**Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in *Drosophila melanogaster***

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**Introduction**

During *Drosophila melanogaster* embryogenesis, hemocytes derive exclusively from head mesoderm at around 2 h after gastrulation (stage 10). From this point of origin, these cells migrate along stereotypical routes to populate the whole embryo by stage 17 (Tepass et al., 1994). It has previously been shown that the developmental migration of these cells is dependent on the expression of the VEGF/PDGF ligands Pvf1, -2, and -3 (Cho et al., 2002). The PDGF/VEGF receptor (PVR) is expressed in hemocytes (Heino et al., 2001), and *pvr* mutant embryos fail to exhibit normal hemocyte migrations, resulting in an accumulation of these cells at their head end (Cho et al., 2002; Sears et al., 2003). A recent study has demonstrated a role of PVR in controlling anti-apoptotic cell survival of embryonic hemocytes (Bruckner et al., 2004) and suggests that the defect in hemocyte distribution observed in the mutant is largely due to high numbers of hemocytes undergoing apoptosis and becoming engulfed by their neighbors. However, this study also showed that Pvr expression within hemocytes is required for the directed migration of a subset of these cells that enter the extended germ during normal development (Bruckner et al., 2004), suggesting that this population of hemocytes may well be using Pvf signals as a chemoattractant to guide their migrations. Additionally, ectopic expression of Pvf2 within the embryo has been shown to be sufficient to induce a chemotactic response from embryonic hemocytes (Cho et al., 2002).

In addition to migrating along developmental pathways, embryonic hemocytes have been shown to migrate toward a laser-induced wound in a process that resembles the vertebrate inflammatory response. For a hemocyte to chemotax toward a chemotactic source, be it a wound or a guidance cue expressed along developmental migration routes, it has to be able to sense a chemotactic gradient and polarize in alignment with that gradient. Studies using *Dictyostelium discoideum* and mammalian neutrophils have demonstrated that the phosphoinositides PtdIns(3,4,5)P3 (PIP3) and PtdIns(3,4)P2 (PIP2) are key signaling molecules that become rapidly and highly polarized in cells that are exposed to a gradient of chemoattractant (Stephens et al., 2002; Weiner, 2002; Merlot and Firtel, 2003). In these actively chemotaxing cells, phosphoinositide 3-kinases (PI3Ks) rapidly translocate from the cytosol to the membrane at the leading edge of the cell, whereas phosphatase and tensin homologue inhibitory drugs, we show that PI3K is essential for hemocyte chemotaxis toward wounds and that Pvf signals and PDGF/VEGF receptor expression are not required for this rapid chemotactic response. Our results demonstrate that at least two separate mechanisms operate in *D. melanogaster* embryos to direct hemocyte migration and show that although PI3K is crucial for hemocytes to sense a chemotactic gradient from a wound, it is not required to sense the growth factor signals that coordinate their developmental migrations along the ventral midline during embryogenesis.
(PTEN) dissociates from the leading edge and becomes restricted to the sides and the rear. The difference in localization of these two enzymes leads to localized PIP3 production at the leading edge of the cell (Funamoto et al., 2002; Iijima and Devreotes, 2002). Down- or up-regulation of PIP3 by deletion of PI3Ks or of PTEN, respectively, results in severely reduced efficiency of chemotaxis (Funamoto et al., 2002). Though PI3K has been shown to be important for cell motility using these model systems, its role for single-cell chemotaxis in vivo in a multicellular organism has yet to be clarified. D. melanogaster has one class I PI3K, Dp110 (Leevers et al., 1996), whose role in cell growth control and cell survival has been well characterized (Leevers et al., 1996; Weinkove et al., 1999; Scanga et al., 2000); however, no role in cell migration and chemotaxis in D. melanogaster for this protein has been shown.

In this study, we have analyzed the developmental migrations of hemocytes and characterized in detail their migration patterns along the ventral midline. Our quantitative analysis shows that ventral midline hemocytes undergo a rapid lateral migration, during which they are highly polarized. We show that Pvf2 and -3 expression in the central nervous system (CNS), and Pvf2 alone in the dorsal vessel, are essential for directing the migration of hemocytes along these structures, and a decrease in expression of these ligands in the CNS is essential for the normal lateral migration of hemocytes in this region. We have also analyzed the function of PI3K in hemocytes. Using both dominant-negative PI3K-expressing hemocytes and the specific PI3K inhibitory drug LY294002, we show that PI3K is not required for the Pvf-dependent normal dispersal of hemocytes during development but is essential for chemotaxis toward wounds. Additionally, we show that hemocyte chemotaxis toward wounds is dependent on actin polymerization but that PI3K is not required for lamellipodial formation and instead appears to be required to sense a chemotactic gradient from a wound and polarize the hemocyte accordingly. Our results demonstrate that at least two separate mechanisms operate in D. melanogaster embryos to direct hemocyte migration and show that although PI3K is crucial for hemocytes to sense a chemotactic gradient from a wound, it is not required to sense the Pvf growth factor signals that coordinate their developmental migrations along the ventral midline and dorsal vessel during embryogenesis.

Results
Developmental dispersal of hemocytes at the embryonic ventral midline follows a segmental pattern
It has previously been shown that in the developing D. melanogaster embryo, hemocytes derive exclusively from the head mesoderm and from this origin migrate along invariant pathways to populate the whole embryo by stage 17 (Tepass et al., 1994). One major migration route is along the ventral midline, where hemocytes come into close contact with the cells of the CNS midline and the neighboring ventral epidermis. To understand more fully the dynamics of hemocytes during their developmental migrations, we analyzed the migration of these ventral midline hemocytes using live time-lapse imaging of embryos expressing GFP.

Figure 1. Developmental dispersal of hemocytes at the ventral midline. (A) Ventral aspect of a pnxGAL4 UAS-GFP embryo at stage 12, showing hemocytes as they migrate along the ventral midline from anterior to posterior. (B) High-magnification detail of hemocytes at this stage reveals that cells are clustered together and exhibit only a few protrusions (arrow). (C) Ventral view of pnxGAL4 UAS-GFP embryos at stage 16. (D) High magnification of the same embryo reveals that these ventrally placed hemocytes are much larger than early hemocytes [compare with B], exhibiting impressive filopodia and lamellipodia. (E) Stills from a time-lapse video show hemocyte dispersal between Stages 14.5 and 15.5 of development. In all images, anterior is up and posterior is down. At the beginning of this video, the majority of hemocytes are positioned in a single line along the midline (i). Over the next 150 min of development, individual hemocytes leave the midline and migrate laterally to form the two more laterally placed populations seen at stage 16 (vi). (F) Superimposing the migratory paths of all hemocytes that leave the midline shows this process to conform to a pattern with hemocytes migrating away from the midline at a 90° angle and leaving from spatially reiterated exit points (i and vi, colored circles). Throughout this movement, midline hemocytes favor these exit points and often clump together at these locations (iii, arrows). A hemocyte can be seen at each of these locations once the migration is complete (vi). Individual hemocytes are labeled with colored numbers as they migrate laterally, with the color corresponding to the exit point from which they left the midline. Elapsed time in minutes is indicated in the lower right corner. Bars, 20 μm.
mocytes aggregate in clusters that correspond to the position of a defined lateral area (Fig. 1 F). While in the ventral midline, hemocytes leave the ventral midline from spatially reiterated exit points before adopting a more random path with constant changes in direction (blue path). This is followed by the lateral migration (red path) displaying high directionality (calculated by applying a ratio between the real distance traversed by the hemocytes and the shortest possible distance shown by the broken white line). On arrival at its destination, the hemocyte returns to a more random directional behavior (yellow path).

The same path represented by dots marking the hemocyte position at each time point shows the corresponding change in velocity of the hemocyte during lateral migration (distance between dots corresponds to velocity). Low-magnification imaging of these midline hemocytes at stage 16 reveals that, in contrast to the early stage hemocytes, these cells exhibit large filopodia and lamellipodia, extending 20 μm away from the cell body (Fig. 1 D).

To investigate more thoroughly the pattern of the characteristic lateral migration observed between stages 14 and 16, we used time-lapse confocal microscopy to track the paths taken by individual hemocytes as they performed this movement. Superimposing all hemocyte tracks from individual embryos shows that this distribution arises from the rapid lateral migration of a subset of midline hemocytes, which individually leave the midline and occupy these more lateral positions (Fig. 1 E and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1). High-magnification imaging of these midline hemocytes at stage 16 reveals that, in contrast to the early stage hemocytes, these cells exhibit large filopodia and lamellipodia, extending 20 μm away from the cell body (Fig. 1 D).

To investigate more thoroughly the pattern of the characteristic lateral migration observed between stages 14 and 16, we used time-lapse confocal microscopy to track the paths taken by individual hemocytes as they performed this movement. Superimposing all hemocyte tracks from individual embryos shows that this movement conforms to a strict segmented pattern where hemocytes leave the ventral midline from spatially reiterated exit points and perform a 90° movement from the midline to arrive at a defined lateral area (Fig. 1 F). While in the ventral midline, hemocytes aggregate in clusters that correspond to the position of the exit points, where cells initiate their lateral migrations (Fig. 1 E). Closer analysis of individual tracks shows that before their lateral movement, hemocytes appear to move up and down the ventral midline between the clusters and after lateral migration, to a lesser extent, along the lateral rows (Fig. 2 A). To further analyze this migration pattern, we quantified the directionality, velocity, and polarity of individual hemocytes while (1) moving within the ventral midline before lateral migration and (2) undergoing the lateral migration. To carry out this quantification, we focused our analysis on the most posterior clusters of hemocytes in each embryo examined (Fig. 1 E, circled area).

The directionality of hemocyte movement was calculated by applying a ratio between the shortest distance possible and the actual distance traversed by hemocytes. As expected from the plotted tracks, the movement during lateral migration is highly directional, showing a mean of 85.6% (±13.1; n = 46) directionality (Fig. 2 A, red track). If the same ratio is applied to the path of the same hemocyte before this lateral migration, while in the ventral midline, the mean directionality observed is much lower (29.6 ± 18.2%; n = 35; Fig. 2 A, blue track). Quantifying the speed of migration, we noted that during the lateral movement, the velocity of migration undergoes a sharp increase, with the mean velocity for lateral migrating hemocytes being 1.8 μm/min (±0.8; n = 46), as opposed to 1.1 μm/min (±0.5; n = 35) for hemocytes within the midline. Laterally migrating
hemocytes not only move faster but also have a more constant speed and rarely stall during the migration, in contrast with hemocytes in the ventral midline clusters, which often pause between periods of intense movement before they migrate laterally or into another cluster (Fig. 2 B).

The increase in speed and directionality of hemocytes during their lateral migration suggests that during this rapid movement, these cells are highly polarized. Actively migrating cells exhibit polarity by extending protrusions in the direction of migration, forming a persistent leading edge with an increased protrusive activity when compared with that of the cell rear. To quantify the polarity of these migrating hemocytes, we artificially divided individual hemocytes into quadrants (corresponding to anterior lateral, anterior medial, posterior lateral, and posterior medial) and measured the protrusive area in each of these quadrants. We found that during the lateral movement, the combined lateral protrusive area of a migrating hemocyte was, on average, 212 μm² (±10; Fig. 2, D and E). This demonstrates that these hemocytes are highly polarized along the medial lateral embryonic axis, displaying a robust and persistent leading edge at their lateral aspect throughout the lateral migration. In contrast, when the same quantitative analysis was performed on hemocytes that remain in the ventral midline, no such persistent polarization was observed. Although cells from the midline are able to extend protrusions similar in dimension to those described for hemocytes migrating laterally, these protrusions rarely remain in the same cell quadrant for >2 min and, consequently, the cell randomly oscillates between different states of polarity (Fig. 2 F). This oscillation correlates with the cell's seemingly random movement as it patrols a small, defined area within the midline. 

Pvf1 and -3 act as hemocyte chemoattractants in the CNS, and Pf2 acts alone in the dorsal vessel

It has previously been reported that hemocyte developmental migrations are dependent on the expression of the PVR tyrosine kinase (PVR in hemocytes) and that the three PDGF/VEGF ligands—Pvf1, -2, and -3—act redundantly as chemotactic factors, directing the migration of hemocytes throughout the embryo (Cho et al., 2002). However, a recent study has suggested that the main role of Pvf signals during hemocyte development is to function as survival factors, as hemocyte-specific expression of the pan-caspase inhibitor p35 in pvr mutants is sufficient to largely restore the hemocyte defects normally observed in mutant embryos (Bruckner et al., 2004). Despite these results, evidence still exists that Pvf ligands may be acting as chemoattractants to hemocytes (Cho et al., 2002). To address more thoroughly the role played by Pvf ligands during hemocyte migrations along the ventral midline, we first observed the detailed expression pattern of all three Pvf family members within this region of the embryo. Consistent with previous studies (Cho et al., 2002), we found that Pvf2 and -3, but not Pvf1, were expressed in the embryonic ventral midline. However, a detailed time course showing the expression pattern within the ventral midline reveals that the timing of expression of these two genes is different (Fig. 3). Pvf3 is expressed along the ventral midline at stage 10, when the germ band is fully extended. This expression subsequently decreases and is almost undetectable by stage 14 (Fig. 3, K–N). In contrast, Pvf2 expression is absent at stage 10 and is only observed from stage 12 onward. Expression appears strongest at stage 14, and over the next 2 h of development, RNA levels in the CNS decrease in a wave from anterior to posterior such that by stage 15...
the ligand is expressed in small, segmentally reiterated points along the posterior region of the ventral nerve cord (Fig. 3, F–I). These points of expression correspond to the locations at which hemocytes cluster in the midline at this stage (Fig. 2 C). We also observed strong expression of Pvf2 in the developing dorsal vessel of stage 14 embryos (Fig. 3, J), whereas no expression of Pvf3 or -1 was detected in this tissue (Fig. 3, E and O).

To further investigate the role of the Pvf ligands, we analyzed in detail hemocyte developmental migrations in pvr mutants. As previously described, pvr mutants exhibit a severe hemocyte migration defect in which the cells are unable to migrate from their origin in the head and undergo apoptosis (Cho et al., 2002; Sears et al., 2003). This defect can be rescued by the hemocyte-specific expression of the pan-caspase inhibitor p35 to prevent apoptosis of these cells (Bruckner et al., 2004). We observed that these rescued hemocytes not only are unable to infiltrate the germ band as previously shown (Bruckner et al., 2004) but also fail to migrate posteriorly along the CNS and the dorsal vessel (Fig. 4, C and D). Because Pvf2 is strongly expressed in both the ventral nerve cord and the dorsal vessel at the same developmental stages that hemocytes are found migrating along these structures, it seemed likely that Pvf2 acts as a chemoattractant to pull hemocytes along these two migration routes. To test this hypothesis, we analyzed hemocyte migration in the pvf2 mutant line vegf27C<br>\(^{6947}\), a homozygous viable piggyBac\( ^{\text{w}^-}\) insertion in the Pvf2 gene (Cho et al., 2002). Consistent with a chemotactic role for Pvf2, we found that these mutants display a complete absence of hemocytes along the dorsal vessel but, curiously, showed only a slight reduction in hemocyte number at the ventral midline (Fig. 4, E and F). srphemoGAL4UASGFP embryos injected with dsRNA against Pvf2 (Fig. 4, G and H), Pvf3 (Fig. 4, I and J), and Pvf2 and -3 together (Fig. 4, K and L). Embryos have been stained using the same antibodies used in A and B. Embryos injected with Pvf2 dsRNA show the same phenotype as the pvf2 mutant (Fig. 4, G and H). Injection of Pvf3 dsRNA had little effect on hemocyte migration (Fig. 4, I and J), but simultaneous silencing of both Pvf2 and -3 prevented hemocytes migrating along both the dorsal vessel (Fig. 4, K) and the ventral nerve cord (Fig. 4, L), mimicking the phenotype observed in rescued UAS-p35-expressing pvr\(^{\text{mutant}}\) embryos (compare L and D). Bar, 50 μm.

Figure 4. Pvf2 and -3 act redundantly as hemocyte chemoattractants in the CNS, and Pvf2 acts alone in the dorsal vessel. (A and B) srphemoGAL4UASGFP embryos stained with anti-armadillo (red) and anti-GFP (green) antibodies to visualize wild-type hemocyte migration pattern. (A) Dorsal aspect of an embryo at stage 14 shows hemocytes migrating posteriorly, along the dorsal vessel (arrows). (B) Ventral aspect of a stage 16 embryo shows hemocytes occupying three parallel lines running anterior to posterior along the embryonic ventral midline. (C and D) pvr\(^{\text{mutant}}\) mutant embryos expressing UAS-p35 and -GFP under the control of the hemocyte-specific srphemoGAL4 driver (Bruckner et al., 2004). (C) Dorsal aspect of a stage 14 embryo shows that although hemocytes are able to migrate away from their origin in the head, no hemocytes are found along the dorsal vessel (arrows) in these mutant embryos. (D) Ventral aspect of a stage 16 embryo shows that despite expression of p35, pvr\(^{\text{mutant}}\) mutant hemocytes are completely absent from the ventral midline and only a small number can be seen in the more lateral positions (arrow). (E and F) pvf2 mutant embryos (vegf27C<br>\(^{6947}\)) double stained with anti-armadillo (red) and anti-croquemort (green). These mutants display a complete absence of hemocytes at the dorsal vessel (E, arrows) but only a slight reduction in hemocyte number at the ventral midline (F). srphemoGAL4UASGFP embryos injected with dsRNA against Pvf2 (G and H), Pvf3 (I and J), and Pvf2 and -3 together (K and L). Embryos have been stained using the same antibodies used in A and B. Embryos injected with Pvf2 dsRNA show the same phenotype as the pvf2 mutant (G and H). Injection of Pvf3 dsRNA had little effect on hemocyte migration (I and J); however, simultaneous silencing of both Pvf2 and -3 prevented hemocytes migrating along both the dorsal vessel (K) and the ventral nerve cord (L), mimicking the phenotype observed in rescued UAS-p35-expressing pvr\(^{\text{mutant}}\) embryos (compare L and D). Bar, 50 μm.
To test this hypothesis, we overexpressed Pvf2 in the ventral migration of a subset of hemocytes away from the midline. A decrease in expression of this ligand is responsible for the lateral migration of hemocytes at the ventral midline (Fig. 5 D). Double staining of these embryos with the antibody simGAL4 UASPvf2 shows that the normal pattern of hemocyte distribution has been drastically disrupted, with only a small number of hemocytes found in lateral positions (Fig. 5 C), demonstrating that wound chemotaxis is independent of PVR expression (Fig. 6, A and B; Rorth, P., personal communication).

We have previously shown that embryonic hemocytes rapidly chemotax toward an epithelial wound (Stramer et al., 2005) in a process resembling the vertebrate inflammatory response. To determine whether Pvf signals play a chemotactic role during this inflammatory response, we made laser ablations to pvr mutants expressing p35. 1 h after wounding, these embryos show a robust wild-type response from the mutant hemocytes, demonstrating that wound chemotaxis is independent of PVR expression (Fig. 6, A and B; Rorth, P., personal communication). To further study the wound chemotactic response, we developed a wound assay using beads that allow the treatment of the wound region with inhibitory drugs. Such beads are routinely used in embryonic studies for the local application of chemical inhibitors in vivo (Kawakami et al., 2003). Implanting a bead into a D. melanogaster embryo creates an epithelial wound approximately the same diameter as the bead (Fig. 6 C). As is the case with laser-induced wounds, application of untreated beads to a stage 16 embryo leads to rapid accumulation of hemocytes at the wound site until, by 30 min, they surround the bead (Fig. 6 D). Like unstimulated midline hemocytes, these cells exhibit large dynamic membrane ruffles and filopodia as they surround and seemingly try to collectively phagocytose the invasive bead (Fig. 6 E and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1). These actin-rich protrusive structures are one of the most distinctive features of moving cells and are generally considered to be critical for single-cell migration. To directly test the requirement of cytokinetic protrusions during hemocyte chemotaxis, we pre-soaked beads in one of two actin polymerization blocking drugs, Cytochalasin D or Latrunculin B, before bead implantation and examined hemocyte recruitment in embryos 1 h after bead insertion. We found that treatment with either drug blocks this chemotactic response so that drug-treated beads show little or no hemocytes in direct contact with the bead and those hemocytes close to the bead exhibit small or no actin protrusions and are much less motile when compared with their wild-type counterparts (Figs. 6, F and G; and Video 2). In both cases, effects of drug treatment can be seen up to 50 μm away from the bead, as hemocytes that lie further away from the bead exhibit normal dynamic lamellipodia (Fig. 6 F). We assume that this reflects a drop off in drug concentration as a result of drug diffusion away from the bead.

PI3K is not required for developmental dispersal but is essential for chemotaxis toward wounds

For a hemocyte to chemotax toward a chemotactic source, it has to be able to rapidly sense a chemotactic gradient and polarize accordingly. Because PI3K has been shown in several systems to be a key mediator for cell polarization and directed cell migration along the dorsal vessel and, together with an earlier expression of Pvf3, orchestrates hemocyte migration patterns along the CNS during embryogenesis.

**Figure 5. Lateral hemocyte migration at the ventral midline requires a down-regulation in Pvf2.** (A–D) Embryos double stained with antibodies against armadillo (red) and croquemort (green). (A) Ventral aspect of a wild-type stage 15 embryo shows the normal pattern of hemocyte distribution with many cells located in lateral positions (arrows), having migrated away from the midline (asterisks). (B) Ventral aspect of a late stage 16 wild-type embryo. Hemocytes can clearly be seen in the characteristic three lines running anterior to posterior along the embryo. (C) Stage 15 embryo in which Pvf2 is being overexpressed in the ventral midline using the driver simGAL4. Antibody staining shows that the normal hemocyte distribution has been drastically disrupted, with only a small number of hemocytes found in lateral positions (arrows) and the majority remaining in clumps along the midline (asterisks; compare with A). (D) By late stage 16, these simGAL4 UASPvf2 embryos show normal lateral hemocyte distribution (arrows) and no trace of the delay in lateral migration can be seen. Bars, 50 μm.

(Cho et al., 2002), demonstrate this ligand to be operating as a hemocyte chemoattractant. The processive down-regulation of Pvf2 RNA from anterior to posterior in the ventral midline observed between stages 14 and 15 correlates temporally with the wave of lateral hemocyte movement occurring during wild-type development. Because Pvf2 is acting as a chemoattractant pulling hemocytes toward the midline, it is possible that the decrease in expression of this ligand is responsible for the lateral migration of a subset of hemocytes away from the midline. To test this hypothesis, we overexpressed Pvf2 in the ventral midline using the CNS midline driver single-mindedGal4 (simGAL4). Double staining of these embryos with the hemocyte-specific anti-croquemort antibody and anti-armadillo showed that the lateral migrations of hemocytes were severely disrupted such that at stage 15 of development, few hemocytes could be seen occupying more lateral positions (Fig. 5 C), demonstrating that a reduction in Pvf2 expression is necessary for normal lateral migrations. Interestingly, by stage 16, these embryos had recovered the defect, and hemocytes could be seen in the usual three lines running anterior to posterior along the midline (Fig. 5 D).

Collectively, these data demonstrate that Pvf2 acts as a chemoattractant signal whose expression mediates hemocyte movement along the CNS midline driver.
migration (Stephens et al., 2002; Weiner, 2002; Merlot and Firtel, 2003), we tested the requirement of PI3K for the directed migration of ventral midline hemocytes during development and their chemotaxis toward wounds. Confocal analysis of embryos expressing a dominant-negative form of the *D. melanogaster* PI3K catalytic subunit *Dp110D954A* (Leevers et al., 1996), specifically in the hemocytes, showed normal hemocyte distribution at all stages of development, and these cells appeared morphologically indistinguishable from their wild-type counterparts exhibiting dynamic lamellipodia and filopodia (Fig. 7, B, D, and F). The same result was observed when the PI3K inhibitor LY294002 was injected into the vitelline space of wild-type embryos during hemocyte migration (unpublished data).

To further characterize any possible role for PI3K during these developmental migrations, we performed the same quantitative tests on the ventral midline hemocytes as on wild type and found that *Dp110D954A*-expressing hemocytes migrate along the ventral midline in a pattern identical to that seen in wild-type embryos (unpublished data), demonstrating velocity (while migrating laterally, 1.6 ± 0.5 μm/min [n = 39]), directionality (while in midline, 33 ± 17.5% [n = 28], and while migrating laterally, 89 ± 8.9% [n = 39]), and polarity (mean lateral protrusive area of 250 vs. 16 μm² for the medial side) equal to wild-type cells, proving that PI3K is not required for these cells to sense and polarize toward the guidance cues that control their developmental dispersal.

Though clearly not required for hemocyte developmental migrations, we wanted to test whether PI3K was required for hemocytes to polarize and chemotax toward a wound site. To test the involvement of PI3K in this process, we made laser ablations in embryos expressing *Dp110D954A* in the hemocytes. In contrast to the wild-type chemotactic response (Fig. 8 A), the mutant cells failed to chemotax toward the wound site and the wound remained largely undetected by the hemocytes up to 1 h after laser ablation (Fig. 8 B). The same result was obtained when beads were implanted in these embryos (Fig. 8 D). To ensure that this phenotype was due to a failure in chemotaxis and not an indirect result of a reduction in hemocyte survival, we counted the number of hemocytes on the ventral side of embryos expressing *Dp110D954A*. We found no significant difference in hemocyte number when compared with wild-type embryos (mean number, 49 ± 9 [n = 17] in *Dp110D954A* and 56 ± 8 [n = 25] in wild type), demonstrating that PI3K plays no significant role in hemocyte survival at this stage of development. This is consistent with previously published data showing that hemocyte-specific expression of *Dp110D954A* does not cause the hemocyte aggregation that is associated with a decrease in cell survival (Bruckner et al., 2004). To further verify our chemotaxis result, we implanted two beads into *pxnGAL4 UAS-GFP* embryos in which the hemocytes were expressing GFP but were otherwise wild type. In these experiments, one bead was pre-soaked in the PI3K inhibitor drug LY294002 (Vanhaesebroeck et al., unpublished data).
and Waterfield, 1999) diluted in DMSO before implantation, and the control was presoaked only in DMSO. 1 h after bead implantation, as expected, the control DMSO bead was surrounded by hemocytes, demonstrating that DMSO alone does not block the chemotactic response of the hemocytes. However, no cells chemotaxed toward the bead soaked in PI3K inhibitor (Fig. 8 F). Time-lapse confocal analysis of hemocytes exposed to LY294002 shows that these cells are actively motile and able to form large, dynamic actin protrusions that look indistinguishable from untreated ventral midline hemocytes that have not been exposed to a wound. However, despite appearing morphologically normal, they are clearly unable to sense the position of the bead and chemotax toward it, even when the bead is implanted only 30 μm away from a large population of hemocytes (Fig. 8 H and Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1).

Discussion

In this study, we have analyzed in detail the developmental dispersal of hemocytes during embryogenesis. We have found that migration of hemocytes along the dorsal vessel requires a chemotactic Pvf2 signal, and migration along the ventral nerve cord is dependent on and orchestrated by chemotactic signals from both Pvf2 and -3 expressed in this tissue. It was previously suggested that the migration routes traversed by all hemocytes during development were controlled by all three Pvf ligands operating redundantly as guidance cues (Cho et al., 2002), and our data support a chemotactic role for both Pvf2 and -3. However, a recent study suggests that the primary role of Pvf signaling in embryonic hemocytes is to control anti-apoptotic cell survival (Bruckner et al., 2004). It is becoming clear that Pvf ligands may well act on hemocytes as both chemoattractants and survival factors. This in itself is not a new idea, and the close relationship between guidance and survival has already been shown to exist in D. melanogaster, where the overexpression of the germ cell chemorepellent Wunen causes excessive germ cell death (Starz-Gaiano et al., 2001). It remains to be seen whether all three Pvf ligands can act as both chemoattractants and survival factors or whether each ligand plays a different role, with Pvf2 and -3 acting primarily as chemoattractants and Pvf1 operating as a survival factor.

Many obvious parallels exist between the migration of hemocytes along the ventral midline CNS and another developmentally regulated migration in D. melanogaster, that of border cell migration. Border cells take ~6 h to migrate a distance of 100 μm, a speed consistent with that we describe during hemocyte migration along the CNS. Successful border cell migration, like hemocyte migration, requires the expression of the Pvr in the migrating cells (Duchek et al., 2001) and, just as we see for hemocytes, the chemotactic signals detected by the PVR in the border cells are not transduced through PI3K (Fulga and Rorth, 2002). Successful migration of border cells does, however, require Rac signaling and the Rac activator myoblastcity (mbc), the D. melanogaster homologue of Dock 180 (Duchek et al., 2001). It has been previously shown that hemocyte-specific expression of dominant-negative RacG12V disrupts all hemocyte developmental migrations, demonstrating that Rac is required for the successful migration of ventral midline hemocytes along the CNS (Paladi and Tepass, 2004; Stramer et al., 2005). Given that Pvr couples to the Dock 180 signaling pathway during border cell migration and that Dock 180 has been shown to be involved in the migration of lymphocytes (Fukui et al., 2001; Reif and Cyster, 2002), Mbc/Dock 180 is a potentially important protein for hemocyte migration. Despite the fact that mbc mutant embryos display a grossly normal pattern of hemocyte dispersal...
(Paladi and Tepass, 2004), it would be interesting to look in detail at the migration of these mutant cells along the ventral nerve cord. More work is needed to investigate what other similarities may exist between border cell migration and ventral midline hemocyte migration. During development, only a subset of the hemocytes present in the embryo respond to the midline Pvf expression and migrate along the CNS accordingly. Other cells follow other migratory pathways. What specifies these cells to migrate along the midline? Important studies in border cell migration have shown that the JAK–Stat signaling pathway signaling through the Domeless receptor (Dome) is necessary and sufficient to transform nonmotile epithelial cells into invasive ones (Silver and Montell, 2001; Beccari et al., 2002; Ghiglione et al., 2002). Whether a similar signaling mechanism is operating to specify future ventral midline hemocytes and initiate their migration remains to be seen.

We demonstrate that from stage 14 onwards, once hemocytes occupy the entire ventral midline, individual cells begin to rapidly leave the midline and occupy more lateral positions. At this stage of development, hemocytes appear to be highly polarized, exhibiting large lamellipodia at their leading edges and migrating at a speed more than three times faster than their earlier midline migration. We have shown that this lateral movement requires a down-regulation in the attractive signal provided...
by Pvf2 in the midline, but is this the only driving force for the lateral movement? One possibility is that a different source of chemotactrant exists in the more lateral positions and that once Pvf2 expression is sufficiently down-regulated, this chemotactrant source operates to pull hemocytes laterally. Alternatively, hemocytes may be actively repelled from the midline or from one another, and the lateral migration observed by a subset of these hemocytes is a consequence of these cells attempting to maximize the distance between one another while maintaining contact with the CNS. It remains to be seen which, if any, of these hypotheses is true, but what is certain is that the guidance of hemocytes along the ventral midline of the embryo is not as simple as was first thought, and more studies are required to determine the exact relationship between this subpopulation of hemocytes and the different structures within the CNS as well as the overlying ectodermal cells, any of which could provide either chemotactrants or repellents for the migrating hemocytes to respond to.

We have demonstrated a requirement of PI3K for the polarization and active chemotaxis of hemocytes toward an epithelial wound. This is the first demonstration of the role of PI3K for single-cell chemotaxis in D. melanogaster and shows a striking correlation with the mechanism of cell chemotaxis used by D. discoideum and mammalian neutrophils (Stephens et al., 2002; Weiner, 2002; Merlot and Firtel, 2003). In these model systems, class I PI3Ks are activated upon stimulation of G protein–coupled chemotactrant receptors and, once activated, PI3Ks catalyze the production of the phosphoinositides PIP3 and PIP2 at the leading edge of the cell. The accumulation of PIP3/PIP2 leads to a rapid and transient recruitment of pleckstrin homology domain–containing proteins, including the serine/threonine kinase Akt/PKB (Funamoto et al., 2001; Wang et al., 2002). Akt/PKB itself becomes activated upon recruitment to the membrane and, in D. discoideum, activates the serine/threonine kinase p21-activated kinase a, which eventually leads to the phosphorylation of Myosin II and subsequent polarization of the cytoskeleton (Chung et al., 2001). Evidence also exists to support a role for the PI3K antagonist PTEN in helping to establish and maintain the intercellular PIP2 gradient required for successful chemotaxis by down-regulating the PIP2 pathway at the rear of the migrating cell (Funamoto et al., 2002; Iijima and Devreotes, 2002). How much of this signaling pathway is operating in chemotaxing hemocytes remains to be seen. Our current study demonstrates the involvement of PI3K, and previous work has shown that the small GTPase Rac is required for efficient hemocyte chemotaxis toward wounds. In neutrophils, PIP2 production has been shown to be autocatalytic and to require Rac but not Cdc42 (Weiner et al., 2002; Srinivasan et al., 2003). In the proposed positive feedback loop, it is thought that PIP2 may stimulate Rac through activation of a specific Rac GEF, which in turn activates PI3K, as well as effectors that mediate lamellipodial protrusion (Weiner et al., 2002). Because Rac is absolutely required for hemocyte chemotaxis and lamellipodia formation, it is tempting to speculate that a similar feedback loop may be operating in D. melanogaster hemocytes. Further work is required to determine the complex relationships operating among PI3K, Rho family small GTPases, and the actin cytoskeleton that coordinate chemotactic migration in these highly motile cells.

The PI3K-dependent mechanism of polarization required for hemocyte chemotaxis toward a wound is extremely fast and perfectly suited for mature, highly motile hemocytes that need to rapidly react to a source of attractive signal, be it a wound, an invading organism, or an apoptotic cell. In contrast, the mechanism to developmentally disperse need not be so rapid, as the aim during development is simply to ensure that hemocytes migrate toward and arrive at their target tissue in a given amount of time and does not require the rapid response to constantly changing environments required for mature hemocytes. The mechanism controlling the developmental migration of hemocytes along the ventral midline is consequently much slower and is dependent on slow-diffusing growth factors of the Pvf family providing short-range guidance information signaling through the receptor tyrosine kinase PVR. These two mechanisms may not be the only ways in which hemocytes are able to chemotax toward an attractive source; indeed, the observation that hemocytes travel different migratory routes in the embryo suggests that they may not all be using the same machinery to polarize and migrate. What does seem to be consistent for both chemotaxis toward developmental signals and toward wounds, like motility in many cell types, is a requirement for Rac signaling and the formation of actin protrusions.

The fact that hemocyte migrations within the embryo are strictly regulated and adhere to a stereotyped pattern is important in a developmental context. Throughout embryogenesis, hemocytes carry out important developmental functions within the embryo, such as the engulfment and removal of apoptotic cells (Franc et al., 1999) and the laying down of many extracellular matrix molecules, including collagen IV and laminin, that compose the basement membrane surrounding internal organs (Fessler and Fessler, 1989). The failure of hemocytes to travel along their normal migratory routes therefore has serious consequences. Such defects have been described in pvr mutants, where a lack of hemocyte migration along the ventral nerve cord results in a failure in CNS condensation (Olofsson and Page, 2005), as well as a disruption in axon patterning (Sears et al., 2003). It is therefore vital for the embryo to ensure that hemocytes arrive at their correct target tissues during development. For this to occur, it is not sufficient to allow these cells to passively disperse throughout the embryo by random migrations; instead, a directed and tightly controlled migration is required.

In this study, we have locally applied drugs to D. melanogaster embryos using bead implantation. The application of drugs has been a powerful tool in cell culture and in vitro cell motility studies but remains largely unused in D. melanogaster. Using our bead assay, we will be able to take advantage of the many useful drugs available to block both specific signaling pathways as well as important cytoskeletal processes. Combined with the powerful genetics available in D. melanogaster and the relative ease of live imaging in this system, the study of D. melanogaster hemocytes provides a powerful model to address the process of cell motility and chemotaxis and will undoubtedly provide a clearer understanding of the regulation and mechanics of single-cell migration in the complex setting of a multicellular organism.
**Materials and methods**

**Fly stocks**
For live studies, GFP was expressed in hemocytes using either the hemocyte-specific Gal4 line serpentHemoGal4 [spHemoGal4; Bruckner et al., 2004] or paxosidasinGal4 [paxGal4; Stramer et al., 2005]. To visualize both hemocytes and epithelial cells, we used a stock homozygous for E-cadherin-GFP (Oda et al., 1998) on the second chromosome and homozygous for a recombinated chromosome carrying both croquemort-GAL4 [crgAL4] and UAS-GFP on the third. To drive sufficient expression of both dominant-negative PI3K and GFP in embryonic hemocytes, w; UASdp110G056A Leeser et al., 1996) flies were crossed to w; pVegf27Ca; UAS-GFP; croquemort-GAL4 flies, generating w; pVegf27Ca; UAS-GFP, UAS-GFP, UAS-GFP/; crqGal4;UAS-GFP/+ progeny. To visualize hemocyte motility in rescued p35-expressing pvf2 mutant embryos (Bruckner et al., 2004), w; spHemoGal4 flies were crossed to w; pV5-actin-GFP; UAS-p35/+ embryos. For Pvf2 overexpression experiments, we crossed w; simGal4 flies to w; UASPv5Fulga, T.A., and P. Rorth. 2002. Invasive cell migration is initiated by guided chemotaxis by Akt/PKB and PI3 kinase through the regulation of PAKa. Genes Dev. 108:865–876.

**Antibody staining and in situ hybridization**
Embryos were fixed in 4% formaldehyde and devitellinized before being hand. Antibody staining on the resulting embryos. The Vegf27Ca(Cr.) (Cho et al., 2002) line was used for Pvf2 mutant analysis.

**Online supplemental material**
Video 1 shows wild-type hemocyte migration at the embryonic ventral midline. Video 2 shows the effect of Cytochalasin D on hemocyte chemotaxis toward wounds. Video 3 demonstrates the effect of the PI3K inhibitor LY294002 on the same process. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200508161/DC1.

**References**


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