Direct sensing of systemic and nutritional signals by haematopoietic progenitors in Drosophila

Jiwon Shim¹, Tina Mukherjee¹ and Utpal Banerjee¹,²,³,⁴,⁵

The Drosophila lymph gland is a haematopoietic organ in which progenitor cells, which are most akin to the common myeloid progenitor in mammals, proliferate and differentiate into three types of mature cell—plasmatocytes, crystal cells and lamellocytes—the functions of which are reminiscent of mammalian myeloid cells¹. During the first and early second instars of larval development, the lymph gland contains only progenitors, whereas in the third instar, a medial region of the primary lobe of the lymph gland called the medullary zone contains these progenitors², and maturing blood cells are found juxtaposed in a peripheral region designated the cortical zone². A third group of cells referred to as the posterior signalling centre functions as a haematopoietic niche³,⁴. Similarly to mammalian myeloid cells, Drosophila blood cells respond to multiple stresses including hypoxia, infection and oxidative stress⁵-⁷. However, how systemic signals are sensed by myeloid progenitors to regulate cell-fate determination has not been well described. Here, we show that the haematopoietic progenitors of Drosophila are direct targets of systemic (insulin and nutritional (essential amino acid) signals), and that these systemic signals maintain the progenitors by promoting Wingless (WNT in mammals) signalling. We expect that this study will promote investigation of such possible direct signal sensing mechanisms by mammalian myeloid progenitors.

It is known that metabolic dysfunction in mammals causes abnormal inflammatory responses in the blood system. However, how metabolic stresses impinge on haematopoiesis is still unclear. Here, we found that starvation of Drosophila larvae leads to blood cell phenotypes. The most striking effect is acceleration of blood cell differentiation both in time and number of cells affected in the lymph gland. Following 24 h of starvation, cells occupying the medullary zone begin to express differentiation markers such as Peroxidasin⁶ (Pxn) and Hemolectin⁷ (Hml) normally restricted to the cortical zone (Fig. 1a–d,g). Corresponding to this increase, a substantial reduction of Domeless (Dome) marking the progenitor population is also evident (Fig. 1e–g). The protein Eater¹⁰, normally expressed at very low levels in the progenitors and at high levels in differentiated cells, is expressed at high levels in all cells on starvation (Fig. 1h,i). These data are schematically summarized in Fig. 1j,k.

The starvation experiments were carried out on either PBS-soaked Whatman paper¹¹ (Supplementary Fig. S1a–c) or a 1% agar plate¹² (Fig. 1a–d,g and Supplementary Fig. S1d–f). Aseptic conditions to control against indirect effects due to bacterial infection were also used (Supplementary Fig. S1g–i). In all controlled experimental conditions, starvation reduced the progenitor population and caused an increase in the number of differentiating cells (Fig. 1j,k), without an obvious alteration in the size of the haematopoietic organ, or the apoptotic profile of its cells (Supplementary Fig. S1j–l).

Similarly to metabolically induced inflammation in mammals¹³, starvation in Drosophila larvae activates NFKB-like transcription factors, assessed by the expression of the reporter D4–LacZ (ref. 14; Fig. 2a,b) and antimicrobial peptides¹⁵ in circulating haemocytes (Fig. 2c) and within the lymph gland (Supplementary Fig. S1m,n). Starvation also causes an increase in the number of circulating blood cells arising from the embryonic head mesoderm (Fig. 2e), infiltration of Pxna++ plasmatocytes into the fat body (Fig. 2d), the Drosophila equivalent of the mammalian liver and adipose tissue, and differentiation of lamellocytes, another hallmark of inflammatory response, in both the lymph gland and in the circulating blood cell population (Fig. 2f–h). Finally, starvation induces the rupture of crystal cells (Fig. 2i,j), a process known to release coagulation and melanization enzymes¹⁶. This rupture depends on JNK signalling (Fig. 2k), a stress signalling pathway required for crystal cell maintenance¹⁶. Thus, starvation alters the homeostatic balance between progenitors and differentiating blood cells through extensive progenitor differentiation, and also activates mature blood cells in a manner that is reminiscent of mammalian inflammatory response.

In Drosophila, the systemic level of glucose is regulated by insulin-like peptides (Dilps) that are produced and secreted by neuroendocrine

¹Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, California 90095, USA. ²Molecular Biology Institute, University of California, Los Angeles, California 90095, USA. ³Department of Biological Chemistry, University of California, Los Angeles, California 90095, USA. ⁴Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, California 90095, USA. ⁵Correspondence should be addressed to U.B. (e-mail: banerjee@mbi.ucla.edu)

Received 13 July 2011; accepted 3 February 2012; published online 11 March 2012; DOI: 10.1038/ncb2453
cells in the brain, much like insulin production by pancreatic β-cells of mammals. As in mammals, insulin signalling in Drosophila plays a conserved role in regulating metabolism and growth, and the levels of nutrients, such as amino acids, regulate secretion of Dilps. We find that starvation blocks Dilp2 release from the IPCs, which will cause an increase in Dilp secretion, suppresses blood cell differentiation under both well-fed and starved conditions. Taken together, we conclude that Dilp2 expression from the IPC neurons is essential for progenitor maintenance and loss of Dilp2 release during starvation results in excessive differentiation of blood cells.

The loss-of-function, heteroallelic combination InR<sup>E19</sup>/InR<sup>GC25</sup> for the Drosophila insulin receptor (InR) is viable and larvae from this genotype also exhibit extensive differentiation of the progenitor population. As Dilp2 is a secreted protein, its target receptor could, in principle, be functional in any tissue that then, in turn, signals to the haematopoietic organ using a secondary pathway. However, we found that disrupting InR function directly in the lymph gland with the use of the lymph-gland-specific driver HHLT-Gal4 (C. Evans and U. Banerjee, unpublished) causes precocious differentiation of the progenitors from an earlier stage in development
directly responds to brain IPC-derived Dilp2 by activating InR signalling, which serves to maintain the progenitor cell population within the lymph gland.

We next examined the function of AKT, which acts downstream of InR as a protein kinase, by downregulating its expression in the progenitors, and this too promotes progenitor differentiation (Fig. 3n), identical to loss of InR (Fig. 3g) and dilp2 (Fig. 3c). Likewise, loss of the TORC1 components dTOR (using the dominant-negative mutant protein dTOR\textsuperscript{DN}; Fig. 3o,p) or Raptor (using RNAi-mediated interference (RNAi); Supplementary Fig. 5h), which together function downstream of AKT, causes loss of progenitors due to their differentiation. Feeding rapamycin, which blocks dTOR function\textsuperscript{22} also phenocopies this effect (Supplementary Fig. 5i). Consistent with a role for this pathway in progenitor maintenance, overexpression of rhh, which activates dTOR (ref. 23), strikingly inhibits differentiation of blood cells under both normally fed and starved conditions (Fig. 3q,r). Interestingly, mammalian haematopoietic stem cells also respond to mTOR signalling\textsuperscript{24}. Overall, it is evident that the canonical Dilp–InR and Rhee–dTOR signalling pathways play a critical role in the maintenance of haematopoietic progenitors, and this maintenance role is overridden during metabolic stress caused by starvation.
Figure 3  Systemic dilp2 is directly sensed by haematopoietic progenitors. The borders of the lymph glands are marked with yellow dotted lines; the progenitors, white dotted lines. In h–k, HHLT-Gal4, UAS-GFP staining, and in l–n, dome-Gal4; UAS-2xYFP staining has been omitted for clarity. Each panel shows a single section from the middle of a Z stack of images from a lymph gland except h–k, which show super-positions of 3 middle sections. Blue indicates the DNA marker ToPro3. Scale bars, 25 µm.

AED, after egg deposition. Quantification of each genotype is presented in Supplementary Fig. S2, and the P values obtained from that quantification for 10 random samples and compared with corresponding wild types are quoted here for each genotype. n is the number of lobes examined. n = the number of lobes examined. a) Neuronal projections from the IPCs (marked by dilp2-Gal4; UAS-GFP, green) are adjacent to the lymph gland, but do not penetrate the lymph gland (n = 10). (b,c) Maturing blood cell marker Pxn (red) staining is increased in the lymph gland when IPCs are ablated in the brain (dilp2–Gal4: UAS-hid; Pxn, red; n = 14; P = 1.8 × 10^{-8}; b) or in dilp2 deficiency, dilp2^{f1}/dilp2^{f1} animals (n = 8; P = 3.3 × 10^{-7}; c; compare with Fig. 1a). (d) Constitutive expression of Dilp2 using elav-Gal4; UAS-dilp2 greatly reduces differentiation (Pxn, red; n = 16; P = 2.3 × 10^{-15}). (e,f) Forced secretion of larval insulins using dilp2–Gal4; UAS-NaChBac, which depolarizes Dilp2-expressing IPCs, under either well-fed (n = 30; P = 4.8 × 10^{-14}; e) or starved conditions (n = 10; P = 6.9 × 10^{-12}; f) also greatly reduces differentiation (Pxn, red). (g) The heteroallelic mutant combination inR^{D14}/inR^{D22} promotes differentiation of maturing blood cells (Pxn, red, n = 14; P = 2.7 × 10^{-7}). (h,i) In early second instar (36 h AED at 29℃), the wild-type lymph gland does not express Pxn (n = 6; h), whereas specific knockdown of InR in the lymph gland (using HHLT-Gal4; UAS-InR^{RNAi}) causes precocious expression of this maturing cell marker (Pxn, red, n = 8; P = 0.0001; i). (j,k) In mid to late second instar (50 h AED at 29℃), maturing cells are first seen in the normal wild-type lymph gland (Pxn, red, n = 8; j). Loss of InR (HHLT-Gal4; UAS-InR^{RNAi}) causes an increased number of Pxn+ cells to appear at this stage (n = 8, P = 0.0001; k). (l–n) Insulin receptor maintains progenitors. Specific knockdown of InR in the progenitor population (dome-Gal4; UAS-InR^{RNAi}) causes a loss of progenitors and a robust differentiation phenotype in the late third instar (n = 40; P = 1.6 × 10^{-8}; l; Pxn, red). RNAi targeting chico (dome–Gal4; UAS–chico ^RNAi; n = 5; P = 1.2 × 10^{-7}; m) or dAKT (dome–Gal4; UAS–dAKT^{RNAi}; n = 14; P = 2.1 × 10^{-5}; n) leads to the same phenotype (Pxn, red). (o,p) dTOR regulates maintenance of progenitors. The heteroallelic mutant combination dTOR^{P2293L}/dTOR^{P2293S}, (n = 14; P = 3.0 × 10^{-4}; o) or expression of dTOR^{homo} (dome–Gal4; UAS–dTOR^{homo}; n = 10; P = 3.1 × 10^{-8}; p) causes increased differentiation (Pxn, red). (q,r) Activation of dTOR by overexpression of Rheb (dome–Gal4; UAS–rheb) greatly inhibits differentiation of maturing cells (Pxn, red) under both fed (n = 8; P = 4.4 × 10^{-8}; q) and starved conditions (n = 8; P = 1.0 × 10^{-3}; r). (s–u) Differential expression profile of pAKT during development of the lymph gland. At 48 h AED (s), pAKT (membrane, red) is uniformly and highly expressed throughout the lymph gland except in a few mature cells at the periphery that are marked by hmp^{D14}/Gal4, UAS–2xEGFP (green). At 72 h AED (t) high levels of pAKT are seen only within the progenitor population (red), and the hmp^{D14}/Gal4, UAS–2xEGFP (green) expressing maturing blood cells have relatively low levels of pAKT. At 96 h AED (u) the expression level of pAKT is significantly reduced and the pattern is even more restricted (red) to the progenitors, as mature blood cells completely exclude pAKT expression.
Dilp2 levels rise during early instars and then gradually decrease during the third instar of larval development\(^2\), indicating a possible mechanism for maintaining InR signalling through the third instar in well-fed larvae. To determine whether InR signalling is modulated during normal development, we assessed levels of phospho-AKT (pAKT) at different developmental stages in the lymph gland. Using this approach, we found two distinct phenomena. First, pAKT expression in progenitor cells is high during the second instar and gradually decreases in these cells during the third instar. Second, pAKT is low, relative to progenitor levels, in differentiating cells at all stages when they are present (Fig. 3s). These observations indicate that during the course of normal development, InR signalling is modulated in progenitors, thereby differentially promoting maintenance at different stages. It is also apparent that once cells are committed to differentiate, little, if any, InR signalling occurs, consistent with lower levels of InR expression (Supplementary Fig. S4c) and the lack of a phenotype associated with InR loss-of-function in these cells (Supplementary Fig. S4a).

In mammals, glucose levels control insulin secretion\(^2\). This is less clear in Drosophila, but it is well established that amino-acid levels are sensed by the fat body through the mediation of the amino-acid transporter protein Slimfast\(^7\) (Slif) and that the fat body indirectly controls insulin secretion from the brain IPCs (ref. 12). As expected, we found that slif\(^\text{anti}\) expressed in the fat body mimics the starvation phenotype in the lymph gland (Fig. 4a), probably owing to decreased Dilp2 secretion from the brain. More interestingly, however, knocking down slif expression directly in the lymph gland (Fig. 4b), but not in the dorsal vessel (Supplementary Fig. S3i,j), and specifically in the progenitor population within the lymph gland (Fig. 4c), accelerates differentiation of mature cells similar to that seen with starvation (Fig. 1b). As with insulin signalling, this result shows that the haematopoietic progenitors themselves directly sense amino-acid levels to maintain their stem-like fate. Taken together, these findings indicate a dual control of haematopoietic homeostasis by systemic levels of insulin and amino acids. Amino acids are sensed by the fat body, which then controls insulin secretion from the brain\(^1\). Insulin is then directly sensed by the blood progenitors. Amino acids are also directly sensed by the blood progenitors to maintain their undifferentiated state (Fig. 5g).
Supplementation of essential amino acids (EAAs) partially restores the progenitor population during an otherwise starved condition (Fig. 4d,h), whereas neither sucrose nor non-essential amino acid (NEAA) supplementation rescues the progenitors from differentiation. Overexpression of Rheb (domeGal4; UASrheb, n=16) is sufficient to restore the progenitors (domeGal4; UAS-EYFP, green) and restrict differentiation of maturing blood cells (Pxn, red) in the lymph gland expressing InR expression (Fig. 4g,h), further establishing that the progenitors directly sense EAA and use the signal to promote their maintenance.

In wild-type lymph glands, the progenitors (expressing Dome; Dome+) rarely overlap with the maturing cells (expressing Pxn; Pxn+); however, downregulation of InR or expression of dominant-negative TOR (TORDN) in the progenitors causes a significant increase in the number of double-positive cells (Dome+ and Pxn+) that are in transition towards differentiation (Supplementary Fig. S5). An increase in this particular cell type is reminiscent of the phenotype seen on downregulation of the wingless (wg) signalling pathway, which has previously been linked to the process of progenitor maintenance (Fig. 4e,f). Wingless (Wg) is dynamically expressed in the lymph gland with higher levels at earlier stages that then decrease during the third instar. Wg is expressed at high levels by progenitors at these stages and is withdrawn from differentiating cells, which is reminiscent of pAKT staining patterns (Fig. 3s–u) and the expression of InR in the third instar (Supplementary Fig. S4c). We found that downregulation of InR (Fig. 5b) or Slif (Fig. 5c) in the progenitors causes a significant decrease in Wg expression, whereas Rheb overexpression significantly increases Wg levels (Fig. 5d) when compared with that seen in wild type (Fig. 5a), indicating that Dilp2–InR and Slif–dTOR activities positively regulate the expression of Wg within the progenitors. Importantly, overexpression of Wg restores the progenitor population in both starvation conditions and in the presence of reduced InR levels (Fig. 5e,f), demonstrating that Wg is likely to be the most direct downstream target of Dilp2–InR signalling, which maintains progenitors within the progenitors. However, our studies do not rule out either direct or indirect involvement of additional pathways downstream of InR–dTOR in this process.

A model describing the systemic and nutritional control of myeloid-like progenitors by insulin and amino acids is shown in Fig. 5g. Our
results demonstrate that metabolic changes are perceived by blood progenitors and this causes alteration of their cell-fate determination program. We find that a major consequence of reduced INR and amino acid levels is the reduction of Wg expression in the lymph gland, which functions to promote progenitor maintenance. In addition to accelerated differentiation of myeloid progenitors, starvation also causes a response similar to the inflammatory response (Fig. 2) typically associated with metabolic disorders. These responses indicate that metabolically induced inflammatory responses in mammals have an ancestral origin that arose to balance an organism's ability to withstand an unfavourable environment and the normal development of myeloid cells. Nutrient/insulin signalling has been linked to the homeostatic control of various Drosophila stem cell populations. Given the highly conserved nature of the blood system in flies and mammals, and the known functional role of metabolism and insulin signalling in myeloid cells, it will be important to determine whether the direct metabolic and nutritional regulation mechanisms uncovered in these studies might also be relevant for the mammalian common myeloid progenitors. Such studies will probably yield insights into chronic inflammation and the myeloid cell accumulation seen in patients with type II diabetes, and other metabolic disorders.

METHODS

Methods and any associated references are available in the online version of the paper at www.nature.com/naturecellbiology

ACKNOWLEDGEMENTS

We thank C. J. Evans, K. T. Jones, other members of the Banerjee laboratory and J. A. Martinez-Agosto for helpful comments and discussions. We thank R. Erdmann for help with the amino-acid supplementation assay and S. Pham for confirming experiments with anti-Hml in different genetic backgrounds. We acknowledge P. Leopold, E. Hafen, E. Rulifson, L. Pick, T. P. Neufeld, R. A. Schulz, A. Courey, J.-M. Reichhart, the VDRC Stock Center and the Bloomington Stock Center for fly stocks, the Developmental Studies Hybridoma Bank (University of Iowa), the Drosophila Genomics Resource Center and J. Fessler for reagents. We also thank M. Crozatiers’ group for providing the in situ hybridization protocol. This work was supported by an NIH grant (5R01 HL076395) to U.B. and the Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research Training Grant at UCLA to J.S.

AUTHOR CONTRIBUTIONS

J.S designed and carried out experiments, and U.B. supervised the project. T.M. carried out experiments. J.S., T.M. and U.B. discussed and analysed results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at www.nature.com/naturecellbiology

Reprints and permissions information is available online at www.nature.com/reprints
METHODS

Drosophila stocks and genetics. The following Drosophila stocks were used in the experiments: 

domains–Gal4 (S. Noselli, Université de Nice, France), peroxidasin–Gal4 (P. Martin, University of Bristol, UK), In[2-3]/TM6B,UAS-GFP (S. Sinenko, UCLA, USA), dilp2–Gal4 (S. Noselli, Université de Nice, France), anti-Gal4/TM3,Sb (S. Cohen, IMCB, Singapore), delta–Gal4 (Bloomington Stock Center) and nsh2–Gal4 (Bloomington Stock Center) are Gal4 drivers used in this study. We obtained UAS–pr, y+; P(UAS–TORb(1)Gal4), y+; P(UAS–Rhb.Pab1), y+; P(UAS–NacBac), In[1-2]/TM4, y+; P(UAS–DR6Gal4), y+; y,w;UAS–Dilp1-Gal4, y,w;UAS–Btc(1), y,w;F47C, P(ubac–Gal80), y,w;FM7c, P(ubac–FLP), P(y,w;UAS–FRT2B P(ubi–GFP)18 P(ubac–FLP) P(y,w;UAS–FRT2B) from Bloomington Stock Center. y+;ilp2(1), y+;ilp3(1) and y+;ilp5(1) were isolated by L. Partridge’s laboratory (UCL, UK) and obtained from Bloomington Stock Center. RNAi lines are from the Vienna Drosophila RNAi Center. y,w;P(UAS–dlip2) was a gift from E. Hafen (ETS-IMS, Switzerland), y,w;UAS–dpnm from P. Leopold (Université de Nice, France), Df[2–3]/TM6B,Tb and Df[2–3]/TM6B,Tb from L. Pick (University of Maryland, USA), y,w;TORb(1)SM6B–TM6b and y,w;TORb(1)SM6B–TM6b from P. Neufeld (University of Minnesota, USA), P(armor–GFP,F9) and eater–GFP from R. A. Schulz (University of Notre Dame, USA), D4–lacZ from A. Courey (UCLA, USA) and dgs–GFP from J-M. Reichhart (Université des Strasbourg, France). Stocks and crosses were maintained at 25 °C, except for those used in RNAi and GAL4/UAS expression experiments, for which crosses were maintained at 29 °C. For GAL80 experiments, crosses were maintained at the permissive temperature 18 °C for 3 days, and shifted to 29 °C. For synchronization of larvae, eggs collected for a week and newly hatched larvae were transferred onto fresh food plates after 24 h and aged for specified time periods at 25 °C. For In[2–3] clones in the lymph gland, we generated FRT28b In[2–3]/TM6b Tb, and confirmed the recombinant with ey–FLP. Larvae containing hy–FLP/FRT28b In[2–3]/FRT28b ubi–GFP were heat-shocked at 38 °C for 40 min in each instar.

Immunohistochemistry and imaging. Lymph glands were dissected and stained as previously described. For fat body samples, anterior fat bodies were gently dissected, and fixed with 3.7% formaldehyde for 20 min. After visualizing DNA with ToPro3, samples were mounted in Vectashield (Vector Labs). For anti-Wg antibody, a modified protocol was used. The following antibodies were used in this study: anti-Px (1:1,500, J. Fessler and L. Fessler, both UCLA, USA), anti-PKT (1:200, Cell Signaling #4054), anti-Wg (1:10, DSHB), anti-L (1:100, L. Ando, BRC, Hungary), mouse anti-βGal (1:200, Promega), anti-Dilp2 (1:1,000, P. Leopold) and anti-PPO (1:250, H. Muller, EMBL, Heidelberg, Germany). Nuclear DNA was visualized with ToPro3 (Invitrogen). Cy3-conjugated secondary antibodies were used (Jackson Laboratory) for immunohistochemistry. Samples were mounted with Vectashield (Vector Laboratories). Images were obtained using a Zeiss LSM700 confocal system using either a ×20 or ×40 oil-immersion objective. Images used for comparison were always captured using identical optical settings.

Quantification of the lymph gland phenotype. Samples were scanned using an LSM700 confocal microscope and captured using Zen 2009 software. Images were processed using ImageJ (NIH). To estimate the expression of differentiation markers within the medullary zone, the middle 3 optical sections from the Z stack were merged into a single section. For wild type, this enables the clearest view of the medullary zone surrounded by the differentiating cells of the cortical zone. Depending on the genotypes studied, the merged image may contain images at two or three different wavelength channels indicating different markers used for the lymph gland analysis. Each individual channel/marker was analysed separately. For the zone-specific markers (Pxn, Hml and Dome), the coloured areas were recalibrated into an identical threshold by using the Binary tool (Process–Binary–Make binary) as an automatic routine. The area with identical threshold was automatically captured with the Wand tool, and the size was measured using the Measure tool (Analyse–Measure). To measure the total size of the lobe, the ToPro3-expressing area was recalibrated by the Threshold tool until overlaid with identical threshold colour. This total area was selected using the Wand tool, and measured. The percentage of differentiation was calculated by dividing the size of the marker-expressing area by the total size of the lobe (ToPro3 area). Ten randomly selected lymph glands were analysed per genotype, and the statistical significance was calculated with a standard t-test.

To quantify the expression of Wingless in different genotypes, we followed the protocol from http://scietechblog.files.wordpress.com/2011/05/measuring-cell-fluorescence-using-imagej.pdf.

Haemocyte collection and counting. For haemocyte collection, staged larvae were washed with water and bled in 20 μl of Schneider’s insect media. The cells were gently redispersed onto 5 mm 14-well slides (Fisher Scientific), allowed to settle for 15 min at room temperature and fixed with 3.7% formaldehyde for 20 min. ToPro3 was used for visualizing DNA (ref.34). For haemocyte counting, cells prepared in 20 μl Schneider’s medium were transferred to a Neubauer improved haemocytometer, and counted and analysed as previously described (ref.38).

Starvation assay, nutrient supplementation assay and rapamycin treatment. Larvae were synchronized and allowed to grow at 25 °C until early third instars (90–96 h after egg deposition (AED)). Staged larvae were collected and washed with water, and finally rinsed with ultrapure distilled water (Gibco). These larvae were then divided into two groups: one group of larvae was placed on regular food media (control) and the second was placed on 1% soft agar media (starvation). Control larvae were handled identically to the ones exposed to starvation conditions. Those raised on agar plates were also maintained with or without yeast to control for any possible effects due to handling and growth on agar plates. Per 60 mm dish, 15–20 larvae were used. Both the standard food media and 1% agar media were treated with 1× antibiotic–antimycotic solution (Invitrogen). The larvae were reared for 24 h at 25 °C, after which they were collected, dissected and processed for antibody staining.

For aseptic starvation, collections of embryos were sterilized by rinsing in 0.25% Chlorox with 0.04% n-alkyl dimethyl benzyl ammonium chloride, washed twice in 70% ethanol following two brief washes in ultrapure distilled water (Gibco) and autoclaved distilled water, and then transferred onto axenic food plates. The animals were staged to 90–96 h AED, and larvae were selected with sterilized forceps and washed with ultrapure distilled water (Gibco). Normally fed larvae were reared on axenic food media with an antibiotic–antimycotic solution, and starved larvae, on a sterilized 1% agar plate with an antibiotic–antimycotic solution as the standard starvation protocol. Both food/agar and homogenized larvae from each plate were plated onto an antibiotic-free bacterial LB agar plate, and assessed for lack of growth to verify axenic conditions. No bacterial colony grew with food/agar or homogenized larva grown in axenic culture conditions.

For nutrient supplementation assays, carbohydrate complexes, lipid complexes and amino acids were added to sterilized 1% agar solution and processed for the standard starvation method as described previously. For yeast supplementation, 34 g l-1 of inactivated yeast powder was added to 1% agar solution, sterilized and processed for the standard starvation method as described above. For rapamycin treatment, 90–96 h AED larvae were transferred to standard fly medium containing 2 μM rapamycin and kept for 24 h.

In situ hybridization. For the anti-InR probe, a 205-base-pair complementary DNA fragment from LD06045 (DGRC, cDNA library) was amplified with InR-forward (5’–CGA TGG CCG TGT TAT GGA GAG-3’) and InR-reverse primers (5’–CGC TCC TTT TCC CGA TGC TGC AGA-3’). Dissection and in situ hybridization procedures were carried out as described previously.

37. Lebestky, T., Chang, T., Hartsenstein, V. & Banerjee, U. Specification of Drosophila hematopoietic lineage by conserved transcription factors. Science 288, 146–149 (2000).
38. Zettervall, C. J. et al. A directed screen for genes involved in Drosophila blood cell activation. Proc. Natl Acad. Sci. USA 101, 14192–14197 (2004).
39. Hennig, K. M., Colombani, J. & Neufeld, T. P. TOR coordinates bulk and targeted endocytosis in the Drosophila melanogaster fat body to regulate cell growth. J. Cell Biol. 173, 963–974 (2006).
Figure S1 Starvation-mediated differentiation and activation of the blood cells.

a-c, As compared to control (a), larvae starved on PBS-soaked Whatman paper (b) exhibit expanded differentiation phenotype (n=8, Pxn, red). Quantification of this phenotype is shown in c (\(*p=3.2\times10^{-9}\) ).

d-f, Similar to well fed control (d), feeding yeast exhibits normal differentiation (n=14) (e), different from the expanded differentiation phenotype seen upon starvation (Fig 1b,d). Quantification of this phenotype is shown in f (n.s.: not significant, \(p=0.15\) ).

g-i, As compared to control (n=20) (g), larvae starved on aseptic condition (h) show expanded differentiation (n=20, Pxn, red). Quantification of this phenotype is shown in i (\(*p=5.4\times10^{-3}\) ).

j-l, Activated Caspase-3 is not detected in either well-fed (j) or starved animals (Caspase-3, red, n=10) (k); as a control, forced expression of pro-apoptotic gene in the lymph gland (using hml-gal4; UAS-hid,rpr) induces active caspase-3 (red) (l).

m-n, As compared to control (n=10) (m), starvation induces D4-LacZ (containing 4 Dorsal/Dif binding sites) expression in the lymph gland (green, n=18) (n).
Figure S2 Quantification of the lymph gland phenotype. Unless otherwise indicated, ten randomly selected samples were analyzed according to the method described in methods section. Asterisks denote statistically significant results. a-d, Mean differentiated area (%) calculated using Pxn as a marker of differentiation. Graphs are grouped according to the genotypes driven with dilp2-gal4 (a), dome-gal4 (b), mutants, HHLT-gal4 (c) and phenotypes in the early stages (d), respectively. Significance was measured by student’s T test, and error bars indicate s.d. Differences in the following genotypes are not significant. (n.s.): dilp2-gal4; UAS-NaChBac fed (shown in Fig 3e) and dilp2-gal4; UAS-NaChBac starved (Fig 3f) (p=0.05), dome-gal4; UAS-rheb fed (Fig 3q) and dome-gal4; UAS-rheb starved (Fig 3r) (p=0.28), HHLT-gal4; UAS-sliF^{100} (Fig 4b) and HHLT-gal4; UAS-sliF^{100}+EAA (Fig 4g) (p=0.06). e, Mean total cell fluorescence of Wg expression for data shown in Figure 5a-d.
**Figure S3** dilp2 and InR are required for maintaining progenitors in the lymph gland. In f-g, HHLT-gal4, UAS-GFP expression is omitted for clarity. a-b, Df[dilp1-5](n=5) (a) and Df(dilp1-3)(n=10; p=8.4x10^{-5}) (b), both of which remove dilp2, show abnormal differentiation (Pxn, red) of the lymph gland. c-d, Neither dilp31 (c) nor dilp51 (d) exhibits enhanced differentiation of maturing blood cells (Pxn, red) as is seen with dilp21 (Fig 2b). e-e', Under well fed condition, Dilp2 (red) is not visible in the IPC cells except for secreted Dilp2 on the projections (arrow) (e); whereas starvation strongly accumulates Dilp2 expression in the brain IPCs (red, yellow dotted) (e') and causes a decrease in secreted Dilp2 expression (arrow). White dotted line indicates brain hemisphere, and images are whole-projections of Z stacks.

f-g, Specific knockdown of InR using InR^{RNAi} (HHLT-gal4; UAS-InR^{RNAi}) (n=48; p=1.6x10^{-11}) (f) and expression of dominant negative InR (InR^{DN}) (HHLT-gal4; UAS- InR^{DN}) (n=12) (g) show enhanced differentiation (Pxn, red), h-i, Cardiac cell specific knockdown of InR (mef2-gal4; UAS-InR^{RNAi}) (n=20) (h) or slif (mef2-gal4; UAS-slif^{Ant}) (n=16) (i) does not cause enhanced differentiation of blood cells (Pxn, red). j-j', Expression patterns of HHLT-gal4 and mef2-gal4 in the lymph gland and dorsal vessel. HHLT-gal4 is expressed both in the lymph gland (GFP, green; white dotted line, LG) and the dorsal vessel (GFP, green; arrow) (j). mef2-gal4 is expressed in the dorsal vessel (arrow) and muscle nuclei (GFP, green; arrowhead), but not in the lymph gland (GFP, green; white dotted line, LG) (j').
Figure S4 MZ as a site of expression and function of InR/dTOR. In a-b, hmlΔ-gal4, UAS-2xeGFP and antp-gal4, UAS-eGFP, and in h, dome-gal4 UAS-2xYFP expressions are omitted for clarity. a-b, Specific knockdown of InR either in the CZ (hmlΔ-gal4; UAS-InRRNAi) (n=6) (a), or in the PSC (antp-gal4; UAS-InRRNAi) (n=6) (b) does not induce abnormal differentiation (Pxn, red) phenotype as is seen with the MZ driver, dome-gal4 (Fig 3l). c-d, InR transcript is enriched in the MZ. CZ shows relatively lower expression except in crystal cells (arrow) (c). Specific knockdown of InR (HHLT-gal4; UAS-InRRNAi) eliminates InR RNA (d). Grey dotted line outlines the lymph gland; white dotted line demarcates, the MZ. e-e”, InRΔ19/InRΔ19 mutant clone (hs-FLP; FRT82B InRΔ19) within the MZ (indicated in the box) is marked by the absence of GFP expression (green) (e); this hypomorphic mutant clone shows differentiation (Pxn, red) (e’.e”). The clone is outlined with yellow dotted line. These clones are small due to the well-known function of InR in growth control. Yellow scale bar: 10 mm. f-f’, InRΔ19 FLP-out clone (Ay-gal4 UAS-GFP; UAS-InRΔ19) in the lymph gland is marked by GFP expression (green) (f); expression of dominant-negative InR clone causes differentiation (f-f’). The clone is outlined with a white dotted line. g-g’, PI3KCAAX clone (HHLT-gal4; UAS-PI3KCAAX) in the lymph gland is marked by the GFP expression (green) (g); these activated PI3K clones are devoid of differentiation (Pxn, red) even for cells at the periphery in the position of the normal Cortical Zone (g-g”). The clone is outlined with a white dotted line. h-i, Inactivation of raptor in the progenitors (dome-gal4; UAS-raptorRNAi) exhibits enhanced differentiation (Pxn, red) (n=10) (h). Feeding 2 μM rapamycin shows abnormal induction of differentiation (Pxn, red) (n=24; p=1.1x10^{-9}) (i).
Figure S5 Inactivation of InR or dTOR often shows cells in transition. a-a'”, In wild-type lymph gland, the progenitors (marked by dome-gal4, UAS-2xEYFP, green) (a) are mutually exclusive with maturing blood cells (marked by Pxn, red) (a’). Merged image is shown in a’’ (The small inset is a magnified view of the cells depicted white dotted box). b-b’”, Specific knockdown of InR in the progenitors (dome-gal4; UAS-InR RNAi) induces cells expressing both with dome-gal4, UAS-2xEYFP (green) (b) and Pxn (red) (b’) as shown in b’’ (The small inset is a magnified view of the cells depicted white dotted box) (therefore, yellow). c-c’”, Lymph gland expressing a dominant negative dTOR (dTORDN) in the progenitors (dome-gal4; UAS-dTORDN) induces cells marked both with dome-gal4, UAS-2xEYFP (green) (c) and Pxn (red) (c’) as shown in c’’ (The small inset is a magnified view of the cells depicted white dotted box) (therefore, yellow).
Supplementary materials

All the lymph glands are outlined by yellow dotted lines, and white dotted lines demarcate the progenitors. Each panel shows a single middle section from a lymph gland derived Z stack. Blue indicates DNA marker, ToPro3. If not otherwise indicated, scale bars: 25 μm. n value indicates the number of samples examined. Quantification of each genotype is presented in Supplementary Fig 2, and the p values obtained from the quantitation for ten random samples and compared with corresponding wild types are quoted here for the genotypes. The number of lobes examined is indicated as n. Error bars indicate s.d.