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Pericardin, a *Drosophila* collagen, facilitates accumulation of hemocytes at the heart

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ABSTRACT

Hematopoietic cell lineages support organismal needs by responding to positional and systemic signals that balance proliferative and differentiation events. *Drosophila* provides an excellent genetic model to dissect these signals, where the activity of cues in the hemolymph or substrate can be traced to determination and differentiation events of well characterized hemocyte types. Plasmatocytes in third instar larvae increase in number in response to infection and in anticipation of metamorphosis. Here we characterize hemocyte clustering, proliferation and transdifferentiation on the heart or dorsal vessel. Hemocytes accumulate on the inner foldings of the heart basement membrane, where they move with heart contraction, and are in proximity to the heart ostia and pericardial nephrocytes. The numbers of hemocytes vary, but increase transiently before pupariation, and decrease by 4 h before pupa formation. During their accumulation at the heart, plasmatocytes can proliferate and can transdifferentiate into crystal cells. Serrate expressing cells as well as lamellocyte-like, Atilla expressing ensheathing cells are associated with some, but not all hemocyte clusters. Hemocyte aggregation is enhanced by the presence of a heart specific Collagen, Pericardin, but not the associated pericardial cells. The varied and transient number of hemocytes in the pericardial compartment suggests that this is not a hematopoietic hub, but a niche supporting differentiation and rapid dispersal in response to systemic signals.

1. Introduction

Genetic models of hematopoiesis provide new avenues to study the origins of blood cell disorders while also proffering a context to understand the stem cell niche. *Drosophila melanogaster* has emerged as a productive model to study blood cell homing and hematopoiesis. Hemocytes of *Drosophila* resemble vertebrate blood cells in various respects such as stem cell regulation, functional subtypes, immune functions, and molecular signaling (Evans et al., 2003; Gold and Bruckner, 2014; Hartenstein, 2006; Martinez-Agosto et al., 2007). The three main types of differentiated hemocytes have immune related functions such as phagocytosis (plasmatocytes), melanization (crystal cells) and encapsulation (lamellocytes) of parasites (Rizki, 1978, 1962; 1957; Rizki et al., 1980; Rizki and Rizki, 1992). Additionally, hemocytes secrete extracellular matrix (ECM) proteins during development (Knibiehler et al., 1987; Kusche-Gullberg et al., 1992; Yasothornsrikul et al., 1997). Besides hemocytes, the fat body of Drosophila larvae also secretes ECM components. Specifically, Pericardin is secreted by the larval fat body and recruited to the cardiac ECM (Drechsler et al., 2013).

Hemocyte types are distinguished by employing molecular and genetic markers. *Hemolectin* (*Hml*) is a marker for most hemocytes. It is expressed in plasmatocytes (Goto et al., 2003; Sinenko and Mathey-Prevot, 2004) which make up 90–95% of all hemocytes (Rizki, 1957). *Lozenge* (*lz*) expression is specific to crystal cells (Lebestky et al., 2000). *Hml* expression persists during transdifferentiation when a plasmatocyte is turning into a crystal cell, but is lost in mature crystal cells (Leitão and Sucena, 2015; Mukherjee et al., 2011).

Drosophila hematopoiesis occurs both in a hematopoietic organ called the lymph gland and also in tissue-resident blood cells (Gold and Bruckner, 2015; Letourneau et al., 2016), which originate from thoracic mesoderm and head mesoderm of the embryo respectively (Holz et al., 2003; Rugendorff et al., 1994; Tepass et al., 1994).

The lymph gland is made up multiple lobes that flank the aorta. This organ develops through larval stages until it releases its hemocytes into circulation at the start of metamorphosis (Lanot et al., 2001). Lymph gland hemocytes will be released into hemolymph during larval stages if

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a larva is infected by a parasitic wasp, but not under normal conditions (Lanot et al., 2001). Primary lobes of the lymph gland include a cortical zone made of maturing hemocytes, a medullary zone containing stem cell like prohemocytes, and a posterior signaling centre (PSC) that regulates the differentiation rate of prohemocytes into hemocytes through various molecular signals (Crozatier et al., 2004; Jung et al., 2005; Lebestky et al., 2003; Mandal et al., 2007; Pennetier et al., 2012; Sinenko et al., 2009). One such signal is Serrate, which regulates crystal cell maturation (Lebestky et al., 2003; Krzemień et al., 2007). Cells of the PSC send thin and elongated cellular processes into the medullary zone (Mandal et al., 2007).

Tissue-resident (sessile) blood cells of *Drosophila* accumulate on the epidermal-muscular pockets of the larval segments (Makhijani et al., 2011). These hematopoietic pockets are found laterally (lateral patch) and dorsally (dorsal stripe) (Lanot et al., 2001; Makhijani et al., 2011; Márkus et al., 2009; Stofanko et al., 2008). Cell exchange is observed between the hematopoietic pockets, which suggests that they are dynamic (Makhijani et al., 2011). Peripheral nervous system (PNS) neurons attract resident hemocytes to these pockets and provide a trophic microenvironment (Makhijani et al., 2011). In the 2nd and early 3rd instar, sessile plasmatocytes proliferate at a higher rate than circulating plasmatocytes (Makhijani et al., 2011). This difference is lost by mid-3rd instar, possibly due to the increased rate of exchange between sessile and circulating hemocytes (Makhijani et al., 2011).

Another hematopoietic feature of sessile plasmatocytes is that they are capable of transdifferentiating into crystal cells (Leitão and Sucena, 2015). Plasmatocytes expressing Serrate signal to adjacent plasmatocytes expressing Notch to turn into crystal cells (Leitão and Sucena, 2015). The Serrate-Notch signaling requires cell-cell contact (Fiúza and Arias, 2007), and sessile plasmatocytes are in close contact with each other through cellular interdigitations (Lanot et al., 2001).

Sessile hemocytes have hematopoietic functions during parasitic wasp infections as well. New lamellocytes are produced from putative prohemocytes found within sessile hemocyte populations or through transdifferentiation of plasmatocytes (Anderl et al., 2016; Márkus et al., 2009).

Hemocytes produced during embryogenesis and larval development can persist into adulthood (Holz et al., 2003; Honti et al., 2014) as do the hemocyte precursors from the larval lymph gland (Ghosh et al., 2015). In the adult, these hemocytes and their precursors are commonly aggregated on the dorsal midline of segments A1-A4 close to the heart (Ghosh et al., 2015). This site is suggested to be a hematopoietic hub of adults, because it harbors plasmatocytes that respond to immune challenges by proliferating and prohemocytes capable of differentiating into crystal cells and plasmatocytes (Ghosh et al., 2015). Nested between the heart and cuticle, this area contains a network of ECM proteins such as Laminin A and Pericardin (Ghosh et al., 2015), similar to how vertebrate bone marrow is enriched with laminin (Gu et al., 2003; Siler et al., 2000) and collagen (Nilsson et al., 1998).

An uncharacterized location of hemocyte accumulation in the larvae is the *Drosophila* heart, called the dorsal vessel (DV), a tubular organ positioned on the dorsal midline (reviewed by Medioni et al., 2009; Tao and Schulz, 2007). Contractile cardiomyocytes form the heart tube to pump hemolymph from posterior to anterior. A series of excretory and non-contractile pericardial cells flank the lateral sides of the heart tube. In fixed larvae, hemocytes are found on pericardial cells (Rizki and Rizki, 1984). The DV has inflow tracks called ostia as well as an intracardiac valve (Lehmacher et al., 2012). Cardiomyocytes and pericardial cells are covered by basement membranes on the abluminal and luminal sides throughout development (Hollfelder et al., 2014; Lehmacher et al., 2012; Rugendorff et al., 1994; Tepass and Hartenstein, 1994; Zhang et al., 2013). Some of the main ECM proteins of the DV include Pericardin, Laminin and Collagen IV (Chartier et al., 2002; Hollfelder et al., 2014; Hughes and Jacobs, 2017; Yarnitzky and Volk, 1996). Pericardin is a *Drosophila* specific collagen-like protein (Chartier et al., 2002) which when absent leads to a disorganized heart tube and detachment of the pericardial cells (Wilmes et al., 2019; Drechsler et al., 2013).

Here we report on a pre-pupariation buildup of hemocytes associated with the heart ECM. Previous reports suggested that hemocytes accumulate at the DV as a result of circulation build-up (Babcock et al., 2008; Makhijani et al., 2011). It was also observed that hemocytes accumulate at the ostia of mosquito adults as a defense against immune attack (King and Hillyer, 2012; Sigle and Hillyer, 2016; 2018a,b). We observed that accumulation and dispersal of hemocytes was temporally consistent, rapid and transient, suggesting a role in metamorphosis, rather than infection or circulatory buildup. In this paper, we characterize the population of DV hemocytes, in comparison to other hematopoietic sites. We note that this population responds to infection, undergoes transdifferentiation and requires a specific microenvironment to become established beside the larval heart.

2. Materials and methods

2.1. Drosophila melanogaster stocks

w; Pxn-Gal4 (courtesy K. Bruckner), *HmlΔ-DsRed* (Makhijani et al., 2011), L*z Gal4; UAS GFP* (Bloomington# (BL): 6313), Mef2 GAL4 (BL: 27390), *prc* ^{MB03017}*/TM6C, Sb* (BL: 23836), *vkg* ^{cc0079}, *yw* (BL: 6598), w^{1118} *PBac{WH}Klf15* ^{f06447} (BL: 18979), fly fucci (Zielke et al., 2014), *w1118; Krlf-1/CyO, dotGal4(BL:26982), UAS-LifeAct-GFP (BL: 35544), P{en1} wgen11; P{UAS-GFP.E2f1.1-230}26 P{UAS-mRFP1.NLS.CycB.1–266} 17/TM6B, Tb* (BL: 55122) UAS- loh,UAS-lohΔTSR1-2 (Drechsler et al., 2013, both courtesy of Achim Paululat), *Ser-lacZ II-9.5Z/CyO* (Bachmann and Knust, 1998, courtesy of Erika Bach).

2.2. Live imaging

For Fig. 1A, a stage 17 embryo was imaged at the confocal microscopy by Dr. Qanber Raza using the hanging drop method (Reed et al., 2009). For Fig. 1E, a 3rd instar was imaged by confocal within 20 min of being anesthetized with 2 doses of 500 µl airborne desflurane (Suprane, Baxter Corp. Mississauga ON) introduced over 20 min. Supplementary Videos were made using an Olympus BX43 Microscope within 20 min after anesthetizing a third instar with 1 dose of 500 µl airborne chloroform (ACP Chemicals C-3300, 99.8%) exposed twice for 24 s with a 3 s interval. Anesthesia protocols are detailed by Cevik et al. (2019).

Supplementary Video V1 and V2. DV hemocytes move with heart contraction. Hemocytes expressing Hml-dsRed (video 2) flank the dorsal vessel outlined with Vkg-GFP (video 1) and move medially with each heart contraction. Hemocyte distribution is not restricted to the locations of the ostia, which are medial to the arrowheads. More laterally, other labeled hemocytes do not move with the heart. These are associated with the ectoderm, in the dorsal stripe (ds). Representative still image combines vkg-GFP and Hml-dsRed during systole. Lateral cluster (arrow) and the dorsal stripe (ds) is faintly visible in the background Genotype: *hmldsred,vkg-GFP/+* Scale bar: 100 µm. hl: heart lumen; ds: dorsal stripe. Frame rate: 20 frames/second.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.ydbio.2019.06.006.

2.3. Immunolabelling

α-βPS (β-Integrin, DF.6g11-s, 1/30) was used to label pericardial cells and α-Talin (1/15) and α-Atilla (L1a,b,c; Kurucz et al., 2007) were used to label putative lamellocytes. α-LanA (Laminin, 1/30) and α-Prc (Pericardin, EC11 anti-Pericardin, 1/30) were used to visualize ECM. β-integrin, Talin and Pericardin antibodies were from the Developmental Studies Hybridoma Bank, Iowa City, IA, USA α-Laminin (Vanderploeg, 2014). Samples immunolabelled with these antibodies were first fixed in 1 ml of 4% paraformaldehyde (Polysciences, Warrington, PA, USA) in phosphate buffered saline (PBS) and immunolabeled according to the protocol adapted from Alayari et al. (2009). α-βGal (Vanderploeg, 2014,

1/1000) was used to detect *ser*-lacZ expression. Alexa 647 or Rhodamine conjugated phalloidin (1:150; Thermo Fisher) were used to visualize the heart tube and the secondary antibodies, Alexa 488 α-chicken and Alexa 647 α -mouse (1:150) were obtained from Thermo Fisher.

Protocols for larval and adult dissections were adapted from Vogler and Ocorr (2009). Dissection plates with magnetic pins and plates with a thin layer of Vaseline were used to dissect larvae and adults respectively. Larval dissections were fixed in 2% formaldehyde in PBS for 2 h at room temperature (RT). Samples were washed with PBT (PBS+0.3% tritonX) for 3 h, changing the solution twice. Samples were blocked for an hour using normal goat serum (NGS, 1/15) in PBT followed by incubating with the primary antibody overnight at 4 °C. Samples were then washed with PBT overnight at 4 °C and blocked again with NGS. Samples were incubated with the secondary antibody at 4 °C overnight or at 25 °C for 4 h, followed by an overnight wash with PBT at 4 °C. Samples were removed from the shaker and left in 50% glycerol in PBS for at least 3 h or overnight at 4 °C before mounting them on a slide with 70% glycerol in PBS. Samples that did not require immunolabelling were fixed for 15 min and washed with PBS for 10 min on a shaker. Samples were left undisturbed for at least 30 min at 4 °C with 50% glycerol before replacing the solution with 70% glycerol overnight.

2.4. Imaging and image processing

Leica SP5 confocal microscope was used for images unless stated otherwise. Frontal sections were taken 1.3 μm apart and projected while transverse cross sections were not projected. Virtual cross sections and 3D projections were constructed using ImageJ (Schneider et al., 2012). Volume of DV hemocytes was estimated using the stereological volume estimation plugin Volumest (Merzin, 2008; Roberts et al., 2000) on ImageJ. The borders of each hemocyte and hemocyte cluster was drawn manually, and Volumest was used to estimate the total volume occupied by hemocytes.

2.5. Collecting embryos, larvae and adults

To collect embryos (Fig. 1A), fly houses were set up with plastic tricorner beakers with pinholes attached to an apple juice agar plate (0.02 gr/ml Agar, 2.5% sucrose, 25% apple juice in water) with a small amount of yeast paste. After 8 h of egg laying, the apple juice agar plate was kept at room temperature (RT, 22 °C) for 12 h and at 4 °C for a maximum of 3 days before fixation.

To collect L1 and L2, fly houses were set up with yeast fly food (Fig. 1B and C). After a few hours of egg laying, plates were kept at RT for about a day or 2 days to collect first and second instars respectively. Instar stage was identified according to larval mouth hook physiology.

Third instars were raised on yeast food in vials (Figs. 3 and 5) or on plates (Figs. 6 and 7). After 2 h of egg laying, adults were removed and the vials were kept at RT to develop. The majority of larvae became 3rd instars (identified by branched spiracles) at 76 h after the beginning of egg laying (AEL). Larvae were dissected every 12 h until 36 h after L3 and then at 39 and 44 (start of pupation) hours. To raise larvae on yeast food plates, a fly house was set up with apple juice agar plates and a small amount of yeast paste. After egg laying for 2 h, adults were removed and embryos on the plates were left to develop at RT. After approximately a day, only the hatched larvae were removed from the apple plate and discarded. Then 25 new larvae were harvested when they hatched over the subsequent hour. They were transferred to a yeast plate and incubated at RT until becoming 3rd instar. Larvae were dissected every 4 h until the start of pupation (44 or 48 h). Dissections for each stage were completed in 90 min.

Adults were raised in yeast food vials and dissected 5–6 days after eclosion.

2.6. Bacterial culture and antibiotic

A bacterial sample from the naturally developing biofilm was obtained from a fly food plate with 25 larvae on it. This sample was placed in a liquid fly food medium (all ingredients except agar). Bacteria were grown in 50 ml liquid fly food medium in an orbital shaker at 37 °C for 21 h until it reached OD_{600} of 35. Small aliquots of bacterial culture were stored at-80 °C with 10% glycerol until needed. To make bacterial culture plates (Fig. 7), 100 μl of glycerol stock was spread on yeast fly food plate. After applying the bacterial culture, plate was left to dry for an hour before larvae were collected. First recorded signs of bacterial colonies appeared the next day, when larvae were at L2. Untreated plates showed signs of bacterial colonies much later, during L3. Antibiotic treatment was delivered as 100 μl of a 25μg/ml chloramphenicol solution, spread on yeast food plates (Fig. 6A–F, 7). After applying the antibiotic, plates were left to dry for an hour before larvae were transferred.

2.7. Statistical treatment

Significance levels for changes in hemocyte volume were determined with the Mann-Whitney *U* test statistic. Bacterial infection effects were first assessed with the Kruskal – Wallis non-parametric test followed by Mann-Whitney tests for between-groups comparisons with the Holm correction for multiple comparisons.

3. Results

3.1. Clusters of hemocytes accumulate inside the ECM networks of the DV during L3

Our studies of heart development in wildtype and mutant *Drosophila* uncovered a dynamic pattern of hemocyte association with the DV. We have characterized the time-course of these interactions in greater detail employing fluorescent markers of hemocyte identity in both living and dissected abdomens. The dynamic pattern of hemocyte migration during embryogenesis has been documented in detail, as their movement reflects the removal of apoptotic cells and the deposition of ECM (Moreira et al., 2010; Sonnenfeld and Jacobs, 1995). Embryonic hemocytes express Collagen IV (Viking), and can be tracked with both the hemolectin > Red-h-Stinger (hml-DsRed; Makhijani et al., 2011) and the Vkg-GFP gene trap. Motile plasmatocytes were visible around the embryonic DV but did not persist or accumulate there (Fig. 1A). In contrast, for all three larval instars, hemocytes were found on the abluminal heart surface, inside the Viking (Vkg) labeled basal lamina of the DV, where pericardial cells reside (Fig. 1B–E). We refer to these hemocytes as **DV hemocytes**. First and second instars have several individual DV hemocytes, but no clusters (Fig. 1B and C). Hemocytes are less often found on the luminal side of the DV in both intact and dissected tissue, which will be termed luminal hemocytes.

Sessile hemocytes in both larvae (dorsal stripes) and adults (hematopoietic hubs) have been documented on the dorsal surface of the heart, immediately under the epidermis or the cuticle (Ghosh et al., 2015; Makhijani et al., 2011). As sessile hemocytes may move or be dislodged during dissection, we sought to clarify their position relative to DV hemocytes in living larva. DV hemocytes move with cardiac contraction and hemocytes of the dorsal stripes do not (Supplementary V1; V2). We briefly stopped the heart of an intact 3rd instar using desflurane to confirm the position of the DV hemocytes in an intact larva (Fig. 1E). Dorsal stripe hemocytes were on the dorsal side of the heart, quite separate from DV hemocytes and the collagen network of the DV.

Hemocyte clustering at the DV is distinct during L3. The clusters are dynamic, can change shape, or persist for hours. Whereas some 3rd instars have no DV hemocyte clusters, others have hemocyte clusters of various sizes. Hemocyte clusters are positioned in close proximity to pericardial cells, within ECM networks that contain Collagen, Pericardin and Laminin (Figs. 1D, 2A-D). As the heart contracts, DV hemocytes move with it. We have not observed DV hemocytes specifically accumulating near the ostia in live larvae, as hemocytes can be positioned anywhere along the DV (Supplemental video V1; Supplemental video V2). DV

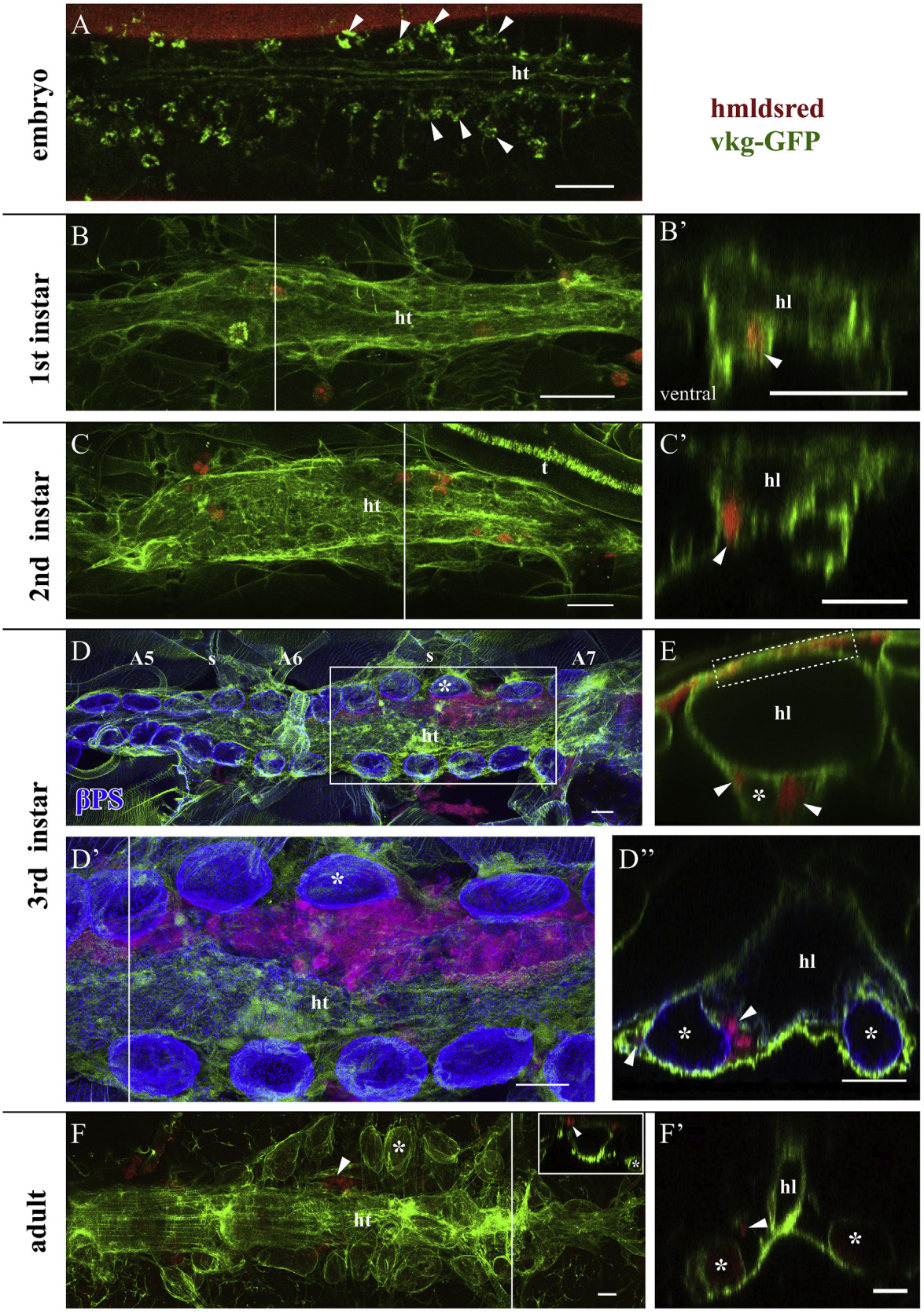

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Fig. 1. Hemocyte accumulation at the dorsal vessel during development. (A) Dorsal view of a late stage live embryo shows hemocytes (arrowheads) migrate close to the DV, but do not accumulate inside the DV. (B, C, D, D′, F) Ventral view of the dissected DVs. (B′, C′, D′′, F′) are cross sections obtained from the location indicated by the vertical line on the image to their left. (B, C) 1st and 2nd instars have very few hemocytes associated with their DVs. (B′, C′) Hemocytes that were associated with the DVs (arrowheads) were located within the collagen network that encases the pericardial cells. (D) 3rd instar have many hemocyte clusters closely associated with the DV. Abdominal segments A5 to A7. (D′) The enlarged view from (D). Cross-section from D′ is shown in D′′. A live larva DV cross-section projection (E) shows hemocyte clusters located within the collagen networks of the DV where pericardial cells reside on the ventral side of the heart (arrowheads), in addition to "dorsal stripe" hemocytes (Makhijani et al., 2011) on the dorsal side of the heart lumen (dashed rectangle). (F) In adults, most hemocytes (arrowhead) near the DV are the extensions of hematopoietic hubs. Inset provides a cross-section from the arrowhead, revealing hemocytes outside the cardiac ECM. (F′) One hemocyte (arrowhead) is found inside the collagen layers of the DV. Posterior is on the right in these and all the subsequent frontal sections. Dorsal side is up in these and all the subsequent cross-sections. Red: Hmldsred (hemocytes); Green: Vkg-GFP (Collagen IV); Blue: anti-βPS (pericardial cells). DV: dorsal vessel; asterisk: pericardial cell; ht: heart tube; hl: heart lumen; s: segment boundary; t: trachea. Genotype: All specimens are *hmldsred,vkg-GFP/+* except (E) which is *hmldsred,vkg-GFP/CyO*. Scale bar: 25 μm.

hemocytes likely enter the DV through ostia, but we have not observed this directly. Movement of hemocyte sized beads through the ostia is documented (Lammers et al., 2017, eg. video-2). Hemocytes clustering at the DV associate closely through interdigitated cell surfaces (Fig. 2E–H), while also surrounded by basement membranes that ensheathe pericardial cells (Fig. 2F). Unidentified cellular sheaths underneath the basement membrane were found wrapped around a subset of hemocyte clusters (Fig. 2F–H). To investigate whether these elongated cells act as support or niche cells, they were labeled for expression of the posterior signaling centre (PSC) marker Serrate (Lebestky et al., 2003) and for lamellocyte markers Talin and Atilla. Similarly located Serrate expressing flat cells with thin projections that did not ensheath plasmatocytes were found near DV clusters in a minority of larvae examined (Fig. 2I). The Serrate expressing cells do not express the plasmatocyte marker *hml*, however there is no direct evidence establishing their role in DV hematopoiesis or shared identity with the ultrastructural cellular sheaths. Additionally, larger cells with low levels of *hml* expression, elevated levels of intracellular Talin, and Atilla were observed with some DV hemocyte clusters (Fig. 2J-L). These cells also extend large cytoplasmic projections comparable to the processes observed in ultrastructure (Fig. 2F–H). These molecular and structural properties are consistent with lamellocyte identity (Stofanko et al., 2010; Kurucz et al., 2007). Although lamellocytes have been identified in the third instar sub-epidermal compartment without immune activation (Márkus et al., 2009), lamellocyte ensheathment of sessile plasmatocytes has not been reported previously, and this may reflect a novel activity.

Adults have relatively few DV hemocytes (Fig. 1F). Most hemocytes found in proximity of the DV are on the dorsal side of the DV, which suggests that they are a part of the hematopoietic hub.

We concluded that clumping of DV hemocytes was a feature of rapid growth in the third instar, perhaps anticipating hemocyte mobilization during metamorphosis. Further study was taken to better characterize this unique phase of hemocyte behaviour.

3.2. DV hemocytes proliferate

According to Makhijani et al. (2011), sessile hemocytes have higher rates of proliferation than circulating hemocytes in early third instar, and this difference in proliferation rate diminishes between the two groups during the stages we observe DV hemocyte clustering. We sought to find which stage of the cell cycle was most prominent in DV hemocyte clusters in late L3. Using Pxn Gal4 and UAS Fly-Fucci (Zielke et al., 2014) we observed cell cycle markers in DV hemocytes 36 h after becoming L3 (Fig. 3). DV hemocytes are capable of proliferation, because a few hemocytes (2%) in these clusters were at S phase. However, the majority of DV hemocytes are either at G2 (79%) or G1 (19%) phase. This is consistent with previously documented sessile hemocyte mitosis in late third instar (Makhijani et al., 2011).

3.3. DV plasmatocytes are capable of transdifferentiating into crystal cells

Larval sessile hemocytes are known to transdifferentiate into crystal cells in a Notch dependent manner (Leitão and Sucena, 2015). At L3, crystal cells and plasmatocytes are mixed uniformly in the DV hemocyte population (Fig. 4A and B). We observed the initiation of a plasmatocyte's transdifferentiation into a crystal cell (Fig. 4C) in a wandering stage L3. The transdifferentiating plasmatocyte expressed both plasmatocyte (*hmldsred*) and increasing levels of crystal cell (*lozenge*-GFP) marker 45 min after expressing only the plasmatocyte marker. If DV plasmatocytes can become crystal cells, then we expect to observe plasmatocytes expressing the Notch ligand Serrate among the DV hemocytes. This is indeed the case, Serrate expressing plasmatocytes are observed within DV hemocyte clusters (Fig. 4D).

3.4. Pericardin is required to retain hemocytes at the DV and dorsal stripe

DV hemocytes are closely associated with ECM networks of the heart and the pericardial cells. We sought to determine whether either the ECM proteins or pericardial cells play a role in attracting or containing the hemocyte clusters. Employing a genetic approach, we dissociated the pericardial cells from the heart tube and depleted the ECM networks that encase the heart. Pericardin is a heart-specific collagen found in ECM pockets where pericardial cells and hemocytes reside (Fig. 2C and D). Pericardin mutants ($prc^{MB03017}$) have a smaller and deformed heart that lacks a well-developed ECM (Fig. 5B). The remaining pericardial cells are distant from the heart, and associate with fibrils of type IV collagen. Few hemocytes are observed attached to these fibers, or near pericardial cells, despite apparent tissue damage. Quantification of the volume of DV hemocytes throughout the third instar revealed growth between 24 and 40 h of age in wildtype, which was more than triple the volume observed in $prc^{MB03017}$ (Fig. 5I), suggesting that the DVs require Pericardin to retain hemocytes. Pericardin or Pericardin-dependent ECM architecture may be a homing factor for hemocyte aggregation.

Sub-epidermal hemocyte stripes, concentrated at the posterior end of the larvae, are dorsal to the DV (Lanot et al., 2001; Makhijani et al., 2011; Márkus et al., 2009; Stofanko et al., 2008), and posterior to the adult location of the "hematopoietic hub" described by (Ghosh et al., 2015). The wildtype "dorsal stripes" are shown in Fig. 5C,E,G and Suppl Fig 1A. In $prc^{MB03017}$ larvae, the dorsal stripes persist, but are smaller and more irregular (Fig. 5D,F,H). The dorsal stripes grow throughout larval development, and therefore the $prc^{MB03017}$ phenotype is most evident 36 h into L3.

During late L3, the body coelom on the lateral sides of the DV develop hemocyte clusters, which appear as ventral elongations from the dorsal stripe. (Fig. 5C, G; Suppl Fig. 1B). They are dorsal to the pericardial cluster, and outside the DV ECM (Suppl Fig. 1C). We refer to these clusters as **lateral extensions**. In +/+ larvae, collagen fibers are visible interspersed between hemocytes of the dorsal stripe, whereas in $prc^{MB03017}$ mutants, such fibers are not visible and the DV is displaced (Fig. 5G and H). Dorsal stripes of the $prc^{MB03017}$ mutants can shift towards the space vacated by the displaced DV.

Pericardin function is required for to establish normal aggregation and location of hemocytes of the dorsal stripe and the DV cluster. To determine whether the spatial pattern of Pericardin deposition was sufficient to organize the location of hemocyte clusters, we directed ectopic deposition of Pericardin with expression of the Pericardin receptor, Lonely heart. The ADAMTS-like protein Lonely heart (Loh) is expressed in the developing heart, and is both necessary and sufficient for the

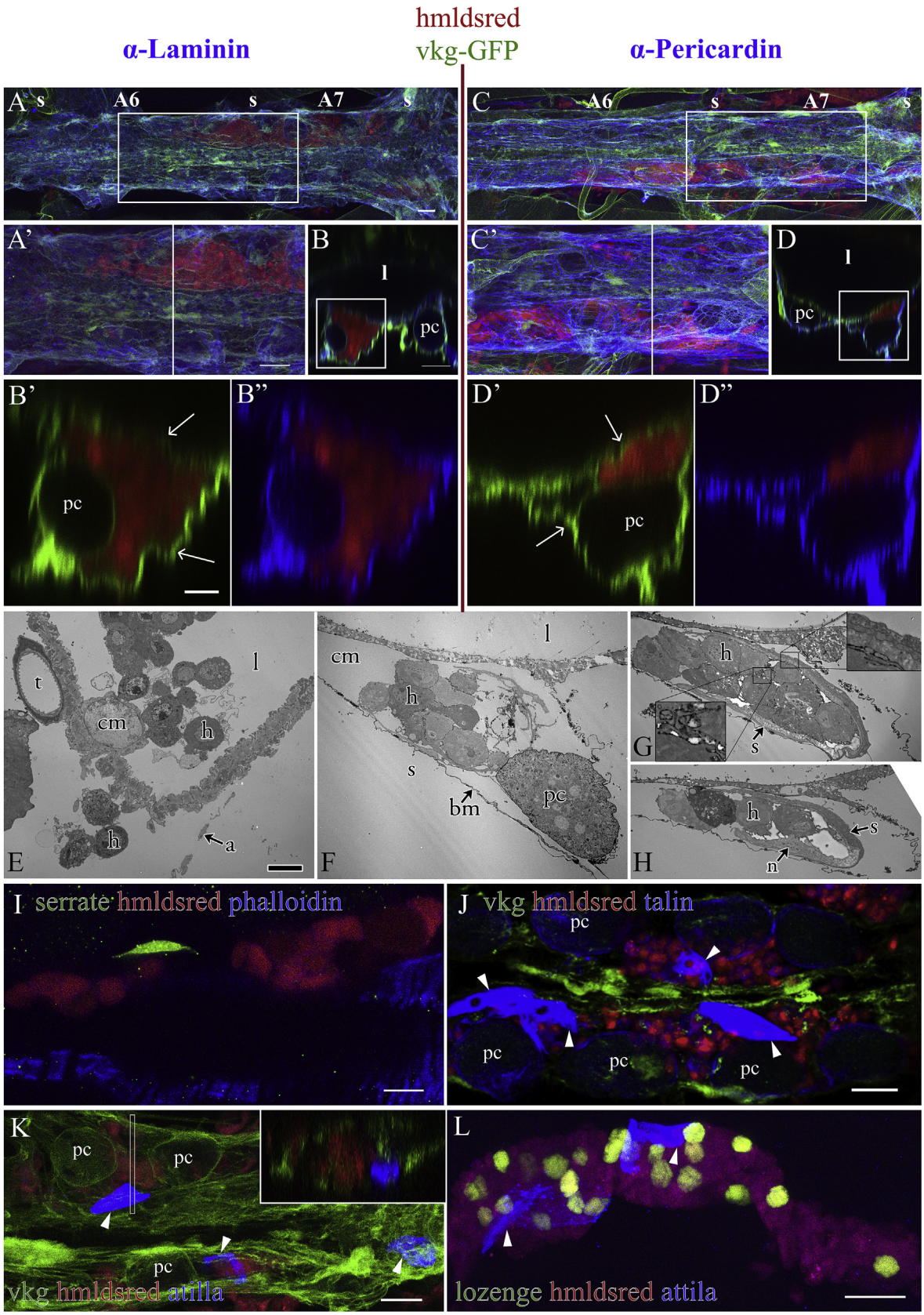

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Fig. 2. Hemocytes associate with pericardial cells and basement membrane. (A, C) Ventral view of dissected migratory 3rd instars labeled for ECM components Laminin (A) or Pericardin (C) reveals the hemocyte clusters are found within this ECM network. The segments are labeled accordingly. (A′,C′) The enlarged views of the boxed areas from A and C. Lines represent the position of the cross sections shown in B and D. (B, D) ECM is detected around the heart lumen and pericardial cells (pc). The enlarged view of the boxed area is shown with vkg-GFP in B′, D′ and with Laminin or Pericardin label in B′′, D′′. (B′, D′) Laminin, Pericardin and Collagen IV (arrows) is visible around the heart lumen and encasing the hemocyte clusters together with the pericardial cells. (E) Hemocytes (h) are found near both the luminal (l) and abluminal surfaces of the heart. (F) Clusters of abluminal hemocytes are closely associated with the basement membrane (bm) and pericardial cells (pc). A hemocyte (h) cluster in contact with a pericardial cell, and surrounded by basement membranes (bm) as well as by unidentified cellular sheaths (s). Panels G and H are a further 10 and 15 μm to the anterior. Larva in F–H was live-labeled with Ruthenium Red to identify extracellular glycoproteins. (I) Optical sections of the DV with parts of the cardiomyocyte (blue) and a Serrate expressing flat cell (green) with thin projections located near a hemocyte cluster (red). (J) Large, Talin expressing cells (blue, arrowheads) are observed near pericardial cells (PC), which extend processes into clusters of hemocytes. (K) Similar cells label also for Atilla; Inset reveals the Atilla cell inside the Cardiac ECM (blue, arrowheads) (L) Flat cells that are labeled with a lamellocyte marker (Atilla, blue) are occasionally found within DV clusters that contain plasmatocytes (Hmldsred) and crystal cells (Lozenge-GFP, green). a: alary muscle fibre; pc: pericardial cells; l: heart lumen; t: trachea; h: hemocyte; bm: basement membrane; cm: cardiomyocyte. (A–D): Red: Hmldsred; Green: vkg-GFP; Blue: anti-Laminin or anti-Pericardin. (I); Green: anti-βgal for Ser.lacZ; Red: Hmldsred; Blue: phalloidin. (J) Green: vkg-GFP; Red: Hmldsred; Blue: anti-Talin. (K) Green: vkg-GFP; Red: Hmldsred; Blue: anti-Atilla. (L) Green: lozenge-GFP; Red: Hmldsred; Blue: anti-Atilla. Genotype: (A-D, J-K) *hmldsred,vkg-GFP/+; (E-H) vkg-GFP/+;* (I) *HmldsRed/Ser.lacZ,* (L) Lz Gal4/+(or Y); UAS GFP/HmldsRed. Scale bar: 25 μm (A-D, J-L); 10 μm (B′,D′,I); 6 μm (E–H).

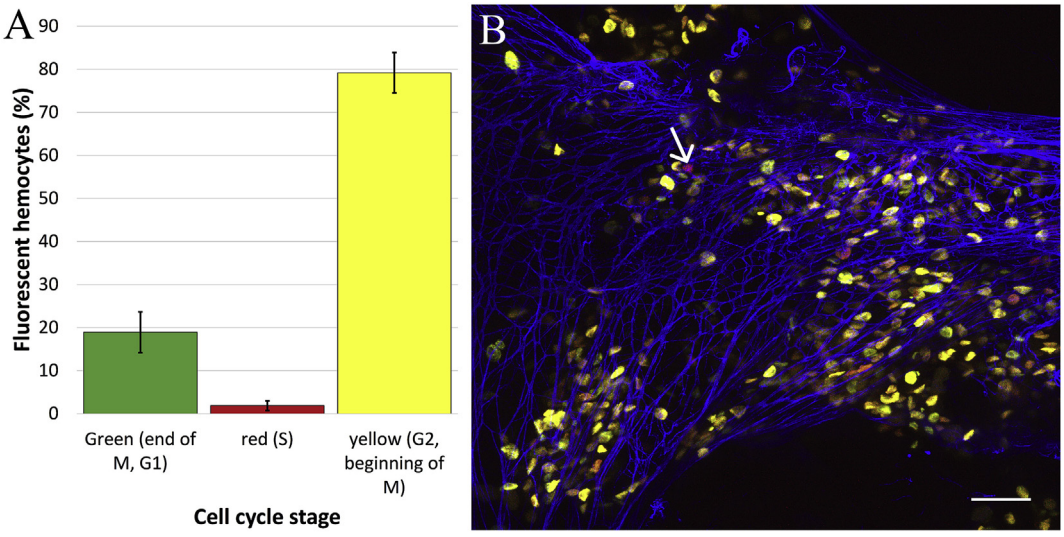


Fig. 3. Most hemocytes at the DV are at G2 stage of the cell cycle. (A) Quantification of DV hemocytes that express Fly-FUCCI indicating their stage in the cell cycle 36 h after becoming 3rd instar. Error bars show standard deviation. (B) Fucci expressing hemocytes at the a7 segment of the DV are visible within the surrounding pericardin (blue) network. Cells at M, G1 or G2 comprise most of heart hemocytes. Cells at S phase (arrow) are rarely visible. n = 10 larva. Blue: anti-Pericardin; Green: end of mitosis, G1; red: synthesis; yellow; G2, beginning of mitosis. Scale bar: 25 μm.

deposition of Pericardin in ECM local to Loh expression (Drechsler et al., 2013; Rotstein et al., 2018). In wildtype larvae, few hemocytes associate with body wall muscles, although clusters of hemocytes are found among muscles after dissection. These hemocytes do not adhere closely to the Muscle ECM (Fig. 6A). Subsequent to ectopic expression of Loh by all muscles, Pericardin accumulates on body wall muscles – but the fibres are thinner than in heart muscle. Isolated hemocytes associate with the muscle ECM – but do not form clusters (Fig. 6B, B'). Loh lacking a thrombospondin repeat (TSR1-2) is much less effective in recruiting Pericardin (Rotstein et al., 2018). Sparse clusters of hemocytes were observed subsequent to expression of *LohΔTST1-2* in muscle (Fig. 6C). These observations suggest hemocyte adhesion to Pericardin containg matrix may prevail over clumping, but that Pericardin is not sufficient to trigger formation of hemocyte hubs.

3.5. Degenerating pericardial cells attract hemocytes

Our evidence suggests Pericardin is required but not sufficient for normal cardiac hemocyte accumulation. Clusters develop near pericardial cells, suggesting a role for these cells in hemocyte homing. We explored this possibility by characterizing DV hemocytes in larvae mutant for *klf15,* in which pericardial cells undergo apoptosis during mid to late larval stages (Ivy et al., 2015). Hemocytes are found recruited to the remaining pericardial cells throughout L3 in klf^{15} mutants (Fig. 7B, D, F). The position of these hemocytes is the same with DV hemocytes that appear under normal circumstances (Fig. 7A, C, E), however, a change in their accumulation pattern was observed. In contrast to tight DV hemocyte clusters seen in klf^{15} heterozygotes (Fig. 7C,G), most DV hemocytes in klf^{15} mutants were in small clusters, distributed or grouped around pericardial cell remnants (Fig. 7F, inset). Pericardial cell markers appear to be inside hemocytes, suggestive of phagocytosis of cell remnants (Fig. 7H-H''). The cardiac ECM of klf^{15} mutants appears wildtype in structure, and contains Collagen IV and Pericardin fibers (Fig. 7E,F,I), attesting that Pericardin is not sufficient to ensure DV hemocyte clustering, and the presence of normal Pericardial cells facilitates hemocyte cluster formation.

3.6. Exposure to Acetobacteraceae and Lactobacillaceae correlates with more DV hemocytes in L3

In normal larvae, there is a major pulse in cardiac hemocyte volume within 8 h of entry to pupariation. This volume declines as pupal formation approaches, and we anticipate this wave is preparatory to pupal metamorphosis. We sought to determine whether the cardiac population participates in other pulses in hemocyte number, such as response to bacterial infection. When raised at low density in feeding medium, bacterial growth and biofilm formation can outpace larval media consumption. We isolated biofilm, containing three species from the Acetobacteraceae and Lactobacillaceae families by 16S rRNA sequence, which develops when wildtype larvae grew at less than 25 per 60 mm

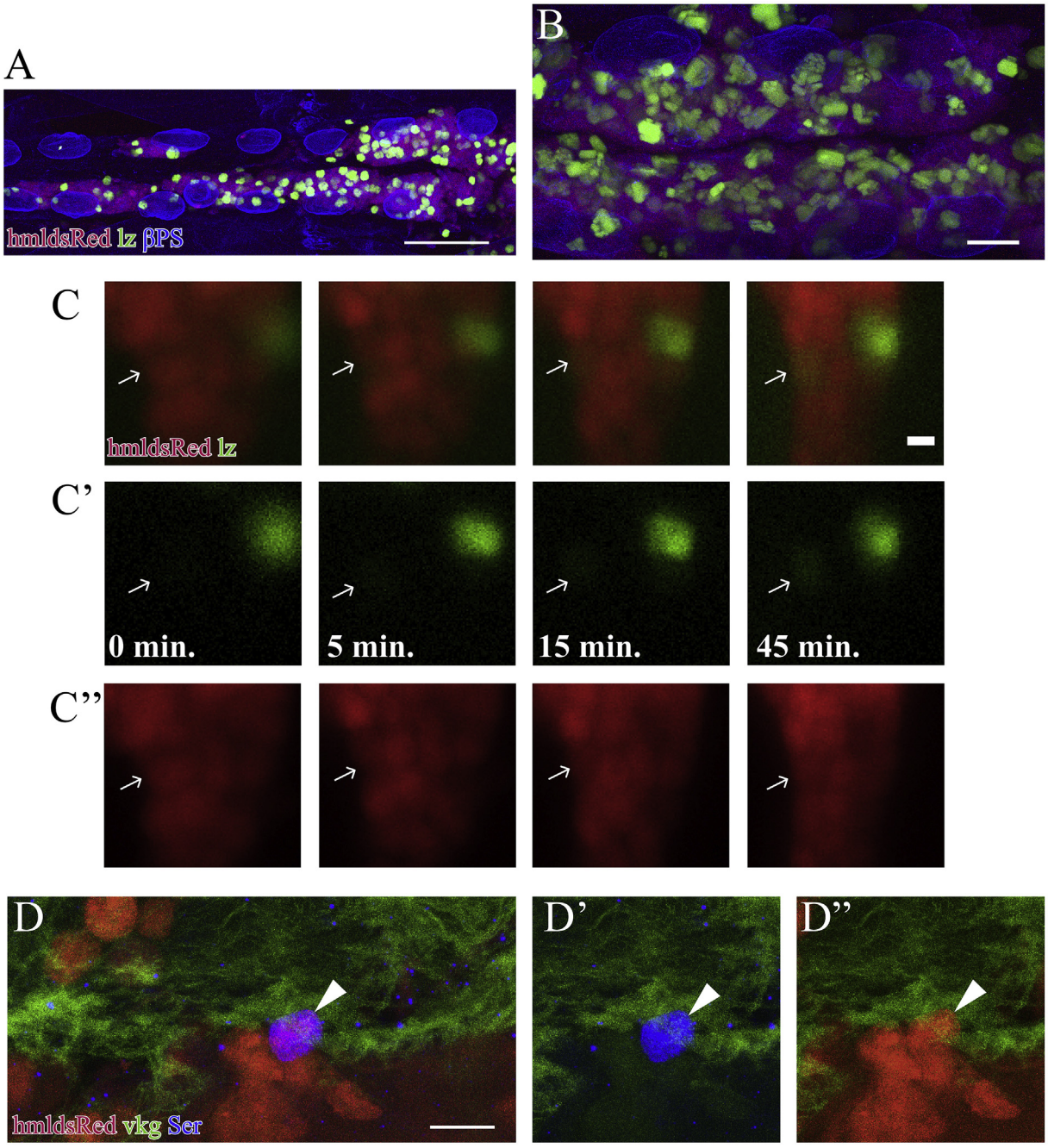
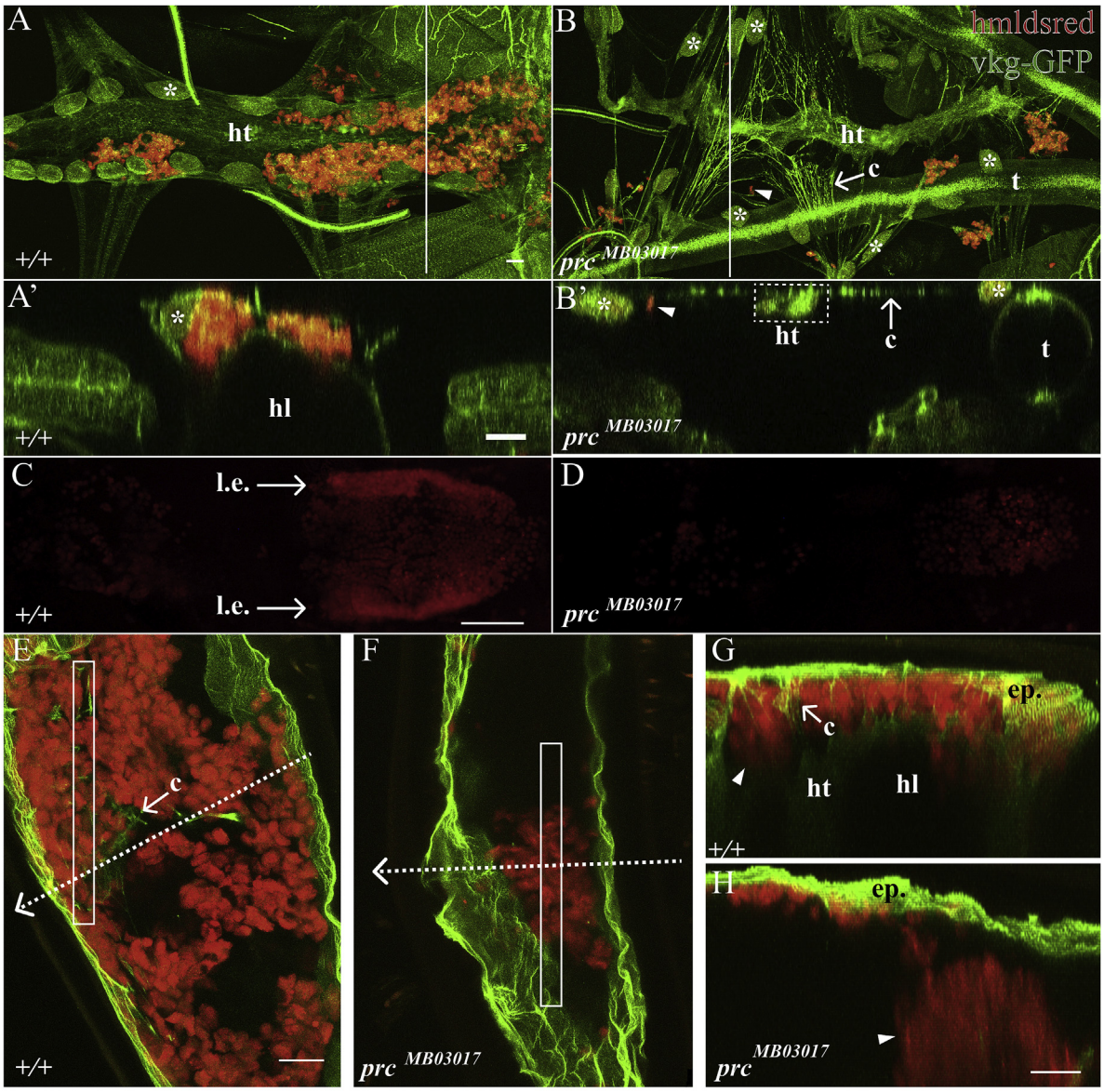


Fig. 4. Transdifferentiation is observed in DV hemocyte clusters. (A) Crystal cells are found together with plasmatocytes throughout the entire DV in migratory 3rd instars. (B) Crystal cells (lozenge GFP) and plasmatocytes (HmldsRed) are closely packed within the DV clusters. (C–C'') Still images from videos taken over 45 min show the initial stages of *lozenge-GFP* expression, reflecting the transdifferentiation of a plasmatocyte (red, arrow) into a crystal cell. Lozenge-GFP (green) and hmldsRed (red) are shown separately in C′ and C". (D) A 5 μm thick projection of the DV showing an Hml and Serrate expressing plasmatocyte (purple) within a DV hemocyte cluster. Serrate (blue) and hmldsRed (red) are shown separately in D′ and D". (A–C) Red: Hmldsred (plasmatocytes); Green: lozenge-GFP (crystal cell); Blue: anti-βPS (pericardial cells). Genotype: Lz Gal4/+(or Y); UAS GFP/HmldsRed. (D) Green: Vkg-GFP (Collagen IV); Blue: anti-βgal for Serrate-lacZ. Genotype: hmldsRed,vkg-GFP/Ser.lacZ. Scale bar: 100 μm (A); 25 μm (B); 5 μm (C); 10 μm (D).

plate. These bacteria are common to the *Drosophila* gut (Staubach et al., 2013), and were traced to unsterilized eggs. Food plates treated with chloramphenicol did not develop bacterial biofilm.

We compared the volume of DV hemocyte clusters in larvae that were raised at low density and the resultant natural biofilm with larvae raised on media inoculated with cultured bacteria at L1 and with a negative control raised on chloramphenicol treated media (Fig. 8). The antibiotic group had a lower DV hemocyte volume than the bacterial groups and a later pupation time (48 h). Comparable with our wildtype study (cf Fig. 5I), DV hemocyte volume rose at 40–48 h in the antibiotic group. Both cultures raised with increased numbers of bacteria had acute DV hemocyte accumulation starting 20 h into L3. Larvae raised on bacterial culture present at L1 did not trigger earlier DV hemocyte accumulation relative to the untreated group with natural bacteria only. This suggests the DV gains the ability to harbour more hemocytes 20 h after becoming L3. We conclude that DV hemocytes participate in the accumulation and dispersal response to immune challenge.



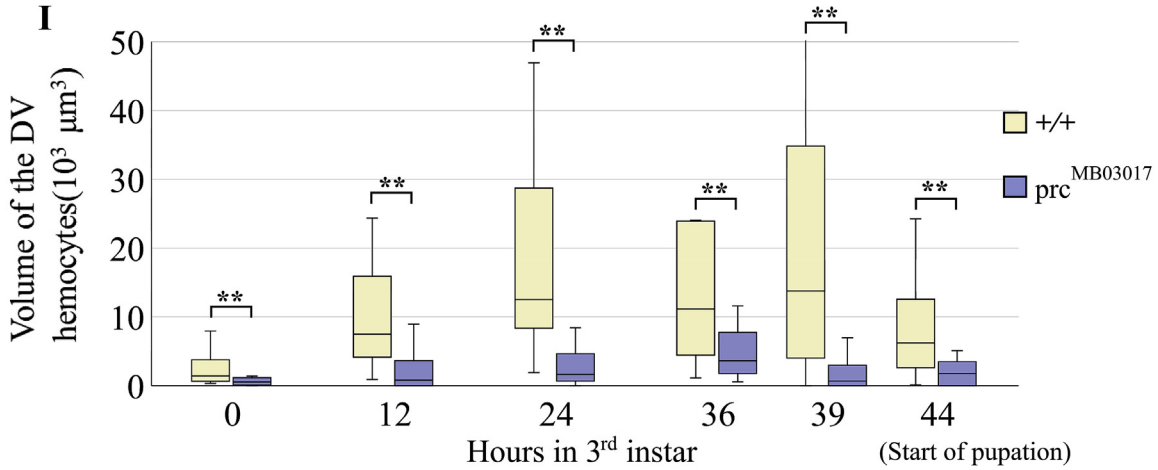


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Fig. 5. *prc*^{MB03017} mutants have DVs that contain fewer hemocytes and a smaller dorsal stripe. (A, B) 36 h after becoming third instar, hemocytes are abundant beside wildtype hearts (A) in contrast to *prc* mutants (B). Line represents the position of the cross-section shown in (A′) and (B′). (B, B′) Homozygous *prc* mutant DVs are suspended by thin, Vkg labeled fibers (c). Few hemocytes associate with the collagen network (arrowheads). Pericardial cells (asterisk) are distant from the heart tube (ht). (C, D) Dorsal view of the dorsal stripes on A6 and A7 of wild-type (C) and *pericardin* mutant (D) larvae at 36 h (75%) of third larval instar. Both A6 and A7 dorsal stripes of the wild-type are more uniform and populated than in *prc*^{MB03017}. (C) Lateral borders of wild-type dorsal stripes extend ventrally (lateral extension, le). (D) These lateral borders are not defined in the *pericardin* mutants. (E, F) Dorsal view of the sections of dorsal stripe embedded underneath the collagen IV (green) containing epidermis. Dashed arrow indicates anterior direction at the midline. Anterior at left. (E) Wild-type dorsal stripe contains fibrous collagen (c) that *prc*^{MB03017} (F) lacks. (G, H) Projected transverse cross-sections of the boxed areas of E and F. (G) In wild-type larvae, the wall of the heart tube (ht) is faintly visible in green. Dorsal stripe hemocytes are located between the DV and the epidermis (ep.). Lateral edge of the dorsal stripe (arrowhead) extends ventrally around the DV. Fibrous collagen IV structures (arrow) that stretch between the DV and the epidermis are interspersed among hemocytes. (H) DV is not located close to the epidermis in the dorsal stripe of *prc*^{MB03017} larvae. Few hemocytes rest on the epidermis and a hemocyte cluster (arrowhead) occupies the space left by dislocated DV. There is no fibrous collagen extending from the epidermis. (I) The growth in volume of DV associated hemocyte clusters seen in wildtype larvae (yellow) is reduced in the absence of Pericardin (blue). Mann-Whitney test, significance **≤0.005, n = 15. Red: Hmldsred; Green: vkg-GFP. c: collagen fibers; ht: heart tube; hl: heart lumen; le: lateral extension; t: trachea; ep: epidermis, asterisk: pericardial cell; arrowhead: hemocyte; arrow: collagen fibres. Genotypes: hmldsred,vkg/+ (+/+); hmldsred/vkg; prc^{MB03017}/prc^{MB03017} (*prc* mutant). Scale bar: All 25 μm except (C, D): 100 μm.

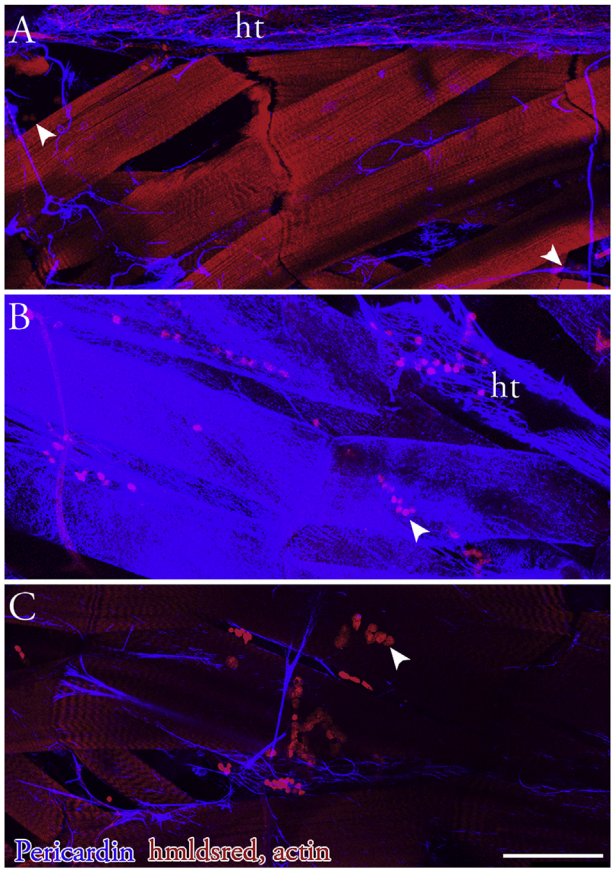

Fig. 6. Ectopic Pericardin is not sufficient to recruit hemocyte clusters. (A) Hemocytes (arrowheads) are sparse, and small clumps associate loosely with wildtype body wall muscles. (B) Expression of Lonely heart (Loh) on body wall muscles under Dmef-Gal4 regulation results in widespread accumulation of Prc. Isolated hemocytes align with the muscle surface (arrowhead). (C) Muscle expression of Loh ΔTSR1-2, which lacks a Prc interaction domain, did not recruit Prc to muscle surfaces, and both clumped and isolated hemocytes were observed. Images were selected for hemocytes and to not reflect normal density. Red signal adjusted to reveal muscle actin. Blue: Pericardin; Red: Hml-dsRed and Rhodamine-Phalloidin. Scale bar: 100 μm.

4. Discussion

The DV of the third instar larva is an organ that enables hemocyte clustering. Here, hemocytes accumulate on the inner foldings of the basement membrane, where they move with heart contraction, and are in proximity to the heart ostia. We have characterized this clustering phenotype, in order to assess the DV as a possible hematopoietic tissue. There are some similarities and differences between the previously identified tissue-resident hematopoietic systems and DV hemocytes.

The DV hemocyte population peaks in late L3 before pupariation, which is after the peak of sessile hemocyte proliferation (Makhijani et al., 2011). We determined that DV hemocyte proliferation can still be detected at this stage. It is probable that hemocytes migrate to the DV from peripheral points of proliferation, such as the dorsal stripe, as their ECM networks intersect. Although hemocytes may be born in the DV hemocyte population, the clusters are well positioned to contribute to hemocyte differentiation.

DV hemocytes are found in tight clusters, similar to sessile hemocytes of the epidermis. Moreover, DV hemocyte clusters are multiple cells thick, which increases the number of cell connections. Crystal cell production in larvae depends on cell-cell signaling, whereby an increase in the number of cells in contact increases the frequency of Lz^{+} cells (Leitão and Sucena, 2015). We have observed the start of *lz* expression in an Hml^{+} cell, reflecting the initiation of the trans-differentiation of a plasmatocyte into a crystal cell. It is not clear if the Lz^{+} cell received the *serrate* signal that caused it to transdifferentiate from a plasmatocyte at a location other than the DV. We describe Hml^{+} Ser^{+} cells at the DV, suggesting that transdifferentiation can be triggered among DV hemocytes.

Unlike hematopoietic compartments of epidermal-muscular pockets (Makhijani et al., 2011), DV hemocytes are not in a location to receive signals from PNS. It is not clear whether there is a signal that attracts hemocytes to the DV. Not all 3rd instars have DV hemocyte clusters and the pattern of clustering can vary. We have observed PSC like cells that are Hml^{-} Ser^{+} and have thin cellular processes. We also describe Atilla^{+}, Hml^{+} cells with elevated levels of cytoplasmic Talin that appear to ensheath some hemocytes. These markers and morphology are consistent with lamellocyte identity, which have been reported in third instar without immune challenge (Márkus et al., 2009). However, lamellocyte ensheathment of sessile plasmatocytes is a novel observation that invites further invbestigation. Nevertheless, as Ser^{+} and Atilla^{+} cells were identified in a minority of larvae, it is likely that they are not essential for hemocyte clustering at the DV.

Pericardin, a Collagen-like protein unique to the DV and dorsal stripe, facilitates hemocyte accumulation at the DV. We observed that larvae lacking Prc recruit fewer hemocytes to the heart as well as the dorsal stripe, suggesting that Prc may be a homing signal for hemocytes. We stimulated Pericardin deposition outside the DV with ectopic expression of its ECM receptor, Lonely heart. Prc deposition on body wall muscle did not did not facilitate the formation of more or larger hemocyte clusters. To the contrary, muscle associated hemocytes were dispersed, rather than the wildtype pattern of small clusters of cells. The role of Pericardin as adhesion factor for hemocytes is worthy of future exploration – our evidence indicates that Prc fibres are necessary but not sufficient to form hemocyte clusters.

Pericardial cells are often closely associated with hemocyte clusters in wildtype larvae and could attract hemocytes. However, Pericardial cells

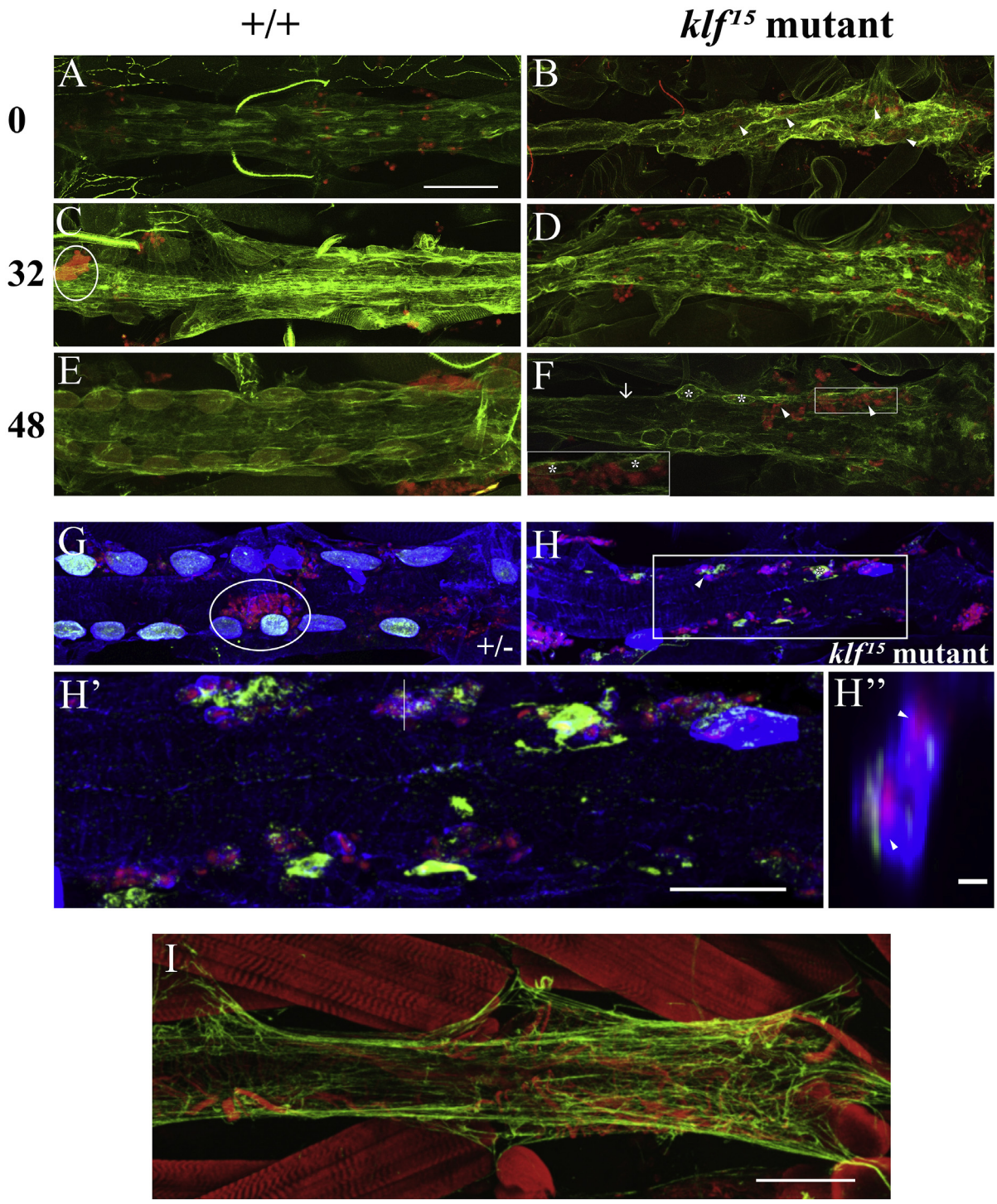

Fig. 7. *klf*^{15} mutation causes hemocyte accumulation at the DV. (A–F) DVs of +/+ and *klf*^{15} mutants (A–B) 0 (C–D) 32 (E–F) 48 h after becoming 3rd instar. Genotype: *Hml-dsRed,Vkg-GFP/+ (+/+); Klf15/Y; Hml-dsRed,Vkg-GFP* (*klf*^{15} mutants). Both groups were raised with chloramphenicol. In *klf*^{15} mutants, hemocytes are observed at the DV throughout development. n = 8. (C) +/+ DV hemocytes are often found in tight clusters (oval), whereas, around pericardial cell remnants in kfl15 mutants (D), DV hemocytes are predominantly separate (arrowheads). (F) Pericardial cell remains (asterisk) are still observed 48 h after becoming 3rd instar as well as where no pericardial cell is observed at all (arrowhead). Enlarged inset in (F) shows that hemocytes accumulate around disintegrating pericardial cells (asterisk). (G–H) DVs of ± and *klf*^{15} mutants during late L3. Genotype of G: *klf15/x: dotGAL4,hmldsRED/+;UAS- LifeActin-GFP/+* (±); H: *klf15/y;dotGAL4,hmldsRed;UAS-LifeActinGFP* (*klf*^{15} mutants). Both groups were raised on yeast plates with no antibiotic. Pericardial cells and the outline of the heart is detected using anti-βPS (blue). LifeActinGFP (green) is expressed in pericardial cells. In *klf15* heterozygotes, hemocytes (red) are closely clustered (oval) around intact wild-type looking pericardial cells, whereas in *klf15* homozygotes, most hemocytes (red, arrowhead) are loosely collected around degrading remnants of pericardial cells. Cluster at lower left is outside the DV ECM. (H′) Enlarged rectangle from (H). (H′′) Cross section of the hemocytes (arrowheads) taken at the line in (H′) shows that pericardial cells label overlap with hemocytes, indicating that hemocytes have engulfed parts of the degraded pericardial cells. (I) A reduced Pericardin network is present in *klf*^{15} mutant hearts. Pericardin (green) and actin (phalloidin red). Scale bar: 100 μm (A–H); 50 μm (H′), 10 μm (H′′).

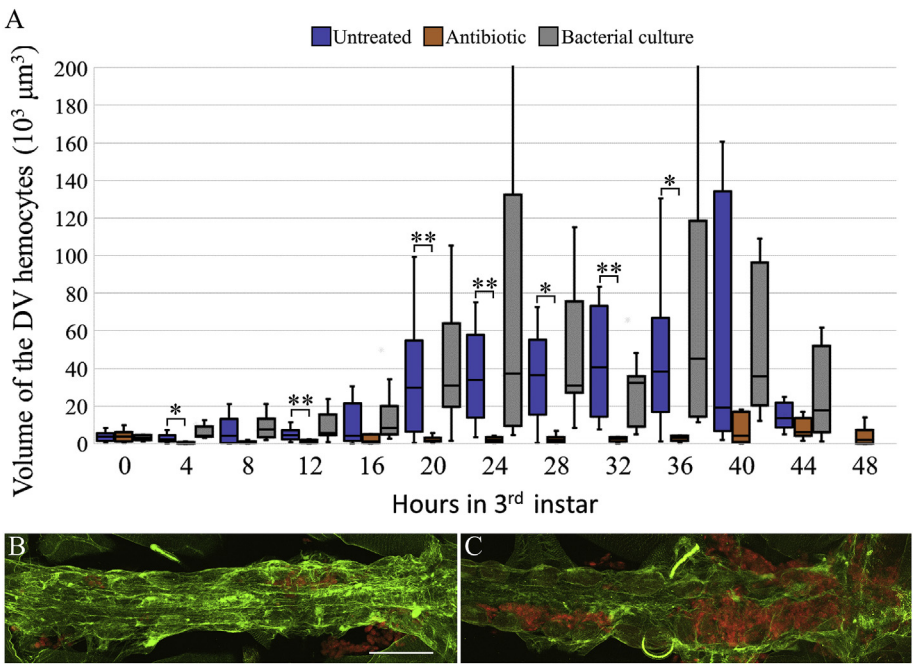

Fig. 8. More hemocytes accumulate at the DV when larvae are exposed to *Acetobacteraceae* and *Lactobacillaceae*. (A) Box-whisker plot of the total DV hemocyte volume throughout the 3rd instar stage. "Untreated" larvae were grown at low density with resultant biofilm production. "Bacterial culture" were first instar larvae inoculated with liquid culture. Medians are shown with lines. The antibiotic (chloramphenicol) group pupated at 48 h after becoming 3rd instar, 4 h later than controls. Mann-Whitney test relative to untreated, significance *≤0.05, **≤0.005. (B, C) Representative untreated group DVs with 43.3k (B) and 170.3k µm^3 (C) of volume occupied by hemocytes, dissected 36 h (B) and 32 h (C) after becoming 3rd instar. n = 10 (untreated, bacterial culture groups), n = 7 (antibiotic group). Genotype: *hmldsred,vkg*/+ Scale bar: 100 µm.

dissociate from the heart in p*rc* mutants, and they do not accumulate hemocytes in their ectopic locations. Furthermore, Pericardial cells degenerate in *klf*^{15} mutants (Ivy et al., 2015), within a normal-looking Pericardin containing ECM. Hemocyte association with the heart remains at normal levels, and they participate in phagocytosis of apoptotic Pericardial cells.

Considering that hemocyte clusters closely associate with basement membranes and *prc* mutants have few DV hemocyte clusters, ECM scaffolding emerges as an important factor that aids hemocyte accumulation at the DV. Subsequent to rupture of the hematopoietic lymph gland, prohemocytes relocate to adult hematopoietic hubs, where, the ECM is also thought to be required for clustering (Ghosh et al., 2015). The DV cluster likely contains many fewer prohemocytes, as it is populated by migrating plasmatocytes. Nevertheless, adult hematopoietic hubs have structural similarities with the larval dorsal stripe (Honti et al., 2014; Makhijani et al., 2011). Cross sections of the dorsal stripe reveal collagen fibers that act as a bridge connecting the DV to the epidermis and hemocytes occupy spaces between these collagen fibers (Makhijani et al., 2011). We found larval DV hemocytes to adhere more to each other through highly interdigitated adhesions, rather than interspersed through the ECM.

Response to infection is an important feature of hematopoietic systems (Márkus et al., 2009; Ghosh et al., 2015; Gold and Bruckner, 2015). We demonstrate a positive correlation between DV hemocyte numbers and environmental bacteria levels. Why are these hemocytes not migrating in surveillance or towards sources of infection, such as the trachea? It is possible that DV clustering behaviour is also affected by systemic cues of infection (Gold and Bruckner, 2015; Yan and Hillyer, 2019.), that affect hemocyte affinity for a Prc rich environment. The heart surface may be key for immune surveillance, as blood flow increases in proximity of the intake ostia. For example, hemocytes accumulate at the intake ostia of the adult mosquito heart in response to infection, and appear to absorb bacteria from the hemolymph from this location (Hillyer, 2015). The pattern of accumulation in the *Drosophila* larva is less discrete, and although not concentrated at the ostia, the DV hemocytes are exposed to incoming hemolymph.

There are other observations that strengthen the relationship between blood flow and hemocyte aggregation. Both mosquito and fruit fly larvae have anterograde (posterior to anterior) direction of hemolymph flow (League et al., 2014; Sláma and Farkaš, 2005). In mosquito larvae, larval ostia exist, but are not functional, which means that hemolymph mainly enters the heart through incurrent openings at the posterior end of the heart (League et al., 2014). These incurrent openings are surrounded by thin, respiratory tubes called tracheal tufts where hemocytes accumulate and kill pathogens (League et al., 2017; League and Hillyer, 2016). Hemocytes are observed in tracheal tufts of other insects such as (Locke, 1997) caterpillars, but *Drosophila* lacks tracheal tufts. Structural differences such as these can be the reason why one finds variation in DV hemocyte accumulation patterns across species or developmental stages.

Developmental age and response to infection both increase hemocyte association with the DV. Other genes likely participate in hemocyte accumulation at the DV. Infection triggers recruitment of hemocytes to the ostia of in the mosquito, which is enhanced by Nimrod family proteins Eater and Draper, as well as complement-like Opsonins that promote phagocytosis (Yan and Hillyer, 2019; Sigle and Hillyer, 2018a,b). A misexpression study in Drosophila identified candidate hemocyte genes that, when over-expressed, led to hemocyte accumulation at the DV in the absence of infection (Stofanko et al., 2008). Further exploration into the functions of these candidate genes may shed light on the mechanisms of hemocyte targeting, and the function of DV hemocytes.

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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.2019.06.006.

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