

The Immune Phenotype of Three *Drosophila* Leukemia Models

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ABSTRACT Many leukemia patients suffer from dysregulation of their immune system, making them more susceptible to infections and leading to general weakening (cachexia). Both adaptive and innate immunity are affected. The fruit fly *Drosophila melanogaster* has an innate immune system, including cells of the myeloid lineage (hemocytes). To study *Drosophila* immunity and physiology during leukemia, we established three models by driving expression of a dominant-active version of the Ras oncogene (*Ras^{V12}*) alone or combined with knockdowns of tumor suppressors in *Drosophila* hemocytes. Our results show that phagocytosis, hemocyte migration to wound sites, wound sealing, and survival upon bacterial infection of leukemic lines are similar to wild type. We find that in all leukemic models the two major immune pathways (Toll and Imd) are dysregulated. Toll-dependent signaling is activated to comparable extents as after wounding wild-type larvae, leading to a proinflammatory status. In contrast, Imd signaling is suppressed. Finally, we notice that adult tissue formation is blocked and degradation of cell masses during metamorphosis of leukemic lines, which is akin to the state of cancer-dependent cachexia. To further analyze the immune competence of leukemic lines, we used a natural infection model that involves insect-pathogenic nematodes. We identified two leukemic lines that were sensitive to nematode infections. Further characterization demonstrates that despite the absence of behavioral abnormalities at the larval stage, leukemic larvae show reduced locomotion in the presence of nematodes. Taken together, this work establishes new *Drosophila* models to study the physiological, immunological, and behavioral consequences of various forms of leukemia.

KEYWORDS

Ras
oncogene
nematodes
insect immunity
hemocyte
Genetics of
Immunity

Immune dysregulation and a general weakness (cachexia or wasting syndrome), including loss of appetite and fatigue, are general features of leukemia (Forconi and Moss 2015). Immune dysregulation affects cells of both the adaptive and the innate branch (Ganan-Gomez *et al.* 2015; Forconi and Moss 2015). Although the mechanisms

for immunosuppression are poorly understood, the leukemic cells may confer part of the suppressive activity themselves. This is due to dysregulation of immune response and to features the leukemic clones share with regulatory B cells (Forconi and Moss 2015). Effects on innate immunity include reduced levels of complement proteins and defects in neutrophils and natural killer cells. In addition, a shift toward nonclassical activation of monocytes to an M2-like phenotype, which is associated with repair and healing functions (Mills and Ley 2014), is observed (Muallem and Hunter 2014; Forconi and Moss 2015). Mutations of the small GTPase, Ras, were shown to occur in several forms of leukemia, similar to other types of tumors (Niemeyer 2014). In general, heterozygosity for mutations of the members in the Ras/Raf/MEK/ERK signaling pathway, collectively called “RASopathies,” predispose to cancer or myeloproliferative disorders during infancy (Niemeyer 2014).

Fewer mutations are sufficient to induce a tumorous or pro-tumorous state in flies compared to mammals (Gateff 1978), which renders

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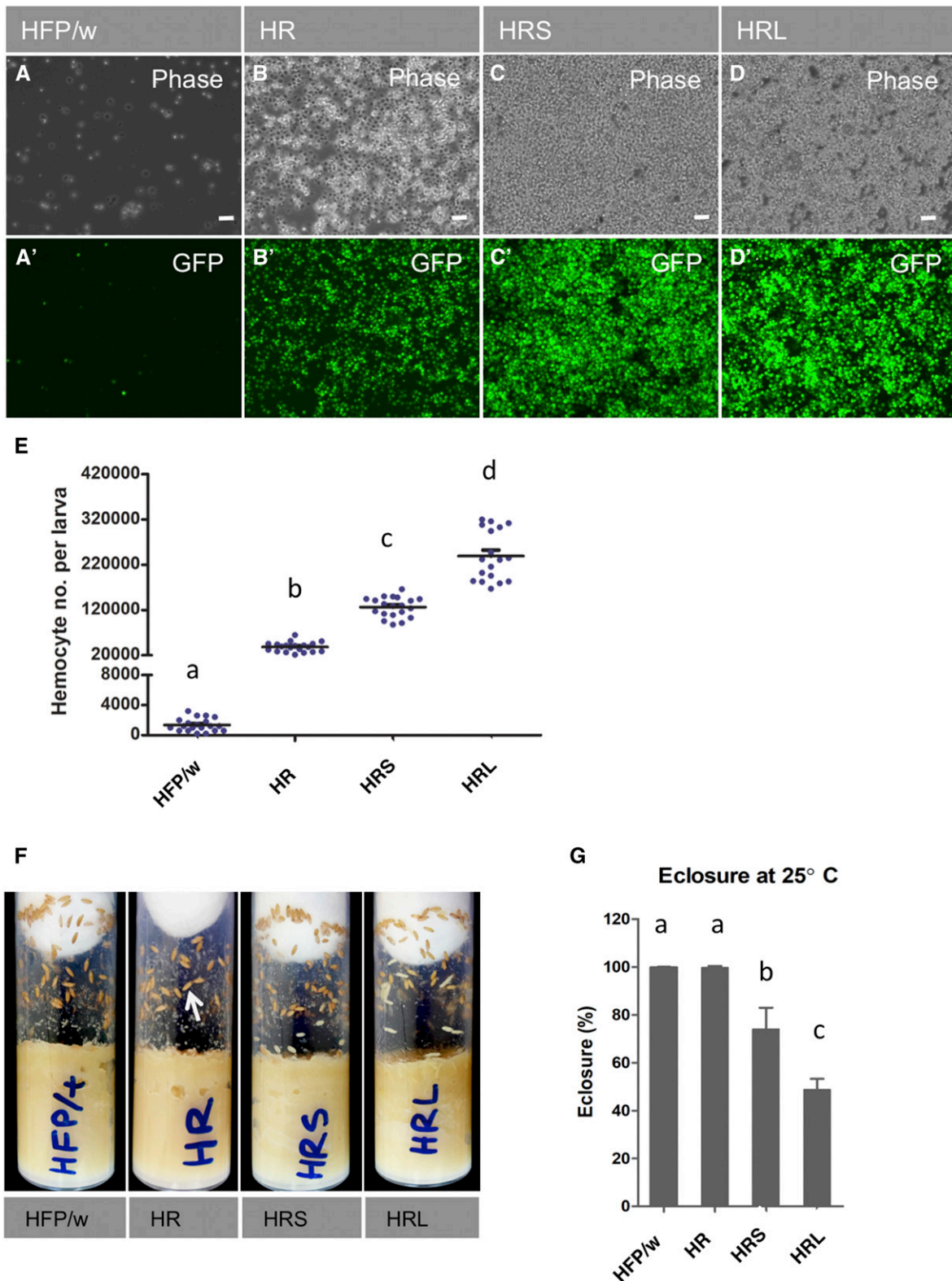


Figure 1 Establishing *Drosophila* leukemia by expressing *Ras*^{V12} alone and in combination with knockdown of tumor suppressors in hemocytes. (A) Larval hemocytes from control crosses expressing eGFP (*w; hml* (Δ)-*GAL4 UAS-eGFP* \times *w*¹¹¹⁸, which is abbreviated to HFP/w). (B–D) The number of hemocytes increases drastically when a dominant active form of Ras (*UAS-Ras*^{V12}) alone (HR) or in combination with RNAi lines of tumor suppressors *scribble* (*scrib*, HRS) or *lethal giant larvae* [*l(l)2*gl, HRL] is expressed using the same driver as in (A). (A–D) show phase contrast exposures while (A'–D') show the corresponding images under fluorescence, revealing eGFP-expressing hemocytes. (E) Quantification of late third-instar larval hemocytes for the genotypes shown in (A–D). (F) Leukemia lines are viable in the prepupal stage (12 hr of metamorphosis) at 25°. (G) Eclosure percentage at 25°C for the genotypes shown in (A–D).

Drosophila melanogaster a suitable model to study tumorigenesis (Bangi 2013). Mutants in single genes can affect the brain and the imaginal discs, as well as blood cells (hemocytes), and lead to hyperplastic and neoplastic growth (Brumby and Richardson 2005; Bangi 2013). *Drosophila* models provide insight into the molecular causes of malignancy and have the potential to identify new therapeutics (Gonzalez 2013). Affecting single genes that are required for hemocyte development can alter their proliferative capacity and lead to a massive increase in hemocyte numbers during larval development (Gateff *et al.* 1980; Crozatier and Vincent 2011). Starting in the early 2000s, the use of molecular genetics allowed induction of hypertrophic and hyperplastic growth in a tissue-specific and clonal manner (Brumby and Richardson 2003; Pagliarini and Xu 2003). This led to the dissection of cell-autonomous and nonautonomous pathways, which enhanced or limited tumor growth [summarized in Pastor-Pareja and Xu (2013); Parisi *et al.* 2014; Tipping and Perrimon 2014]. Expression of a dominant-active form of the Ras oncogene (Ras^{V12}) and mutations of tumor suppressors such as *scribble* (*scrib*) and *lethal giant larvae* (*l(2)gl*) turned out to be very efficient at inducing overproliferation in eye discs (Brumby and Richardson 2003; Pagliarini and Xu 2003). In addition to the fly's short generation cycle, a wide range of molecular tools facilitate whole organism studies of the genes and pathways involved in leukemia progression and modulation (Christofi and Apidianakis 2013; Parisi *et al.* 2014; Hauling *et al.* 2014).

Like all invertebrates, *Drosophila* lack cells of the lymphoid lineage but contains cells that are akin to the myeloid lineage (Gold and Bruckner 2014; Honti *et al.* 2014). These are collectively called hemocytes and the most dominant class of hemocytes in *Drosophila* are the plasmatocytes (Crozatier and Vincent 2011). Plasmatocytes combine the phagocytic function of monocytes/macrophages (Gold and Bruckner 2014) with some of the functions of polymorphonuclear leukocytes (Theopold *et al.* 2014) and display a surprising degree of plasticity (Anderl *et al.* 2016). Ras^{V12} has been used to induce overproliferation of plasmatocytes. Ras^{V12} -expressing plasmatocytes retained normal phagocytic capacity and showed differential expression of cell-cycle regulators, as well as several immune genes (Asha *et al.* 2003). Here, in addition to expressing Ras^{V12} in hemocytes alone, we also increased the protumorous phenotype by coexpressing RNAi constructs targeting *l(2)gl* or *scrib*. At 25°, development and behavior appear normal for all combinations until the pupal stage. However, at higher temperatures, we observe inhibition of adult tissue formation and degradation of cell masses during metamorphosis, which is similar to cancer-associated cachexia. We show that larval hemocytes retain several of their normal activities, such as phagocytic ability and migration to wound sites, in all three leukemic lines. We also find that expression of the Toll reporter Drosomycin (*Drs*) is activated in contrast to Imd reporters, which are suppressed. Although we observe only subtle changes in immune competence, the larvae coexpressing Ras^{V12} with a RNAi construct for the tumor suppressors *l(2)gl* or *scrib* are more sensitive to infection with entomopathogenic nematodes (EPNs), indicating additional layers of immune regulation. We show that locomotion of *scrib*^{RNAi}; Ras^{V12} larvae is reduced in the presence of nematodes, potentially contributing to the increase in larva sensitivity toward EPNs.

MATERIALS AND METHODS

Drosophila stocks and genetics

Fly stocks were kept under standard conditions and crosses were performed at both 25° and 29° (see figure legends for each experimental condition). The *w*; *hml* (Δ)-*GAL4* *UAS-eGFP* (II) stock was a generous gift from Dan Hultmark. *w*; *hml* Δ -*GAL4* *UAS-eGFP* (II) abbreviated to HFP. *Bx*^{MS1096}-*GAL4* and *UAS-Ras85D*^{V12} (III) strains were obtained from the Bloomington *Drosophila* Stock Center, USA. *UAS-Ras85D*^{V12} (III) is referred to as Ras^{V12} in the text. In order to monitor hemocytes, *UAS-GFP* was recombined with *Hml* Δ -*GAL4* on the second chromosome. All RNAi lines for tumor suppressors *Scrib* and *l(2)gl* were received from the Vienna *Drosophila* RNAi Center (Dietzl *et al.* 2007). *UAS-Scrib*. RNAi (v105412/KK); *UAS-Ras85D*^{V12} and *UAS-l(2)gl*. RNAi (v109604/KK); *UAS-Ras85D*^{V12} lines were generated in the laboratory by combining *UAS-Ras85D*^{V12} (III) and RNAi line (II) for tumor suppressor. Abbreviations for control and all leukemia model genotypes are: **HFP/w**- *w*; *hml* (Δ)-*GAL4* *UAS-eGFP* \times *w*¹¹¹⁸, **HR**- HFP/*w*; *UAS-Ras85D*^{V12}/+, **HRS**- HFP/*UAS-Scrib*.RNAi; *UAS-Ras85D*^{V12}/+, and **HRL**-HFP/*UAS-l(2)gl*.RNAi; *UAS-Ras85D*^{V12}/+.

Hemocyte preparation and counting

Late third instar (wandering stage) larvae were washed with 25° tap water. Then larvae were quickly dipped in 70% ethanol, followed by submerging in water to remove ethanol. Larvae were gently brushed to release their tissue-resident sessile hemocytes into circulation (Makhijani *et al.* 2011; Arefin *et al.* 2015) and placed individually in one well of a 12-well slide (Hendley-Essex 12 multislot slides), where each well contained 30 μ l Schneider medium. Anticoagulant phenylthiourea (PTU) was mixed with Schneider medium beforehand to prevent melanization while bleeding. Hemocytes were counted using a hemocytometer. For microscopy, 30 min were allowed to let the hemocytes settle, followed by live imaging.

Phagocytosis assay

Late third-instar larvae were bled in PTU containing Schneider medium and incubated in a humid chamber for 10 min at room temperature (RT). A 10-fold excess of Texas Red-conjugated heat-killed *Escherichia coli* (k-12 strain) was applied to hemocytes and incubated for 20 min at RT. Extracellular fluorescence was quenched by adding trypan blue to a final concentration of 0.2% to the cells just before microscopy (Kurucz *et al.* 2007). The phagocytic index was calculated as the number of hemocytes displaying phagocytosis divided by the total number of hemocytes and multiplied by the average number of phagocytosed bacteria per hemocyte (Kocks *et al.* 2005). The control (HFP \times *w*¹¹¹⁸, referred to as HFP/*w*) was set to 100 and the respective comparisons were relative to the control. Approximately 70–100 hemocytes from six independent larvae were analyzed.

Hemocyte migration and wound sealing assay

White prepupae were gently washed and dried with Kleenex tissue paper. Then prepupae were wounded with a needle (125 μ m in diameter) at the dorsal side of the posterior end and immediately monitored for hemocyte migration to the wound edge [a modified protocol after Regan *et al.* (2013)]. Within a few minutes hemocytes started migrating to the wound edge. For the wound-sealing assay, late third-instar larvae were

No sign of larval lethality is seen in the leukemic lines. White arrow indicates the normal prepupa. (G) Quantification of eclosion rates at 25° for all lines. Expression of Ras^{V12} alone in hemocytes (HR) does not interfere with eclosion rates at 25°; however, reduced eclosion is observed with HRS and HRL. ANOVA followed by Tukey's multiple comparison test was performed. The same and different letters above columns on the graphs indicate nonsignificance [a to a for instance, see (G)] and significance [a to b for example, see (E) and (G)], respectively. Data represent the means with SD; $P < 0.0001$ and $P < 0.01$ was found for (E) and (G), respectively. Bar in (A–D), 50 μ m.

A

| Survival assay- 29° C | | |
|-----------------------|--------------|----------------------------|
| Genotype | Eclosure (%) | When died |
| HFP/w | 100 | - |
| HR | 0 | late pupal stage |
| HRS | 0 | 3rd instar and pupal stage |
| HRL | 0 | 3rd instar and pupal stage |

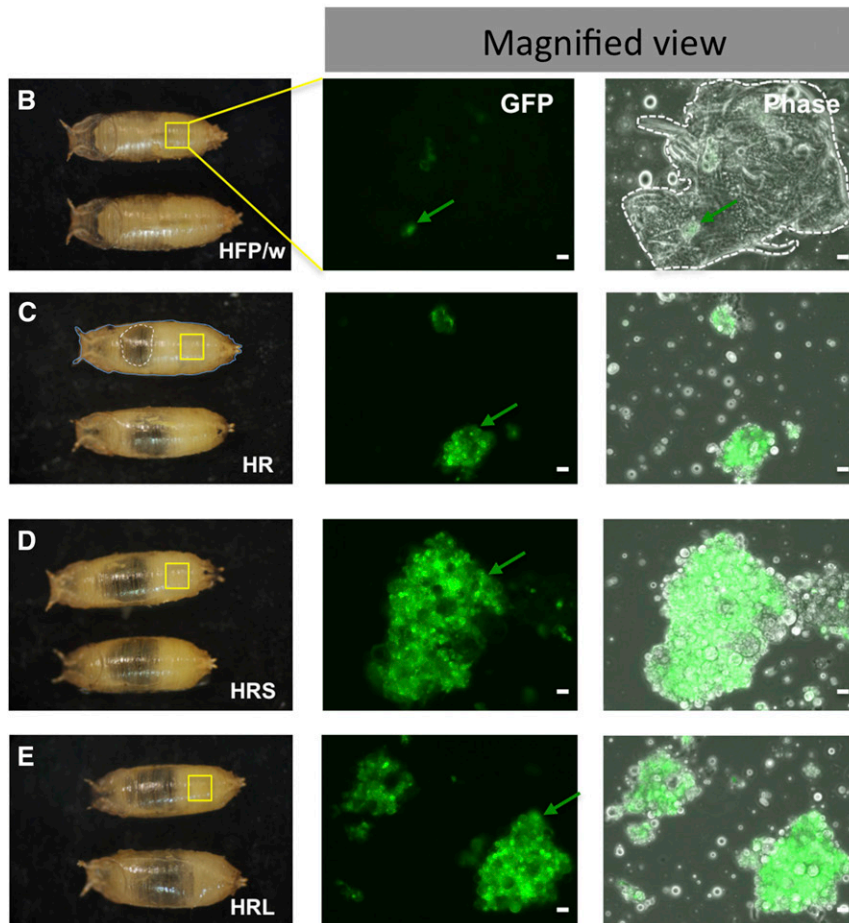


Figure 2 Leukemic lines at higher temperatures die and contain hemocyte aggregates at the pupal stage. (A) At 29°, HR failed to eclose and larval/pupal mortality was observed for HRS and HRL. (B) Control HFP/w pupae. Opening of pupal cage at 46 hr after puparium formation (APF) at 29° showed fewer hemocytes and formation of adult structures (outlined in phase contrast). (C–E) Opening of pupae from leukemic lines showed clusters of hemocytes and an absence of adult structures. The dashed line in (C) outlines the vacuole-like structure (perhaps due to drying out). Green arrows point toward GFP-positive hemocytes. Scale bar, 20 μ m.

wounded and followed until bleeding stopped. Puparium formation was used as an indication for successful sealing and quantified.

RT-qPCR

Late third-instar larvae were washed and dipped in 70% ethanol followed by rinsing with water. Total RNA extraction was performed as previously described (Arefin *et al.* 2014). RNA quality and concentration was determined with NanoDrop 2000 spectrophotometer (Thermo Scientific). A total of 1000 ng of total RNA was used to convert cDNA using SuperScript III Reverse Transcriptase (Invitrogen) and oligo(dT) (20-mer). RT-qPCR was performed using KAPA PROBE FAST Universal qPCR Master Mix (Kapa Biosystems) and TaqMan Expression Assays (Applied Biosystems) for *Drs* and Cecropin A1. Dipterin A1

was synthesized by the custom TaqMan Expression Assays (Applied Biosystems) (Arefin *et al.* 2015; Dantoft *et al.* 2013). A Rotor-gene Q machine (Qiagen) was used to run qPCR. Each sample was run in triplicate. Relative mRNA expression levels were analyzed by normalizing to the reference gene *rpl32* and compared to the control genotype. The data were then transformed to Log₂ fold changes and presented as mean \pm SDs of the mean of at least three independent biological replicates. In the uninfected wounded situation, two independent biological samples were analyzed and both show a similar pattern.

Survival analysis after wounding and bacterial infection

Late third-instar larvae were washed with 25° tap water. Afterward, they were dipped in 70% ethanol followed by rinsing with water to remove

Ex vivo Phagocytosis

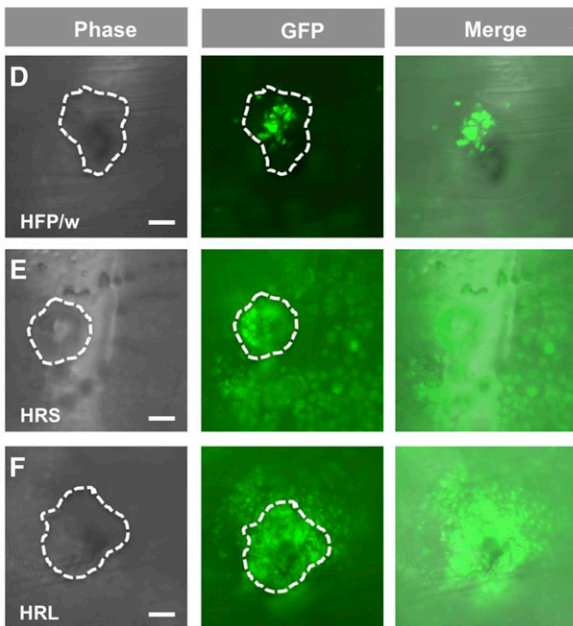
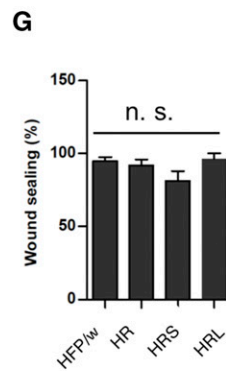
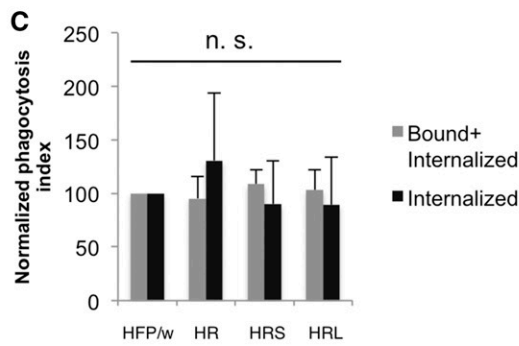
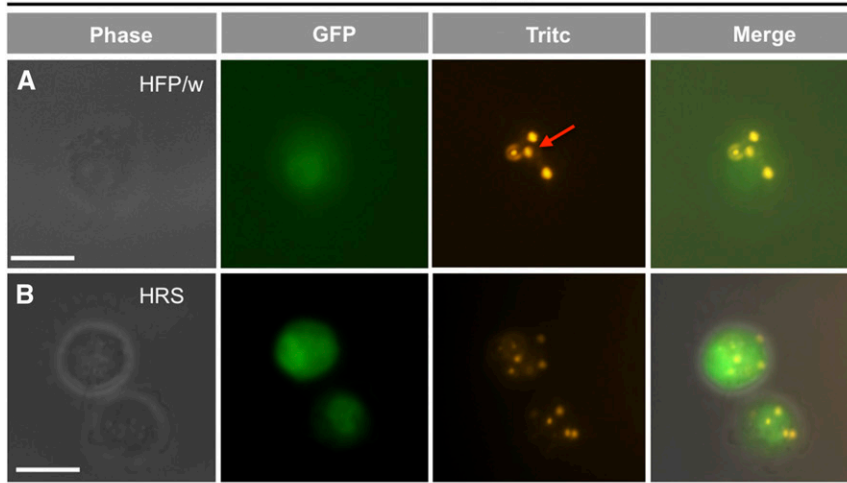
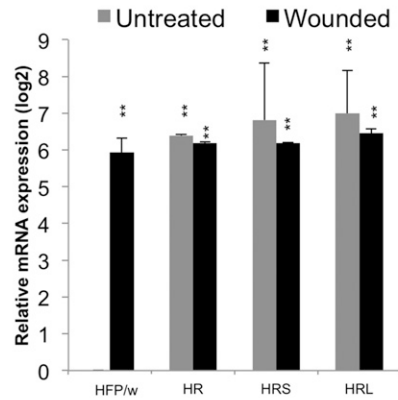


Figure 3 Phagocytosis, hemocyte recruitment to wounds, and wound sealing are not impaired in leukemic lines. (A and B) Ex-vivo phagocytosis of Texas Red-conjugated *E. coli* (K-12 strain). A representative image of the HFP/w and HRS-leukemia lines is shown after phagocytosis. The red arrow indicates phagocytosed *E. coli*. (C) Quantification of the phagocytosis index. All leukemic lines show wild-type levels of phagocytic capacity. (D–F) Hemocyte migration to the wound edge in leukemic situation is comparable to wild type. Dashed line in phase-contrast and the GFP channel encircle the wound edges. (E and F) The increased number of hemocytes in the leukemic lines leads to a more blurry appearance in both GFP and merge channels. (G) Wounds in leukemic lines are sealed equally efficiently as in wild type. Bar in (A) and (B) is 10 μ m, and in (D–F) is 50 μ m. Data represent means of SD; Student's t-test on the normalized data was performed in (C). ANOVA followed by Tukey's multiple comparison test was performed in (G). n.s., not significant.

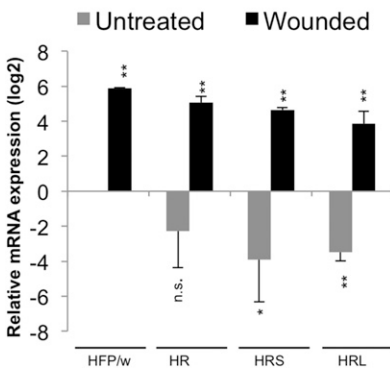
ethanol. Prior to sticking them on tape onto a glass slide, they were dried with Kleenex (Marque) tissue paper. Larval wounding was performed according to Galiko and Krasnow (2004) with a needle (250 μ m in diameter). Wounded larvae were immediately submerged either only in PBS (without microbes) or in highly concentrated microbial suspension (Neyen *et al.* 2014) for 3 min, allowing

microbes to enter via wound. Then they were transferred into specially prepared fly vials (the sponge cap used for fly vials were cut into half and put on top of wet tissue paper on the bottom of vial, and the sponge was made wet to ensure humidity before transferring larvae). Survival was monitored twice a day until eclosion. Data were process in the GraphPad 6.0.

A Drosomycin



B Dipterin A



C Cecropin A1

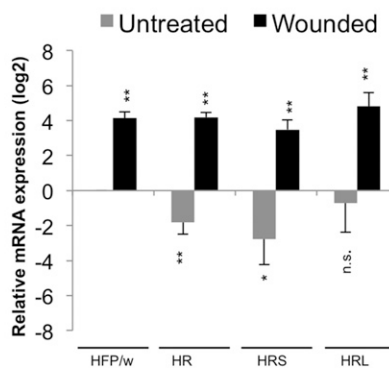


Figure 4 Toll and Imd signaling are regulated in opposite ways in leukemic larvae. Compared to control (HFP/w), expression (A) is found upregulated while *Dpt A* and *Cec A1* (B and C) mRNA levels are downregulated in leukemic larvae compared to untreated larvae (normalized to Rpl32 after RT-qPCR, all levels are shown relative to nontreated controls, which are set to 1). Upon wounding, *Drs* is also upregulated in control larvae to levels similar to nonwounded leukemic lines [HFP/w in (A)]. However, no further *Drs* induction is observed after wounding leukemic larvae. In contrast, *Dpt A* and *Cec A1* levels are induced after wounding in all lines. Data represent means of SD; Student's *t*-test: ** $P < 0.01$, * $P < 0.05$. n.s., not significant.

Nematode infection

EPNs of the species *Heterorhabditis bacteriophora* were used to infect *Drosophila* larvae. Nematode infections were performed according to the protocol described earlier (Arefin *et al.* 2014; Kucerova *et al.* 2015). In short, 62–68-hr-old *Drosophila* larvae were washed in 25° tap water and placed on TORK advanced soft tissue paper. Subsequently, 10 μ l of nematode suspension (dose: 25 nematodes/10 μ l) was added to each well in a 96-well plate followed by quickly adding individual larvae. The plate was covered with parafilm and incubated for 48 hr at 25°. Physically examining larvae by gently touching with forceps scored mortality. Three groups were infected at a time for a genotype where each group contained 48 larvae. In total three independent biological replicates (48 \times 3 \times 3 = 432 larvae) were analyzed to determine whether a fly line is sensitive to nematodes.

Microscopy

To select the correct genotype (GFP enabled), a Leica MZ FLIII fluorescence stereomicroscope coupled with a Panasonic DMC-G2 camera was used. Analysis of samples was performed with a Hamamatsu ORCA-ER camera (C4742-95) associated with a Zeiss Axioplan 2 microscope.

Larval locomotion behavior by FIMTrack

An agarose gel was poured as a crawling surface at a concentration of 0, 8% in deionized water, which produced a solid gel (thickness of 2 mm; see Kunc *et al.* (2017)). To improve illumination properties, the gel was

kept moist by tap water. A salt barrier was poured to prevent larvae from escaping the experimental area by adding 5 M NaCl in 2.5% agarose gel. The gels were surrounded by wet tissue paper to prevent drying out. For image acquisition, a Basler A601f camera was used. FIMTrack v2 Windows (X86) software was downloaded from <http://fim.uni-muenster.de/>. Data obtained from the software were analyzed in Prism 6 (GraphPad).

Statistics

ANOVA followed by Tukey's multiple comparison test, Student's *t*-test (unpaired, two sided), Fisher's LSD test, and Log-rank test on GraphPad 6.0 (survival analysis) were used to determine statistical significance. Data represent means of SD (Figure 1, Figure 2, Figure 3, Figure 4, Figure 5, and Figure 6) and SEM (Figure 7); ** $P < 0.01$, * $P < 0.05$.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

RESULTS

Three *Drosophila* leukemia models

To induce leukemic states in *Drosophila* hemocytes, we expressed a dominant-active form of Ras (*Ras^{V12}*) in hemocytes. To increase hemocyte-specific expression, we relied on the hemolectin driver (*Hml Δ -GAL4*) instead of the previously used collagen IV driver

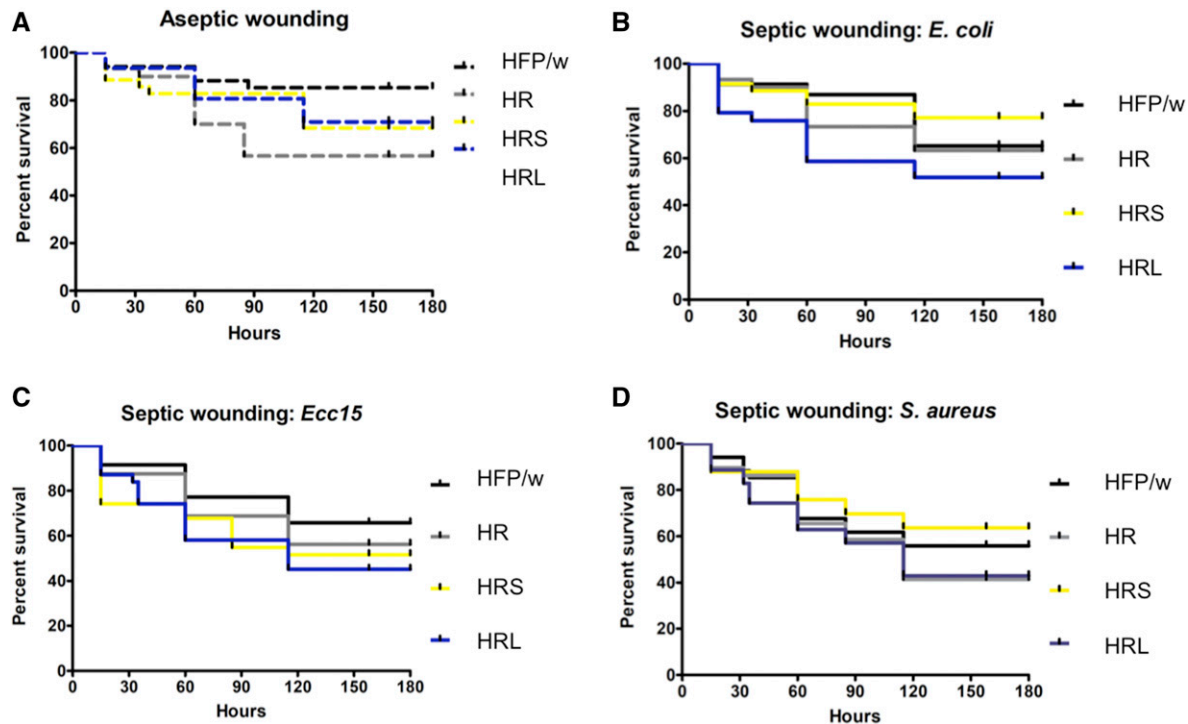


Figure 5 Survival of septic wounded leukemic larvae is similar to wild type. (A) Aseptic wounding: control wild-type larvae show a slight increased survival compared to leukemic larvae; however, this is not significant. (B–D) Septic wounding with both Gram-negative (*E. coli* and *Erwinia carotovora carotovora*) and Gram-positive bacteria (*S. aureus*). None of the septic wounds caused significant increases in mortality for the leukemic lines compared with controls. Survival was monitored until eclosion. Log-rank test on GraphPad 6.0 was performed to determine statistical significance for the survival curves. (A) $P = 0.1182$, (B) $P = 0.2074$, (C) $P = 0.3682$, and (D) $P = 0.3491$.

(*Cg-GAL4*) (Asha *et al.* 2003). To further increase the tumorigenic potential, we combined *Ras^{V12}* with knockdowns of *l(2)gl* and *scrib*, which had previously been used in combination with *Ras^{V12}* to successfully induce tumors in tissues other than hemocytes (Brumby and Richardson 2003; Pagliarini and Xu 2003). We observed a substantial increase in hemocyte counts both on expression of *Ras^{V12}* alone and when coexpressed with one of the RNAi constructs for tumor suppressors (Figure 1, A–E). The increase was ~30-fold for *Ras^{V12}*-overexpression (HR in Figure 1) compared with the control crosses and even more pronounced upon knockdown of tumor suppressors. *L(2)gl^{RNAi}; Ras85D^{V12}*-larvae (HRL) showed the strongest effect with a ~170-fold increase in hemocyte numbers while *scrib^{RNAi}; Ras85D^{V12}* (HRS) larvae contained ~90 times more hemocytes when compared to control (*HFP/w*). At 25°, all combinations were viable up to the pupal stage and migrated along the wall of culture vials to prepare for pupation (Figure 1F), but only *Ras^{V12}*-expressing pupae show eclosion rates comparable to wild type. In contrast, only a fraction of the combinations with tumor suppressors managed to eclose (Figure 1G). When the temperature was raised to 29° to allow for a higher Gal4 activity, all three tumor lines died either at the larval or pupal stage (Figure 2A). While upon bleeding, control pupae were shown to contain precursors for adult structures (Figure 2B), most likely partially unfolded imaginal discs, larvae from all tumor lines were desiccated (Figure 2C) and the remaining hemolymph contained aggregates of GFP-expressing hemocytes (Figure 2, C–E). Moreover, adult tissue formation was not observed in leukemic pupae (Figure 2, C–E).

The immune status of leukemic larvae

To address the physiology and immune status of leukemic larvae we first assessed their phagocytic capacity by counting phagocytosed

Texas red–conjugated bacteria and found that none of the leukemic lines displayed a reduction in phagocytosis (Figure 3, A–C). Similarly, upon wounding, hemocytes were attracted to wound sites in all lines and larvae survived wounding equally well, although there was a trend for HRS larvae to survive slightly worse than the other lines (Figure 3, D–G). To check whether the proliferation of hemocytes had any proinflammatory effects, we analyzed the expression of antimicrobial peptide gene reporters for two major *Drosophila* immune pathways, including *Drs* (a proxy for the Toll pathway, Figure 4A), Diptericin A (*Dpt A*, an Imd reporter, Figure 4B), and Cecropin A1 (*Cec A1*, which receives input from both pathways, Figure 4C). Compared to untreated control larvae, *Drs* expression was activated in leukemic larvae and both *Dpt A* and *Cec A1* reduced, although to a lesser extent. Wounding nonleukemic larvae induced *Drs* to the same degree as the leukemic stage (Figure 4A). *Dpt A* and *Cec A1*, which are more dependent on Imd signaling, were induced to a similar extent upon wounding all leukemic and control lines (Figure 4B). Taken together, this indicates that several aspects of cellular immunity appear normal in leukemic lines but there is a shift in the immune status, which mimics some aspects of wounding.

Leukemic lines survive selected bacterial infections

To assess the immune competence of leukemic lines, we infected larvae with either Gram-positive or Gram-negative bacteria (Figure 5). Leukemic lines survived equally well as controls upon infection with *Staphylococcus aureus*, which is primarily cleared via phagocytosis (Defaye *et al.* 2009). This is in line with our previous observation that phagocytic ability is not affected in leukemic hemocytes (Figure 3, A–C). Similarly, infection with *E. coli* and the natural pathogen *Erwinia carotovora carotovora* failed to induce higher mortality in leukemic larvae.

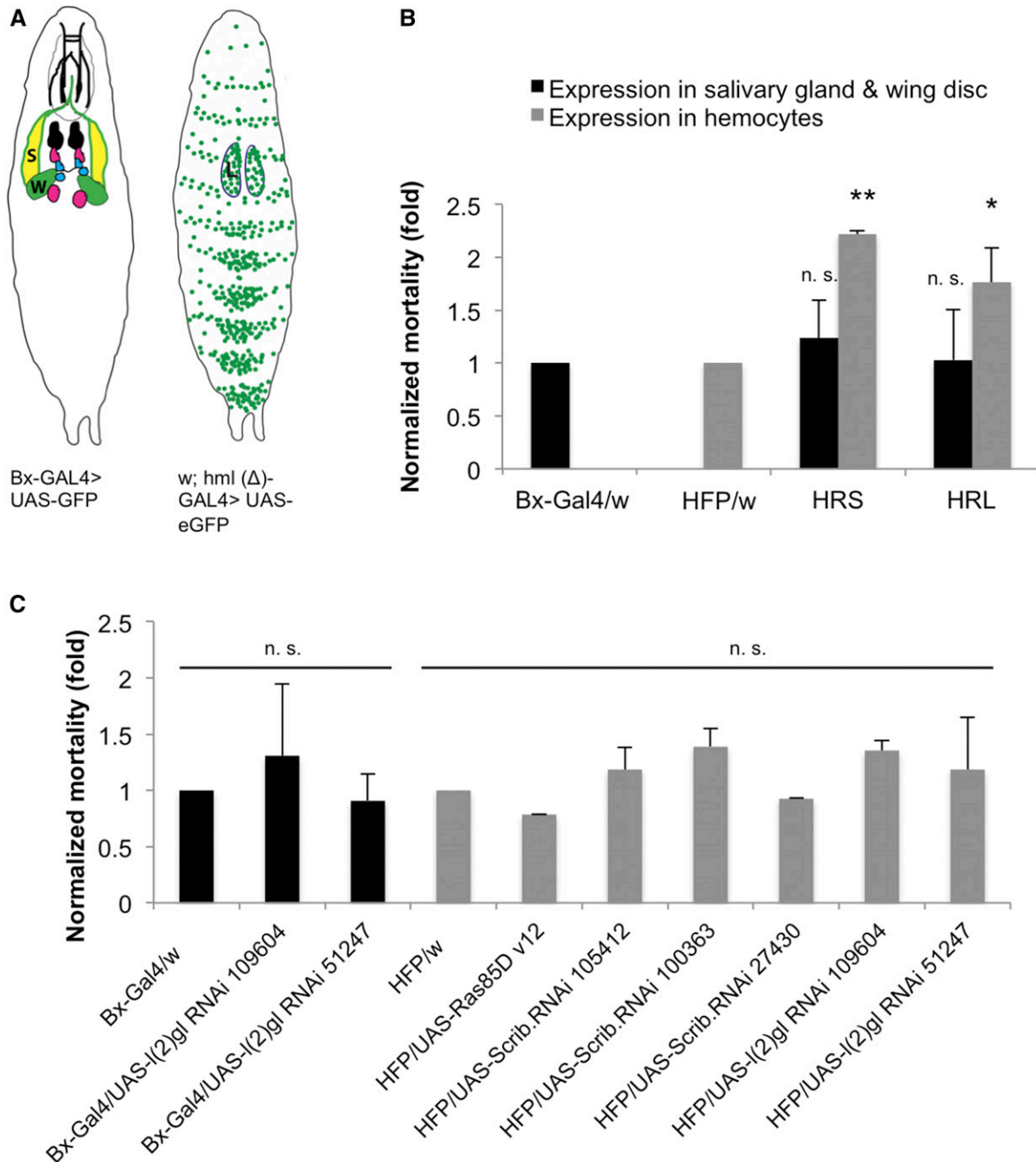


Figure 6 Leukemic lines combined with tumor suppressor knockdowns are more susceptible to nematode infection. (A) Schematic diagrams of the expression pattern of the two different GAL4 drivers used. *Bx-GAL4* expression is in the salivary glands and wing discs, whereas *hml* (Δ)-*GAL4* expression is in the larval lymph glands and in both circulating and sessile hemocytes. (B) Upon infection with entomopathogenic *Heterorhabditis bacteriophora*, the combination of *Ras85D^{V12}* expression and knockdown of tumor suppressors in hemocytes showed increased mortality compared to larvae expressing either construct alone (*UAS-Ras85D^{V12}* and *UAS-RNAi* of tumor suppressors) or in salivary glands and wing discs. Comparisons were made with respective controls (*Bx-GAL4/w* and *HFP/w*). Mortality was normalized to controls which were set to 1. Data represent means of SD; Student's t-test: ** $P < 0.01$, * $P < 0.05$. (C) Knocking down of different RNAi lines using two GAL4 driver lines (*Bx-GAL4* and *HFP*) and expression of *Ras85D^{V12}* alone with *HFP* only. When compared to respective controls, none of them showed significant mortality upon nematode infection. L, lymph gland; n.s., not significant, S, salivary gland; W, wing disc.

Upon knockdown of tumor suppressors, *Ras^{V12}* expressing lines are more susceptible to nematodes

To analyze the immune status of leukemic lines in a more natural setting, we infected larvae with the EPN *H. bacteriophora* and its associated bacterium *Photorhabdus luminescens*. *Heterorhabditis* infects insect

hosts by entering the hemocoel via the cuticle or the gut and subsequently releases *Photorhabdus* into the hemolymph. All three leukemic lines were infected using EPNs as well as control crosses and single knockdown lines for the tumor suppressors. We also included crosses where all combinations were expressed under control of the Beadex

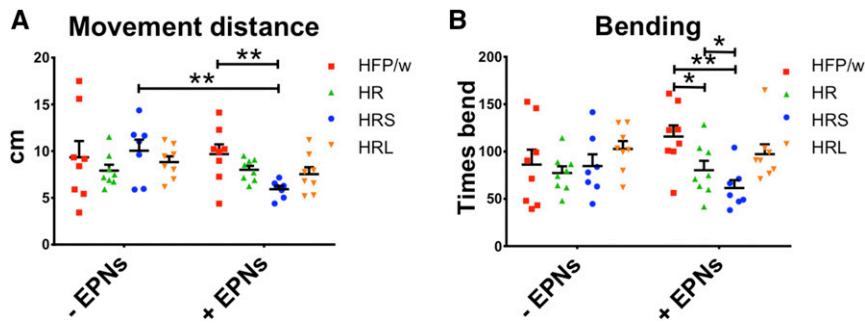


Figure 7 Larval locomotion is impaired in HRS compared to control (HFP/w) in the presence of nematodes. (A) In the absence of nematodes no differences were observed in the distance covered by leukemic lines and control crosses whereas in the presence of nematodes HRS lines showed reduced mobility. (B) Bending frequencies do not differ in the absence of nematodes whereas in the presence of nematodes HRS larvae show reduced bending compared to controls. Each dot represents the mean value for a replicate and the middle line represents the mean of the replicates. Error bar represents SEM; sample size was at least 115 larvae. Fisher's LSD test: ** $P < 0.01$, * $P < 0.05$.

driver (*Bx-GAL4*) in the wing discs and the salivary glands [Figure 6A, see also Hauling *et al.* (2014)]. Although some lines with only one construct showed a trend toward increased susceptibility, none of these were significant (Figure 6C). Only the leukemic lines that expressed *Ras^{V12}* in combination with tumor suppressor knock-downs were consistently more sensitive to nematode infections (Figure 6B). Of note, even crosses with the *Beadex* driver showed similar mortality when compared to their respective control, although immune activation had been observed upon expression of both *Ras^{V12}* alone and in combination with tumor suppressor knock-downs (Hauling *et al.* 2014 and Krautz, R. Khalili, D. Hauling, T. and Theopold, U., unpublished data).

Larvae expressing *Ras^{V12}* and reduced *scrib* show altered behavior in the presence of nematodes

Since we did not detect any gross developmental defects or deficiencies in the cellular response of leukemic larvae, we considered alternative explanations for the increased susceptibility of leukemic lines toward EPNs such as subtle differences in their behavior. To analyze larval locomotion, we used frustrated total internal reflection of infrared light (Risse *et al.* 2013), which we recently adapted for use in infection studies (Kunc *et al.* 2017). When exposed to nematodes, HRS larvae covered shorter distances than larvae from control crosses or the two other leukemic lines. However, no such difference was observed in the absence of nematodes (Figure 7A). The results from measuring the frequency of bending, which is part of larval avoidance behavior, were less clear but they showed that in the presence of nematodes, the bending frequency differed between HR and HRS larvae compared to controls (Figure 7B). This suggests that in the presence of nematodes, leukemic larvae are at least by some measures less mobile, which will decrease their chances of escaping infection.

DISCUSSION

We show that by expressing dominant-active *Ras^{V12}* in hemocytes, a substantial increase in hemocyte numbers is induced, confirming earlier results using a different driver (Asha *et al.* 2003). Hemocyte overproliferation is further exacerbated when tumor suppressors are knocked down (Figure 1). This establishes a set of three *Drosophila* leukemia models which display a graded increase in hemocyte numbers. All models show developmental defects at higher temperature but no such defects at the last larval instar at 25° (Figure 2). The severity of the developmental phenotype positively correlates with the increase in hemocyte numbers and may therefore be at least partially due to a redistribution of resources upon extensive hemocyte overproliferation. This is in line with the

cachexia-like phenotype, which eventually leads to pupal death (Figure 2, C–E).

Upon infection with Gram-positive or Gram-negative bacteria, none of the leukemic lines displayed an increase in mortality and thus appeared immune-competent. Supporting this, phagocytosis and recruitment of hemocytes to wounds were similar in leukemic lines and controls. On the contrary, when induction of the two major immune pathways was checked using pathway-specific reporters, we found that Toll-specific reporters were constitutively activated, while Imd-dependent signaling was reduced. Activation of Toll is in line with several recent findings where activation of this pathway was observed upon damage to cells or tissues. Such settings include the release of intracellular components (Kanoh *et al.* 2015), failure to induce apoptosis (Ming *et al.* 2014), epidermal wounding (Carvalho *et al.* 2014), induction of a protumorous state (Hauling *et al.* 2014; Parisi *et al.* 2014), induction of fibrotic lesions (Zang *et al.* 2015), chromosomal instability (Liu *et al.* 2015), and the deregulation of hemocyte development (Arefin *et al.* 2015).

When larvae were infected with EPNs, those expressing *Ras^{V12}* in combination with either knockdown for tumor suppressors were more susceptible. This was most significant for the *scrib* knockdown. As expected for a complex model such as EPN infections, our data do not fully explain why leukemic lines are more susceptible but the dysregulation of the two major immune pathways provides a partial explanation: precocious activation of Toll-dependent genes may induce a proinflammatory state which hampers the response toward EPNs. Alternatively, or in addition, the repression of Imd targets may delay the response against the symbiotic bacteria that are released by EPNs after their entry into the hemocoel (Hallem *et al.* 2007; Kucerova *et al.* 2015; Arefin *et al.* 2014; Castillo *et al.* 2015). We also observed behavioral changes in the leukemic lines, some of which can be linked to the presence of nematodes (Figure 7) and a trend for decreased survival after injury (Figure 5A), which together may exacerbate the susceptibility toward EPNs. Taken together our data indicate that *Drosophila* leukemia models, which combine overexpression of *Ras^{V12}* with knock-down of tumor suppressors, are more susceptible toward a naturally relevant infection despite a lack of developmental, behavioral, and immune defects in standard assays. This mimics the specific defects observed in some leukemias (Forconi and Moss 2015) and provides useful tools to study subtle interactions between tumor progression, inflammation, and immunity. Further screens for modifiers will permit a detailed genetic dissection of the genes and pathways involved. These will include genetic screens as well as screens for potential chemical modifiers (McCubrey *et al.* 2008) and will address candidate pathways involved in cachexia, stress, and sensors for cellular integrity (Sonoshita and Cagan 2017).

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