCGCTGATCGCATTTT	CACCGGATTTGTCCAGTTCT
Cactus	CTGCTCAACATCCAGAACGA	GCCGAACTTCTCTGTCAAGG
Drom	ATCCTGAAGTGCTGGTGCGAAGGA	ACGTTCATGCTAATTGCTCATGG
dilp6	TGCTAGTCCTGGCCACCTTGTTCG	GGAAATACATCGCCAAGGGCCACC
InR	GACGATGGCTACCCGATG	GCCTCCAATCAGGAAGATC
Chico	GCAAGTTGTCATTCAA	ATCCCAAGACACTTTG
Dp110	GTCCACCTCCACAAGTCGAT	TGTGCAGCGTCAACTGAAAG
dFOXO	GGATGCGGAGTCGATGTCTT	CCCTTTATCCCAAATATGATGCCT
bmm	AATGGCGTCGAATCAGACTT	AACACAGATGGGGATTTGGA
msn	AAGGTGGGTCTCCGCAAATC	ATCAACCGCATGGAAACCCT

3. Objective 1: To study the effect of Methotrexate (MTX) on parasitoid wasp (*Lb-17*) infestation triggered Toll/NF-κB Pathway, in the immune tissues of wild type 3rd instar larvae

3.1. Introduction

Parasitoid wasps lay their eggs in fly larvae or pupae prey on *Drosophila* fruit flies. More than 50% of fruit fly hosts in certain populations are affected, indicating a high selection pressure from the wasps [178]. To eliminate the wasp eggs development, fruit flies mount a physiological immune response known as melanotic encapsulation response[179]. The host's blood cells encapsulate and suffocate the parasite egg's growth in this reaction [118]. But to prevent the fruit flies' immune response, female wasps inject their eggs (100µm), via their ovipositor, which compromises the effectiveness of the host defence mechanism [118]. The fruit fly perishes as a result of the wasp larva eating the host alive. *L. boulardi*, a species of *Leptopilina*, is regarded as a particular type of parasitoid that typically employs *D. melanogaster* as a host. Following infection by *Lb17* (a strain of *L. boulardi*), *D. melanogaster* significantly upregulates several classical immune pathways such as Toll, JAK/STAT, and PO pathways [180]. Also, Toll pathway mutations are used in functional genetic research to show that the Toll pathway is required for the melanotic encapsulation immunological response [181]. This information emphasizes the continuing evolutionary competition between parasitoid wasps and *Drosophila* fruit flies, where the flies evolve immunological defences to thwart the wasps' attempts to conquer them. This encapsulation reaction allows the host to resume its development.

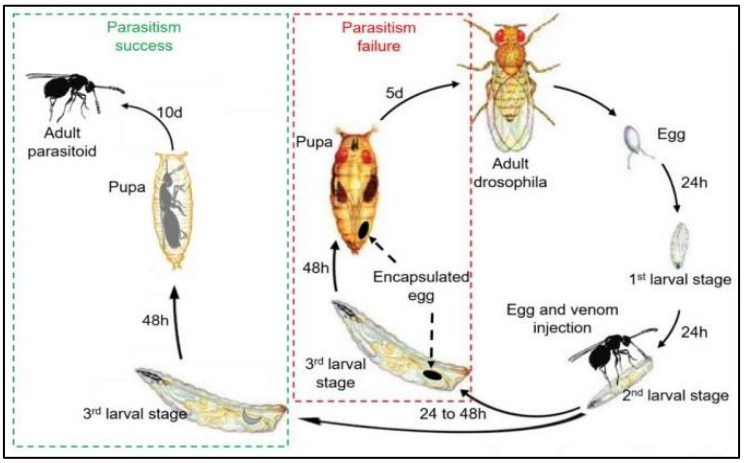


Figure 2.7 *D. melanogaster* and *L. boulardi* life cycle

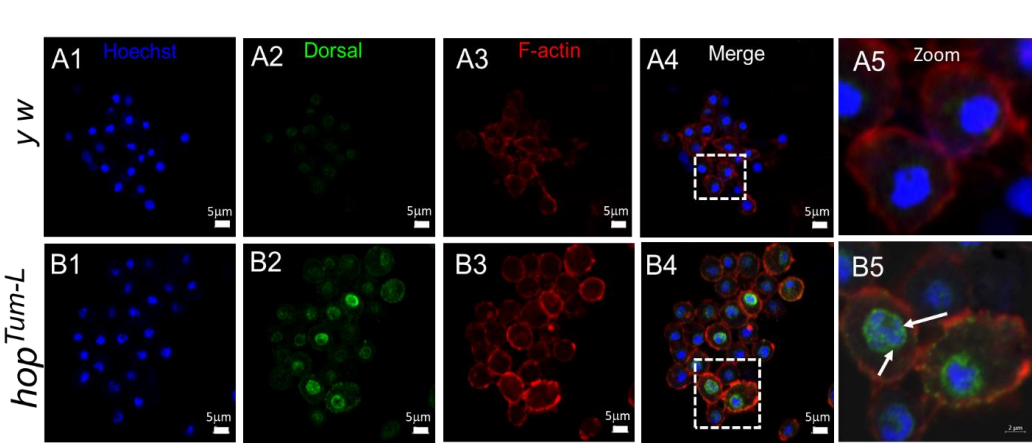
Adapted from Carolina Biological supply company

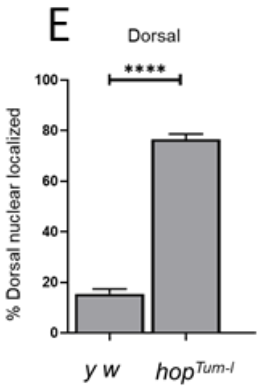
3.2 Result

3.2.1 Status of Toll/NF-κB Pathway in hyperactive JAK/STAT Pathway mutant (*hop*$^{Tum\text{-}l}$) after MTX treatment

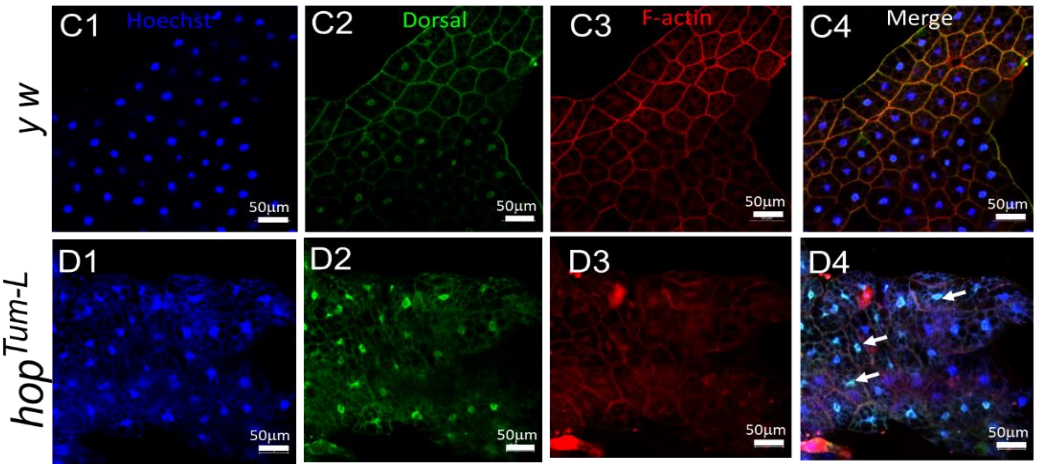
Our earlier research [174] showed the anti-inflammatory effects of MTX on ectopic JAK/STAT pathway activation compelled by wasp infection. In the present investigation, we first discussed the status of the Toll/NF-κB pathway in *hop*$^{Tum\text{-}l}$, a hyperactive JAK/STAT gain-of function mutant. Toll (the transmembrane receptor), Spätzle (the ligand), Spätzle Processing Enzyme (SPE), Myd88 (the adaptor protein), Pelle (the kinase), Tube (the kinase), Cactus (the NF-κB inhibitor), Drosomycin (the readout of the pathway), and Dorsal (the transcription factor) are some of the components of the Toll pathway. To ascertain the status of the Toll pathway's activation, we tested *Spätzle*, *SPE*, *Cactus*, *Drosomycin*, and Dorsal. In the blood cells of *hop*$^{Tum\text{-}l}$ mutants, transcription factor Dorsal's (a homolog of human NF-κB) expression is witnessed both in cytoplasm and nuclear as well (Figure 3.1 B4-B5) and the fat body (Figure 3.1 D4-D5) unlike the wild type *y w* in which it is primarily cytoplasmic (Figure. 3.1 A4-A5, Figure 3.1 C4-C5). We did the statistical counts of Dorsal colocalization with the DAPI nuclear counter stain and found that more than 70% cells showed Dorsal nuclear localization (Figure 3.1 E). Nuclear localization of Dorsal indicates that the Toll pathway is active in *hop*$^{Tum\text{-}l}$ mutants. To further confirm the pathway status, we studied the transcript levels of different components i.e., *Spätzle* (ligand), *Spätzle Processing Enzyme* (*SPE*), *Cactus* (negative regulator) and *Drosomycin* (read out) of the Toll pathway (Figure 3.1 F-I). Consistent with a published microarray dataset, the expression of these genes, was higher in *hop*$^{Tum\text{-}l}$ mutants when compared to wild type, *y w* (Figure 3.1 E, Figure 3.1 F). Our observations indicate that Toll pathway is active in the gain of function mutants of *hop*$^{Tum\text{-}l}$. Therefore, we next wanted to study the effect of MTX on Toll pathway.

BLOOD CELLS





FAT BODY



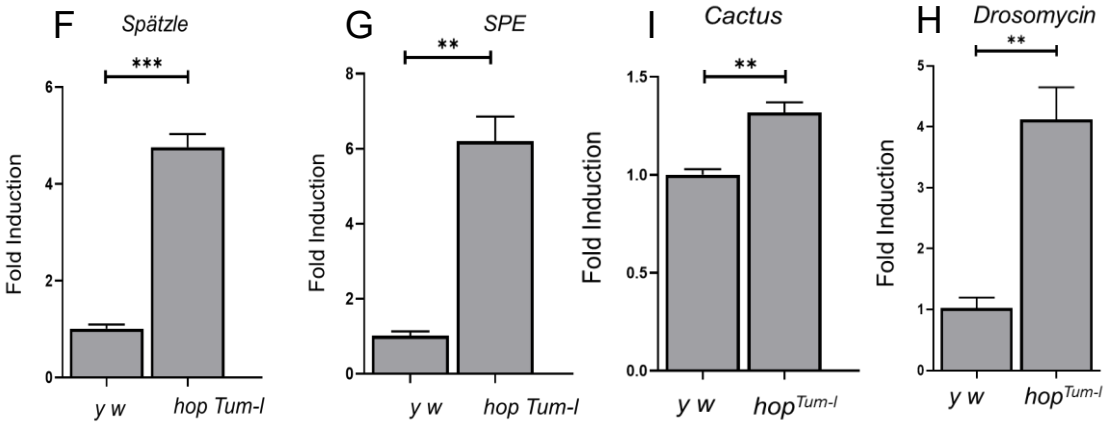


Figure 3.1 Status of Toll/NF-κB pathway in hyperactive JAK/STAT GOF mutant larvae *hop^{Tum-l}*

Dorsal localization in the circulating blood cells of 3rd instar *y w* larvae stained with Hoechst (nuclear stain, blue), Anti-dorsal (dorsal specific antibody, green), polymerized F-actin (cytoskeleton, red).

A1-A5 circulating blood cells in *y w* control

B1-B5 circulating blood cells in *hop^{Tum-l}* larvae with dorsal more of nuclear localized (**Zoom B5**, white arrow indicating) compared to the blood cells in *y w* (**Zoom A5**).

N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Dorsal localization in the fat body cells of 3rd instar *hop^{Tum-l}* larvae stained with Hoechst (nuclear stain, blue), Anti-dorsal (dorsal specific antibody, green), polymerized F-actin (cytoskeleton, red).

C1-C4 Dorsal localization in the fat body cells of *y w* control.

D1-D4 Dorsal localization in the fat body cells of *hop^{Tum-l}*. Dorsal appeared to be more nuclear localized in the fat body cells of *hop^{Tum-l}* (**D4**, white arrow indicating) compared to the fat body cells of *y w* (**C4**). N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

E Graphical representation of % of dorsal nuclear localized in the blood cells of *y w* vs. *hop^{Tum-l}*. Error bars show the standard error mean. Student's t-test (unpaired, two-tailed) showed statistical significance $p<0.0001$(****), N=3, n=30.

F-I Bar graph showing gene expression of *spätzle* (ligand), *SPE* (positive regulator), *Cactus* (negative regulator) *Drosomycin* (read out) components of Toll/NF-B pathway, in the background of JAK/STAT gain of function mutant larvae *hop^{Tum-l}*. Student's t-test showed statistical significance $p < 0.001$(***) & $p<0.01$ (**) on comparison with *y w* control. Graphs was processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C. Bars represent the mean (±SEM) from three independent experiments.

3.2.2 MTX inhibits the *Lb-17* wasp infestation triggered encapsulation response in the immune tissues of wild type *y w* larvae

We utilized the wild type *y w* larvae (Figure 3.2A) and larvae infected with *Lb-17* parasitoid wasps (Figure 3.2B) to study wasp infestation penetrance, expressivity and encapsulation response before and after MTX treatment. We set up four experimental groups; First group represents the untreated in which *y w* larvae without *Lb-17* wasp infestation [**MTX (-) Wasp (-)**] and second group represents untreated *y w* with *Lb-17* wasp infestation [**MTX (-) Wasp (+)**]. Third group represents the MTX treated *y w* larvae without *Lb-17* wasp infestation [**MTX (+) Wasp (-)**] and the fourth group represents the MTX treated *y w* larvae along with *Lb-17* wasp infestation [**MTX (+) Wasp (+)**]. In order to decipher our results, we compared both qualitatively and quantitatively obtained results with the second experimental setup (only infection) condition with the fourth experimental setup (Infection plus MTX treatment). First and foremost, we checked the infestation capacity of the *Lb-17* wasps and found that each host larvae were successfully infected by the wasp implying that there was 100% wasp penetrance with and without MTX treatment (Figure 3.2C). Furthermore, the extent of wasp infestation (wasp infestation expressivity) was investigated i.e., checking and documenting the number of parasitoid bodies per host larvae and to our surprise there was an increase in the number of parasitoid bodies per host larvae after MTX treatment compared to the host larvae that underwent only infection (Figure 3.2D). The appearance of melanised parasitoid bodies (Figure 3.2H**)** were significantly less after MTX treatment (Figure 3.2E). Post MTX treatment we did observe parasitoid bodies that were unencapsulated (Figure 3.2I). and the larval host counter immune response (encapsulation) against the increased parasitoid bodies post MTX treatment was surprisingly inhibited (Figure 3.2F). Overall, there was a 43.10% significant reduction in the encapsulation response upon MTX treatment when compared to 68.06% encapsulation in the untreated *Lb-17* infected background (Figure 3.2F).

We also checked the transcript levels of *SPE*, the positive regulator, *Cactus* (human homologue of IκB) the negative regulator and the target, *Drosomycin* (anti-microbial peptide). Our results did not indicate any significant differences in the transcript levels of untreated and MTX treated wild type larvae. *Lb-17* infected larvae that underwent MTX treatment displayed

downregulation of Toll pathway since the transcript levels of the *SPE* (1.1-fold) and *Drosomycin* (0.94-fold) were lower compared to untreated infected larvae (*SPE* 5.34-fold and *Drosomycin* 6.55-fold) (Figure 3.2J and Figure 3.2L), and cactus whose expression was 3.31-fold in the infected background without MTX treatment, was elevated to 5.27-fold upon drug treatment (Figure 3.2K).

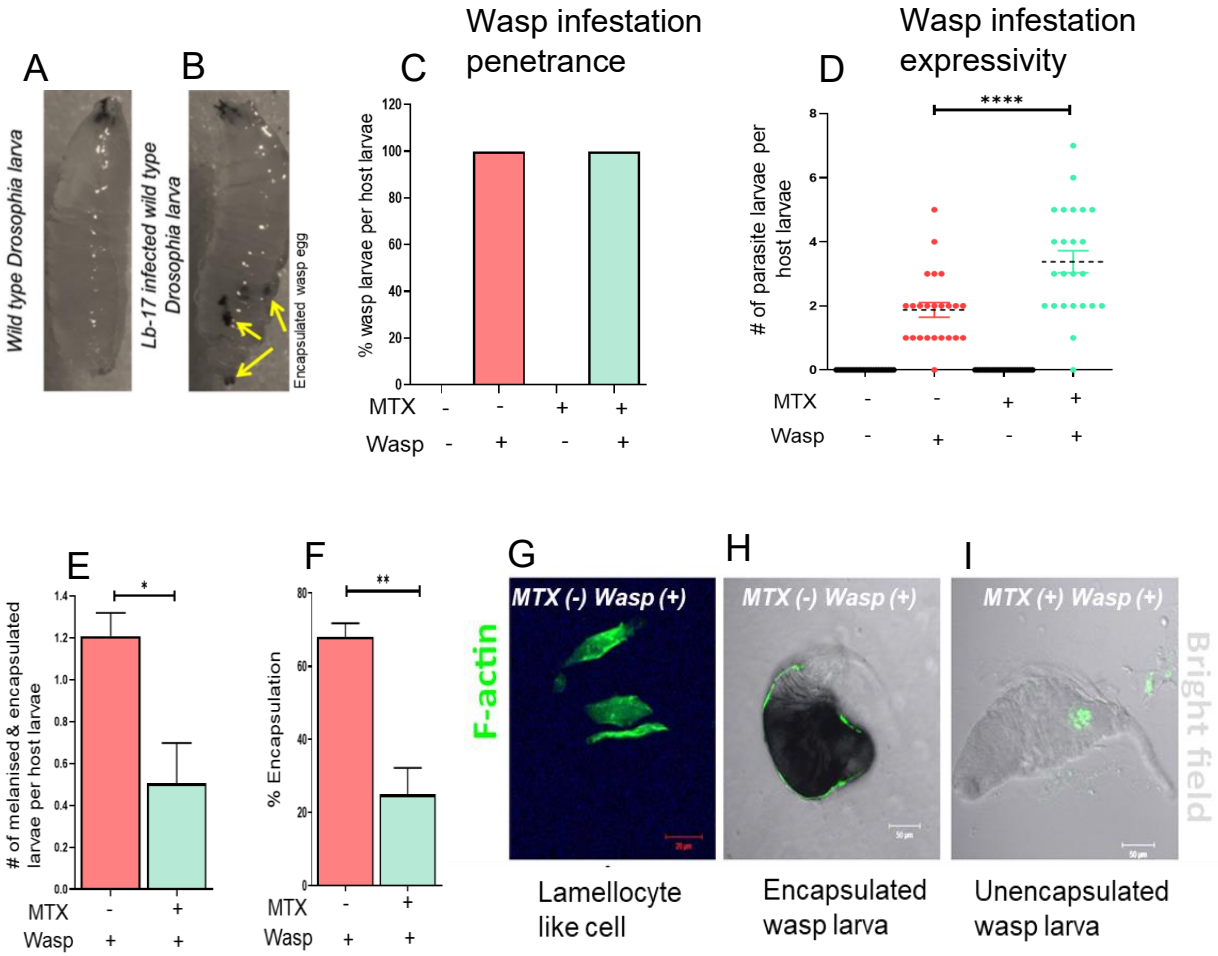


Figure 3.2 Effect of MTX on wasp induced encapsulation response A: Wild type *y w* 3rd instar larvae. **B:** *Lb-17* Infected wild type *y w* larvae. **C:** Graphical representation of % of wasp infestation penetrance (n=50). **D:** Graph showing the wasp infestation expressivity (n=24, ****p<0.0001). **E:** Graph showing the wasp infestation expressivity of melanised and encapsulated parasitoid bodies (n=30, *p<0.0339). **F:** Graphical representation of % of encapsulation response in 3rd instar larvae (n=25, **p<0.0060). **G:** Confocal image showing the lamellocyte like cells (n=12). **H:** Encapsulated and melanised wasp parasite larvae. I: Unencapsulated wasp parasite. Bar graph showing gene expression of **(J)** *SPE* (positive regulator), **(K)** *Cactus* (negative regulator) and **(L)** *Drosomycin* (read out) components of Toll/NF-B pathway. Student's t-test (unpaired, two-tailed) showed statistical significance p<0.05 (*), p<0.01(**), and p<0.0001 (****). Bars represent the mean (±SEM) from three independent experiments. N=3, n=50.

3.2.3 MTX inhibits the nuclear localization of Dorsal in the immune tissues (blood cells and fat body) of wild type *y w* larvae post infestation

We next studied Dorsal (transcription factor) protein expression in both the immune tissues i.e., the blood cells and the fat body postinfestation by the wasp *Lb-17* and as observed in earlier studies it was evidently nuclear localized (Figure 3.3 B3, Figure 3.3 F3) compared to uninfected *y w* samples with (Figure 3.3 C3, Figure 3.4 G3) or without MTX treatment (Figure 3.3 A3, Figure 3.3 E3). This result appeared to be reversed upon treatment with MTX, with fewer cells showing nuclear localization (Figure 3.3 D3, Figure 3.3 H3). To corroborate our qualitative result, we performed the quantification of dorsal nuclear localization specifically in the cells of fat body. We found that 93.85% fat body cells showed dorsal colocalization with nuclear stained DAPI in the *Lb-17* infestation background without any drug treatment while MTX positive parasitoid infected larvae showed only 15.53% colocalization (Figure 3.3 I). Also, our study clearly showed that MTX did not alter the localization of dorsal in the wild type larvae even after MTX treatment.

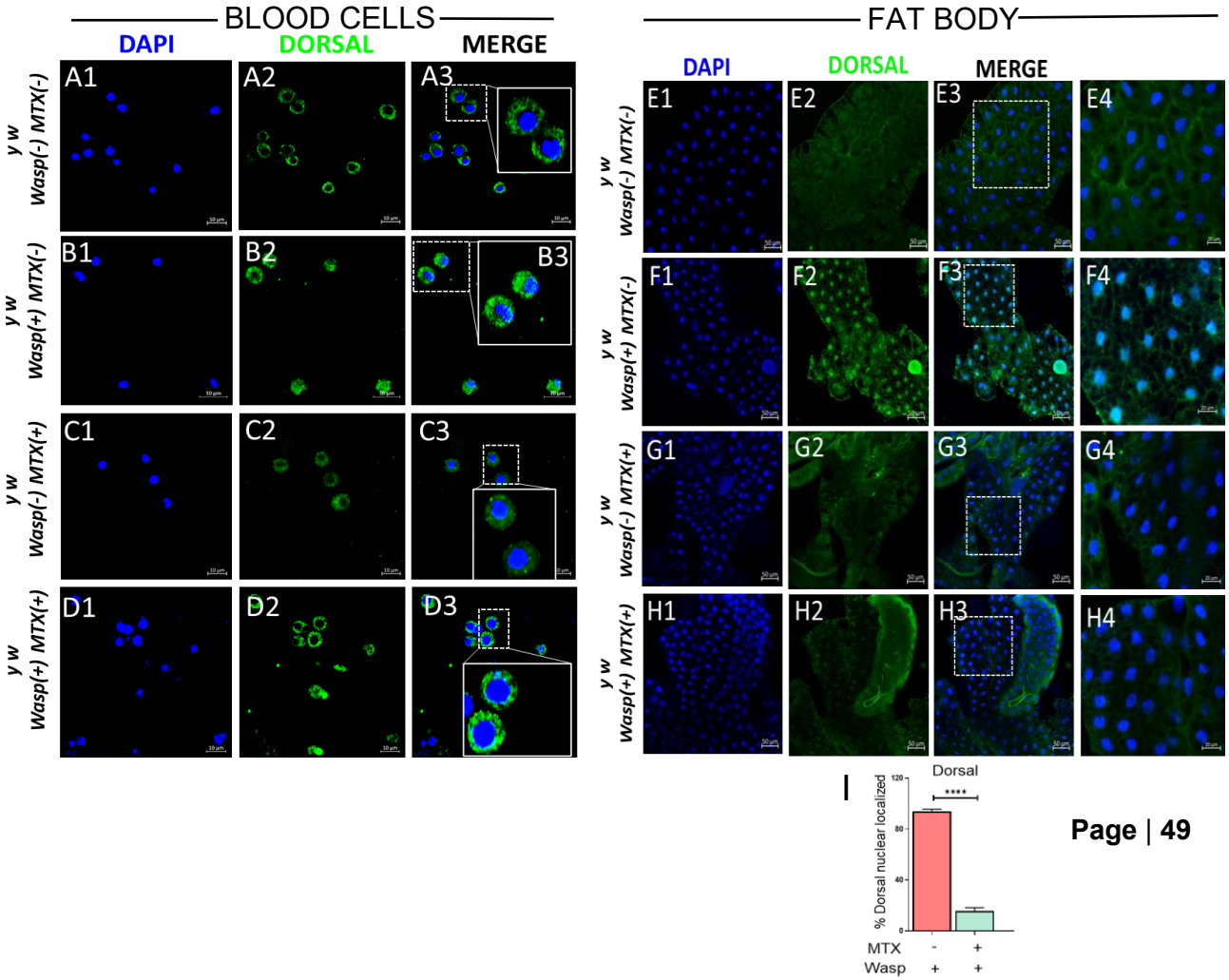


Figure 3.3 Effect of MTX on Toll pathway induced by *Lb-17* parasitoid wasp infestation

Dorsal localization with and without MTX treatment in the circulating blood cells of *y w* 3rd instar larvae induced by *Lb-17* parasitoid wasp infestation stained with DAPI (nuclear stain, blue), Anti-dorsal (dorsal specific antibody, green).

A1-A3 *y w* control untreated and uninfected.

B1-B3 *y w* only wasp infected.

C1-C3 *y w* only drug treated

D1-D3 *y w* infected and drug treated.

N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Dorsal localization with and without MTX treatment in fat body cells of *y w* 3rd instar larvae induced by *Lb-17* parasitoid wasp infestation stained with DAPI (nuclear stain, blue), Anti-dorsal (dorsal specific antibody, green).

E1-E4 *y w* control untreated and uninfected

F1-F4 *y w* only wasp infected

G1-G4 *y w* only drug treated

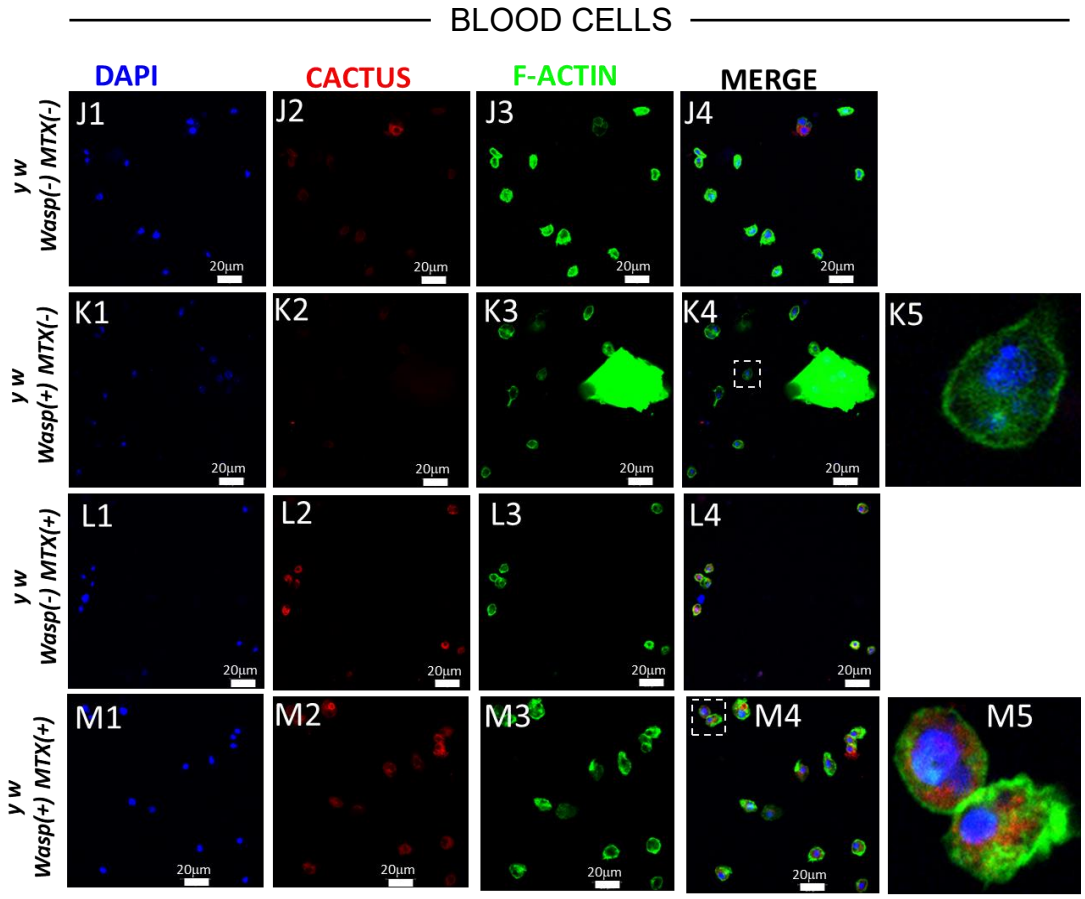
H1-H4 *y w* infected and drug treated

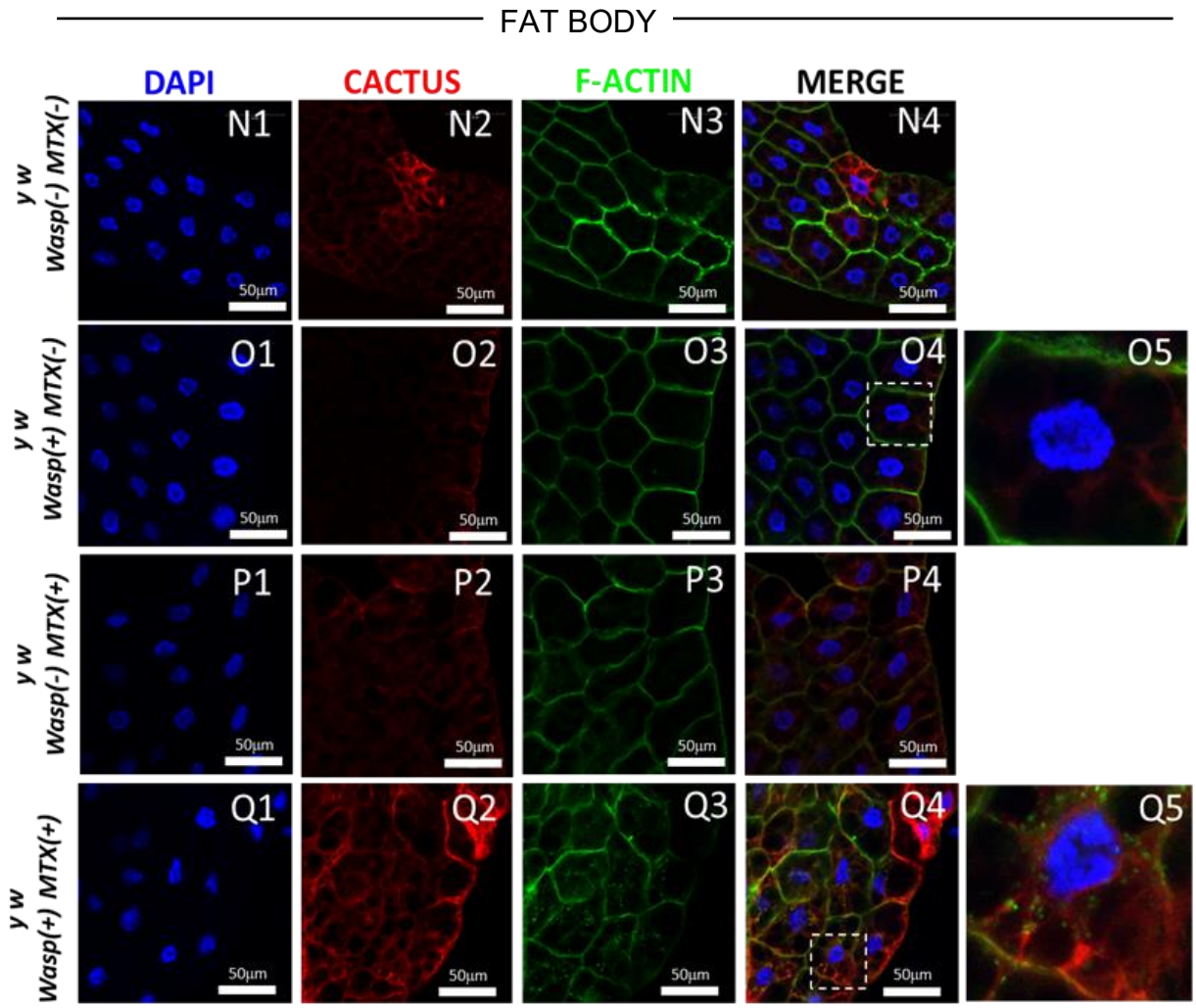
N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

I Graphical representation of % of dorsal nuclear localized wasp infected vs. MTX treated wasp infected. Error bars show the standard error mean. Student's t-test (unpaired, two-tailed) showed statistical significance $p<0.0001$(****), N=3, n=30

3.2.4 MTX inhibits the Cactus protein in both the immune tissues post wasp infestation

Concurrently we also observed the expression of cactus protein in both the immune tissues. Wild type control samples that were uninfected, with (Figure 3.4 L1-L4, Figure 3.4 P1-P4) or without MTX treatment (Figure 3.4 J1-J4, Figure 3.4 N1-N4) showed basal level of expression of cactus protein suggesting that cactus levels did not appear significantly different compared to only infected samples (Figure 3.4 K1-K5, Figure O1-O5). The expression of cactus protein increased in the wasp infected MTX treated sample (Figure 3.4 M1-M5, Figure 3.4 Q1-Q5). Pixel quantification of the fat body cells with anti-cactus antibody indicated that cactus expression in *Lb-17* infected larvae was 20.55% (Figure 3.4 R). Whereas, upon treatment with MTX in the infected background the cactus expression was found to be 32.62% in the fat body (Figure 3.4 R). Put together these results suggest that drug treatment markedly suppressed Toll pathway by affecting the nuclear localization of the dorsal transcription factor, decreasing the transcript levels of the positive regulators *Spz* and *SPE*.




FAT BODY
DAPI
CACTUS
F-ACTIN
MERGE
y w Wasp(-) MTX(-)
y w Wasp(+) MTX(-)
y w Wasp(-) MTX(+)
y w Wasp(+) MTX(+)
N1
N2
N3
N4
O1
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Q5
50µm

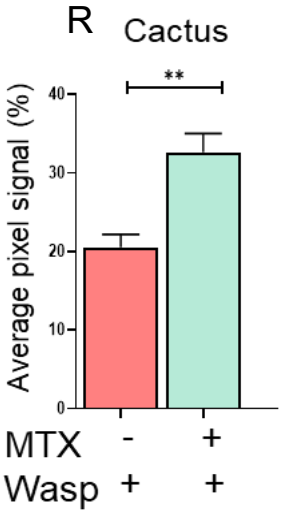

R
Cactus
**
Average pixel signal (%)
40
30
20
10
0
MTX - +
Wasp + +

Figure 3.4 Effect of MTX on the negative regulator of the Toll Pathway, Cactus, localization in the immune tissues post wasp infestation

Status of Cactus protein in the circulating blood cells of *y w* 3rd instar larvae, with and without MTX treatment, induced by *Lb-17* parasitoid wasp infestation stained with DAPI (nuclear stain, blue), Anti-cactus (cactus specific antibody, red), polymerized F-actin (Cytoskeleton, green).

J1-J4 *y w* control untreated and uninfected

K1-K5 *y w* only wasp infected

L1-L4 *y w* only drug treated

M1-M5 *y w* infected and drug treated

N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Status of Cactus protein in the fat body cells of *y w* 3rd instar larvae, with and without MTX treatment, induced by *Lb-17* parasitoid wasp infestation stained with DAPI (nuclear stain, blue), Anti-cactus (cactus specific antibody, red), polymerized F-actin (Cytoskeleton, green).

N1-N4 *y w* control untreated and uninfected

O1-O5 *y w* only wasp infected

P1-P4 *y w* only drug treated

Q1-Q5 *y w* infected and drug treated

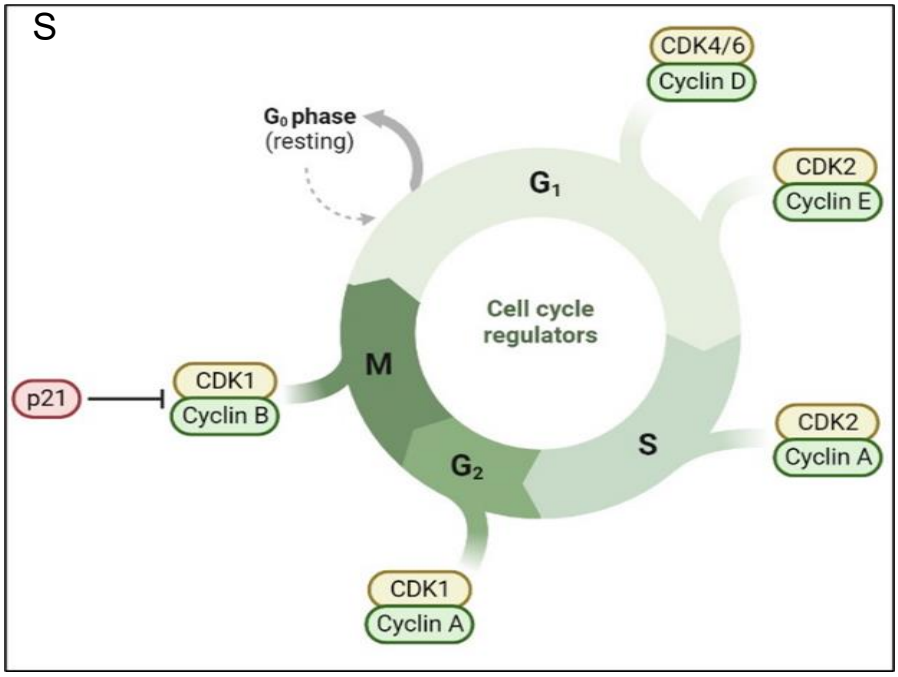
N=3, n=12.Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

R Average Pixel signal of Cactus stain compared between untreated wasp infected and MTX treated wasp infected. Student's t-test (unpaired, two-tailed) showed statistical significance $p<0.01$(**), N=3, n=30.

3.2.5 MTX inhibits the actively proliferating blood cells by inhibiting cell cycle regulators

Since Toll pathway is essential for blood cell proliferation and as drug treatment resulted in decreased encapsulation response we speculated if MTX had any effect on cell cycle regulators considered as potential therapeutic targets in developing cancers [182]. In the current study we evaluated in both the experimental and control backgrounds, the transcript levels of three positive regulators, *Cyclin A*, *Cyclin B*, and *Cyclin D*, that promote cell progression to next phase and one negative regulator, *Dacapo (p21)* that is an inhibitor of cell cycle dependent kinase as depicted in the schematic (Figure 3.5 S). We compared the wasp infected *y w* larvae with and without MTX treatment and found that the transcript levels of these regulatory molecules were affected upon MTX treatment. We observed an upregulation of the three cyclins [*Cyclin A* (9.17-fold), *Cyclin B* (7.54-fold), *Cyclin D* (8.77-fold)] in the wasp infected background (Figure 3.6 T1, Figure 3.5 T2, Figure 3.5 T3) in contrast to *Dacapo* (*p21*) which was downregulated (0.25-fold) (Figure 3.5 T4). The mRNA expression of three different cyclins and the negative regulator, *Dacapo* (*p21*), showed no significant difference in the *y w* controls even with MTX treatment unlike in the wasp infected MTX treated larvae where the expression of all the three cyclins significantly went down [*Cyclin A* (7.31-fold), *Cyclin B* (4.72-fold), *Cyclin D* (7.02-fold)] while *Dacapo* (*p21*) expression was elevated (8.55-fold) (Figure 3.5 T1-T4).

Since cyclins play an essential role in cell cycle progression we tested if MTX treatment affected mitotically active cells. Immunostained blood cells were analysed for phospho-histone-3 (PH3) expression in MTX treated blood cells of wasp infected larvae (Figure 3.5 X1-X5) and were compared to the blood cells from untreated wasp infected larvae (Figure 3.5 U1-U5. MTX treatment resulted in decreased mitotic index to when compared to untreated infected conditions. Of the total blood cells post infestation, 87.30 % of blood cells were phospho-histone-3 positive in untreated larvae whereas MTX treatment significantly reduced the dividing cells to 61.50% (Figure 3.5 Y). Collectively these findings support the notion that MTX hinders blood cell proliferation thereby decreasing the encapsulation response dependent on Toll/NF-κB pathway.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CDK4/6
Cyclin D
G₀ phase
(resting)
G₁
CDK2
Cyclin E
Cell cycle
regulators
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p21
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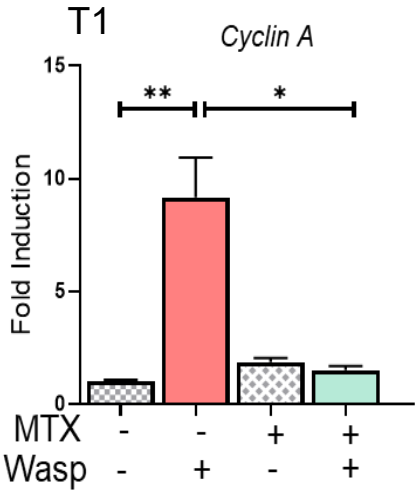
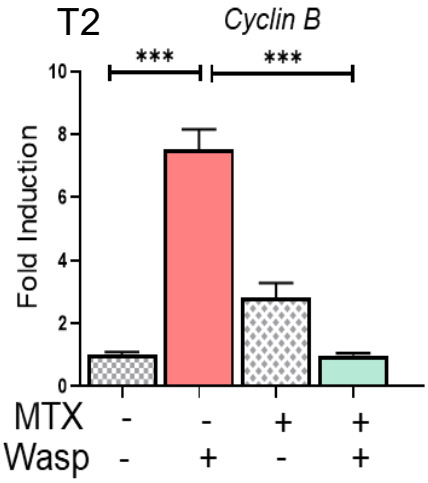
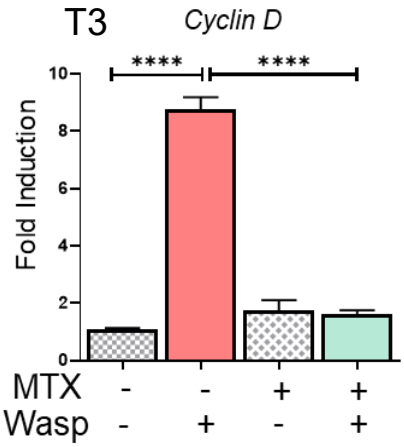
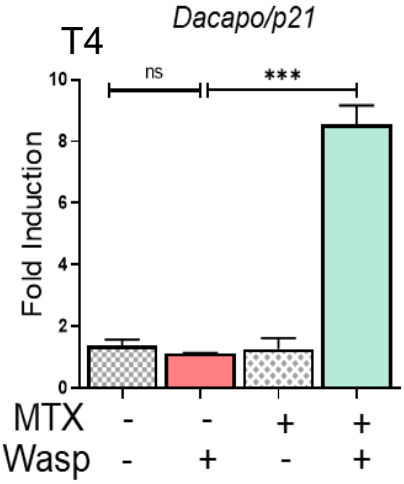
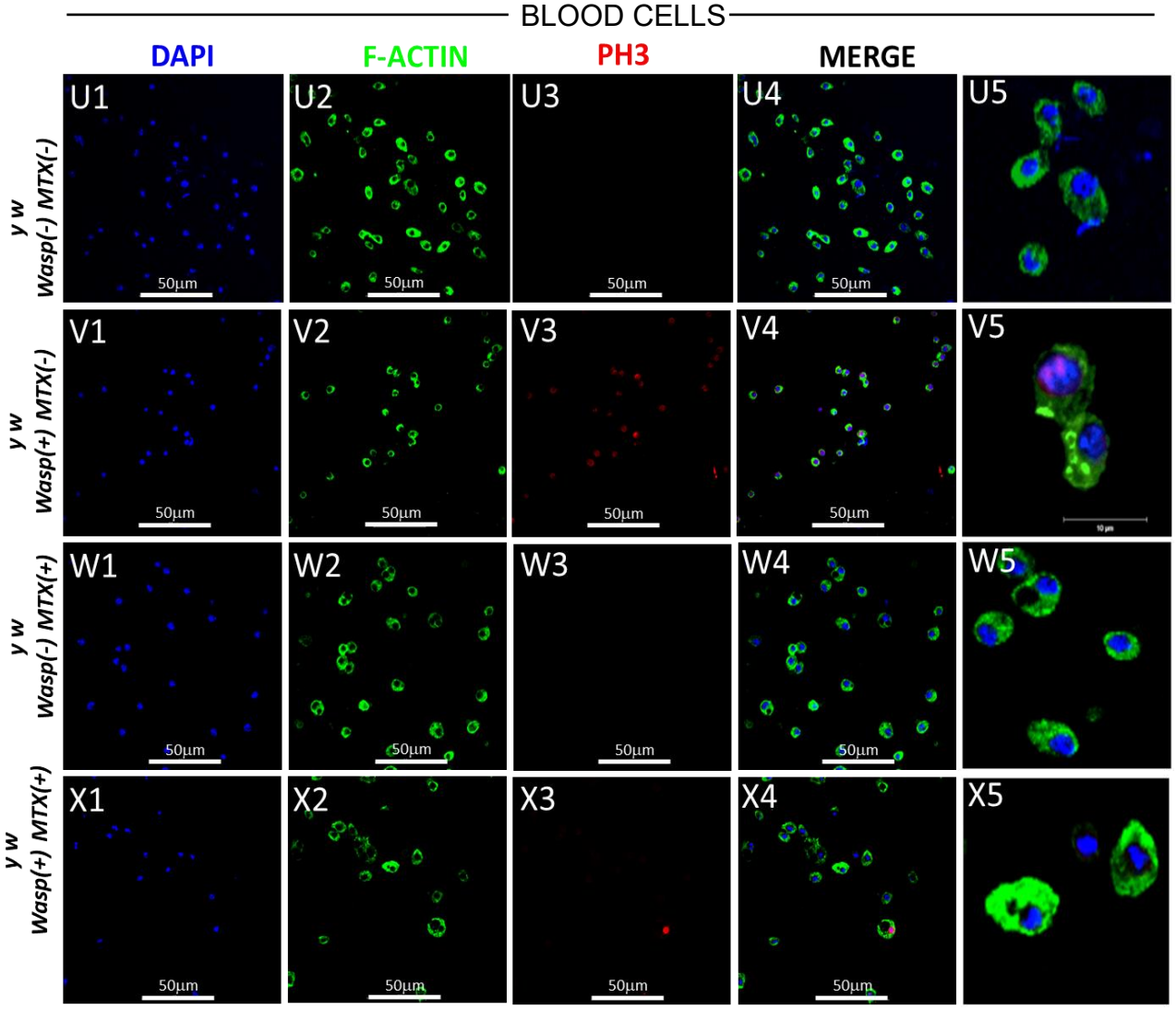
Cyclin B
S
CDK2
Cyclin A
G₂
CDK1
Cyclin A


T1
Cyclin A
Fold Induction
MTX - - + +
Wasp - + - +


T2
Cyclin B
Fold Induction
MTX - - + +
Wasp - + - +


T3
Cyclin D
Fold Induction
MTX - - + +
Wasp - + - +


T4
Dacapo/p21
Fold Induction
MTX - - + +
Wasp - + - +


BLOOD CELLS
DAPI
F-ACTIN
PH3
MERGE
y w Wasp(-) MTX(-)
y w Wasp(+) MTX(-)
y w Wasp(-) MTX(+)
y w Wasp(+) MTX(+)
U1
U2
U3
U4
U5
V1
V2
V3
V4
V5
W1
W2
W3
W4
W5
X1
X2
X3
X4
X5
50µm

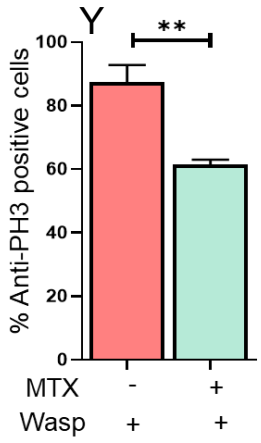

Y
**
% Anti-PH3 positive cells
100
80
60
40
20
0
MTX - +
Wasp + +

Figure 3.5 Effect of MTX on blood cell proliferation

S Schematic representation of cell cycle regulators at G1, S, G2, M phase

T1-T4. Gene expression of cell cycle regulators (*Cyclin A, Cyclin B, Cyclin D*) and cell cycle inhibitor *Dacapo (p21)* in the wasp induced Toll/NF-κB pathway before and after MTX treatment. Student's t-test showed statistical significance $p < 0.05$(*), $p<0.01$ (**), $p<0.001$(***) & $p<0.0001$ (****) on comparison with uninfected untreated, only infected & infected plus treated. N=3, n=50+. Graphs was processed using GraphPad prism version 8.0.2.

Status of mitotically actively proliferating cells (Anti-Phospho-Histone 3 positive cells) in the circulating blood cells of *y w* 3rd instar larvae induced by *Lb-17* parasitoid wasp infestation stained with DAPI (nuclear stain, blue), polymerized F-actin (Cytoskeleton, green), Anti-PH3 (specific to proliferating cells, red).

U1-U4 *y w* control untreated and uninfected

V1-V5 *y w* only wasp infected

W1-W4 *y w* only drug treated

X1-X5 *y w* infected and drug treated

N=3, n=12.Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Y % Of Anti-PH3 cells between Untreated wasp infected and MTX treated wasp infected. Number of anti-PH3 positive cells has gone down after MTX treatment. Student's t-test (unpaired, two-tailed) showed statistical significance *p<0.01(**)*, N=3, n=30.

3.3 Discussion

A vast group of insects known as parasitic wasps frequently prey on other insects. Parasitic wasps are of great commercial importance and can take the place of insecticides to control insect pests due to their complete reliance on their insect victims. In the wild, parasitic wasps are constantly changing to avoid or reduce their hosts' immune reactions. To accomplish this, they secrete substances or create protein complexes with certain molecular functions to prevent encapsulation. Other insects have also reported encapsulation reactions of non-self (wasp egg) or diseased self-tissues (fat body), similar to those seen in *Drosophila* larvae [183]. The reaction is also likely to be similar to mammalian granulomas, which are characterised by various forms of localised nodular inflammation [184]. The host response generated after wasp attack recapitulates the essential key features of mammalian inflammation. We extrapolated this robust host-pathogen interaction to study the conserved innate immune molecular events against MTX. From existing literature, it was quite evident that MTX was successfully able to suppress the JAK/STAT pathway both *in vitro* and *in vivo* [168, 174]. MTX's suppressive effect on hyperactive JAK/STAT pathway in *Drosophila* partially circumvented the larval manifestations such as formation microtumor formation and aggregates. Also, the specific hemocytes (lamellocytes) populations that form these tumors were inhibited as well. This paved a platform for us to check MTXs effects on other essential hematopoietic pathways such as Toll pathway. The fact that *Lb17* attack on its *Drosophila* larval hosts induces the nuclear localization of NF-κB (Dorsal), transient transcriptional upregulation of both *SPE* and *Cactus* leading to the hyperactivity of the Toll/NF-kB pathway. We saw a restoration in the transcript levels of Toll components and cytoplasmic retention of Dorsal after MTX treatment, which relived hyperactivity of the Toll pathway. Another hallmark of wasp infestation i.e., blood cellular proliferation was also limited after the MTX treatment. It was very evident that MTX had an inhibitory effect on the Toll pathway, but the mechanistic approach was still elusive. This non-canonical role of MTX was quite intriguing and was worth extrapolating it to the check on the chronic version of inflammation by pursuing immune-genetic approach.

3. Objective 2A: To study the effect of MTX in larvae with hyperactive Toll/NF-κB pathway via *SPE* overexpression (*Gain-of-function*) specifically in the blood cells

3.1 Introduction

The formation of blood cells (hemocytes) in *Drosophila* depends heavily on the Toll/NF-κB pathway [180]. This route controls immunological reactions and developmental procedures [96, 185-187]. The Toll receptor (Toll 1) is found to be an activator of immune response in vitro conditions [188]. A signaling cascade that results from the activation of the Toll receptor also activates the NF-κB transcription factors [189]. These transcription factors control the expression of many genes involved in the differentiation and maturation of blood cells and also other aspects of blood cell development. The balance between progenitor cell proliferation and differentiation is regulated by the Toll/NF-κB pathway. It encourages blood progenitor cells to differentiate into particular blood cell lineages, like plasmatocytes and crystal cells. Additionally, this route affects how blood cells respond to microbial infections by influencing their immunological activity. Overall, the Toll/NF-κB system is essential for coordinating immune responses and blood cell formation in *Drosophila* [190]. Several key components of the Toll/NF-κB pathway have been found to be important regulators of *Drosophila* blood cell development. Spätzle (Spz) is a ligand that binds to the Toll receptor and starts the Toll signalling cascade. Spz, is basically cysteine knot protein that encodes multiple isoforms [191, 192]. Upstream to Spätzle there is SPE called Spätzle Processing enzyme. The larval fat body and blood cells constitutively express SPE. During signaling conditions SPE actively cleaves and activates the Toll ligand pro-Spätzle (inactive) to Spz (active) [176, 193]. A Toll-dependent positive feedback loop can also be used to induce *SPE* transcription in response to an injury. The induction of *Drosomycin*, readout of the Toll pathway, is hampered by *SPE* loss or depletion [176, 193]. MyD88, or myeloid differentiation factor, it is an adapter protein that is essential for transferring signals from the Toll receptor to components further down the chain [93, 194, 195]. The downstream kinase Pelle and the receptor are connected by MyD88 [195, 196]. Tube: It is an intracellular protein that cooperates with MyD88 to promote Pelle kinase recruitment and activation [196]. Dif and Dorsal are released from the cytoplasmic inhibitory complex as a result of the activation of the activation of Toll signaling, allowing them to go into the nucleus and control target gene expression[197].

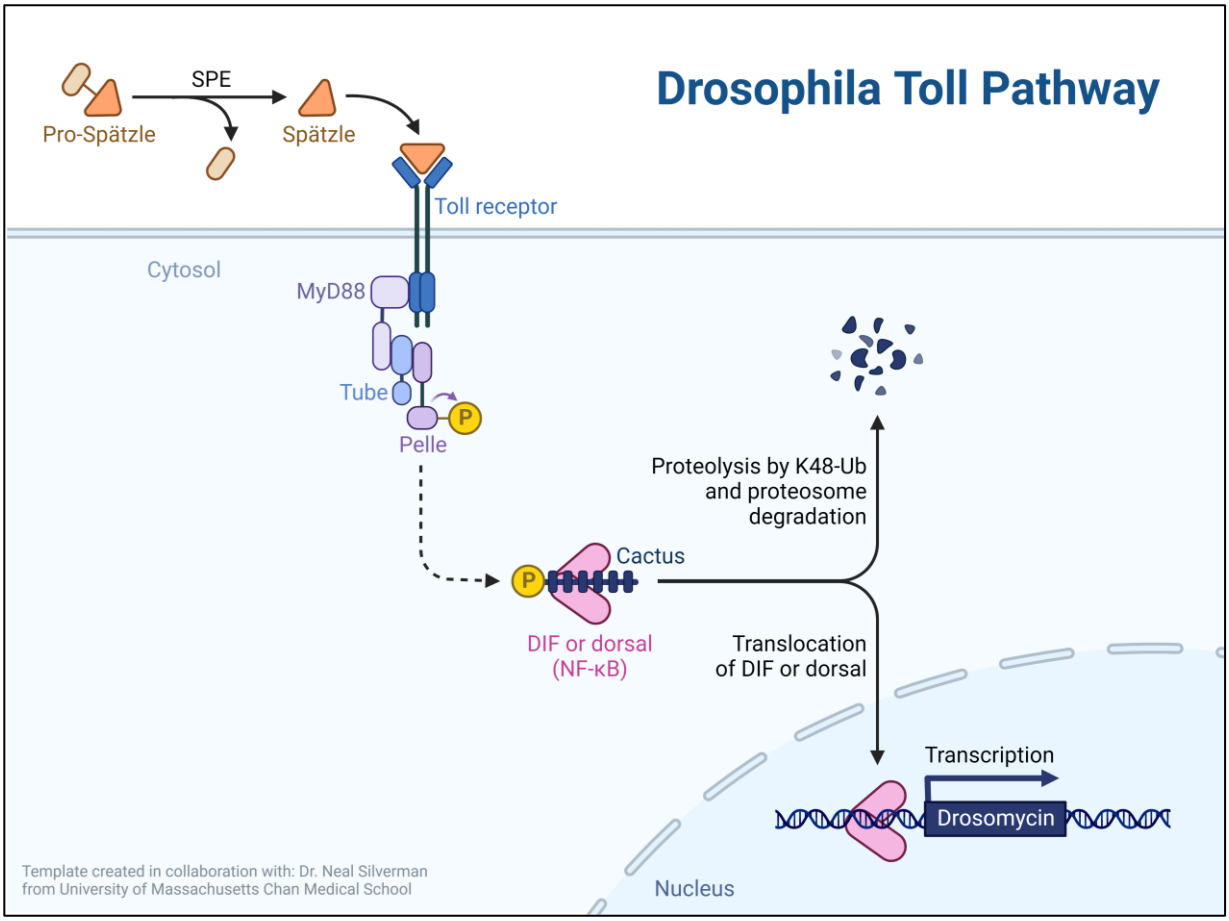


Figure 3.51 Schematic of *Drosophila* canonical Toll/NF-kB pathway

Created in Biorender

3.2 Result

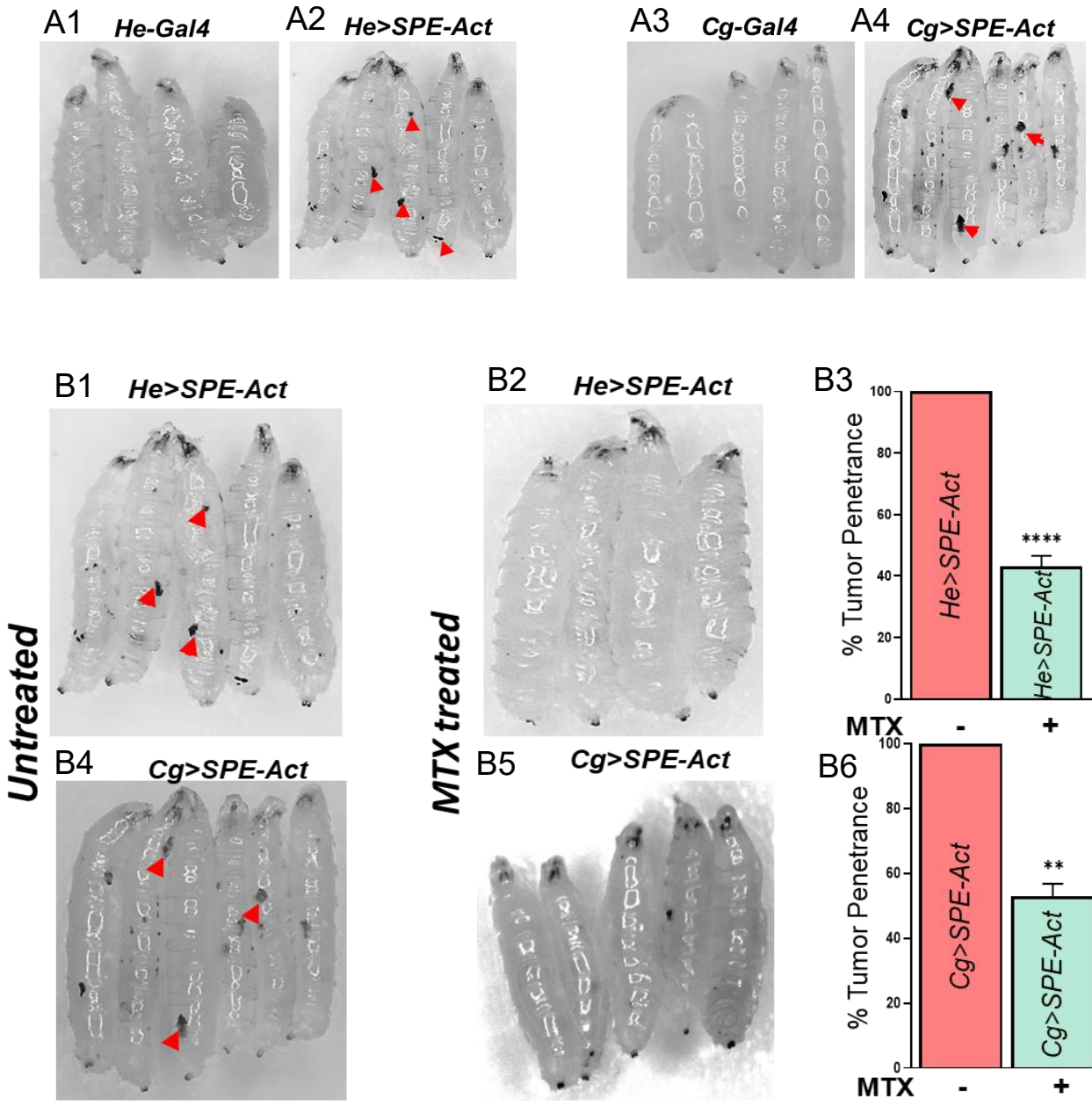
3.2.1 MTX reduces the blood tumor penetrance and blood cell proliferation in larvae with *SPE* overexpression

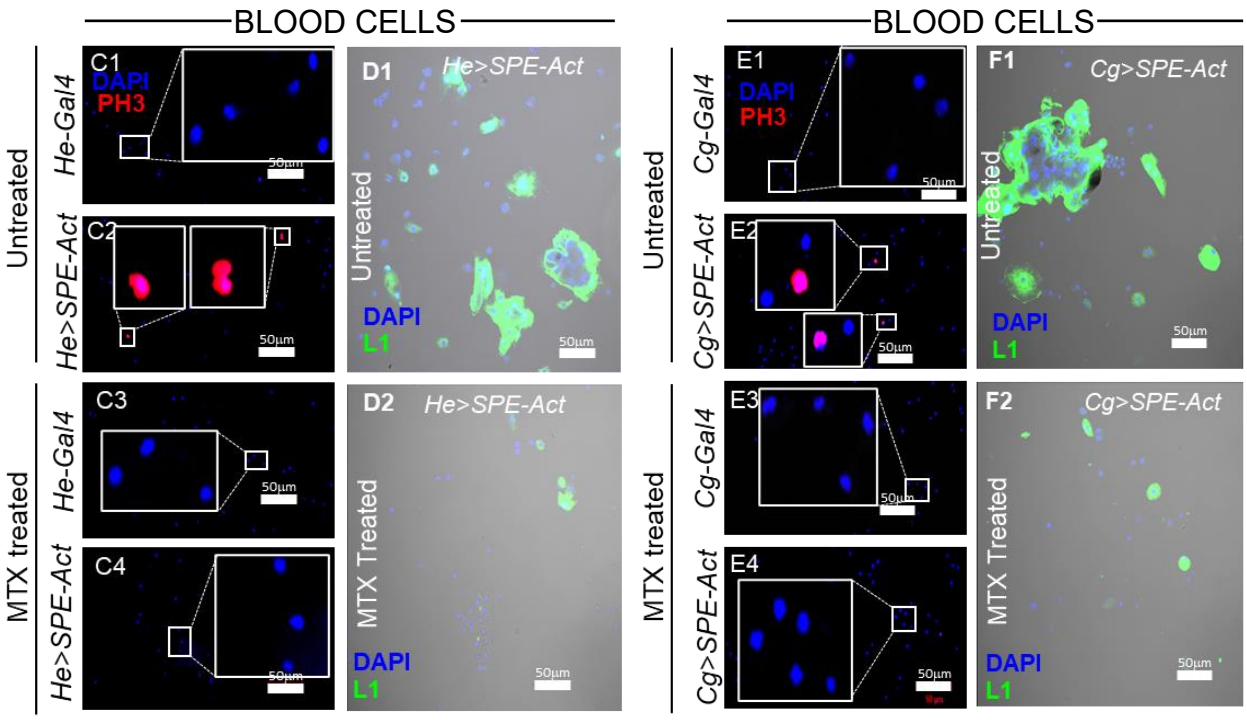
For this part of the study, we utilized two different *Gal4* promoter i.e., *He-Gal4* and *Cg-Gal4* to overexpress *SPE*, in the *Drosophila* larval immune tissues (blood cells and fat body), using typical *UAS/GAL4* system [198]. *He-Gal4* drives the expression of *SPE-Act* exclusively in the blood cells whereas *Cg-Gal4* drives the expression of *SPE-Act* ubiquitously in the fat body and circulating hemocytes. Here, we set up four experimental conditions for each both the *Gal4* driver; Untreated *He-Gal4*, Untreated *He>SPE-Act*, MTX treated *He-Gal4* and MTX treated *He>SPE-Act*. Untreated *Cg-Gal4*, Untreated *Cg>SPE-Act*, MTX treated *Cg-Gal4* and MTX treated *Cg>SPE-Act*. SPE is positive regulator of Toll pathway and its overexpression in the immune tissues results in uncontrolled blood cell proliferation, loss of fat body integrity and melanotic microtumor formation (Paddibhatla et al., 2010). We first checked the phenotype led by the overexpression of *SPE-Act* with both the *Gal4* drivers.

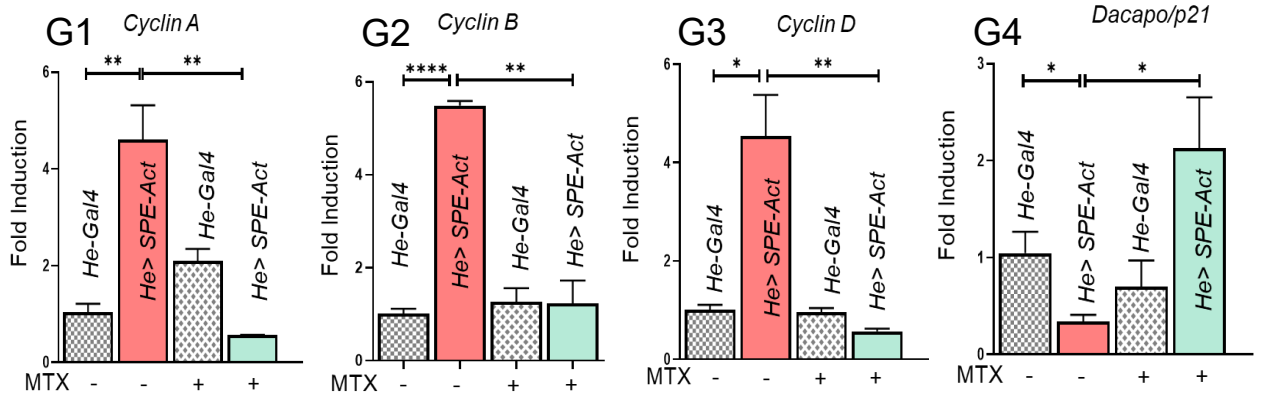
Untreated control *He-Gal4* & *Cg-Gal4* larvae (Figure 3.6 A1, Figure 3.6 A3) and MTX treated control *He-Gal4* & *Cg-Gal4* (data not shown) larvae were devoid of *SPE* overexpression and did not show any melanized microtumor phenotype. Whereas untreated larvae exhibiting *SPE-Act* overexpression with *He-Gal4* and *Cg-Gal4* showed multiple melanized microtumor phenotype (Figure 3.6 B1, Figure 3.6 B4, *red arrow indicating*). To correlate we counted the number of melanized microtumors in the larvae and found that there was 100% tumor penetrance when *SPE-Act* overexpressed via both the *Gal4* driver i.e., each of the larvae was exhibiting melanized microtumor cuticle, visible under the cuticle (Figure 3.6 A2 and Figure 3.6 A4). Interestingly upon MTX treatment we found a significant reduction in the tumor penetrance to 43.21% and 52.81% in the *He>SPE-Act* and *Cg>SPE-Act* larvae respectively (Figure 4.2 B3 and Figure 4.2 B6) compared to untreated larvae (Figure 3.6 B1 and Figure 3.6 B4).

As per the rationale these melanized microtumor formation are the consequence of over proliferation of blood cells due to the hyperactive hematopoietic pathways such as Toll pathway. Earlier research has indicated that often these blood cells (lamellocytes) form such melanized microtumors leading to the formation of melanotic masses.

Therefore, we dissected the hemolymph of 3rd instar untreated and MTX treated *He>SPE-Act* and *Cg>SPE-Act* larvae and compared it with their untreated and treated sibling controls. We used anti-PH3 antibody to trace the mitotically active blood cells and anti-L1-antibody specifically for tracing the lamellocytes lineage. While *He-Gal4* & *Cg-Gal4* with or without MTX treatment did not show the presence of any mitotically active blood cells (Figure 3.6 C3, C1 and Figure 3.6 E3, E1), but in the untreated *He>SPE-Act* and *Cg>SPE-Act* there were appearance of PH3 positive cells (Figure 3.6 C2, Figure 3.6 E2) signifying that *SPE-Act* overexpression leads to increased mitotic activity of blood cells. Upon MTX treatment in the *He>SPE-Act* and *Cg>SPE-Act* the presence of PH3 positive cells were significantly reduced suggesting that MTX is hindering in the blood cell proliferation (Figure 3.6 C4, Figure 3.6 E4). Also, in untreated *He>SPE-Act* and *Cg>SPE-Act* significant L1 positive cells were observed to be forming the part of tumor (Figure 3.6 D1, Figure 3.6 F1). On the contrary, upon MTX treatment we did not observe the presence L1 positive lamellocyte in the *He>SPE-Act* and *Cg>SPE-Act* (Figure 3.6 D2, Figure 3.6 F2). To understand the involvement of cell cycle regulators in the blood cell division we next investigated the status of cell cycle regulators, *Cyclin A*, *Cyclin B* and *Cyclin D* along with an inhibitor *Dacapo* (*p21*) in the control versus experimental backgrounds with and without MTX treatment. In the untreated and MTX treated *He-Gal4* and *Cg-Gal4* the transcript levels of *Cyclin A*, *Cyclin B*, *Cyclin D* and *Dacapo/p21* were ~1 fold. In case of untreated *He>SPE-Act* the levels of *Cyclin A* (4.60-fold), *Cyclin B* (4.75-fold) and *Cyclin D* (4.54-fold) were significantly increased (Figure 4.2 G1-G3) and with untreated *Cg>SPE-Act* also similar significant increase in the levels of *Cyclin A* (3.73-fold), *Cyclin B* (6.12-fold) and *Cyclin D* (3.13-fold) were observed (Figure 3.6 H1-H3). Whereas upon drug treatment, *He>SPE-Act* larvae showed a significant downregulation (Figure 3.6 G1-G3 and Figure 3.6 H1-H3). Unlike *Cyclins*, the gene expression of *Dacapo* (*p21*), in *He>SPE-Act* and *Cg>SPE-Act* appeared to be significantly downregulated but upon MTX treatment the levels were significantly increased (Figure 3.6 G4 and Figure 3.6 H4). Therefore, we think that decrease in the tumor penetrance possibly could be a consequence of reduction in anti-PH3 and L1 positive cells along with the lowered expression of cell cycle regulators due to MTX treatment.


A1
He-Gal4
A2
He>SPE-Act
A3
Cg-Gal4
A4
Cg>SPE-Act
Untreated
B1
He>SPE-Act
B2
He>SPE-Act
MTX treated
B3
% Tumor Penetrance
He>SPE-Act
****
MTX
-
+
B4
Cg>SPE-Act
B5
Cg>SPE-Act
B6
% Tumor Penetrance
Cg>SPE-Act
**
MTX
-
+


BLOOD CELLS
BLOOD CELLS
Untreated
MTX treated
He-Gal4
He>SPE-Act
Cg-Gal4
Cg>SPE-Act
C1
C2
C3
C4
D1
D2
E1
E2
E3
E4
F1
F2
DAPI
PH3
L1
He>SPE-Act
Cg>SPE-Act
MTX Treated
50µm


G1
Cyclin A
G2
Cyclin B
G3
Cyclin D
G4
Dacapo/p21
Fold Induction
He-Gal4
He> SPE-Act
MTX

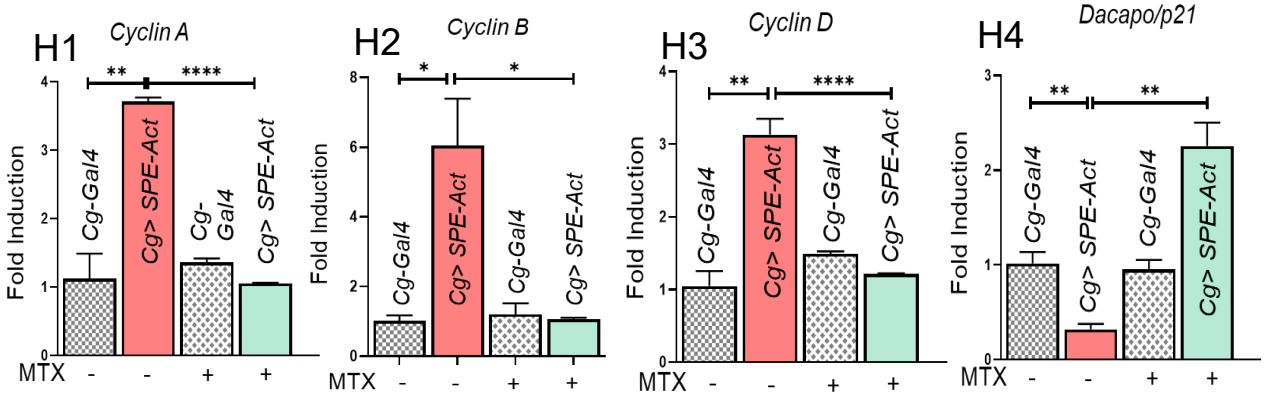

H1
Cyclin A
H2
Cyclin B
H3
Cyclin D
H4
Dacapo/p21
Fold Induction
Cg-Gal4
Cg> SPE-Act
MTX

Figure.3.6 Effect of MTX on the hematopoietic defects manifested by the overexpression of *SPE* in the larval immune tissues

A1 Untreated *He-Gal4* 3rd instar larvae

A2, B1 Untreated *He>SPE-Act* 3rd instar larvae

A3 Untreated *Cg-Gal4* 3rd instar larvae

A4, B4 Untreated *Cg>SPE-Act* 3rd instar larvae

B2 *He>SPE-Act* larvae showing very small melanotic tumors or none visible through cuticle after MTX treatment

B5 *Cg>SPE-Act* larvae showing very small melanotic tumors or none visible through cuticle after MTX treatment

B3 Statistical count of melanotic tumors (% tumor penetrance) in *He>SPE-Act* before and after MTX treatment

B6 Statistical count of melanotic tumors (% tumor penetrance) in *Cg>SPE-Act* before and after MTX treatment N=3, n=50. Graphs were plotted using GraphPad software version 8.0.2. Student's t-test showed statistical significance p<0.0001(****), p<0.01(**). Tumors visible through cuticle as indicated by the red arrow.
Image was captured from trinocular microscope with the help of attached micaps ecocmos510B camera.

Status of Anti-PH3 in the blood cells of 3rd instar wandering larvae of *He>SPE-Act* & *Cg>SPE-Act* stained for DAPI (Nuclear stain, blue), Anti-PH3 (specific to mitotically active cells, red), before and after MTX treatment

C1, E1 Untreated *He-Gal4* control & *Cg-Gal4* control.

C2, E2 Untreated *He>SPE-Act* & *Cg>SPE-Act*.

C3, E3 MTX treated *He-Gal4* control & *Cg-Gal4* control

C4, E4 MTX treated *He>SPE-Act* & *Cg>SPE-Act*. N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Status of L1-positive blood cells in the 3rd instar wandering larvae of *He>SPE-Act* & *Cg>SPE-Act* stained for DAPI (Nuclear stain, blue), Anti-L1 (specific to lamellocyte lineage, green) before and after MTX treatment

D1, F1 Untreated *He>SPE-Act* & *Cg>SPE-Act*

D2, F2 MTX treated *He>SPE-Act* & *Cg>SPE-Act*. N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

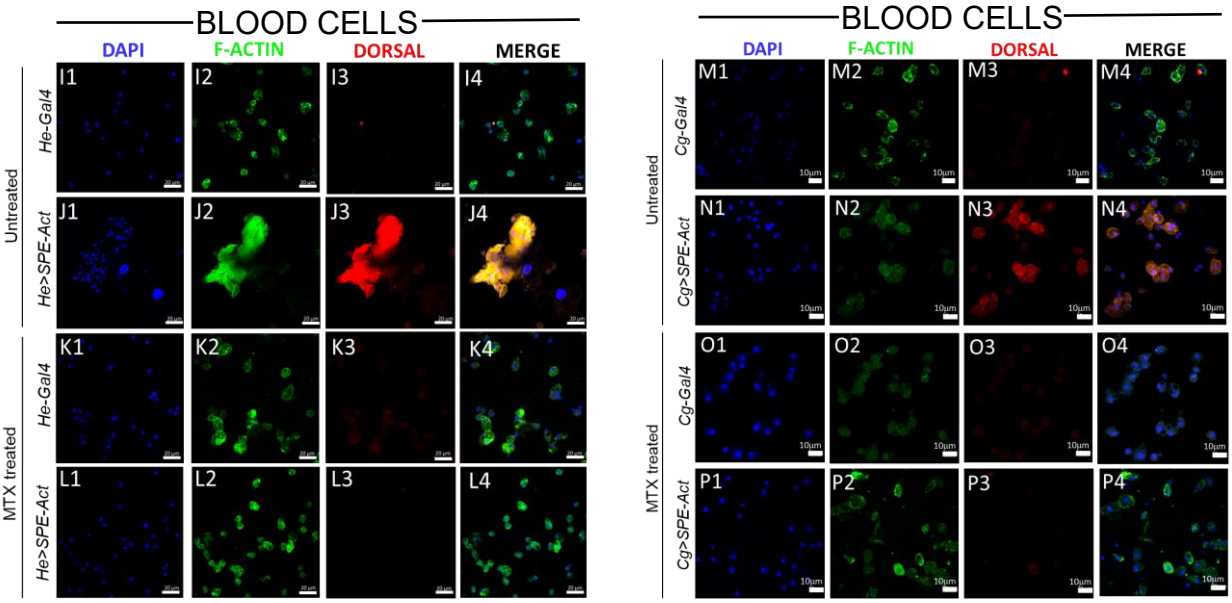
Graphical representation of the transcript levels of **(G1)** *Cyclin A*, **(G2)** *Cyclin B*, **(G3)** *Cyclin D*, and **(G4)** *Dacapo (p21)* in whole larvae (3rd instar) with and without MTX treatment overexpression of *SPE* in the background of *He-Gal4*. N=3, n=50. Graphs were plotted using GraphPad software version 8.0.2. Student's t-test showed statistical significance p<0.0001(****) and p<0.01 (**). Experiments were conducted at 25 °C.

Graphical representation of the transcript levels of **(H1)** *Cyclin A*, **(H2)** *Cyclin B*, **(H3)** *Cyclin D*, and **(H4)** *Dacapo (p21)* in whole larvae (3rd instar) with and without MTX treatment overexpression of *SPE* in the background of

Cg-Gal4. N=3, n=50. Graphs were plotted using GraphPad software version 8.0.2. Student's t-test showed statistical significance $p<0.0001$(****) and $p<0.01$ (**). Experiments were conducted at 25 °C.

3.2.2 MTX hinders the Dorsal localization in the blood cells thereby suppressing the hyperactive Toll/NF-κB pathway in larvae induced with *SPE* overexpression

Since Toll pathway has a role in larval blood cell proliferation, our next approach was to speculate the possibility of MTX hindering the hyperactive Toll pathway. We documented effect of MTX on the active status of Toll pathway in the overexpression backgrounds by determining the cellular localization of the transcription factor Dorsal in hemocytes by performing immunostaining using anti-Dorsal antibody. Blood cells of *He-Gal4* and *Cg-Gal4* showed Dorsal expression predominantly in the cytoplasm (Figure 3.7 I1-I4 and M1-M4) and MTX treatment had no effect on this result (Figure 3.7 K1-K4 and Figure 3.7 O1-O4). Blood cells of untreated *He> SPE-Act* and *Cg>SPE-Act* showed significant nuclear localization of dorsal (Figure 3.7 J1-J4 and Figure 3.7 N1-N4) and this was affected by MTX treatment i.e., drug treatment led to loss of nuclear Dorsal expression retaining the transcription factor in the cytoplasm (Figure 3.7 L1-L4 and Figure 3.7 P1-P4). These results suggest that MTX treatment affected the cellular localization of Dorsal in the blood cells. To understand if the ectopic expression of *SPE-Act* using GAL4 drivers actually led to an increase in the *SPE* expression we first looked into the transcript levels of *SPE*. mRNA levels of *SPE* were significantly increased (*He>SPE-Act* 8.29-fold and *Cg>SPE-Act* 3.41-fold) compared to the controls (*He-Gal4* 1.05-fold and *Cg-Gal4* 1.0-fold) (Figure 3.7 Q1 and Figure 3.7 R1). We next studied the mRNA expression of *Drosomycin* and the negative regulator, *Cactus*. In the untreated *He>SPE-Act* the transcript levels of *Drosomycin* was 30.70-fold and in *Cg>SPE-Act* it was found to be 4.25-fold (Figure 3.7 Q3 and Figure 3.7 R3). Whereas after MTX treatment, there was significant inhibition in the transcript levels of *Drosomycin* to 7.47-fold and 2.39-fold in *He>SPE-Act* and *Cg>SPE-Act* respectively (Figure 3.7 Q3 and Figure 3.7 R3). Unlike *SPE* and *Drosomycin*, the transcript levels of *Cactus*, in untreated *He>SPE-Act* and *Cg>SPE-act*, showed an expression of 2.80-fold and 2.53-fold respectively (Figure 3.7 Q2 and Figure 3.7 R2) which changed to 3.35-fold and 3.23-fold respectively upon treatment with the drug. Combining the results, we comprehend that the suppression of Toll pathway activity could be the possible reason behind loss of hematopoietic defects and tumor penetrance. Furthermore, this possibility is strengthened due to a decrease in the mRNA levels of *SPE*, *Cactus* and *Drosomycin* which are the readouts of the Toll/Dorsal pathway as shown in earlier study [118].


BLOOD CELLS
DAPI
F-ACTIN
DORSAL
MERGE
Untreated
MTX treated
He-Gal4
He>SPE-Act
Cg-Gal4
Cg>SPE-Act
I1
I2
I3
I4
J1
J2
J3
J4
K1
K2
K3
K4
L1
L2
L3
L4
M1
M2
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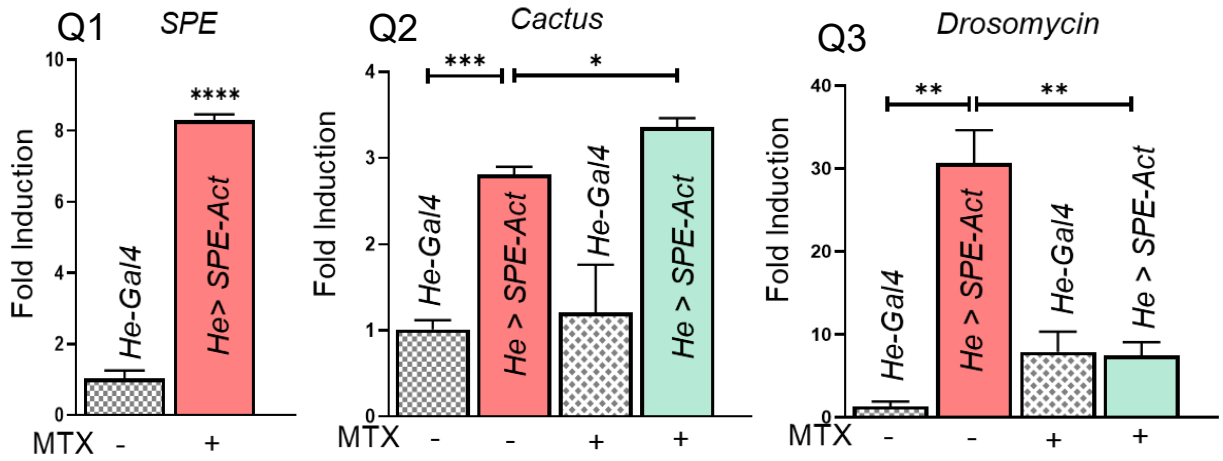
O1
O2
O3
O4
P1
P2
P3
P4


Q1
SPE
Fold Induction
He-Gal4
He> SPE-Act
MTX
Q2
Cactus
He > SPE-Act
Q3
Drosomycin

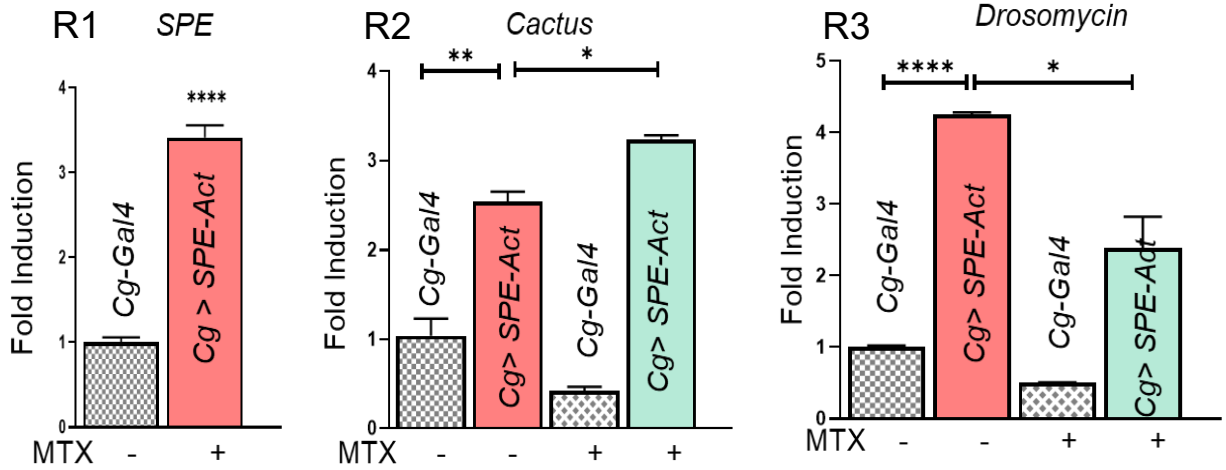

R1
SPE
Fold Induction
Cg-Gal4
Cg > SPE-Act
MTX
R2
Cactus
Cg> SPE-Act
R3
Drosomycin

Fig.3.7 Effect of MTX on Dorsal nuclear localization in the blood cells of 3rd instar wandering larvae of *He->SPE-Act* and *Cg>SPE-Act*

Status of Dorsal nuclear localization in the blood cells of 3rd instar wandering larvae of *He->SPE-Act* stained for DAPI (Nuclear stain, blue), Anti-Dorsal (specific to Dorsal, red), Polymerized F-actin (Cytoskeleton, green)

I1-I4 *He-Gal4* Control untreated shows basal insignificant levels of Dorsal levels.
J1-J4 *He>SPE-Act* untreated showed majority of blood cells with Dorsal colocalizing with nuclear stain DAPI.
K1-K4 MTX treated *He-Gal4* showed similar results with untreated He-Gal4 Control
L1-L4 MTX Treated *He>SPE-Act* reduction in the nuclear localization of Dorsal. N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Status of Dorsal nuclear localization in the blood cells of 3rd instar wandering larvae of *Cg>SPE-Act* stained for DAPI (Nuclear stain, blue), Anti-Dorsal (specific to Dorsal, red), Polymerized F-actin (Cytoskeleton, green)

M1-M4 *Cg-Gal4* Control untreated shows basal insignificant levels of Dorsal levels.
N1-N4 *Cg>SPE-Act* untreated showed majority of blood cells with Dorsal colocalizing with nuclear stain DAPI.
O1-O4 MTX treated *Cg-Gal4* showed similar results with untreated *Cg-Gal4* Control
P1-P4 MTX Treated *Cg>SPE-Act* reduction in the nuclear localization of Dorsal. N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Graphical representation of the transcript levels of **(Q1)** *SPE*, **(Q2)** *Cactus*, and **(Q3)** *Drosomycin* in whole larvae (3rd instar) with and without MTX treatment, overexpression of *SPE* in the background of *He-Gal4*. N=3, n=50. All the data were analysed in GraphPad prism 8.0.2. Student's t-test showed statistical significance $p<0.0001$(****), $p<0.01$ (**) and $p<0.05$ (*)

Graphical representation of the transcript levels of **(R1)** *SPE*, **(R2)** *Cactus*, and **(R3)** *Drosomycin* in whole larvae (3rd instar) with and without MTX treatment, overexpression of *SPE* in the background of *Cg-Gal4*. N=3, n=50. All the data were analysed in GraphPad prism 8.0.2. Student's t-test showed statistical significance $p<0.0001$(****), $p<0.001$(***), and $p<0.05$ (*).

3. Objective 2B: To study the effect of MTX in the immune tissues of larvae with hyperactive Toll/NF-κB pathway in the *Ubc9*$^{-/-}$ mutant model (*Loss-of-function mutant*)

3.1 Introduction

Ubc9 is an E2 SUMO conjugase that negatively regulates Toll pathway in *Drosophila* [125]. *dUbc9* mutants exhibit chronic inflammatory phenotypes that includes increased lamellocytes, blood tumors, hyperactive Toll pathway with nuclear localized dorsal in immune tissues along with elevated expression of the target genes such as *Drosomycin*, *SPE*, and *Cactus*. Existing literature suggests that hyperactive Toll/NF-κB signalling results from the loss of fly IκB or cact, as well as the SUMO conjugase *Ubc9* (also known as *lesswright* or *lwr*)[125, 199, 200]. Both humoral (antimicrobial and immunological peptide gene expression in the fat body [201]) and cellular (blood cell proliferation, aggregation, and microtumor development [125, 200]) responses are continuously activated in *Ubc9*$^{-/-}$ mutant larvae. Similar to how *SPE* is activated, the Toll-Dorsal pathway regulates the transcriptional activation of cactus in response to bacterial assault.

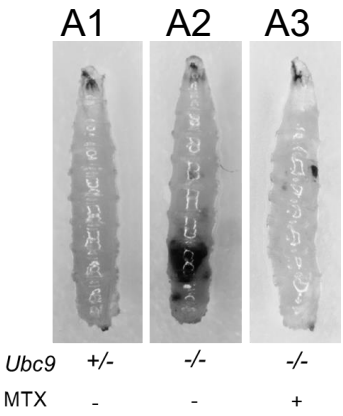
In *Drosophila* melanogaster, the *lwr* protein, a SUMO conjugase, is crucial for controlling larval hematopoiesis. The *lwr* gene is mutated in both dominant and recessive ways, which causes the overproduction of hemocytes in larvae [200]. Lamellocytes made up the majority of aggregated hemocytes in the hemolymph, and these aggregated masses were typically partially to completely melanized. These findings overwhelmingly point to a significant role for lamellocytes in the development of melanotic tumours in *lwr* mutant larvae.

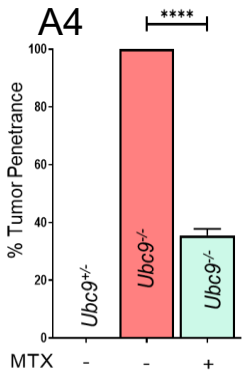
3.2 Result

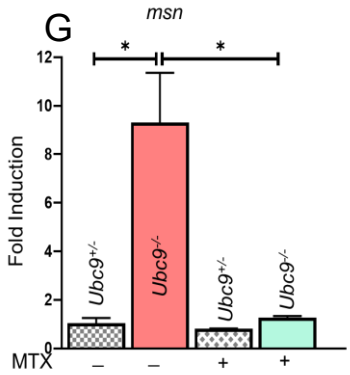
3.2.1 MTX inhibits the tumor penetrance and blood cell proliferation in *Ubc9*$^{-/-}$ mutants

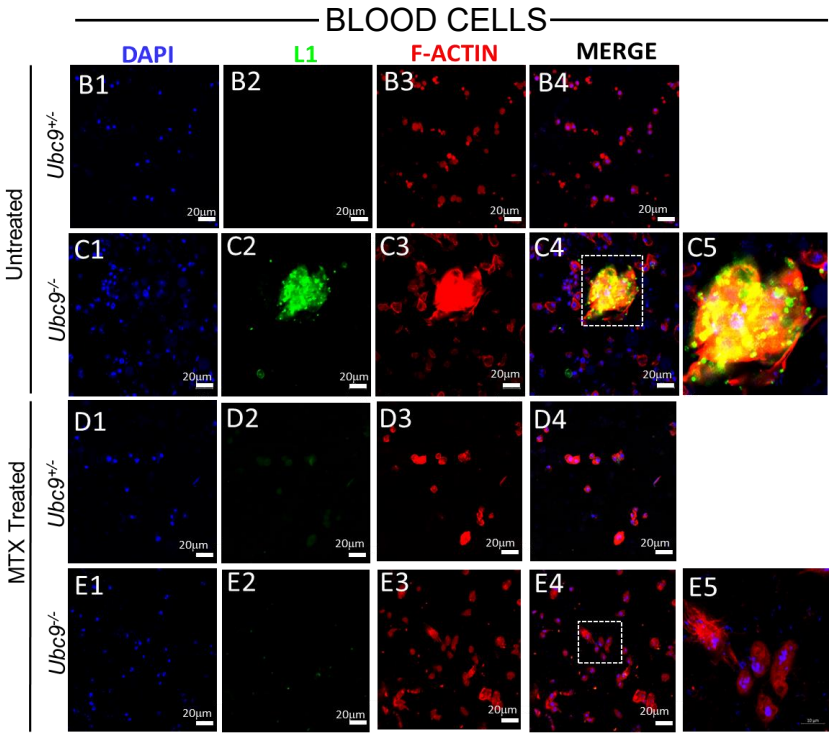
We utilized loss of function mutants of *dUbc9* (negative regulator of Toll pathway) for this part of the study. *Ubc9* is an E2 SUMO conjugase that negatively regulates Toll pathway in *Drosophila* [125]. *dUbc9* mutants exhibit chronic inflammatory phenotypes that includes increased lamellocytes, blood tumors, hyperactive Toll pathway with nuclear localized dorsal in immune tissues along with elevated expression of the target genes such as *Drosomycin*, *SPE*, and *Cactus* [118]. We hypothesized that if the wasp infestation and *SPE* overexpression induced Toll/NF-κB pathway dependent inflammatory phenotypes were mitigated due to MTX, then possibly *Ubc9*$^{-/-}$ mutants should also be relieved of the chronic inflammation associated with the hyperactive Toll pathway upon MTX treatment. As expected upon MTX treatment the melanized tumor were reduced in the *Ubc9*$^{-/-}$ mutants (Figure 3.8 A3) compared to the untreated *Ubc9*$^{-/-}$ mutants (Figure 3.8 A2). Statistically the tumor phenotype accounted for 100% tumor penetrance in *Ubc9*$^{-/-}$ mutants and it went down to 35.55% upon MTX treatment (Figure 3.8 A4). To elucidate the effect of MTX on lamellocytes we studied both the L1 positive cells (using anti-L1 antibody) in the circulating hemolymph and also the expression of *msn* (RT-qPCR), which is one of the known markers for lamellocyte lineage and an enhancer trap allele *misshapen*^{03349} [202, 203]. It is a lamellocyte active-enhancer located within the intron-3 of the *msn* gene [204]. In the untreated and MTX treated *Ubc9*$^{+/-}$ heterozygotes no significant presence of lamellocytes was noticed (Figure 3.8 B1-B4 and D1-D4). On the contrary, in the untreated *Ubc9*$^{-/-}$ mutants there were L1 positive lamellocytes freely circulating (Figure 3.8 C1-C5) while MTX treatment led to a clear reduction in the population of L1 positive lamellocytes (Figure 3.8 E1-E5). As per our statistical analysis, the untreated *Ubc9*$^{-/-}$ mutants had 15.95% increase in the L1 positive cells which was significantly reduced to 2.27% after the drug treatment **(**Figure 3.8 F). We also found that *msn* gene expression in *Ubc9*$^{+/-}$ heterozygotes with and without MTX treatment remained unaffected while *Ubc9*$^{-/-}$ mutants showed an upregulation of 9.31-fold induction which upon with MTX treatment was significantly reduced to 1.27-fold **(**Figure 3.8 G). In a similar approach we also investigated the status of other matured blood cell type (Plasmatocytes) with anti-P1 antibody specific to Plasmatocytes, predominantly the most abundant class of hemocytes in the larva. We did not

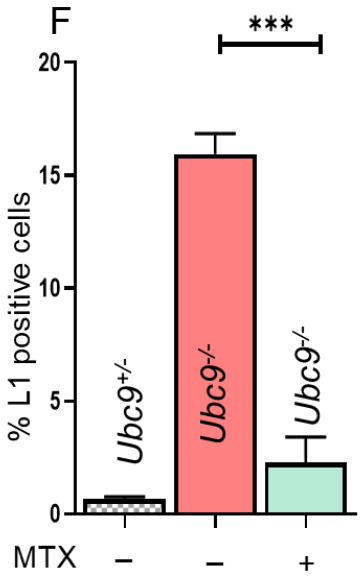
find any significant change in the population of plasmatocyte positive cells in the untreated and MTX treated *Ubc9*$^{+/-}$ heterozygotes (Figure 3.8 H1-H4 and Figure 3.8 J1-J4) and also in the untreated and treated *Ubc9*$^{-/-}$ mutants (Figure 3.8 I1-I4 and Figure 3.8 K1-K4).

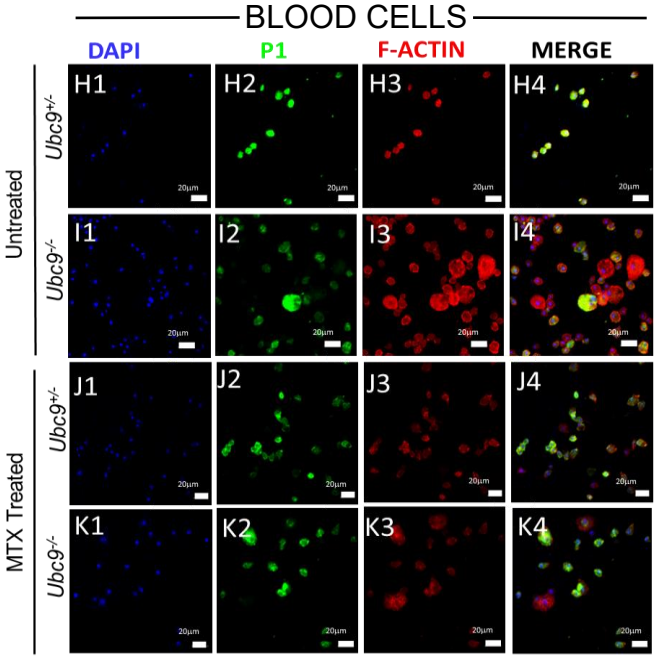

A1
A2
A3
Ubc9 +/- -/- -/-
MTX - - +


A4
****
% Tumor Penetrance
Ubc9+/-
Ubc9-/-
Ubc9-/-
MTX - - +


G
msn
Fold Induction
Ubc9+/-
Ubc9-/-
Ubc9+/-
Ubc9-/-
MTX – – + +


BLOOD CELLS
DAPI
L1
F-ACTIN
MERGE
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
B1
B2
B3
B4
C1
C2
C3
C4
C5
D1
D2
D3
D4
E1
E2
E3
E4
E5
20µm


F
***
% L1 positive cells
Ubc9+/-
Ubc9-/-
Ubc9-/-
MTX – – +


BLOOD CELLS
DAPI
P1
F-ACTIN
MERGE
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
H1
H2
H3
H4
I1
I2
I3
I4
J1
J2
J3
J4
K1
K2
K3
K4
20µm

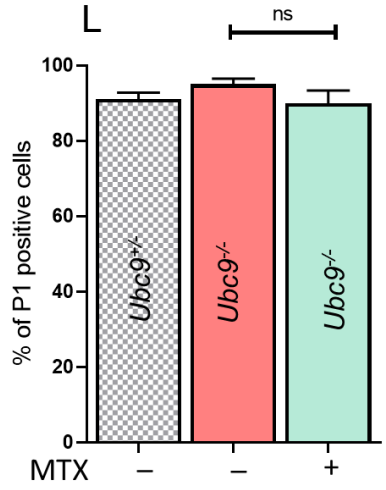

L
ns
% of P1 positive cells
Ubc9+/-
Ubc9-/-
Ubc9-/-
MTX – – +

Fig.3.8 Effect of MTX on blood tumors and blood cells on *Ubc9*$^{-/-}$ mutants 3rd instar larvae

A1-A3 Pictorial representation of Untreated *Ubc9*$^{+/-}$ heterozygote control (**A1,** no tumors), untreated *Ubc9*$^{-/-}$ mutant 3rd instar larva (**A2)** carrying melanotic tumors and MTX treated *Ubc9*$^{-/-}$ mutant larva (**A3**) with reduced melanotic tumors.

A4 Graphical representation of percentage (%) tumor penetrance between untreated *Ubc9*$^{+/-}$ heterozygote control, untreated *Ubc9*$^{-/-}$ mutant and MTX treated *Ubc9*$^{-/-}$ mutant.

Circulating blood cells of 3rd instar *Ubc9*$^{-/-}$ mutant dissected for L1 (lamellocytes) positive cells stained with DAPI (Nuclear stain, blue), Anti-L1 (Lamellocytes, green) Polymerized F-Actin (Cytoskeleton, red)

B1-B4 Untreated *Ubc9*$^{+/-}$ heterozygotes were devoid of L1 positive cells

C1-C5 Untreated *Ubc9*$^{-/-}$ mutants showing significant number of L1 positive cells (B', white arrow indicating)

D1-D4 MTX treated *Ubc9*$^{+/-}$ heterozygotes shows no change in the staining of L1 positive cells compared to untreated *Ubc9*$^{+/-}$ heterozygotes

E1-E5 MTX treated *Ubc9*$^{-/-}$ mutants with reduced L1 positive cells implying that MTX inhibits the L1 positive cells number. N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

F Statistical count of % of L1 positive cells in untreated *Ubc9*$^{-/-}$ mutants vs. MTX treated *Ubc9*$^{-/-}$ mutants. *Ubc9*$^{+/-}$ heterozygotes serves as control. Error bars show the standard error mean. Student's t-test (unpaired, two-tailed) showed statistical significance $p<0.001$(***). N=3, n=10. Graphs were plotted using GraphPad software version 8.0.2

G Bar graph representing the transcript levels of *msn* in *Ubc9*$^{-/-}$ mutants before and after MTX treatment. Student's t-test (unpaired, two-tailed) showed statistical significance $p<0.05$(*). Graphs was processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C.

Circulating blood cells of 3rd instar *Ubc9*$^{-/-}$ mutant dissected for P1 (Plasmatocytes) positive cells stained with DAPI (Nuclear stain, blue), Anti-P1 (Plasmatocytes, green) Polymerized F-Actin (Cytoskeleton, red)

H1-H4 Untreated *Ubc9*$^{+/-}$ heterozygote shows optimum level for P1 positive cells.

I1-I4 Untreated *Ubc9*$^{-/-}$ mutants showing high number of P1 positive cells

J1-J4 MTX treated *Ubc9*$^{+/-}$ heterozygote shows no significant difference in P1 positive cells compared to *Ubc9*$^{+/-}$ heterozygotes

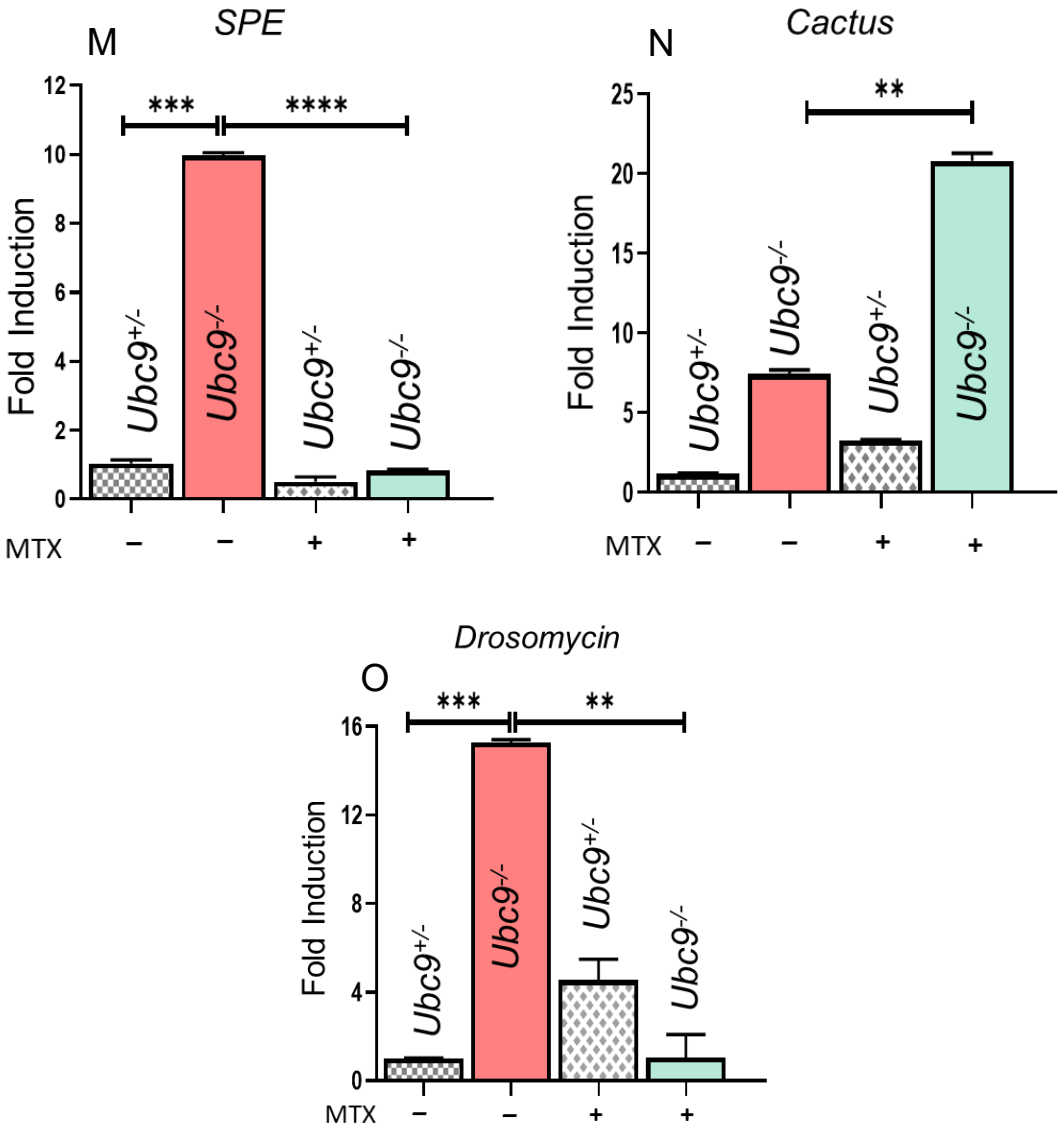
K1-K4 MTX treated *Ubc9*$^{-/-}$ mutants showed basal level of staining for the P1 positive cells. We observed morphological change in the P1 positive cells showing spike like structure (white arrow indicating). N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710)

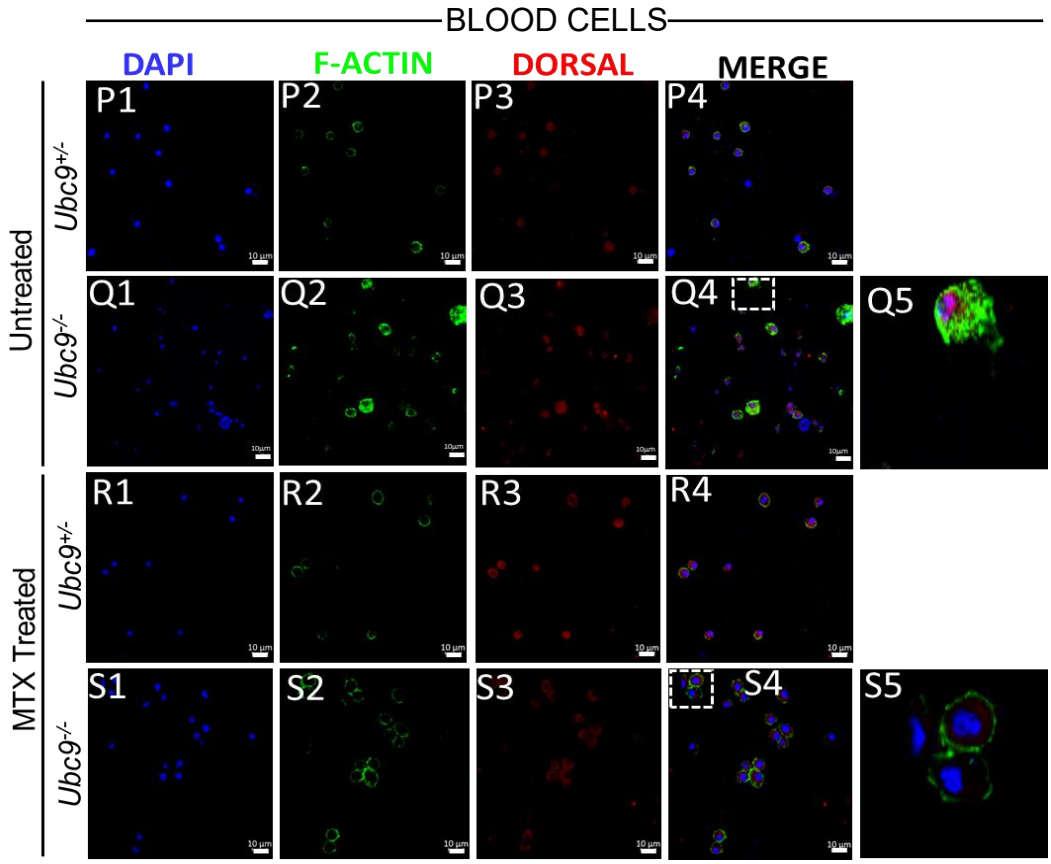
L Statistical count of % of P1 positive cells in untreated *Ubc9*$^{-/-}$ vs. MTX treated *Ubc9*$^{-/-}$. *Ubc9*$^{+/-}$ serves as control. Graphs were plotted using GraphPad software version 8.0.2. Student's t-test showed statistical significance $p<0.1798$ (ns). N=3, n=10.

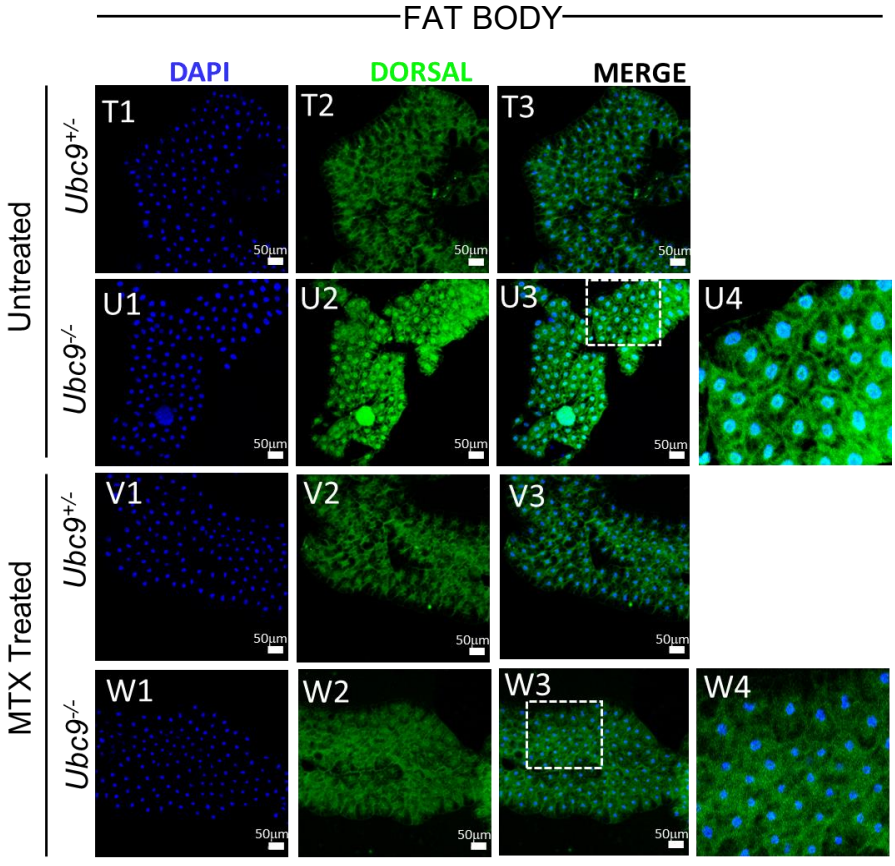
3.2.2 MTX hinders the Toll components and Dorsal localization in *Ubc9*$^{-/-}$ mutants thereby inhibiting the hyperactive Toll/NF-κB pathway

As we knew that blood cells and tumors were significantly affected so our immediate step was to documented MTX effects on transcript levels of Toll pathway components along with Dorsal localization and Cactus protein levels in the immune cells We noticed that the untreated *Ubc9*$^{+/-}$ heterozygotes showed a fold induction of 1.03 and 1.01 for *SPE* and *Drosomycin* respectively and drug treated *Ubc9*$^{+/-}$ heterozygotes showed a fold induction of 0.50 and 4.54 for *SPE* and *Drosomycin* respectively. But in untreated *Ubc9*$^{-/-}$ mutants the transcript levels of *SPE* and *Drosomycin* were approximately 9.98-fold and 15.29-fold respectively, and the treatment resulted in significant downregulation of *SPE* and *Drosomycin* to 0.83-fold and 1.04-fold respectively (Figure 3.9 M and Figure 3.9 O). Transcript levels of *Cactus*, was not significantly affected in *Ubc9*$^{+/-}$ heterozygotes with and without MTX treatment. Whereas upon MTX treatment in *Ubc9*$^{-/-}$ mutants the levels of *Cactus* drastically elevated by approximately 20.8-fold (Figure 3.9 N) compared to untreated *Ubc9*$^{-/-}$ mutants in which it was 7.46-fold. We therefore assessed cellular localization of Dorsal [since *SPE*, *Cactus* and *Drosomycin* are the targets of Dorsal,[118] through immunostaining (using anti-Dorsal antibody) in untreated *Ubc9*$^{+/-}$ heterozygotes, untreated *Ubc9*$^{-/-}$ mutants, MTX treated *Ubc9*$^{+/-}$ heterozygotes, and in MTX treated *Ubc9*$^{-/-}$ mutants. In untreated mutants' Dorsal localization was strikingly nuclear in both immune tissues compared to untreated *Ubc9*$^{+/-}$ heterozygotes in which it was clearly cytoplasmic (blood cells (Figure 3.9 S1-S4) and fat body (Figure 3.9 W1-W3). Upon MTX treatment Dorsal localization was not affected in heterozygotes immune cells but in the mutants, we saw consistency in the hindrance of Dorsal nuclear localization in both the blood cells (Figure 3.9 U1-U4) and fat body (Figure 3.9 Y1-Y3). We statistically correlated this result and found that 66.11% of fat body cells showed colocalization of dorsal with the DAPI (nuclear stain) positive fat body cells implying that dorsal was majorly nuclear localized in fat body cells and after MTX treatment this colocalization was reduced to 7.44% (Figure 3.9 X) suggesting that MTX was significantly inhibiting the dorsal nuclear localization in the fat body cells of *Ubc9*$^{-/-}$ mutants, while heterozygotes *Ubc9*$^{+/-}$ siblings with and without MTX treatment showed no difference in dorsal nuclear localization in fat cells. Similarly, the Cactus protein expression in the blood cells (Figure 3.9 Z1-Z5) and in the fat body (Figure 3.9 B6-B9) of *Ubc9*$^{-/-}$ mutants were relatively less compared to the blood cells (Figure 3.9 Y1-Y4) and fat In MTX treated *Ubc9*$^{+/-}$ heterozygotes

the Cactus protein levels were unaltered in both the blood cells (Figure 3.9 Z6-Z9) and fat body (Figure 3.9 C6-C8) whereas upon MTX treatment, in the *Ubc9*$^{-/-}$ mutants, there was considerable increase in the expression of Cactus in blood cells (Figure 3.9 Z10-Z14) and fat body (Figure 3.9 D6-D9). Altogether, these data suggest that MTX treatment evidently mitigated the hematopoietic aberrations by inhibiting the dorsal localization in the immune cells, decreasing the levels of Cactus in the cytoplasm of blood cells and affecting the transcript levels of Toll pathway components that are also the readouts (*SPE, Cactus* & *Drosomycin*).




BLOOD CELLS
DAPI
F-ACTIN
DORSAL
MERGE
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
P1
P2
P3
P4
Q1
Q2
Q3
Q4
Q5
R1
R2
R3
R4
S1
S2
S3
S4
S5
10 µm


FAT BODY
DAPI
DORSAL
MERGE
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
T1
T2
T3
U1
U2
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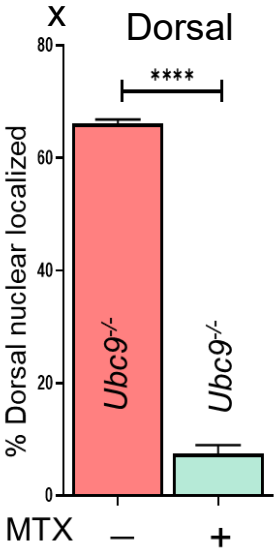
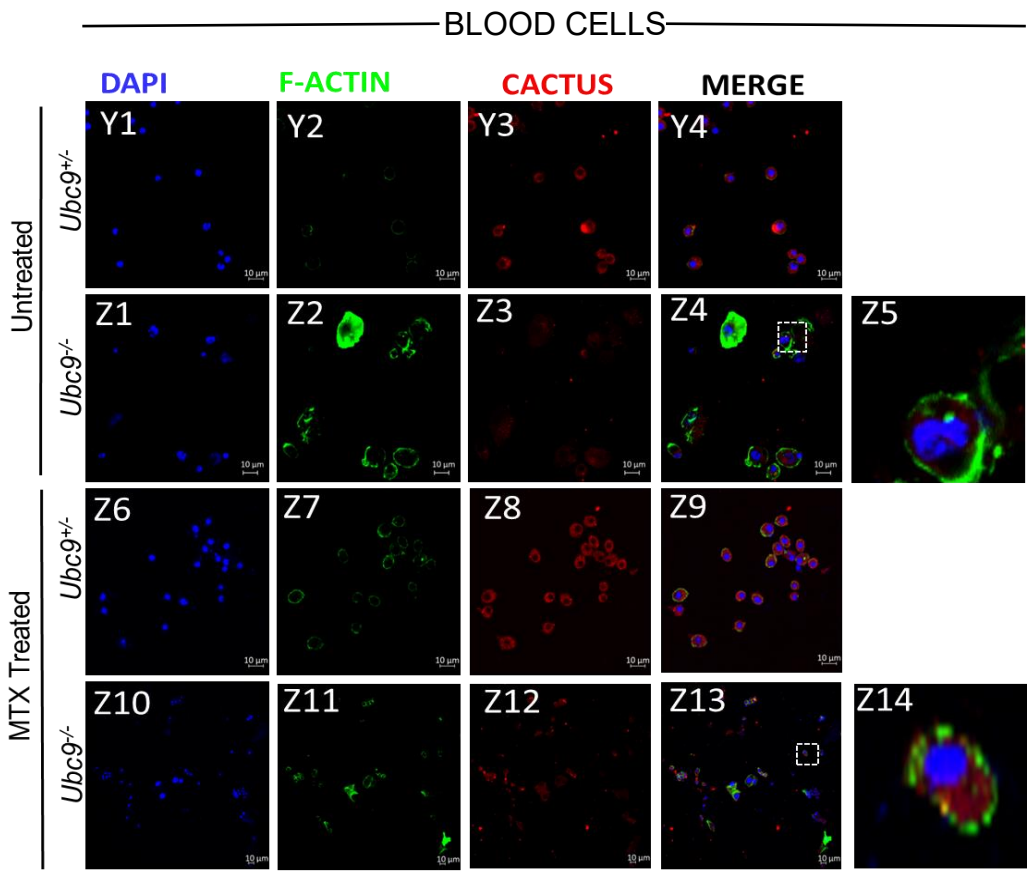
U4
V1
V2
V3
W1
W2
W3
W4
50µm


X
Dorsal
****
% Dorsal nuclear localized
80
60
40
20
0
Ubc9-/-
Ubc9-/-
MTX
–
+


BLOOD CELLS
DAPI
F-ACTIN
CACTUS
MERGE
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
Y1
Y2
Y3
Y4
Z1
Z2
Z3
Z4
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Z9
Z10
Z11
Z12
Z13
Z14
10 µm

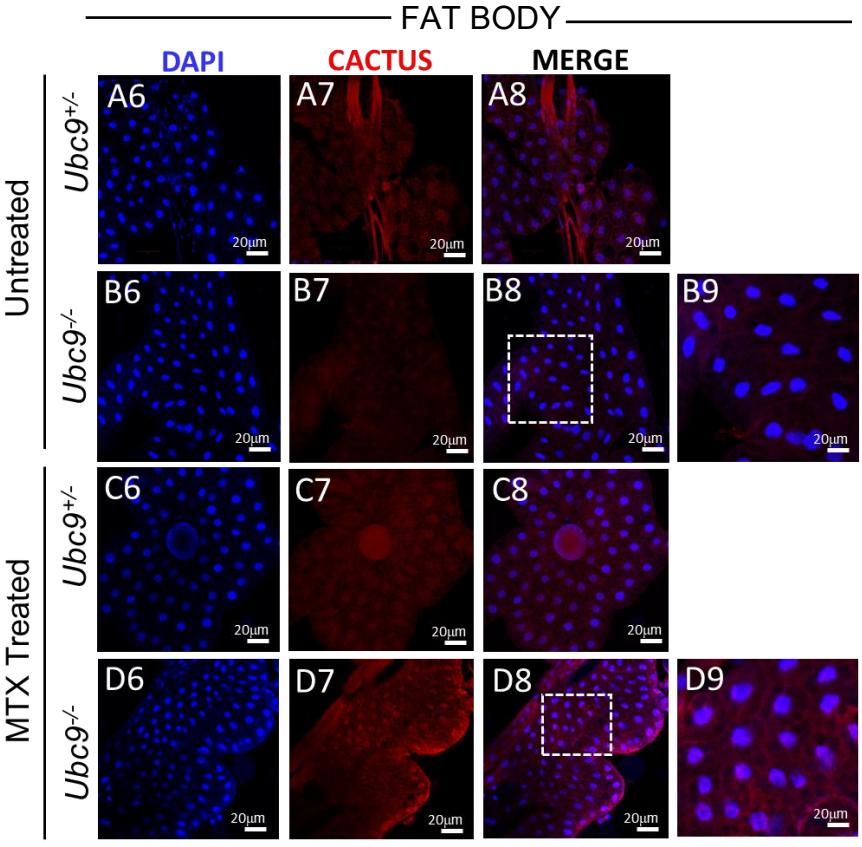

FAT BODY
DAPI
CACTUS
MERGE
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
A6
A7
A8
B6
B7
B8
B9
C6
C7
C8
D6
D7
D8
D9
20µm

Fig. 3.9 Effect of MTX on hyperactive Toll/NF-kB pathway in *Ubc9*$^{-/-}$ mutants

M, N, O Bar graphs representing the transcript levels of *SPE*, *Cactus* and *Drosomycin* in *Ubc9*$^{-/-}$ mutants before and after MTX treatment. Student's t-test (unpaired, two-tailed) showed statistical significance p<0.0001(****), p<0.001(***), and p<0.01(**). Graphs was processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C.

Dorsal localization with and without MTX treatment in the circulating blood cells of 3rd instar *Ubc9*$^{+/-}$ control and *Ubc9*$^{-/-}$ mutant larvae stained with DAPI (nuclear stain, blue), Anti-dorsal (dorsal specific antibody, red), polymerized F-actin (Cytoskeleton, green)

P1-P4 Untreated *Ubc9*$^{+/-}$ heterozygote serves the control with basal levels of Dorsal.

Q1-Q5 Untreated *Ubc9*$^{-/-}$ mutants show Dorsal nuclear localization

R1-R4 MTX treated *Ubc9*$^{+/-}$ heterozygote shows no change in the levels of Dorsal after MTX treatment.

S1-S5 MTX treated *Ubc9*$^{-/-}$ mutants proportionately show more Dorsal cytoplasmic localization after MTX treatment. N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

Dorsal localization with and without MTX treatment in the fat body cells of 3rd instar *Ubc9*$^{+/-}$ control and *Ubc9*$^{-/-}$ mutant larvae stained with DAPI (nuclear stain, blue), Anti-dorsal (dorsal specific antibody, green)

T1-T3 Untreated *Ubc9*$^{+/-}$ heterozygotes.

U1-U4 Untreated *Ubc9*$^{-/-}$ mutants showed evident Dorsal nuclear localization in the fat body cell.

V1-V3 MTX treated *Ubc9*$^{+/-}$ heterozygote shows no change in the levels of Dorsal after MTX treatment.

W1-W4 MTX treated *Ubc9*$^{-/-}$ mutants show more Dorsal cytoplasmic localization after MTX treatment. N=3, n=12. Control and experimental images were taken at identical settings in Confocal microscopy (LSM710).

X Statistical count of Dorsal nuclear localized cells in untreated *Ubc9*$^{-/-}$ vs. MTX treated *Ubc9*$^{-/-}$. Graph was plotted using GraphPad software version 8.0.2. Student's t-test showed statistical significance p<0.0001(****). N=3, n=10

Status of cactus protein in the circulating blood cells of 3rd instar larvae *Ubc9*$^{+/-}$ control and *Ubc9*$^{-/-}$ mutant, with and without MTX treatment, stained with DAPI (nuclear stain, blue), Anti-cactus (cactus specific antibody, red), polymerized F-actin (Cytoskeleton, green).

Y1-Y4 Untreated *Ubc9*$^{+/-}$ heterozygotes serves as control showing basal level of cactus (IκB)

Z1-Z5 Untreated *Ubc9*$^{-/-}$ mutant showing equivalent levels of cactus compared to untreated *Ubc9*$^{+/-}$ heterozygotes before MTX treatment

Z6-Z9 MTX treated *Ubc9*$^{+/-}$ heterozygote shows no change in the levels of cactus after MTX treatment.

Z10-Z14 MTX treated *Ubc9*$^{-/-}$ mutants show increase in the levels of cactus after MTX treatment.

Status of cactus protein in the Fat body cells of 3rd instar larvae *Ubc9*$^{+/-}$ control and *Ubc9*$^{-/-}$ mutant, with and without MTX treatment, stained with DAPI (nuclear stain, blue), Anti-cactus (cactus specific antibody, red).

A6-A8 Untreated *Ubc9*$^{+/-}$ heterozygotes serves as control showing basal level of cactus (IκB)

B6-B9 Untreated *Ubc9*$^{-/-}$ mutant showing equivalent levels of cactus compared to untreated *Ubc9*$^{+/-}$ heterozygotes before MTX treatment

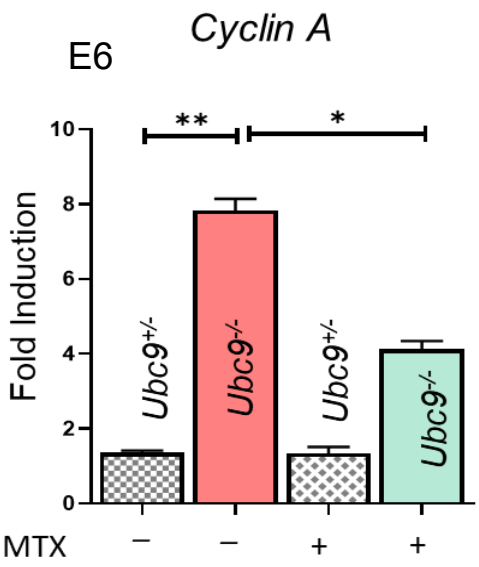
C6-C8 MTX treated *Ubc9*$^{+/-}$ heterozygote shows no change in the levels of cactus after MTX treatment.

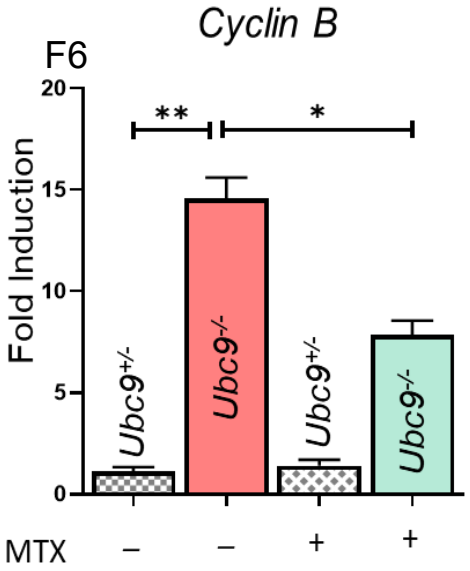
D6-D9 MTX treated *Ubc9*$^{-/-}$ mutants show increase in the levels of cactus after MTX treatment.

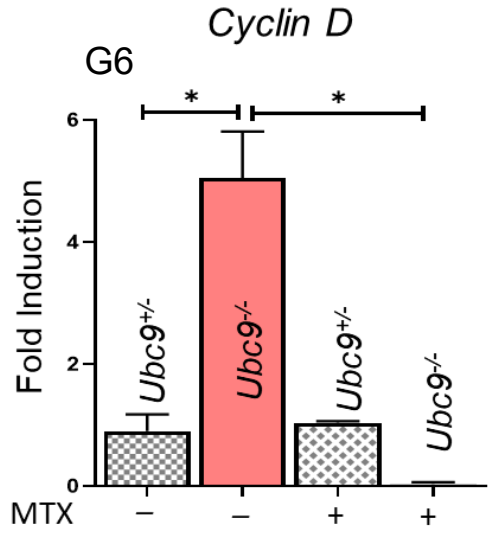
3.2.3 MTX inhibits the blood cell proliferation in *Ubc9*$^{-/-}$ mutants

Since *Ubc9*$^{-/-}$ mutants display over proliferation of blood cells as one of the hematopoietic defects due to constitutive activation of Toll pathway, it was compelling to check the status of cell cycle regulators in these mutants. For consistency and comparative analysis, we used the same cell cycle regulators as in the earlier part of the study i.e., *Cyclin A, Cyclin B and Cyclin D* along with one inhibitor *Dacapo* (*p21*) in the control versus experimental backgrounds of *Ubc9*. We found that in the untreated *Ubc9*$^{+/-}$ heterozygotes with and without MTX treatment had no significant difference in the transcript levels of the *cyclin A*, *cyclin B* and *cyclin D* as they yielded ~1 fold induction. Contrastingly, compared untreated *Ubc9*$^{+/-}$ heterozygotes the transcript levels of *cyclin A*, *cyclin B* and *cyclin D*, in untreated *Ubc9*$^{-/-}$ mutants, shoot up to 7.83-fold, 14.56-fold and 5.05-fold respectively while the drug treatment, in these *Ubc9*$^{-/-}$ mutants, led a significant downregulation to 4.12-fold, 7.86-fold, and 0.04-fold (Figure 3.10 E6, F6, G6). Unlike cyclins, the gene expression of *Dacapo*(*p21*) appeared to be significantly upregulated to 12.39-fold upon MTX treatment in the *Ubc9*$^{-/-}$ mutants in comparison to 0.79-fold observed in *Ubc9*$^{-/-}$ mutants without MTX (Figure 3.10 H6).

Altogether these results indicate that MTX possess strong ability to inhibit the chronic inflammatory phenotypes observed in the *Ubc9*$^{-/-}$ mutant larvae by shutting off the Toll Pathway.


Cyclin A
E6
Fold Induction
10
8
6
4
2
0
**
*
Ubc9+/-
Ubc9-/-
Ubc9+/-
Ubc9-/-
MTX
–
–
+
+


Cyclin B
F6
Fold Induction
20
15
10
5
0
**
*
Ubc9+/-
Ubc9-/-
Ubc9+/-
Ubc9-/-
MTX
–
–
+
+


Cyclin D
G6
Fold Induction
6
4
2
0
*
*
Ubc9+/-
Ubc9-/-
Ubc9+/-
Ubc9-/-
MTX
–
–
+
+

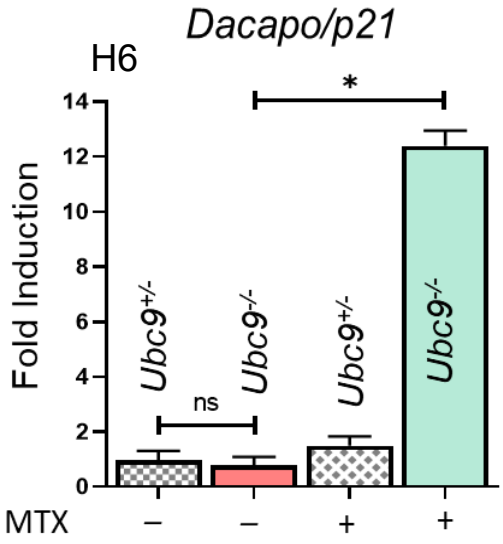

Dacapo/p21
H6
Fold Induction
14
12
10
8
6
4
2
0
*
ns
Ubc9+/-
Ubc9-/-
Ubc9+/-
Ubc9-/-
MTX
–
–
+
+

Fig. 3.10 Effect of MTX on the blood cell proliferation in *Ubc9*$^{-/-}$ mutants

E6-H6 Bar graphs showing the gene expression of cell cycle regulators (*Cyclin A, Cyclin B, Cyclin D*) and cell cycle inhibitor *Dacapo (p21)* in the *Ubc9*$^{+/-}$ control and *Ubc9*$^{-/-}$ mutant of Toll/NF-κB pathway before and after MTX treatment. Student's t-test showed statistical significance $p < 0.05$(*), & $p<0.01$ (**) on comparison with untreated *Ubc9*$^{-/-}$ mutants vs. MTX treated *Ubc9*$^{-/-}$ mutants N=3, n=50+. Graphs was processed using GraphPad prism version 8.0.2.

3. Objective 3: To study effect of MTX on the nutritional metabolism (insulin signaling) in the larvae with hyperactive Toll/NF-κB pathway in the *Ubc9*$^{-/-}$ mutant model

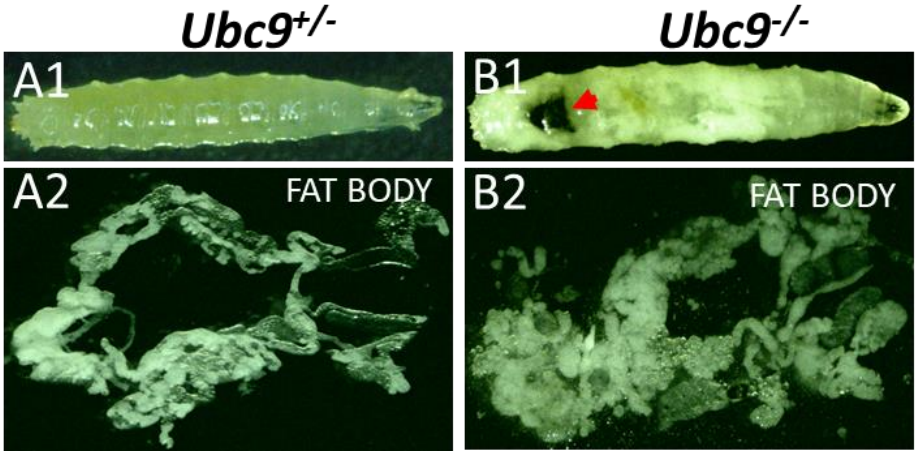
3.1 Introduction:

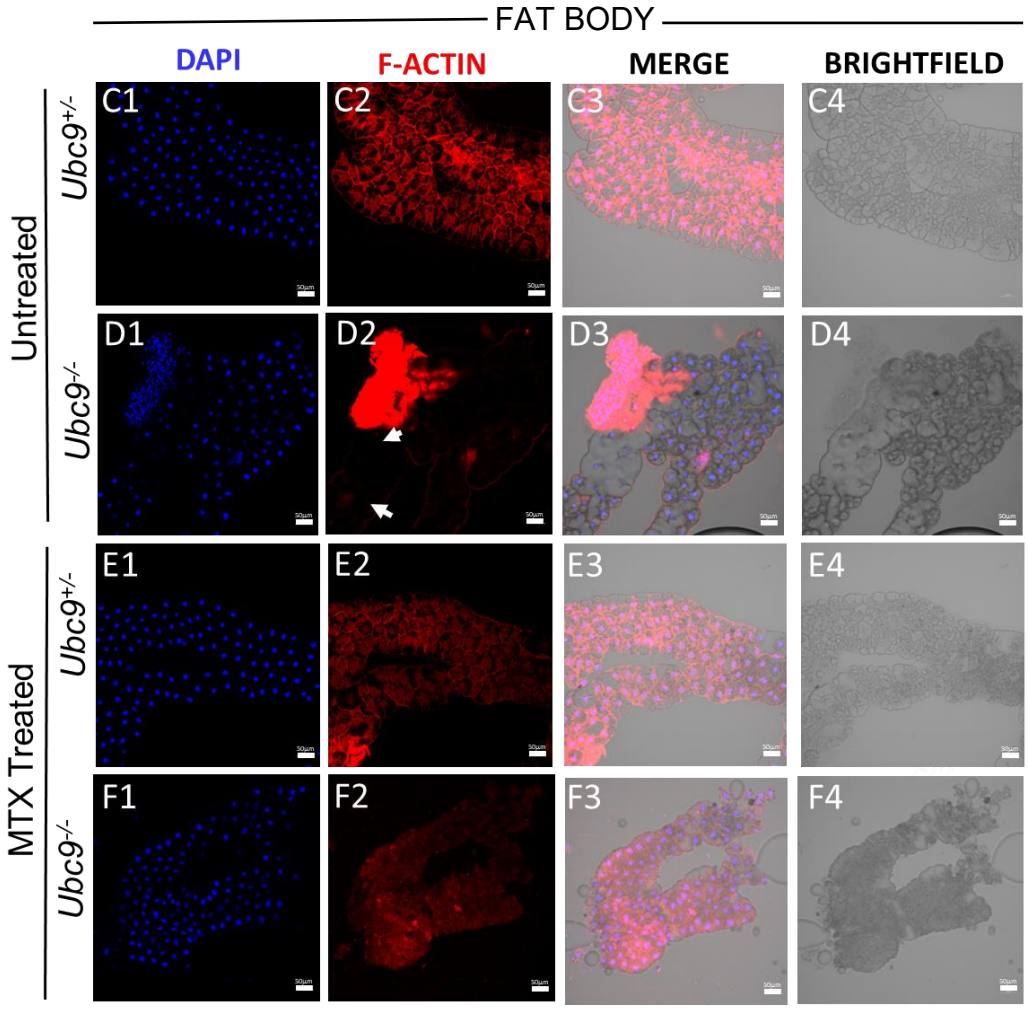
Similar to mammals, insulin signaling in *Drosophila* is critical for metabolism and regulation of energy storage in response to intrinsic and extrinsic cues [205]. PI3′K/PTEN/Akt signaling is critically involved in both the cell growth and as well as survival during different developmental stages of *Drosophila* [206]. In-depth analysis of the role of insulin/PI3K signalling in *Drosophila* has been done in the imaginal discs, which give rise to the fly's wings and eyes. [207-209]. This signaling pathway is mainly regulated by insulin-like peptides (ILPs) which bind to and control the activity of their respective receptor [210]. Till date eight dILPs (dILP1-8) exists with diverse spatiotemporal expression and a range of functions, such as growth, development, ageing, and stress response, that are analogous to the mammalian insulin and IGFs. [211]. The insulin-producing cells, the visceral muscle of the stomach, and the fat body cells all secrete these eight ILPs, also known as dILPs [212-215]. Mammalian insulin can activate the *Drosophila* insulin receptor (Inr), which shares 39% of the ligand binding domain with the human insulin receptor [216-218]. In larval and adult fat bodies, a tissue with properties resembling those of mammalian adipose tissue and the liver, dilp6 is witnessed to be highly expressed. Dilp6 expression in larvae is controlled by dFOXO and is essential for pre-metamorphic growth [150, 219]. Upon binding of dILPs to the *Drosophila* insulin receptor (dInR), the receptor phosphorylates an IRS (CHICO) resulting in a downstream signaling event. Lethal mutations in Dp110 and p60 cause the third instar larva to grow slowly or not at all. [220]. While some combinations of Inr alleles, as well as mutations in Chico or S6K, cause delays in larval development, reduced cell size, and tiny adults, Inr loss is embryonically lethal [218, 221, 222]. However, it has also been suggested that Inr/PI3K signalling may react to intrinsically programmed developmental cues to affect the differential growth that shapes tissues and organs during development. These phenotypes indicate that Inr/PI3K signalling may function in coordinating nutritional conditions with growth rates [223-225].

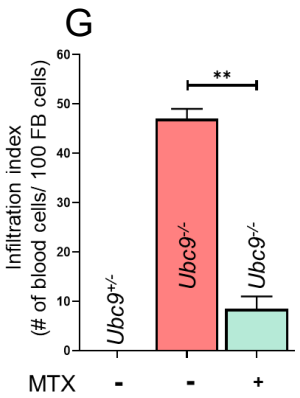
3.2 Result

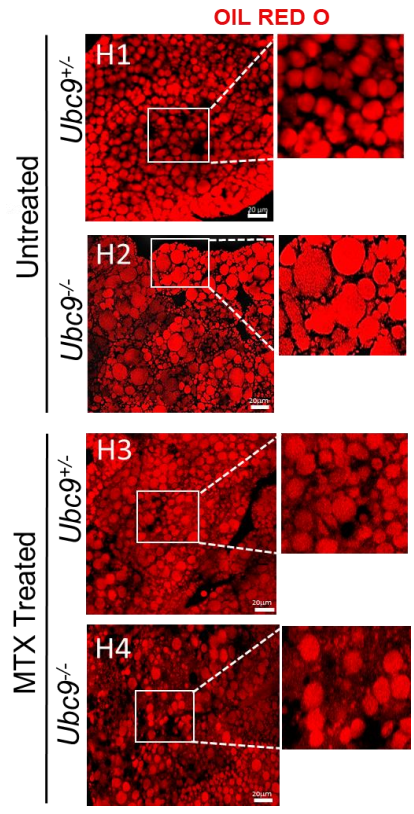
3.2.1 MTX inhibits the high infiltration index and triglyceride content in *Ubc9* mutants' fat body

Consistent with the earlier research findings, we found that the disintegrated fat body structure displayed by LOF (*Ubc9*) mutants were loaded with high fat content (Figure 3.11 B2), compared to their $Ubc9^{+/-}$ heterozygote control siblings (Figure 3.11 A2), and sometimes the disintegrating fat body cells become a part of melanized tumors as well (Figure 3.11 B1, red arrow). Also, the fat body tissue in $Ubc9^{-/-}$ mutant is infiltrated by the blood cells (Figure 3.11 D1-D4) leading to the disintegration and falling apart of fat tissues. So, we wanted to investigate if MTX has the potential to reverse the infiltration and high fat content phenotype in these $Ubc9^{-/-}$. As expected, we found that after MTX treatment in the $Ubc9^{-/-}$ mutants the infiltrated fat tissues by the blood cells were restored (Figure 3.11 F1-F4) and the same was not observed in the heterozygote siblings without (Figure 3.11 C1-C4) and with (Figure 3.11 E1-E4) MTX treatment. We quantified infiltration index by blood cells to the fat body before and after MTX treatment (Figure 3.11 G). Since $Ubc9^{-/-}$ mutants showed high fat content, we speculated further about the fat body phenotype and particularly checked the fat globules. $Ubc9^{-/-}$ mutants' fat globules stained with, *oil red O,* showed bigger lipid globules (Figure 3.11 H2) compared to $Ubc9^{+/-}$ heterozygote control siblings without or with drug treatment (Figure 3.11 H1 and 3.11 H3) but in MTX treated mutants did not show the bigger lipid globules (Figure 3.11 H4). To correlate this qualitative data, we also did the statistical count for small, medium and large lipid droplets and indeed we found that the number of large droplets were significantly reduced post MTX treatment (Figure 3.11 H5). The counting method was adapted from [226] (Panettieri et al., 2019).




FAT BODY
DAPI
F-ACTIN
MERGE
BRIGHTFIELD
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
C1
C2
C3
C4
D1
D2
D3
D4
E1
E2
E3
E4
F1
F2
F3
F4


G
Infiltration index
(# of blood cells/ 100 FB cells)
Ubc9+/-
Ubc9-/-
Ubc9-/-
MTX
-
-
+
**


OIL RED O
Untreated
MTX Treated
Ubc9+/-
Ubc9-/-
H1
H2
H3
H4

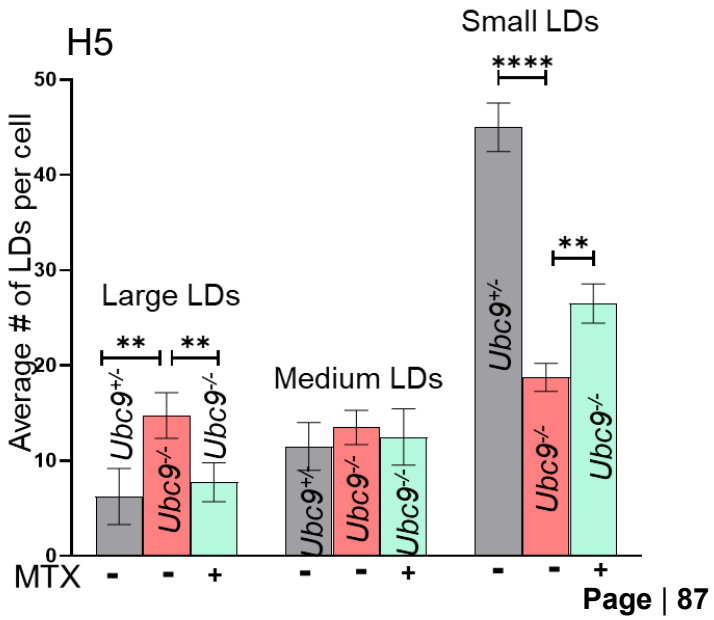

H5
Small LDs
Large LDs
Medium LDs
Average # of LDs per cell
Ubc9+/-
Ubc9-/-
Ubc9-/-
MTX
-
-
+
****
**
**

Figure 3.11 Status of Nutritional pathway in the *Ubc9*$^{-/-}$ animals

A1 3rd instar *Ubc9*$^{+/-}$ heterozygote larva

A2 Total dissected fat content in the *Ubc9*$^{+/-}$ heterozygote larva

B1 3rd instar *Ubc9*$^{-/-}$ mutant larva with tumor phenotype (red arrow indicating)

B2 Total dissected fat content in the *Ubc9*$^{-/-}$ mutant larvae

Effect of MTX on infiltration index of fat body cells in the of *Ubc9*$^{-/-}$
3rd instar wandering larvae dissected fat body tissue. Nuclear stained with DAPI (Blue)
Polymerized F-actin (Cytoskeleton) with Phalloidin red along with brightfield.

C1-C4 Untreated *Ubc9*$^{+/-}$ heterozygote

D1-D4 Untreated *Ubc9*$^{-/-}$ mutant

E1-E4 MTX treated *Ubc9*$^{+/-}$ heterozygote

F1-F4 MTX treated *Ubc9*$^{-/-}$ mutant

G The bar graph represents the statistical count of blood cells infiltrating the fat body cells in untreated *Ubc9*$^{-/-}$ mutants versus MTX treated *Ubc9*$^{-/-}$ mutants. *Ubc9*$^{+/-}$ heterozygote serves as the control. Graphs were plotted using GraphPad software version 8.0.2. Student's t-test showed statistical significance $p<0.01$(**). N=3, n=50

Status of stored triglycerides in the fat body of 3rd instar *Ubc9*$^{+/-}$ heterozygote and
Ubc9$^{-/-}$ mutant larvae before and after MTX treatment stained with oil red (specific to triglycerides, red).

H1 Untreated *Ubc9*$^{+/-}$ heterozygote

H2 Untreated *Ubc9*$^{-/-}$ mutant

H3 MTX treated *Ubc9*$^{+/-}$ heterozygote

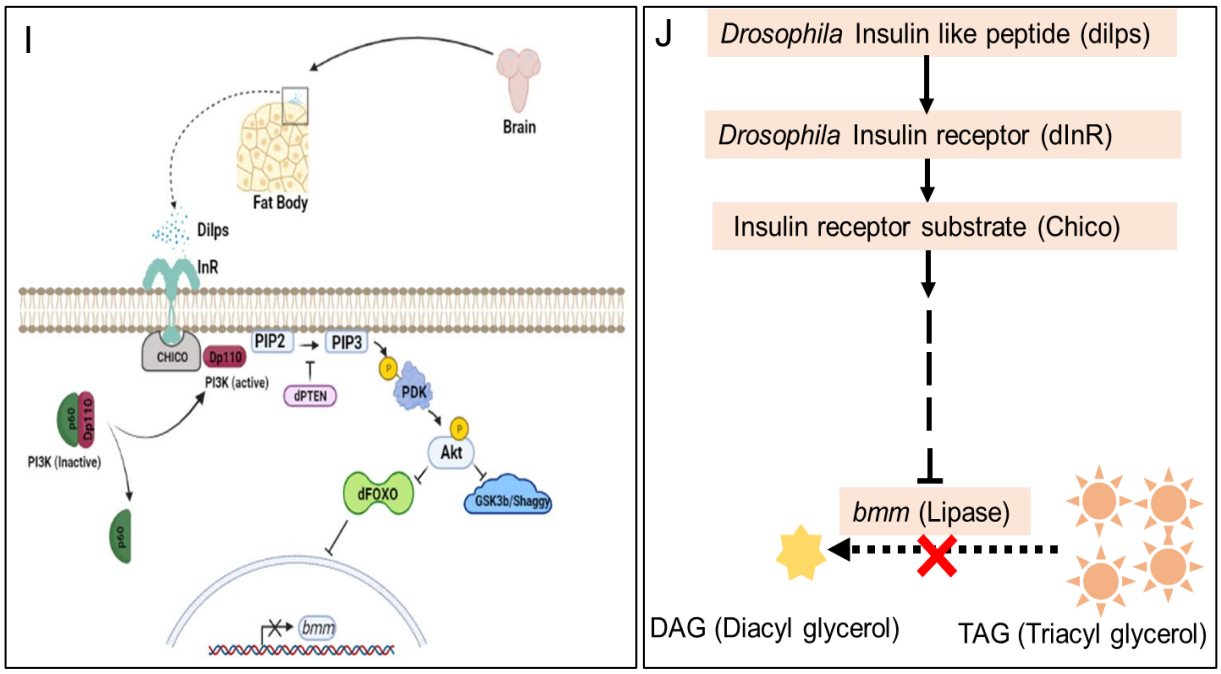
H4 MTX treated *Ubc9*$^{-/-}$ mutant

H5 Average number of large, medium or small LDs per cell from fat bodies of untreated *Ubc9*$^{+/-}$ heterozygote animals, and untreated *Ubc9*$^{-/-}$ mutant and MTX treated *Ubc9*$^{-/-}$ mutant animals. At least 30 fat body cells per animal were scored and mean±s.d. was computed using six animals. Three biological replicates were performed. **P<0.001 (Student's t-test.)

l1-K2 Status of Insulin pathway components (ligand: *dilp6*, receptor: *dInR*, receptor substrate: *chico*, Activated PI3K component: *Dp110*, transcription factor: *dFoXO*, ATGL homologue: *brummer* (*bmm*)) in the 3rd instar *Ubc9*$^{-/-}$mutant larvae before and after MTX treatment. Student's t-test (unpaired, two-tailed) showed statistical significance $p<0.0001$(****), $p<0.001$(***), $p<0.01$(**). Graphs was processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C.

3.2.3 MTX rescues the nutritional signaling components and thereby reducing the triglyceride content in *Ubc9* mutants' fat body

The high triglyceride content in *Ubc9*$^{-/-}$ mutants led us to bridge the connection between the Toll pathway and nutritional metabolism (insulin signaling) suggesting that hyperactive Toll signaling in *Ubc9*$^{-/-}$ might exert an influence on insulin signaling. Past few decades of studies on obesity, type 2 diabetes, and cancer have provided evidences that revealed a clear association between inflammatory and nutritional pathways [227, 228]. *D. melanogaster* shares several components of nutritional pathways with that of mammals. Hence, we studied the link between Toll pathway and triglyceride metabolism in the 3rd instar larvae with hyperactive Toll pathway. We determined the mRNA levels of insulin signaling components (ligand: *dilp6*; membrane receptor: *dInR*; receptor substrate: *Chico*; active component of PI3K: *Dp110*; transcription factor: *dFoxO*; lipase: *bmm* in both the loss of function (*Ubc9*$^{-/-}$ mutants) and gain of function (*Cg>SPE-Act* & *He>SPE-Act*) larvae with constitutively active Toll pathway. *dilp6*, one of the insulin-like peptides and these peptides are conserved proteins predominantly expressed in larval and adult fat body. Our result showed that the transcript levels of *dilp6* & *dInR* are increased in wandering 3rd instar *Ubc9*$^{-/-}$ mutants by 4.3-fold and 1.68-fold (Figure 3.12 K-L) compared to their basal levels in *Ubc9*$^{+/-}$ heterozygotes. Concurrently the expression of *Chico, Dp110,* were also elevated to 2.41-fold, & 1.63-fold induction in *Ubc9*$^{-/-}$ mutants (Figure 3.12 J1-J2) while *dFoxO* and *bmm* showed a decline and were 0.28 & 0.58-fold (Figure 3.12 K1-K2) compared to the normal expression in *Ubc9*$^{+/-}$ heterozygotes. Therefore, all the results put together indicate that the increased triglyceride content in the *Ubc9*$^{-/-}$ mutants' larval adipocytes is likely a consequence of elevated levels of insulin signaling that ultimately leads to the reduction of *bmm* levels. Since we could relieve the *Ubc9*$^{-/-}$ mutants, from loss of fat body integrity by MTX drug treatment we speculated if the recovery of the defective fat body was due to MTX effects on insulin pathway. To comprehend MTX's effect we checked all the insulin components. After MTX treatment we found that elevated transcript levels of *dilp6, dInR, Chico, Dp110* were significantly downregulated whereas the low levels of *dFoxO* and *bmm* were significantly upregulated (Figure 3.12 I1-K2). This led us to conclude that *Ubc9*$^{-/-}$ mutants showing aberrant fat body phenotype is a consequence of upregulated nutritional metabolism that were significantly normalized after MTX treatment.



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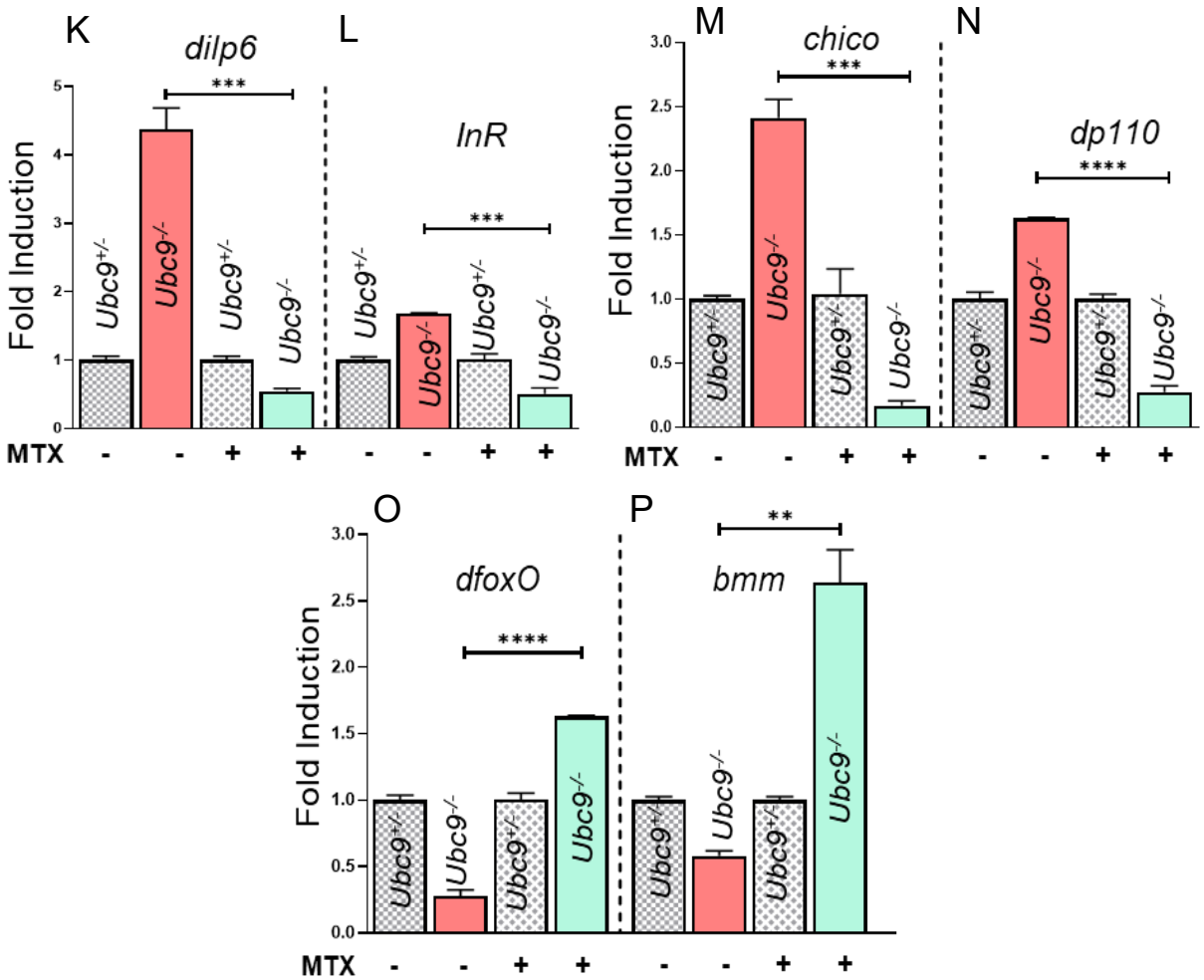


Figure 3.12 Effect of MTX on nutritional metabolism in *Ubc9*$^{-/-}$ mutants

I Schematic showing the nutrient sensing via larval brain by the fat body with Insulin and PI3K/AKT pathway majorly responsible for controlling the nutritional metabolism in *Drosophila* larvae

J Hypothetical flowchart showing the hierarchical components of Insulin pathway in *Ubc9*$^{-/-}$ mutants leading to the inactivation of Lipase enzyme (*bmm*) thereby affecting the conversion of Diacyl glycerol (DAG) to Triacyl glycerol

K-P Status of Insulin pathway components (ligand: *dilp6*, receptor: *dInR*, receptor substrate: *chico*, Activated PI3K component: *Dp110*, transcription factor: *dFoXO*, ATGL homologue: *brummer* (*bmm*)) in the 3rd instar *Ubc9*$^{-/-}$mutant larvae before and after MTX treatment. Student's t-test (unpaired, two-tailed) showed statistical significance $p<0.0001$(****), $p<0.001$(***), $p<0.01$(**). Graphs was processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C.

3.2.4 Genetic rescue of *Ubc9*$^{-/-}$ mutants

We wanted to understand if the hyperactive Toll signaling might exert an influence on insulin signaling. To establish a possible link between Toll pathway and insulin signaling, we performed a genetic rescue of *Ubc9*$^{-/-}$ mutants by overexpressing Cactus, the inhibitor of the transcription factor, Dorsal. In *Ubc9* mutant background we overexpressed a wild type cactus protein (*UAS-Cactus-RFP*) using *Cg-Gal4* promoter. To comprehend if there was a rescue, *Ubc9*$^{4-3/5}$, *Cg>Cact-RFP* were compared to *Ubc9*$^{4-3/5}$ mutants (Figure 3.13 A-C). We documented tumor penetrance (% larvae exhibiting tumor phenotype) and expressivity (number of tumors per larvae), determined the transcript levels of *msn, SPE* and *Drosomycin* to observe the suppression of hyperactive Toll pathway in rescued larvae comparing them to *Ubc9*$^{4-3/5}$ mutants. There was a reduction of 56.55% in the tumor penetrance (Figure 3.13 C) and the expressivity went down to 2.95 tumors per rescued larva compared to 3.59 tumors per mutant larva (Figure 3.13 D). The *msn* transcript levels were induced up to 8.19-fold induction in the *Ubc9*$^{-/-}$ mutants whereas in the genetically rescued larvae the levels significantly dropped to 1.66-fold suggesting a possible reason for the reduction of blood tumor formation that primarily compose of lamellocytes in *Ubc9*$^{-/-}$ mutants (Figure 3.13 E). Expression of *SPE* and *Drs* was upregulated to 6.48-fold and 12.55-fold respectively in *Ubc9*$^{-/-}$ mutants compared to their *Ubc9*$^{+/-}$ heterozygote siblings whereas in the genetic rescued animals (*Ubc9*$^{-/-}$, *Cg>Cact-RFP*) the expression was significantly downregulated 1.14- and 1.35-fold (Figure 3.13 F-G). Since Toll pathway appeared to be restored in rescued animals, we studied the transcript levels of *dilp6, dInR*, and *bmm* to understand if insulin signaling was affected. The insulin signaling components (*dilp6* 4.32-fold and *dInR* 5.04-fold) were upregulated and *bmm* (0.11-fold) transcript levels were downregulated in *Ubc9*$^{-/-}$ mutants. In the larvae of *Ubc9* with overexpression of *Cactus-RFP* the mRNA levels were decreased and appeared closer to the heterozygote siblings. As per our hypothesis in the genetic rescued larvae (*Ubc9*$^{-/-}$, *Cg>Cact-RFP*) the transcript levels of insulin components were restored (Figure 3.13 H-J) thereby maintaining triglycerides in *Drosophila* adipocytes.

Altogether all these results clearly indicate that hyperactive Toll pathway is suppressed by the MTX and it is comparable to the genetic rescue observed in *Ubc9*$^{-/-}$, *Cg>Cact-RFP*. It is also evident from our findings that *Ubc9*$^{-/-}$ mutants display abnormal insulin signaling that is possibly causing the disrupted fat body phenotype. Finally, our investigation clearly shows

the *Ubc9*$^{-/-}$ mutants can restore immune homeostasis upon treatment with MTX as both the over active Toll pathway and the abnormal insulin signaling are affected by MTX treatment.

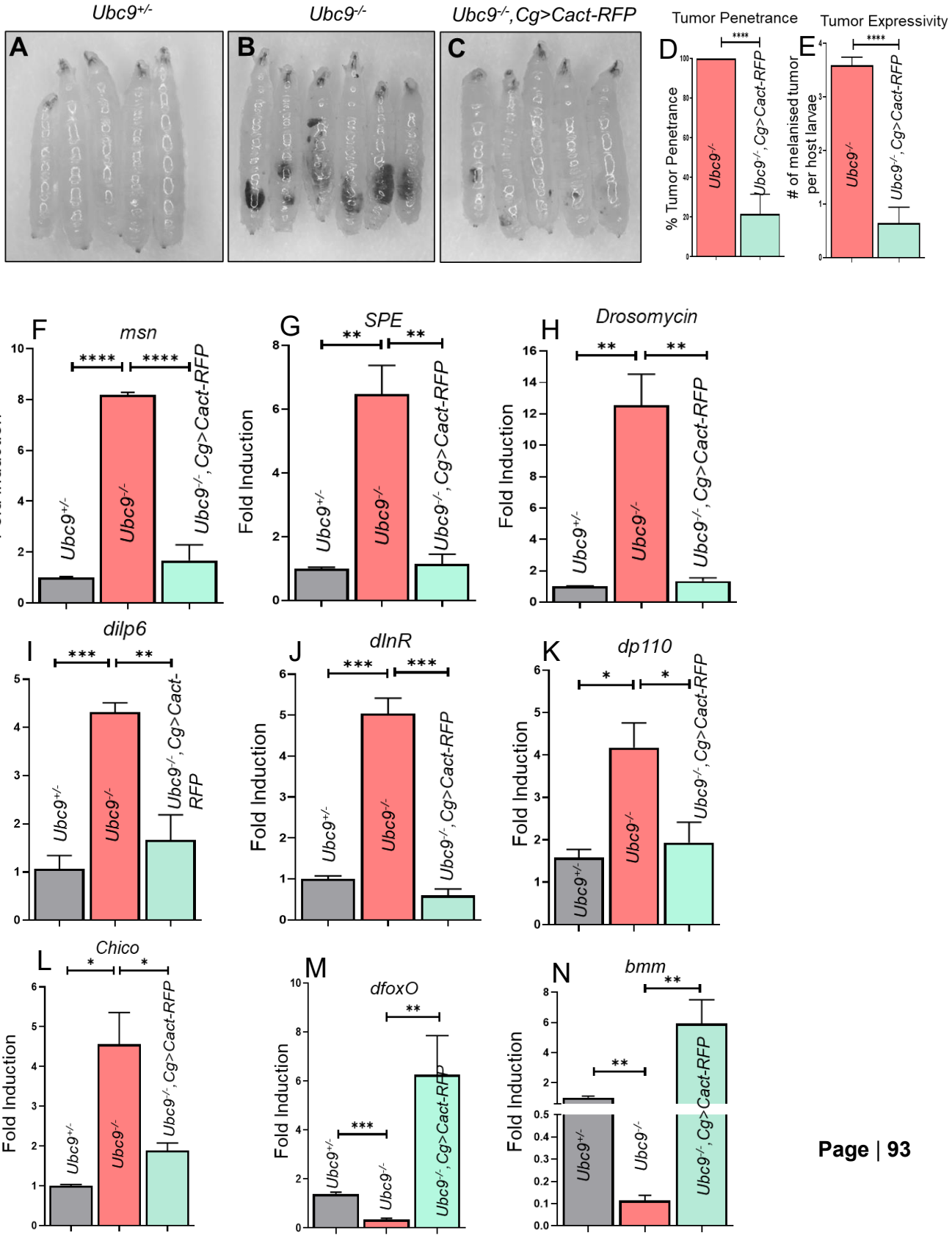


Figure 3.13 Restoring the *Ubc9*$^{-/-}$ defects via genetic rescue by overexpressing cactus

A 3rd instar *Ubc9*$^{+/-}$ heterozygotes larvae control.

B 3rd instar *Ubc9*$^{-/-}$ mutants larvae carrying melanotic tumors.

C 3rd instar *Ubc9*$^{-/-}$ mutants rescued genetically by overexpressing cactus with *Cg-Gal4*.

D-E Graphical representation of percentage (%) tumor penetrance and tumor expressivity between *Ubc9*$^{-/-}$ mutants versus *Ubc9*$^{-/-}$ with genetic rescue. Student's t-test (unpaired, two-tailed) showed statistical significance p<0.0001(****). Graphs was processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C.

E-G Transcript levels of *msn* **(E)**, *SPE* **(F)**, and *Drosomycin* **(G)** in the 3rd instar *Ubc9*$^{-/-}$ mutant larvae and *Ubc9*$^{-/-}$ with genetic rescue, *Ubc9*$^{+/-}$ heterozygote serves as the control. Student's t-test (unpaired, two-tailed) showed statistical significance p<0.0001(****), and p<0.01(**). Graphs were processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C.

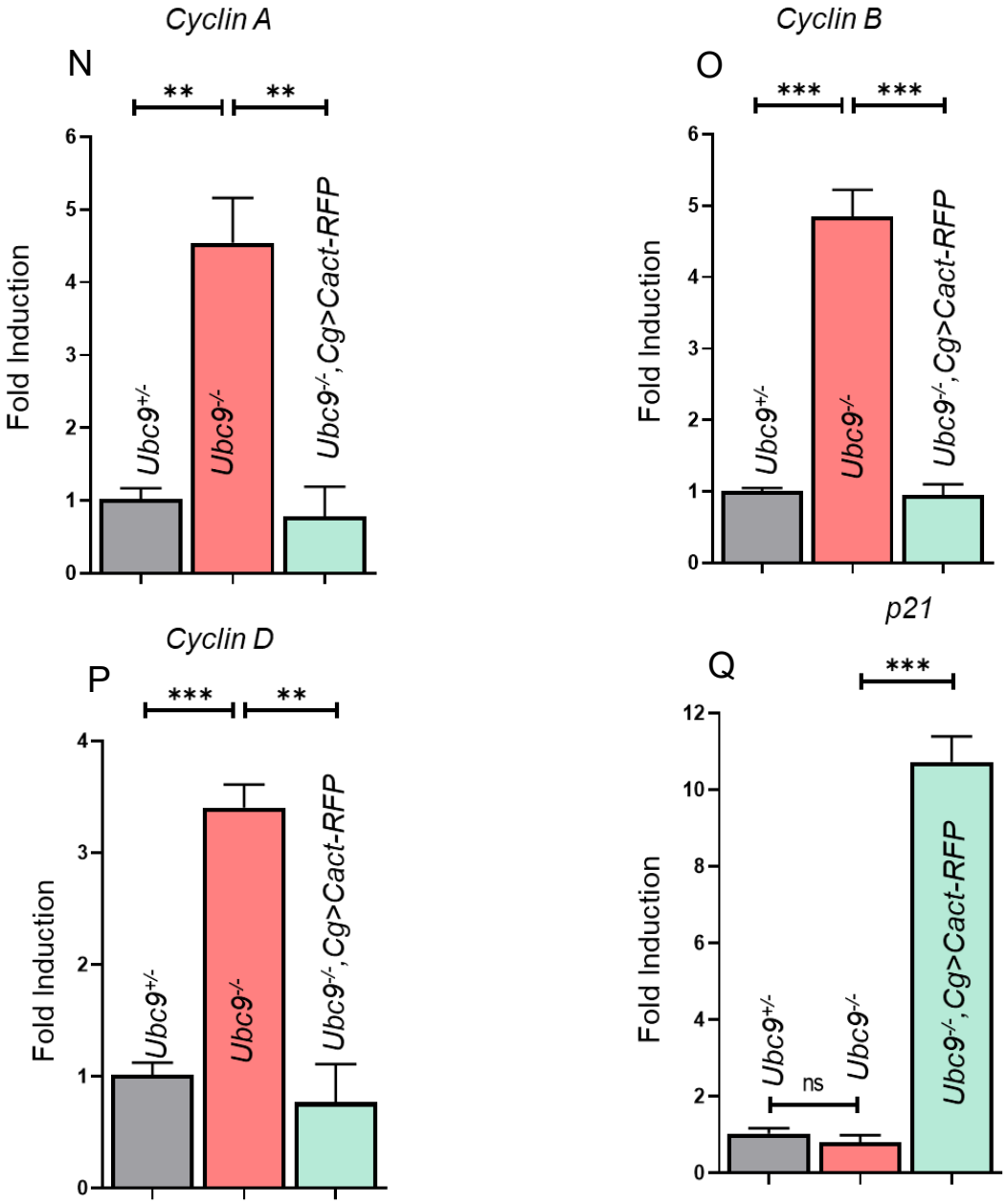
I-N Transcript levels of Insulin components (*dilp6* **(I)**, *dInR* **(J)**, *dp110* **(K)**, *Chico* **(L)**, *dfoxO* **(M),** *bmm* **(N))** in the 3rd instar *Ubc9*$^{-/-}$ mutant larvae and *Ubc9*$^{-/-}$ with genetic rescue. *Ubc9*$^{+/-}$ heterozygote serves as the control Student's t-test (unpaired, two-tailed) showed statistical significance p<0.001(***), and p<0.01(**). Graphs were processed using GraphPad prism version 8.0.2. N=3, n=50. Experiments were conducted at 25 °C.

3.2.5 Genetic rescue of *Ubc9*$^{-/-}$ mutants also rescue the increased cell cycle regulators

After performing genetic rescue in *Ubc9*$^{-/-}$ mutants via overexpression of cactus, not only Toll signaling was rescued we also observed that Insulin components were normalized compared to *Ubc9*$^{-/-}$ mutants. This rescue phenotype was documented as mentioned in the figure 3.13.

Toll/NF-κB signaling has proven role in blood cell proliferation, and also the fact that constitutive activation of Toll pathway (*Ubc9*$^{-/-}$ mutants) leads to the over proliferation of blood cells. With this backdrop we wanted to check the status of cell cycle regulation in the *Ubc9*$^{-/-}$ mutant larvae that are genetically rescued. In order to comprehend the cell cycle regulation in genetically rescued larvae we checked the same set of cyclins *cyclin A*, *Cyclin B*. *Cyclin D* and *p21*(inhibitor or cell cycle) that were used for previous objectives to maintain the consistency throughout the study. All the cyclins were significantly upregulated in the *Ubc9*$^{-/-}$ mutants and the inhibitor of the cyclin *p21*, was significantly downregulated compared to the *Ubc9*$^{-/-}$ heterozygotes (Figure 3.14 N-Q) (consistent with the earlier reported studies). Whereas in the genetically rescued larvae (*Ubc9*$^{-/-}$, *Cg>Gal4, UAS-Cactus RFP*) the upregulated levels of cyclins were significantly restored to the comparable levels of *Ubc9*$^{-/-}$ heterozygotes. Unlike cyclins, the downregulated transcript levels of *p21* in *Ubc9*$^{-/-}$ mutants were significantly upregulated in the genetically rescued larvae (*Ubc9*$^{-/-}$, *Cg>Gal4, UAS-Cactus RFP*) (Figure 3.14 N-Q).

This data strengthened our hypothesis, and had a clear indication that upon performing a genetic rescue in the *Ubc9*$^{-/-}$ mutants not only the Toll signaling dependent insulin signaling was rescued but also the hyper proliferation of blood cells, witnessed as one of the defects associated with *Ubc9*$^{-/-}$ mutants, was also significantly improved thereby reducing the over proliferation of blood cells.


Cyclin A
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Ubc9-/-
Ubc9-/-,Cg>Cact-RFP
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Ubc9-/-,Cg>Cact-RFP

Figure 3.14 Restoring the hyper cell cycle defects via genetic rescue by overexpressing cactus in *Ubc9*$^{-/-}$

N-Q Bar graphs showing the gene expression of cell cycle regulators i.e., (**N)** *Cyclin A*, **(O)** *Cyclin B*, **(P)** *Cyclin D*) and cell cycle inhibitor **(Q)** *Dacapo (p21)* in the *Ubc9*$^{+/-}$ control, *Ubc9*$^{-/-}$ mutant and *Ubc9*$^{-/-}$ with genetic rescue. Student's t-test showed statistical significance $p < 0.05$(*), & $p<0.01$ (**) on comparison with untreated *Ubc9*$^{-/-}$ mutants vs. MTX treated *Ubc9*$^{-/-}$ mutants N=3, n=50+. Graphs was processed using GraphPad prism version 8.0.2.

3.3 Discussion

Chemotherapy, targeted therapy, immunotherapy and hormone therapy are different ways of cancer treatment and differ in their mode of action [229, 230]. The type and extent of cancer treatment depends primarily on the stage of its progression and also on its effect on non-cancerous neighbouring cells but most importantly on the tumor bearing host's immune system. Several (published and ongoing) studies are not only examining the efficacy of the anti-cancer drugs but are also harnessing research to determine ways in which the non-cancerous cells specifically immune cells better tolerate these cancer therapeutics to remain healthy and normal [231-233]. Therefore, it is essential to first decipher the routes of escape by the cancerous cells and secondly to also examine the routes of submission by the non-cancerous cells to cancer treatment. In both of the scenarios the cellular responses are altered by the existing treatments. One of the anti-cancer drugs is Methotrexate which is also used to comprehend immune responses associated with inflammatory diseases [234]. Such studies are an excellent example of experimental approaches addressing drug repurposing or repositioning.

In the current study we used *Drosophila* to elucidate the link between immune deregulation and insulin signaling in *Ubc9*$^{-/-}$ mutant larvae with hyperactive Toll signaling that exhibit abnormal blood cell development, disintegrating fat body and tumorigenesis leading to disturbances in the immune homeostasis. We elucidated how MTX treatment restored this immune balance. In the past decade there were reports from different groups clearly showing a link between Toll pathway and Insulin signaling [235]. Through our study we first demonstrated that upon administration of MTX there was a significant relief in the deteriorating hematopoietic and fat body phenotypes that prevailed in absence of the drug. These findings are supported by inhibition of hyperactive Toll pathway by MTX shown through several experimental results such as a decrease in the nuclear localization of Dorsal in the immune cells, change in the expression Toll pathway target genes *Drosomycin, SPE, Cactus* [118] and decrease in the infiltration of the fat body along with loss of the bigger lipid droplets and finally proper regulation of insulin signaling (**Figure 3.2- Figure 3.12 and Fig.3.15)**. Our final research finding showed that overexpression of Cactus in the constitutively activated Toll pathway mutants, *Ubc9*$^{-/-}$ results in restoration of immune equilibrium by MTX treatment **(Fig.3.13 and Fig.3.14)**. This genetic rescue is comparable to MTX effects on *Ubc9*$^{-/-}$ mutants observed in the study.

Similar results were published where use of an anti-inflammatory drug aspirin relieved the inflammatory phenotypes observed in Toll pathway mutants and *hop*$^{Tum\text{-}l}$ mutants. In previously published research involving the anti-cancer [174] and anti-inflammatory drugs [226] using model systems or in our current study using MTX it is clearly shown that the rescues are not complete with none of the phenotypes completely recovered due to drug treatment. Further studies are therefore mandatory to elucidate the effects of combination of drugs used for such treatment. While novel drugs are constantly used for experimenting in basic research and clinical studies, the need of the hour is also in exploiting the existing drugs by revisiting their effects and side effects.

Throughout in our studies we have utilized both the physiological (wasp induced inflammation) and genetically altered models (LOF and GOF of Toll pathway) that can possibly mimic the molecular events leading to blood cancer or inflammatory phenotypes exhibited by the higher vertebrates. *Drosophila* model system aid this possibility to a greater extent giving us opportunity to test our hypothesis. Our first model, where we actively exploited the host-pathogen interaction thereby creating a disturbance in the *Drosophila* host blood homeostasis leading to the upregulation of several hematopoietic pathways where we specially focussed on Toll pathway. As a consequence, the host response is stimulated such as over proliferation of blood cells (plasmatocytes and lamellocytes) to counteract with the its pathogen. The whole molecular chaos (synonymous to mammalian acute inflammation) in the host was largely was improved and circumvented by the use an anti-cancer drug, MTX, that relieved the host to an extent to survive.

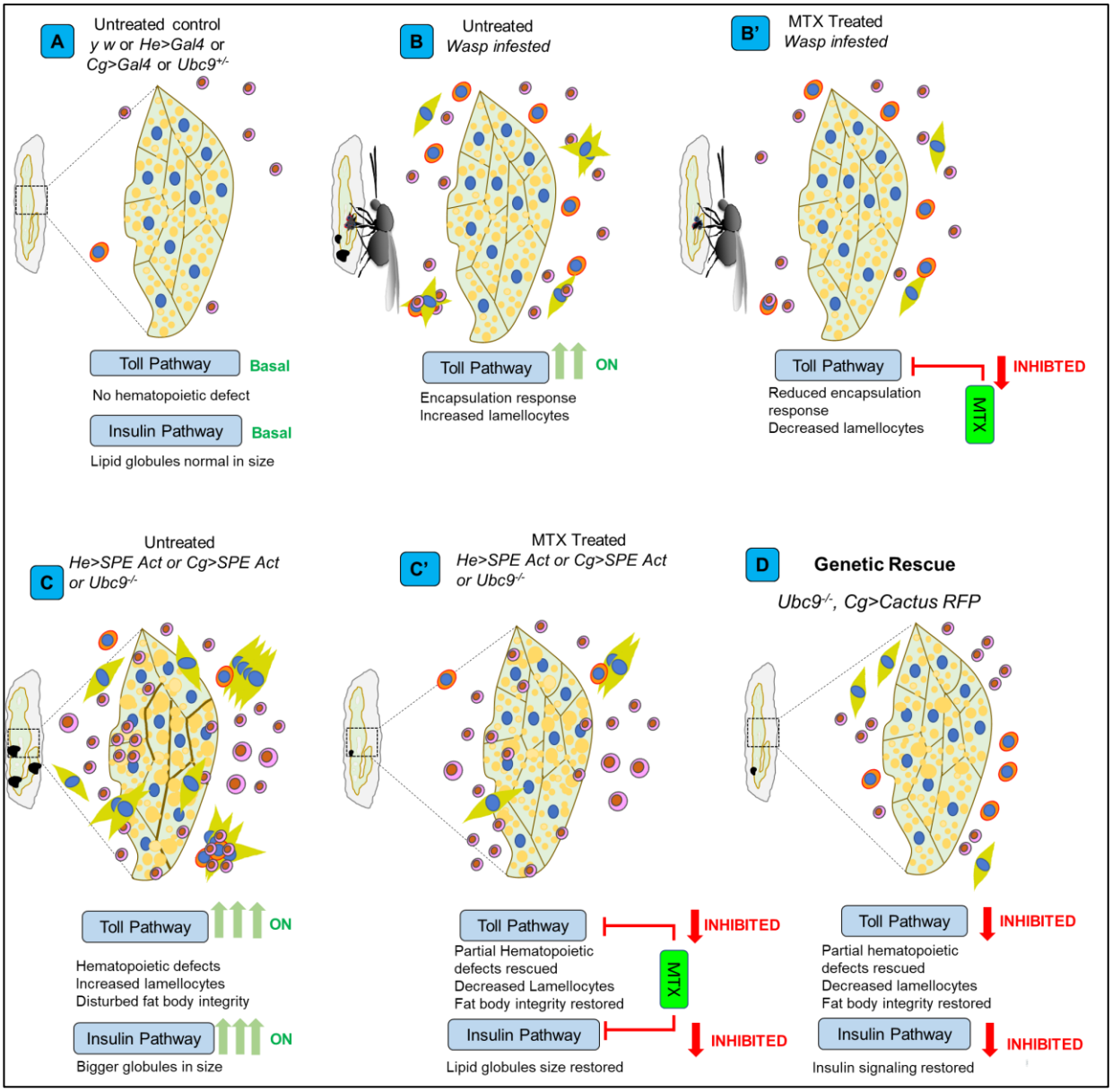
Similarly in the genetically altered models such as knockout of *Ubc9* (LOF) and *SPE* overexpression (GOF) of Toll pathway exhibited the hematopoietic defects with stronger phenotypes, compared to physiological model, that could be synonymous to chronic inflammation. Here also, MTX mitigated the defects associated with LOF and GOF mutants of Toll pathway. In both the models (physiological and genetically altered) the upregulation of one the hematopoietic pathways, Toll pathway, was contributing to the disturbance in the *Drosophila* blood homeostasis. Interestingly, MTX was able to suppress the upregulation of Toll pathway thereby able to maintain the equilibrium. Earlier published research articles including ours have projected the role of MTX effects on hyperactive JAK/STAT pathway. These research articles have extended the possibility to extrapolate the MTX's role in other essential hematopoietic pathways. And here, we unravel the possibility of MTX usage as a novel drug to chase an off target apart from its canonical target (DHFR) in the cell. This whole new mechanistic view can be attributed as drug repositioning or repurposing concept.

Also, we figured out that hyperactive Toll pathway in *Ubc9*$^{-/-}$ mutants was also directly involved in the upregulation of Insulin signaling. And this upregulated insulin signaling resulted in extra storage of triglycerides in the fat tissues of *Ubc9*$^{-/-}$ mutants' larvae. To cross verify the connection of Toll and Insulin signaling we did genetic recuse by putting one of the Toll components back into the *Ubc9*$^{-/-}$ mutants and indeed we found that not only Toll signaling was

rescued but also the insulin signaling was improved. This led us to bridge the connection that Insulin signaling possibly would downstream to Toll pathway.

In a nutshell our research sheds light on (a) the paradigm showing the existing anti-cancer drug MTX repositioning effects on immune defects associated with Toll pathway, (b) contribution of two different cellular pathways, Toll and Insulin for developmental, hematopoietic and immune defects and (c) finally how the genetic rescue of *Ubc9*$^{-/-}$ mutants (with overexpression of *Cactus*) can restore the immune homeostasis disturbed due to hyperactive Toll and Insulin signaling.

4. Summary


A
Untreated control
y w or He>Gal4 or
Cg>Gal4 or Ubc9+/-
Toll Pathway
Basal
No hematopoietic defect
Insulin Pathway
Basal
Lipid globules normal in size
B
Untreated
Wasp infested
Toll Pathway
ON
Encapsulation response
Increased lamellocytes
B'
MTX Treated
Wasp infested
Toll Pathway
INHIBTED
MTX
Reduced encapsulation response
Decreased lamellocytes
C
Untreated
He>SPE Act or Cg>SPE Act or Ubc9-/-
Toll Pathway
ON
Hematopoietic defects
Increased lamellocytes
Disturbed fat body integrity
Insulin Pathway
ON
Bigger globules in size
C'
MTX Treated
He>SPE Act or Cg>SPE Act or Ubc9-/-
Toll Pathway
INHIBITED
MTX
Partial Hematopoietic defects rescued
Decreased Lamellocytes
Fat body integrity restored
Insulin Pathway
INHIBITED
Lipid globules size restored
D
Genetic Rescue
Ubc9-/-, Cg>Cactus RFP
Toll Pathway
INHIBITED
Partial hematopoietic defects rescued
Decreased lamellocytes
Fat body integrity restored
Insulin Pathway
INHIBITED
Insulin signaling restored

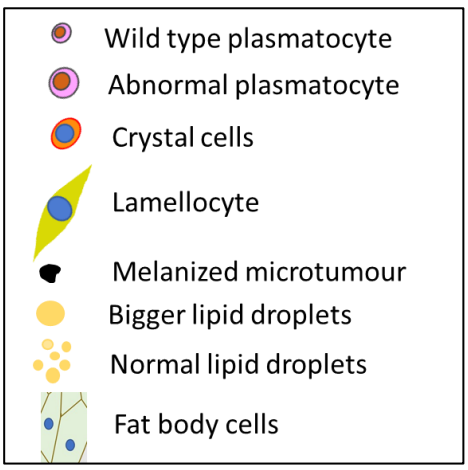

Wild type plasmatocyte
Abnormal plasmatocyte
Crystal cells
Lamellocyte
Melanized microtumour
Bigger lipid droplets
Normal lipid droplets
Fat body cells

Figure 3.15. Schematic of overall summary of the study

A. Schematic showing the fat body and blood cells distribution of wild type *Drosophila* 3rd instar larvae such as *y w* or *He>Gal4* or *Cg>Gal4* or *Ubc9*$^{+/-}$ heterozygote devoid of any hematopoietic defects. Toll and insulin pathway are considered to be switched off.

B. Schematic portraying the *Lb-17* female wasp invading the wild type *y w* 3rd *Drosophila* larval host by injecting its egg inside the hemocoel thereby triggering encapsulation response in the host by the activation of Toll pathway.

B' After MTX treatment the Toll pathway is downregulated with the decrease in encapsulation response and blood cell proliferation

C. Schematic projecting the fat body and blood cells of SPE overexpression (Gain-of-Function) via *He>SPE-Act* and *Cg>SPE-Act*, or *Ubc9*$^{-/-}$ mutants displaying constitutive activation of Toll pathway leading to the hematopoietic and fat body defects with high fat body infiltration by the blood cells. Bigger fat globules are seen as a consequence of enhanced insulin signaling.

C' The hyperactive Toll pathway in He>SPE-Act and Cg>SPE Act, or Ubc9-/- mutants was also mitigated due to MTX treatment and were comparable to their heterozygote control siblings. The fat body integrity was restored. The bigger fat globules were also significantly reduced due to reduction in the Insulin signaling after MTX treatment.

D. The schematic showing the genetic rescue with an extra copy of Cactus into the Ubc9-/- mutants. The genetic rescue was partial and significant as there was reduction in the tumor formation and reduced Toll signaling.

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The list of Publications

First author publications

S.No.	Authors	Title	Journal/Reference
1.	**Gautam DK**, Ahmad Z, Gutti RK, Paddibhatla I	Methotrexate suppresses tumorigenesis by restoring immune equilibrium and hindering insulin signaling via its control on Toll/NF-κB pathway	***Cell Biol Toxicol.*2023** (Undergoing first revision) **IF:6.8**
2.	Yadav RK, **Gautam DK**, Muj C, Gajula Balija MB, Paddibhatla I	Methotrexate negatively acts on inflammatory responses triggered in *Drosophila* larva with hyperactive JAK/STAT pathway	***Dev Comp Immunol***. 2021 Oct; 123:104161. *doi: 10.1016/j.dci.2021.104161* **IF:3.605**
3.	**Gautam DK**, Chimata AV, Gutti RK, Paddibhatla I	Comparative hematopoiesis and signal transduction in model organisms	***J Cell Physiol***. 2021 Aug;236(8):5592-5619. *doi: 10.1002/jcp.30287* **IF:6.513**

Co-author publications

4.	Sharma DS, Paddibhatla I, Raghuwanshi S, Malleswarapu M, Sangeeth A, Kovuru N, Dahariya S, **Gautam DK**, Pallepati A, Gutti RK	Endocannabinoid system: Role in blood cell development, neuroimmune interactions and associated disorders.	***J Neuroimmunol***. 2021 Apr 15; 353:577501. *doi:10.1016/j.jneuroim.2021.577501.* **IF:3.221**
5.	Sharma DS, Raghuwanshi S, Kovuru N, Dahariya S, **Gautam DK**, Paddibhatla I, Gutti RK	Virodhamine, an endocannabinoid, induces megakaryocyte differentiation by regulating MAPK activity and function of mitochondria	***J Cell Physiol***. 2021 Feb;236(2):1445-1453. *doi: 10.1002/jcp.29949.* **IF:6.513**
6.	Raghuwanshi S, Dahariya S, Musvi SS, Gutti U, Kandi R, Undi RB, Sahu I, **Gautam DK**, Paddibhatla I, Gutti RK	MicroRNA function in megakaryocytes	***Platelets***. 2019;30(7):809-816. *doi: 10.1080/09537104.2018.1528343* **IF:4.236**

7.	Paddibhatla I, **Gautam DK**, Mishra RK	SETDB1 modulates the differentiation of both the crystal cells and the lamellocytes in *Drosophila*	***Dev Biol***. 2019 Dec 1;456(1):74-85. *doi: 10.1016/j.ydbio.2019.08.008* **IF:3.148**
8.	**Gautam DK**, Anuradha Venkatakrishnan Chimata, Ravi Kumar Gutti and Indira Paddibhatla	Chapter 2: Investigative Tools to Study Blood Cells: A Focus on Single Cell Isolation and Analysis	**Book:** *Hematopoiesis, Cellular, Molecular and Genomic Perspectives*. Chapter 2 (Accepted for publication) CRC Press Taylor & Francis Group
9.	Swati Dahariya, **Gautam DK**, Aditya Velidandi, Ravi Kumar Gutti and Indira Paddibhatla	Chapter 10: LncRNAs in Leukemia	**Book:** *Hematopoiesis, Cellular, Molecular and Genomic Perspectives*. Chapter 2 (Accepted for publication) CRC Press Taylor & Francis Group
10.	Paddibhatla I, **Gautam DK**, Gutti RK	SETDB1 maintains the inflammatory-immune axis between the blood cells and the fat body by regulating HOX genes in *Drosophila larva*	*Manuscript in preparation*
11.	**Gautam DK**, Bhattacharya S, Nivetha, Aymanns I, Gutti RK Paddibhatla I	Role of SETDB1 in HOX9A regulation in leukemia *in-vitro* and *in-vivo*	*Manuscript in preparation*

Awards and distinctions

S.No.	Name of the award/fellowship	Award given by (organization)	Year
1.	Poster exhibition award	International Congress of BMT 2021& 26th Annual Congress of KSBMT Advancing HSCT through Virtual Collaboration, SOUTH KOREA (Virtual)	2021
2.	Contribution award for poster presentation	ICKSH 2021 Korean Society of Haematology International Conference and 62nd Annual Meeting Seoul, SOUTH KOREA (Virtual)	2021
3.	Travel award for best research work entitled "Methotrexate (MTX) affects NF-κB pathway in *Drosophila melanogaster*"	24th International Congress of APBMT& ICBMT Korean Society of Haematology International Conference, Busan, SOUTH KOREA	2019
4.	Junior Research Fellowship in Life Sciences	Indian Council of Medical Research (ICMR), New Delhi	2017-2022

2021: Poster exhibition award: ICBMT & KSBMT Congress, South Korea

Poster Exhibition Awardees List
Congratulations!

No.	Country	Name
1	Australia	Lawanya Ranjan
2	Belarus	Viktar Kashkevich
3	Belarus	Olga Gerasimovich
4	Belarus	Alena Hlaz
5	China	Cheng Zhang
6	China	Shuwen Wang
7	China	Liping Zhang
8	India	Indira Paddibhatla
9	India	Dushyant Gautam
10	India	Md Aejaz Ahmad Ansari
11	India	Mohammad Imroz
12	India	D. A Johns
13	India	Abdulla Javed
14	India	Preetibala Solanki
15	India	Vikas Kumar
16	India	shilpa anupurba
17	India	Mohammad Tabish
18	India	Harshita Dubey
19	India	anil sharma
20	India	Monika Jain
21	India	Namita Kumari
22	India	Aekta Neha
23	India	Kumari rashmi
24	India	Amar Ranjan
25	India	Rani K Mahkam
26	India	Tanya Prasad
27	India	Saher Khan
28	India	satish meena
29	India	VAIBHAV GUPTA
30	India	NITIN GUPTA
31	India	Rehan Khan
32	India	POONAM GOEL
33	Indonesia	Yusril Yusril
34	Indonesia	M. Valiant Dwinanda
35	Indonesia	Anna Farhana
36	Indonesia	Fikri Gemilang
37	Indonesia	Ratna Sari
38	Indonesia	Indra Suardi
39	Indonesia	Zulfa Saumia
40	Indonesia	Syafri Suardi
41	Indonesia	Haerani Haerani
42	Indonesia	rahmat fauzan
43	Indonesia	Rosinta Hotmaida Pebrianti Purba
44	Indonesia	oktrial budiarto
45	Indonesia	Lingga Wati
46	Indonesia	Alivia Meyrizka Utami
47	Indonesia	Putri Ayu
48	Indonesia	Devi Yulia Rahmi
49	Indonesia	Ardela Iga Pratiwi
50	Indonesia	Fatan Fakihardi
51	Indonesia	Mega Dwi Septivani
52	Indonesia	Alvin Alberta MS
53	Indonesia	Derizal Derizal
54	Indonesia	Elfiany Elfiany
55	Indonesia	Fitri Kurnia
56	Indonesia	Yabes Dwi Nugroho H
57	Indonesia	Maelani Indaswari
58	Indonesia	Ramlah Ramlah
59	Iran	Javad Alizargar
60	Malaysia	Eric Tzyy Jiann Chong
61	Malaysia	CHING SOON TEOH
62	Mongolia	Altanshagai Boldbaatar
63	Mongolia	Baljinnyam Purevsuren
64	Mongolia	Myadagsuren Sukhbaatar
65	Pakistan	AYSHA SULTANA
66	Viet Nam	Van Man Huynh

2021: Poster exhibition award: ICKSH Congress, Seoul, South Korea

ICKSH 2021 & 62nd ANNUAL MEETING | 2021 KOREAN SOCIETY OF HEMATOLOGY INTERNATIONAL CONFERENCE & 62nd ANNUAL MEETING

CONTRIBUTION AWARD

The Korean Society of Hematology hereby certifies that

Dushyant Gautam

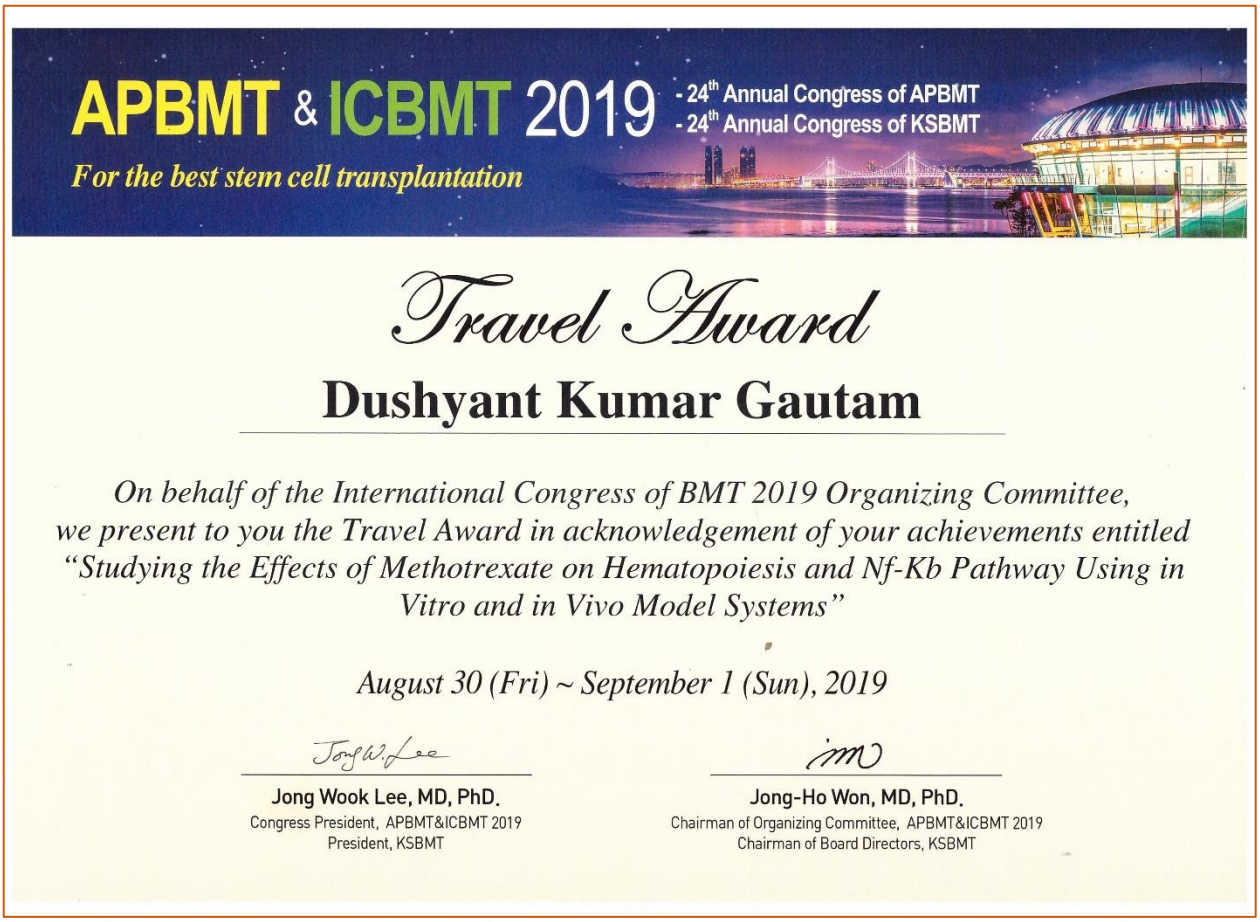
India

has won the contribution award in order to attend
at the 2021 Korean Society of Hematology International Conference
& 62nd Annual Meeting held on April 1 - 3, 2021.

Kyung-Ha Ryu, MD., Ph.D.
Congress Chair
The Korean Society of Hematology

Je-Hwan Lee, MD., Ph.D.
President
The Korean Society of Hematology

2019: Travel award in acknowledgement of the "*Studying the effects of Methotrexate on hematopoiesis and NF-κB pathway using in vitro and in vivo model systems*": APBMT & ICBMT, Busan, Korea



2017: Junior Research Fellowship in Life Sciences: Indian Council of Medical Research (ICMR), New Delhi

Dr. N.C. JAIN
Scientist – G & Head
Human Resource Planning & Development (HRD)

INDIAN COUNCIL OF MEDICAL RESEARCH
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No.3/1/3/JRF-2017/HRD
Dated: 21-08-2017

To,

59239
Dushyant Kumar Gautam
Room No. 218, Men's Hostel-J
South Campus,
University of Hyderabad, Gachibowli
Hyderabad-500046, Telangana
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Subject: ICMR-JRF Examination 2017 held on 16th July 2017.

Dear Research Fellow,

I am pleased to inform you that you have qualified ICMR-JRF Examination 2017 held on 16th July 2017) for the award of Junior Research Fellow under the ICMR Fellowship Scheme. The offer of Junior Research Fellowship is **valid for a period of one year** from the date of issue of award letter. (**No further extension will be considered beyond this period**).

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Yours faithfully,

(N.C. Jain)

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Full Length Article

Methotrexate negatively acts on inflammatory responses triggered in *Drosophila* larva with hyperactive JAK/STAT pathway

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ABSTRACT

Drosophila is a valuable paradigm for studying tumorigenesis and cancer. Mutations causing hematopoietic aberrations and melanotic-blood-tumors found in *Drosophila* mutants are vastly studied. Clear understanding about the blood cells, signaling pathways and the tissues affected during hematopoietic tumor formation provide an opportunity to delineate the effects of cancer therapeutics. Using this simple hematopoietic archetype, we elucidated the effects of the anti-cancer drug, Methotrexate (MTX) on immune responses in two scenarios i.e. against wasp infection and in hematopoietic mutant, $hop^{Tum\text{-}l}$. Through this *in vivo* study we show that MTX impedes the immune responses against wasp infection including the encapsulation response. We further observed that MTX reduces the tumor penetrance in gain-of-function mutants of JAK/STAT pathway, $hop^{Tum\text{-}l}$. MTX is anti-inflammatory as it hinders not only the immune responses of acute inflammation as observed after wasp infestation, but also chronic inflammatory responses associated with constitutively activated JAK/STAT pathway mutant ($hop^{Tum\text{-}l}$) carrying blood tumors.

1. Introduction

Therapeutic agents targeting cancer causing molecules have successfully directed decades of promising research (Sawyers, 2004). Yet, existing cancer therapeutics have failed to satisfy the requirements for an effective treatment with no side effects (Maeda and Khatami, 2018). Chemotherapy is a systemic treatment designed to target exclusively the cancer cells that can either destroy, shrink and/or regulate these cells. But chemotherapy also poses a disadvantage as these drugs are ineffectual in completely distinguishing between cancerous and non-cancerous cells (Bagnyukova et al., 2010; Sutradhar and Amin, 2014). The expected effect of chemotherapy is on metastasizing cancer cells and it ensures that the treatment either controls, cures or is palliative. Understanding how the chemotherapeutic drugs affect the wild type cells can provide insights on the cellular mechanisms perturbed upon its treatment. Past few decades have contributed to the knowledge on drug repositioning and repurposing. In this regard using model organisms is highly beneficial to determine the *in vivo* effects of anti-cancer drugs.

While laboratory mouse is most frequently used to evaluate anti-cancer drugs, past few decades of cancer research using other model organisms such as *Drosophila* and *Danio rerio* proved to be highly advantageous (Gao et al., 2014; Hogenesch and Nikitin, 2012). *Drosophila* shares similarities with mammals in the development of blood cells that are evolutionary conserved (Gautam et al., 2021). *Drosophila* larval hemocytes are found in either circulating hemolymph (originating from head mesoderm) or the hematopoietic organ the lymph gland (originating in the cardiac mesoderm of the embryo) (Banerjee et al., 2019). To date three matured blood cells are detected in the hemolymph and the lymph gland. Blood cells in circulation are either sessile or freely floating in hemolymph. These matured blood cells include plasmatocytes (>95%), crystal cells (>5%) and the lamellocytes (<1%). While acquired immunity does not prevail in *Drosophila*, the conventional innate immune responses including some of the signaling pathways and the mechanisms involved are conserved between fruit flies and mammals (Crozatier and Meister, 2007; Lemaitre and Hoffmann, 2007). Blood cells are immune defensive and in *Drosophila* they orchestrate the anti-inflammatory effects by host defense mechanisms such as

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phagocytosis and synthesis of anti-microbial peptides (Vlisidou and Wood, 2015). In response to wasp infection, in *Drosophila* larva a multifactorial network of signaling cascades is turned on, leading to the constitutive division of hemocytes. Upon wasp infection the host identifies the parasite as foreign body leading to the encapsulation of the parasite. Some of the precursor cells found in both, the circulating hemolymph and the anterior lobes of hematopoietic organ differentiate into lamellocytes. Parasitoid egg is encapsulated by the blood cells necessitating the involvement of the large (>20 μm) lamellocytes (Schlenke et al., 2007; Small et al., 2012). The encapsulated bodies are thereby out-casted within the hemolymph. Increased number of lamellocytes in circulation is observed as a consequence of genetic mutations as well that perturb hematopoietic signaling such as JAK/STAT, Toll/-NF-κB, RAS etc. (Babcock et al., 2008; Banerjee et al., 2019; Zettervall et al., 2004). Loss of function (LOF) mutations of the negative regulators or the gain of function (GOF) mutations in positive regulators of the hematopoietic pathways escalate the rate of blood cell division and melanotic microtumor formation. LOF mutation of *Ubc9* and *Cactus* results in the upregulation of the Toll/NF-κB pathway while in the GOF mutants $hop^{Tum\text{-}l}$ JAK/STAT pathway is constitutively upregulated. Melanotic tumors found in the circulating hemolymph of these hematopoietic mutants, are cellular outgrowths formed from the larval hemocytes with some degree of melanization (Rizki et al., 1957; Rizki and Rizki, 1983, 1984). In *Drosophila* larva JAK-STAT pathway is primarily responsible for the differentiation of lamellocytes (Vlisidou and Wood, 2015). *Tumorous lethal* (*Tum-l*) is an oncogene and larvae carrying this mutation display defective hematopoiesis. $hop^{Tum\text{-}l}$ mutants carrying a dominant mutation in the locus of the hopscotch gene manifest hematopoietic neoplasm due to the uncontrolled blood cell division, differentiation and expanded population of lamellocytes (Hanratty and Dearolf, 1993). Upon injecting the over-proliferating and highly differentiated $hop^{Tum\text{-}l}$ lymph glands into the wild type *Drosophila* adults (females) the injected tissue was capable of inducing melanization and growth of the blood cells. These blood cells shared identities with the parent hematopoietic tissue $hop^{Tum\text{-}l}$ establishing neoplastic nature of these melanotic tumors (Hanratty and Ryerse, 1981).

Various studies depicted the strategies employed by the anti-inflammatory and anti-cancer drugs to target JAK/STAT pathway components for their role in inflammation and tumorigenesis. Aspirin reduced the over-proliferation of blood cells in $hop^{Tum\text{-}l}$ (GOF) mutants and improved their viability (Panettieri et al., 2019). Experimental evidences *(in vitro)* demonstrated that the known anti-folate drug MTX behaved like an anti-JAK/STAT due to its inhibitory effect on the phosphorylation of Stat92E, the transcription factor of the JAK/STAT pathway (Thomas et al., 2015). They further showed that the effect of MTX on JAK/STAT pathway still persisted even in the presence of folinic acid iterating that MTX's potential to reduce the phosphorylation of Stat5 is not completely dependent on folinic acid. These results implicate unidentified mechanism through which MTX exerts its effects in the target cells (Thomas et al., 2015). MTX is one of the Food and Drug Administration (FDA) approved anti-metabolite chemotherapeutic drug (Hannoodee and Mittal, 2021). MTX inhibits Dihydrofolate reductase (DHFR) via competitive inhibition with 1000-fold more affinity compared to folate. DHFR is functionally involved in the conversion of Dihydrofolate to active Tetrahydrofolate (reduced folate factors) (Rajagopalan et al., 2002). HD-MTX (high dose MTX) is used as a chemotherapeutic and LD-MTX (low dose MTX) is shown to be an anti-inflammatory prescribed for immunoinflammatory rheumatological diseases (Malaviya et al., 2010). One of the side effects of low dose MTX (LD-MTX) is cytopenia (Gutierrez-Ureña et al., 1996). Importantly, upon treatment with MTX there is a reduction in the white blood cell (WBC) numbers (Sosin and Handa, 2003). WBCs are required for eliciting the immunoinflammatory responses during infection (Cioffi et al., 1993). Fruit flies fight off infections (bacterial, viral or other foreign pathogens) using plasmatocytes (macrophages) (Gold and Brückner, 2015). While lamellocytes are specialized in encapsulating large foreign bodies that cannot be phagocytized (Rizki and Rizki, 1992). Many key questions related to the drug (anti-cancer or anti-inflammatory) effects on hematopoietic pathways triggered in the blood cells against bacterial or parasitoid infections are yet to be completely understood. Therefore, *Drosophila* is an ideal model to decipher the effects of MTX on blood cells with immune functions involved in fighting infections. We can further benefit from studying the hematopoietic signaling pathways affected in these blood cells upon treatment with MTX. As *Drosophila* larva exhibit a simple hematopoietic system with only three mature blood cells (Wang et al., 2013) understanding the morphological and genetic changes in these blood cells is faster and easier unlike the mammalian model with a complex network of signaling pathways regulating hematopoiesis.

Through this study we bring to focus the significance of MTX on both, the blood cells' mediated inflammatory responses through JAK/STAT pathway and blood tumors observed in JAK/STAT pathway mutants, $hop^{Tum\text{-}l}$. We studied the role of MTX on the immune cells and the inflammatory responses associated with blood tumors. Due to the effects of MTX on JAK/STAT pathway we hypothesized that formation of blood tumors with excessive lamellocytes observed in $hop^{Tum\text{-}l}$ mutants can be intervened by the treatment with the anti-cancer drug, MTX. We therefore elucidate MTX effects in fly hosts with upregulated JAK/STAT pathway causing abnormal hematopoiesis in two different conditions, first scenario is after wasp infestation and the second observed in hematopoietic mutants, $hop^{Tum\text{-}l}$. Parasitization by the *Leptopilina boulardi* (strain *Lb17*) triggers acute inflammatory responses in the host body to combat infection (Hetru and Hoffmann, 2009; Paddibhatla et al., 2010). These include the formation of lamellocytes, activation of Toll/NF-κB signaling pathways in the immune cells and synthesis of AMPs such as *Drosomycin*. These immune responses are categorized under acute inflammation regulated through Toll pathway. After encapsulation of parasitoid wasp egg which is the final step to combat parasitoid infestation these responses are downregulated. While in constitutively activated hematopoietic signaling as observed in mutants of $hop^{Tum\text{-}l}$ and $Ubc9^{-/-}$ the fly larva encounters an autofeedback loop established in the immune cells (fat body and blood cells) due to aberrant signaling synonymous to chronic inflammation (Paddibhatla et al., 2010). Through our study we address for the first time the effects of MTX on these inflammatory responses caused due to hematopoietic deregulation observed after parasitization (acute) and in hematopoietic mutants, $hop^{Tum\text{-}l}$ (chronic).

2. Materials and methods

***Drosophila* stocks:** All the stocks of *Drosophila* were raised on the standard protocol for cornmeal-malt-agar-yeast media at 25 °C under 12h:12h light-dark cycles. We used wild type *Drosophila melanogaster (Canton S)* and $hop^{Tum\text{-}l}$, gain of function mutants of JAK/STAT pathway, [Gift from Dr. Shubha Govind's laboratory, City College, CUNY (Panettieri et al., 2019; Piper et al., 2014).

Wasp stock and infection: *Leptopilina boulardi* (strain *Lb17*). Standard protocol was used for rearing wasps on the wild type (*y, w*) fly strain (Sorrentino et al., 2002). Infection was performed on 3-day-old larvae (after 72 h of egg-laying).

Methotrexate (MTX) drug preparation and dilution: MTX was obtained from TOCRIS biosciences (Biocore Solution, catalog # 1230). Recommended dose of MTX for Acute Human Leukemia (AHL) is 2.5 mg/kg bodyweight. Weight of one wild type *(Canton S)* 3rd instar larva was considered and dose of MTX was calculated accordingly. In this study we used two higher (6.67 μM and 5.67 μM) and four lower MTX concentrations (4.0 μM, 3.5 μM, 3.0 μM and 2.50 μM) than the reference dose (4.8 μM). MTX was mixed in the fly food in 1:3 ratio (1 ml of MTX Solution: 3 ml of fly food) and orally fed to the flies.

Coloring agent assay: To ensure proper food consumption by larvae grown on MTX positive fly food we performed coloring agent assay. Fly food was prepared by adding the food coloring agent (Synthetic food

color preparation 999, Kesar yellow). To ensure that the amount of coloring agent in the food is proportional to the MTX concentration we first added the coloring agent (250 mg) to the MTX solution of 6.67 μM concentration. All the further dilutions were obtained from 6.67 μM concentration MTX and coloring agent mixed together. Therefore, highest MTX concentration (6.67 μM) carried the highest amount of the coloring agent and lowest MTX concentration (2.50 μM) carried the least coloring agent. Control larvae did not receive any food coloring agent. On day six wandering third instar larvae were imaged (Olympus U-TV1X-2 T7 microscope and infinity 1 camera (attached to the microscope) at 5X zoom) and the larvae grown with and without food coloring were compared.

***in vivo* MTX treatments:** 36 h old wild type (*Canton S)* larvae were transferred into food plate mixed with the MTX drug. We referred this as the "First MTX Treatment (FMT)" that lasted for 24 h. Post 36 h all the FMT larvae were transferred into the fresh fly food mixed with the MTX drug. We referred this as the "Second MTX Treatment (SMT)". 24 h after SMT all larvae were finally transferred to normal media food plate for further observation and analysis. Both the MTX treatments (FMT and SMT) were carried at room temperature (25 °C). Untreated control larvae did not receive any MTX drug treatment.

***in vitro* MTX treatments:** Larvae used for experiments were sequentially washed with 1X Phosphate Buffer Saline (1XPBS), double distilled H_2O, 70% Ethanol, double distilled dH_2O, and 1X PBS. Hemolymph containing the blood cells of the wandering third instar larvae were dissected on glass slides in the 1x PBS and after 5 min 1X PBS was removed and the blood cells were incubated with MTX for 2 h at room temperature and later washed with 1X PBS three times with 5 min between each wash. The control blood cells were treated only with 1X PBS. Blood cells were fixed with 4% paraformaldehyde (PFA) and further procedure was followed as mentioned in immunostaining section.

Puparation assay: Egg-to-puparation duration assay" was performed at 25 °C under 12h:12h light-dark cycles. Flies were allowed to lay eggs for 12 h on a fresh food plate (90mmx60mm Petri dish). Post 48 h of egg laying, larvae were collected and transferred into a separate fresh food plate mixed with MTX. A total 8 such plates (one control and seven different MTX concentrations) were used for two rounds of MTX treatment. After both, FMT and SMT all the larvae including untreated controls were incubated at 25 °C until pupariation. MTX treatment plates were covered with conical funnel to provide sufficient space for the crawling and wandering of third instar larvae. When the first pupariation was observed, plates were monitored every 6 h to document the number of pupae formed. The assay was terminated when no newly formed pupa was seen for next 24 h. Data was analyzed with GraphPad prism version 8.0.2 The assay was performed in triplicates. The method was adapted from an earlier publication (Nikhil et al., 2016).

Eclosion Assay: "Egg-to-eclosion duration assay" was performed at 25 °C under 12h:12h light-dark cycles. Flies were allowed to lay eggs on a fresh food plate (90mmx60mm Petri dish) mixed with MTX. Post 48 h of egg laying, larvae were collected and transferred into a separate fresh plate food mixed with MTX dose. A total 8 such plates (one control and seven MTX concentration) were used for two rounds of MTX treatment. After both, first and second MTX treatments, all the larvae of each MTX dose including control were incubated at 25 °C until pupariation. Drug treatment plates were covered with conical funnel to facilitate the eclosion with sufficient space. After the first eclosion was observed, plates were monitored every 6 h to document the number of newly eclosed flies. Freshly eclosed flies were collected every 6 h from same plate for each MTX dose and the assay was terminated when all the flies were eclosed and no eclosion event was seen for next 24 consecutive hours. Data was analyzed with GraphPad prism version 8.0.2 The assay was performed in triplicates. The adapted assay was modified from (Nikhil et al., 2016).

Climbing Assay: Synchronized adult flies were collected shortly after eclosion and separated into cohorts containing 10 flies in each cohort. The assay was performed on flies eclosed from treatment with seven different MTX concentration (10 flies/concentration) and one set of untreated control flies (10 flies). Flies were maintained at 25 °C. Climbing assay was performed in an empty measuring glass cylinder (diameter 2.5 cm X height 50 cm). Flies were acclimatized for >6 min and were landed at bottom of the glass cylinder by gentle tapping. For each trial, the flies successfully crossing 20 cm mark in 20 s was scored and the percentage numbers were used for climbing index graphical representation. Total nine trials were performed for each cohort, with a 2-min recovery period between each trial. Assay was performed in triplicates. The assay was adapted from (Ganetzky and Flanagan, 1978; Taylor and Tuxworth, 2019).

Full eye imaging of the flies: Control (untreated) and MTX treated flies were anaesthetized by CO_2 gas on fly pad facing the eye upside to check the defect on the outer surface of the eye. Whole eye was captured using Olympus U-TV1X-2 T7 microscope and infinity 1 camera (attached to the microscope) at 5X zoom. Ommatidia arrangement and outer surface of the eye was compared between control and MTX treated flies.

Encapsulation response, Tumor Penetrance and Expressivity: *Drosophila* wild type *(Canton S)* flies were allowed to lay eggs in an egg laying chambers for 12 h. After 48 h, (approx. 60 h old) larvae were transferred to the 90 mm × 15 mm Petri dish containing fly food mixed with MTX drug (in 1:3, MTX drug to food ratio). After 12 h (approx. 72 h old) larvae were infected with *Lb17* parasitoid wasps for 12 h duration (1:5, parasite to host ratio) and then wasps were removed (approx. 84 h old). After 36 h (approximately 120 h old) larvae were again transferred to the 90 mm × 15 mm Petri dish containing fly food mixed with MTX drug (in 1:3, MTX drug to food ratio) of desired concentration (we call it Second MTX Treatment). Then, after 24 h (approximately 144 h old), host larvae were dissected to score the number of encapsulated or unencapsulated parasitoid egg/larvae. All the data were analyzed using Graph Pad Prism software (version 8.0.2).

Immunohistochemistry: Ten larvae, developmentally synchronized, were sequentially washed with 1X Phosphate Buffer Saline (1XPBS)→ double distilled H_2O→70% Ethanol→ double distilled dH_2O→ and 1XPBS to avoid contamination. Six-day (6D) old larvae were dissected out for blood smears (circulating hemolymph) using fine forceps. Samples were fixed in 4% paraformaldehyde (PFA) for 15 min at room temperature. After three consecutive washes with 1XPBS (5 min each) fixed samples were incubated in 3% Bovine Serum Albumin (BSA) in 1XPBS for 30 min at room temperature, followed by an overnight incubation with primary antibody (1:100) L1/Atilla (Lamellocyte), gift from Dr. Istvan Ando lab (Kurucz et al., 2007). Following day samples were washed thrice with 1XPBS and incubated with secondary antibody, Anti-mouse FITC (1:200) (Cell Signaling Technologies, catalogue # 4408) for 3 h 45 min. Samples were washed twice with 1XPBS (5 min each) once with 1XPBST (5 min). After washing, samples were stained for polymerized F-actin with Alexa Fluor® 555 Phalloidin (Cell Signaling Technologies, catalogue # 8953S) (1:200) overnight incubation (O/N) at 4 °C or by an overnight incubation with Alexa Fluor® 488 Phalloidin (Cell Signaling Technologies, catalogue # 8878S) (1:200). After overnight incubation, samples were washed thrice with 1X PBS (5 min each) and counterstained with nuclear dye DAPI (Cell Signaling Technologies, catalogue # 4083S) (1:500) for 15 min followed by three washes with 1X PBS (5 min each), then samples for mounted in 50% glycerol. Confocal Imaging was done using Carl Zeiss Laser scanning confocal microscope (LSM710) in 20X and 40X objectives. Final images were processed using Adobe Photoshop CS3 software. Images for control and experimental samples were taken at identical settings.

Sample Collection, RNA Isolation and Real-Time qPCR: Fifty developmentally synchronized larvae were sequentially washed with 1XPBS→ double distilled H_2O→70% Ethanol→ double distilled H_2O→ 1XPBS to avoid contamination. Six-day (6D) old larvae (12 h post wasp infection) were collected for RNA Isolation (TRIzol method, Invitrogen, Carlsbad, CA). RNA was quantified by a NanoDrop 2000 spectrophotometer (Thermo Scientific). 1.5 μg of total RNA served as template for cDNA synthesis ((Iscript TM; Bio-Rad Laboratories, Hercules, CA). Real-

time qPCR was performed running the standard one-step plus PCR program: 1.0 μl of the cDNA sample was mixed KAPA SYBRR FAST Universal (KAPA Biosystems, Lot # 006255-8-1) and primers to set up a 20-μl reaction mix. Transcript levels detected were normalized to rp49 mRNA values. Primers sequence used for real-time PCR were taken from (Paddibhatla et al., 2010).

rp49:
Forward primer (5′) GAC GCT TCA AGG GAC AGT ATC TG (3’)
Reverse primer (5′) AAA CGC GGT TCT GCA TGA G (3’)
upd3:
Forward primer (5′) TAC AAG ATA CTG CCG CGC AA (3’)
Reverse primer (5′): TCA GTT TGG TGA AGA GGG CG (3’)
Tep2:
Forward primer (5’) TCG ACA ACG CGA ACC AAA AC.
Reverse primer (5’) AGG CAG CTA ACT AGG TTA CTT ACA
hop:
Forward primer (5′) CAA GGA TGT GTC CGT GAC GA (3’)
Reverse primer (5′) TAT TGA GCG GAC CAT ACC GC (3’)
Stat92E:
Forward primer (5′) CGG GGG TGC TGT ATA TCG AA (3’)
Reverse primer (5′) GCA GGT GTT GGG GGA AAA AC (3’)
Drosomycin (Drom):
Forward primer (5′) ATC CTG AAG TGC TGG TGC GAA GGA (3’)
Reverse primer (5′) ACG TTC ATG CTA ATT GCT CAT GG (3’)
Tep4.
Forward primer (5′) GGC GAG TCC AAG GAA TCC AA (3′) Reverse primer (5′) CTG AAA CTT ACC CGC ACA CG (3’)
Pro-PO3.
Forward primer (5′) CAA TGC CCA GGG AAT GGT CT (3’)
Reverse primer (5′) GGG CGA CAA GGA GAA TGT CA (3’)
msn.
Forward primer (5′) AAG GTG GGT CTC CGC AAA TC (3’)
Reverse primer (5′) ATC AAC CGC ATG GAA ACC CT (3’)

Statistics: All the samples were included for statistical analysis. For comparisons between two groups, we utilized Student’s t-test (unpaired, two-tailed). All graphs show mean ± SEM. In all cases (not significant) $p > 0.05$, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). Biological repeats (N), sample size (n) and Student’s t-test results are mentioned in the legends section. All the data were analyzed using Graph Pad Prism software (version 8.0.2).

3. Results

3.1. Methotrexate treatment delays development and affects locomotion in Drosophila

To comprehend if the larvae fed with MTX are consuming the fly food we first performed “food coloring assay”. We prepared the fly food with both coloring agent and MTX as mentioned in the material and methods (Coloring agent assay). We used 250 mg of coloring agent and mixed it into a MTX solution of 6.67 μM as starting higher concentration and further concentrations were determined similar to the procedure used to make MTX dilutions. Drug was administered along with the fly food (Figure figs1Supplementary Fig. 1) i.e. we orally fed the larvae with coloring agent along with MTX (seven different chosen concentrations) at two different time points (early second instar and early third instar). The food coloring assay experiment results clearly established that the larvae were consuming the colored food along with MTX, which indicates the consumption of the drug during the experiments.

We next determined the effects of MTX (without coloring agent) on fly development. We initially investigated the pharmacodynamics of MTX in the different developmental stages. To assess the impact of different concentrations on pupariation, we evaluated the time and number of larvae pupariating upon administration of MTX. Number of larvae pupariating were documented for each MTX concentration at every 6 h interval beginning with 0 h as the time of pupariation of the first larvae. Pupariation in wild type *Drosophila* larvae is developmentally synchronized and is observed by the end of day six. Majority of the wild type larvae pupariated (>70%) within a span of 24 h as indicated graphically by the green line (Fig. 1A–G). With the increasing dose we found that the effect on pupariation timing increased and was significantly delayed when larvae were exposed to 4.8 μM, 5.67 μM and 6.67 μM MTX concentrations (Fig. 1E–G). We found that at first 0–6 h interval while 14.33% of total untreated larvae were pupariated, treatment with 2.5 μM, 3.0 μM, 3.5 μM, 4.0 μM, 4.8 μM, 5.67 μM and 6.67 μM of MTX resulted in 18.88%, 4.45%, 5.83%, 0.76%, 0%, 0%, and 1.25% larval pupariation respectively (Fig. 1A–G). In the next 18 h (three 6 h’ time intervals), we noticed 57.79% (18.76%, 24.01% and 15.02%) pupariation in untreated category and with 2.5 μM, 3.0 μM, 3.5 μM MTX treatments similar pattern was observed i.e. 46.79%, 60.75% and 59.07% respectively unlike with higher concentrations 4.0 μM, 4.8 μM, 5.67 μM and 6.67 μM which show minimal pupariation percentage of 30.70%, 18.35%, 27.33% and 22.30% respectively. We further detected that the first three chosen concentration showed comparable graphical pattern to the untreated controls for all the succeeding time intervals while this was not true for higher concentrations. We detected delayed pupariation with higher concentrations extending to 78 h as identified by the red line (Fig. 1D–G). In the next succeeding time intervals, we noticed that all the untreated larvae were pupariated by 54 h whereas with MTX concentration 4.0 μM, 5.67 μM and 6.67 μM the pupariation was prolonged by 18 h implying that all the larvae were pupariated by 72 h. Surprisingly, 4.8 μM MTX showed further more delay with all larvae pupariating by the end of 78th hour. These results underscore the importance of MTX effects on *Drosophila* development.

We next documented the effect of MTX on the time of eclosion followed by pupariation. We observed the number adults eclosed for every 6 h timepoint from the time of first adult’s eclosion in the untreated control category similar to pupariation. When treated with 2.5 μM MTX concentration, the pattern of eclosion time (Fig. 1H, red line) for majority of the adults eclosed is similar to the untreated category (Fig. 1H, green line). With the increasing concentrations of the MTX, the time of eclosion was delayed and went beyond 42 h and extended to 84 h. Treatment with MTX concentration 3.0 μM and 3.5 μM resulted in adults being eclosed by 66 h while higher concentrations 4.0 μM, 4.8 μM, 5.67 μM and 6.67 μM MTX showed even further delay with all the adults being eclosed by the end of 84th hour (Fig. 1I–N).

MTX decreases the folate accessibility to the cancerous cells and is used as anti-cancer drug. But depletion of the folate uptake can also affect other normal cells. We therefore observed for morphological differences in the adults treated with MTX versus untreated adults. We found that MTX did not have any effect on the adult wings’ abdomen and thorax. Although we found that the leg morphology was unaffected, we went ahead to determine whether MTX had any effect on the locomotion. To elucidate the effect of MTX treatment on locomotion, we determined climbing index in adults. Our results indicate that MTX mitigated climbing potential of adults and showed a steady decrease by 16.59%, 32.30%, 37.16%, 48.58%, 58.45%, 73.51% and 81.16% when treated with_2.5 μM, 3.0 μM, 3.5 μM, 4.0 μM, 4.8 μM, 5.67 μM and 6.67 μM concentration respectively, compared to untreated controls showing 100% climbing index (Fig. 2A).

Drosophila Imaginal eye discs are successfully used to decipher not only the tumorous growth and invasion but also to study different cancer genes and to perform chemical screens that contribute to the oncogenicity (Tipping and Perrimon, 2014). We therefore observed the ommatidia in the wild type adult eye with and without MTX treatment. We noticed that increased MTX concentration affected fusion of the ommatidia (Fig. 2B). MTX treated larvae developed into adults with fused ommatidia eye phenotype compared to the untreated flies. Quantification of the number of adult flies showing fusion of ommatidia resulted in incremental percentages i.e.19%, 22.07%, 23.69%, 21.70%, 38.74%, 45.21% and 50.18% when treated with 2.5 μM, 3.0 μM, 3.5 μM, 4.0 μM, 4.8 μM, 5.67 μM and 6.67 μM MTX concentration respectively

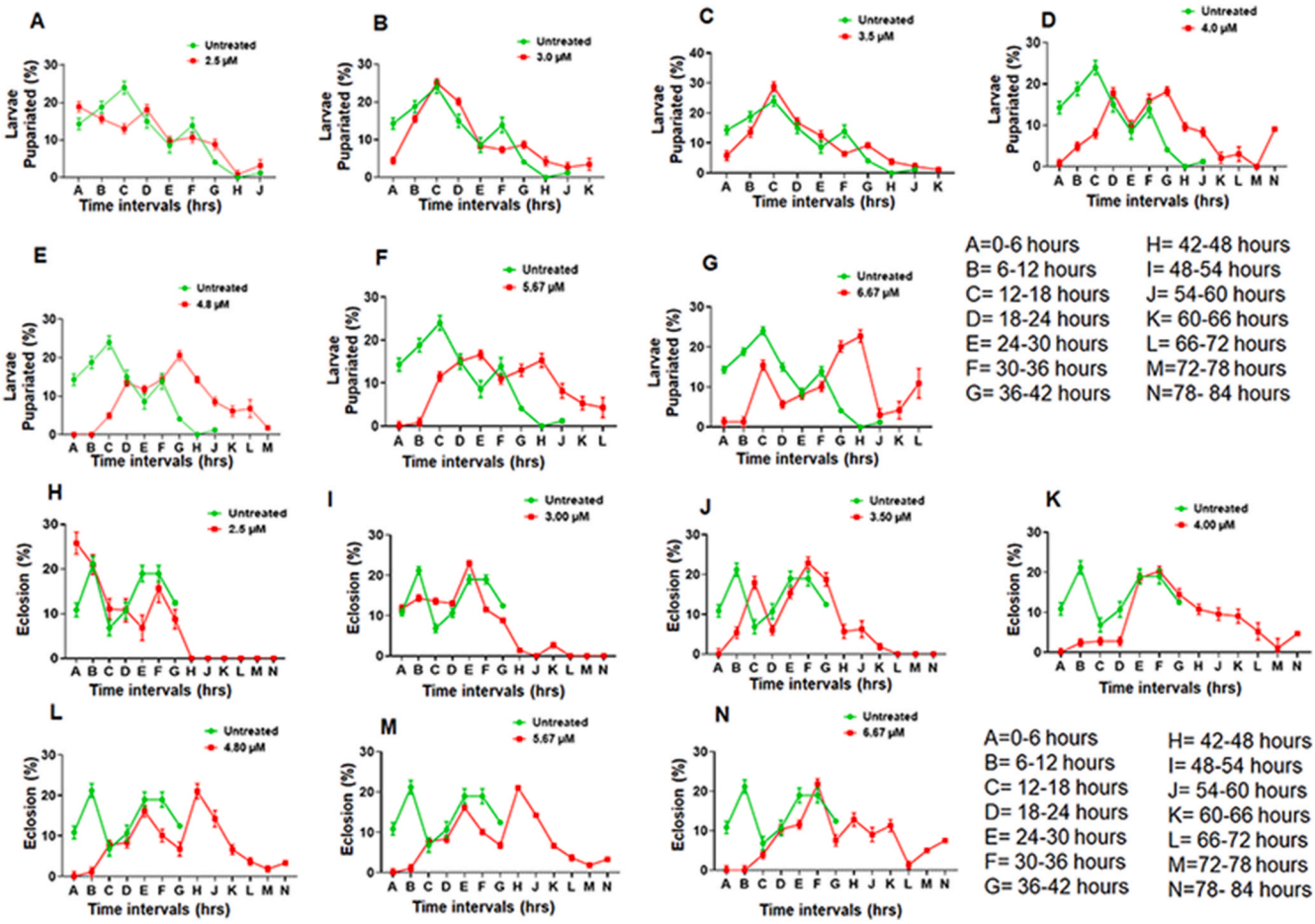

Fig. 1. 1A-G. Effect of MTX on the pupariation of *Drosophila melanogaster*.
Pupariation profile of wild type *D. melanogaster (Canton S)* from 0 to 78 h with different MTX concentrations (2.5 μM, 3.0 μM, 3.5 μM, 4.0 μM, 4.8 μM, 5.67 μM, 6.67 μM). Pupariation profile was scored for every 6 h from 0 to 78 h until the pupariation of each larva. Control set of larvae didn't receive any MTX treatment. Pre-pupa development time in hours is plotted along x-axis, and the percentage of pupariation is plotted along y-axis. All graphs show mean ± SEM. around the mean. N = 3, n = 50.
1H–N.
Eclosion profile of wild type *D. melanogaster (Canton S)* from 0 to 78 h with different MTX concentrations (2.5 μM, 3.0 μM, 3.5 μM, 4.0 μM, 4.8 μM, 5.67 μM, 6.67 μM). Eclosion profile was scored for every 6 h from 0 to 78 h until the eclosion of each fly. Control set of larvae didn't receive any MTX treatment. Adult development time in hours is plotted along x-axis, and the percentage of eclosion is plotted along y-axis. . All graphs show mean ± SEM. N = 3, n = 50.

(Fig. 2C).

3.2. MTX affects circulating blood cells in wandering third instar larva

Drosophila blood cells are under the control of various hematopoietic pathways for their proliferation and differentiation. We investigated the effects of MTX on blood cells and documented the morphology of blood cells with MTX treatment and compared them to the untreated blood cells from wild type. MTX treated blood cells did not appear like any of the progenitor or matured blood cells found in wild type in circulating hemolymph. We noticed a deviation in the morphology of blood cells. Compared to the normal morphology, the blood cells treated with MTX showed altered phenotype with cellular extensions that appeared like "spikes" (Fig. 2D1-D8). The arm like cytoplasmic projections made up of actin filaments, intermediate filaments and microtubules also called pseudopodia, are referred as "cell spike" in this study (Fig. 2D7; red arrows). There was no effect upon treatment with two lower concentrations 2.5 μM and 3.0 μM compared to untreated WT blood cells. The spike phenotype noticeably increased in length with increased MTX concentrations (3.5 μM–6.67 μM) as represented graphically (Fig. 2E). The increase in the spike length of the treated blood cells was significantly higher compared to the WT blood cells' spikes. Treatment with 5.67 μM MTX concentration displayed maximum increase in the average spike length of the blood cells i.e. 5.5 μm compared to average spike length of 1 μm observed in the untreated WT blood cells' category (Fig. 2E).

We next documented penetrance of spike phenotype of untreated and MTX treated larval blood cells. We observed striking differences between the spikes found on the blood cells of larvae treated with lowest MTX concentration (2.5 μM) relative to the spikes present on the blood cells of untreated larvae. In contrast, the other four succeeding higher MTX concentrations 3.0 μM, 3.5 μM, 4.0 μM and 4.8 μM did not demonstrate similar differences compared to the lower concentrations (Fig. 2F). Treatment with the two highest concentrations 5.67 μM and 6.67 μM exhibited increased percentages of blood cells with spike penetrance i.e. 79.16% and 88.73% respectively (Fig. 2F).This data fortifies the role of MTX in the altered morphology of blood cells.

3.3. Methotrexate supresses the inflammatory responses associated with acute

3.3.1. Inflammation

We next explored the therapeutic effects of MTX on the inflammatory phenotypes observed in *Drosophila*. We hypothesized that MTX mitigates

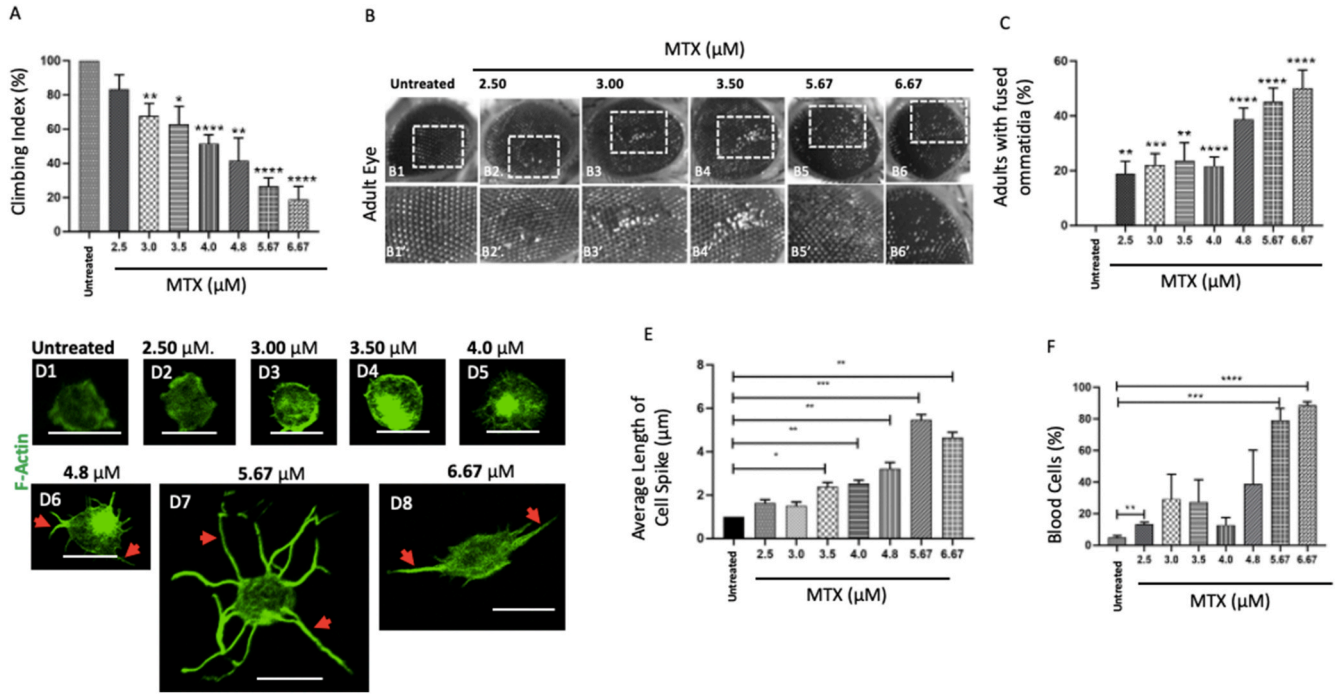

Fig. 2. 2A. Effect of MTX on climbing ability of *Drosophila melanogaster*:
Statistical analysis of climbing index was performed using Student's t-test (unpaired, two-tailed) for independent (between groups) or dependent (within same group) samples (detail mentioned in materials and methods section). The climbing activity was normalized to control flies (no MTX treatment). Error bars show the standard error mean for four independent experiments. Student's t-test (unpaired, two-tailed) showed statistical significance $p < 0.0001$(****), $p < 0.001$(***), $p < 0.01$(**) and $p < 0.05$(*). Graphs were processed using GraphPad prism version 8.0.2. N = 4, n = 20.
2B. Effect of MTX on adult eye phenotype of *Drosophila melanogaster*.
B2–B5 show wild type *(Canton S)* flies' adult eye phenotype (fused ommatidia) due to MTX treatment compared to control fly, B1, (no MTX treatment). B1′-B5′ represent zoomed insets of B1–B5. Micrograph images were obtained from Light Olympus Stereomicroscope and analyzed by infinity analyze software version 7.0.2.920. N = 2, n = 3.
2C.The bar graph shows the percentage of eye phenotype in wild type *(Canton S)* adult flies' due to MTX treatment compared to control flies. Error bars represent the standard error mean. Student's t-test (unpaired, two-tailed) showed statistical significance $p < 0.001$(***) and $p < 0.01$(**) of control vs. MTX treated samples. N = 5, n = 10. Graphs were processed using GraphPad prism version 8.0.2.
2D. Effect of MTX on larval blood cells of *Drosophila melanogaster*.
D1-D8 shows the cytoskeletal projections (spikes) at the periphery of the larval blood cells. Increasing concentration of MTX showed the significant increase in the size of the spikes of larval blood cells (indicated by red arrow) compared to control (Untreated, D1). Polymerized F-Actin of the blood cells of 3rd instar wandering larvae were stained with Alexa flour 488 (FITC), green, and imaged by confocal microscopy. N = 2, n = 8.
2E. Effect of MTX on larval blood cells spike size of *Drosophila melanogaster*:Graphical representation of the average length of the spike size of blood cells of 3rd instar wandering larvae. Increasing concentration of MTX showed increase in the average length (in μm) of size of the spikes of blood cells compared to control. Untreated and MTX treated larvae is plotted along x-axis, and the percentage of average blood cell spikes is plotted along y-axis. MTX concentration, 5.67 μM, showed the highest length of the spike with significance compared to control spike size. The error bars show standard error. Graphs were plotted using GraphPad software version 8.0.2. Student's t-test (unpaired, two-tailed) showed statistical significance $p < 0.001$(***), $p < 0.01$(**) and $p < 0.05$(*). N = 3, n = 20.
2F. Effect of MTX on spike penetrance of *Drosophila melanogaster*.Graphical representation of the percentage of blood cells of 3rd instar wandering larvae with spikes penetrance. MTX concentration above 3.0 μM showed more than 80% of larval blood cells with spikes. Untreated and MTX treated larvae is plotted along x-axis, and the percentage of blood cells with spikes is plotted along y-axis. The error bars are standard error mean. Student's t-test (unpaired, two-tailed) showed statistical significance $p < 0.0001$ (****), $p < 0.001$(***) and $p < 0.01$(**) of control vs. MTX treated samples. Graphs were plotted using GraphPad software version 8.0.2. N = 3, n = 50.

inflammatory responses triggered upon wasp infestation (Fig. 3 A). *Leptopilina boulardi* (*Lb17)* infects the *Drosophila* larval stage to complete its life cycle. Successful infection results in parasite invading the host for its development. Encapsulation response, considered as acute inflammation, is triggered in the host eliciting stronger immune responses. We, therefore, exploited the *Drosophila* inflammatory model system to determine the anti-inflammatory effects.

To determine if MTX treatment had an effect on the wasp infection we first documented the success rate of wasp infection. Any host larvae that carried at least one wasp egg after infection were considered as infection positive. Untreated infected larvae, considered as the control group, showed 100% infection and all the MTX treated infected samples were compared to this control group (Fig. 3B). All the host larvae treated with the chosen MTX concentrations were successfully infected which accounted for 97.5% upon treatment with both 2.5 μM and 5.67 μM drug concentration (Fig. 3B). We next examined if the pattern of wasp infection differed in the untreated versus the treated host larvae. We dissected out the host larvae after wasp infection and accounted for the total number of parasite bodies per host larvae. MTX treatment with 2.5 μM and 5.67 μM concentration affected the rate of infection that resulted in maximum five parasite bodies per host larvae relative to the untreated infected control group that showed maximum two parasite body per host larva (Fig. 3C). This data led us to conclude that MTX treatment resulted in the compromise of the host immunity increasing the rate of infection. Since MTX was having an effect on the rate of wasp infection therefore we also looked into the encapsulated parasite bodies due to the host response. We found that after MTX treatment, 2.5 μM and 5.67 μM, the number of encapsulated parasite bodies reduced to one when compared to control group that showed more than one encapsulated parasite bodies (Fig. 3D). These results strengthened our hypothesis to further

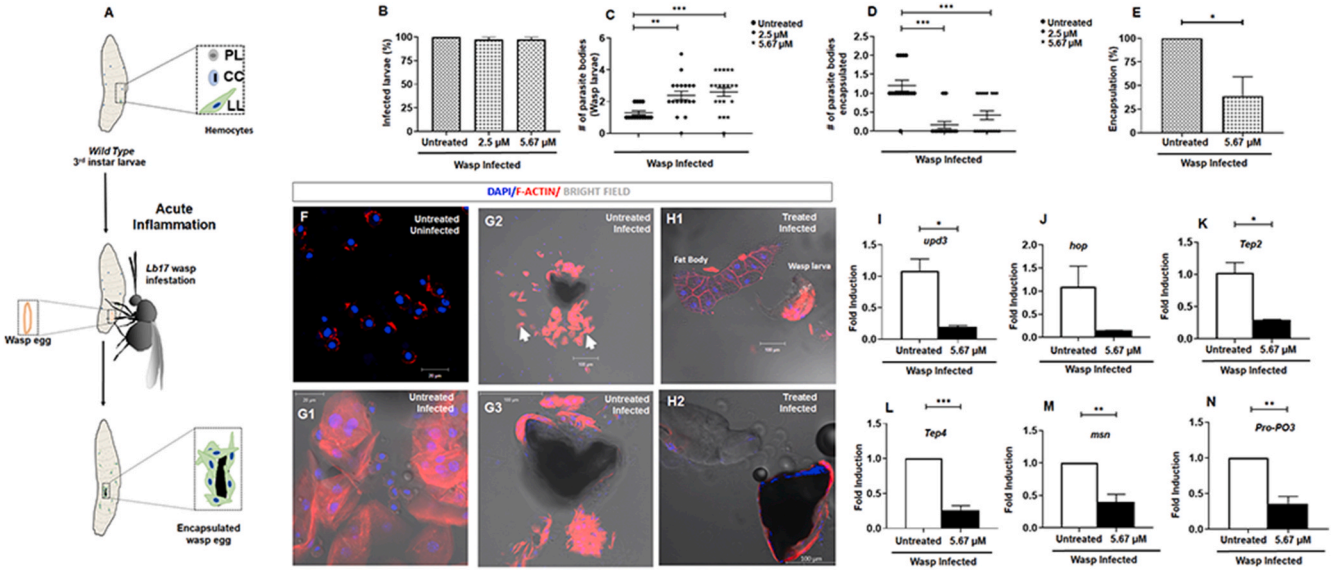

Fig. 3. 3A. Effect of MTX in wasp induced (*Lb17*) inflammation (synonymous to acute inflammation) through blood cells (PL-plasmatocyte, CC-crystal cell, LL-lamellocyte) in *Drosophila* 3rd instar larvae. Egg laid by parasitoid wasp in the *Drosophila* larva, is detected by circulating blood cells leading to the differentiation of lamellocytes. The differentiated lamellocytes encapsulate the wasp egg inhibiting the growth of the parasite inside the host body. MTX possibly could have an effect on the encapsulation event.

3B. Effect of MTX on the success of *Lb17* infection in the *Drosophila*.Wild type *Canton S* control larvae show 100% infection by *Lb17* wasps while infection along with MTX treatment (2.5 µM and 5.67 µM) show 97.5% infection penetrance. Bars show standard mean. Graph were processed using GraphPad prism version 8.0.2. N = 2, n = 50.

3C. Effect of MTX on number of parasite bodies (*Lb17* larvae) in *Drosophila melanogaster*.Dot graph showing number of parasite larvae per host larvae (Expressivity of parasite bodies). Control animals (only infection) on an average showed less than two parasite larvae *(Lb17)* per host larvae *(Canton S)*. In contrast, infection plus MTX treatment (2.5 µM and 5.67 µM) on an average showed more than two parasite larvae per host larvae. Student's t-test (unpaired, two-tailed) showed statistical significance $p < 0.001$(***) and $p < 0.001$(**). Error bars represent standard error mean. Graph were processed using GraphPad prism version 8.0.2. N = 3, n = 50.

3D. Effect of MTX on rate of encapsulation of the *Lb17* parasite bodies in *Drosophila melanogaster*: Dot graph showing number encapsulated parasite bodies in the host larva before and after MTX treatment in an infection background. In control animals (only infection) more than one encapsulated parasite larvae were accounted while in infection plus MTX treatment, encapsulated parasite larvae, reduced to one. N = 3, n = 20.

3E. Effect of MTX on percentage encapsulation in *Drosophila melanogaster*.Bar graph showing encapsulation response in the larva before and after MTX treatment in the *Lb17* infection background. Control (only infection) larvae blood cells showed 100% encapsulation response. Upon MTX treatment the encapsulation response was reduced to more than 60%. Student's t-test showed statistical significance ($p < 0.05$). Student's t-test (unpaired, two-tailed) showed statistical significance $p < 0.05$(*). Graphs were processed using GraphPad prism version 8.0.2.N = 3, n = 20.

3F. Blood cells of wild type wandering 3rd instar larva counter stained with DAPI (nuclei blue) and polymerized F-actin (TRITC, red).3G1-3G3. Circulating lamellocytes (G1), melanized and encapsulated parasite body (*Lb17*) (G2, white arrows pointing to lamellocyte like cells), and zoomed image of encapsulated body (G3) counter stained with DAPI (nuclei blue) and polymerized F-actin (red). 3H1. Unencapsulated wasp larva beside a fat body tissue counter stained with DAPI (nuclei blue) and polymerized F-actin (red). 3H2. Unencapsulated and encapsulated wasp larva (*Lb17*) counter stained with DAPI (nuclei blue) and polymerized F-actin (red). N = 3, n = 8. All the images were taken in Confocal microscopy LSM 7.0. 3I-3L. Effect of MTX on *upd3, hop, Tep2* and *Tep4* in wasp (*Lb17*) induced (acute inflammation) JAK/STAT Pathway: Infection plus MTX treated larvae (TRT INF) showed significant reduction in the *upd3* (I), *hop* (J), *Tep2* (K) and *Tep4* (L) levels compared to only infected larval gene expression. Student's t-test showed statistical significance $p < 0.05$(*) and $p < 0.001$(***) for comparison of control (only Infection) vs. MTX Treated (Infection plus treatment). N = 3, n = 50. Graphs were processed using GraphPad prism version 8.0.2 3M: Effect of MTX on the lamellocyte specific marker, *misshapen, (msn)* on *Lb17* wasp induced inflammation. Infected plus MTX treated host larvae showed significant reduction in the misshapen gene *(msn)* expression, exclusively expressed in LL. Student's t-test showed statistical significance $p < 0.01$(**) compared to control (only Infection) vs. MTX Treated (Infection plus treatment). N = 3, n = 50+. Graphs were processed using GraphPad prism version 8.0.2. 3N: Effect of MTX on the lamellocyte specific *ProPhenoloxidase3* gene expression*, (Pro-PO3*) on *Lb17* wasp induced inflammation. Infected plus MTX treated larvae showed significant decreased gene expression of *Pro-PO3,* compared to only infected larvae, thereby inhibiting the levels of lamellocyte population during wasp infestation. Student's t-test showed statistical significance $p < 0.01$(**) compared to control (only Infection) vs. MTX Treated (Infection plus treatment). N = 3, n = 50+. Graphs were processed using GraphPad prism version 8.0.2.

explore the drug effects on the host triggered encapsulation response. Compared to the control class only 38.86% of MTX (5.67 µM) administered host larvae showed encapsulation response. This established that MTX treatment decreased the encapsulation response in experimental larvae compared to the control group (Fig. 3E). To further assess the *in-vivo* identity of the blood cells differentiated after wasp infection with MTX treatment we stained them for polymerized F-actin and counter-stained with DAPI. Blood cells without wasp Infection and MTX treatment appeared to be morphologically similar to the wild type blood cells (Fig. 3F). While we noticed the prevalence of lamellocytes (Fig. 3G1) and encapsulated parasite larvae (Black mass, Fig. 3G2, 3G3) after wasp infection, we also discovered the presence of parasite larvae unencapsulated in circulating hemolymph (Fig. 3H1, 3H2).

Earlier studies shed light on the requirement of *JAK/STAT* pathway in the differentiation of lamellocytes after wasp infection (Morin-Poulard et al., 2013; Rodrigues et al., 2021). JAK/STAT pathway is linked with the encapsulation process as it helps in the differentiation of lamellocytes. The wasp infection triggered immune responses in *Drosophila* hosts are analogous to the acute inflammation in humans sharing similarities in signaling pathway such as activation of the JAK/STAT and Toll/NF-κB pathway (Chiu et al., 2005; Paddibhatla et al., 2010). This correlation of JAK/STAT pathway and encapsulation response led us to check four different JAK/STAT pathway components i.e. the ligand *(upd3)*, transmembrane *hopscotch* kinase *(hop)* and the readouts the pathway *Tep2 and Tep4* (Fig. 3I–L). MTX decreased the expression of all the four genes (*upd3, hop, Tep2* and *Tep4*) under

scrutiny compared to the untreated controls. This data suggested that MTX is interfering in the encapsulation process by inhibiting the JAK/STAT pathway. Since the encapsulation response was reduced, and the expression of the JAK/STAT pathway components' gene expression was also decreased significantly, we questioned if MTX affected lamellocyte population in the parasitized and MTX treated larvae compared to the untreated and parasitized hosts. We determined the gene expression of two known markers of lamellocyte: *misshapen* (*msn*^{03349}) and *ProPhenoloxidase-3* (*Pro-PO3*). Misshapen (Msn) is a MAPK kinase kinase kinase functionally involved in the production of lamellocytes (Zettervall et al., 2004). *Drosophila* encodes three PPO enzymes, of which Pro-PO1 and Pro-PO2 are predominantly expressed in the crystal cells during larval stages. However, lamellocytes primarily express Pro-PO3 enzyme unlike crystal cells (Honti et al., 2014; Lanot et al., 2001; Rizki et al., 1980). We therefore determined the mRNA levels of these two genes (*msn* and *Pro-PO3*) in hosts that were infected and compared them to hosts that were infected and also MTX treated. Our results indicate that after *Lb17* infection without any MTX treatment the larvae expressed these two genes, and this expression was significant decreased with MTX treatment upon infection. These experimental evidences clearly signify that MTX is negatively affecting the lamellocyte numbers thereby reducing the encapsulation response.

4. Methotrexate impedes chronic inflammation in *hop*$^{Tum\text{-}l}$

Since MTX had an effect on the acute arm of inflammation in *Drosophila* we also wanted to verify the effect of MTX on the other arm of inflammation i.e., chronic inflammation. To decipher this, we utilized *hop*$^{Tum\text{-}l}$ mutants that manifest an uncontrolled over proliferation of blood cells due to constitutively active JAK/STAT pathway. This condition is synonymous to chronic inflammation with the third instar larva having defective blood cell regulation, fat body tissue and melanotic masses observed (Panettieri et al., 2019). *hop*$^{Tum\text{-}l}$ mutant larvae show, in circulating hemolymph, melanotic microtumours observed as black masses under the larval cuticle (Fig. 4B) when compared to healthy wild type larvae (Fig. 4A). Untreated *hop*$^{Tum\text{-}l}$ animals on an average showed up to 88% of tumor penetrance while after MTX treatment, 2.5 μM and 5.67 μM, tumor penetrance was significantly reduced to 41% and 48% respectively (Fig. 4C). We next investigating tumor expressivity i.e. the number of melanotic microtumours in *hop*$^{Tum\text{-}l}$ mutant. We found that untreated larvae on an average had more than 3 melanotic microtumours per host larvae while 2.5 μM and 5.67 μM MTX treated animals exhibited either none or one melanotic microtumour per host larvae (Fig. 4D). This data suggests that MTX successfully inhibits the tumor penetrance and tumor expressivity in *hop*$^{Tum\text{-}l}$ animals. These results instigated us to probe the obvious role of MTX in abating the melanotic tumor formation augmented by the constitutive JAK/STAT pathway in *hop*$^{Tum\text{-}l}$ mutants. We therefore studied the transcript levels of JAK/STAT pathway components: *hopscotch* (kinase), *Stat92e* (transcription factor) *Tep2* and *Tep4* (JAK/STAT pathway target gene) along with an immune defence gene *Drosomycin* (anti-microbial peptide). These genes were highly upregulated in genome wide analysis study on *hop*$^{Tum\text{-}l}$ mutants (Irving et al., 2001). Our results indicate that in MTX treated larvae compared to untreated *hop*$^{Tum\text{-}l}$ larvae the gene expression of *hop, State92E, Tep2 and Tep4* were significantly reduced (Fig. 4E–H) along with significant decrease in the expression of anti-microbial peptide, *Drosomycin* (Fig. 4K). Altogether our data indicates that MTX has an intrinsic potential that can compromise the chronic inflammation induced by constitutive active JAK/STAT pathway. We next wanted to determine if the MTX treatment had any effect on the lamellocyte population as these large blood cells are found in the melanotic tumors and in circulating hemolymph of *hop*$^{Tum\text{-}l}$ mutants. Therefore, we determined the transcript levels of *msn* and *Pro-PO3* and also performed immunostaining using anti-L1/Atilla to understand MTX effect on the lamellocyte population. After MTX treatment we not only found that the transcript levels of *Pro-PO3* and *msn* were significantly downregulated (Fig. 4I–J) but also the number of L1/Atilla positive lamellocytes in circulating hemolymph (white arrow - Green colored cells in Figure 4L2) appeared to be lower compared to untreated larva (Figure 4L1-M4). Taken together these results fortify that MTX has a potential to inhibit lamellocyte lineage thereby reducing in the melanotic tumor phenotype induced by the constitutive JAK/STAT pathway in *hop*$^{Tum\text{-}l}$ mutants.

5. Discussion

Substantial number of studies have explicitly demonstrated the side effects of chemotherapeutics targeting non-cancerous cells. It is imperative to unravel the exact mechanisms involved in chemotherapy that not only inhibit the cancer growth and but also avert side effects. Methotrexate is used for the treatment of leukemia and several other auto-immune diseases such as rheumatoid arthritis (RA), psoriasis, crohn's disease etc. Innumerable studies existing using mammalian models that demonstrated MTX as a potential therapeutic drug for treatment of cancer. Very limited studies prevail that shed light on MTX's effects on blood tumors using invertebrate models. One of the few publications recognized MTX as a teratogen that also affects female fecundity using *Drosophila* model system (Affleck et al., 2006). Another study established MTX's effects on JAK/STAT pathway showing the downregulation of the phosphorylation of STAT transcription factor upon treatment with MTX. JAK/STAT pathway is affected in autoimmune diseases (psoriasis, rheumatoid arthritis, and inflammatory bowel disease) and also in the pathogenesis of inflammation. Our study implicates an anti-inflammatory role of MTX as it mitigates both the encapsulation response and blood tumor formation.

First, we found that MTX affected the morphology of the normal blood cells found in the circulating hemolymph of the wild type larvae along with an effect on the developmental stages. MTX effects on wild type animals include morphological changes in the larval hemocytes with cytoplasmic extensions (referred as spikes), delayed pupariation, delayed eclosion, climbing defects along with adult eye defects. These cellular and developmental effects clearly suggest that MTX affects wild type tissues. Further experiments have to be demonstrated to understand the pharmacodynamics of MTX in experimental models such as fruit flies, zebrafish and C elegans.

Second, the administration of MTX resulted in supernumerary parasitization by *Lb17* compared to the untreated infected larvae. Our study suggests that MTX treatment was the cause for the inability of the parasite to recognize the healthy larvae compared to the larvae with single parasitization leading to supernumerary infection. Our research is the first which addresses the effects of an anti-cancer drug on the inflammatory responses that the parasitoids induce in the host. There exist studies explaining that the supernumerary behavior is associated with virus-like-particles carried by the parasite in its venom gland (Delpuech, 2017). Further studies can provide answers to how MTX treatment leads to supernumerary infection by *Lb17* parasitoid wasps.

Finally, it is evident that MTX downregulated JAK/STAT pathway thereby controlling both acute (parasitoid induced inflammatory responses in the host) and chronic (hematopoietic mutant *hop*$^{Tum\text{-}l}$) inflammatory conditions. Downregulation of the pathway components' gene expression in the larvae i.e. *upd3*, *hop, stat92eE* and the targets *Tep2 and Tep4* in both the scenarios (acute and chronic inflammation) is summarized schematically (Fig. 5). We show that MTX exerts its effects on the expression of the antimicrobial peptide gene, *Drosomycin* and the JAK/STAT pathway. Even though the MTX treatment did not completely inhibit the JAK/STAT pathway it is noteworthy to understand that the expression levels of the pathway components was not sufficient to trigger successfully the encapsulation of all the parasite bodies after *Lb17* infection. Furthermore, in the *hop*$^{Tum\text{-}l}$ mutants even though the JAK/STAT pathway was not completely inhibited, the pathway was not triggered enough for the chronic phenotype to prevail as observed in the untreated *hop*$^{Tum\text{-}l}$ mutants.

Our results provide new avenues to explore the inhibitory effects of

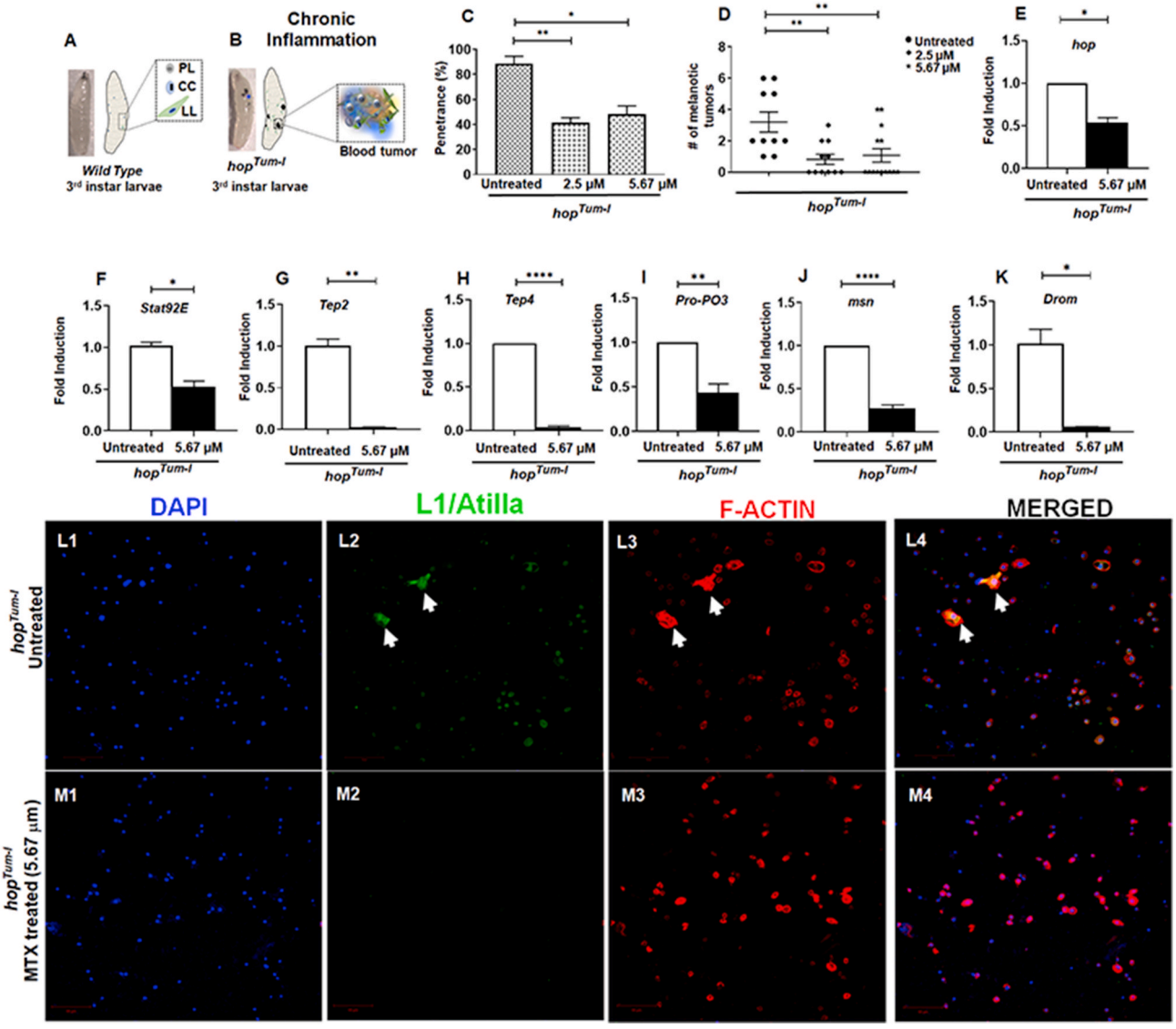

Fig. 4. 4A. Wild type 3rd instar *Canton S* healthy larvae with the circulating blood cells (PL-plasmatocyte, CC- crytsal cell, LL-lamellocyte) devoid of melanotic microtumors. 4B. Control untreated $hop^{Tum\text{-}l}$ 3rd instar wandering larvae showing melanotic microtumors (blue star indicating). MTX having an effect on acute inflammation, could possibly extend its role also in hematopoietic mutants (chronic inflammation). 4C. Effect of MTX on tumor penetrance in hematopoietic mutant, $hop^{Tum\text{-}l}$ Bar graph showing penetrance % in the host larva before and after MTX treatment in the gain-of-function (GOF) mutant in $hop^{Tum\text{-}l}$. Untreated $hop^{Tum\text{-}l}$ 3rd instar wandering larvae scored >80% tumor phenotype. After MTX treatment tumor penetrance was reduced by 50% in the $hop^{Tum\text{-}l}$ larvae. Student's t-test showed statistical significance $p < 0.0001$(****) for comparison of control (only Infection) vs. MTX Treated (Infection plus treatment). Graph was processed using GraphPad prism version 8.0.2. N = 3, n = 20. (Experiments were conducted at 25 °C). 4D. Effect of MTX on number of parasites in the *Drosophila host larva* (tumor expressivity). Dot graph showing tumor expressivity in the host larva before and after MTX treatment in the GOF mutant in $hop^{Tum\text{-}l}$. Untreated $hop^{Tum\text{-}l}$ 3rd instar wandering larvae showed more than 2 melanotic tumors per larvae. After MTX treatment tumor expressivity was reduced to 1 tumor per host in the $hop^{Tum\text{-}l}$ larvae. Student's t-test showed statistical significance $p < 0.0001$(****) for comparison of control (only Infection). Graph was processed using GraphPad prism version 8.0.2. N = 3, n = 20. (Experiments were conducted at 25 °C). 4E-H. Effect of MTX on JAK/STAT Pathway component *hop, Stat92E Tep2* and *Tep4 in* $hop^{Tum\text{-}l}$: *hopscotch kinase (hop)* (E), *Stat92E* (F), *Tep2* (G) and *Tep4* (H) gene expression in $hop^{Tum\text{-}l}$ mutant background was reduced after MTX treatment. Student's t-test showed statistical significance $p < 0.0001$(**) and $p < 0.05$(*) for comparison of control (only Infection N = 3, n = 50. Graph was processed using GraphPad prism version 8.0.2. (Experiments were conducted at 25 °C). 4I-J: Effect of MTX on the LL specific *ProPhenoloxidase3* (*Pro-PO3)* and *misshapen (msn)* gene expression in $hop^{Tum\text{-}l}$. $hop^{Tum\text{-}l}$ larvae upon MTX treatment reduces ProPhenoloxidase3 (Pro-PO3) (I) and msn (J) gene expression. Student's t-test showed statistical significance $p < 0.01$(**) compared to Untreated $hop^{Tum\text{-}l}$ vs. MTX Treated $hop^{Tum\text{-}l}$. N = 3, n = 50+. Graphs were processed using GraphPad prism version 8.0.2. (Experiments were conducted at 25 °C). 4K. Effect of MTX on *Drosomycin (Drom)* in components in $hop^{Tum\text{-}l}$: Low levels of *Drom* in MTX treated $hop^{Tum\text{-}l}$ 3rd instar wandering larvae were detected as compared to Untreated control. Student's t-test showed statistical significance $p < 0.01$(**) for comparison of control (only Infection) N = 3, n = 50. Graph was processed using GraphPad prism version 8.0.2. (Experiments were conducted at 25 °C). 4L1-M4. Circulating blood cells in the hemolymph of $hop^{Tum\text{-}l}$ mutants. Blood cells from the third instar $hop^{Tum\text{-}l}$ mutant larvae stained with anti-L1/Atilla antibody (lamellocyte specific-Green), polymerized F-actin (cytoskeleton-Red) and nuclear dye (Blue), DAPI without MTX treatment (L1-L4) and after MTX (5.67 μM) treatment (M1-M4). White arrows in panel L2 indicate the green lamellocytes positive for the anti-L1/Atilla antibody. (Experiments were conducted at 25 °C).

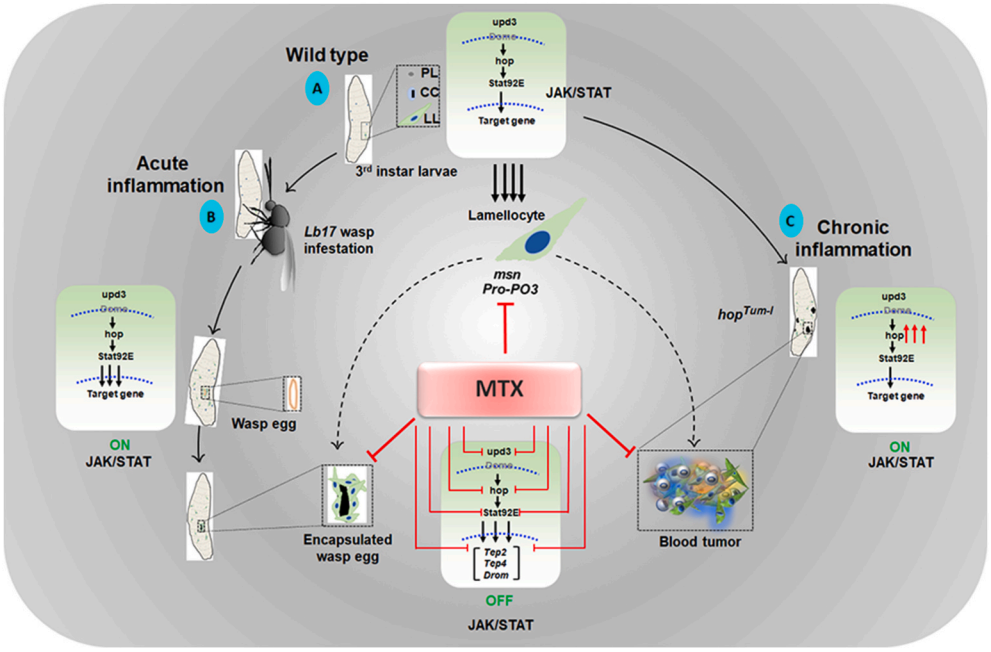

Fig. 5. Schematic representation of MTX's role in both acute (wasp induced) inflammation and chronic inflammation ($hop^{Tum\text{-}l}$) in 3rd instar *Drosophila* larvae.
5A. Wild type 3rd instar larvae with the circulating blood cells (PL-plasmatocyte CC-crystal cell LL-lamellocyte). The wild type larvae show the basal level of JAK/STAT components *(upd3, hop and Stat92E)*. The pathway is crucial for the formation of lamellocytes. The genes, *msn* and *Pro-PO3*, specifically expressed in these cells are inhibited by MTX drug treatment.
5B. The larva challenged with the parasitoid wasp (Acute inflammation) trigger an inflammatory response in the circulating blood cells, that outcast the wasp egg by encapsulating it with lamellocytes. The process involves the activation JAK/STAT pathway *(upd3, hop and Stat92E)*. MTX inhibits the encapsulation response against parasitoid body and also JAK/STAT pathway genes.
5C. The hematopoietic mutant $hop^{Tum\text{-}l}$ larvae shows the melanotic microtumors due to deregulated hematopoieis (Chronic inflammation). $hop^{Tum\text{-}l}$ larvae shows the constitutive activation of JAK/STAT and blood tumors in circulating hemolymph. MTX treatment hinders the JAK/STAT component gene expression *(hop, Stat92E, Tep2)* along with the expression of AMP, *Drosomycin*. The overall schematic depicts the intrinsic potential of MTX that has an inhibitory effect on the encapsulation as well as on blood tumor formation in acute and chronic inflammation respectively.

MTX on the hematopoietic pathways involved in both the encapsulation response and blood tumor formation. Toll/NF-κB pathway is predominantly an inflammatory pathway in the mammalian system that has also gained significance for eliciting *Drosophila* inflammatory responses. Since JAK/STAT pathway is implicated in the *spz* gene expression (Spätzle is the ligand for Toll/NF-κB pathway) (Irving et al., 2001), it is not surprising if MTX affects the NF-κB target gene expression as well i.e. the antimicrobial peptide, *Drosomycin*. Antimicrobial peptides have recently been shown to be anti-tumorous and therefore it is interesting to know how MTX affects other AMPs such as Attacin, Drosocin, Cecropin and Metchinkowin etc. Therefore, it is interesting to explore the effects of MTX on not only Toll pathway but also IMD pathway both of which are involved in AMP expression.

6. Conclusions

Various fields of medicine, pharmacology, biotechnology and therapeutic fields of clinical research are contributing to the immense research addressing various diseases including cancer. Drug discovery is a cumbersome, lengthy and high-investment procedure with various challenges that are difficult to overcome such as lack of knowledge about the pathophysiology of disease under investigation and poorer understanding of heterogeneity of the patients under observation. Past decade shed light on drug repositioning and repurposing which can be highly beneficial in addressing diseases that are life threatening along with those that are therapeutically unaddressed. Through this research investigation we have identified a new application for Methotrexate as a modulator of *Drosophila* larval blood cell differentiation, inflammation and its role in regulating *Drosophila* development. Our study underscores the use of *Drosophila* model to study therapeutic and side effects that can be easily extrapolated due to its shorter life cycle and easy genetic manipulation.

Declaration of competing interest

Authors share no competing or financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104161.

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Author contributions

All the authors contributed sufficiently for the manuscript

preparation. R.K.Y and D.K.G contributed equally. Contributions for conceptualization, methodology, validation, resources, data analysis include I.P., R.K.Y, D.K.G, G.B.M and C.M. Writing - review & editing along with project administration by I.P., R.K.Y, D.K.G, G.B.M.

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REVIEW ARTICLE

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Comparative hematopoiesis and signal transduction in model organisms

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Abstract

Hematopoiesis is a continuous phenomenon involving the formation of hematopoietic stem cells (HSCs) giving rise to diverse functional blood cells. This developmental process of hematopoiesis is evolutionarily conserved, yet comparably different in various model organisms. Vertebrate HSCs give rise to all types of mature cells of both the myeloid and the lymphoid lineages sequentially colonizing in different anatomical tissues. Signal transduction in HSCs facilitates their potency and specifies branching of lineages. Understanding the hematopoietic signaling pathways is crucial to gain insights into their deregulation in several blood-related disorders. The focus of the review is on hematopoiesis corresponding to different model organisms and pivotal role of indispensable hematopoietic pathways. We summarize and discuss the fundamentals of blood formation in both invertebrate and vertebrates, examining the requirement of key signaling nexus in hematopoiesis. Knowledge obtained from such comparative studies associated with developmental dynamics of hematopoiesis is beneficial to explore the therapeutic options for hematopoietic diseases.

KEYWORDS

blood, Drosophila, hematopoiesis, human, mice, model organism, signaling, zebrafish

1 | INTRODUCTION

There exist three theories behind development of human blood cells. To date, the most accepted theory for hematopoiesis is Unitary or Monophyletic theory proposed by Maximow (1909; Konstantinov, 2000). The Monophyletic theory highlights the presence of a common pluripotent stem cell for all the mature blood cells. However, dualistic and trialistic/polyphyletic theories did not gain significant scientific attention. The Dualistic theory proposed by Ehrlich, Schridde, and Naegeli states that all the blood cells are generated from two sources of hematopoietic progenitor cells, that is, the myeloid and the lymphoid. In contrast, Aschoff gave the polyphyletic theory, suggesting that separate stem cells exist for the formation of each type of blood cells.

Hematopoietic stem cells (HSCs) are derived from mesodermal hemangioblast cells (Sabin, 1917). In mammals, these HSCs possess

Abbreviations: AGM, aorta–gonad–mesonephros; AKT, protein kinase B; ALM, anterior lateral mesoderm; Ang-1, angiopoietin 1; BM, bone marrow; BMP, bone morphogenetic protein; CHT, caudal hematopoietic tissue; CZ, cortical zone; DL, delta; DLL, delta Like; dpf, days postfertilization; ECM, extracellular matrix; EMPs, erythromyeloid progenitors; FL, Fetal liver; FoxO-1, transcription factor forkhead boxO-1; GPCR, G-protein-coupled receptor; *hop*, *hopkinase*; hpf, hours postfertilization; HSCs, hematopoietic stem cells; HSPCs, hematopoietic stem progenitor cells; HSs, hemocyte precursor; IKK, IκB kinase; IL, interleukin; Jag, jagged; JAK, Janus Kinases; LG, lymph gland; MZ, medullary zone; NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; NIK, NF-κB-inducing Kinase; OPN, osteopontin; PBI, posterior blood island; PDK-1, 3-phosphoinositide-dependent protein kinase-1; PEAR-1, platelet endothelial aggregation receptor-1; PI3K, phosphoinositide 3-kinase; PLM, posterior lateral mesoderm; PSC, posterior signaling center; PTEN, phosphatase and tensin homolog; P-Sp, para-splanchanopleura; RBI, rostral blood island; RTK, receptor tyrosine kinase; SCF, stem cell factor; Ser, serrate; SETDB1, SET domain bifurcated histone lysine methyltransferase 1; STATs, signal transducer and activator of transcription proteins; TAK1, TGF β-activating Kinase 1; TGF β, transforming growth factor beta; TPO, thrombopoietin; TRAF, tumor necrosis factor receptor-associated factors; *Tum-l*, *Tumorous lethal*; TβR, TGF β-Receptor; YS, Yolk Sac; Znf45l, zinc finger protein 45-like.

repertoires that include quiescence, self-renewal, multipotency, differentiation, egression and mobilization, and apoptosis (Lapid et al., 2008; Orkin & Zon, 2008; Pietras et al., 2011). Self-renewal is a unique property of HSCs, that is, the potential to give rise to all types of blood cells and the ability to divide into daughter cells identical to their parent cell (Orkin & Zon, 2008; Smithgall, 1998). Few daughter cells remain as HSCs committed to stemness also referred as "Hemocytoblasts," maintained through an asymmetric division in the hematopoietic stem cell niche (Morrison & Kimble, 2006). HSC niche is the specific microenvironment in which the quiescence and self-renewal properties of the progenitors are maintained before receiving the signals for differentiation. As the matured blood cells' life span is restricted, they are perpetually replenished by these HSCs (Tavian & Peault, 2005). During ontogeny, in most of the vertebrates, the prime sites for blood cell production are changed along with the course of development (Zon, 1995). In vertebrates, especially in birds and mammals, bone marrow (BM) is regarded as the primary site for new blood cell production (Morin-Poulard et al., 2013). In mammals, the hematopoietic niche is primarily located in the yolk sac and with the course of development the BM niche is responsible for hematopoiesis. Hematopoietic niche maintains the heterogeneity of HSCs and other committed hematopoietic progenitors (Calvi & Link, 2015). Vertebrate blood cells are of multiple types, forming an array of intricate networks, destined for a specific function (Evans et al., 2003). Self-renewal and differentiation properties of HSCs continuously need an appropriate balance, regulated by hematopoietic signal transduction pathways (Chotinantakul & Leeanansaksiri, 2012). HSC quiescence is crucial for its stemness, and failing to maintain this property leads to functional altercations of the blood cells. (Chotinantakul & Leeanansaksiri, 2012; Fleming et al., 1993). This study is the first to discuss the blood cell development and its associated signaling pathways in four different research models. We contemplate the past, the current, and the on-going advancements in hematopoiesis with a comparative perspective on invertebrate and vertebrate model systems, emphasizing the important signaling pathways.

2 | ONTOGENY OF BLOOD IN MODEL ORGANISMS

Stem cells' ability to differentiate into various cells grades them into four different categories, namely totipotent, pluripotent, multipotent, and unipotent (Singh et al., 2016). Initial studies addressed HSCs as multipotent, but in the past decade, scientific evidences contributed to the pluripotent nature of HSCs (Ogawa et al., 2013). In this review, we have used four different model organisms to compare the specific events of ontogeny, the anatomical regions of development, the time points of such developmental events, and the committed hematopoietic lineages. This review seeks attention on HSC's property of multipotency giving rise to various blood cell lineages.

3 | *DROSOPHILA MELANOGASTER* (FRUIT FLY)

Drosophila melanogaster is a versatile model organism with a simple hematopoietic system. Using this model as a typical archetype of invertebrates, we have described below the events during the hematopoietic development and compared it with the higher vertebrate models. Unlike vertebrates, *Drosophila* lacks vasculature with no physical barrier between blood cells and other tissues and organs (Lihui Wang, Kounatidis, & Ligoxygakis, 2013). Blood in the open circulatory system of *Drosophila* is referred as hemolymph. It comprises of three morphologically different types of mature blood cells (Plasmatocytes [>95%], Crystal cells [~5%], and lamellocytes [<1%]) differentiating from a common progenitor (Evans et al., 2003; Lanot et al., 2001; Rizki & Rizki, 1992; Sinenko et al., 2012; Tepass et al., 1994). Along with the hematopoietic precursors and progenitors, all the matured blood cells are actively found in the hemolymph and in the lymph gland (LG). Plasmatocytes are functionally equivalent to mammalian monocytes, macrophages, and neutrophils (Anderl et al., 2016). Plasmatocytes play a role in surveillance of damaged tissue, repair, and defense. Crystal cells are larger in size as compared with plasmatocytes and are functionally equivalent to mammalian platelet-like cells. These cells have paracrystalline inclusions that are involved in melanization reaction and wound healing (Jiravanichpaisal et al., 2006; Vlisidou & Wood, 2015). Lamellocytes are large flat adhesive cells (Lanot et al., 2001). They appear least in healthy larvae, whereas they are absent in the embryonic and adult stages of the fly (Lanot et al., 2001; Sorrentino et al., 2002).

4 | PRIMITIVE AND DEFINITIVE HEMATOPOIESIS IN *DROSOPHILA MELANOGASTER*

Hematopoietic development in an invertebrate model *Drosophila*, which is a metamorphosizing organism, can be studied at different developmental stages, that is, the embryo, the larva, and the adult. Fruit flies have two different anatomical sites for hematopoietic development in larval stages that are not present in the adults. However, in vertebrate model system, the anatomical sites for hematopoietic development remain the same during the fetal and the adult stages. Published literature divides the development of *Drosophila* blood cells into two phases: primitive and definitive. During the primitive phase of hematopoiesis, in the head (procephalic) mesoderm, hemocyte precursors (HSs) arise from an identical set of progenitors known as multipotent hematopoietic progenitors/prohemocytes (Lanot et al., 2001). Furthermore, these HSs give rise to two types of mature blood cells, plasmatocytes and crystal cells, in the early embryo (Figure 1; de Velasco et al., 2006; Tepass et al., 1994; Wood & Jacinto, 2007). As the embryo develops, plasmatocytes populate it totally, found in circulation, whereas crystal cells limit themselves specifically to the proventriculus region (Tepass et al., 1994). The second phase occurs in the larval stages known as "Definitive phase of hematopoiesis." The formation of LG, a small hematopoietic organ, begins in the 16-celled stage of the embryo, but the definitive stages

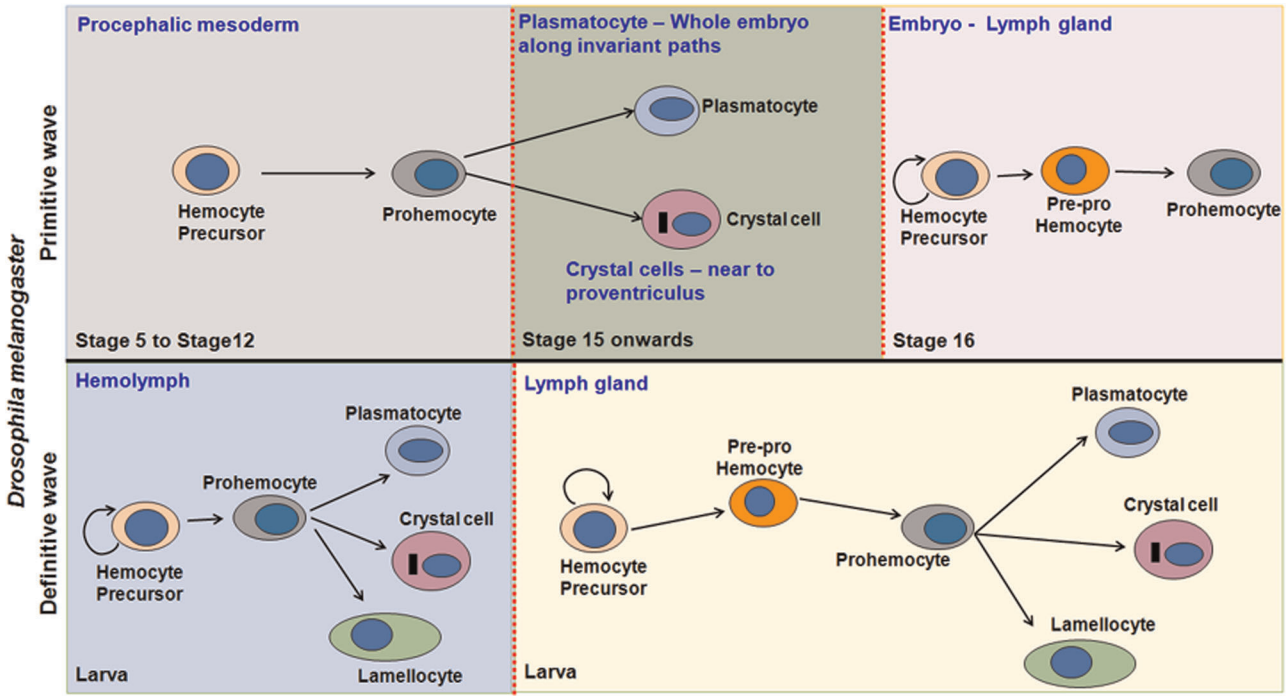

FIGURE 1 Diagrammatic illustration of hematopoiesis in *Drosophila* specifying the stages and tissues of development. (a) A primitive wave occurs at the embryonic stage in head/procephalic mesoderm. Hemocyte precursors gives rise to prohemocytes, and these prohemocytes further differentiate into two types of mature hemocytes: the plasmatocytes in the whole embryo and the crystal cells near proventriculus. The onset of lymph gland (hematopoietic organ) in the embryo occurs at Stage 16. Hemocyte precursors give rise to preprohemocytes, further forming prohemocytes. (b) A definitive wave in larval stages occurs in two different anatomical sites, hemolymph and the lymph gland. In the circulating hemolymph, the hemocyte precursors (HP) give rise to prohemocyte that differentiates into plasmatocytes, crystal cells, and lamellocytes. Hemocyte precursor of the lymph gland, in the larval stages, differentiates into preprohemocyte (PPH) that further gives rise to prohemocytes. These prohemocytes eventually divide to form plasmatocytes, crystal cells, and lamellocytes

occurs only during larval stages, marking the difference between the two stages. During definitive phase, the LG gives rise to all types of hemocytes (Lanot et al., 2001; Lemaitre et al., 1996; Shia et al., 2009). LG is a bilobed structure composed of a single pair of anterior lobes, followed by 2–3 pairs of smaller posterior lobes (Honti et al., 2014) along the dorsal vessel (Jung, 2005; Krzemien et al., 2010a,2010b, 2010c; Lanot et al., 2001). The core of the anterior lobe is known as the medullary zone (MZ) where the undifferentiated hematopoietic progenitor stem cells reside. The cortical zone (CZ), that is, the periphery of the anterior lobe, is mostly occupied with differentiated hemocytes and few intermediate progenitors. The framework of the base of the anterior lobe has the posterior signaling center (PSC; Jung, 2005; Krzemien et al., 2010a, 2010b, 2010c; Lebestky et al., 2003). PSC has a key role in regulating the balance between undifferentiated hematopoietic progenitors (prohemocytes) and their differentiated lineages (Jung, 2005). The existence of stem cells in the hematopoietic organ, LG, was initially determined by clonal studies, that is, by inducing mitotic clones in the hematopoietic cells of the embryos and first instar larvae of *Drosophila* (Minakhina & Steward, 2010). One of the GATA family of transcription factors, serpent (srp) in *Drosophila*, is known to be involved in blood formation apart from gut and fat body development. Srp also regulates the hemocyte precursor formation and is essentially involved in the plasmatocytes and crystal cell specification during larval development (Fossett et al., 2003; Lebestky et al., 2000; Mandal et al., 2004). Like many metazoans, *Drosophila* lacks both the lymphoid lineages and the adaptive immune responses. Nevertheless, *Drosophila* blood cells (plasmatocytes and crystal cells) are considered functional equivalents to mammalian lymphoid lineage for their participation in repair of damaged tissue, macrophagy, wound healing, and innate immune defense mechanisms against pathogens such as microbes and parasitoid wasps (Crozatier & Vincent, 2011; Hartenstein & Mandal, 2006). The parasitoid species strategically attack and utilize their hosts' machinery to pursue their own offspring survival and development, often resulting in death of the host. To circumvent this endoparasite strategy, the host triggers immune responses that include the formation of lamellocytes capable of inducing the encapsulation response. Encapsulation, as a complex cellular immune response against parasitism, results from primarily the increased differentiation of progenitors into lamellocytes (specialized hemocyte produced during wasp infestation), along with the involvement of crystal cells necessary for the melanization process (prophenoloxidase cascade; Geng et al., 2013; Nakhleh et al., 2017; Nappi, 2010; Rizki & Rizki, 1992; Rizki et al., 1990; Sorrentino et al., 2002). Although hematopoiesis in *Drosophila* larva is well established, there is scarce knowledge regarding blood cells and their developmental cues in the adults. *Drosophila* biologists recently identified four diffused "hematopoietic hubs" (hemocyte clusters) in the dorsal abdomen of the

fly. These hematopoietic hubs harbor the progenitors with a potential to differentiate into functional plasmatocytes and crystal cells, suggesting the true nature of hematopoietic organ in adult *Drosophila* (Saikat Ghosh et al., 2015). Therefore, *Drosophila* serves as an excellent model in understanding the immune responses triggered by the hemocytes. Although zebrafish is also a hematopoietic paradigm, the developmental process involved is not simpler as compared with *Drosophila*.

5 | *DANIO RERIO* (ZEBRAFISH)

In the past decades, *Danio rerio* (zebrafish) has emerged to be an appropriate vertebrate model system bridging the barrier between invertebrate and mammalian hematopoiesis. Blood cell development in zebrafish is analogous to hematopoiesis in the higher vertebrates (Berman et al., 2003). Few of the anatomical sites (such as thymus and kidney marrow) of blood cell development in zebrafish are similar to the other vertebrates, whereas these are not found in *Drosophila*. Also, the hematopoietic events observed in zebrafish are conserved across vertebrates and show multiple similarities. In this section, we have discussed and highlighted the importance of zebrafish model in hematopoiesis and compared it with the other paradigms. Zebrafish embryos serve as an ideal model for studying hematopoiesis. This is feasible due to the external fertilization, in vivo visualization of optically transparent embryos, genetic and chemical screening at a large scale, and an ease of genetic manipulation of the embryos (Gore et al., 2018; Jingd & Zon, 2011). The mesoderm germ layer delineates the fate of dorsal and ventral group of cells. Cells with dorsal fate eventually form somites and notochord, whereas cells that attain ventral fate shape into blood, vasculature, and pronephros/kidney. Despite different anatomical tissues assigned for hematopoietic development, zebrafish and mammals share a conserved genetic network regulating the overall hematopoiesis (Avagyan & Zon, 2016). The niche of HSCs in zebrafish is kidney marrow, which is equivalent to BM niche in the mammals (Chen & Zon, 2009; Huang & Zon, 2008; Paik & Zon, 2010).

6 | PRIMITIVE AND DEFINITIVE HEMATOPOIESIS IN *DANIO RERIO*

Zebrafish also has two waves of hematopoiesis, that is, the primitive hematopoiesis and the definitive hematopoiesis. There exist few striking differences that separate this research model from the higher vertebrate. One such example is the absence of anatomical sites like the placenta and the fetal liver (FL) in zebrafish (Jingd & Zon, 2011). Such structures in zebrafish are replaced by dorsal aorta and caudal hematopoietic tissues (CHTs) as sites for blood cells development (Jingd & Zon, 2011). In the next section, we shed light on such differences during hematopoiesis. In case of zebrafish, the primitive hematopoiesis occurs at 11 h postfertilization (hpf) at two different sites: the anterior lateral mesoderm (ALM) and the posterior lateral mesoderm (PLM) within the embryo (intra-embryonic; Figure 2; Davidson et al., 2003; Detrich et al., 1995; Jingd & Zon, 2011). PLM at later stages develop into the intermediate cell mass (ICM) analogous to the extraembryonic blood islands of mammalian origin (Amatruda & Zon, 1999; Galloway & Zon, 2003; Galloway et al., 2005). Apart from PLM, there is evidence for the presence of another anterior hematopoietic site originating from ALM, known as rostral blood islands (RBIs). RBIs generate a population of macrophages, embryonic microglia, and neutrophilic granulocytes (Bennett et al., 2001; Herbomel et al., 1999; Lieschke et al., 2002). Primitive macrophages functionally eliminate the pathogens and apoptotic cells considered as by-product during development (Herbomel et al., 1999). Also, a recent finding suggested a role for macrophages in the establishment of definitive hematopoietic stem cell and progenitor cells (HSPCs; Travnickova et al., 2015). The primitive hematopoiesis in ALM is specifically responsible for producing myeloid lineages (primitive myelopoiesis), whereas erythroid lineages (primitive erythropoiesis) are generated in the ICM (Avagyan & Zon, 2016; Gore et al., 2018). Erythroid cells serve the purpose for tissue oxygenation, ensuring the rapid growth of embryo (Orkin & Zon, 2008). After 24 hpf, ICM diminishes, resulting in the movement of the primitive embryonic erythrocytes into the circulation (Figure 2). Definitive hematopoiesis contributes to the production of all types of blood cells that persist throughout the adulthood of zebrafish (Gore et al., 2018). HSPCs, in definitive hematopoiesis, arise from the ventral wall of the dorsal aorta at the onset of 26 hpf often termed as aorta–gonad–mesonephros (AGM) due to its mammalian counterpart (Burns et al., 2002; Detrich et al., 1995; Kalev-Zylinska et al., 2002; Thompson et al., 1998). These HSPCs, during the adulthood of zebrafish, maintain the pool of erythroid, myeloid, and lymphoid lineages. Definitive hematopoiesis takes place in two sequential events. First, a short intermediate transient phase occurs in the posterior blood island, giving rise to cells expressing GATA-binding factor 1 (*gata-1*) and LIM domain only 2 (rhombotin-like 1; *lmo2*). These cells are known as common erythromyeloid progenitors (EMPs) that eventually differentiate into erythroid and myeloid lineages at the larval stage (Bertrand et al., 2010). Second, around 28–30 hpf, hemogenic endothelial cells gives rise to true multipotent hematopoietic stem cells. A process termed "Endothelial hematopoietic transition" leads to the detachment of HSPCs specified from hemogenic endothelium (Bresciani et al., 2014; Kissa & Herbomel, 2010). This enables the entry of HSPCs into the circulation and targets CHT within 48 hpf, thymus by 3 days postfertilization (dpf), and kidney marrow by 4 dpf as their prime organs for niche (Bertrand et al., 2010; Figure 2). In the mature zebrafish, kidney is considered the primary site for the hematopoiesis, whereas spleen also has a role in the development of HSCs (Al-Adhami & Kunz, 1977; Lam et al., 2010; Murayama et al., 2006). Kidney is the site of origin for the myeloid, erythroid, thromboid, and lymphoid lineages. Thymus, in turn, remains specific for the maturation of T cells throughout the adulthood of zebrafish. Due to the comparable hematopoietic system between zebrafish and mouse, the researchers are generating their chimeric xenograft model to study hematopoiesis and hematopoietic cancer (Parada-Kusz et al., 2018).

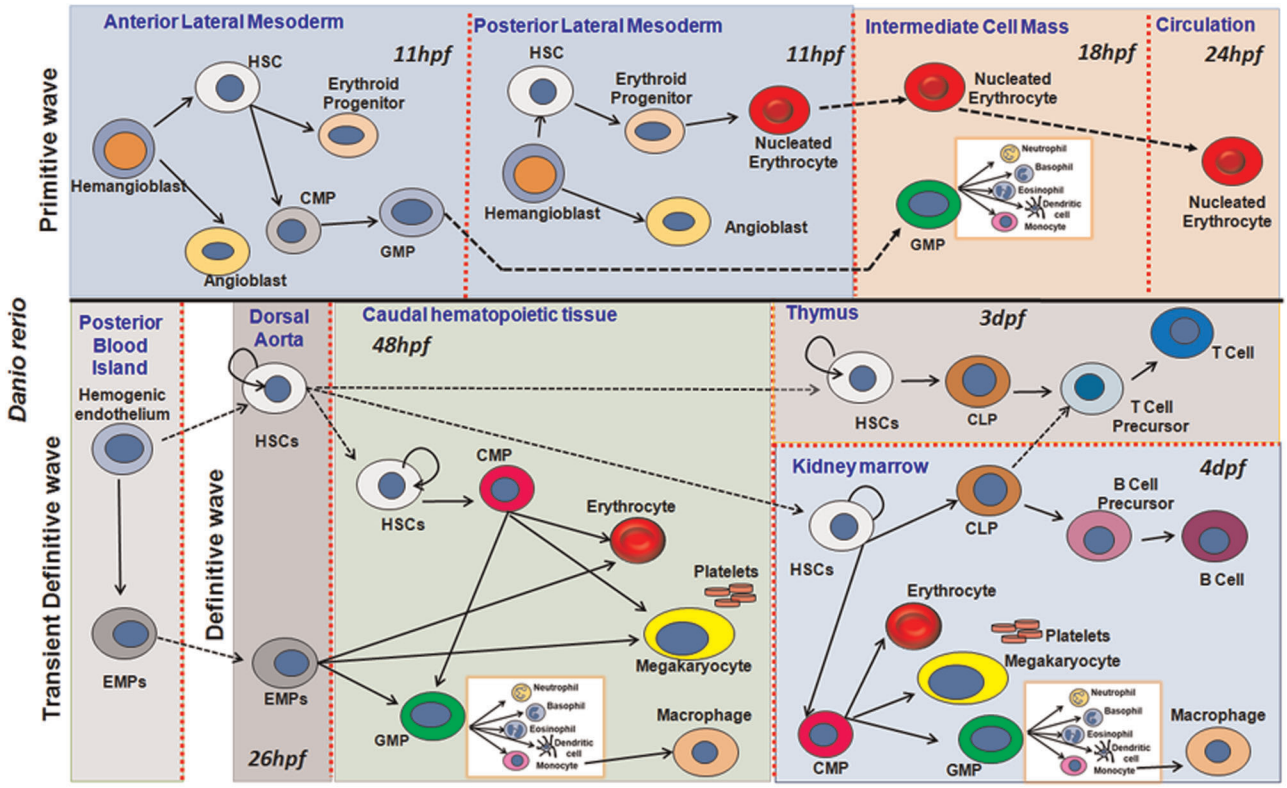

FIGURE 2 Schematic illustration of hematopoietic ontogeny in zebrafish, showing a shift in the anatomical sites with lineage differentiation from their parent progenitors. (a) Primitive hematopoiesis is initiated through hemangioblast simultaneously at the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM) around 11 hpf. Granulocyte/myeloid progenitors (GMPs) from the ALM and nucleated erythrocytes from the PLM enter intermediate cell mass (ICM) at 18 hpf. GMPs in the ICM differentiate into mature blood cells (neutrophils, basophils, eosinophils, dendritic cells, and monocytes). Monocytes further differentiate to form macrophages. Nucleated erythrocytes enter circulation from ICM at around 24 hpf. (b) Definitive hematopoiesis: a short transient wave shows hemogenic endothelium giving rise to erythro-myeloid progenitors (EMPs) in PBI that migrate to dorsal aorta. These EMPs further differentiate giving rise to the erythro-myeloid lineages in the caudal hematopoietic tissues (CHT). The hemogenic endothelium in the PBI gives rise to HSCs found in the dorsal aorta that migrate to and seed the caudal hematopoietic tissue (48 hpf), thymus at around 3 dpf, and kidney marrow at around 4 dpf. HSCs in CHT via common myeloid progenitors (CMP) give rise to erythro-myeloid lineages. HSCs in thymus give rise to common lymphoid progenitors (CLPs) that differentiate into matured T-cells via T cell precursors, and this happens only in the thymus. In kidney marrow, HSCs give rise to erythroid, myeloid, and lymphoid lineages except T cell generation. It is also observed that CLPs migrate from kidney marrow to thymus to give rise to matured T cells. dpf, postfertilization; hpf, postfertilization

7 | *MUS MUSCULUS* (MOUSE)

The house mouse (*Mus musculus*) is viewed as a standout model organism for more than a century. It serves as an essential and vital biomedical model for human genetics and health care. Although mouse genome is 14% less than human genome, more than 99% of the human genes share functional homologs in the mouse genome, establishing it as the best model to study human diseases (Saraswathy & Ramalingam, 2011). In the past few decades, the murine models have gained importance for their contribution in the discovery of self-renewal and lineage commitment of HSCs (Parekh & Crooks, 2013; Schmitt et al., 2014). The concept of HSCs originated in 1960s through transplantation studies of Till and McCullough in mice BM (Becker et al., 1963). Various genetic approaches contributed to identification and understanding of signaling pathways crucial for hematopoiesis (Schmitt et al., 2014). The existing theories suggest that mesodermal precursors (hemangioblast) are the ancestors of the HSCs and endothelial lineages (Choi et al., 1998; Kennedy et al., 1997). Further, they also suggested that the extraembryonic YS and the intraembryonic para-splanchanopluera (P-Sp) and AGM together reconstitute the HSC generation. In the next section, we have recapitulated primitive and definitive events occurring in vertebrate models using mouse as an example. Unlike fruit flies, *Mus musculus* has several well-defined anatomical sites for blood cell development, few of which are also found in zebrafish.

8 | PRIMITIVE AND DEFINITIVE HEMATOPOIESIS IN *MUS MUSCULUS*

Primitive hematopoiesis in mouse is initiated in the extraembryonic yolk sac blood islands during the early embryonic stages. Approximately

between E7 and E7.5, the mesoderm differentiates into a group of cells that eventually give rise to the anatomical site referred as "blood islands" (Figure 3). Murine YS is a bilayer organ made up of an endodermal layer of cells (derived from hypoblasts) and mesodermal layer of cells (derived from epiblasts). The assemblage of these two layers forms a structure called P-Sp (Yamane, 2018; Yoder, 2010). Primitive hematopoiesis generates mainly nucleated erythrocytes. These erythrocytes along with macrophages can be detected at E7 (Yoder, 2010). Also, a group of cells expressing megakaryocytic markers are produced at around E9.8 (Tober et al., 2007). However, in definitive hematopoiesis, all the hematopoietic lineages including definitive erythrocytes (nucleated and enucleated) are produced (Nakano et al., 1996). The circulation of erythroid cells is observed at E8. Mouse embryos devoid of erythrocytes are lethal beyond E11.5 (Fujiwara et al., 1996; Tsang et al., 1998), whereas embryos lacking definitive hematopoiesis are able to survive till E15 (Mucenski et al., 1991). These studies iterate the importance of primitive erythrocytes and their role in the tissue oxygenation of embryos. The transition of EMPs from YS to FL is observed at around 12.5 dpc, and these EMPs maintain the blood cell development through HSCs (Sugiyama et al., 2011). This stage onwards, definitive hematopoiesis is initiated with the end of primitive wave. However, some studies demonstrated that HSCs not only emerge in FL but also from both extraembryonic YS and intraembryonic P-Sp/AGM region. Although placenta serves the purpose of gaseous exchange and fetal nutrition, it also has a role in hematopoiesis during 8.5–13.5 dpc. HSCs generated in placenta are independent of P-Sp/AGM region. HSCs generated in YS, P-Sp/AGM, and placenta migrate to FL. Inside the FL, the HSCs population is well maintained before 12 dpc, after which HSC numbers are increased at a faster rate till 16 dpc (Ema & Nakauchi, 2000; Johnson & Jones, 1973; Johnson & Moore, 1975). Hematopoiesis in FL is regulated by both extrinsic and intrinsic factors. Extrinsic factors such as cytokines (thrombopoietin [TPO], stem cell factor [SCF], angiopoietin-like 3, insulin growth factor 2) and extracellular matrix proteins help in the regulation of hematopoiesis. Intrinsic factor that aid in the expansion and maintenance of HSCs includes PU.1 belonging to the ETS family of transcription factors (Chou & Lodish, 2010; Kim et al., 2004; Swain et al., 2014). However, there exists a debate about the HSCs' capability of self-renewal to be under the influence of cytokines, which stimulate HSC differentiation. An increasing number of HSCs

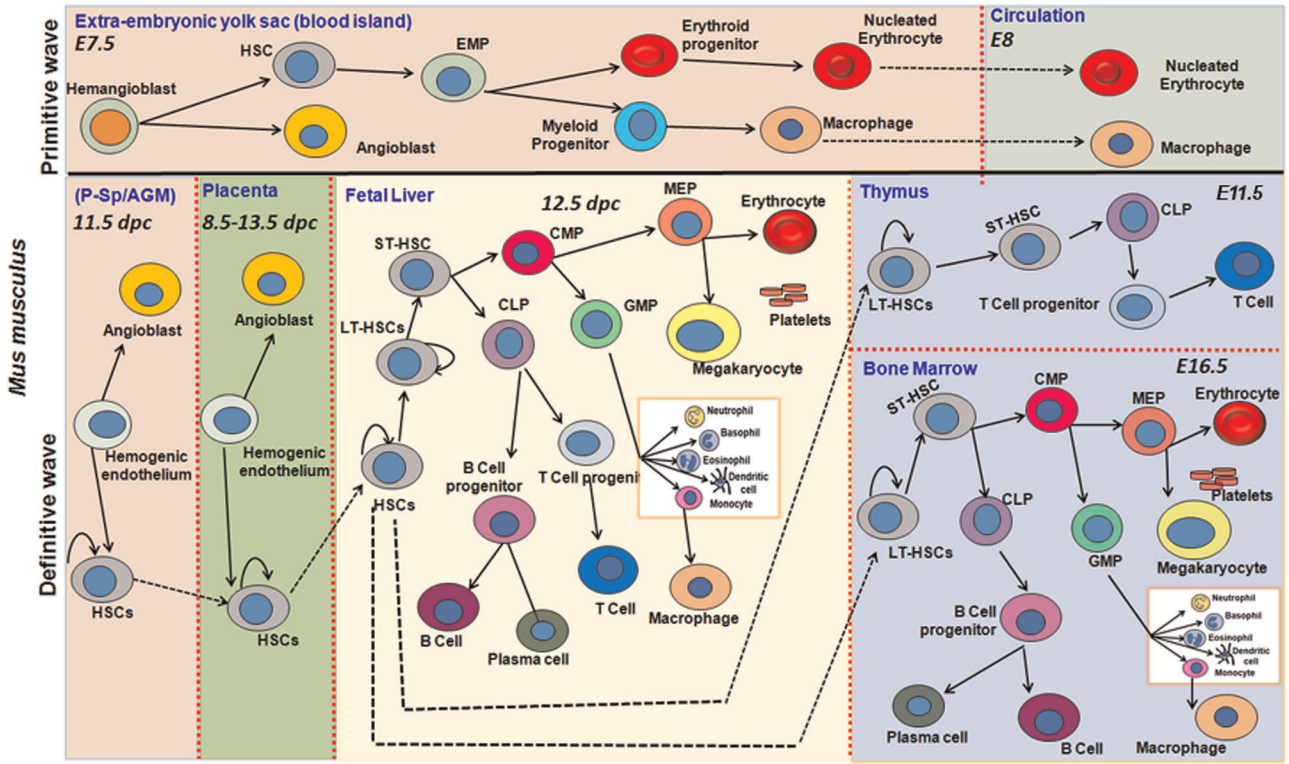

FIGURE 3 Outline of hematopoietic lineage in *Mus musculus* (Mice) showing the anatomical sites and terminally differentiated blood cells. (a) Primitive hematopoiesis in mouse is first seen around E7.5 of gestation in the extraembryonic yolk sac (YS) structure also known as blood islands. HSCs sequentially commit to form erythromyeloid progenitors (EMPs). These bipotent EMPs give rise to primitive nucleated erythrocytes and myeloid progenitor cells that further divide into nucleated erythrocytes and macrophages enter the circulation by stage E8. (b) Definitive hematopoiesis starts around 11.5 dpc in aorta–gonad–mesonephros (AGM). Hemogenic endothelium differentiates into angioblast and self-renewing HSCs that colonize the placenta and later, at around 12.5 dpc, migrate to and seed the fetal liver. The fetal liver serves as a temporary niche for HSCs. This HSCs generate the common myeloid and lymphoid progenitors further differentiating into terminally matured blood cells. HSCs finally shift from fetal liver into the bone marrow (E 16.5) and thymus (E 11.5) as long term-hematopoietic stem cells (LT-HSCs). LT-HSCs in thymus through a sequential lineage lead to the formation of mature T cell. Bone marrow is the permanent niche of HSCs giving rise to erythroid, myeloid, and lymphoid cells

in FL migrate to reside and differentiate temporarily in fetal spleen approximately between 13.5 and 14.5 dpc (Bertrand et al., 2006). Stromal cells derived from fetal spleen support the HSCs to differentiate into macrophages (Bertrand et al., 2006). HSCs in fetal spleen stay for a transient period and later migrate to BM between E10 and E14.5 for the entire lifespan of mice (Ciriza & García-Ojeda, 2010).

9 | *HOMO SAPIENS* (HUMAN)

The vast array of knowledge available for human hematopoiesis is possible from the immense studies conducted with human embryonic and fetal tissues. Although these studies contributed to sufficient knowledge, understanding the events in adults is challenging due to the lack of biological models. The developmental events that were difficult to study in ***Homo sapiens*** were complemented by the knowledge gained using the model organisms. The embryonic development in humans is classified over 23 carnegie stages (CS) occurring from fertilization to approximately Week 8, which is then followed by fetal development occurring until birth (Findlay et al., 2007). In the next section, we discuss the hematopoietic development according to the CS, followed by the events in fetus and adults. In humans, of the two waves of hematopoiesis, the first wave (primitive hematopoiesis) emerges in the YS during the third week of development (Ivanovs et al., 2017; M. Jagannathan-Bogdan & Zon, 2013; Tavian & Peault, 2005). YS is considered the first site for the production of blood cells (Golub & Cumano, 2013; J. Palis & Yoder, 2001). YS is an early extraembryonic structure of endodermal origin attached to the embryo by vitelline duct also known as yolk stalk (M. F. Donovan & Bordoni, 2020). YS is subdivided into two structures, namely the primary YS and the secondary YS. Primary YS is formed by the proliferation and differentiation of endodermal cells typically at 7–8 days after conception, but the hematopoietic function of primary YS has not been clearly defined to date (Christensen, 2018).

10 | PRIMITIVE AND DEFINITIVE HEMATOPOIESIS IN *HOMO SAPIENS*

The purpose of primitive hematopoiesis in humans is to produce red blood cells, which are essential for tissue oxygenation by the early developing embryo (Orkin & Zon, 2008). The primitive wave is of short period during which erythrocytes are derived from the erythroid progenitors (Figure 4). In mammals, the embryonic YS, FL, spleen, and adult BM are the sites of sequential emergence of stem cells (Zon, 1995). In spleen, hematopoiesis occurs only till the fifth month of the fetus, after which spleen does not participate in the development of blood cells. The differentiation of endodermal cells gives rise to mesodermal precursors known as ICM. After 12–15 days of fertilization, YS is collapsed into small vesicles forming secondary YS from the remnants. Primitive hematopoiesis is initiated in the secondary YS 16–19 days postconception (Kelemen et al., 2013; Kennedy et al., 1997; Tavassoli, 1991). Along with the switching of primitive hematopoiesis to definitive hematopoiesis, the anatomical site is also changed, that is, from YS to FL (Figure 4). In case of mice, this switch occurs between the embryonic Days 10–11, whereas in case of humans, it occurs during eighth week of embryonic development. Primitive hematopoiesis is distinguished by the generation of large nucleated erythrocytes that express embryonic globin. Definitive hematopoiesis shows initially nucleated erythrocytes that transform into the enucleated adult-type erythrocytes in humans and mouse, which produce fetal globin and adult globin, respectively (Kawahara, 2007). From the published literature, it is quite evident that definitive hematopoiesis in humans has two distinct sites for the production of differentiated blood cells, similar to the mouse model (Hoggatt & Pelus, 2013; Jagannathan-Bogdan & Zon, 2013; Rieger & Schroeder, 2012). These two sites are referred as P-Sp and AGM. AGM is thought to evolve from P-Sp identified in early embryos of mammals such as human, mice, and nonvertebrates. Definitive HSCs produce all the mature blood cells during the adult life span (Lemischka, 1991). Definitive hematopoiesis in humans is further divided into two waves (Jagannathan-Bogdan & Zon, 2013). One wave has the multipotent progenitors known as the EMPs. The differentiation potential of EMPs is limited to erythrocytes, mega-karyocytes, and myeloid cells (McGrath et al., 2015a, 2015b; James Palis et al., 1999). In the second wave, HSCs attain capabilities such as self-renewal and multipotency all through the life of an individual. These HSCs are also further capable of producing complete adult lineages, including lymphocytes (Figure 4). They are the B- and T-lymphocytes that form in the BM (CS23) and thymus (CS22), respectively (Chen et al., 2009; Farbod Famili et al., 2017). Murine EMPs produce erythrocytes expressing adult globins, and this knowledge created confusion, especially in the in vitro assays seeking to generate HSCs. Also, it is impossible to distinguish EMPs and HSCs on the basis of assays with only an erythro-myeloid readout (Clements & Traver, 2013). HSCs in the placenta are found in Week 6 of the gestation (Gekas et al., 2005). Furthermore, EMPs and HSCs cannot be distinguished by cell surface markers (Clements & Traver, 2013). These issues highlight the need to test hematopoietic precursors for their long-term reconstitution and lymphoid potential. Furthermore, they can help decipher the success of in vitro HSC specification. Several unknown factors and aberrant signaling pathways deregulate the normal process of blood cells' development in hematopoietic diseases. Therefore, the use of different model organisms has benefitted scientific understanding of hematopoietic signal transduction in diseases associated with blood cells.

The study of HSC's behavior and development in primates is still not completely understood due to the reasonable limitation in the experimental assays that include direct observation or competitive repopulation assay along with ethical restrictions on using their embryos as an experimental model (Shepherd et al., 2007; Umeda, 2004). However, studies performed using human embryos uncovered the hematopoietic similarities that primates share with mice (Tavian et al., 1996; Tavian et al., 1999). Existing literature on primitive and definitive hematopoiesis in primates remains to be elusive when compared with the work done on mice hematopoiesis. Humans and old-world monkeys such as cynomolgus monkeys (*Macaca fascicularis*) have similarities in the fetal globin expression. Primate erythrocytes express embryonic (ϵ and ζ), fetal (γ) and adult

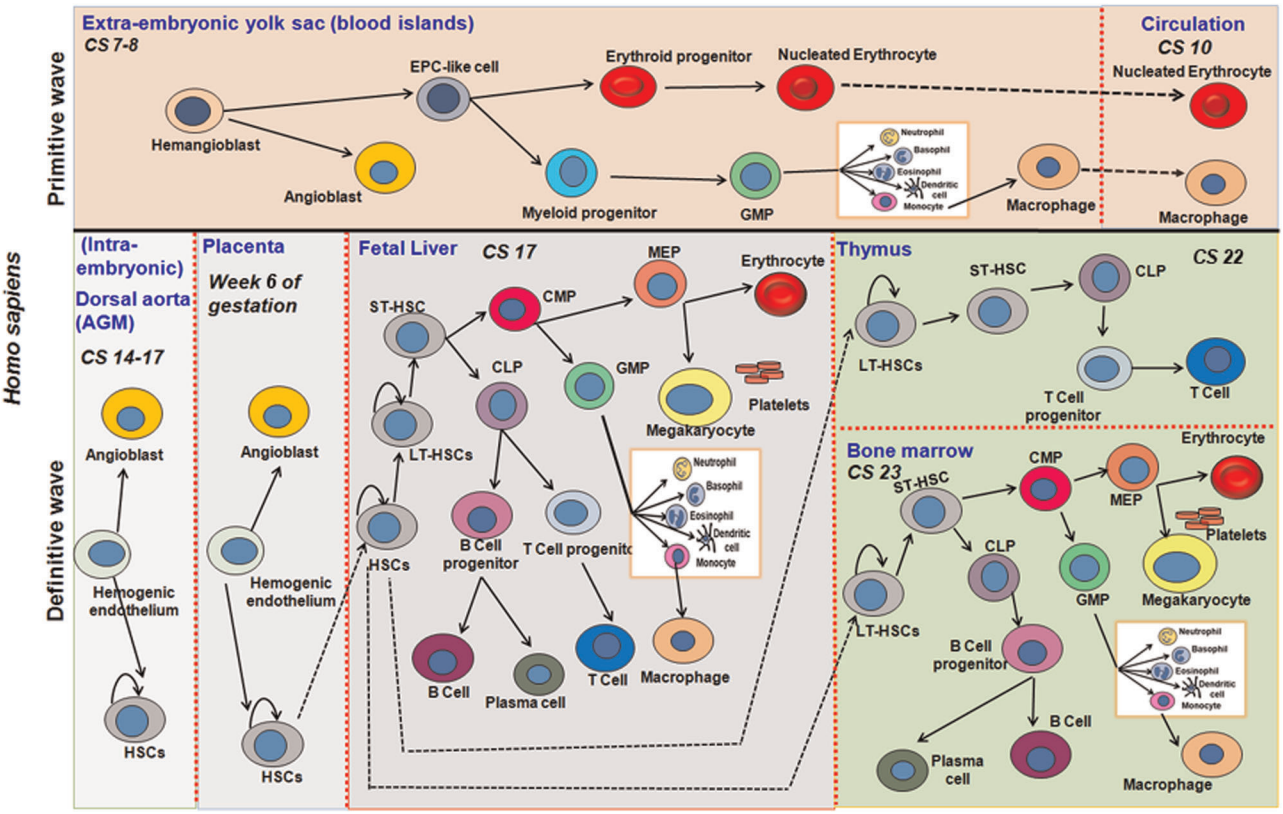

FIGURE 4 The schematic overview of hematopoiesis in *homo sapiens* with the anatomical sites, blood cells, and time points. (a) Primitive hematopoiesis in human occurs in extraembryonic yolk sac (YS). Hemangioblast generates angioblast and primarily endothelial progenitor cell (EPC). EPC through a sequential lineage differentiation forms nucleated erythrocytes and few granulocytes during Carnegie stage (CS) 7–8. These erythrocytes are observed in the circulation during the CS10. (b) Definitive hematopoiesis starts in the intraembryonic aorta–gonad–mesonephros (AGM) region where hemogenic endothelium gives rise to angioblast and self-renewing HSCs that migrate to and seed the placenta during the nineth week of gestation. Placental HSCs migrate to fetal liver, temporary niche of HSCs, generating both the common lymphoid and myeloid progenitors, giving rise to all matured blood cells at around CS17. Fetal liver HSCs, at around CS 23, migrate to and seed the bone Marrow (CS 23) and thymus (CS 22). Bone marrow is the niche of HSCs for the adult life span of an individual. Here, HSCs self-renew and differentiate into the common myeloid and lymphoid progenitors for forming the complete blood cell profile. All blood lineages mature and enter the circulation

(β and α) globin genes sequentially during the course of hematopoietic development (Johnson et al., 2000). This led researchers to exploit embryonic stem cells cocultured with OP9 stromal cells as a potential model to study primate hematopoietic development, leading to the induction of hematopoietic differentiation (Suemori et al., 2001; Thomson et al., 1995, 1996; Thomson, 1998).

We summarized the anatomical sites along with the stages of the blood cells' development in each model organism. We incorporated the aforementioned key points on the hematopoietic ontogeny in the model organisms in a tabular form (Table 1).

11 | HEMATOPOIETIC NICHE AND SIGNAL TRANSDUCTION IN HEMATOPOIETIC REGULATION

Cell–cell signaling is an essential event during cellular processes. This enables cells to perceive and transmit signals for development, growth, and immunity. Stem cells' self-renewal, maturation, differentiation, and regulation properties are fundamentally dependent on their niche microenvironment (Dennis, 2003; Morrison & Scadden, 2014). Crosstalk between the HSCs and the hematopoietic niche establishes homeostasis and steady state of HSCs (Lo Celso & Scadden, 2011). Hematopoietic equilibrium is observed by asymmetrical cell division through which one daughter cell maintains the stem cell identity and the other becomes differentiated (Gómez-López et al., 2014; Inaba & Yamashita, 2012; Morrison & Kimble, 2006). This asymmetry can be achieved by environmental signals, which create prodifferentiation or prorenewal environments. HSC's self-renewal and differentiation properties are critically regulated by cellular signals produced from HSCs' niche as well as systemic environment (Malhotra & Kincade, 2009; Morrison & Scadden, 2014; Seita & Weissman, 2010). CXCL12 (chemokine [C-X-C motif] ligand 12), a chemokine, and SCF, a cytokine, are factors that are implicated in HSC maintenance and retention. Recent studies also shed light on different effects of CXCL12 on blood cells. CXCL12 expressing perivascular region stromal cells are required for HSCs, and this was

TABLE 1 Comparative hematopoietic ontogeny in model organisms

Blood cell	Danio rerio	Mus musculus	Homo sapiens
Hemangioblast	Anterior lateral mesoderm (11 hpf)	Extraembryonic yolk sac/blood island (E 7.5)	Extraembryonic yolk sac/blood island (CS 7–8)
	Posterior lateral mesoderm (11 hpf)		
Angioblast	Anterior lateral mesoderm (11 hpf)	Extraembryonic yolk sac/blood island (E 7.5)	Extraembryonic yolk sac/blood island (CS 7–8)
	Posterior lateral mesoderm (11 hpf)		
		AGM (11.5 dpc)	AGM (CS 14–17)
		Placenta (8.5–13.5 dpc)	Placenta (Week 6 of gestation)
HSC	Anterior lateral mesoderm (11 hpf)	Extraembryonic yolk sac/blood island (E 7.5)	
	Posterior lateral mesoderm (11 hpf)		
	Dorsal aorta (26 hpf)	AGM (11.5 dpc)	AGM (CS 14-17)
		Placenta (8.5–13.5 dpc)	Placenta (Week 6 of gestation)
	Caudal hematopoietic tissue (48 hpf)	Thymus (E 11.5)	Fetal liver (CS 17)
	Thymus (3 dpf)	Fetal liver (12.5 dpc)	Thymus (CS 22)
	Kidney marrow (4 dpf)	Bone marrow (E 16.5)	Bone marrow (CS 23)
Erythroid progenitor	Anterior lateral mesoderm (11 hpf)	Extraembryonic yolk sac/blood island (E 7.5)	Extraembryonic yolk sac/blood island (CS 7–8)
	Posterior lateral mesoderm (11 hpf)		
Myeloid progenitor		Extraembryonic yolk sac/blood island (E 7.5)	Extraembryonic yolk sac/Blood island (CS 7–8)
CMP	Anterior lateral mesoderm (11 hpf)		
	Caudal hematopoietic tissue (48 hpf)	Fetal liver (12.5 dpc)	Fetal liver (CS 17)
	Kidney marrow (4 dpf)	Bone marrow (E 16.5)	Bone marrow (CS 23)
GMP	Anterior lateral mesoderm (11 hpf)		Extraembryonic yolk sac/blood island (CS 7–8)
	Intermediate cell mass (18 hpf)		
	Caudal hematopoietic tissue (48 hpf)	Fetal liver (12.5 dpc)	Fetal liver (CS 17)
	Kidney marrow (4 dpf)	Bone marrow (E 16.5)	Bone marrow (CS 23)
Nucleated erythrocyte	Posterior lateral mesoderm (11 hpf)	Extraembryonic yolk sac/blood island (E 7.5)	Extraembryonic yolk sac/Blood island (CS 7–8)
	Intermediate cell mass (18 hpf)		
	Circulation (24 hpf)	Circulation (E 8)	Circulation (CS 10)
Hemogenic endothelium	Posterior blood island		
		AGM (11.5 dpc)	AGM (CS 14–17)
		Placenta (8.5–13.5 dpc)	Placenta (Week 6 of gestation)
EMP	Posterior blood island	Extraembryonic yolk sac/blood island (E 7.5)	
	Dorsal aorta (26 hpf)		
		Fetal liver (12.5 dpc)	Fetal Liver (CS 17)
	Kidney marrow (4 dpf)	Bone marrow (E 16.5)	Bone marrow (CS 23)
Erythrocyte	Caudal hematopoietic tissue (48 hpf)	Fetal liver (12.5 dpc)	Fetal liver (CS 17)
	Kidney marrow (4 dpf)	Bone marrow (E 16.5)	Bone marrow (CS 23)
Macrophage		Extraembryonic yolk sac/blood island (E 7.5)	Extraembryonic yolk sac/blood island (CS 7–8)
		Circulation (E 8)	Circulation (CS 10)
	Caudal hematopoietic tissue (48 hpf)	Fetal liver (12.5 dpc)	Fetal liver (CS 17)
	Kidney marrow (4 dpf)	Bone marrow (E 16.5)	Bone marrow (CS 23)

(Continues)

TABLE 1 (Continued)

Blood cell	Danio rerio	Mus musculus	Homo sapiens
Megakaryocytes	Caudal hematopoietic tissue (48 hpf) Kidney marrow (4 dpf)	Fetal liver (12.5 dpc) Bone marrow (E 16.5)	Fetal liver (CS 17) Bone marrow (CS 23)
Platelets	Caudal hematopoietic tissue (48 hpf) Kidney marrow (4 dpf)	Fetal liver (12.5 dpc) Bone marrow (E 16.5)	Fetal liver (CS 17) Bone marrow (CS 23)
CLP	 Thymus (3 dpf) Kidney marrow (4 dpf)	Fetal liver (12.5 dpc) Thymus (E 11.5) Bone marrow (E 16.5)	Fetal liver (CS 17) Thymus (CS 22) Bone marrow (CS 23)
T cell Precusor/progenitor	 Thymus (3 dpf)	Fetal liver (12.5 dpc) Thymus (E 11.5)	Fetal liver (CS 17) Thymus (CS 22)
T cell	 Thymus (3 dpf)	Fetal liver (12.5 dpc) Thymus (E 11.5)	Fetal liver (CS 17) Thymus (CS 22)
B cell Precursor/progenitor	 Kidney marrow (4 dpf)	Fetal liver (12.5 dpc) Bone marrow (E 16.5)	Fetal liver (CS 17) Bone marrow (CS 23)
B cell	 Kidney marrow (4 dpf)	Fetal liver (12.5 dpc) Bone marrow (E 16.5)	Fetal liver (CS 17) Bone marrow (CS 23)
Plasma Cell		Fetal liver (12.5 dpc) Bone marrow (E 16.5)	Fetal liver (CS 17) Bone marrow (CS 23)

Note: The table provides the anatomical sites and developmental stages with respect to progenitors, precursors, and matured lineages in the model organism.
Abbreviations: dpf, postfertilization; hpf, postfertilization.

elucidated by deleting CXCL12 function from specific subset of cells (Greenbaum et al., 2013). Similarly, function of SCF in perivascular cells and endothelial cells is also crucial for HSC maintenance (Xu et al., 2018). Past few research publications have established that murine BM niche's cellular constituents interact with the HSCs, leading to their self-renewal or differentiation (Bruns et al., 2014; Zhang et al., 2003). The HSCs localization is often witnessed adjacent to the blood vessels (but not limited to), hinting that HSCs could be maintained in a perivascular niche by endothelial cells (Kiel et al., 2005; T. Sugiyama et al., 2006). A recent study about transcriptomic profiling of murine BM niche at a single cell resolution has precisely determined the understanding of HSCs' self-renewal and differentiation (Tikhonova et al., 2019). Studies on functional significance of E-selectin, a niche factor expressed in the murine BM, established its requirement in quiescence, proliferation, and self-renewal of HSCs (Winkler et al., 2012). The loss of vascular endothelial-expressed Notch ligand (Dll4) in BM narrowed down the HSPCs' differentiation toward myeloid commitment (Tikhonova et al., 2019). BM osteoblast cells interact with HSCs, and in contrast, mice deficient in bone morphogenetic protein (BMP) signaling show an increased number of osteoblast and HSCs (Calvi et al., 2003; Zhang et al., 2003). Adult BM provides sufficient nourishment through the niche predominantly from two cellular compartments, that is, the osteoblast and the vascular endothelial cells (Calvi et al., 2003; Ding et al., 2012; Kiel et al., 2005; Kunisaki et al., 2013; Méndez-Ferrer et al., 2010; T. Sugiyama et al., 2006; Zhang et al., 2003). The vascular cell factors critically important for HSC regulation and maintenance include Notch (Calvi et al., 2003), WNT (Reya et al., 2003), N-cadherin (Wilson, 2004), TPO (Arai et al., 2009), SCF (Ikuta & Weissman, 1992; Miller et al., 1996), angiopoietin-1 (Ang-1; Puri & Bernstein, 2003), and osteopontin (Nilsson et al., 2005). However, factors E-selectin, P-selectin, VCAM1, and ICAM1 are expressed by the BM endothelial cells (Kiel & Morrison, 2006). Together all these facts facilitate hematopoietic homeostasis, stating the dependence of HSCs and their function on the signals and signaling factors from the niche. We discuss below some of these important signaling pathways, with an essential role in hematopoiesis.

12 | NOTCH SIGNALING PATHWAY

Published literature unravels the role of Notch pathway in cell fate choices of hematopoietic stem cells. It also has an effect on progenitors at various stages of development, modulating self-renewal capacity and lineage fate determination during lymphopoiesis, and, to a lesser extent, myelopoiesis (Kojika & Griffin, 2001; MacDonald et al., 2001; Milner & Bigas, 1999; von Boehmer, 2001). Notch receptors are conserved across species and have multidomain proteins (Hori et al., 2013). Mammals possess four Notch transmembrane receptors, Notch 1–4, along with five different ligands of Notch, Delta-like 1, 3, 4, and Jagged (Jag) 1, 2 (Kumar et al., 2016). Notch 1 and Notch 2 are the only Notch receptors expressed in the hematopoietic progenitor cells (Bigas et al., 1998; Milner et al., 1994). Notch 1 has a role in the generation of embryonic hematopoietic stem cells, whereas Notch 2 helps in the development of marginal B cells (Kumano et al., 2003). *Notch 1* conditional knockout mice show deficiency in the development of T cells without affecting other hematopoietic lineages (Radtke et al., 1999). Therefore, Notch is potentially associated with hematological malignancies such as T cell

Lymphoblastic leukemia (Weng, 2004). The Notch signaling pathway plays a central role in generating blood cell diversity at both the level of embryonic development and adult tissue homeostasis (Bigas et al., 2010). Moreover, the Notch pathway is an important hematopoietic pathway in both *Drosophila* and zebrafish (Burns et al., 2005; Gering & Patient, 2005; Lebestky et al., 2003; Mandal et al., 2004). Researchers highlighted the importance of Notch in zebrafish by using Notch mutants that show defects in cell fate decisions during myelopoiesis at the definitive but not primitive stage of hematopoiesis (Bugeon et al., 2011). *Mind bomb* transcribes E3 ubiquitin ligase is involved in the activation of delta (DL) ligands and Jag ligands (Itoh et al., 2003; Yoon et al., 2008). In the ventral floor of dorsal aorta of zebrafish mutants, with *loss of function* of mind bomb, 36 hpf, the expression of HSC marker genes, *cymb* and *runx1*, is lost, leading to loss of HSC number (Burns et al., 2005). Upon overexpression of $Notch^{ICD}$ in these mutants, there was an increase in the populations of HSCs (Burns et al., 2005). In *Drosophila*, the transmembrane receptor, Notch 1, has two ligands DL and Serrate (Ser). In the larval stages, Notch ligand DL is required for the formation of mesodermal clusters constituting the cardiogenic mesoderm, nephrocytes (excretory), cardioblast (vascular), and blood progenitors are formed from the cardiogenic mesoderm. During hematopoiesis, Ser acts as the ligand for Notch for the crystal cell specification. *Loss of function* of Notch signaling leads to reduced crystal cell population (Duvic et al., 2002; Joanna Krzemien et al., 2010; Lebestky et al., 2003; Mandal et al., 2007a). In 2014, a published literature provided evidences for the role of Notch in restricting blood cell progenitors from differentiating into lamellocytes (Small et al., 2014). And in the same study, they showed that an increased $Notch^{ICD}$ expression is adequate for the development of the crystal cells. *Loss-of-function* mutants of *SETDB1* (H3K9 methyl transferase) exhibit defects in blood cell proliferation, leading to the formation of melanotic blood tumors. These mutants show a reduced expression of $Notch^{ICD}$ in the anterior lobes of LG along with the reduction in the crystal cell population (Paddibhatla et al., 2019). Furthermore, studies showed that restricting Notch activity in hematopoietic cells by *Rabex-5* led to (mammalian homologue RABGEF1) hematopoietic homeostasis (Reimels & Pfleger, 2015). From the above information, it is evident that Notch signaling and its components (e.g., Delta/Ser/Jaggered ligands and Notch receptor) are conserved across the species and specifically in all model organisms discussed above. The Notch pathway was initially developed during metazoan evolution and first identified in fruit flies. It has functional significance in HSC maintenance, blood cell lineage specification, and differentiation in both vertebrates and invertebrates. Similar to Notch signaling, Wnt and Hedgehog pathways also play a central role in HSC maintenance and T cell lineage differentiation (Luis et al., 2012).

13 | WNT/β-CATENIN SIGNALING PATHWAY

Various model organisms contributed to the knowledge regarding the significance of the Wnt pathway in hematopoiesis. In mammals, the Wnt pathway essentially contributes to the maintenance, specification, and expansion of hematopoietic stem cells (Lento et al., 2013; Malhotra & Kincade, 2009). *Wingless (wg) and Integrated* together contribute to the name of *Wnt* gene (Baker, 1987). The ligands Wnt3a and Wnt5a modulate canonical and noncanonical pathways, respectively (Grumolato et al., 2010). Wnt signaling assists in HSC self-renewal and repopulates the blood cells apart from recovery of BM (Congdon et al., 2008). Wnt/β-catenin signaling regulates the fine tuning of the hematopoiesis, and its deregulation leads to the development of hematological malignancies, as shown in cancer cells of human and mouse (Kirstetter et al., 2006; Scheller et al., 2006; Undi et al., 2016). Canonical Wnt3a triggered signaling has no role for the thymocyte development; in contrast, Wnt5a ligand overexpression triggered noncanonical signaling, inducing apoptosis in developing thymocytes (Famili et al., 2015). Previous studies suggest that Wnt ligands such as Wnt5a and Wnt10b expressed in the FL of mice upon stimulation expand the fetal hematopoietic cell, manifesting the need for Wnt signaling in FL (Austin et al., 1997). The Wg/Wnt signaling pathway is pivotal for the mammalian hematopoiesis and its deregulation causes leukemogenesis (Reya & Clevers, 2005). Wnt ligands and frizzled receptors are expressed in the murine YS, AGM, and FL, supporting a role for Wnt signaling in developmental hematopoiesis (Corrigan et al., 2009). The loss of Wnt3a or β-catenin in mammalian HSCs significantly impairs their self-renewal capacity, whereas activation of the pathway reverses this phenotype (Fleming et al., 2008; Kirstetter et al., 2006; Reya et al., 2003). In the somites of prehematopoietic mesoderm and dorsal aorta of zebrafish, the early embryonic expression of the noncanonical ligand, Wnt16, is required to initiate definitive hematopoiesis (Clements et al., 2011). Wnt16 specifies HSCs in a β-catenin-independent and in a non-cell autonomous manner in the somites (Clements et al., 2011). Due to the loss of *R-spondin1* (an activator of the Wnt pathway), Wnt16 expression is decreased along with a subsequent decrease in the HSC specification (Genthe & Clements, 2017). The loss of Wnt9a also exhibits a similar phenotype to loss of Wnt16, reiterating the need for Wnt signaling in the formation of HSCs. *Drosophila* Wg expression is not detected in the embryonic stages, but it starts to appear in the first instar larval stage of the hematopoietic organ. Wg expression is predominantly found in the immature blood cells of the LG. The early signs of the CZ formation are clearly delineated by the loss of Wg expression in these cells. Also, like Wg signaling, the JAK/STAT pathway has a noteworthy role in the maintenance of prohemocytes within the MZ of *Drosophila* larval LG (Krzemien et al., 2007; Mandal et al., 2007a). It is noteworthy that similar to Notch, Wnt signaling also has a critical role in the maintenance of the HSCs. Additionally, in humans, aberrations in this pathway manifest as hematological malignancies. As the components of the Wnt pathway are conserved in mammals, zebrafish and *Drosophila* scientific investigations pertaining to their in-depth role in hematopoiesis can be easily and clearly elucidated using the research models discussed in this review.

14 | JAK/STAT SIGNALING PATHWAY

The JAK/STAT pathway acts downstream of a multitude of cytokines and is one of the principal inflammatory and hematopoietic pathways.

Mammals express four JAK family kinases belonging to tyrosine class of family (Jak1, Jak2, Jak3, Tyk2; Ghoreschi et al., 2009). There are seven different STAT transcription factors in humans: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6 (Levy & Darnell, 2002; Wu et al., 2016). JAK kinases are in association with the cytokine receptors (I and II) that include the receptors erythropoietin, interleukin, and interferon. The functional significance of the JAK/STAT pathway was identified upon studying *JAK2V617F* mutants. The *JAK2V617F* mutation is found to be 95% in polycythemia vera patients and 50% of patients suffering from essential thrombocythemia and primary myelofibrosis (Staerk & Constantinescu, 2012; Viny & Levine, 2014). Due to this mutation, HCSs are skewed toward erythroid lineage. Apart from HSCs and erythroid lineage, the JAK/STAT pathway has a hold on the development of T helper subsets. Also, STAT5 is crucial for HSCs' self-renewal in mice and humans. The levels of STAT5 expression modulate the HSC properties, renewal versus differentiation. Recent studies using zebrafish embryos established that JAK2a might have an important role at the level of early hematopoietic progenitor/stem cells before they differentiate into distinct erythroid and myeloid lineages (Zhu et al., 2017). Similar to *JAK2 V617F* mutants, zebrafish *JAK2AV581F* mutants also show polycythemia vera phenotype (Ma et al., 2009). Furthermore, in these zebrafish mutants, the expression of erythropoietin is remarkably decreased, whereas the expression of *gata1* and both the embryonic *α-hemoglobins* and *β-hemoglobins* is enhanced. Therefore, *JAK2AV581F* mutants showed a substantial increase in erythropoiesis (Ma et al., 2009). In *Drosophila*, gain-of-function mutation, tumorous lethal, referred as *hop[tum-l],* leads to constitutive activation of the JAK/STAT pathway. Tumorous lethal is a dominant mutation in the locus of hopscotch gene. This mutation leads to differentiation of immature precursor blood cells into lamellocytes. These mutants carry observable melanotic bodies in both the larvae and the adults (Amoyel et al., 2014; Hanratty & Dearolf, 1993; Hanratty & Ryerse, 1981; Lanot et al., 2001; Minakhina & Steward, 2006). The JAK/STAT pathway, activated by cytokines, is crucial for the development of erythroid and lymphoid lineages, and is thus critical for immune responses. Its role in maintaining immune homeostasis is found to be similar in the model organisms. Interleukin (IL) 12 and IFN-γ are evolutionarily conserved in mammals that trigger the JAK/STAT pathway. Whereas mammalian homologs of JAK exist in all the four model organisms discussed above, homologs of mammalian STAT transcription factors are also found in *Dictyostelium*. Due to such similarities existing among model organisms and presence of extensive research pertaining to the importance of the JAK/STAT pathway in blood cell development, the underlying mechanisms contributing to the hematopoietic disorders and therapeutic studies using these model organisms can be highly beneficial.

15 | TRANSFORMING GROWTH FACTOR BETA (TGF-β) SIGNALING PATHWAY

Mammals include three isoforms of TGF-β ligands (TGF-β1, TGF-β2, and TGF-β3) with two types of ubiquitously expressed TGF-β cell surface receptors (Type 1 TGF-βRI and Type 2 TGF-βRII). However, Type 3 coreceptor TGF-β RIII and endoglin also exist binding to all isoforms of TGF-β, but with the least expression in hematopoietic cells (Bühring et al., 1991; Chen et al., 2002). Most of the HSCs remain in a quiescent cell cycle state to maintain the hematopoietic homeostasis. TGF-β is considered one of the potent inhibitor for the quiescence of HSCs, leading to their early maturation in vitro (Batard et al., 2000; Fortunel et al., 1998, 2000; Garbe et al., 1997; Hatzfeld, 2004; Sitnicka et al., 1996; Soma et al., 1996). TGF-β induces cell cycle arrest in human hematopoietic cells by an upregulation of the cyclin-dependent kinase inhibitor, p57KIP2 (Scandura et al., 2004). Recent publications have established that TGF-β interacts with RUNX1 transcription factor regulating the Ca2+ channels, thereby maintaining the activity of myeloid cell, megakaryocyte, during development in a cell line-based model (Raghuwanshi et al., 2020). Conditional deletion of TGF-β-associated kinase 1 (TAK1) in mice results in hematopoietic failure; however, the connection of TAK1 to TGF-β signaling is yet to be established (Tang et al., 2008). Furthermore, this pathway stimulates the proliferation of myeloid HSCs but inhibits the growth of lymphoid HSCs (Challen et al., 2010). Most of the adult HSCs in mouse stay in a quiescent state for their limited self-renewal potential, which prevents them from exhaustion (Wang & Ema, 2016). TGF-β cytokine regulates this self-renewal potency in the BM niche by slowing down the cell cycle progression. This is achieved via Smad/FoxO signaling regulated in the maintenance of HSCs' pool (Miyamoto et al., 2007; Sitnicka et al., 1996; Tothova et al., 2007; Wahlstrom et al., 2007; Yamazaki et al., 2011). Mouse fed on high-fat diet show altercation in the TGF-β receptors present within the lipid rafts, disrupting the TGF-β signaling. Eventually, this leads to the loss of half of the primitive HSCs in the BM (Hermetet et al., 2019). TβRI knockout mice are embryonically lethal with severe deformities in yolk sac and placental development. However, the mice are involved in continuous development of functional hematopoietic progenitors (Larsson et al., 2001). In contrast, the TβRI conditional knockout in adult mice seldom requires TGF-β signaling for hematopoiesis. The Smad knockout mice are embryonically lethal beyond 7.5 days due to hindered proliferation of ectoderm eventually unable to develop mesoderm (Sirard et al., 1998; Wei et al., 2013; Yang et al., 1998). TGF-β signaling also plays an important role in zebrafish hematopoiesis. Znf45l (zinc finger protein in zebrafish) is expressed at both maternal and zygotic levels throughout early development. Knockdown of *Znf45l* embryos tend to have a reduced expression of hematopoietic maker genes such as *scl*, *lmo2*, *and gata2* (Chen et al., 2014). In *Drosophila*, TGF-β signaling controls the hematopoietic niche size present in the LGs (Pennetier et al., 2012). The signals from the hematopoietic niche help in the sustenance of an equilibrium of the prohemocyte and matured blood cells number. BMP signaling is triggered by three ligands belonging to the TGF-β family. They include decapentaplegic (Dpp), glass bottom boat (GBB), and screw that communicate with the Type I receptors (thick veins and saxophone) and Type II receptors (wishful thinking and punt). BMP signaling in *Drosophila* functions through the collier/early B-cell factor, transcription factor, in the PSC (Pennetier et al., 2012). Therefore, LGs lacking collier activity affects the PSC cell numbers. Deregulation of TGF- β/BMP signaling increases the cell numbers in the niche; as a consequence, hematopoietic progenitor fails to differentiate. Therefore, research pertaining to TGF-β's contribution toward blood cell development using different model organisms enhanced our understanding (Pennetier et al., 2012). Together

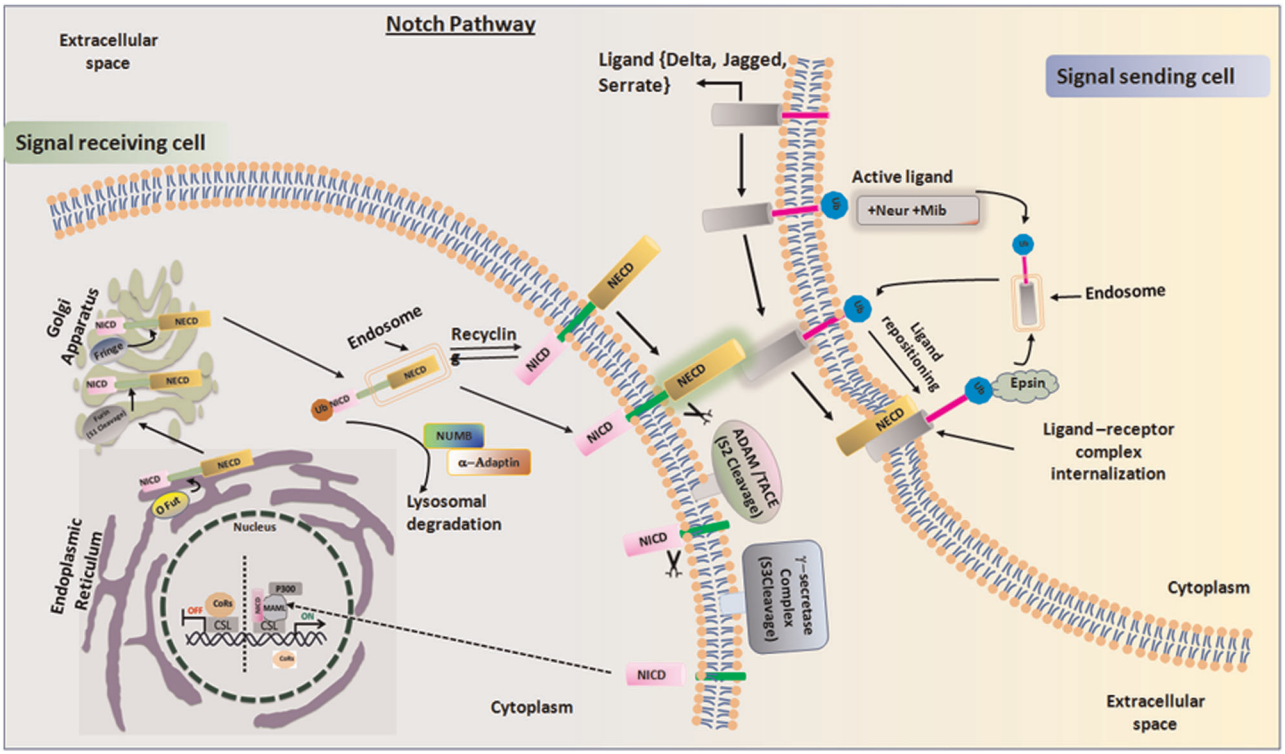

FIGURE 5 Notch Signaling pathway.Notch ligands (Delta, Serrate, Jagged) signal the notch receptor inducing the downstream signaling components. This is achieved when the activated transmembrane ligand from the signal-sending cell interacts with its receptor (Notch) present on the cell membrane of signal-receiving cell. The notch receptor undergoes proteolytic cleavage, leading to separation of NICD and NECD domain. Activation of downstream components allows the nuclear translocation of NICD, which helps in the transcription of its target genes

these findings from different model organisms brought to focus the need for proper TGF-β regulation in blood cell development. Therefore, identical to other pathways, the TGF-β pathway exhibits a wide spectrum of functions and is primarily essential for HSCs niche and blood cells' maintenance. Additionally, this pathway regulates the number and size of the HSCs, besides being critical for differentiation into mature blood cells. Although homologs of the pathway components exist across species, their regulation in hematopoietic disorders is yet to be clearly understood.

16 | NUCLEAR FACTOR KAPPA-LIGHT-CHAIN ENHANCER OF ACTIVATED B CELLS (NF-κB) SIGNALING PATHWAY

The NF-κB pathway is one of the most widely studied signaling pathways in different model organisms. It has various functions that include development of the embryo, formation of cell lineage, apoptosis, inflammation, and oncogenesis along with immune regulation. Mammals include five members of NF-κB family: p65/RelA, RelB, RelC/c-Rel, NF-κB1 (p105, p50), and NF-κB2 (p100, p52; Pahl, 1999). Past four decades of intense and extensive research on the NF-κB pathway provided substantial information regarding its regulation for the specification of blood lineages. This pathway is imperative for the differentiation of blood lineages, that is, myeloid, lymphoid, and erythroid. Deletion of one of the NF-κB subunit (p65/Rel) in the hematopoietic compartment leads to the defects in the functionality of HSCs (Gerondakis et al., 1999; Stein & Baldwin, 2013). Mutants of the NF-κB pathway components *such as* $Relb^{-/-}$ and $I\kappa B\alpha^{-/-}$ mice show hematopoietic abnormalities that affect the myeloid and lymphoid lineages (Burkly et al., 1995). It is two decades since the role of NF-κB was elucidated in restraining the granulocytes from undergoing apoptosis. Both the canonical and the noncanonical NF-κB signaling play a role in the hematopoietic homeostasis (Boyce et al., 2010; Sankar Ghosh & Hayden, 2008; Vallabhapurapu & Karin, 2009). Recently, a scientific group demonstrated that NIK mutant mice showed the regulation of HSPCs through a noncanonical signaling (González-Murillo et al., 2015). NIK phosphorylates IKK-α, which is subsequently degraded by TRAF3, preventing excessive noncanonical NF-κB activation in normal cells (Ling et al., 1998). NIK activation via noncanonical NF-κB signaling instigates HSPCs' self-renewal and BM failure. This alters the expression of the genes involved in various signaling pathways required for hematopoiesis. These genes, *Ecm*, *crispld1*, *CD34*, *Myct1*, *Mpl*, and *Hlf*, contributed to the increased hematopoietic maturation and differentiation along with decrease in stemness and self-renewal properties (Xiu et al., 2017). Utilizing the in vivo visualization technique in transparent zebrafish embryos, it was demonstrated that NF-κB pathway activation is mandatory for HSC specification (Espín-Palazón et al., 2014). Transgenic studies using embryos with the *UAS-dn:iknbaa* tag driven by the promoter hsp70-Gal4 showed impairment of the function

of NF-κB. Deregulation of NF-κB in these embryos 20 hpf down-regulated the NF-κB responsive genes, causing a decrease in the HSC numbers by 40 hpf. Furthermore, another study highlighted the requirement of NF-κB in hematopoiesis using Ing-4 expression, an epigenetic regulator present in the peripheral progenitor cells (CD34+; Thompson et al., 2019). Ing-4 negatively regulates NF-κB signaling in zebrafish. Embryos lacking Ing-4 at 36hpf highly expressed NF-κB readouts such as IL-19, IL1b, and IL-20R (Thompson et al., 2018). Ing-4 was also shown to have a role in the hematopoiesis of both mouse and humans. Past few decades of research provided sufficient evidences for the evolutionarily conserved Toll/NF-κB pathway in regulating *Drosophila* larval hematopoiesis. This signaling is important for proliferation of blood cells and their differentiation into plasmatocytes lineage in larvae (Letourneau et al., 2016). *Drosophila* Dorsal, Dif, and Relish are the homologous proteins of mammalian Rel (Rutschmann et al., 2000). Their overexpression leads to the increased blood cell numbers in the circulating hemolymph. In contrast, double mutants of Dorsal and Dif exhibit a drastic reduction in the number of hemocytes (Matova & Anderson, 2006). Due to constitutively active Toll/NF-κB pathway, melanotic blood tumors are formed along with blood cells infiltrating the fat body in the larvae (Lemaitre et al., 1995). It has been shown that cactus (mammalian homologue of IκB), negative regulator of the Toll/NF-κB pathway, is critically essential for the larval hematopoiesis and works synchronously with Toll, Tube, and Pelle for proper proliferation of blood cells and hematopoietic homeostasis (Qiu et al., 1998). To conclude, the NF-κB pathway is pivotal in the development of all three blood lineages (myeloid, erythroid, and lymphoid), HSC quiescence, and B- and T-cell homeostasis. Aberrations in the pathway fundamentally affects the immune responses in organisms. The mechanisms involved in hematopoietic quiescence and lineage differentiation involving the NF-κB pathway are conserved among animal species along with their pathway components. Due to its regulatory hold on the expression of many of its target genes coding for transcription factors involved in the HSC maintenance (Nakagawa et al., 2018), it is, therefore, not surprising that NF-κB serves as a therapeutic target. Taken together, the scientific knowledge obtained from studying NF-κB from different model organisms strongly fortify central role in regulating hematopoietic homeostasis and immune defense (Figures 5, 7,8,9,10,11).

17 | PI3K/AKT SIGNALING PATHWAY

The PI3K/AKT Pathway has diverse functions in the development of an organism. It has a fundamental role in assisting cell survival, cell proliferation, metabolism, inflammation, and angiogenesis (Engelman et al., 2006; Jiang & Liu, 2009). The PI3K family consists of three classes of PI3K enzymes (Classes I, II, and III; Jean & Kiger, 2014). PI3K class I enzymes are specifically known to have a regulatory role in

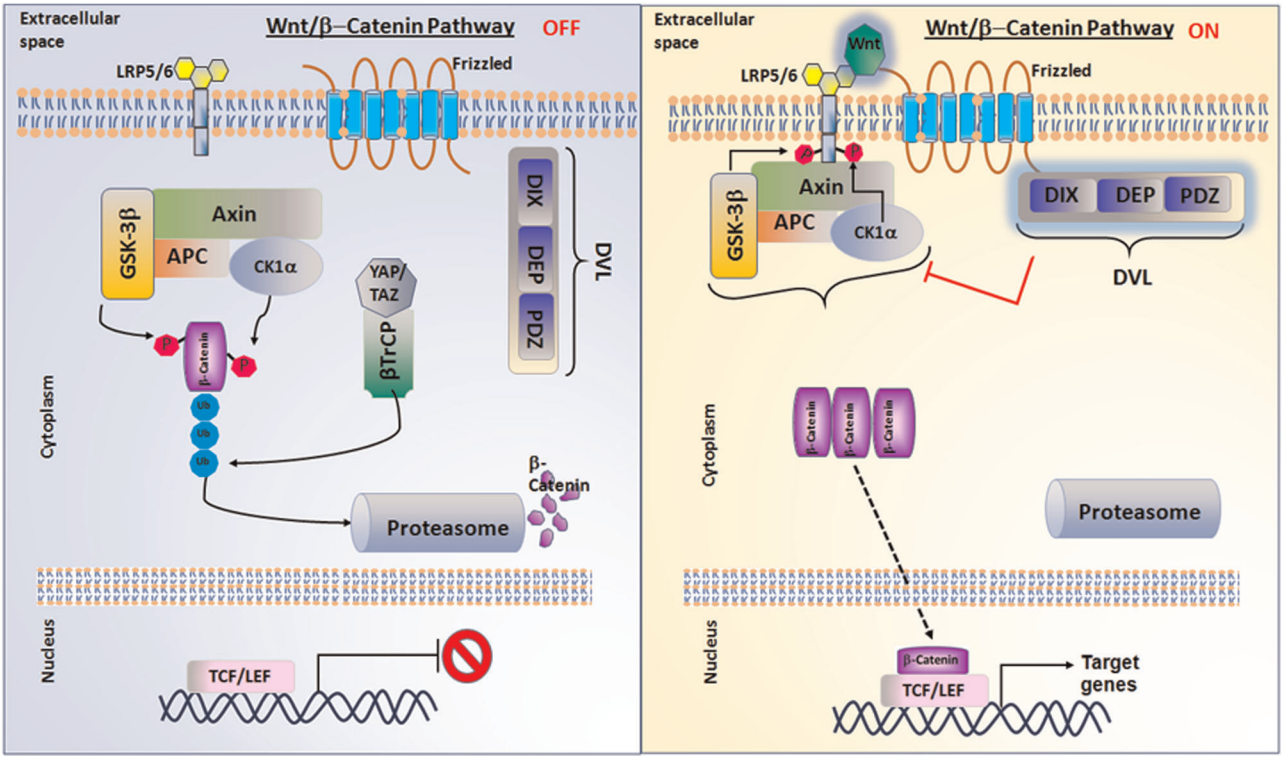

FIGURE 6 Wnt/β-Catenin Signaling Pathway. (a) OFF state: The ligand, Wnt, does not interact with the receptors frizzled and LRP5/6. The inactive disheveled proteins are found in the cytoplasm. The GSK-3β and CK1α of the death complex (GSK-3β, CK1α, APC, Axin) freely phosphorylate the b-catenin, leading to its proteasomal degradation. (b) ON state: Wnt ligand interacts with the receptors frizzled and LRP5/6. Activated disheveled proteins interact with the cytoplasmic domain of the frizzled receptor and inhibit the death complex. As a result, b-catenin is free to enter the nucleus for transcribing its target genes

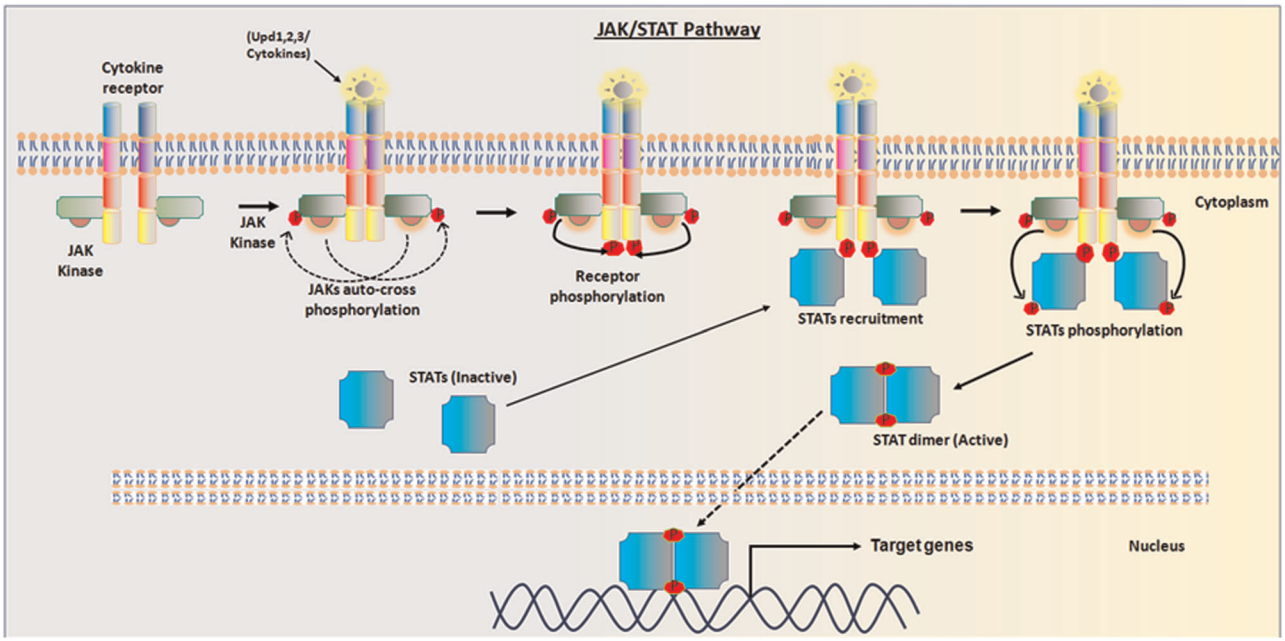

FIGURE 7 JAK/STAT Signaling Pathway. The ligand (Upd,1,2,3/cytokines) binds to its receptors, and their interaction leads to conformational change in the receptor. Kinases (JAK1,2,TYK-2) are activated for cross-phosphorylating themselves and phosphorylating the receptor. Next, STAT transcription factors are recruited to docking site of JAK receptor for phosphorylation. Finally, the phosphorylated STATs are dimerized and translocated to the nucleus targeting the gene expression

hematopoiesis. PI3K/AKT signaling is comprised of various groups of receptors, such as receptor tyrosine kinases, GPCRs, and oncogenes (Ras) effectively activating PI3K (Liu et al., 2009). PI3K converts phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3-intracellular secondary messenger), activating pyruvate dehydrogenase kinase 1 (PDK1) and AKT (PKB) that signal the downstream effectors (Figure 6). PI3K pathway inhibition functionally impairs hematopoiesis (Ghosh & Kapur, 2016). HSCs and other lineage progenitor's functional activities such as differentiation, self-renewal, and survival are controlled by hematopoietic growth factors, like erythropoietin, SCF, FLT3 ligand, fibroblast growth factor, c-kit, and TPO (Caselli et al., 2013; Kumar & Geiger, 2017; Shin et al., 2014). The PI3K pathway is one of the frequently upregulated signaling by the growth factors. Deficiency of p85α subunit (Class IA) of PI3K complex in FL HSCs results in reduced long-term engraftment and differentiation of HSCs in mice (Haneline et al., 2006). Also, inactivation of p85α subunit in mice embryos results in the alteration of fetal erythropoiesis and decrease in the population of FL erythroid progenitors. HSCs in mice deficient in both p85α and p85β reside in the G_0 phase of cell cycle, implying the functional defects in long-term maintenance of hematopoiesis. PI3K and PTEN (Phosphatase and TENsin) are both tightly linked to AKT signaling, critically controlling proliferation, survival, and migration in hematopoietic cells. PTEN is a potential candidate whose inactivation is the significant cause of constitutive activation of PI3K/AKT signaling, leading to the overproliferation of hematopoietic cells (Salmena et al., 2008). This tumor suppressor gene is identified to have a prominent role in blood cell malignancies, specifically in T lineage acute lymphoblastic leukemia (T-ALL). Published literature provides evidences for a role of PTEN in regulating PI3K/AKT signaling. PI3K/PTEN signaling also regulates the megakaryopoiesis under the influence of PEAR-1 expression in vitro and in zebrafish embryos (Kauskot et al., 2013). Therefore, PTEN is a negative regulator of the PI3K/AKT pathway. PTEN deficiency is embryonically lethal not only in mice but also in *Drosophila* (Guigon et al., 2009). In zebrafish, the HSPCs translocate to the CHT, anatomical site, where they populate and differentiate into hematopoietic lineages; concurrently, HSPCs also mobilize to whole kidney marrow to give rise to adult hematopoiesis. There exist two PTEN genes (*ptena* and *ptenb*) in zebrafish (Faucherre et al., 2008). Studies using double mutants of *PTEN* ($ptena^{-/-}ptenb^{-/-}$), 5–6 dpf, showed both hyperplasia and dysplasia, supporting the role of PTEN in HSPC regulation in zebrafish (Faucherre et al., 2008). In *Drosophila*, insulin signaling recruits the PI3K pathway to mediate the effects of insulin (Niswender et al., 2003). Recently, the role of insulin signaling has been identified in the larval hematopoietic organ. Overactive insulin signaling in the hematopoietic niche affects niche size with an increase in the number of cells (Kaur et al., 2019). Fundamentally, PI3K/AKT signaling is imperative for cellular growth and contributes toward cell survival and division, and at the same time inhibits apoptosis during hematopoietic development. PI3K/AKT is shown to operate similarly in mammals by regulating the hematopoietic events. Although there is extensive research on the PI3K pathway in hematopoiesis, we can further benefit from studying this pathway in hematological disorders. Knowledge gained by using model organisms with hematopoietic disorders can be advantageous for therapeutic development targeting the conserved homologs of this pathway.

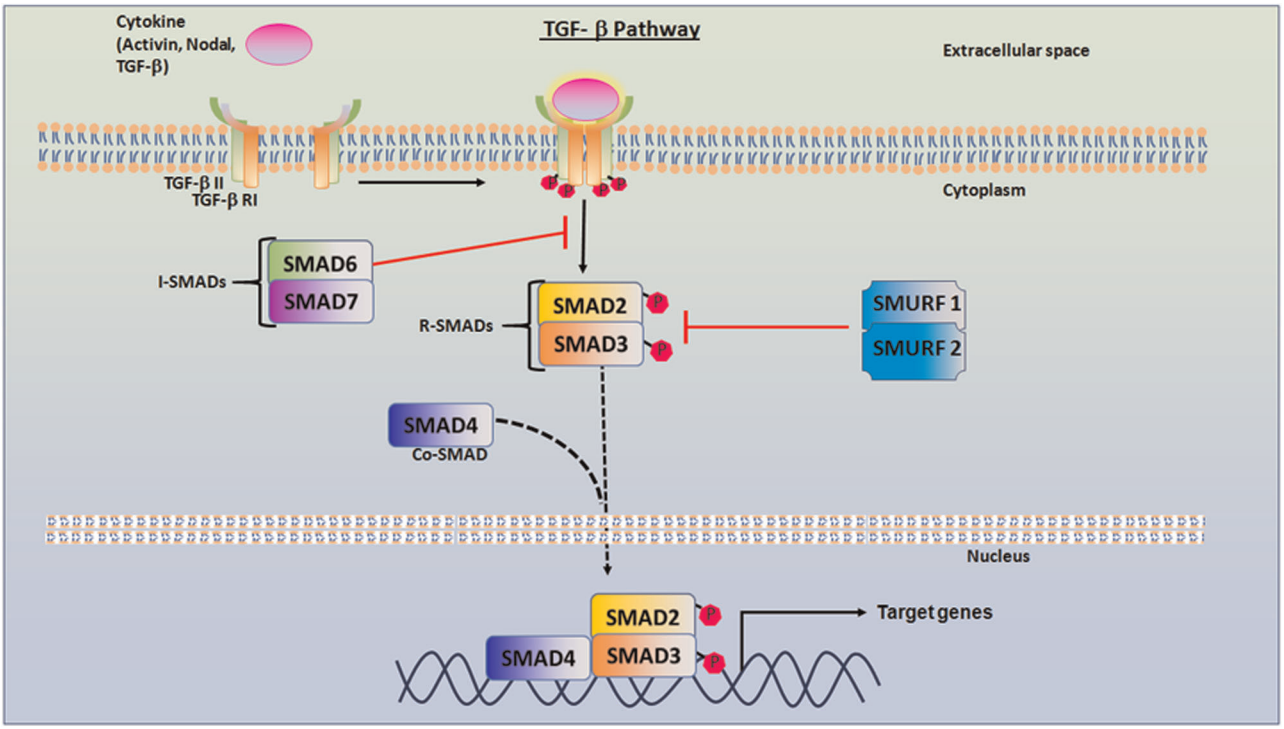

FIGURE 8 TGF-β Signaling Pathway. TGF-β ligands (Activin, Nodal, TGF-β) bind to the hetero-TGF-β receptors RII and RI. Activated receptors, the RII and RI, dimerize, leading to their phosphorylation. Phosphorylated receptors recruit SMAD2/3 (Regulatory SMADs) and phosphorylate them. SMAD2/3 and SMAD4 translocate to the nucleus for target gene expression. TGF-β, transforming growth factor beta

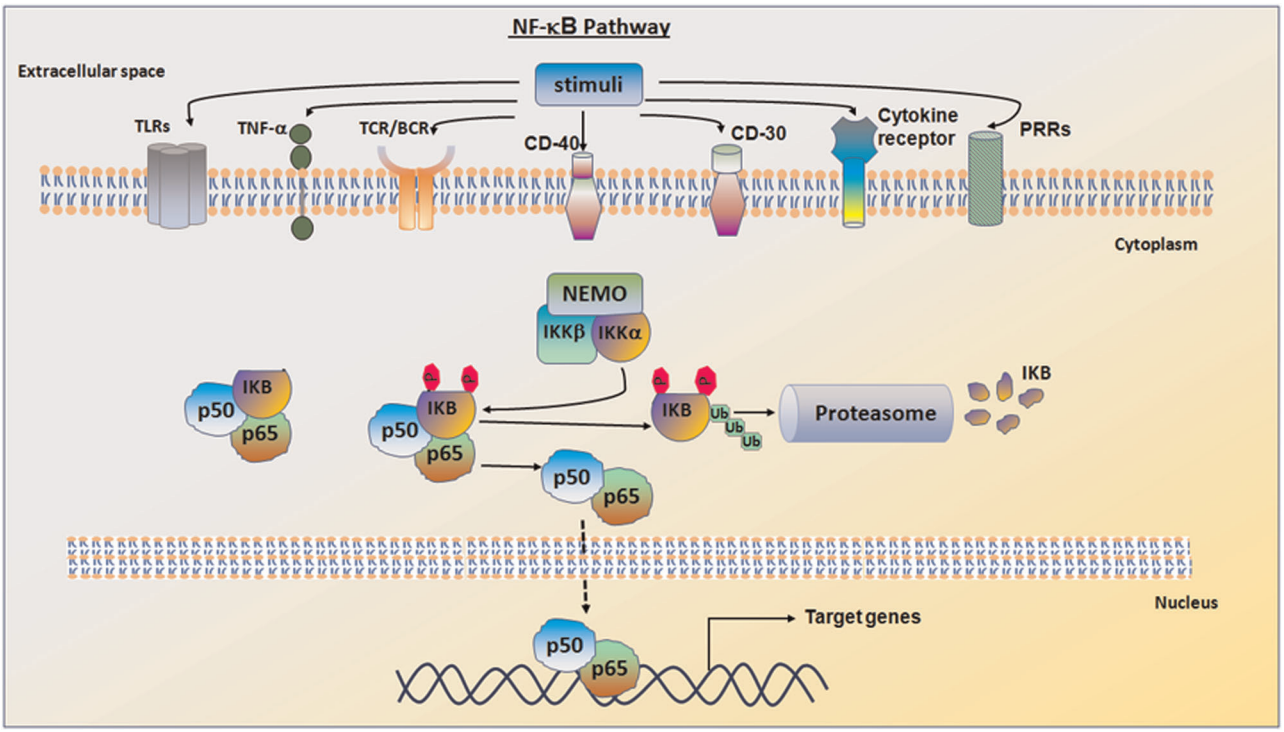

FIGURE 9 NF-κB Signaling Pathway. The transmembrane receptor (TLRs, TNF-α, TCR/BCR, CD40, CD30, Cytokine receptors PRRs) through an external stimulus (ligand binding to receptor) mediates activation of NF-κB. In the cytosol, NF-κB heterodimer (P50/p65) is found in complex with IKB-α in the inactivated state. Upon activation, NF-κB dissociates from the complex, whereas IKB-α is phosphorylated by the IKK-α for proteasomal degradation. Activated NF-κB is enabled for nuclear translocation, resulting in target gene expression.
NF-κB, nuclear factor kappa-light-chain enhancer of activated B cells; TNF-α, tumor necrosis factor alpha

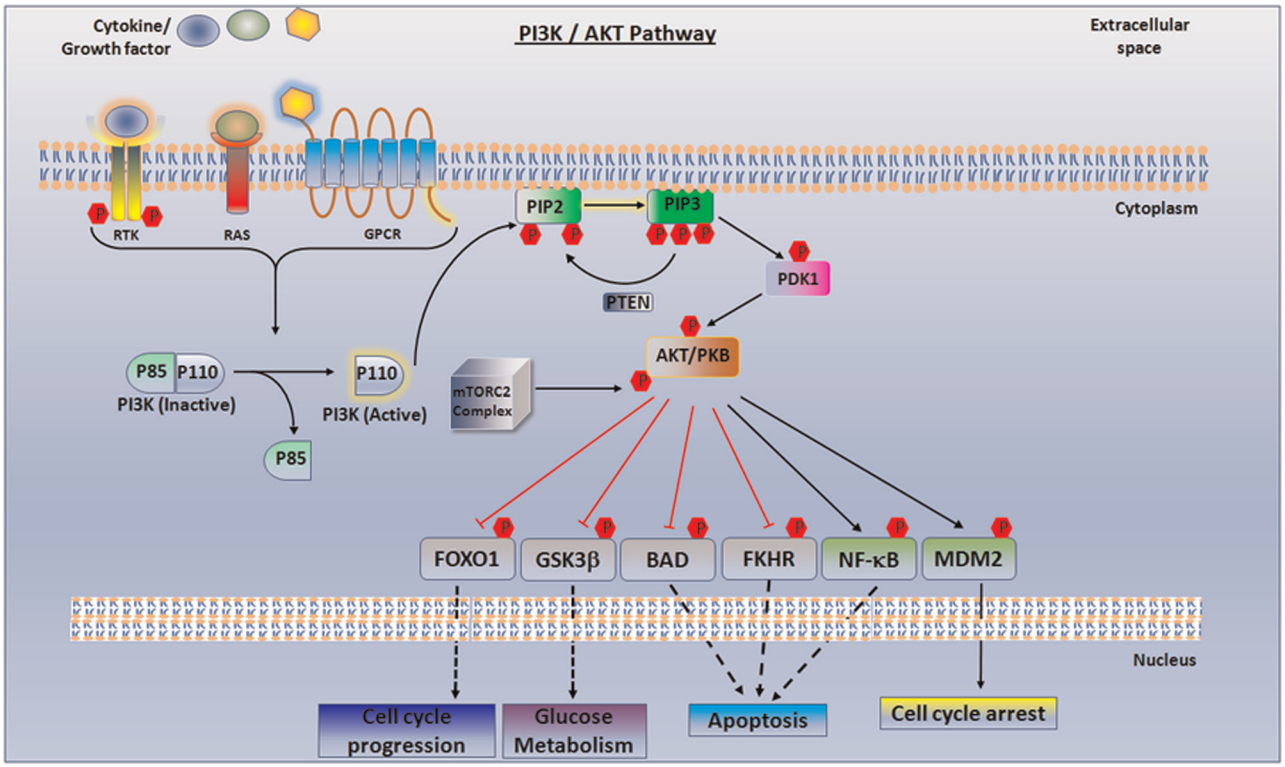

FIGURE 10 PI3K/AKT signaling pathway. Upon the ligand (cytokines, growth factors) binding to any of its receptor, that is, RTK, GPCR, and Ras, the phosphatidylinositol 3-kinase (PI3K) is activated. The activated PI3K phosphorylates PIP2 into PIP3, and phosphorylated PIP3 serves as a second messenger. PIP3 subsequently phosphorylates PDK1 that phosphorylates AKT/PKB. Phosphorylated AKT/PKB targets many cytoplasmic components via phosphorylation that either inhibits or activates the transcription factors, leading to their nuclear localization and target gene expression

18 | RAS SIGNALING PATHWAY

Ras is considered as the core protein in signal transduction pathways, controlling cellular processes such cell proliferation, cell migration, and cell survival. Ras proteins are known to control other signaling pathways that are key regulators of several aspects of normal cell growth and malignant transformation (Bos, 1989). Its role in hematopoiesis is widely studied using different model organisms. The balance between the self-renewal and differentiation of HSCs is regulated by the Ras pathway. There are three isoforms of Ras, namely H-ras, K-ras, and N-ras, of which K-ras isoform is predominantly activated in the cells of BM (Barbacid, 1987; Damnernsawad et al., 2016). Alternative splicing of K-ras results in two isoforms (*K-ras4a* and *K-ras4b*; Schubbert et al., 2007). In contrast to H-ras and N-ras, K-ras knockout mice are lethal beyond Stages E12–E14 due to defect in the FL microenvironment (Esteban et al., 2001; Liu et al., 2008b). *Kras4a*$^{-/-}$ mice are viable but dispensable for hematopoiesis, whereas *Kras4*$^{-/-}$ mice are embryonically lethal at mid gestation period, suggesting a pivotal role of K-ras4b specifically in embryonic hematopoiesis of mice (L.Johnson et al., 1997; Koera et al., 1997). Moreover, *N-Ras*$^{-/-}$ and *H-ras*$^{-/-}$ mice do not display hematopoietic defects (Esteban et al., 2001). However, K-ras conditional knockout mice induce hematopoietic abnormalities including enlarged spleen, extended neutrophilic niche, and reduced B cell population in 9–12-month-adult mice. Furthermore, K-ras deficiency leads to the reduction in LT-HSCs and IT-HSCs compartment, compromising HSCs' self-renewal property, whereas a significant expansion of the ST-HSCs and the MPPs (multi potent progenitors) is observed (Damnernsawad et al., 2016). These knockout studies account specifically the importance of K-ras in the normal hematopoiesis of adult mice. Knockdown of K-ras in zebrafish results in the abnormal functioning of hematopoiesis and angiogenesis. This finding created an understanding that PI3K could be a downstream mediator for K-ras signaling (Liu et al., 2008a, 2008b). Transgenic embryos with activated N-ras isoform show the normal expression of primitive hematopoietic markers (*gata1*, *lmo2*, *pu.1)* but lack the expression of definitive hematopoietic markers *(runx1 and cymb)*. Alghisi and colleagues used Gal4-UAS (upstream activated sequence) binary system to overexpress Hras^{V12G} specifically in endothelial cells. This resulted in hyperproliferation of hematopoietic cells in the CHT of zebrafish (Alghisi et al., 2013). *Drosophila* ras1 is an ortholog of H-ras, K-ras, and N-ras found in mammals. *Ras1* is the Ras oncogene at 85D, whereas *ras2* is the ras oncogene at 64B (*Drosophila* counterpart of mammalian R-ras; Brock, 1987). The function *of Drosophila* ras1 (ras) was delineated in larval hematopoiesis three decades ago. As a consequence of overexpression of activated ras (Ras^{v12}), a tremendous increase in the hemocyte blood cell population is observed in the hemolymph of wandering 3rd instar larvae. In *Drosophila*, ras exerts its effect through the RAF/MAPK pathway. The

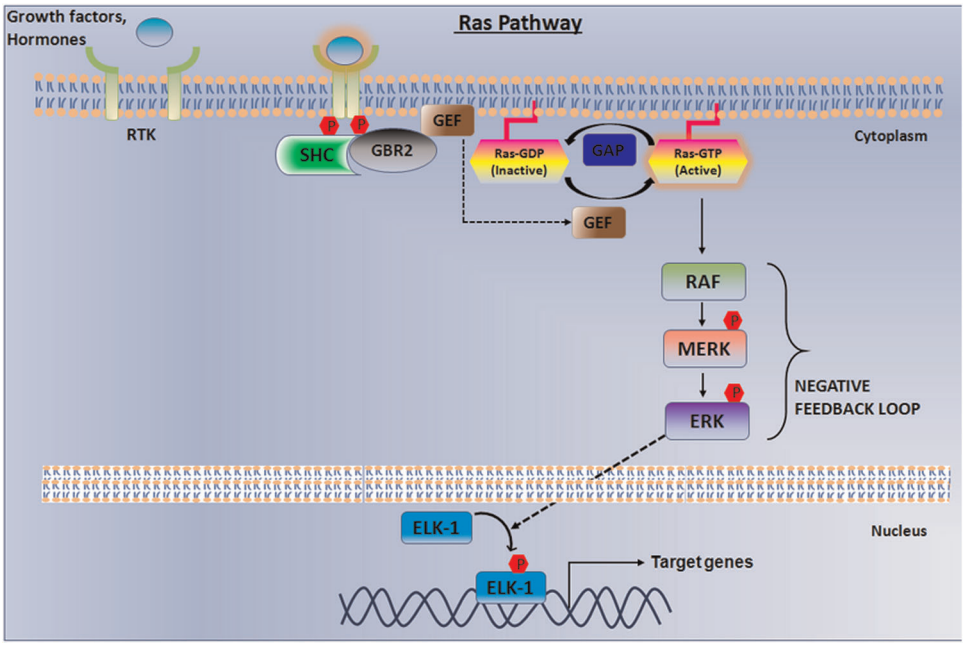

FIGURE 11 Ras Signaling pathway. Binding of the ligand (growth factors, hormones) stimulates the autophosphorylation of the receptor tyrosine kinase (RTK). The phosphorylated receptor recruits an adaptor molecule called GRB2, which, in turn, associates with the guanine nucleotide exchange factor (GEF). GEF activates Ras by exchanging GDP-bound Ras (Ras-GDP) to GTP (Ras-GTP). Activated Ras-GTP recruits and phosphorylates Raf protein. Raf, in turn, initiates the cascade of protein phosphorylation, which eventually leads to its nuclear localization and target gene expression

overexpression of *D-Raf*^{gof} led to a massive increase in the blood cell number (Asha et al., 2003). Furthermore, another study highlighted the requirement of Ras in the hematopoietic organ by studying the overexpression of *ras*^{DN} (dominant negative ras allele). The overexpression of *ras*^{DN} resulted in the reduction of differentiated blood cells, that is, plasmatocytes and crystal cells. Opposing effects were observed upon overexpression of rolled *Drosophila* MAPK with an expansion of prohemocyte population and presence of terminally differentiated plasmatocytes and crystal cells in the anterior lobes of the LG (Dragojlovic-Munther & Martinez-Agosto, 2013). Using all the four discussed model organisms, similar effects were observed when the Ras pathway was upregulated in hematopoietic cells, establishing the need for proper regulation in normal cells. Oncogenic RAS mutations are found in approximately 25% of human cancers showing high frequency in hematopoietic malignancies. (Ward et al., 2012). Therefore, similar to fruit flies, deregulation of the RAS pathway is observed in mammals. It is apparent from the above-discussed literature that the Ras pathway is augmented in various hematopoietic malignancies. Therefore, the scientific input on Ras signaling obtained from the overall studies using various model organisms has highlighted its role in hematopoiesis.

19 | CONCLUSION

Prevailing literature using model organisms has largely imparted knowledge regarding the process and outcome of hematopoiesis. Given the caveats of model organisms (discussed in this review), the knowledge infused from studies using them in hematopoiesis is surprising. Technological advancements are constantly adapted to study blood cell development in model organisms. Information obtained from all these model organisms together, that is, from *Drosophila*, *Danio rerio*, and *Mus musculus*, allowed modeling of hematopoiesis explicitly due to their comparative blood cell biology to humans. However, a better understanding of blood cell genome in these model organisms would make them compelling model systems to bring to focus the perturbations associated with hematopoietic diseases. Although various similarities exist between model organisms for the development of blood cells, significant differences also prevail. Our schematics provide a comparative study of the blood cell development and associated signaling pathways in different model organisms reminiscent of human blood cell development. We discussed in detail how these model organisms share conserved hematopoietic ontogeny along with highly redundant hematopoietic signaling. Elucidating the role of signaling pathways in the induction, migration, and differentiation of all the blood cells could help comprehend the mechanisms required for hematopoietic homeostasis and their responses to injury. However, it is necessary to explore further the mechanistic gears regulating the molecular framework of primitive and definitive hematopoiesis. In this context, the use of model organisms can be further exploited. They are beneficial in determining the aberrant hematopoietic signaling found in blood disorders and in thoroughly examining the therapeutic efficacy of drugs used to treat these hematopoietic defects. The evolutionarily conserved signaling pathways and their

components are an added advantage to use these model organisms for studying not only hematopoiesis and hematological disorders but also other diseases that result in hematopoietic deregulation.

The mouse model is considered to be a major research model due to the striking similarities with humans in pathology, physiology, morphology, genetics, and molecular mechanism underlying the development of blood lineages. Therefore, research on mouse hematopoiesis helped in extrapolating the finer aspects of human blood cell biology. Studies using the mice model were the first to highlight the multipotent nature of HSCs (Becker et al., 1963). Researchers employed them to generate the cells of the blood vessel, bone, and muscle. Unlike the mouse model, zebrafish is an easy paradigm to study hematopoiesis along with tractable genetic manipulation. Genetic and chemical screening at a greater scale is possible using zebrafish as compared with mice. These advantages made zebrafish model an attractive model to study hematopoiesis and helped in identifying novel hematopoietic genes such as *slc25a37*, *slc40a1*, and *glrx5* (Brownlie et al., 1998; Donovan et al., 2000; Liao et al., 2000; Lyons et al., 2002; Shaw et al., 2006; Wingert et al., 2005). We can, therefore, suggest that past two decades of research on zebrafish eventually made it surpass the prime position of mouse in studying hematopoiesis. Compared with mouse and zebrafish models, *Drosophila* has its limitations in studying hematopoiesis such as lack of blood vasculature and presence of fewer types of blood cells (Wang et al., 2014). Yet, functional similarity of plasmatocytes to mammalian myeloid cells exhibits them as an ideal model to study fundamental aspects of myeloid cells' function such as phagocytosis, tissue integrity, and immunity. This review reflects the importance of *Drosophila* model in studying hematopoiesis. We talked about some of the Drosophila hematopoietic gene mutations causing similar defects in other animal models and humans. Henceforth, we iterate the importance of different model organisms to explore and examine the development of blood cells.

20 | FUTURE DIRECTIONS

Primary functions of blood cells include immune surveillance and protection of body cells. Blood cells in any organism incessantly work to attain this target, and failure in performing such duties will lead to defective tissues. Although various in vitro blood cell cultures exist, they do not completely mimic the blood cells in vivo. But in vivo studies are possible using model organisms, which, therefore, should be pursued more aggressively by generating transgenic models of significant human hematopoietic disorders. Future studies can focus on examining the nodes of the hematopoietic signaling nexus involved in the etiology of blood-associated disorders including blood cancer. These nodes can unravel the novel targets that can be studied to develop therapeutics. Information can be elucidated, pertaining to such nodes, by investigating the genes connecting the important hematopoietic signaling networks.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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ORIGINAL RESEARCH ARTICLE

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Virodhamine, an endocannabinoid, induces megakaryocyte differentiation by regulating MAPK activity and function of mitochondria

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Abstract

Endocannabinoids are well-known regulators of neurotransmission by activating the cannabinoid (CB) receptors. Endocannabinoids are being used extensively for the treatment of various neurological disorders such as Alzheimer's and Parkinson's diseases. Although endocannabinoids are well studied in cell survival, proliferation, and differentiation in various neurological disorders and several cancers, the functional role in the regulation of blood cell development is less examined. In the present study, virodhamine, which is an agonist of CB receptor-2, was used to examine its effect on megakaryocytic development from a megakaryoblastic cell. We observed that virodhamine increases cell adherence, cell size, and cytoplasmic protrusions. Interestingly, we have also observed large nucleus and increased expression of megakaryocytic marker (CD61), which are the typical hallmarks of megakaryocytic differentiation. Furthermore, the increased expression of CB2 receptor was noticed in virodhamine-induced megakaryocytic cells. The effect of virodhamine on megakaryocytic differentiation could be mediated through CB2 receptor. Therefore, we have studied virodhamine induced molecular regulation of megakaryocytic differentiation; mitogen-activated protein kinase (MAPK) activity, mitochondrial function, and reactive oxygen species (ROS) production were majorly affected. The altered mitochondrial functions and ROS production is the crucial event associated with megakaryocytic differentiation and maturation. In the present study, we report that virodhamine induces megakaryocytic differentiation by triggering MAPK signaling and ROS production either through MAPK effects on ROS-generating enzymes or by the target vanilloid receptor 1-mediated regulation of mitochondrial function.

KEYWORDS
endocannabinoid, MAPK, megakaryocyte, mitochondria, ROS, virodhamine

1 | INTRODUCTION

Endocannabinoids are endogenous ligand of cannabinoid (CB) receptors and well-known neuromodulators. Endocannabinoids are rigorously studied in neurological disorders such as Alzheimer's and Parkinson's diseases and various other neurological abnormalities. But, there exist limited studies of these endocannabinoids in blood cells. The ability of circulating blood cells to produce

endocannabinoids is well studied (Bisogno, Maurelli, Melck, De Petrocellis, & Di Marzo, 1997), e.g., macrophages and platelets produce and release endocannabinoids (Berdyshev, Schimd, Krebsbach, & Schmid, 2001; Di Marzo et al., 1999).

There are two types of CB receptors, CB1 and CB2 receptor. Though both these receptors localize in the central nervous system, immune cells, and peripheral tissues, their levels of expression vary. CB1 receptor is primarily expressed in the central nervous system; however, CB2 receptor expression is higher in blood cells and peripheral tissue (Mackie, 2008). There are several endocannabinoids such as anandamide (N-arachidonylethanolamine), 2-arachidonyl glycerol, and virodhamine which are the molecules of interest because of their efficacy to modulate biological events such as cell survival, cell differentiation, and cell death (Siegmund et al., 2007). Blood platelets are involved in both the metabolism as well as the release of circulating endocannabinoids; moreover, endocannabinoids are known for their ability to promote platelets activation (reviewed in, Randall, 2007), and so it is possible that circulating endocannabinoids levels might be involved in the regulation of megakaryocyte (MK) development. However, the functional role of endocannabinoid in the regulation of MK development is not studied.

Platelets, an essential component of blood, play an important role in blood clotting and wound healing (Debili et al., 2009; Sim, Poncz, Gadue, & French, 2016), and MKs are immediate precursor of platelets. MKs are large cells residing in the bone marrow. They undergo a unique process called endomitosis and cytoplasmic maturation to generate platelets in circulation (Reems, Pineault, & Sun, 2010; Tijssen & Ghevaert, 2013; Trzeciak-Ryczek, Tokarz-Deptuła, & Deptuła, 2013). Alterations in the platelet counts is a consequence of the increased consumption or destruction of platelets in different disease states such as HIV, dengue infections, multiple myeloma, polycythaemia vera, and thrombocytopenic purpura (Assinger, 2014; Mac Manus et al., 1997). The condition with lower platelet counts is called thrombocytopenia, reported in different disease conditions, viral infections, and as a side-effect of chemotherapies and radiotherapies (Squires, 2015). The most effective treatment of thrombocytopenia is thrombopoietin (TPO) mimetics and platelet transfusion, but its application is very limited due to side effects (Galve-Roperh, Aguado, Rueda, Velasco, & Guzmán, 2006).

Endocannabinoids may have a functional role in MK development in different low platelet conditions (thrombocytopenia), as their metabolism and release is regulated by circulating platelets. In this manuscript, we examined the role of virodhamine in the development of MKs by using megakaryoblastic cell line model. Megakaryopoiesis is a unique process in which megakaryoblast loses its proliferative ability and transforms into a matured MK through endomitosis resulting in a large polyploid nucleus and extension of cytoplasmic protrusions (Guo et al., 2015; Mazzi, Lordier, Debili, Raslova, & Vainchenker, 2018). Present study results indicate that virodhamine is a positive regulator of MK maturation. Herein, we observed that virodhamine triggers mitogen-activated protein kinase (MAPK) activation and ROS production. We have also noticed its effects on the expression of ROS-generating enzyme such as NOX4 and alternative receptors/ion channels such as target vanilloid receptor 1 (TRPV1). On the other hand, we observed depolarized mitochondrial potential. Therefore, in present study we report that virodhamine-induces megakaryocytic differentiation by triggering molecular events which play an important role in megakaryocytopoiesis, such as MAPK activation, alteration of mitochondrial functions, and ROS production.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Human megakaryoblastic cells (Dami cell line) were cultured and maintained in Roswell Park Memorial Institute 1640 (LONZA) medium supplemented with 10% fetal bovine serum (Gibco Invitrogen, Carlsbad, CA) and 1% antibiotics (Life Technologies, Inc.); cells were grown at 37°C under 5% carbon dioxide in humidified incubator. In different experimental conditions, cells were counted by hemocytometer using Trypan Blue stain (Mediatech, Inc.) and used for the analysis of megakaryocytic features and molecular studies. Cell morphological features, such as cells size, adherence, and membrane extension were observed under light microscope (Olympus Pvt., Ltd).

2.2 | Giemsa staining

Giemsa staining was performed to study the nuclei morphology. The cells were harvested on glass slides, dried, and stained with 5% May–Grünwald–Giemsa for 20 min, subsequently washed with distilled water and observed under light microscope.

2.3 | Quantitative analysis of messenger RNA by real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. One microgram (1 μg) of RNA was reverse-transcribed by using abm's superscript first-strand complementary DNA (cDNA) synthesis kit containing a homogenous mixture of oligo (dT). The first-strand cDNA obtained by reverse transcription was used as a template for primer (Table 1) specific amplification using SYBR Green Master Mix (Kappa Biosystems). The thermal cycling parameters were set as follows: initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and extension at 72°C for 30 s. The results presented are from three individual experiments, in which each sample was assayed in triplicate, normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and expressed as relative expression.

2.4 | Cell cycle analysis

Cell cycle analysis was performed using Muse™ Cell Analyzer (Millipore). Cells of different experimental conditions were collected and washed

TABLE 1 List of primers used in present study

Gene	PCR primer sequence (5′-3′)	
CB1 Receptor (CNR1)	F: 5′-TTCCCTCTTGTGAAGGCACT-3′	R: 5′-TCTTGACCGTGCTCTTGATG-3′
CB2 Receptor (CNR2)	F: 5′-CGTGGCTGTGCTCTATCTGA-3′	R: 5′-CACAGAGGCTGTGAAGGTCA-3′
CD61	F: 5′-ACCAGTAACCTGCGGATTGG-3′	R: 5′-TCCGTGACACACTCTGCTTC-3′
TRPV1	F: 5'-ACGGACAGAACACCACCATC-3'	R: 5'-GGCCCTTGTAGTAGCTGTCC-3'
GAPDH	F: 5′-GGAAGGTGAAGGTCGGAGTC-3′	R: 5′-TGAGGTCAATGAAGGGGTCA-3′

Abbreviations: CB, cannabinoid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction; TRPV1, target vanilloid receptor 1.

with phosphate-buffered saline (PBS). Cells were analyzed using Muse® Cell Cycle Kit (EMD Millipore) on Muse™ Cell Analyzer following the manufacturer's instructions.

2.5 | Western blots

Protein from cultured cells was extracted by using a radioimmunoprecipitation assay buffer (Sigma-Aldrich) with protease inhibitor cocktail (Invitrogen). Proteins (50 μg) were separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Blots were blocked using 5% milk powder and incubated overnight with primary antibodies against CB2 receptor protein (1:1,000; rabbit polyclonal to CB2 receptor (H-60), sc-25494; Santa Cruz Biotechnology) and internal control β-actin (Sigma-Aldrich). Detection of the protein-bound primary antibody was carried out with appropriate secondary antibodies conjugated with horseradish peroxidase (Santa Cruz Biotechnology) and developed with enhanced SuperSignal Chemiluminescent Substrate, femtoLucent PLUS-HRP (G-Bioscience). The intensity of the protein band was densitometrically determined by VersaDoc. Finally, the band intensity of CB2 receptor was normalized with the band intensity of β-actin and the normalized CB2 receptor band intensities were displayed in histogram.

2.6 | Immunofluorescence staining of CB2 receptor

Cells of different experimental conditions were collected and washed with ice cold (1×) PBS. Cells were fixed in 2% paraformaldehyde for 15 min, permeabilized with 1% Triton X-100, and blocked with 2% BSA for 60 min. After blocking, cells were incubated overnight with anti-CB2 receptor primary antibody (1:200; sc-25494) and detected in fluorescence microscope with the help of phycoerythrin-conjugated secondary antibody. Images were analyzed with the ImageJ software (NIH, Bethesda, MD) to measure background-corrected "mean gray value" in a visual field selected.

2.7 | MAPK assay

MAPK assay was performed by flow cytometry (Muse™ Cell Analyzer) using the Muse MAPK Activation Dual Detection Kit following the manufacturer's protocol. MAPK Assay Kit includes two directly conjugated antibodies—a phospho-specific antiphospho-ERK1/2 (Thr202/Tyr204, Thr185/Tyr187)-phycoerythrin and an anti-ERK1/2-PECy5 conjugated antibody—to measure total levels of ERK. This two-color kit is designed to measure MAPK phosphorylation relative to the total MAPK expression in any given cell population. By doing this, the levels of both the total and phosphorylated protein can be measured simultaneously in the same cell, resulting in a normalized and accurate measurement of MAPK activation after stimulation.

2.8 | Measurement of intracellular ROS

Intracellular ROS was determined by using 2′,7′–dichlorofluorescin diacetate (DCFDA). ROS levels were measured by incubating cells (1×10^6) from different experimental conditions with 10 μM DCFDA for 40 min at 37°C. Further cells were washed three times with (1×) PBS and transferred to microplate and detected using fluorescence spectroscopy at ex/em 488 nm/525 nm. The experiments were performed in triplicate.

2.9 | Mitochondrial membrane potential

Mitochondrial membrane potential of cells from different experimental conditions was determined by using Muse Mitopotential Assay Kit (Millipore) as per the manufacturer's instructions. The assay utilizes the mitopotential dye, a cationic lipophilic dye to detect the changes in mitochondrial membrane potential and 7-AAD as indicator of cell death. The cells were observed in Muse Cell Analyzer (Millipore).

2.10 | Calcium assay

Fluo-3 fluorescence was utilized to determine intracellular free Ca2+ levels. The assay was performed as reported elsewhere (Raghuwanshi et al., 2020). The emitted fluorescence intensity of Fluo-3 was recorded on a fluorescence-spectrometer at 25°C (excitation 505 nm, emission 526 nm, slit width 5 nm). Further, Fluo-3/AM (3 μM) loaded cells were imaged for monitoring the fluorescence intensity using a fluoresce microscope.

2.11 | Statistical analysis

The significance of the observance was determined using t test for pairwise comparison. The p values of <.05 were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Virodhamine induces megakaryocytic differentiation

Megakaryocytic differentiation is characterized by large nucleus, membrane expansions, and increased expression of megakaryocytic markers (Guo et al., 2015). The present study describes the megakaryocytic effects of virodhamine (CB2 receptor agonist) on a well-established megakaryoblastic cell line (Dami cells). Proliferating Dami cells were grown for 72 hr in the presence of increasing concentrations of virodhamine as well as 50 nM concentration of phorbol 12-myristate 13-acetate (PMA; positive control), a well-known inducer of megakaryocytic differentiation. Much identical to the PMA, virodhamine treatment augmented features associated with megakaryocytic development: cells increased adherence and membrane expansion ($n = 3$; Figure 1a); Giemsa staining showed the increased size of nucleus ($n = 3$; Figure 1b). Moreover, upon virodhamine treatment, the expression level of megakaryocytic marker CD61 was enhanced in a dose-dependent manner ($n = 3$; Figure 1c; $p < .02$), which suggest the functional significance of cannabinoids on megakaryocytic differentiation. Our results are consistent with the previous findings demonstrated positive effects of 2-AG (endocannabinoid) on megakaryocytic maturation of Meg01 (Gasperi et al., 2014).

To check whether the virodhamine affects cell proliferation, upon virodhamine treatment, we have counted cells at different time points up to Day 3 and observed that the cell proliferation was reduced by fourfold over a 3-day period ($n = 3$; Figure 2a). Considering the observed effect of virodhamine on cell proliferation, we performed the cell cycle analysis (i.e., DNA ploidy); virodhamine significantly increased the portion of high ploidy cells as compared to control ($n = 3$; Figure 2b); these results defined that the reduced cell cycling might be because of the virodhamine triggered cells differentiation. This study presents the key aspects of megakaryocytic

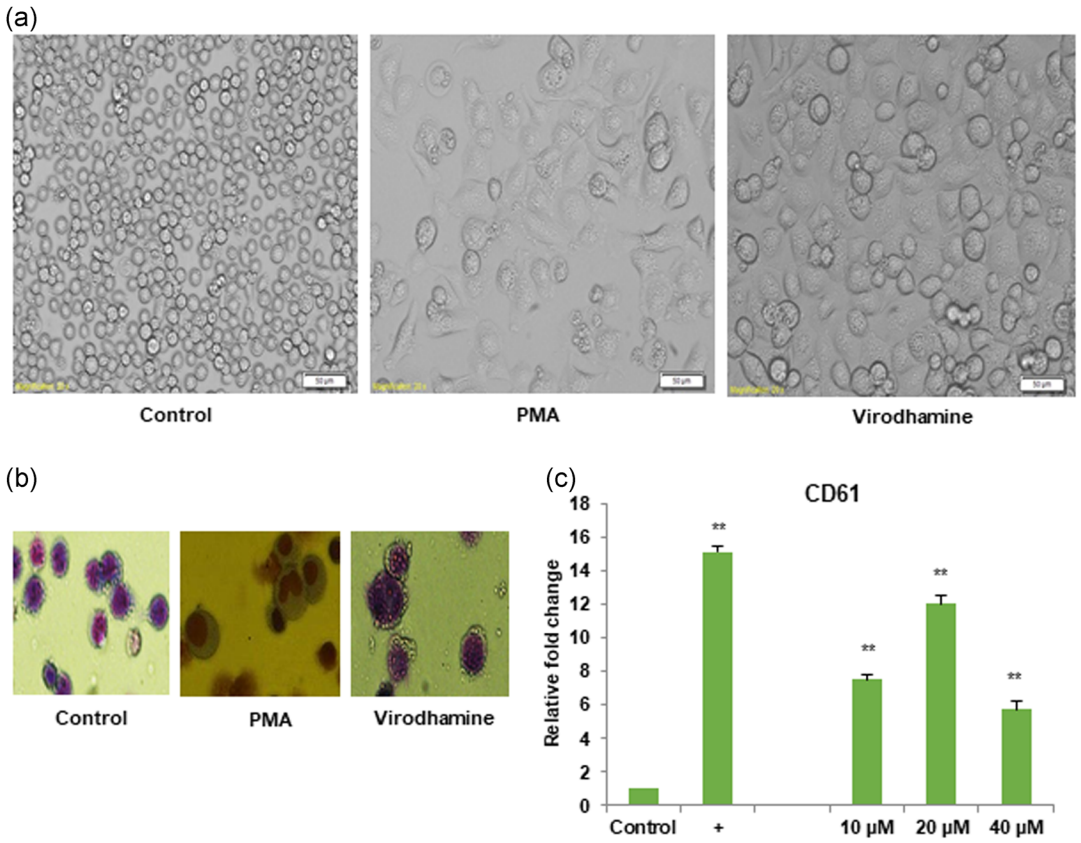

FIGURE 1 Virodhamine induces megakaryocyte differentiation. (a) Representative image of the cells showing megakaryocytic features, such as increased cell size, adherence, and cytoplasmic protrusions, induced upon virodhamine treatment (72 hr). (b) Cells stained with Giemsa showing large nucleus upon PMA or virodhamine treatment (72 hr). (c) qRT-PCR results showing fold induction in the expression of CD61 upon PMA treatment (positive control) or treatment with different concentrations of virodhamine (10, 20, 40 μM) as compared to untreated control ($n = 3$; $^{**}p < .02$). Bars represent mean ± *SD* of three independent experiments. PMA, phorbol 12-myristate 13-acetate; qRT-PCR, quantitative real-time polymerase chain reaction; *SD*, standard deviation

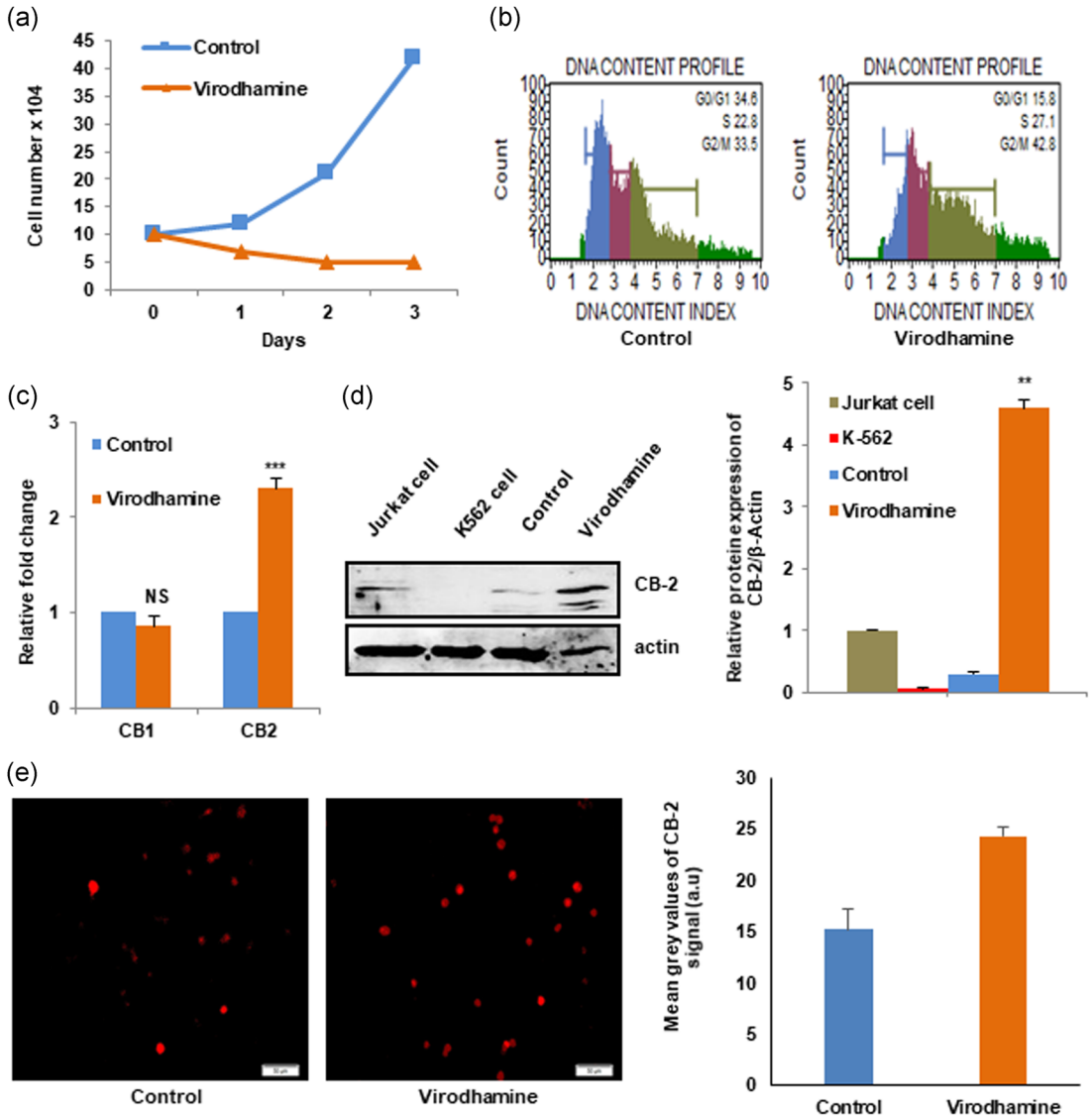

FIGURE 2 Virodhamine regulates cell cycling and induces CB2 receptor expression in megakaryocytes. (a) The graph demonstrates the cell number changed over a 3-day period ($n = 3$) with different treatment conditions. (b) DNA content profile of virodhamine induced and uninduced Dami cells, analyzed by Muse analyzer, the DNA content index was increased upon virodhamine treatment as compared to control ($n = 3$). (c) qRT-PCR results show the expression of CB1 and CB2 receptors upon virodhamine treatment as compared to control ($n = 3$; $^{***}p < .002$). (d) CB2 receptor expression was analyzed by western blot and normalized with β-actin, blots are showing the CB receptor protein band intensity and bar graph shows the actual mean band intensity of blots ($n = 3$; $^{**}p < .02$). (e) Representative CB2 receptor immunofluorescence image of uninduced and virodhamine-induced Dami cells, the histogram displayed mean fluorescence intensity of cells ($n = 3$). Bars represent mean ± *SD* of three independent experiments. *SD*, standard deviation

development and shows that the virodhamine potentially induces megakaryocytic maturation in megakaryoblastic cells. Since there exist two types of CB receptors, CB1 and CB2 receptor (Galve-Roperh et al., 2006), we checked for their expression in Dami cells upon CB2 agonist virodhamine treatment for 72 hr. We found an increase in CB2 receptor expression by qRT-PCR ($n = 3$; $p < .002$; Figure 2c) unlike CB1 receptor expression that was not increased with virodhamine treatment. To detect CB2 receptor protein levels, we performed western blot study with appropriate positive and negative controls. The lysates of Jurkat leukemia cells (Capozzi et al., 2018) and K562 cells (Alberich Jordà et al., 2004) were used, respectively, as the positive and negative controls to test the specificity of CB2 receptor antibody ($n = 3$; Figure 2d). Consistent with the messenger RNA (mRNA) levels, we found that the protein expression of CB2 receptor was significantly increased in virodhamine-induced Dami cells when compared with uninduced control (Dami cells; $n = 3$; $p < .02$; Figure 2d). The immunofluorescence analysis of CB2 receptor in uninduced versus virodhamine-induced Dami cells further confirmed the augmenting effect of virodhamine on CB2 receptor expression ($n = 3$; Figure 2e). The results of the present study clearly defined that the virodhamine selectively enhancing CB2 receptor expression in megakaryocytic cells; however, it did not show any

marked differences in the expression level of CB1 receptor. In previous studies, virodhamine has been defined as a strong CB2 receptor agonist and a partial agonist/antagonist at the CB1 receptor sites (Fezza et al., 2014). In the present study, our results have defined clearly the positive effects of virodhamine on megakaryocytic differentiation, and these megakaryocytic effects are likely mediated through CB2 receptor.

3.2 | CB2 receptor agonist induces MAPK singling and ROS production in megakaryocytic cells

Activation of CB receptor has been shown to induce megakaryocytic differentiation in Meg01 cells (Gasperi et al., 2014). However, the molecular mechanisms involved in this effect are not completely understood yet. We investigated the activity of MAPK, by Muse analyzer using the Muse® MAPK Activation Detection Kit, after CB2 receptor stimulation by virodhamine. The profile showed the reduced percentage of cells with inactivated MAPK; however, the percentage of cells with activated MAPK was increased upon stimulation by virodhamine as compared to unstimulated control ($n = 3$; Figure 3a). The peripheral CB2 is a G protein-coupled receptor that is positively coupled to the MAPK (Bouaboula, Desnoyer, Carayon, Combes, & Casellas, 1999; Herrera, Carracedo, Diez-Zaera, Guzmán, & Velasco, 2005). Moreover, several recent studies have implicated the MAPK pathway in MK differentiation and endomitosis (Rojnuckarin, Drachman, & Kaushansky, 1999).

Furthermore, there is accumulating evidence that shows the functional role of cannabinoids in the modulation of ROS production in different cell types. In the present study, we tested the cellular ROS levels upon virodhamine treatment. Herein, we observed that the ROS production was increased upon CB2 receptor stimulation by virodhamine as compared to control ($n = 3$; $p < .05$; Figure 3b). Earlier, CB1 and/or CB2 receptors have been reported to either stimulate and/or repress the function of enzymes involved in ROS generation. For instance, CB receptor activation acts to suppress the cAMP-dependent activation of PKA, thereby reducing the expression and/or activity of ROS-generating enzymes such as NADPH oxidase. Alternatively, CB receptor stimulation can activate MAPK signaling that is reported to be involved in upregulating the NADPH oxidase expression and/or activity (Lipina & Hundal, 2016). In the present study, we found that NAPDH oxidase NOX4 expression was significantly high in virodhamine stimulated cells as compared to control ($n = 3$; $p < .02$; Figure 3c). It is apparent that in MKs, virodhamine regulates ROS production likely through MAPK/NADPH oxidase axis. On the other hand, we tested the effect of virodhamine on the function of mitochondria by using Muse mitochondrial membrane potential assay. Virodhamine caused a decrease in membrane potential, almost 15% cells were depolarized/live as compared to control where only 4% cells were depolarized/live ($n = 3$; Figure 3d). The depolarization of mitochondrial inner membrane potential indicates that the function of mitochondria is altered. Previously, different research groups have also reported that CB ligands may regulate cellular ROS production by altering mitochondrial functions (Siegmund et al., 2007). Siegmund reported that the CB 2-AG stimulation increases mitochondrial ROS productions and mitochondrial depolarization in hepatic stellate cells. Different other CB receptor agonists (anandamide, phytocannabinoid D-9-tetrahydrocannabinol, and synthetic CB HU 210) have also been reported to decrease mitochondrial membrane potential and increase mitochondrial ROS production (Athanasiou et al., 2007). Interestingly, in a recent report, ROS has been shown to be involved in MK maturation (Raghuwanshi et al., 2020).

Cannabinoids have also been shown to regulate TRPV1 activity, promote intracellular calcium signaling, and increase ROS levels (Lipina & Hundal, 2016; Miyashita, Oyama, Sakuta, Tokuda, & Torii, 2012). Therefore, further we tested the effect of virodhamine on the expression of TRPV1 and intracellular calcium levels in Dami cells. We observed that the expression of TRPV1 mRNA was significantly increased upon virodhamine treatment as compared to control ($n = 3$; $p < .02$; Figure 4a). Intracellular calcium was detected by using Fluo-3 AM dye. We observed that the mean fluorescence intensity of Fluo-3 was increased upon virodhamine treatment as compared to control ($n = 3$; Figure 4b), the same difference was noticed when we visualized the cells under confocal microscope: distribution of Fluo-3 positive cells was increased upon virodhamine treatment ($n = 3$; Figure 4c). It is well known that calcium regulates mitochondrial function and that, in turn, mitochondrial ROS. Hence, it is possible that cannabinoids may regulate ROS levels by targeting alternative receptors/ion channels such as TRPV1. Earlier studies established that calcium and ROS are necessary for megakaryocytic differentiation and functions (Di Buduo et al., 2014; Hirose et al., 2013; Raghuwanshi et al., 2020; Sardina et al., 2010; Tan et al., 2010). Considering the present study results and previous reports, it can be calculated that MAPK signaling and ROS are involved in virodhamine-induced megakaryocytic differentiation.

4 | CONCLUSION

The MK is the hematopoietic cell that produces platelets, essential component of blood system. Platelets are capable to act as fundamental player in blood clotting and wound healing. In different disease states, the blood platelet counts are reduced because of the increased consumption or destruction of platelets. To treat patients with severe thrombocytopenia platelet transfusions is the only preferred treatment option. TPO mimetics are also effective but its application is very limited due to side effects. To overcome these problems, there is a need to define the functional impact of endogenously produced regulatory factors in promoting the process of MK development/platelet biogenesis.

Therefore, to develop novel strategies that can help in inducing the process of MK development, by using the megakaryoblastic cell line model, we have evaluated endocannabinoid virodhamine as one of the inducers of MK development. The present study results, by using an in vitro model, report that virodhamine can stimulate MK

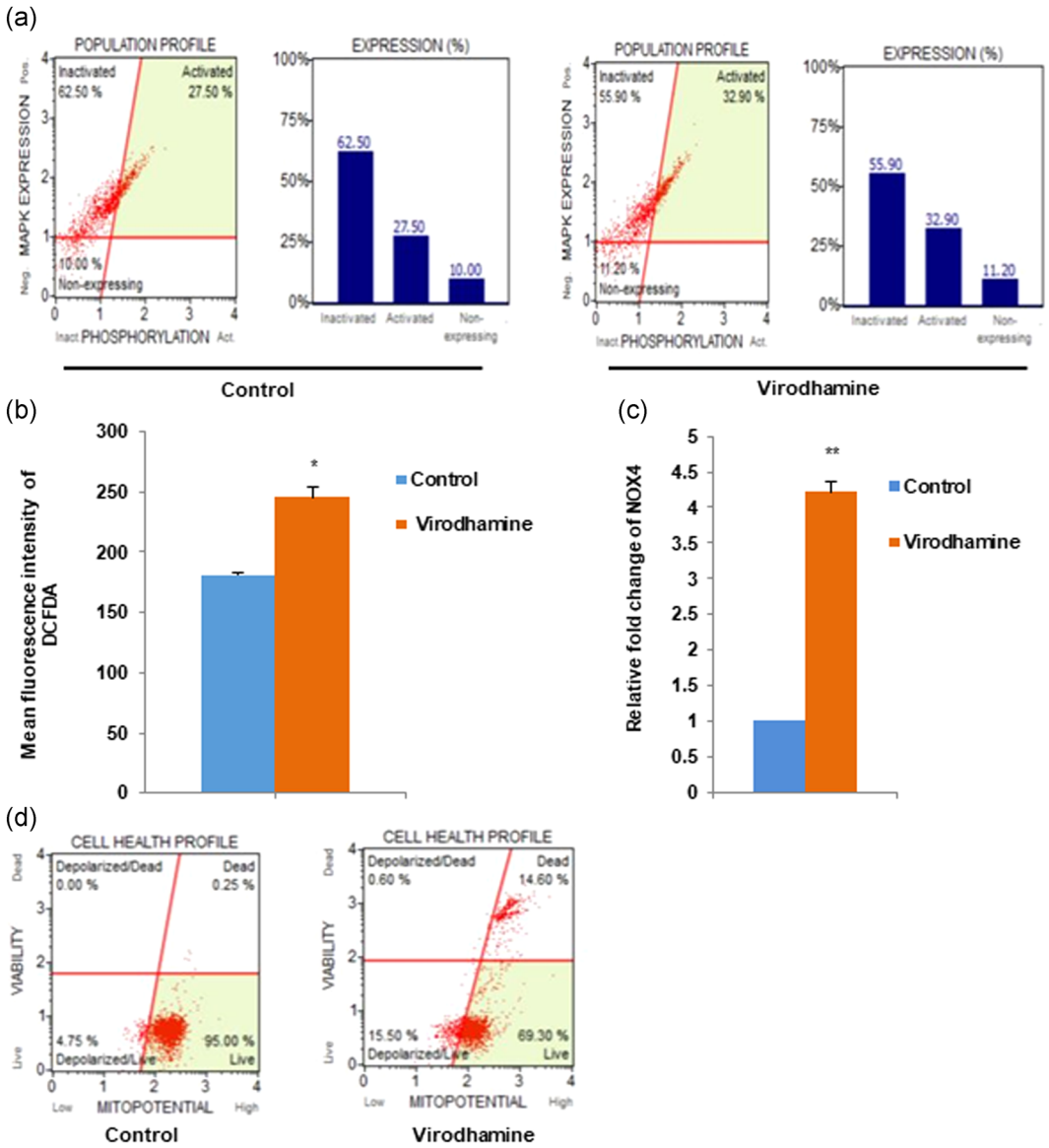

FIGURE 3 Virodhamine triggers MAPK activation and ROS production. (a) Muse flowcytometry showing MAPK expression and activation profile of virodhamine treated and control cells ($n = 3$). (b) ROS production was detected using DCFDA, which is a cell permeable standard probe to detect ROS. Fluorescence was measured by fluorescence spectroscopy at 485 nm excitation and 535 nm emission wavelengths. Graph represents the mean fluorescence intensity of control and virodhamine treated cells ($n = 3$; $^*p < .05$). (c) qRT-PCR results show the differential expression of NOX4 upon virodhamine treatment as compared to control ($n = 3$; $^{**}p < .02$). (d) The health profile of cells showing change in mitochondrial membrane potential upon virodhamine treatment ($n = 3$). Bars represent mean ± *SD* of three independent experiments. MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; *SD*, standard deviation

development. In the present study, megakaryoblastic cells treated with virodhamine underwent MK differentiation process which is characterized by reduced rate of cell proliferation, increased cell size and adherence, and increased expression of megakaryocytic markers. These findings confirm that virodhamine could be a specific signal for the MK development. Furthermore, we report increased MAPK activity and ROS production upon virodhamine treatment. Therefore, the increased MAPK activity and cellular ROS could be involved in the virodhamine-stimulated MK maturation.

In conclusion, our results are in line with previous studies and collectively suggest that virodhamine is inducing megakaryocytic differentiation. This effect is likely triggered by CB2 receptor-mediated MAPK activation and/or ROS production either through CB2/MAPK-regulated ROS-generating enzymes or by the activity of TRPV1 and mitochondria (Figure 4d). Altogether our results define virodhamine as a strong modulator of MK development/platelet production process.

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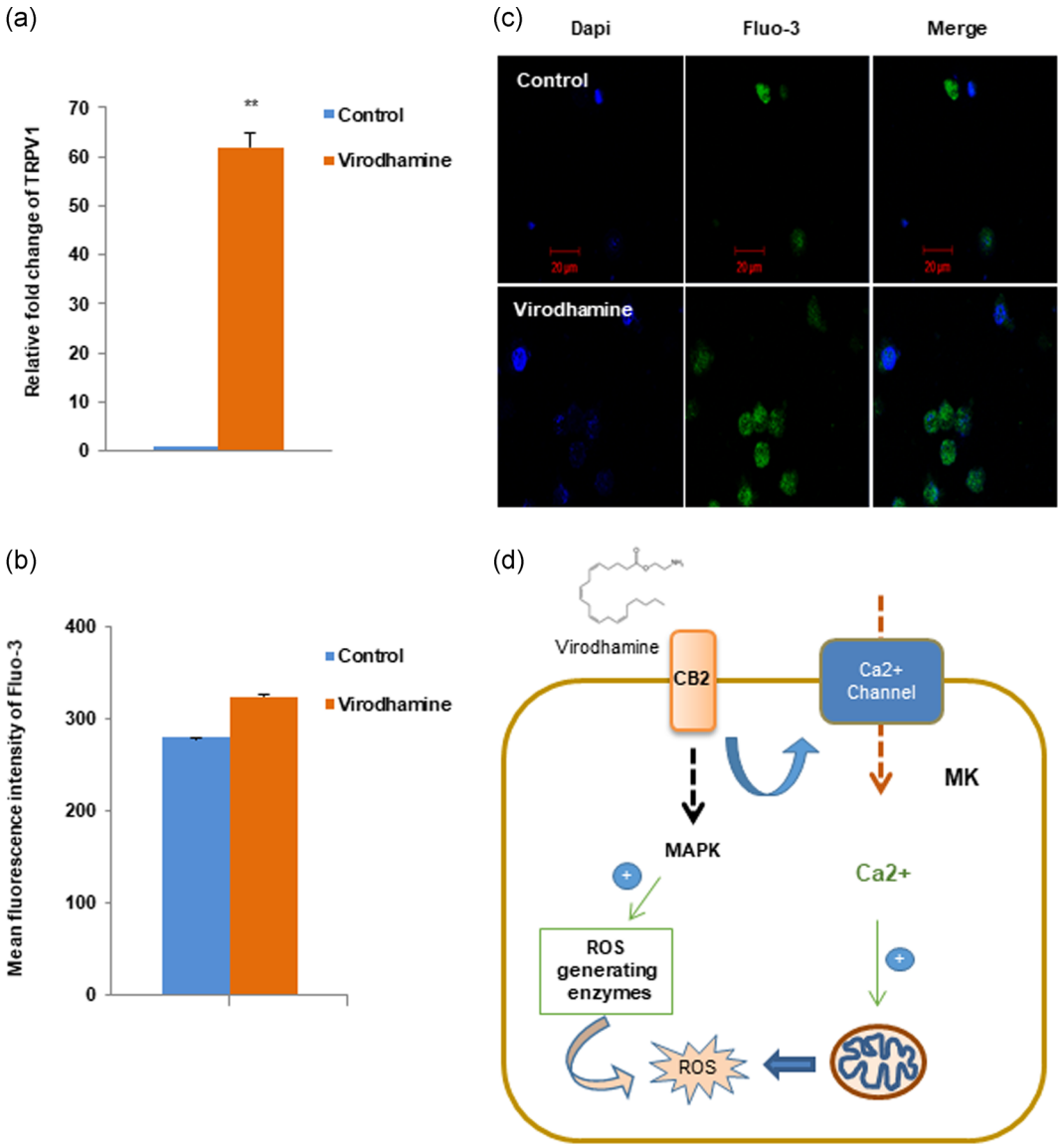

FIGURE 4 Virodhamine increases TRPV1 expression and calcium levels. (a) qRT-PCR results show the effect of virodhamine on TRPV1 mRNA levels ($n = 3$; $^{**}p < .02$). (b) Further, Fluo-3 fluorescence was utilized to determine intracellular calcium activity, the emitted fluorescence intensity of Fluo-3 was recorded on a fluorescence-spectrometer ($n = 3$). (c) Representative immunofluorescence image of Dami cells treated with virodhamine or untreated control. Nuclei stained with DAPI (blue), Fluo-3, calcium dye (green). Bars represent mean ± *SD* of three independent experiments. (d) Schematics showing the effects of virodhamine on the activity of MAPK and ROS production in megakaryocytes. DAPI, 4′,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; *SD*, standard deviation; TRPV1, target vanilloid receptor 1

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

D. S. S. designed and performed experiments. S. R. Contributed to both data analysis and interpretation and prepared the manuscript. K. N., S. D., D. K. G, and I. P. contributed to review and editing. R. K. G. designed and supervised the study.

DATA AVAILABILITY STATEMENT

The data generated during the current study are available from the corresponding author on reasonable request.

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Review Article

Endocannabinoid system: Role in blood cell development, neuroimmune interactions and associated disorders

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ABSTRACT

The endocannabinoid system (ECS) is a complex physiological network involved in creating homeostasis and maintaining human health. Studies of the last 40 years have shown that endocannabinoids (ECs), a group of bioactive lipids, together with their set of receptors, function as one of the most important physiologic systems in human body. ECs and cannabinoid receptors (CBRs) are found throughout the body: in the brain tissues, immune cells, and in the peripheral organs and tissues as well. In recent years, ECs have emerged as key modulators of affect, neurotransmitter release, immune function, and several other physiological functions. This modulatory homoeostatic system operates in the regulation of brain activity and states of physical health and disease. In several research studies and patents the ECS has been recognised with neuro-protective properties thus it might be a target in neurodegenerative diseases. Most immune cells express these bioactive lipids and their receptors, recent data also highlight the immunomodulatory effects of endocannabinoids. Interplay of immune and nervous system has been recognized in past, recent studies suggest that ECS function as a bridge between neuronal and immune system. In several ongoing clinical trial studies, the ECS has also been placed in the anti-cancer drugs spotlight. This review summarizes the literature of cannabinoid ligands and their biosynthesis, cannabinoid receptors and their distribution, and the signaling pathways initiated by the binding of cannabinoid ligands to cannabinoid receptors. Further, this review highlights the functional role of cannabinoids and ECS in blood cell development, neuroimmune interactions and associated disorders. Moreover, we highlight the current state of knowledge of cannabinoid ligands as the mediators of neuroimmune interactions, which can be therapeutically effective for neuro-immune disorders and several diseases associated with neuroinflammation.

1. Introduction

Cannabinoids (CB) are chemical compounds, which are well-known to be derived from the *cannabis sativa* plant; this plant is one of the earliest plants that are cultivated for the clinical use (Russo, 2007). Cannabinoids are a class of biologically active compound that can bind and activate cannabinoid receptors (CBRs). A significant number of studies have proven the biological role of different cannabinoids. This class of compounds is broadly classified in three classes based on their source of production: (1) plant derived cannabinoids (also called Phytocannabinoids), (2) endogenous cannabinoids (called endocannabinoids), and (3) synthetic cannabinoids. Phytocannabinoids are a class of the structurally diverse chemical constituents found in the genus *Cannabis*, they interact with our body receptors and initiate different molecular events leading to psychotropic and therapeutic effects. One such example of Phytocannabinoids is delta-9-tetrahydrocannabinol (Δ^9-THC), which is a well-known psychoactive phytocannabinoid compound explored enormously in clinical and biological investigations for its therapeutic potential (Pertwee, 1999; Pertwee and Ross, 2002; Pertwee, 2006). On the other hand, endocannabinoids (ECs) are defined as endogenous ligands of CBRs, they are the lipid-based molecules derived from long-chain polyunsaturated fatty acids, amides, esters, and ethers, e.g., 2-arachidonoylglycerol (2-AG) and anandamide (Fezza et al., 2014; Pisanti et al., 2013). The ECs bind to G-protein coupled receptor (GPCRs), CB1R and CB2R and exert their action (Morales and Reggio, 2017). Several studies have conformed the regulatory functions

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Table 1
Cannabinoid system in different disease conditions.

Disease/medical condition	Therapeutic effects of CB/ECS	References
Parkinson's Disease (PD)	CB1R agonist could be one of the potential drugs that can cure PD symptoms such as bradykinesia, rigidity, and tremor	Stampanoni Bassi et al., 2017
Huntington's Disease (HD)	Loss of striatal CB1R is recognised as the key pathogenic factor in HD. CB1R as biomarker for monitoring the onset and progression of disease.	Blázquez et al., 2011 Fernández-Ruiz, 2009
Dystonia and Tremor	A synthetic cannabinoid dronabinol seems to be effective in the treatment of Dystonia, Dyskinesias, and Tics.	Koppel, 2015
Seizures/epilepsy	Therapeutic effects of cannabinoids have been shown in preclinical studies in animal models of seizures, epilepsy, epileptogenesis, and epilepsy-related neuroprotection	Jones et al., 2012 Rosenberg et al., 2017
Multiple Sclerosis-related Tremor	Cannabinoids can be an effective treatment for multiple sclerosis spasticity. Studies suggest that CB1R could be the potential therapeutic target for multiple sclerosis related tremor via THC action.	Baker et al., 2000 Hill et al., 2017 Rice and Cameron, 2018
Obesity	CB2R antagonist could be one of the potential therapeutics in obesity.	Agudo et al., 2010
Non-alcoholic fatty liver disease (NAFLD)	CBRs could be the effective targets to reduce liver impairment	Unamuno et al., 2018
Pain	Cannabinoids have the analgesic effect. Cannabinoids could be effective against chronic neuropathic pain.	Hill et al., 2017 Zhao et al., 2010 Jiang et al., 2007 Patinkin et al., 2008

of ECs in health and disease state, these studies have suggested that modulating ECs activity may have therapeutic potential in different disease conditions.

Therapeutic potential of cannabinoids was explored considerably in neurological diseases (Devinsky et al., 2015). Interestingly, in last few years, cannabinoid ligands have also been explored as the crucial regulators of immune and hematopoietic system (Chiurchiù, 2016; Galve-Roperh et al., 2013; Chiurchiù et al., 2015; Chiurchiù et al., 2016). Numerous studies have shown that cannabinoids and CB receptors could be potential therapeutic target for various pathological conditions such as Parkinson's disease, (Table 1) Huntington's disease, (Table 1) Tourette's syndrome, Alzheimer's disease, epilepsy (Table 1) and inflammatory disorders (Fisar, 2009). Cannabinoids affect many biological functions, influencing short-term memory, motor co-ordination to mediating analgesia, and immunosuppression (Hill et al., 2012). In recent years, there is an increasing interest in the medical use of cannabinoids for the treatment of various inflammatory, autoimmune, hematological disorders, and cancers. Various scientific committees that studied the therapeutic potential of cannabinoid and cannabinoid derivatives are House of Lords in Great Britain, Senate Special Committee on Illegal Drugs in Canada and Institute of Medicine in the United States (Nolin et al., 2002). There are multiple reports that evaluates therapeutic potential of cannabinoid ligands in central nervous system, hematopoietic cells and immune cells. This review aims to discuss these reports and provide the current state of knowledge whether cannabinoid ligands could be used as neuro-immune regulator. Further, this review summarizes the literature available for therapeutic application of cannabinoids in hematopoietic and neuronal system.

Table 2
Cannabinoid receptor ligands and their specificities.

Ligand	Specificity	Action	Reference
THC	CB1R	Partial Agonist	Pertwee, 2008
	CB2R	Partial Agonist	
AEA	CB1R	Partial Agonist	Kano, 2014
	CB2R	Partial Agonist	
2-AG	CB1R	full agonist	Kano, 2014
	CB2R	full agonist	
Virodhamine	CB1R	Partial agonist	Porter et al., 2002
	CB2R	Full agonist	
Cannabidiol	CB1R	Antagonist	Thomas et al., 2007
	CB2R	Inverse agonist	
Cannabigerol	CB1R	Partial agonist	Navarro et al., 2018
	CB2R	Partial agonist	
Tetrahydrocannabivarin	CB1R	Antagonist	Thomas et al., 2005
	CB2R	Antagonist	
Cannabinol	CB1R	Partial agonist	Petitet et al., 1998
	CB2R	Partial agonist	

2. Types of cannabinoids

2.1. Phytocannabinoids

Phytocannabinoids are constituents of a structurally diverse class of naturally occurring chemical compounds, these compounds are abundant in the viscous resin produced by the *cannabis* plants. The examples of different phytocannabinoids are Δ^{9}-tetrahydrocannabinol (Δ^{9}-THC), cannabidiol (CBD), cannabichromene (CBC), cannabigerol (CBG), tetrahydrocannabivarin (THCV), and cannabinol (CBN) (Morales et al., 2017). Different studies have shown that Phyto-cannabinoids have differential affinities for CB1R and CB2R receptors (Table 2) (Morales et al., 2017). Apart from CB1R and CB2R, phytocannabinoids are also interact with other receptors such as the opioid or the serotonin receptors, G-protein coupled receptor 55 and/or G-protein coupled receptor 18 (Howlett et al., 2010).

2.2. Endocannabinoids

Endogenous substances, which are capable of binding to cannabinoid receptors and functionally activating them, are referred to as ECs. The ECs is a large group of fatty acids derived active compounds involved in diverse molecular activities in cell (Alexander et al., 2017); examples are 2-AG, virodhamine, anandamide, arachidonyol 20-chloroethylamide (Fezza et al., 2014; Di Marzo et al., 1994; Howlett, 1998).

First endogenous ligand was isolated from pig brain in 1992 and identified as N-arachidonoyl-ethanolamine (AEA, also called anandamide) (Devane et al., 1992). Few years later another ligand called 2-AG was isolated from canine gut. To date, anandamide and 2-AG are the two most studied ECs, they have been studied in various biomolecular processes, such as cell differentiation, proliferation, and maturation (Devane et al., 1992; Turu and Hunyady, 2010). ECs are synthesized in cells on demand. ECs ligands typically binds with either CB1R or CB2R (Table 2). Following the traditional definition of ECs, several EC-like molecules e.g., DHEA and eicosapentaenoic acid analogs were also discovered but the affinity of these EC-like molecules was noticed to be low with CB1R and CB2R, thus DHEA, eicosapentaenoic acid analogs and other more famous endocannabinoid-like compounds, such as palmitoyl ethanolamide (PEA) and oleoyl ethanolamide (OEA), are not considered typical ECs, but rather as amides (Brown et al., 2010; McDougle et al., 2017; Yang et al., 2011; Chiurchiù et al., 2018). Recently, several other receptors, such as TRPV1, GRP119, GRP18, and GRP55 are also recognised as the part of EC system. Moreover, there is a growing interest to discover other potential EC ligands which can bind to typical CBRs or newly recognized EC receptors (TRPV1, GRP119, GRP18 and GRP55). The omega-3 fatty acids derived EC or EC like molecules such as docosahexaenoic acid (DHA) derived N-docosahex-

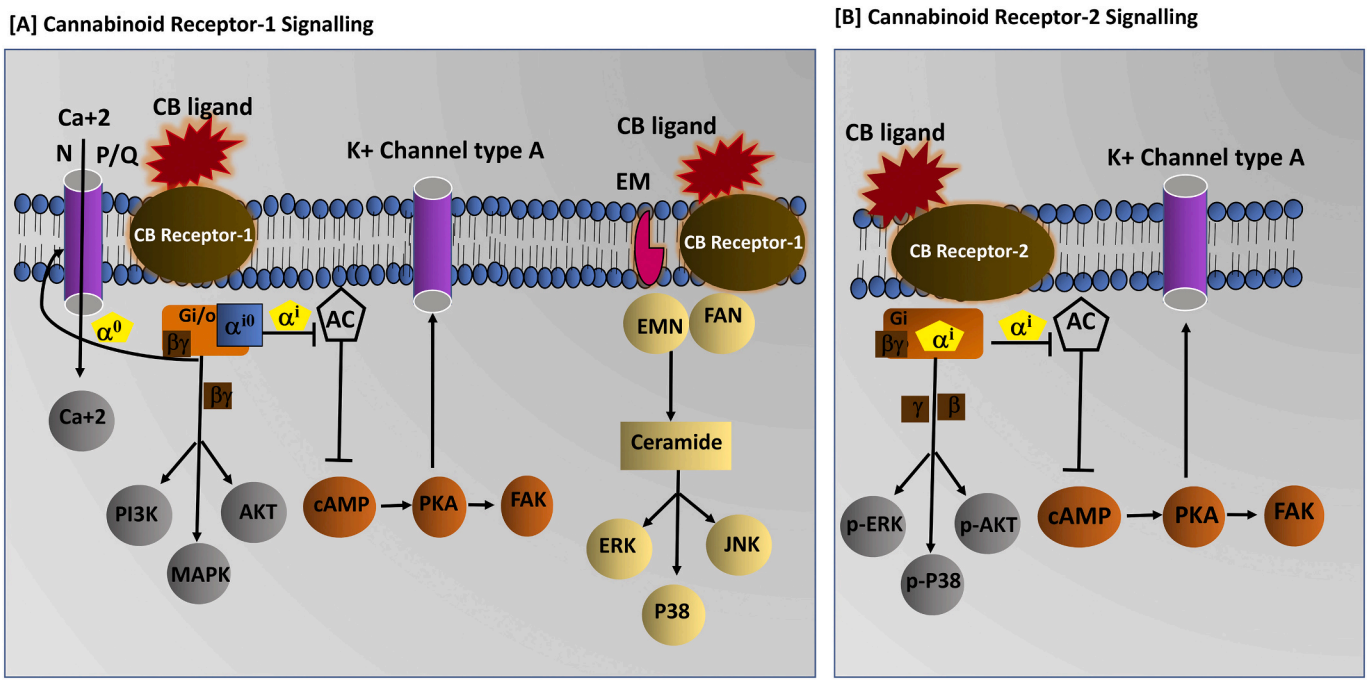

Fig. 1. Schematic representation of the well-known signaling pathways associated with cannabinoid receptors activation. [A] Cannabinoid Receptor-1 Signaling. [B] Cannabinoid Receptor-2 Signaling. AC, adenylyl cyclase; FAN, factor associated with neutral sphingomyelinase activation; voltage-dependent calcium channels type N, P/Q; PKA, protein kinase A; PKB/Akt, protein kinase B; ERK, extracellular signal-regulated kinase; JNK, c-Jun *N*-terminal kinase; FAK, focal adhesion kinase; PI3K, phosphoinositide-3 kinase.

aenoylethanolamide (DHA-EA or DHEA) and eicosapentaenoic acid (EPA) analogs have been recently identified as the molecule of interest because of their novel bioactivity (Di Marzo et al., 2015). In addition, several other fatty acid amide EC or ECs like molecule such as PEA and OEA are also identified as potential biomolecule that can regulate lipid metabolism and inflammation (Chiurchiù et al., 2018). However, more research focus is required to study the functional significance of various ECs in the regulation of different biological processes in both the neuronal and immune system.

2.2.1. *Biosynthesis of endocannabinoids*

Biosynthesis and degradation of ECs is a tightly regulated process. A unique feature of ECs is that they are freshly synthesized upon requirement. This is possible because of the presence of their precursors in cell membranes, these precursors are cleaved by specific enzymes upon requirement. Metabolism and pharmacology of AEA and 2-AG have been thoroughly investigated, and these two cannabinoid compounds are still considered as “major ECs”.

Several different pathways are suggested to be involved in AEA biosynthesis from their corresponding N-acyl phosphatidyl ethanolamine (NAPE) precursor. The most studied pathway of AEA biosynthesis involve NAPE-phospholipase D (NAPE-PLD), which generates AEA from N-arachidonoyl PE (NArPE) precursor (Ahn et al., 2008). Two alternative pathways are also suggested to be involved in AEA biosynthesis, these pathways involve phospholipase-C (PLC)-PTPN22 (Liu et al., 2008) and αβ hydrolase (αβH4)-GDE1 (Egertová et al., 2008). Although, the functional relevance of these multiple pathways of cannabinoids biosynthesis is yet to be determined, the choice of pathway might be depending on precursors availability and/or the cell and the tissue type.

Three major pathways for 2-AG synthesis have been proposed, the most 2-AG comes from membrane phospholipids: One pathway involves the hydrolysis of precursor di-acyl glycerol (DAG) via DAG-lipase (GAGL) (Bisogno et al., 2003; Tanimura et al., 2010), in turn, the DAG is synthesised from PIP2 via phospholipase Cβ (PLCβ) (Farooqui et al., 1989); The second proposed pathway involves lipid precursor 2-Arachidonoyl-LPA, this precursor converted to 2-AG by the activity of lipid phosphatase (2-LPA phosphatase) (Nakane et al., 2002); and the third pathway utilises 2-Arachidonoyl-lysophosphatidylinositol (2-Arachidonoyl-LPI) precursor and the activity of lyso-PLC (Higgs and Glomset, 1994).

Biosynthesis of recently identified ECs, such as virodhamine, noladin ether, and N-arachidonoyl dopamine (NADA) is not completely studied. The enzymes involved in the formation of virodhamine could be phospholipase D which might catalyse fatty acid ethanolamine and arachidonic acid by transphosphotidylation. Previous studies suggest that the biosynthesis of NADA from arachidonic acid and dopamine or tyrosine shares common pathways to those of either arachidonoyl amino acids or of dopamine (Ben, 2006; Steffens et al., 2005). Some other pathways which are not well defined could also be involved in the biosynthesis and degradation of these ECs (Kempe et al., 1996; Fezza et al., 2002; Porter et al., 2002; Huang et al., 2002; Huang et al., 2001).

3. Cannabinoid receptors and their distribution

Cannabinoid receptors are of two types, CB1R and CB2R. To date, only these two CBRs have been identified and cloned. CB1R and CB2R share 44% amino acid identity between themselves (Morales and Reggio, 2017; Ghonghadze et al., 2020; Howlett and Abood, 2017). CB1R is highly conserved in vertebrates and also found in some invertebrates (Elphick and Egertová, 2001; Murphy et al., 2001). The mouse CB1R sequence shows 99% and 97% identity at the amino acid level to rat and human respectively (Pertwee and Ross, 2002; Howlett and Abood, 2017; Lutz, 2002). CB2R shows less homology between the species as compared to CB1R (Shire et al., 1996). For instance, human and mouse CB2R shares 82% amino acid identity (Shire et al., 1996). Abundant expression of CB1R has been found in the neurons of cortex, striatum, hippocampus, amygdala, hypothalamus, cerebellum, brain stem, peripheral neurons, and spinal cord of both rodents and human (Tao et al., 2020). CB2R is predominantly localized in immune cells and organs, such as mononuclear cells, mast cells, natural killer cells, spleen, thymus, and lymph nodes (Schatz et al., 1997; Galiègue et al., 1995). In mammals, CB1R is mainly expressed in central nervous system, whereas

CB2R is present in central nervous system, peripheral tissue, and hematopoietic cells (Elphick and Egertová, 2001; Morales and Reggio, 2017; Liu et al., 2009a, 2009b; Ghonghadze et al., 2020). Several GPCRs have also been suggested to be cannabinoid receptors; currently, only GPR18 and GPR55 have been demonstrated to be the targets of cannabinoid ligands (Morales and Reggio, 2017). Exploration of the distribution pattern of cannabinoid receptors in different tissue is necessary and fundamental to develop clinically significant cannabinoid based therapeutic agents.

4. Cannabinoid receptor signaling

Cannabinoid receptors are G-protein coupled receptor (GPCR) and members of the superfamily of seven-transmembrane-spanning (7-TM) receptors. The signaling pathways initiated by the binding of a cannabinoid ligand to cannabinoid receptors, primarily involves the coupling of the receptor to the inhibitory G proteins Gi and Go (Gi/o), that results in inhibition of adenylate cyclase (AC) and decreased cAMP levels (Fig. 1A) (Howlett, 1998; Liu et al., 2009a, 2009b). In different studies CB1R has been shown as one of the activators of phosphoinositide-3 kinase (PI3K)/ Protein kinase B (AKT) and mitogen activated protein kinase (MAPK) signaling (Fig. 1A) (Turu and Hunyady, 2010). CB1R is known to regulate MAPK and PI3/AKT signaling in cell type and ligand specific manner (Sugiura et al., 2000; Ehrhart et al., 2005; Felder et al., 1995; Sreevalsan and Safe, 2013). Intracellular ceramide biosynthesis is also regulated by the interaction of CB1R with neutral sphingomyelinase (EMN) via interacting protein FAN (factor associated with neutral sphingomyelinase activation) (Howlett et al., 2010). EMN mediates the generation of ceramide from sphingomyelin (EM) in the plasma membrane (Fig. 1A) (Sánchez et al., 1998). It is also involved in other cellular functions, such as the control of cell fate and cell survival (Sánchez et al., 1998; Galve-Roperh et al., 2013).

As noticed for CB1R, CB2R is also observed to inhibit AC and decrease cAMP (Fig. 1B). CB2R have also been reported to stimulate MAPK pathway (Bouaboula et al., 1996), specifically extracellular-signal-regulated kinase (ERK) and P38 MAPK cascade (Bouaboula et al., 1996). Additionally, CB2R known to activate PI3K/AKT pathway (Molina-Holgado et al., 2002). In different studies, Cannabinoid receptors are discussed as the regulators of calcium and potassium channel (Fig. 1) (Atwood et al., 2012). It has also been noticed that different endogenous ligand (ECs) can activate transient receptor potential (TRP) ion channels in cell type- and tissue context-dependent manner (Di Marzo and De Petrocellis, 2010; Hashimotodani et al., 2005; Maejima et al., 2005). For instance, it was noticed that cannabinoid ligands regulate ROS level and target TRPV1 by activation of CB2R (Zhai et al., 2020). Additionally, TRPV1 activation may be driven by Ca^{2+} signalling which is coupled to mitochondrial ROS production (Adam-Vizi and Starkov, 2010; Hofmann et al., 2014; Lipina and Hundal, 2016).

Recent article by Paula Morales et al. (2017) shed light on CB1R and CB2R independent mechanisms. There are orphan-GPCR which can elicit allosteric binding with cannabinoids called biased signaling (Morales et al., 2017). These GPCR selectively activate other signalling cascades independent of classical CB1R/CB2R signalling. This response is reffered as biased agonism and stimulates different physiological response (Ye et al., 2019; Ibsen et al., 2017). Altogether, these advancements in understanding the EC/CBRs signaling pathways identified that there are several signaling pathways which could be activated by different ECs interection with CBRs or orphan-GPCR. However, further development is needed to understand the functional selectivity of CB-ligands and receptors interections, which will give a newer dimension in the use of EC system as the potential theraputic target.

5. Cannabinoids and ECS in blood cell development, neuroimmune interactions and associated disorders

5.1. Cannabinoids in blood cells development and associated disorders

Blood cells development is a tightly regulated physiological process. In recent reports, cannabinoid system has been shown to be involve in the development of hematopoietic cells (Gasperi et al., 2015). Cannabinoid receptors expression is reported in different blood cells such as macrophage (Carlisle et al., 2002), erythrocyte (Bentzen and Lang, 2007), megakaryocyte (Gasperi et al., 2015), platelet (Randall, 2007), mast cell (Samson et al., 2003), B-cell (Tanikawa et al., 2007) and T-cell (Ziring et al., 2006). In vitro studies using anandamide suggests that cannabinoids can stimulate hematopoiesis by the activation of CBRs (Gasperi et al., 2015). Human bone marrow stromal cells release a large amount of ECs, which either alone or along with growth factors exert diverse effects on HSC development, e.g., clonal cell expansion is primarily achieved via activation of CB2 receptor (Keown et al., 2010; Brantl et al., 2014). Previous research, using megakaryoblastic cell line (MEG-01) model provided an evidence of the functional role of 2-AG in megakaryocyte maturation and platelet production (Iannotti et al., 2016; De Angelis et al., 2014; Ligresti et al., 2006). Earlier studies speculated that virodhamine could function as a stimulator of megakaryocyte maturation and platelet production (Brantl et al., 2014). It has also been observed that virodhamine can induce platelet aggregation in blood and platelet rich plasma, however the molecular mechanism is not defined yet (Hall and Degenhardt, 2007; Degenhardt et al., 2010). Recent study by Sharma et al., reported that virodhamine can induce megakaryocyte differentiation (Sharma et al., 2021). Further studies are needed to explore the exact role of cannabinoids in megakaryocyte development and platelet aggregation. The functional role of different cannabinoids has also been reported in platelets activation (Randall, 2007): e.g., Anandamide activates platelets and increases intracellular calcium (Maccarrone et al., 1999); phytocannabinoid THC identified as a risk factor in thromboembolism by initiating abnormal platelet activation (Deusch et al., 2004); 2-AG was also identified for its role in platelet activation (Malorni et al., 2004). Cannabinoid system is also discussed for its role in erythrocyte physiology and survival. In a study, it was observed that anandamide (AEA) trigger apoptosis of nucleated cells and eryptosis of erythrocytes, this effect is mediated through the cycloxygenase dependent formation of PGE2 (Bentzen and Lang, 2007), which in turn activates Ca2+ channels leading to entry of Ca2+, water loss and cell shrinkage (Lang et al., 2003; Myssina et al., 2004). AEA was also reported to induce cell death and decrease parasite burden from human erythrocytes infected with *Plasmodium falciparum* (Bobbala et al., 2010). In another study, AEA was observed to downregulate the inflammatory response of macrophages and mesenchymal stem cells by reducing the cytokine level upon lipopolysaccharide (LPS) treatment (Ruhl et al., 2020).

All immune cells have been shown to express CB2 receptor, but the expression levels vary among all immune cells, the developmental stages, and activation states. B-lymphocytes are known to express highest amounts of CB2 receptor protein among blood cells (Cabral and Griffin-Thomas, 2009). The expression of CB2 receptor in immune cells increases upon activation by various insults and stimuli. It has been reported that cannabinoids can control immune cells recruitment and chemotaxis to the sites of infections and injury (Raborn et al., 2008; Sacerdote et al., 2000). In a study, it was reported that CB2 receptor is involved in the regulation of the chemotactic behaviour of B-lymphocytes towards 2-AG during their activation (Tanikawa et al., 2007). On the other hand, CB2 receptor agonist JWH-015 and JWH-133 were reported to inhibit chemotaxis of peripheral blood T-lymphocytes (Coopman et al., 2007; Ghosh et al., 2006); phytocannabinoid THC was found to be immunosuppressive, it affects splenic T-cell response (Massi et al., 1998). In addition to THC, anandamide was also observed to suppress T-cell proliferation and release of TNFα, IL-2 and IFN-γ

(Cencioni et al., 2010). Altogether these results suggest that cannabinoid system is involved in the regulation of the development and function of immune cells. Further research is needed to uncover the functional role and therapeutic potential of the EC system in immune regulation.

ECs have also been observed to regulate bone cells in terms of cell migration, differentiation and survival. Previous studies have reported that bone cells do express both CB1 and CB2 receptor (Gowran et al., 2013; Idris et al., 2005; Idris et al., 2008; Idris et al., 2009). ECs are also involved in bone cell maintenance. Studies have suggested that CB2 receptor activation is also important in osteogenic differentiation of bone marrow mesenchymal stem cells (BM-MSCs) (Yamaguchi and Levy, 2016). Furthermore, THC has been shown as an effective treatment for the Graft versus Host Disease (GVHD) in mouse semi allogenic model (Pandey et al., 2011). GVHD is caused by bone marrow transplantation (BMT) (Jenq and van den Brink, 2010). BMT is highly effective treatment for number of hematological disorders such as aplastic anaemia, acute leukaemia and multiple myeloma (Blazar et al., 2012; Thomas et al., 1977). Recent studies show that CBD can also be beneficial for GVHD prophylaxis patients (Yeshurun et al., 2015). Activation of cannabinoid receptors reduces the expression of adhesion molecule such as vascular cellular adhesion molecule (VCAM-1), intercellular adhesion molecule -1 (ICAM-1), and P-selectin in the aorta of mice (Zhao et al., 2010). Cannabinoids have also been reported to modulate matrix metalloproteinase-9 (MMP-9) production in multiple cells, such as monocytes and neutrophils (Tauber et al., 2012).

5.2. Cannabinoids as immunomodulators

Cannabinoid have immunomodulatory effects in various immunological diseases. Inflammation is one of possible immune regulation by cannabinoids, e.g., activation of CBRs by 2-AG or AEA can modulate inflammation (Russo, 2008). Cannabinoids modulate inflammation not only by activating cannabinoid receptors but also by regulating the metabolism of bioactive lipids (Turcotte et al., 2015). In different studies it has been observed that cannabinoid system is capable to regulate various functions of immune cell, such as the release of pro-inflammatory mediator cytokines, nitric oxide (NO), and reactive oxygen species (ROS) (Alonso-Alconada et al., 2020). Cytokine produced by macrophages and other immune cells are key molecules that regulates inflammation (Boyman et al., 2007). Klein (2005) reported that THC functions as an anti-inflammatory molecule via inhibiting the production of IFN- γ and IL-12 from helper cells (Klein 2005; Klein and Newton, 2007). Obesity is a complex and challenging public health issue; Previous reports have shown that cannabinoid system is involves in regulation of obesity. Deficiency of CB2R in mice improves insulin sensitivity but increases food intake and obesity with age (Table 1) (Agudo et al., 2010). Type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), and cardiovascular diseases could also be induced due to secretion of pro-inflammatory mediators by adipose tissue. Cannabinoid receptors present in adipose tissue could be an effective target for reducing liver impairment in NAFLD (Table 1) (Unamuno et al., 2018). CB2R selective agonist JWH-133 has been reported to be effective against autoimmune uveitis disease (Xu et al., 2007). They have observed that daily dose of JWH-133 for 7- or 15- days post immunization could be beneficial in improving the severity of disease (Xu et al., 2007). In addition, cannabinoids are also reported to be therapeutically effective for treatment of rheumatoid arthritis (Lowin et al., 2019). Rheumatoid arthritis is an autoimmune disease characterized by the destruction of cartilage, progressive disability and premature death due to over production of chemokine and cytokine from resident fibroblast and macrophages (Bartok and Firestein, 2010; Smolen et al., 2016). Currently, Rheumatoid arthritis therapeutics include reduction of proinflammatory cytokine production (Dhillon, 2017). Cannabinoids were observed to be involve in the reduction of proinflammatory cytokine production and they could be effective for the treatment of Rheumatoid arthritis (Gaul and Mellors, 1975). For example, in collagen-induced arthritis mice model, CB2 activation was observed with reduced arthritis score and degradation of AEA (Kinsey et al., 2011). Previous studies suggest that cannabinoids are also able to regulate cell migration thereby it can modulate communication between endothelial cell and leukocytes. 2-Arachidonoylglycerol was observed to modulate human endothelial cell and leukocyte interaction by controlling P and E-selectin expression through CB1/CB2 receptor (Gasperi et al., 2014a, 2014b). Another study by Julian Jehle et al., suggest that elevated level of 2-AG impair endothelial repair and human coronary artery endothelial cell (HCAEC) proliferation in murine experiment (Jehle et al., 2020). Furthermore, an EC like compound DHEA has also been observed with anti-inflammatory property in 3T3-L1 adipocytes (Balvers et al., 2010).

5.3. Cannabinoids in neurological disorders

As discussed elsewhere, CBRs are expressed in CNS and are well known to regulate neurotransmitters release by regulating adenylate cyclase activity (Sanchez-Mut and Gräff, 2015). Reports suggest that neuronal survival is regulated by CB circuitry (Panlilio et al., 2010). The extracellular deposition of β-amyloid peptides is a typical hallmark of Alzheimer's disease (AD) (Aguilera and Santamaría, 2016), CBs are very effective to reduce the harmful effect of β-amyloid in AD (Aguilera and Santamaría, 2016). It has been reported that 2-AG and anandamide are produced during neuronal damage and responsible for neuroprotective effect in AD (Scotter et al., 2010). Cannabinoids have also been shown with positive effects on Parkinson's disease (PD) (Stampanoni Bassi et al., 2017). It has been observed that the CB1R agonist could be one of the possible drugs that can cure PD symptoms such as bradykinesia, rigidity, and tremor (Table 1) (Stampanoni Bassi et al., 2017). Studies have also suggested antiepileptic effect of cannabinoids. In clinical trials, CBD was noticed with antiepileptic property (Silvestro et al., 2019). Moreover, evidences have shown the inhibitory effects of cannabinoids on brain tumour cells. THC and CBD are also reported to have positive effect on chronic traumatic encephalopathy (CTE) (Mez et al., 2017). *Cannabis* exhibits anticlastic, analgesic, neuroprotective, and anti-inflammatory action (Russo, 2008). They are also effective against some psychiatric diseases such as schizophrenia (Robson et al., 2014), bipolar disorder (Ashton et al., 2005), and anxiety (Black et al., 2019). It has been suggested that cannabinoids can be used as a therapeutic target for various diseases, such as multiple sclerosis (Table 1) (Rice and Cameron, 2018), diabetes (Horváth et al., 2012), and allergic asthma (Vuolo et al., 2019). Δ^9-THC is mostly used as a painkiller for multiple sclerosis patients and spinal injuries (Rice and Cameron, 2018). Multiple sclerosis is driven by activated and autoreactive Th-1 and Th-17 cells (Gentile et al., 2020; Chiurchiù et al., 2013) Dendritic cells are also involved in immunopathogenesis of multiple sclerosis. Anandamide has been observed to play crucial role in immunopathogenesis of multiple sclerosis by effecting myeloid dendritic cells and plasmacytoid dendritic cells (Chiurchiù et al., 2013). In addition, anandamide was observed to modulate immune cells by activation of distinct of Toll-like receptors in multiple sclerosis (Chiurchiù et al., 2016).

Cannabinoids have been shown with anti-atherosclerotic effects in mice (Steffens et al., 2005). Furthermore, it was reported that low-dose of dronabinol may have an additive effect when given with modern antiemetics (Grotenhermen and Müller-Vahl, 2012). Cannabinoids are also effective against chronic neuropathic pain but in patients with acute pain they have very little or no effect (Table 1) (Zhao et al., 2010; Jiang et al., 2007; Patinkin et al., 2008). Additionally, one of the EC like compounds, DHEA was detected in brain and shown to be anti-inflammatory, it was also investigated in synaptogenesis (Kim et al., 2011). Lee et al. (2016) observed that DHEA induces neurogenic differentiation via activating GPR110 (Lee et al., 2016).

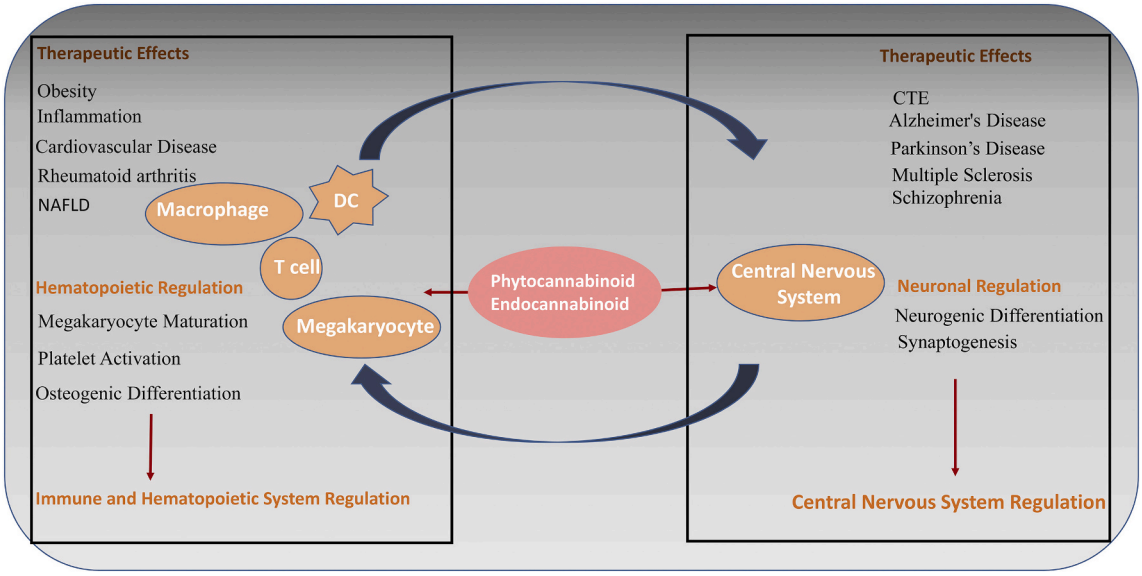

Fig. 2. Cannabinoid system interactions are involved in neuronal and immune regulation. The neuronal and immune system axis have been shown to express cannabinoid receptors and ECs such as anandamide. The endocannabinoid system supports bidirectional communication between neural and immune tissues. Cannabinoids have been reported with therapeutic effects in different neuroimmune diseases.

5.4. Cannabinoids and ECS regulate neuroimmune axis and neuroinflammation

The special properties of ECs as neurotransmitters, their pleiotropic physiological and pathological roles and their impact on the modulation of the immune functions show that the EC system represents a revolving plate of neural and immune interactions (reviewed in, Mestre et al., 2018; Tanasescu et al., 2013). Recently, several studies have reported that CBRs and associated orphan GPCRs are highly expressed in the cells of immune and/or CNS and regulate several neurophysiological events, including key events involved in neuroinflammation. The functions of ECs in neuroimmune axis is of particular of intertest, because the CNS is not only a rich source of ECs but also exquisitely sensitive to inflammation. Several disorders have been identified in which neuroinflammation is a key feature, e.g., multiple sclerosis (MS) and Alzheimer's disease (AD). CBR1/2 and GPR55 receptors are being considered as the emerging targets for the modulation of a variety of events associated with peripheral immune cells and glia (reviewed in, Haugh et al., 2016). It has been shown that the expression levels of CBRs or enzymes of EC system changes in brain regions during neurodegenerative and neuroinflammatory disorders, e.g., it was detected that CB1R expression is more in CNS compared to CB2R, but CB2R can be predominantly expressed more in neuroinflammatory condition (Maresz et al., 2007). Therefore, it is suggested that EC system may play modulating role in the pathophysiological conditions of nervous system via regulating immune function (Wolf and Ullrich, 2008). There are several reports which suggest the neuroprotective role of ECs by controlling the inflammation and apoptosis, because in different brain inflammation conditions the proliferation and differentiation of neural progenitor cells is reduced (Rossi et al., 2010). Therefore, in MS and other neurodegenerative diseases such as AD and PD, neuroinflammation might be controlled by therapeutic strategies involving CBs and EC system. For instance, CB might control the progression of neuronal injury in Parkinson's disease (PD) via influencing local inflammatory events associated with the pathology of the disease (Lastres-Becker and Fernández-Ruiz, 2006). In a study, CB2R was observed with upregulation in microglial cells surrounding beta-amyloid plaques (Ramírez et al., 2005), studies suggest that the differential regulation of EC system is associated with AD pathology and it is under strong influence of the inflammatory CNS environment. In diseases associated with neurodegeneration and neuroinflammation, the increased expression and activation of CB2R is an attempt to halt the activation of microglia, this event is an innate response that try to prevent the inflammatory damage to neurons. On the other hand, this innate response might result in the vulnerability due to the downregulation of CB1R. However, some data, suggest the therapeutic potential of CB stimuli by augmenting the brain innate response (Scotter et al., 2010).

A large amount of data generated in recent years that demonstrated the functional role of cannabinoids in the regulation of the interactions between neurons and immune system. For instance, cannabidiol (CBD), the non-psychotropic cannabinoid derived from Cannabis Sativa, has been demonstrated as a very effective compound because of its antioxidant, anti-inflammatory and anti-apoptotic effects in diverse models of CNS inflammation (Petrosino et al., 2009). A study that discusses the effects of CBD on both in vitro and in vivo models of neuropathologies has shown that CBD exerts its pharmacological activity by targeting reactive astroglia, it efficiently reduces the neuroinflammation in different models of neuropathologies (Iuvone et al., 2009). Furthermore, in a report, CBD was identified as an effective drug to reduce intestinal inflammation via controlling the neuroimmune Axis. In this report, it was shown that CBD regulates the enteroglial-derived S100B protein expression and prevent S100B-mediated amplification of inflammatory/immune response through the involvement of other immune cells (De Filippis et al., 2011).

Different studies have also evaluated the role of CB2R agonists and antagonists in neuroinflammation, e.g., the effects of highly selective CB2R agonist, COR167 were tested on patients suffering from MS, COR167 was observed with immunomodulatory effects on immune cells in both the healthy subject and MS patient in dose dependent manner (Annunziata et al., 2017). It affects the proliferation of PBMNCs and myelin basic protein-reactive T cell lines; it reduces the in vitro migration of immunocompetent cells and reduces the levels of several chemokines (Annunziata et al., 2017). This study suggests CB2R as an effective target to control neuroinflammation. The functional role of CB2R agonist, GP1a was also suggested in the modulation of neuroinflammation (Braun et al., 2018). GP1a reduces anxiety and improve motor function in experimental traumatic brain injury (TBI), this effect is associated with the attenuation of pro-inflammatory M1 macrophage polarization and enhanced anti-inflammatory M2 macrophage polarization (Braun et al., 2018). GP1a was also reported to facilitate recovery

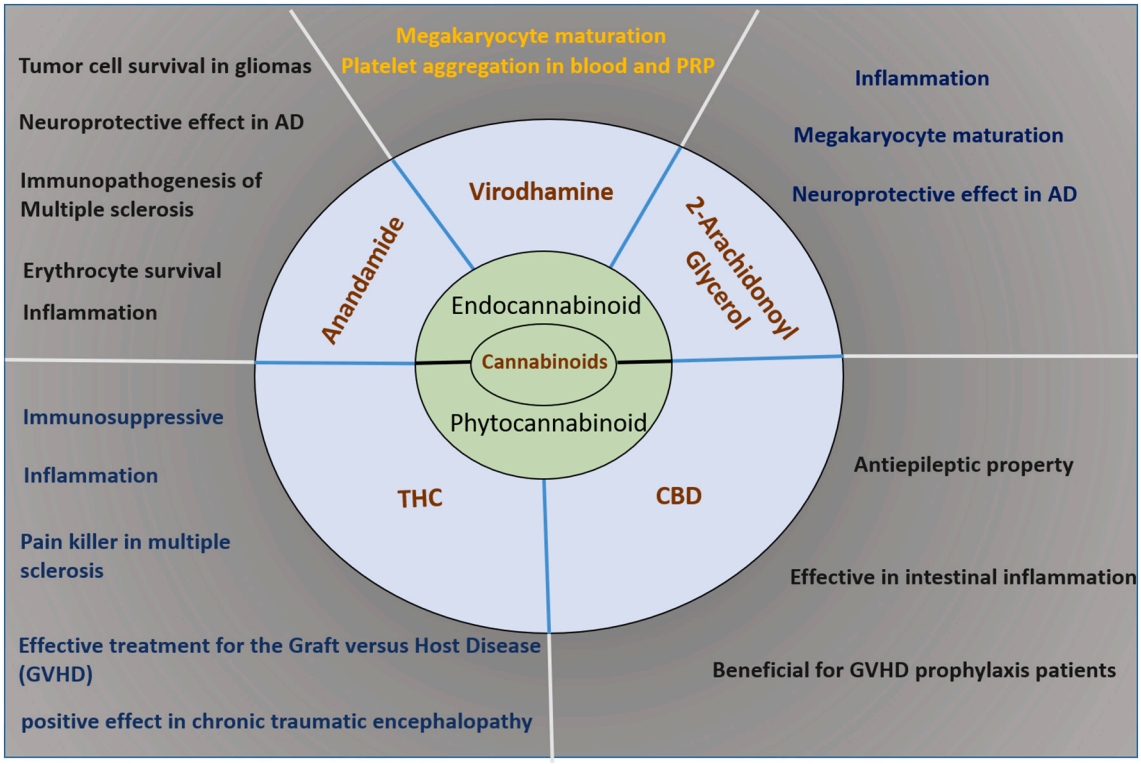

Fig. 3. Schematic representation of the main effects of distinct members of phytocannabinoid and endocannabinoids in system in neuroimmune diseases.

in experimental autoimmune encephalomyelitis (EAE) and long-term reduction in demyelination and axonal loss by reducing the differentiation of Th17 and accumulation of immune cells in CNS (Kong et al., 2014). These are some of the examples defining the functional role of CBs and EC system in the regulation of neuroimmune interactions. However, further research advancements are needed to use EC system and CBs as a potential therapeutic target in different neuroimmune disorders.Figs. 2 and 3

6. Conclusion

In summary, this review represents a report on CBs and EC system from currently available data. In present literature, CBs are classified broadly in two categories, phytocannabinoid and endocannabinoids. Here we reviewed the current available data on the biosynthesis of ECs, CB receptors distribution and well-studied CB receptor signaling pathways. Further, we reviewed recent reports showing cannabinoid system to be involve in the development of hematopoietic cells and the functions of different immune cells. Here we have discussed the immunomodulatory effects of CB-system in blood cells and haematological disorders. Furthermore, we reviewed the involvement of EC system in the development and functions of CNS, because the dysregulation of the ECs is associated with different neurological diseases of brain. The currently available preclinical data suggest the therapeutic potential of CBs and the targets of EC system in different neurological disease such as AD, PD, MS, epilepsy and several psychiatric diseases. In recent reports, the function of neuroimmune axis is discussed in several neuroinflammatory disease. Therefore, this review also discusses recent literature addressing the neuro-immune interactions and their regulation by modulating the CB system in different disease models. The global actions of EC system and its relations with neurons and immune cells is far more complex. Therefore, more advances in the understanding of CB system's efficacy of biased signaling and its modulating role in neuroimmune interactions may provide the basis for future treatments for conditions insufficiently alleviated by current therapies.

Declaration of Competing Interest

Authors have no conflict of interest.

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MicroRNA function in megakaryocytes

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Abstract

Megakaryocytes (MKs), the largest cells in the bone marrow, are generated from hematopoietic stem cells (HSCs) in a sequential process called megakaryocytopoiesis in which HSCs undergo MK-progenitor (MP) commitment and maturation to terminally differentiated MK. Megakaryocytopoiesis is controlled by a complex network of bone marrow niche factors. Traditionally, the studies on megakaryocytopoiesis were focused on different cytokines, growth factors and transcription factors as the regulators of megakaryocytopoiesis. Over the past two decades many research groups have uncovered the key role of microRNAs (miRNAs) in megakaryocytopoiesis. miRNAs are a class of small length non-coding RNAs which play key regulatory role in cellular processes such as proliferation, differentiation and development and are also known to be involved in disease development. This review summarizes the current state of knowledge of miRNAs which have changed expression during megakaryocytopoiesis, also focuses on miRNAs which are differentially regulated during developmental maturation of MKs. Further, we aimed to discuss potential mechanisms of miRNAs-mediated regulation underlying megakaryocytopoiesis and developmental maturation of MKs.

Keywords

Megakaryocyte, miRNA

History

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Introduction

Among the multiple classes of non-coding RNAs (ncRNAs), the most studied ncRNAs are miRNAs. MicroRNAs are approximately 22-nucleotide in length and highly conserved through evolution. They are potent negative regulators of gene expression by degrading mRNA or repressing mRNA translation [1]. The biogenesis of miRNAs has been well reviewed by Krol et al. [2]. To summarize the biogenesis, the pri-miRNA, transcribed from DNA by RNA polymerase II, is processed to pre-miRNA in nucleus by Drosha (type III RNase). The pre-miRNA is exported to cytoplasm and further cleaved by Dicer (another type III RNase) to generate a short, partially double-stranded RNA. One strand of this molecule in the presence of argonaute proteins is loaded into the RNA-induced silencing complex (RISC), the RISC then target mRNA. So far, more than 2694 human miRNAs have been identified and this number is increasing (http://www.mirbase.org/). Recent studies have demonstrated a critical role of miRNAs in normal human hematopoiesis, and deregulated miRNAs have been associated with numerous hematologic diseases (reviewed in [3]). Some of them have been consistently identified in MKs and, together with their targets, they are implicated in MKs development.

Megakaryocytes, the precursors of platelets (thrombocytes), are the largest cells with a polyploidy multi-lobed nucleus in the bone marrow. Megakaryocytes arise from hematopoietic stem cells (HSCs) in a sequential process of development called megakaryocytopoiesis, in which HSCs undergo lineage commitment to become MK-progenitors (MKPs). Further, MKPs generate immature MKs, these immature MKs undergo in the process of endomitosis, cytoplasmic maturation and platelet production [4]. Traditionally, mechanistic studies on megakaryocyte physiology and development biology were primarily focused on identifying functional role of different cytokines and TFs. Over the last decade, multiple reports from different research groups have unveiled molecular and functional roles of miRNAs in megakaryocyte physiology [5].

The essential role of Dicer1 [6] and miRNAs [7] in megakaryocytopoiesis has been established. Recently, Rowley JW et al. [6] revealed that murine MK-specific knockdown of Dicer1 reduces the expression levels of the majority of miRNAs in platelets, that results in altered platelet mRNA expression profiles and mild thrombocytopenia. Many other studies have also addressed various aspects of miRNAs in megakaryocytopoiesis using MKs derived from in vitro cultured CD34+ HSCs or transformed cell lines with megakaryocytic properties, the most abundant miRNAs in megakaryocytes are listed in Table I.

In this review, we bring together most of the miRNAs studies, which were particularly focused on megakaryocytopoiesis and associated miRNAs. From the literature to date, we discuss many miRNAs reported in last two decades in MKs and their functional significance during megakaryocytopoiesis and developmental maturation of MKs.

miRNAs in megakaryocytopoiesis

Megakaryocytopoiesis is a sequential process of MK-lineage commitment, progenitor proliferation and its maturation to

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Table I. miRNAs involved in megakaryocytopoiesis and developmental maturation of MKs.

MicroRNAs involved in earlier steps of megakaryocytopoiesis		
miR-150	Favors MEPs commitment to the MKs lineage by regulating cMyb	[8]
miR-155	Inhibits megakaryocytopoiesis by targeting Ets1 and Meis1	[9]
miR-34a	miR-34a promotes megakaryocyte differentiation of CD34+ cells by regulating MYB expression	[10]
miR-223	miR-223 expression increases during megakaryocytic differentiation of CB-CD34 + cells, it regulates LMO2	[11]
miR-486-3p	Affects HPCs commitment by regulating MAF, it supports the erythropoiesis while restraining the megakaryocytopoiesis	[12]
MicroRNAs involved in later steps of megakaryocytopoiesis		
miR-146a	Observed with down-regulation when human CB derived $CD34^+$ cells induced to differentiate into MKs, and forced over-expression resulted in reduced ploidy levels	[13]
miR-142-3p	miR-142-3p promotes MK maturation and platelet release, it targets a battery of actin cytoskeleton regulators to facilitate proplatelet formation	[14]
MicroRNAs involved in developmental megakaryocytopoiesis		
miR-9	A constitutive reduced expression of miR-9 is observed through MK ontogeny. miR-9 maintain low expression levels of CXCR4 and RUNX1 in neonatal MKs	[15 16 17]
Let-7b	Let-7b was observed with lower expression in neonatal MKs as compared to adult MKs. Lower expression levels of let-7b in neonatal MKs contribute to higher proliferative potential of neonatal MKs	[18]
miR-99a	Observed with higher expression levels in CB CD34+ derived as compared to adult CD34 + derived MK. The high levels of miR-99a maintain the high cell proliferation potential of neonatal MKP by regulating CTDSPL	[19]

terminally differentiated MK. The miRNAs can be broadly classified in three classes: (i) miRNAs involved in earlier steps of megakaryocytopoiesis (ii) miRNAs involved in later steps of megakaryocytopoiesis (iii) miRNAs in developmental maturation of MKs (Table I).

miRNAs involved in earlier steps of megakaryocytopoiesis

miR-150 in lineage commitment

Lu et al. [8] first discussed miR-150 as a critical factor in lineage selection of megakaryocyte-erythrocyte progenitors (MEPs): miRNA expression profiles in MEP, erythroid and megakaryocytic primary cells derived from human umbilical cord blood showed that miR-150 is preferentially expressed in megakaryocytic lineage, further gain- and loss-of-function analysis confirmed that miR-150 drives MEP differentiation towards MKs by regulating *MYB*, a critical target of miR-150. Various *in vitro* and *in vivo* experiments confirmed that miR-150 functionally interacts with the 3′-UTR of *MYB* and down-regulates *MYB* expression, which favors MEPs commitment to the MKs lineage, these observations are consistent with the data showing that low c-Myb levels support megakaryocytopoiesis [20]. Further, Barroga et al. [21] observed that thrombopoietin increased the expression of miR-150, which reduced the c-Myb expression and thereby enhanced thrombopoiesis. In a recent study, miR-150 was identified as an inhibitor of blood count recovery after 5-fluorouracil (5-FU)-induced injury in mice [22]. The forced expression of miR-150 led to negative effects on multiple bone marrow progenitor populations. Whereas, miR-150 knockout mice model observed with normal steady state hematopoiesis, only peripheral platelet counts and spleen megakaryocyte frequency trended lower in the knockouts. However, after 5-FU treatment the number of blood cells was increased in miR-150 knockouts as compared to wild type. In conclusion, the inhibitory effects of miR-150 on murine blood cells recovery after bone marrow damage could be due by its effect on early progenitors. The lower trend of MKs and platelets in miR-150 knockouts shows functional significance of miR-150 in late-stage megakaryocytopoiesis. Taken together, these studies suggest the functional significance of miR-150 in late-stage megakaryocytopoiesis and thrombopoiesis (Figure 1).

miR-155 represses MK differentiation

According to recent study miR-155 is a potent inhibitor of $CD34^+$ hematopoietic stem-progenitor cells (HSPCs) differentiation [23]. In this study, Georgantas et al. [23] performed both the miRNA and mRNA array profiling and the *in silico* prediction from the Transcriptome Interaction Database showed miRNAs that target mRNAs encoding TFs associated with hematopoietic differentiation. Further, functional characterization pointed that miR-155 control on hematopoiesis by regulating expression of multiple hematopoietic differentiation-associated genes, including *C/EBPβ, CREBBP, JUN MEIS1, PU.1, AGTR1, AGTR2*, and *FOS*. MiR-155 is abundantly expressed in early HSPCs and a constitutive reduced expression pattern is observed through MKs differentiation [9,23]. Further, miRNA-mRNA interaction validation studies confirmed the key role of mR-155 in megakaryocytopoiesis by regulating Ets1 and Meis1 [9], which are well known TFs in MK-specific gene promoter regulation. Many MK-specific genes such as *c-MPL* (TPO receptor), play crucial role in megakaryocytopoiesis, the human platelet glycoprotein IIb-IIIa (CD41/CD61) receptor that participates in calcium dependent cell adhesion and normal platelet aggregation, and the platelet glycoprotein IX (CD42a), subunit of the receptor (GPIb/IX/V) for VWF, important for initiating platelet adhesion to damaged blood vessels, are positively controlled by co-operative action of Fli-1, Ets-1 and GATA-1 [24]. The GATA-1 and Ets-1 [25] in combination with Meis-1/Pbx complex [26] transactivate the platelet factor 4 (PF4) gene, MK-lineage specific marker appear in late stage of MK differentiation; MEIS1 is also an essential factor in MK development, MEIS1 deficient embryos reported with extensive bleeding and MK/Platelet deficiency [27]. MiR-155 was also reported for its role in the regulation of chromatin remodeling protein JARID2 [28]. JARID2, the chromatin remodeling protein, is also down regulated by miR-155, which leads to the increased rate of $CD41^+$ MK-precursor formation [28]. Forced over expression of miR-155 inhibits K562 differentiation into MKs and inhibits the CD34+ HSC-derived myeloid and erythroid cell differentiation *in vitro* [23]. Further, O' Connell et al. [29] confirmed that the sustained expression of miR-155 in HSCs cause myeloproliferative disorder (MPD). All together, these data suggest that the miR-155 control megakaryocytopoiesis in a complex manner by regulating gene and TFs having critical role in MK development. MiR-155 with its identified oncogenic potential inhibits megakaryocytopoiesis and its sustained expression cause MPD (Figure 2).

miR-34a in MK differentiation

miR-34a is up-regulated in K562 cells during phorbol ester–induced megakaryocyte differentiation [10,30]. In K562 cells enforced expression of miR-34a induced cell cycle arrest in G1

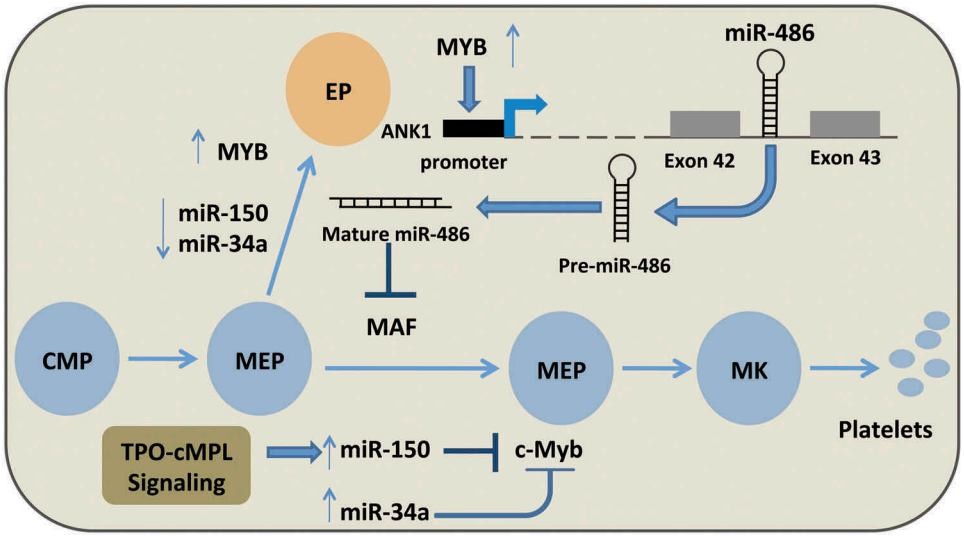

Figure 1. Schematic illustration of the mechanism through which the miRNAs regulate erythroid versus megakaryocyte lineage fate decision. The increased expression levels of MYB favors MEPs commitment to the erythroblast. MYB mediated transactivation of ANK1 up-regulates the expression of miR-486-3p, which in turn targets MAF mRNA and down-regulates its expression. Further, down-regulation of MAF favors the MEP commitment to the erythroid lineage and restrains the MK commitment. Whereas, increased expression levels of miR-150 and miR-34a in earlier steps of megakaryocytopoiesis down-regulates MYB expression, which favors MEP commitment to MK.

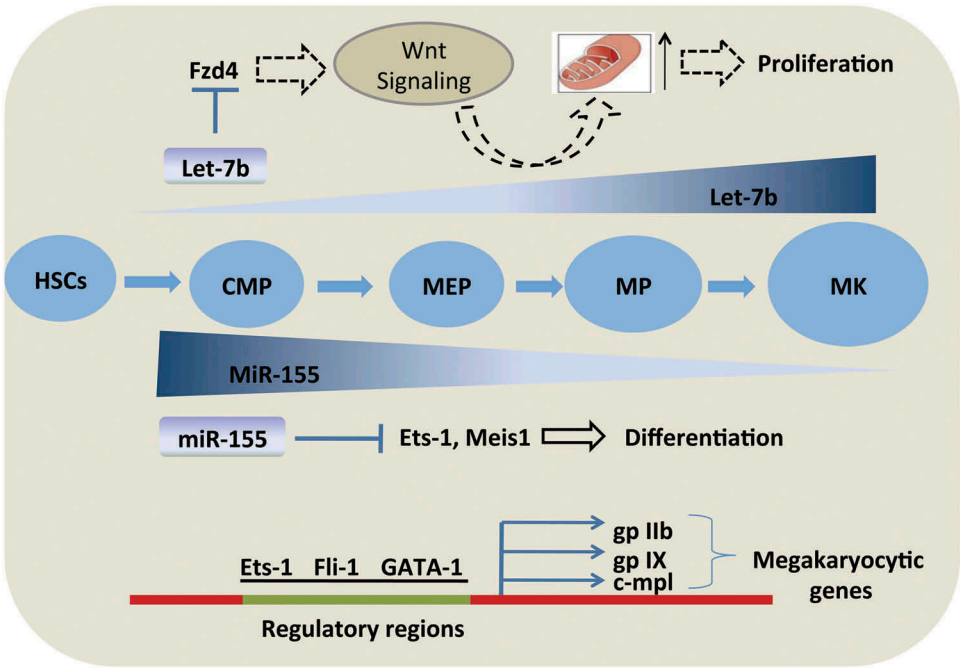

Figure 2. Regulatory role of miR-155 and Let-7b in megakaryocytopoiesis.
Schematic representation of the hematopoietic stem cell lineage commitment to megakaryocyte progenitor and its further maturation to terminally differentiated MKs. The height of triangle indicates the levels of miRNA through HSPCs lineage commitment and differentiation. MiR-155 works as a potent inhibitor of megakaryocytopoiesis by suppressing the Ets-mediated transcription of megakaryocytic gene, whereas Let-7b observed in the regulation of canonical wnt signaling and mitochondrial biogenesis by regulating wnt family protein, Fzd4.

phase and promoted MKs differentiation, further miR-34a was observed with increased rate of megakaryocyte colony formation in TPO-induced differentiation of CD34+ cells, in these cells miR-34a contributes to megakaryocyte differentiation via maintaining the lower levels of MYB (a negative regulator of megakaryocytopoiesis), as well as CDK4 and CDK6 (regulators of the cell cycle) [10]. Another study by Ichimura et al. [30] observed that MEK-ERK pathway in response to PMA strongly activated the transcription of miR-34a, and mitogen-activated protein kinase 1 (MEK1) was identified as a direct target of miR-34a. These studies uncover the functional role of miR-34a in *in vitro* megakaryocyte differentiation. Further, *in vivo* studies need to confirm the role of miR-34a in megakaryocyte physiology (Figure 1).

miR-223 in MK differentiation

miR-223, a fine-tuner of granulocyte differentiation, was observed with a constitutive increased expression during megakaryocytic

Table II. Studies on molecular targets and function of different miRNAs in MK.

miRNA	Targets	Function	
miR-150	cMyb	Favors MEPs commitment to the MKs lineage	[8]
miR-155	Ets1 and Meis1	mR-155 inhibits megakaryocytopoiesis by targeting Ets1 and Meis1	[9]
		Sustained expression of miR-155 in HSCs causes MPD	[29]
		Forced over-expression of miR-155 inhibits K562 differentiation into MKs	[23]
miR-146	CXCR4	miR-146a expression is down-regulated when human CB derived $CD34^+$ cells induced to differentiate into MKs, MiR-146a over-expression impaired megakaryocytopoiesis	[13]
		miR-146a was up-regulated when human and murine HSCs were induced to differentiate into MKs, and forced over-expression observed with no significant effects on megakaryocytopoiesis	[32]
		Knock-down of miR-146a in mouse HSPCs observed with increased platelets in the blood and MKs in the marrow over-expression of miR-146a noticed with no significant effect on platelet and MKs	[33,34]
miR-9	CXCR4 and RUNX1	A constitutive reduced expression of miR-9 through MK ontogeny was noticed	[15 16 17]
		Pre-miR-9 transfection in Meg01 and Dami cells observed with lower levels of megakaryocytic marker-CD61 and increased rate of cell proliferation	[17]
miR-34a	MYB	miR-34a is up-regulated during PMA induced megakaryocytic differentiation of K562 cells Enforced expression of miR-34a promotes MK differentiation of CD34+ cells	[10]
	MEK1	miR-34a inhibits cell proliferation by targeting MEK1 during megakaryocytic differentiation of K562 cells	[30]
miR-130a	MAFB	miR-130a is down regulated in differentiated MKs derived from CD34+ bone marrow cells, and targets MAFB	[7]
miR-10a	HOXA1	miR-10a is down regulated in CD34+ bone marrow progenitor derived MKs, and targets HOXA1	[7]
miR-27a	RUNX1	During megakaryocytic differentiation of K562 cells Runx1 and miR-27a are engaged in a regulatory interplay involving positive regulation of miR-27a expression by Runx1	[35]
miR-181	Lin28	miR-181 targets Lin28, which is a negative regulator of Let-7 miRNAs. Over-expression of miR-181 during TPA induced megakaryocytic differentiation indirectly increased the Let-7 and enhanced differentiation	[36]
miR-223	LMO2	miR-223 expression increases during megakaryocytic differentiation of CB-CD34+ cells, it regulates LMO2	[11]
		Over-expression of miR-223 during PMA induced megakaryocytic differentiation reduces LMO2 levels and increases megakaryocytic differentiation	[31]
Let-7b	Fzl4	Differentially regulated though development, observed with lower expression levels in neonatal as compared with adults MKs. Play role in developmental maturation of MKs	[18]
miR-28a	MPL, E2F6 And MAPK1	Expression of miR-28 in CD34+ derived MKs inhibited terminal differentiation Increased expression levels of miR-28a were noticed in BCR-ABL negative myeloproliferative neoplasms (MPN; polycythemia vera, essential thrombocythemia, primary myelofibrosis) patients	[37]
miR-486-3p	MAF	miR-486-3p regulates MEP commitment, it supports the erythropoiesis while restraining the megakaryocytopoiesis	[12]
miR-142	Wasl, Cfl2, Twf1, Itgav or Grlf1	It supports MK polyploidization, maturation and proplatelet release. It targets a group of actin cytoskeleton regulators and maintains cytoskeleton dynamics	[14]
miR-99a	CTDSPL	miR-99a maintain the high cell proliferation potential of neonatal MKP by regulating CTDSPL miR-99a expression was also reported in megakaryoblastic leukemic cell lines	[19 38]
miR-145	TIRAP	miR145 knockdown in mouse HSPCs results in abnormal megakaryocytopoiesis	[30]
miR-125b		miR-125b is down regulated during megakaryocytopoiesis miR-125b proposed for its function in cell proliferation and survival of neonatal megakaryocytes	[39]

differentiation of CB-CD34+ cells [11]. Further, experimental evidences confirmed that miR-223 directly interacts with the LMO2 3′UTR. Moreover, functional studies showed that the over-expression of miR-223 reduced the LMO2 protein levels and impaired erythroid differentiation. Another study by Yuan JY et al. [31] showed that LMO2 expression sharply reduces during PMA induced megakaryocytic differentiation of K562 cells, and the over-expression of miR-223 reduces the protein levels of LMO2 and increases megakaryocytic differentiation. Thus it is clear that miR-223 may control the MEP commitment process by regulating LMO2 expression (Table II).

miR-486-3p and ankyring (ANK1)

Recent study evidenced the role of transcription factor MYB in erythroid versus MK lineage fate decision through the miR-486-3p mediated down-regulation of MAF, It was demonstrated that MYB regulates the expression of miR-486-3p through the transactivation of its host gene, ANK1, over-expression and down-regulation experiments confirmed that MYB supports the erythropoiesis while restraining the megakaryocytopoiesis through the MYB/miR-486-3p/ANK1 axis [12] (Figure1).

MiRNAs involved in later steps of megakaryocytopoiesis

miR-146a with complex effects on megakaryocytopoiesis

Different research groups demonstrated that miR-146a dramatically changes its expression when HSCs differentiate into MKs, but there is conflicting evidence about the direction of change and the function of miR-146a in megakaryocytopoiesis. Labbaye et al. [13] observed that miR-146a expression is down-regulated when human CB derived $CD34^+$ cells are induced to differentiate into MKs and the forced over-expression of miR-146a significantly reduced the ploidy levels in CB derived MKs. Further, miR-146a down-regulation experiments confirmed that the normal phenotype of CB-derived MKs correlates with the phenotype observed after down-regulation of miR-146a. In normal megakaryocytopoiesis, they identified PLZF/miR-146a/CXCR4 pathway: in MK differentiation from

CB-derived megakaryocytic progenitor, PLZF (promyelocytic leukemia zinc-finger) suppresses miR-146a transcription and thereby activates CXCR4 translation. The CXCR4 was identified as a target negatively regulated by miR-146a that, in turn, under the transcriptional negative control of PLZF [13]. In contrast, Opalinska et al. [32] found the up-regulation of miR-146a when human and murine HSCs were induced to differentiate into megakaryocytes. Further, forced over-expression of miR-146a was observed with no significant effects on megakaryocytopoiesis in both the *in vitro* and *in vivo* studies, also the over expressed miR-146a does not alter circulating platelet number and activation. In addition, two recent studies by Starczynowski et al. [33,34] recognized the expression and function of miR-146a in mouse hematopoietic stem/progenitor cells, but the conflicts were not clarified, as compared with HSPCs, miR-146a expression was lower in MEP, and the knockdown of miR-146a in HSPCs observed with increased platelets in the blood and MKs in the marrow, this observation is consistent with previous findings by Labbaye et al. [13],, whereas the over-expression of miR-146a did not have significant effect on platelet and MKs, similar to observations by Opalinska et al. [32]. All together, the data from different studies suggests the regulatory role of miR-146a in megakaryocytopoiesis, but the effects are complex and vary with different conditions and developmental stage (Table II).

miR-142 in MK maturation

miR-142 is a broadly expressed miRNA in adult mouse hematopoietic lineage, moreover a critically important miRNA for MK maturation and proplatelet release. Loss-of-function mutation of miR-142 was observed in mouse with impaired megakaryocyte maturation, inhibition of polyploidization, abnormal thrombopoiesis, and thrombocytopenia. Further, it was confirmed that miR-142-3p targets a group of actin cytoskeleton regulators (Wasl, Cfl2, Twf1, Itgav or Grlf1) during megakaryocytopoiesis to maintain cytoskeletal dynamics and normal hemostasis [14] (Table II).

miRNAs in developmental maturation of MKs

miR-9 in developmental regulation of RUNX1 and CXCR4

miR-9 has been reported as important regulator of cell fate in hematopoietic development; Bluteau et al. [15] in a miRNA microarray study first noticed the differential expression of miR-9 during MK ontogeny, they observed a constitutive reduced expression of miR-9 through MK ontogeny, whereas the *CXCR4*, miR-9 putative target, was noticed with increased expression through MK ontogeny. The same results were observed by Ferrer-Marin et al. [16], they carried out miR-9 transfection studies in MEG01 cells to investigate whether miR-9 regulates CXCR-4 protein expression and observed significantly lower CXCR-4 protein levels in pre-miR-9 transfected cells. CXCR4, a receptor for SDF-1, is expressed on many hematopoietic cells including MKs. SDF-1 has been shown to stimulate maturation of human-MKs in vitro [40] and murine-MKs in vivo [41]. Interestingly, the administration of SDF-1 in conjunction with fibroblast growth factor-4 (FGF-4), restored thrombopoiesis in Thpo(-/-) and Mpl(-/-) mice [41]. Maturational effects of SDF-1/CXCR-4 axis in MK were thought to be mediated by stimulating the MKP interactions with sinusoidal bone marrow endothelial cells (BMECs), allowing the progenitors to relocate to a microenvironment that is permissive for MK maturation. Recently, in a miRNA-microarray study, we observed that miR-9 is the highest up-regulated miRNA in CB-derived MKs compared with PB-derived MKs. Interestingly these differences are consistent through all the stages of megakaryocytopoiesis [17]. Further, RUNX1 emerged as putative target of miR-9 by several miRNA databases; hypothetically the down-regulated miRNAs through development and differentiation unblock the expression of genes involved in developmental maturation and differentiation of cell. More interestingly, we observed that hsa-miR-9 and RUNX1 expressions levels are inversely related in neonatal versus adult MKs [17]. To confirm the functional significance of miR-9 in RUNX1 regulation in human MKs, we carried out pre-miR-9 transfection studies in Meg01 and Dami cell lines, and observed significant lower expression of RUNX1 protein and its regulated gene CD61 mRNA with increased rate of cell proliferation. RUNX1 is a critically important TF in MKs development, in human, loss of function RUNX1 mutations are common in familial platelet disorder with predisposition to acute myeloid leukemia (FPD/AML), which is characterized by thrombocytopenia and propensity for progression to AML [42]. Moreover, Runx1 conditional knock-out mice phenotype characterized with abundant number of small low ploidy-MKs, which closely resembles normal neonatal megakaryocytopoiesis [43]. Jacob et al. [44] observed impaired niche interactions and increased rate of stem/progenitor cells proliferation with down regulation of *Cxcr4* in *Runx1*$^{-/-}$ mice. The increased hematopoietic stem/progenitor cell fraction with the down-regulation of *Cxcr4* expression in *Runx1*$^{-/-}$ mice indicates the transcriptional regulation of *Cxcr4* expression and niche interaction by Runx1. All together, miR-9 noticed to regulate developmental differences in CXCR4 and RUNX1 expression in neonatal versus adult MKs, also from previous finding it appears that miR-9 may influence CXCR4 expression indirectly via regulating the RUNX1 expression. The differential expression of miR-9 in neonatal versus adult MKs implies its functional role in the developmental differences of neonatal and adult MKs whereas the RUNX1 and CXCR4 could be the downstream factors playing role in niche interaction and maturation of MKs (Figure 3).

miR-99a promotes cell proliferation

Garzon et al. [7] compared the miRNAs expression profile of megakaryoblastic leukemic cell lines with that of CD34+ progenitors derived MKs, data demarcate the up-regulation of miR-99a, miR-101, miR-126, miR-135, and miR-20 in megakaryoblastic leukemic cell lines. Other studies also showed the oncogenic potential of miR-99a [38]. Further, Kandi et al. [19] did a comparative study between neonatal (CB)- and adult (PB)- CD34 + progenitor derived MKs and observed up-regulation of miR-99a in neonatal MKs, whereas miR-99a target, CTDSPL, a phosphatase-like tumor suppressor, was down regulated in neonatal MKs, which in turn led to the phosphorylation of RB and accumulation of E2F1. The E2F1 promotes cell cycle progression by increasing cyclins (CyclinD1, cyclin D2 and Cyclin D3) expression. All together data suggests that the high levels of miR-99a maintain the high cell proliferation potential of neonatal MKP (Figure 4).

Let-7b in developmental maturation of MK

Li X et al. [36] observed an increased expression of Let-7b and other let-7 miRNAs during MK differentiation, Let-7b was also observed as a differentially regulated miRNA through development [18]. Multiple observations support the existence of developmental differences in megakaryocytopoiesis; neonatal MK progenitors are hyperproliferative and give rise to MKs smaller and of lower ploidy than adult MKs. Let-7b was observed with lower expression in neonatal MKs as compared to adult MKs.

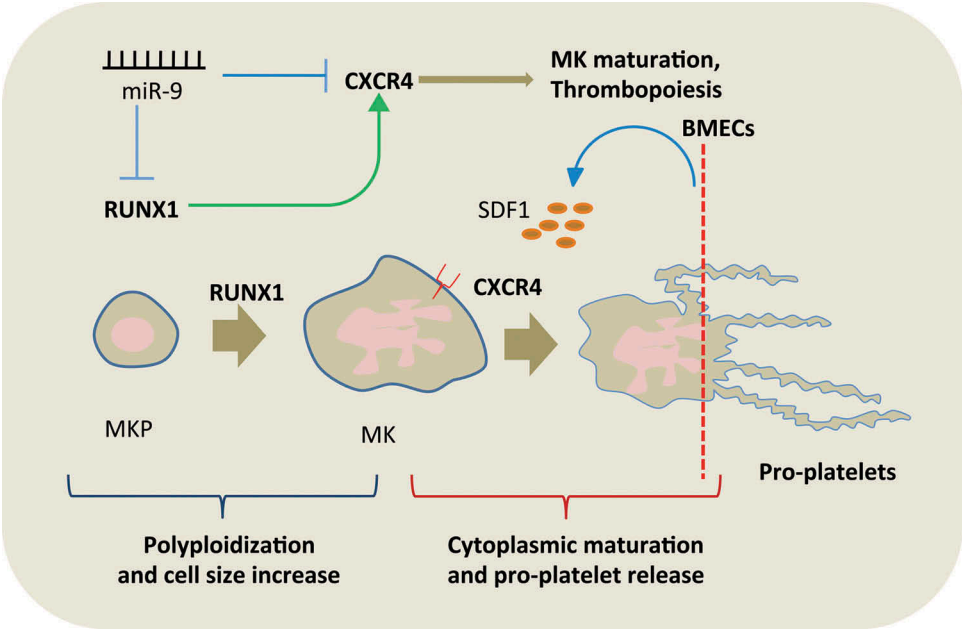

Figure 3. Regulatory role of miR-9 in megakaryocytopoiesis.
The cartoon summarizes key steps, endomitosis and cytoplasmic maturation in megakaryocytopoiesis and platelet production. Extracellular signaling pathway, SDF-1/CXCR-4, is involved in the maturational effects of MK and these effects thought to be mediated by stimulating the MKP interactions with sinusoidal bone marrow endothelial cells (BMECs), allowing the progenitors to relocate to a microenvironment that is permissive for MK maturation and proplatelet release. MiR-9 is a potent inhibitor of MK development. MiR-9 directly targets the expression of RUNX1, having role in MK endomitosis, and CXCR4, SDF-1 receptor involved in MK maturation and proplatelet release.

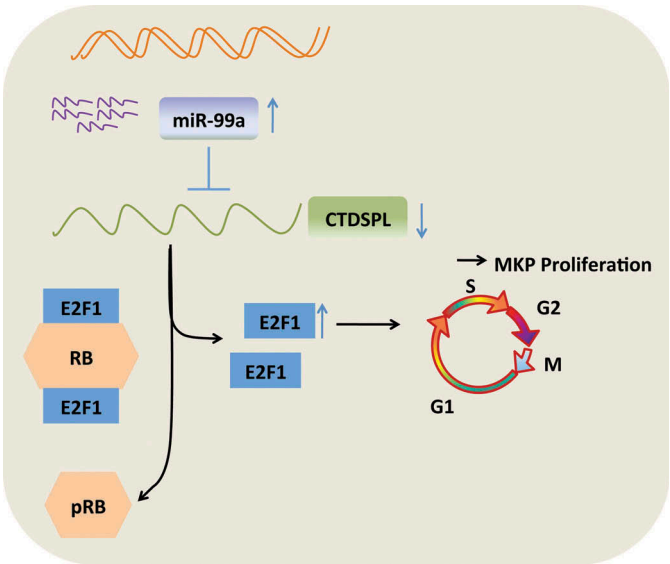

Figure 4. Schematic representation of the pathway modulated by miR-99a in MK.
miR-99a targets CTDSPL, which modulates E2F1, a cell cycle effector protein. miR-99a over-expression induces progression through the G1/S transition and promotes S-phase entry. miR-99a down-regulates the target gene CTDSPL, which in turn results in the phosphorylation of RB and accumulation of E2F1.

Further, Introduction of let-7b oligos in CB-MK progenitors resulted in the reduction of progenitor proliferation [18]. From the above observations it appears that lower expression levels of let-7b in neonatal MKs contribute for higher proliferative potential of neonatal MKs compared to adult-MKs. Further, Let-7b over-expression was observed with down-regulation of Fzd4 (Frizzled-4), a wnt family receptor protein and lower expression levels of wnt signaling molecules. Also Let-7b down regulation observed with induced wnt signaling and mitochondrial biogenesis and its markers PGC-1α and NRF1 [18]. These observations suggest inhibitory effects of Let-7b on Fzd4 expression, which could be a possible regulatory mechanism on wnt signaling pathway and mitochondrial biogenesis. Further experimental evidences are needed to confirm the regulatory role of Let-7b on wnt signaling and mitochondrial biogenesis (Figure 2).

Other miRNAs in megakaryocytes

In addition to those discussed above, many other miRNAs were also noticed in MKs but less well studied in megakaryocytopoiesis. Many research groups observed that most of the miRNAs are down-regulated during MK differentiation [7,32] and development [17,38], hypothetically the down regulation of miRNAs unblocks the expression of target genes involved in MK differentiation and development. Example: in a miRNA profiling study, miR-130a and miR10a were down regulated in *in vitro*-differentiated MKs derived from $CD34^+$ cells, further experimental evidences confirmed that miR-130a targets the *MAFB*, which is involved in the promoter activation of GPIIB, a crucial protein in platelet biology, further *HOXA1* was also observed as direct target of miR-10a [7] (Table II). Ichimura et al. [30] proposed that miR145 can directly target 3′UTR of TIRAP (Toll–interleukin-1 receptor domain– containing adaptor protein), and miR145 Knockdown in mouse HSPCs results in thrombocytosis, neutropenia and megakaryocytic dysplasia (Table II). miR-125b was observed with down regulation during megakaryocytopoiesis, further it was proposed that miR-125b can control cell proliferation and survival of neonatal megakaryocytes [39] (Table II). Thrombopoietin (Tpo) through its receptor, MPL, activates many signaling pathways and can regulate all phases of MK development, interestingly the Tpo receptor is regulated by many miRNA. Girardot et al [37] reported that miR-28a targets the 3′UTR of MPL, inhibiting its translation, as well as other proteins involved in MK differentiation, such as E2F6 and

MAPK1. Expression of miR-28 in CD34+ derived MKs inhibited terminal differentiation (Table II).

Several microRNAs were also observed with increased expression levels during differentiation and development of MKs. For Example, TPA induced megakaryocytic differentiation of K562 cells was observed with increased expression levels of miR-181. miR-181 over-expression affects the stability of Lin28 in K562 cells. In this study a reciprocal regulatory loop between the let-7 miRNAs and Lin28 was observed. The over-expression of miR-181 during TPA induced megakaryocytic differentiation interrupts the Lin28–let-7 reciprocal regulatory loop by targeting Lin28, which resulted in the greater levels of mature Let-7 and enhanced differentiation [36] (Table II). TPA induced megakaryocytic differentiation of K562 cells noticed with the binding of RUNX1 to miR-27a regulatory region and increased expression of miR-27a, that in turn attenuates Runx1 expression. Thus, during megakaryocytopoiesis, Runx1 and miR-27a appear to work in a feedback loop involving positive regulation of miR-27a by Runx1 [35] (Table II).

Conclusion

Several miRNA expression profiles from different research groups in last decade have increased the number of MK derived miRNAs, though all are not functionally characterized for their target selection and regulatory role in MK physiology. Hundreds of miRNAs have been identified yet in MKs but still new miRNAs are emerging. Here we reviewed the expression pattern of different miRNAs and their targets in megakaryocytopoiesis. Moreover, we have reviewed their positive and negative functions during megakaryocytopoiesis and their role in developmental megakaryocytopoiesis. We discussed the current literature on the functions of miRNAs in MKs. A special emphasis was given to miRNAs that have demonstrated a potential in several *in vitro* and *in vivo* studies in MKs. The data from different studies discussed in this review suggests that miRNAs are important regulators of megakaryocytopoiesis, they involved in the regulation of many molecular mechanisms via creating positive and negative feedback loops. Few miRNAs were also observed with more complex effects which vary with different conditions and developmental stage. Also, there are conflicting evidences on the expression and function of some of the miRNAs reported in megakaryocytopoiesis.

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Disclosure of conflicts of interest

The authors declare that they have no conflict of interest.

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SETDB1 modulates the differentiation of both the crystal cells and the lamellocytes in *Drosophila*

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ABSTRACT

Proper genetic and epigenetic regulation is necessary to maintain the identity and integrity of cells. Enzymes involved in post-transcriptional modifications of histones are key factors in epigenetic mechanisms. Such modifications are also gaining importance for their role in growth and development of cancer. SETDB1 catalyzes the epigenetic mark of lysine-9 methylation of histone-3. In this study, we explored the role of SETDB1 in *Drosophila* hematopoiesis. We show that SETDB1 controls the differentiation of matured blood cells in wandering third instar larvae. There are three matured blood cells in wild type *Drosophila melanogaster*: plasmatocytes, crystal cells and lamellocytes. We found that loss-of-function mutants of SETDB1 show hematopoietic defects; increased blood cell proliferation, decreased number of crystal cells, greater differentiation of blood cells into lamellocytes, dysplasia of the anterior lobes of lymph gland and presence of hematopoietic tumors. Cell type specific knockdown of SETDB1 provided similar phenotype i.e., decreased number of crystal cells and an increase in lamellocyte differentiation. In animals with loss of function of SETDB1, *Notch* pathway was downregulated. Further, overexpression of SETDB1 in blood cells resulted in an increase in the number of crystal cells. This increase is accompanied with an increase in the number of $Notch^{ICD}$ expressing cells. We therefore performed genetic rescue using UAS-GAL4 system to rescue loss of function *SETDB1* mutants. Our data show that the rescued larvae carrying a wild type copy of SETDB1 in mutant background are devoid of blood tumors. We have identified a novel dual function of SETDB1 methylatransferase as a critical regulator of two of the matured hemocytes, crystal cells and lamellocytes. We propose a novel role of SETDB1 in modulating the differentiation of crystal cells and lamellocytes from a common progenitor and underscore the importance of SETDB1 in *Drosophila* blood tumor suppression.

1. Introduction

Drosophila melanogaster has a simple hematopoietic system consisting of three known matured blood cells [plasmatocytes (>95%), crystal cells (<5%) and lamellocytes (<1%)] at larval stages (Crozatier and Meister, 2007; Lemaitre and Hoffmann, 2007; Meister and Lagueux, 2003). Blood cells are found in circulating hemolymph, hematopoietic organ, the lymph gland, and in the posterior hematopoietic compartment consisting of sessile cells residing under the cuticle (Crozatier and Meister, 2007; Jung, 2005; Krzemień et al., 2007; Lebestky et al., 2003; Lemaitre and Hoffmann, 2007; R. Markus et al., 2009; Meister and Lagueux, 2003). Some population of sessile cells are seen attached to the imaginal discs, but majority of the population of the sessile cells form "banded patterns" under the larval epidermis. Functional aspect of this posterior hematopietic compartment (PHC) is not yet understood. Previous studies suggest PHC replenishes the progenitor pool in the circulating hemolymph (R Markus et al., 2009) Lymph gland has a pair of anterior lobes and two to six pairs of posterior lobes along the dorsal vessel (Holz, 2003; Jung, 2005; Minakhina and Steward, 2010). Anterior lobes are compartmentalized into three different regions: medullary zone, cortical zone and posterior signaling center (PSC). Hematopoietic niche is considered as the PSC of lymph gland. Lymph gland harbors both mature (cortical zone) and precursor (medullary zone) blood cells, in the anterior lobes. While posterior lobes contain primarily the precursor and quiescent blood cells (Holz, 2003; Jung, 2005; Minakhina and Steward, 2010). Upon receiving signals for both, proliferation and differentiation,

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hematopoietic quiescence is lost and precursor cells differentiate forming matured hemocytes.

Prohemocytes are destined to differentiate into either progenitors or the matured blood cells (plasmatocytes, crystal cells or lamellocytes). Plasmatocyte differentiation occurs twice during *Drosophila* development: embryonic head development and larval development (Holz, 2003; Wood and Jacinto, 2007). Plasmatocytes are phagocytic macrophages required for elimination of bacteria, apoptotic cells and small foreign particles (Crozatier and Meister, 2007; Lemaitre and Hoffmann, 2007). Crystal cells contain paracrystalline inclusions within them. These cells have a role in wound healing and also during melanization reaction (Rizki and Rizki, 1959). Lamellocytes differentiate upon wasp infestation, to encapsulate parasitoid wasp egg (Lemaitre and Hoffmann, 2007; RIZKI and RIZKI, 1994; Small et al., 2012; Sorrentino et al., 2004). There are three prophenol oxidase enzymes know of which ProPO1 and ProPO2 are exclusively expressed in crystal cells and ProPO3 is expressed in blood cells of $hop^{Tum\text{-}l}$ (JAK-STAT mutant) mutants devoid of crystal cells containing increased lamellocytes (Irving et al., 2005; Nam et al., 2008). ProPO3 is shown to function in lamellocyte mediated melanization in *Drosophila* (Nam et al., 2008). Mutants with hyperactive hematopoietic signaling pathways such as JAK-STAT, RAS and NF-κB show increased number of lamellocytes in circulating hemolymph along with abnormal plasmatocytes (Asha et al., 2003; Kalamarz et al., 2012; Minakhina et al., 2007; Minakhina and Steward, 2010; Qiu et al., 1998).

Various existing models have speculated the formation of the three matured blood cells from a common prohemocyte progenitor (Lanot et al., 2001) (Krzemień et al., 2007).

In the past decades experimental evidences provided sufficient information for the existence of bipotent prohemocytes (Fossett, 2013; Gold and Bruckner, 2014; Honti et al., 2014; Kroeger et al., 2012; Owusu-Ansah and Banerjee, 2009; Stofanko et al., 2010; Wang et al., 2014). Previous literature also discuss the differentiation of lamellocytes from plasmatocytes due to over-expression of *Chn* (Stofanko et al., 2010). Gcm, a transcription factor is expressed in all prohemocytes in the early stages of larval development (Bataille et al., 2005). Glial cells missing (*Gcm*) and *Gcm2* are related transcription factors having a crucial role in the differentiation of plasmatocytes (Bernardoni et al., 1999) (Kammerer and Giangrande, 2001) (Alfonso and Jones, 2002). Lack of *Gcm* and *Gcm2* hinders plamatocyte differentiation although crystal cells are not affected. *Gcm* and *Gcm2* double mutants give rise to increased number of crystal cells with a reduction in the number of plasmatocyte population (Williams, 2007). These studies have shed light on the bipotent nature of the prohemocytes. Toll, JAK/STAT, Notch, Pvr, Wg, Hippo, hedgehog and Ras pathway are some of the signaling pathways crucial for the development of blood cells (Asha et al., 2003; Chiu et al., 2005; Duvic et al., 2002; Harrison et al., 1995; Makki et al., 2010; Mandal et al., 2004; Milton et al., 2014; Minakhina et al., 2011, 2007; Mondal et al., 2014; Morin-Poulard et al., 2013; Mukherjee et al., 2011; Qiu et al., 1998; Reimels and Pfleger, 2015; Tokusumi et al., 2010). Hematopoietic pathways are conserved between *Drosophila* and mammals including various genes crucial in deciding the fate of precursors to differentiate into matured blood cells. Some of these comprise Serpent (GATA transcription factor)(Fujiwara et al., 2004; Tokusumi et al., 2010), Lozenge (Acute Myeloid Leukemia-1 like protein)(Canon and Banerjee, 2000; Sinenko et al., 2010), Dorsal (Nuclear transcription Factor-kappa B)(Hayden et al., 2006; Qiu et al., 1998), Gcm (Glial cell missing)(Jacques et al., 2009), Pannier (related to GATA-1) (Minakhina et al., 2011), STAT (Minakhina et al., 2011; Morin-Poulard et al., 2013; Ward et al., 2000), U-Shaped (friend of GATA homologue)(Tokusumi et al., 2010) and Collier (*Drosophila* orthologue of EBF-1)(Krzemień et al., 2007). In the PSC we find expression of genes such as Antennapedia (homeotic gene), Serrate (Ligand for Notch pathway), collier (transcription factor), Hedgehog (segment polarity gene)(Krzemień et al., 2007; Lebestky et al., 2003; Mandal et al., 2007; Tokusumi et al., 2010).

Genetic aberrations affecting NF-κB, JAK/STAT, and Ras pathways show inflammation and dysplasia of blood cells leading to development of blood tumor formation (Asha et al., 2003; Chiu et al., 2005; Harrison et al., 1995; Paddibhatla et al., 2010). Hematopoietic mutants with increased lamellocytes such as $hop^{Tum\text{-}l}$, $Ubc9^{-/-}$show aggregates and melanotic tumors in circulating hemolymph. Aggregates and microtumors are differentiated based on their sizes or number of blood cells found in each of these structures. Tumors show projection area >10,000 μm^2 while aggregates are <10,000 μm^2 (Kalamarz et al., 2012). Hallmarks of cancer arise due to alterations in the genome. Sometimes, epigenetic regulation is also disrupted in cancerous cells. Epigenetic modification such as DNA methylation, histone methylation and acetylation need proper regulation for the functioning of normal cells (Costa, 2010; Grønbæk et al., 2007; Hanahan and Weinberg, 2000) Hanahan and Weinberg, 2011. DNA hypermethylation is a mechanism known to inhibit the expression of tumor suppressor genes (Gou et al., 2010; Vincent et al., 2007; Wajed et al., 2001). Even though research on the signaling pathways affected during cancer growth and development has been ongoing, there exists little evidence for the role of epigenetic regulators, specifically epigenetic mechanisms in melanotic tumor growth and development in *Drosophila*. Inspite of researchers being cognizant about the role of epigenome in hematopoiesis it remains an arena unexplored from the perspective of single gene mutations causing the hematopoietic defects. Various scientific groups shed light on hematopoietic tumors in *Drosophila* (Crozatier and Vincent, 2011; Kalamarz et al., 2012; Minakhina et al., 2007; Minakhina and Steward, 2006; Morán et al., 2015; Paddibhatla et al., 2010; Reimels and Pfleger, 2015). Knowledge gained on epigenetic mechanisms playing a role for hematopoiesis and understanding the perturbations in epigenome of blood cells in *Drosophila* can help to further decipher the steps involved in growth and development of blood tumors.

We explore through this study the role of a histone modifying enzyme, SETDB1 in *Drosophila* hematopoiesis. SETDB1 belongs to the KMT1 family of methyltransferases, methylating histone-3-lysine-9 for which SET domain and the two neighboring pre-SET and post-SET domains are required (Schultz et al., 2002; Stabell et al., 2006). Wild type *Drosophila* express SETDB1 in all the developmental stages of the life cycle (Stabell et al., 2006). Referred also as *eggless*, this gene is expressed during oogenesis in both, germ cells and somatic cells. Preceding studies established the role of SETDB1 for (1) egg chamber formation during oogenesis, (2) wing development and (3) eye development (Clough et al., 2014, 2007; Gou et al., 2010; Li et al., 2006; Lundberg et al., 2013; Seum et al., 2007; Tzeng et al., 2007; Yoon et al., 2008). Apart from these results we also know from earlier literature that SETDB1 is required for silencing *retinoblastoma* gene (Gou et al., 2010). Furthermore it has a significant contribution in silencing chromosome four in *Drosophila* (Seum et al., 2007). We present data through this study on *Drosophila* SETDB1's novel function as a tumor suppressor in larval hematopoiesis.

Based on our experimental evidences we bring to focus a novel role of SETDB1 in (1) differentiation of both crystal cells and lamellocytes (2) in suppressing blood tumor formation.

2. Results

2.1. SETDB1 mutants exhibit hematopoietic defects

To elucidate the importance of SETDB1 in *Drosophila* hematopoiesis we studied loss-of- function mutants of *SETDB1*. Wandering third instar mutant larvae showed melanotic microtumors through the cuticle as depicted by tumor penetrance also in transheterozygotic mutant larvae i.e. $SETDB1^{235/1473}$, $SETDB1^{235/235}$, $SETDB1^{1473/1473}$ (Fig. 1A–D). To determine if the blood cells involved in and contributing to the formation of these melanotic microtumors we counterstained the dissected circulating hemolymph from wandering third instar larvae with nuclear dye (DAPI-blue) and cytoskeletal specific dye (polymerized F-actin-red). Hemolymph in heterozygote larva ($SETDB1^{+/-}$) show typical wild type larval blood cells (Fig. 1E). Compared to heterozygote siblings hemolymph from transheterozygotic mutants ($SETDB1^{235/1473}$) showed

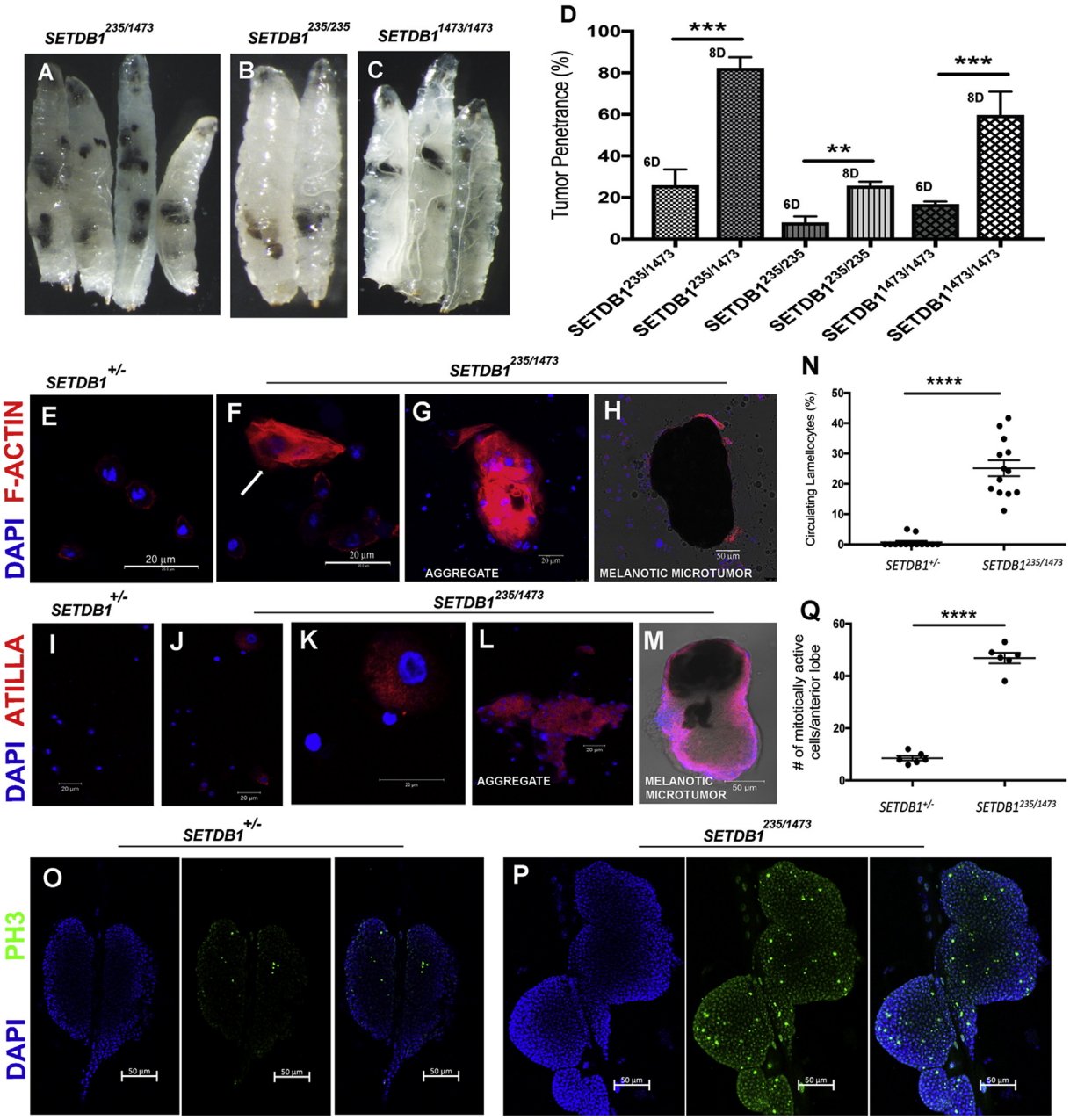

Fig. 1. Loss-of-function mutants of *SETDB1* show melanotic microtumor formation. Melanized microtumors visible through cuticle of wandering third instar whole larva from $SETDB1^{235/1473}$ (A), $SETDB1^{235/235}$ (B), *and* $SETDB1^{1473/1473}$ (C) mutant background. Graphical representation of the tumor penetrance observed in circulating hemolymph in six day old larvae and eight day old mutant larvae [$SETDB1^{235/1473}$ 6D (N=3, n=20) and 8D (N=3, n=22), $SETDB1^{235/235}$ 6D and 8D (N=3, n=19), *and* $SETDB1^{1473/1473}$ 6D (N=3, n=17) and 8D (N=3, n=12). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.01$ (**), $p \leq 0.001$ (***). Circulating blood cells stained for polymerized F-actin (red) and nuclei (blue) from hemolymph of $SETDB1^{+/-}$ heterozygote larva (E) and hemolymph of $SETDB1^{235/1473}$ mutant larva (F). White arrow pointing to lamellocyte. Aggregate (G) and melanotic microtumor (H) from circulating hemolymph of $SETDB1^{235/1473}$ mutant larva stained for polymerized F-actin (red) and nuclei (blue). Circulating blood cells immunostained for lamellocytes using anti-Atilla antibody (red) and nuclei (blue) from hemolymph of $SETDB1^{+/-}$ heterozygote larva (I) and hemolymph of $SETDB1^{235/1473}$ mutant larva (J-K). Aggregate (L) and melanotic microtumor (M) from circulating hemolymph of $SETDB1^{235/1473}$ mutant larva immunostained for lamellocytes using anti-Atilla (red) and nuclei (blue). Graphical representation of the number of lamellocytes (N=3, n1=4, n2=4, n3=5, total n=13 individually plotted data points) in $SETDB1^{+/-}$ and $SETDB1^{235/1473}$ wandering third instar larvae (N). Student t-test to confirm significance shows $p \leq 0.0001$ (****). Anterior lobes of lymph glands stained for mitotically active cells using anti-Phospho-Histone-3 antibody (0-P, green). Graphical representation of mitotically active blood cells per anterior lobe (N=3, n1=2, n2=2, n3=3, total n=6 individually plotted data points) in $SETDB1^{+/-}$ and $SETDB1^{235/1473}$ in early third instar larvae (Q). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****).

abnormal cells that are larger in size like lamellocytes (Fig. 1F-white arrow pointing the differentiated lamellocyte like cell), aggregates (Fig. 1G) and melanotic microtumors (Fig. 1H) composed of blood cells. To identify the blood cell type in the hemolymph of mutants ($SETDB1^{235/1473}$) we stained the hemolymph with a plasmatocyte specific marker (Supplementary Fig. 1, P1/Nimrod C-green). Unlike the plasmatocytes found in wild type larvae, mutant blood cells from circulation showed few abnormal plasmatocytes that were positive for anti-P1 antibody staining. Aggregates found in mutants also show presence of these abnormal plasmatocytes along with region of melanization (Supplementary Fig. 1C, * indicating the region of melanization). While there is increased differentiation of blood cells which appear like lamellocytes in

circulating hemolymph of mutants (Fig. 1F–H) we examined with anti-atilla antibody, the morphology of these blood cells to determine presence of lamellocytes. Our results indicate presence of anti-atilla positive lamellocytes (Fig. 1J–K and N), aggregates (Fig. 1L), and melanotic microtumors (Fig. 1M) in circulating blood cells of mutants, *SETDB1*$^{235/1473}$ compared to heterozygote *SETDB1*$^{+/-}$ controls (Fig. 1I). To ascertain weather loss of function mutants, *SETDB1*$^{235/1473}$ compared to heterozygote *SETDB1*$^{+/-}$ controls have an increased proliferation of blood cells in the anterior lobes of the lymph glands we analyzed the expression of phospho-Histone H3 (H3P) using anti-H3P antibody (M-phase marker) in the anterior lobes. We noticed an increase in the number of mitotically active cells in the anterior lobes of lymph glands with significant differences between the heterozygotes and mutants (Fig. 1O–Q).

To examine the blood cells in the anterior lobes we first dissected out the anterior lobes from day five and day six old larvae. We documented the differences in feeding third instar five day old larva (5D) and

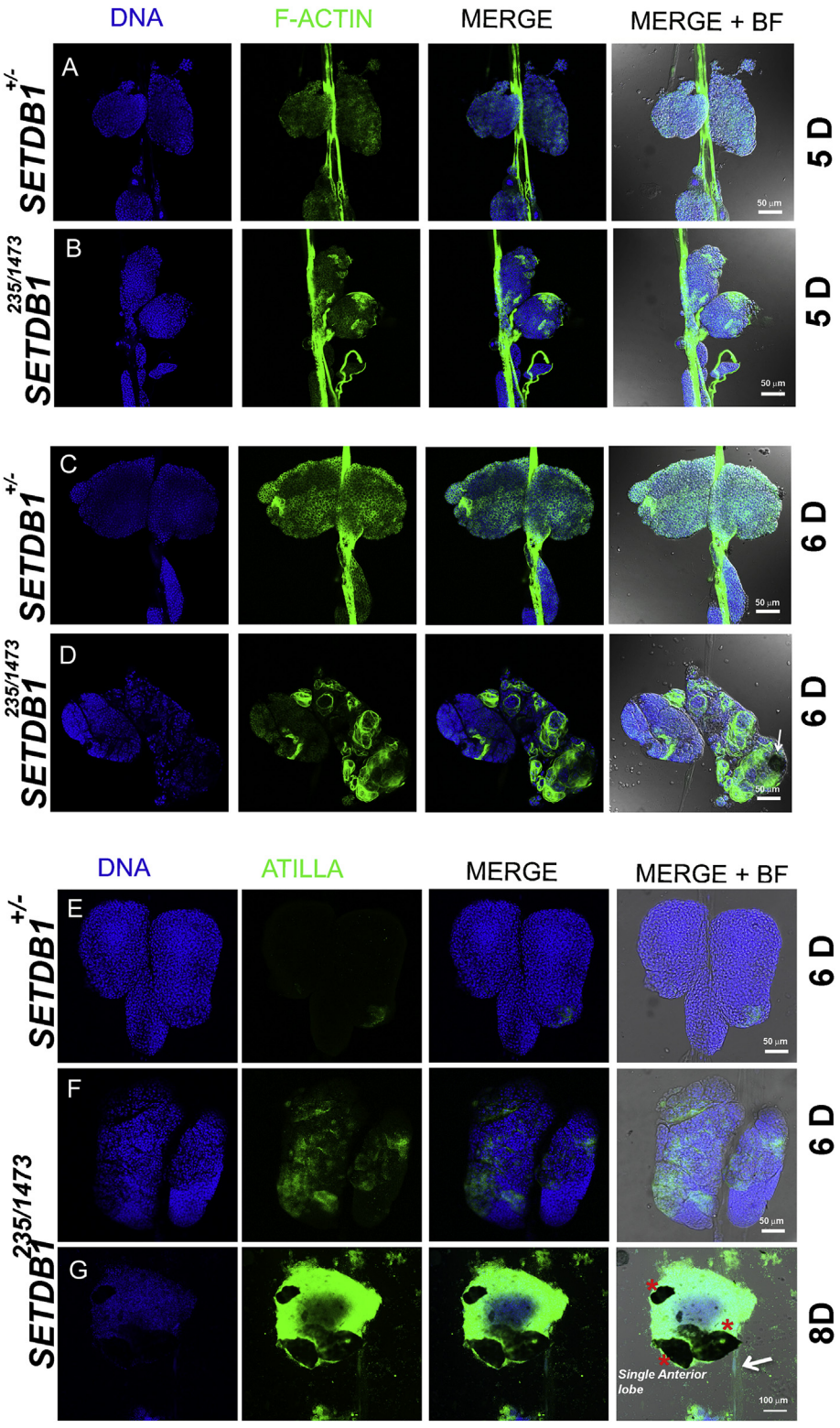

Fig. 2. *SETDB1*$^{235/1473}$ mutants show lymph gland hypertrophy with increased lamellocytes. Anterior lobes (AL) of the lymph glands from both heterozygote (*SETDB1*$^{+/-}$) and mutant (*SETDB1*$^{235/1473}$) siblings dissected from feeding third instar larva (FL3) five day old (5D), wandering third instar larva (WL3) six day old (6D), and mutants from delayed third instar larval stage eight day old (8D). A to D anterior lobes stained for polymerized F-actin (green) and nuclei (blue). White arrow in panels D and G are indicating the regions of melanizationin the anterior lobe. A. *SETDB1*$^{+/-}$ FL3 larva (5D) B. *SETDB1*$^{235/1473}$ FL3 larva (5D) C. *SETDB1*$^{+/-}$ WL3 larva (6D). D. *SETDB1*$^{235/1473}$ WL3 larva (6D). E to G anterior lobes immunostained for lamellocyte marker Atilla (green) and nuclei (blue) E. *SETDB1*$^{+/-}$ WL3 larva (6D) F. *SETDB1*$^{235/1473}$ WL3 larva (6D) G. *SETDB1*$^{235/1473}$ WL3 larva (8D).

wandering third instar six day old larva (6D) of heterozygote (*SETDB1*$^{+/-}$) and *SETDB1*$^{-/-}$ mutants (Fig. 2A–D). The lymph gland anterior lobes of *SETDB1*$^{+/-}$ heterozygote larvae in both the feeding stage and the wandering stage show normal wild type morphology (Fig. 2A, C) while the anterior lobes with regions of intense polymerized F-actin (green) staining is very enhanced in *SETDB1*$^{-/-}$mutants (Fig. 2B, D) indicating presence of differentiated cells. Furthermore, anterior lobe of the lymph gland in wandering third instar (6D) larva of (*SETDB1*$^{-/-}$) mutant showed melanized region (Fig. 2D, star indicating to melanized region). Using lamellocyte specific anti-L1/Atilla antibody we performed immunostaining to identify the lamellocyte population in these mutant' anterior lobes. Indeed as expected we observed increased number of lamellocytes that were Atilla positive in the six-day old mutant *SETDB1*$^{-/-}$ anterior lobes compared to heterozygotes (Fig. 2E and F). We extended the study to document the phenotype of the anterior lobes of eight-day old *SETDB1*$^{-/-}$ mutants to determine if the phenotype deteriorated with time. We noticed lamellocytes were extensively stained for Atilla in the anterior lobes of eight-day old larva and also observed increased regions of melanization (Fig. 2G, * in red indicating regions of melanization). These results clearly establish that the *SETDB1*$^{-/-}$ mutants show dysplasia with enlarged anterior lobes with greater number of lamellocytes and the anterior lobes forming melanotic microtumors within itself.

With the presence of abnormal plasmatocytes and lamellocytes it is indicative that there is hypertrophy of anterior lobes in lymph glands and in the circulating hemolymph of *SETDB1* mutants. This is the first study where anterior lobes of a lymph gland are shown with regions of melanization. Our results suggest a role for SETDB1 in hematopoietic blood cell maintainance.

2.2. Knockdown of SETDB1 contributed to lamellocyte differentiation

To further explore the role of SETDB1 for differentiation of blood cells into lamellocytes we performed RNAi knockdown of SETDB1 using classical UAS-GAL4 system (Brand and Perrimon, 1993). We used TRiP RNAi stock for knockdown of SETDB1 protein (*UAS-SETDB1*^{RNAi}). We utilized two different GAL4 drivers i.e. *Tubulin-Gal4* (ubiquitious) and *MSNF9-Gal4* (lamellocytes) to knockdown SETDB1. A single anterior lobe of control class (*Tubulin-Gal4*) and experimental class (*Tubulin* > *SETDB1*^{RNAi}) were compared through immunostaining experiment using anti-Atilla antibody specific for lamellocytes; those larvae carrying only one component of the expression system, *Tubulin-Gal4* (control class) were compared to the larvae carrying both components of the expression system, *Tubulin* > *SETDB1*^{RNAi} (experimental class). We found differentiated lamellocytes that were Atilla positive in *Tubulin* > *SETDB1*^{RNAi} (Supplementary Fig. 2B-B1, green) compared to control larvae carrying only *Tubulin-Gal4* (Supplementary Fig. 2A). To identify if there were other effects due to ubiquitous loss of expression of SETDB1 (*Tubulin* > *SETDB1*^{RNAi}), we analyzed developmental stages of the growing experimental animals. Larvae underwent through all developmental stages but we found wing defects in adult flies (with 100% penetrance, DNS). We next used lamellocyte specific driver, *MSNF9-GAL4* to study the loss of SETDB1 expression. Anterior lobes of *MSNF9*>*SETDB1*^{RNAi} experimental larva show significant increase in the differentiation of lamellocytes (Fig. 3B-B1 shown via graphical representation (Fig. 3G) compared to anterior lobes of control larva (Fig. 3A).

To elucidate the requirement of SETDB1 for lamellocyte differentiation we subsequently generated heat shock FLP-OUT clones of *SETDB1 [RNAi]* LOF. Experimental larvae carrying heat shock flippase enzyme, *Actin* > *FRT, CD2*>*GAL4, UAS-GFP* and *UAS-SETDB1*^{RNAi} were exposed to heat shock and compared to non-heat shock larva of same genotype. We observed upon heat shock experimental larvae showed differentiated lamellocytes that were atilla positive GFP clones (Fig. 3F-F1, Green and Red positive cells) compared to non-heat shock experimental larva (Fig. 3E) and control larva not carrying *UAS-SETDB1*^{RNAi} (with or without heat shock, Fig. 3C and D) clearly establishing that loss of SETDB1 expression leads to lamellocyte differentiation. We found

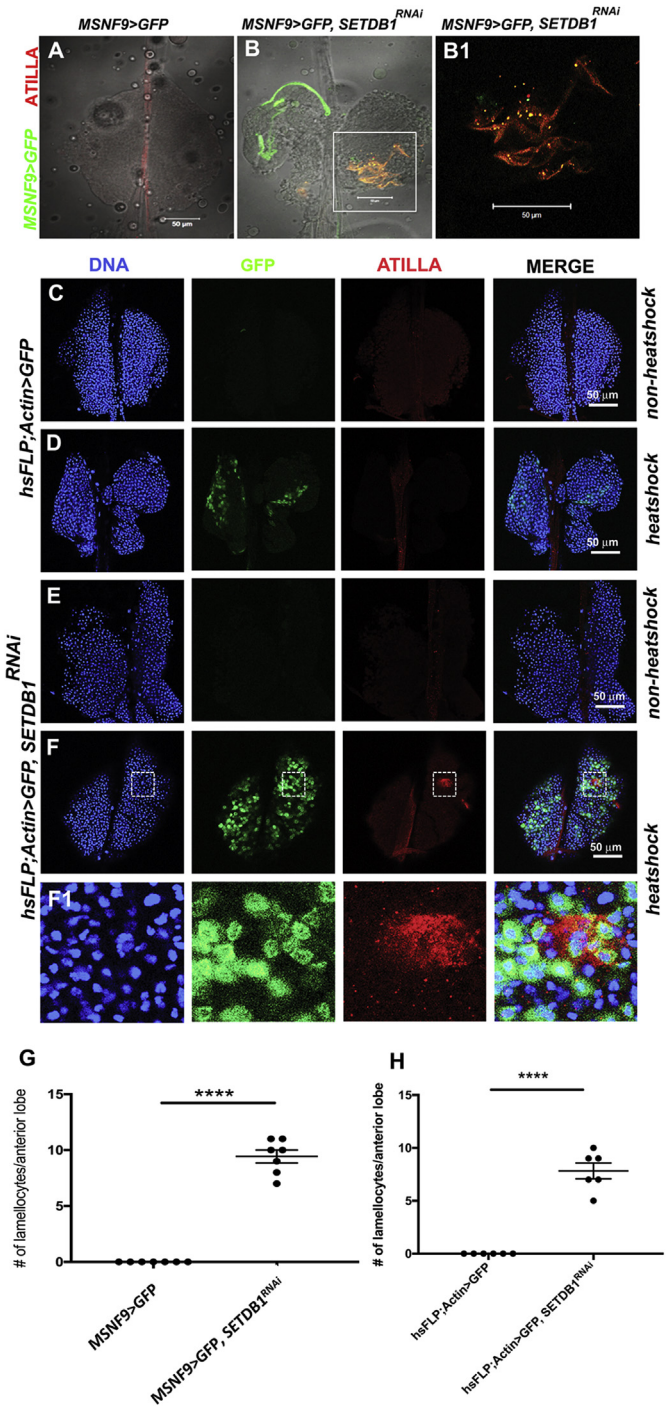

Fig. 3. Knockdown of *SETDB1* leads to lamellocyte production. Anterior lobes of the lymph glands from wandering third instar wild type larva, *MSNF9>GFP* (A), and experimental larva, *MSNF9>GFP, SETDB1*^{RNAi} (B-B1) stained for lamellocytes (anti-Atilla, red). Anterior lobes of the lymph gland stained for lamellocytes (anti-Atilla-red) in control larva *hsFLP;Actin > GFP* without heat shock (C), in experimental larva *hsFLP;Actin > GFP, SETDB1*^{RNAi} without heat shock (E), in control larva *hsFLP;Actin > GFP* with heat shock (D), and in experimental larva *hsFLP;Actin > GFP, SETDB1*^{RNAi} with heat shock (F). High magnification image of a region from F (F1). Graphical representation of number of lamellocytes in *MSNF9>GFP* and *MSNF9>GFP, SETDB1*^{RNAi} (G, n=7) larvae and in *hsFLP;Actin > GFP* and *hsFLP;Actin > GFP, SETDB1*^{RNAi} with heat shock (H, n=6). Student *t-test* (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****).

statistically significant differences after heat shock in the number of lamellocytes as represented graphically (Fig. 3H). These results suggest an autonomous function of SETDB1 in the differentiation of blood cells into lamellocytes (due to presence of Atilla positive cells that were also GFP positive cells). However, it is clear that not all the cells with lack of SETDB1 differentiate into lamellocytes since there exist some GFP cells that are not lamellocytes. Therefore, the requirement of SETDB1 is yet to be confirmed in maintaining few blood cells from differentiating into lamellocytes.

2.3. Loss-of-function of SETDB1 affects crystal cell differentiation

We next wanted to determine the effect of loss-of-function of SETDB1 on crystal cells. Anterior lobes of *SETDB1*$^{235/1473}$ mutants and *SETDB1*$^{+/-}$ controls were stained for crystal cells (anti-ProPO antibody) and cells in the niche (anti-Antp antibody). We observed crystal cells in the anterior lobes of control larva (Fig. 4A, red) that were absent in the anterior lobes of the mutant larva (Fig. 4B). Antennapedia positive niche cells were reduced in mutants (Fig. 4B, green) compared to the heterozygote controls (Fig. 4A, green). These results show the loss of crystal cells in the *SETDB1* mutants with the loss of Antennapedia positive niche cells. We know from published literature that crystal cell differentiation requires Notch signaling pathway in larvae. Apart from Lozenge, Notch also has a role in facilitating the differentiation of crystal cells. Notch undergoes proteolytic cleavage upon receptor activation there by Notch intracellular domain is formed (Bataille et al., 2005; Duvic et al., 2002; Lebestky et al., 2003; Mukherjee et al., 2011; Small et al., 2014). Therefore, we asked if there is a difference in Notch-ICD expression and further determined Notch-ICD protein expression (Notch intracellular domain) along with *Notch* and *Su(H)* gene expression. Su(H) is a transcription factor required for crystal cell development (Lebestky et al., 2003). Our results indicate lower levels of N-ICD expression in anterior lobes of mutants (Fig. 4D-D1, Green) compared to heterozygote controls (Fig. 4C-C1, Green). Consistent with the protein levels we found reduced levels of *Notch* and *Su(H)* gene expression in mutants compared to wild type control larvae (Fig. 4K). We next investigated the requirement of SETDB1 for crystal cell differentiation using *Lozenge*-GAL4 driver carrying *UAS-GFP* (*Lz-Gal4* is specific for crystal cell progenitor and mature crystal cells). To observe N-ICD protein expression we performed immunostaining. Results indicate an increase in the number of crystal cells (Green) with an increased N-ICD expression (red) in anterior lobes of *Lz > SETDB1, GFP* (Fig. 4F) compared to *Lz > GFP* controls (Fig. 4E). There are blood cells exclusively *Lozenge > GFP* (only green), positive for both, *Lozenge > GFP* and Notch^{ICD} (yellow) and positive only for Notch^{ICD} (red) in anterior lobes of both *Lz > GFP* and *Lz > GFP, SETDB1*. Blood cells that were positive for only Notch^{ICD} (red) and for both *GFP* and Notch^{ICD} (yellow) were higher in experimental class when compared to the control anterior lobes, suggestive of SETDB1 playing a role in increased expression of Notch. Upon RNAi knockdown we not only observed a decrease in the number of crystal cells (green) in the anterior lobes of the lymph gland of *Lz > SETDB1*^{RNAi} (Fig. 4G–L) compared to *Lz > GFP* controls (Fig. 4E, L), but also noticed reduced N-ICD expression in experimental *Lz > SETDB1*^{RNAi} compared to *Lz > GFP* controls (Fig. 4E and G, Red). These results clearly signify the role of SETDB1 in the Notch expression. We also performed immunostaining experiments (using anti-Atilla antibody) to determine if the loss of SETDB1 in *Lozenge > SETDB1[RNAi]* larvae can affect blood cells originating from the lymph glands to differentiate into lamellocytes. Our results clearly showed no such effect (data not shown). Apart from SETDB1, in *Drosophila* there exist another histone methyltransferase Su(var)$^{3-9}$ that methylate Histone-3 at lysine-9. Su(var)$^{3-9}$ also plays a role in oogenesis and its function comes into play after SETDB1 (Clough et al., 2014). Therefore, we wanted to explore the possible role of *Su(var)*$^{3-9\ GOF}$ [constitutively expressed gain of function allele (Schotta et al., 2003)] in *Drosophila* larval hematopoiesis. Thus, we determined if Su(var)$^{3-9}$, is essential for blood cell differentiation. We found that unlike SETDB1's function Su(var)$^{3-9}$ does not affect either crystal cells or lamellocytes (Fig. 4H–J, 4M and Supplementary Fig. 3) as shown through graphical representation (Fig. 4M). These results indicate that *Su(var)*$^{3-9}$ is not functionally similar to SETDB1 in hematopoietic tissues of *Drosophila*. To determine the possibility of decreased number of crystal cells in circulating hemolymph we performed cooking assay on third instar larvae with knockdown of SETDB1 expression using the three drivers *Tubulin-Gal4*, *Hemolectin-Gal4* and *Lozenge-Gal4*. Simultaneously, similar experiment was also performed using the heterozygote, *SETDB1*$^{+/-}$ and loss-of-function mutants of SETDB1, *SETDB1*$^{235/1473}$. Our results from cooking assay elucidated the reduced crystal cell numbers in the hemolymph of wandering third instar larvae due to loss of *SETDB1* (Fig. 4N).

The UAS TRiP RNAi stock that was used in our experiments for knockdown of SETDB1 protein has not been used earlier hence we wanted to see if we could reproduce and validate earlier published eye phenotype results with a different *UAS-SETDB1*^{RNAi} stock using the TRiP RNAi line (Gou et al., 2010). Our results confirm previously recognized eye phenotype of *Lozenge > SETDB1*^{RNAi} as we also observed same phenotypes of the eye. Adults show fused ommatidia as documented using scanning electron microscopy (Supplementary Figs. 4A–B).

2.4. Over-expression of SETDB1 affects crystal cell number

We first performed experiments using *Hemolectin-Gal4* driver (*Hml-Gal4,UAS- GFP* represents approximately 66% of the circulating GFP positive cells) to determine the effect of SETDB1 expression on plasmatocytes (Shia et al., 2009). There was no significant difference in the plasmatocyte population (GFP positive cells) stained with anti-P1/Nimrod-C antibody in the anterior lobes of lymph gland in *Hml > GFP, SETDB1* compared to the *Hml > GFP* (Fig. 5A and B). However, an increased crystal cell numbers marked by the crystal cell specific antibody (anti-C4 (Kurucz et al., 2007),) in the anterior lobes was observed in larvae from similar experiment (Fig. 5C and D). In both the experimental and control anterior lobes some of the crystal cells that were C4 positive (red) were also *Hml > GFP* positive. These doubly marked cells were more in *Hml > GFP, SETDB1* anterior lobes (Fig. C1-D2). To find out if similar effect is observed on crystal cells in the circulating hemolymph we performed the cooking assay and we utilized three different drivers, viz., *Tubulin-Gal4, Hml-Gal4* and *Lozenge-Gal4*. Our results from these experiments indicate an increase in the crystal cell population in the last two segments of the whole larva compared to the wild type larvae (Fig. 1E and F). While there is a significant difference between the wild type and experimental class we observed that it is the *Hml > GFP, SETDB1* larvae that showed greater number of crystal cells than the other two experimental classes, *Tubulin > SETDB1* and *Lozenge > GFP, SETDB1*. In *Hml > GFP, SETDB1* we also found crystal cells in the region of anterior lobes of the lymph gland (indicated by *) unlike other experimental larvae.

2.5. Rescue of SETDB1 mutants

To ascertain if the hematopoietic phenotypes observed in mutants of SETDB1 are due to its loss-of-function we performed a genetic rescue using *Tubulin–Gal4*, ubiquitous driver and exogenously expressed SETDB1 in mutants. Heterozygote siblings with only one copy of the mutant allele of *SETDB1* gene (*SETDB1*$^{+/-}$) and heterozygotes along with an exogenously expressed wild type copy (*SETDB1*$^{+/-}$*, Tubulin > SETDB1*) appeared to have normal circulating hemolymph (Fig. 6A and B). As shown earlier, mutants (*SETDB1*$^{235/1473}$) have increased number of lamellocytes, aggregates and melanotic microtumors (Figs. 1 and 6C and Supplementary Fig. 1) but mutants carrying an exogenously expressed wild type copy of SETDB1 (*SETDB1*$^{235/1473}$*, Tubulin > SETDB1*) show loss of lamellocytes and they are devoid of aggregates, and microtumors (Fig. 6D).

These results confirm the essentiality of the gene *SETDB1* in *Drosophila* for two of the matured hematopoietic cell formation i.e.

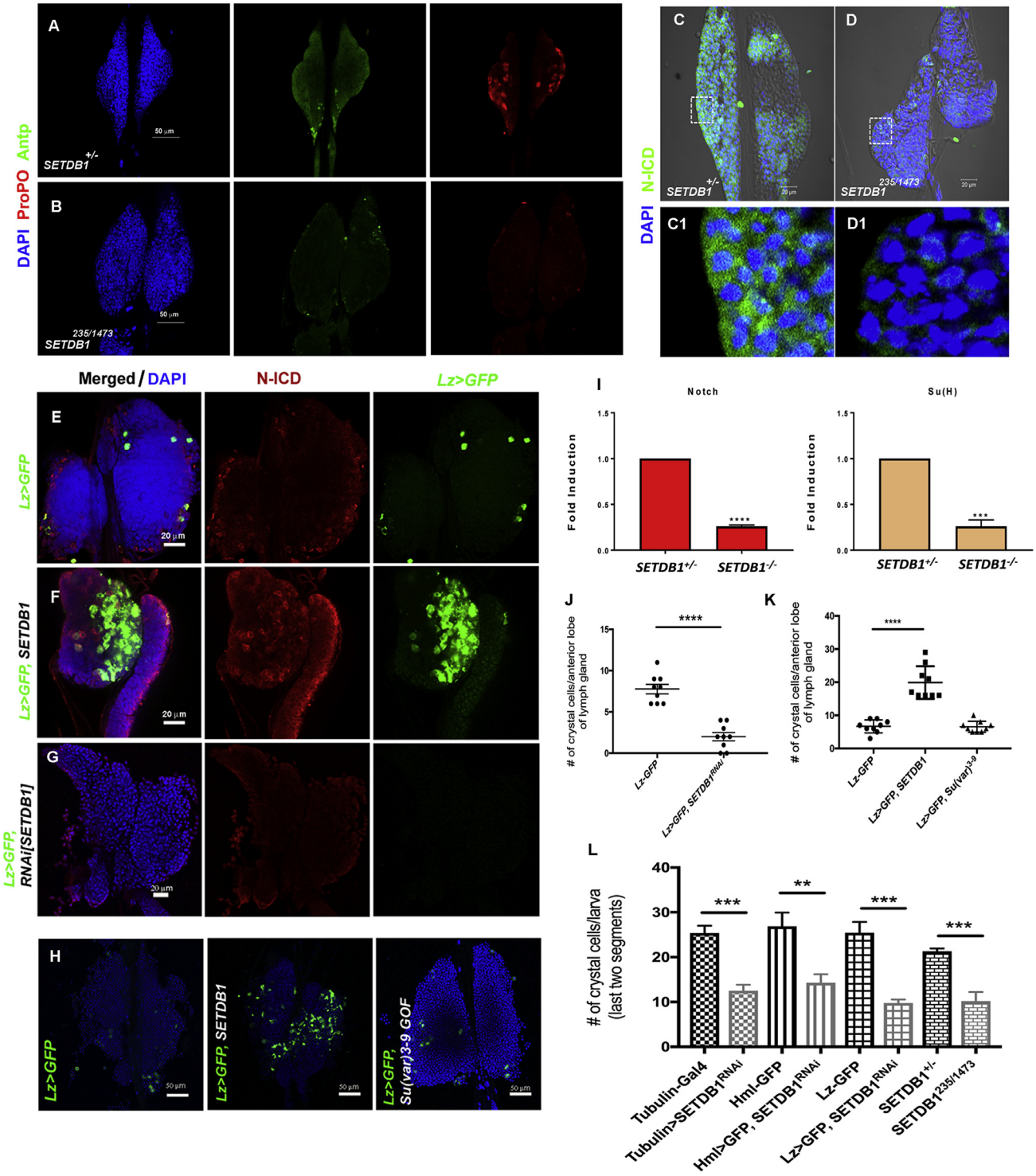

Fig. 4. SETDB1 affects crystal cell differentiation and Notch pathway. Anterior lobes showing expression of both ProPO (red) and Antp (Green) in *SETDB1*$^{+/-}$ (A) and *SETDB1*$^{235/1473}$ (B). Anterior lobes showing expression of N-ICD (Notch-Intra Cellular Domain, Green) in *SETDB1*$^{+/-}$ (C-C1) and *SETDB1*$^{235/1473}$ (D-D1). GFP positive crystal cells in the anterior lobes stained for expression of N-ICD (red) and DNA (blue) dissected from *Lz > GFP* larva (E), from *Lz > GFP, SETDB1* larva (F) and *Lz > GFP, SETDB1*^{RNAi} larva (G). Anterior lobes showing GFP positive crystal cells of *Lz > GFP* larva (H), *Lz > GFP, SETDB1* larva (I) and *Su(var)3–9* GOF, *Lz > GFP* larva (J) stained for nuclei (blue). Graphical representation of the fold induction of *Notch* and *Su(H)* in *SETDB1*$^{+/-}$ and *SETDB1*$^{235/1473}$ larvae (K), (N=3, n=50). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****), $p \leq 0.001$ (***). Graphical representation of the number of crystal cells from single lobe of anterior lobes (n=9) from both *Lz > GFP* and *Lozenge > SETDB1*^{RNAi} (L). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****). Graphical representation of the number (N=3, n=3) of crystal cells per anterior lobe of *Lz > GFP, Lozenge > SETDB1* and *Su(var)3–9* GOF, *Lz > GFP* larva (M). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.0001$ (****). Graphical representation of the number of crystal cells [*Tubulin-Gal4, Tubulin > SETDB1*^{RNAi} (N=3, n=9), *Hml > GFP, Hml > GFP, SETDB1*^{RNAi} (N=3, n=8), *Lz > GFP*, *Lz > GFP*, *SETDB1*^{RNAi} (N=3, n=9), *SETDB1*$^{+/-}$ and *SETDB1*$^{235/1473}$ (N=3, n=9)] in the last two segments of the wandering third instar larvae (E). Student t-test (unpaired, two-tailed) to confirm significance shows $p \leq 0.01$ (**) and $p \leq 0.001$ (***).

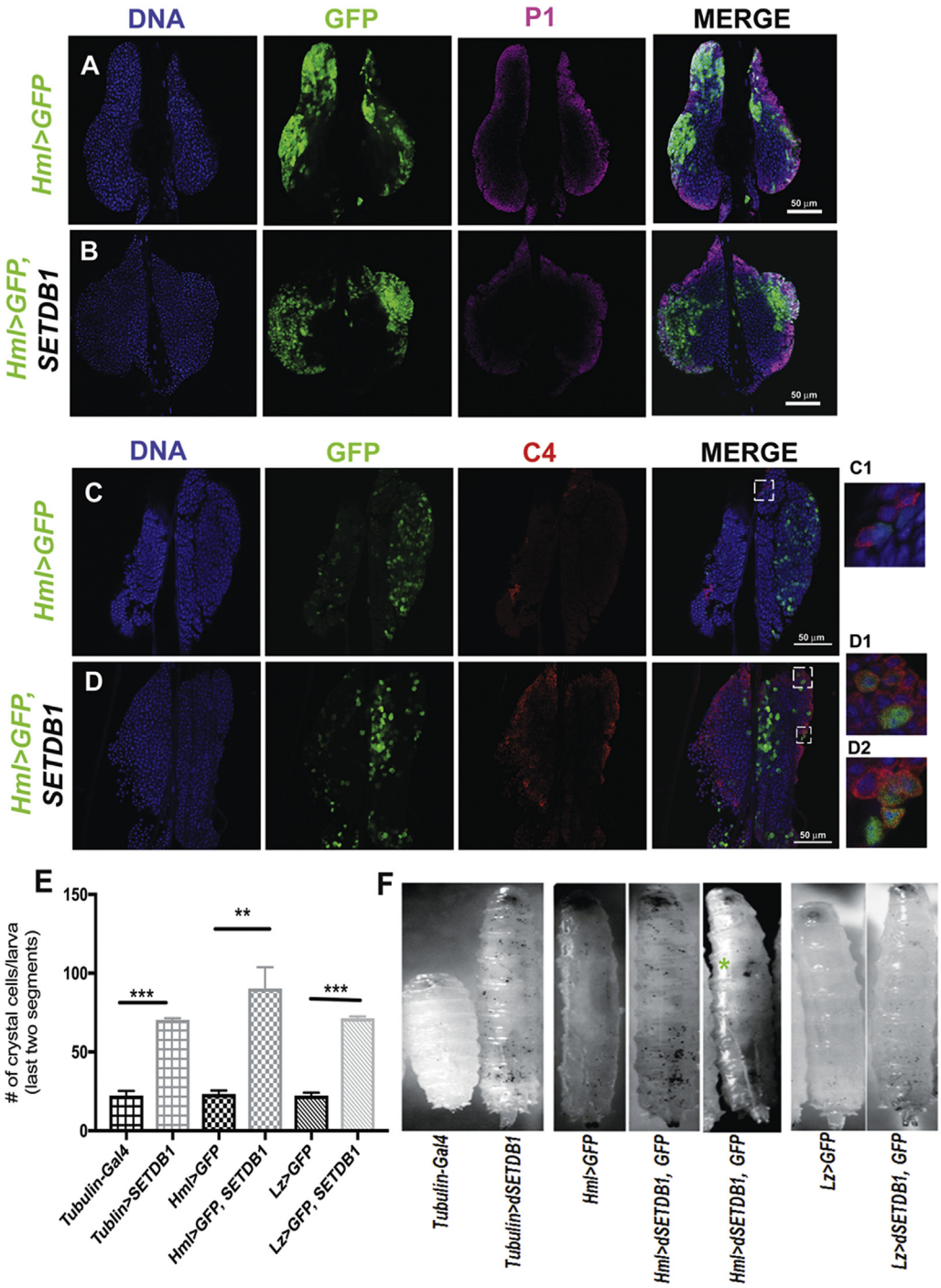

Fig. 5. Overexpression of SETDB1 in blood cells of both hemolymph and the lymph gland results in increased crystal cells. Anterior lobes immunostained for plasmatocyte specific protein expression [P1/Nimrod-C, magenta from *Hemolectin > GFP* (*Hml > GFP*)] (A), and from *Hml > GFP, SETDB1* (B). Anterior lobes immunostained for crystal cell specific protein expression (anti-C4, red) from *Hml > GFP* (C-C1) and from *Hml > GFP, SETDB1* (D-D2). Graphical representation of the number of crystal cells in the last two segments of the [*Tubulin-Gal4*, *Tubulin > SETDB1* (N=3, n=8), *Hml > GFP, Hml > GFP, SETDB1*, *Lozenge > GFP* (*Lz > GFP*) and *Lz > GFP, SETDB1* (N=3, n=9)] third instar larvae (E). Student *t-test*, (unpaired, two-tailed) to confirm significance shows $p \leq 0.01$ (**) and $p \leq 0.001$ (***). Whole larvae from various genotypes showing crystal cells (blackened) after cooking assay (F, green star indicates region of crystal cells found near the anterior lobes of the lymph gland when *SETDB1* is over expressed with *Hml > GFP*).

crystal cells and lamellocytes and further substantiate the role of SETDB1 in blood tumor suppression.

3. Discussion

Drosophila SETDB1 is identical to its human and mouse homologue in its enzymatic activity as a methyltransferase. Studies using vertebrate and mammalian models brought out the function of SETDB1 in accelerating melanoma (human melanoma cells, ovarian cancer, non-small-cell lung cancer, small-cell lung cancer, hepatocellular carcinoma, and breast cancer)(Ceol et al., 2011). Mice, that fail to perform proper histone modifications exhibit hematopoietic defects (Thomas et al., 2006). Recent literature identified the role of SETDB1 in mice for hematopoietic stem cell development (Koide et al., 2016). Various publications contributed to the existing knowledge about *SETDB1*'s role in different developmental stages of *Drosophila*. However, little is known about SETDB1's requirement at tissue level (but for oogenesis) inspite of it being expressed in all developmental stages. Loss of SETDB1 leads to early stage arrest of oogenesis (Clough et al., 2014, 2007). We provide data in the current study taking advantage of fruit flies' genetic system to unravel the function of SETDB1 in hematopoiesis.

Our results indicate for the first time an unidentified function of SETDB1 for the differentiation of crystal cells and lamellocytes from a common progenitor in *Drosophila* larva. Experimental evidences to decipher the regulatory function of SETDB1 for the mature blood cells' differentiation were obtained by performing experiments using classical

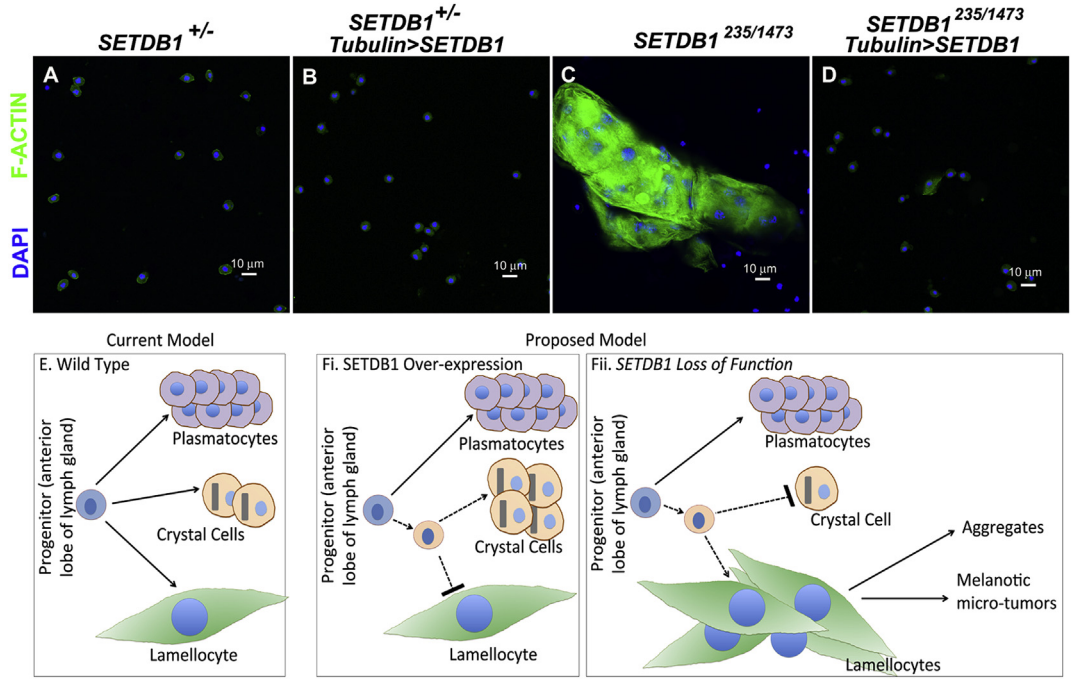

Fig. 6. A-D. Genetic rescue of *SETDB1* mutants with exogenous expression of SETDB1 ubiquitously: Circulating blood cells from hemolymph stained for polymerized F-actin (green) and nuclei (blue) dissected from *SETDB1*$^{+/-}$ heterozygote larva (A), *SETDB1*$^{+/-}$heterozygote larva carrying *Tubulin* > *SETDB1* (B), *SETDB1*$^{235/1473}$ mutant larva with an aggregate (C) and in genetically rescued mutants, *SETDB1*$^{235/1473}$ carrying *Tubulin* > *SETDB1* (D). **E-F. Schematic representing the function of SETDB1 in maintaining the blood cell homeostasis**: Current lineage showing progenitor from the anterior lobe of the lymph gland, differentiating into matured blood cells; plasmatocytes, crystal cells and lamellocytes (E). Proposed lineage shows a progenitor differentiation in two different genetic conditions (Fi-Fii). Upon over-expression of SETDB1, a common progenitor differentiates into increased number of crystal cells inhibiting lamellocytes formation (Fi). While due to loss of function of *SETDB1* a common progenitor differentiates into increased lamellocytes leading to aggregate and melanotic micro-tumor formation with a simultaneous hold on crystal cell differentiation (Fii).

UAS-GAL4 system for both over-expression and knockdown of *SETDB1*. Results obtained from loss-of-function mutants of SETDB1 (*SETDB1*$^{235/1473}$) along with knockdown of SETDB1 (RNAi) using *Tubulin-Gal4*, *Msnf9-Gal4* and *Lozenge-Gal4* clearly establish the requirement of SETDB1 in regulating lamellocyte and crystal cell numbers in wild type. Greater number of lamellocytes, aggregates and microtumors, are found in circulating hemolymph of LOF *SETDB1* mutants. In accordance with the results obtained from circulating hemolymph of LOF mutants we found that morphology of hematopoietic organ in these mutants is also being affected with an abundance of lamellocytes and an enlarged anterior lobe implicating dysplasia of the hematopoietic organ. The morphological changes in the mutant's blood cells eventually led to the formation of neoplastic hematopoietic tumors within the circulating hemolymph and lymph gland anterior lobes which are either non-melanized or melanized. These hematopoietic defects coincide with the phenotypes observed where there is hyperactivation of hematopoietic pathways (for e.g. NF-κB, JAK/STAT and Ras) that show similar blood cell defects in *Drosophila*. To corroborate our findings we further verified by clonal analysis that loss of SETDB1 leads to increased lamellocyte differentiation in wild type. Clonal analysis clearly demonstrates the dependency of few of the progenitor cells on SETDB1 for restricting their differentiation into lamellocytes. Over-expression studies are suggesting a requirement for SETDB1 for the differentiation into crystal cells. Increased number in the crystal cell population was detected in both circulating blood cells and anterior lobes of lymph glands compared to wild type wandering third instar larva due to over-expression of SETDB1 using *Hml-Gal4*, *Lozenge-Gal4* and *Tubulin-Gal4*. Our results indicate a function for SETDB1 in regulating the bipotent progenitor.

Furthermore, we noticed a decrease in the antennapedia expressing cells in mutants with a loss of crystal cells compared to anterior lobes of heterozygotes. Antennapedia is expressed in the niche cells of the lymph gland in wild type maintaining the progenitor population (Mandal et al., 2007). On the basis of these results we understand a requirement for Antp expression in the niche for maintaining progenitor population in *SETDB1* mutants. Consistent with the reduced crystal cell numbers we also noticed a decrease in Notch^{ICD} expression and *Notch*, *Su(H)* gene expression in both experimental conditions i.e. *SETDB1* mutants and also knockdown of *SETDB1* (RNAi). Notch^{ICD} protein expression was reversed upon over-expression of SETDB1 iterating the involvement of SETDB1 in regulating Notch pathway. It is therefore possible that the upsurge in number of crystal cells is a consequence of increased Notch protein in SETDB1 over-expression experiments. We therefore propose the existence of a bipotent progenitor that has the potential to differentiate into either a crystal cell or a lamellocyte without affecting the plasmatocyte population (Fig. 6E-Fii).

Further investigation can shed light on the mechanism involving Notch pathway. It is an oncogenic pathway used as a very strong therapeutic target while designing cancer drugs. Our experimental observations also direct a need to explore the mechanisms, involving SETDB1 in progenitors to decipher its requisite in not only controlling the bipotent progenitor cells' fate from differentiating into lamellocytes, but also in limiting the differentiation of precursors into crystal cells in wild type larva. While SETDB1 has a role in the development of crystal cells and lamellocytes it is crucial to have a better understanding of how SETDB1 is playing such role. It would be interesting and valuable to elucidate the role of SETDB1 in regulating hematopoietic pathways crucial for proliferation and differentiation of precurssor and progenitor cells. Yet to be answered is the connecting link between the genes or signaling pathways that are silenced by SETDB1 implicated in hematopoietic development. Further research needs to be carried out to elucidate the expression of SETDB1 protein in different blood cells and to determine the specific blood cell type in which SETDB1 plays an important role.

Genetic rescue experiments authenticate the requirement of SETDB1 in larval stages in blood tumor suppression. Upon expression of a wild type SETDB1 protein in the SETDB1 mutants' background not only there

is a reduction in the circulating lamellocytes but also melanotic micro-tumors are not formed. This is the first study highlighting the importance of a gene affecting epigenetics (modifying chromatin) involved in hematopoiesis and hematopoietic tumor regulation in *Drosophila*. Whether the effects of SETDB1 in *Drosophila* blood cells are dependent on H3K9me3 is however to be elucidated in future studies for mechanistic purposes. Since the domains involved in methyltransferase enzymatic function are similar in both, humans and fruit flies, further investigations can be carried out to perceive if human SETDB1 can liberate hematopoietic blood tumors in *SETDB1* larval mutants. Previous studies described the requirement of SETDB1 along with HP1 and Dnmt2 in *Drosophila* for DNA methylation for the silencing of target genes like retinoblastoma, a tumor suppressor gene (Gou et al., 2010). Extensive research is required for establishing how different levels of SETDB1 silence oncogenic and tumor suppressor pathways that affect the blood cell progenitor differentiation.

Taken together, current findings support the idea that SETDB1, a histone methyltransferase, has a novel role in regulating differentiation of progenitor cell's fate into either a crystal cell or a lamellocyte. The results obtained from our research lead to further questions in exploring SETDB1s′ contribution in the mechanisms involved for maintaining progenitors thereby limiting the formation of blood tumors.

4. Materials and methods

4.1. Fly stocks

All the stocks and transgenic lines of *Drosophila* were raised at 25 °C on standard media. *Tubulin-Gal4* [*Bloomington Drosophila Stock Center*, BDSC (Mueller et al., 2007)], *Hemolectin-Gal4, UAS-GFP* [Gift from Dr. Utpal Banerjee's Laboratory-UCLA (Sinenko and Mathey-Prevot, 2004)], *Lozenge-Gal4; UAS-GFP* [Gift from Dr. Utpal Banerjee's Laboratory-UCLA (Jung, 2005)], *MSNF9-GAL4* and *y w hs FLP; + / + ; actin > CD2>GAL4, UAS-GFP/TM6 Tb* [Gift from Dr. Shubha Govind, City College, CUNY (Small et al., 2014)], *UAS-SETDB1* [gift from Dr. Andreas Wodarz (Koch et al., 2009)], *UAS-SETDB1^{RNAi}* [Stock # 34803, Expresses dsRNA for RNAi of egg (FBgn0086908) under UAS control. Yale TRiP RNAi at Harvard Medical School (Ni et al., 2011)], *egg^{235}/SM1* [BDSC (Clough et al., 2007)], *egg^{1473}/SM1* [BDSC (Clough et al., 2007)], *Su(var) 3-9^{06}/TM6 Tb* [Dr. Rakesh Kumar Mishra, CCMB (Vasanthi et al., 2013)], *Su(var)3-9^{02}/TM6 Tb* [Dr. Rakesh Kumar Mishra, CCMB (Vasanthi et al., 2013)], *Su(var)3–9* gain-of-function [Dr. Rakesh Kumar Mishra, CCMB (Vasanthi et al., 2013)]

4.2. Mutants

Egg mutants: *egg^{235}/SM1* (null mutant) and *egg^{1473}/SM1* (mutant with loss of SET domain). We replaced SM1 balancer with *CyO GFP* balancers (Prasad, 2003) on chromosome two in both the mutant stocks. To generate heteroallelic combination in trans (transheterozgote mutants) we crossed *egg^{1473}/Cy O GFP* flies to *egg^{235}/Cy O GFP* flies.

4.3. Circulating hemolymph and lymph gland preparations

Crosses were set up and allowed to lay eggs for 6 h. Third instar larvae (5D-feeding third instar [FTI] or 6D and 8D-wandering third instar [WTI]) were collected for washes with 1X phosphate buffer saline, ddH2O, 75% alcohol, ddH2O, and finally in 1xPBS. Blood smears (circulating hemolymph) and hematopoietic organs (lymph glands) were dissected using fine forceps (FST- M5S 11200–14/Inox-Electronic by DUMONT Switzerland) as described in Small et al. (2012) (Small et al., 2012).

4.4. Immunohistochemistry

Antibody staining: Third instarlarval lymph glands or hemolymph were air dried on slides, samples were fixed in 4% paraformaldehyde and incubated with 1% Bovine Serum Albumin in 1x Phosphate Buffer Saline (1x PBS) for 30 min at room temperature, followed by incubation overnight with the primary antibody. After three washes with 1x PBST (15 min each), samples were incubated for 3 h atroom temperature with the respective fluorescently-labeled secondary antibodies (Invitrogen and Jackson Immunochemicals). After three washes in 1X PBST and one final wash with 1x PBS, the samples were counterstained with nuclear dye Hoechst 33258 (1:500) and/or F-actin (phalloidin)(1:200) where specified, then mounted in glycerol containing antifade (N-propylgallate). Primary antibody concentrations used were anti-Nimrod-C (gift from Dr. Istvan Ando) 1:20, anti-Atilla (gift from Dr. Istvan Ando) 1:20, anti-C4 (gift from Dr. Istvan Ando) 1:20 for staining matured blood cells (Kurucz et al., 2007), anti-PH3 (gift from Dr. Krishnaveni Mishra, University of Hyderabad, School of Life Sciences, rabbit, abcam) 1:100 and anti-Notch-ICD (Developmental Studies Hybridoma Bank (DSHB)) 1:10. Flourescently labeled secondary antibodies [TRITC/FITC/Cy5-conjugated goat anti-mouse (1:50) and goat anti-rabbit (1:50)] were commercially obtained from Jackson Immuno Research Laboratories. Anti-ProPO antibody was (gift from Dr. Tina Mukherjee) was diluted 1:100, Anti-Antp (8C11) was commercially obtained from DSHB (1:100).

4.5. Microscopy

Whole larvae: Larvae of interest were washed as mentioned above in "hemolymph sample preparations". Using Zeiss Axio CS microscope larvae were imaged. Immunohistochemistry samples: Circulating hemolymph and lymph gland samples stained and mounted were imaged with LAS AF SP8confocal microscope and images were processed using the LAS AF SP8 software. Final images were processed using Adobe Photoshop CS3 software.

4.6. Flp-out technique

Flp-out technology is used for the spatial and temporal restriction of transgene expression. In this technique there is use of the hybrid flipout (Flp) and GAL4 activation [*hsp70-flp*; *Actin > CD2>GAL4*] and *UAS-NLS-GFP* transgenes or those larvae with an additional *UAS-SETDB1^{RNAi}* transgenes, were heat shocked at 37° in a water bath for 15 min. After removing them from water bath they were placed at 25°. Lymph glands were dissected on day 6 from mid-third instar larvae, 20 h post heat shock. Developmentally synchronized larvae from controls class (without *UAS-SETDB1^{RNAi}* transgenes) and experimental class (with *UAS-SETDB1^{RNAi}* transgenes) both carried *UAS-NLS-GFP* transgenes. These larvae were compared without and with heat shock.

4.7. Crystal cell melanization assay (cooking assay)

Wandering third instar larvae were washed with 1X phosphate buffer saline, ddH2O, 75% alcohol, ddH2O, and finally in 1xPBS. Larvae were then transferred into 1X PBS containing centrifuge tubes. Tubes were placed inside the water bath at 60 °C for 10 min. Both wild type larvaeand experimental larvae were then removed from water bath, and left at roomtemperature for some time. Number of crystal cells in the last two segments of wandering third instar larvae were counted. N=3, n > 8.

4.8. Scanning electron microscopy

Samples were prepared as per the standardprotocols as mentioned. Imaging was done using the Hitachi SEM.

Measurement of crystal cells, lamellocytes, mitotic index and tumor penetrance.

Unless mentioned third instar larvae were used for experiments. All samples under observation for data collection were randomly picked for the experiments (Sample size chosen as noted from earlier published

research studies). Statistical analysiswas performed using GraphPad Prism 7 software. All the samples were included for statistical analysis. For comparisons between two groups, we utilized Student's *t*-test (unpaired, two-tailed). All graphs show mean ± SEM. In all cases (not significant) $p > 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p < 0.001$ and $^{****}p \leq 0.0001$. Biological repeats (N), sample size (n) and *t-test* results are mentioned in the legends section. Crystal cell indexes displayed were *Lozenge > GFP* positive crystal cells counted per anterior lobes. Lamellocyte counts in circulating hemolymph is displayed as a number of Atilla positive cells after staining with anti-Atilla antibody used for staining circulating lamellocytes. Using LAS AF SP8 confocal microscopy lymph glands were laser scanned and Z stack images were collected. Mitotically active cells per anterior lobe were counted from the images obtained. Wandering third instar six day old larvae and eight day old larvae were dissected and observed formicrotumors in circulating hemolymph for tumor penetrance.

4.9. RNA collection and real time-PCR

Fifty synchronized 3rd instar larva of the appropriate genotypes (six days after egg lay) were collected for RNA extraction (Trizol method, Invitrogen, Life technologies, Carlsbad, CA). RNA was quantified by a StepOnePlus ™ System (Applied Biosystem, Thermo Fischer Scientific). 1.5 μg of total RNA was used as template for cDNA synthesis (iscript ™; Bio-rad Laboratories, Hercules, CA). Real time PCR was performed running the standard two-step PCR program: 1 μl of the cDNA sample was mixed with KAPA SYBR^{R} FAST Universal (KAPA Biosystems, Lot # 006255-8-1) and primers to set up a 25-μl reaction mix. Transcript levels detected were normalized to *rp49* mRNA values. Primers used:

Notch: Forward primer (5′) *AGC GAA ATG GAG TCG GTC CCG* (3′); Reverse primer (5′) *GAT GGC GAG CCC AAG TAG GCA* (3′)

SU(H): Forward primer (5′)*AAT GGT CCT TGC AGG TAC GTC* (3′); Reverse primer (5′) *ATC CTC GGC CTG TGT ATT GC* (3′)

RP 49 Forward primer (5') GAC GCT TCA AGG GAC AGT ATC TG (3′); Reverse primer (5′) AAA CGC GGT TCT GCA TGA G (3′)

Author contributions

I. P. conceived and designed the study; I. P. performed the experiments; D. K. G. performed experiments for Fig. 4K. I. P., D. K. G. and R. K. M. analyzed the data and wrote the paper.

Conflict-of-interest disclosure

Authors declare they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.08.008.

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by Dushyant Kumar Gautam

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