# Regulation of Larval Hematopoiesis in *Drosophila melanogaster*: A Role for the *multi sex combs* Gene

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ABSTRACT

Drosophila larval hematopoietic organs produce circulating hemocytes that ensure the cellular host defense by recognizing and neutralizing non-self or noxious objects through phagocytosis or encapsulation and melanization. Hematopoietic lineage specification as well as blood cell proliferation and differentiation are tightly controlled. Mutations in genes that regulate lymph gland cell proliferation and hemocyte numbers in the body cavity cause hematopoietic organ overgrowth and hemocyte overproliferation. Occasionally, mutant hemocytes invade self-tissues, behaving like neoplastic malignant cells. Two alleles of the *Polycomb* group (PcG) gene *multi sex combs* (*mxc*) were previously isolated as such *lethal malignant blood neoplasm* mutations. PcG genes regulate *Hox* gene expression in vertebrates and invertebrates and participate in mammalian hematopoiesis control. Hence we investigated the need for *mxc* in Drosophila hematopoietic organs and circulating hemocytes. We show that *mxc*-induced hematopoietic hyperplasia is cell autonomous and that *mxc* mainly controls plasmatocyte lineage proliferation and differentiation in lymph glands and circulating hemocytes. Loss of the Toll pathway, which plays a similar role in hematopoiesis, counteracted *mxc* hemocyte proliferation but not *mxc* hemocyte differentiation. Several PcG genes tested *in trans* had no effects on *mxc* hematopoietic phenotypes, whereas the *trithorax* group gene *brahma* is important for normal and mutant hematopoiesis control. We propose that *mxc* provides one of the regulatory inputs in larval hematopoiesis that control normal rates of plasmatocyte and crystal lineage proliferation as well as normal rates and timing of hemocyte differentiation.

DROSOPHILA larval circulating blood cells carry out the cellular host defense response through phagocytosis or encapsulation and melanization. Most wild-type circulating hemocytes belong to a monocyte-like phagocytic cell lineage (reviewed in Dearolf 1998; Fossett and Schulz 2001). They are called plasmatocytes, podocytes, or macrophages according to their shape, their adhesion properties, and the stage of development (Gateff 1978; Rizki 1978; Lanot *et al.* 2001; Meister and Govind 2002). Phagocytic cells eliminate small foreign objects and, in pupae, the lysing larval tissues. Less than 1% of circulating hemocytes are flat lamellocytes that encapsulate larger "non-self" objects (Rizki 1978; Rizki and Rizki 1980). Crystal cells represent a second hematopoietic cell lineage (Dearolf 1998; Fossett and Schulz 2001), which is required for melanization of lamellocytes after encapsulation (Rizki 1978). Specification of hemocyte progenitor cells and regulation of lineage commitment and differentiation depend on a series of evolutionarily conserved transcription factors (Lebestky *et al.* 2000; reviewed in Fossett and Schulz 2001). Drosophila blood cells are produced during two successive waves of hematopoiesis in embryos and larvae. All larval hemocytes are produced by the hematopoietic organs or lymph glands. This structure differentiates during late embryogenesis as two lobes along the anterior part of the dorsal vessel (Rugendorff *et al.* 1994) and has in third instar larvae four to seven lobe pairs (Gateff 1978; Rizki 1978). Large anterior lobes contain most types of circulating hemocytes, whereas smaller posterior ones contain undifferentiated blast cells that normally give rise to macrophages at pupariation (Lanot *et al.* 2001). Lymph gland overgrowth, hemocyte proliferation, and hemocyte differentiation are inducible by immune challenge or by external aggression such as infestation by a parasitoid wasp, but they are also observed in certain mutant contexts (Watson *et al.* 1991; Rizki and Rizki 1992; reviewed in Dearolf 1998; Lanot *et al.* 2001). The corresponding larval phenotypes include hypertrophied hematopoietic organs, increased numbers of circulating hemocytes, and abnormal differentiation of lamellocytes that represent up to 50% of the cells. Melanotic masses often occur. They are formed by lamellocyte-covered capsules that contain melanized self-tissue (pseudotumors; Sparrow 1978; Watson *et al.* 1991; Dearolf 1998).

Such abnormal immune response phenotypes are caused by a number of mutations (Watson *et al.* 1991; Torok *et al.* 1993; Dearolf 1998), making these loci potential candidates for genes directly regulating hema-

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topoiesis. Two *lethal (1) malignant blood neoplasm* [*l(1)mbn*] alleles were isolated as causing overproliferation of lymph glands and circulating hemocytes (Gateff 1978; Shrestha and Gateff 1982; Gateff and Mechler 1989). Allelism was later established between *l(1)mbn* and the *Polycomb* group (PcG) gene *multi sex combs* (*mxc*; Santamaria and Randsholt 1995; Saget *et al.* 1998). PcG genes form a conserved group that collectively maintain expression patterns of important selector genes in vertebrates and invertebrates (reviewed in Pirrotta 1998; van Lohuizen 1999; Gebuhr *et al.* 2000; Brock and van Lohuizen 2001; Simon and Tamkun 2002). First isolated in Drosophila as negative *trans*-regulators of the *Hox* genes, PcG genes have been shown to act in conjunction with *trithorax* group (trxG) genes to maintain transcriptional regulation and provide a cellular memory mechanism throughout development, probably by changes in chromatin structure. Several mammalian PcG and trxG members are involved in hematopoiesis control (reviewed in van Lohuizen 1999; Gebuhr *et al.* 2000; Takihara and Hara 2000; Raaphorst *et al.* 2001). PcG genes show stage-specific expression differences in human bone marrow cells (Lessard *et al.* 1998). The mouse PcG gene *embryonic ectoderm development* (*eed*) negatively regulates myeloid and lymphoid progenitor cell proliferation in bone marrow (Lessard *et al.* 1999), whereas targeted disruption of murine PcG genes Bmi-1, Mel 18, Rae23/Mph1, and M33 all lead to loss or hypoproliferation of various hematopoietic tissues. The human *trithorax* homolog MLL is often affected in translocations associated with acute myeloid or lymphoblastic leukemias (reviewed in van Lohuizen 1999; Muller and Leutz 2001), and $MLL^{-}/+$ mice present severe hematopoietic abnormalities (Yu *et al.* 1995). Furthermore, mammalian SWI/SNF chromatin remodeling proteins, which are homologs of the Drosophila trxG protein Brahma (BRM), have been implicated as important cofactors in the regulation of myeloid and erythroid genes (reviewed in Gebuhr *et al.* 2000; Muller and Leutz 2001). Little is known about possible roles of PcG and trxG genes in Drosophila hematopoiesis. The *domino* (*dom*) gene encodes SWI/SNF family DNA-dependent ATPases that interact with PcG products in negative homeotic gene regulation, and *dom* mutations induce hematopoietic disorders. Lymph glands show proliferation defects and the rare *dom* hemocytes that differentiate cannot cross the lymph gland basement membrane (Braun *et al.* 1997, 1998; Ruhf *et al.* 2001). *mxc* is the only Drosophila gene known to cause both abnormal hematopoiesis and homeotic transformations due to HOM/*Hox* gene gain of function (Saget *et al.* 1998). In view of this, we decided to analyze how regulation of larval hematopoiesis and of circulating hemocyte density were affected by *mxc* mutations, alone or in other mutant contexts that also control these processes.

Two signal transduction pathways are well characterized as controlling hemocyte proliferation and steady-state numbers of hemocytes in Drosophila larvae. The conserved Toll/cactus/Rel-NF-κB signaling pathway is one (Qiu *et al.* 1998; reviewed in Dearolf 1998; Mathey-Prevot and Perrimon 1998; Meister and Govind 2002). Toll pathway activation leads to nuclear translocation of Rel/NF-κB transcription factors that regulate hemocyte division and differentiation. Enhanced Toll signaling induces hematopoietic organ hyperplasia, increases in circulating hemocytes, abnormal lamellocyte differentiation, and pseudotumors, whereas larvae with reduced Toll signaling have fewer hemocytes. Toll signaling and Rel/NF-κB proteins also control Drosophila humoral host defense (Govind 1999), and homologous vertebrate Toll/cactus-I-κB/Rel pathways ensure similar functions (Qiu *et al.* 1998; Grossmann *et al.* 1999). Hematopoiesis control likewise involves the Drosophila JAK/STAT signal transduction pathway (reviewed in Dearolf 1998; Mathey-Prevot and Perrimon 1998; Luo and Dearolf 2001). Constitutive activation of the JAK nonreceptor tyrosine kinase encoded by *hopscotch* (*hop*) causes hematopoietic overproliferation, overproduction of lamellocytes, and pseudotumors (Hanratty and Ryerse 1981; Silvers and Hanratty 1984; Hanratty and Dearolf 1993; Harrison *et al.* 1995; Luo *et al.* 1995, 1997; Lanot *et al.* 2001). Gain-of-function *hop* proteins hyperactivate the *s*ignal *t*ransducer and *a*ctivator of *t*ranscription protein encoded by *D-stat*, and haplo-insufficiency for *D-stat* partially suppresses hematopoietic gain-of-function phenotypes of *hop* (Hou *et al.* 1996; Yan *et al.* 1996; Luo *et al.* 1997; reviewed in Dearolf 1998; Luo and Dearolf 2001). Hematopoiesis control by the JAK/STAT pathway is also evolutionarily conserved since alterations of mammalian JAK/STAT affect hematopoietic cell proliferation, differentiation, and apoptosis (Lacronique *et al.* 1997; Nosaka *et al.* 1999; reviewed in Dearolf 1998; Luo and Dearolf 2001).

Here, we describe hematopoietic phenotypes of several increasingly severe *mxc* alleles and confirm that *mxc* directly controls hematopoiesis. Development of plasmatocyte and crystal cell lineages is affected by loss of *mxc*. We compared hematopoietic defects due to mutations of *mxc*, of the Toll, or of the JAK/STAT pathway and analyzed epistatic relations between *mxc* and these mutants. Loss of Toll signal is epistatic to loss of *mxc*. Diminished *D-stat* activity had no effect on *mxc* hematopoietic phenotypes whereas it partially rescued lamellocyte differentiation of *Toll* gain of function and both hemocyte overproliferation and differentiation induced by constitutive JAK activation. Any genetic combination of these proliferation activator contexts caused extreme lymph gland overgrowth together with reductions in circulating hemocyte numbers. Finally, we found that proliferation control by *mxc* is less dependent on other PcG genes than is segmental identity control, whereas the *trithorax* group gene *brahma* is important for normal

and mutant hematopoiesis. We propose that *mxc* provides a hematopoiesis regulatory input that controls normal plasmatocyte and crystal cell lineage development as well as normal rates and timing of hemocyte differentiation.

## MATERIALS AND METHODS

**Fly strains and culture:** Flies were grown on standard culture medium at 25°, unless otherwise stated. *mxc* alleles have been described (Santamaria and Randsholt 1995; Docquier *et al.* 1996). *Dorothy* (*Dot*) encodes an ecdysteroid UDP-glucosyl/UDP glucuronosyl transferase that is expressed in pericardial and lymph gland cells; the *Dot-LacZ* strain was a gift from D. Kimbrell (Rodriguez *et al.* 1996). Reporter line *l(2)113/28* (from M. Meister) expressing β-galactosidase in lamellocytes is described by Braun *et al.* (1997). The ubiquitously β-galactosidase-expressing strain *hsp83-LacZ* contains several *P*[*hsp83-LacZ*] inserts. The β-galactosidase-free strain $\beta gal^{nl}$ was provided by A. Shearn (Woodhouse *et al.* 1998). $hop^{M38}$/*FM7 act-GFP* flies were from M. Lagueux. Other Toll and JAK/STAT mutants were gifts from C. Dearolf or B. Lemaitre. Mutant *domino* (*dom*) phenotypes are described by Ruhf *et al.* (2001); *dom* strains were provided by M. Meister. Balancer chromosomes and all other mutants, including *Black cells* (*Bc*), *tube* (*tub*), *Toll* (*Tl*), *cactus* (*cact*), *hopscotch* (*hop*), *D-stat*, trxG, and PcG genes are described in FlyBase (1999).

**Hemocyte counts:** Wandering third instar larvae were bled in 5 μl of Drosophila Ringer on a hemocytometer. Mean hemocyte numbers per milliliter of hemolymph were estimated by counting the number of cells in a given surface, under a dissection microscope. Each experiment involved an internal, nonmutant control category provided by sibling larvae issued from the same cross. Control larvae were collected from the same vials as experimental larvae and counted under similar conditions (see table legends for controls in each experiment). Hemocytes from at least 10 larvae were counted per genotype and most experiments were repeated. Genotypes were recognized by mouth-hook color, body shape, or the presence of *actin*- or *histone3*-driven *GFP*. *mxc* mutant chromosomes carry $y^1$ and are maintained over *Binsn*. Phagocytic cells were recognized by their ability to absorb particles of India ink injected 2 hr previously into the larva (Lanot *et al.* 2001). Lamellocytes were identified by β-galactosidase expression, using the lamellocytes *LacZ*-reporter *l(2)113/28* (Braun *et al.* 1997). Crystal cells were counted per larva; they were visualized by heating larvae for 10 min at 70° in a water bath. Blackened cells were counted under a dissection microscope. Hemocyte numbers were compared by Student's *t*-tests. Blood cell type distributions and numbers of dividing cells were compared using chi-square tests.

**Lymph gland transplantations:** $y^1$ *mxc*/*Binsn* females were crossed to homozygous *hsp83-LacZ* males at 23°. Lymph glands from wandering third instar progeny, [*yellow*] for experimental and male [$yellow^+$] for control, were dissected in Drosophila Ringer and injected into the abdomen of 2- to 5-day-old $\beta gal^{nl}/\beta gal^{nl}$ females, using a Drummond nanobject automatic injector (Drummond Scientific, Broomall, PA). Injected females were grown overnight at 20°, followed by 5 days at 23° for proliferation tests and 3 weeks for survival tests. Proliferation test females were then dissected and X-Gal stained without prior fixation for 1–3 hr at 37°. Stained tissues were fixed for 10 min in 3.7% formaldehyde in 1× PBS, rinsed twice in 1× PBS, and mounted in PBS:glycerol.

**Genetic interactions:** $Tl^{10b}$ effects on $mxc^{G43}$ were analyzed in male progeny of $y^1$ $mxc^{G43}$/*Binsn* females crossed to $Tl^{10b}$/*His3-GFP* males. Loss of *Toll* function in $mxc^{G43}$ larvae was examined in male progeny of $y^1$ $mxc^{G43}$/*Binsn*;$Tl^{RXA}$/*TM6c* females and *Binsn*/Y;$Tl^{r632}$/*TM6c* males. Other *Toll* pathway effects on $mxc^{G43}$ mutants were examined in male larvae from strains $y^1$ $mxc^{G43}$/*Binsn*;$cact^{A2}$/*CyO act-GFP* and $y^1$ $mxc^{G43}$/*Binsn*;$tub^{238}$/*TM6c*. Interactions between *D-stat* and *hop* were examined in progeny of *y w* $hop^{Tum\text{-}l}$/*FM7* females and *D-stat*$^{6346}$/*His3-GFP* males. For other effects of *D-stat*, $y^1$ $mxc^{G43}$/*Binsn* females were mated to *D-stat*$^{6346}$/*His3-GFP* males, $y^1$ $mxc^{G43}$/*Binsn D-stat*$^{6346}$/*TM6c* females were crossed to *Binsn*/Y;*D-stat*$^{HJ}$/*TM6c* males, and *D-stat*$^{6346}$/*TM6c* females were crossed to $Tl^{10b}$/*His3-GFP* males. Joint Toll signal and JAK gain-of-function effects were evaluated among male progeny from strain *y w* $hop^{Tum\text{-}l}$/*Binsn*;$cact^{A2}$/*CyO act-GFP* or from crosses between *y w* $hop^{Tum\text{-}l}$/*FM7* females and $Tl^{10b}$/*His3-GFP* males. To examine effects of $mxc^{G43}$ and $hop^{Tum\text{-}l}$ together, an X chromosome carrying both mutations was obtained by recombination. PcG- and trxG-*mxc* interactions were tested among male progeny of $y^1$ $mxc^{G43}$/*Binsn* females crossed to $esc^4$/*His3-GFP*, $Psc^{Arp1}$/*His3-GFP*, $Pcl^{XM3}$/*His3-GFP*, $Pc^K$/*His3-GFP*, $Scm^{D1}$/*His3-GFP*, $brm^2$/*His3-GFP*, $brm^2$ $trx^{E2}$/*His3-GFP*, *Df(3R)trx*$^1$/*His3-GFP*, or $mor^1$/*His3-GFP* males.

**X-Gal staining:** Wandering larvae were dissected in 1× PBS; fixed in 1× PBS, 3.7% formaldehyde for 10 min at room temperature; washed in 1× PBS; and stained 4–16 hr as described by Docquier *et al.* (1996). Hemocytes were smeared onto polylysine-coated glass slides, air dried for 5 min, fixed for 30 sec in 3.7% formaldehyde in 1× PBS, washed twice in 1× PBS, and then stained as other imaginal tissues.

**Immunohistochemistry:** Wandering larvae of adequate genotypes were dissected in 1× PBS; tissues were fixed in 1× PBS, 3.7% formaldehyde for 15 min at room temperature and then immunostained as described by LaJeunesse and Shearn (1996). Circulating hemocytes were smeared on polylysine-coated slides, air dried and fixed for 5 min in 3.7% formaldehyde, and then immunostained as other imaginal tissues. Anti-phosphohistone H3 antibody (Upstate Biotechnology, Lake Placid, NY) was used at 1:10,000 without prior incubation. Staining was revealed after incubation with secondary biotinylated anti-rabbit antibody (Roche, Indianapolis; 1:400) using the Vectastain ABC (Elite) kit. Apoptosis in imaginal tissues was visualized using TUNEL (White *et al.* 1994) with modifications. Dissected imaginal tissues were fixed for 15 min in 1× PBS, 3.7% formaldehyde and for additional 15 min in 1× PBS, 3.7% formaldehyde, 0.6% Triton X-100 and then washed three times in PTW (1× PBS, 1% Tween 20) and three times in 1× PBS. Tissues were incubated 30 min in $H_2O_2$:methanol (3:1000); washed three times in 1× PBS; incubated 4 min with proteinase K in 1× PBS; washed in PTW glycine (2 mg/ml); fixed again for 15 min in 3.7% formaldehyde, 0.2% gluteraldehyde, 1× PBS; washed in PTW; and incubated for 90 min with terminal transferase and biotinylated dUTP. Label was revealed using DAB and the Vectastain ABC (Elite) kit. Tissues were mounted in PBS:glycerol.

## RESULTS

***mxc* mutations cause lymph gland overgrowth and hemocyte overproliferation:** Two early pupal lethal *mxc*/*l(1)mbn* mutants were isolated as showing severe hematopoietic neoplasia (Gateff 1978; Shrestha and Gateff 1982; Gateff and Mechler 1989). To determine whether such defects were particular for these alleles, we examined lymph glands of four hypomorphic *mxc* mutants with increasingly severe homeotic and developmental phenotypes that range from viable to larval le-

**TABLE 1**

**Lymph glands and hemocytes in wild-type and *mxc* mutants**

| Genotype (*N*) | Phenotype[a] | Lymph glands | Hemocyte[b] cells/ml ($10^6$) | Plasmatocytes (%) | Podocytes (%) | Lamellocytes (%) | Crystal cells per larva (*N*) |
|---|---|---|---|---|---|---|---|
| $y^1\ ac^1\ z^1$ (10) | | | 1.9 ± 1.2 | >99 | 0.2 | 0.2 | 103 ± 80 (25) |
| *Binsn*/Y (35) | | | 1.2 ± 0.5 | >99 | <0.1 | 0.2 | 133 ± 93 (16) |
| $mxc^{G46}$/Y (16) | Viable; HT; S | Overgrown | 1.4 ± 0.8 | >99 | <0.1 | 0.7 | 72 ± 63[d] (24) |
| $mxc^{G43}$/Y (16) | Pharate lethal; HT | Overgrown | 4.7 ± 1.8[c] | 95 | 2.7 | 2.4 | 73 ± 70[d] (25) |
| $mxc^{mbm1}$/Y (19) | Pupal lethal; SD | Overgrown | 6.1 ± 2.3[c] | 93 | 4.3 | 2.3 | 46 ± 42[c,e] (21) |
| $mxc^{16a\text{-}1}$/Y (24) | Larval/pupal lethal; SD | Overgrown | 3.6 ± 2.1[c] | 95 | 3.3 | 1.7 | 52 ± 42[c,e] (10) |

Two wild-type controls are included: *Binsn*/Y male sibling larvae of *mxc*/Y individuals and $y^1\ ac^1\ z^1$/Y males. $mxc^{G46}$ and $mxc^{G43}$ were induced on the $y^1\ ac^1\ z^1$ chromosome. For *Binsn*/Y males, data from all the experimental series were pooled. *N*, no. of animals used for analysis of this genotype.

[a] Viability and phenotypes of *mxc* mutants. HT, adult homeotic transformations; S, male and female sterile; SD, small imaginal discs.

[b] Hemocyte counts of larvae from outcrossed balanced *mxc* stocks. Results are mean blood cells/milliliter ± SE.

[c] Significantly different from the *Binsn* control ($P < 0.01$).

[d] Significantly different from the *Binsn* wild-type control ($P < 0.05$).

[e] Significantly different from the $y^1\ ac^1\ z^1$ wild-type control ($P < 0.05$).

thal. Their characteristics are summarized in Table 1. *mxc* mutant larvae develop pseudotumors mainly when raised under crowded conditions. In that case, <10% of $mxc^{G43}$ larvae exhibit pseudotumors while up to 25% older $mxc^{mbm1}$ larvae and most older $mxc^{16a\text{-}1}$ larvae do (Sparrow 1978; Saget *et al.* 1998). Effects of *mxc* on lymph glands were observed in X-Gal-stained *mxc*/Y;*Dot-LacZ*/+ late third instar larvae, compared to *Binsn*/Y;*Dot-LacZ*/+ siblings (Rodriguez *et al.* 1996). All four mutants exhibit overgrown lymph glands (Table 1; Figure 1). In $mxc^{G46}$/Y, only some second and third lymph gland lobes showed hypertrophy, whereas more severe alleles induced stronger overgrowth in more posterior lobe pairs. To determine whether overgrowth was associated with increased cell divisions, we stained lymph glands with anti-phosphohistone H3 antibody, which labels cells undergoing mitosis. Lymph gland lobes from all *mxc* mutants exhibit increased mitotic activity as compared to wild type (Figure 2).

We examined numbers and relative proportions of circulating hemocytes in *mxc* larvae (Table 1; Figure 3). Numbers and ratios of plasmatocytes or macrophages, lamellocytes, and crystal cells in wild type have been described (Rizki 1978; Lanot *et al.* 2001; see Introduction). All control larvae showed mean hemocyte concentrations per milliliter that were within the range of previously published values for animals of comparable age (Gateff 1978; Brehelin 1982; Silvers and Hanratty 1984; Luo *et al.* 1995, 1997; Table 1). The same was true

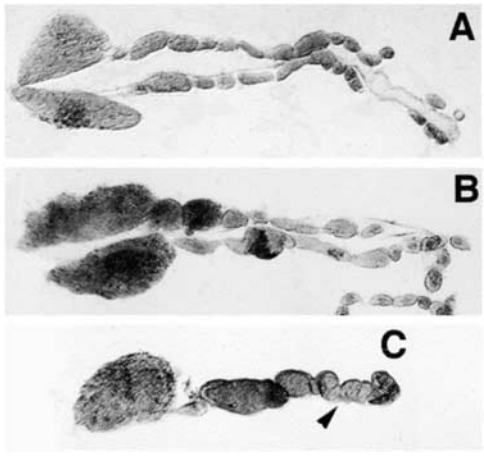


Figure 1.—Overgrown hematopoietic organs of *mxc* larvae. Lymph glands from wild-type and *mxc* late third instar larvae carrying a *Dot-LacZ* enhancer trap were stained with X-Gal. Anterior lobes are to the left. (A) Wild type; (B) $mxc^{G46}$; (C) $mxc^{G43}$. Note that posterior lobes show stronger hypertrophy in C (arrowhead) than in B.

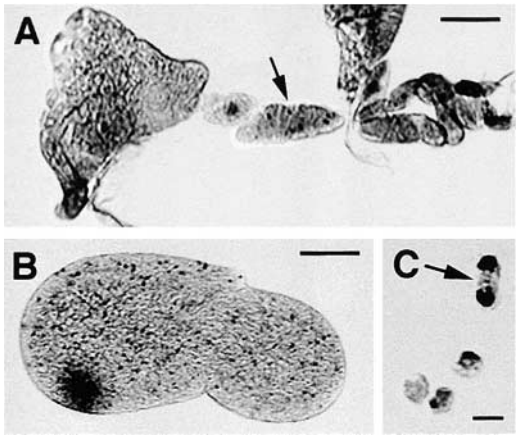


Figure 2.—Cell division in lymph glands and circulating hemocytes. Cell division was visualized with anti-phosphohistone H3 antibody. (A) Lymph gland chain from *Binsn*/Y larva. Dividing cells are detected in posterior lobes (arrow). Bar, 100 μm. (B) First and second lymph gland lobes from $mxc^{G43}$/Y larva. Numerous dividing cells are present in both. Bar, 100 μm. (C) Mitotic figure (arrow) in circulating hemocyte from $mxc^{G43}$/Y larva. Bar, 10 μm.

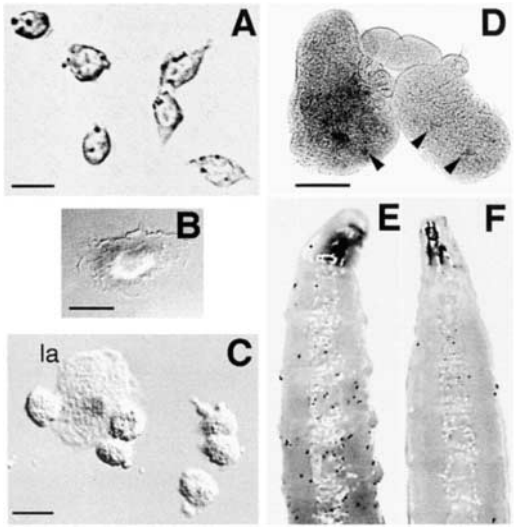


FIGURE 3.—Circulating blood cell types in *mxc* larvae. Third instar larvae were dissected in Drosophila Ringer solution and hemocytes were visualized with a Leitz DMRD photomicroscope. (A) Plasmatocytes and spindle-shaped podocytes from $mxc^{G43}$/Y larva absorb particles of India ink injected 2 hr previously; (B) macrophage; (C) the *l(2)113/28* reporter line drives nuclear β-galactosidase expression in lamellocytes (la), here from $mxc^{G43}$/Y larva. Bars in A–C, 10 μm. (D) X-Gal staining of $mxc^{G43}$/Y;*l(2)113/28*/+ reveals differentiated lamellocytes inside the lymph glands (arrowheads). Bar, 100 μm. (E and F) Crystal cells, visualized by heat treatment, are more frequent in control *Binsn*/Y (E) than in $mxc^{16a\text{-}1}$/Y (F) larvae.

for the number of crystal cells per control larva (Table 1; LANOT *et al.* 2001; SORRENTINO *et al.* 2002). $mxc^{G43}$, $mxc^{mbn1}$, and $mxc^{16a\text{-}1}$ larvae exhibited three- to fivefold increases in circulating hemocytes (Table 1). *mxc* blood cell types appeared similar to wild type with one possible exception: mutant larvae exhibited a significant increase (4–5% compared to <0.1% in controls) in a cell type with large pseudopod-like extensions. The cells appeared pear-shaped or spindle-shaped and were different from round plasmatocytes, even with cytoplasmic filaments, or from flattened macrophages (Figure 3). Like phagocytic plasmatocytes, these cells absorbed particles of India ink injected into the larva (LANOT *et al.* 2001; Figure 3); they presented numerous cytoplasmic organelles and vacuoles and resembled $mxc^{mbn1}$ podocytes (GATEFF 1978; SHRESTHA and GATEFF 1982). Accordingly, we considered them as phagocytes, possibly with altered adhesion capacities, and call them podocytes hereafter. Mutant larvae contained 2–3% lamellocytes, whereas such cells often represented <0.1% in wild type (Table 1). Lamellocytes were already differentiated within *mxc* mutant lymph glands, as revealed by the lamellocyte-specific enhancer trap line *l(2)113/28* (Figure 3). Staining of hemocytes from wandering larvae with anti-phosphohistone H3 antibody revealed increased mitoses in *mxc* mutants (Table 2; Figure 2). Among wild-type hemocytes 0.64% showed mitotic figures in good agreement with previous data (RIZKI 1978; QIU *et al.* 1998), whereas mitoses were up to four times as frequent in *mxc* mutants. Hence increased proliferation is present in lymph glands and in circulating blood cells. We compared crystal cell numbers per mutant larva with two controls: the $y^1\ ac^1\ z^1$ strain in which $mxc^{G46}$ and $mxc^{G43}$ were induced (SANTAMARIA and RANDSHOLT 1995) and the balancer chromosome *Binsn*. Crystal cells, visualized by heat treatment, were fewer in all *mxc* contexts than in wild-type controls (Table 1; Figure 3). Similar results were observed when black cells were compared in *Binsn*/Y;*Bc*/+ and *mxc*/Y;*Bc*/+ animals (not shown). The reduction was statistically significant for $mxc^{mbn1}$ and $mxc^{16a\text{-}1}$ larvae when compared to both controls (Table 1). $mxc^{G46}$ and $mxc^{G43}$ crystal cell numbers were statistically different only from the *Binsn*/Y control.

**TABLE 2**

**Cell division in circulating hemocytes**

| Genotype | Plasmatocytes | Dividing cells | % dividing cells |
|---|---|---|---|
| *Binsn*/Y[a] | 2509 | 16 | 0.64 |
| $mxc^{G43}$/Y | 1516 | 23 | 1.52* |
| $mxc^{mbn1}$/Y | 2033 | 38 | 1.86* |
| $mxc^{16a\text{-}1}$/Y | 1930 | 48 | 2.48* |

*$P < 0.01$; significantly different from *Binsn*/Y.
[a] Genotype of sibling larvae, which served as controls in this experiment.

Together the data show that all four *mxc* mutants affect lymph gland and circulating hemocyte proliferation and differentiation. Loss-of-function alterations of *mxc* result in abnormal numbers of circulating larval hemocytes of both the plasmatocyte and the crystal cell hematopoietic lineages.

***mxc* hematopoietic tissue is intrinsically overproliferating and invasive:** *mxc* hematopoietic phenotypes could reflect a defense response to apoptosis, which occurs in *mxc* imaginal discs (reviewed in DEAROLF 1998; SAGET *et al.* 1998). Alternatively, *mxc* could directly control prohemocyte and hemocyte proliferation and differentiation since *l(1)mbn* lymph gland cells can divide after transplantation into larvae or adults: they invade healthy tissues and can, according to GATEFF and MECHLER (1989), kill the host. To choose between these alternatives, we first compared the number of apoptotic cells in wild-type and *mxc* mutant lymph glands. TUNEL labeling of apoptotic cells revealed hardly any increase in cell death in lymph glands from *mxc* mutant animals (Figure 4). Hence *mxc* lymph gland overproliferation is likely not induced to compensate for cell death in the hematopoietic organs.

We performed a series of transplantation experiments, since transplantation of Drosophila cells with mutations in tumor suppressor genes into wild-type adult hosts can reveal their capacity for autonomous, uncontrolled proliferation (HANRATTY and RYERSE

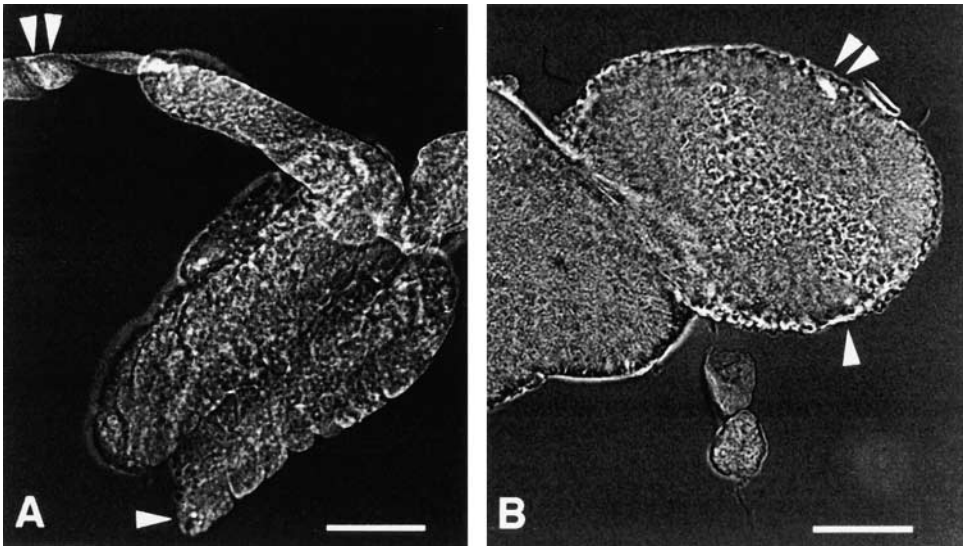


FIGURE 4.—Cell death in wild-type and *mxc* mutant lymph glands. Dark-field photograph of apoptotic cells, revealed by Tunel. (A) First lymph gland lobe pair and second lymph gland lobe from *Binsn*/Y wandering larva. Rare apoptotic cells are detected (arrowheads). (B) Detail of hypertrophic posterior lymph gland lobes from $mxc^{G43}$/Y larva. Arrowheads show apoptotic cells. Bars, 100 μm.

1981; WOODHOUSE *et al.* 1998). $mxc^{G43}$, $mxc^{mbn1}$, or $mxc^{16a\text{-}1}$ lymph gland tissues that were all *hsp83-LacZ*/+ were injected into β$gal^{nl}$/β$gal^{nl}$ females. We found no differences in viability between females transplanted with *mxc* or with wild-type lymph gland tissue, even 3 weeks after injection. To test for growth, a number of transplanted females were observed 24 hr after injection. This provided sizes of the original transplants. The rest were allowed to grow for 5 days. β-Galactosidase-expressing cells were revealed by X-Gal staining. Results are summarized in Table 3. From 50 to 100% of the transplants were detected after 5 days of growth. Furthermore, 60–80% of *mxc* lymph gland transplants multiplied in the hosts, whereas none of the 46 control transplants did so. Growing transplanted cells either remained localized in the abdomen of the host or invaded from part to all of the body cavity (Figure 5). Hence overproliferation of *mxc* lymph gland tissue is an autonomous and intrinsic characteristic of these cells. We conclude that *mxc* wild-type product is directly involved in control of lymph gland and hemocyte proliferation in larvae.

**TABLE 3**

**Transplantation of *mxc* lymph glands into wild-type hosts**

| Donor cells[a] | No. of hosts[c] | No. of transplants[d] (%) | Growth[e] (%) |
|---|---|---|---|
| $y^1$ $mxc^{mbn1}$/Y | 111 | 79 (70) | 49 (60) |
| *Binsn*/$Y^b$ | 29 | 23 (80) | 0 |
| $y^1$ $mxc^{G43}$/Y | 20 | 10 (50) | 7 (70) |
| *Binsn*/$Y^b$ | 10 | 5 (50) | 0 |
| $y^1$ $mxc^{16a\text{-}1}$/Y | 24 | 19 (80) | 15 (80) |
| *Binsn*/$Y^b$ | 21 | 21 (100) | 0 |

[a] Genotype of transplanted *hsp83-LacZ* lymph glands.
[b] Internal control *Binsn*/Y siblings for each experimental series.
[c] Transplanted β$gal^{nl}$/β$gal^{nl}$ females.
[d] Transplants expressing β-galactosidase 5 days after transplantation.
[e] Transplants showing increased size after 5 days.

***mxc* and Toll signaling in hematopoiesis:** The Toll pathway controls insect cellular defense responses, and *Toll* gain of function (g.o.f.) causes hematopoietic overgrowth (QIU *et al.* 1998). We compared hematopoietic phenotypes of $mxc^-$ and of Toll signal g.o.f., due to either constitutive receptor activation by $Tl^{10b}$ or loss of the cytoplasmic inhibitor encoded by *cactus* in $cact^{A2}$/$cact^{A2}$ hypomorphs (Table 4). QIU *et al.* (1998) reported a low incidence of pseudotumors along with a 2-fold hemocyte increase in $Tl^{10b}$/+ larvae and a >10-fold one in the strong $cact^{S1}$/$cact^{S1}$ mutant. We found that $Tl^{10b}$/+ larvae exhibited a 3- to 5-fold increase in hemocyte numbers and that 99% presented pseudotumors; hypomorphic $cact^{A2}$/$cact^{A2}$ larvae showed a 3-fold hemocyte increase (Table 4). These values are in the same range as *mxc*-induced overproliferation. In contrast to even the most severe *mxc* mutants (Table 1), which exhibit only 3–5% lamellocytes, both gain-of-Toll signal contexts were associated with higher lamellocyte ratios of, respectively, 13.8 and 17.0% (similar to previous $Tl^{10b}$/+ data; LANOT *et al.* 2001). Spindle-shaped podocytes were also present, but in lesser amounts (4.0 and 1.1% of circulating cells, respectively).

To address how *mxc* related to Toll signaling in hemocyte control, we constructed $mxc^{G43}$/Toll pathway double mutants. Loss of the pathway was obtained in either heteroallelic $Tl^{RXA}$/$Tl^{r632}$ larvae or $tub^{238}$/$tub^{238}$ animals where cytoplasmic transduction of Toll signal is blocked (GOVIND 1999). Results are summarized in Table 4. In agreement with QIU *et al.* (1998), we found that diminished Toll signaling reduces hemocyte numbers (68 and 77% of sibling *Tl*/+ or $tub^{238}$/+ controls, respectively). Furthermore, both Toll signal loss-of-function (l.o.f.) contexts counteracted the effects of $mxc^{G43}$ l.o.f. on hemocyte production. Mean hemocyte numbers of $mxc^{G43}$/Y;$Tl^{RXA}$/$Tl^{r632}$ or $mxc^{G43}$/Y;$tub^{238}$/$tub^{238}$ lar-

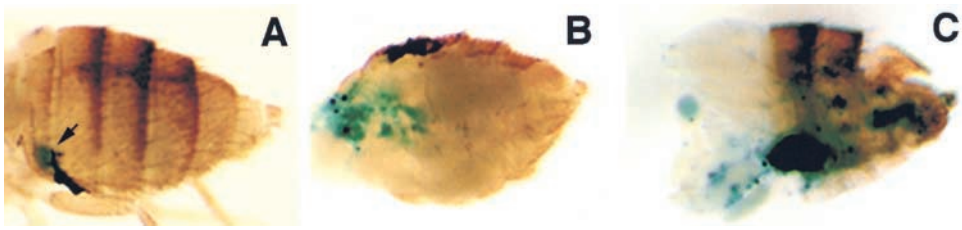


FIGURE 5.—*mxc* lymph gland cells proliferate after transplantation into wild-type hosts. Lymph glands were dissected from third instar *Binsn*/Y;*hsp83-LacZ*/+ or *mxc*/Y;*hsp83-LacZ*/+ larvae and transplanted into the abdomen of $\beta gal^{nl}/\beta gal^{nl}$ females. Hosts were dissected 5 days after injection and stained with X-Gal. (A) Transplanted wild-type cells show localized β-galactosidase expression (arrow); (B) transplanted $mxc^{G43}$/Y cells; (C) transplanted $mxc^{mbm1}$/Y cells. The dissected abdomen in C shows that β-galactosidase-expressing cells have partially invaded the body cavity.

vae were not statistically different from the corresponding *Binsn*/Y;*Tl*/+ or *Binsn*/Y;$tub^{238}$/+ internal controls, whereas $mxc^{G43}$/Y;*Tl*/+ and $mxc^{G43}$/Y;$tub^{238}$/+ sibling larvae always had more circulating hemocytes ($P < 0.01$). Podocytes and lamellocytes, on the other hand, were still more numerous in $mxc^{G43}$/Y;$Tl^{RXA}/Tl^{r632}$ and $mxc^{G43}$/Y;$tub^{238}/tub^{238}$ larvae compared to *Binsn*/Y;*Tl*/+ or *Binsn*/Y;$tub^{238}$/+ controls. Hence for hemocyte density, loss of Toll signal is epistatic to the loss of *mxc*. We conclude that loss of *mxc* can increase body cavity hemocyte numbers only when the Toll pathway is functional.

***mxc* and JAK/STAT control of hematopoiesis:** Gain of function of the JAK kinase encoded by *hopscotch* can be lethal and can induce pseudotumors, strong lymph gland and hemocyte overgrowth, abnormal differentiation of lamellocytes, and reduced crystal cell numbers (HANRATTY and RYERSE 1981; HANRATTY and DEAROLF 1993; HARRISON *et al.* 1995; LUO *et al.* 1995, 1997; LANOT *et al.* 2001). Some of these effects are mediated by *JAK* overactivation of *D-stat* product and can be rescued by loss of *D-stat* (HOU *et al.* 1996; YAN *et al.* 1996; LUO *et al.* 1997). As for the Toll pathway, we compared the effects of *mxc* mutations on hematopoiesis to those of loss or gain of function of the *JAK/STAT* pathway. Loss of *hop* reduces cell proliferation in larval tissues such as imaginal discs and brain (PERRIMON and MAHOWALD 1986), but no data are, to our knowledge, available concerning hemocyte numbers and ratios in *hop* amorphic animals that are larval/pupal lethals. We examined hemocyte production in amorphic $hop^{VA275}$ and $hop^{M38}$ males raised at 25°. Interestingly, both *hop* null alleles exhibit hemocyte numbers and cell type distributions that were no different from control *Binsn*/Y or *FM7*/Y siblings (Table 5). This result suggests that, contrary to loss of Toll signaling, loss of *hop*/JAK might have no effect on the number of circulating larval hemocytes.

**TABLE 4**

**Hemocytes in Toll pathway and *mxc* mutants**

| Genotype | *N* | Hemocytes cells/ml ($10^6$)[b] | Plasmatocytes (%) | Podocytes (%) | Lamellocytes (%) |
|---|---|---|---|---|---|
| *TM3*/+[a] | 10 | 1.3 ± 0.6 | 100 | <0.1 | <0.1 |
| $Tl^{10b}$/+ | 12 | 3.1 ± 2.1 | 82.2 | 4.0 | 13.8 |
| $cact^{A2}/CyO\ y^{+}$[a] | 10 | 1.7 ± 0.7 | 99 | 0.2 | 0.8 |
| $cact^{A2}/cact^{A2}$ | 10 | 5.1 ± 1.5 | 81.9 | 1.1 | 17.0 |
| *Binsn*/Y;*TM6c*/$Tl^{a}$ | 10 | 2.5 ± 1.1 | >99 | <0.1 | <0.1 |
| *Binsn*/Y;$Tl^{RXA}/Tl^{r632}$ | 11 | 1.7 ± 0.8 | 99.5 | 0.4 | <0.1 |
| $y^1\ mxc^{G43}$/Y;*TM6c*/*Tl* | 11 | 6.4 ± 2.9* | 95.2 | 2.8 | 2.0 |
| $y^1\ mxc^{G43}$/Y;$Tl^{RXA}/Tl^{r632}$ | 9 | 3.2 ± 1.9** | 95.3 | 3.0 | 1.7 |
| *Binsn*/Y;*TM6c*/$tub^{238}$[a] | 14 | 1.3 ± 1.0 | >99 | 0.2 | <0.1 |
| *Binsn*/Y;$tub^{283}/tub^{238}$ | 13 | 1.0 ± 0.7 | >99 | 0.3 | <0.1 |
| $y^1\ mxc^{G43}$/Y;*TM6c*/$tub^{238}$ | 10 | 4.5 ± 1.4* | 90.4*** | 6.9*** | 2.7*** |
| $y^1\ mxc^{G43}$/Y;$tub^{283}/tub^{238}$ | 10 | 1.0 ± 0.3** | >97*** | 1.1*** | 1.8*** |

Effects of loss of *Toll* were evaluated among progeny of $y^1\ mxc^{G43}/Binsn;Tl^{RXA}/TM6c$ females and *Binsn*/Y;*TM6c*/$Tl^{r632}$ males. Effects of loss of *tube* were evaluated among progeny of $y^1\ mxc^{G43}/Binsn;tub^{238}/TM6c$ females crossed to *Binsn*/Y;$tub^{238}$/*TM6c* males. *$P < 0.01$; significantly different from *Binsn*/Y control. **$P < 0.01$; significantly different from internal $mxc^{G43}$ control category in this experiment. ***$P < 0.01$; cell type distribution significantly different from *Binsn*/Y.

[a] Genotype of sibling larvae, which served as internal controls in this experimental series. *N*, no. of animals used for this genotype.

[b] Mean hemocyte density per milliliter of hemolymph ± SE.

**TABLE 5**

**Hemocyte phenotypes induced by loss of *hop* and loss of *D-stat***

| Genotype | *N* | Cells/ml ($10^6$)[b] | Plasmatocytes (%) | Podocytes (%) | Lamellocytes (%) |
|---|---|---|---|---|---|
| *FM7act-GFP*/Y[a] | 11 | 2.5 ± 1.0 | >99 | <0.1 | <0.1 |
| *y w hop$^{M38}$*/Y | 14 | 2.2 ± 1.0 | >99 | 0.1 | <0.1 |
| *Binsn*/Y[a] | 10 | 2.6 ± 2.1 | 98.3 | 1.5 | 0.2 |
| *y hop$^{VA275}$*/Y | 11 | 2.8 ± 2.1 | >99 | 0.6 | <0.1 |
| *FM7c*/Y;*His3-GFP*/+[a] | 17 | 1.1 ± 0.4 | >99 | <0.1 | 0.1 |
| *FM7c*/Y;*D-stat$^{6346}$*/+ | 17 | 1.0 ± 0.6 | >99 | 0.2 | 0.2 |
| *hop$^{Tum\text{-}l}$*/Y;*His3-GFP*/+ | 22 | 15.1 ± 7.6 | 63.2 | 11.4 | 25.4 |
| *hop$^{Tum\text{-}l}$*/Y;*D-stat$^{6346}$*/+ | 23 | 6.2 ± 3.6* | 77.1** | 5.6** | 17.3** |

*hop$^{Tum\text{-}l}$/FM7c* females were mated to +/Y;*D-stat$^{6346}$*/;*His3-GFP* males. Data from two independent experiments were pooled. *$P < 0.01$; significantly different from *hop$^{Tum\text{-}l}$*/Y;*His3-GFP*/+. **$P < 0.01$; cell type distribution significantly different from *hop$^{Tum\text{-}l}$*/Y;*His3-GFP*/+.

[a] Genotype of sibling larvae, which served as internal wild-type controls in this experiment. *N*, no. of animals used for this genotype.

[b] Mean hemocyte density per milliliter, ± SE.

Hou *et al.* (1996), Yan *et al.* (1996), and Luo *et al.* (1997) have reported partial suppression of g.o.f. *hop*-associated lethality and pseudotumors by mutations of *D-stat*, mainly at 29°. Furthermore, Luo *et al.* (1997) reported inhibition of *hop$^{Tum\text{-}l}$*/Y-associated lamellocyte differentiation by loss of a *D-stat* copy. To establish whether similar effects are observed at 25°, we examined plasmatocyte and lamellocyte production in a *hop$^{Tum\text{-}l}$*/Y;*D-stat$^{6346}$*/+ context at this temperature. In agreement with the authors cited above, we found that *hop$^{Tum\text{-}l}$*/Y induced strong hemocyte overproduction and that 25% of these were lamellocytes; pseudotumors were less frequent in female *hop$^{Tum\text{-}l}$*/+;*D-stat$^{6346}$*/+ larvae compared to their *hop$^{Tum\text{-}l}$*/+;*His3-GFP*/+ siblings at this semirestrictive temperature (10 and 24%, respectively). Interestingly, larvae raised at 25° revealed a suppressive effect of loss of *D-stat* on *hop$^{Tum\text{-}l}$*/Y hemocyte numbers since *hop$^{Tum\text{-}l}$*/Y;*D-stat$^{6346}$*/+ males had significantly fewer circulating blood cells than did their *hop$^{Tum\text{-}l}$*/Y;*His3-GFP*/+ siblings (Table 5). Hence *D-stat* product could be required for both hemocyte proliferation and hemocyte differentiation in response to *hop$^{Tum\text{-}l}$*-encoded product. *D-stat$^{6346}$* null mutants are larval/pupal lethals and exhibit small imaginal discs, indicating that *D-stat* plays a role in control of imaginal cell proliferation (Hou *et al.* 1996). Our data suggest that this role extends to hemocyte proliferation.

We first looked for *mxc* and JAK/STAT pathway interactions in *mxc$^{G43}$*/Y;*D-stat$^{6346}$*/+ larvae. Total circulating hemocyte numbers as well as lamellocyte numbers were similar in *mxc$^{G43}$*/Y;*TM6c*/+ and *mxc$^{G43}$*/Y;*D-stat$^{6346}$*/+ larvae (Table 6). Similar results were obtained for *mxc$^{G43}$*/Y;*D-stat$^{HJ}$*/*D-stat$^{6346}$* larvae compared to *mxc$^{G43}$*/Y;*D-stat*/+ animals (data not shown). Hence *mxc* effects on blood cell numbers are likely not modified by loss of *D-stat*. We wondered whether the same held true for *Tl$^{10b}$* and assessed this by looking for effects of *D-stat$^{6346}$ in trans* with *Tl$^{10b}$*. Heterozygosity for *D-stat$^{6346}$* reduced lamellocyte differentiation in *Tl$^{10b}$*/*D-stat$^{6346}$* compared to *Tl$^{10b}$*/*His3-GFP* larvae (Table 6). Therefore the strong induction of lamellocyte differentiation caused by gain of Toll requires *D-stat*.

To conclude, our data indicate that hemocyte production and lamellocyte differentiation in various genetic and experimental contexts show different sensitivities to changes in *D-stat* dosage. *D-stat* quantity is apparently limiting for the strong hemocyte production induced by constitutive *hop*/JAK activation at 25°, whereas the lesser blood cell productions of *mxc$^{G43}$* or *Tl$^{10b}$* are not affected by *D-stat* hemizygosity. Furthermore, as previously reported for *hop$^{Tum\text{-}l}$* lamellocyte production (Luo *et al.* 1997), we find that the strong lamellocyte differentiation in *Tl$^{10b}$* also depends on *D-stat* product, whereas the lesser lamellocyte production of *mxc$^{G43}$* is unaffected in both a *D-stat$^{6346}$*/+ and a *D-stat$^{HJ}$*/*D-stat$^{6346}$* context.

**Lymph gland proliferation and circulating hemocytes in double inductive conditions:** We examined the effects on hemocyte production of several double mutant contexts that each alone increase hemocyte numbers. Interestingly, *mxc$^{G43}$*/Y;*Tl$^{10b}$*/+ and *mxc$^{G43}$*/Y;*cact$^{A2}$*/*cact$^{A2}$* larvae had fewer hemocytes than did *Tl$^{10b}$*/+, *mxc$^{G43}$*/Y, or *cact$^{A2}$*/*cact$^{A2}$* animals (Table 7; $P < 0.01$). Furthermore, joint activation of the Toll and the JAK/STAT pathways had comparable effects in *hop$^{Tum\text{-}l}$*/Y;*Tl$^{10b}$*/+ and *hop$^{Tum\text{-}l}$*/Y;*cact$^{A2}$*/*cact$^{A2}$* larvae (not shown). *mxc$^{G43}$ hop$^{Tum\text{-}l}$*/Y larvae had $3.1 \times 10^6$ (SD ± $1.3 \times 10^6$) hemocytes/ml, which represents an increase compared to the internal wild-type control ($0.7 \pm 0.4 \times 10^6$ hemocytes/ml), but is considerably less than the added effects of *mxc$^{G43}$* and *hop$^{Tum\text{-}l}$* alone. All such double mutant animals contained

**TABLE 6**

**Hemocytes in *mcx* and *D-stat*/+ or *D-stat/Tl*$^{10b}$ *trans*-heterozygotes**

| Genotype | *N* | Cells/ml ($10^6$)[b] | Plasmatocytes (%) | Podocytes (%) | Lamellocytes (%) |
|---|---|---|---|---|---|
| *Binsn*/Y;*His3-GFP*/+[a] | 10 | 0.6 ± 0.2 | >99 | <0.1 | <0.1 |
| *Binsn*/Y;*D-stat*$^{6346}$/+ | 8 | 0.5 ± 0.3 | >99 | <0.1 | <0.1 |
| *y*$^1$ *mxc*$^{G43}$/Y;*His3-GFP*/+ | 11 | 1.4 ± 0.6* | 89.5 | 8.5 | 2.0 |
| *y*$^1$ *mxc*$^{G43}$/Y;*D-stat*$^{6346}$/+ | 11 | 1.8 ± 0.9* | 92.5 | 6.5 | 1.0 |
| | | | | | |
| +/Y;*TM6c*/*His3-GFP*[a] | 14 | 0.4 ± 0.3 | >99 | <0.1 | <0.1 |
| +/Y;*Tl*$^{10b}$/*TM6c* | 16 | 2.2 ± 0.9* | 84.0 | 0.1 | 15.8 |
| +/Y;*D-stat*$^{6346}$/*His3-GFP* | 15 | 0.3 ± 0.2 | >99 | <0.1 | <0.1 |
| +/Y;*Tl*$^{10b}$/*D-stat*$^{6346}$ | 16 | 3.5 ± 1.0* | 95.6** | 1.4** | 3.3** |

Effects of *D-stat*$^{6346}$ on *mxc*$^{G43}$ were examined in male progeny from +/Y;*D-stat*$^{6346}$/;*His3-GFP* males mated with *y*$^1$ *mxc*$^{G43}$/*Binsn* females. *D-stat*$^{6346}$ and *Tl*$^{10b}$ interactions were examined among male progeny of +/Y;*Tl*$^{10b}$/*His3-GFP* males and +/+;*D-stat*$^{6346}$/*TM6c* females. *N*, no. of animals utilized for this genotype. *$P < 0.01$; significantly different from internal control. **$P < 0.01$; blood cell type distribution significantly different from +/Y;*Tl*$^{10b}$/*TM6c*.

[a] Genotype of sibling larvae, which served as internal wild-type control in this experiment.

[b] Mean hemocyte no. per milliliter ± SE.

abnormally large hemocytes. To understand this phenomenon, we examined lymph glands from *mxc*$^{G43}$ *hop*$^{Tum-l}$/Y and *mxc*$^{G43}$/Y;*cact*$^{A2}$/*cact*$^{A2}$ animals. Such hematopoietic organs were very fragile and difficult to dissect. When the lymph glands could be isolated, they showed severe overgrowth and contained high numbers of differentiated blood cells (*mxc*$^{G43}$/Y;*cact*$^{A2}$/*cact*$^{A2}$ in Figure 6). This was also associated with intense mitotic activity, as revealed by staining with anti-phosphohistone H3 antibody (Figure 6). TUNEL label revealed no increase in apoptotic cell death in these lymph glands (*mxc*$^{G43}$/Y;*cact*$^{A2}$/*cact*$^{A2}$ example in Figure 6). Lamellocytes differentiated within the hematopoietic organs; in some larvae, the glands were covered by lamellocytes, as shown by the lamellocyte-specific reporter *l(2)113/28*, and progressively melanized (Figure 6). Together these data suggest that differentiated blood cells in the dramatically overgrown lymph gland lobes are not released into the hemolymph. The lymph glands could end by being recognized as non-self and encapsulated by the cellular host defense system of the animal.

***mxc*, PcG, and trxG genes and larval hemocyte control:** During Drosophila and vertebrate development, trxG and PcG genes control the expression of many targets, including the *Hox* genes. Furthermore, mammalian hematopoiesis is a target of trxG and PcG regulation (reviewed in Takihara and Hara 2000; Muller and Leutz 2001; Raaphorst *et al.* 2001). Hence, we asked whether Drosophila larval hematopoiesis depended on PcG and trxG gene products to the same extent as ho-

**TABLE 7**

**Hemocytes in *mxc* and Toll signal g.o.f. double mutants**

| Genotype | *N* | Cells/ml ($10^6$)[b] | Plasmatocytes (%) | Podocytes (%) | Lamellocytes (%) |
|---|---|---|---|---|---|
| *Binsn*/Y;*cact*$^{A2}$/*CyO act-GFP*[a] | 10 | 1.7 ± 0.7 | >99 | 0.1 | 0.6 |
| *y*$^1$ *mxc*$^{G43}$/Y;*cact*$^{A2}$/*CyO act-GFP* | 10 | 5.0 ± 2.0* | 92.9 | 5.0 | 2.1 |
| *Binsn*/Y;*cact*$^{A2}$/*cact*$^{A2}$ | 10 | 5.1 ± 1.5* | 88.8 | 1.3 | 9.9 |
| *y*$^1$ *mxc*$^{G43}$/Y;*cact*$^{A2}$/*cact*$^{A2}$ | 10 | 1.9 ± 0.9 | 86.3 | 8.5 | 6.2 |
| | | | | | |
| *Binsn*/Y;*His3-GFP*/+[a] | 10 | 0.7 ± 0.3 | >99 | <0.1 | <0.1 |
| *y*$^1$ *mxc*$^{G43}$/Y;*His3-GFP*/+ | 15 | 3.0 ± 1.4* | 88.8 | 8.6 | 2.6 |
| *Binsn*/Y;*Tl*$^{10b}$/+ | 9 | 3.9 ± 1.3* | 93.0 | 1.0 | 6.0 |
| *y*$^1$ *mxc*$^{G43}$/Y;*Tl*$^{10b}$/+ | 13 | 1.8 ± 1.0 | 73.8 | 23.8 | 2.4 |

*y*$^1$ *mxc*$^{G43}$;*cact*$^{A2}$ animals were progeny of *y*$^1$ *mxc*$^{G43}$/*Binsn*;*cact*$^{A2}$/*CyO act-GFP* females and *Binsn*/Y;*cact*$^{A2}$/*CyO act-GFP* males. *mxc*$^{G43}$/Y;*Tl*$^{10b}$/+ larvae were progeny of *y*$^1$ *mxc*$^{G43}$/*Binsn* females and +/Y;*Tl*$^{10b}$/*His3-GFP* males. *N*, no. of larvae utilized for this genotype. *$P < 0.01$; significantly different from internal control.

[a] Genotype of sibling larvae, which served as internal wild-type controls in this experimental series.

[b] Mean hemocyte no. per milliliter ± SE.

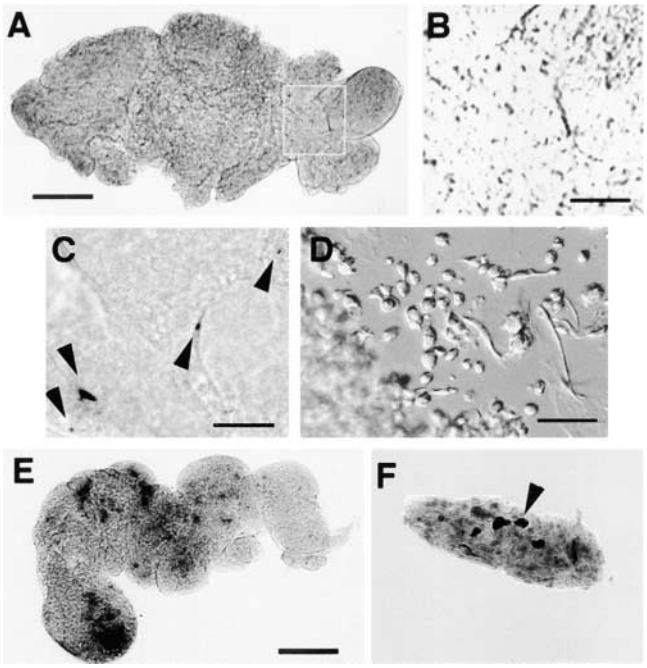


FIGURE 6.—Lymph glands in *mxc;cact* and *mxc;hop$^{Tum\text{-}l}$* double mutants. (A) Whole view of dramatically overgrown lymph gland chain from *mxc$^{G43}$*/Y;*cact$^{A2}$*/*cact$^{A2}$* larva, stained with anti-phosphohistone H3 antibody. (B) Enlarged view of detail of lymph gland in A reveals anti-phosphohistone H3 antibody label in dividing cells. (C) Detail of Tunel-labeled *mxc$^{G43}$ hop$^{Tum\text{-}l}$*/Y lymph gland. Apoptotic cells are indicated by arrowheads. (D) *mxc$^{G43}$hop$^{Tum\text{-}l}$* lymph glands contain differentiated hemocytes; lymph glands were squashed with a coverslip after dissection in 1× PBS. (E) Lamellocytes differentiate inside overgrown *mxc$^{G43}$hop$^{Tum\text{-}l}$*/Y lymph glands, revealed by β-galactosidase expression of the *l(2)113/28* reporter line. (F) X-Gal-stained *mxc$^{G43}$hop$^{Tum\text{-}l}$*/Y;*l(2)113/28* /+ lymph gland lobe, covered by lamellocytes and partially melanized (arrowhead); same scale as E. Bars in A and E, 150 μm; bars in B, C, and D, 40 μm.

meotic genes do for identity specification along the anterior/posterior body axis. Indeed, homeotic transformations of single PcG mutants are synergistically enhanced by adding a second PcG mutation *in trans* (JÜRGENS 1985), whereas trxG mutations *in trans* suppress PcG mutant phenotypes (KENNISON and TAMKUN 1988). Hemocytes were counted for *mxc$^{G43}$*/Y *in trans* with alleles of *Sex comb on Midleg* (*Scm*), *Polycomb-like* (*Pcl*), *Polycomb* (*Pc*), and *Posterior sex combs* (*Psc*), which strongly enhance adult homeotic transformations of the viable *mxc$^{M1}$* allele (SAGET *et al.* 1998). We also tested loss of *extra sex combs* (*esc*). None of the five mutations affected control or *mxc$^{G43}$* hemocyte numbers or ratios (data not shown). This suggests that Drosophila PcG genes are required differently in larval hemocyte production and in HOM/*Hox* gene regulation. Indeed, *mxc*, *Pcl*, *Pc*, *Scm*, and *Psc* exert common negative control on the latter process, whereas *mxc* alone seems critically required for the former.

Implication of trxG products in hematopoiesis was tested in *trans*-heterozygous *mxc*;trxG genetic contexts, using *trithorax* (*trx*), *moira* (*mor*), and *brahma* (*brm*) mutations that counteract PcG homeotic phenotypes *in trans* (FLYBASE 1999). *trx* and *mor* mutations had no effect on wild-type or *mxc* hemocyte numbers and ratios. Interestingly, whereas lymph glands of *brm$^{2}$*/+ were no different from wild type (not shown), loss of *brm* reduced circulating hemocytes in *brm$^{2}$*/+ and in *y$^{1}$ mxc$^{G43}$*/Y;*brm$^{2}$*/+ larvae (Table 8). Similar results were observed for *brm$^{2}$ trx$^{E2}$*/+ and *y$^{1}$ mxc$^{G43}$*/Y;*brm$^{2}$ trx$^{E2}$*/+ larvae, indicating that *brm* alone affects circulating hemocyte numbers. The trxG protein BRM is similar to yeast SWI/SNF chromatin-remodeling proteins. The Drosophila gene *domino* also encodes SWI/SNF chromatin proteins that are, like their mammalian homologs, involved in control of cell proliferation (reviewed in GEBUHR *et al.* 2000; RUHF *et al.* 2001). To further characterize the role of *brm* in hemocyte control, we examined hemocyte numbers in *Tl$^{10b}$*/+ and *hop$^{Tum\text{-}l}$*/Y larvae carrying a *brm$^{2}$* allele (Table 8). The *brm$^{2}$*/+ context had no effect on *Tl$^{10b}$*/+ phenotype, whereas *hop$^{Tum\text{-}l}$*/Y;*brm$^{2}$*/+ larvae contained significantly fewer hemocytes than did their *hop$^{Tum\text{-}l}$*/Y;*His3-GFP*/+ siblings ($P < 0.01$). Lamellocyte ratios remained unchanged (respectively, 28.3 and 28.4%); hence *brm$^{2}$* does not block *hop$^{Tum\text{-}l}$*-induced lamellocyte differentiation. Together, these data show, first, that two Drosophila SWI/SNF proteins, BRM and DOM, participate in hematopoiesis control and, second, that upregulation of hemocyte production by Toll is less sensitive to the level of *brm* product than upregulation either by activation of JAK or by loss of *mxc*.

## DISCUSSION

***mxc* controls blood cell proliferation and differentiation:** Here, we confirm that wild-type *mxc* directly regulates hemocyte proliferation and differentiation in Drosophila larvae. GATEFF and MECHLER (1989) reported modification of these processes by *mxc l(1)mbn* alleles, which were screened as causing hyperplasia and neoplasia. We examined hematopoietic phenotypes of three mutants that were isolated on the basis of other criteria (SANTAMARIA and RANDSHOLT 1995; DOCQUIER *et al.* 1996). Hematopoiesis was not analyzed in strong *mxc* hypomorphic or amorphic embryos, since the maternal *mxc* component allows normal development until the second instar. Nor did we study *mxc*/+ females alone or *in trans* with other mutations, since no haplo-insufficiency has ever been observed for any set of *mxc* phenotypes. We show that overproliferation and premature lamellocyte differentiation take place in the hyperplastic lymph gland lobes and that increased proliferation is also seen in circulating cells. Lymph gland overgrowth is progressively stronger from *mxc$^{G46}$* to *mxc$^{l6a\text{-}1}$* (Table 1) and affects more and more posterior lobes, in good agreement with SHRESTHA and GATEFF (1982) who described the whole hematopoietic organ of *mxc$^{mbn1}$* as hy-

**TABLE 8**

**Hemocytes of *mxc*, *Tl*, *hop*$^{Tum\text{-}l}$, and *brm trans*-heterozygotes**

| Genotype | *N* | Cells/ml ($10^6$)[b] | Plasmatocytes (%) | Podocytes (%) | Lamellocytes (%) |
|---|---|---|---|---|---|
| *Binsn*/Y;*His3-GFP*/+[a] | 13 | 0.8 ± 0.4 | >99 | <0.1 | <0.1 |
| *Binsn*/Y;*brm*$^2$/+ | 24 | 0.4 ± 0.3* | >99 | <0.1 | <0.1 |
| *y*$^1$ *mxc*$^{G43}$/Y;*His3-GFP*/+ | 18 | 3.4 ± 0.8 | 93.5 | 5.0 | 1.5 |
| *y*$^1$ *mxc*$^{G43}$/Y;*brm*$^2$/+ | 25 | 1.4 ± 0.7*·*** | 93.3 | 3.9 | 2.8 |
| +/Y;*His3-GFP/TM6c*[a] | 11 | 0.7 ± 0.5 | >99 | <0.1 | <0.1 |
| +/Y;*brm*$^2$/*His3-GFP* | 13 | 0.4 ± 0.3 | >99 | <0.1 | <0.1 |
| +/Y;*Tl*$^{10b}$/*TM6c* | 12 | 1.4 ± 0.8* | 81.4 | 7.0 | 12.6 |
| +/Y;*brm*$^2$/*Tl*$^{10b}$ | 14 | 1.4 ± 0.9* | 77.8 | 7.8 | 14.6 |
| *FM7c*/Y;+/*TM6*[a] | 16 | 1.0 ± 0.3 | >99 | <0.1 | <0.1 |
| *FM7c*/Y;*brm*$^2$/+ | 19 | 0.7 ± 0.5* | >99 | <0.1 | <0.1 |
| *hop*$^{Tum\text{-}l}$/Y;+/*TM6c* | 15 | 5.7 ± 4.2** | 42.2 | 29.4 | 28.4 |
| *hop*$^{Tum\text{-}l}$/Y;*brm*$^2$/+ | 17 | 2.7 ± 1.7**·***** | 48.0 | 23.7 | 28.3 |

For interactions with *brm*, *y*$^1$ *mxc*$^{G43}$/*Binsn* females were crossed to +/Y;*brm*$^2$/*His3-GFP* males; results from two experiments were pooled. *brm*$^2$/*TM6c* females were crossed to *Tl*$^{10b}$/*His3-GFP* males. *hop*$^{Tum\text{-}l}$/*FM7c* females were mated with *brm*$^2$/*TM6c* males and data from two experiments were pooled. *$P < 0.05$; significantly different from wild-type control. **$P < 0.01$; significantly different from wild-type control. ***$P < 0.01$; significantly different from *mxc*$^{G43}$/Y;*His3-GFP*/+. ****$P < 0.05$; significantly different from *hop*$^{Tum\text{-}l}$/Y;+/*TM6c*.

[a] Genotype of sibling larvae, which served as internal wild-type controls in this experiment. *N*, no. of animals analyzed for this genotype.

[b] Mean no. of hemocytes per milliliter ± SE.

perplastic. Lanot *et al.* (2001) proposed that posterior lymph gland lobes contain hematopoietic blast cells, similar to bone marrow cells, which can be solicited to differentiate into given blood cell lines. Our data support the notion that anterior lobe prohemocytes are more readily solicited to divide and differentiate than are posterior ones.

Interestingly, the stronger *mxc* alleles showed lower numbers of circulating crystal cells that are about 10 times less frequent in *mxc*$^{mbn1}$ and *mxc*$^{16a\text{-}1}$ larvae compared to wild type. It has been suggested that crystal cell numbers might also be reduced in *hop*$^{Tum\text{-}l}$/Y animals at nonpermissive temperatures (Lanot *et al.* 2001). This could in both cases reflect recruitment of crystal cells in the melanotic capsules of mutant larvae. Still, such capsules were far from always detected in *mxc* larvae, since only up to 25% *mxc*$^{mbn1}$/Y animals developed pseudotumors. Hence *mxc* might affect crystal cell development. During hematopoiesis, plasmatocytes and crystal cells develop from a common pool of cells expressing the GATA protein Serpent (reviewed in Fossett and Schulz 2001; Meister and Govind 2002). The plasmatocyte cell lineage is specified by the *g*lial *c*ells *m*issing (GCM) conserved transcription factor; crystal cell lineage development depends on expression of the Acute Myeloid Leukemia 1 (AML-1)-like transcription factor Lozenge and is repressed by the friend of GATA protein U-shaped (USH; Lebestky *et al.* 2000; Fossett *et al.* 2001; Fossett and Schulz 2001). As some crystal cells are still present in the most severe *mxc* mutant, we propose that procrystal cell proliferation or differentiation into crystal cells, rather than crystal cell lineage specification *per se*, might be affected. Future investigation will determine whether modifications of *mxc* product levels also affect crystal cell precursors in the lymph glands and, if so, through which targets.

**Phagocytic cell types in *mxc* mutants:** Circulating hemocytes in *mxc* larvae consistently exhibited several percent of phagocytic cells, which we called podocytes (Figure 2), like the phagocytic cells described by Shrestha and Gateff (1982) as a prevailing cell type in *mxc*$^{mbn1}$ larvae. Like lamellocytes, such podocytes are rarely seen in wild-type larvae (<0.1% of circulating cells). We examined wild-type hemocytes from mid-third instar until 20 hr after pupariation without finding large amounts of similar cells. Changes in phagocytic cells from rounded plasmatocytes to flattened macrophages with membranous extensions and changed adhesion capacities normally occur at the end of the third instar and can be induced before by increasing ecdysone titer (Lanot *et al.* 2001). As suggested by Rizki (1978), podocytes might represent an intermediary form between these phagocytic cell types, which would differentiate in greater numbers in *mxc* mutants before pupariation. Their spindle shape suggests changed adhesion features and raises the question whether these cells could play an invasive role in *mxc* mutants.

***mxc* invasiveness and neoplasia:** *mxc* hematopoietic tissue from different alleles divides autonomously when transplanted into a wild-type host. Proliferative capaci-

ties of *mxc* lymph glands appear higher than those of larval brain or imaginal disc cells mutated for tumor suppressor genes. Indeed, growth of *lethal giant larvae*, *brain tumor*, or *discs large* transplants required 9–12 days at 25° (Woodhouse *et al.* 1998), whereas *mxc* transplants increased after 5 days at 23°. $hop^{Tum\text{-}l}$ lymph gland cells, grown at the restrictive temperature of 29° before and after transplantation, also increase after 3–5 days in wild-type females (Hanratty and Ryerse 1981). This might reflect differences in proliferation control mechanisms between the loosely structured lymph gland tissue where cell division is inducible and the highly structured larval disc and brain tissue (Harrison *et al.* 1995). Such differences in growth control might explain why *mxc* and other hematopoiesis mutants exhibit blood cell overproliferation together with reduced imaginal discs and brains (Watson *et al.* 1991; Torok *et al.* 1993; Saget *et al.* 1998).

Contrary to the data reported by Gateff and Mechler (1989), we found no lessened viability in females transplanted with *mxc* lymph glands, even 3 weeks after transplantation. This result was unexpected for the $mxc^{mbn1}$ and $mxc^{16a\text{-}1}$ pupal lethal alleles and less so for $mxc^{G43}$, which shows partial viability at 23°. As *mxc* mutations are temperature sensitive (Saget *et al.* 1998), 23° may not induce sufficient proliferation to endanger the transplanted hosts. Indeed, at 29° $hop^{Tum\text{-}l}$ is lethal and transplanted $hop^{Tum\text{-}l}$ lymph gland tissue kills a wild-type host, whereas $hop^{Tum\text{-}l}$ animals are viable at 25°. We propose that loss of *mxc* function leads to hematopoietic neoplasia, invasiveness, and altered blood cell composition but not necessarily to a lethal malignant transformation.

**Hematopoietic defects and other *mxc* phenotypes:** Severity of hematopoietic defects was correlated with that of other *mxc* phenotypes. Previous genetic studies showed $mxc^{G46}$ as a viable and $mxc^{G43}$ as a medium severe allele, whereas $mxc^{mbn1}$ is a very strong and $mxc^{16a\text{-}1}$ an even stronger hypomorph. *mxc* adult males exhibit ANT-C and BX-C gene gain-of-function-like homeotic transformations, with increasing penetrance and expressivity from $mxc^{M1}$ via $mxc^{G46}$ to $mxc^{G43}$ (Santamaria and Randsholt 1995). $mxc^{mbmSO}$, $mxc^{mbn1}$, and $mxc^{16a\text{-}1}$ larvae die before the pharate stage with ectopic homeotic gene expression in many discs (Saget *et al.* 1998), hence strong HOM gene deregulation is also present in these mutants. Severity of germline proliferation defects increases similarly from partial $mxc^{M1}$ sterility to reduced $mxc^{G43}$ gonads in males and to almost no detectable gonad development in $mxc^{mbn}$ larvae (Docquier *et al.* 1996). *mxc* animals show increasing developmental delays from $mxc^{M1}$ to $mxc^{16a\text{-}1}$ (the strongest allele that reaches the third larval instar), whereas imaginal discs and brains of some $mxc^{mbn1}$ and all $mxc^{16a\text{-}1}$ larvae are reduced in size and amorphic *mxc* alleles are lethal in clones (Saget *et al.* 1998). Mitotic figures or metaphase chromosome morphology are not visibly affected by loss of *mxc* (Saget *et al.* 1998), hence $mxc^{+}$ could have a more subtle effect on cell division rates, possibly through the cell cycle. Interestingly, recent data suggest several links between PcG function and regulation of the cell cycle (reviewed in Brock and van Lohuizen 2001).

For pseudotumor formation, lymph gland overgrowth, and mitosis rate in circulating hemocytes, we found increasing severity from $mxc^{G46}$, $mxc^{G43}$, $mxc^{mbn1}$ to $mxc^{16a\text{-}1}$. The exception was the lower number of circulating hemocytes in $mxc^{16a\text{-}1}$ compared to $mxc^{mbn1}$ larvae (Table 1). Proliferation and survival of larval imaginal cells are more affected by $mxc^{16a\text{-}1}$ than by $mxc^{mbn1}$ (Saget *et al.* 1998), hence this lower hemocyte density might reflect the final outcome of increased lymph gland and hemolymph cell division together with lesser survival of circulating hemocytes. Alternatively, $mxc^{16a\text{-}1}$ might affect hemocyte identity in the lymph glands more than $mxc^{mbn1}$, and fewer $mxc^{16a\text{-}1}$ cells would consequently be released into the body cavity (see below).

***mxc* function in relation to the Toll pathway:** Several mutant phenotypes suggested that *mxc* and the Toll pathway could regulate a common set of processes. First, both gain of Toll signaling and *mxc* mutations induce lymph gland and hemocyte overproliferation. Furthermore, *mxc* and *cact* mutants exhibit decreased adhesion among fat body cells, while overexpression of the target of Toll signal Dorsal/Rel and loss of *mxc* both induce salivary gland atrophy (Qiu *et al.* 1998; O. Saget, personal communication). Our genetic analysis shows that loss of Toll signal is epistatic over loss of *mxc* in circulating hemocyte production, indicating that increased hemocyte numbers in *mxc* mutants require the Toll pathway. But Toll signal g.o.f. and *mxc* phenotypes also show striking differences since lamellocyte differentiation is more strongly induced and pseudotumors are much more frequent in Toll g.o.f. larvae. A further difference is that crystal cell numbers are not affected in Toll g.o.f. mutants (Lanot *et al.* 2001). We interpret these differences as an indication that Toll signal and *mxc* represent different inputs, which both regulate wild-type hemocyte production. Loss of the Toll input could render lymph gland cells unable to respond to loss of *mxc*.

***hop* and *D-stat* in hematopoiesis control:** Contrary to a study of larvae with lessened Toll signal (Qiu *et al.* 1998), we found no modification of circulating hemocyte numbers in wandering larvae with total loss of *hop*. Amorphic *hop* larvae survive until the late larval/pupal stage because of maternal *hop* product perdurance (Perrimon and Mahowald 1986). Hence wandering $hop^{-}$ larvae may not be sufficiently depleted of *hop*/JAK to show defective hemocyte production. Alternatively, this result could support the view (Luo *et al.* 1997) that in plasmatocytes *hop*/JAK regulates larval capacity to respond to proliferative and differentiative signals. Therefore, only the mutant, overactive forms of *hop*/JAK encoded by $hop^{Tum\text{-}l}$ and $hop^{T42}$ (Luo *et al.* 1997) would affect hemocyte production. Loss of *hop* might not change

basic hemocyte production but could render the system less able to respond to an infection or immune challenge.

Our comparison between $hop^{Tum\text{-}l}$/Y and $hop^{Tum\text{-}l}$/Y; $D\text{-}stat^{6346}$/+ males (as well as $hop^{Tum\text{-}l}$/+ and $hop^{Tum\text{-}l}$/+; $D\text{-}stat^{6346}$/+ females) revealed that *D-stat* product is involved in *hop* g.o.f. plasmatocyte overproliferation. This possibility was previously suggested by Zeidler *et al.* (2000) and is upheld by the fact that D-STAT can bind to the *D-raf* promoter and activate its transcription (Kwon *et al.* 2000). Luo *et al.* (2002) recently reported that JAK g.o.f.-mediated hemocyte overproliferation and lamellocyte differentiation both require the *D-Raf*/D-MEK/mitogen-activated protein kinase pathway, linking again in a common regulatory network these two aspects of Drosophila hematopoiesis. If active in both, then D-STAT functions like its mammalian homolog STAT5, which regulates both proliferation and differentiation of hematopoietic cells (Nosaka *et al.* 1999; reviewed in Luo and Dearolf 2001). We observed a critical requirement for *D-stat* product on hemocyte numbers only in the *hop* g.o.f. context at 25°; indeed, loss of *D-stat* in $D\text{-}stat^{HJ}/D\text{-}stat^{6346}$ larvae had no effect on hemocyte numbers of $mxc^{G43}$/Y, and loss of one *D-stat* copy had no effect on plasmatocyte numbers of $Tl^{10b}$/Y larvae. Gain of *hop* induced a >10-fold increase in circulating hemocytes whereas the gain-of-Toll or loss-of-*mxc* contexts that we examined induced but 3- to 4-fold increases. Therefore, this apparent difference in sensitivity to *D-stat* dosage could reflect a threshold situation, where only stronger hemocyte overproductions are visibly affected when *D-stat* is reduced.

**Lamellocyte differentiation and *D-stat*:** Mutation of *D-stat* partially suppresses pseudotumors and lamellocyte differentiation in *hop* g.o.f. (Hou *et al.* 1996; Yan *et al.* 1996; Luo *et al.* 1997; this article). Lamellocyte differentiation induced in $Tl^{10b}$/+ larvae also depends on *D-stat* dosage (Table 5). We were unable to establish whether $Tl^{10b}$-induced lamellocyte differentiation depends on *hop*/JAK, since $hop^{VA275}$/Y;$Tl^{10b}$/+ larvae, in which JAK is absent, died before the third instar. As heterozygosity for *D-stat* suppresses $+/Tl^{10b}$ lamellocyte production, we propose that the Toll pathway is upstream of JAK/STAT signaling in this process. This would confirm the hypothesis of Mathey-Prevot and Perrimon (1998) who speculated that Toll might be upstream of JAK/STAT in hemocyte differentiation. Lagueux *et al.* (2000) have recently shown similar sequential effects of these two pathways on induction of a complement-like protein, which could have important roles in defense response to infection of Drosophila larvae. As argued above, the lack of effect of *D-stat* mutation in $mxc^{G43}$ larvae could indicate that lamellocyte production in this mutant was too low to be sensitive to a reduction in *D-stat* product.

**Lymph glands and circulating hemocytes in double stimulated hematopoiesis conditions:** Double-mutant contexts, which each alone increase hemocyte production, yielded intriguing results of reduced hemocyte numbers compared to single mutants. This was true for combinations associating *mxc* with gain of function of Toll or of JAK, but also for double Toll signal and JAK g.o.f. contexts. The double-mutant animals contained abnormally large hemocytes with numerous inclusions or vacuoles and showed delayed development (24–48 hr in wandering larvae). This could reflect a lesser circulating hemocyte production, as in severe *domino* mutants (Braun *et al.* 1997, 1998; Ruhf *et al.* 2001). Alternatively, hemocytes could be attached to the imaginal discs and involved in phagocytosis, as in *proliferation disruptor* (*prod*) mutants that show delayed development and intense imaginal cell death (Torok *et al.* 1997). Lymph glands from $mxc^{G43}$ $hop^{Tum\text{-}L}$/Y and $mxc^{G43}$/Y;$cact^{A2}/cact^{A2}$ larvae showed dramatic overgrowth and extreme fragility, associated with intense mitotic activity and the presence of numerous differentiated hemocytes in the glands. The enlarged lymph glands were sometimes encapsulated by lamellocytes and melanized, recalling phenotypes of medium severe *domino* mutants that show massive overgrowth and blackening of the lymph glands together with rare, abnormally large circulating hemocytes (Ruhf *et al.* 2001). Recently, Luo *et al.* (2002) reported that *hop* g.o.f. animals without a functional *D-raf* pathway also exhibit overgrown lymph glands and dramatic reductions in circulating blood cell numbers and attributed this phenotype to a requirement of *D-raf* signaling for cell survival in the lymph glands, downstream of *hop* g.o.f.-induced proliferation. All these phenotypes illustrate that the final number of circulating larval hemocytes depends on several processes in the lymph glands, including control of prohemocyte division rates and of hemocyte differentiation (*hop*, *D-stat*, *Toll*, and *mxc*) but also hemocyte survival (*dom*, *D-raf*; Braun *et al.* 1997, 1998; Luo *et al.* 2002) and hemocyte capacity to cross the basement membrane (*dom*). A common explanation for all these data could be that strong deregulation of one or joint deregulation of any two of these processes could lead to production of cells whose modified characteristics (cell surfaces) hinder their passage into the body cavity or whose modified identities prevent survival. Overgrown $mxc^{G43}$ $hop^{Tum\text{-}l}$/Y or $mxc^{G43}$/Y;$cact^{A2}/cact^{A2}$ lymph glands showed numerous differentiated hemocytes and no enhanced cell death, suggesting that basement membrane passage rather than cell survival is affected in these animals. On the other hand, hemocyte identities would be so modified in strong $dom^{1}$ mutant larvae that cell survival is impossible, hence the lack of circulating hemocytes and melanized lymph glands containing abnormal dying cells in these animals (Braun *et al.* 1997). This agrees with the fact that $dom^{1}$ is not rescued by stimulated hemocyte production, as in $dom^{1}/dom^{1}$;$Tl^{10b}$/+, $hop^{Tum\text{-}l}$/Y;$dom^{1}/dom^{1}$ and $dom^{1}/dom^{1}$;$cact^{A2}/cact^{A2}$ animals (Braun *et al.* 1998) or in

*mxc*$^{GA3}$;*dom*$^{1}$/*dom*$^{1}$ and *mxc*$^{mbn1}$;*dom*$^{1}$/*dom*$^{1}$ larvae (M. Meister, personal communication).

***mxc*, PcG genes, and trxG genes in Drosophila hematopoiesis control:** Several lines of evidence suggest that mammalian hematopoiesis depends on *Hox* genes. First, blood stem cells express a number of *Hox* genes; second, disruption of specific *Hox* genes in mice causes hematopoietic defects; finally, overexpression of individual *Hox* genes in hematopoietic cells can induce leukemogenesis (reviewed in Magli *et al.* 1997; Chiba 1998). Furthermore, murine PcG genes—M33, Bmi-1, Mel 18, and *eed*—that maintain *Hox* gene expression during development also participate in hematopoiesis regulation (reviewed in Takihara and Hara 2000; Raaphorst *et al.* 2001). An attractive, simple hypothesis would be that loss of mammalian PcG genes affects hematopoiesis through deregulation of *Hox* genes in hematopoietic cells, as PcG mutations modify anterior-posterior identity specification (reviewed in Pirrotta 1998; van Lohuizen 1999). In view of this, we asked whether other PcG mutations associated with *mxc* could affect hematopoiesis. We observed no hematopoietic phenotype interactions between *mxc* and *Scm*, *Pcl*, *Pc*, or *Psc* mutations, although these genes together regulate Drosophila HOM-*Hox* genes (Saget *et al.* 1998) and include the M33 homolog *Pc* and the Bmi-1 and Mel 18 homolog *Psc*. Furthermore, no interaction was found between the *eed* homolog *esc* and *mxc*, although *eed* represses murine hematopoietic cell proliferation (Lessard *et al.* 1999) as *mxc* does in Drosophila larvae. Thus *mxc* hematopoiesis defects behave like *mxc* germline proliferation defects, which according to genetic analysis are also independent of other PcG gene products (Docquier *et al.* 1996). Some of these PcG genes may play a role in larval hematopoiesis, since we examined only *trans*-heterozygous mutant contexts. But in contrast to their role in segmental identity specification, no haplo-insufficiency for such an effect was detected in hematopoiesis. Hence Drosophila requirements for PcG gene products are different in A/P identity specification and in hematopoiesis. As mammalian hematopoiesis involves specification of many different cell lineages and differentiation of many tissues and cell types (reviewed in Orkin 2000), PcG genes may well have been recruited during evolution to control a number of these steps, which have no Drosophila equivalents. In agreement with this, a recent review (Raaphorst *et al.* 2001) underlined the role of PcG genes as regulators of mammalian lymphopoiesis.

Three trxG genes were tested for effects on hemocyte production. *trx* mutations had no effect on hemocyte numbers either, although overexpression of the human *trithorax* homolog MLL is associated with many acute myeloid or lymphoblastic leukemias (reviewed in van Lohuizen 1999) and MLL$^{-}$/+ mice suffer severe hematopoietic defects (Yu *et al.* 1995). We found that overproduction of hemocytes in *mxc* larvae depends on the transcriptional activator BRM. BRM is homologous to yeast SWI2, a DNA-stimulated ATPase that is part of the large SWI/SNF protein complex that modifies target transcription by changes in chromatin structure (Tamkun *et al.* 1992; Dingwall 1995; Vignali *et al.* 2000). In larvae, BRM activates HOM gene expression and is required for imaginal disc cell viability (Elfring *et al.* 1998). BRM has many targets, so our results could reflect a general requirement for BRM dosage on cell division. Still, *brm*$^{2}$/+ animals show no developmental delay and loss of a *brm*$^{+}$ copy had no effect on hematopoietic overproliferation induced by gain of Toll signal. Hence *brm* could well have a positive part in blood cell number or division control. *brm* could, as a trxG gene, be required at the same level as but antagonistic to *mxc*. Alternatively, since hemocyte phenotypes induced by loss of *mxc* and by activation of JAK are both partially suppressed by loss of *brm*, *brm*, *mxc*, and *hop* could all provide separate regulatory inputs, which together control hematopoietic cell divisions and cell density and cell survival in the larva.

*moira* encodes a Drosophila homolog of human and yeast chromatin-remodeling factors; *mor* and *brm* interact genetically and MOR interacts physically with BRM in the same large chromatin-remodeling protein complex in the embryo (Crosby *et al.* 1999). Yet *mor* mutations *in trans* had no effect on hematopoiesis regulation. One possible explanation might be that composition of the SWI/SNF protein complex is different in embryos and in the lymph glands. Alternatively, as previously argued for the PcG, haploidy for *mor* (or for *trx*) may not reduce gene products enough to cause a mutant phenotype.

We found that three genes, *mxc*, *brm*, and *dom*, required for maintenance of HOM gene expression patterns are involved in control of Drosophila hematopoiesis. *dom*, like *brm*, encodes SWI2/SNF2 family DNA-dependent ATPases involved in gene expression control through modulation of chromatin structure (Ruhf *et al.* 2001). Interestingly, hypomorphic *dom* phenotypes indicate that *dom*, like *mxc*, negatively regulates lymph gland cell proliferation and maintains lymph gland cell identities (Braun *et al.* 1997, 1998; Ruhf *et al.* 2001), whereas *brm* activates proliferation. These effects recall *dom* and *mxc* repression and *brm* activation of HOM gene expression (Ruhf *et al.* 2001), suggesting the possibility that in the lymph glands, *dom*, *mxc*, and *brm* could participate in a common mechanism of proliferation and identity maintenance, which could involve modulation of chromatin structure.

***mxc* controls blood cell proliferation and differentiation but not lineage specification:** We have shown that *mxc* functions as a cell autonomous regulator of cell divisions in the lymph glands, as well as in circulating blood cells, and that loss of *mxc* favors differentiation of plasmatocyte lineage-specific cells such as podocytes and lamellocytes. All blood cell types found in *mxc* mutants, even podocytes, are found in wild type. Crystal

cells, although fewer, were always present in *mxc* mutants. We interpret this as meaning that loss of *mxc* does not change hemocyte lineage specification in the lymph glands. Rather, *mxc* controls steady-state hemocyte numbers in the body cavity of the larva. *mxc* and *brm* could both provide regulatory inputs in this process, together with *domino*, the JAK and Toll pathways, and other products, including the cell cycle regulated Pendulin protein, the Drosophila homolog of the mammalian S6 riboprotein, and the *l(3)mbn*-encoded plasma membrane protein (reviewed in Dearolf 1998). Together our data indicate that wild-type *mxc* product in larval hematopoiesis would maintain the normal rates of plasmatocyte proliferation and of crystal cell formation, as well as the normal timing of differentiation into self-recognizing macrophages. Under this hypothesis, *mxc* function in hematopoiesis would still be similar to PcG function in segmental identity specification in that both ensure that normal structures develop at the right time and place.

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